CYTOKININS AS CENTRAL REGULATORS OF PLANT GROWTH AND DEVELOPMENT

EDITED BY: Jan Hejatko, Wolfram G. Brenner, Klára Hoyerová and G. Eric Schaller

PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88974-277-6
DOI 10.3389/978-2-88974-277-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

CYTOKININS AS CENTRAL REGULATORS OF PLANT GROWTH AND DEVELOPMENT

Topic Editors:
Jan Hejatko, Central European Institute of Technology (CEITEC), Czechia
Wolfram G. Brenner, Leipzig University, Germany
Klára Hoyerová, Academy of Sciences of the Czech Republic, Czechia
G. Eric Schaller, Dartmouth College, United States

Citation: Hejatko, J., Brenner, W. G., Hoyerová, K., Schaller, G. E., eds. (2022). Cytokinins as Central Regulators of Plant Growth and Development. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-277-6

Table of Contents

frontiers in Plant Science

MINI REVIEW
published: 05 June 2020
doi: 10.3389/fpls.2020.00741

New Insights Into the Metabolism and Role of Cytokinin *N*-Glucosides in Plants

Klára Hoyerová and Petr Hošek*

Institute of Experimental Botany of the Czech Academy of Sciences, Prague, Czechia

Cytokinin (CK) *N*-glucosides are the most abundant group of CK metabolites in many species; however, their physiological role *in planta* was for a long time perceived as irreversible storage CK forms only. Recently, a comprehensive screen showed that only vascular plants form CK *N*-glucosides in contrast to mosses, algae, and fungi. The formation of CK *N*-glucosides as biologically inactive CK conjugates thus represents an evolutionarily young mechanism for deactivation of CK bases. Even though CK *N*-glucosides are not biologically active themselves due to their inability to activate the CK perception system, new data on CK *N*-glucoside metabolism show that *trans*-zeatin (tZ) N7- and N9-glucosides are metabolized *in vivo*, efficiently releasing free CK bases that are most probably responsible for the biological activities observed in a number of bioassays. Moreover, CK *N*-glucosides' subcellular localization as well as their abundance in xylem both point to their possible plasma membrane transport and indicate a role also as CK transport forms. Identification of the enzyme(s) responsible for the hydrolysis of tZ N7- and N9-glucosides, as well as the discovery of putative CK *N*-glucoside plasma membrane transporter, would unveil important parts of the overall picture of CK metabolic interconversions and their physiological importance.

Keywords: cytokinin *N*-glucoside, isopentenyladenine N7-glucoside, isopentenyladenine N9-glucoside, zeatin N7-glucoside, zeatin N9-glucoside, cytokinin metabolism, cytokinin transport, UGT

OPEN ACCESS

Edited by:
Rujin Chen,
Lanzhou University, China

Reviewed by:
Georgy A. Romanov
Timiryazev Institute of Plant Physiology (RAS)
Moscow, Russia
Ivo Frébort,
Palacký University Olomouc, Czechia
Wendy A. Stirk,
University of KwaZulu-Natal,
South Africa

****Correspondence:***
Klára Hoyerová
hoyerova@ueb.cas.cz

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *09 March 2020*
Accepted: *08 May 2020*
Published: *05 June 2020*

Citation:
Hoyerová K and Hošek P (2020) New Insights Into the Metabolism and Role of Cytokinin N-Glucosides in Plants. Front. Plant Sci. 11:741. doi: 10.3389/fpls.2020.00741

INTRODUCTION

Cytokinins (CKs), being one of the most important groups of regulators of plant growth, development and adaptability, are present in plant tissues in numerous forms that differ in their biological activity due to differing affinities of the CK sensing system to particular CK derivatives. Modifications and interconversions of the CK forms, their distribution in plant/cellular compartments, their transportability and degradability through cytokinin oxidase/dehydrogenase (CKX) thus form a complex net of tightly controlled CK signaling. Among others, CK bases – most abundant among them being *trans*-zeatin (tZ) – are recognized to be the most potent regulators of physiological processes while CK glucose conjugates, especially when bound at the N7 or N9 position of the purine ring, are believed to serve as irreversible deactivation products (Kieber and Schaller, 2018).

N-glucosides are formed through the activity of enzymes transferring nucleotide-diphosphate-activated sugars, usually UDP-glucose. The glucosyltransferase (UGT) superfamily consists of 107 genes in Arabidopsis (Yang et al., 2018) but only two specific CK *N*-glucosyltransferases UGT76C1

and UGT76C2 have been identified so far (Hou et al., 2004). Their involvement in N7- and N9-glucoside formation was then confirmed *in planta* (Wang et al., 2011) and the dominant activity of UGT76C2 compared to UGT76C1 in maintaining CK homeostasis was revealed (Šmehilová et al., 2016). However, no enzyme responsible for the release of CK bases from N7- and N9-glucosides has yet been identified in Arabidopsis despite the Zm-p60.1 enzyme isolated from maize having been shown to hydrolyze tZ N9-glucoside (albeit at very low velocity) (Filipi et al., 2012).

In vascular plants, *N*-glucosides can accumulate to greater extents compared to other CK metabolites, both under natural conditions and especially in response to CK overabundance. Following overexpression of a CK biosynthetic gene – isopentenyltransferase (IPT) – or after exogenous application of CKs, increase in CK content is immediately followed by the formation of N7-glucosides, serving probably as the most feasible mechanism for fast attenuation of active CK levels (radish: Parker et al., 1972; tobacco, potato, yellow lupine: Fox et al., 1973; Arabidopsis: Galichet et al., 2008; Hošek et al., 2020; maize: Šmehilová et al., 2016). On the contrary, the natural distribution of CK metabolites in young maize leaves is mostly lacking CK *N*-glucosides (Gajdošová et al., 2011; Hluska et al., 2016) suggesting a possibly distinct CK metabolism concerning *N*-glucosides in monocotyledonous compared to dicotyledonous plants.

Here, the current state of knowledge on the distribution of CK *N*-glucosides, their metabolism, as well as their physiological role is reviewed, opening a new view on the otherwise old topic.

EVOLUTIONARILY DEPENDENT OCCURRENCE OF CYTOKININ *N*-GLUCOSIDES

CK *N*-glucosides were detected in plant material as early as in the early 1970s and were suggested to be formed in a wide range of plants (radish: Parker et al., 1972; tobacco, potato, yellow lupine: Fox et al., 1973). Soon after that, an enzyme responsible for the *N*-glucosylation, cytokinin 7-glucosyltransferase was reported (Entsch et al., 1979) and another enzyme involved in N9-glucoside production was probably also present in the radish enzyme assay (Entsch and Letham, 1979). However, a comprehensive screen across the plant kingdom for *N*-glucoside occurrence was published only recently. Climbing up the evolutionary tree, CK metabolic profiles in fungi (Morrison et al., 2015; Trdá et al., 2017), algae (Stirk et al., 2013; Žižková et al., 2017), and bryophytes (Drábková et al., 2015) revealed none or barely detectable concentrations of CK *N*-glucosides, in contrast to vascular plants, where *N*- or *O*-glucosides represent the prevailing CK forms (Gajdošová et al., 2011). *N*-glucosylation thus seems to be an evolutionarily recent mechanism to inactivate biologically active CKs. In vascular plants however, the abundance of CK *N*-glucosides is not proportional to the evolutionary age of the species and neither is their distribution with respect to monocotyledonous or dicotyledonous plants since the prevalence of *N*-glucosides occurs in a wide variety of evolutionarily distinct species such as *Petunia hybrida*, *Nicotiana tabacum*, *Musa acuminata*, *Lilium elodie*, *Anthurium andreanum* (Gajdošová et al., 2011), *Arabidopsis thalina* (Jiskrová et al., 2016; Šmehilová et al., 2016), *Centaurium erythraea* (Trifunović-Momčilov et al., 2016), *Hordeum vulgare* (Jiskrová et al., 2016), *Raphanus sativus* (Blagoeva et al., 2004), *Solanum lycopersicum* (Žižková et al., 2015) and others. The opposite is the case in e.g., *Manihot esculenta*, *Zea mays*, *Triticum aestivum*, *Phragmites australis*, *Avena sativa* (Gajdošová et al., 2011) with only a minor portion of CK *N*-glucosides in whole CK spectrum.

THE LEVELS OF CYTOKININ *N*-GLUCOSIDES CHANGE DURING ONTOGENESIS

CK content changes over the lifespan in a number of species. CK *N*-glucoside content was inspected in four stages of Arabidopsis ontogenesis: green fully developed leaves from a non-flowering rosette, green fully developed leaves from a flowering rosette, green fully developed leaves from a plant with maturing siliques and senescent leaves from a plant with maturing siliques (Šmehilová et al., 2016). The abundance of CK *N*-glucosides shows an increasing trend with a pronounced elevation in senescent leaves caused predominantly by the accumulation of iP-N7G followed to a lesser extent by tZ-N7G. Similarly, in tobacco the levels of *N*-glucosides (predominantly iP-N9G and tZ-N7G) is higher in mature non-senescing leaves compared to younger ones (Benková et al., 1999). The content of *N*-glucosides (iP-N9G and tZ-N9G) in maize exhibited a growing trend in 3-month-old roots compared to 7-day-old ones (Hluska et al., 2016). The same study (Hluska et al., 2016) also showed similar trends of increasing CK *N*-glucoside levels in reproductive organs (ovules, kernels, and silks), where the content of tZ-N9G, DHZ-N9G, and iP-N9G grew gradually over time following pollination. Apart from these detailed studies, it is generally observed that the content of *N*-glucosides in 2-week-old Arabidopsis seedlings – commonly used experimental material – is lower (Šimura et al., 2018; Hošek et al., 2020 and others) compared to mature plants (35 day-old leaf rosettes: Galichet et al., 2008; 45 day-old leaf rosettes: Jiskrová et al., 2016). It could be concluded that CK *N*-glucosides gradually accumulate during the life span of both monocotyledonous and dicotyledonous plants.

METABOLISM OF CYTOKININ *N*-GLUCOSIDES

Based on the longevity of benzyladenine N7-glucoside (BAP-N7G) in plant cells due to its resistance to enzymes that degrade CK molecules by side chain cleavage, CK-N7Gs were suggested to be either active or simply detoxification or storage forms of CKs more than 40 years ago (Fox et al., 1973). Their role as terminal products of irreversible deactivation was then supported by a study showing that N7- and N9-glucosides are not efficiently cleaved by β-glucosidase (Brzobohatý et al., 1993), and their possible hydrolysis was

further questioned based on their enormous accumulation in comparison to active CKs (Šmehilová et al., 2016). On the contrary, BAP-N7G conversion to its base was demonstrated in tobacco cells (Laloue et al., 1977). Also, direct release of CK bases from CK *N*-glucosides was demonstrated in both monocotyledonous and dicotyledonous plants. It was shown that N9-glucosides of 3-methoxyBAP, BAP and dihydrozeatin (DHZ) are converted back to their active forms in maize roots (Podlešáková et al., 2012). Further, Zm-p60.1 maize enzyme was able to hydrolyze tZ N9-glucoside but not tZ N7-glucoside *in vitro* (Filipi et al., 2012). Using a barley line overexpressing *AtCKX1* with predominant expression in roots, Jiskrová et al. (2016) observed a 60% decrease of tZ-N9G levels in leaf extracellular space compared to control. Expecting CKX preference for tZ-N9G in roots, transport of root-synthesized tZ-N9G via xylem to leaves is implicated, also calling into question their role as irreversibly inactivated metabolites that accumulate in older tissues. Recently, hydrolysis of both tZ-N7G and tZ-N9G was shown in 2-week-old Arabidopsis seedlings (Hošek et al., 2020). After short time treatments with exogenously applied sugar conjugates, both tZ N7- and N9-glucosides were efficiently converted into tZ free base within 10 min. Contrary to this, no conversion of iP *N*-glucosides was observed as high levels of both iP-N7G and iP-N9G were accumulated with no increase of other CK metabolites. The same result was then observed with radioactively labeled iP N9- and tZ N9-glucosides exogenously applied to Arabidopsis cell cultures. In this study, the differences between the metabolism of iP *N*-glucosides and tZ *N*-glucosides in the Arabidopsis seedling were also shown by their differing native concentrations (iP-type *N*-glucosides being significantly more abundant than tZ-type *N*-glucosides), and by the fact that after application of CK bases, iP was predominantly converted to its N7-glucoside, while for tZ phosphoribosylation prevailed (Hošek et al., 2020).

In Arabidopsis, the activity of putative glucosidase(s) with the ability to cleave CK *N*-glucoconjugates thus seems to be CK-type-specific and not affected solely by the position of the sugar on the adenine skeleton. This is, however, not the case in monocotyledonous maize where tZ N9-glucoside was efficiently hydrolyzed in contrast to tZ N7-glucoside (Filipi et al., 2012), suggesting distinct strategies for *N*-glucoside management in maize compared to Arabidopsis (**Table 1**).

TABLE 1 | Isopentenyladenine (iP) and *trans*-zeatin (tZ) N7- and N9-glucosides characteristics observed in dicotyledonous and monocotyledonous plants.

Arabidopsis thaliana (tobacco, zucchini, soya) (dicotyledonous)					
iP N7-GLUCOSIDE			**tZ N7-GLUCOSIDE**		
Content in seedlings	Highest, iP7N >> CKs	Wang et al., 2011; Šimura et al., 2018; Hošek et al., 2020	Content in seedlings	Medium high	Wang et al., 2011; Šimura et al., 2018; Hošek et al., 2020
CKX substrate	No	Galuszka et al., 2007	CKX substrate	No	Galuszka et al., 2007
Conversion to base	No	Wang et al., 2011; Šimura et al., 2018; Hošek et al., 2020	Conversion to base	Yes	Hošek et al., 2020
Localization	Apoplast, vacuole, (chloroplast)	Benková et al., 1999; Jiskrová et al., 2016	Localization	Apoplast, vacuole, (chloroplast)	Benková et al., 1999; Jiskrová et al., 2016
iP N9-GLUCOSIDE			**tZ N9-GLUCOSIDE**		
Content in seedlings	High, iP9N < iP7N	Hošek et al., 2020	Content in seedlings	Medium high	Hošek et al., 2020
CKX substrate	Yes	Galuszka et al., 2007	CKX substrate	Yes	Galuszka et al., 2007
Conversion to base	No	Hošek et al., 2020	Conversion to base	Yes	Hošek et al., 2020
Localization	Apoplast, vacuole, (chloroplast)	Benková et al., 1999; Jiskrová et al., 2016	Localization	Apoplast, vacuole, (chloroplast)	Benková et al., 1999; Jiskrová et al., 2016
Maize (barley, oat, wheat) (monocotyledonous)					
iP N7-GLUCOSIDE			**tZ N7-GLUCOSIDE**		
Content in seedlings	–	–	Content in seedlings	–	–
CKX substrate	No	Zalabák et al., 2014	CKX substrate	No	Zalabák et al., 2014
Conversion to base	–	–	Conversion to base	No	Filipi et al., 2012
Localization	(Apoplast, chloroplast)	Benková et al., 1999; Jiskrová et al., 2016	Localization	(Apoplast, chloroplast)	Benková et al., 1999; Jiskrová et al., 2016
iP N9-GLUCOSIDE			**tZ N9-GLUCOSIDE**		
Content in seedlings	Medium	Vyroubalová et al., 2009; Hluska et al., 2016	Content in seedlings	Medium	Vyroubalová et al., 2009; Hluska et al., 2016
CKX substrate	Yes	Zalabák et al., 2014	CKX substrate	Yes	Zalabák et al., 2014
Conversion to base	–	–	Conversion to base	Yes	Filipi et al., 2012
Localization	(Apoplast, chloroplast)	Benková et al., 1999; Jiskrová et al., 2016	Localization	(Apoplast, chloroplast)	Benková et al., 1999; Jiskrová et al., 2016

CKX ACTIVITY AND SUBSTRATE SPECIFICITY TOWARD CYTOKININ *N*-GLUCOSIDES

The only enzyme known to be responsible for the degradation of cytokinins is cytokinin oxidase/dehydrogenase (CKX; EC 1.5.99.12), which selectively removes the isoprenoid side chain from unsaturated CKs converting them to adenine derivatives and the corresponding unsaturated aldehydes (Paces et al., 1971; Galuszka et al., 2001). The CKX enzymes from Arabidopsis (Galuszka et al., 2007) and maize (Zalabák et al., 2014) were studied extensively and substrate preferences of particular isozymes were described. In Arabidopsis, AtCKX1 and AtCKX7 exhibit high preference for iP N9-glucoside and AtCKX1 also for tZ N9-glucoside under weakly acidic conditions *in vitro* (Galuszka et al., 2007). Kowalska et al. (2010) further reported a 40-fold higher degradation rate of iP N9-glucoside by AtCKX1 and AtCKX7 compared to iP. In maize, all four isoforms that are targeted to the apoplast – namely ZmCKX2, ZmCKX3, ZmCKX4a, and ZmCKX4b, degraded iP N9-glucoside preferentially. In addition, iP N7-glucoside is resistant to degradation by Arabidopsis and maize CKXs (Galuszka et al., 2007; Zalabák et al., 2014). This is in line with the general observation that iP-N9G is less abundant compared to iP-N7G in Arabidopsis. In conclusion, both Arabidopsis and maize CKXs are able to cleave CK N9-glucosides but not CK N7-glucosides. Considering also that the number of CKX genes and isozymes varies between monocotyledonous and dicotyledonous plants (monocotyledonous species tend to have more; Mameaux et al., 2012; Zalabák et al., 2014), the differences between CK N7- and N9-glucoside metabolism can be explained by the different CKX affinities in combination with distinct substrate-enzyme intracellular localization, without the need for different activities of the UGTs producing the *N*-glucosides.

Sensitivity of CK Signaling Cascade Toward *N*-Glucosides

At the level of CK signaling, both tZ N7- and tZ N9-glucosides were tested. Both glucosides were biologically active in an Arabidopsis reporter gene assay but showed no activity in an *E. coli* expression assay. The authors speculated that this might be due to their rapid metabolism in Arabidopsis (Spíchal et al., 2004). It was later demonstrated that the 3D structure of the ligand-binding site of the cytokinin receptor AHK4 prevents any hormonal activity of tZ ribosylated at the N9 position since the riboside moiety does not fit into the binding pocket (Hothorn et al., 2011). Since the same molecular structural feature is valid for all CK *N*-glycosides it might be concluded that the CK sensing system does not recognize CK *N*-glucosides. Lomin et al. (2015) later showed that only free bases are biologically active, and the observation was supported by molecular modeling of cytokinin-receptor interaction including AHK3 and ZmHK1. Furthermore, iP-N7G, iP-N9G, tZ-N7G, and tZ-N9G were analyzed for their inhibition potency on AHK3 and AHK4 receptors. No inhibition was detected for any of the tested glucosides (Šmehilová et al., 2016), thus confirming that CK *N*-glucosides do not interact directly with the CK perception system.

COMPARTMENTATION AND TRANSPORT OF CYTOKININ *N*-GLUCOSIDES

The compartmentation of CK metabolites and enzymes involved in CK metabolism represent a major factor for achieving precisely regulated levels of active CK derivatives available to particular CK receptors, subsequently triggering the cognate physiological response. Specific localization of Arabidopsis CK metabolic enzymes with respect to both tissues and cellular compartments suggested possible places of CK origin as well as the ways of CK metabolite re-localization at the cellular and also the whole-plant level. Recently, Jiskrová et al. (2016) identified 25 CK metabolites in isolated vacuoles and protoplasts as well as in whole leaf tissue to calculate a distribution pattern of individual CK forms in the cell interior, exterior and vacuoles of Arabidopsis and barley leaves. Interestingly, tZ-N7G, iP-N7G, and tZ-N9G were found predominantly in the apoplast and to a lesser extent in vacuoles, where DHZ-N7G was also detected. No CK *N*-glucosides were detected in the cytosol despite the fact that both CK *N*-glucosidases UGT76C1 and UGT76C2 are confirmed to localize solely to cytosol (Hou et al., 2004; Šmehilová et al., 2016; **Figure 1**). The addition of a sugar onto a lipophilic acceptor makes the compound more polar and thus prevents it from diffusing freely across lipid bilayer membranes. On the other hand, the glucose conjugates are more hydrophilic and therefore more easily accessible to membrane-bound transporters that recognize glycosyl residues. Such carriers were found in tonoplast membranes, and the possible involvement of ABC pumps in the transport of glycosylated small lipophilic molecules has also been suggested (reviewed in Bowles et al., 2006). The question on the existence of CK *N*-glucoside-specific plasma membrane/tonoplast transporter(s) thus arises. The question is probably even more complex since CK *N*-glucosides were found in chloroplasts of both tobacco and wheat leaves (Benková et al., 1999).

PHYSIOLOGICAL IMPORTANCE OF CYTOKININ *N*-GLUCOSIDES

CK *N*-glucosides are biologically inactive CK forms not recognized by the CK sensing system (Hothorn et al., 2011; Lomin et al., 2015; Šmehilová et al., 2016). Their function seems to be in rapid reversible or irreversible inactivation, depending probably on the particular species and the specific CK type.

N-glucosides of both iP and tZ were reported to be highly abundant in the extracellular space and also found in xylem (Jiskrová et al., 2016) suggesting their export from the cytoplasm. In their study on the CK plasma membrane transporter ABCG14, Zhang et al. (2014) showed that *abcg14* mutant plants retain [$^{13}C_5$]tZ7G and [$^{13}C_5$]tZ9G

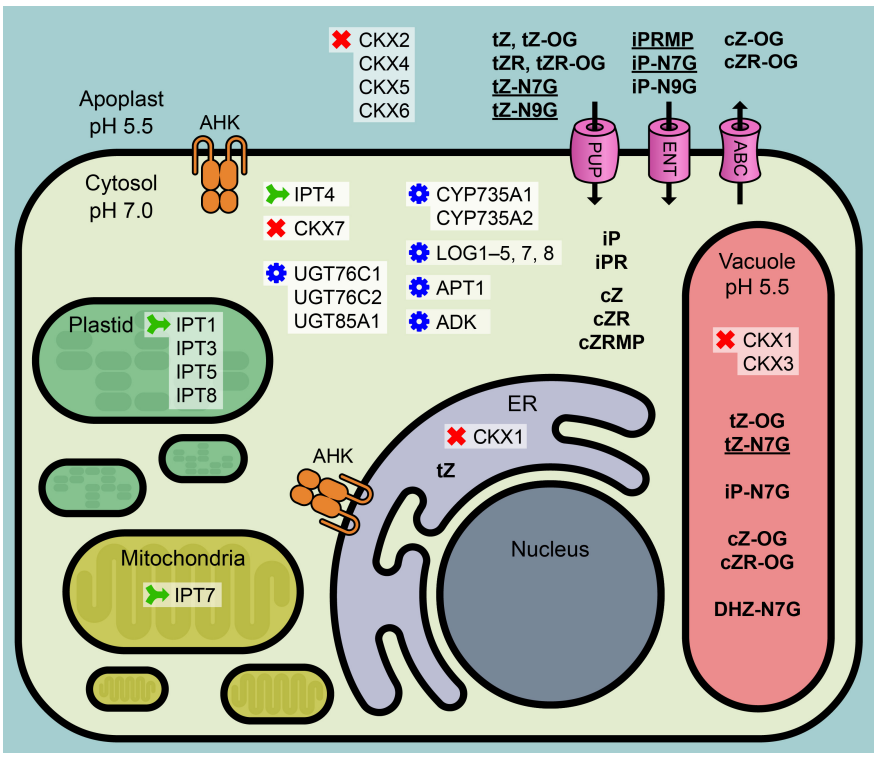


FIGURE 1 | Condensed overview of CK-related metabolic mechanisms in Arabidopsis cell. CK metabolic enzymes: green – biosynthesis, red – degradation, blue – conversion. IPT, isopentenyltransferase; UGT, UDP-glucosyltransferase; LOG, Lonely Guy, CK phosphoribohydrolase; CYP735A, cytochrome P450 mono-oxygenase; APT, adenine phosphoribosyltransferase; ADK, adenosine kinase; CKX, cytokinin oxidase/dehydrogenase. Pink – possible plasma membrane transporters: PUP, purine permeases; ENT, equilibrative nucleoside transporters; ABC, ABC transport proteins. Orange mark: AHK, Arabidopsis histidine kinase; ER, endoplasmic reticulum. Bold: CK metabolites; bold underlined: dominant CK metabolites. Based on the data published in Moffatt et al. (2000), Werner et al. (2003), Kasahara et al. (2004), Takei et al. (2004), Galuszka et al. (2007), Lizák et al. (2008), Kuroha et al. (2009), Zhang et al. (2013), Jiskrová et al. (2016), and Romanov et al. (2018).

in roots compared to wild type plants, indicating active transport of tZ *N*-glucosides via ABCG14. Even though the mechanism of PM transport of CK glucoconjugates still needs to be elucidated, their extracellular localization indicates a possible function in the facilitation of short- as well as long-distance transport.

Biological activity of CK *N*-glucosides observed in a number of assays is rather difficult to interpret since the assays usually assess slow processes (callus growth, leaf senescence) while CK metabolism in plant cells and tissues is considerably more rapid. Physiological activities of CK *N*-glucosides can thus probably be attributed to their hydrolysis and subsequent release of the free base. That was already suggested by Van Staden and Drewes (1991) in a comprehensive study of the biological activity of 28 natural and synthetic CKs in the soybean callus bioassay. It was shown that iP derivatives including iP-N9G are the least active, which is in agreement with the high levels of biologically inactive iP *N*-glucosides that are commonly seen soon after iP overabundance – since the iP *N*-glucosides seem not to be hydrolyzed and simply accumulate in the tissue (if not degraded by CKX) with no physiological effects.

In contrast, both tZ-N9G and DHZ-N9G had a comparable or even higher stimulatory effect on cell division than tZ, pointing to efficient hydrolysis of tZ (DHZ) *N*-glucosides producing the respective biologically active bases (Van Staden and Drewes, 1991).

Another possible explanation for the biological effects observed following CK *N*-glucoside overabundance can be their allosteric effect on certain enzymes of CK metabolism, for example, inhibition of the activity of distinct CKX. In such case, the levels of CK bases would increase due to a change in cell metabolism (CK decay inhibition), thus leading to the same situation and physiological response without direct hydrolysis of CK *N*-glucosides.

The involvement of enzymes that glucosylate CKs in control of CK homeostasis was extensively studied using Arabidopsis *ugt76c2* mutant plants as well as *UGT76C2* overexpressing lines (Wang et al., 2011). As expected, while the content of CK *N*-glucosides (iP-N7G, iP-N9G, tZ-N7G, tZ-N9G) was substantially lower in *ugt76c2* mutant compared to control plants, the overexpression of this gene led to increased production of iP-N7G, iP-N9G, tZ-N7G, and tZ-N9G. Cytokinin activity assays were then used to monitor the physiological response in the *ugt76c2* mutant and *UGT76C2* overexpressing lines.

Assays for primary root elongation, lateral root formation, chlorophyll retention as well as anthocyanin accumulation (all with exogenously applied benzylaminopurine) revealed cytokinin-deficient phenotypes for *UGT76C2* overexpressing lines (longer main root, higher lateral root density, lower chlorophyll and anthocyanin contents) while the opposite was true for the *ugt76c2* mutant. Furthermore, it was demonstrated that the *ugt76c2* mutant produces smaller seeds with reduced weights compared to wild type, while no difference was observed in the *UGT76C2* overexpressing lines (Wang et al., 2011).

CONCLUSION

CK *N*-glucosides are evolutionarily advanced players in the maintenance of CK homeostasis. iP-type *N*-glucosides represent products of irreversible deactivation serving to rapidly and efficiently reduce the active CK pool in cases of sudden overabundance or overproduction of iP-type CKs. iP *N*-glucosides thus accumulate in plant tissues to high levels that might be affected only by the specific activity of CKX. On the contrary, tZ (DHZ) *N*-glucosides are commonly hydrolyzed by an as yet unknown enzyme releasing the active base tZ (DHZ) that is probably responsible for the biological activities of tZ *N*-glucosides observed in many assays, since no CK *N*-glucoside has yet been shown to interact with the CK perception system. This mode of *N*-glucoside action seems to be distinct in dicotyledonous (both tZ N7- and N9-glucoside being metabolized) and monocotyledonous plants (where only tZ N9-glucoside is hydrolyzed). The differences observed between the accumulations of N7- and N9-glucosides may be attributed to distinct CKX activities toward particular CK *N*-glucosides in a species- and tissue-specific manner or to the differential activity of the suggested enzyme(s) responsible for CK *N*-glucoside hydrolysis, rather than to the activity of UGTs, which seem to act non-specifically. The occurrence of CK *N*-glucosides in the extracellular space strongly suggests their ability to cross the PM with the help of an unidentified PM transporter, thus presupposing that CK *N*-glucosides might act as CK transport forms as well (see the summaries in **Figure 1** and **Table 1**).

AUTHOR CONTRIBUTIONS

KH wrote the first draft of the manuscript. PH wrote sections of the manuscript and drew the figure. KH and PH contributed to manuscript revision, read and approved the submitted version.

FUNDING

The work was supported by the Czech Science Foundation grant no. 16-19557S and by The European Regional Development Fund-Project "Centre for Experimental Plant Biology" (No. CZ.02.1.01/0.0/0.0/16_019/0000738).

REFERENCES

Benková, E., Witters, E., Van Dongen, W., Kolár, J., Motyka, V., Brzobohatý, B., et al. (1999). Cytokinins in tobacco and wheat chloroplasts. Occurrence and changes due to light/dark treatment. *Plant Physiol.* 121, 245–252. doi: 10.1104/pp.121.1.245

Blagoeva, E., Dobrev, P. I, Malbeck, J., Motyka, V., Strnad, M., Hanuš, J., et al. (2004). Cytokinin N-glucosylation inhibitors suppress deactivation of exogenous cytokinins in radish, but their effect on active endogenous cytokinins is counteracted by other regulatory mechanisms. *Physiol. Plantarum* 121, 215–222. doi: 10.1111/j.1399-3054.2004.00320.x

Bowles, D., Lim, E., Poppenberger, B., and Vaistij, E. (2006). Glycosyltransferases of lipophilic small molecules. *Annu. Rev. Plant Biol.* 57, 567–597. doi: 10.1146/annurev.arplant.57.032905.105429

Brzobohatý, B., Moore, I., Kristoffersen, P., Bako, L., Campos, N., Schell, J., et al. (1993). Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. *Science* 262, 1051–1054. doi: 10.1126/SCIENCE.8235622

Drábková, L. Z., Dobrev, P. I., and Motyka, V. (2015). Phytohormone profiling across the bryophytes. *PLoS One* 10:e0125411. doi: 10.1371/journal.pone.0125411

Entsch, B., and Letham, D. S. (1979). Enzymic glucosylation of the cytokinin, 6-benzylaminopurine. *Plant Sci. Lett.* 14, 205–212. doi: 10.1016/0304-4211(79)90061-0

Entsch, B., Parker, C. W., Letham, D. S., and Summons, R. E. (1979). Preparation and characterization, using high-performance liquid chromatography, of an enzyme forming glucosides of cytokinins. *Biochim. Biophys. Acta Enzymol.* 570, 124–139. doi: 10.1016/0005-2744(79)90207-9

Filipi, T., Mazura, P., Janda, L., Kiran, N. S., and Brzobohatý, B. (2012). Engineering the cytokinin-glucoside specificity of the maize β-d-glucosidase Zm-p60.1 using site-directed random mutagenesis. *Phytochemistry* 74, 40–48. doi: 10.1016/j.phytochem.2011.10.008

Fox, J., Cornette, J., Deleuze, G., Dyson, W., Giersak, C., Niu, P., et al. (1973).). The formation, isolation, and biological activity of a cytokinin 7-glucoside. *Plant Physiol.* 52, 627–632. doi: 10.1104/pp.52.6.627

Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P. I., et al. (2011). Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants. *J. Exp. Bot.* 62, 2827–2840. doi: 10.1093/jxb/erq457

Galichet, A., Hoyerová, K., Kamínek, M., and Gruissem, W. (2008). Farnesylation directs AtIPT3 subcellular localization and modulates cytokinin biosynthesis in *Arabidopsis*. *Plant Physiol.* 146, 1155–1164. doi: 10.1104/pp.107.107425

Galuszka, P., Frébort, I., Šebela, M., Sauer, P., Jacobsen, S., and Peč, P. (2001). Cytokinin oxidase or dehydrogenase? *Eur. J. Biochem.* 268, 450–461.

Galuszka, P., Popelková, H., Werner, T., Frébortová, J., Pospíšilová, H., Mik, V., et al. (2007). Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. *J. Plant Growth Regul.* 26, 255–267. doi: 10.1007/s00344-007-9008-5

Hluska, T., Dobrev, P. I., Tarkowská, D., Frébortová, J., Zalabák, D., Kopečný, D., et al. (2016). Cytokinin metabolism in maize: novel evidence of cytokinin abundance, interconversions and formation of a new trans-zeatin metabolic product with a weak anticytokinin activity. *Plant Sci.* 247, 127–137. doi: 10.1016/j.plantsci.2016.03.014

Hošek, P., Hoyerová, K., Kiran, N. S., Dobrev, P. I., Zahajská, L., Filepová, R., et al. (2020). Distinct metabolism of N-glucosides of isopentenyladenine and trans-zeatin determines cytokinin metabolic spectrum in *Arabidopsis*. *New Phytol.* 225, 2423–2438. doi: 10.1111/nph.16310

Hothorn, M., Dabi, T., Chory, J., Jolla, L., and Jolla, L. (2011). Structural basis for cytokinin recognition by *Arabidopsis thaliana* histidine kinase 4. *Nat. Chem. Biol.* 7, 766–768. doi: 10.1038/nchembio.667.Structural

Hou, B., Lim, E. K., Higgins, G. S., and Bowles, D. J. (2004). N-glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. *J. Biol. Chem.* 279, 47822–47832. doi: 10.1074/jbc.M409569200

Jiskrová, E., Novák, O., Pospíšilová, H., Holubová, K., Karády, M., Galuszka, P., et al. (2016). Extra- and intracellular distribution of cytokinins in the leaves of monocots and dicots. *N. Biotechnol.* 33, 735–742. doi: 10.1016/j.nbt.2015.12.010

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., et al. (2004). Distinct isoprenoid origins of cis- and trans-zeatin biosyntheses in *Arabidopsis*. *J. Biol. Chem.* 279, 14049–14054. doi: 10.1074/jbc.M314195200

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:dev149344. doi: 10.1242/dev.149344

Kowalska, M., Galuszka, P., Frébortová, J., Šebela, M., Béres, T., Hluska, T., et al. (2010). Vacuolar and cytosolic cytokinin dehydrogenases of *Arabidopsis thaliana*: heterologous expression, purification and properties. *Phytochemistry* 71, 1970–1978. doi: 10.1016/j.phytochem.2010.08.013

Kuroha, T., Tokunaga, H., Kojima, M., Ueda, N., Ishida, T., Nagawa, S., et al. (2009). Functional analyses of LONELY GUY cytokinin-activating enzymes reveal the importance of the direct activation pathway in *Arabidopsis*. *Plant Cell* 21, 3152–3169. doi: 10.1105/tpc.109.068676

Laloue, M., Terrine, C., Guern, J., Laloue, M., Terrine, C., and Guern, J. (1977). Cytokinins: metabolism and biological activity of N6- (A12- Isopentenyl) adenosine and N6- (42-Isopentenyl) adenine in tobacco cells and callus1. *Plant Physiol.* 59, 478–483. doi: 10.1104/pp.59.3.478

Lizák, B., Csala, M., Benedetti, A., and Bánhegyi, G. (2008). The translocon and the non-specific transport of small molecules in the endoplasmic reticulum (Review). *Mol. Membr. Biol.* 25, 95–101. doi: 10.1080/09687680701670481

Lomin, S. N., Krivosheev, D. M., Steklov, M. Y., Arkhipov, D. V., Osolodkin, D. I., Schmülling, T., et al. (2015). Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. *J. Exp. Bot.* 66, 1851–1863. doi: 10.1093/jxb/eru522

Mameaux, S., Cockram, J., Thiel, T., Steuernagel, B., Stein, N., Taudien, S., et al. (2012). Molecular, phylogenetic and comparative genomic analysis of the cytokinin oxidase/dehydrogenase gene family in the Poaceae. *Plant Biotechnol. J.* 10, 67–82. doi: 10.1111/j.1467-7652.2011.00645.x

Moffatt, B. A., Wang, L., Allen, M. S., Stevens, Y. Y., Qin, W., Snider, J., et al. (2000). Adenosine kinase of *Arabidopsis*. Kinetic properties and gene expression. *Plant Physiol.* 124, 1775–1785. doi: 10.1104/pp.124.4.1775

Morrison, E. N., Knowles, S., Hayward, A., Thorn, R. G., Saville, B. J., and Emery, R. J. N. (2015). Detection of phytohormones in temperate forest fungi predicts consistent abscisic acid production and a common pathway for cytokinin biosynthesis. *Mycologia* 107, 245–257. doi: 10.3852/14-157

Paces, V., Werstiuk, E. V. A., and Hall, R. H. (1971). Conversion of N6-(Δ2-Isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. *Plant Physiol.* 48, 775–778. doi: 10.1104/pp.48.6.775

Parker, C., Letham, D. S., Cowley, D. E., and MacLeod, J. K. (1972). Raphanatin, an unusual purine derivative and a metabolite of zeatin. *Biochem. Biophys. Res. Commun.* 49, 460–466. doi: 10.1016/0006-291X(72)90433-0

Podlešáková, K., Zalabák, D., Ěudejková, M., Plíhal, O., Szüèová, L., Doležal, K., et al. (2012). Novel cytokinin derivatives do not show negative effects on root growth and proliferation in submicromolar range. *PLoS One* 7:e39293. doi: 10.1371/journal.pone.0039293

Romanov, G. A., Lomin, S. N., and Schmülling, T. (2018). Cytokinin signaling: from the ER or from the PM? That is the question! *New Phytol.* 18, 41–53. doi: 10.1111/nph.14991

Šimura, J., Antoniadi, I., Široká, J., Tarkowská, D., Strnad, M., Ljung, K., et al. (2018). Plant hormonomics: multiple phytohormone profiling by targeted metabolomics. *Plant Physiol.* 177, 476–489. doi: 10.1104/pp.18.00293

Šmehilová, M., Dobrůšková, J., Novák, O., Takáč, T., and Galuszka, P. (2016). Cytokinin-specific Glycosyltransferases possess different roles in cytokinin homeostasis maintenance. *Front. Plant Sci.* 7:1264. doi: 10.3389/fpls.2016.01264

Spíchal, L., Rakova, N. Y., Riefler, M., Mizuno, T., Romanov, G. A., Strnad, M., et al. (2004). Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, Differ in their ligand specificity in a bacterial assay. *Plant Cell Physiol.* 45, 1299–1305. doi: 10.1093/pcp/pch132

Stirk, W. A., Ördög, V., Novák, O., Rolčík, J., Strnad, M., Bálint, P., et al. (2013). Auxin and cytokinin relationships in 24 microalgal strains. *J. Phycol.* 49, 459–467. doi: 10.1111/jpy.12061

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., et al. (2004). AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in *Arabidopsis*. *Plant Cell Physiol.* 45, 1053–1062. doi: 10.1093/pcp/pch119

Trdá, L., Barešová, M., Šašek, V., Nováková, M., Zahajská, L., Dobrev, P. I., et al. (2017). Cytokinin metabolism of pathogenic fungus *Leptosphaeria maculans* involves isopentenyltransferase, adenosine kinase and cytokinin oxidase/dehydrogenase. *Front. Microbiol.* 8:e01374. doi: 10.3389/fmicb.2017.01374

Trifunović-Momčilov, M., Motyka, V., Dragićević, I., Petrić, M., Jevremović, S., Malbeck, J., et al. (2016). Endogenous phytohormones in spontaneously regenerated centaurium erythraea rafn. plants grown in vitro. *J. Plant Growth Regul.* 35, 543–552. doi: 10.1007/s00344-015-9558-x

Van Staden, J., and Drewes, F. E. (1991). The biological activity of cytokinin derivatives in the soybean callus bioassay. *Plant Growth Regul.* 10, 109–115. doi: 10.1007/BF00024957

Vyroubalová, S., Vaclavikova, K., Tureckova, V., Novak, O., Smehilova, M., Hluska, T., et al. (2009). Characterization of new maize genes putatively involved in cytokinin metabolism and their expression during osmotic stress in relation to cytokinin levels. *Plant Physiol.* 151, 433–447. doi: 10.1104/pp.109.142489

Wang, J., Ma, X. M., Kojima, M., Sakakibara, H., and Hou, B. K. (2011). N-glucosyltransferase UGT76C2 is involved in cytokinin homeostasis and cytokinin response in *Arabidopsis thaliana*. *Plant Cell Physiol.* 52, 2200–2213. doi: 10.1093/pcp/pcr152

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmuelling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15, 2532–2550. doi: 10.1105/tpc.014928.)

Yang, M., Fehl, C., Lees, K. V., Lim, E. K., Offen, W. A., Davies, G. J., et al. (2018). Functional and informatics analysis enables glycosyltransferase activity prediction. *Nat. Chem. Biol.* 14, 1109–1117. doi: 10.1038/s41589-018-0154-9

Zalabák, D., Galuszka, P., Mrízová, K., Podlešáková, K., Gu, R., and Frébortová, J. (2014). Biochemical characterization of the maize cytokinin dehydrogenase family and cytokinin profiling in developing maize plantlets in relation to the expression of cytokinin dehydrogenase genes. *Plant Physiol. Biochem.* 74, 283–293. doi: 10.1016/j.plaphy.2013.11.020

Zhang, K., Novak, O., Wei, Z., Gou, M., Zhang, X., Yu, Y., et al. (2014). *Arabidopsis* ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. *Nat Commun.* 5:3274. doi: 10.1038/ncomms4274

Zhang, X., Chen, Y., Lin, X., Hong, X., Zhu, Y., Li, W., et al. (2013). Adenine phosphoribosyl transferase 1 is a key enzyme catalyzing cytokinin conversion from nucleobases to nucleotides in *Arabidopsis*. *Mol. Plant* 6, 1661–1672. doi: 10.1093/mp/sst071

Žižková, E., Dobrev, P. I., Muhovski, Y., Hošek, P., Hoyerová, K., Haisel, D., et al. (2015). Tomato (*Solanum lycopersicum* L.) SlIPT3 and SlIPT4 isopentenyltransferases mediate salt stress response in tomato. *BMC Plant Biol.* 15:85. doi: 10.1186/s12870-015-0415-7

Žižková, E., Kubeš, M., Dobrev, P. I., Přibyl, P., Šimura, J., Zahajská, L., et al. (2017). Control of cytokinin and auxin homeostasis in cyanobacteria and algae. *Ann. Bot.* 119, 151–166. doi: 10.1093/aob/mcw194

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 October 2020
doi: 10.3389/fpls.2020.590337

Barley Root Proteome and Metabolome in Response to Cytokinin and Abiotic Stimuli

*Miroslav Berka[1], Markéta Luklová[1], Hana Dufková[1], Veronika Berková[1], Jan Novák[1], Iñigo Saiz-Fernández[1], Aaron M. Rashotte[2], Břetislav Brzobohatý[1,3] and Martin Černý[1]**

[1] Department of Molecular Biology and Radiobiology, Faculty of AgriSciences, Mendel University in Brno, Brno, Czechia, [2] Department of Biological Sciences, Auburn University, Auburn, AL, United States, [3] Central European Institute of Technology, Faculty of AgriSciences, Mendel University in Brno, Brno, Czechia

Cytokinin is a phytohormone involved in the regulation of diverse developmental and physiological processes in plants. Its potential in biotechnology and for development of higher-yield and more resilient plants has been recognized, yet the molecular mechanisms behind its action are far from understood. In this report, the roots of barley seedlings were explored as a new source to reveal as yet unknown cytokinin-responsive proteins for crop improvement. Here we found significant differences reproducibly observed for 178 proteins, for which some of the revealed cytokinin-responsive pathways were confirmed in metabolome analysis, including alterations phenylpropanoid pathway, amino acid biosynthesis and ROS metabolism. Bioinformatics analysis indicated a significant overlap between cytokinin response and response to abiotic stress. This was confirmed by comparing proteome and metabolome profiles in response to drought, salinity or a period of temperature stress. The results illustrate complex abiotic stress response in the early development of model crop plant and confirm an extensive crosstalk between plant hormone cytokinin and response to temperature stimuli, water availability or salinity stress.

Keywords: ***Hordeum vulgare*, zeatin, proteome, metabolome, abiotic stress, phenylpropanoid biosynthesis, root, ROS**

OPEN ACCESS

Edited by:
Wolfram G. Brenner, Universität Leipzig, Germany

Reviewed by:
Branka Salopek Sondi, Rudjer Boskovic Institute, Croatia
Soulaiman SAKR, Agrocampus Ouest, France

***Correspondence:**
Martin Černý
martincerny83@gmail.com

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *01 August 2020*
Accepted: *05 October 2020*
Published: *28 October 2020*

Citation:
Berka M, Luklová M, Dufková H, Berková V, Novák J, Saiz-Fernández I, Rashotte AM, Brzobohatý B and Černý M (2020) Barley Root Proteome and Metabolome in Response to Cytokinin and Abiotic Stimuli. Front. Plant Sci. 11:590337. doi: 10.3389/fpls.2020.590337

INTRODUCTION

Cytokinin is a multifaceted plant hormone that plays major roles in plant growth and development as well as interacting with both biotic and abiotic factors, including light, temperature, drought, and salinity signals (as reviewed in Argueso et al., 2009; Kieber and Schaller, 2014; Zwack and Rashotte, 2015; Pavlů et al., 2018; Cortleven et al., 2019). The canonical cytokinin signal transduction pathway is a multistep phosphorelay with sensor hybrid histidine kinase that phosphorylates histidine-containing phosphotransfer proteins which are then translocated into the nucleus, where they transfer the phosphate to type-B response regulators. The previous proteome-wide analyses have also revealed a transcription-independent rapid response to cytokinin targeting predominantly plastidial proteins (Chen et al., 2010; Černý et al., 2011, 2014; Žďárská et al., 2013; Hallmark et al., 2020).

Seed germination and early seedling establishment is a complex biological process that includes drastic morphological, physiological, and biochemical changes, and represents a period of life

when a plant is highly susceptible to both biotic and abiotic stressors (Bewley et al., 2013; Kaur et al., 2017). The early germination phase is thought of as predominantly governed by the phytohormones abscisic acid and gibberellin, yet other growth regulators are also known to participate in the complex process. The role of cytokinin in seedling establishment is indisputable, including regulation of shoot and root apical meristems, development of lateral roots or vasculature system, light signaling, chloroplast development and de-etiolation and leaf development (Chory et al., 1994; Kieber and Schaller, 2014; Skalák et al., 2019). Early reports of Arabidopsis cytokinin mutants showed that a receptor mutation or an enhanced cytokinin degradation resulted in more rapid germination (Riefler et al., 2006). In contrast, later studies revealed that cytokinin antagonizes abscisic acid by downregulating ABI5 and promoting its degradation (Wang et al., 2011; Guan et al., 2014) and found that plants overexpressing cytokinin response regulators type-A were insensitive to abscisic acid (Huang et al., 2017).

Barley refers to the cereal *Hordeum vulgare* subsp. *vulgare* and belongs to the Triticeae tribe of grasses. The tribe comprises about 350 species among them the important cereals are wheat (*Triticum* spp.), rye (*Secale cereale*) and triticale (x*Triticosecale*), many forage grasses and ecologically important taxa of temperate grasslands (Blattner, 2018). Barley is the fourth most important cereal grain with a worldwide production exceeding 141 million tonnes (FAOSTAT, year 2018; http://faostat.fao.org) and an excellent crop model species with a sequenced genome (Mayer et al., 2012). Barley has a diploid genome (2n) with only 14 chromosomes making it more suitable for analyses than its close relative from Triticeae tribe, wheat. There are over 400,000 barley accessions conserved in collections world-wide (Dawson et al., 2015) and biotechnology tools for transformation have been developed (e.g., Harwood, 2019). Previous research on cytokinin signaling has successfully developed transgenic barley lines with an enlarged root system, higher yield and improved drought resilience (Zalewski et al., 2010; Mrízová et al., 2013; Pospíšilová et al., 2016; Vojta et al., 2016; Holubová et al., 2018; Ramireddy et al., 2018), but analyses targeting molecular mechanisms underlying cytokinin response in barley are limited. The Arabidopsis Information Resource lists 142 genes associated with cytokinin. In contrast, only 20 and 30 genes are associated with the cytokinin signaling or metabolism in the reference barley genomes IBSC and Morex, respectively (plants.ensembl.org; barlex.barleysequence.org).

Cytokinin is one of the principal players in the process of root organogenesis (e.g., Laplaze et al., 2007; Müller and Sheen, 2008; Bielach et al., 2012; Kurepa et al., 2019) and roots are thus an excellent target for the cytokinin response analysis. Further, the detection of plant proteins is often hindered by the presence of Ribulose bisphosphate carboxylase/oxygenase (RubisCO) which may represent more than 50% of the plant protein extracts (e.g., Righetti and Boschetti, 2016). Techniques have been developed to eliminate or deplete this highly abundant enzyme (Gupta et al., 2015) but the root tissue, being devoid of RubisCO, does not require this additional step. We hypothesized that barley seedling root could be an excellent model for analysis of cytokinin-responsive proteins and that comprehensive, integrated and high-resolution analyses could extend current understanding of cytokinin role in early plant development. To elicit the cytokinin response, we selected the treatment with trans-zeatin, which is together with cis-zeatin the dominant active cytokinin in barley (Powell et al., 2013).

RESULTS

Barley Root Proteome

Barley root proteome was extracted from roots of four-day-old seedlings as described in Materials and Method. Root proteome profiling identified over 10,000 peptides corresponding to more than 2,600 proteins represented by 1,735 protein families. The gene ontology (GO) analysis revealed that most proteins are identified as being involved in biosynthetic processes, including amino acid biosynthesis, proteosynthesis and the production of secondary metabolites (**Figures 1A,B**). Proportionally, the most abundant proteins representing 80% of the analyzed root proteome comprised of only 460 proteins and the majority of these was formed by carbohydrate metabolism enzymes (15.2%), ribosomal proteins and proteins involved in proteosynthesis (11.3%), energy metabolism (10.3%), protein folding (9.1%), histones (6.1%) and reactive oxygen species (ROS) metabolism (5.4%). There was also a noticeable amount of jasmonic acid (JA)-responsive proteins and JA biosynthetic enzymes (12-oxophytodienoate reductase, multiple lipoxygenases), representing more than 2.3% of all detected proteins.

Cytokinin at Micromolar Range Does Not Have a Negative Impact on Barley Germination

To elicit the cytokinin response, barley seedlings were treated with 1 μM of the cytokinin, trans-zeatin for 24 h. This is a similar concentration to that used in previous cytokinin transcriptomics and proteomics analyses but far above the natural endogenous levels (Černý et al., 2016). The addition of higher cytokinin concentrations may induce a hypersensitive-like response and promote cell death (Vescovi et al., 2012; Novák et al., 2013), and thus it cannot be excluded that some of the observed cytokinin-responsive proteins found here could represent a response to toxicity. However, as illustrated in **Figure 2**, the barley germination rate in the presence of 1 μM trans-zeatin was similar to that of mock-treated grains, and the percentage of germinated grains after 48 h was unaffected. This standard assay evaluates only the emergence of the radicle, but it is likely that barley seedlings can counter any negative effect of cytokinin at this concentration and duration by triggering its degradation, which is supported by the fact that cytokinin-treated roots accumulated a sufficient amount of cytokinin dehydrogenase (CKX6) to facilitate its detection in an untargeted proteome-wide analysis (**Supplementary Table S2**).

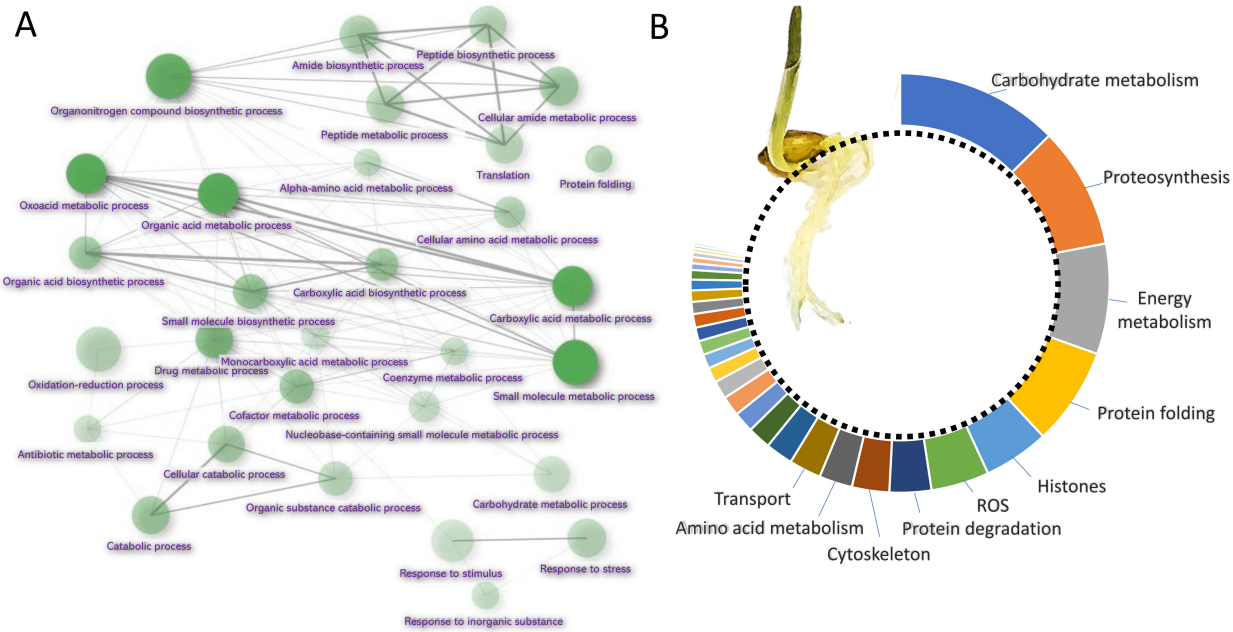


FIGURE 1 | Barley root proteome composition. **(A)** Enrichment analysis based on hypergeometric distribution followed by FDR correction. Two pathways are connected if they share 20% or more proteins. Darker and bigger nodes are more significantly enriched and larger sets, respectively. Analyzed by ShinyGO 0.61 (Ge et al., 2020); **(B)** The function of the most abundant proteins representing 80% of the barley root proteome. For the sake of clarity, only ten most abundant categories are labeled.

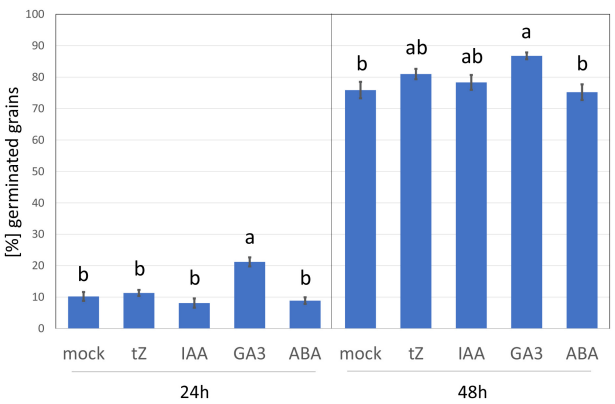


FIGURE 2 | Barley germination in response to different growth regulators. Percentage of germinated seeds 24 and 48 h after imbibition in 4 ml water supplemented with 5×10^{-4}% (v/v) dimethyl sulfoxide (mock buffer) or 1 μM phytohormone (tZ, trans-Zeatin; IAA, Indole-3-acetic acid; GA3, Gibberellic acid; ABA, abscisic acid) in dimethyl sulfoxide (final concentration, as for the mock). Different letters indicate significant differences (Kruskal-Wallis test, $p < 0.05$).

Cytokinin-Responsive Proteins in Barley Root Proteome

A comparison of proteome profiles between cytokinin and mock-treated roots showed significant separation (**Figure 4A**) and revealed 350 candidate cytokinin-responsive proteins ($p < 0.05$), including the above-mentioned enzyme CKX6 (found only in cytokinin-treated roots). Next, candidates failing stringent identification and quantitation criteria were removed and only differentially abundant proteins with at least 10 spectral matches in a biological replicate, more than one unique peptide and absolute fold-change of at least 1.4 were selected, limiting the set to 178 differentially abundant cytokinin-responsive proteins (**Tables 1**, **2**). In total, 63 and 115 cytokinin-responsive proteins representing more than 12% of the total root proteome were found as significantly increased and decreased, respectively. The most numerous categories also matched the total proteome composition (carbohydrate metabolism, ribosomal proteins and proteosynthesis, energy metabolism; **Figure 3D**). Predicted cellular localization showed that the cytokinin-responsive proteins were found predominantly in the cytosol (35%), extracellular space (16%), plastids (14%), nucleus (10%), and mitochondrion (9%). Comparisons of cytokinin-responsive localization to the whole proteome dataset showed that mitochondrial and nuclear proteins were underrepresented (**Figures 3B,C**). The set of cytokinin-induced proteins included enzymes of phenylpropanoid biosynthesis, components of cytoskeleton and enzymes of lipid metabolism. The metabolic pathway enrichment revealed that proteins with a significant depletion were enriched in ribosomal proteins (11), enzymes of biosynthesis of secondary metabolites (17) or protein processing (8). About one-third of the cytokinin-repressed proteins was associated with a stress response, indicating a putative connection between cytokinin-induced alleviation or attenuation of the stress. Not all identified differentially abundant proteins are novel cytokinin-responsive proteins *per se*. The comparison to

previously known cytokinin-responsive proteins identified at least 26 barley root proteins orthologs that were found with a similar response to cytokinin (Lochmanová et al., 2008; Liu et al., 2011; Černý et al., 2011, 2014; Zhang et al., 2012; Žďárská et al., 2013; Hallmark et al., 2020) and 36 cytokinin-responsive genes from *Arabidopsis thaliana* (Hoth et al., 2003; Brenner et al., 2005; Kiba et al., 2005; Nemhauser et al., 2006; Argyros et al., 2008; Li et al., 2010; Brenner and Schmulling, 2012; Nishiyama et al., 2012; Bhargava et al., 2013).

Cytokinin-Responsive Metabolites in Roots of Barley Seedling

A GC-MS metabolome analysis for barley seedling roots provided quantitative data for 80 different root metabolites, giving a snapshot of the key primary metabolism pathways as well as phenylpropanoid biosynthesis or polyamine production (**Figure 4A**, **Supplementary Table S3**). The cytokinin treatment induced a significant accumulation of N-acetyl serine, mannitol, pyroglutamic acid, coumaric acid, polyamine spermidine, and tryptamine. The accumulation of an osmoprotective compound (mannitol), the product of glutathione degradation (pyroglutamic acid), phenolic compound (coumaric acid), and an important precursor for many secondary metabolites (tryptamine) was matching the observed accumulation of stress-responsive proteins. In parallel, the set of 22 metabolites that were significantly depleted in response to cytokinin fully supported observed changes on the proteome level. Namely, the depletion of oxaloacetate and aspartate correlated with a decrease in aspartate aminotransferase and asparagine synthase. The depletion of aminoacylase and alanine aminotransferase coincided with a decrease in arginine and proline. And, finally, a decrease in lysine was likely the result of diaminopimelate aminotransferase repression. The observed accumulation of coumaric acid, depletion of aromatic amino acids phenylalanine and tyrosine matched the accumulation of enzymes of phenylpropanoid pathway (**Figures 3E**, **4A**).

Role of Cytokinin-Responsive Proteins in Response to Abiotic Stressors

The list of cytokinin-responsive proteins and metabolites indicated a similarity between cytokinin and an abiotic stimuli. Next, a set of experiments was designed to explore this putative crosstalk, including the response to salinity, temperature and drought. The same stringent protein quantitation criteria were applied, resulting in the identification of 308 stress-responsive proteins (**Supplementary Table S1**). Notably, the overlap between abiotic stress and cytokinin response was high, and only 76 cytokinin-responsive proteins were not considered to be differentially abundant in response to any of the abiotic stimuli (**Supplementary Figure S1**).

Salinity and Cytokinin Share Similar Response in Barley Root Proteome

The functional analysis (**Figure 3D**) indicated that 17 barley cytokinin-responsive proteins could be induced by salinity stress. In order to validate this potential crosstalk, seedlings were put for 24h into medium supplemented with 80 mM NaCl to elicit a salinity stress. The consecutive proteomics analyses revealed a statistical significant separation of salinity- and cytokinin-treated roots (**Figure 4C**), and a depletion and accumulation of 55 and 59 root proteins, respectively (**Supplementary Table S1**). The accumulated proteins included multiple glutathione S-transferases, glutathione reductase and superoxide dismutase, indicating an increase in the ROS metabolism. Roots under salinity stress also accumulated enzymes of phenylpropanoid biosynthetic pathway (phenylalanine ammonia-lyase, HORVU0Hr1G016330; alcohol dehydrogenase, HORVU6Hr1G035420), enzymes of energy metabolism, sulfur metabolism, and an ortholog of lipocalin that confers resistance to high salt in poplar (Abo-Ogiala et al., 2014). A decrease was found for proteins associated with cell wall organization or biogenesis, including several peroxidases or chitinase. In total, only four out of 17 putative salinity-responsive proteins were significantly changed under salinity stress, but the comparison with the response to cytokinin showed 26 similar changes, including an accumulation of a jasmonic acid biosynthetic enzyme (HORVU2Hr1G077220), phenylpropanoid biosynthetic enzymes, potassium channel subunit and a putative regulator of cell elongation (HORVU1Hr1G045630). A contrasting response was found for 15 proteins, including nuclease S1 and acid phosphatase (HORVU2Hr1G112830, HORVU4Hr1G085050; positive response to cytokinin).

Cytokinin Treatment Elicited Drought-stress Response in Barley Root Proteome

The cytokinin treatment resulted in a decrease of TCTP protein (HORVU4Hr1G033200) with a putative role in drought tolerance (Kim et al., 2012), and an increase in an osmoprotective compound mannitol and at least five proteins associated with the drought stress response. Next, in a parallel experiment, the three-day-old seedlings were deprived of water for 24 h, reaching the relative water content 83.2 $\pm$ 0.4% of control roots, and the identified cytokinin-responsive proteins were quantified (**Tables 1**, **2**, **Supplementary Table S1**). The results confirmed that cytokinin-responsive proteins significantly overlapped with the drought-responsive subset which is well in line with the previous reports suggesting that cytokinin may induce osmotic stress hypersensitivity (Karunadasa et al., 2020). In total, out of 70 identified drought-responsive proteins, 39 were cytokinin-responsive and the response to cytokinin and drought was similar for 35 of these, including an accumulation of the subunit of a voltage-gated potassium channel (HORVU6Hr1G091250), annexin (HORVU6Hr1G074440) and depletion of ferredoxin–nitrite reductase (HORVU6Hr1G080750) and multiple enzymes of carbohydrate and amino acid metabolism.

Temperature-stress Response in Barley Root Proteome

Cytokinin signaling is an integral part of temperature-stress response, including heat stress, cold stress and temperature acclimation (Jeon et al., 2010; Macková et al., 2013; Černý et al., 2014; Danilova et al., 2016; Skalák et al., 2016). Here, 16 cytokinin-responsive proteins were associated with a

TABLE 1 | Proteins with a positive response to cytokinin.

	Accession	Name	AGI	CK	tZ	D	C	H	S
Amino acid metabolism	HORVU5Hr1G104700	Glutamate dehydrogenase 1	N/A		↑	NR	NR	NR	NR
	HORVU2Hr1G029870	Phospho-2-dehydro-3-deoxyheptonate aldolase 2	AT1G22410		↑	↑	NR	NR	↑
Biosynthesis of secondary metabolites	HORVU3Hr1G015640	Alkenal reductase	AT5G16990		↑	NR	NR	NR	NR
	HORVU6Hr1G035420	Cinnamyl alcohol dehydrogenase 5	AT3G19450		↑	NR	NR	NR	↑
	HORVU7Hr1G082280	O-Methyltransferase 1	AT5G54160	×	↑	NR	NR	NR	NR
	HORVU6Hr1G058820	Phenylalanine ammonia-lyase 2	AT2G37040	×	↑	NR	NR	↑	↑
	HORVU0Hr1G016330	Phenylalanine ammonia-lyase 2	AT2G37040	×	↑	NR	NR	↑	↑
	HORVU2Hr1G117770	Sulfotransferase 2A	AT5G07010	×	↑	NR	↓	NR	NR
Carbohydrate metabolism	HORVU7Hr1G001040	Acid β-fructofuranosidase	AT1G12240		↑	↑	NR	NR	↓
	HORVU6Hr1G074200	Dihydroorotate dehydrogenase (quinone)	AT3G17810		↑	NR	NR	NR	NR
	HORVU3Hr1G002780	Fructose-bisphosphate aldolase 2	AT2G21330	×	↑	NR	NR	↑	NR
	HORVU7Hr1G013600	Transketolase	AT2G45290		↑	NR	NR	↑	NR
Cell cycle	HORVU4Hr1G072010	Cell division cycle 48	AT5G03340		↑	NR	NR	↑	NR
Cell wall	HORVU1Hr1G054240	Expansin B2	AT1G65680		↑	↑	NR	NR	NR
Cytoskeleton	HORVU1Hr1G081280	Tubulin α-4 chain	AT4G14960	×	↑	NR	NR	NR	NR
	HORVU3Hr1G078940	Tubulin β chain 2	AT5G12250	×	↑	NR	NR	NR	NR
	HORVU4Hr1G002530	Tubulin β chain 4	AT2G29550	×	↑	NR	NR	NR	NR
	HORVU5Hr1G100900	Tubulin β chain 5	AT1G20010	×	↑	NR	NR	NR	NR
Energy metabolism	HORVU6Hr1G088420	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase	AT1G49390		↑	NR	NR	NR	NR
	HORVU1Hr1G083470	5-Dehydro-2-deoxygluconokinase	AT4G10260		↑	NR	NR	NR	NR
	HORVU1Hr1G090720	Malate dehydrogenase	AT1G53240	×	↑	NR	↑	NR	NR
	HORVU5Hr1G111620	Methylenetetrahydrofolate reductase 2	AT2G44160		↑	↑	NR	NR	NR
Histone	HORVU1Hr1G074340	Histone superfamily protein	AT1G09200		↑	NR	NR	NR	NR
	HORVU6Hr1G031580	Histone superfamily protein	AT1G09200		↑	NR	NR	NR	NR
Chaperon	HORVU2Hr1G092190	Hsp70-Hsp90 organizing protein	AT1G62740		↑	↑	↑	NR	NR
Lipid metabolism	HORVU7Hr1G119060	GDSL esterase/lipase	AT3G04290	×	↑	NR	NR	NR	NR
	HORVU6Hr1G074940	Lipase/lipooxygenase	AT4G39730		↑	NR	NR	NR	NR
	HORVU1Hr1G012140	PATATIN-like protein 4	AT2G26560	×	↑	NR	NR	NR	NR
	HORVU7Hr1G036460	PATATIN-like protein 4	AT2G26560	×	↑	NR	NR	NR	NR
Nucleic acid and nucleotide metabolism	HORVU3Hr1G077000	Adenine nucleotide α hydrolase	AT5G54430		↑	NR	NR	NR	NR
	HORVU6Hr1G079150	Adenine nucleotide α hydrolase	AT3G03270	×	↑	NR	↑	NR	NR
	HORVU5Hr1G109340	ATP-dependent RNA helicase DED1	AT2G42520		↑	NR	NR	↑	NR
	HORVU2Hr1G003110	Ectonucleoside triphosphate diphosphohydrolase 5	AT3G04080	×	↑	NR	↓	NR	NR
	HORVU4Hr1G087230	Ectonucleoside triphosphate diphosphohydrolase 5	AT3G04080		↑	NR	↓	↓	↓
	HORVU2Hr1G112830	Nuclease S1	AT1G68290		↑	NR	↓	NR	↓

(Continued)

TABLE 1 | Continued

	Accession	Name	AGI	CK	tZ	D	C	H	S
Phosphatase	HORVU4Hr1G087310	Acid phosphatase	N/A		↑	NR	NR	NR	NR
	HORVU4Hr1G085050	Purple acid phosphatase 27	AT5G50400		↑	NR	↓	NR	↓
Protease	HORVU7Hr1G009410	Eukaryotic aspartyl protease	AT1G01300	×	↑	NR	NR	NR	NR
	HORVU6Hr1G081640	Subtilisin-like protease	AT3G14067		↑	NR	NR	NR	↓
ROS	HORVU6Hr1G008640	Catalase 1	AT1G20620		↑	NR	NR	NR	NR
	HORVU7Hr1G121700	Catalase 2	AT4G35090	×	↑	NR	NR	NR	NR
	HORVU4Hr1G081100	Glutathione S-transferase family protein	AT3G03190	×	↑	NR	NR	NR	NR
	HORVU1Hr1G016660	Peroxidase superfamily protein	AT4G11290		↑	↓	↓	↓	↓
	HORVU2Hr1G124970	Peroxidase superfamily protein	AT1G71695	×	↑	NR	NR	↓	↓
Signaling	HORVU2Hr1G077220	12-Oxophytodienoate reductase 2	AT1G09400		↑	NR	NR	NR	↑
	HORVU4Hr1G005920	Lipoxygenase 1	AT1G55020	×	↑	NR	↓	NR	NR
	HORVU5Hr1G093770	Lipoxygenase 1	AT1G17420	×	↑	NR	NR	NR	NR
Stress response	HORVU6Hr1G083960	Dehydrin	AT3G50970	×	↑	NR	NR	NR	NR
	HORVU5Hr1G010880	Germin-like protein 2	AT5G38910	×	↑	NR	NR	NR	NR
	HORVU0Hr1G011720	Major pollen allergen bet v 1-B	AT1G35260		↑	NR	NR	NR	NR
	HORVU5Hr1G023720	Pathogenesis-related protein STH-2	AT1G35260		↑	↑	NR	NR	NR
	HORVU2Hr1G120530	Wound-induced protein	AT3G04720		↑	NR	NR	NR	NR
	HORVU3Hr1G113620	Wound-induced protein	AT3G04720		↑	NR	NR	NR	NR
Transport	HORVU6Hr1G074440	Annexin 7	AT5G10230		↑	↑	↓	↑	↑
	HORVU6Hr1G091250	Voltage-gated potassium channel subunit β-1	AT1G04690	×	↑	↑	NR	NR	↑
Unknown	HORVU7Hr1G021430	HXXXD-type acyl-transferase family protein	AT1G31490	×	↑	↑	↓	NR	↓
	HORVU7Hr1G039930	NAD(P)-linked oxidoreductase superfamily protein	AT1G60690		↑	NR	NR	NR	NR
	HORVU1Hr1G043910	Plant basic secretory protein	AT2G15130		↑	NR	NR	NR	NR
	HORVU1Hr1G043920	Plant basic secretory protein	AT2G15220	×	↑	NR	NR	NR	NR
	HORVU6Hr1G013710	Plant basic secretory protein	AT2G15220	×	↑	NR	NR	NR	↓
	HORVU2Hr1G081080	UBX domain-containing protein	AT4G04210		↑	NR	NR	NR	NR
	HORVU5Hr1G073650	Unknown	AT1G09310		↑	NR	NR	NR	NR
	HORVU5Hr1G024040	Zinc-binding dehydrogenase family protein	AT3G03080		↑	↑	NR	NR	NR

AGI, Arabidopsis best match; CK – (×) found with a similar response to cytokinin in previous analyses; Arrows indicate protein accumulation (↑) or depletion (↓) in response to cytokinin (tZ), drought (D), period of cold (C) or heat (H) stress or salinity (S). See ***Supplementary Tables S1, S2*** *for details.*

TABLE 2 | Proteins with a negative response to cytokinin.

	Accession	Name	AGI	CK	tZ	D	C	H	S
Amino acid metabolism	HORVU7Hr1G051070	Aminoacylase-1	AT4G38220		↓	NR	↓	↓	NR
	HORVU4Hr1G056240	Asparagine synthetase [glutamine-hydrolyzing] 1	AT3G47340		↓	↑	NR	NR	↑
	HORVU3Hr1G073220	Aspartate aminotransferase 3	AT5G11520	×	↓	NR	NR	NR	NR
	HORVU5Hr1G048890	Carbamoyl-phosphate synthase small chain	AT3G27740		↓	↓	NR	NR	NR
	HORVU4Hr1G016410	Methylthioribose-1-phosphate isomerase	AT2G05830		↓	NR	NR	NR	NR
Biosynthesis of secondary metabolites	HORVU2Hr1G086080	6,7-Dimethyl-8-ribityllumazine synthase	AT2G44050		↓	↓	↓	NR	NR
	HORVU5Hr1G100910	Inosine-5′-monophosphate dehydrogenase	AT1G16350		↓	NR	NR	↓	NR
	HORVU3Hr1G032400	D-Alanine aminotransferase	AT5G57850		↓	↓	↓	NR	↓
Carbohydrate metabolism	HORVU1Hr1G080480	6-Phosphogluconate dehydrogenase, decarboxylating 1	AT1G64190		↓	↓	↓	NR	NR
	HORVU7Hr1G000250	Acid β-fructofuranosidase	AT1G12240		↓	NR	↓	NR	↑
	HORVU1Hr1G070310	Aldose reductase	AT2G37760		↓	↓	NR	↓	NR
	HORVU1Hr1G088560	GDP-D-mannose 3′,5′-epimerase	AT5G28840	×	↓	NR	NR	NR	NR
	HORVU5Hr1G069850	Glucose-6-phosphate isomerase	AT4G24620		↓	↓	↓	NR	↓
	HORVU3Hr1G073780	α-1,4-Glucan-protein synthase [UDP-forming]	AT3G02230	×	↓	NR	NR	NR	NR
	HORVU6Hr1G078330	α-Amylase	AT4G25000		↓	NR	↓	NR	NR
	HORVU6Hr1G080790	α-Amylase	AT4G25000		↓	NR	↓	NR	NR
	HORVU5Hr1G113880	α-Mannosidase	AT3G26720		↓	NR	NR	↓	↓
	HORVU1Hr1G057680	β-1,3-Glucanase	AT3G57260	×	↓	NR	NR	↓	NR
	HORVU6Hr1G075010	β-D-xylosidase 4	AT1G78060	×	↓	↓	NR	NR	↓
	HORVU5Hr1G095080	β-Glucosidase C	AT5G20950	×	↓	↓	NR	NR	NR
Cell cycle	HORVU4Hr1G033200	Translationally controlled tumor protein	AT3G16640		↓	NR	NR	NR	NR
Cytoskeleton	HORVU5Hr1G113060	Actin depolymerizing factor 4	AT1G01750	×	↓	NR	NR	NR	NR
	HORVU4Hr1G047580	H/ACA ribonucleoprotein complex subunit 1-like protein 2	AT5G07760		↓	NR	NR	NR	NR
	HORVU7Hr1G052350	Myosin heavy chain	AT4G31340	×	↓	NR	↓	NR	NR
Energy metabolism	HORVU5Hr1G078540	2Fe-2S Ferredoxin-like superfamily protein	AT3G07480		↓	↓	NR	↓	↓
	HORVU2Hr1G070090	2-Oxoglutarate dehydrogenase	AT5G65750		↓	NR	↓	NR	NR
	HORVU4Hr1G080640	Aconitate hydratase 1	AT2G05710	×	↓	NR	↓	NR	NR
	HORVU4Hr1G016810	Alcohol dehydrogenase 1	AT1G64710	×	↓	NR	NR	↑	NR
	HORVU5Hr1G112850	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	AT3G25860		↓	NR	↓	NR	NR

(Continued)

TABLE 2 | Continued

	Accession	Name	AGI	CK	tZ	D	C	H	S
	HORVU2Hr1G006250	Dihydrolipoyllysine-residue succinyltransferase	N/A		↓	NR	NR	NR	NR
	HORVU2Hr1G103180	L-Lactate dehydrogenase	N/A		↓	NR	↓	NR	↓
	HORVU1Hr1G066240	NAD(P)H dehydrogenase (quinone)	AT5G54500		↓	↓	NR	NR	NR
	HORVU3Hr1G077250	NAD(P)H dehydrogenase (quinone)	AT5G54500		↓	↓	↓	↓	↓
	HORVU0Hr1G013850	Succinate dehydrogenase	AT5G66760		↓	NR	NR	NR	NR
Histone	HORVU5Hr1G082190	Histone H2A 6	AT1G51060	×	↓	NR	NR	NR	NR
LEA protein	HORVU4Hr1G051780	LEA protein 1	AT2G36640	×	↓	NR	NR	↓	NR
	HORVU3Hr1G039050	LEA protein-like	AT2G36640	×	↓	NR	NR	NR	NR
Nitrogen metabolism	HORVU6Hr1G080750	Ferredoxin–nitrite reductase	AT2G15620		↓	↓	↓	NR	NR
Nucleic acid and nucleotide metabolism	HORVU2Hr1G085880	Lupus La protein homolog A	AT4G32720		↓	NR	↓	NR	NR
	HORVU4Hr1G016440	Multiple organellar RNA editing factor 1	AT4G20020		↓	↓	NR	NR	NR
	HORVU7Hr1G063970	Polyadenylate-binding protein 2	AT4G34110		↓	NR	↓	NR	NR
	HORVU5Hr1G110370	RNA-binding protein 1	AT5G61030	×	↓	↓	↓	NR	↓
	HORVU6Hr1G091860	rRNA/tRNA 2'-O-methyltransferase fibrillarin-like protein 1	AT5G52470		↓	NR	NR	NR	NR
	HORVU4Hr1G005100	Tudor domain-containing protein 1	AT5G07350		↓	NR	↓	NR	NR
Protein degradation	HORVU4Hr1G010300	Cathepsin B-like cysteine proteinase 5	AT1G02300		↓	NR	NR	NR	NR
	HORVU5Hr1G076130	Proteasome subunit β type-1	AT3G60820		↓	NR	NR	NR	NR
	HORVU0Hr1G040340	Proteasome subunit β type-2	AT4G14800		↓	NR	NR	NR	NR
	HORVU5Hr1G078810	Proteasome subunit β type-4	AT1G56450	×	↓	NR	NR	NR	NR
	HORVU5Hr1G065170	Related to ubiquitin 1	AT1G31340		↓	NR	NR	NR	NR
	HORVU1Hr1G089380	Subtilisin-like protease	AT1G32960		↓	NR	↓	↓	↓
	HORVU1Hr1G023660	Ubiquitin 5	AT1G23410		↓	NR	NR	NR	NR
Protein folding and chaperons	HORVU5Hr1G062310	10 kDa chaperonin	AT5G20720		↓	NR	NR	↓	NR
	HORVU5Hr1G125130	60 kDa chaperonin 2	AT5G18820		↓	NR	NR	NR	NR
	HORVU7Hr1G082540	Copper chaperone	AT1G66240		↓	NR	NR	NR	NR
	HORVU1Hr1G087070	DnaJ homolog subfamily B member 13	AT2G20560		↓	↓	↑	NR	NR
	HORVU5Hr1G078400	HSP 70 C	AT5G42020	×	↓	NR	NR	NR	NR
	HORVU4Hr1G012460	Chaperone protein DnaK	AT4G24280		↓	NR	NR	NR	NR
	HORVU4Hr1G089090	Chaperone protein DnaK	AT4G24280		↓	NR	NR	NR	NR
	HORVU5Hr1G075490	Peptidyl-prolyl cis-trans isomerase	AT3G25220	×	↓	↓	NR	↓	NR
	HORVU6Hr1G077340	Peptidyl-prolyl cis-trans isomerase	AT5G64350	×	↓	NR	↑	↓	NR
	HORVU3Hr1G020490	sHSP 17.6 kDa class II	AT5G12020		↓	NR	↑	NR	NR
	HORVU3Hr1G020500	sHSP 17.6 kDa class II	AT5G12020		↓	NR	↑	NR	NR
	HORVU3Hr1G020520	sHSP 17.6 kDa class II	AT1G54050		↓	NR	↑	NR	NR
	HORVU4Hr1G063350	sHSP 21	AT4G27670		↓	↓	↑	↓	NR
Protein inhibitor	HORVU5Hr1G111920	Serpin-Z7	AT1G47710		↓	NR	NR	NR	NR
Peptide metabolism	HORVU6Hr1G084960	Leucine aminopeptidase 1	N/A		↓	NR	↓	NR	↓

(Continued)

TABLE 2 | Continued

	Accession	Name	AGI	CK	tZ	D	C	H	S
Proteosynthesis	HORVU4Hr1G023570	30S ribosomal protein S9	AT2G09990		↓	NR	NR	↑	NR
	HORVU1Hr1G042220	40S ribosomal protein S17-4	AT5G04800		↓	NR	NR	NR	↑
	HORVU3Hr1G115820	40S ribosomal protein S28	AT3G10090		↓	NR	↑	↓	NR
	HORVU4Hr1G070370	40S ribosomal protein S3a	AT4G34670		↓	NR	NR	NR	NR
	HORVU2Hr1G029890	40S ribosomal protein S6	AT5G10360	×	↓	NR	NR	NR	NR
	HORVU4Hr1G055230	40S ribosomal protein S7	AT3G02560		↓	NR	NR	NR	NR
	HORVU1Hr1G028820	50S ribosomal protein L5	AT2G42740		↓	NR	NR	NR	NR
	HORVU7Hr1G063280	60S ribosomal protein L27-3	AT4G15000		↓	NR	NR	NR	↑
	HORVU3Hr1G038950	60S ribosomal protein L30	N/A		↓	NR	NR	NR	NR
	HORVU7Hr1G031850	60S ribosomal protein L35a-3	AT1G74270		↓	NR	NR	NR	NR
	HORVU1Hr1G021130	Asparagine–tRNA ligase	AT5G56680		↓	↑	↓	↑	NR
	HORVU7Hr1G080870	Elongation factor Ts	AT4G11120		↓	NR	NR	NR	NR
	HORVU5Hr1G122350	Elongation factor Tu	AT4G02930		↓	NR	NR	NR	NR
	HORVU4Hr1G050630	Eukaryotic translation initiation factor 2 β subunit	AT5G20920	×	↓	NR	NR	NR	NR
	HORVU6Hr1G012010	Eukaryotic translation initiation factor 3 subunit J-A	AT5G37475		↓	NR	NR	↓	↓
	HORVU1Hr1G057970	Nascent polypeptide-associated complex subunit α-like protein 3	AT3G12390		↓	↓	NR	↓	↓
	HORVU3Hr1G001140	Ribosomal protein L6 family	AT1G33120		↓	NR	NR	NR	NR
	HORVU5Hr1G034770	Ribosomal protein S3 family protein	AT5G35530		↓	↑	NR	↑	NR
ROS	HORVU5Hr1G103180	Ferredoxin–NADP reductase	AT1G30510	×	↓	↓	NR	↓	NR
	HORVU3Hr1G023970	Glutaredoxin 4	AT3G15660		↓	NR	NR	NR	NR
	HORVU6Hr1G080770	Lipid-binding protein	AT5G42890	×	↓	NR	NR	↓	NR
	HORVU2Hr1G083170	Peptide methionine sulfoxide reductase MsrA	AT5G07470		↓	NR	NR	↓	↑
	HORVU2Hr1G125200	Peroxidase superfamily protein	N/A		↓	↓	NR	NR	NR
	HORVU3Hr1G112350	Peroxidase superfamily protein	AT1G71695	×	↓	NR	NR	NR	NR
	HORVU7Hr1G010280	Peroxidase superfamily protein	AT1G05260	×	↓	NR	NR	↑	NR
	HORVU3Hr1G037720	Peroxiredoxin-2D	AT3G06050		↓	↓	↓	NR	NR
	HORVU7Hr1G046280	Thioredoxin domain-containing protein 17	AT5G42850		↓	↓	↓	↓	↓
Signaling	HORVU1Hr1G001850	12-oxophytodienoate reductase 2	AT1G17990		↓	NR	↓	↓	↑
	HORVU5Hr1G067710	Jasmonate-induced protein	AT3G21380		↓	↓	↓	NR	↓
	HORVU1Hr1G045630	Membrane steroid binding protein 1	AT5G52240		↓	NR	↓	NR	↓
Storage protein	HORVU1Hr1G008130	11S seed storage protein	AT2G28680	×	↓	NR	↓	NR	NR
	HORVU5Hr1G104630	Vicilin	AT3G22640		↓	NR	NR	NR	NR
	HORVU4Hr1G070970	Vicilin-like antimicrobial peptides 2-2	AT2G18540		↓	NR	NR	NR	NR
Stress response	HORVU6Hr1G089570	Germin-like protein 5	AT1G02335		↓	NR	NR	NR	↓
	HORVU6Hr1G089510	Germin-like protein 5	AT1G02335		↓	NR	↓	NR	↓
	HORVU4Hr1G054920	LL-Diaminopimelate aminotransferase	AT2G13810		↓	NR	NR	NR	NR

(Continued)

TABLE 2 | Continued

	Accession	Name	AGI	CK	tZ	D	C	H	S
Transport	HORVU6Hr1G070780	ADP,ATP carrier protein, mitochondrial	AT5G13490	×	→	NR	→	→	→
	HORVU3Hr1G009370	Non-specific lipid-transfer protein 4.1	AT5G59310		→	NR	→	→	NR
	HORVU7Hr1G054440	Nuclear transport factor 2A	AT1G27970		→	NR	→	NR	NR
	HORVU2Hr1G109500	Protein transport protein SEC13 homolog A	AT2G30050		→	NR	→	NR	NR
Unknown	HORVU5Hr1G042310	ACT domain-containing protein	AT5G04740	×	→	NR	NR	NR	NR
	HORVU1Hr1G000940	Copper ion binding protein	AT4G12340		→	→	NR	→	NR
	HORVU1Hr1G080980	Hyaluronan/mRNA binding family	AT4G17520		→	NR	←	NR	NR
	HORVU5Hr1G073450	NAD(P)-binding Rossmann-fold superfamily protein	AT5G10730	×	→	NR	NR	→	NR
	HORVU7Hr1G003710	RNA-binding (RRM/RBD/RNP motifs) family protein	AT5G16840		→	NR	NR	NR	NR
	HORVU2Hr1G056740	Small nuclear ribonucleoprotein associated protein B	AT4G20440		→	NR	NR	NR	NR
	HORVU2Hr1G098860	Unknown	AT2G32240	×	→	→	→	→	→
	HORVU7Hr1G080350	Unknown	N/A		→	NR	NR	NR	NR
	HORVU1Hr1G082820	Unknown	AT3G53040		→	NR	NR	→	NR

AGI, Arabidopsis best match; CK, (×) found with a similar response to cytokinin in previous analyses; Arrows indicate protein accumulation (↑) or depletion (↓) in response to cytokinin (tZ), drought (D), period of cold (C) or heat (H) stress or salinity (S). See ***Supplementary Tables S1, S2*** *for details.*

temperature-stress. To provide evidence for this putative link, three-day old seedlings in magenta boxes were put onto an ice-bath or heated block, and temperature in the growth medium was kept for 120 min at 4 ± 2 and 30 ± 1°C to emulate cold- and heat-stress, respectively. The root tissue was harvested after 22 h of recovery at 20°C. Proteomics analysis revealed 152 and 108 cold- and heat-stress responsive proteins, respectively. Surprisingly, only 27 proteins were found to be differentially abundant in the both datasets and only seven of these manifested a temperature-dependent pattern, namely ribosomal protein S28 (HORVU3Hr1G115820), glutathione S-transferase (HORVU5Hr1G103420), peptidyl-prolyl cis-trans isomerase (HORVU6Hr1G077340), small heat shock protein (HORVU4Hr1G063350), aldehyde dehydrogenase 12A1 (HORVU1Hr1G080320), annexin 7 (HORVU6Hr1G074440) and asparagine–tRNA ligase (HORVU1Hr1G021130). The comparison with cytokinin-responsive proteins revealed a modest overlap, with 54 and 42 differentially abundant proteins for plants after a period of cold- and heat-stress, respectively. In total, out of 178 cytokinin-responsive proteins, 81 were found in the set of temperature-stress-responsive proteins, and most shared a similar response between temperature-stress and cytokinin treatment (**Tables 1**, **2**, **Supplementary Table S1**, **Supplementary Figure S1**).

Response to Abiotic Stress and Cytokinin - Similarities in Proteome and Metabolome Patterns

Metabolome profiling showed a surprising similarity between cytokinin treatment and abiotic stress (**Figures 4A,C**). Notable similarity was found for the depletion of oxaloacetic acid and amino acids, including serine and proline. Proline is a well-known osmoprotectant, and its content should gradually increase upon prolonged stress. However, it has been shown that the initial free proline content in barley roots is very low and at least 48 h of salinity stress are required for any positive changes to be manifested (Ueda et al., 2007). The most similar metabolome response to cytokinin of the abiotic stresses examined was found in samples that experienced a period of cold stress, sharing 23 of 29 differentially abundant metabolites. Not all of the observed changes in roots under abiotic stress were deemed statistically significant (compared to the mock-treated controls; $p < 0.05$), but analysis of principal components confirmed that all treatments were distinct from the control, but not indistinguishable from each other (**Figure 4B**). In contrast, the comparison of proteome profiles by independent component analysis (**Figure 4C**) showed statistically significant separation of cytokinin treatment, salinity stress (IC1), and cold stress (IC2) from each other and both drought and heat stress that were similar to the mock control treatment. In total, 46 proteins were found with a similar and statistically significant response in at least three different treatments. These proteins included an enzyme of ascorbate biosynthesis (HORVU5Hr1G079230; positive response to temperature stress and drought), Hsp70-Hsp90 organizing protein (HORVU2Hr1G092190; positive response to cytokinin, cold- and drought-stress), annexin (HORVU6Hr1G074440; positive response to cytokinin, heat, drought and salinity),

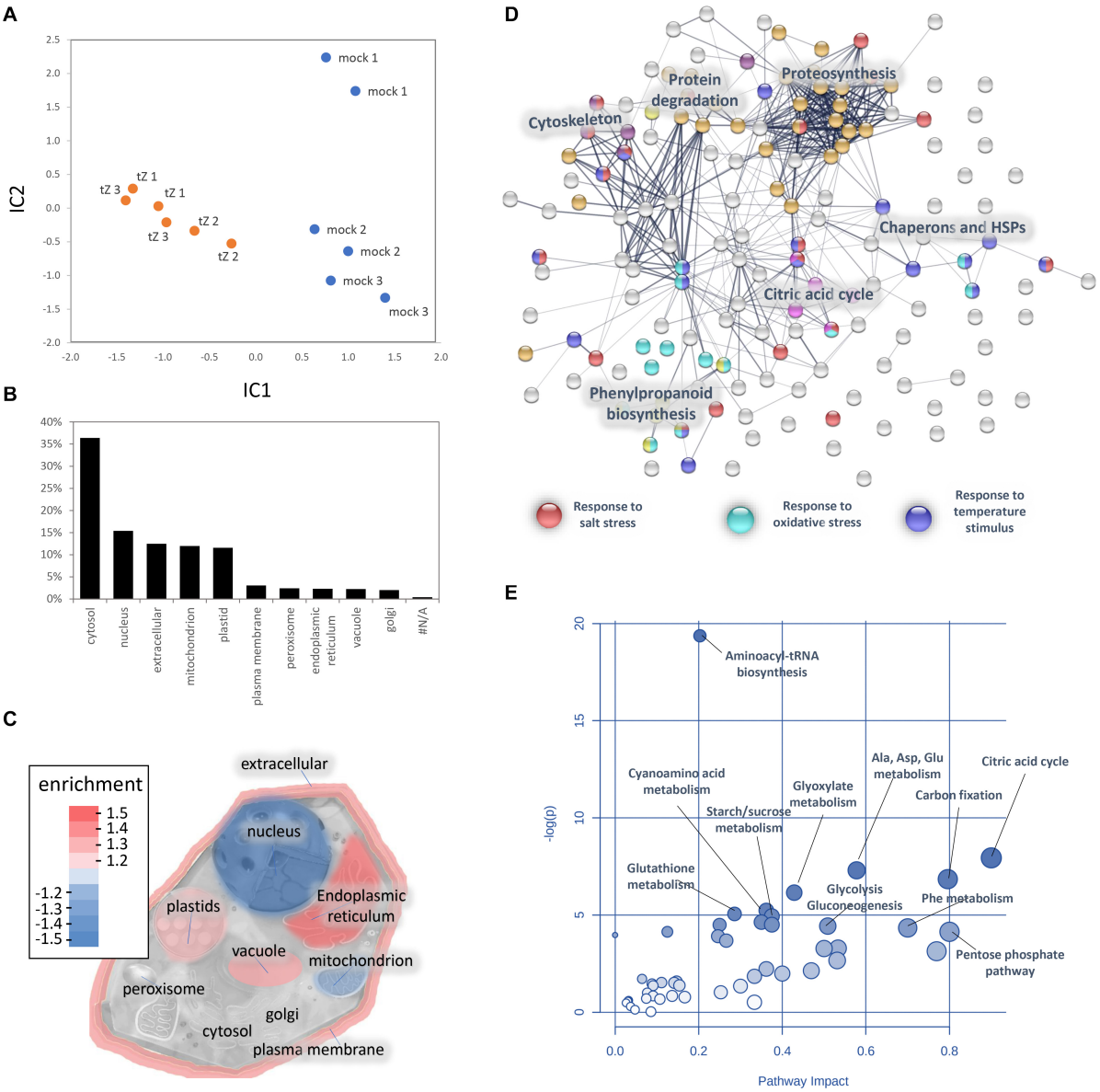


FIGURE 3 | Cytokinin impact on the barley root proteome and metabolome. **(A)** The proteome profile separation of cytokinin- and mock-treated samples. Independent component analysis based on quantitative data of 972 proteins. Results of three biological replicates represent proteins with at least 10 matched spectra in a biological replicate and more than > 95% of the detected root proteome. **(B)** Expected localization of quantified barley root proteins and **(C)** visualization of organellar enrichment in cytokinin-responsive proteins. Localization based on data prediction by CropPal and SUBA4.0 (Hooper et al., 2016, 2017); **(D)** Interactions and functional clusters of cytokinin-responsive proteins highlighted by String (Szklarczyk et al., 2019). The selected highlighted categories are represented by at least five and ten proteins for 'Biological Processes' and 'KEGG Pathways,' respectively; **(E)** Cytokinin impact on barley root metabolism. Highlighted pathways were identified by an integrative analysis of identified cytokinin-responsive proteins and metabolites (MetaboAnalyst; Pang et al., 2020). Results (D-E) are based on data available for putative Arabidopsis orthologs.

phenyl ammonium lyase (HORVU0Hr1G016330; positive response to cytokinin, heat and salinity) and a subunit of potassium channel (HORVU6Hr1G091250; positive response to cytokinin, drought, salinity). Only three proteins were found with a similar and statistically significant response in all experiments indicating that they have a role in the general response to external stimuli, namely thioredoxin-like protein (HORVU7Hr1G046280; depleted), NAD(P)H dehydrogenase (HORVU3Hr1G077250; depleted) and unknown protein HORVU2Hr1G098860 (depleted).

Cytokinin Treatment Reduced Hydrogen Peroxide Content in Roots

The measurement of aqueous hydrogen peroxide showed that the cytokinin treatment significantly reduced hydrogen peroxide content in barley roots by more than 25% (**Figure 5A**). The

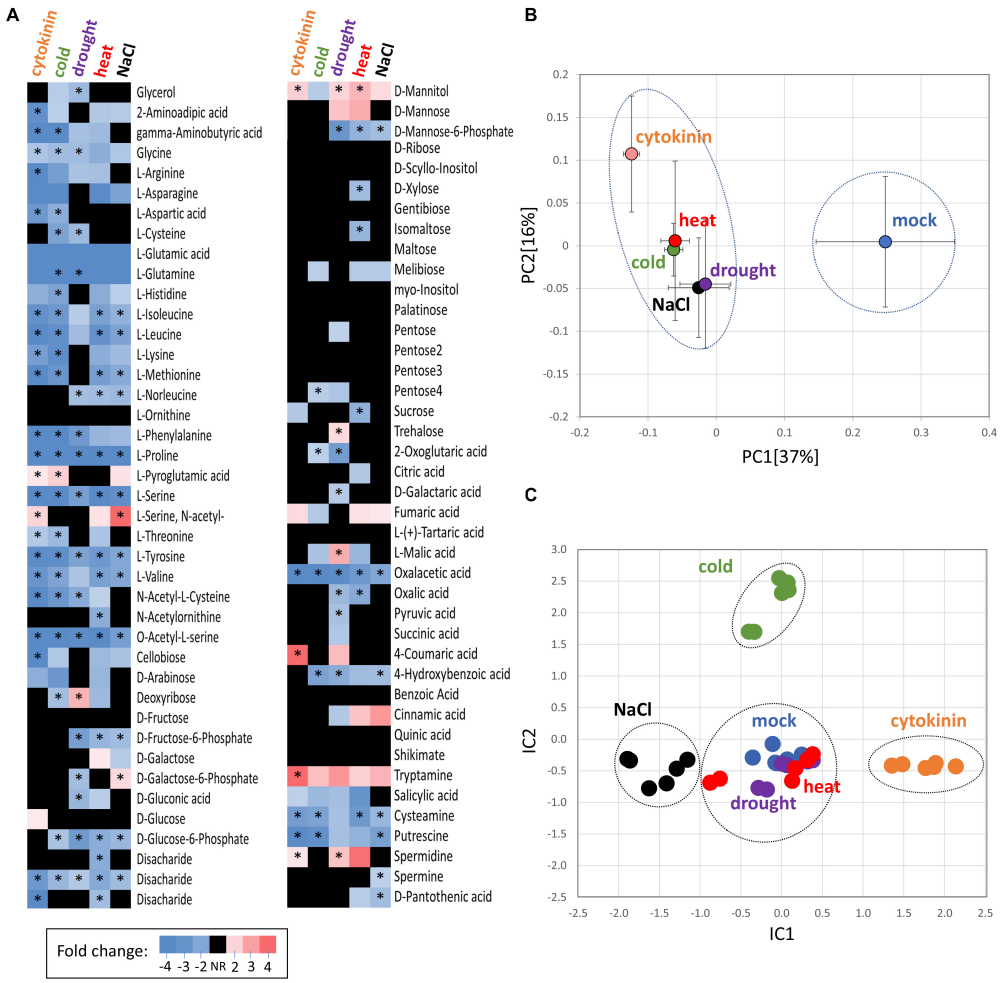


FIGURE 4 | Comparison of cytokinin- and abiotic stress-induced changes in roots of barley. **(A)** Heat map visualization of detected root metabolites. Asterisks indicate statistically significant changes ($p < 0.05$); **(B)** Similarities in the average metabolome composition visualized by PCA. Presented data are means and standard error ($n = 6$); **(C)** Clusters of treatments obtained from independent component analysis of protein profiles; Dashed circles represent statistically significant separation (Kruskal-Wallis test, $p < 0.05$).

histochemical staining indicated that the reduction in the root tips could be even higher and a similar response was found for superoxide radicals (**Figures 5B–E**).

DISCUSSION

Cytokinin Modulates ROS Metabolism in Barley Root

Changes in ROS metabolism enzymes have been previously reported in response to all major phytohormones (Černý et al., 2016), and it has been demonstrated that the ROS metabolism does not only represent a general stress response, but that it is required for mediating growth. For instance, phytohormone auxin promotes ROS production to facilitate cell wall loosening and cell elongation (Huan et al., 2016; Voxeur and Höfte, 2016). Here, out of more than 90 proteins involved in ROS metabolism, 11 were significantly differentially abundant in response to cytokinin, indicating an increase in the ROS production. Cytokinin application induced accumulation of all detected catalase isoforms (HORVU6Hr1G008640, HORVU7Hr1G121700), peroxidases (HORVU2Hr1G124970, HORVU1Hr1G016660), an enzyme that predicted to act on lipid peroxide-derived reactive aldehydes (HORVU3Hr1G015640), and a minor isoform of glutathione S-transferase (HORVU4Hr1G081100; less than 2% of the amount of all detected isoforms). Peroxiredoxin (HORVU3Hr1G037720) together with glutaredoxin (HORVU3Hr1G023970) and thioredoxin (HORVU7Hr1G046280) were depleted, as well as two peroxidases (HORVU2Hr1G125200, HORVU3Hr1G112350). The induction of ROS catabolism resulted in a decrease of hydrogen peroxide and superoxide radicals in the roots (**Figure 5**). This induction was likely triggered by a transient increase in the ROS production, which could correlate with an increase in spermidine and a depletion of its precursor

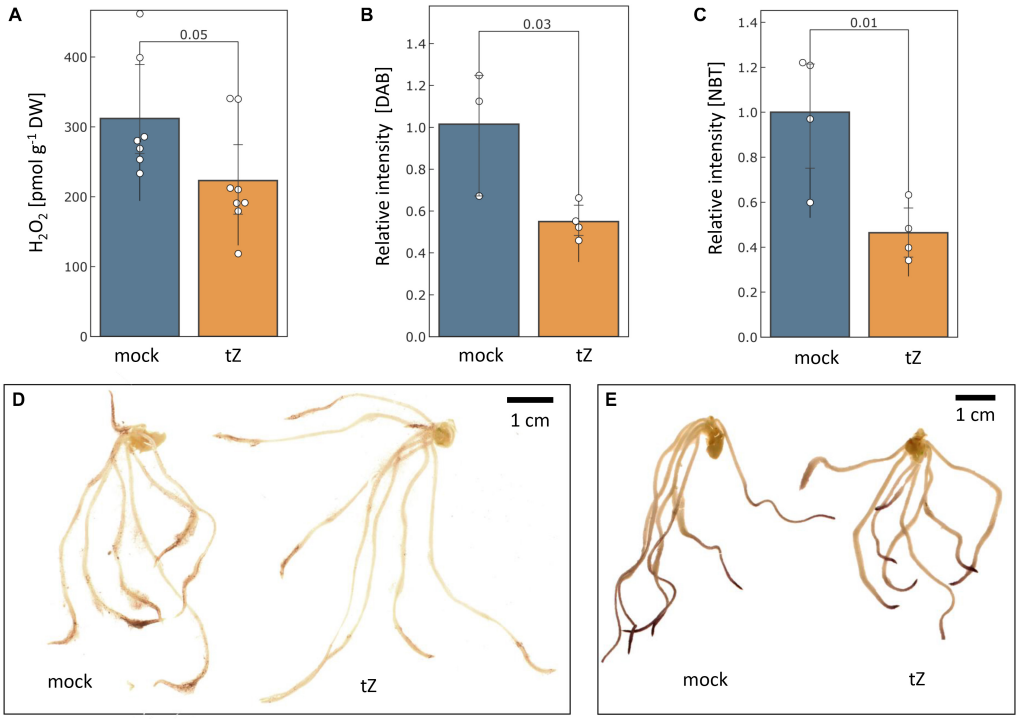


FIGURE 5 | Cytokinin impact on the ROS production. The estimated mean content of hydrogen peroxide in barley roots determined by **(A)** PeroxiDetect Kit and **(B)** 3,3′-diaminobenzidine (DAB) staining; **(C)** Cytokinin impact on superoxide radical production as estimated by histochemical staining with nitroblue tetrazolium (NBT); Representative images of cytokinin- and mock-treated roots stained with DAB **(D)** and NBT **(E)**. Presented data are means and standard deviation of at least three biological replicates; Statistically significant differences (Student's *t*-test) are indicated.

putrescine (**Figure 4A**). Spermidine is a precursor of spermine (no difference in response to cytokinin detected) and both these polyamines reportedly mediate protection against oxidative damage caused by hydrogen peroxide (Rider et al., 2007). Polyamine production is also reportedly linked with an increase activity of catalase and glutathione S-transferase (Seifi and Shelp, 2019) which could coincide with the observed changes at the proteome level. Metabolomic data also indicate a higher turnover rate for glutathione, with a decrease of its precursor O-acetyl-serine and an increase in pyroglutamic acid (glutathione degradation product). However, a decrease in O-acetyl-serine could also be the result of cytokinin inactivation by zeatin 9-aminocarboxyethyltransferase which converts O-acetyl-serine and trans-zeatin into lupinic acid (Entsch et al., 1983). The existence of this enzyme found in Fabaceae has not yet been confirmed in barley, but the available data cannot exclude this possibility.

Salinity and Drought Stress Elicit Cytokinin-like Response in Barley Root Proteome

Drought and salinity are globally the most frequent abiotic stresses, and both significantly impair crop yields. Barley is more resilient to salinity than other cereals with some cultivars tolerating up to 250 mM NaCl (Hanin et al., 2016). Here, we employed 80 mM NaCl that showed only a mild effect on the leaf area (less than 10% reduction at a prolonged treatment; results of preliminary experiments, data not shown) but still elicited a more potent response on the seedling root proteome than the water deprivation (**Supplementary Table S1** and **Supplementary Figure S1**). Modulations of cytokinin metabolism or signaling can improve drought and salt tolerance, and it has been demonstrated that both the depletion of cytokinin and an increase in the cytokinin pool can promote plant growth under abiotic stress (e.g., Nishiyama et al., 2012; Prerostova et al., 2018). The exact mechanisms are not yet fully understood, but at least a part of the cytokinin promoted stress alleviation could reflect the priming of antioxidant systems (Nakabayashi et al., 2014). The similar priming was found here (as discussed above). Further, abundances of 48 cytokinin-responsive root proteins were found with a similar response under salinity stress or water deprivation (**Supplementary Figure S1**). It is thus tempting to speculate that this cytokinin-induced priming could be responsible for an enhanced resilience found in plants with modulated cytokinin pool.

Cytokinin Has a Variable Role in Temperature Stress Response in Barley Root Proteome

The proteome profiling revealed that ca. 5% of the root proteome is formed by heat shock proteins. Heat shock proteins are ubiquitous and widely spread proteins across all taxonomic

kingdoms. These proteins were first discovered in the response to an increase in temperature, but accumulated evidence indicates that they are involved in diverse processes. Besides their chaperon functions, HSPs participate in proteasomal degradation, protein-protein interactions and may also play a role in signaling cascades (e.g., McLoughlin et al., 2019; ul Haq et al., 2019; Tichá et al., 2020). Here, seven heat shock proteins (representing ca. 10% of the total identified HSP protein amount) were significantly depleted in response to cytokinin, including three and four representatives of the HSP70 and sHSP family, respectively. Proteins HSP70-6 (an ortholog of HORVU4Hr1G089090 and HORVU4Hr1G012460) and HSP21 (HORVU4Hr1G063350) were found to be essential for chloroplast development and thermotolerance in germinating seeds of Arabidopsis (Latijnhouwers et al., 2010; Zhong et al., 2013). In general, the exogenous cytokinin treatment elicited (at least partially) a low-temperature response in barley roots, which was manifested in the observed significant changes at the metabolome level and 37 proteins with a cytokinin-like response under cold stress. However, all four cytokinin-responsive sHSP in barley roots were accumulated after a period of cold-stress and not affected or depleted in response to a period of heat. This indicates a variable role of cytokinin in temperature stress response and is well in line with the previous contrasting reports of cytokinin role in cold- and heat-shock response and temperature acclimation.

Cytokinin May Act as a Switch in the Phenylpropanoid Pathway

Cytokinin suppressed membrane steroid binding protein 1 (HORVU1Hr1G045630), an ortholog of Arabidopsis MSBP1 which regulates lignin biosynthesis, negatively regulates brassinosteroid signaling and cell elongation (Yang et al., 2005; Gou et al., 2018). A downregulation of MSBP in *Arabidopsis* resulted in a lower lignin deposition, and the accumulation of soluble phenolics in the monolignol branch (Gou et al., 2018). This would explain the observed significant accumulation of 4-coumaric acid which was specific for cytokinin treatment (**Figure 4A**). It has been previously reported that cytokinin signaling could result in a reduced lignification (reviewed in Didi et al., 2015), and results reported here indicate that MSBP could be its direct target. There are at least two plausible explanations for this cytokinin effect supported in the barley root dataset. First, lowering lignification could coincide with the cytokinin-induced accumulation of expansin B2 (HORVU1Hr1G054240) which is expected (by similarity) to cause loosening and extension of plant cell walls. Alternatively (or in parallel), cytokinin-induced reduction of lignin biosynthesis could be a switch in the phenylpropanoid pathway, promoting anthocyanin production, which is a well-known cytokinin response (Deikman and Hammer, 1995). This alternative is supported by the accumulation of phenylpropanoid biosynthetic enzymes, including two isoforms of phenylalanine ammonia-lyase 1 (HORVU0Hr1G016330, HORVU6Hr1G058820) or flavone 3′-O-methyltransferase 1 (HORVU7Hr1G082280).

CONCLUSION

The cytokinin response has been extensively characterized in the model plant *Arabidopsis thaliana*, but an equivalent study of a crop plant has been largely missing. This work provided the first insight into the cytokinin-responsive proteins and metabolites in developing roots of barley seedling. The observed overlap with known cytokinin-responsive genes and proteins showed that this could be an excellent model for identifying hormone-responsive proteins and for the analysis of intensive crosstalk between plant hormones and abiotic signaling pathways.

MATERIALS AND METHODS

Plant Material

Grain samples of *Hordeum vulgare* L. sensu lato variety (Sebastian) were obtained from field grown plants from the breeding station Stupice (Czechia) in 2016 and were stored in a sealed container at 4°C. For germination assay, grains were imbibed in 4 ml water supplemented with 5×10^{-4}% (v/v) dimethyl sulfoxide (mock buffer) or 1 μM phytohormone (tZ - trans-zeatin; IAA - indole-3-acetic acid; GA3 - gibberellic acid; ABA – abscisic acid; Duchefa) in dimethyl sulfoxide (final concentration, as for the mock) and incubated at 20°C for 48 h in dark. For root proteome and metabolome analysis, grains were surface-sterilized (2% hypochlorite), imbibed and stratified at 4°C for 48 h. Stratified seeds were transferred onto half-strength Murashige & Skoog medium and placed in a growth chamber providing 20°C and 16/8 h light/dark cycles with 100 μmol m^{-2} s^{-1} photon flux density during light periods. After 72 h, sets of 10 germinated seedlings were exposed to 1 μM trans-zeatin or an abiotic stress by exposure to: 30°C or 4°C temperature for 2 h followed by a 22 recovery period at 20°C; medium supplemented with 80 mM NaCl (final concentration); or drought (by transfer to a dry Magenta box). 24 h after each treatment, the root tissue was dissected from the shoot, frozen and stored at −80°C. All experiments were carried out in at least three biological replicates, each consisting of 10 seedlings per sample.

Protein Extraction and LC-MS Proteome Profiling

Total protein extracts were prepared as previously described (Hloušková et al., 2019) employing a combination of phenol/acetone/TCA extraction. Portions of samples corresponding to 5 μg of peptide were analyzed by nanoflow reverse-phase liquid chromatography-mass spectrometry using a 15 cm C18 Zorbax column (Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) and a qTOF maXis Impact mass spectrometer (Bruker) as previously described (Dufková et al., 2019). Peptides were eluted with up to a 120-min, 4% to 40% acetonitrile gradient. MS spectra were acquired at 2 Hz, while MS/MS spectra were acquired between 10–20 Hz using an intensity-dependent mode with a total cycle time of 7 s. The acquired spectra were recalibrated and searched against the reference barley (Mascher et al., 2017) by Proteome Discoverer 2.0, employing Sequest HT

with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, N-terminal acetylation, Met-loss (protein N-terminus), Met-loss + Acetylation (protein N-terminus); MS1 tolerance −35 ppm, MS2 tolerance −0.1 Da (Sequest). The quantitative differences were determined by the spectral counting method, followed by normalization and t-test (compared to the mock-treated roots; p-value < 0.05). For selected candidate proteins, the corresponding peptide peak areas were evaluated in Skyline (Pino et al., 2020). The proteomic data acquired have been deposited to the ProteomeXchange Consortium[1] via the PRIDE partner repository (Vizcaíno et al., 2016) with the dataset identifier PXD020627.

Metabolite Extraction and Analysis

Polar metabolites were extracted as previously described with few modifications (Cerna et al., 2017) and measured using a Q Exactive GC Orbitrap GC-tandem mass spectrometer and Trace 1300 Gas chromatograph (Thermo Fisher). Samples were injected using the split mode (inlet temperature 250°C, splitless time 0.8 min, purge flow 5.0 ml/min, split flow 6.0 ml/min) onto TG-5SILMS GC Column (Thermo Fisher, 30 m × 0.25 mm × 0.25 μm) with helium as a carrier gas at a constant flow of 1.2 ml/min. Metabolites were separated with a 28 min gradient (70°C for 5 min followed by 9°C per min gradient to 320°C and finally 10 min hold time) and ionized using the electron ionization mode (electron energy 70 eV, emission current 50 μA, transfer line and ion source temperature 250°C). The MS operated in the full scan mode, 60000 resolution, scan range 50–750 m/z, automatic maximum allowed injection time with automatic gain control set to 1e6, and lock mass [m/z]: 207.0323. Data were analyzed by TraceFinder 4.1 with Deconvolution Plugin 1.4 (Thermo) and searched against NIST2014, GC-Orbitrap Metabolomics library and inhouse library. Only metabolites fulfilling identification criteria (score ≥ 75 and ΔRI < 2%) were included in the final list.

Determination of Hydrogen Peroxide and ROS Production

The lyophilized root tissue was homogenized and aliquots corresponding to 30–40 mg were analyzed using PeroxiDetect Kit (Sigma-Aldrich) according to the manufacturer's instructions. The distribution of hydrogen peroxide and superoxide radical was visualized by endogenous peroxidase-dependent histochemical staining using 3,3′-diaminobenzidine (e.g., Novák et al., 2013) and nitroblue tetrazolium, respectively (Zhang et al., 2014). The staining intensity was quantified using ImageJ 1.53e (Schneider et al., 2012).

Statistical Analyses

The reported statistical tests were generated and implemented using Instant Clue (Nolte et al., 2018), Rapid Miner (www.rapidminer.com; Mierswa et al., 2006) and Proteome Discoverer. Significant differences refer to $p < 0.05$.

[1]http://proteomecentral.proteomexchange.org

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/pride/archive/, PXD020627.

AUTHOR CONTRIBUTIONS

MC and MB designed research. MC, MB, and HD performed research. MC, MB, ML, JN, IS-F, AR, and BB analyzed data. MC prepared figures and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Funding support for this work was provided by: the Ministry of Education, Youth and Sports of the Czech Republic (CR) to MC, MB, and BB (grant no. CZ.02.1.01/0.0/0.0/16_019/0000738) with support from the European Regional Development Fund "Centre for Experimental Plant Biology"; Mendel University in Brno grants (AF-IGA2019-IP081 and AF-IGA2020-IP048) to ML and MB; and the LQ1601 (CEITEC 2020) project, with financial contribution by the Ministry of Education, Youths and Sports of CR from National Programme for Sustainability II funds to BB.

ACKNOWLEDGMENTS

We thank Vladěna Liberdová and Simona Menšíková for their technical assistance throughout the project, Dr. Vratislav Psota for providing barley grains and Brno City Municipality for the Brno Ph.D. Talent Scholarship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.590337/full#supplementary-material

Supplementary Figure 1 | Differentially abundant proteins in response to cytokinin or abiotic stimuli. Venn diagram summarizing **(A)** all identified differentially abundant proteins; **(B)** significantly accumulated proteins; and **(C)** significantly depleted proteins.

Supplementary Table 1 | Differentially abundant proteins in response to cytokinin or abiotic stimuli. Supplementary table to **Figures 1**, **3**, **4** and **Tables 1**, **2**.

Supplementary Table 2 | Results of proteome profiling. All identified proteins and peptides. Supplementary table to **Figure 1** and **Figure 3**.

Supplementary Table 3 | Results of metabolome profiling. Supplementary table to **Figure 4**.

REFERENCES

Abo-Ogiala, A., Carsjens, C., Diekmann, H., Fayyaz, P., Herrfurth, C., Feussner, I., et al. (2014). Temperature-induced lipocalin (TIL) is translocated under salt stress and protects chloroplasts from ion toxicity. *J. Plant Physiol.* 171, 250–259. doi: 10.1016/j.jplph.2013.08.003

Argueso, C. T., Ferreira, F. J., and Kieber, J. J. (2009). Environmental perception avenues: the interaction of cytokinin and environmental response pathways. *Plant. Cell Environ.* 32, 1147–1160. doi: 10.1111/j.1365-3040.2009.01940.x

Argyros, R. D., Mathews, D. E., Chiang, Y.-H., Palmer, C. M., Thibault, D. M., Etheridge, N., et al. (2008). Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. *Plant Cell* 20, 2102–2116.

Bewley, J. D., Bradford, K. J., Hilhorst, H. W. M., and Nonogaki, H. (2013). *Seeds*, 3rd Edn. New York, NY: Springer, doi: 10.1007/978-1-4614-4693-4

Bhargava, A., Clabaugh, I., To, J. P., Maxwell, B. B., Chiang, Y.-H., Schaller, E. G., et al. (2013). Identification of cytokinin responsive genes using microarray meta-analysis and RNA-seq in *Arabidopsis thaliana*. *Plant Physiol* 162, 272-294.

Bielach, A., Podlešáková, K., Marhavý, P., Duclercq, J., Cuesta, C., Müller, B., et al. (2012). Spatiotemporal regulation of lateral root organogenesis in arabidopsis by cytokinin. *Plant Cell* 24, 3967–3981. doi: 10.1105/tpc.112.103044

Blattner, F. R. (2018). "Taxonomy of the Genus Hordeum and Barley (Hordeum vulgare)," in *The Barley Genome*, eds N. Stein and G. J. Muehlbauer (New York, NY: Springer), 11–23. doi: 10.1007/978-3-319-92528-8_2

Brenner, W. G., Romanov, G. A., Köllmer, I., Bürkle, L., and Schmülling, T. (2005). Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. *Plant J.* 44, 314–333. doi: 10.1111/j.1365-313X.2005.02530.x

Brenner, W. G., and Schmulling, T. (2012). Transcript profiling of cytokinin action in Arabidopsis roots and shoots discovers largely similar but also organ-specific responses. *BMC Plant Biol.* 12:112. doi: 10.1186/1471-2229-12-112

Cerna, H., Černý, M., Habánová, H., Šafářová, D., Abushamsiya, K., Navrátil, M., et al. (2017). Proteomics offers insight to the mechanism behind *Pisum sativum* L. response to pea seed-borne mosaic virus (PSbMV). *J. Proteomics* 153, 78–88. doi: 10.1016/j.jprot.2016.05.018

Černý, M., Dyčka, F., Bobál'ová, J., and Brzobohaty, B. (2011). Early cytokinin response proteins and phosphoproteins of *Arabidopsis thaliana* identified by proteome and phosphoproteome profiling. *J. Exp. Bot.* 62, 921–937. doi: 10.1093/jxb/erq322

Černý, M., Jedelský, P. L., Novák, J., Schlosser, A., and Brzobohatý, B. (2014). Cytokinin modulates proteomic, transcriptomic and growth responses to temperature shocks in Arabidopsis. *Plant Cell Environ.* 37, 1641–1655. doi: 10.1111/pce.12270

Černý, M., Novák, J., Habánová, H., Cerna, H., and Brzobohatý, B. (2016). Role of the proteome in phytohormonal signaling. *Biochim. Biophys. Acta Prot. Proteom.* 1864, 1003–1015. doi: 10.1016/j.bbapap.2015.12.008

Chen, Y., Hoehenwarter, W., and Weckwerth, W. (2010). Comparative analysis of phytohormone-responsive phosphoproteins in *Arabidopsis thaliana* using TiO2-phosphopeptide enrichment and mass accuracy precursor alignment. *Plant J.* 63, 1–17.

Chory, J., Reinecke, D., Sim, S., Washburn, T., and Brenner, M. (1994). A role for cytokinins in De-etiolation in Arabidopsis (det mutants have an altered response to cytokinins). *Plant Physiol.* 104, 339–347. doi: 10.1104/pp.104.2.339

Cortleven, A., Leuendorf, J. E., Frank, M., Pezzetta, D., Bolt, S., and Schmülling, T. (2019). Cytokinin action in response to abiotic and biotic stresses in plants. *Plant. Cell Environ.* 42, 998–1018. doi: 10.1111/pce.13494

Danilova, M. N., Kudryakova, N. V., Doroshenko, A. S., Zabrodin, D. A., Vinogradov, N. S., and Kuznetsov, V. V. (2016). Molecular and physiological responses of *Arabidopsis thaliana* plants deficient in the genes responsible for ABA and cytokinin reception and metabolism to heat shock. *Russ. J. Plant Physiol.* 63, 308–318. doi: 10.1134/S1021443716030043

Dawson, I. K., Russell, J., Powell, W., Steffenson, B., Thomas, W. T. B., and Waugh, R. (2015). Barley: a translational model for adaptation to climate change. *New Phytol.* 206, 913–931. doi: 10.1111/nph.13266

Deikman, J., and Hammer, P. E. (1995). Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. *Plant Physiol.* 108, 47–57. doi: 10.1104/pp.108.1.47

Didi, V., Jackson, P., and Hejátko, J. (2015). Hormonal regulation of secondary cell wall formation. *J. Exp. Bot.* 66, 5015–5027. doi: 10.1093/jxb/erv222

Dufková, H., Berka, M., Luklová, M., Rashotte, A. M., Brzobohatý, B., and Černý, M. (2019). Eggplant germination is promoted by hydrogen peroxide and temperature in an independent but overlapping manner. *Molecules* 24:4270. doi: 10.3390/molecules24234270

Entsch, B., Parker, C. W., and Letham, D. S. (1983). An enzyme from lupin seeds forming alanine derivatives of cytokinins. *Phytochemistry* 22, 375–381. doi: 10.1016/0031-9422(83)83008-8

Ge, S. X., Jung, D., and Yao, R. (2020). ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* 36, 2628–2629. doi: 10.1093/bioinformatics/btz931

Gou, M., Ran, X., Martin, D. W., and Liu, C. J. (2018). The scaffold proteins of lignin biosynthetic cytochrome P450 enzymes. *Nat. Plants* 4, 299–310. doi: 10.1038/s41477-018-0142-9

Guan, C., Wang, X., Feng, J., Hong, S., Liang, Y., Ren, B., et al. (2014). Cytokinin antagonizes abscisic acid-mediated inhibition of cotyledon greening by promoting the degradation of ABSCISIC ACID INSENSITIVE5 protein in Arabidopsis. *PLANT Physiol.* 164, 1515–1526. doi:10.1104/pp.113.234740

Gupta, R., Wang, Y., Agrawal, G. K., Rakwal, R., Jo, I. H., Bang, K. H., et al. (2015). Time to dig deep into the plant proteome: a hunt for low-abundance proteins. *Front. Plant Sci* 6:22. doi: 10.3389/fpls.2015.00022

Hallmark, H. T., Černý, M., Brzobohatý, B., and Rashotte, A. M. (2020). trans-Zeatin-N-glucosides have biological activity in *Arabidopsis thaliana*. *PLoS One* 15:e0232762. doi: 10.1371/journal.pone.0232762

Hanin, M., Ebel, C., Ngom, M., Laplaze, L., and Masmoudi, K. (2016). New insights on plant salt tolerance mechanisms and their potential use for breeding. *Front. Plant Sci* 7:1787. doi: 10.3389/fpls.2016.01787

Harwood, W. A. (2019). An introduction to barley: the crop and the model. *Methods Mol. Biol.* 1900, 1–5. doi: 10.1007/978-1-4939-8944-7_1

Hloušková, P., Černý, M., Kořínková, N., Luklová, M., Minguet, E. G., Brzobohatý, B., et al. (2019). Affinity chromatography revealed 14-3-3 interactome of tomato (*Solanum lycopersicum* L.) during blue light-induced de-etiolation. *J. Proteomics* 193, 44–61. doi: 10.1016/j.jprot.2018.12.017

Holubová, K., Hensel, G., Vojta, P., Tarkowski, P., Bergougnoux, V., and Galuszka, P. (2018). Modification of barley plant productivity through regulation of cytokinin content by reverse-genetics approaches. *Front. Plant Sci.* 9:1676. doi: 10.3389/fpls.2018.01676

Hooper, C. M., Castleden, I. R., Aryamanesh, N., Jacoby, R. P., and Millar, A. H. (2016). Finding the subcellular location of barley, wheat, rice and maize proteins: the compendium of crop proteins with Annotated Locations (cropPAL). *Plant Cell Physiol.* 57:e9. doi: 10.1093/pcp/pcv170

Hooper, C. M., Castleden, I. R., Tanz, S. K., Aryamanesh, N., and Millar, A. H. (2017). SUBA4: the interactive data analysis centre for Arabidopsis subcellular protein locations. *Nucleic Acids Res.* 45, D1064–D1074. doi: 10.1093/nar/gkw1041

Hoth, S., Ikeda, Y., Morgante, M., Wang, X., Zuo, J., Hanafey, M. K., et al. (2003). Monitoring genome-wide changes in gene expression in response to endogenous cytokinin reveals targets in *Arabidopsis thaliana*. *FEBS Lett.* 554, 373–380. doi: 10.1016/S0014-5793(03)01194-3

Huan, C., Jiang, L., An, X., Yu, M., Xu, Y., Ma, R., et al. (2016). Potential role of reactive oxygen species and antioxidant genes in the regulation of peach fruit development and ripening. *Plant Physiol. Biochem.* 104, 294–303. doi: 10.1016/j.plaphy.2016.05.013

Huang, Y., Sun, M.-M., Ye, Q., Wu, X.-Q., Wu, W.-H., and Chen, Y.-F. (2017). Abscisic Acid modulates seed germination via ABA INSENSITIVE5-mediated PHOSPHATE1. *Plant Physiol.* 175, 1661–1668. doi: 10.1104/pp.17.00164

Jeon, J., Kim, N. Y., Kim, S., Kang, N. Y., Novák, O., Ku, S. J., et al. (2010). A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in Arabidopsis. *J. Biol. Chem.* 285, 23371–23386. doi: 10.1074/jbc.M109.096644

Karunadasa, S. S., Kurepa, J., Shull, T. E., and Smalle, J. A. (2020). Cytokinin-induced protein synthesis suppresses growth and osmotic stress tolerance. *New Phytol.* 227, 50–64. doi: 10.1111/nph.16519

Kaur, N., Erickson, T. E., Ball, A. S., and Ryan, M. H. (2017). A review of germination and early growth as a proxy for plant fitness under petrogenic

contamination — knowledge gaps and recommendations. *Sci. Total Environ.* 603, 728–744. doi: 10.1016/j.scitotenv.2017.02.179

Kiba, T., Yamashino, T., Naito, T., Koizumi, N., Sakakibara, H., and Mizuno, T. (2005). Combinatorial microarray analysis revealing araidopsis genes implicated in cytokinin responses through the His-to-Asp phosphorelay circuitry. *Plant Cell Physiol.* 46:S48.

Kieber, J. J., and Schaller, G. E. (2014). Cytokinins. *Arab. B.* 12:e0168.

Kim, Y. M., Han, Y. J., Hwang, O. J., Lee, S. S., Shin, A. Y., Kim, S. Y., et al. (2012). Overexpression of arabidopsis translationally controlled tumor protein gene AtTCTP enhances drought tolerance with rapid ABA-induced stomatal closure. *Mol. Cells* 33, 617–626. doi: 10.1007/s10059-012-0080-8

Kurepa, J., Shull, T. E., and Smalle, J. A. (2019). Antagonistic activity of auxin and cytokinin in shoot and root organs. *Plant Dir.* 3:e00121. doi: 10.1002/pld3.121

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., et al. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. *Plant Cell* 19, 3889–3900. doi: 10.1105/tpc.107.055863

Latijnhouwers, M., Xu, X. M., and Møller, S. G. (2010). Arabidopsis stromal 70-kDa heat shock proteins are essential for chloroplast development. *Planta* 232, 567–578. doi: 10.1007/s00425-010-1192-z

Li, X. G., Su, Y. H., Zhao, X. Y., Li, W., Gao, X. Q., and Zhang, X. S. (2010). Cytokinin overproduction-caused alteration of flower development is partially mediated by CUC2 and CUC3 in Arabidopsis. *Gene* 450, 109–120.

Liu, M.-S., Li, H.-C., Chang, Y.-M., Wu, M.-T., and Chen, L.-F. O. (2011). Proteomic analysis of stress-related proteins in transgenic broccoli harboring a gene for cytokinin production during postharvest senescence. *Plant Sci.* 181, 288–299. doi: 10.1016/j.plantsci.2011.06.005

Lochmanová, G., Zdráhal, Z., Konečná, H., Koukalová, Š, Malbeck, J., Souček, P., et al. (2008). Cytokinin-induced photomorphogenesis in dark-grown Arabidopsis: a proteomic analysis. *J. Exp. Bot* 59, 3705–3719. doi: 10.1093/jxb/ern220

Macková, H., Hronková, M., Dobrá, J., Turečková, V., Novák, O., Lubovská, Z., et al. (2013). Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. *J. Exp. Bot.* 64, 2805–2815. doi: 10.1093/jxb/ert131

Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S. O., Wicker, T., et al. (2017). A chromosome conformation capture ordered sequence of the barley genome. *Nature* 544, 427–433. doi: 10.1038/nature22043

Mayer, K. F. X., Waugh, R., Langridge, P., Close, T. J., Wise, R. P., Graner, A., et al. (2012). A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491, 711–716. doi: 10.1038/nature11543

McLoughlin, F., Kim, M., Marshall, R. S., Vierstra, R. D., and Vierling, E. (2019). HSP101 interacts with the proteasome and promotes the clearance of ubiquitylated protein aggregates. *Plant Physiol.* 180, 1829–1847. doi: 10.1104/pp.19.00263

Mierswa, I., Wurst, M., Klinkenberg, R., Scholz, M., and Euler, T. (2006). "YALE," in *Proceedings of the 12th ACM SIGKDD International Conference On Knowledge Discovery and Data Mining - KDD '06*. New York, NY: Association for Computing Machinery, 935. doi: 10.1145/1150402.1150531

Mrízová, K., Jiskrová, E., Vyroubalová, Š, Novák, O., Ohnoutková, L., Pospíšilová, H., et al. (2013). Overexpression of Cytokinin Dehydrogenase Genes in Barley (Hordeum vulgare cv. Golden Promise) fundamentally affects morphology and fertility. *PLoS One* 8:e79029. doi: 10.1371/journal.pone.0079029

Müller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453, 1094–1097. doi: 10.1038/nature06943

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., et al. (2014). Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *Plant J.* 77, 367–379. doi: 10.1111/tpj.12388

Nemhauser, J. L., Hong, F., and Chory, J. (2006). Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell* 126, 467–475.

Nishiyama, R., Le, D. T., Watanabe, Y., Matsui, A., Tanaka, M., Seki, M., et al. (2012). Transcriptome analyses of a salt-tolerant cytokinin-deficient mutant reveal differential regulation of salt stress response by cytokinin deficiency. *PLoS One* 7:e32124. doi: 10.1371/journal.pone.0032124

Nolte, H., MacVicar, T. D., Tellkamp, F., and Krüger, M. (2018). Instant clue: a software suite for interactive data visualization and analysis. *Sci. Rep.* 8:12648. doi: 10.1038/s41598-018-31154-6

Novák, J., Pavlù, J., Novák, O., Nožková-Hlaváčková, V., Špundová, M., Hlavinka, J., et al. (2013). High cytokinin levels induce a hypersensitive-like response in tobacco. *Ann. Bot.* 112, 41–55. doi: 10.1093/aob/mct092

Pang, Z., Chong, J., Li, S., and Xia, J. (2020). MetaboAnalystR 3.0: toward an optimized workflow for global metabolomics. *Metabolites* 10:186. doi: 10.3390/metabo10050186

Pavlů, J., Novák, J., Koukalová, V., Luklová, M., Brzobohatý, B., and Černý, M. (2018). Cytokinin at the crossroad of abiotic stress signalling pathways. *Int. J. Mol. Sci.* 19:2450.

Pino, L. K., Searle, B. C., Bollinger, J. G., Nunn, B., MacLean, B., and MacCoss, M. J. (2020). The Skyline ecosystem: informatics for quantitative mass spectrometry proteomics. *Mass Spectrom. Rev.* 39, 229–244. doi: 10.1002/mas.21540

Pospíšilová, H., Jiskrová, E., Vojta, P., Mrízová, K., Kokáš, F., Čudejková, M. M., et al. (2016). Transgenic barley overexpressing a cytokinin dehydrogenase gene shows greater tolerance to drought stress. *N. Biotechnol.* 33, 692–705. doi: 10.1016/J.NBT.2015.12.005

Powell, A. F., Paleczny, A. R., Olechowski, H., and Emery, R. J. N. (2013). Changes in cytokinin form and concentration in developing kernels correspond with variation in yield among field-grown barley cultivars. *Plant Physiol. Biochem* 64, 33-40. doi: 10.1016/j.plaphy.2012.12.010

Prerostova, S., Dobrev, P. I., Gaudinova, A., Knirsch, V., Körber, N., Pieruschka, R., et al. (2018). Cytokinins: their impact on molecular and growth responses to drought stress and recovery in Arabidopsis. *Front. Plant Sci.* 9:655. doi: 10.3389/fpls.2018.00655

Ramireddy, E., Hosseini, S. A., Eggert, K., Gillandt, S., Gnad, H., von Wirén, N., et al. (2018). Root engineering in barley: increasing cytokinin degradation produces a larger root system, mineral enrichment in the shoot and improved drought tolerance. *Plant Physiol.* 177, 1078–1095. doi: 10.1104/pp.18.00199

Rider, J. E., Hacker, A., Mackintosh, C. A., Pegg, A. E., Woster, P. M., and Casero, R. A. (2007). Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. *Amino Acids* 33, 231–240. doi: 10.1007/s00726-007-0513-4

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18, 40–54. doi: 10.1105/tpc.105.037796

Righetti, P. G., and Boschetti, E. (2016). Global proteome analysis in plants by means of peptide libraries and applications. *J. Proteomics* 143, 3–14. doi: 10.1016/j.jprot.2016.02.033

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671-675. doi: 10.1038/nmeth.2089

Seifi, H. S., and Shelp, B. J. (2019). Spermine differentially refines plant defense responses against biotic and abiotic stresses. *Front. Plant Sci* 10:117. doi: 10.3389/fpls.2019.00117

Skalák, J., Černý, M., Jedelský, P., Dobrá, J., Ge, E., Novák, J., et al. (2016). Stimulation of ipt overexpression as a tool to elucidate the role of cytokinins in high temperature responses of *Arabidopsis thaliana*. *J. Exp. Bot.* 67, 2861–2873. doi: 10.1093/jxb/erw129

Skalák, J., Vercruyssen, L., Claeys, H., Hradilová, J., Černý, M., Novák, O., et al. (2019). Multifaceted activity of cytokinin in leaf development shapes its size and structure in Arabidopsis. *Plant J* 97, 805-824. doi: 10.1111/tpj.14285

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 47, D607–D613. doi: 10.1093/nar/gky1131

Tichá, T., Samakovli, D., Kuchařová, A., Vavrdová, T., and Šamaj, J. (2020). Multifaceted roles of heat shock protein 90 molecular chaperones in plant development. *J. Exp. Bot.* 71, 3966–3985. doi: 10.1093/jxb/eraa177

Ueda, A., Yamamoto-Yamane, Y., and Takabe, T. (2007). Salt stress enhances proline utilization in the apical region of barley roots. *Biochem. Biophys. Res. Commun.* 355, 61–66. doi: 10.1016/j.bbrc.2007.01.098

ul Haq, S., Khan, A., Ali, M., Khattak, A. M., Gai, W.-X., Zhang, H.-X., et al. (2019). Heat shock proteins: dynamic biomolecules to counter plant biotic and abiotic stresses. *Int. J. Mol. Sci.* 20:5321. doi: 10.3390/ijms20215321

Vescovi, M., Riefler, M., Gessuti, M., Novak, O., Schmulling, T., and Lo Schiavo, F. (2012). Programmed cell death induced by high levels of cytokinin in Arabidopsis cultured cells is mediated by the cytokinin receptor CRE1/AHK4. *J. Exp. Bot.* 63, 2825–2832. doi: 10.1093/jxb/ers008

Vizcaíno, J. A., Csordas, A., Del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., et al. (2016). 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* 44, D447–D456.

Vojta, P., Kokáš, F., Husičková, A., Grúz, J., Bergougnoux, V., Marchetti, C. F., et al. (2016). Whole transcriptome analysis of transgenic barley with altered cytokinin homeostasis and increased tolerance to drought stress. *N. Biotechnol.* 33, 676–691. doi: 10.1016/j.nbt.2016.01.010

Voxeur, A., and Höfte, H. (2016). Cell wall integrity signaling in plants: "To grow or not to grow that's the question". *Glycobiology* 26, 950–960. doi: 10.1093/glycob/cww029

Wang, Y., Li, L., Ye, T., Zhao, S., Liu, Z., Feng, Y. Q., et al. (2011). Cytokinin antagonizes ABA suppression to seed germination of Arabidopsis by downregulating ABI5 expression. *Plant J.* 68, 249–261. doi: 10.1111/j.1365-313X.2011.04683.x

Yang, X. H., Xu, Z. H., and Xue, H. W. (2005). Arabidopsis membrane steroid binding protein 1 is involved in inhibition of cell elongation. *Plant Cell* 17, 116–131. doi: 10.1105/tpc.104.028381

Zalewski, W., Galuszka, P., Gasparis, S., Orczyk, W., and Nadolska-Orczyk, A. (2010). Silencing of the HvCKX1 gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to higher plant productivity. *J. Exp. Bot.* 61, 1839–1851. doi: 10.1093/jxb/erq052

Žďárská, M., Zatloukalová, P., Benítez, M., Šedo, O., Potěšil, D., Novák, O., et al. (2013). Proteome analysis in Arabidopsis reveals shoot- and root-specific targets of cytokinin action and differential regulation of hormonal homeostasis. *Plant Physiol.* 161, 918–930.

Zhang, Y., Chen, B., Xu, Z., Shi, Z., Chen, S., Huang, X., et al. (2014). Involvement of reactive oxygen species in endosperm cap weakening and embryo elongation growth during lettuce seed germination. *J. Exp. Bot.* 65, 3189–3200. doi: 10.1093/jxb/eru167

Zhang, Y., Liu, S., Dai, S. Y., and Yuan, J. S. (2012). Integration of shot-gun proteomics and bioinformatics analysis to explore plant hormone responses. *BMC Bioinformatics* 13(Suppl. 1):S8. doi: 10.1186/1471-2105-13-S15-S8

Zhong, L., Zhou, W., Wang, H., Ding, S., Lu, Q., Wen, X., et al. (2013). Chloroplast small heat shock protein HSP21 interacts with plastid nucleoid protein pTAC5 and is essential for chloroplast development in arabidopsis under heat stress. *Plant Cell* 25, 2925–2943. doi: 10.1105/tpc.113.111229

Zwack, P. J., and Rashotte, A. M. (2015). Interactions between cytokinin signalling and abiotic stress responses. *J. Exp. Bot.* 66, 4863–4871. doi: 10.1093/jxb/erv172

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 05 November 2020
doi: 10.3389/fpls.2020.560696

An *Arabidopsis* Cytokinin-Modifying Glycosyltransferase UGT76C2 Improves Drought and Salt Tolerance in Rice

Yanjie Li[1,2†], Fangfei Liu[1,2†], Pan Li[3], Ting Wang[1,2], Chengchao Zheng[4] and Bingkai Hou[1,2]*

[1] The Key Laboratory of Plant Development and Environment Adaptation Biology, Ministry of Education, Shandong University, Qingdao, China, [2] School of Life Sciences, Shandong University, Qingdao, China, [3] College of Pharmacy, Liaocheng University, Liaocheng, China, [4] State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, China

OPEN ACCESS

Edited by:
Klára Hoyerová,
Academy of Sciences of the Czech Republic, Czechia

Reviewed by:
Aaron M. Rashotte,
Auburn University, United States
Marketa Pernisova,
Masaryk University, Czechia
Karel Dolezal,
Academy of Sciences of the Czech Republic, Czechia

***Correspondence:**
Bingkai Hou
bkhou@sdu.edu.cn

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: 15 June 2020
Accepted: 09 October 2020
Published: 05 November 2020

Citation:
Li Y, Liu F, Li P, Wang T, Zheng C and Hou B (2020) An Arabidopsis Cytokinin-Modifying Glycosyltransferase UGT76C2 Improves Drought and Salt Tolerance in Rice. Front. Plant Sci. 11:560696. doi: 10.3389/fpls.2020.560696

Drought and salt stresses are common environmental threats that negatively affect rice development and yield. Here we report that the overexpression of *AtUGT76C2*, a cytokinin glycosyltransferase, in rice modulates cytokinin homeostasis and confers the plants an eminent property in drought and salt tolerance. The transgenic plants exhibit sensitivity to salt and drought stress as well as abscisic acid during the germination stage and the postgermination stage while showing enhanced tolerance to drought and salinity at the young seedling stage and the mature stage. The overexpression of *UGT76C2* decreases the endogenous cytokinin level and enhances root growth, which greatly contributes to stress adaptation. In addition, the transgenic plants also show enhanced ROS scavenging activity, reduced ion leakage under salt stress, smaller stomatal opening, and more proline and soluble sugar accumulation, which demonstrate that UGT76C2 acts as an important player in abiotic stress response in rice. To explore the molecular mechanism of UGT76C2 in response to stress adaptation, the expressions of eight stress-responsive genes including *OsSOS1*, *OsPIP2.1*, *OsDREB2A*, *OsCOIN*, *OsABF2*, *OsRAB16*, *OsP5CR*, and *OsP5CS1* were detected, which showed notable upregulation in *UGT76C2* overexpression plants under salt and drought stresses. Our results reveal that the ectopic expression of *AtUGT76C2* confers the transgenic rice many traits in improving drought and salt stress tolerance in both developmental and physiological levels. It is believed that *AtUGT76C2* could be a promising candidate gene for cultivating saline- and drought-tolerant rice.

Keywords: abscisic acid, *AtUGT76C2*, drought stress, salt stress, glycosyltransferase, rice

INTRODUCTION

Drought and salinity are the most common abiotic threats limiting plant growth and crop yield. As the most consumed staple food in the world, rice is extremely sensitive to these stresses. Rice requires sufficient water during the whole life cycle, especially at the grain filling stage. Until now, drought and salinity have become major factors causing rice yield loss. Thus, a study exploring

critical saline- and drought-tolerant genes as well as revealing their molecular mechanisms is a critical prerequisite for cultivating saline- and drought-tolerant rice *via* molecular design breeding.

Until now, many stress-regulated genes have been identified to improve stress resistance in plants, which can be grouped into four types: genes involved in the synthesis of osmotic regulators, such as proline biosynthesis gene *OsP5CS1* and *OsP5CR* (Hu et al., 1992; Sripinyowanich et al., 2013); genes involved in ion transportation, such as *SOS1* (Shi et al., 2000); antioxidant-related genes, such as *CAT* (catalase), *APX* (ascorbate peroxidase), and *SOD* (superoxide dismutase) (Mhamdi et al., 2010; Verma et al., 2019); and genes regulating signaling cascades, such as protein kinases (Kang et al., 2017), transcription factors, and so on (Xiong et al., 2014; Chen et al., 2015). However, to enrich the present knowledge and uncover the molecular mechanism on stress regulation in every aspect, more pathways still need to be elucidated.

Cytokinins (CK) are N6-substituted adenine derivatives which are originally defined as a key regulator participating in various plant developmental activities, including organ formation, apical dominance, and leaf senescence (El-Showk et al., 2013). Nowadays, increasing evidence also indicates the action of cytokinin in response to stresses (Hai et al., 2020). A recent cytokinin-responsive transcriptome analysis in rice revealed that a large number of genes are involved in both biotic and abiotic stresses (Raines et al., 2016). It is also reported that cytokinin is required for responding to a series of environmental factors including temperature, drought, osmotic stress, salinity, nutrient stress, plant pathogens, and herbivores (Raines et al., 2016; Cortleven et al., 2019). Genetic studies by modifying cytokinin level in plants indicate that it generally serves as a negative player in stress response (Nishiyama et al., 2011). For instance, the overexpression of the cytokinin-degrading enzyme cytokinin oxidase/dehydrogenase gene enhanced the drought and heat stress tolerance in transgenic tobacco plants (Macková et al., 2013). Previous studies found that AHK2, AHK3, and AHK4 are negative regulators of dehydration and salt tolerance, and the *ahk2* and *ahk3* mutants displayed higher survival rates under severe water deprivation (Tran et al., 2007; Kang et al., 2012). However, some studies found that cytokinin could also function as a positive regulator in drought stress adaptation (Hai et al., 2020). For example, the ectopic expression of the isopentenyltransferase gene (*IPT*) that encodes a rate-limiting enzyme in cytokinin biosynthesis increases endogenous cytokinin levels as well as improves drought stress tolerance in transgenic cotton (Kuppu et al., 2013), creeping bentgrass (Xu et al., 2016), eggplant (Xiao et al., 2017), and tropical maize (Leta et al., 2016). It might be attributed to the choice of promoter, which led to the regulation of different pathways.

Plants tend to fine-tune the developmental process and environmental responses *via* crosstalk between phytohormones. A number of studies have revealed the interplay between cytokinin and abscisic acid (ABA), which act antagonistically in regulating stress response. In contrast to cytokinin, ABA synthesis will be induced in response to water loss, which represents a protective role in response to adverse conditions *via* arresting seed germination and postgermination growth (Lopez-Molina et al., 2002), modulating stomata closure (Zhang et al., 1987) as well as regulating the expression of stress-responsive genes (Zhu, 2002). An earlier study found that trans-zeatin riboside decreases significantly in sunflower plants upon exposure to drought stress, while the endogenous ABA content was increased (Hansen and Dörffling, 2003). Jeon et al. (2010) reported that AHK2, AHK3, and A-type ARRs act as negative regulators in cold stress signaling pathway *via* inhibiting ABA response. It is illustrated that both the *ahk2 ahk3* double mutant and the *arr7* single mutant showed enhanced freezing tolerance, while they are hypersensitive to ABA. Although cytokinin and ABA antagonistically regulate many processes, the molecular mechanisms are poorly known. Recently, Huang et al. (2018) reported that the ABA-activated SnRK2s could phosphorylate the negative regulator type-A response regulator 5 and enhance its stability upon drought stress, by which amplifying the ABA-mediated stress response. This study provides insights into the molecular mechanism of how ABA and cytokinin interplay antagonistically in response to stress.

The levels of endogenous cytokinin is closely related to small molecular modification by the family 1 UDP glycosyltransferases (UGTs), which transfer sugar moieties to small acceptor molecules (Bowles et al., 2006; Lairson et al., 2008). The *Arabidopsis* UGT76C1 and UGT76C2 have been identified to finely tune the glycosylation of cytokinins, which deactivate the molecule and play crucial roles in regulating cytokinin homoestasis in plants (Hou et al., 2004). In our previous study, we revealed that *UGT76C2* is repressed by ABA, osmotic stress, and drought stress. The ectopic expression of *UGT76C2* led to sensitivity to ABA and mannitol during germination stage and tolerance to drought stress as established big plants. It is typically an ABA-dependent pathway in resisting abiotic stresses. We demonstrated that the involvement of UGT76C2 to water deprivation response can be mediated *via* cytokinin and ABA correlations in *Arabidopsis* (Li et al., 2015).

Many identified stress-regulated UGTs were also heterologously expressed in other plant species and bring special properties to the transgenic plants. For example, the ectopic expression of the *Arabidopsis UGT85A5* in transgenic tobacco enhanced salt tolerance in the plants (Sun et al., 2013). Li et al. (2017) found that a barley UDP-glucosyltransferase HvUGT13248, which modify deoxynivalenol and nivalenol, provides *Fusarium* head blight resistance in transgenic wheat. Although a number of rice UGTs are putatively stress-responsive upon examination on Genevestigator[1], no stress-related UGTs have been functionally characterized in rice, and none of the known UGTs have been introduced into rice yet. To see what effect will a stress-related UGT bring to rice, here we transferred the *Arabidopsis* UGT76C2 into rice and further explored its role. Our results showed that AtUGT76C2 greatly enhanced the plant tolerance to drought and salt in transgenic rice in both developmental and physiological levels. This study laid a theoretical foundation that *UGT76C2* could be a promising

[1]https://genevestigator.com/

candidate gene for cultivating saline- and drought-tolerant plants in both dicots and monocots.

MATERIALS AND METHODS

Plant Materials, Vector Construct, and Plant Transformation

Oryza japonica was used in this study. Seedlings were grown on MS plates or soil under SD (10 h light/14 h dark) condition at 28°C in a growth chamber. To generate *UGT76C2* overexpression vector driven by a ubiquitin promoter, the coding region of *Arabidopsis UGT76C2* (At5g05860) was PCR-amplified and sequenced to be right. Then, the fragment was inserted into pUN1301 binary vector by *Kpn*I and *Bam*HI digestion and T_4 ligation. Rice transformation was performed by Biorun biological company[2].

HPLC and LC–MS Analysis

For analyzing the reaction activity toward cytokinin, total protein was extracted from wild-type (WT), OE2, and OE41 plants. The glycosyltransferase activity assay toward trans-zeatin was carried out with 0.1 mg total protein, 1 mM trans-zeatin, 5 mM UDP-glucose, 50 mM HEPES (pH 7.0), 2.5 mM $MgSO_4$, 10 mM KCl, and 14.4 mM β-mercaptoethanol in 100 μl reaction mix. The reaction mix was incubated at 30°C for 3 h. The total protein was extracted in three biological replicates. For analyzing cytokinin glycosides produced in the plants, 7-day-old WT, *UGT76C2OE2*, and *UGT76C2OE41* were treated with 150 μmol trans-zeatin for 24 h, and the total glycosides were extracted with 80% methanol. The treatment and the total glycoside extraction were done in three biological replicates. High-performance liquid chromatography (HPLC) was performed on a Shimadzu HPLC system (Japan). Then, a 10 μl-sample was loaded onto a 5-μm C18 column (150 mm × 4.6 mm; Welch, Ultimate). A linear gradient with increasing methanol (solvent A) against 0.1% triethylamine acetate (solvent B) at a flow rate of 1 ml/min over 40 min was used. Both solutions contained 0.1% H_3PO_4. The peak of trans-zeatin was monitored at 245 nm. For liquid chromatography–mass spectrometry (LC–MS) analysis (Shimadzu), the methods and the mobile phases were similar to the HPLC condition. The mass spectrometer was operated in a positive electrospray ionization mode with 50 eV and a probe voltage of 5.0 kV. The dry heater was set to 180°C. Data acquisition and analysis were performed with Xcalibur software (version 2.0.6).

Stress Assays

For calculating seed germination rates under stresses, full and same-size rice seeds were surface-sterilized in 75% ethanol for 2 min and then in 0.1% mercuric chloride solution for 3 min and rinsed three or four times. The sterilized seeds were imbibed at 28°C in the presence of water (control) and a hydroponic solution of 100 mM NaCl, 7.5% PEG8000, and 150 mM mannitol. For the ABA treatment, ABA was firstly dissolved in a small amount of ethanol and then diluted into the hydroponic solution with 2 and 5 μM ABA. The seeds were germinated in SD (10 h light/14 h dark) condition at 28°C in a growth chamber and were regarded as germinated when the radicles protrude from the seed coat over 2 mm. For each replicate, 50 seeds were calculated. The subsequent seedling growth was observed 5 days later and photographed. For drought stress treatment, watering of 2-week-old rice seedlings growing in soil was stopped until they wilted, followed by re-irrigation. After recovery, the performance of the plants in stress tolerance was observed and photographed. For salt stress, the WT and overexpression lines were irrigated with 200 mM NaCl until they show differences in stress tolerance. Each stress treatment experiment contained three biological replicates. After the treatment, the survival rate was calculated. The recovered plants were regarded as survivors.

Activities of ROS-Scavenging Enzymes

The leaves were detached from 1-week-old rice plants after 200 mM NaCl and 15% PEG8000 treatments for 12 h, and the activities of ROS-scavenging enzymes such as APX, CAT, and SOD activities were determined according to a previously described method (Cai et al., 2015). In this assay, three independent samples were collected, and three technical replicates were done for evaluating enzyme activities.

Diaminobenzidine and Nitrobluetetrazolium Staining, Determination of H_2O_2 Content

For the determination of H_2O_2 and superoxide accumulation under abiotic stress conditions, 4-week-old rice plants were exposed to 200 mM NaCl and 15% PEG8000 for 24 h, respectively, and then were harvested for diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining. Briefly, the leaves were firstly infiltrated by vacuum for 1 h and then subjected to 0.1% DAB staining for 24 h (pH = 5.8), followed by incubation in the de-staining buffer (ethanol/lactic acid/glycerol = 3:1:1). DAB staining was performed according to Du et al. (2008) to determine the H_2O_2 content. NBT staining for superoxide detection was conducted as described by Wohlgemuth et al. (2002). At least five independent plants were subjected to staining for each line. To determine the content of H_2O_2, Catalase Assay Kit (S0051, Beyotime, Shanghai, China) was used according to the user's manual. In brief, the 4-week-old rice plants were treated under salt and drought stress for 24 h, and 0.1 g of tissue was harvested and homogenized with reagents in the kit. Being catalyzed by the catalase, the accumulated H_2O_2 was changed into a red product that can be determined by a spectrophotometer at A_{520}. In the assay, H_2O_2 was determined at three biological replicates.

Determination of Cytokinin Content

To determine the cytokinin content, 0.1 g of fresh leaves of different lines were weighed, and for each line, three independent samples were collected as three biological replicates. The leaf tissues were homogenized in 900 μl phosphate-buffered saline (PBS; pH 7.2–7.4) and centrifuged for 20 min at the speed of

[2]http://www.biorun.net/

3,000 rpm, and then the supernatant was collected. For the quantification of cytokinin, ELISA Kit (MM-3259201)[3] was used. The concentration of cytokinin in the samples is determined by a microplate reader (Tecan, InfiniteTMM200 PRO).

Determination of Proline and Soluble Sugar Content

To determine the content of proline and soluble sugar, 4-week-old rice plants growing under normal condition were treated with 200 mM NaCl and 15% PEG8000 for 24 h, respectively, and the untreated plants served as control. For each line, three independent samples with 0.1 g harvested leaf tissues were collected. The samples were homogenized in 3% aqueous sulphosalicylic acid and centrifuged. The supernatant was collected, and equal volumes of glacial acetic acid and ninhydrin were added. The content of proline was determined at 520 nm (Nakazawa et al., 1982). To examine the content of soluble sugar, 0.1 g of harvested leaves was crushed into powder in liquid nitrogen and homogenized in 80% ethanol, followed by incubation at 95°C for 1 h. The supernatant was collected by centrifugation, dried at 80°C for 2 h, and then dissolved in 10 ml distilled water. Quantification of soluble sugar was performed as described by Li et al. (2004).

Calculations on Ion Leakage, Water Loss, and Water Content of Detached Leaves

Upon exposure to 200 mM NaCl for 7 days, rice leaves were collected from 2-week-old rice seedlings to measure the electrolyte leakage as described by Tang et al. (2017). For calculating water loss, fresh leaves detached from 3-week-old plants were firstly weighed (FW) and then dried naturally in open air. The drying leaves were weighed (DW) every 30 min. Water loss was calculated as (FW − DW)/time * FW. For each assay, three biological replicates were performed.

Observations of Stomatal Opening by Scanning Electron Microscopy

Leaves of 3-week-old *UGT76C2* transgenic rice and WT plants were detached and kept in air for 2 h to allow water loss and stimulate stomatal closure; then, the leaf tissues were fixed in 2.5% glutaraldehyde at 4°C for 12 h and then washed five times with 0.1 mol/L phosphate buffer, followed by dehydration with gradient ethanol. The samples were bonded with a drying instrument on the HCP-2 critical point, followed by spraying gold with an IB-v ion sputtering device and photographed with a scanning electron microscope (FEI Quanta250 FEG). For each line, at least 100 stomata were observed.

Determination of ABA Content

Leaves of 3-week-old *UGT76C2* transgenic rice and WT plants were detached and kept in air for 2 h. For each line, three independent samples were collected, at approximately 50 mg leaf tissue for each sample. The samples were rapidly frozen with liquid nitrogen, followed by homogenization in PBS (pH 7.4). The samples were centrifuged for 20 min, and the supernatant was collected carefully for determination. The ABA concentration was assayed according to the instructions provided by the plant hormone abscisic acid ELISA Kit (Shanghai Fusheng Industrial Co., Ltd., catalog number: A112641-96T).

[3]http://www.mmbio.cn/

Quantitative and Semi-Quantitative RT-PCR

For quantitative and semi-quantitative RT-PCR, total RNA was extracted from rice samples with Trizol reagent (Vazyme), and 5 μg RNA was reverse-transcribed with the PrimeScript RT reagent kit with gDNA Eraser (Vazyme) according to the supplier's manual. Real-time PCR was done with a real-time thermal cycling system (Bio-Rad). SYBR-Green was used to detect gene abundances. Each reaction was done with three biological replicates. Data were analyzed using Bio-Rad CFX Manager software. For evaluating *UGT76C2* expression in transgenic rice, semi-quantitative RT-PCR was employed. The synthesized cDNA was diluted five times (1:5), and 2 μl was used for analyzing the transcript level. *OsActin1* was used as an internal reference gene. PCR reactions were performed in 25 μl of total reaction volume, with 25 cycles for amplifying *OsActin1* and 32 cycles for amplifying *UGT76C2*. The primers for the RT-PCR assay are included in **Supplementary Table S1**.

Statistical Analysis

Student's *t*-test with one-tailed test was performed to compare the performance of WT and *UGT76C2* transgenic lines. For each assay and experiment, data from at least three biological replicates were subjected to statistical analysis. The minimum significance levels were set at $^{*}p < 0.05$ and $^{**}p < 0.01$.

RESULTS

Overexpression of the *Arabidopsis UGT76C2* in Rice Generates More Cytokinin Glycosides

To determine the role of the *Arabidopsis* UGT76C2 in rice, transgenic plants overexpressing *AtUGT76C2* under the control of the maize ubiquitin (ubi) promoter were generated, and the homozygous lines were selected with hygromycin. qRT-PCR analysis indicated that the transcripts of *AtUGT76C2* could be detected in transgenic plants, but not in WT plants (**Figure 1A**). The *UGT76C2* overexpression lines OE2 and OE41 with relatively higher *UGT76C2* abundances were chosen for all assays in this study. To determine the role of UGT76C2 in catalyzing cytokinins in rice, firstly, we purified the total protein from WT, OE2, and OE41 plants and assessed their activity in catalyzing trans-zeatin. It was found that more trans-zeatin-7-N-Glc and trans-zeatin-9-N-Glc were produced after incubation with total protein purified from OE2 and OE41 than that from WT (**Figures 1B1–3**). The identification of 7 and 9-N-Glc was based on their retention time as reported by Hou et al. (2004) and by LC–MS confirmation (**Figure 1C**). To further investigate

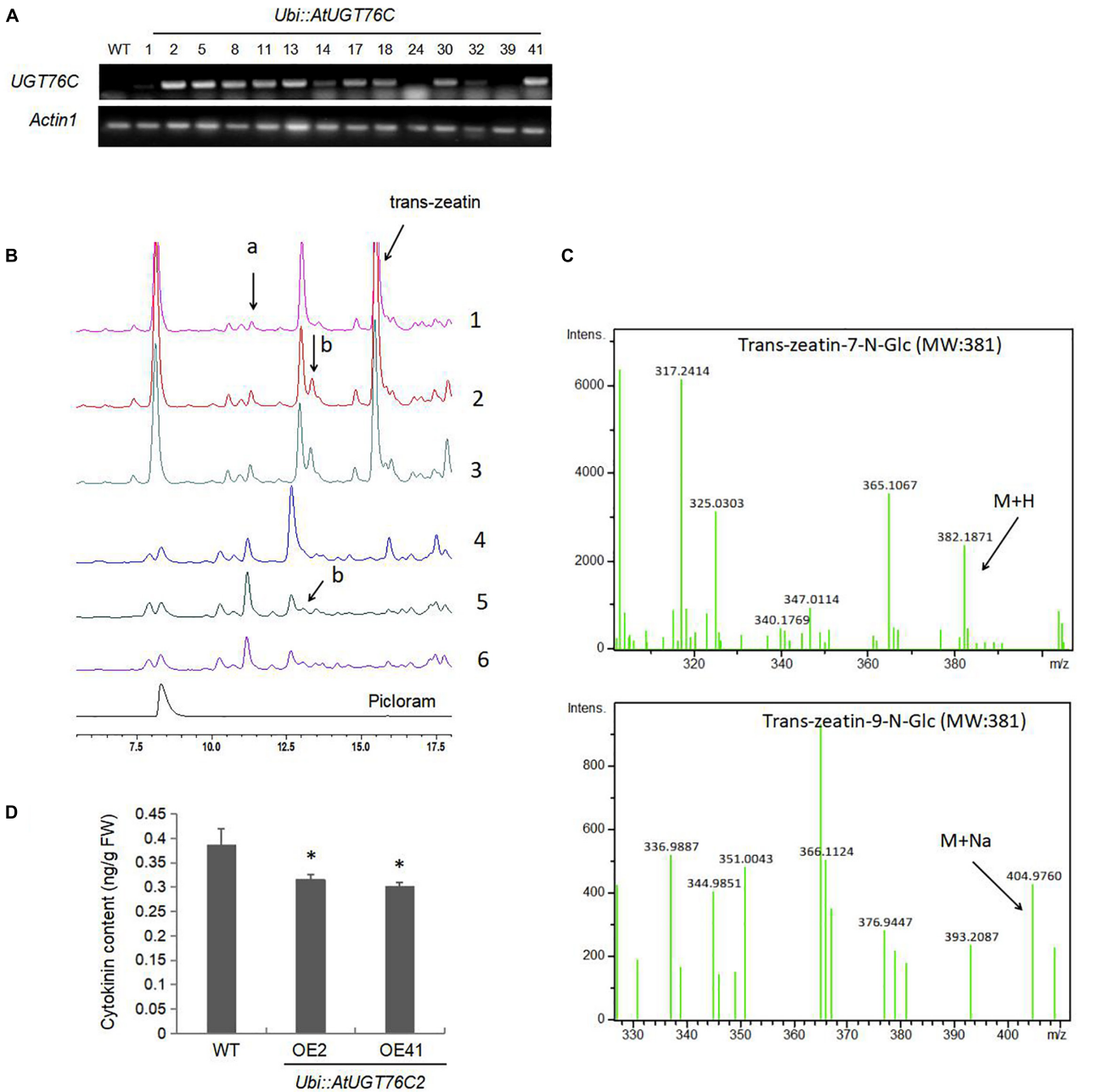


FIGURE 1 | The Arabidopsis UGT76C2 glycosylates cytokinin in transgenic rice. **(A)** RT-PCR analysis of the mRNA levels of *UGT76C2* in transgenic rice plants. **(B)** Trans-zeatin catalyzing activity of the total proteins extracted from WT (1), OE2 (2), OE41 (3), and analysis of total glycosides extracted from WT (4), OE2 (5) and OE41 (6) after 150 n mol trans-zeatin treatment for 24 h. The product (a) indicates trans-zeatin-7-N-Glc, (b) indicates trans-zeatin-9-N-glc. The experiments were conducted in three biological replicates. **(C)** LC-MS confirmation trans-zeatin-7-N-Glc and trans-zeatin-9-N-glc. **(D)** The content of cytokinin in 2-week-old seedlings of WT and *UGT76C2* transgenic rice. Bars show standard deviations of three biological replicates. Asterisks indicate significant differences between the wild type and the overexpression lines evaluated with Student's *t*-test (*$P < 0.05$).

UGT76C2 activity toward trans-zeatin in rice, 7-day-old WT, *UGT76C2*OE2, and *UGT76C2*OE41 seedlings were treated with 150 μmol trans-zeatin for 24 h. Then, total glycosides were extracted from the three plants and subjected to HPLC and LC–MS analysis. Consistent with a previous study (Hou et al., 2004), more 7-N-Glc was generated than 9-N-Glc in plants (**Figures 1B4–6**), which further demonstrates that UGT76C2 has a higher activity toward 7-N of trans-zeatin. However, for the total protein-catalyzed reaction *in vitro*, it seems that more 9-N-Glc was produced than 7-N-Glc (**Figures 1B1–3**). It is likely that UGT76C2 activity is affected by other proteins in the mix. These results showed that *UGT76C2* overexpression in

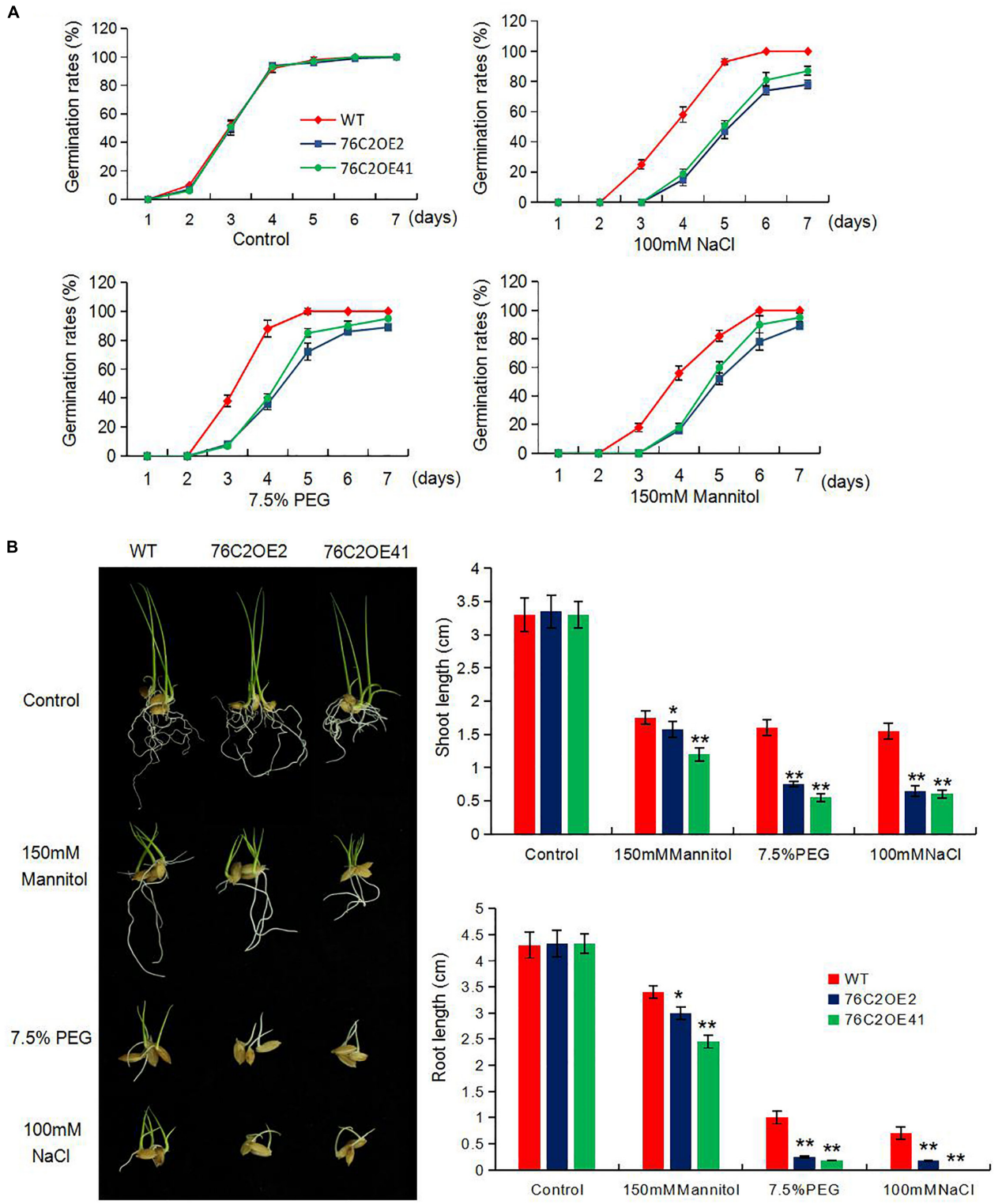


FIGURE 2 | Overexpression of AtUGT76C2 increased the plant sensitivity to abiotic stresses during germination and post-germination growth. **(A)** Rice seeds were imbibed at 28°C in the presence of water (Control) and hydroponic solution of 100 mM NaCl, 7.5% PEG and 150 mM mannitol. Germination (based on radicles>2 mm) of at least 50 seeds were recorded for each biological replicates, and three biological replicates were done for calculating germination rates **(B)**. Post-germination growth of the seedlings under abiotic stresses, and shoot and root lengths were recorded. Bars show standard deviations of three independent replicates. Asterisks indicate significant differences between the wild type and the overexpression lines evaluated with Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

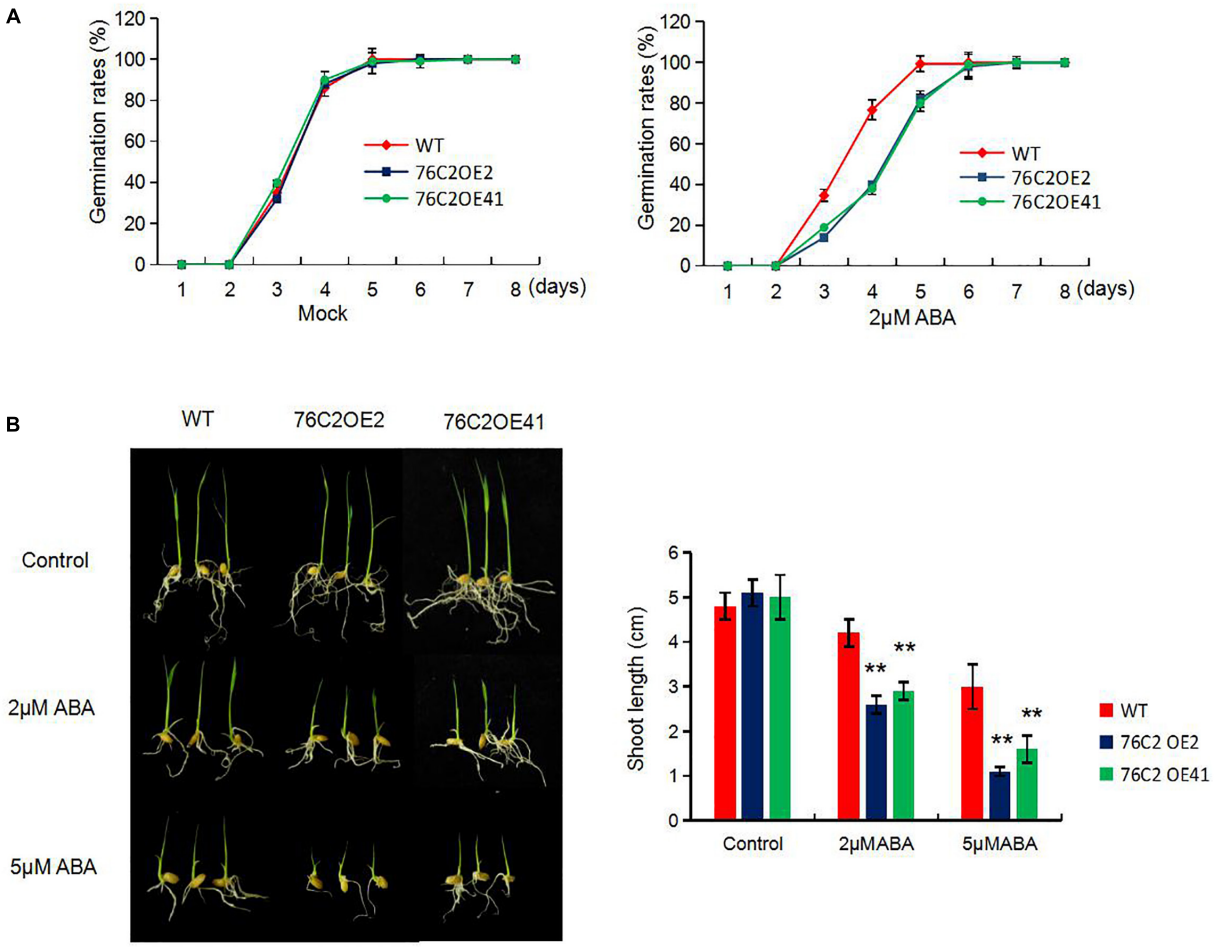


FIGURE 3 | Overexpression of *AtUGT76C2* increased the plant sensitivity to abscisic acid (ABA) during germination and post-germination growth. **(A)** Rice seeds were imbibed at 28°C in hydroponic solution with 2 μM ABA (dissolved with little ethonal) and without ABA (Mock, with equal amount of ethonal). Germination (based on radicles>2 mm) of at least 50 seeds were recorded for each biological replicates, and three biological replicates were done for calculating germination rates **(B)**. Post-germination growth of the seedlings under ABA, and shoot lengths were recorded. Bars show standard deviations of three independent replicates. Asterisks indicate significant differences between the wild type and the overexpression lines evaluated with Student's *t*-test (**$P < 0.01$).

rice could catalyze the glycosylation of cytokinin. In addition, we also evaluated the endogenous cytokinin content in the three plants and found that it decreased in the two UGT76C2 overexpression lines (**Figure 1D**), demonstrating that UGT76C2 affected cytokinin homeostasis in transgenic rice.

Overexpression of the *AtUGT76C2* in Rice Leads to Sensitivity to Abiotic Stresses and ABA at Germination and Postgermination Stages

To see the performance of the *UGT76C2* transgenic lines in response to abiotic stresses, seeds of wild type, *UGT76C2*OE2, and *UGT76C2*OE41 were exposed to 100 mM NaCl, 150 mM mannitol, and 7.5% PEG for germination, and the germination rates were recorded in the following 8 days. As illustrated in **Figure 2A**, OE2 and OE41 showed lower germination rates compared to WT under these adverse conditions. The subsequent growth of OE2 and OE41 seedlings was also more affected under abiotic stresses. The two OE lines were observed to have shorter shoots and roots than that of WT when kept growing under mannitol, NaCl, and PEG conditions for 1 week more after germination. Especially the shoot growth of *76C2*OE lines is significantly inhibited under PEG and NaCl treatments (**Figure 2B**). Next, we also examined the response of *UGT76C2* overexpression lines upon ABA treatment in terms of seed germination and shoot growth. It was observed that OE2 and OE41 exhibited lower germination rates as well as slower shoot growth (**Figure 3**).

Expression of *AtUGT76C2* Improves Stress Adaptation and Enhances Root Growth at Seedling Stage

To see the performance of *AtUGT76C2* overexpression plants under salt stress, the soil-growing 2-week-old WT,

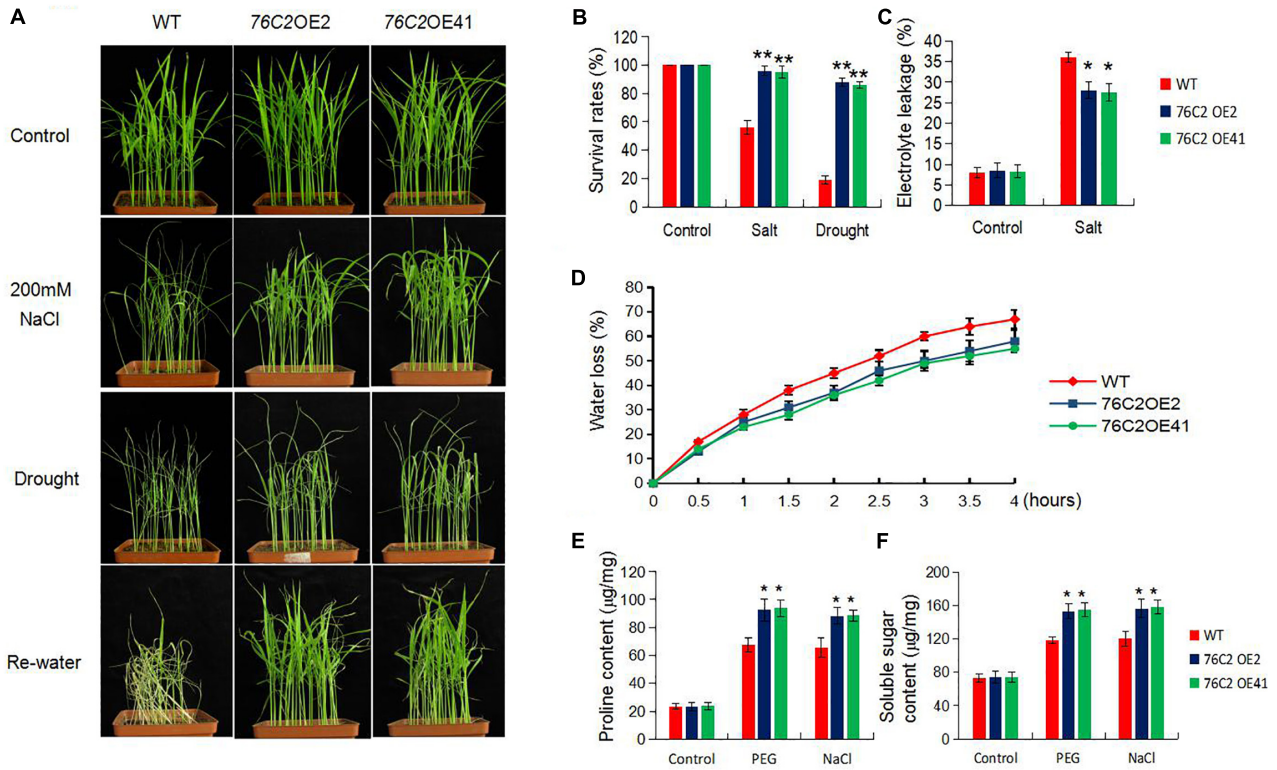


FIGURE 4 | Overexpression of *AtUGT76C2* in rice enhanced salt and drought tolerance in the adult transgenic plants. **(A)** For salt treatment, 2-week-old rice seedlings were exposed to 200 mM NaCl for 7 days. For drought treatment, the plants were dried for 1 week, and then allowed for recovery for 7 days. **(B)** Survival rates of the transgenic and the control rice after salt and drought treatment. **(C)** Electrolyte leakage of the plants after salt treatment. **(D)** Water loss rate of detached leaves during 4 h. Proline **(E)** and soluble sugar **(F)** content under PEG and NaCl treatments. Each assay was done at three independent biological replicates. Asterisks indicate significant differences between the wild type and the overexpression lines evaluated with Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

*UGT76C2*OE2, and *UGT76C2*OE41 seedlings were exposed to 200 mM NaCl for 2 weeks, and then the WT plants became withered, while the transgenic lines were more tolerant to salt stress (**Figure 4A**). Their tolerance to salt stress was also assessed in terms of survival rate and electrolyte leakage. The survival rates of WT, OE2, and OE41 were 53.6, 93.3, and 92.1%, respectively (**Figure 4B**), and the two transgenic lines OE2 and OE41 exhibited less electrolyte leakage under salt stress than WT, indicating that less damage is caused in the *UGT76C2* overexpression plants (**Figure 4C**). For drought stress treatment, 2-week-old WT, OE2, and OE41 were dried for 7 days and then re-irrigated. It was observed that most of the WT plants wilted (**Figure 4A**), while the two OE lines were still vigorous and showed higher survival rates, 86.2 for *76C2*OE2 and 85.3% for 76C2OE41, respectively. However, the survival rate of WT plants was only 18.8% (**Figure 4B**). To make a further investigation, we also detected the water loss rates of the detached leaves from the three plants in 4 h. The result indicates that the two transgenic rice lines exhibited lower water loss than that of WT. After 4 h, the water loss rates of WT, OE2, and OE41 were 67.1, 58.0, and 55.2%, respectively (**Figure 4D**). Additionally, the proline and soluble sugars, which serve as osmotic regulators, were also accumulated more in OE2 and OE41 plants (**Figures 4E,F**). Above all, from both visual phenotype and assessment of physiological indexes, drought and salt stress tolerance is greatly improved in *UGT76C2*OE rice plants.

Interestingly, it was also observed that, with the growth of the rice seedlings, root growth was enhanced upon *UGT76C2* overexpression in the transgenic lines. For seedlings as young as 4 days old, they showed little difference in root length. However, for 7, 10, and 15-days-old seedlings, the roots of the UGT76C2 overexpression lines were obviously longer than those of WT (**Figure 5**), which also contributes to the drought and salt stress tolerance of the UGT76C2 overexpression lines.

Overexpression of *AtUGT76C2* in Rice Reduced Stomatal Opening in Response to Drought Stress

When suffering from drought stress, plants will soon reduce the transpiration rate and water loss, for example, by controlling stomata movement. Therefore, we compared the stomatal opening of 4-week-old rice leaves of WT, *UGT76C2*OE2, and *UGT76C2*OE41. The degrees of stomatal opening are shown in **Figure 6A**. Under the non-treatment condition, the proportion of closed stomata showed a little difference in WT and transgenic plants at 20.2, 17, and 18.7% for WT, OE2, and

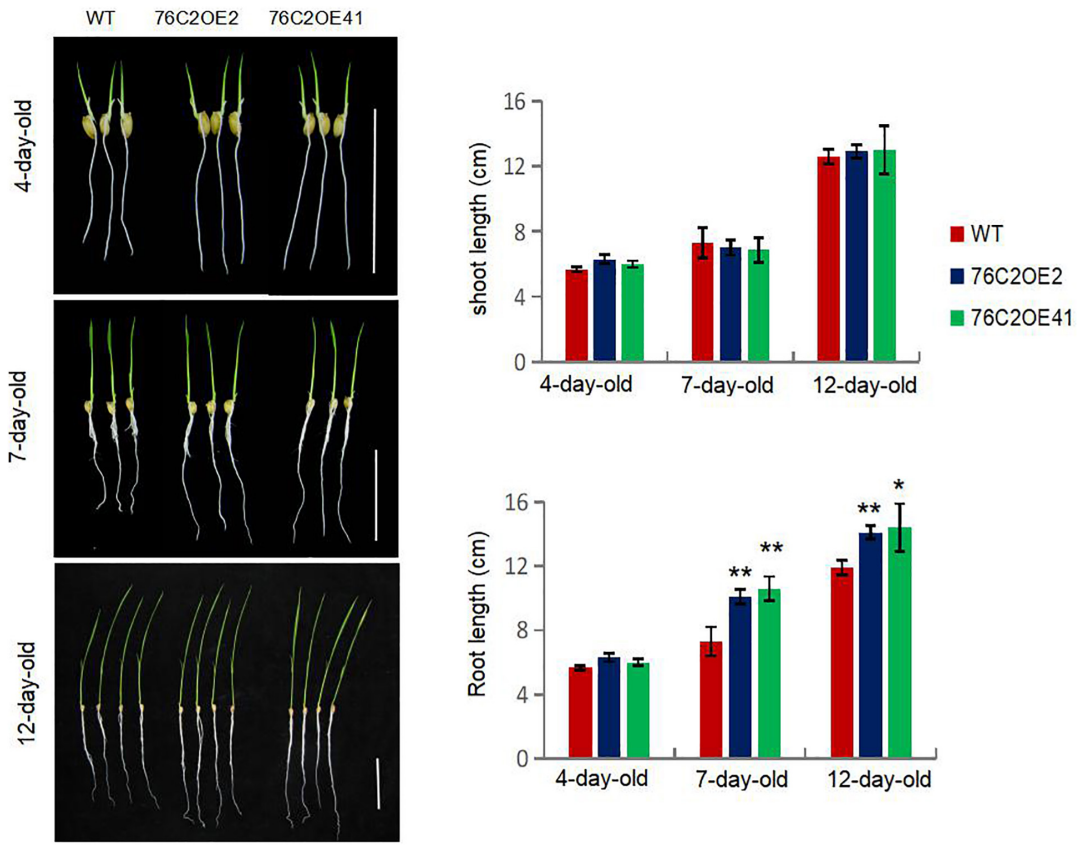


FIGURE 5 | Overexpression of *AtUGT76C2* in rice enhanced root growth (bar = 5 cm). Bars show standard deviations of at least 10 seedlings. Asterisks indicate significant differences between wild type and the overexpression lines evaluated with Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

OE41, respectively. However, the proportion of completely open stomata was more in WT than in OE plants and that of partially open stomata was more in OE than in WT. After exposing the detached leaves in open air for 2 h, the number of closed stomata increased and that of completely open stomata substantially decreased. More completely closed stomata and less completely/partially open stomata were observed in OE2 and OE41 leaves compared with WT. The proportion of completely closed stomata accounts for 28.4, 48.8, and 46.5% for WT, OE2, and OE41, respectively. The completely open stomata for WT, OE2, and OE41 plants account for 64, 47.2, and 49.6%, respectively, and the partially open stomata were 7.6, 3.9, and 4% for WT, OE2, and OE41 (**Figure 6B**). These results suggested that the elevated water saving capacity and the drought resistance in *AtUGT76C2* transgenic rice were largely due to reduced stomatal opening.

Stomatal movement is known to be closely related to ABA level or ABA signaling. Here we determined the ABA contents in WT and *UGT76C2OE* plants under normal and drought stress conditions. It was observed that the endogenous ABA levels in both *AtUGT76C2* overexpression and WT seedlings were more accumulated in response to drought stress; however, we did not see any significant differences in ABA levels between transgenic and WT seedlings under both normal and drought stress conditions (**Figure 6C**). These results imply that *UGT76C2* overexpression might have affected ABA signaling instead of ABA synthesis.

Overexpression of *AtUGT76C2* Enhanced ROS Scavenging in Transgenic Plants

It is known to all that stresses cause an accumulation of reactive oxygen species (ROS) that can damage the membrane systems. Here, we also investigated ROS levels in *UGT76C2OE* and WT plant under stress condition. DAB and NBT staining indicated that ROS accumulated more in WT than in *UGT76C2* transgenic lines (**Figure 7**). To explain the differential ROS production, we firstly examined the expression of genes encoding ROS scavenging enzymes, such as catalase isozyme A (CAT-A), catalase isozyme B (CAT-B), ascorbate peroxidase 2 (APX2), iron-superoxide dismutase b (Fe-SODb), and copper/zinc-superoxide dismutase (SODCc2). Quantitative RT-PCR showed that all the five genes were upregulated by salinity and drought and were more upregulated in the *AtUGT76C2* transgenic lines (**Figure 8A**). Besides, we also examined the antioxidant enzyme activities of CAT, APX and SOD in WT and transgenic plant under normal and stress conditions, and found that they were higher in transgenic lines than in WT in response to

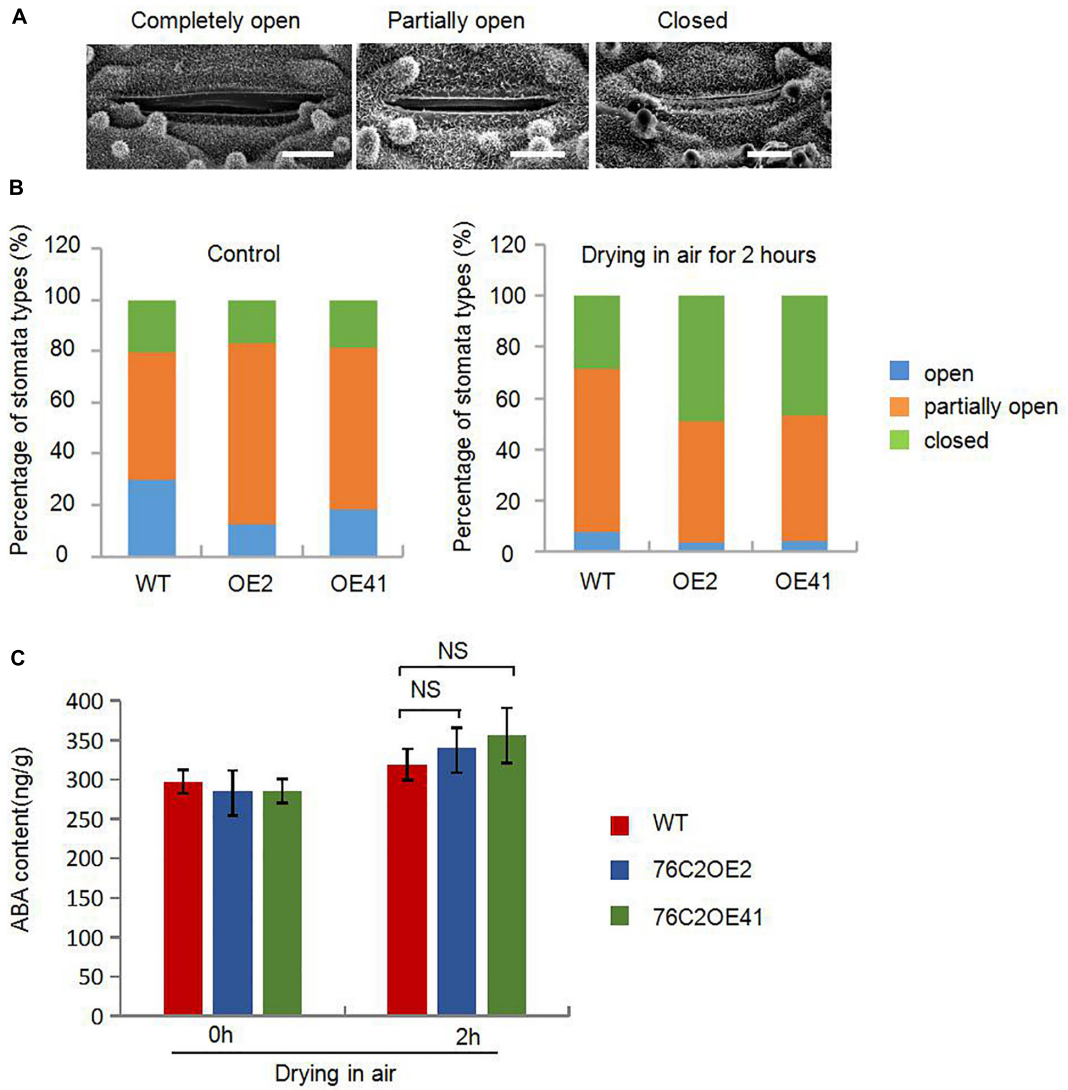


FIGURE 6 | AtUGT76C2 enhanced the plant stomata closure in rice in response to water loss. **(A)** The opening of stomata observed with a scanning electron microscope. Bar = 5 urn. **(B)** The proportion of three stomata openings in WT and *AtUGT76C2* overexpression lines ($n > 100$). **(C)** ABA contents in WT and *AtUGT76C2* overexpression lines which were measured at three biological replicates.

abiotic stresses, whereas showed no difference under control condition (**Figure 8B**).

Expressions of Stress-Related Genes Were Affected Upon *UGT76C2* Overexpression in Rice

To investigate the potential molecular pathways affected by UGT76C2 in regulating stress tolerance, we then monitored the expressions of eight abiotic stress-related genes including *OsSOS1*, *OsPIP2.1*, *OsDREB2A*, *OsCOIN*, *OsABF2*, *OsRAB16*, *OsP5CR*, and *OsP5CS1*. Two-week-old wild type and *UGT76C2*OE plants were exposed to NaCl and PEG, respectively, for 12 h, and qPCR was performed. The results showed that all these genes were induced at different degrees by salt and drought and were more highly expressed in the overexpression lines compared with that in WT plants (**Figure 9**). For instance, in response to salt stress, the expression of *OsSOS1*, *OsPIP2.1*, and *OsRAB16* were significantly induced, more than 15-folds in transgenic lines, while less induced in WT plants. Upon exposure to PEG, *DREB2A* was substantially elevated in the two overexpression lines, up to 25-fold, while less than 20-fold in WT plants. The differential expression of these stress-regulated genes also accounted for the stress tolerance of *76C2*OE plants.

DISCUSSION

Rice is the most consumed staple food in the world that feeds more than half of the world's population. However, with the

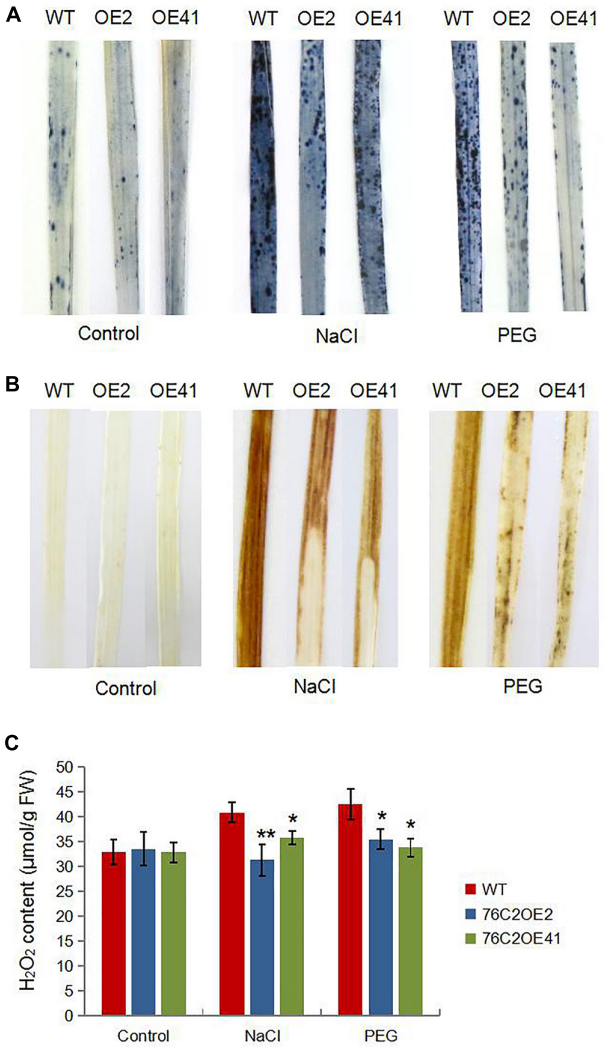


FIGURE 7 | ROS production in the transgenic plants in response to salt and drought conditions. Four-week-old WT and *AtUGT76C2* overexpression rice plants were subjected to 200 mM NaCl and 15% PEG8000 treatments for 12 h, respectively. Then rice leaves were subjected to NBT **(A)** and DAB staining **(B)**, and H_2O_2 contents were quantitatively measured **(C)**. For each staining and H_2O_2 determination, leaves were harvested from at least five independent plants, and the pictures were representative of most samples. Asterisks indicate significant differences between wild type and the overexpression lines evaluated with Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

deterioration of the current global environment and uneven precipitation, the area of soil drying is gradually increasing. As a semi-aquatic crop, rice is greatly threatened by drought and salt stresses. In order to cultivate rice varieties with good traits, it is important to explore candidate genes that can be applied into rice breeding. To cope with adverse environmental stimuli, scientists are devoted to identifying stress-responsive genes and generate stress-adaptive crops by molecular breeding or gene engineering. Adverse environmental factors affect many aspects of plants but will finally lead to disturbance of the metabolic process that closely associated with plant growth and development. UDP-glycosyltransferases, as the manager of cellular homeostasis in plants (Lim and Bowles, 2004; Bowles et al., 2005), likely provide new strategies in cultivating stress-resistant crops.

In this study, we introduced the Arabidopsis UGT76C2 into rice, which affects cytokinin homeostasis (**Figure 1**) and enhances stress tolerance in the transgenic rice (**Figure 4**). We demonstrate that UGT76C2 contributes to stress tolerance in several aspects. First, overexpression of *UGT76C2* decreased the level of cytokinins (**Figure 1D**), which is generally regarded as a negative factor in plant abiotic responses (Nishiyama et al., 2011). *UGT76C2* overexpression also enhanced root growth in the transgenic lines (**Figure 5**). This finding is consistent with several former studies indicating the role of cytokinin in modulating root development (Zalabák et al., 2013). For instance, overexpression of *CKX* in barley led to cytokinin deficiency, which enlarged the root system and increased root biomass (Zalewski et al., 2010). Pineda Rodó et al. (2008) found that constitutive expression of a zeatin-O-glycosyltransferase in maize resulted in more branched roots with greater biomass. A former study in our group also reported that overexpression of *UGT76C2* enhanced the lateral root density in Arabidopsis (Wang et al., 2011). A strong root system is a critical trait in adapting to water deficit. A recent study found that the ZmPTF1 transcription factor enhanced drought tolerance by promoting root growth (Li et al., 2019). Additionally, according to Li et al.'s (2018), overexpression of *RCc3* in rice increased the root system and enhanced salt tolerance. Thus, it is believed that enhanced root growth in *UGT76C2* overexpression lines greatly contribute to plant tolerance to drought and salt.

It is well known that abiotic stresses cause an excess production of reactive oxygen species. Cellular ROS are usually maintained at a relatively low level through a wide range of scavenging and detoxification mechanisms. More and more studies have identified the connection between cytokinin and oxidative stress. Wang et al. (2015) showed that the overproduction of cytokinin by overexpressing *AtIPT8* increased ROS production and decreased the CAT and POD activity in response to salt stress, which thus declined salt stress resistance. Additionally, Zwack and Rashotte (2015) showed that cytokinin response factor 6 functions in mediating oxidative stress response *via* attenuating cytokinin signaling. In our study, we found that overexpression of *AtUGT76C2* in rice, which decreased cytokinin level, resulted in less ROS accumulation under drought and salt stresses. Furthermore, the transcripts of ROS-scavenging enzymes such as CAT, SOD, and POD were upregulated, and their enzyme activities were also enhanced upon UGT76C2 overexpression (**Figure 8**). These findings further suggest that cytokinin plays a negative role for the plant to cope with oxidative stress, which is in agreement with former studies (Wang et al., 2015; Zwack and Rashotte, 2015).

We also found that the ectopic expression of *UGT76C2* impeded seed germination and postgermination growth under drought and salt stress (**Figures 2**, **3**) while improving stress tolerance in transgenic rice seedlings (**Figure 4**), suggesting that the role of *AtUGT76C2* is likely related to ABA signaling. ABA plays pivotal roles in response to abiotic stresses. When the plants are challenged by water deficit, ABA is soon synthesized

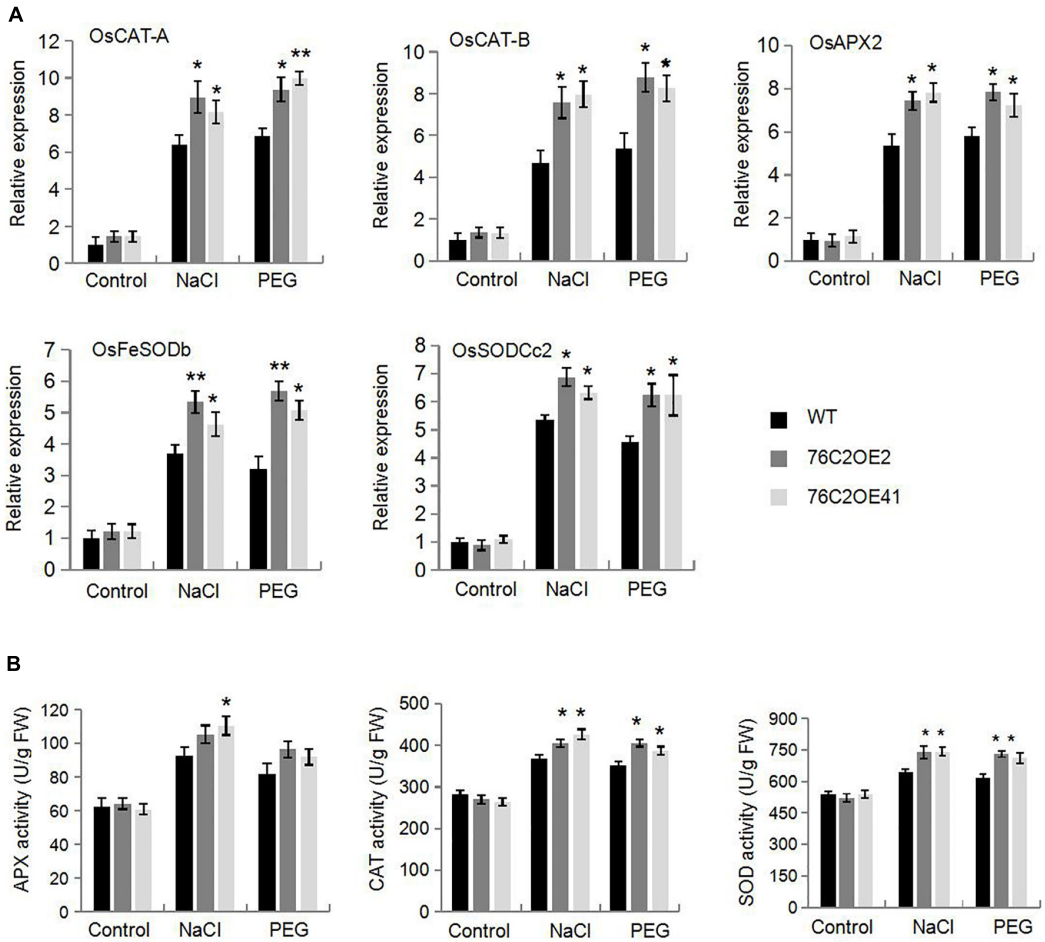


FIGURE 8 | Overexpression of *AtUGT76C2* enhanced ROS scavenging in the transgenic plants. Four-week-old WT and *AtUGT76C2* overexpression rice plants were subjected to 200 mM NaCl and 250 mM PEG treatments for 12 h, respectively. Then the transcript levels **(A)** and activities of ROS scavenging enzymes **(B)** were determined. These experiments were done at three independent biological replicates. Asterisks indicate significant differences between the wild type and the overexpression lines evaluated with Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

and triggers the expression of many ABA-dependent genes in coping with stresses. During the germination and the postgermination stages, ABA helps to arrest the germination and the postgermination growth, which is actually a protective mechanism to young seedlings (Lopez-Molina et al., 2002). After the plants grow up into adult plants, ABA on one side contributes to induce stomatal closure in response to drought stress and on the other side activates the expression of some stress-responsive genes to cope with adverse conditions. Consistently, in our study, more closed stomata were observed in *UGT76C2*-overexpressed rice than in WT plants when confronting drought stress (**Figure 6B**). Although the ABA level was not affected in *UGT76C2*OE plants in response to dehydration (**Figure 6C**), it is speculated that ABA signaling is enhanced. This point could also be approved by the upregulation of ABA signaling or responsive genes in *UGT76C2*OE plants. For instance, OsABF2 and OsRAB16 are positive regulators in ABA signaling pathway (Lu et al., 2009; Hossain et al., 2010), and both genes were more upregulated upon *UGT76C2* overexpression in response to drought and salt. *OsPIP2.1* is strongly induced by ABA, and *PIP2.1* activation was required for stomatal closure in response to ABA treatment in *Arabidopsis* (Rodrigues et al., 2017; Ding et al., 2019). *OsCOIN* is induced by ABA as well as cold, salt, and drought stresses. The overexpression of *OsCOIN* could increase tolerance to chilling, salt, and drought and enhance proline level in rice (Liu et al., 2007). The expression of the proline biosynthesis genes *OsP5CS1* and *OsP5CR*, which are responsive to osmotic and salt stresses and ABA (Hu et al., 1992; Sripinyowanich et al., 2013), was also upregulated in *UGT76C2* overexpressors under salt and drought (**Figure 9**).

UGT76C2 recognizes all cytokinins and modifies the hormones at the N7 and N9 positions (Hou et al., 2004). The cytokinin glucosides are generally regarded as biologically inactive CK forms in CK signaling (Hothorn et al., 2011; Lomin et al., 2015). Interestingly, there is evidence indicating that the N-linked (N6 and/or N9) cytokinin glycosides show antisenescent and antioxidant activity (Hönig et al., 2018). The effects might be explained by the presence of electronegative

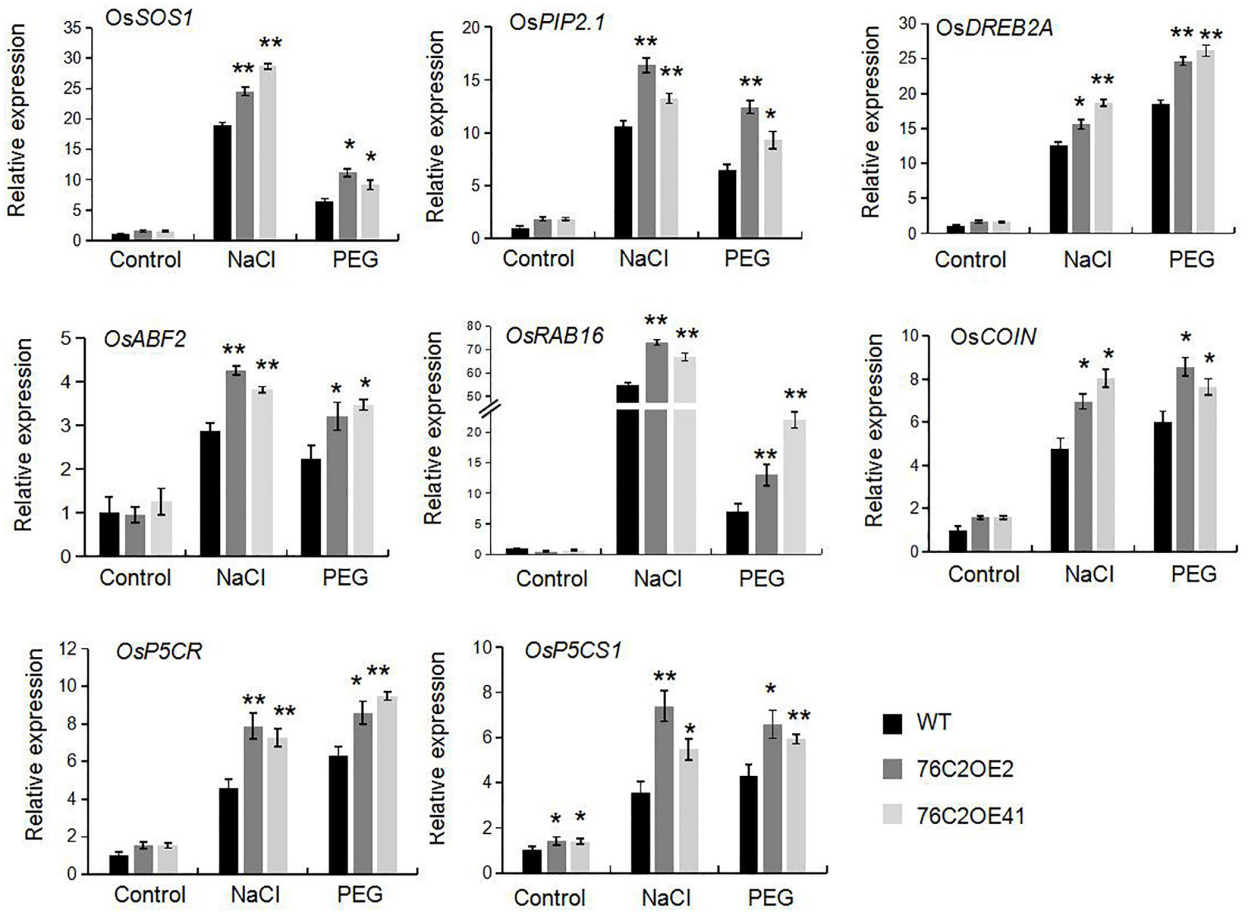


FIGURE 9 | Expression of abiotic stress related genes in wild type, *UGT76C2* overexpression plants in response to stresses. The experiments included three biological replicates, each with three technical replicates. Asterisks indicate significant differences between the wild type and the overexpression lines evaluated with Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

atoms, which are near the N6 and/or N9 atoms of purine (Hönig et al., 2018). A recent study also reported that some types of N-linked CK glucosides, such as transzeatin (tZ) N7- and N9-glucosides, efficiently release free CK bases that are probably responsible for the biological activities (Hoyerová and Hošek, 2020). Additionally, in a latest study, Hallmark et al. (2020) reported that the exogenous application of trans-Zeatin-N-glucosides could lead to CK response. All these findings updated our understanding in the role of cytokinin glycosides. It can be inferred that the heterologous expression of *Arabidopsis UGT76C2* into rice might also cause some other effects in the biochemical and molecular levels that are beyond our study here. Furthermore, cytokinin signaling under abiotic stress is acting as an inter-cellular communication network, which is essential to crosstalk with other types of phytohormones except ABA. It was demonstrated that cytokinin and auxin play complementary actions in regulating a series of plant developmental processes, which also work together in plant response to stresses (Bielach et al., 2017). A link also exists between cytokinin and SA signaling pathway *via* ARR2 and TGA3 in stress response (Verma et al., 2016). From these points of view, cytokinin-regulated stress response is far more complicated than we have revealed here. The detailed mechanisms remain elusive and require further investigation for more insights into cytokinin involvement in plant defense systems. Anyway, however, we reveal that the overexpression of *UGT76C2* in rice brings good traits in coping with stresses, which lays a theoretical foundation that *UGT76C2* could serve as a promising candidate gene for cultivating saline- and drought-tolerant crops.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YL, FL, and PL carried out the experiments. YL, TW, and CZ analyzed the data. YL and BH conceived and designed the research. YL and FL wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was supported by the Key R&D Program Projects in Shandong Province (No. 2018GNC110019) and the National Natural Science Foundation of China (No. 31970290).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.560696/full#supplementary-material

REFERENCES

Bielach, A., Hrtyan, M., and Tognetti, V. B. (2017). Plants under stress: involvement of auxin and cytokinin. *Int. J. Mol. Sci.* 18:1427. doi: 10.3390/ijms18071427

Bowles, D., Isayenkova, J., Lim, E. K., and Poppenberger, B. (2005). Glycosyltransferases: managers of small molecules. *Curr. Opin. Plant Biol.* 8, 254–263. doi: 10.1016/j.pbi.2005.03.007

Bowles, D., Lim, E.-K., Poppenberger, B., and Vaistij, F. E. (2006). Glycosyltransferases of lipophilic small molecules. *Annu. Rev. Plant Biol.* 57, 567–597. doi: 10.1146/annurev.arplant.57.032905.105429

Cai, W., Liu, W., Wang, W. S., Fu, Z. W., Han, T. T., and Lu, Y. T. (2015). Overexpression of rat neurons nitric oxide synthase in rice enhances drought and salt tolerance. *PLoS One* 10:e0131599. doi: 10.1371/journal.pone.0131599

Chen, M., Zhao, Y., Zhuo, C., Lu, S., and Guo, Z. (2015). Overexpression of a NF-YC transcription factor from bermudagrass confers tolerance to drought and salinity in transgenic rice. *Plant Biotechnol. J.* 13, 482–491. doi: 10.1111/pbi.12270

Cortleven, A., Leuendorf, J. E., Frank, M., Pezzetta, D., Bolt, S., and Schmülling, T. (2019). Cytokinin action in response to abiotic and biotic stresses in plants. *Plant Cell Environ.* 42, 998–1018. doi: 10.1111/pce.13494

Ding, L., Uehlein, N., Kaldenhoff, R., Guo, S., Zhu, Y., and Kai, L. (2019). Aquaporin PIP2;1 affects water transport and root growth in rice (Oryza sativa L.). *Plant Physiol. Biochem.* 139, 152–160. doi: 10.1016/j.plaphy.2019.03.017

Du, Y. Y., Wang, P. C., Chen, J., and Song, C. P. (2008). Comprehensive functional analysis of the catalase gene family in *Arabidopsis* thaliana. *Plant Physiol.* 050, 1318–1326. doi: 10.1111/j.1744-7909.2008.00741.x

El-Showk, S., Ruonala, R., and Helariutta, Y. (2013). Crossing paths: cytokinin signalling and crosstalk. *Development* 140, 1373–1383. doi: 10.1242/dev.086371

Hai, N. N., Chuong, N. N., Tu, N. H. C., Kisiala, A., Hoang, X. L. T., and Thao, N. P. (2020). Role and regulation of cytokinins in plant response to drought stress. *Plants* 9:422. doi: 10.3390/plants9040422

Hallmark, H. T., Černý, M., Brzobohatý, B., and Rashotte, A. M. (2020). trans-Zeatin-N-glucosides have biological activity in *Arabidopsis thaliana*. *PLoS One* 15:e0232762. doi: 10.1371/journal.pone.0232762

Hansen, H., and Dörffling, K. (2003). Root-derived trans-zeatin riboside and abscisic acid in drought-stressed and rewatered sunflower plants: interaction in the control of leaf diffusive resistance? *Funct. Plant Biol.* 30, 365–375. doi: 10.1071/FP02223

Hönig, M., Plíhalová, L., Husičková, A., Nisler, J., and Doležal, K. (2018). Role of cytokinins in senescence, antioxidant defence and photosynthesis. *Int. J. Mol Sci.* 19:4045. doi: 10.3390/ijms19124045

Hossain, M. A., Cho, J. I., Han, M., Ahn, C. H., Jeon, J. S., An, G., et al. (2010). The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. *J. Plant Physiol.* 167, 1512–1520. doi: 10.1016/j.jplph.2010.05.008

Hothorn, M., Dabi, T., and Chory, J. (2011). Structural basis for cytokinin recognition by *Arabidopsis thaliana* histidine kinase 4. *Nat. Chem. Biol.* 7, 766–768. doi: 10.1038/nchembio.667

Hou, B., Lim, E. K., Higgins, G. S., and Bowles, D. J. (2004). N-glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. *J. Biol. Chem.* 279, 47822–47832. doi: 10.1074/jbc.M409569200

Hoyerová, K., and Hošek, P. (2020). New insights into the metabolism and role of cytokinin N-glucosides in plants. *Front. Plant Sci.* 11:741. doi: 10.3389/fpls.2020.00741

Hu, C. A., Delauney, A. J., and Verma, D. P. (1992). A bifunctional enzyme (delta 1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. *Proc. Natl. Acad. Sci. U.S.A.* 89, 9354–9358. doi: 10.1073/pnas.89.19.9354

Huang, X., Hou, L., Meng, J., You, H., Li, Z., Gong, Z., et al. (2018). The antagonistic action of abscisic acid and cytokinin signaling mediates drought stress response in *Arabidopsis*. *Mol .Plant.* 11, 970–982. doi: 10.1016/j.molp.2018.05.001

Jeon, J., Kim, N. Y., Kim, S., Kang, N. Y., Novák, O., Ku, S. J., et al. (2010). A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in *Arabidopsis*. *J. Biol. Chem.* 285, 23371–23386. doi: 10.1074/jbc.M109.096644

Kang, J., Li, J., Gao, S., Tian, C., and Zha, X. (2017). Overexpression of the leucine-rich receptor-like kinase gene LRK2 increases drought tolerance and tiller number in rice. *Plant Biotechnol. J.* 15, 1175–1185. doi: 10.1111/pbi.12707

Kang, N. Y., Cho, C., Kim, N. Y., and Kim, J. (2012). Cytokinin receptor-dependent and receptor-independent pathways in the dehydration response of *Arabidopsis thaliana*. *J. Plant Physiol.* 169, 1382–1391. doi: 10.1016/j.jplph.2012.05.007

Kuppu, S., Mishra, N., Hu, R., Sun, L., Zhu, X., Shen, G., et al. (2013). Water-deficit inducible expression of a cytokinin biosynthetic gene IPT improves drought tolerance in cotton. *PLoS One* 8:e64190. doi: 10.1371/journal.pone.0064190

Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008). Glycosyltransferases: structures, functions, and mechanisms. *Annu. Rev. Biochem.* 77, 521–555. doi: 10.1146/annurev.biochem.76.061005.092322

Leta, T. B., Miccah, S. S., Steven, M. R., Wondyifraw, T., and Francis, W. (2016). Drought tolerant tropical maize (Zea mays L.) developed through genetic transformation with isopentenyltransferase gene. *Afr. J. Biotechnol.* 15, 2447–2464. doi: 10.5897/AJB2016.15228

Li, W., Li, M., Zhang, W., Welti, R., and Wang, X. (2004). The plasma membrane–bound phospholipase Dδ enhances freezing tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol.* 22, 427–433. doi: 10.1038/nbt949

Li, X., Chen, R., Chu, Y., Huang, J., Jin, L., Wang, G., et al. (2018). Overexpression of RCc3 improves root system architecture and enhances salt tolerance in rice. *Plant Physiol. Biochem.* 130, 566–576. doi: 10.1016/j.plaphy.2018.08.008

Li, X., Michlmayr, H., Schweiger, W., Malachova, A., Shin, S., Huang, Y., et al. (2017). A barley UDP-glucosyltransferase inactivates nivalenol and provides fusarium head blight resistance in transgenic wheat. *J. Exp. Bot.* 68, 2187–2197. doi: 10.1093/jxb/erx109

Li, Y. J., Wang, B., Dong, R. R., and Hou, B. K. (2015). AtUGT76C2, an *Arabidopsis* cytokinin glycosyltransferase is involved in drought stress adaptation. *Plant Sci.* 236, 157–167. doi: 10.1016/j.plantsci.2015.04.002

Li, Z., Liu, C., Zhang, Y., Wang, B., Ran, Q., and Zhang, J. (2019). The bHLH family member ZmPTF1 regulates drought tolerance in maize by promoting root development and abscisic acid synthesis. *J. Exp. Bot.* 70, 5471–5486. doi: 10.1093/jxb/erz307

Lim, E. K., and Bowles, D. J. (2004). A class of plant glycosyltransferases involved in cellular homeostasis. *EMBO J.* 23, 2915–2922. doi: 10.1038/sj.emboj.7600295

Liu, K., Wang, L., Xu, Y., Chen, N., Ma, Q., and Chong, L. K. (2007). Overexpression of OsCOIN, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. *Planta* 226, 1007–1016. doi: 10.2307/23389762

Lomin, S. N., Krivosheev, D. M., Steklov, M. Y., Arkhipov, D. V., Osolodkin, D. I., Schmülling, T., et al. (2015). Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. *J. Exp. Bot.* 66, 1851–1863. doi: 10.1093/jxb/eru522

Lopez-Molina, L., Mongrand, S. B., Mclachlin, D. T., Chait, B. T., and Chua, N. H. (2002). ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *Plant J.* 32, 317–328. doi: 10.1046/j.1365-313X.2002.01430.x

Lu, G., Gao, C., Zheng, X., and Han, B. (2009). Identification of OsbZIP72 as a positive regulator of ABA response and drought tolerance in rice. *Planta* 229, 605–615. doi: 10.1007/s00425-008-0857-3

Macková, H., Hronková, M., Dobrá, J., Turečková, V., Novák, O., Lubovská, Z., et al. (2013). Enhanced drought and heat stress tolerance of tobacco plants with

ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. *J. Exp. Bot.* 64, 2805–2815. doi: 10.1093/jxb/ert131

Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., Van Breusegem, F., and Noctor, G. (2010). Catalase function in plants: a focus on *Arabidopsis* mutants as stress-mimic models. *J. Exp. Bot.* 61, 4197–4220. doi: 10.1093/jxb/erq282

Nakazawa, K., Tanaka, H., and Arima, M. (1982). Rapid, simultaneous and sensitive determination of free hydroxyproline and proline in human serum by high-performance liquid chromatography. *J. Chromatogr. Biomed. Appl.* 233, 313–316. doi: 10.1016/S0378-4347(00)81759-4

Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D. T., Kojima, M., Werner, T., et al. (2011). Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. *Plant Cell.* 23, 2169–2183. doi: 10.1105/tpc.111.087395

Pineda Rodó, A., Brugière, N., Vankova, R., Malbeck, J., Olson, J. M., Haines, S. C., et al. (2008). Over-expression of a zeatin O-glucosylation gene in maize leads to growth retardation and tasselseed formation. *J. Exp. Bot.* 59, 2673–2686. doi: 10.1093/jxb/ern137

Raines, T., Blakley, I. C., Tsai, Y. C., Worthen, J. M., Franco-Zorrilla, J. M., Solano, R., et al. (2016). Characterization of the cytokinin-responsive transcriptome in rice. *BMC Plant Biol.* 16:260. doi: 10.1186/s12870-016-0932-z

Rodrigues, O., Reshetnyak, G., Grondin, A., Saijo, Y., Leonhardt, N., Maurel, C., et al. (2017). Aquaporins facilitate hydrogen peroxide entry into guard cells to mediate ABA- and pathogen-triggered stomatal closure. *Proc. Natl. Acad. Sci. U.S.A.* 114, 9200–9205. doi: 10.1073/pnas.1704754114

Shi, H., Ishitani, M., Kim, C., and Zhu, J. K. (2000). The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6896–6901. doi: 10.1073/pnas.120170197

Sripinyowanich, S., Klomsakul, P., Boonburapong, B., Bangyeekhun, T., Asami, T., Gu, H., et al. (2013). Exogenous ABA induces salt tolerance in indica rice (Oryza sativa L.): the role of OsP5CS1 and OsP5CR gene expression during salt stress. *Environ. Exp. Bot.* 86, 94–105. doi: 10.1016/j.envexpbot.2010.01.009

Sun, Y. G., Wang, B., Jin, S. H., Qu, X. X., Li, Y. J., and Hou, B. K. (2013). Ectopic expression of *Arabidopsis* glycosyltransferase UGT85A5 enhances salt stress tolerance in tobacco. *PLoS One* 8:e59924. doi: 10.1371/journal.pone.0059924

Tang, Y., Liu, K., Ju, Z., Li, X., Xu, K., Yi, Z., et al. (2017). JcDREB2, a physic Nut AP2/ERF gene, alters plant growth and salinity stress responses in transgenic rice. *Front. Plant Sci.* 8:306. doi: 10.3389/fpls.2017.00306

Tran, L. S., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., et al. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20623–20628. doi: 10.1073/pnas.0706547105

Verma, D., Lakhanpal, N., and Singh, K. (2019). Genome-wide identification and characterization of abiotic-stress responsive SOD (superoxide dismutase) gene family in *Brassica juncea* and *B. rapa*. *BMC Genomics*. 20:227. doi: 10.1186/s12864-019-5593-5

Verma, V., Ravindran, P., and Kumar, P. P. (2016). Plant hormone-mediated regulation of stress responses. *BMC Plant Biol.* 16:86. doi: 10.1186/s12870-016-0771-y

Wang, J., Ma, X. M., Kojima, M., Sakakibara, H., and Hou, B. K. (2011). N-Glucosyltransferase UGT76C2 is Involved in cytokinin homeostasis and cytokinin response in *Arabidopsis thaliana*. *Plant Cell Physiol.* 52, 2200–2213. doi: 10.1093/pcp/pcr152

Wang, Y., Shen, W., Chan, Z., and Wu, Y. (2015). Endogenous cytokinin overproduction modulates ROS homeostasis and decreases salt stress resistance in *Arabidopsis thaliana*. *Front. Plant Sci.* 6:1004. doi: 10.3389/fpls.2015.01004

Wohlgemuth, H., Mittelstrass, K., Kschieschan, S., Bender, J., and Sandermann, H. J. (2002). Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. *Plant Cell Environ.* 25, 717–726. doi: 10.1046/j.1365-3040.2002.00859.x

Xiao, X. O., Zeng, Y. M., Cao, B. H., Lei, J. J., and Cheng, Y. J. (2017). PSAG12-IPT overexpression in eggplant delays leaf senescence and induces abiotic stress tolerance. *J. Hortic. Sci. Biotech.* 92, 1–9. doi: 10.1080/14620316.2017.1287529

Xiong, H., Li, J., Liu, P., Duan, J., Zhao, Y., Guo, X., et al. (2014). Overexpression of OsMYB48-1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. *PLoS One* 9:e92913. doi: 10.1371/journal.pone.0092913

Xu, Y., Burgess, P., Zhang, X., and Huang, B. (2016). Enhancing cytokinin synthesis by overexpressing ipt alleviated drought inhibition of root growth through activating ROS-scavenging systems in *Agrostis stolonifera*. *J. Exp. Bot.* 67, 1979–1992. doi: 10.1093/jxb/erw019

Zalabák, D., Pospíšilová, H., Šmehilová, M., Mrízová, K., Frébort, I., and Galuszka, P. (2013). Genetic engineering of cytokinin metabolism: prospective way to improve agricultural traits of crop plants. *Biotechnol. Adv.* 31, 97–117. doi: 10.1016/j.biotechadv.2011.12.003

Zalewski, W., Galuszka, P., Gasparis, S., Orczyk, W., and Nadolska-Orczyk, A. (2010). Silencing of the HvCKX1 gene decreases the cytokinin oxidase/dehydrogenase level in barley and leads to higher plant productivity. *J. Exp. Bot.* 61, 1839–1851. doi: 10.1093/jxb/erq052

Zhang, J., Schurr, U., and Davies, W. J. (1987). Control of stomatal behaviour by abscisic acid which apparently originates in the roots. *J. Exp.Bot.* 38, 1174–1181. doi: 10.1093/jxb/38.7.1174

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53, 247–273. doi: 10.1146/annurev.arplant.53.091401.143329

Zwack, P. J., and Rashotte, A. M. (2015). Interactions between cytokinin signalling and abiotic stress responses. *J. Exp. Bot.* 66, 4863–4871. doi: 10.1093/jxb/erv172

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 November 2020
doi: 10.3389/fpls.2020.577676

Functional Analysis of the Rice Type-B Response Regulator RR22

Maria V. Yamburenko[1†], Jennifer M. Worthen[1†], Asyia Zeenat[1,2], Beenish J. Azhar[1,2], Swadhin Swain[1], Adam R. Couitt[1], Samina N. Shakeel[1,2], Joseph J. Kieber[3] and G. Eric Schaller[1]*

[1]Department of Biological Sciences, Dartmouth College, Hanover, NH, United States, [2]Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan, [3]Department of Biology, University of North Carolina, Chapel Hill, NC, United States

The phytohormone cytokinin plays a critical role in regulating growth and development throughout the life cycle of the plant. The primary transcriptional response to cytokinin is mediated by the action of the type-B response regulators (RRs), with much of our understanding for their functional roles being derived from studies in the dicot Arabidopsis. To examine the roles played by type-B RRs in a monocot, we employed gain-of-function and loss-of-function mutations to characterize *RR22* function in rice. Ectopic overexpression of *RR22* in rice results in an enhanced cytokinin response based on molecular and physiological assays. Phenotypes associated with enhanced activity of *RR22* include effects on leaf and root growth, inflorescence architecture, and trichome formation. Analysis of four *Tos17* insertion alleles of *RR22* revealed effects on inflorescence architecture, trichomes, and development of the stigma brush involved in pollen capture. Both loss- and gain-of-function *RR22* alleles affected the number of leaf silica-cell files, which provide mechanical stability and improve resistance to pathogens. Taken together, these results indicate that a delicate balance of cytokinin transcriptional activity is necessary for optimal growth and development in rice.

Keywords: type-B response regulator, cytokinin, rice, panicle, grain yield, trichome, root architecture, silica cell

OPEN ACCESS

Edited by:
Patrick H. Masson, University of Wisconsin-Madison, United States

Reviewed by:
Jan Hejatko, Central European Institute of Technology (CEITEC), Czechia
Jan Smalle, University of Kentucky, United States
Edgar P. Spalding, University of Wisconsin-Madison, United States

***Correspondence:**
G. Eric Schaller
george.e.schaller@dartmouth.edu

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *29 June 2020*
Accepted: *14 October 2020*
Published: *10 November 2020*

Citation:
Yamburenko MV, Worthen JM, Zeenat A, Azhar BJ, Swain S, Couitt AR, Shakeel SN, Kieber JJ and Schaller GE (2020) Functional Analysis of the Rice Type-B Response Regulator RR22. Front. Plant Sci. 11:577676. doi: 10.3389/fpls.2020.577676

INTRODUCTION

The phytohormone cytokinin plays a critical role in regulating growth and development throughout the life cycle of the plant, including the regulation of cell proliferation, shoot and root architecture, seed yield, senescence, and stress responses (Sakakibara, 2006; Hwang et al., 2012; Kieber and Schaller, 2014; Jameson and Song, 2016). Much of our understanding of the mechanisms that underlie metabolism and perception of cytokinin come from studies of the model dicot Arabidopsis (Hwang et al., 2012; Kieber and Schaller, 2014). More recently, such studies have been extended to monocots due to their agronomic importance, with rice being a preferred monocot species for such studies in part because of its small genome and ease of transformation (Devos and Gale, 2000). Of particular interest has been the discovery that cytokinin plays a key role in regulating the development and architecture of the rice inflorescence, alterations in cytokinin levels accounting for differences in the yield for several rice varieties (Ashikari et al., 2005; Kurakawa et al., 2007; Gu et al., 2015; Yeh et al., 2015).

The cytokinin signal is transduced through a multi-step phosphorelay that incorporates cytokinin receptors with histidine-kinase (HK) activity, histidine-containing phosphotransfer

proteins (AHPs), and type-B response regulators (RRs; Ito and Kurata, 2006; Pareek et al., 2006; Du et al., 2007; Schaller et al., 2007; Werner and Schmülling, 2009; Tsai et al., 2012; Kieber and Schaller, 2014). Activation of the receptors initiates the transfer of phosphate from one signaling element to the next, thereby relaying the cytokinin signal from membrane to nucleus. Within the nucleus, the type-B RRs function as transcription factors to regulate the expression of cytokinin primary-response genes. Among the primary-response genes are a second family of RRs, the type-A RRs, which function as negative feedback regulators to desensitize the plant to cytokinin (To et al., 2004; Kieber and Schaller, 2014). The KMD family of F-box proteins targets the type-B RRs for degradation (Kim et al., 2013a,b). In rice, as in most plants, these signaling elements are encoded by multi-gene families, there being 13-type-B *RR*s and 13 type-A *RR*s in the rice genome (Pareek et al., 2006; Du et al., 2007; Schaller et al., 2007; Tsai et al., 2012).

According to this model for the cytokinin signal transduction pathway, the type-B RRs play a key role in regulating the initial transcriptional response to cytokinin. The type-B RRs contain two conserved signaling motifs: a receiver domain that is phosphorylated on a conserved aspartate residue to regulate their activity, and a long C-terminal extension with a Myb-like DNA-binding domain (Imamura et al., 1999; Hosoda et al., 2002). Multiple lines of evidence support the role of type-B RRs as transcription factors (Sakai et al., 2000, 2001; Hwang and Sheen, 2001; Imamura et al., 2001, 2003; Lohrmann et al., 2001; Hosoda et al., 2002; Mason et al., 2004, 2005; Rashotte et al., 2006; Liang et al., 2012; Tsai et al., 2012), and our recent analyses using protein-binding microarrays reveal similar DNA-binding motifs for the Myb-like domains of the type-B RRs from rice and Arabidopsis (Raines et al., 2016; Zubo et al., 2017). The type-B RRs fall into five subfamilies based on phylogenetic analysis of rice and Arabidopsis (Tsai et al., 2012), with subfamily-1 being the only subfamily to contain type-B RR members from both rice and Arabidopsis. Genetic analysis in Arabidopsis indicates that subfamily-1 plays the most prominent role in cytokinin signaling (Mason et al., 2005; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008).

We have employed several approaches to functionally characterize the role of the rice type-B *RR*s. Initially, we determined that the rice *RR22* of subfamily-1 can complement an Arabidopsis type-B *RR* mutant (*arr1/12*), consistent with rice subfamily-1 also mediating cytokinin responses (Tsai et al., 2012). More recently, we employed a CRISPR/Cas9 gene editing approach to target the four most abundant type-B *RR*s of rice subfamily-1: *RR21*, *RR22*, *RR23*, and *RR24*. Results from this analysis revealed functional overlap as well as subfunctionalization within the type-B *RR* gene family, the *rr21/22/23* triple mutant exhibiting decreased cytokinin sensitivity and a variety of defects in growth and development, including effects on shoot and root growth, panicle architecture, flower development, and trichome formation (Worthen et al., 2019). Here, we employ a gain-of-function approach to characterize the effects of ectopic overexpression of *RR22* in rice. To complement this gain-of-function approach, we also examined loss-of-function *Tos17* insertion alleles of *RR22*. Results from these analyses indicate that a delicate balance of cytokinin transcriptional activity is necessary for optimal growth and development in rice.

MATERIALS AND METHODS

Plant Materials

For overexpression of *RR22*, a GFP-tagged version was driven by the *ZmUbi* promoter (Mann et al., 2012) in the pCAMBIA1300 vector (GenBank Accession Number AF234296). For this purpose, *sGFP* along with the *NOS* terminator were amplified by PCR from the plasmid pGWB5 (Nakagawa et al., 2007), and this sGFP-tNOS fragment inserted into the Pst I and Hind III sites of pCambia1300 by an In-Fusion reaction according to the manufacturer (Takara Bio United States, Inc.), creating the vector pCambia1300-MCS-GFP. The ZmUbi1 promoter was amplified from the pANIC6A vector (Mann et al., 2012), and a genomic version of *RR22* amplified from rice genomic DNA, and both fragments cloned into the Xma I and Pst I sites of the pCambia1300-MCS-GFP vector by an In-Fusion reaction. Primers used for cloning are listed in **Supplementary Table 1**. The resulting pZmUbi1:RR22-GFP:tNOS vector was confirmed by restriction digest and sequencing. Transformation of *Oryza sativa ssp. japonica* cv. Kitaake rice with pZmUbi1:RR22-GFP:tNOS vector in the EHA105 strain of *Agrobacterium* was performed by the Plant Transformation Facility at Iowa State University.[1] Three RR22-OX transgenic lines, characterized by a single T-DNA insertion and high expression of the transgene, were selected for further characterization. Wild-type (WT) siblings isolated from segregating populations for each of the transgenic lines were retained as controls.

For isolation of *RR22* lines containing *Tos17* insertions, seed was obtained from the *Tos17* Rice Insertion Mutant (TRIM) collection at the National Institute of Agrobiological Sciences (NIAS) in Japan[2] (Miyao et al., 2003; Hirochika et al., 2004). Four *Tos17* insertion alleles for *RR22* designated *rr22-1* (NF0017), *rr22-2* (ND3038), *rr22-3* (NF6804), and *rr22-4* (NG4931) were obtained for *Oryza sativa ssp. japonica* cv. Nipponbare. PCR-based screening of DNA isolated from mature leaves by the CTAB extraction method (Clarke, 2009) was used to identify homozygous *rr22* mutant lines and their WT siblings, using the primers listed in **Supplementary Table 1**. The locations for the *Tos17* insertions were confirmed by sequencing (**Supplementary Table 2**). The *rr21/22/23* CRISPR-Cas9 derived line has been previously described (Worthen et al., 2019).

Characterization of Adult Plant Growth Parameters

Plants were grown in a greenhouse at 30°C during the day and 25°C at night, using a 10-h light/14-h dark cycle as described (Worthen et al., 2019). Panicle parameters were

[1]https://www.biotech.iastate.edu/ptf/
[2]https://tos.nias.affrc.go.jp/

quantified as described, using the largest panicle from each plant characterized (Worthen et al., 2019). To visualize stigma brush hairs and trichomes, spikelets and hulls were dissected from plants right before anthesis and images captured using a Leica MZ16 microscope with a Spot Idea camera. To quantify stigma brush hair length for each line, we imaged 10 stigma brushes, with two or three stigmas isolated from each of four plants, and measured the five longest branches on each stigma brush using ImageJ. To quantify length of hull trichomes for each line, we imaged 10 hull tips, with two or three hulls imaged from each of four plants after anthesis, and measured the length of the five longest trichomes on each image.

To characterize leaf epidermal cells, flag leaves from the central tillers of mature plants were chosen, and nail polish impressions made of the abaxial side from the central region of the leaf. Approximately 1.5 cm of the leaf surface from one side of the central vein was brushed to cover with a single layer of a transparent nail polish, left on the living plant for 16 h, and then removed with a scalpel. Impressions were made of two leaves from each of five plants for each line characterized. Impressions were imaged with a Nikon 90i microscope in DIC mode and a 20x objective. Measurements of cell length and width were made with ImageJ software for 50 epidermal long cells (LC1; two different cell files from each of five specimens, with five consecutive cells analyzed per cell file; Luo et al., 2012). Cell file counts for the silica/cork cell files were performed using a Zeiss Axioscope, and all files present in the impressions were counted.

Characterization of Seedling Growth Parameters

For growth of seedlings, seeds were sterilized and germinated as described (Worthen et al., 2019), then transferred to plastic mesh floating on Yoshida medium (Yoshida et al., 1976) supplemented with 50 μg/ml carbenicillin to control bacteria contamination in a dark plastic container. Seedlings were grown for 8 days at 30/28°C with a 12-h light/12-h dark cycle (light = 400 μE). To assess cytokinin sensitivity, the Yoshida medium was supplemented with NaOH as a vehicle control, 10 nM BA, or 100 nM BA.

Gene Expression Analysis

For RNA sample preparation from shoots of RR22-OX lines and their corresponding WT siblings, germinated seeds were grown in sterile Kimura media as described (Worthen et al., 2019) in Solo Sundae cups (TS5R) with lids (DLR100) at 30/28°C with a 12-h light/12-h dark cycle (light = 100 μE) for 7 days. Seedlings were then removed and treated with 1 μM BA or a NaOH vehicle control for 1 h in liquid Kimura media by placing roots into the solution. Sample preparation from the *Tos17 rr22* carpels and seedlings was as described (Worthen et al., 2019). Samples were flash frozen in liquid nitrogen, ground with a tissue homogenizer (Mixer Mill 400, Retsch), and total RNA extracted using the E.Z.N.A Plant RNA Kit (Omega Bio-Tek) as described (Worthen et al., 2019). DNase treatment, first-strand cDNA synthesis, and RT-qPCR with three biological replicates, along with technical replicates, were performed as described (Worthen et al., 2019). Primers used for qRT-PCR are listed in **Supplementary Table 1**.

Immunoblot Analysis

Leaf samples were flash frozen in liquid nitrogen, ground with a tissue homogenizer (Mixer Mill 400, Retsch), and total protein isolated by resuspending 50 mg of leaf powder in 500 μl of SDS-PAGE loading buffer composed of 0.12 M Tris-HCl (pH 6.8), 0.1 M EDTA, 4% (w/v) SDS, 10% (v/v) ß-mercaptoethanol, 5% (v/v) glycerol, and 0.005% (w/v) bromophenol blue. The suspension was incubated at 90°C for 10 min, centrifuged at 14,000 *g* for 2 min to pellet insoluble plant debris, proteins in the supernatant fractionated by SDS-PAGE, then electroblotted to Immobilon-P PVDF membrane (MilliporeSigma) and immundetection performed as described (Gao et al., 2008). Ponceau S staining of the nylon membrane was used to visualize proteins as a loading control (Salinovich and Montelaro, 1986). An anti-GFP antibody (B-2) from Santa Cruz Biotechnology was used as the primary antibody for detection of the RR22-GFP fusion proteins.

Statistical Analysis

ANOVA-based analyses were performed using an online calculator.[3] *T*-tests were performed in Excel.

RESULTS

Generation of *RR22* Gain-of-Function and Loss-of-Function Lines

To functionally characterize the role of a type-B *RR* in rice, we focused on *RR22* based on its relatively high expression level among the rice type-B *RR*s, prior indication that *RR22* plays a role in mediating cytokinin signaling, and the availability of insertion alleles through public resources (Kim et al., 2012; Tsai et al., 2012; Yamburenko et al., 2017; Worthen et al., 2019). Lines for the ectopic overexpression of *RR22* (*RR22-OX* lines) were generated by expressing a genomic copy of the *RR22* coding region from the *Zea mays UBI1* promoter. Sequence encoding a GFP tag was incorporated at the 3'-end of the *RR22* gene to allow for *in planta* visualization and assessment of relative protein levels of the *RR22-OX* transgenes (**Figure 1A**). Out of 15 independent transgenic lines obtained for this construct in *Oryza sativa ssp. japonica* cv. Kitaake, we selected three lines (lines L4, L5, and L9) for further characterization, based on their having a single T-DNA insertion and also exhibiting a high level of protein expression (**Figure 1C**). We note that the *RR22-GFP* transgenes had a tendency to decrease in expression and silence in subsequent generations, and so

[3]http://astatsa.com/OneWay_Anova_with_TukeyHSD/

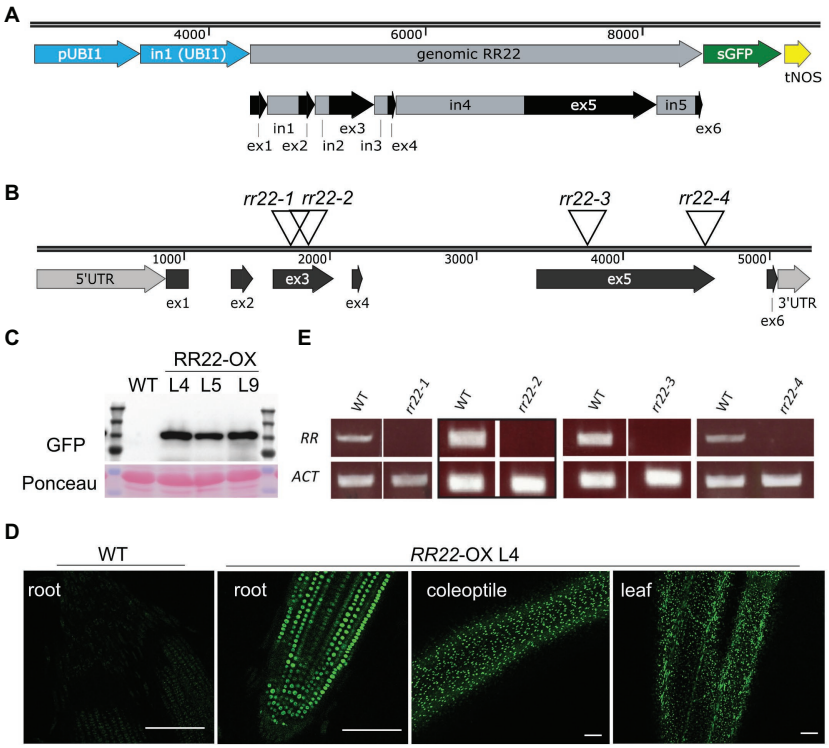


FIGURE 1 | *RR22* gain-of-function and loss-of-function lines. **(A)** Structural design of the *RR22-OX* cassette. A genomic version of *RR22* was expressed from the *Zea mays UBI1* promoter incorporating the first intron of the *UBI1* gene. A sGFP fluorescent tag was encoded at the 3'-end of *RR22*. The intron/exon structure of the *RR22* gene is shown below the cassette diagram. **(B)** Insertional positions for the four *Tos17* alleles of *rr22*. **(C)** Relative protein expression levels of RR22-GFP in three independent *RR22*-OX lines as assessed by immunoblot using an anti-GFP antibody. A non-transgenic wild-type (WT) extract serves as a negative control. Ponceau S staining was used as a protein loading control. **(D)** Distribution of RR22-GFP fluorescent signal in root, dark-grown coleoptile, and leaf of *RR22*-OX L4. The WT root is a negative control. Scale bar = 100 μm. **(E)** Transcript levels of *rr22* and *ACT1* in the *Tos17 rr22* mutant lines and corresponding WT siblings assessed by semiquantitative qPCR.

following their protein levels was important for maintaining ectopic overexpression in these lines. From the segregating populations, we isolated WT siblings for each of the *RR22-OX* lines to serve as controls. Based on fluorescence microscopy, RR22-GFP was expressed in root and in shoot tissues, and the protein localized in nuclei, consistent with what was observed following transient expression in rice protoplasts and with its function as a transcription factor (**Figure 1D**; **Supplementary Figure 1A**; Tsai et al., 2012).

To assist in the functional characterization of *RR22*, we obtained *Tos17* insertional mutant alleles from publicly available sources (Miyao et al., 2003; Hirochika et al., 2004; **Figure 1B**). The *Tos17* lines are in *Oryza sativa ssp. japonica* cv. Nipponbare, prior loss-of-function analyses having been performed through the CRISPR-Cas9 approach in *Oryza sativa ssp. japonica* cv. Kitaake (Worthen et al., 2019). Four independent *Tos17* alleles for *RR22* were brought to homozygosity from the segregating populations, and their WT siblings isolated as controls. The locations for the *Tos17* insertions were confirmed by sequencing (**Figure 1B**; **Supplementary Table 2**). All four alleles disrupt the coding region of *RR22*: the Tos17 insertions for *rr22-1* and *rr22-2* are in exon 3, and for *rr22-3* and *rr22-4* are in exon 5 (**Figure 1B**). None of the four *Tos17 rr22* mutants produced full-length transcripts, consistent with their being loss-of-function alleles (**Figure 1E**).

Ectopic Overexpression of *RR22* Results in Cytokinin Hypersensitivity

The primary role established for type-B RRs is in mediating the transcriptional response to cytokinin (Imamura et al., 1999; Hosoda et al., 2002; Raines et al., 2016; Zubo et al., 2017). Therefore, increased expression of *RR22* is predicted to result in cytokinin hypersensitivity, which can include increased induction of cytokinin-inducible genes. To this end, we examined the expression of *RR9/10* and *CKX5*, which we previously identified as potential cytokinin primary response genes based on their rapid induction by cytokinin in multiple tissues as well as the presence of extended type-B RR binding motifs in their promoters (Raines et al., 2016; Worthen et al., 2019). We compared gene expression in shoots for the *RR22-OX* lines to their WT siblings following a 1-h treatment with 1 μM BA or a vehicle control. These treatment conditions were chosen because they result in only a modest increase in cytokinin-dependent gene expression in the WT siblings, such that the response is not saturated under these conditions (**Figure 2A**). All three *RR22-OX* lines exhibited enhanced induction of

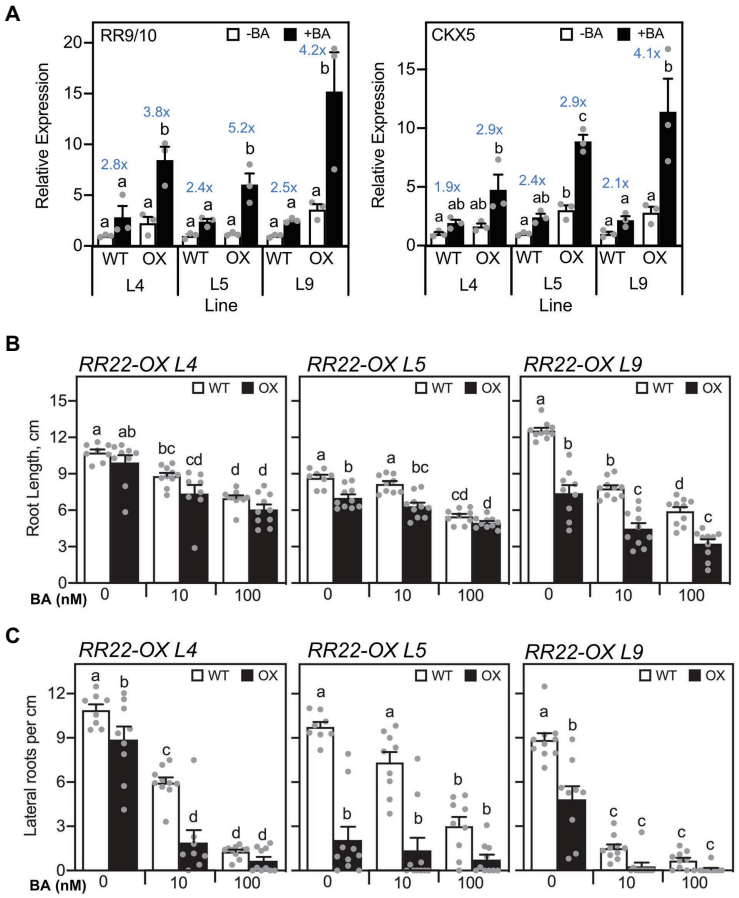


FIGURE 2 | Cytokinin hypersensitive response of *RR22-OX* lines. **(A)** Expression of cytokinin-dependent genes based on qRT-PCR. Seven-day-old seedlings were treated with 1 μM BA or a NaOH vehicle control for 1 h, and gene expression examined in the shoots of the *RR22-OX* lines and their WT siblings ($n = 3$). Expression of the *ACT1* gene was used for normalization. The fold change in gene expression is indicated in blue lettering for each BA-treated to untreated sample. **(B,C)** Root growth response to cytokinin. Seminal root length **(B)** and lateral root density **(C)** were determined for 7-day-old seedlings grown on Yoshida hydroponics medium supplemented with a vehicle control, 10, or 100 nM BA ($n \geq 8$). For statistical analyses, ANOVA with a *post hoc* Holm multiple comparison calculation was performed for each line, involving *RR22-OX* and the WT sibling of the line. Different letters indicate significant differences at $p < 0.05$.

expression of *RR9/10* and *CKX5* in response to cytokinin as compared to their WT siblings, consistent with a heightened transcriptional response to cytokinin.

The enhanced transcriptional response of the *RR22-OX* lines to cytokinin suggests that these lines should also exhibit an enhanced response to cytokinin in growth assays. For this purpose, we examined the root growth response to cytokinin, both for seminal root length as well as for lateral root production, both of which are inhibited to various extents by cytokinin (Rani Debi et al., 2005). As shown in **Figure 2B**, cytokinin significantly inhibits growth of the WT seminal root, when comparing the untreated to 100 nM BA-treated roots. The roots of all three *RR22-OX* lines are shorter than their WT siblings in the absence and presence of BA, lines L5 and L9 significantly so, a result most consistent with an enhanced response to endogenous cytokinin. A more pronounced difference between the *RR22-OX* lines and their WT siblings is observed when examining lateral root production (**Figure 2C**). All three *RR22-OX* lines exhibit significantly reduced production of lateral roots in the absence of added BA, by 18, 78, and 45% for *RR22-OX* lines L4, L5, and L9, respectively, consistent with an enhanced response to endogenous cytokinin. In addition, lines L4 and L9 also exhibit an enhanced responsiveness to exogenous cytokinin, their production of lateral roots being reduced by 68 and 81%, respectively, when compared to their WT siblings when grown in the presence of 10 nM BA. The enhanced transcriptional response to cytokinin of the *RR22-OX* lines, based on gene expression and growth assays, is consistent with the abundance of rice type-B RRs as being rate-limiting for cytokinin signal transduction.

Effects of *RR22* Ectopic Overexpression on Rice Growth and Development

When grown on soil, mature *RR22-OX* lines were dwarfed in comparison to their WT siblings (**Figure 3**). On average, the

plant height for the three *RR22-OX* lines decreased 43% compared to their WT siblings. As noted earlier, the *RR22-GFP* transgene had a tendency toward silencing, and we observed a correlation between the level of RR22-GFP protein and the stature of the *RR22-OX* plants, a higher level of RR22-GFP protein being observed in the plants that exhibited the most substantial dwarfing (**Supplementary Figure 1B**). The *RR22-OX* lines also exhibited an elevated number of tillers (**Figures 3A,B**), and we observed a delay of approximately 2 weeks in flowering time compared to their WT siblings.

All three lines of the *RR22-OX* plants have shorter and narrower leaves compared to their WT siblings (**Figure 3C**), accounting at least in part for the reduced stature of the *RR22-OX* plants. To determine whether the decrease in leaf size of the *RR22-OX* lines was due to changes in cell proliferation and/or cell size, we examined epidermal long cells (LC1) on the abaxial surface of the flag leaf. Both length and width of the cells are reduced in the *RR22-OX* lines compared to their WT siblings (**Figure 3D**). The decrease in cell length (~18%) closely parallels the reduction of flag leaf length (~26%), suggesting that this decrease is primarily due to the decrease in cell length rather than any reduction in cell proliferation. Although a decrease in cell width also occurs in the *RR22-OX* lines (~20%), the decrease is not as proportionately great as that observed in flag leaf width (~38%), suggesting that other factors such as cell proliferation may also play a role in this leaf dimension.

In addition to changes in cell size, we also observed effects of the *RR22-OX* lines on the development of specific cell types. The abaxial side of the rice leaf epidermis has a distinct pattern consisting of several different cell files (Luo et al., 2012). We observed effects on the number of files that contain the

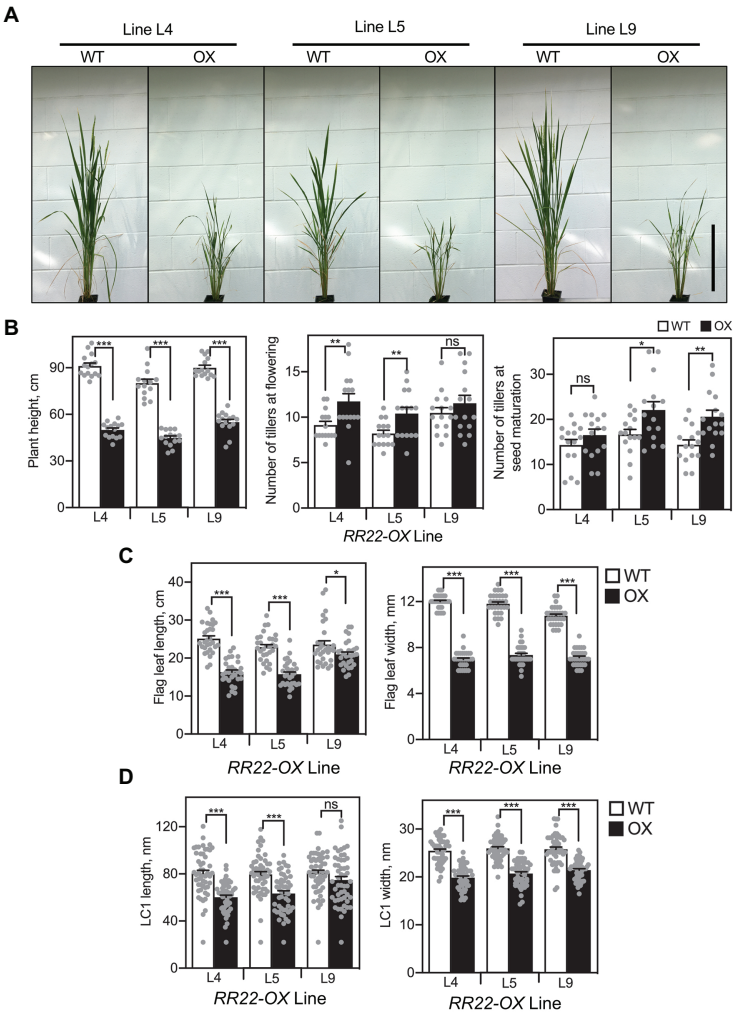


FIGURE 3 | Shoot growth phenotypes of *RR22-OX* lines. **(A)** Shoots of *RR22-OX* lines and their WT siblings, taken at the initiation of flowering in the WT plants. Scale bar = 30 cm. **(B)** Plant height at flowering, number of tillers at flowering and at seed maturation. **(C)** Flag leaf length and width. **(D)** Epidermal long cell 1 (LC1) length and width of the flag leaves. Error bars show SE. The *T*-test was used for statistical comparison of each *RR22-OX* line to its WT sibling (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant).

silica and phellem (cork) cells in the *RR22-OX* lines. In the silica/cork cell files, dumbbell-shaped silica cells alternate with cork cells and provide mechanical stability for rice leaves and improve rice resistance to pathogens (Kaufman et al., 1985). In WT plants, the silica/cork cells are typically arranged in a single file (**Figure 4**). However, we observed much greater variability in the number of adjacent silica/cork cell files in the *RR22-OX* lines: generally, the rows of adjacent files increased (**Figure 4**), although we also observed instances in which the file was no longer present (**Figure 4B**). In addition, we observed effects on the number of stomata files (**Figure 4A**; **Supplementary Figure 2**). In the WT flag leaves, stomata were typically arranged in two adjacent files. However, in the flag leaves of the *RR22-OX* lines, stomata were typically arranged in a single file (**Figure 4A**; **Supplementary Figure 2**), although in some cases, we also observed the sporadic appearance of stomata in a neighboring file.

Overexpression of *RR22* also altered development of the rice inflorescence. Rice produces a panicle-type inflorescence, which is determinate and branched, with a defined architecture derived from the organization of the reproductive meristem (Itoh et al., 2005; Prusinkiewicz et al., 2007). The rice panicle consists of a central axis with several primary and secondary branches, each of which produces spikelets that encapsulate the rice flower. Panicles of the *RR22-OX* lines were smaller and had reduced branching compared to those of their WT siblings (**Figure 5**). In this respect, all three of the *RR22-OX* lines exhibited significant reductions in panicle length, primary branch number, and especially secondary branch number. The effects of the *RR22-OX* lines on panicle architecture resulted in a significant decrease in the number of spikelets per panicle (**Figure 5**). Seed set was also reduced in the *RR22-OX* lines and this, combined with the decreased number of spikelets, severely reduced yield based on the number of grains per panicle (**Figure 5**).

Cytokinin and the rice type-B *RR*s have also been implicated in trichome initiation and elongation (Maes et al., 2008; Maes and Goossens, 2010; Worthen et al., 2019). The *RR22-OX* lines produced shorter trichomes on the grain hulls than their WT siblings (**Supplementary Figure 3**). On the other hand, the *RR22-OX* lines had no apparent effect on the production and growth of the stigma brush hairs (**Supplementary Figure 3**), which are trichome-related structures involved in pollen capture whose development has been shown to be affected by cytokinin signaling (Worthen et al., 2019).

Effects of *RR22* Loss-of-Function Mutants on Rice Growth and Development

Four *Tos17* insertion mutations of *RR22* (*rr22-1*, *rr22-2*, *rr22-3*, and *rr22-4*) were identified (**Figure 1A**) and characterized. These did not exhibit any prominent growth phenotypes for the shoots and roots, nor a consistent change in sensitivity for the induction of cytokinin primary response genes (**Supplementary Figure 4**). This is likely due to functional redundancy of *RR22* with other members of the type-B *RR* family of rice, as we previously found that the triple mutant

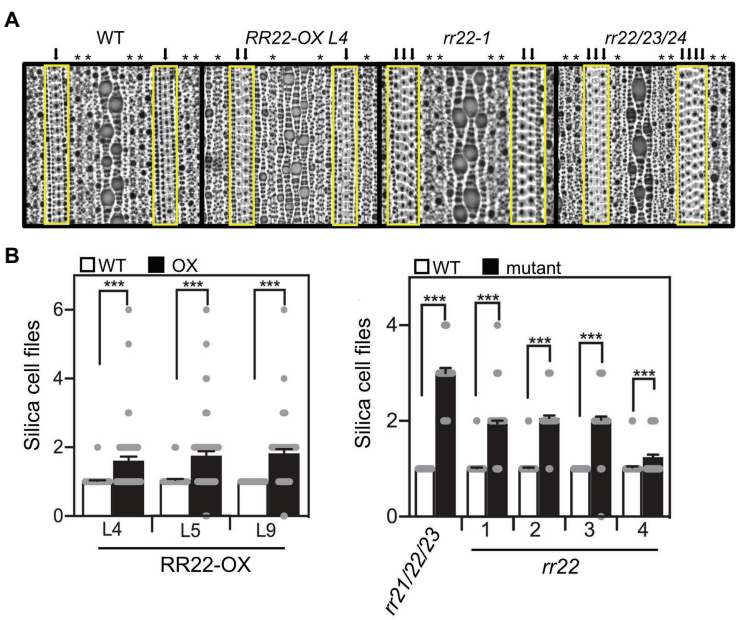


FIGURE 4 | Leaf cell file phenotypes of *RR22-OX*, *rr22*, and *rr21/22/23* mutant lines. **(A)** Representative images of the abaxial epidermis of the flag leaf. The region containing the silica/cork cell files is highlighted with a yellow box, and each silica/cork cell file indicated with a black arrow. Each stomata file is indicated with an asterisk (*). **(B)** Quantification for the number of rows of adjacent silica/cork cell files. Impressions from five plants (two per plant) were examined and the number of rows of adjacent silica/cork cell files determined ($n > 47$). Error bars show SE. The *T*-test was used for statistical comparison of each mutant line to its WT sibling (***$p < 0.001$).

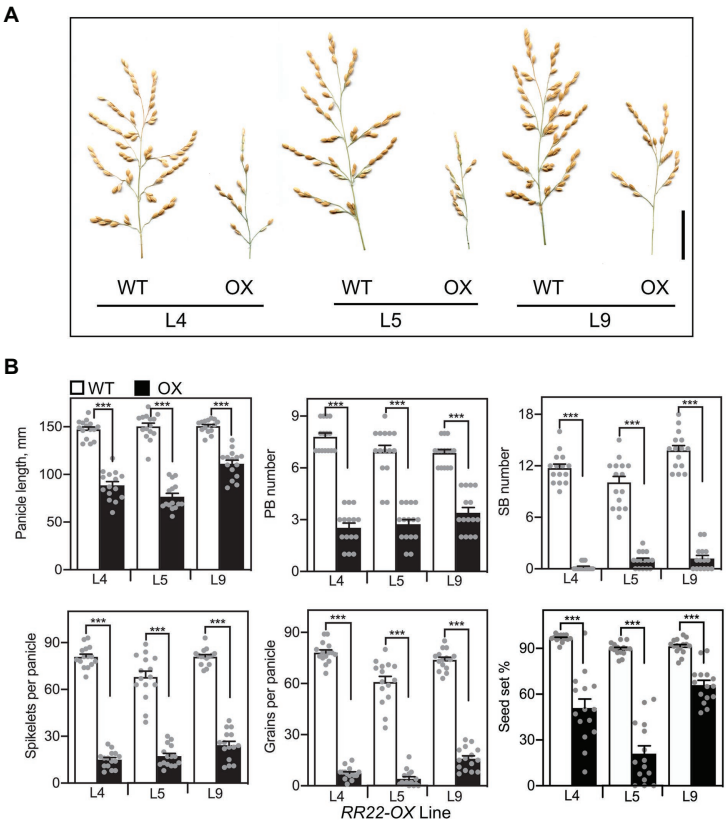


FIGURE 5 | Panicle phenotypes of *RR22-OX* lines. **(A)** Panicles of *RR22-OX* lines and their WT siblings. Scale bar = 5 cm. **(B)** Yield parameters of the *RR22-OX* lines, including panicle length, primary branch (PB) and secondary branch (SB) number, spikelets and grains per panicle, and percent seed set (n = 15). Error bars show SE. The *T*-test was used for statistical comparison of each *RR22-OX* line to its WT sibling (***$p < 0.001$).

rr21/22/23 is of reduced stature and exhibits a reduced capacity for the induction of cytokinin primary response genes (Worthen et al., 2019). However, as described below, we were able to uncover effects of the single *rr22* mutants on the development of silica/cork files, panicle architecture, and trichomes.

As shown in **Figure 4**, WT leaves generally produce a single file containing alternating silica and cork cells. In contrast, the single mutants *rr22-1*, *rr22-2*, and *rr22-3* produced two adjacent files of silica/cork cells; *rr22-4* was more similar to the WT phenotype, but still produced several instances with two adjacent files of silica/cork cells. Analysis of the triple mutant *rr21/22/23* revealed an even greater increase in the rows of adjacent silica/cork files, there being three rows on average (**Figure 4**). Based on the number of these cell files in *rr21/22/23*, along with additional internal silica/cork cells appearing within these expanded rows, the increase in cell files appears due to increased latitudinal cell proliferation.

The panicles of the *rr22* mutants were smaller than those of their WT siblings, primarily due to a reduced branching complexity (**Figure 6**). Analysis of various parameters associated with panicle architecture revealed that three out of four *rr22* alleles exhibited significant decreases in panicle length. The *rr22* mutants produced similar numbers of primary branches to their WT siblings but, strikingly, all four *rr22* mutant alleles exhibited a significant reduction in secondary branch number (**Figure 6**). These effects on panicle architecture result in a significant decrease in the total number of spikelets per panicle compared to WT. Due to fewer spikelets per panicle, coupled to a decrease in seed set, the *rr22* mutants produce fewer grains per panicle than their WT siblings (**Figure 6**).

The *rr22* mutants exhibited defects in the development of several types of trichomes (**Figure 7**). We previously found that the triple type-B *RR* mutant *rr21/22/23* was defective in the development of macro trichomes on leaves and grain hulls, as well as in the production of the stigma brush hairs (Worthen et al., 2019). We therefore examined the length of hull trichomes and stigma brush hairs in the *Tos17 rr22* lines, finding these significantly reduced in length when compared to those of their WT siblings (**Figures 7A, B**). Additionally, stigma-enriched genes exhibited reduced expression in carpels of the *rr22* lines when compared to their WT siblings (**Figure 7C**; **Supplementary Figure 5**). Expression of *GL3A*, which encodes a rice homolog of the transcription factor GLABRA3 of Arabidopsis, and of *EXPA6* was reduced in all four *rr22* lines (**Figure 7C**). Expression of two other stigma-enriched genes (*GH3.1* and *WDA1*) was less consistently reduced

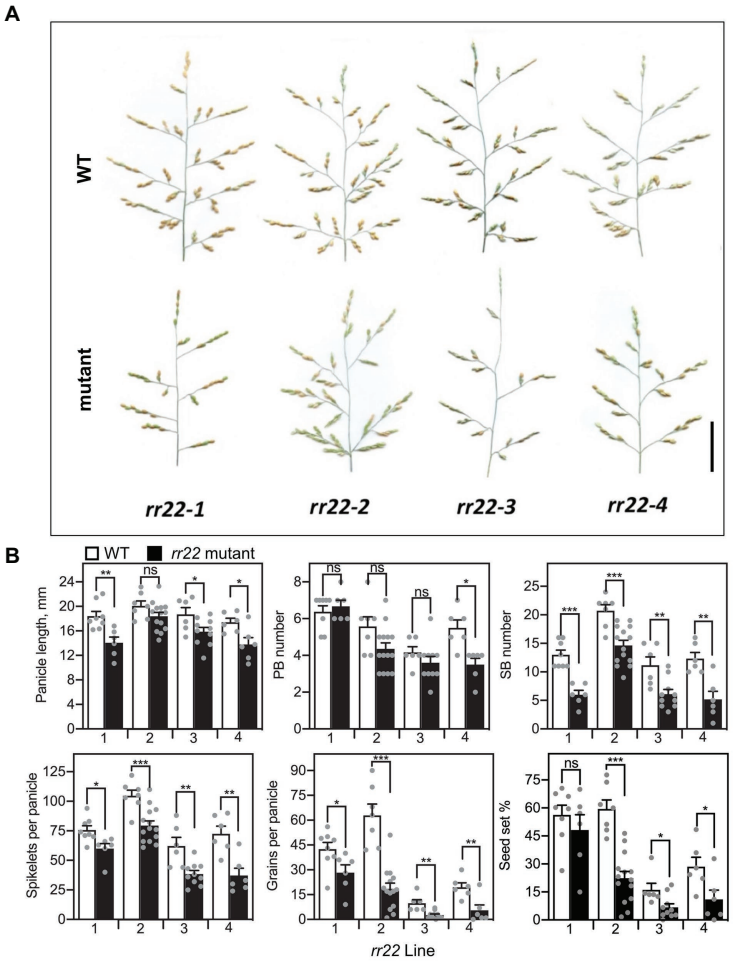


FIGURE 6 | Panicle phenotypes of *Tos17 rr22* lines. **(A)** Panicles of *rr22-1*, *rr22-2*, *rr22-3*, and *rr22-4* lines and their WT siblings. Scale bar = 5 cm. **(B)** Yield parameters of the *rr22* lines, including panicle length, PB and SB number, spikelets and grains per panicle, and percent seed set ($n \geq 6$). The *T*-test was used for statistical comparison of each *rr22* line to its WT sibling (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant).

(**Supplementary Figure 5**). All four of these genes were previously found to be significantly downregulated in the *rr21/22/23* mutant, which exhibits a more severe stigma brush hair defect than the *rr22* single mutants; the more variable effects on downregulation of these genes in the *Tos17 rr22* lines are thus likely due to the intermediate mutant phenotype for the brush hairs and functional overlap with the other type-B *RR*s in the regulation of their development.

DISCUSSION

We recently employed a CRISPR/Cas9 gene editing approach to target the four most abundant type-B *RR*s of rice (*RR21*, *RR22*, *RR23*, and *RR24*), results from that analysis confirming a role in cytokinin signaling and revealing various roles in the growth and development (Worthen et al., 2019). As a complement to this CRISPR/Cas9-based loss-of-function approach, we have now employed a gain-of-function approach to characterize the effects of ectopic overexpression of *RR22* in rice, as well as characterized four *Tos17* loss-of-function alleles of *RR22*. Below, we discuss these results in relationship to cytokinin sensitivity, inflorescence architecture, grain yield, the production of trichome-related structures, and the growth and development of leaves and roots.

Genetic analysis of the type-B *RR*s indicates that they can be rate-limiting for cytokinin signal transduction in rice and Arabidopsis. There is functional overlap among members of the type-B *RR* family such that the overall abundance of the type-B *RR*s correlates reasonably well with the effect of loss-of-function mutations on cytokinin-dependent gene expression as well as on some physiological responses in both rice (Tsai et al., 2012; Yamburenko et al., 2017; Worthen et al., 2019) and Arabidopsis (Mason et al., 2005; Argyros et al., 2008; Hill et al., 2013). Furthermore, increased expression of *RR22* in rice, as we show here, and of the Arabidopsis type-B *RR*s *ARR1* and *ARR10* can confer cytokinin hypersensitivity on plants (Sakai et al., 2001; Zubo et al., 2017). However, although

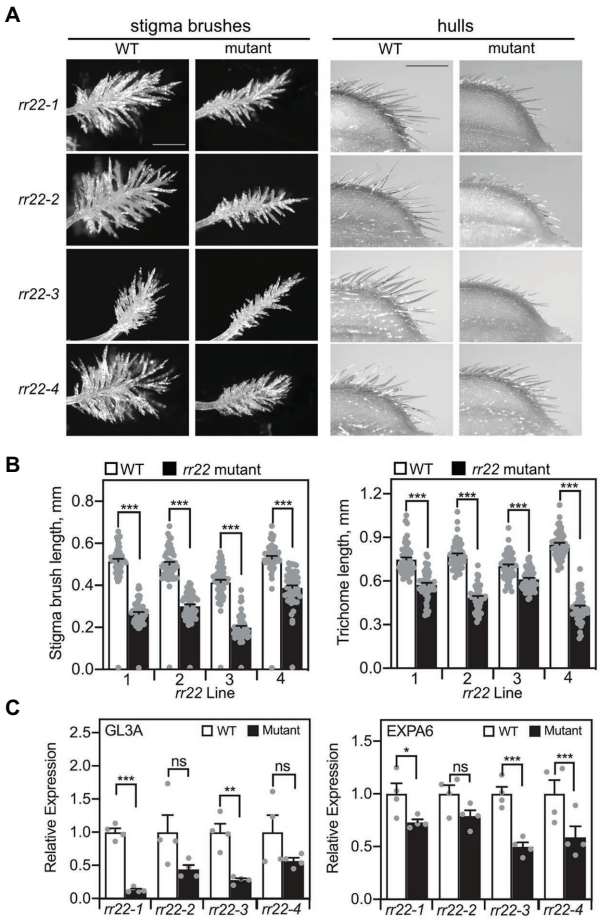


FIGURE 7 | Trichome-related phenotypes of *Tos17 rr22* lines. **(A)** Stigma brushes (scale bar = 0.5 mm) and trichomes on grain hulls (scale bar = 1 mm). **(B)** Stigma brush hair length and hull trichome length, with the five longest hairs or trichomes measured per stigma or hull, respectively (n = 50). **(C)** Expression analysis by qRT-PCR of the stigma-enriched genes *GL3A* and *EXPA6* in carpels of *rr22* mutants and their WT siblings (n = 4). Expression of the *UBQ5* gene was used for normalization. Error bars are for SE. The *T*-test was used for statistical comparison of each *rr22* line to its WT sibling (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant).

cytokinin sensitivity can be modulated by altering the expression levels of type-B RRs, the degree to which these alterations affect growth and development can vary depending on the process, tissue, and organ involved, as discussed in more detail below.

Prior studies emphasize the critical role that cytokinin levels play in regulating meristematic activity and inflorescence architecture in rice (Ashikari et al., 2005; Kurakawa et al., 2007; Gu et al., 2015; Yeh et al., 2015). Rice and Arabidopsis both produce inflorescences; however, the architecture of these inflorescences is quite different and arises from differences in the development of their inflorescence meristems (Itoh et al., 2005; Prusinkiewicz et al., 2007). Rice produces a panicle-type inflorescence, which is determinate and branched, whereas Arabidopsis produces a simple raceme-type inflorescence, which is indeterminate and unbranched. The rice panicle consists of a central stem with several primary and secondary branches, each of which produces the floral structures, called spikelets (Itoh et al., 2005). The branches are produced by the primary and secondary branch meristems and the spikelets by spikelet meristems. Disruption of the *LOG* gene, involved in cytokinin biosynthesis, produces a smaller panicle and reduced grain yield due to a failure to maintain meristematic cells in both inflorescence and spikelet meristems (Kurakawa et al., 2007; Gu et al., 2015). Of particular interest has been the discovery that a reduction in the expression of the cytokinin oxidase gene *CKX2* results in a larger panicle due to an elevation in cytokinin levels, suggesting that grain yield can be manipulated by modulating cytokinin activity (Ashikari et al., 2005; Yeh et al., 2015).

Our analyses of loss- and gain-of-function type-B *RR* mutants of rice emphasize the sensitivity of panicle architecture to alterations in cytokinin signal transduction. *RR21*, *RR22*, and *RR23* are all expressed during the initial stages of panicle development when primary and secondary branch meristems are produced (Yamburenko et al., 2017), and we find that the loss of a single type-B *RR* compromises the panicle architecture. This is evident in all four *Tos17 rr22* loss-of-function mutants, the most pronounced effect being a reduction in secondary branch production, a similar architectural defect also occurring in CRISPR/Cas9 generated loss-of-function mutants of *RR21* and *RR23* (Worthen et al., 2019). Interestingly, we did not observe such a reduction in panicle size for the CRISPR/Cas9 generated mutant of *RR22*, this mutant in fact exhibiting an increase in secondary branch number (Worthen et al., 2019). This phenotypic difference between the *Tos17* and CRISPR/Cas9 alleles may arise due differences in the rice varieties examined (Nipponbare for the *Tos17* lines; Kitaake for the CRISPR-Cas9 lines) and/or functional compensation of the single *rr22* mutant by another type-B *RR* family member. The latter is a possibility because the triple *rr21/22/23* CRISPR/Cas9-generated mutant exhibited substantially smaller panicles with a reduction in both primary and secondary branch production, indicating functional overlap among the type-B *RR*s in the regulation of panicle architecture (Worthen et al., 2019).

The results from *CKX2* mutants (Ashikari et al., 2005; Yeh et al., 2015), in which an increase in cytokinin levels resulted in a larger more branched panicles might suggest that increased activity of signal transduction components could phenocopy this effect. However, this was not the case based on our analysis of the *RR22-OX* lines. Instead, the *RR22-OX* lines exhibited substantially smaller panicles due to reductions in both primary branch and secondary branch production, the production of secondary branches being almost eliminated. This may suggest a heightened cytokinin response due to overexpression is inhibitory, and/or that an expanded zone of cytokinin activity due to ectopic expression results in a lack of proper spatial temporal coordination of signaling within the meristematic tissues of the panicle.

Grain yield per panicle is reduced in the *rr22* loss-of-function mutants due to a reduction in spikelet production

coupled to a reduction in seed set. The reduced seed set is likely due in part to a defect in stigma brush development, because the stigma brushes of grasses play an important role in pollen capture, hydration, and guidance of the pollen tube toward the ovary (Heslop-Harrison et al., 1984; Heslop-Harrison and Reger, 1988). Each brush hair in rice is multicellular and branched, and our prior analysis indicates that type-B RR mutants severely affect cell proliferation of the brush hairs but not their cell expansion (Worthen et al., 2019). Our results from the *Tos17 rr22* mutants confirm a predominant role for *RR22* in mediating this effect on stigma brush development. The stigma brush is an epidermal trichome-related structure and we also observed that the *Tos17 rr22* mutants exhibited reduced growth of trichomes on their seed hulls, consistent with a general role for *RR22* in modulating trichome growth and development. Interestingly, the *RR22-OX* lines affected growth of the hull trichomes, but had no apparent effect on growth and development of the stigma brush structure.

Alterations in type-B *RR* activity also affect the growth and development of rice leaves. A recent study reported that the overexpression of the rice type-B RR *ORR2*, which is equivalent to *RR22* from our study (Schaller et al., 2007), reduced plant height by approximately 20% but reported no additional effects on growth and development (Shi et al., 2020). We observed a substantially greater decrease in plant height of over 40% in our overexpression lines, potentially due to higher levels of *RR22* expression in our study. The decrease in plant height of the over-expression lines, based on our study, can be largely attributed to a decrease in leaf growth, this primarily arising due to a decrease in cell size rather than cell proliferation. This effect of type-B *RR* overexpression contrasts with that found in the triple mutant *rr21/22/23* of rice, which like the *RR22-OX* lines also has shorter flag leaves, but due to a decrease in longitudinal cell proliferation rather than a decrease in cell size (Worthen et al., 2019). Interestingly, ectopic overexpression of RR22 in rice did not recapitulate what was found from the ectopic overexpression of the type-B RR *ARR10* of Arabidopsis, which induced leaf cell proliferation, although this was also sometimes accompanied by a decrease in cell size (Zubo et al., 2017).

Alterations in type-B *RR* activity affected the development of rice leaves along with affecting their growth. The architecture of grass leaves differs from that of dicots such as Arabidopsis, with various cell files serving distinct functions related to the development of diagnostic cell types (Kaufman et al., 1985; Luo et al., 2012). Both loss- and gain-of-function type-B *RR* mutants affected the number of silica/cork cell files, the *rr* mutants resulting in an increase in the number of adjacent files, likely due to an increase in lateral cell proliferation, whereas ectopic overexpression of *RR22* resulted in a greater variability in the number of adjacent cell files. Ectopic overexpression of *RR22* also resulted in a decrease in the number of adjacent stomatal cell files. This effect on stomata development may involve the genes *SHR* and *SCR*, because these partner transcription factors positively regulate the formation of stomatal cell files in rice (Schuler et al., 2018; Buckley et al., 2019; Wu et al., 2019) and, based on studies in Arabidopsis, cytokinin can inhibit *SCR* expression (Zhang et al., 2013). These results suggest that, in addition to affecting cell size and proliferation, that alterations in the cytokinin transcriptional response can also affect the developmental architecture of the rice leaf.

Our results are also consistent with a role for cytokinin in regulating root architecture in rice. Overexpression of *RR22* inhibited seminal root growth and had an even more pronounced effect on lateral root formation. Such an effect is consistent with cytokinin and the type-B RRs serving as negative regulators of primary root growth and lateral root development, based on studies in dicots such as Arabidopsis and tobacco as well as the monocot rice (Mason et al., 2005; Dello Ioio et al., 2007; Werner et al., 2010; Hill et al., 2013; Zubo et al., 2017). We did not note any obvious developmental abnormalities in roots of the *rr22* mutants, although *RR22* is one of the more abundantly expressed members of the type-B *RR* family in the root, shoot, and inflorescence of rice (Tsai et al., 2012; Yamburenko et al., 2017). The lack of *rr22* root phenotypes is likely due to functional overlap of *RR22* with the other type-B *RR*s based on our prior analyses of the CRISPR/Cas9-generated type-B *RR* mutants (Worthen et al., 2019), that study indicating that a triple *rr21/22/23* mutant exhibited defects in root development as well as root growth sensitivity to exogenous cytokinin. The *rr22* single mutant phenotypes we do observe in inflorescence architecture, stigma brush development, and silica/cork cell production may arise due to the sensitivity of those tissues to small changes in cytokinin activity and/or a relatively greater role for *RR22* compared to other type-B *RR*s in their developmental regulation.

In conclusion, altered expression of the type-B RRs, mediated either by loss-of-function or ectopic overexpression, results in compromised growth and development in rice. Ectopic overexpression of *RR22* in rice appears to hamper growth and development more severely than is the case when a similar study was performed in Arabidopsis (Zubo et al., 2017). The ectopic overexpression of ARR10 in Arabidopsis closely parallels expectations for increased cytokinin signaling, resulting in increased cell proliferation in the shoot and the inhibition of root growth, the opposite of what is found with loss-of-function mutants of the type-B RRs, and with no major defects in inflorescence development. In rice, on the other hand, ectopic overexpression compromises growth of the leaves and the inflorescence in a manner reminiscent to what is observed in type-B *RR* loss-of-function mutations (Worthen et al., 2019). Two possibilities, not mutually exclusive, may explain these phenotypic observations. First, that an optimal level of cytokinin activity is required to mediate these responses, with either a decrease or increase in activity compromising growth and development. Second, due to the substantial differences in inflorescence and leaf development between rice and Arabidopsis, rice is more sensitive to expanded zones of cytokinin activity arising from the ectopic expression of the type-B RR. Regardless, these studies demonstrate that a delicate balance of cytokinin transcriptional activity is necessary for optimal growth and development in rice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GS, JK, MY, and JW designed the research. GS and SNS provided support and oversaw the research. MY, JW, AZ, BA, and AC performed the experiments. MY, JW, AZ, BA, and SS analyzed the data. GS, MY, and JW wrote the manuscript with contributions from all authors. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Science Foundation grant nos. IOS-1238051 and MCB-1856248 (GS and JK), by the Agriculture and Food Research Initiative competitive grant nos. 2019-67013-29191 (GS) and 2016-67011-24723 (JW) from the USDA National Institute of Food and Agriculture, and the International Research Support Initiative Program of Higher Education Commission of Pakistan (AZ).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.577676/full#supplementary-material

REFERENCES

Argyros, R. D., Mathews, D. E., Chiang, Y. -H., Palmer, C. M., Thibault, D. M., Etheridge, N., et al. (2008). Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. *Plant Cell* 20, 2102–2116. doi: 10.1105/tpc.108.059584

Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., et al. (2005). Cytokinin oxidase regulates rice grain production. *Science* 309, 741–745. doi: 10.1126/science.1113373

Buckley, C. R., Caine, R. S., and Gray, J. E. (2019). Pores for thought: can genetic manipulation of stomatal density protect future rice yields? *Front. Plant Sci.* 10:1783. doi: 10.3389/fpls.2019.01783

Clarke, J. D. (2009). Cetyltrimethyl ammonium bromide (CTAB) DNA miniprep for plant DNA isolation. *Cold Spring Harb Protoc* 2009:pdb.prot5177. doi: 10.1101/pdb.prot5177

Dello Ioio, R., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., et al. (2007). Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. *Curr. Biol.* 17, 678–682. doi: 10.1016/j.cub.2007.02.047

Devos, K. M., and Gale, M. D. (2000). Genome relationships: the grass model in current research. *Plant Cell* 12, 637–646. doi: 10.1105/tpc.12.5.637

Du, L., Jiao, F., Chu, J., Jin, G., Chen, M., and Wu, P. (2007). The two-component signal system in rice (*Oryza sativa* L.): a genome-wide study of cytokinin signal perception and transduction. *Genomics* 89, 697–707. doi: 10.1016/j.ygeno.2007.02.001

Gao, Z., Wen, C. -K., Binder, B. M., Chen, Y. -F., Chang, J., Chiang, Y. -H., et al. (2008). Heteromeric interactions among ethylene receptors mediate signaling in Arabidopsis. *J. Biol. Chem.* 283, 23081–23810. doi: 10.1074/jbc.M800641200

Gu, B., Zhou, T., Luo, J., Liu, H., Wang, Y., Shangguan, Y., et al. (2015). An-2 encodes a cytokinin synthesis enzyme that regulates awn length and grain production in rice. *Mol. Plant* 8, 1635–1650. doi: 10.1016/j.molp.2015.08.001

Heslop-Harrison, Y., and Reger, B. J. (1988). Tissue organisation, pollen receptivity and pollen tube guidance in normal and mutant stigmas of the grass *Pennisetum typhoides* (Burm.) Stapf et Hubb. *Sex. Plant Reprod.* 1, 182–193. doi: 10.1007/BF00193749

Heslop-Harrison, Y., Reger, B. J., and Heslop-Harrison, J. (1984). The pollen-stigma interaction in the grasses. 5. Tissue organization and cytochemistry of the stigma ('silk') of *Zea mays* L. *Acta Bot. Neerl.* 33, 81–99. doi: 10.1111/j.1438-8677.1984.tb01774.x

Hill, K., Mathews, D. E., Kim, H. J., Street, I. H., Wildes, S. L., Chiang, Y. H., et al. (2013). Functional characterization of type-B response regulators in the Arabidopsis cytokinin response. *Plant Physiol.* 162, 212–224. doi: 10.1104/pp.112.208736

Hirochika, H., Guiderdoni, E., An, G., Hsing, Y. I., Eun, M. Y., Han, C. D., et al. (2004). Rice mutant resources for gene discovery. *Plant Mol. Biol.* 54, 325–334. doi: 10.1023/B:PLAN.0000036368.74758.66

Hosoda, K., Imamura, A., Katoh, E., Hatta, T., Tachiki, M., Yamada, H., et al. (2002). Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the Arabidopsis response regulators. *Plant Cell* 14, 2015–2029. doi: 10.1105/tpc.002733

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. *Nature* 413, 383–389. doi: 10.1038/35096500

Hwang, I., Sheen, J., and Müller, B. (2012). Cytokinin signaling networks. *Annu. Rev. Plant Biol.* 63, 353–380. doi: 10.1146/annurev-arplant-042811-105503

Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Kiba, T., et al. (1999). Compilation and characterization of *Arabidopsis thaliana* response regulators implicated in his-asp phosphorelay signal transduction. *Plant Cell Physiol.* 40, 733–742. doi: 10.1093/oxfordjournals.pcp.a029600

Imamura, A., Kiba, T., Tajima, Y., Yamashino, T., and Mizuno, T. (2003). In vivo and in vitro characterization of the ARR11 response regulator implicated in the his-to-asp phosphorelay signal transduction in *Arabidopsis thaliana*. *Plant Cell Physiol.* 44, 122–131. doi: 10.1093/pcp/pcg014

Imamura, A., Yoshino, Y., and Mizuno, T. (2001). Cellular localization of the signaling components of Arabidopsis his-to-asp phosphorelay. *Biosci. Biotechnol. Biochem.* 65, 2113–2117. doi: 10.1271/bbb.65.2113

Ishida, K., Yamashino, T., Yokoyama, A., and Mizuno, T. (2008). Three type-B response regulators, ARR1, ARR10, and ARR12, play essential but redundant roles in cytokinin signal transduction throughout the life cycle of *Arabidopsis thaliana*. *Plant Cell Physiol.* 49, 47–57. doi: 10.1093/pcp/pcm165

Ito, Y., and Kurata, N. (2006). Identification and characterization of cytokinin-signalling gene families in rice. *Gene* 382, 57–65. doi: 10.1016/j.gene.2006.06.020

Itoh, J., Nonomura, K., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., et al. (2005). Rice plant development: from zygote to spikelet. *Plant Cell Physiol.* 46, 23–47. doi: 10.1093/pcp/pci501

Jameson, P. E., and Song, J. (2016). Cytokinin: a key driver of seed yield. *J. Exp. Bot.* 67, 593–606. doi: 10.1093/jxb/erv461

Kaufman, P. B., Dayanandan, P., Franklin, C. I., and Takeoka, Y. (1985). Structure and function of silica bodies in the epidermal system of grass shoots. *Ann. Bot.* 55, 487–507. doi: 10.1093/oxfordjournals.aob.a086926

Kieber, J. J., and Schaller, G. E. (2014). Cytokinins. *Arabidopsis Book* 12:e0168. doi: 10.1199/tab. 0168

Kim, H. J., Chiang, Y. H., Kieber, J. J., and Schaller, G. E. (2013a). SCF(KMD) controls cytokinin signaling by regulating the degradation of type-B response regulators. *Proc. Natl. Acad. Sci. U. S. A.* 110, 10028–10033. doi: 10.1073/pnas.1300403110

Kim, H. J., Kieber, J. J., and Schaller, G. E. (2012). Overlapping and lineage-specific roles for the type-B response regulators of monocots and dicots. *Plant Signal. Behav.* 7, 1110–1113. doi: 10.4161/psb.21293

Kim, H. J., Kieber, J. J., and Schaller, G. E. (2013b). The rice F-box protein KISS ME DEADLY2 functions as a negative regulator of cytokinin signalling. *Plant Signal. Behav.* 8:e26434. doi: 10.4161/psb.26434

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., et al. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445, 652–655. doi: 10.1038/nature05504

Liang, Y., Wang, X., Hong, S., Li, Y., and Zuo, J. (2012). Deletion of the initial 45 residues of ARR18 induces cytokinin response in Arabidopsis. *J. Genet. Genomics* 39, 37–46. doi: 10.1016/j.jgg.2011.12.004

Lohrmann, J., Sweere, U., Zabaleta, E., Baurle, I., Keitel, C., Kozma-Bognar, L., et al. (2001). The response regulator ARR2: a pollen-specific transcription factor involved in the expression of nuclear genes for components of mitochondrial complex I in Arabidopsis. *Mol. Gen. Genomics*. 265, 2–13. doi: 10.1007/s004380000400

Luo, L., Zhou, W. -Q., Liu, P., Li, C. -X., and Hou, S. -W. (2012). The development of stomata and other epidermal cells on the rice leaves. *Biol. Plant.* 56, 521–527. doi: 10.1007/s10535-012-0045-y

Maes, L., and Goossens, A. (2010). Hormone-mediated promotion of trichome initiation in plants is conserved but utilizes species- and trichome-specific regulatory mechanisms. *Plant Signal. Behav.* 5, 205–207. doi: 10.4161/psb.5.2.11214

Maes, L., Inze, D., and Goossens, A. (2008). Functional specialization of the TRANSPARENT TESTA GLABRA1 network allows differential hormonal control of laminal and marginal trichome initiation in Arabidopsis rosette leaves. *Plant Physiol.* 148, 1453–1464. doi: 10.1104/pp.108.125385

Mann, D., Lafayette, P., Abercrombie, L., King, Z., Mazarei, M., Halter, M., et al. (2012). Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species. *Plant Biotechnol. J.* 10, 226–236. doi: 10.1111/j.1467-7652.2011.00658.x

Mason, M. G., Li, J., Mathews, D. E., Kieber, J. J., and Schaller, G. E. (2004). Type-B response regulators display overlapping expression patterns in Arabidopsis. *Plant Physiol.* 135, 927–937. doi: 10.1104/pp.103.038109

Mason, M. G., Mathews, D. E., Argyros, D. A., Maxwell, B. B., Kieber, J. J., Alonso, J. M., et al. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. *Plant Cell* 17, 3007–3018. doi: 10.1105/tpc.105.035451

Miyao, A., Tanaka, K., Murata, K., Sawaki, H., Takeda, S., Abe, K., et al. (2003). Target site specificity of the Tos17 retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome. *Plant Cell* 15, 1771–1780. doi: 10.1105/tpc.012559

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., et al. (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* 104, 34–41. doi: 10.1263/jbb.104.34

Pareek, A., Singh, A., Kumar, M., Kushwaha, H. R., Lynn, A. M., and Singla-Pareek, S. L. (2006). Whole genome analysis of *Oryza sativa* L. reveals similar architecture of two-component-signaling-machinery with Arabidopsis. *Plant Physiol.* 142, 380–397. doi: 10.1104/pp.106.086371

Prusinkiewicz, P., Erasmus, Y., Lane, B., Harder, L. D., and Coen, E. (2007). Evolution and development of inflorescence architectures. *Science* 316, 1452–1456. doi: 10.1126/science.1140429

Raines, T., Blakley, I. C., Tsai, Y. C., Worthen, J. M., Franco-Zorrilla, J. M., Solano, R., et al. (2016). Characterization of the cytokinin-responsive transcriptome in rice. *BMC Plant Biol.* 16:260. doi: 10.1186/s12870-016-0932-z

Rani Debi, B., Taketa, S., and Ichii, M. (2005). Cytokinin inhibits lateral root initiation but stimulates lateral root elongation in rice (*Oryza sativa*). *J. Plant Physiol.* 162, 507–515. doi: 10.1016/j.jplph.2004.08.007

Rashotte, A. M., Mason, M. G., Hutchison, C. E., Ferreira, F. J., Schaller, G. E., and Kieber, J. J. (2006). A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. *Proc. Natl. Acad. Sci. U. S. A.* 103, 11081–11085. doi: 10.1073/pnas.0602038103

Sakai, H., Aoyama, T., and Oka, A. (2000). Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. *Plant J.* 24, 703–711. doi: 10.1046/j.1365-313x.2000.00909.x

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., et al. (2001). ARR1, a transcription factor for genes immediately responsive to cytokinins. *Science* 294, 1519–1521. doi: 10.1126/science.1065201

Sakakibara, H. (2006). Cytokinins: activity, biosynthesis, and translocation. *Annu. Rev. Plant Biol.* 57, 431–449. doi: 10.1146/annurev.arplant.57.032905.105231

Salinovich, O., and Montelaro, R. C. (1986). Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* 156, 341–347. doi: 10.1016/0003-2697(86)90263-0

Schaller, G. E., Doi, K., Hwang, I., Kieber, J. J., Khurana, J. P., Kurata, N., et al. (2007). Letter to the editor: nomenclature for two-component signaling elements of *Oryza sativa*. *Plant Physiol.* 143, 555–557. doi: 10.1104/pp.106.093666

Schuler, M. L., Sedelnikova, O. V., Walker, B. J., Westhoff, P., and Langdale, J. A. (2018). SHORTROOT-mediated increase in stomatal density has no impact on photosynthetic efficiency. *Plant Physiol.* 176, 757–772. doi: 10.1104/pp.17.01005

Shi, F., Wang, M., and An, Y. (2020). Overexpression of a B-type cytokinin response regulator (OsORR2) reduces plant height in rice. *Plant Signal. Behav.* 15:1780405. doi: 10.1080/15592324.2020.1780405

To, J. P., Haberer, G., Ferreira, F. J., Deruere, J., Mason, M. G., Schaller, G. E., et al. (2004). Type-a Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* 16, 658–671. doi: 10.1105/tpc.018978

Tsai, Y. C., Weir, N. R., Hill, K., Zhang, W., Kim, H. J., Shiu, S. H., et al. (2012). Characterization of genes involved in cytokinin signaling and metabolism from rice. *Plant Physiol.* 158, 1666–1684. doi: 10.1104/pp.111.192765

Werner, T., Nehnevajova, E., Kollmer, I., Novak, O., Strnad, M., Kramer, U., et al. (2010). Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in Arabidopsis and tobacco. *Plant Cell* 22, 3905–3920. doi: 10.1105/tpc.109.072694

Werner, T., and Schmülling, T. (2009). Cytokinin action in plant development. *Curr. Opin. Plant Biol.* 12, 527–538. doi: 10.1016/j.pbi.2009.07.002

Worthen, J. M., Yamburenko, M. V., Lim, J., Nimchuk, Z. L., Kieber, J. J., and Schaller, G. E. (2019). Type-B response regulators of rice play key roles in growth, development and cytokinin signaling. *Development* 146:dev174870. doi: 10.1242/dev.174870

Wu, Z., Chen, L., Yu, Q., Zhou, W., Gou, X., Li, J., et al. (2019). Multiple transcriptional factors control stomata development in rice. *New Phytol.* 223, 220–232. doi: 10.1111/nph.15766

Yamburenko, M. V., Kieber, J. J., and Schaller, G. E. (2017). Dynamic patterns of expression for genes regulating cytokinin metabolism and signaling during rice inflorescence development. *PLoS One* 12:e0176060. doi: 10.1371/journal.pone.0176060

Yeh, S. Y., Chen, H. W., Ng, C. Y., Lin, C. Y., Tseng, T. H., Li, W. H., et al. (2015). Down-regulation of cytokinin oxidase 2 expression increases tiller number and improves rice yield. *Rice* 8:36. doi: 10.1186/s12284-015-0070-5

Yokoyama, A., Yamashino, T., Amano, Y., Tajima, Y., Imamura, A., Sakakibara, H., et al. (2007). Type-B ARR transcription factors, ARR10 and ARR12, are implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of *Arabidopsis thaliana*. *Plant Cell Physiol.* 48, 84–96. doi: 10.1093/pcp/pcl040

Yoshida, S., Forno, D. A., Cock, J. H., and Gomez, K. A. (1976). *Laboratory manual for physiological studies of Rice*. Los Banos, Laguna, Philippines: The International Rice Research Institute.

Zhang, W. J., Swarup, R., Bennett, M., Schaller, G. E., and Kieber, J. J. (2013). Cytokinin induces cell division in the quiescent center of the Arabidopsis root apical meristem. *Curr. Biol.* 23, 1979–1989. doi: 10.1016/J.Cub.2013.08.008

Zubo, Y. O., Blakley, I. C., Yamburenko, M. V., Worthen, J. M., Street, I. H., Franco-Zorrilla, J. M., et al. (2017). Cytokinin induces genome-wide binding of the type-B response regulator ARR10 to regulate growth and development in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 114, E5995–E6004. doi: 10.1073/pnas.1620749114

Conflict of Interest: The reviewer JH is currently organizing a Research Topic with one of the authors GS. The review process met the standards of a fair and objective review.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 17 November 2020
doi: 10.3389/fpls.2020.604489

Cytokinin Signaling Downstream of the His-Asp Phosphorelay Network: Cytokinin-Regulated Genes and Their Functions

*Carlo K. Kroll and Wolfram G. Brenner**

General and Applied Botany, Institute of Biology, Faculty of Life Sciences, Universität Leipzig, Leipzig, Germany

The plant hormone cytokinin, existing in several molecular forms, is perceived by membrane-localized histidine kinases. The signal is transduced to transcription factors of the type-B response regulator family localized in the nucleus by a multi-step histidine-aspartate phosphorelay network employing histidine phosphotransmitters as shuttle proteins across the nuclear envelope. The type-B response regulators activate a number of primary response genes, some of which trigger in turn further signaling events and the expression of secondary response genes. Most genes activated in both rounds of transcription were identified with high confidence using different transcriptomic toolkits and meta analyses of multiple individual published datasets. In this review, we attempt to summarize the existing knowledge about the primary and secondary cytokinin response genes in order to try connecting gene expression with the multitude of effects that cytokinin exerts within the plant body and throughout the lifespan of a plant.

Keywords: cytokinin, signal transduction, downstream signaling, signaling crosstalk, feed-forward signaling, feed-back signaling

OPEN ACCESS

Edited by:
Catherine Bellini,
Umeå University, Sweden

Reviewed by:
Sabine Carpin,
Université d'Orléans, France
François Héricourt,
Université d'Orléans, France

***Correspondence:**
Wolfram G. Brenner
wolfram.brenner@uni-leipzig.de

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *09 September 2020*
Accepted: *26 October 2020*
Published: *17 November 2020*

Citation:
Kroll CK and Brenner WG (2020) Cytokinin Signaling Downstream of the His-Asp Phosphorelay Network: Cytokinin-Regulated Genes and Their Functions. Front. Plant Sci. 11:604489. doi: 10.3389/fpls.2020.604489

INTRODUCTION

The plant hormone cytokinin, regulates a wide range of processes in plants, ranging from development (growth, meristem activity, vascular development) over metabolism and physiology (source–sink relationships, secondary metabolism) to environmental interactions (both biotic and abiotic) (Mok and Mok, 2001; Argueso et al., 2009; Werner and Schmülling, 2009; Kieber and Schaller, 2014, 2018; Cortleven et al., 2019; Wybouw and De Rybel, 2019).

The immediate-early cytokinin signaling network is an extended version of the two-component signaling system known in prokaryotes. Besides having receptors (histidine kinases, HK) and transcription factors (type-B response regulators, RRB) like the original prokaryotic system, this plant-specific multi-step His-Asp phosphorelay system is augmented by mobile signaling components traveling between cytosol and nucleus (histidine phosphotransmitters, HPT), and negative feedback regulators (type-A response regulators, RRA) (Heyl et al., 2013). The RRB transcription factors are transcriptional activators, and numerous transcriptomic studies have not found genes that are consistently negatively regulated in response to cytokinin at very early time points (Brenner et al., 2012; Brenner and Schmülling, 2015), suggesting that RRBs have no repressive function.

Among the cytokinin-induced genes, there are numerous signal transduction components such as transcription factors, protein kinases, F-box proteins, etc. (Rashotte et al., 2003; Brenner et al., 2005, 2012; Bhargava et al., 2013; Brenner and Schmülling, 2015). Some of them are immediate-early response genes, the transcripts of which have started to accumulate as early as 15 min after cytokinin treatment. Others are induced at later time points, indicating that several subsequent rounds of gene expression happen after cytokinin treatment. Additionally, few genes transducing downstream branches of the cytokinin signal were found by other means.

Some of the cytokinin-regulated signal transduction genes have been functionally characterized, a few of them in great detail, and in part focusing on aspects other than cytokinin. This review aims at summarizing the accumulated knowledge about selected signaling components downstream of the phosphorelay signaling system, at finding functional interactions between them, and at presenting hypotheses resulting from mechanistic and functional considerations.

The regulation of gene expression is obviously not the only means by which signals are transduced. However, other means such as different post-translational protein modifications are not as easily detected in a comprehensive manner. Thus, this review focuses on genes transcriptionally regulated by cytokinin and mentions other means of signal transduction only if they have been shown in the context of the respective gene.

In the following paragraphs, the collected knowledge of selected cytokinin-regulated genes will be presented in order to derive ideas about their functions and their contribution to the hormonal effects of cytokinin. The selection of genes (**Table 1**) is largely based on their frequency of occurrence in transcriptomic investigations of the global gene expression response to the hormone in the model plant *Arabidopsis thaliana*.

GENES INVOLVED IN CYTOKININ HOMEOSTASIS

Cytokinin Biosynthesis and Activation

CYP735A2 is a gene encoding a cytochrome p450 family protein with trans-hydroxylation enzyme activity forming *trans*-zeatin (tZ)-type cytokinins from $N^6(\Delta^2$-isopentenyl) adenine (iP)-type cytokinins (Takei et al., 2004b; Kiba et al., 2013). This change of the side-chain structure is relevant for the biological activity of the respective cytokinin derivative (Schmitz et al., 1972; Mok et al., 1978) and their route of transportation via phloem or xylem (Takei et al., 2001a; Hirose et al., 2007; Kudo et al., 2010; Kiba et al., 2013). Previous studies showed that the *CYP735A2* transcript is induced by all forms of active cytokinins including the synthetic cytokinin BA, while the transcript of the paralog *CYP735A1* is insensitive to cytokinin (Takei et al., 2004b; Brenner et al., 2012; Bhargava et al., 2013). The CYP735A2 promoter harbors several core motifs and one extended motif binding type-B RRs (Brenner et al., 2012; Brenner and Schmülling, 2015), linking it with immediate-early cytokinin signaling network. Both CYP735A enzymes can be inhibited by uniconazole (Sasaki et al., 2013).

CYP735A2 is mainly expressed in roots (Takei et al., 2001b, 2004b; Schmid et al., 2005). Higher expression levels were also found during petal differentiation, in hypocotyls and in the leaf-forming structures of the shoot apical meristem (Schmid et al., 2005). The encoded protein is predicted to be localized in mitochondria and extracellular regions. A proteomic study has found the protein in the plasmodesmata (Fernandez-Calvino et al., 2011).

CYP735A2 is regarded as one of the major genes in maintaining the homeostasis of active cytokinins (Wang et al., 2013). This was concluded after studies with an *ugtc76c1* mutant showed attenuated N-glycosylation of tZ and iP, but stable tZ and iP content and normal developmental phenotypes. The upregulation of *CYP735A2* is the likely reason for that stable homeostasis.

CYP735A2 is strongly upregulated by increases of the nitrate concentration in the medium (Ramireddy et al., 2014). In contrast, increased phosphate availability, acidity, and osmotic stress downregulate *CYP735A2* expression (Ramireddy et al., 2014). The CYP735A2 enzyme produces tZ-type cytokinins predominantly in the root, which are then transported to the shoot, promoting shoot growth (Takei et al., 2004b; Hirose et al., 2007; Kiba et al., 2013). Cytokinin has been proven to be one of the systemic signals of nitrogen availability in the soil (Krouk et al., 2011; Ruffel et al., 2011; Poitout et al., 2018; Vega et al., 2019). Therefore, it is concluded that *CYP735A2* may be the main regulator of that systemic signal (Ramireddy et al., 2014). However, CYP735A2-produced tZ is not the only root-to-shoot nitrate signal since cytokinin-independent signaling by mobile peptides has also been found (Ruffel et al., 2016). In addition to being a long-distance signal promoting shoot growth in the presence of nitrate, cytokinin directly influences root system architecture by suppressing root growth and branching (Ramireddy et al., 2014).

CYP735A2 is strongly upregulated by cytokinin in roots. This can be regarded as part of a feed-forward loop. Such feed-forward loops tend to increase the signal through itself. On the other hand, cytokinin signaling involves numerous feedback loops mediated through type-A response regulators or cytokinin-degrading enzymes (e.g., *CKX4*, *UGT76C2*). Temporally separated counteracting feed-forward and feed-back loops are frequently observed in developmental biology as they help establish patterning by promoting steeper gradients of morphogens between different domains. In this scenario, a feed forward loop may help establish a state of no return, fixing the developmental fate of a cell or a group of cells. In terms of long-distance signaling, a feed-forward loop may conceptually be a signal enhancing mechanism to increase the speed of signal propagation. In this case, that concept would be realized by a process in which tZ-activated CYP735A2 successively synthesizes tZ in the tissue at the arriving tZ signal. Such a mechanism may be faster than the process relying on transport with the water stream in the xylem, which is dependent on water evaporation of the

TABLE 1 | Cytokinin-regulated genes reviewed in this article.

Gene ID	Gene name	Cited in[a]	Function (named in publications)	Crosstalk with major pathways
AT1G67110	*CYP735A2*	3, 6, 8, 9, 10, 11, 13, 15, 16	Conversion of iP-type cytokinins to tZ-type cytokinins	
AT4G29740	*CKX4*	2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 15, 16	Irreversible degradation of cytokinins by cleavage	
AT5G05860	*UGT76C2*	4, 6, 8, 12, 15, 16	Irreversible inactivation of cytokinins by *N*-glucosylation Drought and osmotic stress	
AT4G23750	*CRF2*	1, 3, 4, 6, 7, 8, 9, 12, 15, 16	*De novo* SAM generation in calli Lateral root elongation Root architecture in the cold	Auxin
AT2G46310	*CRF5*	3, 4, 6, 7, 8, 9, 15, 16	Development of embryos, cotyledons and leaves	Auxin
AT3G61630	*CRF6*	12, 13, 15	Leaf senescence	Auxin
AT1G68360	*GIS3*	8, 10, 12, 13, 15, 16	Trichome cell differentiation Root hair differentiation	Gibberellin
AT1G16530	*LBD3/ASL9*	3, 6, 8, 10, 11, 15, 16	Leaf development	
AT1G31320	*LBD4*	13, 15, 16	Cambium activity, vascular development, xylem differentiation	
AT2G21650	*RL2/MEE3/RSM1*	3, 6, 8, 15	Endosperm development Gravitropism Photomorphogenesis Embryo development Floral development Seed germination Response to salinity	Auxin ABA
AT4G26150	*CGA1/GNL/GATA22*	5, 6, 8, 15	Chloroplast biogenesis Chloroplast proliferation Chlorophyll biosynthesis Leaf senescence Flowering time Phyllotaxis Branching Floral development Silique length Nitrate assimilation	Light Gibberellin Auxin
AT1G13740	*AFP2*	5, 8, 13, 15	Seed dormancy Flowering time	ABA
AT3G44326	*CFB*	14, 15, 16	Sterol biosynthesis	
AT1G78580	*TPS1*	3, 8, 15	Trehalose-6-phosphate homeostasis Primary metabolism Sink–source relations	
AT2G22860	*PSK2*	3, 5	Cell division and proliferation Adventitious organ formation Pollen germination and growth Chlorophyll biosynthesis Differentiation of tracheary elements Stress-induced senescence	
AT2G17500	*PILS5*	7, 12, 13, 15, 16	Negative regulator of auxin signaling	Auxin
At2g34350		3, 15	Biotic and abiotic stress (?)	JA
At1g11670	*DTX36*	15, 16	Toxin and heavy metal efflux (?) Cell cycle (?)	Phytochrome
AT2G17820	*AHK1/HK1*	14, 15	Osmosensor	ABA
At3G51660	*MDL3*	5, 15, 16	Response to pathogens (?) Response to stress (?)	

[a] *Numbers refer to the following publications: 1 Che et al., 2002; 2 Hoth et al., 2003; 3 Rashotte et al., 2003; 4 Kiba et al., 2004; 5 Brenner et al., 2005; 6 Kiba et al., 2005; 7 Rashotte et al., 2006; 8 Lee et al., 2007; 9 Taniguchi et al., 2007; 10 Yokoyama et al., 2007; 11 Argyros et al., 2008; 12 Heyl et al., 2008; 13 Brenner and Schmülling, 2012; 14 Brenner et al., 2012; 15 Bhargava et al., 2013; 16 Brenner and Schmülling, 2015.*

upper shoot and may therefore, under conditions of little evaporation, be quite slow.

Cytokinin Deactivation and Degradation

CKX4 encodes one of the seven cytokinin oxidases/dehydrogenases in Arabidopsis (Werner et al., 2001, 2003, 2006), and is the only one whose transcript levels are strongly induced by cytokinin treatment (Rashotte et al., 2003; Brenner et al., 2005). CKX enzymes degrade cytokinins irreversibly by cleaving the adenine or adenosine moiety from the respective side chain. Thus, the induction of the *CKX4* gene by cytokinin may be regarded as another negative feedback mechanism superimposed to the negative feedback by type-A response regulators at the signaling level.

CKX4 is predominantly expressed in the root cap, but also in meristemoid cells of the leaf epidermis forming stomata (Werner et al., 2003). Other authors found *CKX4* expression in a wide variety of other tissues, including the shoot apex (Schmid et al., 2005; Obulareddy et al., 2013) and in the endo-reduplicating cells of developing trichomes and stipules (Werner et al., 2006). No exact function of the *CKX4* gene could be established by analysis of single mutants as it obviously has overlapping functions with other *CKX* genes. Overexpression of *CKX4* as well as other *CKX* genes appeared to increase tolerance to drought, heat, or salt stress. Apparently, decreased levels of iP and tZ, which are the main substrates of CKX4 (Gajdošová et al., 2011), play a major role in establishing drought, heat, or salt stress tolerance (Wang et al., 2020). It was also determined that CKX4 plays a role in the pathogen-induced reduction of cytokinin levels after inoculation with *Pseudomonas syringae* pv. Tomato DC3000 since the gene is induces by the phytotoxin coronatine delivered through the type III secretion system, thereby downregulating the plant defense system (Thilmony et al., 2006). Lastly, *CKX4* expression is down-regulated by IAA (Bilyeu et al., 2001; Wang et al., 2020), underlining the importance of this gene in auxin–cytokinin crosstalk.

Although the main expression domain of *CKX4* is in the root cap, it has most likely also a function in the shoot apical meristem:

A *ckx3 ckx4* double mutant showed a significant increase of the meristem activity manifesting in a higher number of flowers and siliques (Bartrina et al., 2011). In the shoot apical meristem, cytokinin oxidases/dehydrogenases are involved in cytokinin homeostasis to maintain meristem activity at sustainable levels, and *CKX4* may be an active negative feedback regulator in this signaling circuitry due to its responsiveness to cytokinin.

The CKX4 protein is most likely secreted into the apoplast as it has corresponding sequence features and is also secreted when expressed in the yeast *P. pastoris* (Bilyeu et al., 2001; Werner et al., 2003). Computational localization predicts the protein also to localize in the ER. Other CKX proteins were predicted to be localized in the mitochondria (Schmülling et al., 2003), or found in the vacuole (Šmehilová et al., 2009; Kowalska et al., 2010), and in the cytoplasm (Zürcher and Müller, 2016). iP-ribotides and tZ-ribotides are the predominant long-range transport forms of cytokinin, but their respective locations of biosynthesis and directions of transport differ fundamentally: While tZ-type cytokinins move from the root to the shoot in the xylem, iP-type cytokinins are transported rootward through symplastic connections in the phloem (Takei et al., 2001b; Corbesier et al., 2003; Matsumoto-Kitano et al., 2008; Shimizu-Sato et al., 2008; Kudo et al., 2010; Bishopp et al., 2011). Given their different subcellular locations, it is likely that different CKX genes are specialized in degrading different types of cytokinins with differing functions.

CKX5 is another cytokinin oxidase/dehydrogenase gene mainly expressed in the testa and in old leaves and primarily appears to have functions in germination, senescence and flowering (Gajdošová et al., 2011; Klepikova et al., 2016). Like CKX4, the CKX5 protein is localized in the ER and secreted into the apoplast (Werner et al., 2003; Zürcher and Müller, 2016). CKX5 is not very specific with regards to its substrate and metabolizes, in contrast to CKX4, *cis*-zeatin and *cis*-zeatin riboside quite efficiently (Gajdošová et al., 2011). These findings underline the assumption that different CKX enzymes are degrading different forms of cytokinin. Differential tissue-specific expression patterns suggest that the degradation of specific cytokinins happens in specific parts of the plant. Complex glycosylation patterns were found, and it has been speculated that these may be responsible for the regulation of enzymatic activity, protein stability, pH optimum, or subcellular localization (Schmülling et al., 2003; Werner et al., 2003; Gajdošová et al., 2011).

Another cytokinin-deactivating gene transcriptionally induced by cytokinin is *UGT76C2*, which encodes a cytokinin N- glycosyltransferase of *Arabidopsis thaliana* (Wang J. et al., 2011; Li et al., 2015; Šmehilová et al., 2016). It is one out of three UGTs having the ability to deactivate cytokinin *in vivo*. *UGT76C2* is an immediate-early cytokinin response gene (Kiba et al., 2004, 2005; Lee et al., 2007; Heyl et al., 2008), and its gene product was shown to be located in the cytosol (Šmehilová et al., 2016). The gene shows a spatio-temporal expression pattern in plants with high expression levels in roots, hypocotyls, cotyledons, young leaves, young lateral roots and immature seeds, but low expression levels in inflorescences and other tissues (Wang J. et al., 2011).

In comparison to the wild type, the amount of cytokinin N-glycosides is reduced in *ugt76c2* loss-of-function mutant plants and increased in plants overexpressing *UGT76C2*. The content of active cytokinins is increased in *ugt76c2* mutant plants, which is reflected by pertinent phenotypes in roots (root length and lateral root density), leaves (chlorophyll retention in detached leaves kept in the dark), and seeds (seed size) correlating with typical cytokinin functions (Wang J. et al., 2011). Being a cytokinin-deactivating gene, *UGT76C2* influences the expression of other cytokinin homeostasis and signaling genes: In *UGT76C2*-deficient plants, the positive regulators of the cytokinin status *AHK2*, *AHK3*, *ARR1*, and *IPT5* are down-regulated, while the negative regulator *CKX3* is upregulated (Wang J. et al., 2011). Generally, loss of UGT enzyme activity tends to be compensated by an increased *CKX* gene activity (Šmehilová et al., 2016). Transgenic plants overexpressing the *UGT76C2* gene show enhanced tolerance to water deficit suggesting a function of UGT76C2 in drought stress adaptation (Li et al., 2015).

In summary, *CKX4*, *CKX5,* and *UGT76C2* show crosstalk signaling with other cytokinin homeostasis and signaling genes, such as the receptor genes *AHK2*, *AHK3*, *AHK4* and the response regulator genes *ARR1* and *ARR2*, suggesting a complex network of balancing feed-forward and feed-back loops, and signal attenuation events that may be differentially shaped depending on cell type, tissue or the underlying conditions.

TRANSCRIPTION FACTOR GENES REGULATED BY CYTOKININ

The Arabidopsis genome harbors more than 1,600 genes encoding transcription factors, more than 5% of the protein-coding genes. Based on their phylogenetic relationship they can be grouped into at least 11 major families. Members of at least four families, ERF/AP2, zinc finger, LBD/ASL, and MYB, are directly or indirectly transcriptionally regulated by cytokinin.

Cytokinin-Responsive *CRF* Genes Have Roles in Diverse Areas Such as Stress Response and Development

According to sequence similarity, *CRF2*, *CRF5*, and *CRF6* are the three cytokinin-responsive genes of a group of six identified as the CRF (Cytokinin Response Factor) subset of ERF/AP2 transcription factor genes (Rashotte et al., 2006; Rashotte and Goertzen, 2010; Cutcliffe et al., 2011; Jeon et al., 2016). Among other functions, they play a major role in establishing adjustments to pathogens, wounding and cold (Müller and Munné-Bosch, 2015; Sun X. et al., 2020). All three of the encoded proteins contain a highly conserved DNA-binding AP2 domain in the central region. This domain is around 60 amino acids long (Weigel, 1995; Cutcliffe et al., 2011) and directly binds to the GCC-Box, which appears to be the key motif in the promoters of ethylene-responsive genes (Hao et al., 1998; Sakuma et al., 2002; Rashotte and Goertzen, 2010; Sun X. et al., 2020). Additionally, they have a C-terminal MAPK phosphorylation site

and an N-terminal CRF domain. Deletion constructs lacking the C-terminal domains of CRF5 demonstrated that the AP2 domain is required for target gene transcription (Cutcliffe et al., 2011; Striberny et al., 2017).

CRF proteins form dimers among each other, with the CRF domain functioning as the sole dimerization domain (Cutcliffe et al., 2011). They also interact with all histidine phosphotransmitter proteins and some of the type-A and type-B response regulator proteins of the TCS pathway, probably also by means of the CRF domain, but with none of the cytokinin receptors. Specific interactions between response regulators and CRF proteins were reported for CRF2 with ARR1, ARR7, ARR10, and ARR12, for CRF5 with ARR1 and ARR12, and for CRF6 with ARR6, ARR9, ARR10, and ARR11 (Rashotte et al., 2006; Cutcliffe et al., 2011; Jeon et al., 2016; Zwack et al., 2016). For *CRF2*, and *CRF5*, multiple type-B RR binding motifs were found in the 5′ region (Brenner et al., 2012; Brenner and Schmülling, 2015).

Outside of the cytokinin signaling network, CRFs influence the auxin transport machinery. Transcription of the two auxin efflux carrier genes *PIN1* and *PIN7* is directly up-regulated by CRFs binding to PIN CYTOKININ RESPONSE ELEMENTs (PCREs) in the promoter regions of *PIN1* and *PIN7*. Consequently, plants lacking CRF activity show aberrations in developmental patterning consistent with abnormal auxin distribution. Investigations of the root suggested that CRFs fine-tune root growth and development (Šimášková et al., 2015).

As demonstrated by mutant phenotypes, the CRF proteins act as developmental regulators in embryos, leaves, and cotyledons (Rashotte et al., 2006). Gene expression data suggest that *CRF2* is important for root development (Schlereth et al., 2010), highly expressed in seeds imbibed for 1 day, and moderately expressed in cotyledons and roots of 1-day-old seedlings, young leaves, seed forming organs and developing seeds (Klepikova et al., 2016). *CRF5* appears to have it highest expression rate in the shoot apex, in the female floral organs (particularly in the ovules), in mature seeds, in the root, and in the axis of the inflorescence. *CRF6* has its highest expression levels in petals, carpels, the first internode and in the mature leaves. It is not expressed in the embryo so that the first expression of *CRF6* is shown in the cotyledon of a 1-day-old seedling. Of all three cytokinin-regulated CRFs, *CRF2* has the highest expression level (Klepikova et al., 2016).

There is strong evidence that CRF2 plays a crucial role in the MONOPTEROS (MP) signaling pathway during *de novo* shoot apical meristem (SAM) generation in calli. The transcription factor MP directly binds to the *CRF2* promoter and positively regulates its expression, positioning CRF2 as a downstream signaling molecule of MP (Schlereth et al., 2010; Ckurshumova et al., 2014). Loss of function of *CRF2* totally abolished the increased shoot formation present in calli expressing a constitutively active variant of MP (Ckurshumova et al., 2014). The finding that CRF2 is a strong positive regulator of shoot regeneration from calli, more precisely the *de novo* establishment of SAMs, strongly suggests a role as a mediator of the cytokinin signal in this cytokinin-dependent process.

Besides its role in fine-tuning root growth and *de novo* SAM generation, the transcription factor CRF2 is involved in lateral root (LR) elongation. Together with CRF3, it promotes LR elongation, which is strongly reduced under cold stress in *crf2 crf3* double mutants (Jeon et al., 2016). Interestingly, cold-induced up-regulation of the *CRF2* transcript is partially dependent on the two-component signaling system (Jeon et al., 2016), indicating convergence of multiple signaling pathways upstream of the *CFR2* promoter. Whereas cytokinin inhibits LR initiation (Riefler et al., 2006; Laplaze et al., 2007; Bielach et al., 2012; Chang et al., 2015), it is involved in LR elongation, and cytokinin-responsive genes, among them CRF2, are expressed in emerging lateral roots. In summary, CRF2 is involved in shaping root system architecture in response to cold, and is probably also involved in the cellular signal transduction of other root growth responses mediated by cytokinin.

Cytokinin has a major function in delaying leaf senescence (Werner and Schmülling, 2009). Recently it was found that *CRF6* has a major role in dark-induced and stress-induced senescence and is most likely part of a fine-tuning system between both senescence pathways. Among the receptors, the main mediator of this response is *AHK3* (Kim et al., 2006). Furthermore, the CRF6 protein acts as a negative regulator in developmental leaf senescence and senescence caused by oxidative stress (Zwack and Rashotte, 2013; Zwack et al., 2016). From experiments with H_2O_2 it was concluded that *CRF6* has a function as a transcriptional suppressor repressing the expression of the type-A RRs *ARR6*, *ARR9*, and of the type-B RR *ARR11* in terms of signaling, *LOG7* in terms of cytokinin biosynthesis and *ABCG14* in terms of cytokinin transport (Zwack et al., 2016). The role of cytokinin and its downstream signaling components in alleviating diverse stresses is still not fully investigated. Research into this topic may lead to findings of potential importance in agriculture when applied in green biotechnology (Gan and Amasino, 1995).

In summary, cytokinin-regulated CRFs appear to mediate a number of cytokinin-related plant responses in different organs. As most of these CRFs are also regulators in other signaling pathways, they can be regarded as hubs for crosstalk and signal integration between cytokinin and stress-related signaling.

GIS3: A Link Between Cytokinin and Trichome/Root Hair Development

The protein encoded by *GIS3* (AT1G68360, *GLABROUS INFLORESCENCE STEMS*) is a member of the C2H2-type Zinc finger family of transcription factors and is induced by cytokinin as early as 15 min (Bhargava et al., 2013; Brenner and Schmülling, 2015). It is a direct target of the type-B response regulator ARR10 (Zubo et al., 2017). The C2H2 subfamily of zinc finger transcription factors contains the GIS subfamily made up of *ZFP5*, *ZFP6*, *ZFP8*, *GIS*, *GIS2*, and *GIS3* (Sun et al., 2015), two of which (*ZFP6*, *ZFP8*) were also reported as direct targets of ARR10 (Zubo et al., 2017). The main function of *GIS3* is in trichome development where it is a positive regulator of trichome cell differentiation and morphogenesis (Sun et al., 2015; Han et al., 2020). *GIS3* is primarily expressed in these tissues but additionally in roots (Schmid et al., 2005; Sun et al., 2015).

Cytokinin is a positive regulator of trichome formation in Arabidopsis, and it is even able to induce trichome formation when applied to organs that normally do not form trichomes, such as floral organs (Greenboim-Wainberg et al., 2005). Cytokinin signaling promoting trichome differentiation

is transduced through two other C2H2 zinc finger proteins, ZFP8 and GIS2, the latter being a cytokinin-inducible gene itself (Gan et al., 2007b). GIS3 acts upstream of GIS, GIS2 and ZFP8 to induce trichome development by binding to their promoters (Gan et al., 2007a,b; Sun et al., 2015). It was shown that the Gibberellin-activated signaling pathway plays a key role for trichome development (Gan et al., 2007b; Sun et al., 2015). Thus, GIS3 appears to be the signaling component that feeds the cytokinin signal into the module consisting of GIS, ZFP8, and GIS2 to integrate cytokinin and gibberellin signaling.

Another function of certain C2H2 zinc finger transcription factors regarding the development of epidermal layers is in root hair development in Arabidopsis, integrating cytokinin and gibberellin signals (Han et al., 2020). Consequently, the gene is mainly expressed in root hair cells (Zhou Z.-Y. et al., 2011; An et al., 2012) but additionally it is involved in initiation of inflorescence trichomes in response to gibberellin (Zhou Z.-Y. et al., 2011). Similar to trichome development, *GIS3* functions upstream of GIS, GIS2 and ZFP8, the latter being again directly targeted by GIS3 (Zhou Z.-Y. et al., 2011).

The involvement of virtually identical signaling molecules in the development of (unicellular) trichomes and root hairs in Arabidopsis underlines the idea that both structures are developmentally related. Consequently, cytokinin plays the same promoting role in the formation of both trichomes and root hairs.

Cytokinin-Regulated Genes Encoding LOB Domain Proteins Involved in Secondary Growth and Vascular Development

LATERAL ORGAN BOUNDARY DOMAIN (*LBD*) genes encode a plant-specific transcription factor family whose first discovered member *LOB* shows a ring-shaped expression pattern around the sites where lateral organs emerge from an axis (Shuai et al., 2002). These genes are also referred to as *ASL* (*AS2-like*) genes, based on their sequence similarity to ASYMMETRIC LEAVES2 (Iwakawa et al., 2002).

The *LBD3*/*ASL9* transcript was found to be induced by cytokinin in a number of transcriptomic studies (Rashotte et al., 2003; Kiba et al., 2005; Bhargava et al., 2013), but by no other hormone (Naito et al., 2007). Consistent with that, its promoter contains type-B response regulator binding sites (Brenner and Schmülling, 2015). No other LBD gene was rapidly responsive to cytokinin (Naito et al., 2007).

Another LBD gene regulated by cytokinin, albeit at later stages of the response (2 h after induction and later), is *LBD4* (AT1G31320). Unlike most cytokinin-responsive genes, which are regulated in the same way in root and shoot, *LBD4* is specifically upregulated in the root but not in the shoot (Brenner and Schmülling, 2012). *LBD4* was identified as part of a feed-forward loop in a transcriptional network analysis to identify signaling mechanisms controlling vascular development (Smit et al., 2020). This network transduces the signal of TDIF, a mobile CLE peptide perceived by the PXY receptor, leading to the upregulation of several WOX genes (Hirakawa et al., 2008, 2010; Etchells et al., 2013; Morita et al., 2016; Zhang et al., 2016). The ligand-receptor pair of TDIF and PXY is part of a regulatory loop between phloem and cambium controlling xylem differentiation through another transcription factor, BES1 (Kondo et al., 2014).

Both *LBD3* and *LBD4*, the two closest relatives among the LBD genes, are redundantly involved in vascular development as shown by mutant and overexpression analysis (Smit et al., 2020). Remarkably, *LBD4* is expressed at the phloem-procambium boundary, in accordance with the general expression pattern of genes of the LBD family at organ boundaries. It is hypothesized that LBD4 may function as a boundary regulator or as an enhancer of cell divisions at the phloem side of the procambium, or to have both functions. Since redundancy of *LBD3* and *LBD4* was determined, it is very likely that LBD3 acts in the same way. Both genes may act as a signaling hub feeding the cytokinin signal into the system controlling vascular development and cambial activity with *LBD4* acting as a factor differentiating between root and shoot.

MEE3: Signaling Hub Coupling Cytokinin With Auxin and ABA Signal Transduction

MEE3 (AT2G21650, *MATERNAL EFFECT EMBRYO ARREST 3*), also known as *RSM1* or *ATRL2*, belongs to the family of MYB-related transcription factors. It is a gene responding at a later time point to a cytokinin pulse, probably not being directly coupled to the phosphorelay network by the type-B response regulators.

MEE3 is essential for endosperm development, gravitropism, and photomorphogenesis. Additionally, it may have roles in embryo development, plant hormone interaction, floral development, and response to stress, and modulates seed germination and seedling development in response to abscisic acid and salinity (Riechmann and Ratcliffe, 2000; Pagnussat et al., 2005; Baxter et al., 2007; Hamaguchi et al., 2008; Yang et al., 2018).

During early photomorphogenesis, *MEE3* may be implicated in HOOKLESS1 (HLS1)-mediated auxin signaling, negatively regulating this pathway as a feedback regulator by a mechanism that is so far unknown (Hamaguchi et al., 2008). This observation may reflect part of the antagonistic effect of cytokinin on auxin action. The mutually inhibitory influence of the two hormones on each other's action is known since a long time from phenotypical observations and is realized on the molecular level through hormone homeostasis and signal inhibition (Dello Ioio et al., 2008; Müller and Sheen, 2008; Schaller et al., 2015). Thus, *MEE3* may be another piece to be added to the multi-faceted auxin–cytokinin interaction network.

MEE3 binds to the *ABI5* promoter driving the expression of a transcription factor negatively regulating seed germination, major mediator of abscisic acid (ABA) signal transduction and abiotic stress response (Finkelstein, 1994; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Nakamura et al., 2001; Yang et al., 2018). In addition, MEE3 physically interacts with the transcription factor HY5, which promotes photomorphogenesis and activates *ABI5* expression (Alabadí and Blázquez, 2008; Yang et al., 2018). This interaction between MEE3, HY5, and the *ABI5* promoter appears to modulate the sensitivity of several abscisic acid (ABA)-dependent processes to the hormone. This

way, cytokinin signaling couples into the ABA and abiotic stress response pathway by means of regulating *MEE3* expression.

All of the above leads to the conclusion that regulation of ABA-, auxin- and abiotic stress response may be partially mediated by *MEE3* as a secondary response gene of cytokinin during early morphogenesis (Hamaguchi et al., 2008; Yang et al., 2018).

LLM Domain-Containing GATA Transcription Factors Mediate Multiple Developmental Processes and Promote Chloroplast Development

GATA transcription factors belong to one of four subfamilies of the C2C2 zinc finger proteins. Characteristically, they bind to the consensus sequence (T/A)GATA(G/A), which was found in the promoters of many light-regulated genes (Teakle et al., 2002; Reyes et al., 2004). Among them, *CGA1*/*GNL*/*GATA22* was found to be transcriptionally regulated by cytokinin in a number of transcriptomic studies (Kiba et al., 2004; Brenner et al., 2005; Bhargava et al., 2013) as an early-responding gene, probably directly activated by type-B response regulators. In addition to cytokinin, the *CGA1* transcript is also regulated by nitrate (Price et al., 2004; Scheible et al., 2004; Wang et al., 2004; Bi et al., 2005), (red) light (Manfield et al., 2007), and sugar (Wang et al., 2003; Price et al., 2004; Scheible et al., 2004), and is under the control of the circadian clock (Harmer et al., 2000; Alabadì et al., 2002; Manfield et al., 2007). Although *CGA1* is co-regulated with seven other GATA transcription factor genes (*GATA15*, *GATA16*, *GATA17*, *GATA17L*, *GATA21*/*GNC*), all of which contain an LLM (Leu-Leu-Met) domain (Ranftl et al., 2016), it is special with regards to its particularly strong reaction to cytokinin. Of these, the two paralogs *GATA21*/*GNC* and *GATA22*/*GNL*/*CGA1* are repressed by the homeotic floral organ identity transcription factors AP3 and PI (Mara and Irish, 2008). Higher-order mutants of these transcription factor genes showed defects in several cytokinin-regulated developmental processes such as phyllotaxis, cytokinin-induction of leaf greening and suppression of chlorophyll degradation during leaf senescence, branching and plant height, the number of floral organs and silique length (Ranftl et al., 2016).

CGA1 has multiple roles in plant development and physiology. In terms of crosstalk with other hormones, it represses gibberellin signaling downstream of the DELLA proteins and PIFs (Richter et al., 2010), enabling a negative regulation of gibberellin signaling by cytokinin. Consistently, plants overexpressing CGA1 show an altered timing of numerous developmental events such as germination, leaf production, flowering and senescence (Hudson et al., 2011). CGA1 was suspected as a point of convergence of cytokinin, light, and gibberellin signaling (Köllmer et al., 2011). The repressive effect of *CGA1* on flowering time is mediated by direct transcriptional repression of the flowering time regulator *SOC1*, simultaneously influencing greening (Bastakis et al., 2018) and cold tolerance (Richter et al., 2013a). In addition, auxin signaling converges at *CGA1*, repressing its expression through ARF7 (Richter et al., 2013b).

Mutant analysis revealed that CGA1 promotes chlorophyll biosynthesis by modulating the expression of a number of chlorophyll biosynthesis genes (Mara and Irish, 2008; Hudson et al., 2011). However, not only chlorophyll biosynthesis is regulated by *CGA1*, but chloroplast proliferation in all aspects, development, growth, and division. For these processes, *CGA1* was assigned the role of a master regulator because overexpression causes ectopic chloroplast development even in roots or in darkness (Chiang et al., 2012; Zubo et al., 2018). From the analysis of mutants, it was also concluded that the positive effect of cytokinin on chloroplasts is at least partially transduced through CGA1. During wound-induced root greening, *CGA1* and *GNC* are important factors transducing the cytokinin signal, but the exact way how CGA1 and other GATA transcription factors induce the transcription of photosynthesis-related genes is not known (Kobayashi and Iwase, 2017; Kobayashi et al., 2017). These findings are consistent with the observation that cytokinin shifts the root transcriptome toward a more shoot-like profile, which may be largely due to chloroplast genes becoming expressed in the root after an extended period of cytokinin treatment (Brenner and Schmülling, 2012).

In terms of metabolism, CGA1 positively regulates the expression of *GLU1* encoding the chloroplast-localized GLUTAMATE SYNTHASE1, the primary enzyme controlling nitrogen assimilation in green tissue and providing substrate for chlorophyll biosynthesis (Hudson et al., 2011). This may be another section of nitrate signaling mediated by cytokinin, coupling into processes related to greening and photosynthesis.

SIGNALING BY TARGETED PROTEIN DEGRADATION

AFP2: An ABA Signaling Component Targeting ABI5 for Proteasomal Degradation

AFP2 (AT1G13740, *ABI FIVE-BINDING PROTEIN*) belongs to a small family of five genes in Arabidopsis (Garcia et al., 2008), whose members bind to the transcription factor and key regulator of the ABA response ABI5, thereby attenuating the ABA response by targeting ABI5 for ubiquitin-mediated degradation (Lopez-Molina et al., 2003). All these proteins share three conserved domains of unknown function (Garcia et al., 2008). Additionally, the transcriptional repression of ABI5 target genes may be mediated by recruitment of a co-repressor of the TOPLESS family (Pauwels et al., 2010; Causier et al., 2012; Lynch et al., 2017). AFP proteins also interact with themselves and other members of the AFP family, and, remarkably, also with histone deacetylases, providing another level of gene regulation by chromatin modification. AFP2 has also emerged as a regulator for breaking heat-induced secondary seed dormancy (Chang et al., 2018) and as a factor delaying flowering time (Chang et al., 2019).

Cytokinin negatively regulates ABA-dependent responses such as drought and salt tolerance (Tran et al., 2007). Thus, cytokinin-induced upregulation of AFP2 may be one of the molecular links mediating the negative influence of cytokinin on ABA signaling. Another mechanism of cytokinin-ABA signaling crosstalk is the direct interaction of several type-A RRs with ABI5, inhibiting its function as a transcription factor (Wang Y. et al., 2011).

CFB: A Cytokinin-Regulated Gene Directly Interfering With a Key Enzyme of Sterol Biosynthesis

CFB (At3G44326, *CYTOKININ-REGULATED F-BOX PROTEIN*) has emerged as one of the most robustly upregulated genes after cytokinin treatment in meta analyses of microarray experiments and RNA-Seq transcriptomics (Bhargava et al., 2013; Brenner and Schmülling, 2015). It is an early-responding gene and as such probably directly activated by type-B response regulators. It encodes an F-box protein belonging to a small group of three related proteins in Arabidopsis (Brenner et al., 2017). Orthologs exist in all land plants. The group of CFB-like proteins is characterized by an F-box carrying the unique motif ILTRLDG not found in the F-box domain of any other F-box protein. In addition, the proteins possess five domains of unknown function, two highly conserved sequence motifs, and a C-terminal transmembrane domain.

The CFB protein interacts with the only cycloartenol synthase enzyme in Arabidopsis, CAS1, thereby downregulating a bottleneck step in plant sterol biosynthesis. The resulting accumulation of 2,3-oxidosqualene in young shoot tissue causes a disturbed and delayed development of chloroplasts resulting in white shoot tips. In which tissues and for what purpose a possible downregulation of sterol biosynthesis by cytokinin may be relevant for plant development or other processes is not known.

SMALL DOWNSTREAM EFFECTORS

TPS1 and Trehalose-6-Phosphate: Cytokinin Influencing Primary Metabolism

Trehalose-6-phosphate (T6P) is a major signaling molecule in plants regulating sucrose levels, hence it is referred to as "the plant insulin." Levels of free sucrose in tissues are regulated by the formation or degradation of starch. This regulation is governed by T6P, the levels of which are highly positively correlated to sucrose levels, leading to the formation of a homeostatic feedback regulatory circuit referred to as the sucrose-T6P nexus (Figueroa and Lunn, 2016).

T6P homeostasis is governed by two enzymatic activities, trehalose-6-phosphate synthase (TPS) for synthesis, and trehalose-6-phosphate phosphatase (TPP) for degradation. Transcriptomic experiments have revealed that genes encoding these two types of enzymes are regulated in a reciprocal manner by cytokinin: Upon cytokinin treatment, *TPS* transcripts are more abundant and *TPP* transcripts are less abundant, while in cytokinin-deficient plants the opposite is true (Brenner et al., 2005). Thus, T6P levels are likely to be increased under cytokinin treatment while T6P levels are probably reduced in cytokinin-deficient plants. T6P directs primary metabolism toward a more consumptive mode, thus a cytokinin-induced increase would be consistent with the generally proliferative action of the hormone. However, it is not clear whether *TPS1* (AT1G78580), which appears to encode the major T6P biosynthetic enzyme (Fichtner et al., 2020), and the other enzymes involved in T6P homeostasis are directly regulated by the cytokinin-dependent TCS signaling network or whether the homeostatic regulation mentioned above is an indirect effect of altered sucrose levels due to cytokinin modulating carbohydrate consumption by, e.g., growth processes. Motifs that are demonstrated to bind type-B response regulators (Franco-Zorrilla et al., 2014) or that are enriched in cytokinin-responsive promoters (Brenner and Schmülling, 2015) are present in the promoter region of TPS1, favoring the idea of direct manipulation of T6P homeostasis and the associated changes in primary metabolism by cytokinin.

PSK2: Phytosulfokine as a Downstream Signal of Cytokinin Leading to Its Proliferating and Chloroplast-Promoting Action?

Phytosulfokine (PSK) is a 5 aa-long peptide sulfated at two tyrosine residues that was first identified in conditioned medium of plant cell cultures, where it is the primary signal molecule for cell-cell communication promoting callus growth. Due to that property, PSK can be regarded as a plant growth factor. There are at least five PSK precursor genes in Arabidopsis, of which the *PSK2* gene (AT2G22860) is induced by cytokinin (Rashotte et al., 2003; Brenner et al., 2005). Genes encoding proteases and tyrosylprotein sulfotransferases processing the PSK precursor proteins were also identified in the Arabidopsis genome, as well as respective receptors (Matsubayashi et al., 2006a,b).

Besides its effects on callus proliferation, PSK is also associated with a number of events associated with growth and proliferation in whole plants. The PSK transcripts in rice are highly expressed in the proliferating zones of the root and shoot meristems (Yang H. et al., 1999). PSK promotes adventitious bud formation in *Antirrhinum* (Yang G. et al., 1999), adventitious root formation from hypocotyls in cucumber (Yamakawa et al., 1998b), somatic embryogenesis (Kobayashi et al., 1999; Hanai et al., 2000; Igasaki et al., 2003), and pollen germination and growth (Chen et al., 2000; Stührwohldt et al., 2015). It also enhances chlorophyll biosynthesis in the dark such as during the night or under etiolating conditions (Yamakawa et al., 1998a; 1999). Finally, PSK promotes the differentiation of tracheary elements (Matsubayashi et al., 1999) and retards stress-induced senescence (Yamakawa et al., 1999).

Many of these PSK functions overlap with the effects observed by cytokinin and are in accordance with the generally proliferative, growth-promoting and anti-senescence action of the hormone. Thus, it is tempting to speculate that PSK may be an important downstream signal of cytokinin. The *PSK2*

promoter contains several motifs either found to be bound by type-B response regulators or enriched in the promoters of other cytokinin-induced genes, encouraging investigations into the *PSK2* gene as part of the downstream signaling network of cytokinin.

TRANSPORT ACROSS MEMBRANES

PILS5, a Player in Cytokinin–Auxin Interactions

PILS5 (AT2G17500, *PIN-LIKES 5*) encodes a PIN transporter-like auxin efflux carrier protein and is induced by cytokinin during the late response (≥120 min) (Brenner et al., 2005; Brenner and Schmülling, 2015). There are seven members in the PIN-LIKES family. PILS proteins have predicted topological similarities to PIN-FORMED proteins, despite the circumstance that they only share 10–18% of their sequence (Feraru et al., 2012; Sun L. et al., 2020). PILS family members were identified by the presence of the auxin carrier domain spanning nearly the whole length of the PILS protein. Due to that domain, PILS proteins are predicted to have auxin transport function (Barbez et al., 2012). However, it is difficult to pinpoint functional residues within the domain. Moreover, nothing is known about possible post-translational modifications, but generic phosphorylation sites, kinase specific phosphorylation sites and isoform variations were predicted (Blom et al., 1999, 2004). Furthermore, different numbers of serine, threonine and tyrosine phosphorylation sites were used to assign three different classes of PILS proteins. *PILS5* was grouped into class one because it has less than 10 phosphorylation sites (Feraru et al., 2012).

Interestingly and in contrast to the proper PIN transporters, the subcellular localization of PILS proteins is in the ER (Barbez et al., 2012). For that reason, expression of PILS transporters results in a retention of auxin within cells. They sequester auxin at the ER, limiting active auxin availability in the nucleus, thereby attenuating auxin signaling and decreasing cellular sensitivity to auxin (Barbez et al., 2012; Feraru et al., 2012, 2019; Béziat et al., 2017). Furthermore, it affects auxin homeostasis and signaling by regulating the auxin conjugation rate and its intracellular accumulation. Consequently, *PILS5* gain-of-function results in multiple phenotypic changes consistent with a low auxin status regarding root organ growth (lateral root formation positively, root-hair elongation negatively), growth regulation in general, as well as seedling growth and development (Barbez et al., 2012; Dal Bosco et al., 2012; Feraru et al., 2012; Barbez and Kleine-Vehn, 2013; Sun L. et al., 2020).

Phylogenetic analyses revealed that PILS proteins are probably older than PIN-FORMED proteins, hence intracellular auxin accumulation is evolutionary older PIN dependent auxin transport (Feraru et al., 2012). Nearly all family members except for PILS4 originate from lineage specific duplications. They are grouped into three different clades with PILS5 grouped into Clade III (Feraru et al., 2012).

Transcription of *PILS5* is strongly dependent on auxin, cytokinin and brassinosteroid levels (Sun L. et al., 2020). Additionally, abiotic factors such as light and temperature, repress *PILS5* expression, leading to growth effects reminiscent of a higher auxin status (Feraru et al., 2012; Béziat et al., 2017; Sun L. et al., 2020). The gene is expressed during all developmental stages, specifically in mature pollen (Klepikova et al., 2016), seedling, cauline leaves, and flowers (Barbez et al., 2012). Through the well-known antagonistic action between auxin and cytokinin, *PILS5* indirectly affects homeostasis and signaling of cytokinin (Kuderová et al., 2008; Naseem and Dandekar, 2012). That antagonism may be accomplished by auxin mediated shifts in pH that regulate cytokinin receptor activity (Werner and Schmülling, 2009). A more direct signaling mechanism is the upregulation of certain type-A response regulator genes by the auxin signal transduction (Müller and Sheen, 2008). AUXIN RESPONSE FACTOR3 represses cytokinin biosynthesis and signaling at multiple levels (Zhang et al., 2018). During plant development these interactions are important, e.g., for cell specification, growth and size of plant structures both below-ground and above-ground (Müller and Sheen, 2007; Taniguchi et al., 2007; Dello Ioio et al., 2008).

In summary, *PILS5* promotes auxin accumulation at the ER, thereby repressing auxin signaling (Barbez et al., 2012; Feraru et al., 2012; Sun L. et al., 2020). As cytokinin supposedly increases PILS5 activity by transcriptionally activating the corresponding gene, PILS5 may be one of the players that mediate the negative influence of cytokinin on auxin signaling, making it a factor in mediating crosstalk of cytokinin and auxin.

DTX36: A Transmembrane Export Protein Probably Involved in Abiotic Stress Response

DTX36 (At1g11670, *DETOXIFICATION 36*) encodes a MATE-related efflux protein located in membranes, particularly in the plasma membrane (Li et al., 2002; Gaudet et al., 2011). It is part of a gene family of at least 56 members mediating the efflux of endo- and exogenous toxic compounds and heavy metals (Li et al., 2002). Upregulated at 15 min after cytokinin treatment, *DTX36* is an early cytokinin response gene (Bhargava et al., 2013; Brenner and Schmülling, 2015), probably directly activated by type-B response regulators. Furthermore, the gene is regulated by the cell cycle peaking in the G1 phase (Menges et al., 2002). Another process during which *DTX36* is regulated is photomorphogenesis induced by the phytochrome pathway: *fhy3* and *far1* mutants show reduced *DTX36* expression (Hudson et al., 2003).

DTX36 is expressed in nearly every structure from seed, root, shoot, leaves, to inflorescence structures (Schmid et al., 2005; Obulareddy et al., 2013). The highest expression levels were found in seeds after 3 days of soaking and in the root apex of seedlings, whereas the lowest expression levels were found in dry seeds (Klepikova et al., 2016). Generally, the expression in the roots was higher than in aboveground organs.

Cytokinin has been implicated in stress responses in numerous studies (Naseem et al., 2014; Bielach et al., 2017; Yang and Li, 2017; Huang et al., 2018; Kieber and Schaller, 2018; Cortleven et al., 2019). Here, an unspecific stress response gene is induced by cytokinin in an immediate-early fashion, further

corroborating the function of cytokinin as a hormone involved in stress response.

At2g34350: A Nodulin-Like Major Facilitator Superfamily Gene With Links to Biotic and Abiotic Stress

According to sequence similarity, the gene At2g34350 is a Nodulin-like major facilitator superfamily protein. As a member of this family, it is probably involved in transmembrane transport of hydrophilic molecules or water itself. Genes of this family are mainly associated with the response to abiotic stress, but also to biotic stress (Bezerra-Neto et al., 2019).

The gene is primarily expressed in the root apex (Klepikova et al., 2016) and is induced by cytokinin as a late (120 min) response gene (Rashotte et al., 2003; Bhargava et al., 2013). Its cytokinin-dependent expression pattern was further confirmed in plants overexpressing *ARR22*, a negative regulator of cytokinin signaling (Wallmeroth et al., 2017, 2019), where its transcript levels were lower (Kiba et al., 2004).

Furthermore, the gene is also regulated by salt stress (Sottosanto et al., 2004) and the jasmonate signaling pathway (Chini et al., 2007), corroborating the idea that it has a role in biotic and abiotic stress response. Its exact function, however, has not yet been investigated.

GENES WITH OTHER FUNCTIONS

AHK1: A Probable Osmosensor

Another gene induced by cytokinin is *AHK1* (AT2G17820, *ARABIDOPSIS HISTIDINE KINASE 1*) (Brenner and Schmülling, 2012; Bhargava et al., 2013). The gene encodes a member of the histidine kinase family and is involved in response to osmotic stress, response to water deprivation, seed maturation and stomatal complex patterning (Tran et al., 2007; Wohlbach et al., 2008; Kumar et al., 2013). Unlike the three cytokinin receptors AHK2, AHK3, and AHK4, which belong to the same family, AHK1 is an osmosensor, but – lacking the cytokinin-binding CHASE domain – not a cytokinin sensor. Just like the cytokinin receptors, AHK1 acts according to the principle of histidine phosphotransfer (Urao et al., 1999).

The gene is expressed in nearly every plant structure in relatively even levels (Schmid et al., 2005; Obulareddy et al., 2013; Klepikova et al., 2016). The subcellular localization of the Arabidopsis protein and at least one of the poplar orthologs is in the plasma membrane (Caesar et al., 2011; Héricourt et al., 2013, 2016).

AHK1 is suggested to be a positive regulator in stress response through ABA-dependent and ABA-independent signaling pathways. Furthermore, it has a major role in plant growth (Tran et al., 2007). Additionally, it is a necessary player to prevent desiccation during seed development and also in vegetative tissues (Wohlbach et al., 2008). How water limitation is actually sensed is not finally clarified, however, the predicted extracellular domain is essential for its activity (Urao et al., 1999). AHK1 most likely integrates mechanisms such as sensing of cell volume, shape, turgor pressure, or macromolecular crowding to a downstream signal that is so far unknown (Kumar et al., 2013). Interestingly, it was shown in poplar that the cytokinin receptors are also able to interact with those histidine phosphotransmitter proteins through which the poplar orthologs of AHK1 signal (Héricourt et al., 2019). Conversely, the poplar orthologs of AHK1 are unable to interact with a subset of histidine phosphotransfer proteins that are uniquely interacting with the cytokinin receptors. Thus, in poplar, crosstalk can happen from cytokinin into the osmosensing pathway, but not vice versa. Similar investigations in Arabidopsis are missing.

MDL3: A Gene of Unknown Function With Links to Diverse Abiotic Stresses

The *MDL3* gene (At3G51660, MACROPHAGE MIGRATION INHIBITORY FACTOR/D-DOPACHROME TAUTOMERASE-LIKE PROTEIN 3) is expressed in nearly every Arabidopsis plant structure including even plant sperm cells and guard cells (Schmid et al., 2005; Obulareddy et al., 2013; Klepikova et al., 2016). The highest expression levels were found in the petioles of senescent leaves and in the pods of older siliques (Klepikova et al., 2016). The encoded protein is an LS1-like protein belonging to the tautomerase/MIF superfamily and is localized in the peroxisomes (Reumann et al., 2007).

Proteins of this family are found in mammalian and non-mammalian organisms and are known as upstream mediators of various immune responses. In plants it most likely integrates intracellular effects and induces precursor proteins which are part of the secondary plant metabolite signaling pathway (Panstruga et al., 2015; Sparkes et al., 2017). The transcript is induced as early as 15 min after cytokinin treatment (Brenner et al., 2005; Brenner and Schmülling, 2015), by cold stress (Mori et al., 2018), osmotic stress, wounding, and UV-B radiation (Panstruga et al., 2015). The protein is most likely a part of self-protection of plants in response to pathogens and environmental stress (Reumann et al., 2007; Ascencio-Ibáñez et al., 2008; Panstruga et al., 2015; Mori et al., 2018) and therefore possibly also part of cytokinin-mediated stress responses. Its subcellular localization in the peroxisome substantiates a possible function in defense and/or detoxification mechanisms (Reumann et al., 2007).

DISCUSSION

In the previous paragraphs, accumulated knowledge about a selection of cytokinin-regulated genes was collected and summarized (**Table 1**). The selection of genes was based on the number of occurrences primary literature about cytokinin-related transcriptomic studies (Brenner et al., 2012; Bhargava et al., 2013; Brenner and Schmülling, 2015). These genes can be regarded as a subset of the most reliably cytokinin-regulated genes. We focused our selection on signaling genes, but included genes with other functions as well if significant knowledge was found in the literature.

Collecting information available in the literature has revealed numerous functional connections between cytokinin and processes such as plant development, primary metabolism, biotic and abiotic stress response, cytokinin homeostasis and

phytohormone crosstalk. These connections between cytokinin-regulated genes and plant processes known to be controlled by them are summarized in **Figure 1**. The scheme shows how the cytokinin signal splits up into several strands, each one transduced by its own component to result in the different hormonal actions.

One conspicuous observation is that although the percentage of kinase-encoding genes in the Arabidopsis genome is >3%, there was only one cytokinin-regulated (0.05%) kinase found among the set of genes considered as the most reliably cytokinin regulated ones outside of the phosphorelay signaling system. The activity of kinases is usually regulated by means other than their mere abundance, and the transcript levels of genes encoding kinases are often quite stable under a wide range of conditions. Kinases are rather regulated by other means such as the presence of ligands or posttranslational modification. This way, transcriptomic experiments do not necessarily shed light on the kinase parts of signaling pathways, limiting the approach using transcriptomic data in this respect. On the other hand, transcriptomic data are easy to generate and may serve as a starting point for in-depth investigations leading to the discovery of signaling chains involving events other than transcriptional regulation such as kinase activities.

Feed-Forward and Feed-Back Loops Must Be Spatially and Temporally Separated

It is known that there is an immediate feed-back loop built into the phosphorelay system in the form of the type-A response regulators (Kiba et al., 2003; To et al., 2004; Lee et al., 2007). The existence of feed-back at the level of hormone homeostasis by deactivating and degrading enzymes has also been noted (Kieber and Schaller, 2014). Generally, feed-back loops are a frequently emerging theme in developmental biology, and their roles are exhaustingly covered.

In contrast, the role of a feed-forward mechanism at the level of cytokinin homeostasis has not found much attention. It is generally contradicting the paradigm of the self-limiting action of the hormone in order to maintain stable developmental processes. One scenario that requires escalating a signal by a feed-forward mechanism is rapid long-distance signaling as it happens, for instance, during the propagation of the electrical signal in the axons of nerve cells followed by a delayed feed-back mechanism.

It is certain that cytokinin is the long-distance signal to transmit nitrogen availability to the shoot tip in order to control a sustainable growth rate of the shoot (Miyawaki et al., 2004; Takei et al., 2004a). The cytokinins transported shootward in the xylem belong to the tZ type, the members of which are catalytically formed by the CYP735A enzymes from iP-type cytokinins (Takei et al., 2004b; Kiba et al., 2013). The *CYP735A2* gene is responsive to both nitrate and cytokinin and mainly expressed in the root, but it has not been determined in which cell types it is expressed. It is tempting to speculate that CYP735A2 induced in cells neighboring xylem elements (e.g., xylem parenchyma cells) may lead to a rapid increase of tZ-type cytokinins in the xylem vessels, even more so as the protein is predicted to be localized in the apoplast. The increase of active tZ would in turn trigger the induction of CYP735A2 activity in cells further upstream, releasing more tZ into the xylem in the shootward direction. This mechanism could speed up the migration of the tZ signal beyond the velocity of the xylem stream. Thus, the signal would travel at a speed largely independent of the velocity of the water stream in the xylem vessels, which strongly depends on the transpiration rate, and would be driven by the feed-forward loop of biosynthesis, perception and signaling, rapidly propagating over long distances. To this end, *CYP735A2* should be expected to be expressed in cells neighboring the xylem vessels, such as xylem parenchyma cells. This hypothesis, however, remains to be tested.

Feed-forward and feed-back mechanisms have to be carefully controlled as they may form a wasteful short-circuit if they are active at the same time or place. Thus, it is to be expected to find feed-forward components distinctly from feed-back components. In certain situations, there may be a temporal succession of a feed-forward phase followed by a feed-back phase to first escalate the cytokinin signal before seeking homeostasis.

Multi-Layered Cytokinin–Auxin Interplay

Auxin and cytokinin action are closely interwoven and each of the two hormone influences the status of the other at multiple layers and through multiple signaling pathways, mostly in an antagonistic fashion (Dello Ioio et al., 2008; Müller and Sheen, 2008). Components of that largely unknown network continue to be discovered, such as *SYAC1* very recently (Hurný et al., 2020).

Some of the cytokinin–auxin crosstalk components, however, have been characterized. Polar auxin transport may be influenced via CRFs transcriptionally regulating PIN expression (Šimášková et al., 2015). The cytokinin-induced MYB-related transcription factor MEE3 is a negative regulator of the HOOKLESS1-dependent auxin signaling pathway during early seedling morphogenesis (Hamaguchi et al., 2008). Cytokinin-stimulated *PILS5* expression may sequester auxin into the ER, removing it from the nucleus where it is supposed to exhibit its activity (Barbez et al., 2012; Feraru et al., 2012; Sun L. et al., 2020). All three examples of crosstalk show a negative influence of cytokinin to auxin action. On the other hand, cytokinin upregulates auxin biosynthesis by increasing *TAA1* and *YUCCA8* expression (Jones et al., 2010; Zhou Z. et al., 2011; Schaller et al., 2015; Di et al., 2016). The crucial function of the two hormones acting in complementary patterns in many developing structures of the plant has been reviewed to great detail (Schaller et al., 2015).

Downstream Effectors Mediate Part of the Cytokinin Action

Despite the discovery of multiple signaling hubs mediating crosstalk between cytokinin and other pathways for hormone, environmental, and developmental signals, it is still not understood how the multitude of hormonal effects comes into action. Light may be shed on parts of these unknown links by investigating how cytokinin affects the levels and activities of downstream effectors. Cytokinin has been implicated in the regulation of sink–source relationships (Werner et al., 2008; Kieber and Schaller, 2014). The finding that genes responsible

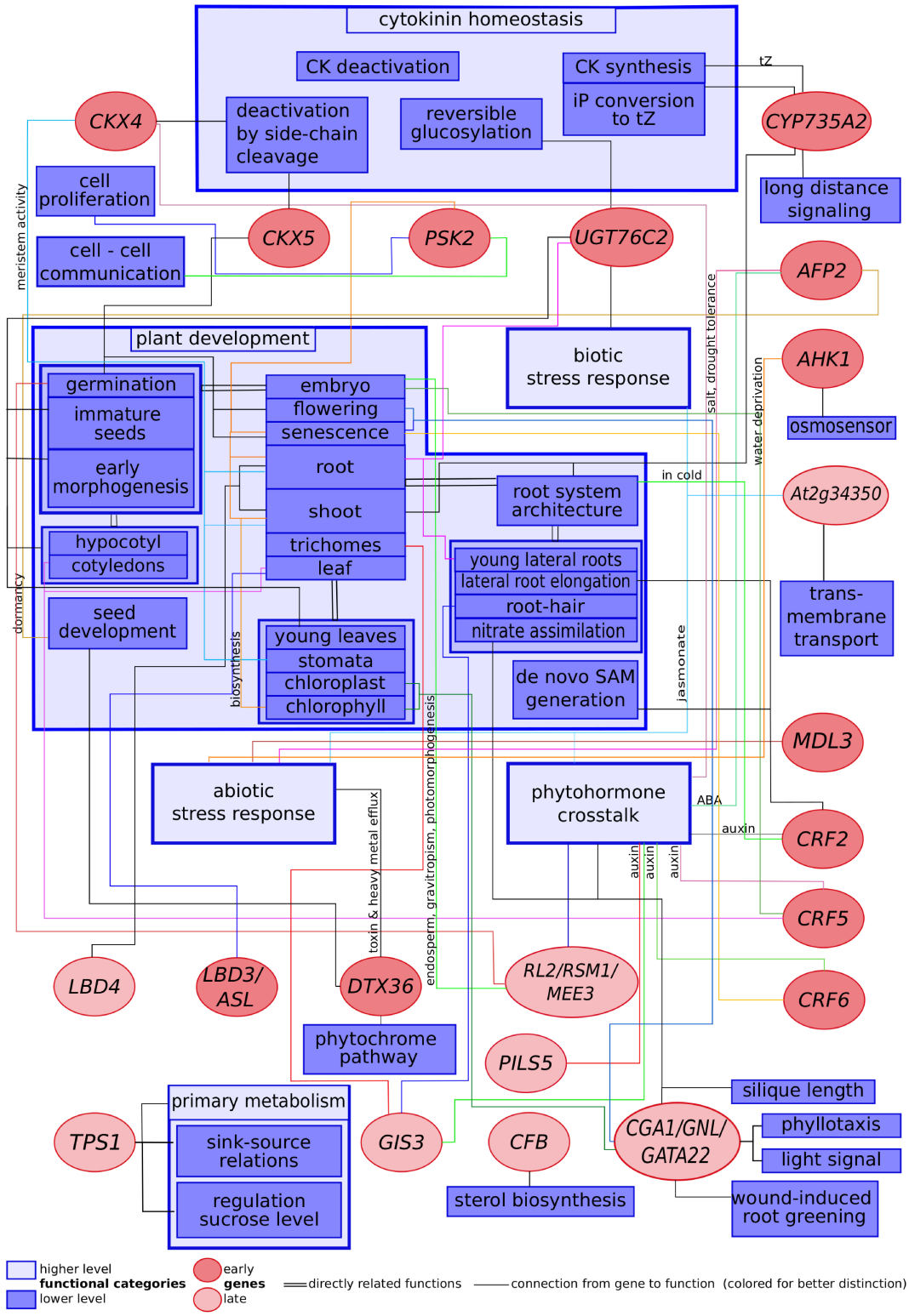


FIGURE 1 | Scheme showing selected cytokinin-regulated genes related to their functions described in published literature.

for the homeostasis of a master regulator of primary metabolism, trehalose-6-phosphate, are differentially regulated in cytokinin-treated and cytokinin-deficient plants may give a clue on how the cytokinin signal is integrated into the control of primary metabolism.

The general proliferative effect of cytokinin may be mediated by phytosulfokine (PSK), as one of the five PSK precursor genes is positively regulated by the hormone. Not only is PSK regarded as the plant growth factor, but it is also implicated in chloroplast development and other processes driven by cytokinin. Thus, it is tempting to speculate that PSK is a downstream regulator for a significant part of the hormonal action of cytokinin regarding plant development. However, a conclusive loss-of-function experiment is missing due to difficulties obtaining a pertinent mutant.

AUTHOR CONTRIBUTIONS

CK selected the genes according to their frequency of occurrence in published transcriptomic studies, collected published information about genes and wrote part of the manuscript. WB initiated the research, supervised the writing process, collected published information about genes, wrote part of the manuscript, including introduction and discussion. Both authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We acknowledge support from Leipzig University for Open Access Publishing.

REFERENCES

Alabadí, D., and Blázquez, M. A. (2008). Integration of light and hormone signals. *Plant Signal. Behav.* 3, 448–449. doi: 10.4161/psb.3.7.5558

Alabadı̀, D., Yanovsky, M. J., Más, P., Harmer, S. L., and Kay, S. A. (2002). Critical role for CCA1 and LHY in maintaining circadian rhythmicity in *Arabidopsis*. *Curr. Biol.* 12, 757–761. doi: 10.1016/S0960-9822(02)00815-1

An, L., Zhou, Z., Sun, L., Yan, A., Xi, W., Yu, N., et al. (2012). A zinc finger protein gene ZFP5 integrates phytohormone signaling to control root hair development in *Arabidopsis*. *Plant J.* 72, 474–490. doi: 10.1111/j.1365-313X.2012.05094.x

Argueso, C. T., Ferreira, F. J., and Kieber, J. J. (2009). Environmental perception avenues: the interaction of cytokinin and environmental response pathways. *Plant Cell Environ.* 32, 1147–1160. doi: 10.1111/j.1365-3040.2009.01940.x

Argyros, R. D., Mathews, D. E., Chiang, Y.-H., Palmer, C. M., Thibault, D. M., Etheridge, N., et al. (2008). Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. *Plant Cell* 20, 2102–2116. doi: 10.1105/tpc.108.059584

Ascencio-Ibáñez, J. T., Sozzani, R., Lee, T.-J., Chu, T.-M., Wolfinger, R. D., Cella, R., et al. (2008). Global analysis of *Arabidopsis* gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol.* 148, 436–454. doi: 10.1104/pp.108.121038

Barbez, E., and Kleine-Vehn, J. (2013). Divide Et Impera—cellular auxin compartmentalization. *Curr. Opin. Plant Biol.* 16, 78–84. doi: 10.1016/j.pbi.2012.10.005

Barbez, E., Kubeš, M., Rolčík, J., Béziat, C., Pěnčík, A., Wang, B., et al. (2012). A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. *Nature* 485, 119–122. doi: 10.1038/nature11001

Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmülling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. *Plant Cell* 23, 69–80. doi: 10.1105/tpc.110.079079

Bastakis, E., Hedtke, B., Klermund, C., Grimm, B., and Schwechheimer, C. (2018). LLM-Domain B-GATA transcription factors play multifaceted roles in controlling greening in *Arabidopsis*. *Plant Cell* 30, 582–599. doi: 10.1105/tpc.17.00947

Baxter, C. E. L., Costa, M. M. R., and Coen, E. S. (2007). Diversification and co-option of RAD-like genes in the evolution of floral asymmetry. *Plant J.* 52, 105–113. doi: 10.1111/j.1365-313X.2007.03222.x

Bezerra-Neto, J. P., Czekalski, de Araújo, F., Ferreira-Neto, J. R. C., da Silva, M. D., Pandolfi, V., et al. (2019). Plant aquaporins: diversity, evolution and biotechnological applications. *Curr. Protein Pept. Sci.* 20, 368–395. doi: 10.2174/1389203720666181102095910

Béziat, C., Barbez, E., Feraru, M. I., Lucyshyn, D., and Kleine-Vehn, J. (2017). Light triggers PILS-dependent reduction in nuclear auxin signalling for growth transition. *Nat. Plants* 3:17105. doi: 10.1038/nplants.2017.105

Bhargava, A., Clabaugh, I., To, J. P., Maxwell, B. B., Chiang, Y.-H., Schaller, G. E., et al. (2013). Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-Seq in *Arabidopsis*. *Plant Physiol.* 162, 272–294. doi: 10.1104/pp.113.217026

Bi, Y.-M., Zhang, Y., Signorelli, T., Zhao, R., Zhu, T., and Rothstein, S. (2005). Genetic analysis of *Arabidopsis* GATA transcription factor gene family reveals a nitrate-inducible member important for chlorophyll synthesis and glucose sensitivity. *Plant J.* 44, 680–692. doi: 10.1111/j.1365-313X.2005.02568.x

Bielach, A., Hrtyan, M., and Tognetti, V. B. (2017). Plants under stress: involvement of auxin and cytokinin. *Int. J. Mol. Sci.* 18:1427. doi: 10.3390/ijms18071427

Bielach, A., Podlešáková, K., Marhavı, P., Duclercq, J., Cuesta, C., Müller, B., et al. (2012). Spatiotemporal regulation of lateral root organogenesis in *Arabidopsis* by cytokinin. *Plant Cell* 24, 3967–3981. doi: 10.1105/tpc.112.103044

Bilyeu, K. D., Cole, J. L., Laskey, J. G., Riekhof, W. R., Esparza, T. J., Kramer, M. D., et al. (2001). Molecular and biochemical characterization of a cytokinin oxidase from maize. *Plant Physiol.* 125, 378–386. doi: 10.1104/pp.125.1.378

Bishopp, A., Lehesranta, S., Vatén, A., Help, H., El-Showk, S., Scheres, B., et al. (2011). Phloem-transported cytokinin regulates polar auxin transport and maintains vascular pattern in the root meristem. *Curr. Biol.* 21, 927–932. doi: 10.1016/j.cub.2011.04.049

Blom, N., Gammeltoft, S., and Brunak, S. (1999). Sequence and structure-based prediction of eukaryotic protein phosphorylation sites1 1edited by F. E. Cohen. *J. Mol. Biol.* 294, 1351–1362. doi: 10.1006/jmbi.1999.3310

Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4, 1633–1649. doi: 10.1002/pmic.200300771

Brenner, W. G., Leuendorf, J. E., Cortleven, A., Martin, L. B. B., Schaller, H., and Schmülling, T. (2017). Analysis of CFB, a cytokinin-responsive gene of *Arabidopsis thaliana* encoding a novel F-box protein regulating sterol biosynthesis. *J. Exp. Biol.* 68, 2769–2785. doi: 10.1093/jxb/erx146

Brenner, W. G., Ramireddy, E., Heyl, A., and Schmülling, T. (2012). Gene regulation by cytokinin in *Arabidopsis*. *Front. Plant Sci.* 3:8. doi: 10.3389/fpls.2012.00008

Brenner, W. G., Romanov, G. A., Köllmer, I., Bürkle, L., and Schmülling, T. (2005). Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. *Plant J.* 44, 314–333. doi: 10.1111/j.1365-313X.2005.02530.x

Brenner, W. G., and Schmülling, T. (2012). Transcript profiling of cytokinin action in *Arabidopsis* roots and shoots discovers largely similar but also organ-specific responses. *BMC Plant Biol.* 12:112. doi: 10.1186/1471-2229-12-112

Brenner, W. G., and Schmülling, T. (2015). Summarizing and exploring data of a decade of cytokinin-related transcriptomics. *Front. Plant Sci.* 6:29. doi: 10.3389/fpls.2015.00029

Caesar, K., Thamm, A. M. K., Witthöft, J., Elgass, K., Huppenberger, P., Grefen, C., et al. (2011). Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. *J. Exp. Bot.* 62, 5571–5580. doi: 10.1093/jxb/err238

Causier, B., Ashworth, M., Guo, W., and Davies, B. (2012). The TOPLESS interactome: a framework for gene repression in *Arabidopsis*. *Plant Physiol.* 158, 423–438. doi: 10.1104/pp.111.186999

Chang, G., Wang, C., Kong, X., Chen, Q., Yang, Y., and Hu, X. (2018). AFP2 as the novel regulator breaks high-temperature-induced seeds secondary dormancy through ABI5 and SOM in *Arabidopsis thaliana*. *Biochem. Biophys. Res. Commun.* 501, 232–238. doi: 10.1016/j.bbrc.2018.04.222

Chang, G., Yang, W., Zhang, Q., Huang, J., Yang, Y., and Hu, X. (2019). ABI5-BINDING PROTEIN2 coordinates CONSTANS to delay flowering by recruiting the transcriptional corepressor TPR2. *Plant Physiol.* 179, 477–490. doi: 10.1104/pp.18.00865

Chang, L., Ramireddy, E., and Schmülling, T. (2015). Cytokinin as a positional cue regulating lateral root spacing in *Arabidopsis*. *J. Exp. Bot.* 66, 4759–4768. doi: 10.1093/jxb/erv252

Che, P., Gingerich, D. J., Lall, S., and Howell, S. H. (2002). Global and hormone-induced gene expression changes during shoot development in *Arabidopsis*. *Plant Cell* 14, 2771–2785. doi: 10.1105/tpc.006668

Chen, Y. F., Matsubayashi, Y., and Sakagami, Y. (2000). Peptide growth factor phytosulfokine-α contributes to the pollen population effect. *Planta* 211, 752–755. doi: 10.1007/s004250000370

Chiang, Y.-H., Zubo, Y. O., Tapken, W., Kim, H. J., Lavanway, A. M., Howard, L., et al. (2012). Functional characterization of the GATA transcription factors GNC and CGA1 reveals their key role in chloroplast development, growth, and division in *Arabidopsis*. *Plant Physiol.* 160, 332–348. doi: 10.1104/pp.112.198705

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J. M., Lorenzo, O., et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448, 666–671. doi: 10.1038/nature06006

Ckurshumova, W., Smirnova, T., Marcos, D., Zayed, Y., and Berleth, T. (2014). Irrepressible MONOPTEROS/ARF5 promotes de novo shoot formation. *New Phytol.* 204, 556–566. doi: 10.1111/nph.13014

Corbesier, L., Prinsen, E., Jacqmard, A., Lejeune, P., Van Onckelen, H., Périlleux, C., et al. (2003). Cytokinin levels in leaves, leaf exudate and shoot apical meristem of Arabidopsis thaliana during floral transition. *J. Exp. Bot.* 54, 2511–2517. doi: 10.1093/jxb/erg276

Cortleven, A., Leuendorf, J. E., Frank, M., Pezzetta, D., Bolt, S., and Schmülling, T. (2019). Cytokinin action in response to abiotic and biotic stresses in plants. *Plant Cell Environ.* 42, 998–1018. doi: 10.1111/pce.13494

Cutcliffe, J. W., Hellmann, E., Heyl, A., and Rashotte, A. M. (2011). CRFs form protein–protein interactions with each other and with members of the cytokinin signalling pathway in *Arabidopsis* via the CRF domain. *J. Exp. Bot.* 62, 4995–5002. doi: 10.1093/jxb/err199

Dal Bosco, C., Dovzhenko, A., Liu, X., Woerner, N., Rensch, T., Eismann, M., et al. (2012). The endoplasmic reticulum localized PIN8 is a pollen-specific auxin carrier involved in intracellular auxin homeostasis. *Plant J.* 71, 860–870. doi: 10.1111/j.1365-313X.2012.05037.x

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., et al. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. *Science* 322, 1380–1384. doi: 10.1126/science.1164147

Di, D.-W., Wu, L., Zhang, L., An, C.-W., Zhang, T.-Z., Luo, P., et al. (2016). Functional roles of *Arabidopsis* CKRC2/YUCCA8 gene and the involvement of PIF4 in the regulation of auxin biosynthesis by cytokinin. *Sci. Rep.* 6:36866. doi: 10.1038/srep36866

Etchells, J. P., Provost, C. M., Mishra, L., and Turner, S. R. (2013). *WOX4* and *WOX14* act downstream of the PXY receptor kinase to regulate plant vascular proliferation independently of any role in vascular organisation. *Development* 140, 2224–2234. doi: 10.1242/dev.091314

Feraru, E., Feraru, M. I., Barbez, E., Waidmann, S., Sun, L., Gaidora, A., et al. (2019). PILS6 is a temperature-sensitive regulator of nuclear auxin input and organ growth in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 116, 3893–3898. doi: 10.1073/pnas.1814015116

Feraru, E., Vosolsobì, S., Feraru, M., Petrášek, J., and Kleine-Vehn, J. (2012). Evolution and structural diversification of PILS putative auxin carriers in plants. *Front. Plant Sci.* 3:227. doi: 10.3389/fpls.2012.00227

Fernandez-Calvino, L., Faulkner, C., Walshaw, J., Saalbach, G., Bayer, E., Benitez-Alfonso, Y., et al. (2011). *Arabidopsis* plasmodesmal proteome. *PLoS One* 6:e18880. doi: 10.1371/journal.pone.0018880

Fichtner, F., Olas, J. J., Feil, R., Watanabe, M., Krause, U., Hoefgen, R., et al. (2020). Functional features of TREHALOSE-6-PHOSPHATE SYNTHASE1, an essential enzyme in *Arabidopsis*. *Plant Cell* 32, 1949–1972. doi: 10.1105/tpc.19.00837

Figueroa, C. M., and Lunn, J. E. (2016). A tale of two sugars: Trehalose 6-Phosphate and sucrose. *Plant Physiol.* 172, 7–27. doi: 10.1104/pp.16.00417

Finkelstein, R. R. (1994). Maternal effects govern variable dominance of two abscisic acid response mutations in *Arabidopsis thaliana*. *Plant Physiol.* 105, 1203–1208. doi: 10.1104/pp.105.4.1203

Finkelstein, R. R., and Lynch, T. J. (2000). The *Arabidopsis* abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. *Plant Cell* 12, 599–609. doi: 10.1105/tpc.12.4.599

Franco-Zorrilla, J. M., López-Vidriero, I., Carrasco, J. L., Godoy, M., Vera, P., and Solano, R. (2014). DNA-binding specificities of plant transcription factors and their potential to define target genes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2367–2372. doi: 10.1073/pnas.1316278111

Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P. I., et al. (2011). Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants. *J. Exp. Bot.* 62, 2827–2840. doi: 10.1093/jxb/erq457

Gan, S., and Amasino, R. M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* 270, 1986–1988. doi: 10.1126/science.270.5244.1986

Gan, Y., Liu, C., Yu, H., and Broun, P. (2007a). Integration of cytokinin and gibberellin signalling by *Arabidopsis* transcription factors GIS, ZFP8 and GIS2 in the regulation of epidermal cell fate. *Development* 134, 2073–2081. doi: 10.1242/dev.005017

Gan, Y., Yu, H., Peng, J., and Broun, P. (2007b). Genetic and molecular regulation by DELLA proteins of trichome development in *Arabidopsis*. *Plant Physiol.* 145, 1031–1042. doi: 10.1104/pp.107.104794

Garcia, M. E., Lynch, T., Peeters, J., Snowden, C., and Finkelstein, R. (2008). A small plant-specific protein family of ABI five binding proteins (AFPs) regulates stress response in germinating *Arabidopsis* seeds and seedlings. *Plant Mol. Biol.* 67, 643–658. doi: 10.1007/s11103-008-9344-2

Gaudet, P., Livstone, M. S., Lewis, S. E., and Thomas, P. D. (2011). Phylogenetic-based propagation of functional annotations within the gene ontology consortium. *Brief. Bioinform.* 12, 449–462. doi: 10.1093/bib/bbr042

Greenboim-Wainberg, Y., Maymon, I., Borochov, R., Alvarez, J., Olszewski, N., Ori, N., et al. (2005). Cross talk between gibberellin and cytokinin: the *Arabidopsis* GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. *Plant Cell* 17, 92–102. doi: 10.1105/tpc.104.028472

Hamaguchi, A., Yamashino, T., Koizumi, N., Kiba, T., Kojima, M., Sakakibara, H., et al. (2008). A small subfamily of *Arabidopsis* RADIALIS-LIKE SANT/MYB genes: a link to HOOKLESS1-mediated signal transduction during early morphogenesis. *Biosci. Biotechnol. Biochem.* 72, 2687–2696. doi: 10.1271/bbb.80348

Han, G., Lu, C., Guo, J., Qiao, Z., Sui, N., Qiu, N., et al. (2020). C2H2 Zinc finger proteins: master regulators of abiotic stress responses in plants. *Front. Plant Sci.* 11:115. doi: 10.3389/fpls.2020.00115

Hanai, H., Matsuno, T., Yamamoto, M., Matsubayashi, Y., Kobayashi, T., Kamada, H., et al. (2000). A secreted peptide growth factor, phytosulfokine, acting as a stimulatory factor of carrot somatic embryo formation. *Plant Cell Physiol.* 41, 27–32. doi: 10.1093/pcp/41.1.27

Hao, D., Ohme-Takagi, M., and Sarai, A. (1998). Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *J. Biol. Chem.* 273, 26857–26861. doi: 10.1074/jbc.273.41.26857

Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H.-S., Han, B., Zhu, T., et al. (2000). Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290, 2110–2113. doi: 10.1126/science.290.5499.2110

Héricourt, F., Chefdor, F., Bertheau, L., Tanigawa, M., Maeda, T., Guirimand, G., et al. (2013). Characterization of histidine-aspartate kinase HK1 and identification of histidine phosphotransfer proteins as potential partners in a *Populus* multistep phosphorelay. *Physiol. Plant.* 149, 188–199. doi: 10.1111/ppl.12024

Héricourt, F., Chefdor, F., Djeghdir, I., Larcher, M., Lafontaine, F., Courdavault, V., et al. (2016). Functional divergence of poplar histidine-aspartate kinase HK1

paralogs in response to osmotic stress. *Int. J. Mol. Sci.* 17:2061. doi: 10.3390/ijms17122061

Héricourt, F., Larcher, M., Chefdor, F., Koudounas, K., Carqueijeiro, I., Lemos Cruz, P., et al. (2019). New insight into HPts as hubs in poplar cytokinin and osmosensing multistep phosphorelays: cytokinin pathway uses specific HPts. *Plants* 8:591. doi: 10.3390/plants8120591

Heyl, A., Brault, M., Frugier, F., Kuderova, A., Lindner, A.-C., Motyka, V., et al. (2013). Nomenclature for members of the two-component signaling pathway of plants. *Plant Physiol.* 161, 1063–1065. doi: 10.1104/pp.112.213207

Heyl, A., Ramireddy, E., Brenner, W. G., Riefler, M., Allemeersch, J., and Schmülling, T. (2008). The transcriptional repressor ARR1-SRDX suppresses pleiotropic cytokinin activities in *Arabidopsis*. *Plant Physiol.* 147, 1380–1395. doi: 10.1104/pp.107.115436

Hirakawa, Y., Kondo, Y., and Fukuda, H. (2010). TDIF peptide signaling regulates vascular stem cell proliferation via the *WOX4* homeobox gene in *Arabidopsis*. *Plant Cell* 22, 2618–2629. doi: 10.1105/tpc.110.076083

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., et al. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15208–15213. doi: 10.1073/pnas.0808444105

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., and Sakakibara, H. (2007). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *J. Exp. Bot.* 59, 75–83. doi: 10.1093/jxb/erm157

Hoth, S., Ikeda, Y., Morgante, M., Wang, X., Zuo, J., Hanafey, M. K., et al. (2003). Monitoring genome-wide changes in gene expression in response to endogenous cytokinin reveals targets in *Arabidopsis thaliana*. *FEBS Lett.* 554, 373–380. doi: 10.1016/s0014-5793(03)01194-3

Huang, X., Hou, L., Meng, J., You, H., Li, Z., Gong, Z., et al. (2018). The antagonistic action of abscisic acid and cytokinin signaling mediates drought stress response in *Arabidopsis*. *Mol. Plant* 11, 970–982. doi: 10.1016/j.molp.2018.05.001

Hudson, D., Guevara, D., Yaish, M. W., Hannam, C., Long, N., Clarke, J. D., et al. (2011). GNC and CGA1 modulate chlorophyll biosynthesis and glutamate synthase (GLU1/Fd-GOGAT) expression in *Arabidopsis*. *PLoS One* 6:e26765. doi: 10.1371/journal.pone.0026765

Hudson, M. E., Lisch, D. R., and Quail, P. H. (2003). The FHY3 and FAR1 genes encode transposase-related proteins involved in regulation of gene expression by the phytochrome A-signaling pathway. *Plant J.* 34, 453–471. doi: 10.1046/j.1365-313x.2003.01741.x

Hurný, A., Cuesta, C., Cavallari, N., Ötvös, K., Duclercq, J., Dokládal, L., et al. (2020). SYNERGISTIC ON AUXIN AND CYTOKININ 1 positively regulates growth and attenuates soil pathogen resistance. *Nat. Commun.* 11:2170. doi: 10.1038/s41467-020-15895-5

Igasaki, T., Akashi, N., Ujino-Ihara, T., Matsubayashi, Y., Sakagami, Y., and Shinohara, K. (2003). Phytosulfokine stimulates somatic embryogenesis in *Cryptomeria japonica*. *Plant Cell Physiol.* 44, 1412–1416. doi: 10.1093/pcp/pcg161

Iwakawa, H., Ueno, Y., Semiarti, E., Onouchi, H., Kojima, S., Tsukaya, H., et al. (2002). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana*, required for formation of a symmetric flat leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. *Plant Cell Physiol.* 43, 467–478. doi: 10.1093/pcp/pcf077

Jeon, J., Cho, C., Lee, M. R., Van Binh, N., and Kim, J. (2016). CYTOKININ RESPONSE FACTOR2 (CRF2) and CRF3 regulate lateral root development in response to cold stress in *Arabidopsis*. *Plant Cell* 28, 1828–1843. doi: 10.1105/tpc.15.00909

Jones, B., Gunnerås, S. A., Petersson, S. V., Tarkowski, P., Graham, N., May, S., et al. (2010). Cytokinin regulation of auxin synthesis in *Arabidopsis* involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. *Plant Cell* 22, 2956–2969. doi: 10.1105/tpc.110.074856

Kiba, T., Aoki, K., Sakakibara, H., and Mizuno, T. (2004). Arabidopsis response regulator, ARR22, ectopic expression of which results in phenotypes similar to the wol cytokinin-receptor mutant. *Plant Cell Physiol.* 45, 1063–1077. doi: 10.1093/pcp/pch128

Kiba, T., Naitou, T., Koizumi, N., Yamashino, T., Sakakibara, H., and Mizuno, T. (2005). Combinatorial microarray analysis revealing *Arabidopsis* genes implicated in cytokinin responses through the His→Asp phosphorelay circuitry. *Plant Cell Physiol.* 46, 339–355. doi: 10.1093/pcp/pci033

Kiba, T., Takei, K., Kojima, M., and Sakakibara, H. (2013). Side-chain modification of cytokinins controls shoot growth in *Arabidopsis*. *Dev. Cell* 27, 452–461. doi: 10.1016/j.devcel.2013.10.004

Kiba, T., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T., et al. (2003). The Type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in *Arabidopsis thaliana*. *Plant Cell Physiol.* 44, 868–874. doi: 10.1093/pcp/pcg108

Kieber, J. J., and Schaller, G. E. (2014). Cytokinins. *Arabid. Book* 12:e0168. doi: 10.1199/tab.0168

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:dev149344. doi: 10.1242/dev.149344

Kim, H. J., Ryu, H., Hong, S. H., Woo, H. R., Lim, P. O., Lee, I. C., et al. (2006). Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 814–819. doi: 10.1073/pnas.0505150103

Klepikova, A. V., Kasianov, A. S., Gerasimov, E. S., Logacheva, M. D., and Penin, A. A. (2016). A high resolution map of the *Arabidopsis thaliana* developmental transcriptome based on RNA-seq profiling. *Plant J.* 88, 1058–1070. doi: 10.1111/tpj.13312

Kobayashi, K., and Iwase, A. (2017). Simultaneous but spatially different regulation of non-photosynthetic callus formation and photosynthetic root development after shoot removal. *Plant Signal. Behav.* 12:e1338999. doi: 10.1080/15592324.2017.1338999

Kobayashi, K., Ohnishi, A., Sasaki, D., Fujii, S., Iwase, A., Sugimoto, K., et al. (2017). Shoot removal induces chloroplast development in roots via cytokinin signaling. *Plant Physiol.* 173, 2340–2355. doi: 10.1104/pp.16.01368

Kobayashi, T., Eun, C.-H., Hanai, H., Matsubayashi, Y., Sakagami, Y., and Kamada, H. (1999). Phytosulphokine-α, a peptidyl plant growth factor, stimulates somatic embryogenesis in carrot. *J. Exp. Bot.* 50, 1123–1128. doi: 10.1093/jxb/50.336.1123

Köllmer, I., Werner, T., and Schmülling, T. (2011). Ectopic expression of different cytokinin-regulated transcription factor genes of *Arabidopsis thaliana* alters plant growth and development. *J. Plant Physiol.* 168, 1320–1327. doi: 10.1016/j.jplph.2011.02.006

Kondo, Y., Ito, T., Nakagami, H., Hirakawa, Y., Saito, M., Tamaki, T., et al. (2014). Plant GSK3 proteins regulate xylem cell differentiation downstream of TDIF–TDR signalling. *Nat. Commun.* 5:3504. doi: 10.1038/ncomms4504

Kowalska, M., Galuszka, P., Frébortová, J., Šebela, M., Béres, T., Hluska, T., et al. (2010). Vacuolar and cytosolic cytokinin dehydrogenases of *Arabidopsis thaliana*: heterologous expression, purification and properties. *Phytochemistry* 71, 1970–1978. doi: 10.1016/j.phytochem.2010.08.013

Krouk, G., Ruffel, S., Gutiérrez, R. A., Gojon, A., Crawford, N. M., Coruzzi, G. M., et al. (2011). A framework integrating plant growth with hormones and nutrients. *Trends Plant Sci.* 16, 178–182. doi: 10.1016/j.tplants.2011.02.004

Kuderová, A., Urbánková, I., Válková, M., Malbeck, J., Brzobohatı, B., Némethová, D., et al. (2008). Effects of conditional IPT-dependent cytokinin overproduction on root architecture of *Arabidopsis* seedlings. *Plant Cell Physiol.* 49, 570–582. doi: 10.1093/pcp/pcn029

Kudo, T., Kiba, T., and Sakakibara, H. (2010). Metabolism and long-distance translocation of cytokinins. *J. Integ. Plant Biol.* 52, 53–60. doi: 10.1111/j.1744-7909.2010.00898.x

Kumar, M. N., Jane, W.-N., and Verslues, P. E. (2013). Role of the putative osmosensor *Arabidopsis* Histidine Kinase1 in dehydration avoidance and low-water-potential response. *Plant Physiol.* 161, 942–953. doi: 10.1104/pp.112.209791

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., et al. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. *Plant Cell* 19, 3889–3900. doi: 10.1105/tpc.107.055863

Lee, D. J., Park, J.-Y., Ku, S.-J., Ha, Y.-M., Kim, S., Kim, M. D., et al. (2007). Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. *Mol. Genet. Genomics* 277, 115–137. doi: 10.1007/s00438-006-0177-x

Li, L., He, Z., Pandey, G. K., Tsuchiya, T., and Luan, S. (2002). Functional cloning and characterization of a plant efflux carrier for multidrug and heavy metal detoxification. *J. Biol. Chem.* 277, 5360–5368. doi: 10.1074/jbc.M108777200

Li, Y. J., Wang, B., Dong, R. R., and Hou, B. K. (2015). AtUGT76C2, an *Arabidopsis* cytokinin glycosyltransferase is involved in drought stress adaptation. *Plant Sci.* 236, 157–167. doi: 10.1016/j.plantsci.2015.04.002

Lopez-Molina, L., Mongrand, S., and Chua, N.-H. (2001). A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4782–4787. doi: 10.1073/pnas.081594298

Lopez-Molina, L., Mongrand, S., Kinoshita, N., and Chua, N.-H. (2003). AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. *Genes Dev.* 17, 410–418. doi: 10.1101/gad.1055803

Lynch, T. J., Erickson, B. J., Miller, D. R., and Finkelstein, R. R. (2017). ABI5-binding proteins (AFPs) alter transcription of ABA-induced genes via a variety of interactions with chromatin modifiers. *Plant Mol. Biol.* 93, 403–418. doi: 10.1007/s11103-016-0569-1

Manfield, I. W., Devlin, P. F., Jen, C.-H., Westhead, D. R., and Gilmartin, P. M. (2007). Conservation, convergence, and divergence of light-responsive, circadian-regulated, and tissue-specific expression patterns during evolution of the *Arabidopsis* GATA gene family. *Plant Physiol.* 143, 941–958. doi: 10.1104/pp.106.090761

Mara, C. D., and Irish, V. F. (2008). Two GATA transcription factors are downstream effectors of floral homeotic gene action in *Arabidopsis*. *Plant Physiol.* 147, 707–718. doi: 10.1104/pp.107.115634

Matsubayashi, Y., Ogawa, M., Kihara, H., Niwa, M., and Sakagami, Y. (2006a). Disruption and overexpression of *Arabidopsis* phytosulfokine receptor gene affects cellular longevity and potential for growth. *Plant Physiol.* 142, 45–53. doi: 10.1104/pp.106.081109

Matsubayashi, Y., Shinohara, H., and Ogawa, M. (2006b). Identification and functional characterization of phytosulfokine receptor using a ligand-based approach. *Chem. Rec.* 6, 356–364. doi: 10.1002/tcr.20090

Matsubayashi, Y., Takagi, L., Omura, N., Morita, A., and Sakagami, Y. (1999). The endogenous sulfated pentapeptide phytosulfokine-α stimulates tracheary element differentiation of isolated mesophyll cells of zinnia. *Plant Physiol.* 120, 1043–1048. doi: 10.1104/pp.120.4.1043

Matsumoto-Kitano, M., Kusumoto, T., Tarkowski, P., Kinoshita-Tsujimura, K., Václavíková, K., Miyawaki, K., et al. (2008). Cytokinins are central regulators of cambial activity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20027–20031. doi: 10.1073/pnas.0805619105

Menges, M., Hennig, L., Gruissem, W., and Murray, J. A. H. (2002). Cell cycle-regulated gene expression in *Arabidopsis*. *J. Biol. Chem.* 277, 41987–42002. doi: 10.1074/jbc.M207570200

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant J.* 37, 128–138. doi: 10.1046/j.1365-313X.2003.01945.x

Mok, D. W., and Mok, M. C. (2001). Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 89–118. doi: 10.1146/annurev.arplant.52.1.89

Mok, M. C., Mok, D. W. S., and Armstrong, D. J. (1978). Differential cytokinin structure-activity relationships in *Phaseolus*. *Plant Physiol.* 61, 72–75. doi: 10.1104/pp.61.1.72

Mori, K., Renhu, N., Naito, M., Nakamura, A., Shiba, H., Yamamoto, T., et al. (2018). Ca2+-permeable mechanosensitive channels MCA1 and MCA2 mediate cold-induced cytosolic Ca2+ increase and cold tolerance in *Arabidopsis*. *Sci. Rep.* 8:550. doi: 10.1038/s41598-017-17483-y

Morita, J., Kato, K., Nakane, T., Kondo, Y., Fukuda, H., Nishimasu, H., et al. (2016). Crystal structure of the plant receptor-like kinase TDR in complex with the TDIF peptide. *Nat. Commun.* 7:12383. doi: 10.1038/ncomms12383

Müller, B., and Sheen, J. (2007). Advances in cytokinin signaling. *Science* 318, 68–69. doi: 10.1126/science.1145461

Müller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453, 1094–1097. doi: 10.1038/nature06943

Müller, M., and Munné-Bosch, S. (2015). Ethylene response factors: a key regulatory hub in hormone and stress signaling. *Plant Physiol.* 169, 32–41. doi: 10.1104/pp.15.00677

Naito, T., Yamashino, T., Kiba, T., Koizumi, N., Kojima, M., Sakakibara, H., et al. (2007). A link between cytokinin and ASL9 (ASYMMETRIC LEAVES 2 LIKE 9) that belongs to the AS2/LOB (LATERAL ORGAN BOUNDARIES) family genes in *Arabidopsis thaliana*. *Biosci. Biotechnol. Biochem.* 71, 1269–1278. doi: 10.1271/bbb.60681

Nakamura, S., Lynch, T. J., and Finkelstein, R. R. (2001). Physical interactions between ABA response loci of *Arabidopsis*. *Plant J.* 26, 627–635. doi: 10.1046/j.1365-313x.2001.01069.x

Naseem, M., and Dandekar, T. (2012). The role of auxin-cytokinin antagonism in plant-pathogen interactions. *PLoS Pathog.* 8:e1003026. doi: 10.1371/journal.ppat.1003026

Naseem, M., Kunz, M., and Dandekar, T. (2014). Probing the unknowns in cytokinin-mediated immune defense in *Arabidopsis* with systems biology approaches. *Bioinform. Biol. Insights* 8, 35–44. doi: 10.4137/bbi.S13462

Obulareddy, N., Panchal, S., and Melotto, M. (2013). Guard cell purification and RNA isolation suitable for high-throughput transcriptional analysis of cell-type responses to biotic stresses. *Mol. Plant Microbe Interact.* 26, 844–849. doi: 10.1094/MPMI-03-13-0081-TA

Pagnussat, G. C., Yu, H.-J., Ngo, Q. A., Rajani, S., Mayalagu, S., Johnson, C. S., et al. (2005). Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. *Development* 132, 603–614. doi: 10.1242/dev.01595

Panstruga, R., Baumgarten, K., and Bernhagen, J. (2015). Phylogeny and evolution of plant macrophage migration inhibitory factor/D-dopachrome tautomerase-like proteins. *BMC Evol. Biol.* 15:64. doi: 10.1186/s12862-015-0337-x

Pauwels, L., Barbero, G. F., Geerinck, J., Tilleman, S., Grunewald, W., Pérez, A. C., et al. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* 464, 788–791. doi: 10.1038/nature08854

Poitout, A., Crabos, A., Petøík, I., Novák, O., Krouk, G., Lacombe, B., et al. (2018). Responses to systemic nitrogen signaling in *Arabidopsis* roots involve *trans*-Zeatin in shoots. *Plant Cell* 30, 1243–1257. doi: 10.1105/tpc.18.00011

Price, J., Laxmi, A., St. Martin, S. K., and Jang, J.-C. (2004). Global transcription profiling reveals multiple sugar signal transduction mechanisms in *Arabidopsis*. *Plant Cell* 16, 2128–2150. doi: 10.1105/tpc.104.022616

Ramireddy, E., Chang, L., and Schmülling, T. (2014). Cytokinin as a mediator for regulating root system architecture in response to environmental cues. *Plant Signal. Behav.* 9:e27771. doi: 10.4161/psb.27771

Ranftl, Q. L., Bastakis, E., Klermund, C., and Schwechheimer, C. (2016). LLM-domain containing B-GATA factors control different aspects of cytokinin-regulated development in *Arabidopsis thaliana*. *Plant Physiol.* 170, 2295–2311. doi: 10.1104/pp.15.01556

Rashotte, A. M., Carson, S. D. B., To, J. P. C., and Kieber, J. J. (2003). Expression profiling of cytokinin action in *Arabidopsis*. *Plant Physiol.* 132, 1998–2011. doi: 10.1104/pp.103.021436

Rashotte, A. M., and Goertzen, L. R. (2010). The CRF domain defines cytokinin response factor proteins in plants. *BMC Plant Biol.* 10:74. doi: 10.1186/1471-2229-10-74

Rashotte, A. M., Mason, M. G., Hutchison, C. E., Ferreira, F. J., Schaller, G. E., and Kieber, J. J. (2006). A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11081–11085. doi: 10.1073/pnas.0602038103

Reumann, S., Babujee, L., Ma, C., Wienkoop, S., Siemsen, T., Antonicelli, G. E., et al. (2007). Proteome analysis of *Arabidopsis* leaf peroxisomes reveals novel targeting peptides, metabolic pathways, and defense mechanisms. *Plant Cell* 19, 3170–3193. doi: 10.1105/tpc.107.050989

Reyes, J. C., Muro-Pastor, M. I., and Florencio, F. J. (2004). The GATA family of transcription factors in *Arabidopsis* and rice. *Plant Physiol.* 134, 1718–1732. doi: 10.1104/pp.103.037788

Richter, R., Bastakis, E., and Schwechheimer, C. (2013a). Cross-repressive interactions between SOC1 and the GATAs GNC and GNL/CGA1 in the control of greening, cold tolerance, and flowering time in *Arabidopsis*. *Plant Physiol.* 162, 1992–2004. doi: 10.1104/pp.113.219238

Richter, R., Behringer, C., Zourelidou, M., and Schwechheimer, C. (2013b). Convergence of auxin and gibberellin signaling on the regulation of the GATA transcription factors *GNC* and *GNL* in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 13192–13197. doi: 10.1073/pnas.1304250110

Richter, R., Behringer, C., Müller, I. K., and Schwechheimer, C. (2010). The GATA-type transcription factors GNC and GNL/CGA1 repress gibberellin signaling downstream from DELLA proteins and phytochrome-interacting factors. *Genes Dev.* 24, 2093–2104. doi: 10.1101/gad.594910

Riechmann, J. L., and Ratcliffe, O. J. (2000). A genomic perspective on plant transcription factors. *Curr. Opin. Plant Biol.* 3, 423–434. doi: 10.1016/s1369-5266(00)00107-2

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18, 40–54. doi: 10.1105/tpc.105.037796

Ruffel, S., Krouk, G., Ristova, D., Shasha, D., Birnbaum, K. D., and Coruzzi, G. M. (2011). Nitrogen economics of root foraging: transitive closure of the nitrate–cytokinin relay and distinct systemic signaling for N supply vs. demand. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18524–18529. doi: 10.1073/pnas.1108684108

Ruffel, S., Poitout, A., Krouk, G., Coruzzi, G. M., and Lacombe, B. (2016). Long-distance nitrate signaling displays cytokinin dependent and independent branches. *J. Integ. Plant Biol.* 58, 226–229. doi: 10.1111/jipb.12453

Sakuma, Y., Liu, Q., Dubouzet, J. G., Abe, H., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002). DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem. Biophys. Res. Commun.* 290, 998–1009. doi: 10.1006/bbrc.2001.6299

Sasaki, E., Ogura, T., Takei, K., Kojima, M., Kitahata, N., Sakakibara, H., et al. (2013). Uniconazole, a cytochrome P450 inhibitor, inhibits trans-zeatin biosynthesis in *Arabidopsis*. *Phytochemistry* 87, 30–38. doi: 10.1016/j.phytochem.2012.11.023

Schaller, G. E., Bishopp, A., and Kieber, J. J. (2015). The Yin-Yang of hormones: cytokinin and auxin interactions in plant development. *Plant Cell* 27, 44–63. doi: 10.1105/tpc.114.133595

Scheible, W.-R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., et al. (2004). Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiol.* 136, 2483–2499. doi: 10.1104/pp.104.047019

Schlereth, A., Möller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E. H., et al. (2010). MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature* 464, 913–916. doi: 10.1038/nature08836

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., et al. (2005). A gene expression map of *Arabidopsis thaliana* development. *Nat. Genet.* 37, 501–506. doi: 10.1038/ng1543

Schmitz, R. Y., Skoog, F., Playtis, A. J., and Leonard, N. J. (1972). Cytokinins: synthesis and biological activity of geometric and position isomers of zeatin. *Plant Physiol.* 50, 702–705. doi: 10.1104/pp.50.6.702

Schmülling, T., Werner, T., Riefler, M., Krupková, E., and Bartrina y Manns, I. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. *J. Plant Res.* 116, 241–252. doi: 10.1007/s10265-003-0096-4

Shimizu-Sato, S., Tanaka, M., and Mori, H. (2008). Auxin–cytokinin interactions in the control of shoot branching. *Plant Mol. Biol.* 69, 429–435. doi: 10.1007/s11103-008-9416-3

Shuai, B., Reynaga-Peña, C. G., and Springer, P. S. (2002). The lateral organ boundaries gene defines a novel, plant-specific gene family. *Plant Physiol.* 129, 747–761. doi: 10.1104/pp.010926

Šimášková, M., O'Brien, J. A., Khan, M., Van Noorden, G., Ötvös, K., Vieten, A., et al. (2015). Cytokinin response factors regulate *PIN-FORMED* auxin transporters. *Nat. Commun.* 6:8717. doi: 10.1038/ncomms9717

Šmehilová, M., Dobrùšková, J., Novák, O., Takáč, T., and Galuszka, P. (2016). Cytokinin-specific glycosyltransferases possess different roles in cytokinin homeostasis maintenance. *Front. Plant Sci.* 7:1264. doi: 10.3389/fpls.2016.01264

Šmehilová, M., Galuszka, P., Bilyeu, K. D., Jaworek, P., Kowalska, M., Šebela, M., et al. (2009). Subcellular localization and biochemical comparison of cytosolic and secreted cytokinin dehydrogenase enzymes from maize. *J. Exp. Bot.* 60, 2701–2712. doi: 10.1093/jxb/erp126

Smit, M. E., McGregor, S. R., Sun, H., Gough, C., Bågman, A.-M., Soyars, C. L., et al. (2020). A PXY-mediated transcriptional network integrates signaling mechanisms to control vascular development in *Arabidopsis*. *Plant Cell* 32, 319–335. doi: 10.1105/tpc.19.00562

Sottosanto, J. B., Gelli, A., and Blumwald, E. (2004). DNA array analyses of *Arabidopsis thaliana* lacking a vacuolar Na+/H+ antiporter: impact of AtNHX1 on gene expression. *Plant J.* 40, 752–771. doi: 10.1111/j.1365-313X.2004.02253.x

Sparkes, A., De Baetselier, P., Roelants, K., De Trez, C., Magez, S., Van Ginderachter, J. A., et al. (2017). The non-mammalian MIF superfamily. *Immunobiology* 222, 473–482. doi: 10.1016/j.imbio.2016.10.006

Striberny, B., Melton, A. E., Schwacke, R., Krause, K., Fischer, K., Goertzen, L. R., et al. (2017). Cytokinin response factor 5 has transcriptional activity governed by its C-terminal domain. *Plant Signal. Behav.* 12:e1276684. doi: 10.1080/15592324.2016.1276684

Stührwohldt, N., Dahlke, R. I., Kutschmar, A., Peng, X., Sun, M.-X., and Sauter, M. (2015). Phytosulfokine peptide signaling controls pollen tube growth and funicular pollen tube guidance in *Arabidopsis thaliana*. *Physiol. Plant.* 153, 643–653. doi: 10.1111/ppl.12270

Sun, L., Feraru, E., Feraru, M. I., Waidmann, S., Wang, W., Passaia, G., et al. (2020). PIN-LIKES coordinate brassinosteroid signaling with nuclear auxin input in *Arabidopsis thaliana*. *Curr. Biol.* 30, 1579.e6–1588.e6. doi: 10.1016/j.cub.2020.02.002

Sun, X., Malhis, N., Zhao, B., Xue, B., Gsponer, J., and Rikkerink, E. H. A. (2020). Computational disorder analysis in ethylene response factors uncovers binding motifs critical to their diverse functions. *Int. J. Mol. Sci.* 21:74. doi: 10.3390/ijms21010074

Sun, L., Zhang, A., Zhou, Z., Zhao, Y., Yan, A., Bao, S., et al. (2015). GLABROUS INFLORESCENCE STEMS3 (GIS3) regulates trichome initiation and development in *Arabidopsis*. *New Phytol.* 206, 220–230. doi: 10.1111/nph.13218

Takei, K., Sakakibara, H., and Sugiyama, T. (2001a). Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J. Biol. Chem.* 276, 26405–26410. doi: 10.1074/jbc.M102130200

Takei, K., Sakakibara, H., Taniguchi, M., and Sugiyama, T. (2001b). Nitrogen-dependent accumulation of cytokinins in root and thetranslocation to leaf: implication of cytokinin species that induces geneexpression of maize responseregulator. *Plant Cell Physiol.* 42, 85–93. doi: 10.1093/pcp/pce009

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., et al. (2004a). AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in *Arabidopsis*. *Plant Cell Physiol.* 45, 1053–1062. doi: 10.1093/pcp/pch119

Takei, K., Yamaya, T., and Sakakibara, H. (2004b). Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-zeatin. *J. Biol. Chem.* 279, 41866–41872. doi: 10.1074/jbc.M406337200

Taniguchi, M., Sasaki, N., Tsuge, T., Aoyama, T., and Oka, A. (2007). ARR1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions. *Plant Cell Physiol.* 48, 263–277. doi: 10.1093/pcp/pcl063

Teakle, G. R., Manfield, I. W., Graham, J. F., and Gilmartin, P. M. (2002). Arabidopsis thaliana GATA factors: organisation, expression and DNA-binding characteristics. *Plant Mol. Biol.* 50, 43–56. doi: 10.1023/A:1016062325584

Thilmony, R., Underwood, W., and He, S. Y. (2006). Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. tomato DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* 46, 34–53. doi: 10.1111/j.1365-313X.2006.02725.x

To, J. P. C., Haberer, G., Ferreira, F. J., Deruère, J., Mason, M. G., Schaller, G. E., et al. (2004). Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* 16, 658–671. doi: 10.1105/tpc.018978

Tran, L.-S. P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., et al. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20623–20628. doi: 10.1073/pnas.0706547105

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., et al. (1999). A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell* 11, 1743–1754. doi: 10.1105/tpc.11.9.1743

Vega, A., O'Brien, J. A., and Gutiérrez, R. A. (2019). Nitrate and hormonal signaling crosstalk for plant growth and development. *Curr. Opin. Plant Biol.* 52, 155–163. doi: 10.1016/j.pbi.2019.10.001

Wallmeroth, N., Anastasia, A. K., Harter, K., Berendzen, K. W., and Mira-Rodado, V. (2017). *Arabidopsis* response regulator 22 inhibits cytokinin-regulated gene

transcription in vivo. *Protoplasma* 254, 597–601. doi: 10.1007/s00709-016-0944-4

Wallmeroth, N., Jeschke, D., Slane, D., Nägele, J., Veerabagu, M., Mira-Rodado, V., et al. (2019). ARR22 overexpression can suppress plant two-component regulatory systems. *PLoS One* 14:e0212056. doi: 10.1371/journal.pone.0212056

Wang, J., Ma, X.-M., Kojima, M., Sakakibara, H., and Hou, B.-K. (2011). N-Glucosyltransferase UGT76C2 is involved in cytokinin homeostasis and cytokinin response in *Arabidopsis thaliana*. *Plant Cell Physiol.* 52, 2200–2213. doi: 10.1093/pcp/pcr152

Wang, Y., Li, L., Ye, T., Zhao, S., Liu, Z., Feng, Y.-Q., et al. (2011). Cytokinin antagonizes ABA suppression to seed germination of *Arabidopsis* by downregulating ABI5 expression. *Plant J.* 68, 249–261. doi: 10.1111/j.1365-313X.2011.04683.x

Wang, J., Ma, X.-M., Kojima, M., Sakakibara, H., and Hou, B.-K. (2013). Glucosyltransferase UGT76C1 finely modulates cytokinin responses via cytokinin N-glucosylation in *Arabidopsis thaliana*. *Plant Physiol. Biochem.* 65, 9–16. doi: 10.1016/j.plaphy.2013.01.012

Wang, R., Okamoto, M., Xing, X., and Crawford, N. M. (2003). Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, Trehalose-6-Phosphate, iron, and sulfate metabolism. *Plant Physiol.* 132, 556–567. doi: 10.1104/pp.103.021253

Wang, R., Tischner, R., Gutiérrez, R. A., Hoffman, M., Xing, X., Chen, M., et al. (2004). Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. *Plant Physiol.* 136, 2512–2522. doi: 10.1104/pp.104.044610

Wang, X., Lin, S., Liu, D., Gan, L., McAvoy, R., Ding, J., et al. (2020). Evolution and roles of cytokinin genes in angiosperms 1: do ancient IPTs play housekeeping while non-ancient IPTs play regulatory roles? *Horticult. Res.* 7:28. doi: 10.1038/s41438-019-0211-x

Weigel, D. (1995). The APETALA2 domain is related to a novel type of DNA binding domain. *Plant Cell* 7, 388–389. doi: 10.1105/tpc.7.4.388

Werner, T., Holst, K., Pörs, Y., Guivarc'h, A., Mustroph, A., Chriqui, D., et al. (2008). Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. *J. Exp. Bot.* 59, 2659–2672. doi: 10.1093/jxb/ern134

Werner, T., Köllmer, I., Bartrina, I., Holst, K., and Schmülling, T. (2006). New insights into the biology of cytokinin degradation. *Plant Biol.* 8, 371–381. doi: 10.1055/s-2006-923928

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15, 2532–2550. doi: 10.1105/tpc.014928

Werner, T., Motyka, V., Strnad, M., and Schmülling, T. (2001). Regulation of plant growth by cytokinin. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10487–10492. doi: 10.1073/pnas.171304098

Werner, T., and Schmülling, T. (2009). Cytokinin action in plant development. *Curr. Opin. Plant Biol.* 12, 527–538. doi: 10.1016/j.pbi.2009.07.002

Wohlbach, D. J., Quirino, B. F., and Sussman, M. R. (2008). Analysis of the *Arabidopsis* histidine kinase ATHK1 reveals a connection between vegetative osmotic stress sensing and seed maturation. *Plant Cell* 20, 1101–1117. doi: 10.1105/tpc.107.055871

Wybouw, B., and De Rybel, B. (2019). Cytokinin – a developing story. *Trends Plant Sci.* 24, 177–185. doi: 10.1016/j.tplants.2018.10.012

Yamakawa, S., Matsubayashi, Y., Sakagami, Y., Kamada, H., and Satoh, S. (1998a). Promotion by a peptidyl growth factor, phytosulfokine, of chlorophyll formation in etiolated cotyledon of cucumber. *Biosci. Biotechnol. Biochem.* 62, 2441–2443. doi: 10.1271/bbb.62.2441

Yamakawa, S., Sakuta, C., Matsubayashi, Y., Sakagami, Y., Kamada, H., and Satoh, S. (1998b). The promotive effects of a peptidyl plant growth factor, phytosulfokine-α, on the formation of adventitious roots and expression of a gene for a root-specific cystatin in cucumber hypocotyls. *J. Plant Res.* 111, 453–458. doi: 10.1007/BF02507810

Yamakawa, S., Matsubayashi, Y., Sakagami, Y., Kamada, H., and Satoh, S. (1999). Promotive effects of the peptidyl plant growth factor, phytosulfokine-α, on the growth and chlorophyll content of *Arabidopsis* seedlings under high night-time temperature conditions. *Biosci. Biotechnol. Biochem.* 63, 2240–2243. doi: 10.1271/bbb.63.2240

Yang, B., Song, Z., Li, C., Jiang, J., Zhou, Y., Wang, R., et al. (2018). RSM1, an *Arabidopsis* MYB protein, interacts with HY5/HYH to modulate seed germination and seedling development in response to abscisic acid and salinity. *PLoS Genet.* 14:e1007839. doi: 10.1371/journal.pgen.1007839

Yang, C., and Li, L. (2017). Hormonal regulation in shade avoidance. *Front. Plant Sci.* 8:1527. doi: 10.3389/fpls.2017.01527

Yang, G., Shen, S., Kobayashi, T., Matsubayashi, Y., Sakagami, Y., and Kamada, H. (1999). Stimulatory effects of a novel peptidyl plant growth factor, phytosulfokine-α, on the adventitious bud formation from callus of *Antirrhinum majus*. *Plant Biotechnol.* 16, 231–234. doi: 10.5511/plantbiotechnology.16.231

Yang, H., Matsubayashi, Y., Nakamura, K., and Sakagami, Y. (1999). *Oryza sativa PSK* gene encodes a precursor of phytosulfokine-α, a sulfated peptide growth factor found in plants. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13560–13565. doi: 10.1073/pnas.96.23.13560

Yokoyama, A., Yamashino, T., Amano, Y.-I., Tajima, Y., Imamura, A., Sakakibara, H., et al. (2007). Type-B ARR transcription factors, ARR10 and ARR12, are implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of *Arabidopsis thaliana*. *Plant Cell Physiol.* 48, 84–96. doi: 10.1093/pcp/pcl040

Zhang, H., Lin, X., Han, Z., Wang, J., Qu, L.-J., and Chai, J. (2016). SERK family receptor-like kinases function as co-receptors with PXY for plant vascular development. *Mol. Plant* 9, 1406–1414. doi: 10.1016/j.molp.2016.07.004

Zhang, K., Wang, R., Zi, H., Li, Y., Cao, X., Li, D., et al. (2018). Auxin response factor3 regulates floral meristem determinacy by repressing cytokinin biosynthesis and signaling. *Plant Cell* 30, 324–346. doi: 10.1105/tpc.17.00705

Zhou, Z.-Y., Zhang, C.-G., Wu, L., Zhang, C.-G., Chai, J., Wang, M., et al. (2011). Functional characterization of the *CKRC1/TAA1* gene and dissection of hormonal actions in the *Arabidopsis* root. *Plant J.* 66, 516–527. doi: 10.1111/j.1365-313X.2011.04509.x

Zhou, Z., An, L., Sun, L., Zhu, S., Xi, W., Broun, P., et al. (2011). Zinc finger protein5 is required for the control of trichome initiation by acting upstream of zinc finger protein8 in *Arabidopsis*. *Plant Physiol.* 157, 673–682. doi: 10.1104/pp.111.180281

Zubo, Y. O., Blakley, I. C., Franco-Zorrilla, J. M., Yamburenko, M. V., Solano, R., Kieber, J. J., et al. (2018). Coordination of chloroplast development through the action of the GNC and GLK transcription factor families. *Plant Physiol.* 178, 130–147. doi: 10.1104/pp.18.00414

Zubo, Y. O., Blakley, I. C., Yamburenko, M. V., Worthen, J. M., Street, I. H., Franco-Zorrilla, J. M., et al. (2017). Cytokinin induces genome-wide binding of the type-B response regulator ARR10 to regulate growth and development in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 114, E5995–E6004. doi: 10.1073/pnas.1620749114

Zürcher, E., and Müller, B. (2016). "Cytokinin synthesis, signaling, and function—advances and new insights," in *International Review of Cell and Molecular Biology*, ed. K. W. Jeon (Cambridge, MA: Academic Press), 1–38. doi: 10.1016/bs.ircmb.2016.01.001

Zwack, P. J., De Clercq, I., Howton, T. C., Hallmark, H. T., Hurny, A., Keshishian, E. A., et al. (2016). Cytokinin response factor 6 represses cytokinin-associated genes during oxidative stress. *Plant Physiol.* 172, 1249–1258. doi: 10.1104/pp.16.00415

Zwack, P. J., and Rashotte, A. M. (2013). Cytokinin inhibition of leaf senescence. *Plant Signal. Behav.* 8:e24737. doi: 10.4161/psb.24737

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 December 2020
doi: 10.3389/fpls.2020.599228

Hormopriming to Mitigate Abiotic Stress Effects: A Case Study of N^9-Substituted Cytokinin Derivatives With a Fluorinated Carbohydrate Moiety

Magdaléna Bryksová[1], Andrea Hybenová[1], Alba E. Hernándiz[1], Ondřej Novák[2], Aleš Pěnčík[2], Lukáš Spíchal[1], Nuria De Diego[1] and Karel Doležal[1,2]*

[1] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czechia, [2] Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany, Czech Academy of Sciences, Olomouc, Czechia

OPEN ACCESS

Edited by:
Wolfram G. Brenner, Universität Leipzig, Germany

Reviewed by:
Aaron M. Rashotte, Auburn University, United States
Sibu Simon, Central European Institute of Technology (CEITEC), Czechia

***Correspondence:**
Nuria De Diego
nuria.de@upol.cz

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *26 August 2020*
Accepted: *16 November 2020*
Published: *10 December 2020*

Citation:
Bryksová M, Hybenová A, Hernándiz AE, Novák O, Pěnčík A, Spíchal L, De Diego N and Doležal K (2020) Hormopriming to Mitigate Abiotic Stress Effects: A Case Study of N^9-Substituted Cytokinin Derivatives With a Fluorinated Carbohydrate Moiety. Front. Plant Sci. 11:599228. doi: 10.3389/fpls.2020.599228

Drought and salinity reduce seed germination, seedling emergence, and early seedling establishment, affect plant metabolism, and hence, reduce crop yield. Development of technologies that can increase plant tolerance of these challenging growth conditions is a major current interest among plant scientists and breeders. Seed priming has become established as one of the practical approaches that can alleviate the negative impact of many environmental stresses and improve the germination and overall performance of crops. Hormopriming using different plant growth regulators has been widely demonstrated as effective, but information about using cytokinins (CKs) as priming agents is limited to only a few studies using kinetin or 6-benzylaminopurine (BAP). Moreover, the mode of action of these compounds in improving seed and plant fitness through priming has not yet been studied. For many years, BAP has been one of the CKs most commonly applied exogenously to plants to delay senescence and reduce the impact of stress. However, rapid endogenous N^9-glucosylation of BAP can result in negative effects. This can be suppressed by hydroxylation of the benzyl ring or by appropriate N^9 purine substitution. Replacement of the 2′ or 3′ hydroxyl groups of a nucleoside with a fluorine atom has shown promising results in drug research and biochemistry as a means of enhancing biological activity and increasing chemical or metabolic stability. Here, we show that the application of this chemical modification in four new N^9-substituted CK derivatives with a fluorinated carbohydrate moiety improved the antisenescence properties of CKs. Besides, detailed phenotypical analysis of the growth and development of Arabidopsis plants primed with the new CK analogs over a broad concentration range and under various environmental conditions revealed that they improve growth regulation and antistress activity. Seed priming with, for example, 6-(3-hydroxybenzylamino)-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine promoted

plant growth under control conditions and alleviated the negative effects of the salt and osmotic stress. The mode of action of this hormopriming and its effect on plant metabolism were further analyzed through quantification of the endogenous levels of phytohormones such as CKs, auxins and abscisic acid, and the results are discussed.

Keywords: abiotic stress, antisenescence, Arabidopsis, cytokinin analogs, hormopriming, plant biostimulant characterization index

INTRODUCTION

Global climate change is increasing the severity of drought and soil salinity, with deleterious effects on already-stressed agricultural ecosystems. Moreover, predictions for the future indicate that the areas affected by these two types of stress are going to expand and as a consequence the productivity of many plant species will be reduced (Savvides et al., 2016; Uddin et al., 2016; Pavlů et al., 2018). The development of biotechnological approaches that increase plant tolerance and assure the maintenance of yield under these challenging growth conditions is therefore one of the main aims of plant scientists and breeders.

One of the technologies that attracts a high level of interest nowadays is "seed priming" (Savvides et al., 2016). Seed priming is an effective pre-sowing technology in which seeds are treated with small doses of certain agents just prior to germination. Unlike un-primed seeds, primed seeds are able to respond to very low levels of specific stimuli, which helps plants to prepare their metabolism for better defense responses to stress factors (Conrath, 2011; Paparella et al., 2015). Thus, priming can improve seed performance, ensure higher uniformity among the seeds, result in faster and better synchronized germination, and enhance plant growth (Gamir et al., 2014; Ibrahim, 2016; Lutts et al., 2016). Several methods of seed priming, including hydropriming, osmopriming, hormopriming, biopriming, and chemical priming, have been developed (Jisha et al., 2013; Paparella et al., 2015). Hormopriming consists in the exogenous application of plant growth regulators or phytohormones that can stimulate seed imbibition and modify seed metabolism. The plant growth regulators most often used in this way are abscisic acid (ABA), gibberellins, cytokinins (CKs), auxins, ethylene, and polyamines (reviewed by De Diego and Spíchal, 2020).

In plants, CKs are involved in many biological processes: regulating sink/source relationships (Roitsch and Ehneß, 2000; Werner et al., 2008), nutrient uptake (Sakakibara, 2006; Criado et al., 2009), leaf senescence (Jordi et al., 2000; Marchetti et al., 2018), and responses to abiotic stress (Bielach et al., 2017). Since the discovery of the first CK, kinetin, by Skoog, Miller, and associates in 1955 (Miller et al., 1955), the number of chemicals fitting the definition of CK has grown to include a large array of natural and synthetic compounds, among which are adenine and phenylurea derivatives (Mok and Mok, 2001). Depending on their chemical structure, natural CKs are adenine derivatives with an isoprenoid or aromatic N^6-side chain (Mok and Mok, 2001). CKs are present in plants in the forms of free bases, glucosides, nucleosides and nucleotides, at very low concentrations [pmol g^{-1} fresh weight (FW)] (Strnad, 1997). The precursor nucleotides, namely N^9-riboside-5′- mono-, di-, and tri-phosphates, are endogenously synthesized *de novo* and converted to active free bases. The bases can be subsequently conjugated with glucose at positions N^3, N^7, and N^9 of the purine ring and at the hydroxyl group of the side chain, which can be also conjugated with xylose (Frébort et al., 2011). Addition of sugar moieties to the N^9 position of the purine ring can also form N^9-riboside-glucoside (George et al., 2008). While the *O*-glycosylated forms can be converted back into active CKs, *N*-glycosylation occurs primarily at positions N^7 or N^9 of the purine ring, and is thought to be irreversible (Brzobohatý et al., 1993), except in the case of the *t*Z forms (Hošek et al., 2020). Furthermore, it has been demonstrated that some of these conjugates may have significant CK activity, especially in the case of N^9-riboside analogs (Doležal et al., 2007).

The aromatic CK benzylaminopurine (BAP) is considered the most effective and the cheapest CK, which has led to its widespread use in biotechnology. However, many disadvantages associated with its applications have been reported (Werbrouck et al., 1995; Bairu et al., 2009). Negative effects can be caused by natural N^9-glucosylation of the applied purine based CK, leading to extensive accumulation of non-active CK glucosides (Werbrouck et al., 1995). Moreover, N^9-glucosides can activate ethylene production and the ethylene signaling pathway causing inhibition of root elongation (Podlešáková et al., 2012). One way of avoiding the negative effects of N^9-glucosylation is to suppress it by appropriate N^9 purine substitution in BAP or by hydroxylation of its benzyl ring (Plíhal et al., 2013).

Fluorination has a long tradition in nucleoside chemistry and the replacement of the 2′ or 3′ hydroxyl group of a nucleoside with a fluorine atom causes only a minor change in the overall structure, but significantly affects the stereoelectronic properties of the sugar moiety (Thibaudeau et al., 1998). It has been reported that important factors in the substitution of fluorine for hydrogen are the comparable size of the two atoms and the powerful electron withdrawing properties of fluorine relative to hydrogen, as well as the increased stability of the carbon-fluorine bond relative to the carbon-hydrogen bond. Hence, replacement of hydrogen by fluorine in a bioactive molecule is expected to cause minimal steric perturbations with respect to the molecule's mode of binding to receptors or enzymes (Pitzer, 1960). Moreover, replacement of the hydrogen by fluorine causes not only changes in biological activity, but also increases the chemical and metabolic stability of nucleosides. The conformation of the sugar

Abbreviations: BAP, 6-benzylaminopurine, ABA, abscisic acid, CKs, cytokinins, IAA, indole-3-acetic acid, iP, N^6-isopentenyladenine, *c*Z, *cis*-zeatin, DHZ, dihydrozeatin, *t*Z, *trans*-zeatin, HTS, high-throughput screening, PBC, plant biostimulant characterization index.

moiety of these analogs is strongly affected by the presence of the fluorine substituent and is different from that of natural deoxynucleosides (Pankiewicz et al., 1992).

Nucleosides bearing fluorine or fluorinated substituents within the carbohydrate moiety have been widely used in biochemical research and therapeutic treatment (Meng and Qing, 2006; Hagmann, 2008; Kirk, 2008). However, to date only few fluorinated CK derivatives have been prepared and their biological activity tested in plants (Clemenceau et al., 1996; Doležal et al., 2007). Only recently, several 6-benzylaminopurines substituted with β-D-arabinose at the N^9-position with similar structures were synthesized in our laboratory, and subsequently patented as powerful antisenescence compounds compared with BAP, but the activity of these compounds were only tested in a detached wheat leaves senescence bioassay (Patent No. US 10,100,077 B2, 2018). Here, we present a new class of N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine derivatives which show not only high levels of antisenescence activity but also promise as seed priming agents due to their high efficiency as plant growth promotors and plant stress alleviators. Their mode of action as priming agents is also discussed.

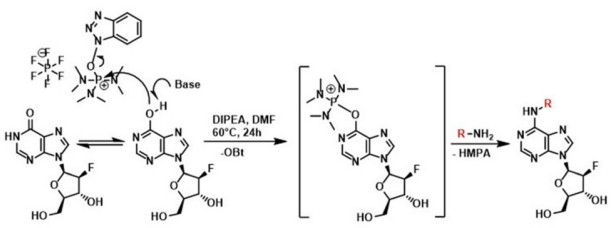


FIGURE 1 | Scheme for the preparation of substituted 6-benzylamino-2′-deoxy-2-′fluoro-9-(β)-D-arabinofuranosylpurine derivatives by a method using Castro's reagent and Hunig base.

MATERIALS AND METHODS

General Synthesis of N^6-Substituted-2′-Deoxy-2′-Fluoro-9-(β)-D-Arabinofuranosylpurine Derivatives

All the compounds presented here were prepared by a slightly modified one-step synthesis (Wan et al., 2005) of 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)hypoxanthine with benzylamine or isopentenylamine hydrochloride as appropriate in the presence of BOP and DIPEA in DMF (**Figure 2**). Firstly 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)hypoxanthine (200 mg, 1 equiv.) and BOP (396 mg, 1.2 equiv.) were mixed together in DMF (4 mL) and subsequently DIPEA (194 μL, 1.5 equiv.) and benzylamine (1–3) or isopentenylamine hydrochloride (4) (1.2 equiv.) as the last component were added. Each reaction mixture was stirred under an argon atmosphere in an oil bath at a temperature of 55–60°C for 24 h and the effectiveness of the reaction was checked by Thin-layer chromatography (TLC) ($CHCl_3$/MeOH 4:1). The reaction mixture was evaporated using a vacuum rotary evaporator to give a specifically colored gel. The resulting residue was carefully purified by column chromatography (1 and 3) or by preparative HPLC (2 and 4) to give the desired product, which in some cases (1 and 3) could be crystallized from various solvents.

General Procedures

The chromatographic purity and mass spectra of the compounds described were characterized using the HPLC-PDA-MS method. Samples (10 μL of 3×10^{-5} M in 1% methanol) were injected onto a reverse-phased column (Symmetry C18, 5 μm, 150 mm × 2.1 mm; Waters, Milford, MA, United States) tempered at 25°C. Solvent (A) consisted of 15 mM ammonium formate adjusted to pH 4.0 and solvent (B) consisted of methanol. The flow-rate was set to 200 μL min^{-1}. A binary gradient was used: 0 min, 10% of B; 24 min; 90% of B; 34 min; 90% of B; 45 min; 10% of B using a Waters Alliance 2695 Separations Module (Waters, Manchester, United Kingdom). Then the effluent was introduced to a Waters 2996 PDA detector (Waters, Manchester, United Kingdom) (scanning range 210–700 nm with 1.2 nm resolution) and a tandem mass analyzer Q-Tof micro Mass Spectrometer (Waters, Manchester, United Kingdom) with an electrospray. The cone voltage was set to 20 V. Exact mass was determined by QTOF-MS (Synapt G2-Si, Waters, United Kingdom) operating in positive ion mode and recorded as $(M + H)^+$. Melting points were determined on a Büchi Melting Point B-540 apparatus and are uncorrected. ^{1}H NMR spectra were analyzed on a Jeol 500 SS spectrometer operating at a temperature of 300 K and a frequency of 500.13 MHz. The samples were prepared by dissolving in DMSO-d_6. Tetramethylsilane (TMS) was used as an internal standard. TLC was carried out using silica gel 60 WF_{254} plates (Merck). Purification *via* column chromatography was performed using silica gel Davisil R LC60A 40-63 micron.

HPLC-MS Purification

A preparative HPLC-MS chromatography machine (Agilent 1290 Infinity II) was used coupled to a UV-VIS detector with a mass LC/MSD detector (Agilent InfinityLab) and an Agilent Prep-C18 column (5 μm, 21.2 mm × 50 mm, Waters, Milford, MA, United States) to obtain the final products. Analyzed samples were dissolved in 50% MeOH before injection. The mobile phase was methanol (A):H_2O (B) with a flow rate of 20 mL min^{-1} and linear gradients (0 min, 10% B; 0–12 min; 90% B) were used.

HRMS Conditions

Samples (5 μL) were characterized using the HPLC-PDA-MS method. They were injected onto a reversed-phase column (Symmetry C18, 5 μm, 150 mm × 2.1 mm; Waters, Milford, MA, United States) incubated at 40°C. Solvent A was 15 mM ammonium formate adjusted to pH 4.0. Solvent B was methanol. The following linear gradient was used at a flow rate of 250 μL min^{-1}: 0 min, 10% B; 0–15 min, 90% B. The effluent was introduced to a DAD detector (scanning range 210–400 nm with 1.2 nm resolution) and then to an electrospray source (source temperature 150°C, desolvation temperature 550°C, capillary

voltage 1 kV, cone voltage 25 V). Nitrogen was used as the cone gas (50 L h^{-1}) and the desolvation gas (1000 L h^{-1}). Data acquisition was performed in full-scan mode (50–1000 Da) with a scan time of 0.5 s and collision energy of 4 eV; argon was used as the collision gas (optimized pressure of 5×10^{-3} mbar). Analyses were performed in positive mode (ESI^+), therefore protonated molecules $(M + H)^+$ were collected in each MS spectrum. For exact mass determination experiments, external calibration was performed using lock spray technology and a mixture of leucine/encephalin (50 pg μL^{-1}) in an acetonitrile and water (1:1) solution with 0.1% formic acid as a reference. Accurate masses were calculated and used to determine the elemental composition of the analytes with a fidelity better than 1.0 ppm.

Cytokinin Bioassays

Cytokinin bioassays, including Amaranthus, tobacco callus and senescence bioassays, were carried out as previously described by Holub et al. (1998), using BAP as a positive control for all three classical CK bioassays. Results were recorded to define the highest activities of the four compounds prepared. All of them were dissolved in 0.5% DMSO and tested at five concentrations (from 10^{-8} to 10^{-4} M).

Plant Phenotyping – Rosette Growth of Seedlings From Arabidopsis Hormoprimed Seeds

The four compounds synthesized were tested as priming agents under optimal and two different stress conditions. Arabidopsis seeds (*Arabidopsis thaliana* accession Col-0) were sterilized and germinated as described by Ugena et al. (2018). During germination the compounds were added at four different concentrations (from 10^{-7} to 10^{-4} M) to germination medium containing 0.5× MS (pH 5.7) supplemented with a gelling agent (0.6% Phytagel; Sigma-Aldrich, Germany). Three days after germination, seedlings of similar size were transferred under sterile conditions into 48-well plates (Jetbiofil, Guangzhou, China). One seedling was transferred to each well filled with 850 mL 1× MS medium (pH 5.7; supplemented with 0.6% Phytagel), without stress treatment (optimal conditions) or containing 100 mM NaCl (as salt stress) or 100 mM mannitol (as osmotic stress), and the plates were sealed with perforated transparent foil allowing gas and water exchange. Hormopriming of 10^{-8} M BAP was also used as positive control for all tested growth conditions.

The 48-well plates containing the transferred Arabidopsis seedlings were placed in an OloPhen platform,[1] which uses the PlantScreen^{TM} XYZ system installed in a growth chamber with a controlled environment, and cool-white LED and far-red LED lighting (Photon Systems Instruments, Brno, Czechia). The conditions were set to simulate a long day with a temperature regime of 22/20°C in a 16/8 h light/dark cycle, an irradiance of 120 μmol photons of PAR m^2 s^{-1} and a relative humidity of 60%. The PlantScreen^{TM} XYZ system consists of a robotically driven arm holding an RGB camera with customized lighting panel and growing tables. The XYZ robotic arm was automatically moved above the plates to take RGB images of single plates from the top. The imaging of each 48 well plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days as described in Ugena et al. (2018). As outcome, the individual image of 48 Arabidopsis seedlings per variant (treatment vs. growth condition) as biological replicates were used for the analyzed phenotyping traits.

Different traits were determined from the RGB images: Arabidopsis rosette growth curves [as changes in the green area (Pixels)], relative (RGR) and absolute (AGR) growth rate and final rosette size. All these traits were then used to define the mode of action of the compound under test. Using the traits, the plant biostimulant characterization (PBC) index was determined as described by Ugena et al. (2018). The PBC index was calculated as the sum of the values obtained from each phenotyping trait calculated as the differences (as the log2 of the ratio in each case) between the controls and treatment variants (compound and concentration) under the same growth conditions.

Determination of Arabidopsis Rosette Color Indices

To estimate the greenness of the Arabidopsis seedlings, and changes in leaf color, three vegetation indices (NGRDI, VARI, and GLI), which have been shown to be correlated with plant biomass, nutrient status, or tolerance to abiotic stress (Gitelson et al., 2002; Perry and Roberts, 2008; Hunt et al., 2013), were used. The images captured on the seventh day of an Arabidopsis rosette growth assay subjected to HTS were segmented for the extraction of leaf rosettes using software described in our previous report (De Diego et al., 2017). The values corresponding to particular color channels (red, R; green, G; and blue, B) were then extracted for each pixel within the plant mask, and the vegetation indices were calculated as described by Ugena et al. (2018). Subsequently, indices representing particular seedlings were determined by calculating the mean values for each plant mask. The mean and the standard error (SE) values for each 48-well plate were then calculated and represented in a graph.

Plant Hormone Quantification

Four independent biological replicates consisted in four individual pools from 12 Arabidopsis seedlings per variant were collected for the hormonal analysis. After purification and extraction, the concentration of each analyte was calculated using the standard isotope dilution method (Rittenberg and Foster, 1940). Briefly, as the first step a micro solid-phase extraction (μSPE) based on StageTip (STop And Go Extraction Tip) technology was used to purify the plant tissue samples. The μSPE protocol used in CK extraction and purification was applied as described by Svačinová et al. (2012), whereas auxins and ABA were isolated as described by Pěnčík et al. (2018). CKs were determined using ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (an Acquity UPLC

[1] http://www.plant-phenotyping.org/db_infrastructure#/tool/57

I-Class System coupled with a Xevo TQ-S MS, Waters). Quantification of auxins and ABA was performed and the concentration of each analyte was calculated using the standard isotope dilution method on a 1260 Infinity II system coupled with a 6495B Triple Quadrupole LC/MS system (Agilent Technologies).

Statistical Analysis

To assess differences between treatments (compound and concentration) values for each non-invasive trait extracted by means of image analysis, a non-parametric (Dunn's test after Kruskal–Wallis' test parametric) method and a parametric method (Tukey's HSD test after two-way ANOVA) were applied using the packages *multcomp, FSA,* and *agricolae* in RStudio (Version 1.1.463 – 2009-2018 RStudio, Inc.). Multivariate statistical analyses, including heatmap and principal component (PC) analysis, were also performed in RStudio using the packages *gplots*, *cluster*, *tidyverse*, *factoextra*, *heatmap.plus*, *ggpubr*, *factoextra*, *FactoMineR,* and *corrplot*.

RESULTS

Synthesis of Four N^9-Substituted CK Derivatives With a Fluorinated Carbohydrate Moiety

In this work, a group of three N^9-substituted aromatic or one isoprenoid CK derivatives with a fluorinated carbohydrate moiety were synthesized (**Figure 1**) and their biological activity was investigated. The compounds prepared were characterized by ^{1}H NMR, elemental analysis, melting points, TLC and ESI + MS. The purity of the prepared derivatives was confirmed by high-performance liquid chromatography (HPLC-UV) (**Table 1**).

First, the synthesis of a 2-fluoropentose from a pentoside precursor followed by its conversion into 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine was performed as reported in 1969 (Wright et al., 1969). Subsequently, 3-deoxy-3-fluoro-D-glucose was synthesized and converted into 2-deoxy-2-fluoro-D-arabinose via oxidation by sodium metaperiodate as described by Reichman et al. (1975). The compound 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine (F-ara-A) has previously been prepared by condensation of 6-chloropurine with 2-deoxy-2-fluoro-D-arabinofuranosyl bromide followed by conversion of the purine into adenine, but the reaction produced a mixture of four isomers and only a very low yield of the desired isomer could be isolated (Marquez et al., 1990). Later, a three-step synthesis of 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine was carried out via displacement of the 2′-hydroxyl group of 03′,05′,N^6-tritrityladenosine and 03′,05′-ditritylinosine with diethylaminosulfur trifluoride, as published by Pankiewicz et al. (1992). The importance of introducing a fluorine at the 2′(S)(ara) site of purine deoxynucleosides has been highlighted, since 2′-deoxy-2′-fluoroarabinosides have been found to be biologically active and chemically stable against hydrolysis catalyzed both chemically and by purine nucleoside phosphorylase (Chu et al., 1989).

TABLE 1 | Physico-chemical properties of four new synthesized compounds: elemental analysis, melting temperature, mass of positively charged molecular ions analyzes by HPLC-MS, purity, and HRMS mass analysis.

Compound	Elemental analysis calculated/found			Mp (°C)	ES-MS ($M + H^+$)	HPLC (%)	HRMS			
	% of C	% of H	% of N				Exact mass	Theoretical monoisotopic mass	Difference (ppm)	Elementary analysis ($M + H^+$)
1	56.82/56.18	5.05/5.02	19.49/19.28	179–180	360	99^+	359.1393	359.1394	−0.23	$C17H_{19}FN_5O_3$
2	54.40/53.61	4.83/5.00	18.66/18.23	101–103	376	99^+	375.1341	375.1343	−0.48	$C_{17}H_{19}FN_5O_4$
3	55.52/56.05	5.18/4.86	17.99/19.24	212–213	390	99^+	389.1502	389.1499	0.74	$C_{18}H_{21}FN_5O_4$
4	53.40/54.94	5.98/5.80	20.76/18.73		338	99^+	337.1552	337.155	0.4	$C_{15}H_{21}FN_5O_3$

In the present study, all the aforementioned steps were followed and finally the synthesis of new compounds was performed as previously reported by Wan et al. (2005) with some modifications. Typically, the synthesis of purine nucleosides is based on the protection of hydroxyl groups, which prolongs this method to a four-step process with low yield. This transformation usually causes cleavage of the glycosyl bond, therefore only acid-labile protecting groups must be used. In our new simple one-step unprotected synthesis, BOP was used to activate the formation of a C-N bond. Subsequently, substitution by appropriate amines led to the formation of final products, after elimination of hexamethylphosphoramide (HMPA). However, the nucleophilic substitution of unprotected purine nucleosides with amines required longer reaction times compared with their protected counterparts (Wan et al., 2005).

CK-Like Activity of the New N^9-Substituted CK Derivatives With a Fluorinated Carbohydrate Moiety in Cytokinin Bioassays

To evaluate the CK activities of the newly synthesized compounds, three classical *in vitro* CK bioassays were used. Despite the fact that all four of the new compounds are derived from CKs with known high levels of activity in all three bioassays, their 2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosyl purine derivatives showed decreases in activity in the Amaranthus and tobacco callus bioassays (**Supplementary Table 1**). On the other hand, high antisenescence activity was recorded in the bioassay based on evaluating the effect of the compound on retention of chlorophyll in excised wheat leaves kept in the dark (**Table 2**). The greatest ability to prevent chlorophyll degradation was shown by compounds 1 and 2, which reached, respectively, 277 and 267% of the values for the positive control BAP at concentrations of 10^{-4} M, followed by compound 3, which showed 179% of the BAP activity. Compound 4 had comparable activity to BAP (**Table 2**). Overall, these results showed that substitution at the N^9 position with a fluorinated carbohydrate moiety selectively influences the CK-like activity, specifically improving the antisenescence properties of CKs modified in this way. This suggests that such CK analogs could activate plant processes related to stress responses and would therefore have antistress properties when applied to plants.

TABLE 2 | Relative CK activities of four new synthesized compounds in the senescence bioassay.

Compounds	Senescence bioassay	
	Optimal concentration (M)	Relative activity (%)
1	10^{-4}	277 (±9)
2	10^{-4}	267 (±17)
3	10^{-4}	179 (±3)
4	10^{-4}	95 (±6)

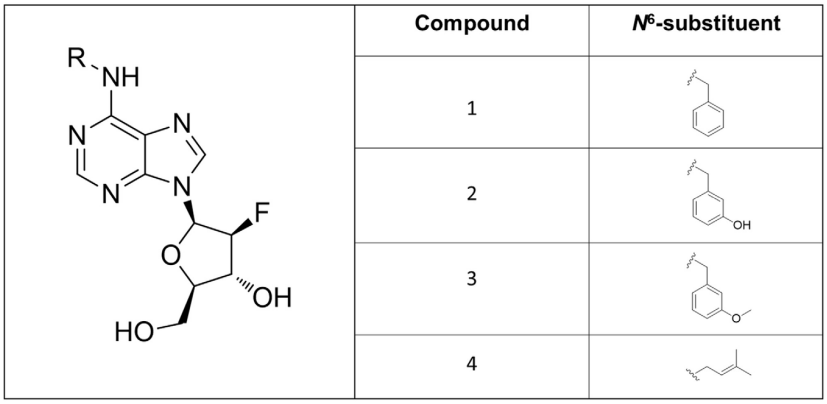
The optimal concentration for compounds 1–3 was compared with the activity of benzylaminopurine (BAP), where 100% means 10^{-4} M BAP. The optimal concentration for compound 4 was compared with the activity of isopentenyladenine (iP), where 100% means 10^{-4} M iP.

Priming With N^9-Substituted CK Derivatives With a Fluorinated Carbohydrate Moiety Improves the Growth of Arabidopsis Under Both Optimal and Stress Conditions

To corroborate the involvement of these compounds in plant stress tolerance and better define the mode of action of our four newly synthesized compounds, we tested their effects on Arabidopsis growth and development under optimal and stress conditions using a complex multi-trait high-throughput screening approach (Ugena et al., 2018). The four compounds were used as seed priming agents at four concentrations (from 10^{-7} to 10^{-4} M). Non-primed and primed seeds were germinated under optimal conditions and then the seedlings were transferred into 48 well plates with 1× MS alone, or supplemented with 100 mM NaCl or 100 mM mannitol to induce salt or osmotic stress, respectively. First, we evaluated how the priming affected early seedling establishment. To do so, the rosette area of the seedlings transferred to control conditions (1× MS) at day 1 was determined. Here, we saw a clear interaction between compound and concentration affecting early seedling establishment (**Figure 3** and **Supplementary Figure 1**). The seedlings developed from seeds primed with all the compounds except compound 1 had increased rosette area. The largest rosettes were observed after priming with the highest concentrations of compound 2 and 3, or lower concentrations of compound 4. Interestingly, priming with the highest concentration (10^{-4} M) of compound 4 caused strong growth inhibition, leading to seedlings reaching only half the size of the control (MOCK) seedlings (**Figure 3** and **Supplementary Figure 1**).

The rosette areas of the seedlings were further analyzed twice a day for an additional 6 days to record a growth curve (**Supplementary Figure 2**). All four compounds improved Arabidopsis seedling growth under control and stress conditions at some of the concentrations tested and there was significant interaction between compound concentration and growth conditions according to ANOVA. On the other hand, the highest concentration of compound 4 (10^{-4} M) showed inhibitory activity under all three of the conditions tested and the rosette areas were significantly reduced to 20, 60, and 48% of those in the non-primed control (MOCK) seedlings under, respectively, control, salt, and osmotic stress (**Supplementary Figure 2**).

Other traits such as relative growth rate (RGR) and absolute growth rate (AGR) were also calculated. For better visualization, these traits together with early seedling establishment and final rosette area (at day 7, **Supplementary Figure 3**) are presented in a parallel coordinate plot shown in **Figure 3**. To construct this, the differences between the controls and variants (compound and concentration) under the same growth conditions were calculated as the log2 of the ratio. The value obtained for each trait is shown in twelve independent parallel coordinate plots, one per compound (a total of 4) for optimal conditions, salt stress,



Compound	N^6-substituent
1	
2	
3	
4	

FIGURE 2 | Structures of the newly synthesized 6-benzylamino-2′-deoxy-2-′fluoro-9-(β)-D-arabinofuranosylpurine derivatives.

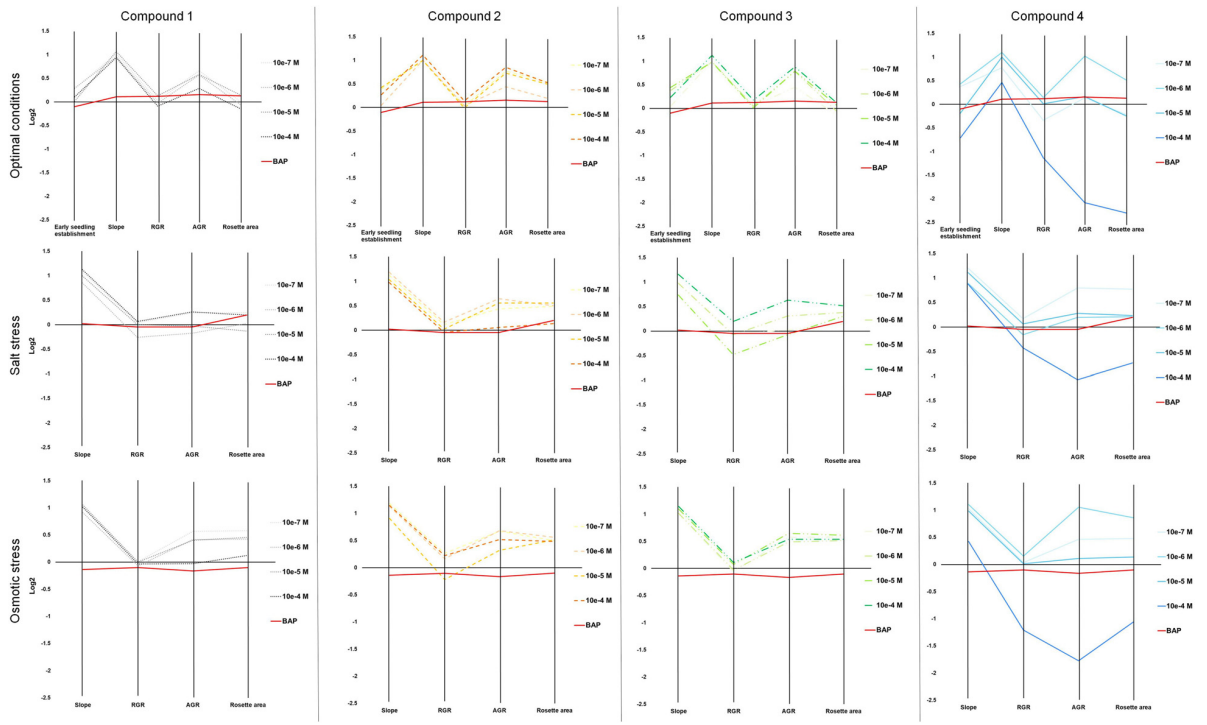


FIGURE 3 | Parallel coordinate plot of the traits (Early seedling establishment, slope of the growing curve, RGR, AGR and the final rosette area) obtained from multi-trait high-throughput screening of Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) and grown under optimal (upper panels), or salt (100 mM NaCl, middle panels) or osmotic (100 mM mannitol, bottom panels) stress conditions (N = 48). BAP at 10^{-8} M was used as positive control.

and osmotic stress (**Figure 3**). Additionally, the priming effect of 10^{-8} BAP was evaluated as a positive control. Interestingly, in the parallel plot the three N^9-substituted aromatic had similar profile whereas isoprenoid CK derivative showed different response (**Figure 3**). Under optimal growth conditions, priming with the new CK analogs improved some growth related traits analyzed (early seedling establishment, the slope of the curve, and AGR). At the assay end-point, mainly the seedlings primed with almost all concentrations of compound 2 had larger rosettes compared to the non-primed seedlings (MOCK) or those primed with the positive control (BAP) (**Figure 3**). These plants also presented higher homogeneity of the population (represented by coefficient of variance = standard deviation/Mean, %) compared with the MOCK variant (28.81 and 38.40, respectively) (**Figure 4**).

Importantly, hormopriming improved the tolerance of the Arabidopsis seedlings to salt and mannitol induced stress by increasing the values of the slope of the curve, RGR, ARG and final rosette size compared to the negative and positive

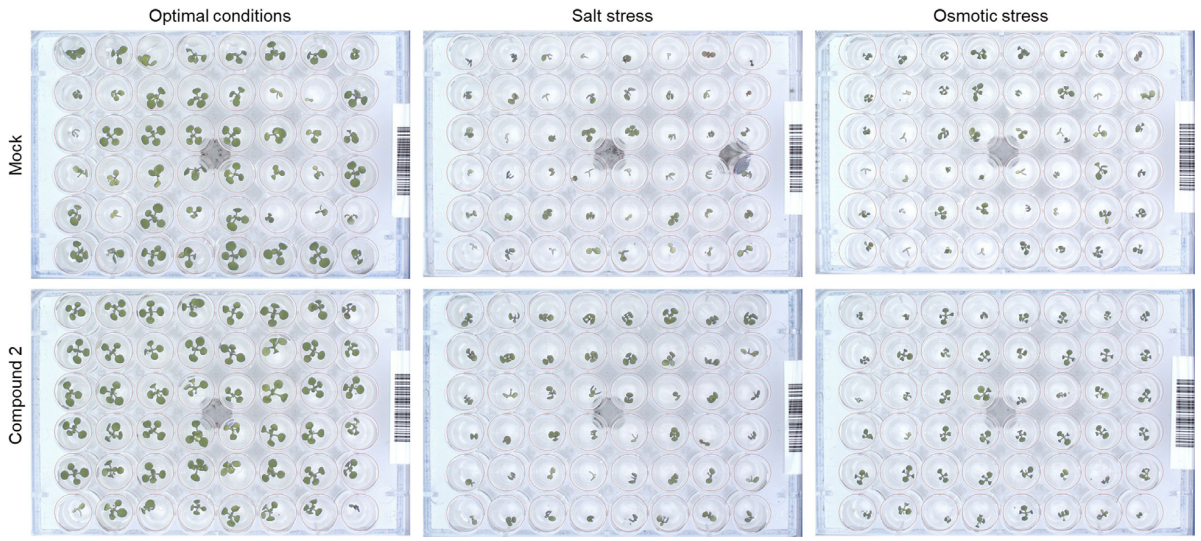


FIGURE 4 | RGB images of the non-primed Arabidopsis seedlings (MOCK) and those primed with the best performing concentration of compound 2 (according to the PBC index, **Table 3**) grown under optimal growth condition (left images), salt (100 mM NaCl, middle images) or osmotic (100 mM mannitol, right images) stress.

controls (**Figure 3**). In both cases, low concentrations of compound 4 (10^{-7} M for salt stress and 10^{-6} M for osmotic stress) resulted in the highest increases in the traits analyzed, whereas a concentration of 10^{-4} M inhibited plant growth under all growth conditions (**Figure 3** and **Supplementary Figures 1**, **2**). Conversely, plants primed with compound 2 showed improvements in all traits under both control and stress conditions (**Figure 4**). All these results were then combined to calculate the PBC index, which helps to simplify and sum up the overall outcomes in order to define the mode of action of a biostimulant (Ugena et al., 2018). As listed in **Table 3**, all compounds worked as plant growth promotors and stress alleviators at some of the concentrations tested, all with higher efficiency than the control CK BAP. The most efficient plant growth promotor was compound 4 followed by compound 2. However, whereas compound 2 improved growth at all concentrations tested and growth conditions (working as strong plant growth promotor and stress alleviator), compound 4 was highly toxic at the highest concentration (10^{-4} M), at which it showed a growth inhibitory effect (**Table 3**). Overall, we conclude that priming with the newly prepared N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines had positive effects on Arabidopsis growth and, importantly, improved tolerance to salt and osmotic stress, with a stronger effect in the latter case (**Figures 3**, **4** and **Table 3**).

Hormopriming With N^6-Substituted-2′-Deoxy-2′-Fluoro-9-(β)-D-Arabinofuranosylpurines Maintains Seedling Greenness

To gain a further understanding of priming with compound 2 (N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines), changes in seedling color after 7 days under different growth conditions (optimal, salt, or osmotic stress) were determined. The degradation of chlorophyll, manifested as a change in Arabidopsis rosette color, represents one of the most important symptoms of stress (Ugena et al., 2018). Three different indices (NGRDI, VARI, and GLI) were calculated and presented in **Figure 5**. Significant differences were observed between seedlings from non-primed and primed seeds, especially regarding NGRDI and VARI indices under all growth conditions (**Figure 4**). Under optimal conditions, the highest values were obtained when the compound 2 was applied at 10^{-4} M, a concentration that also resulted in the highest PBC index (**Table 3**). However, under salt and osmotic stress, the highest NGRDI and VARI indices were observed when 10^{-5} M and 10^{-6} M were used (**Figure 5**). Taken together, these results corroborated the aforementioned antisenescence effect of this compound observed in the CK-like bioassays (**Table 2**).

Hormopriming With N^6-Substituted-2′-Deoxy-2′-Fluoro-9-(β)-D-Arabinofuranosylpurines Improves Arabidopsis Growth and Stress Tolerance by Altering the Hormonal Profile

To understand the molecular nature of the mode of action of priming by the N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines, the hormonal profile of Arabidopsis seedlings primed with the best performing compound 2 was analyzed at the end of the phenotyping experiment. The endogenous levels of CKs (**Table 4**), some auxins and ABA were quantified using LC/MS (**Supplementary Table 2**). For better visualization and interpretation, all metabolites were analyzed together using a heatmap (**Figure 6A**). The results separated the variants (treatments and growth conditions) into two clusters

TABLE 3 | Plant biostimulant characterization (PBC) index calculated by summing the relative changes (log2) obtained for the parallel coordinate plot (**Figure 4**) for each synthetized compound (four different N^9-substituted CK derivatives with a fluorinated carbohydrate moiety) at four concentration (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) and growth condition; optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) (*N* = 48).

	Optimal growth condition				**100 mM NaCl**				**100 mM mannitol**			
	Concentration (M)				**Concentration (M)**				**Concentration (M)**			
	10^{-7}	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-7}**	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-7}**	**10^{-6}**	**10^{-5}**	**10^{-4}**
Comp_1	2.00	1.73	1.95	1.11	0.53	−0.40	−0.16	0.52	1.16	0.77	0.86	0.06
Comp_2	2.73	1.73	2.64	2.90	1.04	1.31	1.13	0.15	1.43	1.40	0.62	1.22
Comp_3	1.40	2.14	2.29	2.54	0.12	0.62	−0.25	1.35	1.09	0.98	1.33	1.19
Comp_ 4	0.74	3.22	0.75	−5.77	1.74	0.26	0.58	−2.22	0.97	2.07	0.26	−4.04
BAP	0.41				0.10				−0.37			
Comp_1	**Strong growth promotor**				Weak stress alleviator				Medium stress alleviator			
Comp_2	**Strong growth promotor**				**Strong stress alleviator**				**Strong stress alleviator**			
Comp_3	**Strong growth promotor**				Medium growth promotor				**Strong stress alleviator**			
Comp_4	Growth promotor and inhibitor				Stress alleviator and inhibitor				Stress alleviator and inhibitor			
BAP	Weak growth promotor				Weak stress alleviator				Stressor			

BAP at 10^{-8} M was used as positive control. Bold terms indicate the best performing compounds at the indicated growth conditions. Blue shades indicates positive effect, white no effect and red shade indicates negative effect.

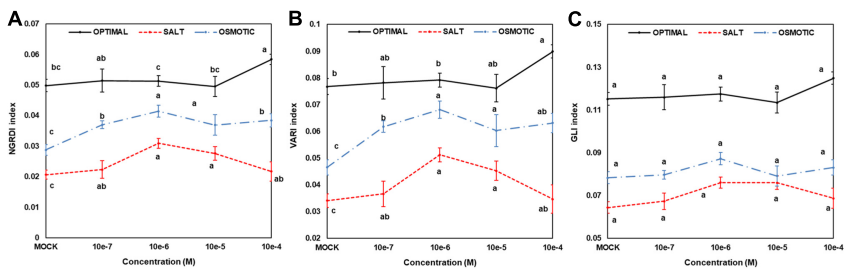


FIGURE 5 | Color indices [NGRDI **(A)**, VARI **(B)**, or GLI **(C)**] used as greenness parameters for Arabidopsis seedlings from non-primed (MOCK) seeds or seeds primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) grown under optimal (upper panel), or salt (100 mM NaCl, middle panel), or osmotic (100 mM mannitol, bottom panel) stress conditions for 7 days (*N* = 48). Different letters mean significant differences among treatments (priming effect) for each growth conditions according to Dunn's test after Kruskal–Wallis' test.

using the Spearman correlation as the distance method: one for the plants grown under salt stress and the control for osmotic stress, and a second cluster for the rest. Additionally, the first group was separated into two subclusters in which all plants grown under salt stress showed, in general, a reduction in content of the CK nucleotides, total auxin, and ABA (**Figure 6A**). On the other hand, the variants represented in the second cluster showed increased levels of these metabolites and of some *N*- and *O*-glucosides (DHZ7G, DHZ9G, *t*Z7G, and *t*Z9G), and IAA conjugated with glucose (IAAGlu). On the other hand, they reduced content of total CKs, bases, and ribosides, especially in the case of the hormoprimed seedlings under optimal and osmotic stress conditions (**Figure 6A**). Similar results were obtained when the distance among variants was determined (**Supplementary Figure 4**), in which the hormopriming seedlings grown under optimal and osmotic stress were separated by a short distance (close to 0; similar behavior), but the distance was longer for the primed plants grown under salt stress conditions.

To extend the analysis, a PC analysis was also performed (**Figure 6B**). The components PC1 and PC2 accounted for 60.5% of the total variance of the model. In PC1, there was clear evidence of contrasting behavior between all plants grown under optimal conditions and hormoprimed seedlings under salinity stress (blue ellipses). Thus, whereas the first group was positively correlated with the phenotyping traits and the CK nucleotides (synthesized *de novo*), which also showed a strong relationship (**Supplementary Figure 5**), the second group had a higher content of total CKs due to an increase in ribosides and *N*-glucosides (iP7G and iP9G) and *O*-Glucosides (*c*ZOG and *c*ZROG) (**Figure 6B**). Interestingly, hormopriming with the highest concentration (10^{-4} M) of compound 2 (as shown by PC2, red ellipses) induced similar contents of total auxins and the degradation form 2-oxindole-3-acetic acid (oxIAA) independent of growth conditions (**Figure 6B**), a pattern opposite to that in the MOCK variant under salt and osmotic stress. Overall, we demonstrated that in general hormopriming with the N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurines presented here induced changes in the hormonal content of Arabidopsis seedlings, thus conditioning the final phenotype, with the changes depending on the concentration of the compound and on growth conditions.

TABLE 4 | Changes in CK levels (pmol g^{-1} FW) of 10-day-old *Arabidopsis thaliana* seedlings from non-primed seeds or seeds hormoprimed with compound 2 grown at four different concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) under optimal conditions, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) for 7 days.

Conditions	Cytokinins	Optimal conditions				
		MOCK	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Optimal conditions	Total CKs	104.73 ± 18.27	108.33 ± 14.54	97.85 ± 14.04	114 ± 21.56	114.54 ± 16.29
	Bases	0.08 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.018
	Ribosides	18.65 ± 3.53	19.96 ± 4.08	16.98 ± 4.72	20.1 ± 5.28	16.77 ± 2.88
	Nucleotides	15.35 ± 5.90	12.25 ± 0.97	12.98 ± 1.44	14.73 ± 2.41	18.51 ± 3.29
	O-glucosides	7.53 ± 1.30	7.94 ± 1.21	6.65 ± 1.56	7.63 ± 2.02	6.49 ± 0.91
	N-glucosides	63.12 ± 9.19	68.11 ± 9.45	61.15 ± 7.43	71.46 ± 14.07	72.7 ± 9.93
	iP-types	47.40 ± 8.85	47.29 ± 5.40	45.92 ± 6.3	54.05 ± 9.58	56.09 ± 8.43
	iP	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	11.92 ± 2.64	12.3 ± 2.99	10.81 ± 3.08	13.3 ± 3.71	11.49 ± 2.23
	iPRMP	6.43 ± 1.43	6.05 ± 1.04	7.01 ± 0.62	7.23 ± 1.42	10.22 ± 1.72
	iP7G	25.02 ± 4.29	25.48 ± 3.05	24.99 ± 2.85	29.69 ± 5.79	29.84 ± 4.42
	iP9G	4.03 ± 0.68	3.46 ± 0.59	3.12 ± 0.39	3.83 ± 0.67	4.54 ± 0.49
	*t*Z-types	25.41 ± 4.52	32.79 ± 6.04	28.94 ± 3.01	32.33 ± 6.57	30.42 ± 6.23
	*t*Z	0.08 ± 0.01	0.07 ± 0.02	0.08 ± 0.023	0.07 ± 0.016	0.07 ± 0.018
	*t*ZR	2.27 ± 0.33	4.41 ± 1.32	4.07 ± 1.03	4.09 ± 0.89	3.06 ± 0.61
	*t*ZRMP	1.45 ± 0.25	2.1 ± 0.47	2.26 ± 0.22	2.12 ± 0.39	2.76 ± 0.65
	*t*ZOG	1.89 ± 0.33	2.92 ± 0.42	2.37 ± 0.54	2.94 ± 0.66	2.17 ± 0.37
	*t*ZROG	0.73 ± 0.20	0.32 ± 0.05	0.2 ± 0.03	0.35 ± 0.09	0.23 ± 0.02
	*t*Z7G	7.79 ± 1.82	10.17 ± 0.97	12.45 ± 2.59	13.53 ± 2.30	10.68 ± 2.86
	*t*Z9G	3.13 ± 0.61	9.79 ± 1.07	10.32 ± 2.66	8.6 ± 2.44	5.99 ± 1.37
	DHZ-types	2.96 ± 0.51	4.36 ± 0.74	3.46 ± 0.94	4.09 ± 0.90	4.42 ± 0.53
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.15 ± 0.04	0.17 ± 0.07	0.12 ± 0.04	0.15 ± 0.05	0.09 ± 0.01
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZ7G	1.5 ± 0.37	3.20 ± 0.90	3.80 ± 0.83	4.18 ± 0.48	1.49 ± 0.44
	DHZ9G	0.07 ± 0.02	0.13 ± 0.03	0.14 ± 0.03	0.17 ± 0.03	0.07 ± 0.02
	*c*Z-types	28.97 ± 7.73	23.89 ± 3.33	19.53 ± 4.8	23.52 ± 4.90	23.61 ± 2.97
	*c*Z	<LOD	<LOD	<LOD	<LOD	<LOD
	*c*ZR	4.33 ± 1.24	3.07 ± 0.47	1.98 ± 0.64	2.57 ± 0.66	2.16 ± 0.42
	*c*ZRMP	7.48 ± 5.00	4.1 ± 1.36	3.71 ± 1.04	5.37 ± 0.67	5.53 ± 1.02
	*c*ZOG	1.02 ± 0.22	0.58 ± 0.11	0.52 ± 0.1	0.59 ± 0.14	0.55 ± 0.13
	*c*ZROG	3.90 ± 0.86	4.12 ± 0.69	3.57 ± 1.02	3.76 ± 1.21	3.54 ± 0.77
	*c*Z7G	14.46 ± 3.81	9.16 ± 2.50	10.69 ± 2.64	11.32 ± 1.35	13.79 ± 3.38
	*c*Z9G	0.57 ± 0.14	0.59 ± 0.16	0.55 ± 0.14	0.51 ± 0.09	0.64 ± 0.20
Salt stress (100 mM NaCl)	Total CKs	157.34 ± 35.63	173.13 ± 27.9	208.79 ± 19.85	244.29 ± 61.86	164.18 ± 22.6
	Bases	0.13 ± 0.03	0.49 ± 0.09	0.46 ± 0.12	0.20 ± 0.05	0.20 ± 0.06
	Ribosides	75.35 ± 17.82	53.94 ± 16.41	76.5 ± 16.27	95.05 ± 28.28	44.51 ± 11.92
	Nucleotides	4.51 ± 1.06	4.25 ± 1.43	6.8 ± 0.96	9.36 ± 5.12	8.35 ± 2.36
	O-glucosides	8.48 ± 1.47	14.33 ± 3.63	15.66 ± 2.17	16.32 ± 3.80	14.5 ± 3.38
	N-glucosides	68.90 ± 17.88	100.11 ± 7.04	109.36 ± 13.89	123.37 ± 25.39	96.63 ± 18.40
	iP-types	67.33 ± 15.565	73.08 ± 7.08	95.7 ± 11.8	117.44 ± 25.00	80.28 ± 8.09
	iP	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	30.56 ± 5.83	27.47 ± 8.71	36.77 ± 10.13	47.19 ± 13.93	26.68 ± 8.57
	iPRMP	1.88 ± 0.49	1.7 ± 0.47	2.59 ± 0.56	3.12 ± 0.82	4.32 ± 0.51
	iP7G	30.49 ± 8.87	39.03 ± 5.18	51.16 ± 10.73	61.20 ± 9.55	45.16 ± 8.81
	iP9G	4.41 ± 1.09	4.88 ± 1.29	5.18 ± 0.19	5.93 ± 1.33	5.2 ± 1.42

(Continued)

TABLE 4 | Continued

Conditions	Cytokinins	Optimal conditions				
		MOCK	**10^{-7} M**	**10^{-6} M**	**10^{-5} M**	**10^{-4} M**
	*t*Z-types	24.3 ± 6.30	57.99 ± 10.36	42.58 ± 9.15	41.62 ± 11.39	19.86 ± 4.25
	*t*Z	0.13 ± 0.03	0.49 ± 0.09	0.46 ± 0.12	0.20 ± 0.05	0.20 ± 0.06
	*t*ZR	4.19 ± 1.10	15.19 ± 4.67	7.14 ± 1.73	7.93 ± 2.54	2.25 ± 0.66
	*t*ZRMP	0.50 ± 0.12	0.43 ± 0.08	1.18 ± 0.26	1.73 ± 0.53	0.78 ± 0.18
	*t*ZOG	1.72 ± 0.51	5.5 ± 1.38	3.48 ± 0.97	3.65 ± 1.13	1.58 ± 0.44
	*t*ZROG	0.77 ± 0.21	0.7 ± 0.18	0.65 ± 0.13	0.68 ± 0.17	0.32 ± 0.11
	*t*Z7G	10.87 ± 2.14	19.13 ± 4.16	17.41 ± 4.46	9.97 ± 1.76	10.52 ± 2.24
	*t*Z9G	6.04 ± 1.36	10.54 ± 2.45	10.03 ± 2.59	4.76 ± 1.54	6.81 ± 1.89
	DHZ-types	2.04 ± 0.51	3.46 ± 0.81	4.06 ± 0.69	4.33 ± 1.06	2.22 ± 0.5
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.58 ± 0.11	0.57 ± 0.12	1.02 ± 0.26	0.93 ± 0.25	0.32 ± 0.09
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZ7G	2.59 ± 0.54	2.9 ± 0.54	3.27 ± 0.84	1.76 ± 0.52	2.65 ± 0.66
	DHZ9G	0.12 ± 0.01	0.14 ± 0.02	0.13 ± 0.03	0.13 ± 0.04	0.13 ± 0.04
	*c*Z-types	63.67 ± 15.175	38.6 ± 10.84	66.44 ± 9.76	80.9 ± 25.69	61.83 ± 10.99
	*c*Z	<LOD	<LOD	<LOD	<LOD	<LOD
	*c*ZR	40.02 ± 12.19	10.72 ± 3.1	31.58 ± 6.52	39 ± 12.4	15.25 ± 3.84
	*c*ZRMP	2.13 ± 0.55	2.12 ± 1.02	3.03 ± 0.57	4.51 ± 3.81	4.34 ± 1.22
	*c*ZOG	1.90 ± 0.59	2.02 ± 0.53	1.87 ± 0.19	3.08 ± 0.76	2.43 ± 0.75
	*c*ZROG	4.10 ± 0.90	6.12 ± 1.58	9.67 ± 1.38	8.91 ± 1.95	10.17 ± 2.52
	*c*Z7G	14.49 ± 2.53	19.28 ± 2.58	24.63 ± 7.6	28.43 ± 8.26	16.72 ± 4.28
	*c*Z9G	0.73 ± 0.17	1.03 ± 0.16	0.78 ± 0.25	1.21 ± 0.39	0.86 ± 0.28
Osmotic stress (100 mM mannitol)	Total CKs	83.87 ± 18.50	112.08 ± 8.93	103.89 ± 9.47	148.80 ± 15.57	138.51 ± 10.90
	Bases	0.056 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.03	0.23 ± 0.08
	Ribosides	16.83 ± 3.28	15.71 ± 2.19	13.91 ± 3.03	21.62 ± 2.88	25.41 ± 4.07
	Nucleotides	5.64 ± 1.52	10.41 ± 1.25	9.43 ± 1.44	15.15 ± 2.53	16.63 ± 0.98
	O-glucosides	6.44 ± 1.27	11.41 ± 1.34	8.71 ± 1.13	11.30 ± 1.15	12.15 ± 1.75
	N-glucosides	54.92 ± 14.04	74.45 ± 6.65	71.77 ± 6.45	100.63 ± 10.93	84.09 ± 10.49
	iP-types	41.23 ± 9.84	49.60 ± 2.20	46.33 ± 3.91	70.72 ± 6.57	61.58 ± 6.30
	iP	<LOD	<LOD	<LOD	<LOD	<LOD
	iPR	10.83 ± 2.35	11.25 ± 1.62	9.02 ± 1.97	15.12 ± 1.64	16.54 ± 2.72
	iPRMP	2.10 ± 0.63	4.93 ± 1.25	3.8 ± 0.75	6.81 ± 1.36	6.04 ± 1.26
	iP7G	25.38 ± 7.08	30.05 ± 1.13	30.24 ± 1.85	44.51 ± 4.67	35.41 ± 5.77
	iP9G	2.94 ± 0.82	3.38 ± 0.53	3.28 ± 0.72	4.28 ± 0.74	3.58 ± 0.33
	*t*Z-types	16.33 ± 3.34	28.25 ± 3.95	24.96 ± 2.68	31.01 ± 6.53	28.95 ± 3.19
	*t*Z	0.056 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.026	0.23 ± 0.08
	*t*ZR	2.04 ± 0.46	2.78 ± 0.61	2.29 ± 0.32	3.21 ± 0.69	4.15 ± 0.26
	*t*ZRMP	0.565 ± 0.15	1.47 ± 0.41	1.08 ± 0.27	1.66 ± 0.28	1.96 ± 0.38
	*t*ZOG	1.595 ± 0.31	2.69 ± 0.52	2.25 ± 0.44	3.18 ± 0.56	2.58 ± 0.36
	*t*ZROG	0.32 ± 0.05	0.41 ± 0.06	0.37 ± 0.07	0.33 ± 0.08	0.41 ± 0.11
	*t*Z7G	11.815 ± 2.56	11.59 ± 2.08	13.41 ± 2.63	11.21 ± 1.53	4.01 ± 1.07
	*t*Z9G	6.26 ± 1.51	7.3 ± 1.15	9.12 ± 2.94	8.42 ± 1.37	2.19 ± 0.65
	DHZ-types	2.08 ± 0.50	3.14 ± 0.24	3.32 ± 0.79	4.33 ± 1.08	4.58 ± 0.66
	DHZ	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZR	0.15 ± 0.04	0.11 ± 0.03	0.07 ± 0	0.15 ± 0.05	0.22 ± 0.07
	DHZRMP	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZOG	<LOD	<LOD	<LOD	<LOD	<LOD
	DHZROG	<LOD	<LOD	<LOD	<LOD	<LOD

(Continued)

TABLE 4 | Continued

Conditions	Cytokinins	Optimal conditions				
		MOCK	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
	DHZ7G	1.92 ± 0.33	3.17 ± 0.76	4 ± 1.01	4.2 ± 0.67	1.06 ± 0.34
	DHZ9G	0.11 ± 0.03	0.11 ± 0.02	0.17 ± 0.05	0.17 ± 0.03	0.05 ± 0.01
	*c*Z-types	24.23 ± 5.10	31.09 ± 2.9	29.28 ± 2.93	42.74 ± 6.71	43.4 ± 1.98
	*c*Z	<LOD	<LOD	<LOD	<LOD	<LOD
	*c*ZR	3.83 ± 0.97	1.57 ± 0.17	2.56 ± 0.75	3.14 ± 0.91	4.5 ± 1.23
	*c*ZRMP	2.98 ± 0.935	4.01 ± 0.52	4.55 ± 0.63	6.68 ± 1.68	8.63 ± 1.23
	*c*ZOG	0.87 ± 0.19	1.4 ± 0.26	0.75 ± 0.16	0.96 ± 0.3	1.12 ± 0.25
	*c*ZROG	3.67 ± 0.835	6.91 ± 0.72	5.34 ± 0.99	6.84 ± 1.37	8.04 ± 1.15
	*c*Z7G	14.20 ± 2.68	15.53 ± 2.02	24.35 ± 4.67	20.14 ± 2.4	7.87 ± 2.07
	*c*Z9G	0.67 ± 0.18	0.55 ± 0.16	0.79 ± 0.23	0.96 ± 0.14	0.34 ± 0.07

Mean and SD.

DISCUSSION

In this work, a group of four N^9-substituted CK derivatives with a fluorinated carbohydrate moiety (three with an aromatic and one with isoprenoid N^6 side chain) were synthesized (Wan et al., 2005) by a slightly modified one-step reaction of 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)hypoxanthine with the appropriate amine or amine hydrochloride in the presence of BOP and DIPEA in DMF (**Figure 1** and **Table 1**). Nucleosides bearing fluorine or fluorinated substituents within the carbohydrate moiety have been used successfully in many biochemical research studies and therapeutic treatments. As an example, the ability of 9-(2-deoxy-β-D-arabinofuranosyl)adenine to completely inhibit the protozoan parasite *Trichomonas vaginalis* (Shokar et al., 2012), as well as its antibacterial (Gao et al., 2015) and antitrypanosomal (Ranjbarian et al., 2017) effect, have been reported. Significant antiviral activity was also confirmed for their dideoxy analogs. Montgomery et al. (1992) proved that 2-fluoro-9-(2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)adenine had anti-HIV properties, and 9-(2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)adenine was identified as an anti-HBV agent (Maruyama et al., 1999). However, their effects on plant species have never been investigated. Only few works presenting positive bioassay results for kinetin and isopentenyladenine analogs with N^9-substituted with short aliphatic chains been published (Mik et al., 2011a,b). Similar to these results, our newly synthesized compounds (especially compound 2) also showed high levels of antisenescence activity. Moreover, the low activity of the N^6-substituted-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine derivatives in the callus bioassay confirms that fluorination of the sugar moiety prevents its hydrolysis to free bases and makes these compounds metabolically stable. Due to the strong effect of the CK analogs with a fluorinated carbohydrate moiety on the retention of chlorophyll in excised wheat leaves in the dark (**Table 2**), we hypothesized that the compounds would have antistress properties and analyzed their mode of action and their potential use as priming agents.

For many years, it has been shown that seed priming with certain hormones or other compounds improves seed germination and fitness in many plants (Van Hulten et al., 2006; Hussain et al., 2016). Seed priming improves stress tolerance through "priming memory," which is established during priming and can be recruited later when seeds are exposed to stresses during germination (Chen and Arora, 2013). The beneficial effect of seed priming with CKs has been previously described under a range of growth conditions for many plant species, such as spring wheat (*Triticum aestivum* L.) (Iqbal and Ashraf, 2005; Iqbal et al., 2006) or basil (*Ocimum basilicum* L) (Bagheri et al., 2014). Despite this, there is not always a clear positive effect of priming, and it may also have a negative effect (Miyoshi and Sato, 1997; Sneideris et al., 2015; Willams et al., 2016), depending on the type of compound, the concentration used for priming, or the plant species and cultivars tested (reviewed by De Diego and Spíchal, 2020). In this work, hormopriming with the new N^9-substituted CK derivatives improved early seed establishment and plant growth in *A. thaliana* under optimal and stress growth conditions (**Figure 3**), mainly by making the population more homogeneous, maintaining plant greenness (less chlorophyll degradation) and better nutrient status as defined by higher color indices (**Figure 5**). However, this response was concentration dependent. The best-performing compound was 6-(3-hydroxybenzylamino)-2′-deoxy-2′-fluoro-9-(β)-D-arabinofuranosylpurine, which was a good growth promotor under optimal growth conditions and a stress alleviator under both salt and osmotic stress at almost all concentrations tested, according to the PBC index (**Table 3**). As an exception, compound 4 at 10^{-4} M showed a strong growth inhibitory (toxic) effect. However, lower concentrations (10^{-7} or 10^{-6} M) improved plant growth under different growth conditions (**Table 3**). This underlines the importance of testing chemicals over broad concentration ranges and under different growth conditions. This is possible through initial high-throughput approaches using model plants such as Arabidopsis, followed by studies in the targeted species and specific growth conditions (Rouphael et al., 2018).

To understand better how these new compounds modify plant metabolism when they are used as priming agents, the endogenous levels of some plant hormones (CK, auxins, and ABA) were quantified. It was clear that hormopriming

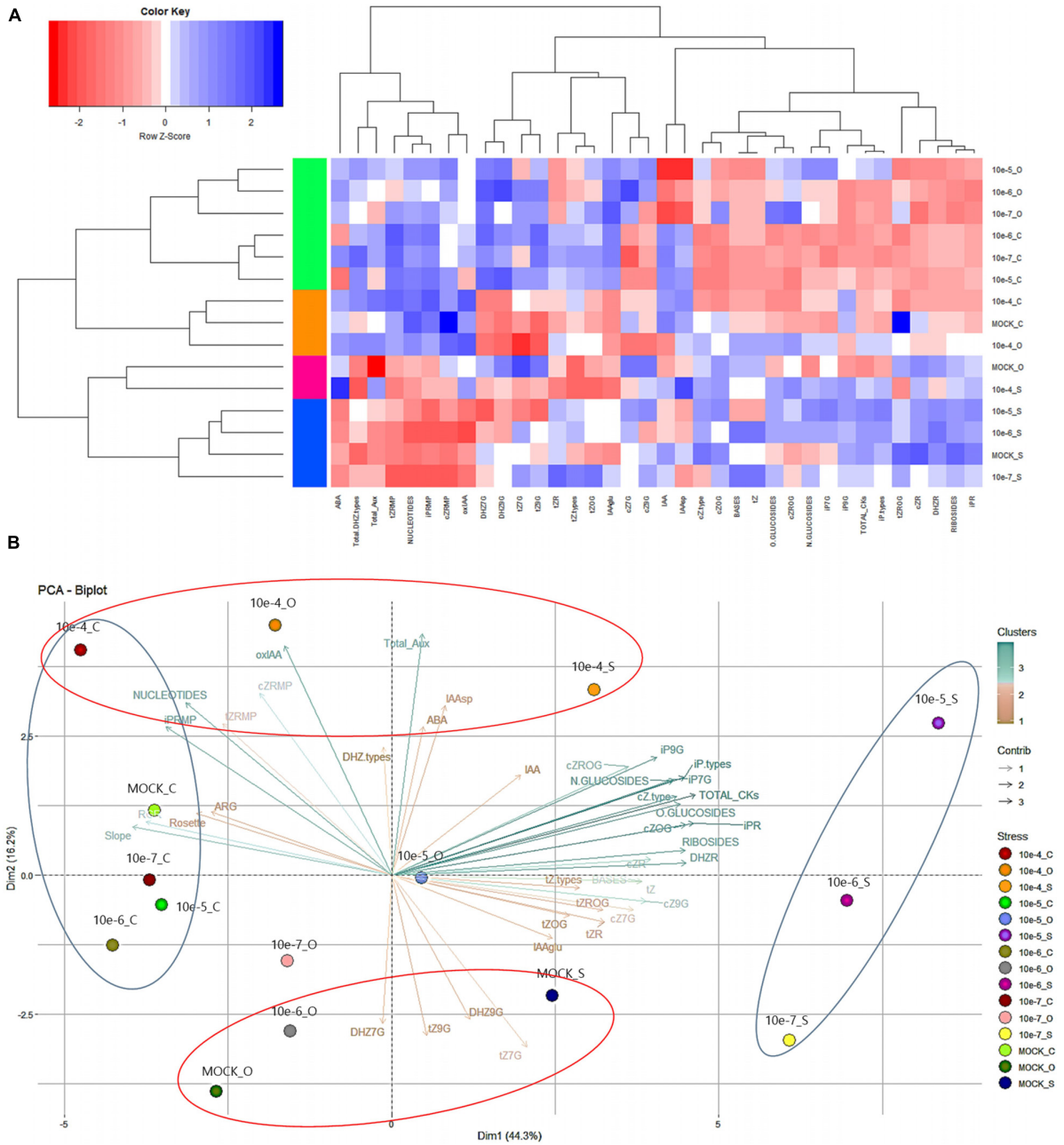


FIGURE 6 | Heatmap **(A)** representing the changes in CKs, auxins, and ABA for Arabidopsis seedlings from non-primed (MOCK) seeds or seeds primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) grown under optimal (black line), or salt (100 mM NaCl, red line) or osmotic (100 mM mannitol, blue line) stress conditions for 7 days. Principal component analysis (Dimension, Dim) **(B)** of the same Arabidopsis seedlings.

with compound 2 disrupted the plants' hormonal homeostasis (**Figure 6B**). However, the changes varied depending on the conditions under which the plants were grown. Thus, different behaviors were observed between hormoprimed seedlings under optimal and osmotic stress conditions, and those under salt stress (**Supplementary Figure 4**). For example, under optimal conditions, primed Arabidopsis seedlings accumulated higher levels of ribotides (precursors), which were positively correlated with phenotypic traits such as AGR, RGR, slope of the growing curve and final rosette area (**Supplementary Figure 5A**). However, primed plants grown under salt stress conditions elevated their total CK content by increasing the amounts of conjugated forms including ribosides (iPR. DHZR and *c*ZR), *O*-glucosides (*c*ZOG and *c*ZROG), and *N*-glucosides (iP7G and iP9G). It has been reported that riboside accumulation under stress conditions can be a defense mechanism, helping plants to

deal with stress (Veerasamy et al., 2007; Man et al., 2011; De Diego et al., 2015). This may be because they play a crucial role in CK-mediated leaf longevity, and hence senescence, through phosphorylation of the CK response regulator ARR2 (reviewed by Hönig et al., 2018). For several years the *c*Z-type CKs and the base *c*Z were considered to be low-activity forms. However, in recent years, it has been proved that *c*Z-type CKs play important roles during plant development and in environmental interactions (Schäfer et al., 2015; Lacuesta et al., 2018). Thus, in primed Arabidopsis plants high levels of accumulation of *c*ZOG and *c*ZROG could be a strategy for maintaining plant growth under salt stress conditions. In support of this, it has been reported that the content of *c*Z-type CKs changes rapidly during maize seedling growth, and that *c*Z catabolism and glycosylation by *c*Z *O*-glucosyl transferases work synergistically to fine-tune *c*Z levels during plant development (Zalabák et al., 2014). Finally, in these primed plants there was also considerable accumulation of iP7G and iP9G. These two iP derivates are the terminal products of iP metabolism (Hošek et al., 2020). The iP metabolites including iP-N9G are the least active CKs, which seem not to be hydrolyzed and simply accumulate in the tissue (if not degraded by CKX) with no physiological effects (Hoyerová and Hošek, 2020). Overall, it is clear that priming with CK analogs modifies CK metabolism, but these changes are dependent on plant growth conditions. The results also pointed to the iP-type and *c*Z-type CKs as the main metabolites regulating the alleviation of salt stress in primed Arabidopsis seedlings.

Regarding auxins, levels of oxIAA mainly increased when compound 2 was applied at a high concentration (10^{-4} M) (**Figure 6** and **Supplementary Table 2**). In recent years it has been proved that oxidizing IAA into oxIAA is of major physiological significance in the regulation of plant growth and development (Stepanova and Alonso, 2016). However, changes in other auxin-related metabolites did not show any correlation with the phenotypical changes in plants primed with compound 2.

CONCLUSION

In summary, in this case study we showed that hormopriming with N^9-substituted CK derivatives with a fluorinated carbohydrate moiety seems to be a promising biotechnological approach for improving early seedling establishment and plant growth under both control and stress conditions. This is due to changes in plant hormone metabolism (especially of CKs and auxins) that differs according to growth conditions. Moreover, we believe that we have shown here that a complex approach is needed for selection of suitable compounds, by employing strategies allowing simultaneous testing of a broad range of concentrations and different growth conditions to define the conditions in which they are most efficient as priming agents.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MB and KD synthesized the compounds. AH, AEH, LS, and ND designed and performed the phenotyping experiments. AH, AP, and ON carried out the metabolite quantification. AH and ND performed the data analysis. MB, AH, LS, KD, and ND wrote the manuscript. All authors discussed the results.

FUNDING

This work was funded by the project "Plants as a tool for sustainable global development" (registration number: CZ.02.1.01/0.0/0.0/16_019/0000827) within the program Research, Development and Education (OP RDE) and the Internal Grant Agency of Palacký University (IGA_PrF_2020_010).

ACKNOWLEDGMENTS

We thank sees-editing for the English revision.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.599228/full#supplementary-material

Supplementary Figure 1 | Early seedling establishment of Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives each with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown under control conditions (N = 48). Mean ± SE. Different letters mean significant differences among variants according to Tukey's HSD test after ANOVA.

Supplementary Figure 2 | Growth curves for Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives each with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) conditions (N = 48). Mean ± SE.

Supplementary Figure 3 | Maximum rosette size of Arabidopsis seedlings non-primed (MOCK) or primed with four different N^9-substituted CK derivatives each with a fluorinated carbohydrate moiety at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) conditions (N = 48). Mean ± SE. Different letters mean significant differences among variants according to Tukey's HSD test after ANOVA.

Supplementary Figure 4 | Distance between Arabidopsis seedlings non-primed (MOCK) or primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal (C), salt stress (100 mM NaCl, S) or osmotic stress (100 mM mannitol, O) conditions.

Supplementary Figure 5 | Correlation matrix **(A)** and contribution of the loadings to each PC (Dim) **(B)** according to multivariate statistical analyses of traits and metabolites in Arabidopsis seedlings non-primed (MOCK) or primed with compound 2 at four concentrations (10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M) grown for 7 days under optimal, salt stress (100 mM NaCl), or osmotic stress (100 mM mannitol) conditions (N = 48).

REFERENCES

Bagheri, A., Bagherifard, A., Saborifard, H., Ahmadi, M., and Safarpoor, M. (2014). Effects drought, cytokinins and GA3 on seedling growth of Basil (*Ocimum basilicum*). *Int. J. Adv. Biol. Biomed. Res.* 2, 489–493. doi: 10.1017/CBO9781107415324.004

Bairu, M. W., Jain, N., Stirk, W. A., Doležal, K., and Van Staden, J. (2009). Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. *South Afr. J. Bot.* 75, 122–127. doi: 10.1016/j.sajb.2008.08.006

Bielach, A., Hrtyan, M., and Tognetti, V. B. (2017). Plants under stress: involvement of auxin and cytokinin. *Int. J. Mol. Sci.* 18:1427. doi: 10.3390/ijms18071427

Brzobohatý, B., Moore, I., Kristoffersen, P., Bako, L., Campos, N., and Schell, J. (1993). Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. *Science* 262, 1051–1054. doi: 10.1126/science.8235622

Chen, K., and Arora, R. (2013). Priming memory invokes seed stress-tolerance. *Environ. Exp. Bot.* 94, 33–45. doi: 10.1016/j.envexpbot.2012.03.005

Chu, C. K., Matulic-Adamic, J., Huang, J.-T., Chou, T.-C., Burchanal, J. H., Fox, J. J., et al. (1989). Nucleotides. CXXXV. Synthesis of some 9-(2-Deoxy-2-fluoro-(β-D-arabinofuranosyl)-9H-purines and their biological activities. *Chem. Pharm. Bull.* 37, 336–339.

Clemenceau, D., Cousseau, J., Martin, V., Molines, H., Wakselman, C., Mornet, R., et al. (1996). Synthesis and cytokinin activity of two fluoro derivatives of N^6-isopentenyladenine. *J. Agric. Food Chem.* 44, 320–323. doi: 10.1021/jf9501148

Conrath, U. (2011). Molecular aspects of defence priming. *Trends Plant Sci.* 16, 524–531. doi: 10.1016/J.TPLANTS.2011.06.004

Criado, M. V., Caputo, C., Roberts, I. N., Castro, M. A., and Barneix, A. J. (2009). Cytokinin-induced changes of nitrogen remobilization and chloroplast ultrastructure in wheat (*Triticum aestivum*). *J. Plant Physiol.* 166, 1775–1785. doi: 10.1016/j.jplph.2009.05.007

De Diego, N., Fürst, T., Humplík, J. F., Ugena, L., Podlešáková, K., and Spíchal, L. (2017). An automated method for high-throughput screening of *Arabidopsis* rosette growth in multi-well plates and its validation in stress conditions. *Front. Plant Sci.* 8:1702. doi: 10.3389/fpls.2017.01702

De Diego, N., Saiz-Fernández, I., Rodríguez, J. L., Pérez-Alfocea, P., Sampedro, M. C., Barrio, R. J., et al. (2015). Metabolites and hormones are involved in the intraspecific variability of drought hardening in radiata pine. *J. Plant Physiol.* 188, 64–71. doi: 10.1016/j.jplph.2015.08.006

De Diego, N., and Spíchal, L. (2020). "Use of plant metabolites to mitigate stress effects in crops," in *The Chemical Biology of Plant Biostimulants*, eds D. Geelen and L. Xu (Hoboken, NJ: Wiley), 261–300. doi: 10.1002/9781119357254.ch11

Doležal, K., Popa, I., Hauserová, E., Spíchal, L., Chakrabarty, K., Novák, O., et al. (2007). Preparation, biological activity and endogenous occurrence of N^6-benzyladenosines. *Bioorganic Med. Chem.* 15, 3737–3747. doi: 10.1016/j.bmc.2007.03.038

Frébort, I., Kowalska, M., Hluska, T., Frébortová, J., and Galuszka, P. (2011). Evolution of cytokinin biosynthesis and degradation. *J. Exp. Bot.* 62, 2431–2452. doi: 10.1093/jxb/err004

Gamir, J., Sánchez-Bel, P., and Flors, V. (2014). Molecular and physiological stages of priming: how plants prepare for environmental challenges. *Plant Cell Rep.* 33, 1935–1949. doi: 10.1007/s00299-014-1665-9

Gao, J., Li, W., Niu, L., Cao, R., and Yin, W. (2015). Isolation and structural elucidation of novel antimicrobial compounds from maggots of *Chrysomyis megacephala* Fabricius. *Nat. Prod. Res.* 29, 239–246. doi: 10.1080/14786419.2014.948875

George, E. F., Hall, M. A., and De Klerk, G. J. (2008). "Plant growth regulators II: cytokinins, their analogues and antagonists," in *Plant Propagation by Tissue Culture*, 3rd Edn, eds E. F. George, M. A. Hall, and G. J. D. Klerk (Dordrecht: Springer). doi: 10.1007/978-1-4020-5005-3_6

Gitelson, A. A., Kaufman, Y. J., Stark, R., and Rundquist, D. (2002). Novel algorithms for remote estimation of vegetation fraction. *Remote Sens. Environ.* 80, 76–87. doi: 10.1016/S0034-4257(01)00289-9

Hagmann, W. K. (2008). The many roles for fluorine in medicinal chemistry. *J. Med. Chem.* 51, 4359–4369. doi: 10.1021/jm800219f

Holub, J., Hanuš, J., Hanke, D. E., and Strnad, M. (1998). Biological activity of cytokinins derived from Ortho–and Meta-Hydroxybenzyladenine. *Plant Growth Regul.* 26, 109–115.

Hönig, M., Plíhalová, L., Husičková, A., Nisler, J., and Doležal, K. (2018). Role of cytokinins in senescence, antioxidant defence and photosynthesis. *Int. J. Mol. Sci.* 19, 1–23. doi: 10.3390/ijms19124045

Hošek, P., Hoyerová, K., Kiran, N. S., Dobrev, P. I., Zahajská, L., Filepová, R., et al. (2020). Distinct metabolism of *N*−glucosides of isopentenyladenine and *trans*−zeatin determines cytokinin metabolic spectrum in *Arabidopsis*. *New Phytol.* 225, 2423–2438. doi: 10.1111/nph.16310

Hoyerová, K., and Hošek, P. (2020). New insights into the metabolism and role of cytokinin *N*-glucosides in plants. *Front. Plant Sci.* 11:741. doi: 10.3389/fpls.2020.00741

Hunt, E. R., Doraiswamy, P. C., McMurtrey, J. E., Daughtry, C. S. T., Perry, E. M., and Akhmedov, B. (2013). A visible band index for remote sensing leaf chlorophyll content at the canopy scale. *Int. J. Appl. Earth Obs. Geoinf.* 21, 103–112. doi: 10.1016/J.JAG.2012.07.020

Hussain, S., Khan, F., Cao, W., Wu, L., and Geng, M. (2016). Seed priming alters the production and detoxification of reactive oxygen intermediates in rice seedlings grown under sub-optimal temperature and nutrient supply. *Front. Plant Sci.* 7:439. doi: 10.3389/fpls.2016.00439

Ibrahim, E. A. (2016). Seed priming to alleviate salinity stress in germinating seeds. *J. Plant Physiol.* 192, 38–46. doi: 10.1016/j.jplph.2015.12.011

Iqbal, M., and Ashraf, M. (2005). Presowing seed treatment with cytokinins and its effect on growth, photosynthetic rate, ionic levels and yield of two wheat cultivars differing in salt tolerance. *J. Integr. Plant Biol.* 47, 1315–1325. doi: 10.1111/j.1744-7909.2005.00163.x

Iqbal, M., Ashraf, M., and Jamil, A. (2006). Seed enhancement with cytokinins: changes in growth and grain yield in salt stressed wheat plants. *Plant Growth Regul.* 50, 29–39. doi: 10.1007/s10725-006-9123-5

Jisha, K. C., Vijayakumari, K., and Puthur, J. T. (2013). Seed priming for abiotic stress tolerance: an overview. *Acta Physiol. Plant.* 35, 1381–1396. doi: 10.1007/s11738-012-1186-5

Jordi, W., Schapendonk, A., Davelaar, E., Stoopen, G. M., Pot, C. S., De Visser, R., et al. (2000). Increased cytokinin levels in transgenic P(SAG12)-IPT tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. *Plant Cell Environ.* 23, 279–289. doi: 10.1046/j.1365-3040.2000.00544.x

Kirk, K. L. (2008). Fiuorination in medical chemistry: methods, strategies, and recent development. *Org. Process Res. Dev.* 12:305. doi: 10.1021/op700134j

Lacuesta, M., Saiz-Fernández, I., Podlešáková, K., Miranda-Apodaca, J., Novák, O., Doležal, K., et al. (2018). The *trans* and *cis* zeatin isomers play different roles in regulating growth inhibition induced by high nitrate concentrations in maize. *Plant Growth Regul.* 85, 199–209. doi: 10.1007/s10725-018-0383-7

Lutts, S., Benincasa, P., Wojtyla, L., Kubala, S., Pace, R., Lechowska, K., et al. (2016). "Seed priming: new comprehensive approaches for an old empirical technique," in *New Challenges in Seed Biology–Basic and Translational Research Driving Seed Technology*, eds S. Araujo, and A. Balestrazzi (London: InTech). Available online at: https://www.intechopen.com/books/new-challenges-in-seed-biology-basic-and-translational-research-driving-seed-technology/seed-priming-new-comprehensive-approaches-for-an-old-empirical-technique. doi: 10.5772/64420

Man, D., Bao, Y. X., Han, L. B., and Zhang, X. (2011). Drought tolerance associated with proline and hormone metabolism in two tall fescue cultivars. *HortScience* 46, 1027–1032. doi: 10.21273/hortsci.46.7.1027

Marchetti, C. F., Škrabišová, M., Galuszka, P., Novák, O., and Causin, H. F. (2018). Blue light suppression alters cytokinin homeostasis in wheat leaves senescing

under shading stress. *Plant Physiol. Biochem.* 130, 647–657. doi: 10.1016/j.plaphy.2018.08.005

Marquez, V. E., Tseng, C. K. H., Kelley, J. A., Ford, H., Roth, J. S., Driscoll, J. S., et al. (1990). Acid-stable 2′-fluoro purine dideoxynucleosides as active agents against HIV. *J. Med. Chem.* 33, 978–985. doi: 10.1021/jm00165a015

Maruyama, T., Takamatsu, S., Kozai, S., Satoh, Y., and Izawa, K. (1999). Synthesis of 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine bearing a selectively removable protecting group. *Chem. Pharm. Bull.* 47, 966–970. doi: 10.1248/cpb.47.966

Meng, W.-D., and Qing, F.-L. (2006). Fluorinated nucleosides as antiviral and antitumor agents. *Curr. Top. Med. Chem.* 6, 1499–1528. doi: 10.2174/156802606777951082

Mik, V., Szüčová, L., Šmehilová, M., Zatloukal, M., Doležal, K., Nisler, J., et al. (2011a). N9-substituted derivatives of kinetin: effective anti-senescence agents. *Phytochemistry* 72, 821–831. doi: 10.1016/j.phytochem.2011.02.002

Mik, V., Szüčová, L., Spíchal, L., Plíhal, O., Nisler, J., Zahajská, L., et al. (2011b). N9-Substituted N^6-[(3-methylbut-2-en-1-yl)amino]purine derivatives and their biological activity in selected cytokinin bioassays. *Bioorganic Med. Chem.* 19, 7244–7251. doi: 10.1016/j.bmc.2011.09.052

Miller, C. O., Skoog, F., Von Saltza, M. H., and Strong, F. M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. *J. Am. Chem. Soc.* 77:1392. doi: 10.1021/ja01610a105

Miyoshi, K., and Sato, T. (1997). The effects of kinetin and gibberellin on the germination of dehusked seeds of indica and japonica rice (*Oryza sativa* L.) under anaerobic and aerobic conditions. *Ann. Bot.* 80, 479–483. doi: 10.1006/anbo.1997.0470

Mok, D., and Mok, M. (2001). Cytokinin metabolism and action. *Annu. Rev. Plant Biol.* 52, 89–118.

Montgomery, J. A., Shortnacy-Fowler, A. T., Clayton, S. D., Riordan, J. M., and Secrist, J. A. (1992). Synthesis and biological activity of 2'-fluoro-2-halo derivatives of 9-β-D-Arabinofuranosyladenine. *J. Med. Chem.* 35, 397–401. doi: 10.1021/jm00080a029

Pankiewicz, K. W., Krzeminski, J., Ciszewski, L. A., Ren, W. Y., and Watanabe, K. A. (1992). A synthesis of 9-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)adenine and hypoxanthine. An Effect of C3′-endo to C2′-endo conformational shift on the reaction course of 2′-hydroxyl group with DAST. *J. Org. Chem.* 57, 553–559. doi: 10.1021/jo00028a030

Paparella, S., Araújo, S. S., Rossi, G., Wijayasinghe, M., Carbonera, D., and Balestrazzi, A. (2015). Seed priming: state of the art and new perspectives. *Plant Cell Rep.* 34, 1281–1293. doi: 10.1007/s00299-015-1784-y

Pavlů, J., Novák, J., Koukalová, V., Luklová, M., Brzobohatý, B., and Černý, M. (2018). Cytokinin at the crossroads of abiotic stress signalling pathways. *Int. J. Mol. Sci.* 19, 1–36. doi: 10.3390/ijms19082450

Pěnčík, A., Casanova-Sáez, R., Pilaøová, V., Žukauskaite, A., Pinto, R., Micol, J. L., et al. (2018). Ultra-rapid auxin metabolite profiling for high-throughput mutant screening in *Arabidopsis*. *J. Exp. Bot.* 69, 2569–2579. doi: 10.1093/jxb/ery084

Perry, E. M., and Roberts, D. A. (2008). Sensitivity of narrow-band and broad-band indices for assessing nitrogen availability and water stress in an annual crop. *Agron. J.* 100:1211. doi: 10.2134/agronj2007.0306

Pitzer, K. S. (1960). The nature of the chemical bond and the structure of molecules and crystals: an introduction to modern structural chemistry. *J. Am. Chem. Soc.* 82:4121. doi: 10.1021/ja01500a088

Plíhal, O., Szüčová, L., and Galuszka, P. (2013). N^9-substituted aromatic cytokinins with negligible side effects on root development are an emerging tool for *in vitro* culturing. *Plant Signal. Behav.* 8:e24392. doi: 10.4161/psb.24392

Podlešáková, K., Zalabák, D., Čudejková, M., Plíhal, O., Szüčová, L., Doležal, K., et al. (2012). Novel cytokinin derivatives do not show negative effects on root growth and proliferation in submicromolar range. *PLoS One* 7:e39293. doi: 10.1371/journal.pone.0039293

Ranjbarian, F., Vodnala, M., Alzahrani, K. J. H., Ebiloma, G. U., De Koning, H. P., and Hofer, A. (2017). 9-(2'-Deoxy-2-Fluoro-β-D-Arabinofuranosyl) adenine is a potent antitrypanosomal adenosine analogue that circumvents transport-related drug resistance. *Antimicrob. Agents Chemother.* 61:e02719-16. doi: 10.1128/AAC.02719-16

Reichman, U., Watanabe, K. A., and Fox, J. J. (1975). A practical synthesis of 2-deoxy-2-fluoro-D-arabinofuranose derivates. *Carbohydr. Res.* 42, 233–240.

Rittenberg, D., and Foster, G. L. (1940). A new procedure for quantitative analysis by isotope dilution, with application to the determination of amino acids and fatty acids. *R. Soc. Open Sci.* 5:181322.

Roitsch, T., and Ehneß, R. (2000). Regulation of source / sink relations by cytokinins. *Plant Growth Regul.* 32, 359–367. doi: 10.1023/A:1010781500705

Rouphael, Y., Spíchal, L., Panzarová, K., Casa, R., and Colla, G. (2018). High-throughput plant phenotyping for developing novel biostimulants: from lab to field or from field to lab? *Front. Plant Sci.* 9:1197. doi: 10.3389/fpls.2018.01197

Sakakibara, H. (2006). CYTOKININS: activity, biosynthesis, and translocation. *Annu. Rev. Plant Biol.* 57, 431–449. doi: 10.1146/annurev.arplant.57.032905.105231

Savvides, A., Ali, S., Tester, M., and Fotopoulos, V. (2016). Chemical priming of plants against multiple abiotic stresses: mission possible? *Trends Plant Sci.* 21, 329–340. doi: 10.1016/j.tplants.2015.11.003

Schäfer, M., Brütting, C., Meza-Canales, I. D., Großkinsky, D. K., Vankova, R., Baldwin, I. T., et al. (2015). The role of *cis*-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. *J. Exp. Bot.* 66, 4873–4884. doi: 10.1093/jxb/erv214

Shokar, A., Au, A., An, S. H., Tong, E., Garza, G., Zayas, J., et al. (2012). S-Adenosylhomocysteine hydrolase of the protozoan parasite *Trichomonas vaginalis*: potent inhibitory activity of 9-(2-deoxy-2-fluoro-β,d- arabinofuranosyl)adenine. *Bioorganic Med. Chem. Lett.* 22, 4203–4205. doi: 10.1016/j.bmcl.2012.03.087

Sneideris, L. C., Gavassi, M. A., Campos, M. L., D'amico-Damião, V., and Carvalho, R. F. (2015). Effects of hormonal priming on seed germination of pigeon pea under cadmium stress. *An. Acad. Bras. Cienc.* 87, 1847–1852. doi: 10.1590/0001-3765201520140332

Stepanova, A. N., and Alonso, J. M. (2016). Auxin catabolism unplugged: role of IAA oxidation in auxin homeostasis. *Proc. Natl. Acad. Sci. U.S.A.* 113, 10742–10744. doi: 10.1073/pnas.1613506113

Strnad, M. (1997). The aromatic cytokinins. *Physiol. Plant* 101, 674–688. doi: 10.1111/j.1399-3054.1997.tb01052.x

Svačinová, J., Novák, O., Plačková, L., Lenobel, R., Holík, J., Strnad, M., et al. (2012). A new approach for cytokinin isolation from *Arabidopsis* tissues using miniaturized purification: pipette tip solid-phase extraction. *Plant Methods* 8:17. doi: 10.1186/1746-4811-8-17

Thibaudeau, C., Plavec, J., and Chattopadhyaya, J. (1998). A new generalized Karplus-type equation relating vicinal proton-fluorine coupling constants to H-C-C-F Torsion Angles. *J. Org. Chem.* 63:4967. doi: 10.1021/jo980144k

Uddin, M. N., Hossain, M. A., and Burritt, D. (2016). "Salinity and drought stress: similarities and differences in oxidative responses and cellular redox regulation," in *Water Stress and Crop Plants: A Sustainable Approach*, ed. P. Ahmad (Hoboken, NJ: Wiley), 86–101. doi: 10.1002/9781119054450.ch7

Ugena, L., Hýlová, A., Podlešáková, K., Humplík, J. F., Doležal, K., De Diego, N., et al. (2018). Characterization of biostimulant mode of action using novel multi-trait high-throughput screening of *Arabidopsis* germination and rosette Growth. *Front. Plant Sci.* 9:1327. doi: 10.3389/fpls.2018.01327

Van Hulten, M., Pelser, M., Van Loon, L. C., Pieterse, C. M. J., and Ton, J. (2006). Costs and benefits of priming for defense in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5602–5607. doi: 10.1073/pnas.0510213103

Veerasamy, M., He, Y., and Huang, B. (2007). Leaf senescence and protein metabolism in creeping bentgrass exposed to heat stress and treated with cytokinins. *J. Am. Soc. Hortic. Sci.* 132, 467–472. doi: 10.21273/jashs.132.4.467

Wan, Z. K., Binnun, E., Wilson, D. P., and Lee, J. (2005). A highly facile and efficient one-step synthesis of N^6-adenosine and N^6-2′-deoxyadenosine derivatives. *Org. Lett.* 7, 5877–5880. doi: 10.1021/ol052424+

Werbrouck, S. P. O., van der Jeugt, B., Dewitte, W., Prinsen, E., Van Onckelen, H. A., and Debergh, P. C. (1995). The metabolism of benzyladenine in *Spathiphyllum floribundum* "Schott Petite" in relation to acclimatisation problems. *Plant Cell Rep.* 14, 662–665. doi: 10.1007/BF00232734

Werner, T., Holst, K., Pörs, Y., Guivarc'h, A., Mustroph, A., Chriqui, D., et al. (2008). Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. *J. Exp. Bot.* 59, 2659–2672. doi: 10.1093/jxb/ern134

Willams, F. S. B., Fábio, S., Leandro, C. M., de, O., Paulo, H., and Menezes, C. (2016). Comparison of seed priming techniques with regards to germination

and growth of watermelon seedlings in laboratory condition. *African J. Biotechnol.* 15, 2596–2602. doi: 10.5897/ajb2016.15279

Wright, J. A., Taylor, N. F., and Fox, J. J. (1969). Nucleosides. LX. Fluorocarbohydrates. XXII. Synthesis of 2-Deoxy-2-fluoro-D-arabinose and 9-(2-Deoxy-2-fluoro-α- and -β-D-arabinofuranosyl)adenines. *J. Org. Chem.* 34, 2632–2636. doi: 10.1021/jo01261a031

Zalabák, D., Galuszka, P., Mrízová, K., Podlešáková, K., Gu, R., and Frébortová, J. (2014). Biochemical characterization of the maize cytokinin dehydrogenase family and cytokinin profiling in developing maize plantlets in relation to the expression of cytokinin dehydrogenase genes. *Plant Physiol. Biochem.* 74, 283–293. doi: 10.1016/j.plaphy.2013.11.020

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

PERSPECTIVE
published: 21 December 2020
doi: 10.3389/fpls.2020.613624

Global View on the Cytokinin Regulatory System in Potato

*Sergey N. Lomin, Yulia A. Myakushina, Oksana O. Kolachevskaya, Irina A. Getman, Ekaterina M. Savelieva, Dmitry V. Arkhipov, Svetlana V. Deigraf and Georgy A. Romanov**

Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia

Cytokinins (CKs) were earlier shown to promote potato tuberization. Our study aimed to identify and characterize CK-related genes which constitute CK regulatory system in the core potato (*Solanum tuberosum*) genome. For that, CK-related genes were retrieved from the sequenced genome of the *S. tuberosum* doubled monoploid (DM) Phureja group, classified and compared with Arabidopsis orthologs. Analysis of selected gene expression was performed with a transcriptome database for the *S. tuberosum* heterozygous diploid line RH89-039-16. Genes responsible for CK signaling, biosynthesis, transport, and metabolism were categorized in an organ-specific fashion. According to this database, CK receptors StHK2/3 predominate in leaves and flowers, StHK4 in roots. Among phosphotransmitters, StHP1a expression largely predominates. Surprisingly, two pseudo-phosphotransmitters intended to suppress CK effects are hardly expressed in studied organs. Among B-type *RR* genes, *StRR1b*, *StRR11*, and *StRR18a* are actively expressed, with *StRR1b* expressing most uniformly in all organs and *StRR11* exhibiting the highest expression in roots. By cluster analysis four types of prevailing CK-signaling chains were identified in (1) leaves and flowers, StHK2/3→StHP1a→StRR1b/+; (2) shoot apical meristems, stolons, and mature tubers, StHK2/4→StHP1a→StRR1b/+; (3) stems and young tubers, StHK2/4→StHP1a→StRR1b/11/18a; and (4) roots and tuber sprouts, StHK4→StHP1a→StRR11/18a. CK synthesis genes *StIPT3/5* and *StCYP735A* are expressed mainly in roots followed by tuber sprouts, but rather weakly in stolons and tubers. By contrast, CK-activation genes *StLOGs* are active in stolons, and *StLOG3b* expression is even stolon-confined. Apparently, the main CK effects on tuber initiation are realized via activity of *StLOG1/3a/3b/7c/8a* genes in stolons. Current advances and future directions in potato research are discussed.

Keywords: potato, cytokinin, two-component system, receptor, response regulator, gene expression, signaling chains, multistep phosphorelay

OPEN ACCESS

Edited by:
Wolfram G. Brenner, Universität Leipzig, Germany

Reviewed by:
Takatoshi Kiba, Nagoya University, Japan
Adam Solti, Eötvös Loránd University, Hungary

***Correspondence:**
Georgy A. Romanov
gromanov@yahoo.com

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *02 October 2020*
Accepted: *19 November 2020*
Published: *21 December 2020*

Citation:
Lomin SN, Myakushina YA, Kolachevskaya OO, Getman IA, Savelieva EM, Arkhipov DV, Deigraf SV and Romanov GA (2020) Global View on the Cytokinin Regulatory System in Potato. Front. Plant Sci. 11:613624. doi: 10.3389/fpls.2020.613624

INTRODUCTION

Potato tubers (*Solanum tuberosum* L.) are well known and widespread sources of food, feed, and technical substances (starches). Cytokinins (CKs), classical plant hormones, are known to promote potato tuber formation, at least in conventional *in vitro* systems (Palmer and Smith, 1970; Aksenova et al., 2000; Romanov et al., 2000; Cheng et al., 2020). CKs are also involved in the formation of artificial "tuberoids" on tobacco and tomato shoots (Guivarc'h et al., 2002; Eviatar-Ribak et al., 2013)

as well as tuber-shaped rounded tumors on agrobacteria-infected plants (Dodueva et al., 2020). In addition to stimulating the formation of tubers, CKs contribute to their sprouting (Hartmann et al., 2011; Aksenova et al., 2013). The morphogenic effect of CKs certainly exploits their ability to induce cell divisions (Miller et al., 1955; Sakakibara, 2006; Romanov, 2009; Kieber and Schaller, 2014), although is not limited to this. All the above emphasizes the importance of CK regulatory system for such a valuable tuber-producing crop as potatoes.

Over the past decade, a prominent progress has been achieved in potato research. First of all, this concerns the sequencing of the complete genome of *S. tuberosum* group Phureja doubled monoploid DM1-3 516 R44 (DM) by the Potato Genome Sequencing Consortium [PGSC] (2011). Thereafter, a set of genes/proteins controlling tuberization was uncovered (Dutt et al., 2017; Hannapel et al., 2017). It became clear that the regulation of tuberization is based on a complex crosstalk between numerous hormonal and non-hormonal factors (Aksenova et al., 2012, 2014). In our research, we focused on the hormonal part of this regulatory network. On the basis of our experimental data (Kolachevskaya et al., 2015, 2017, 2018, 2019a) and data from recent literature, an updated hypothesis of hormonal regulation of potato tuberization was advanced (Kolachevskaya et al., 2017, 2019b), where CKs play an important role, especially at the tuber induction and initiation stages. Furthermore, the DNA sequence coding for CK receptors (sensor histidine kinase) and basic CK-signaling machinery were identified in the sequenced DM genome and analyzed by means of bioinformatics tools (Lomin et al., 2018b). In parallel, a suite of genes encoding sensor histidine kinases from tetraploid cultivar "Désirée" were cloned and expressed, giving rise to individual CK receptors. These receptors were studied in-depth, including their phylogenetics, conserved domains, 3D-structures, ligand-binding properties, organ-specificity of expression and responsiveness to CKs, regulatory *cis*-elements in their promoters, subcellular localization, homo- and heterodimerization, and mode of interaction with phosphotransmitters (Lomin et al., 2018b, 2020; Arkhipov et al., 2019).

Receptors are considered key proteins in hormone signaling, but they cannot signal by themselves. Regulation by any hormone *in planta* requires dozens of genes/proteins that function in various aspects of the given hormonal system. Like any other hormonal system, the CK regulatory system can be conventionally divided into a relatively conserved central part (CK signaling, synthesis, metabolism, and transport) and a more variable periphery—mainly CK-responsive regulatory genes (Bhargava et al., 2013; Brenner and Schmülling, 2015). For example, the central part of the model Arabidopsis plant distinguished by small genome size comprises, according to the latest estimates, some 80 genes (**Supplementary Figure 1**). Among them, 34 genes belong to the so-called two-component system (TCS) and constitute a signaling pathway termed multistep phosphorelay (MSP) from transmembrane receptors to primary response genes in the nucleus (Heyl and Schmülling, 2003; Mizuno, 2005; Kieber and Schaller, 2014, 2018; Pekárová et al., 2016, 2018; Arkhipov et al., 2019; Hallmark and Rashotte, 2019). Other involved genes have been classified as genes for CK synthesis (20 genes in total) (Kamada-Nobusada and Sakakibara, 2009), catabolism, and reversed inactivation (12 genes) (Schmülling et al., 2003; Hoyerová and Hošek, 2020), as well a transport (eight genes) (Liu et al., 2019). The CK regulatory system in potatoes is far less studied than in Arabidopsis. Here we intend to gain insight into the genome-wide composition and functioning of the central part of CK regulatory system in potatoes, with a particular focus on tuber formation. The prospects for using current knowledge to improve potato yield are discussed below.

CK-RELATED GENES IN POTATOES

Current molecular studies of potatoes are based on the sequenced DM genome representing the core genome of this widespread crop. Nevertheless, the size of even this minimal genome (844 Mb) is many times larger than the genome of the model Arabidopsis plant (135 Mb). However, this difference essentially disappears when we compare the numbers of protein-coding genes: 27,029 in *Arabidopsis thaliana* "Columbia" (Swarbreck et al., 2008) and 39,031 in DM potato (Potato Genome Sequencing Consortium [PGSC], 2011). In this case, the size of the one genome is now only reduced to only 1.44 times that of the other. Consequently, a large dissimilarity in numbers of genes of CK regulatory systems between these two species seems unlikely. **Table 1** demonstrates that the core potato genome encodes orthologs of nearly all gene families involved in the central part of the CK system in Arabidopsis. And indeed, the sizes of orthologous gene families are rather close in Arabidopsis and potato (**Supplementary Figure 1**; Lomin et al., 2018b). For CK synthesis, potatoes possess 16 genes (compare to 20 in Arabidopsis), encoding six *isopentenyl adenine transferases* (*StIPT*), one cytochrome P450 monooxygenase *StCYP735A*, and nine *lonely guy* (*StLOG*) orthologs. For CK perception, potatoes have three receptor histidine kinases (StHK2–4) similarly to Arabidopsis; 8, 7, and 2 response regulators of class A, B, and C, respectively, as well as three cytokinin response factor (StCRF) proteins. In this regard, potatoes markedly differ from Arabidopsis in the proportion of TCS-like genes. In potatoes, half (three out of six) of the members of the phosphotransfer protein family (StHP) lack conserved phospho-accepting amino acids and are considered putative inhibitors of the MSP. By contrast, in Arabidopsis only one such member (AHP6, 1 from 6) is a true MSP inhibitor. In Arabidopsis, type B response regulators were divided into three groups, from which only ARR-Bs of the first group (ARR-BI) were proven to participate in CK signaling. Interestingly, ARR-Bs of these groups are not the closest neighbors on the phylogenetic tree but are interspersed with the APRR2 and APRR6 groups of pseudo-RRs. Similarly to Arabidopsis, most (7) of potato *RR-B* genes are homologous to RR-BI group, whereas three *StRR-B* genes are beyond this phylogenetic branch (**Supplementary Figure 2A**). The encoded three StRR-B proteins (StRR25, 27, and 28), along with conserved phospho-accepting aspartate, contain abnormal DD- and K-motifs in their

TABLE 1 | Proven or high-probable components of the CK regulatory system in DM potatoes.

Gene	GenBank			PGSC	
	Gene ID	Protein	Amino acids	Primary transcript	Location
CHK					
StHK2	LOC102591086	XP_015158747.1	1,263	PGSC0003DMT400015729	ST4.03ch07:44284533.44287254 R
StHK3	LOC102587294	XP_006352176.1	1,032	PGSC0003DMT400084727	ST4.03ch05:14358273.14361236 F
StHK4	LOC102603756	XP_006355050.1	992	PGSC0003DMT400075775	ST4.03ch04:2774233.2781847 R
HK					
StCKI1	LOC102594786	XP_006349947.1	765	PGSC0003DMT400011818	ST4.03ch12:60313883.60322789 F
StETR1	LOC102588584	XP_00634996.1/97.1	754/751	PGSC0003DMT400020285	ST4.03ch12:1112062.1121655 R
HPt					
StHP1a	LOC102590747	XP_00636569.1/70.1/71.1	151	PGSC0003DMT400081746	ST4.03ch01:60730670.60732462 R
StHP1b	LOC102603297	XP_006352793.1	152	PGSC0003DMT400051833	ST4.03ch06:59207849.59211570 F
StHP4a	LOC102589200	XP_006364721.1/XP_015159552.1	136/112	PGSC0003DMT400077451	ST4.03ch08:38158555.38161451 R
PHPt					
StPHP4b	LOC102584884	XP_015170906.1	137	PGSC0003DMT400047799	ST4.03ch11:42457958.42462239 R
StPHP4c	LOC102606269	XP_00634303 9.1/40.1	152		
StPHP6	LOC102601463	XP_006364219.1	156	PGSC0003DMT400001706	ST4.03ch03:56593561.56594955 R
RR-B I					
StRR1a	LOC102578736	XP_00636357 9.1/80.1	675	PGSC0003DMT400065835	ST4.03ch01:11998173.12002053 F
StRR1b	LOC102586468	XP_006345976.1	663	PGSC0003DMT400060506	ST4.03ch05:50113044.50119581 F
StRR1c	LOC102596771	XP_006349953.1	556	PGSC0003DMT400090747	ST4.03ch12:60090199.60093910 R
StRR11	LOC102593308	XP_01516176 4.1/67.1/68.8	481/581/581	PGSC0003DMT400031260	ST4.03ch05:11541271.11546411 F
StRR14	LOC102606335	XP_00635505 8.1/59.1	656/653	PGSC0003DMT400075907	ST4.03ch04:2709257.2714486 F
StRR18a	LOC102598455	XP_006343681.1	681	PGSC0003DMT400008290	ST4.03ch07:402301.406809 F
StRR18b	LOC102587717	XP_006350077.1	707	PGSC0003DMT400020233	ST4.03ch12:2023372.2028700 F
RR-A					
StRR4	LOC102602758	XP_015168830.1	248	PGSC0003DMT400058306	ST4.03ch05:6752023.6754796 R
StRR9a	LOC102590336	XP_006355595.1	163	PGSC0003DMT400007977	ST4.03ch02:30409061.30409639 R
StRR9b	LOC102588738	XP_01517032 .1/33.1	214/211	PGSC0003DMT400007618	ST4.03ch04:24951499.24954972 f
StRR9c	LOC102599826	XP_006351272.1	226	PGSC0003DMT400076726	ST4.03ch10:58221853.58223696 F
StRR9d	LOC102601166	XP_006351276.1	226	PGSC0003DMT400076758	ST4.03ch10:58094625.58096547 R
StRR15	LOC102605280	XP_006344995.1	202	PGSC0003DMT400063187	ST4.03ch03:53710748.53712289 R
StRR17a	LOC102583233	XP_006357298.1	156	PGSC0003DMT400070964	ST4.03ch06:34954518.34956370 F
StRR17b	LOC102579353	XP_006358744.1	148	PGSC0003DMT400042922	ST4.03ch06:34247673.34249935 F
RR-C[1]					
StRR22a	LOC107059982	XP_015162643.1	137	PGSC0003DMT400092899	ST4.03ch03:23672034.23672546 R
StRR22b	LOC102580685	XP_006361623.2	115	PGSC0003DMT400096803	ST4.03ch03:37709010.37709447 R
CRF					
StCRF1	LOC102599019	XP_006343893.1	338	PGSC0003DMT400032058	ST4.03ch08:55617019.55618035 F
StCRF3a	LOC102605010	XP_006360182.1	374	PGSC0003DMT400006041	ST4.03ch06:38128680.38129804 F
StCRF3b	LOC102591238	XP_006350598.1	389	PGSC0003DMT400086922	ST4.03ch03:44865282.44866451 F
ATP/ADP-IPT					
StIPT1a	LOC102595379	XP_006360459	334	PGSC0003DMT400015411	ST4.03ch04:1541542.1542378 F
StIPT1b	LOC102604151	XP_006344131	345	PGSC0003DMT400037749	ST4.03ch05:2800989.2801840 R
StIPT3	LOC102579012	XP_006355868	323	PGSC0003DMT400002509	ST4.03ch09:46852324.46853283 R
StIPT5	LOC102599418	XP_006339408	330	PGSC0003DMT400083203	ST4.03ch01:61389842.61390834 R
tRNA-IPT					
StIPT9	LOC102605568	XP_006358948.1/49.1/XP_015169686.1	450/368/398	PGSC0003DMT400039795	ST4.03ch12:270799.276885 F
StIPT2	LOC102594223	XP_006349089/XP_015164944	467/466	PGSC0003DMT400068271	ST4.03ch11:39838731.39844810 R
CYP735A					
StCYP735A	LOC102603015	XP_006363117.1	517	PGSC0003DMT400032989	ST4.03ch02:41114766.41117388 F
LOG					
StLOG1a	LOC102584678	XP_006340180.1	228	PGSC0003DMT400020890	ST4.03ch11:41500955.41505580 F
StLOG1b	LOC102598045	XP_006365730.2	216		

(Continued)

TABLE 1 | continued

Gene	GenBank			PGSC	
	Gene ID	Protein	Amino acids	Primary transcript	Location
StLOG3a/LOG1	LOC102581470	XP_006339070.1	220	PGSC0003DMT400027157	ST4.03ch10:54947968.54951688 F
StLOG3b	LOC102592821	XP_006354329.1	218	PGSC0003DMT400055525	ST4.03ch09:5646817.5650774 F
StLOG7a/LOG2	LOC102583076	XP_006348482.1	218	PGSC0003DMT400042349	ST4.03ch01:3283634.3289271 F
StLOG7b	LOC102587326	XP_006339321.1	217	PGSC0003DMT400072345	ST4.03ch10:56042745.56046560 R
StLOG7c/LOG3	LOC102592408	XP_006342033.1	225	PGSC0003DMT400009551	ST4.03ch04:70714467.70717363 F
StLOG8a	LOC102597227	XP_006351590.1	213	PGSC0003DMT400021223	ST4.03ch08:35250415.35253629 R
StLOG8b	LOC102595783	XP_015167145.1/XP_006354132.1	206/205	PGSC0003DMT400081828	ST4.03ch01:1308627.1312950 F
CKX					
StCKX1a	LOC102577758	NP_001275030.1	543	PGSC0003DMT400033123	ST4.03ch04:11108702.11111248 R
StCKX1b	LOC102605765	XP_006351290.1	536		
StCKX3	LOC102577888	NP_001275401.1	527	PGSC0003DMT400000752	ST4.03ch12:3650404.3656647 F
StCKX5	CKX4	NP_001274957.1	526	PGSC0003DMT400009621	ST4.03ch04:70221912.70227296 R
StCKX6	CKX3	NP_001275006.1/XP_015163315.1/16.1	533/509/533	PGSC0003DMT400017390	ST4.03ch01:64568740.64570801 F
StCKX7a	LOC102605861	XP_006367057.1	542	PGSC0003DMT400080680	ST4.03ch08:33930753.33933803 R
StCKX7b	CKX5	NP_001275121.1	513	PGSC0003DMT400080679	ST4.03ch08:33888758.33891903 R
ABCG14					
StABCG14a	LOC102579805	XP_006359961.1	646	PGSC0003DMT400004973	ST4.03ch08:47265148.47267889 R
StABCG14b	LOC102600750	XP_00636127.1/28.1	662/659	PGSC0003DMT400047547	ST4.03ch08:3599414.3602415 R
ENT					
StENT3a	LOC102583617	XP_006347184.1	415		
StENT3b	LOC102601592	XP_006347157.1/XP_015164176.1	421	PGSC0003DMT400057543	ST4.03ch02:35070810.35072418 R
StENT3c	LOC102592247	XP_00635297.1/98.1	448/421	PGSC0003DMT400029352	ST4.03ch10:520474.523484 F
StENT3d	LOC102604121	NP_001275018.1	418	PGSC0003DMT400059645	ST4.03ch02:10149698.10151875 F
PUP					
StPUP1_1	LOC102593174	XP_006338103.1	354	PGSC0003DMT400005470	ST4.03ch04:64474151.64477355 F
StPUP1_2	LOC102592621	XP_006358992.1	358		
StPUP1_3	LOC102592834	XP_006338102.1	352	PGSC0003DMT400005467	ST4.03ch04:64481580.64482969 F
StPUP1_4	LOC102580279	XP_006366415.1	355	PGSC0003DMT400071864	ST4.03ch06:36382676.36385224 R
StPUP1_5	LOC102581289	XP_006364706.1	363		
StPUP1_6	LOC102590236	XP_006357923.1	341		
StPUP1_7	LOC102590918	XP_015169158.1	351	PGSC0003DMT400036604	ST4.03ch06:36309239.36311744 R
StPUP1_8	LOC102591268	XP_006357925.1	343		
StPUP1_9	LOC102580805	XP_006360266.1	364	PGSC0003DMT400045772	ST4.03ch12:55367629.55371917 F
StPUP1_10	LOC102581812	XP_006360267.2	363		

Gene families (left column) are marked bold. Slash separated and underlined end numbers in the Protein column corresponds to different splice forms. [1]This family contains non-expressing genes in the displayed organ set (StRR22a) or in the overall organism (StRR22b).

receiver (REC-) domains (**Supplementary Figure 2B**), which most likely render these proteins inactive. Other potato non-canonical genes are listed in the **Supplementary Table 1** as they hardly contribute to CK action. Notably, the potato genome contains genes encoding type C pseudo RRs (*PRR* type C) which are lacking in Arabidopsis. At least an essential portion of Arabidopsis PRR orthologs harbor CCT motif and take part in the photoperiodic flowering control unrelated to the CK system and to being subject to circadian rhythms (Mizuno, 2005). Any other role of the remaining PRRs (**Supplementary Table 1**) in CK action cannot be completely excluded but is very questionable.

We included in **Table 1** non-CK receptor histidine kinases (CKI1, ETR1) as they may play a role in CK signaling. When one such kinase (AtCKI1) was spontaneously overexpressed, the mutated phenotype mimicked the effect of massive CK treatment (Kakimoto, 1996). The receiver domain of another histidine kinase, ethylene receptor ETR1, also can interact with MSP signaling intermediates acting downstream from CK receptors (Zdarska et al., 2019). The background TCS activity of these proteins seems to be sufficient to rescue the basic phenotype of Arabidopsis triple mutants lacking all three CK receptors and no longer responding to CKs (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Romanov, 2009). All this indicates the possible role of CKI1 and ETR1 in MSP signaling. In total, the estimated number of genes directly involved in CK signaling (MSP) in DM potatoes is 31.

For CK degradation, potatoes possess seven CKX orthologs (**Table 1**). Genes for putative CK conjugation are included only in **Supplementary Table 1** because potato *StUGT*s have

no direct homology to Arabidopsis *UGT* genes responsible for CK *O*- and *N*-glucosylation (**Supplementary Figure 3**). Also, genes encoding CK transporters (Liu et al., 2019)—*StPUP* (10), *StENT* (4), and *StABCG14* (2) [possibly also *StABCI19-21* and *StAZG1,2* (Kim et al., 2020; Tessi et al., 2020), see **Supplementary Table 1**]—have active orthologs in Arabidopsis and are obviously of particular importance. The exceptions are gene-orthologs of *PUP14* transporters of Arabidopsis that are missing in potatoes (**Supplementary Figure 4**). Overall, the central part of the CK regulatory system in the core potato genome comprises 70 genes, the number close to that in Arabidopsis (**Supplementary Figure 1**). Among the potato genes, 28 (40%) are TCS homologs, whereas the remaining genes are not TCS-related.

THE EXPRESSION PATTERN OF CK-RELATED GENES

To elucidate the molecular events underlying potato growth, productivity and stress tolerance, the list of families of paralog genes is a useful but insufficient characteristic. Knowledge of absolute and relative spatiotemporal gene expression is necessary to address this issue. The expression pattern of genes involved in the CK action in the diploid potato line RH89-039-16[1] is shown in **Figure 1**. Clearly, most of these genes are expressed differently depending on the organ and stage of development. Among CHK receptors, the expression of *StHK2/3* predominates in leaves and flowers, while *StHK4* is mostly expressed in roots. In the latter organ, *StHK2/4* genes are quite active. Among phosphotransmitters, *StHP1a* expression predominates in every organ, following by *StHP1b*, which is also expressed quasi-constitutively but to a much lesser extent. Interestingly, all three genes for the phosphotransfer-like proteins StPHP4b, 4c, 6, which are supposed to suppress CK signaling, are hardly expressed in RH89-039-16 line. As for RR-B transcription factors, *StRR1b* is expressed most uniformly in all organs, *StRR1a* acts similarly but much more weakly, and *StRR1c* is almost not expressed. Among all B-type *RR* genes, *StRR1a*, *StRR1b*, *StRR11*, and *StRR18a* are the most strongly expressed. Roots represent the site where almost all *RR-B* genes are expressed, dominated by *StRR11*.

By means of cluster analysis of the organ-specific expression of genes encoding receptors and StRRs type B (**Supplementary Figure 5**), we identified four types of prevailing signaling chains: (1) in leaves and flowers, StHK2/3→StHP1a→StRR1b/+; (2) in shoot apical meristems, stolons, and mature tubers, StHK2/4→StHP1a→StRR1b/+; (3) in stems and young tubers, StHK2/4→StHP1a→StRR1b/11/18a; and (4) in roots and tuber sprouts, StHK4→StHP1a→StRR11/18a.

Cytokinins synthesis genes *StIPT3/5* and *StCYP735A* are expressed mainly in roots (similarly to Arabidopsis). CK-perception and synthesis genes (*StHKs*, *StIPTs*, and *StCYP735A*) are also actively expressed in tuber sprouts, where *StHK4* transcripts prevail over transcripts of other CK receptor genes. A special group of CK-activation genes termed *StLOGs* are active in stolons, and *StLOG3b* expression is mainly restricted to this organ (**Figure 1**). It is noteworthy that organs (leaves, stolons) in which *StLOG* genes are strongly expressed, are mostly devoid of transcripts of other CK-synthesizing genes (*StIPT*, *StCYP735A*) (**Figure 1**). The above observations, based on the gene expression data[1] for the diploid potato RH89-039-16, are generally

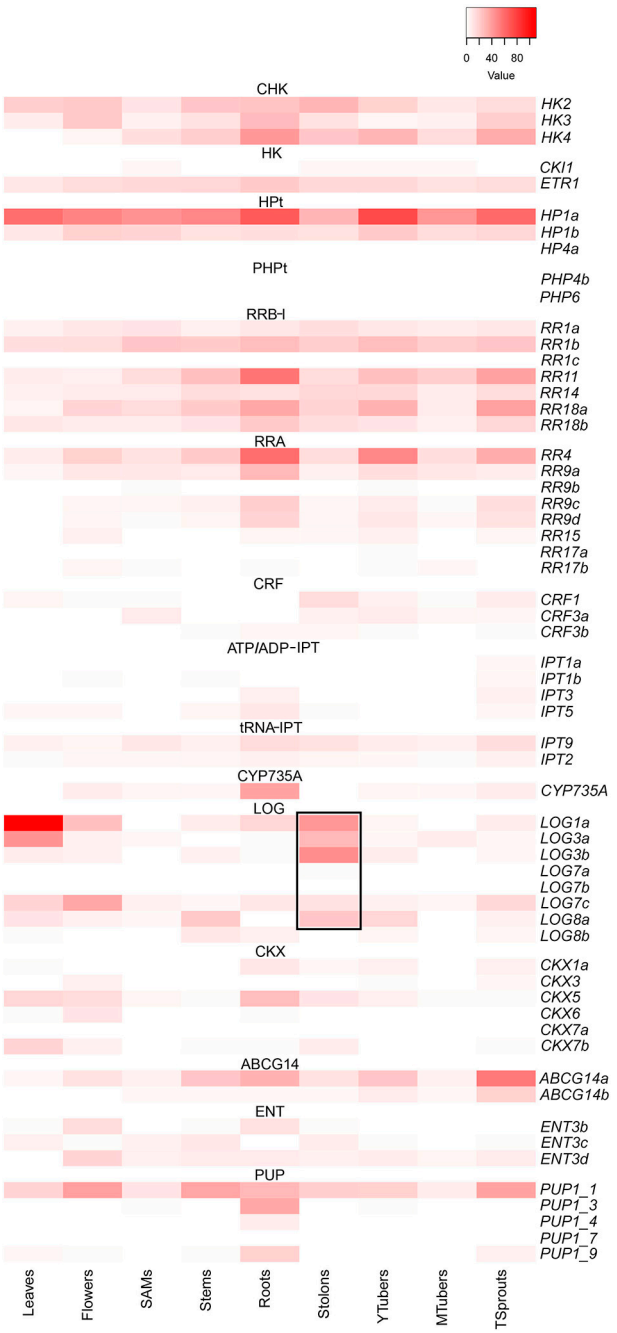


FIGURE 1 | Expression pattern of cytokinin system genes of potato generated with the use of Heatmapper service (http://www.heatmapper.ca/expression/) (Babicki et al., 2016). The *LOG* gene expression pattern in the stolon is framed. SAMs, shoot apical meristems; YTubers and MTubers, young and mature tubers, respectively; TSprouts, tuber sprouts.

[1]http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml

consistent with organ-specific analysis of the transcriptome in the commercial tetraploid potatoes "Désirée" (Lomin et al., 2018b, and data not shown). However, the organ/tissue patterns of the expressed genes in different potato lines/cultivars (DM, RH89-039-16, "Désirée") were not strictly identical, indicating some cultivar-specificity of gene functioning in potatoes. For example, RH89-039-16 and "Désirée" share similar organ-dependent expression patterns for *StHP1a, StPHP4b, StPHP6, StRR1b, StRR14, StRR18a, StRR9a, StCYP735A*, and *StABCG14a*, although marked differences in these patterns were observed for *StRR11, StRR9c, StIPT5*, and *StCKX3*. The expression patterns of remaining CK-related genes coincide moderately.

DISCUSSION

Here we present a global view on the CK regulatory system in potatoes. Generally, each hormonal regulatory system, in particular the cytokinin one (Romanov, 2009), includes complex units ensuring hormone biosynthesis, translocation, perception, and inactivation, as well as the primary response structures to targeted signal transduction. These units consist of corresponding proteins encoded by cognate genes. Here, the CK-related gene sequences were retrieved from the annotated core genome of the doubled monoploid DM1-3 516 R44 potato Phureja (the genes whose role in the CK system is proven or highly probable, **Table 1**) and thus represent the minimal gene set of the potato CK system. There is no doubt that commercial varieties of potatoes, mostly tetraploids, possess much many genes related to CK system. This is corroborated by data on the tetraploid "Désirée" variety, in which at least six authentic CK receptors have been identified (Lomin et al., 2018b), twice as many (3) CK receptors encoded by the DM genome. However, additional genes are close paralogs of the "core" genes, so the number of gene clades remains unchanged. Other genes that could theoretically acquire the status of CK "core" genes are listed in **Supplementary Table 1**, but their involvement requires detailed research and seems unlikely at this time.

In terms of nomenclature, we propose to follow the tradition of naming gene/protein according to the best homology to its Arabidopsis counterpart. Thereby, the potato genes were named according to their closest orthologs in Arabidopsis (see **Supplementary Figures 2–4**) in **Supplementary Data**. When many potato genes turned out to be orthologous to the same Arabidopsis gene, the former are marked with an additional letter at the end (a, b, c, etc.). Such nomenclature has already been used to designate potato CK-receptors (Lomin et al., 2012; Steklov et al., 2013) and other potato genes/proteins (Lomin et al., 2018b). The exceptions are *StPUP* genes since their homology to any of Arabidopsis *PUPs* is not obvious. In any case, this compiled gene set (**Table 1**) can serve as a convenient basis for studying genes, constituting the basic part of the CK system in potato.

As expected, most of CK-related enzymatic activities are encoded in the core potato genome by small (mostly 2–10 members) gene families. At the moment, 16 such families can be counted, of which eight families correspond to intracellular signal transduction (MSP), totally 31 genes encoding 41 proteins (**Table 1**). Among these proteins, only three (StHKs) have contact directly with hormonal ligands, while others obviously do not, though this has not been definitely proven. In fact, this part of the global CK system includes 28 authentic TCS homologs, which generate or affect signal transmission through His-Asp phosphorelay. The exceptions are proteins with degenerated TCS homology, which cannot directly participate in MSP because of structural deficiency. These non-functioning in MSP genes and proteins are evidently not *bona fide* members of the central part of the CK system. Some of them are displayed in **Supplementary Table 1** containing not yet excluded but possible candidates for the CK system in potatoes. In fact, CKs almost monopolized the MSP system, using it as a signaling part of their global regulatory circuit. This statement is supported by the fact that every functional phosphotransmitter is promiscuous, i.e., is able to transmit "hot" phosphate from any CK receptor to any RR-B in the nucleus (Hutchison et al., 2006; Dortay et al., 2008; Lomin et al., 2018a; Arkhipov et al., 2019). Other hormones—in particular, ethylene, whose receptors are also TCS homologs—had to switch to a signal transduction pathway other than MSP (Pekárová et al., 2018).

Data on the relative expression of selected genes allowed us to outline the most plausible protein chains transmitting the CK signal from receptors to primary response genes. This corresponds to the activity of the prevailing potato MSP. We suggested four types of main signaling chains, which are delineated above. These chains are partially redundant, especially in relation to the transfer stage from the cytosol to the nucleus, where phosphotransmitter StHP1a predominates in all organs of the diploid potato. However, predominant receptors and B-type response regulators may differ depending on the organ, and this CK-signaling specificity should be taken into account when researchers manipulate the potato genome. To date, we consider the stolon as the most promising organ for engineering potato productivity and early maturation. Apparently, this is where the most important events leading to tuber initiation should take place. The most noticeable feature of the transcriptome of the CK system in stolons is the increased activity of the *LOG*-genes (*StLOG1/3a/3b/7c/8a*) without visible changes in the expression of other CK-related gene families. Moreover, the expression of *StLOG3b* is stolon-specific. In contrast, the activity of other CK synthesis genes, *IPTs* and *CYP735A*, was much weaker here than in other organs. Thus, stolons are likely sites where CK precursors (phosphoribosides) are exported and activated to functional CK bases by LOG enzymes. Recently, it was found that three *LOG* genes (*StLOG3a, 7a,* and *7c*) are activated by the tuberigen StBEL5, most likely through the direct interaction of this transcription factor with the corresponding *cis*-elements in the promoters of these genes (Sharma et al., 2016). It is noteworthy that the ectopic expression of only one of the tomato *LOG* genes was sufficient for the formation of tuber-like structures from the lateral meristems in tomato (Eviatar-Ribak et al., 2013). Thus, we assume that CKs are involved in the tuberization process through the activation of *LOG* genes by the StBEL5 pathway. The contribution of the stolon identity pathway is also possible due to the only partial overlap of *LOG* expression patterns induced by StBEL5 and inherent in stolons.

Collectively, since CKs were known to be implicated in many aspects of potato growth and productivity, the presented genome-wide data characterizing the CK-regulatory system of potatoes can be useful for the exploration and breeding of this important crop.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml.

AUTHOR CONTRIBUTIONS

SL, YM, OK, IG, ES, SD, and DA presented and analyzed their experimental and/or bioinformatic results. SL prepared main illustrative materials. GR wrote the version of the manuscript. All authors contributed to the manuscript editing, developed the idea and outline of the manuscript, and read and approved the manuscript final version before submission.

FUNDING

Supported by a grant of the Russian Science Foundation No. 17-74-20181. We also thank the Ministry of Science and Higher Education of the Russian Federation for generous subsidies used to pay for the main Institute facilities as prerequisites for the successful experimental work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.613624/full#supplementary-material

REFERENCES

Aksenova, N. P., Konstantinova, T. N., Golyanovskaya, S. A., Kossmann, J., Willmitzer, L., and Romanov, G. A. (2000). Transformed potato plants as a model for studying the hormonal and carbohydrate regulation of tuberization. *Russ. J. Plant Physiol.* 47, 370–379.

Aksenova, N. P., Konstantinova, T. N., Golyanovskaya, S. A., Sergeeva, L. I., and Romanov, G. A. (2012). Hormonal regulation of tuber formation in potato plants. *Russ. J. Plant Physiol.* 59, 451–466. doi: 10.1134/S1021443712040024

Aksenova, N. P., Sergeeva, L. I., Kolachevskaya, O. O., and Romanov, G. A. (2014). "Hormonal regulation of tuber formation in potato," in *Bulbous Plants: Biotechnology*, eds K. Ramavat, and J. Mérillon (Boca Raton, FL: Taylor & Francis Group), 3–36. doi: 10.1201/b16136-3

Aksenova, N. P., Sergeeva, L. I., Konstantinova, T. N., Golyanovskaya, S. A., Kolachevskaya, O. O., and Romanov, G. A. (2013). Regulation of potato tuber dormancy and sprouting. *Russ. J. Plant Physiol.* 60, 301–312. doi: 10.1134/S1021443713030023

Arkhipov, D. V., Lomin, S. N., Myakushina, Y. A., Savelieva, E. M., Osolodkin, D. I., and Romanov, G. A. (2019). Modeling of protein-protein interactions in cytokinin signal transduction. *Int. J. Mol. Sci.* 20:2096. doi: 10.3390/ijms20092096

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., et al. (2016). Heatmapper: web-enabled heat mapping for all. *Nucl. Acids Res.* 44, W147–W153. doi: 10.1093/nar/gkw419

Bhargava, A., Clabaugh, I., To, J. P., Maxwell, B. B., Chiang, Y. H., Schaller, G. E., et al. (2013). Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-Seq in Arabidopsis. *Plant Physiol.* 162, 272–294. doi: 10.1104/pp.113.217026

Brenner, W. G., and Schmülling, T. (2015). Summarizing and exploring data of a decade of cytokinin-related transcriptomics. *Front. Plant Sci.* 6:29. doi: 10.3389/fpls.2015.00029

Cheng, L., Wang, D., Wang, Y., Xue, H., and Zhang, F. (2020). An integrative overview of physiological and proteomic changes of cytokinin-induced potato (*Solanum tuberosum* L.) tuber development *in vitro*. *Physiol. Plant.* 168, 675–693. doi: 10.1111/ppl.13014

Dodueva, I. E., Lebedeva, M. A., Kuznetsova, K. A., Gancheva, M. S., Paponova, S. S., and Lutova, L. A. (2020). Plant tumors: a hundred years of study. *Planta* 251:82.

Dortay, H., Gruhn, N., Pfeifer, A., Schwerdtner, M., Schmülling, T., and Heyl, A. (2008). Toward an interaction map of the two-component signaling pathway of *Arabidopsis thaliana*. *J. Proteome Res.* 7, 3649–3660. doi: 10.1021/pr0703831

Dutt, S., Manjul, A. S., Raigond, P., Singh, B., Siddappa, S., Bhardwaj, V., et al. (2017). Key players associated with tuberization in potato: potential candidates for genetic engineering. *Crit. Rev. Biotechnol.* 37, 942–957. doi: 10.1080/07388551.2016.1274876

Eviatar-Ribak, T., Shalit-Kaneh, A., Chappell-Maor, L., Amsellem, Z., Eshed, Y., and Lifschitz, E. (2013). A cytokinin-activating enzyme promotes tuber formation in tomato. *Curr. Biol.* 23, 1057–1064. doi: 10.1016/j.cub.2013.04.061

Guivarc'h, A., Rembur, J., Goetz, M., Roitsch, T., Noin, M., Schmülling, T., et al. (2002). Local expression of the *ipt* gene in transgenic tobacco (*Nicotiana tabacum* L. cv. SR1) axillary buds establishes a role for cytokinins in tuberization and sink formation. *J. Exp. Bot.* 53, 621–629. doi: 10.1093/jexbot/53.369.621

Hallmark, H. T., and Rashotte, A. M. (2019). Review - Cytokinin Response Factors: responding to more than cytokinin. *Plant Sci.* 289, 110251. doi: 10.1016/j.plantsci.2019.110251

Hannapel, D. J., Sharma, P., Lin, T., and Banerjee, A. K. (2017). The multiple signals that control tuber formation. *Plant Physiol.* 174, 845–856. doi: 10.1104/pp.17.00272

Hartmann, A., Senning, M., Hedden, P., Sonnewald, U., and Sonnewald, S. (2011). Reactivation of meristem activity and sprout growth in potato tubers require both cytokinin and gibberellin. *Plant Physiol.* 155, 776–796. doi: 10.1104/pp.110.168252

Heyl, A., and Schmülling, T. (2003). Cytokinin signal perception and transduction. *Curr. Opin. Plant Biol.* 6, 480–486. doi: 10.1016/s1369-5266(03)00087-6

Higuchi, M., Pischke, M. S., Mähönen, A. P., Miyawaki, K., Hashimoto, Y., Seki, M., et al. (2004). *In planta* functions of the Arabidopsis cytokinin receptor family. *Proc. Nat. Acad. Sci. U.S.A.* 101, 8821–8826. doi: 10.1073/pnas.0402887101

Hoyerová, K., and Hošek, P. (2020). New insights into the metabolism and role of cytokinin *N*-glucosides in plants. *Front. Plant Sci.* 11:741. doi: 10.3389/fpls.2020.00741

Hutchison, C. E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M. W., et al. (2006). The *Arabidopsis* histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* 18, 3073–3087. doi: 10.1105/tpc.106.045674

Kakimoto, T. (1996). CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274, 982–985. doi: 10.1126/science.274.5289.982

Kamada-Nobusada, T., and Sakakibara, H. (2009). Molecular basis for cytokinin biosynthesis. *Phytochem.* 70, 444–449. doi: 10.1016/j.phytochem.2009.02.007

Kieber, J. J., and Schaller, G. E. (2014). Cytokinins. *Arabidopsis Book* 12:e0168. doi: 10.1199/tab.0168

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:dev149344. doi: 10.1242/dev.149344

Kim, A., Chen, J., Khare, D., Jin, J. Y., Yamaoka, Y., Maeshima, M., et al. (2020). Non-intrinsic ATP-binding cassette proteins ABCI19, ABCI20 and ABCI21

modulate cytokinin response at the endoplasmic reticulum in *Arabidopsis thaliana*. *Plant Cell Rep.* 39, 473–487. doi: 10.1007/s00299-019-02503-0

Kolachevskaya, O. O., Alekseeva, V. V., Sergeeva, L. I., Rukavtsova, E. B., Getman, I. A., Vreugdenhil, D., et al. (2015). Expression of auxin synthesis gene *tms1* under control of tuber-specific promoter enhances potato tuberization *in vitro*. *J. Integr. Plant Biol.* 57, 734–744. doi: 10.1111/jipb.12314

Kolachevskaya, O. O., Lomin, S. N., Arkhipov, D. V., and Romanov, G. A. (2019a). Auxins in potato: molecular aspects and emerging roles in tuber formation and stress resistance. *Plant Cell Rep.* 38, 681–698. doi: 10.1007/s00299-019-02395-0

Kolachevskaya, O. O., Lomin, S. N., Kojima, M., Getman, I. A., Sergeeva, L. I., Sakakibara, H., et al. (2019b). Tuber-specific expression of two gibberellins oxidase transgenes from Arabidopsis regulates over wide ranges the potato tuber formation. *Russ. J. Plant Physiol.* 66, 984–991. doi: 10.1134/S1021443720010094

Kolachevskaya, O. O., Sergeeva, L. I., Floková, K., Getman, I. A., Lomin, S. N., Alekseeva, V. V., et al. (2017). Auxin synthesis gene *tms1* driven by tuber-specific promoter alters hormonal status of transgenic potato plants and their responses to exogenous phytohormones. *Plant Cell Rep.* 36, 419–435. doi: 10.1007/s00299-016-2091-y

Kolachevskaya, O. O., Sergeeva, L. I., Getman, I. A., Lomin, S. N., Savelieva, E. M., and Romanov, G. A. (2018). Core features of the hormonal status in *in vitro* grown potato plants. *Plant Signal. Behav.* 13:e1467697. doi: 10.1080/15592324.2018.1467697

Liu, C. J., Zhao, Y., and Zhang, K. (2019). Cytokinin transporters: multisite players in cytokinin homeostasis and signal distribution. *Front. Plant Sci.* 10:693. doi: 10.3389/fpls.2019.00693

Lomin, S. N., Krivosheev, D. M., Steklov, M. Y., Osolodkin, D. I., and Romanov, G. A. (2012). Receptor properties and features of cytokinin signaling. *Acta Nat.* 4, 31–45. doi: 10.32607/20758251-2012-4-3-31-45

Lomin, S. N., Myakushina, Y. A., Arkhipov, D. V., Leonova, O. G., Popenko, V. I., Schmülling, T., et al. (2018a). Studies of cytokinin receptor–phosphotransmitter interaction provide evidences for the initiation of cytokinin signalling in the endoplasmic reticulum. *Funct. Plant Biol.* 45, 192–202. doi: 10.1071/FP16292

Lomin, S. N., Savelieva, E. M., Arkhipov, D. V., and Romanov, G. A. (2020). Evidences for preferential localization of cytokinin receptors of potato in the endoplasmic reticulum. *Biochem. (Moscow) Suppl. Ser. A Membr. Cell Biol.* 14, 146–153. doi: 10.1134/S1990747820010079

Lomin, S. N., Myakushina, Y. A., Kolachevskaya, O. O., Getman, I. A., Arkhipov, D. V., Savelieva, E. M., et al. (2018b). Cytokinin perception in potato: new features of canonical players. *J. Exp. Bot.* 69, 3839–3853. doi: 10.1093/jxb/ery199

Miller, C. O., Skoog, F., Von Saltza, M. H., and Strong, F. M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. *J. Am. Chem. Soc.* 77:1392. doi: 10.1021/ja01610a105

Mizuno, T. (2005). Two-component phosphorelay signal transduction systems in plants: from hormone responses to circadian rhythms. *Biosci. Biotechnol. Biochem.* 69, 2263–2276. doi: 10.1271/bbb.69.2263

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. *Plant Cell* 16, 1365–1377. doi: 10.1105/tpc.021477

Palmer, C. E., and Smith, O. E. (1970). Effect of kinetin on tuber formation on isolated stolons of *Solanum tuberosum* L. cultured *in vitro*. *Plant Cell Physiol.* 11, 303–314. doi: 10.1093/oxfordjournals.pcp.a074511

Pekárová, B., Szmitkowska, A., Dopitová, R., Degtjarik, O., Žídek, L., and Hejátko, J. (2016). Structural aspects of multistep phosphorelay-mediated signaling in plants. *Mol. Plant* 9, 71–85. doi: 10.1016/j.molp.2015.11.008

Pekárová, B., Szmitkowska, A., Houser, J., Wimmerova, M., and Hejátko, J. (2018). "Cytokinin and ethylene signaling," in *Plant Structural Biology: Hormonal Regulations*, eds J. Hejátko, and T. Hakoshima (Basel: Springer). doi: 10.1007/978-3-319-91352-0_10

Potato Genome Sequencing Consortium [PGSC] (2011). Genome sequence and analysis of the tuber crop potato. *Nature* 475, 189–195. doi: 10.1038/nature10158

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18, 40–54. doi: 10.1105/tpc.105.037796

Romanov, G. A. (2009). How do cytokinins affect the cell? *Russ. J. Plant Physiol.* 56, 268–290. doi: 10.1134/S1021443709020174

Romanov, G. A., Aksenova, N. P., Konstantinova, T. N., Golyanovskaya, S. A., Kossmann, J., and Willmitzer, L. (2000). Effect of indole-3-acetic acid and kinetin on tuberisation parameters of different cultivars and transgenic lines of potato in vitro. *Plant Growth Regul.* 32, 245–251. doi: 10.1023/A:1010771510526

Sakakibara, H. (2006). Cytokinins: activity, biosynthesis, and translocation. *Annu. Rev. Plant Biol.* 57, 431–449. doi: 10.1146/annurev.arplant.57.032905.105231

Schmülling, T., Werner, T., Riefler, M., Krupková, E., and Bartrina y Manns, I. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. *J. Plant Res.* 116, 241–252. doi: 10.1007/s10265-003-0096-4

Sharma, P., Lin, T., and Hannapel, D. J. (2016). Targets of the StBEL5 transcription factor include the FT ortholog StSP6A. *Plant Physiol.* 170, 310–324. doi: 10.1104/pp.15.01314

Steklov, M. Y., Lomin, S. N., Osolodkin, D. I., and Romanov, G. A. (2013). Structural basis for cytokinin receptor signaling: an evolutionary approach. *Plant Cell Rep.* 32, 781–793. doi: 10.1007/s00299-013-1408-3

Swarbreck, D., Wilks, C., Lamesch, P., Berardini, T. Z., Garcia-Hernandez, M., Foerster, H., et al. (2008). The Arabidopsis Information Resource (TAIR): gene structure and function annotation. *Nucleic Acids Res.* 36, D1009–D1014. doi: 10.1093/nar/gkm965

Tessi, T. M., Brumm, S., Winklbauer, E., Schumacher, B., Pettinari, G., Lescano, I., et al. (2020). Arabidopsis AZG2 transports cytokinins *in vivo* and regulates lateral root emergence. *New Phytol.* doi: 10.1111/nph.16943 [Epub ahead of print].

Zdarska, M., Cuyacot, A. R., Tarr, P. T., Yamoune, A., Szmitkowska, A., Hrdinová, V., et al. (2019). ETR1 integrates response to ethylene and cytokinins into a single multistep phosphorelay pathway to control root growth. *Mol. Plant* 12, 1338–1352. doi: 10.1016/j.molp.2019.05.012

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 22 December 2020
doi: 10.3389/fpls.2020.618560

Endoplasmic Reticulum-Localized PURINE PERMEASE1 Regulates Plant Height and Grain Weight by Modulating Cytokinin Distribution in Rice

Yunhua Xiao[1,2], Junwen Zhang[1], Guiyuan Yu[1], Xuedan Lu[1], Wentao Mei[1], Huabing Deng[1], Guilian Zhang[1], Guihua Chen[1], Chengcai Chu[2], Hongning Tong[3] and Wenbang Tang[1]**

[1] Southern Regional Collaborative Innovation Center for Grain and Oil Crops in China, College of Agriculture, Hunan Agricultural University, Changsha, China, [2] State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Innovative Academy of Seed Design, Chinese Academy of Sciences, Beijing, China, [3] National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China

OPEN ACCESS

Edited by:
Wolfram G. Brenner, Universität Leipzig, Germany

Reviewed by:
Georgy A. Romanov, Timiryazev Institute of Plant Physiology (RAS), Russia
Radomira Vankova, Institute of Experimental Botany (LCAS), Czechia

***Correspondence:**
Hongning Tong
tonghongning@caas.cn
Wenbang Tang
tangwenbang@163.com

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *17 October 2020*
Accepted: *03 December 2020*
Published: *22 December 2020*

Citation:
Xiao Y, Zhang J, Yu G, Lu X, Mei W, Deng H, Zhang G, Chen G, Chu C, Tong H and Tang W (2020) Endoplasmic Reticulum-Localized PURINE PERMEASE1 Regulates Plant Height and Grain Weight by Modulating Cytokinin Distribution in Rice. Front. Plant Sci. 11:618560. doi: 10.3389/fpls.2020.618560

Cytokinins (CKs) are a class of phytohormones playing essential roles in various biological processes. However, the mechanisms underlying CK transport as well as its function in plant growth and development are far from being fully elucidated. Here, we characterize the function of PURINE PERMEASE1 (OsPUP1) in rice (*Oryza sativa* L.). *OsPUP1* was predominantly expressed in the root, particularly in vascular cells, and CK treatment can induce its expression. Subcellular localization analysis showed that OsPUP1 was predominantly localized to the endoplasmic reticulum (ER). Overexpression of *OsPUP1* resulted in growth defect of various aerial tissues, including decreased leaf length, plant height, grain weight, panicle length, and grain number. Hormone profiling revealed that the CK content was decreased in the shoot of *OsPUP1*-overexpressing seedling, but increased in the root, compared with the wild type. The CK content in the panicle was also decreased. Quantitative reverse transcription-PCR (qRT-PCR) analysis using several CK type-A *response regulators* (*OsRRs*) as the marker genes suggested that the CK response in the shoot of *OsPUP1*-overexpressing seedling is decreased compared to the wild type when CKs are applied to the root. Genetic analysis revealed that BG3/OsPUP4, a putative plasma membrane-localized CK transporter, overcomes the function of OsPUP1. We hypothesize that OsPUP1 might be involved in importing CKs into ER to unload CKs from the vascular tissues by cell-to-cell transport.

Keywords: purine permease, cytokinin, plant height, grain weight, rice (*Oryza sativa* L.)

INTRODUCTION

Cytokinins are a class of phytohormones playing important roles in various biological processes including cell division and differentiation, shoot/root balance, nutrient relocation, seed number as well as stress responses (Sakakibara, 2006). In plants, CKs exist naturally either in free-base forms, including N^6-(Δ^2-isopentenyl)-adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin

(DHZ), or in conjugated forms, although the ratio varies with plant species (Sakakibara, 2006; Osugi and Sakakibara, 2015). A series of synthetic enzymes, including isopentenyltransferases (IPTs), CK-specific cytochrome P450 (CYP735As), and LONELY GUY/LOG LIKE phosphoribohydrolases (LOG/LOGLs), have been identified, while uridine diphosphate glucosyltransferases (UGTs) and CK oxidase/dehydrogenases (CKXs) are involved in the CK inactivation and degradation (Sakakibara, 2006; Kurakawa et al., 2007). IPTs preferably utilize adenosine triphosphate (ATP) or adenosine diphosphate (ADP) as isoprenoid acceptors to synthesize isopentenyladenine riboside 5′-triphosphate (iPRTP) and isopentenyladenine riboside 5′-diphosphate (iPRDP), respectively (Kakimoto, 2001); CYP735As convert iP nucleotides into the corresponding tZ nucleotides (Takei et al., 2004); LOG/LOGLs catalyze the transition from inactive CK derivatives to bioactive CK nucleobases (Kurakawa et al., 2007; Kuroha et al., 2009); UGTs deactivate CK nucleobases by conjugation at *O*- and *N*- position with a sugar moiety, mostly glucose (Martin et al., 1999a,b; Šmehilová et al., 2016). CKXs catabolize CKs to adenine or adenosine (Galuszka et al., 2001).

The signal pathway of CK involves a His-Asp phosphorelay system from receptor histidine kinases (HKs) to histidine-containing phosphotransfer proteins (HPTs), then to the transcriptional factor type-B response regulators (RRs) (Werner and Schmülling, 2009; Hwang et al., 2012; Kieber and Schaller, 2018). Hybrid HKs sense CKs via the cyclases/histidine kinases associated sensory extracellular (CHASE) domain for CK-binding, which reside both in plasma membrane (PM) and ER, and have been suggested to mainly happen in ER lumen (Caesar et al., 2011; Lomin et al., 2011, 2018; Wulfetange et al., 2011; Hwang et al., 2012; Ding et al., 2017; Romanov et al., 2018; Kubiasová et al., 2020). Type-B RRs contain DNA-binding domain and mediate CK-dependent transcriptional activation (Sakai et al., 2000, 2001; Hwang and Sheen, 2001; Hutchison et al., 2006). Type-B RRs regulate the expression of target genes in response to the hormone (Sakai et al., 2000, 2001). Among the target genes, type-A RRs are induced by CK and play negative roles through competing with type-B RRs for phosphoryl group (Werner and Schmülling, 2009; Hwang et al., 2012; Kieber and Schaller, 2018).

Cytokinins regulate various agronomic traits, such as grain number, grain size, and plant height. Loss-of-function of *LOG* decreases shoot apical meristem and reduces grain number (Kurakawa et al., 2007). Knockout of *CYP735A4* decreases plant height (Gao et al., 2019). Decreased expression of *OsCKX2/Gn1a* increases the grain number (Ashikari et al., 2005; Li et al., 2013). *OsCKX2* also negatively regulates grain weight (Yeh et al., 2015). The knockout mutants of *OsCKX11* display delayed leaf senescence and increased grain number (Zhang W. et al., 2020). Overexpression of another CK oxidase/dehydrogenase gene *OsCKX4* significantly decreases grain number, grain weight, and plant height (Gao et al., 2014). *TaCKX6-D1*, a wheat ortholog of rice *OsCKX2*, has been shown to be significantly associated with grain weight, and haplotype of the gene is associated with higher grain weight (Zhang L. et al., 2012). Knockdown of *TaCKX2.4* increases grain numbers per spike (Li et al., 2018). However, it has been suggested that *TaCKX2.1* and *TaCKX2.2* expressions are positively correlated with grain number per spike (Zhang J. et al., 2011).

Cytokinins function not only as local paracrine signal, but also as long-distance signal through translocating in vascular tissues (Sakakibara, 2006; Hirose et al., 2008; Osugi and Sakakibara, 2015; Liu et al., 2019). Trace experiments with the help of isotope-labeled CKs have demonstrated the movement of CK among tissues *in planta* (Bishopp et al., 2011; Kiba et al., 2013; Sasaki et al., 2014; Zhang K. et al., 2014). Due to the tissue-specific expression pattern of CK biosynthetic genes such as *CYP735As* which are mainly expressed in the roots for synthesis of tZ-type CKs, CK species are unevenly produced in different tissues (Takei et al., 2004; Hirose et al., 2008). tZ-type CKs are mainly distributed in xylem sap, while iP-type CKs mainly present in the phloem sap (Hirose et al., 2008). Moreover, it has been demonstrated that the shoot-derived and root-derived CKs could have specific function in regulating plant growth and development (Matsumoto-Kitano et al., 2008; Kiba et al., 2013; Sasaki et al., 2014). The *Arabidopsis atipt1;3;5;7* quadruple mutant with reduced CK content does not form cambium and displays reduced thickness of the stem and root (Matsumoto-Kitano et al., 2008). Reciprocal grafting the shoot and root of the quadruple mutant and the wild-type plant recover the growth-deficient phenotypes of the mutant (Matsumoto-Kitano et al., 2008). The *Arabidopsis cyp735a1 cyp735a2* double mutant with severely reduced tZ-type CK content but unchanged total CK quantity has retardation of the shoot growth, which can be recovered to the wild-type phenotype by applying exogenous tZ but not iP (Kiba et al., 2013). The shoot phenotype can also be complemented with the recovery of tZ-type CK content by grafting the shoot of the double mutant onto the wild-type stock (Kiba et al., 2013).

There are at least four types of proteins reported to be involved in CK traffic and translocation. One type is ATP-binding cassette (ABC) transporter subfamily. *AtABCG14* is expressed in cells of vascular tissues and localized to the plasma membrane and it functions as an efflux transporter for loading CK into xylem, and plays a crucial role in the long distance transport of root-derived CKs (Ko et al., 2014; Zhang K. et al., 2014). A rice homolog, *OsABCG18*, has been shown to play a similar role (Zhao et al., 2019). Loss-of-function of either *AtABCG14* in *Arabidopsis* or *OsABCG18* in rice leads to the retention of tZ-type CKs in the roots, resulting in reduced growth of the shoots (Ko et al., 2014; Zhang K. et al., 2014; Zhao et al., 2019). Another type is equilibrative nucleoside transporter (ENT) family, which has been suggested to selectively translocate CK nucleosides (Hirose et al., 2005, 2008). *OsENT2* is expressed in the scutellum of germinating seeds and the vascular tissues of germinated seedlings, and predominantly expressed in the roots in mature plants (Hirose et al., 2005). It has been suggested that OsENT2 participates in retrieving endosperm-derived nucleosides through the germinating embryo and in the long-distance transport of nucleosides in growing plants (Hirose et al., 2005). Three homologs in *Arabidopsis*, AtENT3, AtENT6, and AtENT8, are also suggested to be involved in transporting CK nucleoside (Sun et al., 2005; Hirose et al., 2008).

Very recently, AZG2, a member of AZA-GUANINE RESISTANT (AZG) purine transporter family, is reported to have the ability to transports purines and CK with high affinity (Tessi et al., 2020). The forth type is purine permease (PUP) family. Three genes, *AtPUP1*, *AtPUP2*, and *AtPUP14*, are supposed to mediate CK nucleobase uptake in *Arabidopsis* (Bürkle et al., 2003; Zürcher et al., 2016). *AtPUP1* is expressed in the epithem of hydatodes and the stigma surface of silique, and localized to the plasma membrane, whereas *AtPUP2* is expressed in the phloem of leaves (Bürkle et al., 2003; Szydlowski et al., 2013). AtPUP14 is also localized to the plasma membrane, and has the ability to import CK nucleobase into cell (Zürcher et al., 2016). AtPUP14 is proposed to diminish the spatiotemporal active CK sink in the apoplast for perception by plasma membrane-localized CK receptor (Zürcher et al., 2016). In rice, there are 12 PUP family members (Qi and Xiong, 2013). OsPUP4 and OsPUP7 are localized to the plasma membrane and endoplasmic reticulum (ER), respectively, though they are both expressed in vascular tissues (Qi and Xiong, 2013; Xiao et al., 2019). OsPUP4 and OsPUP7 are assumed to be involved in long-distance transport and local allocation of CK in a cell-to-cell way (Xiao et al., 2019).

In this study, we identified another PUP homolog OsPUP1. The gene was expressed highly in the root, predominantly in vascular cells, and the protein was predominantly localized to ER. Overexpression of *OsPUP1* led to altered distribution of CKs, and resulted in growth defect in the shoot. Further analyses suggested that the CK response in *OsPUP1*-overexpressing seedling plant is altered. We hypothesize that OsPUP1 might be involved in importing CKs into ER to mediate CK transport and CK response.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Japonica* cultivar Zhonghua11 was used as the wild type in this study. For the analysis at the reproductive stage, rice plants were grown in the field under natural condition. For seedling analysis, rice plants were grown hydroponically in a growth chamber at 28°C with a 12-h-day/12-h-night cycle, light intensity of 30000 lux, and humidity of 70%. Modified Kimura B (pH 5.8) solution (Ma et al., 2001) was supplied as nutrient medium containing the following macronutrients (mM): $(NH_4)_2SO_4$ (0.36), $MgSO_4.7H_2O$ (0.54), KNO_3 (0.18), $Ca(NO_3)_2$ (0.36), K_2SO_4 (0.09), KH_2PO_4 (0.18), and $Na_2SiO_3.9H_2O$ (1.6); and micronutrients (μM): $MnCl_2.4H_2O$ (9.14), H_3BO_3 (46.2), H_2MoO_4 (0.56), $ZnSO_4.7H_2O$ (0.76), $CuSO_4.5H_2O$ (0.32), and Fe(II)-EDTA (20).

Vector Construction and Plant Transformation

The full-length coding sequence of *OsPUP1* was cloned into pCAMBIA2300-Actin and pCAMBIA2300-35S:GFP to generate the constructs for overexpression and protein subcellular localization analysis, respectively. The 2,091 bp promoter sequence upstream the start codon of *OsPUP1* was cloned into pCAMBIA2391Z to generate the construct for GUS staining analysis. Sequences were cloned into vectors by recombination fusion strategy. To create knockout mutants, *OsPUP1* was edited by targeting 5′-GTCGTGCTTCGTGTACGCGCTGG-3′ in the coding sequence as described previously (Lu et al., 2017). The transgenic plants were produced using Zhonghua11 as the receptor by *Agrobacterium tumefaciens*-mediated transformation following the previously described method (Liu et al., 2007). T_0 and T_1 lines of *pOsPUP1:GUS* transgenic plants, and T_3 and higher lines of *OsPUP1*-overexpressing and knockout homozygous plants were used for analyses.

Total RNA Isolation and qRT-PCR Analysis

Total RNA was isolated using TRIzol (Code No. 15596026, Invitrogen). The cDNA was synthesized using a kit named "PrimeScript™ RT reagent Kit with gDNA Eraser" (Code No. RR047A, TaKaRa) following the product instructions. qRT-PCR using SYBR Green PCR mix (Code No. RR820A, TaKaRa) was performed on a real-time PCR detection system (Bio-Rad CFX96) according to the manufacturer's instructions. The rice *Ubiquitin2* gene was used as an internal reference for all analyses. The primers used for qRT-PCR are listed in **Supplementary Table 2**.

GUS Staining

Root from plants at the seedling stage and other tissues from plants at the reproductive stage were sampled for GUS staining according to a previously described method (Jefferson, 1989). The stained tissues were observed and the images were taken using a stereomicroscope (Olympus SZX16) with a digital camera (Canon EOS 600D).

Hormone Treatment

For responsive analyses of *OsPUP1* to CK as well as other phytohormones, the roots of 8-day-old wild-type seedlings were treated with iP, tZ, or cZ at 1 μM concentrations for 2 h, or treated with other phytohormones, including brassinolide (BL), gibberellin (GA_3), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA), and jasmonic acid (JA), at 10 μM concentrations for 4 h. The materials for the analyses were used as the same as in our previous work (Xiao et al., 2019). For CK transport analysis, the roots of 10-day-old wild-type seedlings and *OsPUP1*-overexpressing seedlings were treated using iP, tZ, or cZ at 0.01 μM concentrations for 4 h. After treatments, the shoots and roots of the plants were separately harvested for expression analyses of *OsPUP1*, *OsRR1*, *OsRR2*, and *OsRR4*.

Measurement of CKs

Shoots and roots of 10-day-old rice seedlings grown in a growth chamber and 18–20 cm length panicles of plants grown in the field under natural condition were harvested and used for measurement of CKs as described previously (Cai et al., 2014).

Subcellular Localization Analysis of OsPUP1

pCAMBIA2300-35S:GFP-OsPUP1 was transformed alone or co-transformed with endoplasmic reticulum-red fluorescent protein (ER-RFP) into rice protoplasts using a previously described method (Zhang Y. et al., 2011). The same vector was introduced into *Nicotiana benthamiana* leaves by *Agrobacterium tumefaciens*-mediated transformation following the method described previously (Sparkes et al., 2006). After incubating for 18 h in rice protoplasts and 48 h in tobacco leaves, fluorescent signals were detected using a confocal laser scanning microscopy (Leica TCS SP5).

Phylogenetic Analysis

PUP genes in *Arabidopsis*, coffee, and rice are numbered according to previous studies (Qi and Xiong, 2013; Zürcher et al., 2016; Kakegawa et al., 2019). Gene information referred to websites for *Arabidopsis*[1], coffee[2], and rice[3]. Protein sequences were used to construct the phylogenetic tree by software MEGA X (Kumar et al., 2018) using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

[1]https://www.arabidopsis.org/

[2]http://coffee-genome.org/

[3]http://rice.plantbiology.msu.edu/

Accession Numbers

Sequence data from this article can be found in the Rice Genome Annotation Project (see text footnote 3) under the following accession numbers: *OsPUP1* (LOC_Os03g08880), *BG3/OsPUP4* (LOC_Os01g48800), *OsRR1* (LOC_Os04g36070), *OsRR2* (LOC_Os02g35180), *OsRR4* (LOC_Os01g72330), and *Ubiqutin2* (LOC_Os02g06640).

RESULTS

Molecular Characteristics of OsPUP1

We previously identified two OsPUPs, BG3/OsPUP4, and OsPUP7, being involved in long-distance transport of CK (Xiao et al., 2019). OsPUP1 is a close homolog of OsPUP4 and OsPUP7. In addition, it has been shown that both OsPUP1 and OsPUP4 have increased expression in *OsPUP7*-overexpressing plant (Qi and Xiong, 2013). We thus selected OsPUP1 for further analysis in order to explore its potential role in CK transport. We firstly evaluated the expression pattern of *OsPUP1* in different tissues of the wild-type plant by qRT-PCR. The results showed that *OsPUP1* was evidently expressed in all tissues tested, including mature

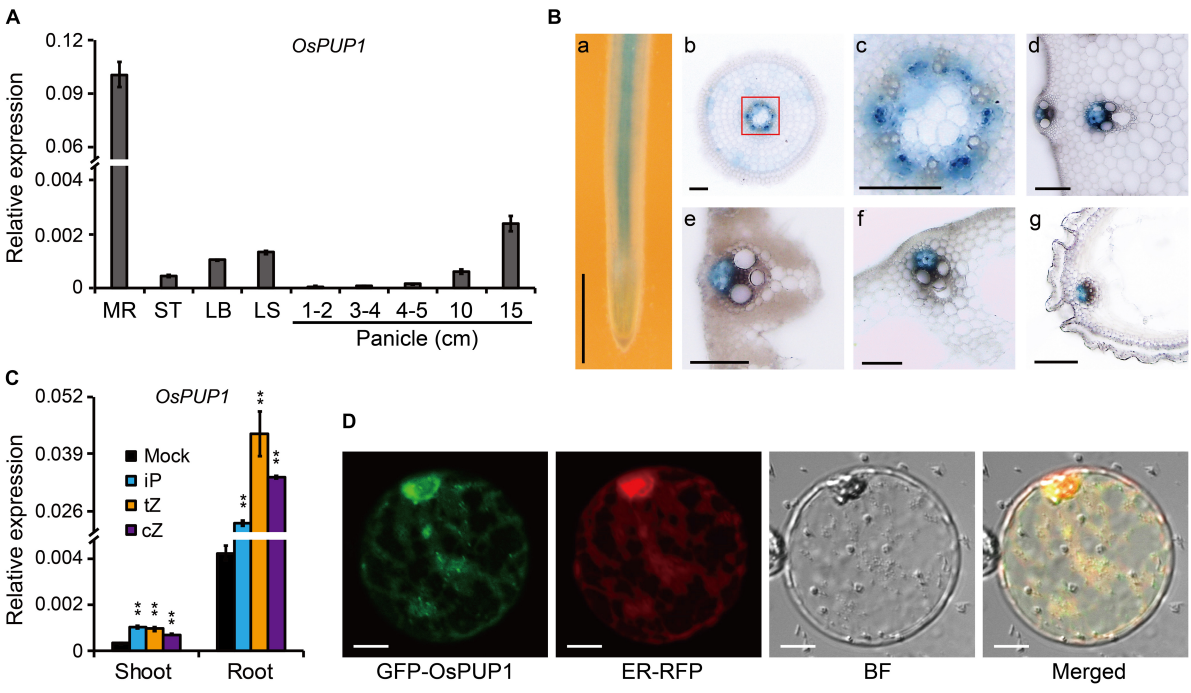


FIGURE 1 | Molecular characteristics of OsPUP1. **(A)** Expression pattern of *OsPUP1* in various tissues of the wild-type plant. MR, mature root; ST, stem; LB, leaf blade; LS, leaf sheath. *Ubiquitin2* gene was used as the internal reference. $n = 3$, bar = SD. **(B)** GUS staining analyses of the various tissues of the *pOsPUP1:GUS* transgenic plants. **(a)** young root; **(b)** cross section of young root; **(c)** amplification of the red-framed regions in **(b)** to better show the signals in phloem; cross section of culm **(d)**, leaf blade **(e)**, leaf sheath **(f)**, and grain husk **(g)**. Scale bar: 1 mm in **(a)**, 50 μm in **(b–g)**. **(C)** Inductive effect of different CK nucleobases on *OsPUP1* expression in the shoots and roots of 8-day-old wild-type seedlings, of which roots were treated by iP, tZ, and cZ at 1 μM concentrations for 2 h. *Ubiquitin2* gene was used as the internal reference. $n = 3$, bar = SD, $^{**}P < 0.01$ in Student's *t*-test. **(D)** Subcellular localization of OsPUP1 in rice protoplast. ER, endoplasmic reticulum; BF, bright field. Scale bar, 10 μm.

root, stem, leaf blade, leaf sheath, and panicles with different length (**Figure 1A**). However, the expression level was much higher in the root than those of other tissues (**Figure 1A**). In reproductive tissues, *OsPUP1* expression was gradually increased along with the panicle development (**Figure 1A**). This expression pattern was somewhat similar with those of *OsPUP4* and *OsPUP7* (Xiao et al., 2019), indicating that OsPUP1 could also play a role in panicle growth and development.

To further dissect the expression pattern of *OsPUP1*, we constructed a plasmid with GUS driven by the promoter of *OsPUP1* and introduced it into the wild-type plant. Histochemical staining of various tissues, including root, stem, leaf blade, leaf sheath, young panicle, and husk, showed that *OsPUP1* was predominantly expressed in vascular tissues, and turned to be specific in phloem (**Figure 1B**). The expression was also detected in other cells such as parenchymal cells, but to a much lesser extent (**Figure 1B**).

To test whether *OsPUP1* is responsive to CK, we analyzed the *OsPUP1* expression under CK treatment. iP, tZ, and cZ are three type of active CKs that can be endogenously synthesized in rice. When the roots of the wild-type seedling were treated with these different CKs, respectively, *OsPUP1* was always significantly induced in both shoots and roots (**Figure 1C**), suggesting that CKs can positively regulate the expression of *OsPUP1*. We further examined the expression of *OsPUP1* under other phytohormone treatments. Interestingly, *OsPUP1* could also be induced by brassinolide (BL), gibberellin (GA), 1-aminocyclopropane-1-carboxylic acid (ACC), and jasmonic acid (JA), but was suppressed by abscisic acid (ABA) and indole-3-acetic acid (IAA) (**Supplementary Figure 1**). Thus, it appears that OsPUP1 as a potential CK transporter is involved in response to various phytohormones.

Subcellular localization of a protein is important for its function, and OsPUP4 has been shown to be localized on plasma membrane for CK transport (Xiao et al., 2019). We thus tagged OsPUP1 with a green fluorescent protein (GFP) tag at the N-terminus of the protein and then introduced the corresponding vector into either rice protoplast or tobacco epidermal cells for analysis. Observation with a confocal laser scanning microscopy showed that OsPUP1 was apparently not localized to plasma membrane in protoplast (**Supplementary Figure 2**), but appeared to be localized to the endoplasmic reticulum (ER), as the nuclei were surrounded by the fluorescence signal (**Supplementary Figure 3**), which is thought to be a typical characteristic of ER localization (Sparkes et al., 2006). To confirm this result, we co-expressed the fusion protein with an ER marker (ER-RFP) in rice protoplasts, and found the fluorescence signals of the two fusion proteins are highly overlapped, demonstrating that OsPUP1 was predominantly localized to ER.

Overexpression of *OsPUP1* Suppresses Plant Height, Grain Weight and Grain Number

To study the function of OsPUP1 in regulating growth and development in rice, we overexpressed *OsPUP1* under the control of *ACTIN1* promoter in the wild-type plants, and obtained a number of independent transgenic plants. Compared with the wild-type plant, homozygous *OsPUP1*-overexpressing plants (designated as *OE* for short) exhibited remarkably reduced growth of various tissues. In detail, the grain size and grain weight were significantly decreased, which could be mainly attributed to the reduction of grain width (**Figures 2A–D**). The plant height was also decreased both at the reproductive stage and at the seedling stage (**Figures 2E,F** and **Supplementary Figure 4A**). In addition, the tiller number of the transgenic plants was slightly less than the wild type (**Supplementary Figure 4B**). Both the leaf blade and leaf sheath were shorter than the wild type (**Figure 2G** and **Supplementary Figure 4C**). Moreover, the panicle length, the primary and secondary branches were all decreased, which finally resulted in a decreased grain number (**Supplementary Figure 5**). The severity of the above-mentioned phenotypes were well consistent with the expression level of *OsPUP1* (**Figure 2H**).

We also generated knockout mutants of *OsPUP1* using CRISPR/Cas9 gene-editing technology. Two independent homozygous lines, both containing frameshift mutations with 1 bp insertion in the coding region and thus should be knockout alleles, were selected for phenotypic analysis (**Supplementary Figure 6A**). The mutation seems to have no effect on gene transcription since the expression of *OsPUP1* was not changed in both mutants (**Supplementary Figure 6B**). However, no clear phenotypic difference was observed compared with the wild-type plant, suggesting the existence of functional redundancy among PUP members.

Since *ospup1* mutant is phenotype-silent, we next focused on the analysis of the overexpressing plants for dissection of potential functions of OsPUP1. Considering that *OE1* showed a weak phenotype and even had no statistically significant difference in some terms compared with the wild type (**Supplementary Figures 4**, **5**), whereas *OE2* presented a very typical and marked phenotype, we majorly used *OE2* as a representative line for the following analyses.

OsPUP1-Overexpressing Plants Have Reduced CK Levels in Shoot and Panicle

Given the potential role of PUPs in CK transport, we asked whether the marked phenotypic changes of *OsPUP1*-overexpressing plants are associated with alteration of CK contents. To this end, we directly quantified various CK forms in both the shoot and the root of *OsPUP1*-overexpressing seedlings, respectively. CK nucleobases are thought to be solely active CK forms (Sakakibara, 2006; Hothorn et al., 2011; Lomin et al., 2015), and CK nucleosides can be easily transformed *in vivo* to CK nucleobases (Yonekura-Sakakibara et al., 2004; Hwang et al., 2012). Compared to the wild-type plants, the content of iP, tZ, and DHZ, three kinds of CK nucleobases, and the nucleoside form of tZ (tZR) were reduced, but cZ and other nucleoside forms tested were not markedly changed in the shoot of *OsPUP1*-overexpressing

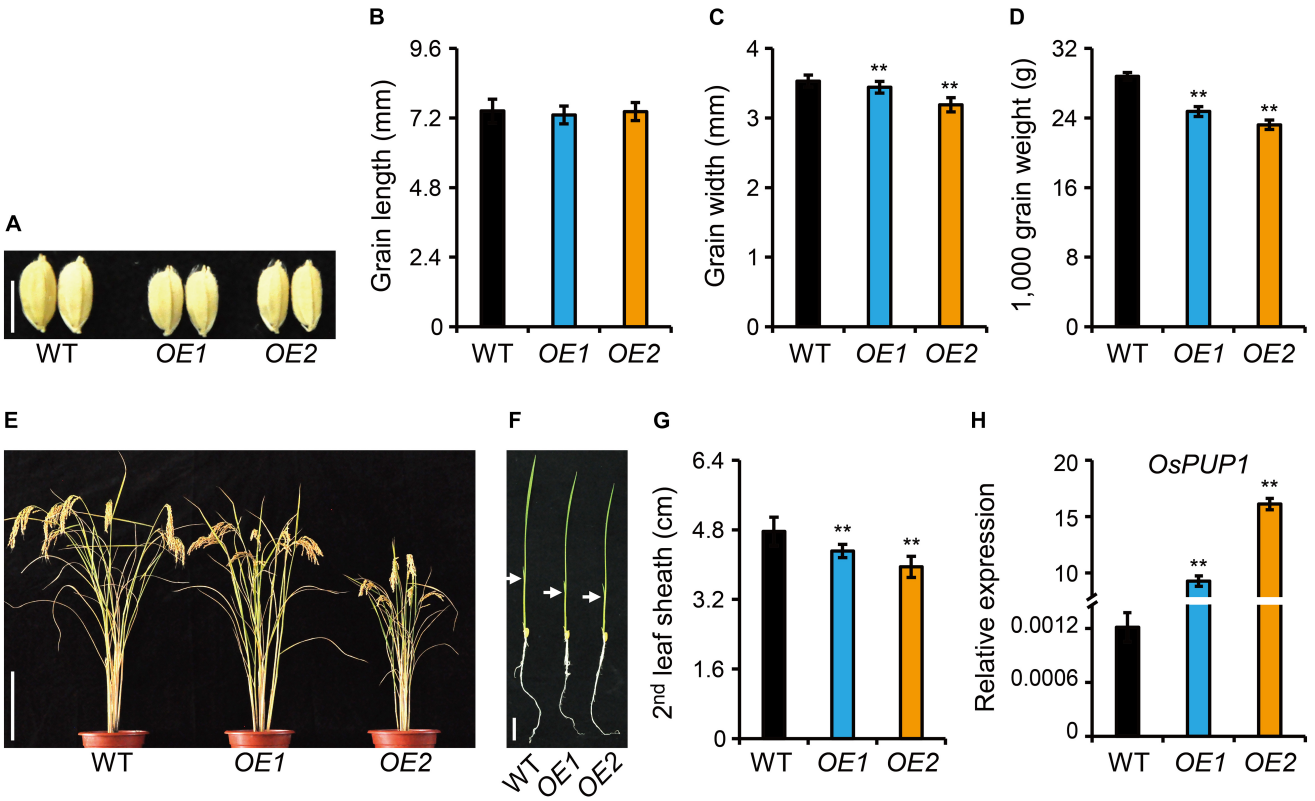


FIGURE 2 | Phenotype analyses of *OsPUP1*-overexpressing plants. **(A)** Comparison of the grain morphology of the wild-type plant (WT) and two representative *OsPUP1*-overexpressing plants, designated as *OE1* and *OE2* for short. Scale bar, 5 mm. **(B–D)** Statistical data of the grain length **(B)**, grain width **(C)**, and grain weight **(D)** in **(A)**. n = 20 in **(B,C)**, n = 3 in **(D)**, bar = SD, $^{**}P < 0.01$ in Student's t-test. **(E,F)** Gross morphology of 140-day-old plants at the reproductive stage **(E)** and 7-day-old plants at the seedling stage **(F)**. Scale bar: 20 cm in **(E)** and 2 cm in **(F)**. White arrowheads in **(F)** mark the second leaves. **(G)** Statistical data of the second leaf sheath length in **(F)**. n = 9, bar = SD, $^{**}P < 0.01$ in Student's t-test. **(H)** Relative expression of *OsPUP1* in shoots of 10-day-old *OsPUP1*-overexpressing seedlings tested by qRT-PCR, compared with WT. *Ubiquitin2* gene was used as the internal reference. n = 3, bar = SD, $^{**}P < 0.01$ in Student's t-test.

plants (**Figure 3A**). However, cZ, cZR, and DHZR were increased, tZR was decreased, and other biologically active forms were not markedly changed in the root (**Figure 3C**). Interestingly, the most abundant inactive form *cis*-zeatin *O*-glucoside (cZOG) increased in both shoots and roots (**Supplementary Table 1**). Nevertheless, the significant reduction of the total CK nucleobases and nucleosides in the shoot might explain the dwarfism phenotype of *OsPUP1*-overexpressing seedling plant (**Figure 3B**).

It has been shown that CKs also promote grain size and grain number in rice (Ashikari et al., 2005; Gao et al., 2014; Xiao et al., 2019; Yin et al., 2020). To test whether the decrease of grain size and grain number in *OsPUP1*-overexpressing plants are associated with the alteration of CK content, we further quantified the CK content in the panicles (**Figures 3E,F** and **Supplementary Table 1**). Compared with the wild-type plants, iP, iPR, and cZR were all significantly decreased in the transgenic plants, but DHZ and DHZR were slightly increased, while other biologically active forms were not significantly changed (**Figure 3E**). Thus, the decreased grain weight of *OsPUP1*-overexpressing plants might be caused by the reduction of the total content of CK nucleobases and nucleosides in the panicles (**Figure 3F**).

Shoot of *OsPUP1*-Overexpressing Plants Exhibits Decreased Response to CK Application in Root

The levels of tZ and tZR, two CK forms mainly synthesized in the root (Takei et al., 2004; Xiao et al., 2019), were decreased in the shoot of *OsPUP1*-overexpressing seedlings, and the significant increase of cZ and cZR in the root did not lead to the accordingly increase of the two forms in the shoot (**Figure 3A**). Given that OsPUP1 could be a CK transporter, we hypothesized that the root-to-shoot transport of CK was impaired in the transgenic plants. To test this possibility, we treated the roots of both *OsPUP1*-overexpressing plants and the wild-type with three kinds of CK nucleobases, including iP, tZ, and cZ, at 0.01 μM concentrations for 4 h, and then tried to compare the CK response in the shoots. If the root-to-shoot transport of CK were altered in the plant, the hormone response in the shoot should be accordingly altered in response to the CK treatment in the root. Three A-type *OsRR* genes, *OsRR1*, *OsRR2*, and *OsRR4*, which are

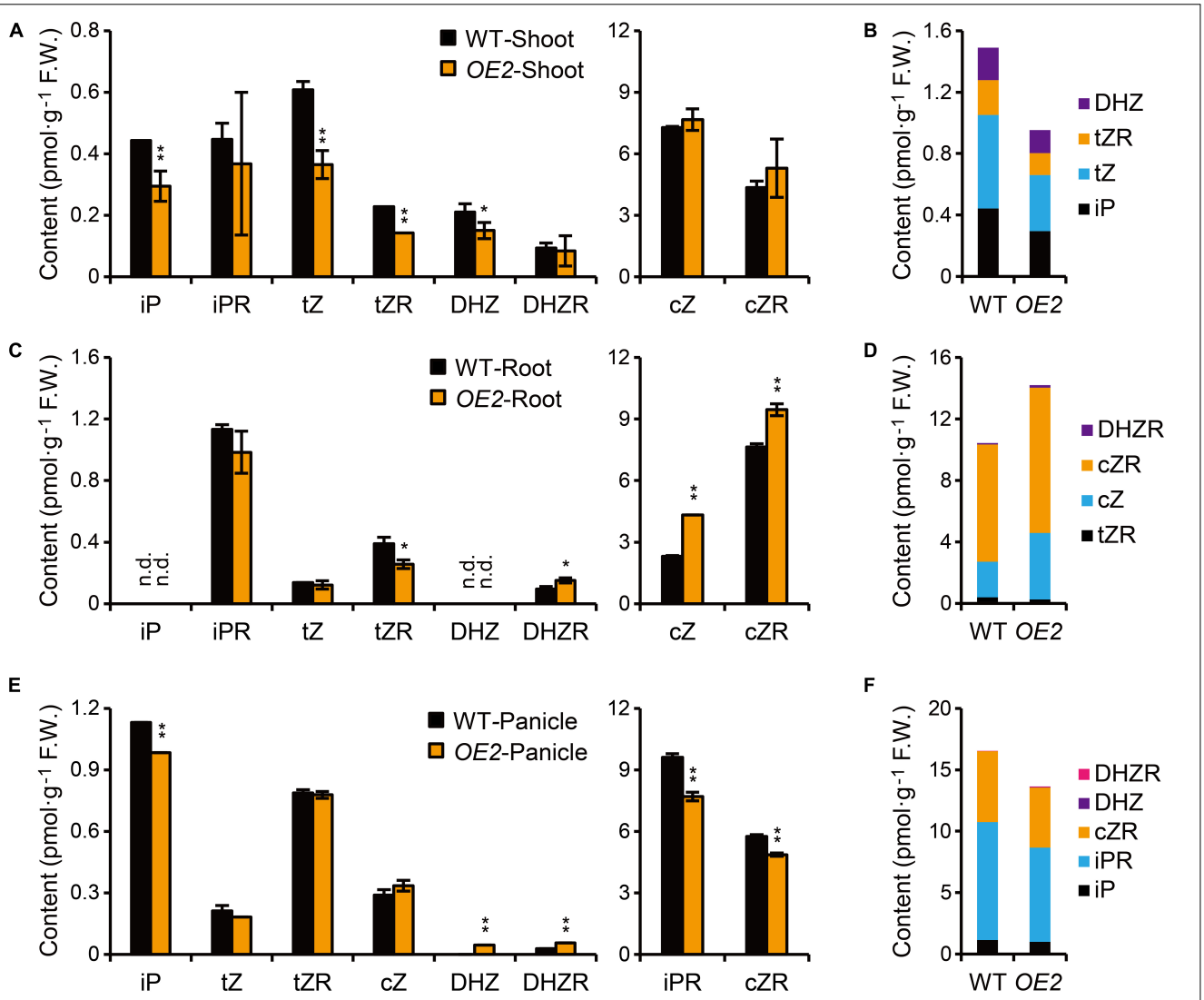


FIGURE 3 | Quantification of CKs in *OsPUP1*-overexpressing plants. **(A,C,E)** Quantification of CKs in shoots **(A)** and roots **(C)** of 10-day-old seedlings and panicles **(E)** with 18–20 cm length of the wild-type (WT) and *OsPUP1*-overexpressing plants (*OE2*). See **Supplementary Table 1** for individual values. Different CK forms were grouped according to the content. $n = 3$, bar = SD. n.d., not detected. $^{*}P < 0.05$ and $^{**}P < 0.01$ in Student's *t*-test. Asterisks indicate statistically significant difference of *OE2*, compared with WT. **(B,D,F)** Total amounts of the biologically active forms of CKs with statistical significance in shoots **(B)**, roots **(D)**, and panicles **(F)**. FW, fresh weight.

sensitively induced by CK (Kudo et al., 2012; Tsai et al., 2012; Xiao et al., 2019), were used as the marker genes to analyze the CK response in the shoot and root, respectively, and thus to indicate the activity of CK signal transduction. Without treatment, the expression levels of these *OsRR* genes were decreased in the shoot of *OsPUP1*-overexpressing plants, consistent with the decreased CK level, but unchanged in the root, compared with the wild-type (**Figure 4**). Upon CK treatment in the roots, the expression levels were significantly induced in the roots of both *OsPUP1*-overexpressing plants and the wild type (**Figure 4**). In the shoots, the expression levels of the three genes were induced in both *OE2* and the wild type under either tZ or cZ treatment, however, the induction extents are much lower in *OE2* than those in the wild type (**Figure 4**). For iP treatment, similar tendency was also observed, although the extent was much lower compared to those in tZ and cZ treatment (**Figure 4**). Taken together, these results strongly suggested that the long-distance transport of CK from root to shoot is reduced in *OsPUP1*-overexpressing plants.

BG3/OsPUP4 Overcomes OsPUP1 Function

Phylogenetic analysis of PUPs involving three different plant species, including *Arabidopsis*, coffee, and rice, showed that OsPUP1 is close to BG3/OsPUP4 as well as OsPUP7 and OsPUP8 in rice (**Supplementary Figure 7**). However, our results clearly revealed that overexpression of *BG3/OsPUP4* and *OsPUP1* led to significantly different or even opposite

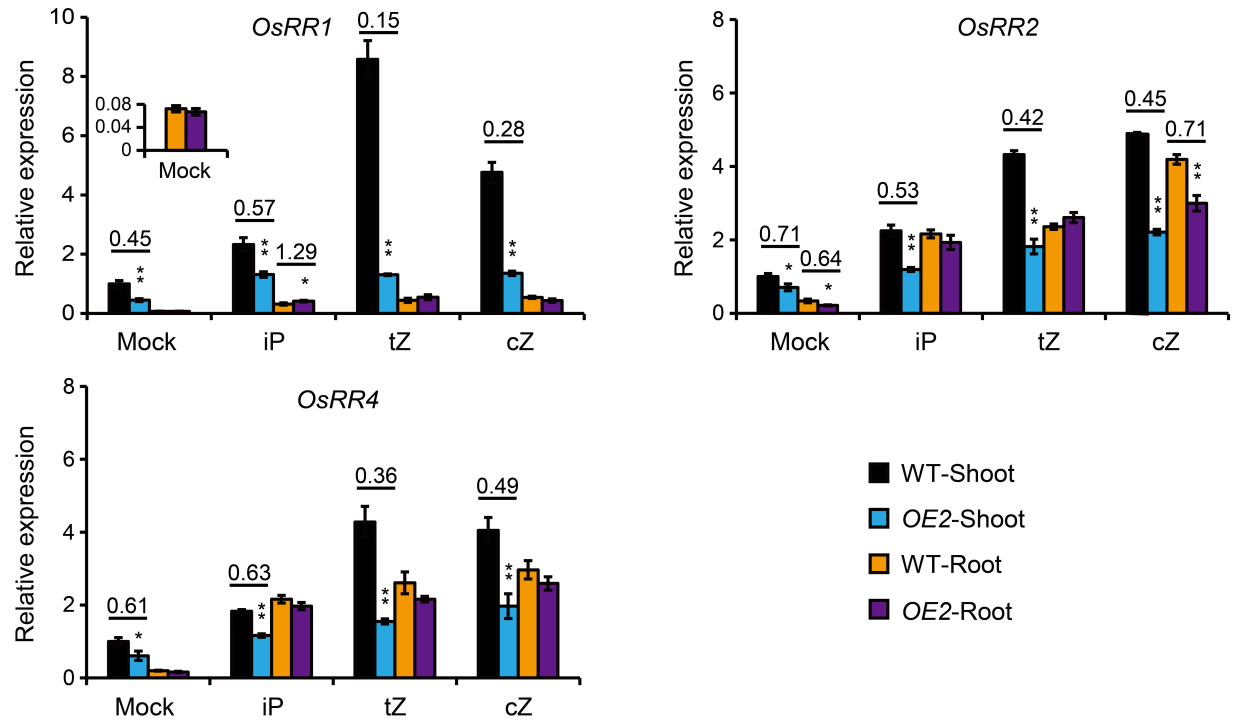


FIGURE 4 | Expression analyses of *OsRR* genes in the root and shoot of the seedlings treated by different forms of CKs in the root. The roots of 10-day-old seedlings of *OsPUP1*-overexpressing plant (*OE2*) and the wild type (WT) were treated by mock solution, or different CK nucleobases at 0.01 μM concentrations for 4 h, and then the shoot and root tissues were separately collected for gene expression analyses. *Ubiquitin2* gene was used as the internal reference. Genes and tissues are marked in each panel. $n = 3$, bar = SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ in Student's *t*-test. The values of WT shoots without CK treatment (Mock) were set to 1, and other values were the relative values compared to them. Asterisks indicate statistically significant difference of *OE2*, compared with WT. The pairs with statistically significant difference were calculated for the ratios of the relative expression of *OE2* to WT.

phenotypes (Xiao et al., 2019). To study the relationship between the two genes, we crossed *bg3-D* mutant, in which *BG3/OsPUP4* expression is activated (Xiao et al., 2019), with *OsPUP1*-overexpressing plant. At the reproductive stage, the F_1 plants with both *BG3/OsPUP4* and *OsPUP1* overexpressed exhibited a similar plant height as the wild type (**Figures 5A,B** and **Supplementary Figure 8**). Similar result was obtained at the seedling stage, as the F_1 plants showed a plant height like *bg3-D* (**Figures 5C,D**), which is taller than the wild type due to longer leaves (Xiao et al., 2019), suggesting that activation of *BG3* may mask the effect of *OsPUP1*-overexpression. In addition, the F_1 plants also showed increased grain weight (**Figure 5E**). Taken together, these results strongly suggested that, although the two genes function antagonistically in regulating plant growth and development, BG3/OsPUP4 can somehow overcome the role of OsPUP1.

DISCUSSION

Plasmodesmata provide efficient channels for molecules to move from cell-to-cell via the ER lumen (Barton et al., 2011). As overexpression of *OsPUP1* resulted in phenotypes almost contrary to those of *BG3/OsPUP4*- or *OsPUP7*-overexpressing plants, especially regarding the plant height and grain weight, we hypothesized that, while BG3/OsPUP4 and OsPUP7 function in loading CK into vascular tissues (Xiao et al., 2019), OsPUP1 might be involved in unloading CK out from vascular tissues (**Figure 6**). The ER-localized OsPUP1 might function as an influx transporter together with other CK transporters in importing CKs from cytoplasm into ER of cells in vascular tissues. Considering the role of root-derived tZ in promoting the shoot growth (Takei et al., 2004; Gao et al., 2014; Ko et al., 2014; Zhang K. et al., 2014), the reduced content of tZ and tZR in the shoot might be the reason for the dwarfism of *OsPUP1*-overexpressing plants. It should be mentioned that *OsPUP1* was expressed much more higher in the root than other tissues in the shoot (**Figure 1A**), which is quite different from the expression pattern of *BG3/OsPUP4* and *OsPUP7*. The root-preferential expression of *OsPUP1* suggests the potential role in unloading shoot-derived or phloem-transported CK for root growth and development. The decreased efficiency of the root-to-shoot transport of root-applied CKs in *OsPUP1*-overexpressing plants also supported this hypothesis. As *OsPUP1* was predominantly expressed in vascular tissues, particular in phloem (**Figure 1B**), the gene might play a role in unloading the systemic transport of CKs to regulate shoot growth and development. Thus, the identification of OsPUP1 could represent a distinct CK transporter, whose functions differ from those of OsPUP4 and OsPUP7. Apparently, these two types of CK transporters collaborated with each other contributing to the

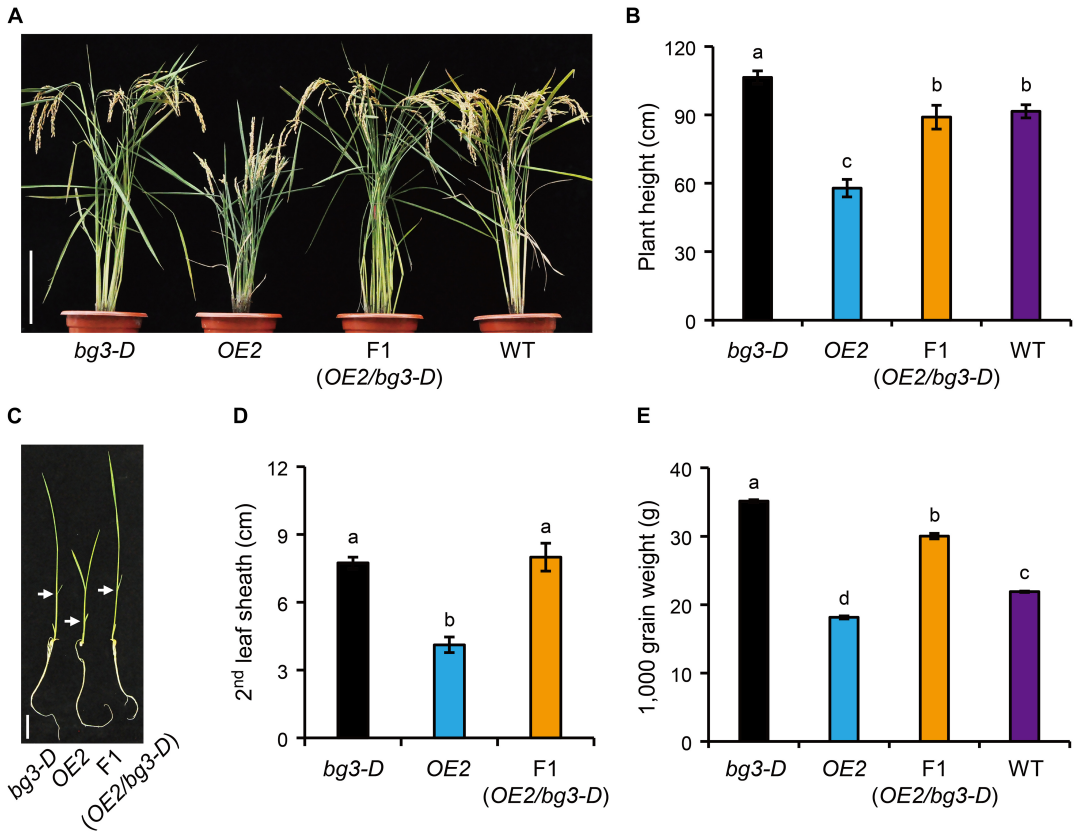


FIGURE 5 | Analysis of the relationship between BG3/OsPUP4 and OsPUP1. **(A,C)** Comparison of the gross morphology of the *bg3-D* mutant, *OsPUP1*-overexpressing plant, and their F_1 progeny, in which both of *BG3/OsPUP4* and *OsPUP1* were overexpressed, at 128-day-old reproductive stage **(A)** and 12-day-old seedling stage **(C)**. White arrowheads in **(C)** mark the second leaves. Scale bar: 20 cm in **(A)**, 4 cm in **(B)**. **(B,D,E)** Statistical data of plant height in **(A)**, the second leaf sheath length **(D)** in **(C)**, and grain weight **(E)**. $n = 8$ in **(B)**, $n = 5$ in **(D)**, $n = 3$ in **(E)**, bar = SD. Different letters above the columns indicate statistically significant differences between groups (*t* Test LSD, $P < 0.05$).

efficient hormone transportation. Together with many other additional homologs, they may form an efficient loading and unloading system to fulfill the long transport of CK.

Cytokinin receptors are suggested to be mainly localized in ER, and the perceiving CHASE domain is supposed to be exposed to the ER lumen (Caesar et al., 2011; Lomin et al., 2011, 2018; Wulfetange et al., 2011; Hwang et al., 2012; Ding et al., 2017; Romanov et al., 2018; Kubiasová et al., 2020). Considering the subcellular localization of OsPUP1 in ER and expression in cells other than those of vascular tissues (**Figures 1B,D**), OsPUP1 might play a role in importing CKs from cytoplasm into ER, somewhat like the role of AtPUP14 for transporting CK from apoplast to cytoplasm (Zürcher et al., 2016), to regulate the CK pool for signal perception. If OsPUP1 imports CK into ER, overexpression of *OsPUP1* might increase the CK signal transduction. However, the signal extents reflected by the expression level of *OsRRs* were not markedly changed in the roots of *OsPUP1*-overexpressing seedling (**Figure 4**). One possibility is that the cells producing active CKs might not be the cells containing effective CK receptors. In this case, overexpression of *OsPUP1* restricts CKs out from the cells responsible for active CK synthesis to cells responsible for CK perception. Thus, the functions of OsPUP1 may depend on cells, tissues, actual developmental stages, as well as environmental conditions.

In rice, cZ-type CKs account for the largest proportion of CKs (**Supplementary Table 1**; Kudo et al., 2012; Kamada-Nobusada et al., 2013; Osugi and Sakakibara, 2015). It has been reported that cZ can induce CK-dependent responses (**Figure 4**; Kudo et al., 2012; Silva-Navas et al., 2019). In our study, the total content of CK nucleobases and nucleosides in the roots of *OsPUP1*-overexpressing plants were increased, mainly due to the increase of cZ and cZR (**Figure 3D**). However, the CK response was not markedly changed, as indicated by the expression of *OsRR* genes in the roots without CK treatment (**Figure 4**). In addition, the content of cZOG was significantly increased in both shoots and roots of *OsPUP1*-overexpressing seedlings, but has little change in the panicles (**Supplementary Table 1**). Since the physiological significance and homeostasis of cZ and its conjugated forms have not been fully elucidated so far, the reason underlying these intriguing observations remain unclear.

Although application of either of the iP, tZ or cZ in root is able to induce the CK response in the shoot, the extents are quite different, that is, the induction effect of iP is much lighter than

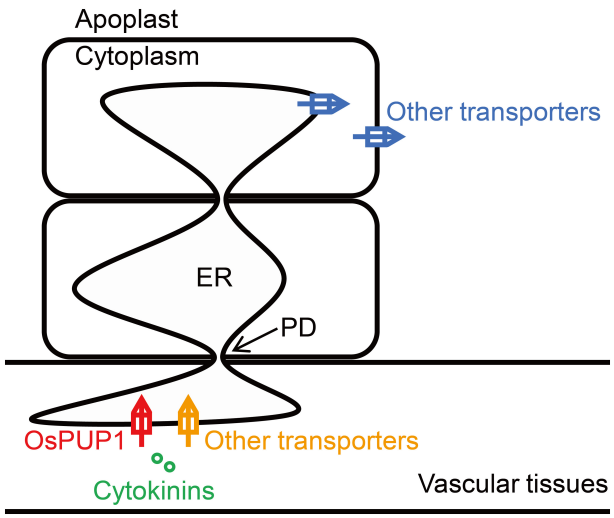


FIGURE 6 | Proposed working model for the function of OsPUP1 in CK transport system. OsPUP1 is localized to endoplasmic reticulum (ER) in the cells of vascular tissues, and might functions as an influx transporter together with other CK transporters in importing CKs from cytoplasm into ER, which might facilitate the cell-to-cell movement of CK through the plasmodesmata (PD).

those of tZ and cZ (**Figure 4**). These results imply that the root-to-shoot efficiency of the translocation of iP could be lower than those of tZ and cZ, which might result from the low recognition efficiency of iP or affinity of the responsible transporters for loading iP into the vascular tissues in the root. As the CK receptors in the shoots are suggested to be usually less sensitive to iP (Heyl et al., 2012; Lomin et al., 2012), another possibility is that CK receptors have a relatively low-affinity binding to iP in the shoots.

Overexpression of *OsPUP1* caused marked morphological alterations, while the knockout mutants showed no phenotypic change. Similar observation has been reported in our previous studies, showing that both the single and the double mutants of *OsPUP4* and *OsPUP7* are phenotypically silent (Xiao et al., 2019). Thus, there should be existed strong functional redundancy among PUP members in plant, which, on the other hand, implies the importance of the hormone transportation system (Zürcher et al., 2016). Further efforts uncovering this complicated system are significant for understanding hormone functions in plant growth and development. Given the crucial roles of the OsPUP1 as well as OsPUP4 and OsPUP7 in regulating several key agronomic traits, comprehension of the hormone transport certainly has a great potential for crop improvement as has been exemplified in a recent study (Yin et al., 2020).

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

AUTHOR CONTRIBUTIONS

YX performed most of the experiments with the assistance of JZ, GY, XL, WM, HD, GZ, GC, and HT. YX, HT, CC, and WT designed the study, analyzed the data, and wrote the manuscript. CC and WT conceived and supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (grants 31901528 and 31871599) and the Natural Science Foundation of Hunan Province (grant 2019JJ50243).

ACKNOWLEDGMENTS

We thank Shouyun Cao and Gupo Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for plant transformation and field management, Yangwen Qian (Biogle Co., Ltd.) for producing gene edited plants and plant transformation, and Yuqi Feng and Baodong Cai (Wuhan University) for CK measurements.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.618560/full#supplementary-material

Supplementary Figure 1 | Effect of different kinds of phytohormones on *OsPUP1* expression in shoots and roots of the wild-type seedlings.

Supplementary Figure 2 | Subcellular localization analyses of OsPUP1 in rice protoplasts.

Supplementary Figure 3 | Subcellular localization analyses of OsPUP1 in tobacco epidermal cells.

Supplementary Figure 4 | Comparison of the plant height, tiller number, and leaf length of wild-type and *OsPUP1*-overexpressing plants.

Supplementary Figure 5 | Panicle characteristics of *OsPUP1*-overexpressing plants.

Supplementary Figure 6 | Mutation information in the knockout mutants of *OsPUP1*.

Supplementary Figure 7 | Phylogenetic analysis of PUP family proteins in *Arabidopsis* (At), coffee (Cc), and rice (Os).

Supplementary Figure 8 | Gene expression analyses of OsPUP1 and BG3/OsPUP4.

Supplementary Table 1 | Quantification of various CK forms in shoots, roots, and panicles of the wild-type (WT) and *OsPUP1*-overexpressing plants (*OE2*).

Supplementary Table 2 | Primers used for analyses.

REFERENCES

Ashikari, M., Sakakibara, H., Lin, S. Y., Yamamoto, T., Takashi, T., Nishimura, A., et al. (2005). Cytokinin oxidase regulates rice grain production. *Science* 309, 741–745. doi: 10.1126/science.1113373

Barton, D. A., Cole, L., Collings, D. A., Liu, D. Y. T., Smith, P. M. C., Day, D. A., et al. (2011). Cell-to-cell transport via the lumen of the endoplasmic reticulum. *Plant J.* 66, 806–817. doi: 10.1111/j.1365-313X.2011.04545.x

Bishopp, A., Lehesranta, S., Vatén, A., Help, H., El-Showk, S., Scheres, B., et al. (2011). Phloem-transported cytokinin regulates polar auxin transport and maintains vascular pattern in the root meristem. *Curr. Biol.* 21, 927–932. doi: 10.1016/j.cub.2011.04.049

Bürkle, L., Cedzich, A., Döpke, C., Stransky, H., Okumoto, S., Gillissen, B., et al. (2003). Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of *Arabidopsis*. *Plant J.* 34, 13–26. doi: 10.1046/j.1365-313X.2003.01700.x

Caesar, K., Thamm, A. M., Witthöft, J., Elgass, K., Huppenberger, P., Grefen, C., et al. (2011). Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. *J. Exp. Bot.* 62, 5571–5580. doi: 10.1093/jxb/err238

Cai, B., Zhu, J., Gao, Q., Luo, D., Yuan, B., and Feng, Y. (2014). Rapid and high-throughput determination of endogenous cytokinins in *Oryza sativa* by bare Fe_3O_4 nanoparticles-based magnetic solid-phase extraction. *J. Chromatogr.* 1340, 146–150. doi: 10.1016/j.chroma.2014.03.030

Ding, W., Tong, H., Zheng, W., Ye, J., Pan, Z., Zhang, B., et al. (2017). Isolation, characterization and transcriptome analysis of a cytokinin receptor mutant *Osckt1* in rice. *Front. Plant Sci.* 8:88. doi: 10.3389/fpls.2017.00088

Galuszka, P., Frébort, I., Sebela, M., Sauer, P., Jacobsen, S., and Pec, P. (2001). Cytokinin oxidase or dehydrogenase? Mechanism of cytokinin degradation in cereals. *Eur. J. Biochem.* 268, 450–461. doi: 10.1046/j.1432-1033.2001.01910.x

Gao, S., Fang, J., Xu, F., Wang, W., Sun, X., Chu, J., et al. (2014). *CYTOKININ OXIDASE/DEHYDROGENASE4* integrates cytokinin and auxin signaling to control rice crown root formation. *Plant Physiol.* 165, 1035–1046. doi: 10.1104/pp.114.238584

Gao, S., Xiao, Y., Xu, F., Gao, X., Cao, S., Zhang, F., et al. (2019). Cytokinin-dependent regulatory module underlies the maintenance of zinc nutrition in rice. *New Phytol.* 224, 202–215. doi: 10.1111/nph.15962

Heyl, A., Riefler, M., Romanov, G. A., and Schmülling, T. (2012). Properties, functions and evolution of cytokinin receptors. *Eur. J. Cell Biol.* 91, 246–256. doi: 10.1016/j.ejcb.2011.02.009

Hirose, N., Makita, N., Yamaya, T., and Sakakibara, H. (2005). Functional characterization and expression analysis of a gene, *OsENT2*, encoding an equilibrative nucleoside transporter in rice suggest a function in cytokinin transport. *Plant Physiol.* 138, 196–206. doi: 10.1104/pp.105.060137

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., and Sakakibara, H. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *J. Exp. Bot.* 59, 75–83. doi: 10.1093/jxb/erm157

Hothorn, M., Dabi, T., and Chory, J. (2011). Structural basis for cytokinin recognition by *Arabidopsis thaliana* histidine kinase 4. *Nat. Chem. Biol.* 7, 766–768. doi: 10.1038/nchembio.667

Hutchison, C. E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M. W., et al. (2006). The *Arabidopsis* histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* 18, 3073–3087. doi: 10.1105/tpc.106.045674

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* 413, 383–389. doi: 10.1038/35096500

Hwang, I., Sheen, J., and Müller, B. (2012). Cytokinin signaling networks. *Annu. Rev. Plant Biol.* 63, 353–380. doi: 10.1146/annurev-arplant-042811-105503

Jefferson, R. A. (1989). The GUS reporter gene system. *Nature* 342, 837–838. doi: 10.1038/342837a0

Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci.* 8, 275–282. doi: 10.1093/bioinformatics/8.3.275

Kakegawa, H., Shitan, N., Kusano, H., Ogita, S., Yazaki, K., and Sugiyama, A. (2019). Uptake of adenine by purine permeases of *Coffea canephora*. *Biosci. Biotechnol. Biochem.* 83, 1300–1305. doi: 10.1080/09168451.2019.1606698

Kakimoto, T. (2001). Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. *Plant Cell Physiol.* 42, 677–685. doi: 10.1093/pcp/pce112

Kamada-Nobusada, T., Makita, N., Kojima, M., and Sakakibara, H. (2013). Nitrogen-dependent regulation of *de novo* cytokinin biosynthesis in rice: the role of glutamine metabolism as an additional signal. *Plant Cell Physiol.* 54, 1881–1893. doi: 10.1093/pcp/pct127

Kiba, T., Takei, K., Kojima, M., and Sakakibara, H. (2013). Side-chain modification of cytokinins controls shoot growth in *Arabidopsis*. *Dev. Cell* 27, 452–461. doi: 10.1016/j.devcel.2013.10.004

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:dev149344. doi: 10.1242/dev.149344

Ko, D., Kang, J., Kiba, T., Park, J., Kojima, M., Do, J., et al. (2014). *Arabidopsis* ABCG14 is essential for the root-to-shoot translocation of cytokinin. *Proc. Natl. Acad. Sci. U.S.A.* 111, 7150–7155. doi: 10.1073/pnas.1321519111

Kubiasová, K., Montesinos, J. C., Šamajová, O., Nisler, J., Mik, V., Semerádová, H., et al. (2020). Cytokinin fluoroprobe reveals multiple sites of cytokinin perception at plasma membrane and endoplasmic reticulum. *Nat. Commun.* 11:4285. doi: 10.1038/s41467-020-17949-0

Kudo, T., Makita, N., Kojima, M., Tokunaga, H., and Sakakibara, H. (2012). Cytokinin activity of *cis*-zeatin and phenotypic alterations induced by overexpression of putative *cis*-zeatin-*O*-glucosyltransferase in rice. *Plant Physiol.* 160, 319–331. doi: 10.1104/pp.112.196733

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). Mega X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., et al. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445, 652–655. doi: 10.1038/nature05504

Kuroha, T., Tokunaga, H., Kojima, M., Ueda, N., Ishida, T., Nagawa, S., et al. (2009). Functional analyses of *LONELY GUY* cytokinin-activating enzymes reveal the importance of the direct activation pathway in *Arabidopsis*. *Plant Cell* 21, 3152–3169. doi: 10.1105/tpc.109.068676

Li, S., Zhao, B., Yuan, D., Duan, M., Qian, Q., Tang, L., et al. (2013). Rice zinc finger protein DST enhances grain production through controlling *Gn1a/OsCKX2* expression. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3167–3172. doi: 10.1073/pnas.1300359110

Li, Y., Song, G., Gao, J., Zhang, S., Zhang, R., Li, W., et al. (2018). Enhancement of grain number per spike by RNA interference of cytokinin oxidase 2 gene in bread wheat. *Hereditas* 155:33. doi: 10.1186/s41065-018-0071-7

Liu, C. J., Zhao, Y., and Zhang, K. (2019). Cytokinin transporters: multisite players in cytokinin homeostasis and signal distribution. *Front. Plant Sci.* 10:693. doi: 10.3389/fpls.2019.00693

Liu, X., Bai, X., Wang, X., and Chu, C. (2007). OsWRKY71, a rice transcription factor, is involved in rice defense response. *J. Plant Physiol.* 164, 969–979. doi: 10.1016/j.jplph.2006.07.006

Lomin, S. N., Krivosheev, D. M., Steklov, M. Y., Arkhipov, D. V., Osolodkin, D. I., Schmülling, T., et al. (2015). Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. *J. Exp. Bot.* 66, 1851–1863. doi: 10.1093/jxb/eru522

Lomin, S. N., Krivosheev, D. M., Steklov, M. Y., Osolodkin, D. I., and Romanov, G. A. (2012). Receptor properties and features of cytokinin signaling. *Acta Naturae* 4, 31–45.

Lomin, S. N., Myakushina, Y. A., Arkhipov, D. V., Leonova, O. G., Popenko, V. I., Schmülling, T., et al. (2018). Studies of cytokinin receptor-phosphotransmitter interaction provide evidences for the initiation of cytokinin signalling in the endoplasmic reticulum. *Funct. Plant Biol.* 45, 192–202. doi: 10.1071/FP16292

Lomin, S. N., Yonekura-Sakakibara, K., Romanov, G. A., and Sakakibara, H. (2011). Ligand-binding properties and subcellular localization of maize cytokinin receptors. *J. Exp. Bot.* 62, 5149–5159. doi: 10.1093/jxb/err220

Lu, Y., Ye, X., Guo, R., Huang, J., Wang, W., Tang, J., et al. (2017). Genome-wide targeted mutagenesis in rice using the CRISPR/Cas9 system. *Mol. Plant* 10, 1242–1245. doi: 10.1016/j.molp.2017.06.007

Ma, J. F., Goto, S., Tamai, K., and Ichii, M. (2001). Role of root hairs and lateral roots in silicon uptake by rice. *Plant Physiol.* 127, 1773–1780. doi: 10.1104/pp.010271

Martin, R. C., Mok, M. C., and Mok, D. W. (1999a). A gene encoding the cytokinin enzyme zeatin *O*-xylosyltransferase of *Phaseolus vulgaris*. *Plant Physiol.* 120, 553–558. doi: 10.1104/pp.120.2.553

Martin, R. C., Mok, M. C., and Mok, D. W. (1999b). Isolation of a cytokinin gene, *ZOG1*, encoding zeatin *O*-glucosyltransferase from *Phaseolus lunatus*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 284–289. doi: 10.1073/pnas.96.1.284

Matsumoto-Kitano, M., Kusumoto, T., Tarkowski, P., Kinoshita-Tsujimura, K., Václavíková, K., Miyawaki, K., et al. (2008). Cytokinins are central regulators of cambial activity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20027–20031. doi: 10.1073/pnas.0805619105

Osugi, A., and Sakakibara, H. (2015). Q&A: how do plants respond to cytokinins and what is their importance? *BMC Biol.* 13:102. doi: 10.1186/s12915-015-0214-5

Qi, Z., and Xiong, L. (2013). Characterization of a purine permease family gene *OsPUP7* involved in growth and development control in rice. *J. Integr. Plant Biol.* 55, 1119–1135. doi: 10.1111/jipb.12101

Romanov, G. A., Lomin, S. N., and Schmülling, T. (2018). Cytokinin signaling: from the ER or from the PM? That is the question! *New Phytol.* 218, 41–53. doi: 10.1111/nph.14991

Sakai, H., Aoyama, T., and Oka, A. (2000). *Arabidopsis* ARR1 and ARR2 response regulators operate as transcriptional activators. *Plant J.* 24, 703–711. doi: 10.1046/j.1365-313x.2000.00909.x

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., et al. (2001). *ARR1*, a transcription factor for genes immediately responsive to cytokinins. *Science* 294, 1519–1521. doi: 10.1126/science.1065201

Sakakibara, H. (2006). Cytokinins: activity, biosynthesis, and translocation. *Annu. Rev. Plant Biol.* 57, 431–449. doi: 10.1146/annurev.arplant.57.032905.105231

Sasaki, T., Suzaki, T., Soyano, T., Kojima, M., Sakakibara, H., and Kawaguchi, M. (2014). Shoot-derived cytokinins systemically regulate root nodulation. *Nat. Commun.* 5:4983. doi: 10.1038/ncomms5983

Silva-Navas, J., Conesa, C. M., Saez, A., Navarro-Neila, S., Garcia-Mina, J. M., Zamarreño, A. M., et al. (2019). Role of *cis*-zeatin in root responses to phosphate starvation. *New Phytol.* 224, 242–257. doi: 10.1111/nph.16020

Šmehilová, M., Dobrùšková, J., Novák, O., Takáè, T., and Galuszka, P. (2016). Cytokinin-specific glycosyltransferases possess different roles in cytokinin homeostasis maintenance. *Front. Plant Sci.* 7:1264. doi: 10.3389/fpls.2016.01264

Sparkes, I. A., Runions, J., Kearns, A., and Hawes, C. (2006). Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc.* 1, 2019–2025. doi: 10.1038/nprot.2006.286

Sun, J., Hirose, N., Wang, X., Wen, P., Xue, L., Sakakibara, H., et al. (2005). *Arabidopsis SOI33/AtENT8* gene encodes a putative equilibrative nucleoside transporter that is involved in cytokinin transport *in planta*. J. Integr. Plant Biol. 47, 588–603. doi: 10.1111/j.1744-7909.2005.00104.x

Szydlowski, N., Bürkle, L., Pourcel, L., Moulin, M., Stolz, J., and Fitzpatrick, T. B. (2013). Recycling of pyridoxine (vitamin B6) by PUP1 in *Arabidopsis*. *Plant J.* 75, 40–52. doi: 10.1111/tpj.12195

Takei, K., Yamaya, T., and Sakakibara, H. (2004). *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. *J. Biol. Chem.* 279, 41866–41872. doi: 10.1074/jbc.M406337200

Tessi, T. M., Brumm, S., Winklbauer, E., Schumacher, B., Pettinari, G., Lescano, I., et al. (2020). *Arabidopsis* AZG2 transports cytokinins in *vivo* and regulates lateral root emergence. *New Phytol.* 66, 1851–1863. doi: 10.1111/nph.16943

Tsai, Y. C., Weir, N. R., Hill, K., Zhang, W. J., Kim, H. J., Shiu, S. H., et al. (2012). Characterization of genes involved in cytokinin signaling and metabolism from rice. *Plant Physiol.* 158, 1666–1684. doi: 10.1104/pp.111.192765

Werner, T., and Schmülling, T. (2009). Cytokinin action in plant development. *Curr. Opin. Plant Biol.* 12, 527–538. doi: 10.1016/j.pbi.2009.07.002

Wulfetange, K., Lomin, S. N., Romanov, G. A., Stolz, A., Heyl, A., and Schmülling, T. (2011). The cytokinin receptors of *Arabidopsis* are located mainly to the endoplasmic reticulum. *Plant Physiol.* 156, 1808–1818. doi: 10.1104/pp.111.180539

Xiao, Y., Liu, D., Zhang, G., Gao, S., Liu, L., Xu, F., et al. (2019). *Big Grain3*, encoding a purine permease, regulates grain size via modulating cytokinin transport in rice. *J. Integr. Plant Biol.* 61, 581–597. doi: 10.1111/jipb.12727

Yeh, S. Y., Chen, H. W., Ng, C. Y., Lin, C. Y., Tseng, T. H., Li, W. H., et al. (2015). Down-regulation of cytokinin oxidase 2 expression increases tiller number and improves rice yield. *Rice* 8:36. doi: 10.1186/s12284-015-0070-5

Yin, W., Xiao, Y., Niu, M., Meng, W., Li, L., Zhang, X., et al. (2020). ARGONAUTE2 enhances grain length and salt tolerance by activating *BIG GRAIN3* to modulate cytokinin distribution in rice. *Plant Cell* 32, 2292–2306. doi: 10.1105/tpc.19.00542

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T., and Sakakibara, H. (2004). Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to *cis*-zeatin. *Plant Physiol.* 134, 1654–1661. doi: 10.1104/pp.103.037176

Zhang, J., Liu, W., Yang, X., Gao, A., Li, X., Wu, X., et al. (2011). Isolation and characterization of two putative cytokinin oxidase genes related to grain number per spike phenotype in wheat. *Mol. Biol. Rep.* 38, 2337–2347. doi: 10.1007/s11033-010-0367-9

Zhang, K., Novak, O. R., Wei, Z., Gou, M., Zhang, X., Yu, Y., et al. (2014). *Arabidopsis* ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. *Nat. Commun.* 5:3274. doi: 10.1038/Ncomms4274

Zhang, L., Zhao, Y. L., Gao, L. F., Zhao, G. Y., Zhou, R. H., Zhang, B. S., et al. (2012). *TaCKX6-D1*, the ortholog of rice *OsCKX2*, is associated with grain weight in hexaploid wheat. *New Phytol.* 195, 574–584. doi: 10.1111/j.1469-8137.2012.04194.x

Zhang, W., Peng, K., Cui, F., Wang, D., Zhao, J., Zhang, Y., et al. (2020). Cytokinin oxidase/dehydrogenase OsCKX11 coordinates source and sink relationship in rice by simultaneous regulation of leaf senescence and grain number. *Plant Biotechnol. J.* doi: 10.1111/pbi.13467

Zhang, Y., Su, J., Duan, S., Ao, Y., Dai, J., Liu, J., et al. (2011). A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant Methods* 7:30. doi: 10.1186/1746-4811-7-30

Zhao, J., Yu, N., Ju, M., Fan, B., Zhang, Y., Zhu, E., et al. (2019). ABC transporter OsABCG18 controls the shootward transport of cytokinins and grain yield in rice. *J. Exp. Bot.* 70, 6277–6291. doi: 10.1093/jxb/erz382

Zürcher, E., Liu, J. C., di Donato, M., Geisler, M., and Müller, B. (2016). Plant development regulated by cytokinin sinks. *Science* 353, 1027–1030. doi: 10.1126/science.aaf7254

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 February 2021
doi: 10.3389/fpls.2020.608711

Light Regulates the Cytokinin-Dependent Cold Stress Responses in *Arabidopsis*

*Sylva Prerostova[1†], Martin Černý[2†], Petre I. Dobrev[1], Vaclav Motyka[1], Lucia Hluskova[1], Barbara Zupkova[1], Alena Gaudinova[1], Vojtech Knirsch[1], Tibor Janda[3], Bretislav Brzobohatý[2,4] and Radomira Vankova[1]**

[1] Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany, Czech Academy of Sciences, Prague, Czechia, [2] Department of Molecular Biology and Radiobiology, Faculty of AgriSciences, Mendel University in Brno, Brno, Czechia, [3] Department of Plant Physiology, Agricultural Institute, Centre for Agricultural Research, Martonvasar, Hungary, [4] CEITEC MENDELU: Central European Institute of Technology, Faculty of AgriSciences, Mendel University in Brno, Brno, Czechia

To elucidate the effect of light intensity on the cold response (5°C; 7 days) in *Arabidopsis thaliana*, we compared the following parameters under standard light (150 μmol m^{-2} s^{-1}), low light (20 μmol m^{-2} s^{-1}), and dark conditions: membrane damage, photosynthetic parameters, cytokinin oxidase/dehydrogenase (CKX) activity, phytohormone levels, and transcription of selected stress- and hormone-related genes and proteome. The impact of cytokinins (CKs), hormones directly interacting with the light signaling pathway, on cold responses was evaluated using transformants overexpressing CK biosynthetic gene isopentenyl transferase (*DEX:IPT*) or CK degradation gene *HvCKX2* (*DEX:CKX*) under a dexamethasone-inducible promoter. In wild-type plants, cold treatment under light conditions caused down-regulation of CKs (in shoots) and auxin, while abscisic acid (ABA), jasmonates, and salicylic acid (SA) were up-regulated, especially under low light. Cold treatment in the dark strongly suppressed all phytohormones, except ABA. *DEX:IPT* plants showed enhanced stress tolerance associated with elevated CK and SA levels in shoots and auxin in apices. Contrarily, *DEX:CKX* plants had weaker stress tolerance accompanied by lowered levels of CKs and auxins. Nevertheless, cold substantially diminished the impact from the inserted genes. Cold stress in dark minimized differences among the genotypes. Cold treatments in light strongly up-regulated stress marker genes *RD29A*, especially in roots, and *CBF1-3* in shoots. Under control conditions, their levels were higher in *DEX:CKX* plants, but after 7-day stress, *DEX:IPT* plants exhibited the highest transcription. Transcription of genes related to CK metabolism and signaling showed a tendency to re-establish, at least partially, CK homeostasis in both transformants. Up-regulation of strigolactone-related genes in apices and leaves indicated their role in suppressing shoot growth. The analysis of leaf proteome revealed over 20,000 peptides, representing 3,800 proteins and 2,212 protein families (data available via ProteomeXchange, identifier PXD020480). Cold stress induced proteins involved in ABA and jasmonate metabolism, antioxidant enzymes, and enzymes of flavonoid and glucosinolate biosynthesis. *DEX:IPT* plants

OPEN ACCESS

Edited by:
G. Eric Schaller, Dartmouth College, United States

Reviewed by:
Hsu-Liang Hsieh, National Taiwan University, Taiwan
Branka Salopek Sondi, Rudjer Boskovic Institute, Croatia

***Correspondence:**
Radomira Vankova
vankova@ueb.cas.cz

[†] *These authors share first authorship*

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *21 September 2020*
Accepted: *24 December 2020*
Published: *04 February 2021*

Citation:
Prerostova S, Černý M, Dobrev PI, Motyka V, Hluskova L, Zupkova B, Gaudinova A, Knirsch V, Janda T, Brzobohatý B and Vankova R (2021) Light Regulates the Cytokinin-Dependent Cold Stress Responses in Arabidopsis. Front. Plant Sci. 11:608711. doi: 10.3389/fpls.2020.608711

up-regulated phospholipase D and MAP-kinase 4. Cold stress response at the proteome level was similar in all genotypes under optimal light intensity, differing significantly under low light. The data characterized the decisive effect of light–CK cross-talk in the regulation of cold stress responses.

Keywords: acclimation, cold stress, cytokinin, cytokinin oxidase/dehydrogenase, isopentenyl transferase, karrikin, light intensity, phytohormone

INTRODUCTION

Light represents one of the most important environmental signals for plants. As a source of energy, it strongly affects plant growth and development. Plants sense light via photoreceptors and rapidly adapt their gene expression and metabolism to follow changing light availability (Cejudo et al., 2019).

Plant photosystems are uniquely adaptable to low light intensities (Demmig-Adams et al., 2018). Nevertheless, low light intensity diminishes the photosynthesis rate, which is associated with delayed cell division and organ development, and slows and prolongs entire developmental programs. Low light intensity also results in the gradual spontaneous deactivation of phytochrome B due to the lack of red light (Galvao and Fankhauser, 2015), which allows up-regulation of phytochrome-interacting factors (PIFs). These factors modulate auxin biosynthesis, transport, and signal transduction (de Wit et al., 2014). Simultaneously, gibberellins (GA), brassinosteroids, and ethylene are elevated under low light conditions. In leaves, auxin stimulates the expression of the main cytokinin (CK) degrading enzyme – cytokinin oxidase/dehydrogenase (CKX), which leads to down-regulation of CK levels (de Wit et al., 2014).

Low light intensity has usually been studied in combination with shading by neighboring plants (Ballare and Pierik, 2017). Apart from the reduction in overall light intensity, neighboring plants also reflect the far-red, thus reducing the red/far-red light ratio. This reduction accelerates a decrease of the active form of phytochrome B, leading to the subsequent activation of PIFs. The shade avoidance response stimulates the elongation of hypocotyl, internodes, and petioles as well as suppression of leaf growth (Yang and Li, 2017). Prolonged shade exposure leads to diminished branching, probably by elevating abscisic acid (ABA). The response to a low red/far-red light ratio, however, is distinguishable from the general effect of low light intensity (Yang and Li, 2017).

Energy deprivation during dark treatment arrests the growth of meristematic tissues and up-regulates starvation genes, especially in the shoot apex (Mohammed et al., 2018). Prolonged dark exposure results in G_1 being arrested irrespective of the sugar content. The auxin response is relatively high in shoot apices (Ulmasov et al., 1997), but its transporter PIN1 (pin-formed 1), is internalized, which negatively affects auxin transport toward the tips of leaf primordia and rib meristem, so preventing the formation of auxin maxima (Yoshida et al., 2011). Ethylene signal transduction [via transcription factor EIN3 (ethylene-insensitive 3)] is consistently elevated in the dark. The expression of several photosynthesis-related genes is down-regulated, e.g., marker genes for chloroplast biogenesis [GC1 (giant chloroplast 1) and ARC5 (accumulation and replication of chloroplast 5)] and vasculature-related genes. Photomorphogenesis-related transcripts, including transcription factor HY5 (elongated hypocotyl 5), are targeted by DET1 (de-etiolated 1) and COP1 (constitutive photomorphogenic 1) for degradation. Enzyme activities are affected by the redox state, the reduced forms being active in light, while inactivation by oxidation occurs in the dark (Cejudo et al., 2019). Enzyme reduction can be catalyzed in chloroplasts by ferredoxin-dependent thioredoxin reductases, which utilize the photosynthetic electron transport chain for reducing equivalents. Their function is associated with light. In contrast, NADPH-dependent thioredoxin reductases can use NADPH as a source of reducing power. In chloroplasts, some reductases have a joint domain (NtrC).

Cytokinins are among the plant hormones that have a close, positive link to light. They may have similar effects to light on plant growth and development (Miller et al., 1956). CKs can promote photomorphogenesis even in the absence of light, inhibiting chlorophyll degradation in the dark and stimulating chloroplast differentiation (Chory et al., 1994). CKs may up-regulate the expression of light-related genes in the absence of light (Cortleven and Schmulling, 2015). The CK signaling pathway also has an important function in the dark, when it maintains the levels of transcripts of several plastid genes, which enable plants to respond quickly to subsequent light (Doroshenko et al., 2016). However, the lack of hormone activation of plastid-encoded genes results in suppression of the chloroplast protein synthesis in the dark.

A sufficient light intensity is required for effective acclimation to cold stress (Szalai et al., 2009; Janda et al., 2014). Energy is necessary for activation of defense and synthesis of protective substances, especially polyamines and dehydrins. Hormonal signals may also be involved in regulation of the cold acclimation processes, as demonstrated in wheat plants (Majlath et al., 2012). However, the exact mechanisms are still poorly understood. The main questions addressed by the present study, using *Arabidopsis thaliana* as the model, are as follows: (i) How dependent on light intensity is the cold stress response? (ii) What is the role

Abbreviations: ABA, abscisic acid; C-D, cold and dark treatment; C-LL, cold and low light treatment; C-NL, cold and normal light treatment; CK, cytokinin; CKX, cytokinin oxidase/dehydrogenase; cZ, *cis*-zeatin; cZR, *cis*-zeatin riboside; cZRMP, *cis*-zeatin riboside monophosphate; DEX, dexamethasone; DMSO, dimethyl sulfoxide; F_v/F_m, maximum quantum yield of photosystem II; GA, gibberellin; IAA, indole-3-acetic acid; ICA, independent component analysis; iP, N^6-(Δ^2-isopentenyl)adenine; IPT, isopentenyl transferase; JA, jasmonic acid; MDA, malondialdehyde; NPQ_{Lss}, the steady-state non-photochemical quenching; QY_{Lss}, the steady-state PSII quantum yield; ROS, reactive oxygen species; SA, salicylic acid; tZ, *trans*-zeatin; tZR, *trans*-zeatin riboside; tZRMP, *trans*-zeatin riboside monophosphate; WT, wild-type.

of CKs in cold stress responses? (iii) Is it possible to affect the cold stress response in different light conditions by modulating the endogenous CK content?

To answer these questions, we examined the effect of optimal, low, and absent light intensity on membrane damage, photosynthetic activity, hormone levels, proteome, and the transcription profiles of selected stress- and hormone-related genes, using *Arabidopsis* plants. The impact of CK levels on the cold response was evaluated by comparing the reaction of wild-type (WT) plants to those of transformants with either enhanced CK levels [achieved by expression of the CK biosynthetic gene *IPT* (*isopentenyl transferase*) driven by the dexamethasone (DEX)-inducible promoter] or with down-regulated CK levels (using plants expressing *CKX* under the DEX-inducible promoter).

MATERIALS AND METHODS

Experimental Setup

Transformant lines used in this study originated from *A. thaliana* ecotype Columbia (Col-0): DEX-inducible lines *CaMV35S* > GR > *ipt* expressing *ipt* from *Agrobacterium tumefaciens* (pOp^{BK}-*ipt*; *DEX:IPT*; Craft et al., 2005) and *CaMV35S* > GR > *HvCKX2* expressing *HvCKX2* from *Hordeum vulgare* (*DEX:CKX*; Černý et al., 2013). Transformed and WT plants were cultivated in a climate chamber (Percival AR41-L2) at 20°C, 60% RH, 8/16 h light/dark, under the optimal light intensity of 150 μmol m^{-2} s^{-1} using an Araponics hydroponic system consisting of 1.7-L tanks filled with 1/4 Hoagland solution. The medium was aerated every 3 h and changed after 3 weeks of cultivation and at the beginning of the cold stress.

The DEX stock solution (20 mM) was prepared in dimethyl sulfoxide (DMSO). DEX (final concentration, 10 μM) or the corresponding amount of DMSO (850 μL) was added to the hydroponic solution to 26-day-old plants. In order to evaluate the impact of DMSO, some WT plants were grown in medium without DEX or DMSO. After 24 h, plants were incubated in a fresh, pre-cooled medium supplemented with DEX/DMSO. Plants were exposed for 7 days to the stress conditions described in **Table 1**: cold (5°C) combined with a normal light intensity of 150 μmol m^{-2} s^{-1} (C-NL), cold (5°C) with a low light intensity of 20 μmol m^{-2} s^{-1} (C-LL), or cold (5°C) and dark conditions (C-D). Control (DEX-treated as well as DMSO-treated) plants of all tested genotypes (WT, *DEX:IPT*, and *DEX:CKX*) were kept at 20°C under 150 μmol m^{-2} s^{-1}.

TABLE 1 | The specification of experimental conditions and the abbreviations of the respective experimental variants.

Abbreviation	Conditions	Photosynthetic photon flux density (μmol m^{-2} s^{-1})	Temperature (°C)
Control	Control conditions	150	20
C-NL	Cold at normal light intensity	150	5
C-LL	Cold at low light intensity	20	5
C-D	Cold at dark	0	5

Four-week-old plants were exposed for 7 days to the treatments.

Fresh mass of shoots and roots of all experimental variants is shown in **Supplementary Figure 1**. Data were obtained from the total mass of ca 60 plants grown in two hydroponics vessels within one experiment, divided by the number of collected plants. Three independent biological experiments were analyzed ($n = 3$).

Samples of developed leaves, roots, and the shoot apical meristem with the four youngest leaf primordia (apex) were collected, frozen in liquid nitrogen, and stored at −80°C. The three independent biological experiments were performed, and number of biological replicates is specified for each analysis.

Lipid Peroxidation

Lipid peroxidation was determined in frozen leaves as malondialdehyde (MDA) content by the thiobarbituric acid reactive substances (TBARS) method. The MDA level was used as a general marker of oxidative damage to cell membranes. The spectrophotometric measurement was performed at 532 nm, and the absorbance of interfering compounds was taken into consideration (Hodges et al., 1999). The calculation was done according to Landi (2017).

Chlorophyll Fluorescence

Developed leaves were cut and dark adapted for 15 min at the same temperature as the respective experimental variant. After adaptation, chlorophyll fluorescence was measured using a Handy FluorCam FC 1000-H (PSI). The Kautsky characteristic was analyzed in Pulse-Amplitude-Modulated Mode (40 s dark relaxation after the saturating pulse followed by 10 min duration of actinic light 200 μmol m^{-2} s^{-1} with 15 light flashes). The maximum quantum yield of photosystem II in the dark-adapted state (F_v/F_m) and the steady-state PSII quantum yield in the light (QY_{Lss}) were calculated according to Genty et al. (1989). The steady-state non-photochemical quenching in the light (NPQ_{Lss}) was measured according to Horton and Ruban (1992).

Activity of CK Oxidase/Dehydrogenase Enzyme

The CKX was extracted from frozen developed leaves and partially purified according to Motyka and Kaminek (1992, 1994), as modified in Motyka et al. (2003). The enzyme activity was determined by *in vitro* radioisotope assays based on the conversion of tritium-labeled N^6-(Δ^2-isopentenyl)adenine (iP) (prepared by the Isotope Laboratory, Institute of Experimental Botany of the Czech Academy of Sciences, Prague, Czech Republic) to adenine. The assay mixture (final volume 50 μL) comprised 100 mM TAPS-NaOH buffer containing 75 μM 2,6-dichloroindophenol (pH 8.5), 2 μM substrate ($^2H_3$-iP), and enzyme preparation equivalent to 16.6 or 50 mg of tissue FW. After incubation (1 h at 37°C), the reaction was terminated by adding 10 μL of Na_4EDTA (200 mM) and 120 μL of 95% (v/v) ethanol. The substrate was separated from the product of the enzyme reaction by HPLC, as described in Gaudinova et al. (2005).

Phytohormone Analysis

Phytohormones were extracted from frozen samples (ca 10 mg FW) by 100 µl 50% acetonitrile solution in water. Isotope labeled standards (10 pmol/sample) were added to samples: $^{13}C_6$-IAA, $^{2}H_4$-OxIAA, and $^{2}H_4$-OxIAA-GE (Cambridge Isotope Laboratories); $^{2}H_4$-SA and $^{2}H_2$-GA_{19} (Sigma-Aldrich); $^{2}H_3$-PA and $^{2}H_3$-DPA (NRC-PBI); and $^{2}H_6$-ABA, $^{2}H_5$-JA, $^{2}H_5$-tZ, $^{2}H_5$-tZR, $^{2}H_5$-tZRMP, $^{2}H_5$-tZ7G, $^{2}H_5$-tZ9G, $^{2}H_5$-tZOG, $^{2}H_5$-tZROG, $^{15}N_4$-cZ, $^{2}H_3$-DZ, $^{2}H_3$-DZR, $^{2}H_3$-DZ9G, $^{2}H_3$-DZRMP, $^{2}H_7$-DZOG, $^{2}H_6$-iP, $^{2}H_6$-iPR, $^{2}H_6$-iP7G, $^{2}H_6$-iP9G, and $^{2}H_6$-iPRMP (Olchemim). Samples were homogenized with zirconia beads (1.5 mm diameter) in FastPrep-24^{TM} 5G Instrument (MP Biomedicals) for 40 s at 6 m/s. The extracts were centrifuged at 4°C, 30,000 *g*. The supernatant was applied to the SPE Oasis HLB 96-well column plate (10 mg/well; Waters) activated with 100 µL of methanol and eluted with 100 µL of 50% acetonitrile using a Pressure+ 96 manifold (Biotage). The sediment was re-extracted in 100 µL of 50% acetonitrile, centrifuged, and applied again to the column plate.

Phytohormones from the eluate were separated on a Kinetex EVO C_{18} column (2.6 µm, 150 × 2.1 mm, Phenomenex). Mobile phases consisted of A – 5 mM ammonium acetate and 2 µM medronic acid in water, and B – 95:5 acetonitrile:water (v/v). The following gradient program was applied: 5% B in 0 min, 7% B in 0.1 to 5 min, 10 to 35% in 5.1 to 12 min, 100% B in 13 to 14 min, and 5% B in 14.1 min. Hormone analysis was performed with a LC/MS system consisting of UHPLC 1290 Infinity II (Agilent) coupled to a 6495 Triple Quadrupole Mass Spectrometer (Agilent). MS analysis was done in MRM mode, using the isotope dilution method. Data acquisition and processing were performed with Mass Hunter software B.08 (Agilent).

RT-qPCR

Frozen samples (up to 100 mg) were homogenized with zirconia beads in a cooled ball mill MM301 (Retsch) for 150 s at 25 Hz. RNA was isolated using RNeasy Plant Mini Kit (Qiagen). Samples were DNased using DNase I recombinant (Roche Applied Science). Total mRNA was converted to complementary DNA (cDNA) using M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant, Promega), oligo-dT primers, and Protector RNase Inhibitor (Roche Applied Science). Final cDNA was diluted 20-fold with RNase-free water, and 2.5 µL of the solution was mixed with 5 µL of GoTaq qPCR Master Mix (Promega) and specific primers (see **Supplementary Table 1**) to a final volume of 10 µl. The PCR program (primer denaturation: 10 s at 95°C; annealing and elongation: 30 s at 60°C) was performed by Light Cycler 480 (Roche Applied Science). Ubiquitin UBQ10 was selected as the reference gene with stable transcription in all treatments, genotypes, and tissues, which is in accordance with the Genevestigator database (Hruz et al., 2008). The relative content of RNA was calculated by the ddCt method (Livak and Schmittgen, 2001).

Proteomic Analysis

Proteins were analyzed in mixed leaf samples in three independent biological replicates. Total protein extracts were prepared as described previously (e.g., Hloušková et al., 2019). Digested and desalted peptides were analyzed by nanoflow C_{18} chromatography using a 15-cm Zorbax nanocolumn (0.1 mm inner diameter; Agilent) and a Dionex Ultimate 3000 RSLC nano-UHPLC system (Thermo). The LC was directly coupled to the Nanospray Flex (1,700 V, ion transfer tube temperature 250°C) and the Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo). Peptides were eluted with a 60-min, 4 to 40% acetonitrile gradient. Spectra were acquired using the default settings for peptide identification in data-dependent mode with a cycle time of 3 s. MS: resolution 60,000, scan range 375–1,500 m/z, maximum allowed injection time 50 ms, automatic gain control 4e5, 1 microscan; MS_2: resolution 15,000, automatic gain control 5e4, maximum injection time 30 ms, 1 microscan; quadrupole isolation, HCD activation (30% Collision energy), the dynamic exclusion for 60 s (5 ppm tolerance). The measured spectra were recalibrated and searched against the reference Arabidopsis Araport 11 protein database by Proteome Discoverer 2.4, employing Sequest HT or MS Amanda 2.0 with the following parameters: Enzyme – trypsin, max two missed cleavage sites; Modifications – up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, *N*-terminal acetylation, *N*-terminal Met-loss; MS_1 tolerance – 5 ppm; MS_2 tolerance – 0.02 Da (MS Amanda), 0.1 Da (Sequest). Only proteins with at least two unique peptides were considered for the quantitative analysis. The quantitative differences were determined by Minora, employing precursor ion quantification followed by normalization and a background-based *t* test. Interactions and functional clusters were evaluated by String (Szklarczyk et al., 2019). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD020480.

Statistical Analysis

The effect of DMSO and DEX was evaluated by Student's *t* test in the case of MDA, CKX, activity, and chlorophyll fluorescence measurement. The evaluation of proteome data was performed by statistical tests, which were generated using Instant Clue (Nolte et al., 2018), the Real Statistics Resource[1] Pack software for MS Excel (Release 6.8; Copyright 2013–2020; Charles Zaiontz), Rapid Miner[2] (Mierswa et al., 2006), and Proteome Discoverer. The data from other analyses were evaluated using Prism 8 (GraphPad). The independent component analyses (ICA) were performed by OriginPro 2020b (OriginLab).

RESULTS

The present study focused on elucidation of the impact of different light intensities on cold stress responses (**Table 1**). The applied stresses imposed significant effects on fresh mass of shoots and roots in WT, *DEX:IPT*, as well as *DEX:CKX* plants (**Supplementary Figure 1**). Under control conditions, the activation of *ipt* had negative effect on the root growth,

[1]www.real-statistics.com

[2]www.rapidminer.com

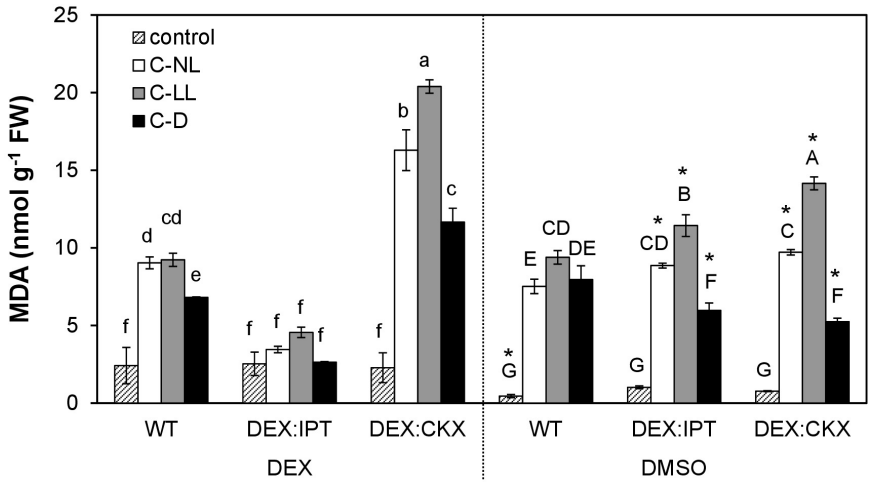


FIGURE 1 | Lipid peroxidation expressed as malondialdehyde (MDA) content in leaves of wild-type (WT) and transformants *DEX:IPT* and *DEX:CKX*. See **Table 1** for the description of experimental conditions. Plants were either activated with dexamethasone (DEX; diluted in DMSO) or treated with DMSO only. Means ± SD are shown. Biological samples from three independent experiments were analyzed ($n = 3$). The differences between DEX and DMSO treatments within each experimental variant were evaluated by Student's *t* test (significant differences at $p < 0.05$ are indicated with an asterisk). The comparison among all experimental variants within the DEX (lowercase letters) or DMSO (capital letters) treatments was evaluated by one-way ANOVA with Tukey's post hoc test ($p < 0.05$).

while activation of *HvCKX2* negatively affected shoot growth and positively root growth. Under stress conditions, the impact of the inserted genes was diminished. The comparison of phenotypes after 7-day stress treatments is shown in **Supplementary Figure 2**.

Lipid Peroxidation Induced by Cold Stress Was Diminished by CKs

The stress effect of cold treatment at different light intensities was estimated as peroxidation of membrane lipids via MDA determination (**Figure 1**). A significant negative effect on membrane structure was observed under C-NL conditions in WT with almost a 20-fold increase in MDA. The stress impact was even more pronounced under C-LL conditions when MDA was elevated to ca 23 times that of the control. In contrast, under C-D conditions, the cold stress impact, although present, was rather lower than under C-NL and C-LL. These data are from plants treated with DMSO, but these results did not significantly differ from those of plants grown in medium without DEX or DMSO (data not shown).

The impact of CK content on stress tolerance was tested using transformants with elevated CK levels (DEX-inducible transformant with CK biosynthetic gene *ipt* – *DEX:IPT* plants) or with diminished CK levels (DEX-inducible transformant with gene coding for CK degradation enzyme *HvCKX2* – *DEX:CKX* plants). Comparison of MDA levels in WT as well as in the non-induced transformants with plants exhibiting DEX-stimulated CK biosynthesis (*DEX:IPT*) and profound CK degradation (*DEX:CKX*) allowed evaluation of CK effect at different light intensities. DEX slightly increased the membrane damage in WT under control conditions. *DEX:IPT* transformants with activated expression of the CK biosynthetic gene showed no significant impact of cold on membrane damage. By contrast, the DEX-activated transformant with enhanced CK degradation (*DEX:CKX*) exhibited a much higher level of MDA than all the other variants. In summary, higher levels of CKs suppressed the negative effect of cold stress, while down-regulation of CK content promoted membrane damage. Membrane lipid peroxidation also depended on light intensity.

Distinct Effect of Dark on Photosynthetic Parameters in Comparison With Light at a Wide Range of Intensities

Under the experimental conditions employed here, cold had little effect on photosynthetic parameters characterizing the photosystem II state and activity, the maximum quantum yield (F_v/F_m), the actual quantum yield (QY_{Lss}), and the non-photochemical quenching (NPQ_{Lss}) in steady state (**Table 2**). Only minor differences were observed among the tested genotypes. Mild but statistically significant inhibition of maximum quantum yield (F_v/F_m) was detected under C-NL conditions, especially in *DEX:IPT* plants. At the same time, the NPQ_{Lss} was suppressed (to similar extent in all genotypes). With the C-LL treatment, F_v/F_m and QY_{Lss} did not generally differ from control conditions. C-D stress conditions resulted in significant down-regulation of F_v/F_m and QY_{Lss} in all genotypes. This treatment led to the highest increase of NPQ_{Lss}, especially in the case of *DEX:CKX* plants.

Phytohormones

CK Down-Regulation by Cold Correlates With Diminished Light Intensity

In WT, the highly active CK *trans*-zeatin (tZ) and its riboside (tZR) were lowered in apices and leaves in the following order of treatments: C-NL > C-LL > C-D (**Figure 2** and **Supplementary Table 2**). The content of the precursor *trans*-zeatin riboside

TABLE 2 | Photosynthetic parameters.

	DEX						DMSO					
	WT		**DEX:IPT**		**DEX:CKX**		**WT**		**DEX:IPT**		**DEX:CKX**	
F_v/F_m												
Control	0.850 ± 0.001	a	0.847 ± 0.005	ab	0.838 ± 0.004	ab	0.846 ± 0.005	A	0.849 ± 0.004	A	0.847 ± 0.005	A*
C-NL	0.837 ± 0.005	bc	0.823 ± 0.009	d	0.828 ± 0.005	cd	0.839 ± 0.007	A	0.821 ± 0.015	B	0.822 ± 0.008	B
C-LL	0.848 ± 0.004	ab	0.845 ± 0.005	ab	0.842 ± 0.008	ab	0.853 ± 0.005	A	0.840 ± 0.007	A	0.847 ± 0.005	A
C-D	0.820 ± 0.009	d	0.809 ± 0.007	e	0.805 ± 0.008	e	0.814 ± 0.011	B	0.810 ± 0.011	BC	0.798 ± 0.015	C
QY_{Lss}												
Control	0.521 ± 0.027	bc	0.599 ± 0.016	a	0.562 ± 0.022	ab	0.601 ± 0.009	A*	0.563 ± 0.043	ABC	0.548 ± 0.056	ABC
C-NL	0.604 ± 0.026	a	0.563 ± 0.031	ab	0.578 ± 0.040	ab	0.597 ± 0.025	AB	0.523 ± 0.061	BC	0.551 ± 0.054	ABC
C-LL	0.607 ± 0.014	a	0.560 ± 0.026	ab	0.582 ± 0.008	ab	0.577 ± 0.036	ABC	0.582 ± 0.032	AB	0.591 ± 0.027	AB
C-D	0.481 ± 0.043	c	0.474 ± 0.055	c	0.466 ± 0.039	c	0.499 ± 0.071	C	0.520 ± 0.053	ABC	0.504 ± 0.050	C
NPQ_{Lss}												
Control	1.199 ± 0.188	abc	0.817 ± 0.070	cde	0.802 ± 0.117	cde	0.823 ± 0.099	BC*	1.044 ± 0.186	ABC*	1.113 ± 0.268	AB*
C-NL	0.701 ± 0.046	e	0.611 ± 0.124	e	0.649 ± 0.114	e	0.629 ± 0.055	C*	0.769 ± 0.130	BC*	0.691 ± 0.119	BC
C-LL	0.785 ± 0.052	de	0.857 ± 0.154	bcde	0.750 ± 0.088	e	0.869 ± 0.136	BC	0.792 ± 0.193	BC	0.777 ± 0.087	BC
C-D	1.391 ± 0.320	a	1.162 ± 0.345	abcd	1.210 ± 0.334	ab	1.308 ± 0.541	A	0.947 ± 0.310	ABC	1.028 ± 0.241	ABC

*The maximum quantum yield of photosystem II in the dark-adapted state (F_v/F_m), the steady-state quantum yield of photosystem II in the light (QY_{Lss}), and the non-photochemical quenching in steady state (NPQ_{Lss}) of photosystem II in developed leaves of selected genotypes exposed to experimental conditions. See **Table 1** for the description of experimental variants and **Figure 1** for statistics. Six independent leaves were evaluated (n = 6).*

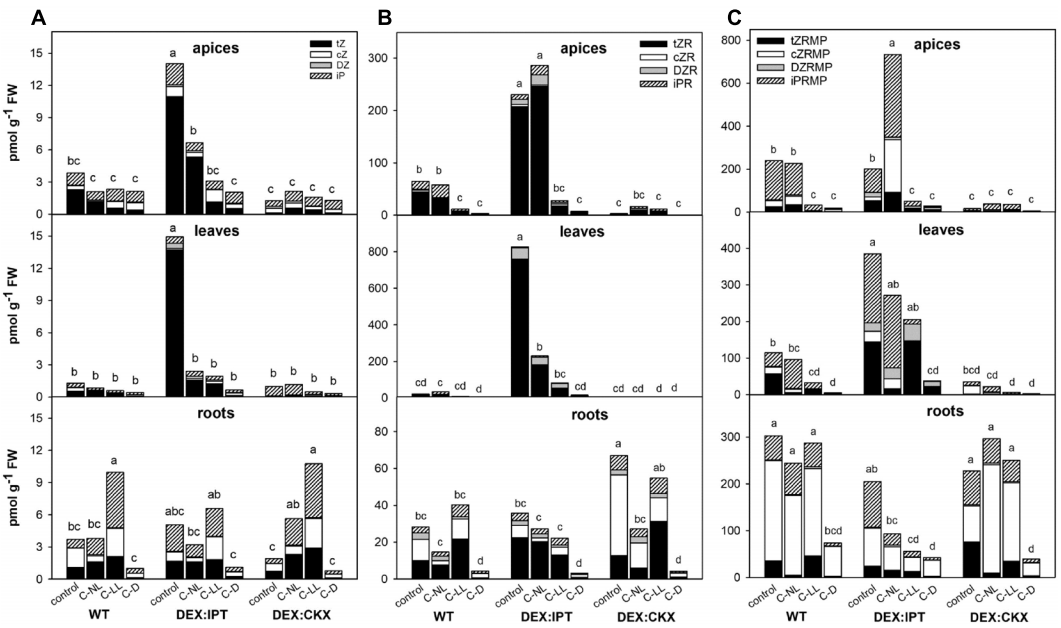


FIGURE 2 | The levels of **(A)** CK bases [*trans*-zeatin (tZ), *cis*-zeatin (cZ), dihydrozeatin (DZ), and N^6-(Δ^2-isopentenyl)adenine (iP)]; **(B)** CK ribosides [*trans*-zeatin riboside (tZR), *cis*-zeatin riboside (cZR), dihydrozeatin riboside (DZR), and N^6-(Δ^2-isopentenyl)adenosine (iPR)]; and **(C)** CK phosphates [*trans*-zeatin riboside monophosphate (tZRMP), *cis*-zeatin riboside monophosphate (cZRMP), dihydrozeatin riboside monophosphate (DZRMP), and N^6-(Δ^2-isopentenyl)adenosine monophosphate (iPRMP)] in apices, leaves, and roots of wild-type (WT) and transformants with high CK synthesis after activation by dexamethasone (*DEX:IPT*), and high CK degradation after activation by dexamethasone (*DEX:CKX*) exposed to cold treatments under different light conditions (see **Table 1** for the description). The expression of the transgenes was activated by dexamethasone (DEX). The statistical analyses of the total CK bases/ribosides/phosphates content were performed among all experimental variants within each tissue by one-way ANOVA with Tukey's post hoc test in four independent biological replicates (significant differences at $p < 0.05$, $n = 4$, are indicated by different letters). Means ± SD and detailed statistics of individual CKs are shown in **Supplementary Table 2**.

monophosphate (tZRMP) was preserved in apices under C-NL. In contrast, the levels of tZ and tZR were slightly up-regulated in roots under C-NL and especially C-LL. Under the latter treatment, we observed iP in roots to be highly elevated. The levels of the stress-related CKs *cis*-zeatin (cZ), its riboside (cZR), and phosphate (cZRMP) were high in roots of control plants and

those under C-LL. The C-D treatment resulted in low levels of all CKs in all tissues (with the exception of CK bases in apices).

Under control conditions, stimulation of the inserted genes had a profound effect on the levels of CKs. *DEX:IPT* transformant produced very high amounts of tZ, tZR, and tZRMP in apices, leaves, and, to a lower extent, roots as well (**Figure 2** and **Supplementary Table 2**). The strong up-regulation of tZ content was partially compensated by stimulation of deactivation mechanisms such as the formation of CK *N*- and *O*-glucosides (**Supplementary Table 2**), as well as enhancement of CKX activity (see **Figure 5** hereafter). Activated *DEX:CKX* transformant exhibited low levels of tZ in all tissues and those of tZR and tZRMP in apices and leaves (**Figure 2** and **Supplementary Table 2**). The CK decrease caused by activated *HvCKX2* expression was compensated by an up-regulated synthesis of the precursor tZRMP and cZR in roots, by suppressed CK deactivation via glucosylation as well as by down-regulation of endogenous *CKX* expression (**Figures 2**, as well as see **Figure 6** and **Supplementary Table 2**). Due to a large amount of data, only values determined in DEX-treated plants are shown.

In *DEX:IPT*, CK repression by cold was much more profound than in WT, but the levels of tZ and tZR remained under C-NL, and under C-LL in the case of leaves, higher than in WT plants grown under control conditions. C-NL led to an increase of the levels of the precursor N^6-(Δ^2-isopentenyl)adenosine monophosphate, especially in the case of apices (**Figure 2** and **Supplementary Table 2**). The content of cZ was enhanced under C-LL in apices and roots, and that of cZRMP was enhanced only in apices (**Figure 2** and **Supplementary Table 2**). In *DEX:CKX* plants, the C-NL treatment had a slightly positive impact on CK content (tZ, iP, and cZRMP) in comparison with control conditions; even the CK *O*-glucosylation producing storage forms was supported (**Figure 2** and **Supplementary Table 2**). C-D treatment maintained low levels of all CKs in all tissues regardless of the genotype.

Auxins Were Down-Regulated by Cold Under Normal Light Conditions

The C-NL treatment suppressed the production of auxin indole-3-acetic acid (IAA) in WT plants (**Figure 3** and **Supplementary Table 2**). C-LL only had a minor effect on IAA levels. In contrast, strong IAA suppression was observed under C-D conditions. Under C-NL treatment, IAA was predominantly converted to the reversible storage form (its glucose ester) in all tissues, under C-LL only in roots (**Supplementary Table 2**). Under C-D conditions, IAA was partially deactivated to the irreversible amino acid conjugates, IAA-aspartate and IAA-glutamate (**Supplementary Table 2**).

In comparison with WT, *DEX:IPT* plants exhibited high IAA elevation in apices and also, but to a lesser extent, in roots (**Figure 3**). In this genotype, IAA under control conditions was actively converted to its major primary catabolite 2-ox-indole-3-acetic acid in roots (**Supplementary Table 2**). In comparison with the other genotypes, *DEX:CKX* plants had, under control conditions, lower IAA content in leaves and especially in roots.

Cold treatments had similar effects on *DEX:IPT* plants as on WT, leading to a significant decrease of IAA under C-NL, while only a minor effect was found under C-LL. However, under C-D, relatively high IAA levels were detected in apices. *DEX:CKX* plants maintained the same IAA concentration under cold treatments as under control conditions, with the exception of a mild elevation under C-LL, especially in roots.

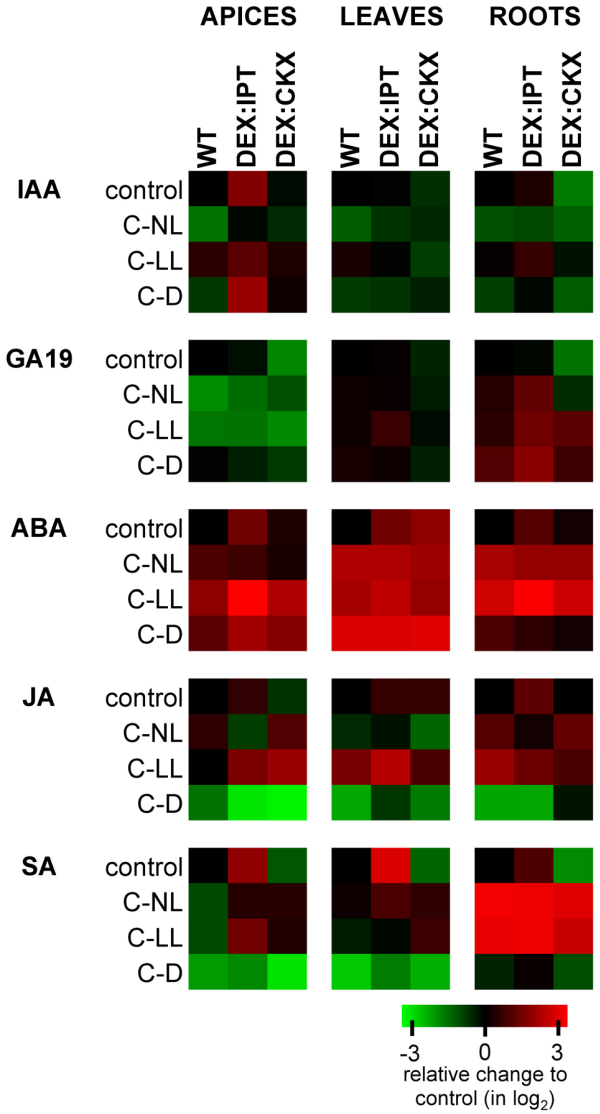


FIGURE 3 | The relative levels of phytohormones in apices, leaves, and roots of WT, *DEX:IPT*, and *DEX:CKX* plants expressed as a $\log_2$ change of the ratio between the genotype under individual treatments and the WT under control conditions within each tissue separately. The expression of the transgenes was activated by dexamethasone (DEX). See **Table 1** for the description of experimental variants. ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid; GA19, gibberellin GA_{19}; IAA, auxin indole-3-acetic acid. The means ± SD and detailed statistics of hormones are shown in **Supplementary Table 2**.

Gibberellins Were Affected Predominantly in Apices

Suppression of the GA precursor GA_{19} indicated down-regulation of GAs in the cold at two levels of illumination (C-NL

and C-LL) in apices of WT as well as *DEX:IPT* (**Figure 3** and **Supplementary Table 2**). Up-regulation of GA_{19} was detected in roots of the latter genotype in all cold treatments, especially in the dark (C-D). Under control conditions, down-regulation of CKs in *DEX:CKX* plants led to a decrease of the levels of GA_{19} in apices and leaves. During cold treatments, this genotype exhibited levels of GAs similar to those in WT.

ABA Was Up-Regulated by all Cold Treatments

After 7-day treatment under C-NL, only moderate ABA elevation was observed in WT apices (**Figure 3** and **Supplementary Table 2**). In leaves and roots, ABA was significantly elevated. Under C-LL, ABA increased substantially in all WT tissues. Under C-D, ABA increased especially in leaves.

In control conditions, *DEX:IPT* plants had basal levels of ABA ca 2.5 times higher than WT in all tissues, while in *DEX:CKX* plants, this only occurred in leaves. In comparison with WT, ABA increase was prominent in all *DEX:IPT* tissues under C-LL. Under C-D, all genotypes exhibited similar high ABA up-regulation in leaves. The ABA catabolites – phaseic acid, dihydrophaseic acid, and 9-hydroxy-abscisic acid (**Supplementary Table 2**) – remained at low levels in apices and leaves.

Jasmonates Were Stimulated by Cold at Low Light Conditions

Jasmonic acid (JA) and its active isoleucine conjugate were moderately up-regulated under C-NL in WT roots and highly up-regulated under C-LL in leaves and roots (**Figure 3** and **Supplementary Table 2**). Under C-D conditions, the contents of JA and its isoleucine conjugate were strongly suppressed in all tissues. *DEX:IPT* plants had higher JA basal levels in all tissues, while *DEX:CKX* had lower levels in apices. Under C-D, *DEX:IPT* plants exhibited a strong JA down-regulation in roots, similarly to WT.

Salicylic Acid Content Correlated With CKs

Both cold treatments with light caused a moderate decrease of salicylic acid (SA) levels in apices, a negligible effect in leaves, and a strong positive effect in roots (**Figure 3** and **Supplementary Table 2**). SA content correlated well with CK levels in all organs. Under control conditions, the *DEX:IPT* genotype contained high SA levels in all tissues, while in *DEX:CKX*, they were lower. Both C-NL and C-LL treatments enhanced the synthesis of SA in roots to similar levels in all genotypes and, to a minor extent, also in *DEX:IPT* and *DEX:CKX* apices and leaves. The C-D treatment was associated with very low SA content in all tissues of all genotypes.

Phytohormone Changes in Summary

Independent component analyses of total phytohormones in apices and leaves (**Figure 4A**) has clearly shown the divergence of the *DEX:IPT* genotype from the other two genotypes. The response of this transformant to cold stress treatments was the most distinct from control conditions. In general, C-NL stressed plants differed from both C-LL- and C-D-treated plants at the hormonal levels in all tissues (separation on IC2 axis). C-D-treated plants showed the same behavior regardless of genotype. The *DEX:CKX* genotype exhibited a phytohormone profile similar to dark-treated plants, except in the roots, which indicates the intensive stress response of this tissue.

CK Oxidase/Dehydrogenase Activity Increased in Both Transformants and Cold Treatments in Light

Under C-NL, WT exhibited a strong up-regulation of CKX activity in leaves (**Figure 5**). Under C-LL conditions, CKX activity was close to the control levels. Low CK content under C-D was associated with very low CKX activity.

Cytokinin oxidase/dehydrogenase activity in transformants treated with DMSO did not differ significantly from WT plants. After DEX treatment, CKX activity was substantially elevated in leaves of *DEX:IPT* plants grown in control conditions, probably due to the plant efforts to re-establish CK homeostasis. CKX activity also increased, but to a lower extent, in *DEX:CKX* plants, because of the activation of *HvCKX2* expression. Under C-NL conditions, an intense stimulation of CKX activity was detected, slightly above the levels of DEX-activated *DEX:IPT* transformant under control conditions. Under C-LL conditions CKX activity was only moderately stimulated, and to a similar degree in all genotypes. CKX activity under C-D was very low in all genotypes, although slightly higher in *DEX:CKX*.

Gene Transcription

Transformant Characterization

The expression of both introduced genes (*ipt* from *A. tumefaciens* and *HvCKX2* from *H. vulgare*) was, after activation with DEX, verified by RT-qPCR (**Figure 6** and **Supplementary Table 3**; data from DMSO treatment not shown). The transcription of *ipt* in *DEX:IPT* plants did not change with experimental conditions except in apices under C-D conditions. In contrast, the activated *DEX:CKX* plants exhibited high transcription of *HvCKX2* under control conditions, which was significantly down-regulated by all cold treatments, especially in the dark.

Light Intensity Affected Cold Responsiveness of Stress-Related Genes

The transcription profiles of stress-related genes are shown in **Figure 6** and **Supplementary Table 3**. Under control conditions, the transcription of *RD29A* (*responsive to desiccation 29A*) was most profound in *DEX:CKX* plants, less so in *DEX:IPT*. However, C-NL stress up-regulated *RD29A* transcription after 7 days to the highest extent in the apices and roots of *DEX:IPT* plants. The C-LL treatment substantially elevated *RD29A* transcription, less so in *DEX:CKX* apices and leaves. A very high increase of its transcription was detected in roots of all genotypes. Under C-D, the most profound transcription was found in *DEX:IPT* leaves. The transcription of *COR47* (*cold-regulated 47*) had, under control conditions, a similar profile to that of *RD29A*. Under C-NL, the stimulation of *COR47* transcription was most profound in WT apices and leaves, while, compared with the control, it was enhanced only in roots of *DEX:IPT* where it reached the level of WT. The most substantial *COR47*

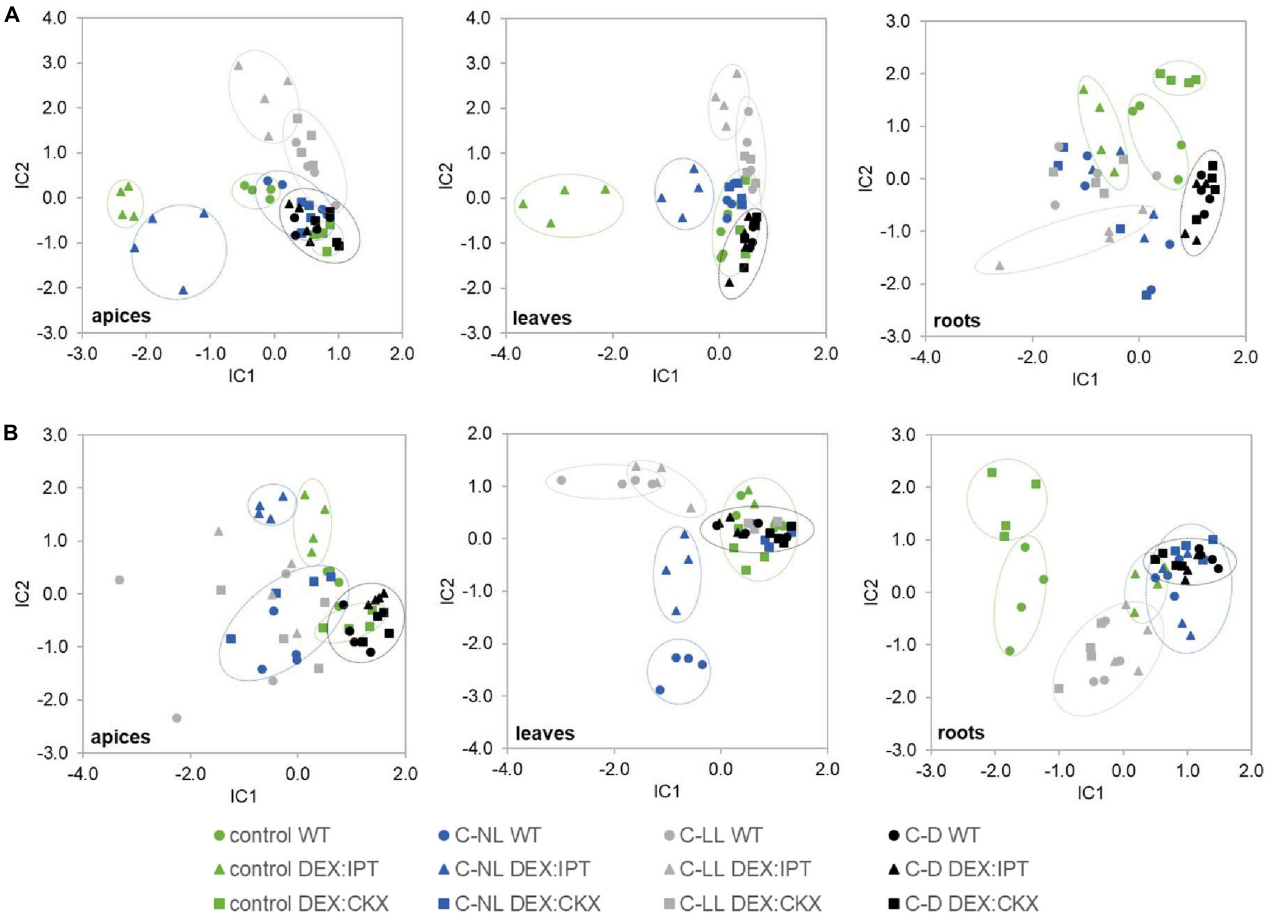


FIGURE 4 | The independent component analysis (ICA) of **(A)** hormonal and **(B)** transcriptomic data in apices, leaves, and roots of WT, *DEX:IPT*, and *DEX:CKX* plants exposed to cold stress under different light conditions (see **Table 1** for the description of variants). All samples are shown as individual points (four biological replicates of each variant). Groups with high diversity are highlighted by circles. In the case of transcriptomic data, the inserted genes were excluded from the analysis.

up-regulation was found in the C-LL-treated plants, especially in WT plants. Under C-D, *COR47* transcription was also up-regulated, predominantly in the case of *DEX:CKX* apices and *DEX:IPT* leaves, where it was more than double that under C-LL conditions.

The key transcription factors associated with cold stress are C-repeat binding factors (CBFs). The transcription of *CBF1*, *2*, and *3* was up-regulated by all cold treatments in apices and leaves, substantially in the case of C-NL, except in *DEX:CKX* leaves. *CBF1* transcription was also considerably enhanced under C-D, especially in leaves of *DEX:IPT*. In roots, the expression was generally low.

Elongated hypocotyl 5 (HY5) is the hub, light-related transcription factor, which coordinates light and hormone signaling pathways. Under control conditions, *HY5* transcription was relatively low in *DEX:IPT* plants in comparison with other genotypes. All 7-day cold treatments (especially C-D) diminished its transcription, with the exception of apices and leaves of *DEX:IPT* plants under C-NL, when it was promoted in comparison with *DEX:IPT* control.

Low light intensity responses are associated with PIFs. In apices, *PIF3* transcription was significantly down-regulated after prolonged C-D treatment. In leaves, both C-NL and C-LL conditions resulted in the up-regulation of *PIF3* and *4* in WT and *DEX:IPT* plants. In roots, C-NL was accompanied by *PIF3* down-regulation, while *PIF4* was highly transcribed under C-D conditions, but to a lesser extent in *DEX:CKX* plants.

Hormone-Related Genes

The transcription changes of hormone-related genes in DEX-treated plants are shown in **Figure 6** and **Supplementary Table 3**.

Cytokinins

The basal transcription of the CK biosynthetic gene, *IPT3*, was strongly suppressed in *DEX:IPT* plants, while it was highly promoted in the *DEX:CKX* transformant. Similarly, transcription of another CK biosynthetic gene, *IPT5*, was stimulated in *DEX:CKX* leaves and roots. C-NL conditions reduced *IPT3* transcription in *DEX:CKX* plants, which remained, however, higher in apices and leaves than in WT plants. By contrast, the

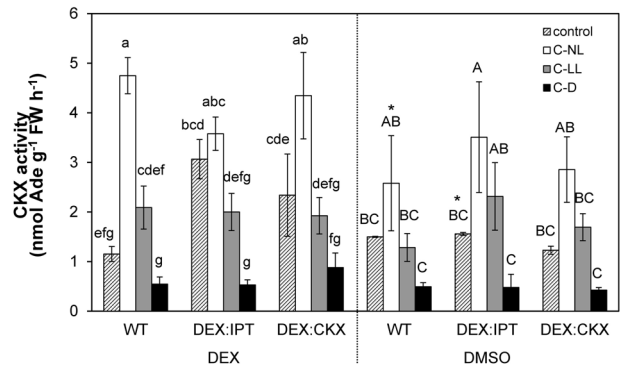


FIGURE 5 | The activity of cytokinin oxidase/dehydrogenase (CKX) enzyme in leaves of WT, *DEX:IPT*, and *DEX:CKX* plants exposed to cold treatments under different light conditions. Plants were activated by dexamethasone diluted in DMSO (DEX) or treated by pure DMSO. See **Table 1** for the description of experimental variants and **Figure 1** for statistics (n = 3).

transcription in *DEX:IPT* roots under C-NL was up-regulated compared with the control. The *IPT3* transcription was higher in apices of all genotypes under C-LL conditions than under C-NL, while under C-D, it was very low, close to the detection limit. By contrast, *IPT5* was substantially up-regulated in apices and leaves, especially in *DEX:CKX*.

Opposite to *IPTs*, transcription of the gene for the CK degradation enzyme, *CKX5*, was, under control conditions, elevated in apices and leaves of *DEX:IPT* and strongly diminished in apices and roots of *DEX:CKX*. It was also generally enhanced in *DEX:IPT* plants exposed to cold treatments. C-NL promoted *CKX5* transcription in WT leaves but diminished it in apices and roots. C-D conditions were associated with the lowest abundance of *CKX5*.

The CK signal transduction via type-B *ARR10* (*Arabidopsis response regulator 10*) was promoted in control conditions especially in roots of *DEX:CKX* plants. C-NL and C-LL diminished this up-regulation, resulting in transcription levels comparable with the other genotypes. C-D suppressed *ARR10* transcription in all tissues of all genotypes. The transcription profiles of type-A response regulators *ARR4*, *5*, and *7* were similar. Under control conditions, the expression correlated with CK levels in apices and leaves. C-NL treatment inhibited the transcription of these regulators in all tissues, except for *DEX:IPT* apices and leaves, which maintained high transcript levels. In the case of C-LL, the up-regulation of type-A response regulators was found in apices and leaves of WT and *DEX:IPT*, while it was kept low in the *DEX:CKX* transformant. C-D resulted in low transcription of these regulators, being the highest in the case of leaves of the *DEX:IPT* genotype.

The transcription levels of *CRF3* and *4* (*cytokinin response factors*) were generally lower in *DEX:CKX* plants in comparison with other genotypes. The most significant response was up-regulated transcription under C-NL in apices and leaves (although there was no response in *DEX:CKX* leaves) and down-regulation under C-D. In roots, all stress treatments were associated with *CRF3* and *4* suppression. *CRF3* exhibited a more profound response under C-NL, while *CRF4* transcription was most prevalent under C-LL.

Abscisic acid

The transcription level of the gene coding the rate-limiting ABA biosynthetic enzyme *NCED3* (*9-cis-epoxycarotenoid dioxygenase 3*) was highest under control conditions in apices and leaves of *DEX:CKX* plants. A mildly increased level was also detected in apices of the *DEX:IPT* transformant. The most prominent change in leaves was an increase in *NCED3* in WT under C-NL, while in *DEX:CKX* plants, *NCED3* transcription was down-regulated. In roots, C-LL and C-D treatments elevated its transcription in *DEX:CKX* plants. The transcription of the ABA receptor *PYL6* (*PYR-like 6*) was diminished in the *DEX:IPT* genotype under control conditions, but it was elevated in apices and leaves under both C-NL and C-LL conditions. Darkness repressed *PYL6* transcription since low *PYL6* levels were detected in roots under C-D stress.

Gibberellins

Transcription of the gene for the GA deactivating enzyme *GA2ox1* (*gibberellin 2 oxidase 1*) was low in the case of apices and leaves of *DEX:CKX* plants under control conditions. Cold treatments inhibited its transcription in these tissues in all genotypes (except C-NL treatment in *DEX:IPT* apices). In roots, *GA2ox1* was down-regulated only in the dark (C-D). Under control conditions, transcription of DELLA proteins *RGA1*, *RGA2* (*repressors of GA*), and *RGL3* (*RGA-like 3*) was much lower in apices of *DEX:CKX* plants than in the other genotypes. By contrast, *DELLAs* were less transcribed in *DEX:IPT* roots. C-NL conditions elevated *RGA1* and *2* transcription in *DEX:CKX* apices to the levels of the other genotypes. Significant elevation of *RGA1* transcription was found in leaves of WT and *DEX:IPT* plants under C-LL. C-D diminished the transcription of *RGA1* and *2* in all tissues of all genotypes, except roots of the *DEX:CKX* transformant, which maintained high transcription even in the dark. *RGL3* exhibited up-regulation under all stress conditions in apices of all genotypes (except in *DEX:IPT* under C-D). In leaves, significant elevation was observed under C-D in WT and *DEX:IPT* and in the latter genotype also under C-LL.

Strigolactones

Transcription of strigolactone receptor *MAX2* (*more axillary branches 2*) exhibited up-regulation under C-NL and C-LL in apices of all genotypes. Its transcription was also enhanced in leaves of *DEX:IPT* under all cold treatments (because of low basal transcription under control conditions). In roots, a sharp *MAX2* down-regulation was observed under C-NL. Strigolactone co-receptor *D14* (*dwarf 14*) transcription was also diminished in *DEX:IPT* under control conditions. It was increased under C-LL only in WT apices and leaves and, to a lesser extent, in *DEX:IPT* leaves. Transcription of strigolactone repressor *SMXL6* (*SMAX1-like 6*) was high under C-LL in leaves of *DEX:IPT* plants and in roots of all genotypes. C-D conditions generally down-regulated *SMXL6* transcription. As *MAX2* may function also as a karrikin receptor, transcription of karrikin co-receptor *KAI2* (*karrikin insensitive 2*) and karrikin repressor *SMAX1* (*suppressor of MAX2 1*) was also followed. Significant up-regulation of *KAI2*

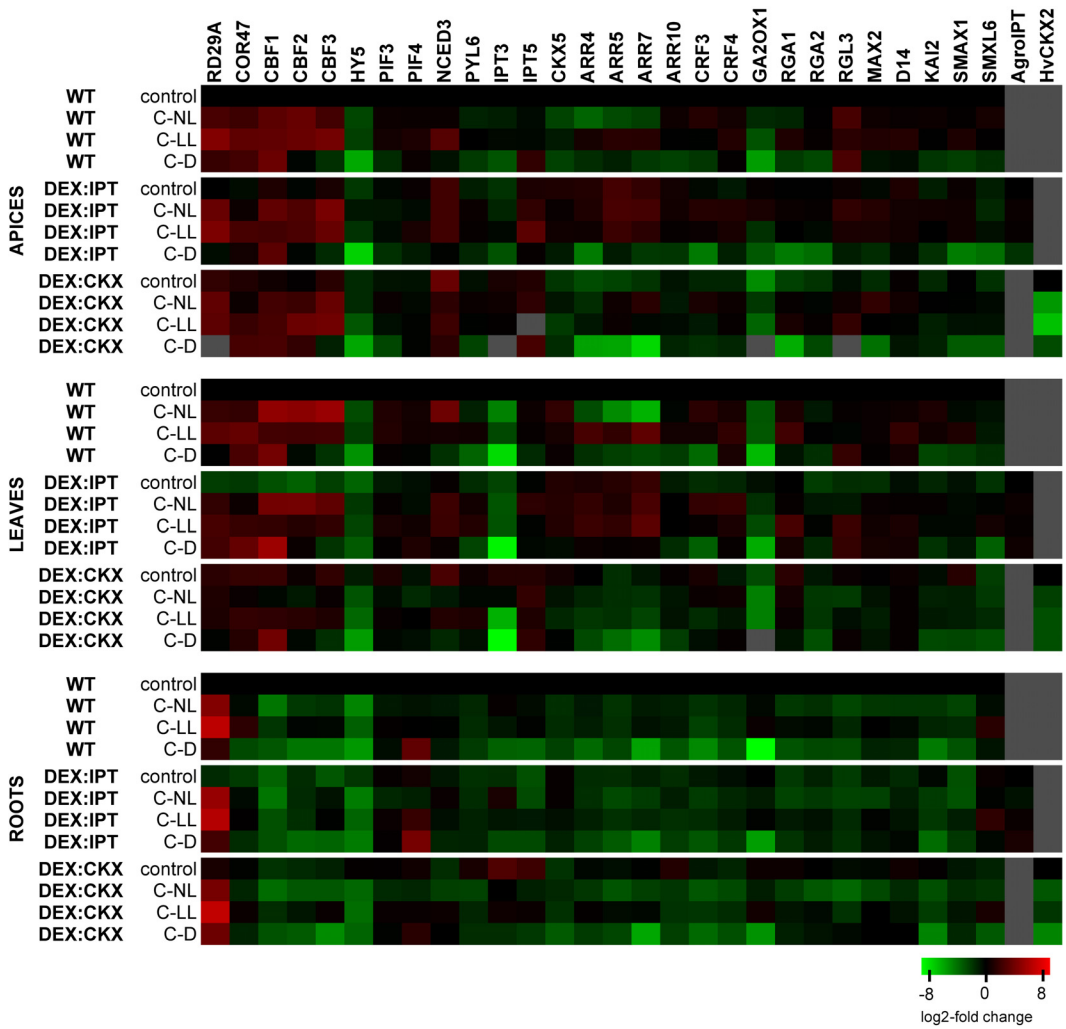


FIGURE 6 | The transcription changes of selected genes related to WT in control conditions within each tissue separately. Means ± SD and detailed statistics of gene transcription are shown in **Supplementary Table 3**. Gray squares indicate the transcription level to be below the detection limit. The expression of the transgenes was activated by dexamethasone (DEX).

was found in apices and leaves of *DEX:IPT* plants exposed to C-NL and C-LL. By contrast, all stress treatments suppressed its transcription in roots, especially under C-D. *SMAX1* had very low transcript levels in apices of the C-D-treated plants. Moderate elevation was found under C-LL in WT leaves and in *DEX:IPT* roots.

Transcriptome Changes in Summary

Independent component analyses of transcriptomic changes (**Figure 4B**) revealed that the changes between cold stress under normal light (C-NL) and low light (C-LL) conditions were similar in apices, indicating the protective efforts of plants in maintaining growth processes in this tissue. By contrast, in leaves, the responses to both cold treatments under light clearly separated, especially in the case of WT. In the case of *DEX:CKX* plants, the changes in gene transcription were similar regardless of conditions. Under cold stress the transcriptomic changes in roots were distinct from those of control plants; however, the response under C-LL still differed from the other two stress treatments. The only exception was the *DEX:IPT* transformant, which resembled C-NL and C-D plants in showing distinct responses under control conditions in comparison with WT and *DEX:CKX* plants. As in the case of phytohormones, all genotypes in all tissues reacted similarly to cold in the dark (C-D).

Proteome

Proteome Cold Stress Response in *Arabidopsis*

Analysis of C-NL conditions on WT revealed an accumulation of 202 and depletion of 35 proteins. The set of cold-induced proteins contained 41 well-known stress-responsive proteins, including enzymes of the ABA-biosynthetic pathway: zeaxanthin epoxidase (AT5G67030) and phytoene desaturase (AT4G14210); reactive oxygen species (ROS) metabolism

(e.g., peroxidase PRXR1, AT4G21960); and chloroplastic enzymes of carbohydrate metabolism, e.g., phosphoglucomutase (AT5G51820), α-glucan water dikinase 1 (AT1G10760), or chalcone synthase (AT5G13930).

Low light intensity and darkness significantly altered the WT cold response at the proteome level. Of 237 cold-responsive proteins under C-NL, only 57 and 41 showed a similar trend under C-LL and C-D treatments, respectively. The functional enrichment analyses revealed mutual enrichment in the abiotic stress response, biosynthesis of secondary metabolites, amino acids, photosynthesis, and ribosomes. In contrast, carotenoid and flavonoid biosynthetic pathways were enriched only under C-NL, whereas a higher proportion of ROS metabolism, JA metabolism, and glucosinolate biosynthesis were found only under C-LL or C-D. Statistically significant changes were found for 173 proteins, and trends of most of these (94) were similar for C-LL and C-D treatments. The set of proteins specifically responsive under C-LL included enzymes of ROS metabolism, ascorbate peroxidase (AT4G35000, accumulated), and peroxidases (AT4G21960, AT4G33420, depleted); JA biosynthesis (allene oxide cyclase 2, AT3G25770, accumulated); and tryptophan and histidine biosynthesis (AT4G26900, AT1G07780, accumulated). The C-D-specific response included depletion of nine stress-responsive proteins, an increase in the ubiquitin–proteasome pathway (UBC8, AT5G41700; UBC35, AT1G78870; proteasome subunit AT1G79210), or protein required for vesicular transport (AT3G56190).

Modulation of CK Content Affected Proteome

The analysis of total leaf proteome allowed us to identify a total of over 20,000 peptides, representing more than 3,800 proteins and 2,212 protein families. DEX-induced expression of *ipt* and *HvCKX2* under control conditions resulted in a significant change of 232 proteins. A CK-dependent protein accumulation was found for 31 proteins, including mitochondrial chaperone ClpB4 (casein lytic proteinase B4, AT2G25140, negative response to CK); lipid transfer protein LPT5 (AT3G51600, negative); chloroplastic hydroxyacyl-glutathione hydrolase 2 (AT1G06130, negative); and alcohol dehydrogenase (AT1G77120, negative). A similar response of both transformants (*DEX:IPT* and *DEX:CKX*) was found for 24 proteins, including EIN2 interacting protein AT4G24800 (accumulated); a stress-induced protein KIN2 (kinase 2, AT5G15970, accumulated); amidase 1 (AT1G08980, depleted); and a splicing factor subunit AT1G14650 (depleted).

The functional enrichment analysis showed that of 112 proteins differentially abundant only in the *DEX:IPT* genotype, the most numerous categories were enzymes of amino acid biosynthesis (10 proteins), citrate cycle (6 enzymes), and ribosomal proteins (12 proteins). A notably significant depletion was found for a protein of thermotolerance TIL1 (temperature-induced lipocalin 1, AT5G58070), and enzymes spermidine synthase (AT1G70310) and arginase (AT4G08870), indicating a decrease in polyamine biosynthesis. Important proteins accumulated in the *DEX:IPT* transformant were phospholipase D (AT3G15730), MAP-kinase 4 (AT4G01370), and a mitochondrial protein prohibitin-3 (AT5G40770) that is required for the ethylene-mediated signaling (Christians and Larsen, 2007).

The *DEX:CKX* line-specific changes were only found for 65 proteins: emphasizing cold-regulated protein (AT2G42540, depleted); JA biosynthetic enzyme 12-oxophytodienoate reductase (AT2G06050, accumulated); sulfite oxidase (AT3G01910, accumulated); and histone H4 (AT5G59970, accumulated).

Cold Stress Response of Proteome Under Optimal Light Intensity Was Similar Among Genotypes

When the cold-responsive proteins were characterized in plants with a modulated CK pool, some of the observed changes were not statistically significant within the set of replicates, but of 237 cold-responsive proteins, 178 and 180 proteins showed a similar trend to that seen in the leaves of activated *DEX:IPT* and *DEX:CKX* plants, respectively. In accord with that observation, only 38 proteins that showed no cold response in WT were significantly changed in *DEX:IPT* and *DEX:CKX* plants under C-NL stress. The high similarity between plants with a modulated CK pool and WT plants under C-NL is demonstrated by ICA (**Figure 7**). The following enzymes were significantly accumulated in *DEX:IPT* and *DEX:CKX* plants under cold stress: FtsH protease (AT1G50250); tryptophan-tRNA ligase (AT3G04600); potassium/sodium channel protein (AT1G51100); and enzyme releasing ABA from its conjugate BGLU18 (β-glucosidase 18, AT1G52400).

Proteins accumulated specifically in leaves of cold-stressed *DEX:IPT* plants included glutathione S-transferase L3 (AT5G02790) and peptidyl-prolyl *cis-trans* isomerase CYP19-4 (AT2G29960). Photoreceptor phototropin-2 (AT5G58140) was significantly depleted, in contrast to control conditions, when it was in *DEX:IPT* plants substantially higher than in WT.

The set of differentially abundant proteins in cold-stressed *DEX:CKX* plants included a protein involved in programmed cell death NUDT7 (nudix hydrolase homolog 7, AT4G12720), a positive regulator of abiotic stress tolerance and ABA target lipoxygenase PLAT1 (AT4G39730), and a thioredoxin-family protein (AT4G29670).

The Impact of Low Light Intensity on Proteome During Cold Stress Response

In total, 188 proteins that were not found to be cold-responsive in WT in our test conditions were significantly changed in the *DEX:IPT* plants. The comparison of the sets from C-LL and C-D showed a higher overlap of the cold-accumulated proteins, with ca 27 and 16% shared accumulated and depleted proteins, respectively. The mutual C-LL and C-D accumulated proteins in *DEX:IPT* included ribosomal proteins; hydroxyacyl-glutathione hydrolase (AT1G06130); an enzyme with a putative role in sucrose mobilization (FRUCT4, fructosidase 4, AT1G12240); NADPH-dependent thioredoxin reductase (NTRB, AT4G35460); pectin acetylesterase 11 (AT5G45280); and methyltransferase FIB2 (fibrillarin 2, AT4G25630). In *DEX:IPT* plants, the set of depleted proteins common to both C-LL and C-D treatments contained phospholipase D (AT3G15730) and glucan endo-1,3-β-glucosidase. C-LL alone resulted in accumulation of

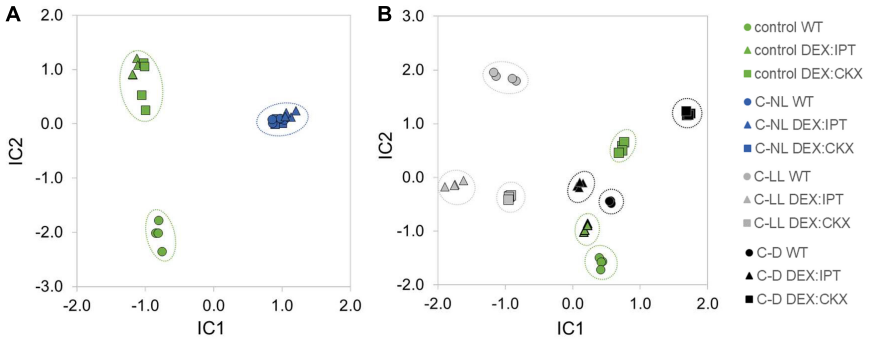


FIGURE 7 | The independent component analysis (ICA) of proteome profiles in leaves of WT, *DEX:IPT*, and *DEX:CKX* plants. **(A)** Cold stress response at standard light intensity (C-NL) masks the effect of altered cytokinin pool. Results represent the quantitative analysis of 2,067 proteins in four replicates. **(B)** The response to cold stress under low light (C-LL) and dark (C-D) conditions in comparison with plants grown under control conditions. The ICA separates light intensity and cytokinin effects in the IC1 and IC2 axes, respectively. The results represent the quantitative analysis of 1,950 proteins in four replicates.

citric acid cycle enzymes (AT5G40650, AT1G54220); 14-3-3 protein (AT2G42590); actin 1 (AT2G37620); and magnesium-chelatase subunit (AT5G45930). The set of *DEX:IPT* proteins depleted under C-LL conditions included NAD-dependent malic enzyme (AT4G00570); a negative regulator of ABA signaling nodulin-related protein 1 (AT2G03440); a subunit of DNA-directed RNA polymerase (ATCG00740); and chaperones and ribosomal proteins. Dark-specific (C-D) proteome changes in the *DEX:IPT* transformant included an accumulation of histone H2B.11 (AT5G59910); enzymes of amino acid biosynthesis and glutathione metabolism; an increase in HSP 90-6 (AT3G07770); and protein calnexin (AT5G61790) that may participate in glycoprotein assembly. Three citric acid cycle components were depleted in *DEX:IPT* under C-D: a decrease was found in the case of HSP70-14 (AT1G79930) and its interacting protein HIP1 (AT4G22670); SGT1 protein (AT4G11260) required for degradation of auxin-responsive proteins; plastid lipid-associated proteins (AT3G26070, AT3G58010, AT3G23400); proteasome subunits; and ribosomal proteins.

In total, 154 proteins that were not found to be cold-responsive in WT under C-LL or C-D were significantly changed in those plants overexpressing *HvCKX2*. The comparison of the sets of C-LL- and C-D-affected proteins in *DEX:CKX* showed a limited overlap of 16 and 14 accumulated and depleted proteins, respectively. These included auxin biosynthetic enzyme AMI1 (amidase 1, AT1G08980, accumulated); cold-regulated protein COR15A (AT2G42540, accumulated); importin subunit (AT1G09270, accumulated); ABA receptor PYL1 (AT5G46790, depleted); and growth-limiting enzyme of guanine biosynthesis (AT1G16350, depleted). The positive response in the *DEX:CKX* transformant under C-LL was found in the case of ribosomal proteins; enzymes of carbohydrate metabolism; mannose-binding lectin (AT3G16470); chalcone synthase (AT5G13930); ABA biosynthesis enzyme zeaxanthin epoxidase (AT5G67030); histone H3-like (AT5G65350); malate dehydrogenase (AT5G43330); and HHL1 (hypersensitive to high light 1, AT1G67700). Negative responses of *DEX:CKX* plants under C-LL were found for two enzymes involved in aromatic amino acid biosynthesis (AT5G38530, AT1G48850); receptor AT4G23170 similar to NPR1 (positive regulator of SA signaling); two enzymes of sulfur metabolism (AT1G79230, AT3G01910); glutathione-*S*-transferase (AT1G78380); and thioredoxin reductase 2 (AT2G17420). The cold-stress-specific response of *DEX:CKX* plants under C-D included an accumulation of lipid peroxidation protectant lipocalin (AT3G47860); enzymes of fatty acid biosynthesis (AT1G65290, AT5G10160); HSP70-15 protein (AT1G79920); actin-depolymerizing factor 3 (AT5G59880); and tubulin (AT5G19780). In contrast, tubulin (AT1G20010) was depleted under C-D; a decrease was found also for multiple ribosomal proteins, enzymes of porphyrin and chlorophyll metabolism (AT4G03205, AT4G03205), thioredoxin X (AT1G50320), and a protein involved in multivesicular body formation CHMP1A (charged multivesicular body protein/chromatin modifying protein 1A, AT1G73030).

Proteome Changes in Summary

The modulation of CK pool had a significant impact on cold stress-responsive proteins in leaves of all tested genotypes. The ICA separated light intensity and CK effects in the IC1 and IC2, respectively (**Figure 7**). WT plants showed the most distinct responses to stresses at the proteome level. In contrast to hormonome and transcription analyses, proteome data clustered all genotypes grown under C-NL stress together. More differences were found under C-LL conditions, especially when transformants were compared with WT. C-D and control conditions resembled each other (as in the case of hormonome and transcriptome). C-D had a much higher impact on *DEX:CKX* plants than on the other two genotypes; however, its proteome was likewise shifted also under control conditions.

DISCUSSION

The Impact of Light Intensity on Cold Stress Responses

The first goal of this study was to investigate the effect of different light intensities on cold stress responses in *A. thaliana* WT. The impact of cold stress under normal light (C-NL,

150 μmol m^{-2} s^{-1}), low light (C-LL, 20 μmol m^{-2} s^{-1}), and dark (C-D, 0 μmol m^{-2} s^{-1}) was evaluated after 1-week exposure to reveal long-term stress responses. The comparison of phenotypes is shown in **Supplementary Figure 2**. At standard light intensity (C-NL), apices showed preferential protection against cold stress, exhibiting milder reaction in comparison with leaves. At low light intensity (C-LL), similar stress responses were observed in these tissues. Dark in combination with cold stress (C-D) arrested plant growth.

Cold Stress Under Normal Light Conditions

Cold stress under normal light conditions resulted in significant membrane damage and moderate photosynthesis suppression, but plants were simultaneously stimulated to form protective compounds (e.g., flavonoids and chaperones), including antioxidant enzymes (peroxidases) (**Figure 1**, **Table 2**). The observed negative effect of cold stress on photosynthesis agrees with reports of Janda et al. (2014) and Prinzenberg et al. (2020). This cold stress response was associated with significant transcriptome changes (**Figure 6**) including the transcription of stress-related genes *RD29A*, *COR47*, and *CBF1–3*. The transcription of *CRF3* and *4* was induced by C-NL in above-ground tissues but suppressed in roots, which is in accordance with Zwack et al. (2016), who reported their cold induction and positive function in freezing tolerance. The transcription of *PIFs* was enhanced in leaves (but diminished in roots), which conforms with their function in cold stress responses as a part of CBFs-PIF3-phyB regulatory module (Jiang et al., 2020). Under cold stress, CBFs interact with PIF3 to facilitate the degradation of PIF1, PIF4, and PIF5, which repress transcription of COR genes (Xu and Deng, 2020).

Prolonged cold was associated with down-regulation of bZIP transcription factor *HY5*, especially in roots. This is in apparent contradiction with the fact that transcription of *HY5* is both cold- and light-inducible. However, the reason may be *HY5* dynamics, as its maximum has been reported to occur 3 h after cold stress initiation (Catala et al., 2011). HY5 is a convergence point between light and hormone signaling pathways (Lau and Deng, 2010). It suppresses auxin signaling via activation of its negative regulators [e.g., IAA7 (indole-3-acetic acid inducible 7)], promotes ABA signaling by interaction with ABI5 (ABA insensitive 5), and stimulates GA deactivation (Lau and Deng, 2010). CKs enhance the stability of HY5 protein by diminishing its degradation by COP1 (Vandenbussche et al., 2007), while GAs have the opposite effect (Alabadi et al., 2008).

Cold stress led to significant changes in hormone pools (see **Figures 2**, **3**, **5**, **6**). The content of ABA increased in all tissues, as well as *NCED3* transcription and abundance of ABA-biosynthetic enzymes in leaves. The ABA sensitivity (estimated by *PYL6* transcription) was elevated in apices and leaves. Cold stress diminished tZ and tZR levels in WT apices, the degree depending on light intensity (C-NL > C-LL > C-D). C-NL strongly stimulated *CKX* transcription as well as the enzyme activity in leaves, indicating a high rate of CK degradation. Nevertheless, *CKX5* transcription was diminished in apices and roots, which could indicate that CK down-regulation in these tissues was caused by other CKX isozymes, such as CKX4 or 6 (Wang et al., 2020), or by a low rate of CK biosynthesis. The level of iP precursor [N^6-(Δ^2-isopentenyl)adenosine monophosphate] was increased in leaves, which may suggest the replacement of highly active tZ with less active iP (Spichal et al., 2004). Decrease of transcription of type-A ARRs suggests that suppression of CK signal transduction was eliminated after prolonged stress, probably to allow plant acclimation, associated with CK content increase (Kosova et al., 2012). This is in accordance with Jeon et al. (2010), who found that overexpression of *ARR7* resulted in reduced cold tolerance. Decrease of CKs was accompanied by inactivation of auxins to reversible storage forms, which were found up-regulated in all tissues (**Supplementary Table 2**). C-NL seems to be associated with down-regulation of GAs in apices, as indicated by up-regulated transcript levels of GA repressor *RGL3*. Up-regulation of strigolactone receptor *MAX2* in apices suggests suppression of primordia development. Inhibition of branching or tillering is one of the most distinct strigolactone physiological functions (Waldie et al., 2014). Down-regulation of *MAX2* transcription in roots may be connected with inhibition of root growth in cold stress. Strigolactones have been reported to enhance tolerance to several abiotic stresses, e.g., drought and salinity (Mostofa et al., 2018); thus, it is possible that they also play a role in cold stress responses. Moreover, Koltai et al. (2011) reported a positive correlation between light intensity and strigolactone levels in tomato roots. The parallel karrikin pathway was suppressed by cold in general. This is in apparent contradiction with the studies of Zhao et al. (2012) and Zheng et al. (2015), who suggested that karrikin has a role in cold or freezing stress responses in *Chorispora bungeana* and *Camellia sinensis*, respectively. However, both studies determined transcription profiles at the early phase of cold stress (after 4–8 h and 24 h, respectively). Karrikin has been studied predominantly in relation to germination promotion (e.g., Bunsick et al., 2020).

Cold Stress Under Low Light Conditions

Cold stress under low light conditions caused substantial membrane damage (**Figure 1**). The response to cold stress under low light differed significantly from that under normal light at the level of transcriptome, hormonome, and proteome (**Figures 4**, **7**). Cold treatment at low light intensity has been found to result in much lower acquired freezing tolerance than at normal light conditions (Szalai et al., 2009; Janda et al., 2014). Nevertheless, transcription of the stress marker genes *RD29A* and *COR47* was stimulated much more than in the case of C-NL (*RD29A* in roots, *COR47* in all tissues; **Figure 6**). On the other hand, *CBF1–3* genes were less up-regulated under C-LL than under C-NL. Elevation of *PIF* transcription in leaves under C-LL and C-NL was similar.

Plants exposed to C-LL had ABA and jasmonate levels up-regulated in all tissues, as well as SA content in roots (**Figure 3**). CK content under C-LL was lower than under C-NL (**Figure 2** and **Supplementary Table 2**). C-LL was the only treatment that stimulated production of the less active CK cZ (and its riboside), which has been connected with stress responses, preserving certain CK functions under conditions of suppressed growth (Gajdosova et al., 2011). CK signal transduction during C-LL, estimated via *ARR10* transcription, was maintained or

slightly up-regulated in all tissues (**Figure 6**). Significant up-regulation of the transcription of negative regulators (*ARR4*, *5*, and *7*) was, however, detected in leaves. C-LL was associated with only minor changes in IAA content (**Figure 3**) compared with control, which agrees with a reported positive auxin function at skotomorphogenesis (Ballare and Pierik, 2017). Simultaneously, storage conjugate glucose ester of IAA was formed in roots (**Supplementary Table 2**). Prolonged C-LL stimulated transcription of GA repressors *RGA1* in leaves and, to a minor extent, of *RGL3* in apices (**Figure 6**). Up-regulation of strigolactone co-receptor *D14* in apices and leaves (as in the case of C-NL), together with a decrease of the strigolactone repressor *SMXL6* transcript in roots, seems to indicate that strigolactones participate in cold stress responses at low light intensity. These data agree with Xie et al. (2020) who found that, in shade, strigolactones stimulate SMXL6/7/8 degradation, promoting the expression of transcription factor *BRC1*, which inhibits bud outgrowth.

Cold stress under low light resulted in increased abundance of proteins associated with the abiotic stress response, as well as the biosynthesis of secondary metabolites such as glucosinolates (**Figure 8**). The low light-specific cold-responsive (C-LL) proteins included enzymes of ROS metabolism, e.g., ascorbate peroxidase.

Cold Stress Under Dark Conditions Caused Plant Growth Arrest

Absence of light during cold stress (C-D) partially diminished the stress impact on membrane peroxidation in comparison with the other cold treatments, C-NL or C-LL (**Figure 1**). In spite of the relatively low level of oxidative damage, C-D conditions led to the most significant reduction in both the maximal and actual quantum efficiency values (**Table 2**), indicating the presence of a secondary, dark-induced stress. However, it must also be mentioned that the lowest F_v/F_m values were still around 0.8, indicating that the stress was indeed mild, and at least the photosynthetic machinery was in a relatively good physiological state. Transcription of the stress-related gene *COR47* was only moderately up-regulated in apices and leaves, while *RD29A* was elevated considerably less than in the light treatments (**Figure 6**). *CBF1* (in contrast to *CBF2* and especially *CBF3*) was up-regulated to a similar extent as in the light conditions, indicating its dependence on temperature rather than light intensity. Transcription of *CRF4* and especially of *CRF3* was low in comparison with other cold treatments. *HY5* transcription was strongly diminished in the dark, which is in agreement with its association with photomorphogenesis (Lau and Deng, 2010). *PIF4* was highly stimulated in roots of C-D-treated plants, which could be related to its role in skotomorphogenesis (Ballare and Pierik, 2017).

Abscisic acid production was stimulated only in above-ground tissues, but it was not accompanied with the promotion of the levels of deactivation metabolites or transcription of *NCED3* and *PYL6* (**Figures 3**, **6** and **Supplementary Table 2**). On the other hand, jasmonates were down-regulated in all tissues. C-D resulted in very low levels of all CKs, their metabolites, as well as low *IPT3* and *ARR10* transcription, which consequently led to low transcription of type-A *ARRs* and CKX activity (**Figures 2**, **5**, **6** and **Supplementary Table 2**). CK suppression is in accordance with negative function of CKs in skotomorphogenesis, described by Ballare and Pierik (2017). Nevertheless, transcription of *IPT5*, the enzyme predominant in roots, was substantially elevated in leaves and roots of dark-treated plants. This accords with the finding that a functional CK signaling pathway is necessary also in the dark in order to maintain transcripts of some plastid genes, to enable plants to keep their photosynthetic apparatus ready in case of environmental changes (Doroshenko et al., 2016).

Auxins were degraded to irreversible conjugates (**Supplementary Table 2**). C-D treatment did not significantly affect the content of precursor GA_{19}, but transcription of GA repressors *RGA1* and *2* was down-regulated (**Figures 3**, **6**). This may indicate that plants in the dark had high level of GAs, which could be connected with their role in skotomorphogenesis (Ballare and Pierik, 2017). Repression of the strigolactone signaling pathway via SMXL6 was inhibited, but the karrikin pathway was diminished most in all treatments.

The evidence of attenuated metabolism under C-D conditions was also evident from proteome analysis showing the up-regulation of the ubiquitin–proteasome pathway and vesicular transport (**Figure 8**).

The Effect of Modulated CK Levels

Modulation of CK Levels Changed Hormone Pools as Well as Basal Metabolism in Plants

To investigate the effect of modified content of CKs on cold stress responses, transformants with increased CK biosynthesis (*DEX:IPT*) or degradation (*DEX:CKX*) were used. In order to avoid changes in plant morphology, expression of introduced genes was activated by DEX, 24 h before stress initiation. The effect of DEX on plants was checked at the level of membrane damage, chlorophyll fluorescence, and CKX activity (**Figures 1**, **5** and **Table 2**). DEX had only a mild (but significant) impact on all abovementioned parameters under control conditions, being of negligible importance in comparison with the intensity of changes imposed by modulated CK content or the cold stress.

Elevated endogenous CK levels caused by the introduced *ipt* gene from *A. tumefaciens* (*DEX:IPT* plants) substantially changed metabolism and signaling processes in plants. The transcription of the crucial CK biosynthetic enzyme *IPT3* was strongly suppressed, while CKX expression and activity as well as CK deactivation via CK *N*- and *O*-glucosylation pathways were elevated in order to down-regulate high CK levels and re-establish (at least partially) CK homeostasis (**Figures 2**, **5**, **6** and **Supplementary Table 2**). CK signaling in apices and leaves was also regulated by stimulation of the transcription of negative response regulators (*ARR4*, *5*, and *7*).

Due to the hormone cross-talk, levels of IAA, ABA, SA, and JA were also moderately elevated (**Figure 3**). Auxin up-regulation in apices may support, together with CKs, meristem growth. The level of ABA, as well as *NCED3* transcription in apices (**Figures 3**, **6**), was high in *DEX:IPT* compared with WT, which could indicate the maintenance of an optimal ABA:CK ratio, as described in previous studies (e.g., Dobra et al., 2015;

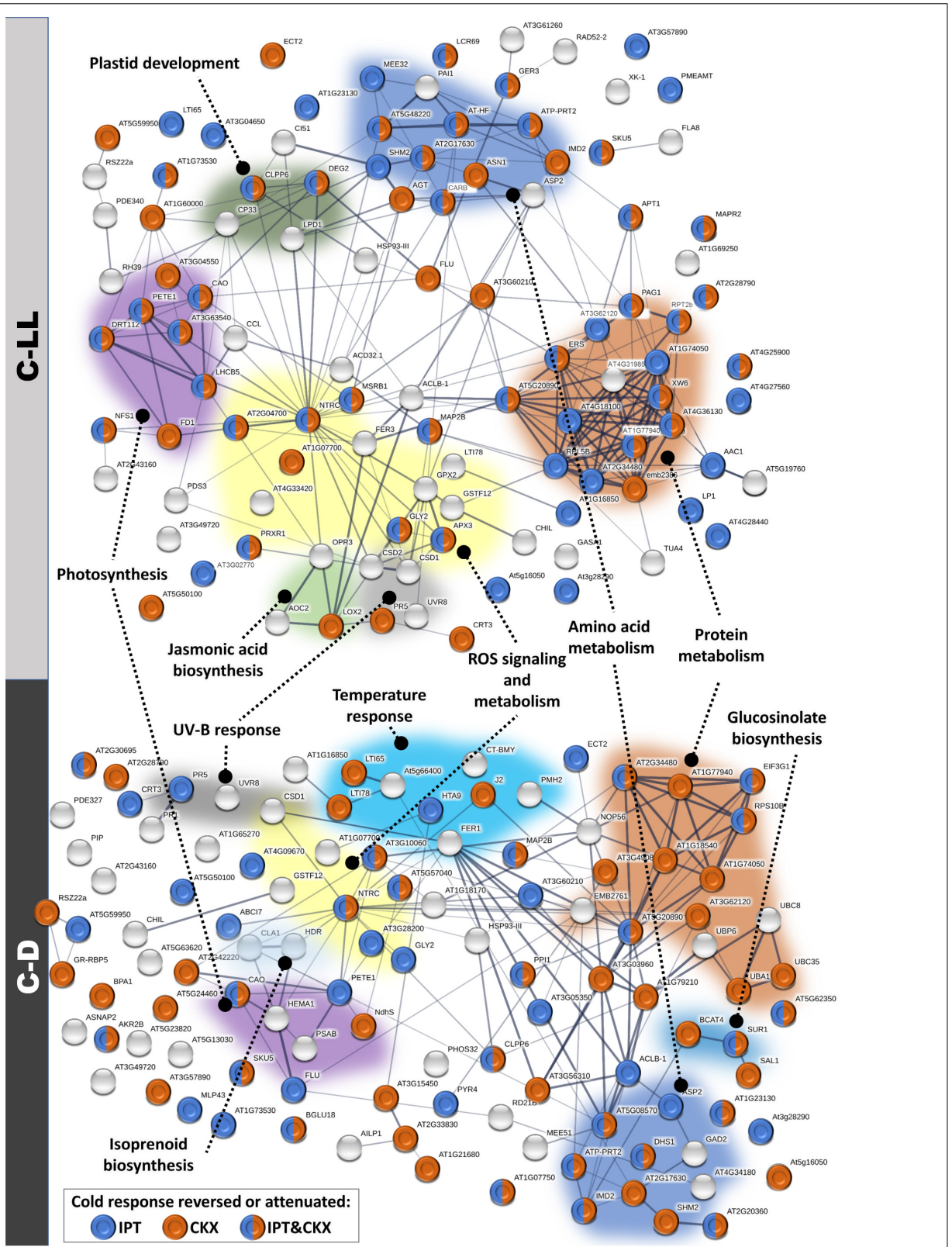


FIGURE 8 | Modulation of cold-responsive proteins in plants with an altered cytokinin pool. Interactions and functional clusters of identified cold-responsive proteins (compared to control, 20°C, 150 μmol m^{-2} s^{-1}; $p < 0.05$) were highlighted by String (Szklarczyk et al., 2019). The line thickness indicates the strength of data support, and the minimum required interaction score is 0.4 (medium confidence). See proteomic data in ProteomeXchange for details.

Skalak et al., 2016; Pavlu et al., 2018). However, ABA perception via PYL6 was down-regulated under control conditions. SA levels correlated well with CK content, as the *DEX:IPT* transformant had a higher basal level of SA (**Figures 2**, **3**). Signaling pathways of both hormones can interact, forming a complex between the type-B response regulator ARR2 and transcription factor TGA3, which increases plant tolerance to pathogen attack (Choi et al., 2010). Moderate JA elevation agrees with the reported positive CK effect on JA levels upon wounding (Sano et al., 1996). *DEX:IPT* plants showed low *MAX2* and *D14* transcription compared with WT, which may reflect antagonistic hormone relationship, as CKs and strigolactones regulate by opposing a number of physiological processes, e.g., bud or root growth (Dun et al., 2012).

Elevated content of CKs boosted synthesis of amino acids, sugar metabolism, and transcription (up-regulation of ribosomal proteins). These plants also contained more phototropin-2, which may reflect the CK link with light signaling (Werner and Schmulling, 2009). In summary, improved growth, but diminished transcription of stress-related genes *RD29A* and *COR47*, and translation of stress-related proteins in *DEX:IPT* plants support the hypothesis that plants with higher CK levels are delayed in sensing stress (Havlova et al., 2008).

Decreased endogenous CK levels caused by the introduced *HvCKX2* gene from *H. vulgare* (*DEX:CKX* plants) suppressed growth and increased the level of stress-related compounds under control conditions. CKX activity was moderately elevated, but this increase did not correlate with high *HvCKX2* transcription, probably due to attenuation of CKX protein function or its rapid degradation (**Figures 2**, **5**). Moreover, this genotype exhibited down-regulated CK deactivation through CK glucosylation and transcription of endogenous CKXs (e.g., *CKX5*), while transcription of CK biosynthetic enzymes (*IPTs*) and positive response regulators (*ARR10*) was significantly enhanced (**Figure 6** and **Supplementary Table 2**). Negative regulation of CK signaling via type-A ARRs (*ARR4*, *5*, and *7*) was diminished. A high content of tZRMP (precursor of tZ) in roots was accompanied by synthesis of cZR, which is readily convertible to the low active cZ, which could in turn be set aside as the substrate of the HvCKX2 enzyme.

DEX:CKX exhibited a generally lower transcription of *CRF3* and *4*, which corresponds with their lower cold tolerance as indicated by higher MDA levels after cold treatments (**Figures 1**, **6**). Low CK levels down-regulated the production of auxin IAA, which might contribute to plant growth suppression (**Figure 3**). Correlation of SA content with CK levels was also observed in this genotype. By contrast, *NCED3* transcription and ABA content were stimulated in apices and leaves (**Figures 3**, **6**). Subsequently, *RD29A* transcription and translation of stress-related proteins (including a JA biosynthetic enzyme) were enhanced. *DEX:CKX* plants had low levels of GA_{19} precursor indicating low GA content leading to growth retardation (Ross et al., 2001).

Independent component analyses of phytohormone pools (**Figure 4A**) showed distinct responses of *DEX:IPT* plants, especially in apices and leaves. *DEX:CKX* plants differed from WT under control conditions significantly in roots. ICA of transcriptional data (**Figure 4B**) showed the most distinct differences among genotypes in roots. A clear separation of the genotypes at the proteome level (**Figure 7**) reflected the higher distance of *DEX:CKX* in comparison with WT and *DEX:IPT*.

Elevated CK Content Up-Regulated Stress Tolerance

The transcription of the *ipt* transgene was generally stable regardless of cold stress (**Figure 6**). Only the C-D treatment partially down-regulated *ipt* transcription. The huge amount of CKs was diminished by cold treatments, but the levels in *DEX:IPT* apices were, under C-NL and C-LL, still higher than in WT under control conditions (**Figure 2**). The elevated CK content in *DEX:IPT* plants maintained up-regulated negative feedback on CK signaling in apices and leaves via up-regulation of transcription of type-A response regulators in all stress treatments (**Figure 6**). The positive effect on IAA levels in apices was also preserved during cold stress treatments, as it was under control conditions (**Figure 3**). ABA levels in *DEX:IPT* plants were high under cold stress, especially in apices and roots under C-LL. The ABA content also seems to correlate with a higher abundance of enzyme releasing ABA from storage conjugates under C-NL. ABA signaling via PYL6 was enhanced in apices and leaves under both C-NL and C-LL, and SA elevation was partially maintained under the same conditions in apices. In roots, high SA levels were observed in all genotypes. In contrast, C-NL and C-LL stressed plants suppressed the strigolactone signaling pathway in *DEX:IPT* plants. This finding may reflect the fact that strigolactones exhibit antagonistic relationships with CKs (Dun et al., 2012). The karrikin pathway seemed to be enhanced.

Despite low basal transcription of *RD29A* under control conditions, *DEX:IPT* plants showed its highest elevation under C-NL stress, which may contribute to the diminished negative impact of cold stresses on the level of membrane peroxidation in *DEX:IPT* plants (**Figures 1**, **6**). A further contribution to this effect might arise from changes in leaf proteome, since C-NL stress stimulated in *DEX:IPT* plant proteins associated with correct folding (CYP19-4) as well as the production of protective compounds (glutathione *S*-transferase). C-LL conditions regulated transcription as well as saccharide metabolism (**Figure 8**). *DEX:IPT* plants exposed to C-LL showed moderately elevated transcription of *HY5* in comparison with other genotypes (**Figure 6**), which might arise from a synergy between light and CK signals. Despite the fact that C-D conditions elicited similar responses in both WT and the transformants, *DEX:IPT* plants still exhibited a higher transcription of negative CK signaling regulators as well as *RD29A*, *COR47*, and *CBF1* in leaves. Under C-D, *DEX:IPT* plants were able to maintain the higher translation of proteins involved in amino acid biosynthesis, transcription activity (histone accumulation, ribosomal proteins), protein folding (HSPs), and glycoprotein assembly (**Figure 8**).

Our data are in accord with reported positive effects of elevated CK contents on cold stress tolerance, e.g., in the callus of tall fescue transformed with *ipt* under maize ubiquitin promoter (Hu et al., 2005), or in *COR15a:ipt* sugarcane transformant (Belintani et al., 2012). It seems that despite lower preparedness

of *DEX:IPT* plants to stress, they are able to stimulate stress-defense mechanisms protecting against cold stress effectively. Nevertheless, the impact of cold stress on CK levels as well as on CKX activity in the transformants indicate that cold stress is a predominant regulatory factor in comparison with transgene activation (see ICA at **Figures 4**, **7**).

Independent component analyses showed very distinct cold stress responses at different light intensities (**Figures 4**, **7**). ICA of hormone pools showed a clear separation of *DEX:IPT* plants between C-NL and C-LL especially in apices, the distance in the leaves being lower, while a close proximity was observed in roots. ICA of transcription data showed clear separation between C-NL and C-LL in leaves and roots. Proteome ICA revealed quite close proximity under C-NL, while under C-LL and C-D, distinct differences were observed among genotypes as well as treatments.

Low CK Content Had a Negative Impact on Cold Stress Tolerance of Plants

Because *DEX:CKX* plants exhibited intensive suppression of the transgene activity under all cold stresses, especially in apices of plants exposed to C-LL (**Figure 6**), low CK content seems to be a serious disadvantage during cold stress responses. In comparison with control conditions, tZ and iP were significantly up-regulated under C-NL in *DEX:CKX* apices and roots (**Figure 2**). The cZRMP in roots of this genotype reached a higher level than in stressed WT. Even up-regulation of storage metabolites CK *O*-glucosides was detected (**Supplementary Table 2**). The overexpression of *HvCKX2* led to stimulation of the transcription of *IPT3* gene in leaves and roots (**Figure 6**). C-LL stressed plants also had diminished transcription of negative regulators of CK signaling (*ARR4*, *5*, and *7*) in leaves. Despite the intensive regulation of endogenous CKs, lower CK content strengthened the negative impact of cold stress on membrane damage (**Figure 1**). This finding may also be due to the fact that transcription of *RD29A* in apices and leaves was not as high as it was in the other genotypes (**Figure 6**). Also, C-NL and C-LL responses were associated with lower up-regulation of *CBFs*, suggesting a weaker cold stress reaction by this genotype.

The C-NL treatment diminished transcription of ABA biosynthetic gene *NCED3* in leaves of *DEX:CKX* plants; however, the enzyme releasing ABA from storage conjugates was up-regulated; thus, ABA content remained high, comparable to values in other genotypes (**Figures 3**, **6**). Stress-related proteins connected with programmed cell death, thioredoxin, or ABA targeting lipoxygenase were exclusively up-regulated in *DEX:CKX* plants exposed to C-NL. This cold treatment also led to the increase of transcription of DELLA proteins in apices. Both light treatments (C-NL and C-LL) were associated with the elevation of SA content in roots to a level comparable with other genotypes.

The enhanced abundance of auxin biosynthetic enzyme amidase 1 under C-LL and C-D conditions may be responsible for IAA elevation in apices and roots under C-LL, and in apices only, under C-D (**Figures 3**, **8**). Up-regulation of ABA biosynthetic enzymes (NCED3, zeaxanthin epoxidase) in leaves and roots of *DEX:CKX* plants under C-LL may be the cause of relatively high ABA levels in all tissues, comparable with WT (**Figures 3**, **6**, and **8**). C-LL also led to an increase of SA comparable to that in WT. Jasmonates were maintained at high levels in roots during C-D stress, higher than in the other genotypes. *DEX:CKX* plants had diminished levels of glutathione-S-transferase and thioredoxin. In leaves, high levels of proteins associated with cytoskeleton re-organization, lipid peroxidation protector (lipocalin), and fatty acid synthesis were found. Surprisingly, HHL1 protein, which is involved in protection against high light stress, was up-regulated under low light conditions (C-LL) in leaves of *DEX:CKX* plants. C-D treatment had a slightly more negative impact on photosynthetic capacity (F_v/F_m) than in WT, which was in agreement with decrease in proteins of chlorophyll synthesis and translation (**Figure 8** and **Table 1**). These results correlate well with reported CK functions in the synthesis and stabilization of chlorophyll (Werner and Schmulling, 2009).

CONCLUSION

Light intensity strongly affected the cold stress response. At low light intensity, the negative effects of cold on photosynthesis were diminished. A combination of dark and cold arrested the growth of plants regardless of genotype, resulting in a strong suppression of CK levels.

Cold stress strongly affected CK levels in transformants with high (*DEX:IPT*) or low (*DEX:CKX*) CK contents, considerably diminishing the effect of inserted genes. The influence was predominant in roots, the primary location of CK production. Nevertheless, modulation of CK levels still had a significant effect on cold stress responses. Although *DEX:IPT* plants under control conditions predominantly targeted available energy to growth, exhibiting only a low level of transcription of stress-related genes (especially in leaves and roots), they were able, upon cold stress, to acclimate well to unfavorable conditions. On the other hand, *DEX:CKX* plants exhibited better "stress adjustment" under control conditions, but their ability to acclimate in prolonged stress was worse.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/pride/archive/projects/PXD020480.

AUTHOR CONTRIBUTIONS

RV designed the experiment. VK and SP prepared plant material. PD, AG, VK, and SP analyzed phytohormone contents. SP, BZ, and LH determined gene transcription. MČ and BB performed proteome analyses. VM determined CKX activity. TJ analyzed photosynthetic parameters. SP, RV, and MČ evaluated results and prepared the publication. All authors contributed to manuscript revision, and read and approved the submitted version.

FUNDING

This work was supported by Czech Science Foundation projects nos. 17-04607S and 19-12262S and the Ministry of Education, Youth and Sports of CR from European Regional Development Fund-Project "Centre for Experimental Plant Biology": No. CZ.02.1.01/0.0/0.0/16_019/0000738.

ACKNOWLEDGMENTS

We thank Eva Kobzová and Marie Korecká for their invaluable technical assistance. For English corrections, we also thank Joelle Hoggan from Sees-Editing Ltd.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.608711/full#supplementary-material

Supplementary Figure 1 | Shoot and root mass.

Supplementary Figure 2 | Plant phenotypes at different experimental conditions. Proteomic data are available via ProteomeXchange (http://www.proteomexchange.org/) with identifier PXD020480. For the purpose of peer review, the data are accessible under the username reviewer41015@ebi.ac.uk and the password qFxVQj3A.

Supplementary Table 1 | List of qPCR primers.

Supplementary Table 2 | Phytohormones.

Supplementary Table 3 | Transcriptomic data.

REFERENCES

Alabadi, D., Gallego-Bartolome, J., Orlando, L., Garcia-Carcel, L., Rubio, V., Martinez, C., et al. (2008). Gibberellins modulate light signaling pathways to prevent *Arabidopsis* seedling de-etiolation in darkness. *Plant J.* 53, 324–335. doi: 10.1111/j.1365-313X.2007.03346.x

Ballare, C. L., and Pierik, R. (2017). The shade-avoidance syndrome: multiple signals and ecological consequences. *Plant Cell Environ.* 40, 2530–2543. doi: 10.1111/pce.12914

Belintani, N. G., Guerzoni, J. T. S., Moreira, R. M. P., and Vieira, L. G. E. (2012). Improving low-temperature tolerance in sugarcane by expressing the ipt gene under a cold inducible promoter. *Biol. Plant* 56, 71–77. doi: 10.1007/s10535-012-0018-1

Bunsick, M., Toh, S., Wong, C., Xu, Z., Ly, G., McErlean, C. S., et al. (2020). SMAX1-dependent seed germination bypasses GA signalling in *Arabidopsis* and *Striga*. *Nat. Plants* 6, 646–652. doi: 10.1038/s41477-020-0653-z

Catala, R., Medina, J., and Salinas, J. (2011). Integration of low temperature and light signaling during cold acclimation response in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16475–16480. doi: 10.1073/pnas.1107161108

Cejudo, F. J., Ojeda, V., Delgado-Requerey, V., Gonzalez, M., and Perez-Ruiz, J. M. (2019). Chloroplast redox regulatory mechanisms in plant adaptation to light and darkness. *Front. Plant Sci.* 10:380. doi: 10.3389/fpls.2019.00380

Černý, M., Kuklova, A., Hoehenwarter, W., Fragner, L., Novak, O., Rotkova, G., et al. (2013). Proteome and metabolome profiling of cytokinin action in *Arabidopsis* identifying both distinct and similar responses to cytokinin down- and up-regulation. *J. Exp. Bot.* 64, 4193–4206. doi: 10.1093/jxb/ert227

Choi, J., Huh, S. U., Kojima, M., Sakakibara, H., Paek, K. H., and Hwang, I. (2010). The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in *Arabidopsis*. *Dev. Cell* 19, 284–295. doi: 10.1016/j.devcel.2010.07.011

Chory, J., Reinecke, D., Sim, S., Washburn, T., and Brenner, M. (1994). A role for cytokinins in de-etiolation in *Arabidopsis* (det mutants have an altered response to cytokinins). *Plant Physiol.* 104, 339–347. doi: 10.1104/pp.104.2.339

Christians, M. J., and Larsen, P. B. (2007). Mutational loss of the prohibitin AtPHB3 results in an extreme constitutive ethylene response phenotype coupled with partial loss of ethylene-inducible gene expression in *Arabidopsis* seedlings. *J. Exp. Bot.* 58, 2237–2248. doi: 10.1093/jxb/erm086

Cortleven, A., and Schmulling, T. (2015). Regulation of chloroplast development and function by cytokinin. *J. Exp. Bot.* 66, 4999–5013. doi: 10.1093/jxb/erv132

Craft, J., Samalova, M., Baroux, C., Townley, H., Martinez, A., Jepson, I., et al. (2005). New pOp/LhG4 vectors for stringent glucocorticoid−dependent transgene expression in *Arabidopsis*. *Plant J.* 41, 899–918. doi: 10.1111/j.1365-313X.2005.02342.x

de Wit, M., Lorrain, S., and Fankhauser, C. (2014). Auxin-mediated plant architectural changes in response to shade and high temperature. *Physiol. Plant* 151, 13–24. doi: 10.1111/ppl.12099

Demmig-Adams, B., Stewart, J. J., Baker, C. R., and Adams, W. W. (2018). Optimization of photosynthetic productivity in contrasting environments by regulons controlling plant form and function. *Int. J. Mol. Sci.* 19, 872. doi: 10.3390/ijms19030872

Dobra, J., Černý, M., Storchova, H., Dobrev, P., Skalak, J., Jedelsky, P. L., et al. (2015). The impact of heat stress targeting on the hormonal and transcriptomic response in *Arabidopsis*. *Plant Sci.* 231, 52–61. doi: 10.1016/j.plantsci.2014.11.005

Doroshenko, A. S., Danilova, M. N., Kudryakova, N. V., Soloviev, A. A., and Kusnetsov, V. V. (2016). Cytokinin membrane receptors participate in regulation of plastid genome expression in the skotomorphogenesis. *Dokl. Biochem. Biophys.* 469, 294–297. doi: 10.1134/S1607672916040153

Dun, E. A., de Saint Germain, A., Rameau, C., and Beveridge, C. A. (2012). Antagonistic action of strigolactone and cytokinin in bud outgrowth control. *Plant Physiol.* 158, 487–498. doi: 10.1104/pp.111.186783

Gajdosova, S., Spichal, L., Kaminek, M., Hoyerova, K., Novak, O., Dobrev, P. I., et al. (2011). Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants. *J. Exp. Bot.* 62, 2827–2840. doi: 10.1093/jxb/erq457

Galvao, V. C., and Fankhauser, C. (2015). Sensing the light environment in plants: photoreceptors and early signaling steps. *Curr. Opin. Neurobiol.* 34, 46–53. doi: 10.1016/j.conb.2015.01.013

Gaudinova, A., Dobrev, P. I., Solcova, B., Novak, O., Strnad, M., Friedecky, D., et al. (2005). The involvement of cytokinin oxidase/dehydrogenase and zeatin reductase in regulation of cytokinin levels in pea (*Pisum sativum* L.) leaves. *J. Plant Growth Regul.* 24, 188–200. doi: 10.1007/s00344-005-0043-9

Genty, B., Briantais, J. M., and Baker, N. R. (1989). The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990, 87–92. doi: 10.1016/S0304-4165(89)80016-9

Havlova, M., Dobrev, P. I., Motyka, V., Storchova, H., Libus, J., Dobra, J., et al. (2008). The role of cytokinins in responses to water deficit in tobacco plants over−expressing trans−zeatin O−glucosyltransferase gene under 35S or SAG12 promoters. *Plant Cell Environ.* 31, 341–353. doi: 10.1111/j.1365-3040.2007.01766.x

Hloušková, P., Černý, M., Kořínková, N., Luklová, M., Minguet, E. G., Brzobohatý, B., et al. (2019). Affinity chromatography revealed 14-3-3 interactome of tomato (Solanum lycopersicum L.) during blue light-induced de-etiolation. *J. Proteomics* 193, 44–61. doi: 10.1016/j.jprot.2018.12.017

Hodges, D. M., DeLong, J. M., Forney, C. F., and Prange, R. K. (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207, 604–611. doi: 10.1007/s004250050524

Horton, P., and Ruban, A. V. (1992). Regulation of photosystem-II. *Photosynth. Res.* 34, 375–385. doi: 10.1007/BF00029812

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., et al. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinform.* 2008, 420747. doi: 10.1155/2008/420747

Hu, Y., Jia, W., Wang, J., Zhang, Y., Yang, L., and Lin, Z. (2005). Transgenic tall fescue containing the *Agrobacterium tumefaciens* ipt gene shows enhanced cold tolerance. *Plant Cell Rep.* 23, 705–709. doi: 10.1007/s00299-004-0863-2

Janda, T., Majlath, I., and Szalai, G. (2014). Interaction of temperature and light in the development of freezing tolerance in plants. *J. Plant Growth Regul.* 33, 460–469. doi: 10.1007/S00344-013-9381-1

Jeon, J., Kim, N. Y., Kim, S., Kang, N. Y., Novak, O., Ku, S. J., et al. (2010). A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in *Arabidopsis*. *J. Biol. Chem.* 285, 23371–23386. doi: 10.1074/jbc.M109.096644

Jiang, B., Shi, Y., Peng, Y., Jia, Y., Yan, Y., Dong, X., et al. (2020). Cold-induced CBF-PIF3 interaction enhances freezing tolerance by stabilizing the phyB thermosensor in *Arabidopsis*. *Mol. Plant* 13, 894–906. doi: 10.1016/j.molp.2020.04.006

Koltai, H., Cohen, M., Chesin, O., Mayzlish-Gati, E., Becard, G., Puech, V., et al. (2011). Light is a positive regulator of strigolactone levels in tomato roots. *J. Plant Physiol.* 168, 1993–1996. doi: 10.1016/j.jplph.2011.05.022

Kosova, K., Prasil, I. T., Vitamvas, P., Dobrev, P., Motyka, V., Flokova, K., et al. (2012). Complex phytohormone responses during the cold acclimation of two wheat cultivars differing in cold tolerance, winter Samanta and spring Sandra. *J. Plant Physiol.* 169, 567–576. doi: 10.1016/j.jplph.2011.12.013

Landi, M. (2017). Commentary to: "Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds" by Hodges et al., Planta (1999) 207: 604-611. *Planta* 245, 1067–1067. doi: 10.1007/s00425-017-2699-3

Lau, O. S., and Deng, X. W. (2010). Plant hormone signaling lightens up: integrators of light and hormones. *Curr. Opin. Plant Biol.* 13, 571–577. doi: 10.1016/j.pbi.2010.07.001

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Majlath, I., Szalai, G., Soos, V., Sebestyen, E., Balazs, E., Vankova, R., et al. (2012). Effect of light on the gene expression and hormonal status of winter and spring wheat plants during cold hardening. *Phys. Plant.* 145, 296–314. doi: 10.1111/j.1399-3054.2012.01579.x

Mierswa, I., Wurst, M., Klinkenberg, R., Scholz, M., and Euler, T. (2006). "Yale: rapid prototyping for complex data mining tasks," in *Proceedings of the 12th ACM SIGKDD International Conference on Knowledge Discovery and Data Mining*, (New York, NY: Association for Computing Machinery), 935–940. doi: 10.1145/1150402.1150531

Miller, C. O., Skoog, F., Okumura, F. S., von Saltza, M. H., and Strong, F. M. (1956). Isolation, structure and synthesis of kinetin, a substance promoting cell division. *J. Am. Chem. Soc.* 78, 1375–1380. doi: 10.1021/ja01588a032

Mohammed, B., Bilooei, S. F., Doczi, R., Grove, E., Railo, S., Palme, K., et al. (2018). Converging light, energy and hormonal signaling control meristem activity, leaf initiation, and growth. *Plant Physiol.* 176, 1365–1381. doi: 10.1104/pp.17.01730

Mostofa, M. G., Li, W., Nguyen, K. H., Fujita, M., and Tran, L. S. P. (2018). Strigolactones in plant adaptation to abiotic stresses: an emerging avenue of plant research. *Plant Cell Environ.* 41, 2227–2243. doi: 10.1111/pce.13364

Motyka, V., and Kaminek, M. (1992). "Characterization of cytokinin oxidase from tobacco and poplar callus cultures," in *Physiology and Biochemistry of Cytokinins in Plants*, eds M. Kaminek, D. W. S. Mok, and E. Zazimalova (The Hague: SPB Academic Publishing), 33–39.

Motyka, V., and Kaminek, M. (1994). Cytokinin oxidase from auxin- and cytokinin-dependent callus cultures of tobacco (*Nicotiana tabacum* L.). *J. Plant Growth Regul.* 13, 1–9. doi: 10.1007/BF00210700

Motyka, V., Vankova, R., Capkova, V., Petrasek, J., Kaminek, M., and Schmulling, T. (2003). Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. *Physiol. Plant* 117, 11–21. doi: 10.1034/j.1399-3054.2003.1170102.x

Nolte, H., MacVicar, T. D., Tellkamp, F., and Kruger, M. (2018). Instant clue: a software suite for interactive data visualization and analysis. *Sci. Rep.* 8, 1–8. doi: 10.1038/s41598-018-31154-6

Pavlu, J., Novak, J., Koukalova, V., Luklova, M., Brzobohatý, B., and Černý, M. (2018). Cytokinin at the crossroads of abiotic stress signalling pathways. *Int. J. Mol. Sci.* 19, 2450. doi: 10.3390/ijms19082450

Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D. J., et al. (2019). The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* 47, D442–D450. doi: 10.1093/nar/gky1106

Prinzenberg, A. E., Campos-Dominguez, L., Kruijer, W., Harbinson, J., and Aarts, M. G. (2020). Natural variation of photosynthetic efficiency in *Arabidopsis thaliana* accessions under low temperature conditions. *Plant Cell Environ.* 43, 2000–2013. doi: 10.1111/pce.13811

Ross, J. J., O'Neill, D. P., Wolbang, C. M., Symons, G. M., and Reid, J. B. (2001). Auxin-gibberellin interactions and their role in plant growth. *J. Plant Growth Regul.* 20, 346–353. doi: 10.1007/s003440010034

Sano, H., Seo, S., Koizumi, N., Niki, T., Iwamura, H., and Ohashi, Y. (1996). Regulation by cytokinins of endogenous levels of jasmonic and salicylic acids in mechanically wounded tobacco plants. *Plant Cell Physiol.* 37, 762–769. doi: 10.1093/oxfordjournals.pcp.a029011

Skalak, J., Černý, M., Jedelsky, P., Dobra, J., Ge, E., Novak, J., et al. (2016). Stimulation of *ipt* overexpression as a tool to elucidate the role of cytokinins in high temperature responses of *Arabidopsis thaliana*. *J. Exp. Bot.* 67, 2861–2873. doi: 10.1093/jxb/erw129

Spichal, L., Rakova, N. Y., Riefler, M., Mizuno, T., Romanov, G. A., Strnad, M., et al. (2004). Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. *Plant Cell Physiol.* 45, 1299–1305. doi: 10.1093/pcp/pch132

Szalai, G., Pap, M., and Janda, T. (2009). Light-induced frost tolerance differs in winter and spring wheat plants. *J. Plant Physiol.* 166, 1826–1831. doi: 10.1016/j.jplph.2009.04.016

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 47, D607–D613. doi: 10.1093/nar/gky1131

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T. J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* 9, 1963–1971. doi: 10.1105/tpc.9.11.1963

Vandenbussche, F., Habricot, Y., Condiff, A. S., Maldiney, R., Straeten, D. V. D., and Ahmad, M. (2007). HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. *Plant J.* 49, 428–441. doi: 10.1111/j.1365-313X.2006.02973.x

Waldie, T., McCulloch, H., and Leyser, O. (2014). Strigolactones and the control of plant development: lessons from shoot branching. *Plant J.* 79, 607–622. doi: 10.1111/tpj.12488

Wang, X., Ding, J., Lin, S., Liu, D., Gu, T., Wu, H., et al. (2020). Evolution and roles of cytokinin genes in angiosperms 2: do ancient CKXs play housekeeping roles while non-ancient CKXs play regulatory roles? *Hortic. Res.* 7, 1–15. doi: 10.1038/s41438-020-0246-z

Werner, T., and Schmulling, T. (2009). Cytokinin action in plant development. *Curr. Opin. Plant Biol.* 12, 527–538. doi: 10.1016/j.pbi.2009.07.002

Xie, Y., Liu, Y., Ma, M., Zhou, Q., Zhao, Y., Zhao, B., et al. (2020). Arabidopsis FHY3 and FAR1 integrate light and strigolactone signaling to regulate branching. *Nat. Commun.* 11, 1–13. doi: 10.1038/s41467-020-15893-7

Xu, D., and Deng, X. W. (2020). CBF-phyB-PIF module links light and low temperature signaling. *Trends Plant Sci.* 25, 952–954. doi: 10.1016/j.tplants.2020.06.010

Yang, C., and Li, L. (2017). Hormonal regulation in shade avoidance. *Front. Plant Sci.* 8:1527. doi: 10.3389/fpls.2017.01527

Yoshida, S., Mandel, T., and Kuhlemeier, C. (2011). Stem cell activation by light guides plant organogenesis. *Genes Dev.* 25, 1439–1450. doi: 10.1101/gad.631211

Zhao, Z., Tan, L., Dang, C., Zhang, H., Wu, Q., and An, L. (2012). Deep-sequencing transcriptome analysis of chilling tolerance mechanisms of a subnival alpine plant, *Chorispora bungeana*. *BMC Plant Biol.* 12:222. doi: 10.1186/1471-2229-12-222

Zheng, C., Zhao, L., Wang, Y., Shen, J., Zhang, Y., Jia, S., et al. (2015). Integrated RNA-Seq and sRNA-Seq analysis identifies chilling and freezing responsive key molecular players and pathways in tea plant (*Camellia sinensis*). *PLoS One* 10:e0125031. doi: 10.1371/journal.pone.0125031

Zwack, P. J., Compton, M. A., Adams, C. I., and Rashotte, A. M. (2016). Cytokinin response factor 4 (CRF4) is induced by cold and involved in freezing tolerance. *Plant Cell Rep.* 35, 573–584. doi: 10.1007/s00299-015-1904-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 17 February 2021
doi: 10.3389/fpls.2021.644823

Signal Integration in Plant Abiotic Stress Responses via Multistep Phosphorelay Signaling

Jan Skalak[1†], Katrina Leslie Nicolas[1†], Radomira Vankova[2] and Jan Hejatko[1]*

[1] CEITEC - Central European Institute of Technology and National Centre for Biomolecular Research, Masaryk University, Brno, Czechia, [2] Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany, Czech Academy of Sciences, Prague, Czechia

Plants growing in any particular geographical location are exposed to variable and diverse environmental conditions throughout their lifespan. The multifactorial environmental pressure resulted into evolution of plant adaptation and survival strategies requiring ability to integrate multiple signals that combine to yield specific responses. These adaptive responses enable plants to maintain their growth and development while acquiring tolerance to a variety of environmental conditions. An essential signaling cascade that incorporates a wide range of exogenous as well as endogenous stimuli is multistep phosphorelay (MSP). MSP mediates the signaling of essential plant hormones that balance growth, development, and environmental adaptation. Nevertheless, the mechanisms by which specific signals are recognized by a commonly-occurring pathway are not yet clearly understood. Here we summarize our knowledge on the latest model of multistep phosphorelay signaling in plants and the molecular mechanisms underlying the integration of multiple inputs including both hormonal (cytokinins, ethylene and abscisic acid) and environmental (light and temperature) signals into a common pathway. We provide an overview of abiotic stress responses mediated via MSP signaling that are both hormone-dependent and independent. We highlight the mutual interactions of key players such as sensor kinases of various substrate specificities including their downstream targets. These constitute a tightly interconnected signaling network, enabling timely adaptation by the plant to an ever-changing environment. Finally, we propose possible future directions in stress-oriented research on MSP signaling and highlight its potential importance for targeted crop breeding.

Keywords: multistep phosphorelay (MSP), cytokinin, ethylene, abscisic acid, light signaling, temperature, abiotic stress, Arabidopsis

OPEN ACCESS

Edited by:
Alejandro Ferrando, Universitat Politècnica de València, Spain

Reviewed by:
Borja Belda-Palazon, Instituto Gulbenkian de Ciência (IGC), Portugal
Sabine Carpin, Université d'Orléans, France

***Correspondence:**
Jan Hejatko
hejatko@sci.muni.cz

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *21 December 2020*
Accepted: *26 January 2021*
Published: *17 February 2021*

Citation:
Skalak J, Nicolas KL, Vankova R and Hejatko J (2021) Signal Integration in Plant Abiotic Stress Responses via Multistep Phosphorelay Signaling. Front. Plant Sci. 12:644823. doi: 10.3389/fpls.2021.644823

PROLOG: HORMONES AND STRESS SIGNAL TRANSDUCTION – THE CHICKEN OR THE EGG?

Under natural conditions, plants are need to continuously maintain a balance between growth and defense or adaptive responses that are dictated by the severity, duration, and developmental timing at which any particular stress occurs (Claeys and Inzé, 2013). The essential question fundamental to understanding the mechanisms of hormonal control over any stress response is what is the very first action of the plant in response to (a sudden or long-term) change in environmental conditions? Does the stress response begin by activating the expression of defense/stress-responsive gene(s) that then subsequently impacts the hormonal status, or do plant hormones mediate the expression of

stress-responsive transcription factors (TFs), or do hormonal signaling and metabolism act either synergistically with or independently on stress response gene expression?

The answer is not trivial, simply because stress responses employ mutually interconnected molecular cascades and networks, and a detailed description of these networks is outside the scope of this review. Recently available evidence suggests a combination of all the above response mechanisms. For instance, numerous studies have been published that show stress-controlled expression of key members of the MSP pathway, while genome-wide and proteomic studies of cytokinin (one of the key plant hormones and a major regulator of MSP activity, see below) action have revealed that cytokinin modulates levels of stress-related genes/proteins (Brenner et al., 2005, 2012; Bhargava et al., 2013; Černý et al., 2014; Brenner and Schmülling, 2015; Raines et al., 2016). On the other hand, the metabolism of stress-associated plant hormones abscisic acid (ABA) and cytokinins are also rapidly modulated by stress conditions. Stress initiation results in a rapid (under an hour) increase of ABA content but reduced endogenous levels of cytokinins together with attenuated expression of cytokinin receptor genes (Dobrá et al., 2015). Induction of jasmonic acid (JA) production was observed to occur within seconds after wounding (root excision), suggesting that in this case, regulation of endogenous hormonal levels is the primary stress response (Zhou et al., 2019). Down- and up-regulation of signaling components acting downstream of cytokinins and ABA, respectively, suggests a combined effect of hormone metabolism and signal transduction on the regulation of heat-stress responses (Skalák et al., 2016). In the case of ethylene, multiple environmental stresses stimulate its biosynthesis to act antagonistically with ABA in the regulation of shoot and root growth under water-limiting conditions (Sharp and Lenoble, 2002; Skirycz et al., 2011; Dubois et al., 2018). The expression of *ETHYLENE RESPONSE FACTOR 1* (*ERF1*) was gradually induced by drought stress treatment over 12 h (reaching a maximum after 1 h), and this plays a positive role in drought, salt, and heat stress tolerance via stress-specific gene regulation (Cheng et al., 2013).

All these findings (and many others described below) show how important plant hormone signaling (including the MSP) and metabolism are during early stress responses. In the next few sections, we attempt to summarize recent observations on the biological relevance of MSP-mediated stress adaptation strategies in plants (mostly in *Arabidopsis thaliana*).

THE MULTISTEP PHOSPHORELAY CASCADE – HORMONAL AND ENVIRONMENTAL SIGNAL TRANSDUCTION AND CROSSTALK

The Cytokinin Signaling Pathway

Mainly due to historic reasons, much of the information about MSP has been on cytokinin signaling. Cytokinins are recognized by membrane-bound ARABIDOPSIS HISTIDINE KINASES (AHK2, AHK3, and AHK4/CRE1/WOL), located both at the plasma membrane and the endoplasmic reticulum (Caesar et al., 2011; Wulfetange et al., 2011). Recently, the quantitatively minor fraction of plasma membrane-located AHKs was shown to be functional in mediating the extracellular cytokinin signal (Antoniadi et al., 2020; Kubiasová et al., 2020). According to the current model (**Figure 1**), cytokinins bind to the CHASE domain (Hothorn et al., 2011) and activate intracellular histidine kinase (HK) activity possibly by changing receptor conformation; this then leads to sensor autophosphorylation (Hutchison and Kieber, 2002; Kieber and Schaller, 2018). The presence of phospho-His triggers downstream His-to-Asp-to-His-to-Asp phosphorelay. In the first (intramolecular) step, the phosphoryl group is passed from a conserved His residue of the HK domain to the conserved Asp residue located in the receiver domain of the receptor (Hwang and Sheen, 2001; Müller and Sheen, 2007). The small and mobile cytosolic proteins, ARABIDOPSIS HISTIDINE-CONTAINING PHOSPHOTRANSMITTERs (AHPs) 1-5, which shuttle between the cytosol and the nucleus, mediate the next step (Hwang and Sheen, 2001; Hutchison et al., 2006; **Figure 1**) and serve as a substrate for the phosphorylation of the terminal phosphate acceptors, the nuclear-located type-B ARABIDOPSIS RESPONSE REGULATORS (B-ARRs). Once the type B-ARRs are phosphorylated, inhibition on their GARP DNA-binding domain is released. This initiates the transcription of cytokinin-regulated genes, including the type-A *RESPONSE REGULATORS* (*A-ARRs*), the cytokinin primary-response genes, which also function as negative feedback regulators of the signaling pathway (Kieber and Schaller, 2018). In tandem with type-B ARRs, CYTOKININ RESPONSE FACTORS (CRFs) – closely related members of the Arabidopsis *APETALA 2/ETHYLENE RESPONSE FACTOR* (*AP2/ERF*) gene family, mediate a large fraction of the transcriptional response to cytokinins, affecting a set of cytokinin-responsive genes that largely overlaps with targets of type-B ARRs (Rashotte et al., 2006). For more details we would like to point the reader to some excellent comprehensive reviews, e.g., those by Zürcher and Müller (2016) or Kieber and Schaller (2018).

Canonical Ethylene Signaling and Its Crosstalk With Multistep Phosphorelay

Ethylene is recognized by sensors belonging to subfamily 1, consisting of ETHYLENE RESPONSE 1 (ETR1) and ETHYLENE RESPONSE SENSOR 1 (ERS1) with a functional HK domain, and the HK-like sensor Ser/Thr kinases ETR2, ERS2, and ETHYLENE INSENSITIVE 4 (EIN4), that make up subfamily 2 (Etheridge et al., 2006). Ethylene binds to the hydrophobic pocket of the receptors via a copper cofactor, delivered there by the action of copper transporter, RESPONSE TO ANTAGONIST 1 (RAN1); (Schaller and Bleecker, 1995; Hirayama et al., 1999; Binder et al., 2010). Ethylene binding causes inactivation of both the receptor and the downstream Raf-like Ser/Thr protein kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a negative regulator of ethylene signaling (Kieber et al., 1993; Huang et al., 2003). In the absence of ethylene (in air), CTR1 phosphorylates and inhibits ETHYLENE INSENSITIVE 2 (EIN2), an ER membrane-localized Nramp homolog and

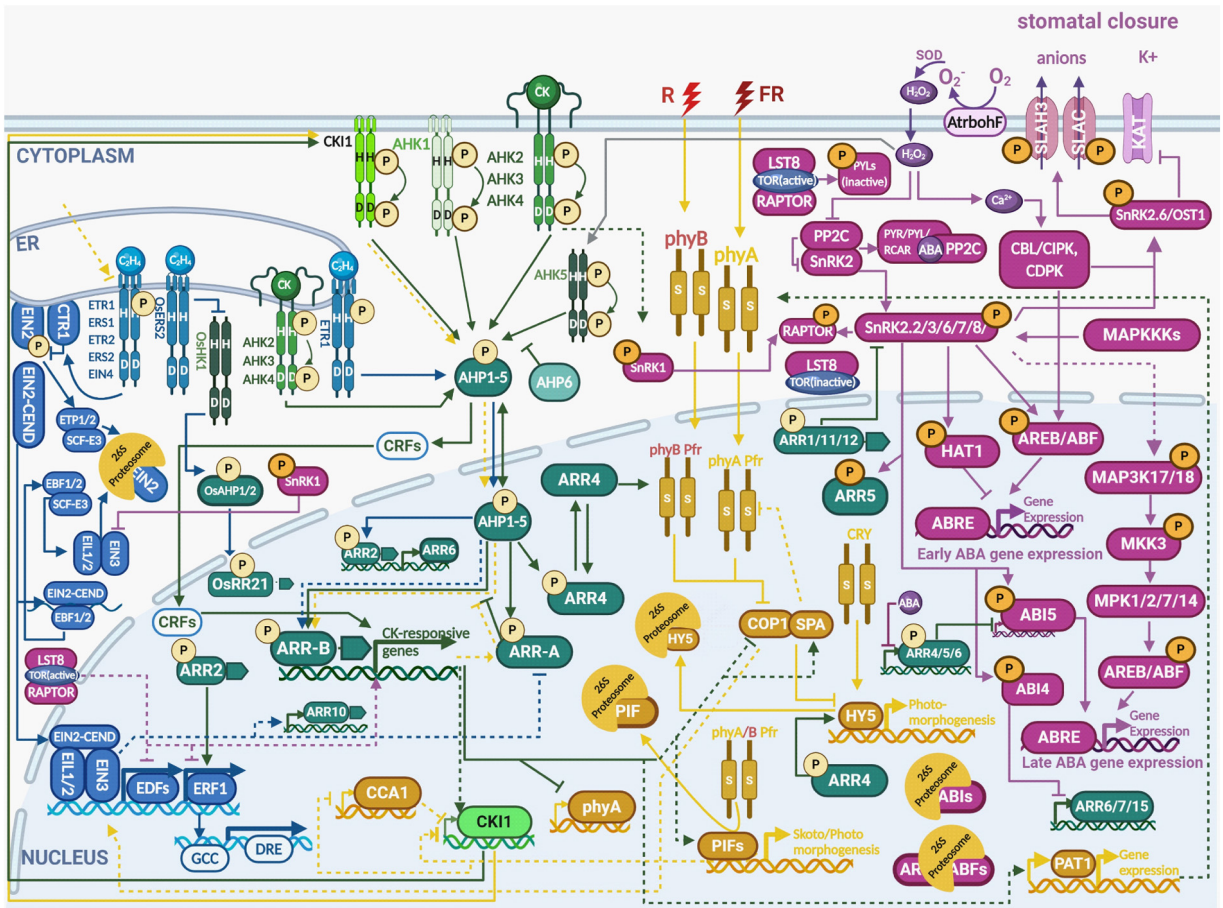


FIGURE 1 | Multistep phosphorelay integrates hormonal and environmental signaling during abiotic stress responses. The output of multistep phosphorelay is affected by crosstalk with a number of individual signaling pathways. The crosstalk takes place either at the level of AHPs phosphorylation by the receptors with varying signal specificities or through interaction of the response regulators (both A-ARRs and B-ARRs) with various transcription factors [e.g., ELONGATED HYPOCOTYL 5 (HY5), CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTORS (ABFs)], as well as SNF1-RELATED PROTEIN KINASE 2 (SnRK2) and phytochromes. See the main text for a detailed description of each signaling pathway. Color code: MSP signaling is depicted in green, ethylene signaling in blue, light signaling in ochre, abscisic acid in purple. The His/Asp phosphorylation is depicted by circled P in bright yellow, Ser/Thr/Tyr phosphorylation in orange. Created with BioRender.com.

a positive regulator of ethylene responses (Ju et al., 2012). That allows Skp1/Cullen/F-box (SCF) E3 ubiquitin ligase complexed with the EIN2-TARGETING PROTEIN 1 (ETP1) and ETP2 F-box proteins to target EIN2 for 26S proteasome-mediated degradation (Qiao et al., 2009). In parallel, the TFs and positive regulators of ethylene-induced gene expression EIN3, ETHYLENE-INSENSITIVE3-LIKE 1 (EIL1) and EIL2 are targeted for ubiquitination by an EBF1- and EBF2- (F-box proteins) containing SCF E3 complex (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Binder et al., 2007; An et al., 2010). The ethylene-induced inactivation of all ethylene sensors leads to the attenuation of CTR1 kinase activity, dephosphorylation of EIN2 and cleavage of its C-terminus (EIN2-CEND). In the cytoplasm, EIN2-CEND inhibits translation of EBF1 and EBF2 by targeting their mRNAs to the processing bodies and subsequent degradation (Li et al., 2015; Merchante et al., 2015). This then upregulates the levels of their targets EIN3, EIL1 and EIL2. EIN2-CEND is also translocated to the nucleus (Qiao et al., 2012; Wen et al., 2012), where it recognizes EIN2 NUCLEAR ASSOCIATED PROTEIN 1 (ENAP1) and promotes EIN3 binding to specific (ENAP1-recognized) chromatin regions by upregulating histone acetylation (Zhang et al., 2017). Specific histone posttranslational modifications seem to be important for ethylene-regulated gene expression (Binder, 2020; and references therein). EIN2-CEND-stabilized EIN3, EIL1 and EIL2 upregulate the expression of ethylene induced genes, including the TFs ETHYLENE RESPONSE DNA BINDING FACTOR 1 (EDF1), EDF2, EDF3 and EDF4 and ETHYLENE RESPONSE FACTORs (ERF)s. Activated ERFs initiate a transcriptional cascade leading to the activation of many ethylene-responsive genes by binding to specific *cis*-acting GCC box and DRE elements depending on whether the stress conditions are abiotic or biotic (reviewed in Müller and Munné-Bosch, 2015). For more

detailed information about the ethylene signaling pathway, see the recent review by Binder (2020).

The fact that two ethylene sensors belong to HKs indicates possible signaling crosstalk with the MSP pathway. However, ethylene sensors from both subfamilies mediate ethylene signal transduction primarily through the CTR1/EIN2/EIN3 pathway and it took nearly two decades until evidence pointing to intense cytokinin/ethylene signaling crosstalk was revealed (**Figure 1**). ETR1 (containing a receiver domain) was shown to interact with AHP1, AHP2, AHP3 and AHP5 (Urao et al., 2000; Scharein et al., 2008; Zdarska et al., 2019). ETR1-dependent phosphorylation of type-B ARR2 and activation of the type-A ARR6 promoter was demonstrated in Arabidopsis protoplasts (Hass et al., 2004; Cho and Yoo, 2007). The *arr2* mutant is less sensitive to treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) probably due to a positive effect of ARR2 on the expression of *ERF1,* an ethylene-responsive member of the ERF/AP2 transcription factor family (Hass et al., 2004). A recent study showed that ETR1-mediated ethylene signaling controls RAM size via ARR3 independently of EIN2 and in a distinct way from canonical CTR1/EIN2/EIN3 signaling (Street et al., 2015; Zdarska et al., 2019). Importantly, canonical ethylene signaling seems to control the sensitivity of MSP to cytokinins, possibly via ethylene-regulated expression of type-B *ARR10* (Zdarska et al., 2019). In rice, the ethylene receptor OsERS2 physically interacts with cytosolic HK MHZ1/OsHK1 (homolog of Arabidopsis AHK5, see below) through its GAF domain in an ethylene-dependent manner and this interaction inhibits MHZ1/OsHK1 kinase activity. OsHK1 phosphorylates the response regulator OsRR21 via the phosphotransfer proteins OsAHP1 and OsAHP2, which are both required for ethylene-mediated root-growth inhibition (Zhao et al., 2020). Considering the strong regulation of ethylene biosynthesis by cytokinins (reviewed in Zdarska et al., 2015) MSP output must be considered as a combined output of both cytokinin and ethylene signals.

Crosstalk Between Light Signaling and Multistep Phosphorelay

Light is an essential environmental factor influencing plant development and adaptive responses. Photochemically active light receptors such as phytochromes, cryptochromes, phototropins, ZEITLUPE (ZTL) and the UV-B RESISTANCE 8 (UVR8) family proteins affect plant behavior in response to both quantity and quality of light. The individual signaling branches mediating responses to a wide range of wavelengths as well as their interplay has been extensively studied (e.g., Quail et al., 1995; Rockwell et al., 2006; Li et al., 2011; Wang Q. et al., 2018). Since light signaling is a very broad topic, we focus here only on the direct interaction of phytochrome-mediated light signaling components with MSP.

Phytochromes are light-regulated Ser/Thr kinases, mediating plant response to red (R) and far-red (FR) light. Phytochromes are synthesized as light-insensitive apoproteins and the functional, i.e., light photoswitchable holoproteins (phys) are assembled following binding with the light-sensitive, linear tetrapyrrole chromophore cofactor phytochromobilin, that is imported to the cytosol from plastids (Terry et al., 2002; Wang, 2015). Upon absorbing R-light the phytochromes switch from the inactive (R-light absorbing) Pr form to the active (FR-light absorbing) Pfr form. The only phy activated by FR (thanks to partial overlap between Pr and Pfr absorption spectra) in Arabidopsis is phyA (Casal et al., 2014; Ballaré and Pierik, 2017). Activated phys translocate to the nucleus either alone [as for phytochrome B (phyB)] or complexed to phy-transporting proteins (as in the case of phyA). The nuclear-located phys control light-responsive genes through two main routes. The first is based on phosphorylating PHY-INTERACTING FACTORs (PIFs), thus targeting them for subsequent degradation via the $CUL4^{COP1-SPAE3}$ ubiquitin ligase (Zhu et al., 2015). PIFs control both skotomorphogenesis (by activating genes expressed during the dark phase) and photomorphogenesis (by inhibiting light-induced genes). The second pathway is controlled by phytochrome-mediated inhibition of transcriptional repressors CONSTITUTIVE PHOTOMORPHOGENIC (COP), DE-ETIOLATED (DET), and FUSCA (FUS; Huang et al., 2014). COP1 interacts with SUPPRESSOR OF PHYA (SPA) proteins, forming an E3 ubiquitin ligase complex that targets light-responsive TFs for 26S proteasome-mediated degradation. Activated phys inhibit the COP1-SPA, allowing accumulation of photomorphogenesis-promoting TFs including ELONGATED HYPOCOTYL 5 (HY5) (Huang et al., 2014; Lu et al., 2015; Sheerin et al., 2015; **Figure 1**). More details on the complex issue of phytochrome signaling and its interaction with hormonal signaling can be found in excellent reviews by Wang (2015); Xu et al. (2015) and De Wit et al. (2016).

One of the first pieces of evidence for direct crosstalk between MSP and light signaling was presented by Sweere et al. (2001). ARR4, a type-A ARR, interacts with phyB and stabilizes its active Pfr form, preventing dark reversion to the inactive Pr form, thus making plants more sensitive to red light (Sweere et al., 2001). ARR4 action on phyB and ARR4-controlled regulation of photomorphogenesis is dependent on its conserved phosphorylatable Asp 95, and this ARR4-mediated stabilization of phyB is reversed in the presence of cytokinins, providing a direct functional link between MSP and light signaling (Mira-Rodado et al., 2007).

Expression of the constitutively active HK *CKI1* was found to be under the control of phyA, probably via PHY-INTERACTING FACTOR 1 (PIF1) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1). Plants defective in phyA and HEME OXYGENASE1 (HO1), whose product is involved in the production of phytochromobilin, show misregulation of *CKI1* expression, associated with attenuation of MSP responsiveness to cytokinins and occurrence of cytokinin-related phenotypes (Dobisova et al., 2017). Light-controlled phyA-dependent transcriptional regulation of *CKI1* expression is a conceptually novel signaling mechanism in plants. Here, an environmental regulatory signal (light) controls the activity of (MSP) signaling pathway by transcriptional regulation of the constitutively active HK, thus controlling MSP sensitivity to its primary signal, cytokinin (Dobisova et al., 2017). On the other hand, exogenous cytokinins were reported to negatively modulate phyA expression (Cotton et al., 1990; Brenner et al., 2012).

The negative impact of cytokinins on phyA transcription is fine-tuned by up-regulating both PHYTOCHROME A SIGNAL TRANSDUCTION 1 (PAT1), a positive regulator of phyA signaling and SPA1, its negative regulator (Brenner et al., 2005, 2012). Furthermore, cytokinins suppress the function of the E3 ubiquitin-protein ligase COP1, which mediates ubiquitination and subsequent proteasomal degradation of HY5, associated with repression of photomorphogenesis in darkness (Srivastava et al., 2015) and increased accumulation of the bHLH family proteins PIFs and EIN3 (Alabadí and Blázquez, 2008; Zhong et al., 2009; **Figure 1**). HY5 acts as a downstream intermediate in the CRYPTOCHROME 1 (CRY1; photoreceptor absorbing blue light) signaling pathway (Vandenbussche et al., 2007). Both the cytokinin (via ARR4) and CRY pathways upregulate HY5 protein levels that consequently regulates the transcription of many genes by binding directly to their *cis*-regulatory elements. Thus, regulation of the stability of HY5 protein has been suggested to be a point of convergence between both signaling pathways (Shin et al., 2007, 2013; Vandenbussche et al., 2007; Gangappa and Botto, 2016).

Apart from the mutual light-MSP interactions, a positive role was shown for light in ethylene signaling, as well as light-dependent stimulation of ETR1 and EIN4 expression. On the other hand, *ETR2* and *ERS2* gene expression was attenuated during the exposure to light (Grefen et al., 2008). We saw earlier how the ethylene receptor (ETR1) and downstream MSP components (AHP1, AHP2, AHP3, and AHP5) (Hass et al., 2004; Scharein et al., 2008; Street et al., 2015; Zdarska et al., 2019; Zhao et al., 2020) are linked. Thus, the light-controlled expression of ethylene sensor-encoding genes might represent another way of MSP-mediated integration of light with plant hormone signaling pathways.

Histidine Kinases Not Responsive to Hormone Signals

In *Arabidopsis thaliana*, several histidine kinases have been identified, namely AHK1, AHK5, and CYTOKININ-INDEPENDENT 1 (CKI1) that do not mediate hormone responses (**Figure 1**). The sensory histidine kinase AHK1 was previously shown to transduce the external osmolarity signal (Urao et al., 1999; Tran et al., 2007) and its exact role in cytokinin signaling has not been elucidated. CKI1 was identified by Kakimoto (1996) using activation mutagenesis, and was proposed to act as a cytokinin sensor. Later on, however, CKI1 was found to be a constitutively-active HK, activating MSP signaling in a cytokinin-independent fashion (Hwang and Sheen, 2001; Yamada et al., 2001; Hejátko et al., 2009), and controlling many aspects of plant development including root growth, vascular tissue formation in the inflorescence stem and female gametophyte development (Hwang and Sheen, 2001; Pischke et al., 2002; Hejátko et al., 2003, 2009; Deng et al., 2010; Yuan et al., 2016; Liu et al., 2017). AHK5 has been shown to play a key role in integrating multiple signals in guard cells – independent of ABA signaling via H_2O_2 homeostasis – possibly acting as redox sensor (Desikan et al., 2008). In terms of expression patterns, *CKI1* is expressed in female gametophytes, developing seeds, shoot apical meristem, shoot and root vasculature and the lateral root cap, whereas *AHK1* transcript levels are most abundant in roots and is transcriptionally regulated by osmotic stress (Urao et al., 1999; Pischke et al., 2002; Hejátko et al., 2003, 2009; Dobisova et al., 2017). The AHK5 transcript was present in roots, flowers and siliques, and at low levels in leaves, where it plays a unique role in stomatal signaling (Desikan et al., 2008; Horak et al., 2011). Several researchers have shown that these non-hormone responsive HKs are necessary for modulating the plant responses to stress, and is described in detail below in Section "Sensing Abiotic Stresses via Multistep Phosphorelay."

Canonical ABA Signaling

Abscisic acid has been known for decades as an essential plant hormone for plant development and stress responses; for instance, ABA is elevated when plants suffer from decreased water potential, as well as during seed maturation and fruit ripening (Wright, 1977; Rodríguez-Gacio Mdel et al., 2009). The ABA signaling network includes the receptors PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE (PYLs)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCARs), which, upon ABA binding, form a complex with TYPE 2C PROTEIN PHOSPHATASES (PP2Cs), resulting in the PP2C inactivation (Ma et al., 2009; Park et al., 2009). This, in turn, releases members of SNF1-RELATED PROTEIN KINASEs 2 (SnRK2s) subfamily (see below), which get activated by autophosphorylation (Belin et al., 2006) and further phosphorylate several transcription factors such as ABA INSENSITIVE 4 (ABI4), ABI5, ABA-RESPONSIVE ELEMENT BINDING FACTORS (ABFs) as well as membrane ion channel proteins like SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1) and potassium channel protein 3-KETO-ACYL-COA THIOLASE 1 (KAT-1) as well as MITOGEN-ACTIVATED PROTEIN KINASE1 (MPK1) and MPK2 (reviewed in Fujita et al., 2013; Yu et al., 2016; Dejonghe et al., 2018) (**Figure 1**). Based on their ability to be activated by ABA, SnRK2s could be divided into three subclasses: Subclass I – not responsive to ABA (SnRK2.1, SnRK2.4, SnRK2.5, SnRK2.9, and SnRK2.10), Subclass II - weakly activated by ABA (SnRK2.7 and SnRK2.8) and Subclass III - strongly activated by ABA (SnRK2.2, SnRK2.3, and SnRK2.6) (Ng et al., 2014). Subclass III kinases are essential components of ABA signaling pathway, as indicated by impaired ABA responses in triple mutants lacking SnRK2.2, SnRK2.3, and SnRK2.6 (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009).

Another regulatory circuit affecting SnRK activity employs TARGET OF RAPAMYCIN (TOR) kinase (Wang P. et al., 2018; Belda-Palazón et al., 2020). Under unstressed (growth favorable) conditions, TOR signaling complex represses ABA-mediated stress signaling via phosphorylation of PYL receptors. This phosphorylation leads to SnRK2 kinase inactivation, which associates with disruption of PYL and PP2C phosphatase effector function, thus promoting plant growth. In the presence of stress, ABA signaling represses function of TOR complex via phosphorylation of RAPTOR, the regulatory subunit of TOR, leading to stress responses and growth inhibition (Wang P. et al., 2018). Interestingly, the growth promoting role has

been attributed to SnRK2s, too. In the absence of ABA, SnRK2s-containing complexes recognize SnRK1, a negative regulator of TOR. That allows TOR activation and growth under non-stress (growth-promoting) conditions (Belda-Palazón et al., 2020). SnRK1, via its α subunit, has been shown to interact and phosphorylate RAPTOR1B, one of two *Arabidopsis* RAPTOR/KOG1 homologs, which seems to control energy-demanding processes including translation. Surprisingly, SnRK1 might also be involved in regulating photosynthesis via phosphorylaton of chloroplast proteins (Nukarinen et al., 2016). The ability of SnRK1 to act upstream of TOR has been also proposed in stress- (nutrient or energy deficiency) induced autophagy (Soto-Burgos and Bassham, 2017).

The ABA-signaling pathway is an essential regulator of stomatal movement which is either dependent or independent on Ca^{2+} levels (McAinsh and Hetherington, 1998; Webb et al., 2001; Siegel et al., 2009). Previously was reported that increases in Ca^{2+} are the components of the guard cell ABA-nuclear as well as ABA-turgor signaling pathway (Webb et al., 2001). However, part of Ca^{2+} signaling components are found in the guard cells to act as a separate Ca^{2+}-independent ABA signaling pathway (Allan et al., 1994; Blatt and Grabov, 1997; Webb et al., 2001). Ca^{2+} elevation can accelerate the stomata closure by enhancing plasma membrane SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) and cytoskeletal rearrangement (Waidyarathne and Samarasinghe, 2018). Nevertheless, Ca^{2+} increase seems not to be the only essential component of this process, thus the exact role of Ca^{2+} in ABA signaling needs to be explained (Waidyarathne and Samarasinghe, 2018).

Similarly to cytokinin and ethylene, ABA responses also include chromatin-mediated control of gene expression by regulation of chromatin-remodeling complexes by, for instance, the SWI/SNF subgroup complexes forming around the chromatin-remodeling ATPase BRAHMA (BRM) (Chinnusamy and Zhu, 2009; Yaish et al., 2011; Han and Wagner, 2014; Han et al., 2015). SnRK2s and PP2Cs directly target conserved phosphorylation site in the C-terminal region of BRM (Peirats-Llobet et al., 2016). Whereas PP2CA dephosphorylates BRM, likely as a repressive mechanism of BRM-dependent modulation of ABA responses, SnRK2s phosphorylate BRM to release BRM-mediated repression of *ABI5* expression (Peirats-Llobet et al., 2016).

Since ABA interacts tightly with MSP signaling during the abiotic stress response, the mutual crosstalk might occur at several levels including regulation of phosphorylation of individual signaling components as well as regulation of dynamic chromatin state (Chinnusamy and Zhu, 2009; Yaish et al., 2011; Han and Wagner, 2014). These interactions are described further in the text.

Altogether, the MSP pathway is not just the cytokinin signaling transduction system, but it acts as a signaling hub, integrating both intrinsic (mostly hormonal) regulation with that from environmental signals, including light, osmoregulation, and temperature (**Figure 1**). The importance of integration of cytokinin, ethylene, ABA, and light signaling pathways is what underlies the ability of plants to display such plasticity in continual adjustment to the changing environment.

SENSING ABIOTIC STRESSES VIA MULTISTEP PHOSPHORELAY

Stress, in general, affects the metabolism of cytokinins, ABA, and ethylene, which in turn interact with specific kinases to regulate many stress-related biological processes ranging from regulation of shoot growth under stress to stomata movement. Drought results in the reduction of cytokinin content by modulating cytokinin metabolism and stimulating ABA and ethylene contents in a wide range of terrestrial plants (e.g., Dobra et al., 2010; Guo and Gan, 2011; Nishiyama et al., 2011; Macková et al., 2013; Todaka et al., 2017; **Figure 2**). Even though there is a correlation between hormone metabolism and MSP activity, a significant set of stress responses take place independently of the hormonal pool (O'Brien and Benková, 2013). In this review, we focus on the role of individual MSP components and molecular links in pathways for abiotic stress responses (**Table 1**). Detailed descriptions of the stress-related changes in hormonal metabolism are left out as they are beyond the scope of this review.

Hormonal Crosstalk in Drought, Osmotic and Cold Stress

Abscisic acid mediates one of the fastest plant adaptations to drought, i.e., stomata closure to control the trade-off between CO_2 uptake and water loss by transpiration (Sah et al., 2016; Vishwakarma et al., 2017). However, ABA controls also mid- and long-term plant adaptations to abiotic stresses including control of plant architecture, wherein ABA tightly interacts with cytokinins. The current model predicts that cytokinin-regulated MSP attenuates the expression of ABA-inducible genes involved in the stress response. Stress-induced ABA accumulation in turn downregulates cytokinin biosynthesis via the MYB2 TF, relieving the repression on MSP and activation of ABA- and stress-inducible genes (Li et al., 2016). ABA-mediated inhibition of cytokinin signaling initiates reshaping of the plant body by downregulating growth in the shoot but accelerating root growth. That allows the plant to diminish water loss while increasing water uptake from deeper layers of the soil (Li et al., 2016). In line with that, MSP signaling mutants, including mutants defective in cytokinin sensors AHK2, AHK3, and AHK4 and type-B ARRs ARR1, ARR10, and ARR16 are hypersensitive to ABA and show higher drought resistance (Tran et al., 2007, 2010; Nguyen et al., 2016). Both ABA and drought were shown to downregulate expression of *ARR1, ARR10,* and *ARR12* (Nguyen et al., 2016). ABA also downregulates *ARR2* but not *AHK3* or *AHK4* (Takatsuka and Umeda, 2019), and a possible role for ABA in the control of nucleocytoplasmic partitioning of AHP2 was reported (Marchadier and Hetherington, 2014). Interestingly, the osmosensor AHK1 is not a negative, but rather a positive regulator of the ABA-mediated stress response (Tran et al., 2007), suggesting certain specificity at the level of signals initiating MSP-regulated (drought) stress response (Hai et al., 2020).

The negative interaction between ABA and MSP activity is mediated not only at the level of ABA-controlled downregulation of cytokinin biosynthesis, but also at the level of interaction

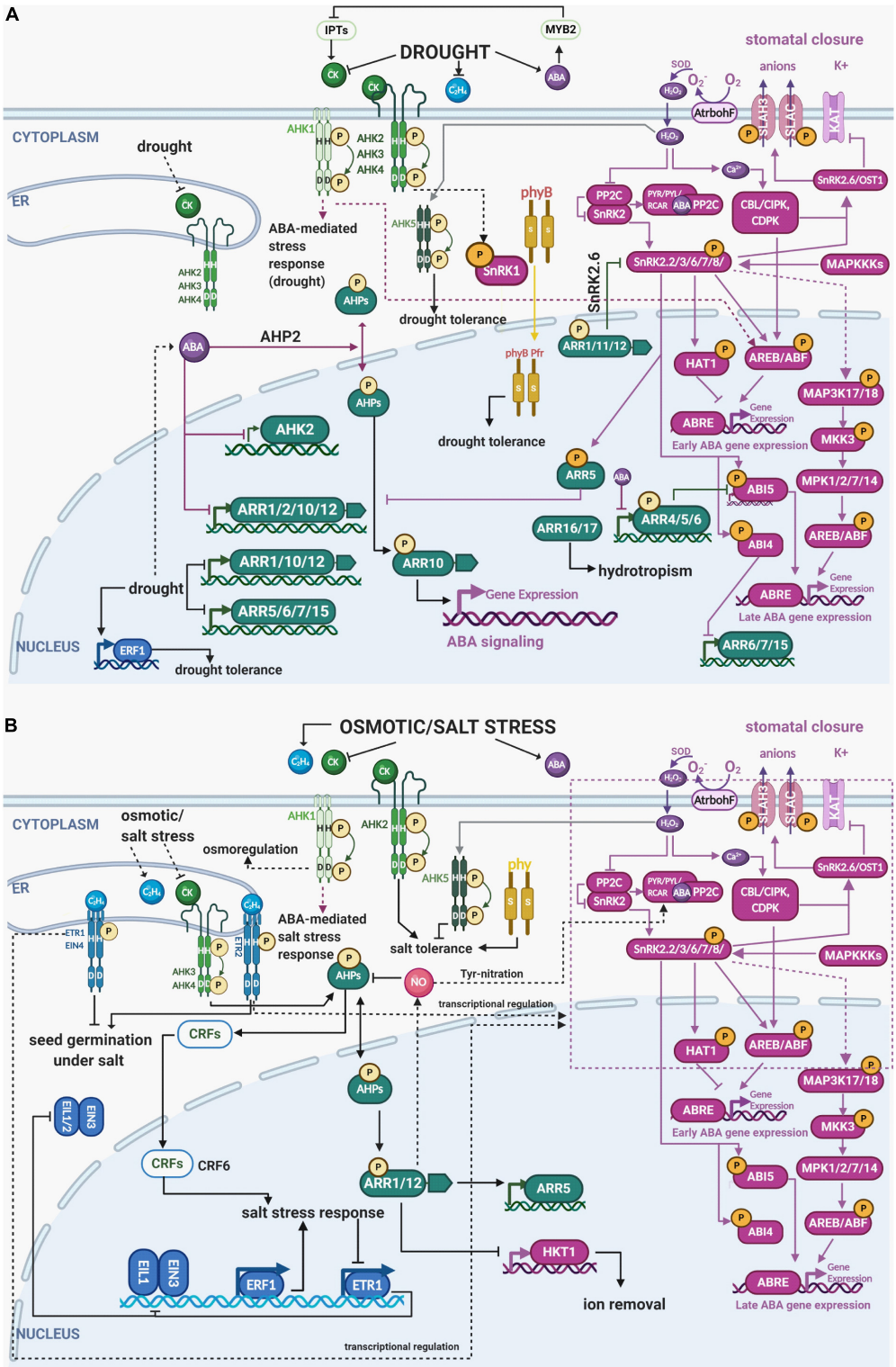


FIGURE 2 | Integration of the different components of ethylene, cytokinin, ABA and light signaling in multistep phosphorelay during drought and osmotic/salt stress. **(A)** Drought stress stimulates ABA and ethylene content while reducing the endogenous levels of cytokinins. Both cytokinin-responsive HKs AHK2, AHK3, and AHK4 as well as cytokinin-independent AHK1 and AHK5 contribute to the drought-regulated MSP activity. Strong crosstalk (mostly mutually negative) occurs between MSP and canonical ABA signaling. Ethylene response factors (ERFs) are also induced and play mostly positive role in stress responses. **(B)** Like drought, osmotic/salt stress also stimulates ABA and ethylene production, thus activating ABA and ethylene signaling components, while downregulating cytokinin

(Continued)

FIGURE 2 | Continued
levels and the expression of its downstream signaling components. Also, expression of *ETR1* is reduced, enabling the continued activity and participation of downstream components of ethylene signaling (EIL1, EIN3, and ERFs) in the activation of different response factors necessary for the osmotic/salt stress response. Phytochromes are also implicated to be involved in drought and osmotic/salt stress responses, particularly phyB which was shown to enhance osmotic and drought tolerance. See the main text for a detailed description of each signaling pathway and their crosstalk in response to stress. Color code: MSP signaling is depicted in green, ethylene signaling in blue, light signaling in ochre, abscisic acid in purple. The His/Asp phosphorylation is depicted by circled P in bright yellow, Ser/Thr/Tyr phosphorylation in orange. Created with BioRender.com.

TABLE 1 | Role of MSP components from *Arabidopsis thaliana* L. in various environmental stimuli.

Gene ID	AGI code	Description	Regulatory function in stress response	Type of stress	Regulatory function in ABA response	References
AHK1	AT2G17820	Histidine kinase	Positive	Drought, salt	Positive	Tran et al., 2007
AHK2	AT5G35750	Cytokinin receptor kinase	Negative	Cold, drought, salt	Negative	Tran et al., 2007; Jeon et al., 2010
AHK3	AT1G27320	Cytokinin receptor kinase	Negative	Cold, drought, salt	Negative	Tran et al., 2007; Jeon et al., 2010
AHK4/ CRE1	AT2G01830	Cytokinin receptor kinase	Negative	Salt, drought	Negative	Tran et al., 2007
AHK5	AT5G10720	Histidine kinase	Negative	Drought, salt		Desikan et al., 2008; Pham and Desikan, 2012
ARR1	AT3G16857	Transcription factor (Type B-ARRs)	Positive	Cold	Unknown	Jeon and Kim, 2013
ARR1, ARR12	AT3G16857, AT2G25180	Transcription factors (Type B-ARRs)	Negative	Salt	Unknown	Mason et al., 2010
ARR1, ARR10, ARR12	AT3G16857 AT4G31920 AT2G25180	Transcription factors (Type B-ARRs)	Negative	Drought	Negative	Nguyen et al., 2016
ARR5	AT3G48100	Primary response genes (Type A-ARRs)	Negative Positive	Drought, salt	Positive Positive	Huang et al., 2018 Mason et al., 2010
ARR7	AT1G19050	Primary response genes (Type A-ARRs)	Negative	Cold, drought	Negative	Jeon et al., 2010
ARR5	AT3G48100	Primary response genes (Type A-ARRs)	Negative	Cold	None	Jeon et al., 2010
ARR6	AT5G62920	Primary response genes (Type A-ARRs)	Negative	Cold	Negative	Jeon et al., 2010
ARR7	AT1G19050	Primary response genes (Type A-ARRs)	Negative	Cold	Negative	Jeon et al., 2010
ARR16 ARR17	AT2G40670 AT3G56380	Primary response genes (Type A-ARRs)	Positive	Root hydrotropism	–	Chang et al., 2019

between components of canonical ABA signaling and MSP. ABA-activated ABI4 binds the promoters and downregulates *ARR6, ARR7,* and *ARR15*; single and multiple mutant lines *arr4*, *arr6, arr7,* and *arr15* were shown to be hypersensitive to ABA (Jeon et al., 2010; Wang et al., 2011; Huang et al., 2017). SnRK2.2, SnRK2.3, and SnRK2.6 phosphorylate several Ser residues of ARR5, the type-A ARR and negative regulator of MSP signaling. This leads to stabilization of the ARR5 protein, thus enhancing ABA response and drought tolerance by suppressing the cytokinin signaling (Huang et al., 2018; **Figure 2**). On the other hand, the phosphoproteomic study performed by Dautel et al. (2016) proposed an AHK2/AHK3-dependent phosphorylation of Thr6 and Tyr19 of KIN10, one of the two subunits of SnRK1, acting in energy stress (Baena-González and Sheen, 2008). SnRK1 down-regulation has been previously linked to cytokinin and auxin signaling based on global gene regulation by KIN10 (Radchuk et al., 2006; Baena-González et al., 2007), whereas ethylene signaling was negatively regulated by SnRK1 phosphorylation-mediated inactivation of EIN3 (Kim et al., 2017) leading to a growth-defense trade-offs model via cell cycle regulations (Eichmann and Schäfer, 2015; Filipe et al., 2018). The negative relationship between ABA and cytokinin levels/signaling is bi-directional. Upregulation of cytokinin biosynthesis via upregulation of *AtIPT8* resulted in ABA insensitivity in seed germination (Wang et al., 2011). Furthermore, when endogenous cytokinins are elevated, ABA was unable to downregulate genes for type-A ARRs ARR4, ARR5, and ARR6 that physically interact with ABI5 and downregulate *ABI5* expression (Wang et al., 2011). The lower sensitivity to ABA under high endogenous cytokinin levels is likely mediated by the cytokinin-responsive type-B ARRs ARR1, ARR11, and ARR12 that physically interact with SnRK2s and repress the kinase activity of SnRK2.6 (Huang et al., 2018).

At the transcriptional level, the cytokinin-dependent regulation of ABA signaling might be mediated through ARR10, which was found to bind promoters of numerous members of ABA signaling (Zubo et al., 2017). Since canonical ABA signaling stimulates the expression of many transcription factors essential for stress responses and seed development (reviewed in Nakashima and Yamaguchi-Shinozaki, 2013; Kumar et al., 2019), the interaction with MSP components contributes to fine-tuning of the final stress response and regulates developmental processes (Kieber et al., 1993; Clark et al., 1998; Ju et al., 2012).

In the response to salt stress, cytokinins act through ARR1 and ARR12 to repress expression of the Arabidopsis HIGH-AFFINITY K^+ TRANSPORTER 1;1 (AtHKT1;1) which is responsible for removing sodium ions from root xylem. Cytokinins were also shown to regulate salt stress−induced expression of the type−A response regulator *ARR5* predominantly via ARR1 and ARR12, revealing the action of particular MSP components in the roots to control sodium accumulation in the shoots (Mason et al., 2010).

Interestingly, cold transiently stimulates the expression of *ARR5, ARR6, ARR7,* and *ARR15* similarly to dehydration (Jeon et al., 2010; Kang et al., 2012), most probably to attenuate cytokinin signal transduction and suppress growth. Shi et al. (2012) found that ethylene biosynthesis and signaling negatively regulates the freezing stress response in *Arabidopsis* by repressing cold-inducible C-REPEAT BINDING FACTORs (CBFs) and the type-A ARR genes *ARR5, ARR7,* and *ARR15*. This ethylene-mediated repression was supposed to be mediated by direct binding of EIN3 to the promoters of type-A ARRs, thus potentially representing another mechanistic link between canonical ethylene signaling and MSP during plant desiccation (**Figures 2**, **3**).

Hormone-Independent Abiotic Stress Responses

AHK1 gain-of-function mutations revealed that the osmo-responsive AHK1 was a positive regulator of drought and salt stress responses acting upstream of ABA signaling (Tran et al., 2007) (**Figure 2**). Disturbed up-regulation in the *ahk1* mutant of many stress- or ABA-inducible genes, including ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING PROTEIN 1 (AREB1), NAC DOMAIN CONTAINING PROTEIN (ANAC), and DRE-BINDING PROTEIN 2A (DREB2A) transcription factors and their downstream genes may explain the increased stress tolerance of *AHK1* overexpressing plants (Tran et al., 2007). Moreover, AHK1 was shown to control stomatal density as well as the transcription of several stress-responsive genes. *ahk1* mutants have a higher transpiration rate and reduced ABA sensitivity, resulting in higher susceptibility to uncontrolled soil drying (Kumar et al., 2013).

Another experiment showed attenuated expression of *AHK2* in the first hours after exposure to reduced water potential (**Figure 2**; Kumar and Verslues, 2015). The examination of *ahk2-2* mutant lines showed hypersensitive response to salt stress, while root elongation in *ahk3-3* mutants was increased following transfer to low water potential media when compared to wild type (Kumar and Verslues, 2015). These results show the specific role of individual HKs in osmotic stress acclimation and provide evidence for the specificity of individual MSP components to different types of stress.

AHK5 was shown to mediate ABA-independent stomata closure in response to H_2O_2 produced by the NADPH oxidase AtrbohF (Desikan et al., 2006), which is induced by abiotic stimuli including darkness, nitric oxide (NO), and ethylene (Desikan et al., 2008). AHK5 inhibits root elongation by negatively regulating the ABA and ethylene signaling pathways (Iwama et al., 2007). On the other hand, AHK5 was also demonstrated to be a negative regulator of salinity tolerance, while providing plants with drought tolerance by promoting ABA-independent stomatal closure by maintaining H_2O_2 homeostasis in guard cells (Desikan et al., 2008; Pham and Desikan, 2012; **Figure 2**). Moreover, AHK5 is required for full immunity toward the necrotrophic fungus *Botrytis cinerea* and the virulent bacterium *Pseudomonas syringae* pv. tomato DC3000. It thus acts as an integrator of both abiotic and biotic stress responses in *Arabidopsis* (Pham and Desikan, 2012; Pham et al., 2012).

Nitric oxide has been recently characterized as a plant growth regulator involved in the control of many important developmental and adaptive responses, including the abiotic stress response (reviewed e.g., in Sami et al., 2018). Cytokinins induce NO production via MSP signaling, and cytokinin-induced NO contributes substantially toward plant morphological responses to cytokinin (Tun et al., 2008). Importantly, NO was shown to *S*-nitrosylate the conserved Cys 115 of AHP1, thus impairing its ability to mediate phosphotransfer to ARR1. This is an example of a mechanism by which the redox signal is integrated into cytokinin signaling – highlighting the complex coordination underlying plant growth and development (Feng et al., 2013). NO seems to antagonize not only cytokinin, but also ABA signaling. NO biosynthetic mutant *nia1 nia2 noa1-2* is hypersensitive to ABA, leading to highly efficient ABA-mediated control over stomata closure (Lozano-Juste and León, 2010a) and NO production under growth-favorable conditions represses inhibition of seed developmental transitions by ABA (Lozano-Juste and León, 2010b). Increase in NO levels post-translationally modifies members of the ABA receptor PYR/PYL/RCAR family by *S*-nitrosylation or tyrosine nitration at cysteine residues (Castillo et al., 2015). Tyrosine nitration under conditions in which NO is produced was proposed as a rapid mechanism limiting ABA signaling (Castillo et al., 2015). On the other hand, ABA signaling in guard cells includes H_2O_2 and NO production, which seems to be necessary for the ABA-induced stomata closure (Castillo et al., 2015). Thus, NO appears as a multifaceted plant growth regulator mediating direct modifications of key components in several hormone signaling pathways.

The ability of the root to grow toward higher water potential (hydrotropism) is an important physiological adaptation trait of plants to drought. The recent findings of Chang et al. (2019) have uncovered the importance of type-A ARRs in controlling root hydrotropism. ARR16 and ARR17 were found to be upregulated at the side of the root tip that "faces" lower water potential, resulting in increased cell division in the meristem zone and

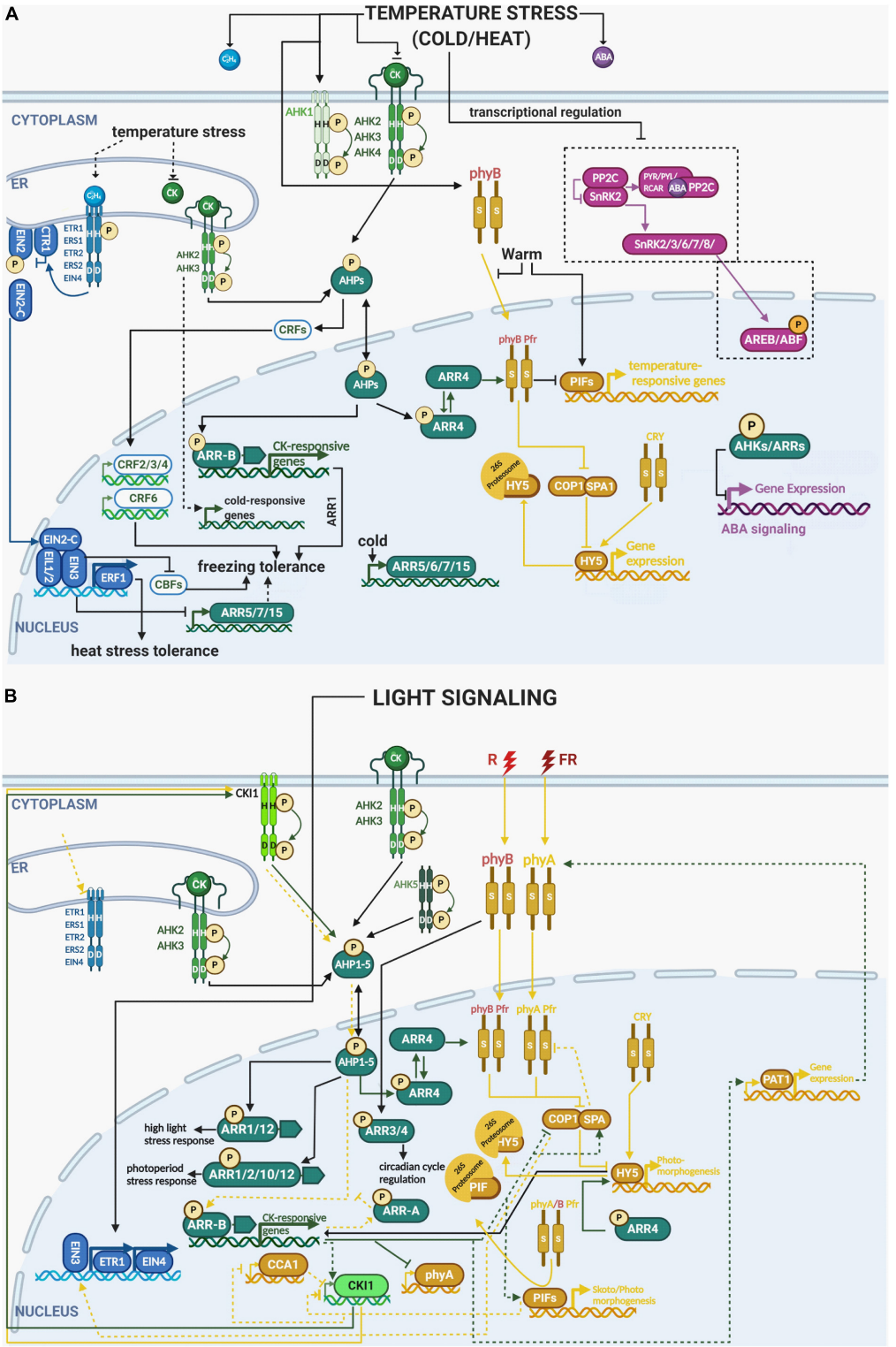


FIGURE 3 | Integration of the different components of ethylene, cytokinin, ABA and light signaling pathways in multistep phosphorelay in temperature and light responses. **(A)** Heat stress: similarly to drought and osmotic stress, heat stress also enhances ABA and ethylene levels while downregulating cytokinin levels and the expression of cytokinin receptors (AHK2, 3, 4). ETR1 and ERF1 play a positive role in heat tolerance; however, downstream components of ABA (PYR/PYL/RCAR, PP2Cs, SnRK2s, and ABFs) are downregulated. **(B)** Cold stress: low temperature reduces the levels of cytokinin, but activates cytokinin receptors (AHKs), putatively in a cytokinin-independent manner. Several downstream components of the cytokinin signaling pathway are also activated (AHP2,3,5, B-ARR 1, A-ARR 5, 6, 7, 15)

(Continued)

FIGURE 3 | Continued
by cold temperature and correlated with enhanced cold tolerance in *Arabidopsis thaliana* (modified from Zdarska et al., 2015). phyB was demonstrated to be a temperature sensor, which interacts with ARR4, the key regulator providing a molecular link between MSP and light (and possibly temperature) signaling. Light response: light can also attenuate the levels of ethylene. ETR1 and EIN4 expression are dependent on light stimulation. ETR1 also genetically interacts with both phyA and phyB that govern germination and the growth response to various light regimes. Cytokinins were also shown to maintain photoprotective mechanisms during high light stress, mediated by AHK2 and AHK3. See the main text for a detailed description of each signaling pathway and their crosstalk in response to stress. Color code: MSP signaling is depicted in green, ethylene signaling in blue, light signaling in ochre, abscisic acid in purple. The His/Asp phosphorylation is depicted by circled P in bright yellow, Ser/Thr/Tyr phosphorylation in orange. Created with BioRender.com.

consequent bending of the roots toward the opposite side – in the direction of higher water potential.

Furthermore, various types of abiotic stresses including cold, osmotic stress, salt, and drought differentially regulate the expression of several individual MSP components (Argueso et al., 2009). As mentioned above, some of these regulations can be attributed to stress-induced ABA, however, the molecular mechanisms behind most of these regulations remain to be clarified.

Ethylene and ABA Crosstalk During Osmotic Stress

Ethylene signaling too affects salt and osmotic stress responses. The receptors ETR1 and EIN4 inhibit, and ETR2 stimulates, seed germination under salt stress (Zhao and Schaller, 2004; Wilson et al., 2014b). It has been demonstrated that seeds of the loss-of-function *etr1-6* and *etr1-7* mutants have a higher germination rate than wild-type seeds in the dark or following FR illumination (that inhibits seed germination in the WT) indicating a negative role for ETR1 in the control of germination (Wilson et al., 2014a). The authors suggest that ETR1 may genetically interact with phyA and phyB to govern germination and growth under various light conditions (Wilson et al., 2014a; **Figure 3**). The ETR1 receptor may modulate ABA sensitivity even when ethylene does not bind to this receptor (Wilson et al., 2014a,b). ETR1 and ETR2 indirectly affect the expression of ABA signaling genes, without a requirement for canonical ethylene signaling (Bakshi et al., 2015, 2018). These findings show that ETR1 and ETR2 act independently of ethylene signaling to affect seed germination under salt stress by interacting with ABA signaling (Wilson et al., 2014b). Another crosstalk between ABA and ethylene was associated with TOR protein kinase (Dong et al., 2015). Inhibition of TOR kinase function led to increased expression of ethylene signaling components such ethylene response factors and ethylene responsive element-binding factors while down-regulating type-A response regulators (Dong et al., 2015). In line with that, TOR protein kinase has been shown to act as a central regulator of cell growth by integrating nutrient, energy, and growth factors in photosynthetic organisms (Schepetilnikov and Ryabova, 2018; Jamsheer et al., 2019).

Early osmotic stress response is integrated by ABA signaling through the hyperosmotic activation of RAF-like mitogen-activated protein kinases (MAPKKKs) that further activate downstream ABA-unresponsive and ABA-activated SnRK2s (Katsuta et al., 2020; Lin et al., 2020; Lozano-Juste et al., 2020; Soma et al., 2020). Fast response to osmotic stress is, however, also mediated by ABA-unresponsive subclass I and III SnRK2s independently to ABA (Boudsocq et al., 2007).

MSP-Mediated Responses to Ambient Temperature Changes

Temperature is another fundamental environmental factor, with 10–30°C being optimum for the growth of most higher plants (Źróbek-Sokolnik, 2012). Temperatures below and above this optimum range are referred to as cold or heat stress, respectively (Larkindale et al., 2007; Miura and Furumoto, 2013). Both extremes may have fatal consequences for the plant (for details see, e.g., Xiong et al., 2002; Sung et al., 2003; Kaplan et al., 2004; Ahuja et al., 2010; Johnová et al., 2016).

In a response to adverse ambient temperatures, plants reprogram their transcriptome, metabolome, and proteome as well as their hormonal status to adapt their growth to the unfavorable temperature conditions (Stavang et al., 2009; Mittler et al., 2012). Cytokinin content is generally reduced by cold shock, which seems to be related to the reallocation of energy resources from growth toward defense. Prolonged cold treatment (for more than 3–7 days) is accompanied by plant acclimation (e.g., in winter wheat cultivars), associated with elevation of cytokinin content (Kosová et al., 2012). This finding is in line with the positive effect of exogenous cytokinins on freezing tolerance of *Arabidopsis* (Jeon et al., 2010; Maruyama et al., 2014). Thus, the role of cytokinins in cold responses and freezing tolerance seems to depend on the severity and phase of the stress response. Additionally, environmental stimuli such as temperature and water stress alter the phosphorylation status of MSP components partly independent of hormonal status suggesting that histidine kinases (and/or thermo-responsive phytochromes, see below) function as abiotic stress sensors, as shown for AHK1 (Tran et al., 2007; Jeon et al., 2010; O'Brien and Benková, 2013).

Even if enhanced freezing tolerance of *ahk2 ahk3* and *ahk3 ahk4* double mutants seems to be in accordance with the decreased cytokinin content under cold stress, it may be related, at least partially, to their low growth rate (Jeon et al., 2010). Cytokinin-independent (but nevertheless requiring functional cytokinin-responsive AHKs) upregulation of genes encoding the type-A response regulators ARR5, ARR6, ARR7, and ARR15 was reported as an early cold stress response (Jeon et al., 2010). Interestingly, the enhanced freezing tolerance observed in *arr7* and *ahk2 ahk3*, *ahk3 ahk4* mutants seems to act independently of the C-REPEAT-BINDING FACTOR/DRE-BINDING FACTORs (CBF/DREBs)-mediated cold acclimation pathway. Based on the hypersensitive response of *ahk2, ahk3,* and *arr7* mutants, as well as the insensitivity of *ARR7* overexpression

line to ABA, the authors propose that the MSP-mediated cold response is mediated via negative regulation of ABA signaling by AHK/ARRs, thus following the general scheme of negative cytokinin/ABA interaction as in the drought response described above (**Figure 1**). Moreover, ARR1 was shown to function as a positive factor for cold signaling, acting downstream of AHK2, AHK3, and AHPs (Jeon and Kim, 2013; **Figure 3**). Interestingly, the majority of cold-responsive genes that are differentially regulated in *ahk2 ahk3* compared to WT, do not seem to be regulated via CBF3 or ARR1, suggesting the existence of yet unidentified (either direct or indirect) regulatory mechanisms, mediating the AHK2/3-dependent cold response (Jeon and Kim, 2013).

The CRF signaling branch of MSP is also involved in the response to cold stress, but also to heat, salt and oxidative stress, as well as elevated hydrogen peroxide content. All these stresses stimulate the activity of the CRF6 promoter. Overexpression of *CRF6* diminished oxidative stress impact, improving photosynthesis and stimulating root growth in *Arabidopsis* and tomato plants (Inzé et al., 2012; Zwack et al., 2013, 2016; Gupta and Rashotte, 2014). Other CRFs were also observed to be regulated by cold, enabling freezing tolerance by either inducing CRF4 or altering lateral root development in response to low temperature through CRF2 and CRF3 (Shi et al., 2014; Zwack and Rashotte, 2015; Jeon et al., 2016).

Light-Related Stress Responses Through Multistep Phosphorelay

Light irradiance is an essential environmental factor influencing a wide range of physiological and growth aspects of the plant, and is tightly linked to plant hormonal status. An intimate link between light and cytokinins has been recognized for a long time now (reviewed in Cortleven and Schmülling, 2015; Zdarska et al., 2015). However, light can also act as a stressor, affecting both abiotic and biotic stress responses in plants (Roeber et al., 2020).

Cytokinins were shown to play an important role in photoinhibition induced by high dose irradiation. AHK2 and AHK3 and their downstream targets ARR1 and ARR12 were suggested to mediate cytokinin-dependent protection of the photosynthetic apparatus during high light stress (Cortleven et al., 2014; Cortleven and Schmülling, 2015). Cytokinins (through AHK3) were also found to modulate a novel type of stress called photoperiod stress, identified in plants with reduced endogenous cytokinin content or disturbed cytokinin signaling, and in circadian clock mutants (Nitschke et al., 2017). Photoperiod stress occurs due to abrupt changes in the alternation of light and dark periods, inducing symptoms of stress in *Arabidopsis* plants, including JA-dependent cell death and downregulation of the key circadian clock regulators CCA1 and LATE ELONGATED HYPOCOTYL [(LHY), (**Figure 3**; Nitschke et al., 2016, 2017). Recently it was found that the root-derived cytokinin *trans*-zeatin, acting via AHK2 and AHK3 in cooperation with AHK5, and the downstream type-B ARRs ARR2, ARR10, and ARR12, has a protective role against photoperiod stress, associated with an oxidative burst-like response (Abuelsoud et al., 2020).

Metaphorically, sensor kinases serve as guards at the gate to the cell or nucleus, enabling the perception of various environmental signals. Besides HKs, acting as an integral part of canonical MSP signaling, other sensor kinases integrated into MSP signaling are phytochromes (see above, **Figure 1**). Besides their major role in the light perception, phytochromes are also involved in osmotic-stress (Balestrasse et al., 2008) and drought responses (Kidokoro et al., 2009; D'Amico-Damião et al., 2015) (**Figure 2**). For instance, phyB was reported to positively affect drought tolerance in Arabidopsis (González et al., 2012). However, in other studies, phytochrome mutants exhibited improved drought tolerance (Liu et al., 2012), altered responses to high temperature (Njimona et al., 2014), and sensitivity to prolonged UV-B light radiation (Boccalandro et al., 2001; Rusaczonek et al., 2015). phys are necessary also for effective cold acclimation (Crosatti et al., 1999). phyB is stabilized at low temperature by the main cold-inducible TFs CBFs (Jiang et al., 2020). As phyB promotes degradation of PIF1, 4, and 5, which (especially PIF4) repress CBF genes at cold stress (Lee and Thomashow, 2012), phyB enhances freezing tolerance. Positive effect on freezing tolerance was found also in the case of phyA, especially at low R:FR ratio (Wang et al., 2016).

Importantly, phyB and its downstream signaling partner PIF7 were found to act also as thermosensors (Jung et al., 2016; Legris et al., 2016; Chung et al., 2020). PIF7 protein abundance was shown to accumulate in response to warm temperature further stimulating the expression of genes involved in thermomorphogenesis such as the auxin biosynthetic gene YUCCA8 (Chung et al., 2020). Interestingly, secondary structure of *PIF7* mRNA forms an RNA hairpin within the 5′UTR region which changes its conformation at higher temperature and stimulating the *PIN7* mRNA translation. This unique temperature-controlled mechanism of protein translation was identified also for WRKY22 and heat-shock regulator HSFA2 suggesting that as a conserved mechanism used by plants as a fast response to sudden temperature changes (Chung et al., 2020). Another rapid temperature-regulated mechanism was identified in case of phyB itself. phyB was postulated to be a temperature sensor in plants through its temperature-dependent reversion from the active Pfr state to the inactive Pr state, which affects its association with the promoters of key target genes in a temperature-dependent manner (Jung et al., 2016; Legris et al., 2016). Here, both temperature-dependent transcriptional regulation of *PIF4* together and phyB-mediated post-transcriptional regulation of PIF4 ability to activate transcription of target genes was proposed (Jung et al., 2016). The active Pfr form of phyB was shown to be stabilized by ARR4 *in planta*, which was confirmed by increased hypersensitivity to red light in *ARR4* overexpressing transgenic lines (Sweere et al., 2001; Fankhauser, 2002; see above). ARR3 and ARR4 seem to control both length of the period and its phase (Salomé et al., 2006). The loss of ARR4 and its closest homolog ARR3, prolongs the circadian cycle period even in the absence of light, suggesting that ARR3 and ARR4 control the circadian period independently

of active phyB through an as yet unknown mechanism. In white light, *arr3* and *arr4* mutants show a leading phase (i.e., an earlier peak in the expression of genes that oscillate with the circadian rhythm) similar to *phyB* mutants, demonstrating that circadian light input is modulated by the interaction of phyB with ARR3 and ARR4. Considering the absence of any correlation between sensitivity to cytokinins in higher-order arr mutants and the period phenotype, as well as the distinct (concentration-dependent) effects of exogenous cytokinins, ARR3 and ARR4 seem to control both the length of the period (independent of phyB) and the phase of the period (through phyB) independently of cytokinins (Salomé et al., 2006). Since phyB acts as a light and temperature sensor and type-A *ARRs* expression responds to temperature fluctuations independently of cytokinin levels (see above), we may say that type-A ARRs could represent an intersection for light and temperature signaling to fine-tune photomorphogenesis under adverse environmental conditions (**Figure 3**).

Considering that stress responses are also modulated by cryptochromes (D'Amico-Damião et al., 2015), further research should be focused on the interconnection between blue light perception and MSP. Data about blue light – MSP crosstalk in abiotic stress responses is rather rudimentary. One of the potential mechanisms of blue light-cytokinin crosstalk might be mutual regulation of HY5 protein levels, as described above (Gangappa and Botto, 2016).

CONCLUSION AND FUTURE OUTLINES

In our opinion one of the most critical roles of plant hormones is to act as a "universal" transducer between a constantly changing environment and control of the immediate physiological and developmental status of the plant. This transduction is then manifested as the final growth and developmental response. Multistep phosphorelay seems to be an ideal mechanism, allowing for the integration of multiple signaling inputs into a molecular response, characterized by quantitative and readily tunable changes in the transcriptional profile. In the light of several recent findings, implicating cytokinin-regulated MSP signaling (through type-B ARRs) in the control over context-dependent chromatin accessibility (Potter et al., 2018), the well-documented (cytokinin-modulated) role of ethylene in histone acetylation and ABA-dependent regulation of chromatin-remodeling complexes (see above), we can venture to say that epigenetic regulations form part of the repertoire of transcription regulatory tools available to MSP (and interconnected signaling pathways), possibly mediating environmental "memory" used by the plants in their adaptation to short- and medium-term environmental fluctuations. In line with this, Samsonová et al. (2020) showed that cytokinins occupy a prominent position among other hormones as unique ecotype identifiers. A positive correlation between mean ABA content and ABA/cytokinin ratio in the shoots with mean temperature at the site of origin is in line with existing data on the involvement of cytokinin signaling in the cold response (see above). This then means that control over cytokinin metabolism is one of the mechanisms involved in long-term adaptation. Also, the large variability in the ABA/cytokinin ratios observed among the ecotypes in both shoots and roots suggests hormonal plasticity, which might contribute to and/or determine stress responses in particular geographical regions (Samsonová et al., 2020).

The conclusions and implications we summarize above point toward a number of important questions. How is the combination of individual inputs translated into the final MSP signaling output? How is signaling fidelity maintained? In other words, how does the plant recognize and discern the particular origin of the signal, given that a number of sensory kinases recognizing the different type of signals result in phosphorylation of AHPs, and act as a signaling hub within the pathway? Is there any variability in the sensitivity of MSP signaling to individual types of MSP inputs associated with ecotype-specific adaptation to the site of its origin? And finally, what are the mechanisms acting downstream of MSP signaling in the stress response, either independently or in concert with other signaling pathways?

Future studies aimed at potential modification of the MSP signaling output at the level of the regulated genes and characterization of the underlying molecular mechanisms will be necessary to answer some of these questions. Studying the existing pool of natural genetic variability in MSP signaling affecting the responsiveness of the pathway to various inputs might provide important evidence on the potential role of MSP signaling and its downstream targets. They would also go a long way in shedding light on the ability of plants to successfully face environmental stresses which are after all an everyday reality of plant life. In combination with tools for targeted genome editing of plant genomes that have become available recently (Jinek et al., 2012; Anzalone et al., 2019), this type of knowledge will be a powerful enabler for rapid and targeted introduction of valuable alleles into the elite varieties being used in contemporary crop breeding programs.

AUTHOR CONTRIBUTIONS

JS, KN, and JH performed the literature search. KN and JS created the figures. JS, KN, RV, and JH wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the European Regional Development Fund project "SINGING PLANT" (No. CZ.02.1.01/0.0/0.0/16_026/0008446), which received a financial contribution from the Ministry of Education, Youth and Sports of the Czechia in the form of special support through the National Programme for Sustainability II program, "Centre for Experimental Plant Biology" (No. CZ.02.1.01/0.0/0.0/16_019/0000738), LTAUSA18161 and the Czech Science Foundation (19-24753S).

REFERENCES

Abuelsoud, W., Cortleven, A., and Schmülling, T. (2020). Photoperiod stress induces an oxidative burst-like response and is associated with increased apoplastic peroxidase and decreased catalase activities. *J. Plant Physiol.* 253, 153252. doi: 10.1016/j.jplph.2020.153252

Ahuja, I., de Vos, R. C. H., Bones, A. M., and Hall, R. D. (2010). Plant molecular stress responses face climate change. *Trends Plant Sci.* 15, 664–674. doi: 10.1016/j.tplants.2010.08.002

Alabadí, D., and Blázquez, M. A. (2008). Integration of light and hormone signals. *Plant Signal. Behav.* 3, 448–449. doi: 10.4161/psb.3.7.5558

Allan, A. C., Fricker, M. D., Ward, J. L., Beale, M. H., and Trewavas, A. J. (1994). Two transduction pathways mediate rapid effects of abscisic acid in commelina guard cells. *Plant Cell* 6, 1319–1328. doi: 10.2307/3869829

An, F., Zhao, Q., Ji, Y., Li, W., Jiang, Z., Yu, X., et al. (2010). Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in arabidopsis. *Plant Cell* 22, 2384–2401. doi: 10.1105/tpc.110.076588

Antoniadi, I., Novák, O., Gelová, Z., Johnson, A., Plíhal, O., Simerský, R., et al. (2020). Cell-surface receptors enable perception of extracellular cytokinins. *Nat. Commun.* 11:4284. doi: 10.1038/s41467-020-17700-9

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., et al. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157. doi: 10.1038/s41586-019-1711-4

Argueso, C. T., Ferreira, F. J., and Kieber, J. J. (2009). Environmental perception avenues: the interaction of cytokinin and environmental response pathways. *Plant Cell Environ.* 32, 1147–1160. doi: 10.1111/j.1365-3040.2009.01940.x

Baena-González, E., Rolland, F., Thevelein, J. M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signalling. *Nature* 448, 938–942. doi: 10.1038/nature06069

Baena-González, E., and Sheen, J. (2008). Convergent energy and stress signaling. *Trends Plant Sci.* 13, 474–482. doi: 10.1016/j.tplants.2008.06.006

Bakshi, A., Piy, S., Fernandez, J. C., Chervin, C., Hewezi, T., and Binder, B. M. (2018). Pethylene receptors signal via a noncanonical pathway to regulate abscisic acid responses. *Plant Physiol.* 176, 910–929. doi: 10.1104/pp.17.01321

Bakshi, A., Wilson, R. L., Lacey, R. F., Kim, H., Wuppalapati, S. K., and Binder, B. M. (2015). Identification of regions in the receiver domain of the ETHYLENE RESPONSE1 ethylene receptor of arabidopsis important for functional divergence. *Plant Physiol.* 169, 219–232. doi: 10.1104/pp.15.00626

Balestrasse, K. B., Zilli, C. G., and Tomaro, M. L. (2008). Signal transduction pathways and haem oxygenase induction in soybean leaves subjected to salt stress. *Redox Rep.* 13, 255–262. doi: 10.1179/135100008X308966

Ballaré, C. L., and Pierik, R. (2017). The shade-avoidance syndrome: multiple signals and ecological consequences. *Plant Cell Environ.* 40, 2530–2543. doi: 10.1111/pce.12914

Belda-Palazón, B., Adamo, M., Valerio, C., Ferreira, L. J., Confraria, A., Reis-Barata, D., et al. (2020). A dual function of SnRK2 kinases in the regulation of SnRK1 and plant growth. *Nat. Plants* 6, 1345–1353. doi: 10.1038/s41477-020-00778-w

Belin, C., De Franco, P. O., Bourbousse, C., Chaignepain, S., Schmitter, J. M., Vavasseur, A., et al. (2006). Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiol.* 141, 1316–1327. doi: 10.1104/pp.106.079327

Bhargava, A., Clabaugh, I., To, J. P., Maxwell, B. B., Chiang, Y. H., Schaller, G. E., et al. (2013). Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-seq in *Arabidopsis*. *Plant Physiol.* 162, 272–294. doi: 10.1104/pp.113.217026

Binder, B. M. (2020). Ethylene signaling in plants. *J. Biol. Chem.* 295, 7710–7725. doi: 10.1074/jbc.REV120.010854

Binder, B. M., Rodríguez, F. I., and Bleecker, A. B. (2010). The copper transporter RAN1 is essential for biogenesis of ethylene receptors in *Arabidopsis*. *J. Biol. Chem.* 285, 37263–37270. doi: 10.1074/jbc.M110.170027

Binder, B. M., Walker, J. M., Gagne, J. M., Emborg, T. J., Hemmann, G., Bleecker, A. B., et al. (2007). The Arabidopsis EIN3 binding F-box proteins EBF1 and EBF2 have distinct but overlapping roles in ethylene signaling. *Plant Cell* 19, 509–523. doi: 10.1105/tpc.106.048140

Blatt, M. R., and Grabov, A. (1997). Signal redundancy, gates and integration in the control of ion channels for stomatal movement. *J. Exp. Bot.* 48, 529–537. doi: 10.1093/jxb/48.special_issue.529

Boccalandro, H. E., Mazza, C. A., Mazzella, M. A., Casal, J. J., and Ballaré, C. L. (2001). Ultraviolet B radiation enhances a phytochrome-B-mediated photomorphogenic response in *Arabidopsis*. *Plant Physiol.* 126, 780–788. doi: 10.1104/pp.126.2.780

Boudsocq, M., Droillard, M. J., Barbier-Brygoo, H., and Laurière, C. (2007). Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant Mol. Biol.* 63, 491–503. doi: 10.1007/s11103-006-9103-1

Brenner, W. G., Ramireddy, E., Heyl, A., and Schmülling, T. (2012). Gene regulation by cytokinin in *Arabidopsis*. *Front. Plant Sci.* 3:8. doi: 10.3389/fpls.2012.00008

Brenner, W. G., Romanov, G. A., Köllmer, I., Bürkle, L., and Schmülling, T. (2005). Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. *Plant J.* 44, 314–333. doi: 10.1111/j.1365-313X.2005.02530.x

Brenner, W. G., and Schmülling, T. (2015). Summarizing and exploring data of a decade of cytokinin-related transcriptomics. *Front. Plant Sci.* 6:29. doi: 10.3389/fpls.2015.00029

Caesar, K., Thamm, A. M. K., Witthöft, J., Elgass, K., Huppenberger, P., Grefen, C., et al. (2011). Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. *J. Exp. Bot.* 62, 5571–5580. doi: 10.1093/jxb/err238

Casal, J. J., Candia, A. N., and Sellaro, R. (2014). Light perception and signalling by phytochrome A. *J. Exp. Bot.* 65, 2835–2845. doi: 10.1093/jxb/ert379

Castillo, M. C., Lozano-Juste, J., González-Guzmán, M., Rodriguez, L., Rodriguez, P. L., and León, J. (2015). Inactivation of PYR/PYL/RCAR ABA receptors by tyrosine nitration may enable rapid inhibition of ABA signaling by nitric oxide in plants. *Sci. Signal.* 8:ra89. doi: 10.1126/scisignal.aaa7981

Černý, M., Jedelský, P. L., Novák, J., Schlosser, A., and Brzobohatý, B. (2014). Cytokinin modulates proteomic, transcriptomic and growth responses to temperature shocks in *Arabidopsis*. *Plant Cell Environ.* 37, 1641–1655. doi: 10.1111/pce.12270

Chang, J., Li, X., Fu, W., Wang, J., Yong, Y., Shi, H., et al. (2019). Asymmetric distribution of cytokinins determines root hydrotropism in *Arabidopsis thaliana*. *Cell Res.* 29, 984–993. doi: 10.1038/s41422-019-0239-3

Cheng, M.-C., Liao, P.-M., Kuo, W.-W., and Lin, T.-P. (2013). The *Arabidopsis* ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. *Plant Physiol.* 162, 1566–1582. doi: 10.1104/pp.113.221911

Chinnusamy, V., and Zhu, J. K. (2009). Epigenetic regulation of stress responses in plants. *Curr. Opin. Plant Biol.* 12, 133–139. doi: 10.1016/j.pbi.2008.12.006

Cho, Y. H., and Yoo, S. D. (2007). ETHYLENE RESPONSE 1 histidine kinase activity of arabidopsis promotes plant growth. *Plant Physiol.* 143, 612–616. doi: 10.1104/pp.106.091504

Chung, B. Y. W., Balcerowicz, M., Di Antonio, M., Jaeger, K. E., Geng, F., Franaszek, K., et al. (2020). An RNA thermoswitch regulates daytime growth in *Arabidopsis*. *Nat. Plants* 6, 522–532. doi: 10.1038/s41477-020-0633-3

Claeys, H., and Inzé, D. (2013). The agony of choice: how plants balance growth and survival under water-limiting conditions. *Plant Physiol.* 162, 1768–1779. doi: 10.1104/pp.113.220921

Clark, K. L., Larsen, P. B., Wang, X., and Chang, C. (1998). Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5401–5406. doi: 10.1073/pnas.95.9.5401

Cortleven, A., Nitschke, S., Klaumünzer, M., AbdElgawad, H., Asard, H., Grimm, B., et al. (2014). A novel protective function for cytokinin in the light stress response is mediated by the ARABIDOPSIS HISTIDINE KINASE2 and ARABIDOPSIS HISTIDINE KINASE3 receptors. *Plant Physiol.* 164, 1470–1483. doi: 10.1104/pp.113.224667

Cortleven, A., and Schmülling, T. (2015). Regulation of chloroplast development and function by cytokinin. *J. Exp. Bot.* 66, 4999–5013. doi: 10.1093/jxb/erv132

Cotton, J. L. S., Ross, C. W., Byrne, D. H., and Colbert, J. T. (1990). Down-regulation of phytochrome mRNA abundance by red light and benzyladenine in etiolated cucumber cotyledons. *Plant Mol. Biol.* 14, 707–714. doi: 10.1007/BF00016503

Crosatti, C., De Laureto, P. P., Bassi, R., and Cattivelli, L. (1999). The interaction between cold and light controls the expression of the cold-regulated barley gene

cor14b and the accumulation of the corresponding protein. *Plant Physiol.* 119, 671–680. doi: 10.1104/pp.119.2.671

D'Amico-Damião, V., Cruz, F. J. R., Gavassi, M. A., Santos, D. M. M., Melo, H. C., and Carvalho, R. F. (2015). Photomorphogenic modulation of water stress in tomato (*Solanum lycopersicum* L.): the role of phytochromes A, B1, and B2. *J. Hortic. Sci. Biotechnol.* 90, 25–30. doi: 10.1080/14620316.2015.11513149

Dautel, R., Wu, X. N., Heunemann, M., Schulze, W. X., and Harter, K. (2016). The sensor histidine kinases AHK2 and AHK3 proceed into multiple serine/threonine/tyrosine phosphorylation pathways in *Arabidopsis thaliana*. *Mol. Plant* 9, 182–186. doi: 10.1016/j.molp.2015.10.002

De Wit, M., Galvão, V. C., and Fankhauser, C. (2016). Light-mediated hormonal regulation of plant growth and development. *Annu. Rev. Plant Biol.* 67, 513–537. doi: 10.1146/annurev-arplant-043015-112252

Dejonghe, W., Okamoto, M., and Cutler, S. R. (2018). Small molecule probes of ABA biosynthesis and signaling. *Plant Cell Physiol.* 59, 1490–1499. doi: 10.1093/pcp/pcy126

Deng, Y., Dong, H., Mu, J., Ren, B., Zheng, B., Ji, Z., et al. (2010). Arabidopsis histidine kinase CKI1 acts upstream of histidine phosphotransfer proteins to regulate female gametophyte development and vegetative growth. *Plant Cell* 22, 1232–1248. doi: 10.1105/tpc.108.065128

Desikan, R., Horák, J., Chaban, C., Mira-Rodado, V., Witthöft, J., Elgass, K., et al. (2008). The histidine kinase AHK5 integrates endogenous and environmental signals in *Arabidopsis* guard cells. *PLoS One* 3:e2491. doi: 10.1371/journal.pone.0002491

Desikan, R., Last, K., Harrett-Williams, R., Tagliavia, C., Harter, K., Hooley, R., et al. (2006). Ethylene-induced stomatal closure in *Arabidopsis* occurs via AtrbohF-mediated hydrogen peroxide synthesis. *Plant J.* 47, 907–916. doi: 10.1111/j.1365-313X.2006.02842.x

Dobisova, T., Hrdinova, V., Cuesta, C., Michlickova, S., Urbankova, I., Hejatkova, R., et al. (2017). Light controls cytokinin signaling via transcriptional regulation of constitutively active sensor histidine kinase CKI1. *Plant Physiol.* 174, 387–404. doi: 10.1104/pp.16.01964

Dobrá, J., Černý, M., Štorchová, H., Dobrev, P., Skalák, J., Jedelský, P. L., et al. (2015). The impact of heat stress targeting on the hormonal and transcriptomic response in *Arabidopsis*. *Plant Sci.* 231, 52–61. doi: 10.1016/j.plantsci.2014.11.005

Dobra, J., Motyka, V., Dobrev, P., Malbeck, J., Prasil, I. T., Haisel, D., et al. (2010). Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. *J. Plant Physiol.* 167, 1360–1370. doi: 10.1016/j.jplph.2010.05.013

Dong, P., Xiong, F., Que, Y., Wang, K., Yu, L., Li, Z., et al. (2015). Expression profiling and functional analysis reveals that TOR is a key player in regulating photosynthesis and phytohormone signaling pathways in *Arabidopsis*. *Front. Plant Sci.* 6:677. doi: 10.3389/fpls.2015.00677

Dubois, M., Van den Broeck, L., and Inzé, D. (2018). The pivotal role of ethylene in plant growth. *Trends Plant Sci.* 23, 311–323. doi: 10.1016/j.tplants.2018.01.003

Eichmann, R., and Schäfer, P. (2015). Growth versus immunity-a redirection of the cell cycle? *Curr. Opin. Plant Biol.* 26, 106–112. doi: 10.1016/j.pbi.2015.06.006

Etheridge, N., Hall, B. P., and Schaller, G. E. (2006). Progress report: ethylene signaling and responses. *Planta* 223, 387–391. doi: 10.1007/s00425-005-0163-2

Fankhauser, C. (2002). Light perception in plants: cytokinins and red light join forces to keep phytochrome B active. *Trends Plant Sci.* 7, 143–145. doi: 10.1016/S1360-1385(02)02228-8

Feng, J., Wang, C., Chen, Q., Chen, H., Ren, B., Li, X., et al. (2013). S-nitrosylation of phosphotransfer proteins represses cytokinin signaling. *Nat. Commun.* 4:1529. doi: 10.1038/ncomms2541

Filipe, O., De Vleesschauwer, D., Haeck, A., Demeestere, K., and Höfte, M. (2018). The energy sensor OsSnRK1a confers broad-spectrum disease resistance in rice. *Sci. Rep.* 8:3864. doi: 10.1038/s41598-018-22101-6

Fujii, H., and Zhu, J.-K. (2009). *Arabidopsis* mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8380–8385. doi: 10.1073/pnas.0903144106

Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., et al. (2009). Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in *Arabidopsis*. *Plant Cell Physiol.* 50, 2123–2132. doi: 10.1093/pcp/pcp147

Fujita, Y., Yoshida, T., and Yamaguchi-Shinozaki, K. (2013). Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. *Physiol. Plant* 147, 15–27. doi: 10.1111/j.1399-3054.2012.01635.x

Gagne, J. M., Smalle, J., Gingerich, D. J., Walker, J. M., Yoo, S. D., Yanagisawa, S., et al. (2004). Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 6803–6808. doi: 10.1073/pnas.0401698101

Gangappa, S. N., and Botto, J. F. (2016). The multifaceted roles of HY5 in plant growth and development. *Mol. Plant* 9, 1353–1365. doi: 10.1016/j.molp.2016.07.002

González, C. V., Ibarra, S. E., Piccoli, P. N., Botto, J. F., and Boccalandro, H. E. (2012). Phytochrome B increases drought tolerance by enhancing ABA sensitivity in *Arabidopsis thaliana*. *Plant Cell Environ.* 35, 1958–1968. doi: 10.1111/j.1365-3040.2012.02529.x

Grefen, C., Städele, K., Růžiěka, K., Obrdlik, P., Harter, K., and Horák, J. (2008). Subcellular localization and in vivo interactions of the *Arabidopsis thaliana* ethylene receptor family members. *Mol. Plant* 1, 308–320. doi: 10.1093/mp/ssm015

Guo, H., and Ecker, J. R. (2003). Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* 115, 667–677. doi: 10.1016/s0092-8674(03)00969-3

Guo, Y., and Gan, S. (2011). AtMYB2 regulates whole plant senescence by inhibiting cytokinin-mediated branching at late stages of development in *Arabidopsis*. *Plant Physiol.* 156, 1612–1619. doi: 10.1104/pp.111.177022

Gupta, S., and Rashotte, A. M. (2014). Expression patterns and regulation of SlCRF3 and SlCRF5 in response to cytokinin and abiotic stresses in tomato (*Solanum lycopersicum*). *J. Plant Physiol.* 171, 349–358. doi: 10.1016/j.jplph.2013.09.003

Hai, N. N., Chuong, N. N., Tu, N. H. C., Kisiala, A., Hoang, X. L. T., and Thao, N. P. (2020). Role and regulation of cytokinins in plant response to drought stress. *Plants* 9:422. doi: 10.3390/plants9040422

Han, S. K., and Wagner, D. (2014). Role of chromatin in water stress responses in plants. *J. Exp. Bot.* 65, 2785–2799. doi: 10.1093/jxb/ert403

Han, S. K., Wu, M. F., Cui, S., and Wagner, D. (2015). Roles and activities of chromatin remodeling ATPases in plants. *Plant J.* 83, 62–77. doi: 10.1111/tpj.12877

Hass, C., Lohrmann, J., Albrecht, V., Sweere, U., Hummel, F., Yoo, S. D., et al. (2004). The response regulator 2 mediates ethylene signalling and hormone signal integration in *Arabidopsis*. *Embo J.* 23, 3290–3302. doi: 10.1038/sj.emboj.7600337

Hejátko, J., Pernisová, M., Eneva, T., Palme, K., and Brzobohatý, B. (2003). The putative sensor histidine kinase CKI1 is involved in female gametophyte development in *Arabidopsis*. *Mol. Genet. Genomics* 269, 443–453. doi: 10.1007/s00438-003-0858-7

Hejátko, J., Ryu, H., Kim, G. T., Dobešová, R., Choi, S., Choi, S. M., et al. (2009). The Histidine kinases cytokinin-independent1 and arabidopsis histidine kinase2 and 3 regulate vascular tissue development in arabidopsis shoots. *Plant Cell* 21, 2008–2021. doi: 10.1105/tpc.109.066696

Hirayama, T., Kieber, J. J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., et al. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. *Cell* 97, 383–393. doi: 10.1016/S0092-8674(00)80747-3

Horak, J., Janda, L., Pekarova, B., and Hejatko, J. (2011). Molecular mechanisms of signalling specificity via phosphorelay pathways in *Arabidopsis*. *Curr. Protein Pept. Sci.* 12, 126–136. doi: 10.2174/1389211213488452037

Hothorn, M., Dabi, T., and Chory, J. (2011). Structural basis for cytokinin recognition by *Arabidopsis thaliana* histidine kinase 4. *Nat. Chem. Biol.* 7, 766–768. doi: 10.1038/nchembio.667

Huang, X., Hou, L., Meng, J., You, H., Li, Z., Gong, Z., et al. (2018). The antagonistic action of abscisic acid and cytokinin signaling mediates drought stress response in *Arabidopsis*. *Mol. Plant* 11, 970–982. doi: 10.1016/j.molp.2018.05.001

Huang, X., Ouyang, X., and Deng, X. W. (2014). Beyond repression of photomorphogenesis: role switching of COP/DET/FUS in light signaling. *Curr. Opin. Plant Biol.* 21, 96–103. doi: 10.1016/j.pbi.2014.07.003

Huang, X., Zhang, X., Gong, Z., Yang, S., and Shi, Y. (2017). ABI4 represses the expression of type-A ARRs to inhibit seed germination in *Arabidopsis*. *Plant J.* 89, 354–365. doi: 10.1111/tpj.13389

Huang, Y., Li, H., Hutchison, C. E., Laskey, J., and Kieber, J. J. (2003). Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in *Arabidopsis*. *Plant J.* 33, 221–233. doi: 10.1046/j.1365-313X.2003.01620.x

Hutchison, C. E., and Kieber, J. J. (2002). Cytokinin signaling in *Arabidopsis*. *Plant Cell* 14, S47–S59. doi: 10.1105/tpc.010444

Hutchison, C. E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M. W., et al. (2006). The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* 18, 3073–3087. doi: 10.1105/tpc.106.045674

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* 413, 383–389. doi: 10.1038/35096500

Inzé, A., Vanderauwera, S., Hoeberichts, F. A., Vandorpe, M., van Gaever, T., and van Breusegem, F. (2012). A subcellular localization compendium of hydrogen peroxide-induced proteins. *Plant Cell Environ.* 35, 308–320. doi: 10.1111/j.1365-3040.2011.02323.x

Iwama, A., Yamashino, T., Tanaka, Y., Sakakibara, H., Kakimoto, T., Sato, S., et al. (2007). AHK5 histidine kinase regulates root elongation through an ETR1-dependent abscisic acid and ethylene signaling pathway in *Arabidopsis thaliana*. *Plant Cell Physiol.* 48, 375–380. doi: 10.1093/pcp/pcl065

Jamsheer, M. K., Jindal, S., and Laxmi, A. (2019). Evolution of TOR–SnRK dynamics in green plants and its integration with phytohormone signaling networks. *J. Exp. Bot.* 70, 2239–2259. doi: 10.1093/jxb/erz107

Jeon, J., Cho, C., Lee, M. R., Van Binh, N., and Kim, J. (2016). CYTOKININ RESPONSE FACTOR2 (CRF2) and CRF3 regulate lateral root development in response to cold stress in *Arabidopsis*. *Plant Cell* 28, 1828–1843. doi: 10.1105/tpc.15.00909

Jeon, J., and Kim, J. (2013). Arabidopsis response regulator1 and arabidopsis histidine phosphotransfer protein2 (AHP2), AHP3, and AHP5 function in cold signaling. *Plant Physiol.* 161, 408–424. doi: 10.1104/pp.112.207621

Jeon, J., Kim, N. Y., Kim, S., Kang, N. Y., Novák, O., Ku, S. J., et al. (2010). A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in *Arabidopsis*. *J. Biol. Chem.* 285, 23371–23386. doi: 10.1074/jbc.M109.096644

Jiang, B., Shi, Y., Peng, Y., Jia, Y., Yan, Y., Dong, X., et al. (2020). Cold-Induced CBF–PIF3 interaction enhances freezing tolerance by stabilizing the phyB thermosensor in *Arabidopsis*. *Mol. Plant* 13, 894–906. doi: 10.1016/j.molp.2020.04.006

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829

Johnová, P., Skalák, J., Saiz-Fernández, I., and Brzobohatý, B. (2016). Plant responses to ambient temperature fluctuations and water-limiting conditions: a proteome-wide perspective. *Biochim. Biophys. Acta Proteins Proteomics* 1864, 916–931. doi: 10.1016/j.bbapap.2016.02.007

Ju, C., Yoon, G. M., Shemansky, J. M., Lin, D. Y., Ying, Z. I., Chang, J., et al. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19486–19491. doi: 10.1073/pnas.1214848109

Jung, J. H., Domijan, M., Klose, C., Biswas, S., Ezer, D., Gao, M., et al. (2016). Phytochromes function as thermosensors in *Arabidopsis*. *Science* 354, 886–889. doi: 10.1126/science.aaf6005

Kakimoto, T. (1996). CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274, 982–985. doi: 10.1126/science.274.5289.982

Kang, N. Y., Cho, C., Kim, N. Y., and Kim, J. (2012). Cytokinin receptor-dependent and receptor-independent pathways in the dehydration response of *Arabidopsis thaliana*. *J. Plant Physiol.* 169, 1382–1391. doi: 10.1016/j.jplph.2012.05.007

Kaplan, F., Kopka, J., Haskell, D. W., Zhao, W., Schiller, K. C., Gatzke, N., et al. (2004). Exploring the temperature-stress metabolome. *Plant Physiol.* 136, 4159–4168. doi: 10.1104/pp.104.052142.1

Katsuta, S., Masuda, G., Bak, H., Shinozawa, A., Kamiyama, Y., Umezawa, T., et al. (2020). Arabidopsis Raf-like kinases act as positive regulators of subclass III SnRK2 in osmostress signaling. *Plant J.* 103, 634–644. doi: 10.1111/tpj.14756

Kidokoro, S., Maruyama, K., Nakashima, K., Imura, Y., Narusaka, Y., Shinwari, Z. K., et al. (2009). The phytochrome-interacting factor PIF7 negatively regulates dreb1 expression under circadian control in *Arabidopsis*. *Plant Physiol.* 151, 2046–2057. doi: 10.1104/pp.109.147033

Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A., and Ecker, J. R. (1993). CTR1, a negative regulator of the ethylene response pathway in arabidopsis, encodes a member of the Raf family of protein kinases. *Cell* 72, 427–441. doi: 10.1016/0092-8674(93)90119-B

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:dev149344. doi: 10.1242/dev.149344

Kim, G. D., Cho, Y. H., and Yoo, S. D. (2017). Regulatory functions of cellular energy sensor SNF1-Related Kinase1 for leaf senescence delay through ETHYLENE- INSENSITIVE3 repression. *Sci. Rep.* 7:3193. doi: 10.1038/s41598-017-03506-1

Kosová, K., Prášil, I. T., Vítámvás, P., Dobrev, P., Motyka, V., Floková, K., et al. (2012). Complex phytohormone responses during the cold acclimation of two wheat cultivars differing in cold tolerance, winter Samanta and spring Sandra. *J. Plant Physiol.* 169, 567–576. doi: 10.1016/j.jplph.2011.12.013

Kubiasová, K., Montesinos, J. C., Šamajová, O., Nisler, J., Mik, V., Semerádová, H., et al. (2020). Cytokinin fluoroprobe reveals multiple sites of cytokinin perception at plasma membrane and endoplasmic reticulum. *Nat. Commun.* 11:4285. doi: 10.1038/s41467-020-17949-0

Kumar, M., Kesawat, M. S., Ali, A., Lee, S. C., Gill, S. S., and Kim, H. U. (2019). Integration of abscisic acid signaling with other signaling pathways in plant stress responses and development. *Plants* 8:592. doi: 10.3390/plants8120592

Kumar, M. N., Jane, W. N., and Verslues, P. E. (2013). Role of the putative osmosensor *Arabidopsis* histidine kinase1 in dehydration avoidance and low-water-potential response. *Plant Physiol.* 161, 942–953.

Kumar, M. N., and Verslues, P. E. (2015). Stress physiology functions of the *Arabidopsis* histidine kinase cytokinin receptors. *Physiol. Plant* 154, 369–380. doi: 10.1111/ppl.12290

Larkindale, J., Mishkind, M., and Vierling, E. (2007). "Plant responses to high temperature," in *Plant Abiotic Stress*, eds A. Muhammed, R. Aksel, and R. C. von Borstel (Boston, MA: Springer).

Lee, C.-M., and Thomashow, M. F. (2012). Photoperiodic regulation of the C-repeat binding factor (CBF) cold acclimation pathway and freezing tolerance in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15054–15059. doi: 10.1073/pnas.1211295109

Legris, M., Klose, C., Burgie, E. S., Rojas, C. C., Neme, M., Hiltbrunner, A., et al. (2016). Phytochrome B integrates light and temperature signals in *Arabidopsis*. *Science* 354, 897–900. doi: 10.1126/science.aaf5656

Li, J., Li, G., Wang, H., and Wang Deng, X. (2011). Phytochrome signaling mechanisms. *Arab. B.* 9, e0148. doi: 10.1199/tab.0148

Li, W., Herrera-Estrella, L., and Tran, L.-S. P. (2016). The Yin–Yang of cytokinin homeostasis and drought acclimation/adaptation. *Trends Plant Sci.* 21, 548–550. doi: 10.1016/j.tplants.2016.05.006

Li, W., Ma, M., Feng, Y., Li, H., Wang, Y., Ma, Y., et al. (2015). EIN2-directed translational regulation of ethylene signaling in *Arabidopsis*. *Cell* 163, 670–683. doi: 10.1016/j.cell.2015.09.037

Lin, Z., Li, Y., Zhang, Z., Liu, X., Hsu, C. C., Du, Y., et al. (2020). A RAF-SnRK2 kinase cascade mediates early osmotic stress signaling in higher plants. *Nat. Commun.* 11:613. doi: 10.1038/s41467-020-14477-9

Liu, J., Zhang, F., Zhou, J., Chen, F., Wang, B., and Xie, X. (2012). Phytochrome B control of total leaf area and stomatal density affects drought tolerance in rice. *Plant Mol. Biol.* 78, 289–300. doi: 10.1007/s11103-011-9860-3

Liu, Z., Yuan, L., Song, X., Yu, X., and Sundaresan, V. (2017). AHP2, AHP3, and AHP5 act downstream of CKI1 in *Arabidopsis* female gametophyte development. *J. Exp. Bot.* 68, 3365–3373. doi: 10.1093/jxb/erx181

Lozano-Juste, J., Alrefaei, A. F., and Rodriguez, P. L. (2020). Plant osmotic stress signaling: MAPKKKs Meet SnRK2s. *Trends Plant Sci.* 25, 1179–1182. doi: 10.1016/j.tplants.2020.09.003

Lozano-Juste, J., and León, J. (2010a). Enhanced abscisic acid-mediated responses in nia1nia2noa1-2 triple mutant impaired in NIA/NR- and AtNOA1-dependent nitric oxide biosynthesis in *Arabidopsis*. *Plant Physiol.* 152, 891–903. doi: 10.1104/pp.109.148023

Lozano-Juste, J., and León, J. (2010b). Nitric oxide modulates sensitivity to ABA. *Plant Signal. Behav.* 5, 314–316. doi: 10.4161/psb.5.3.11235

Lu, X. D., Zhou, C. M., Xu, P. B., Luo, Q., Lian, H. L., and Yang, H. Q. (2015). Red-light-dependent interaction of phyB with SPA1 promotes COP1-SPA1

dissociation and photomorphogenic development in *Arabidopsis*. *Mol. Plant* 8, 467–478. doi: 10.1016/j.molp.2014.11.025

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., et al. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324, 1064–1068. doi: 10.1126/science.1172408

Macková, H., Hronková, M., Dobrá, J., Turečková, V., Novák, O., Lubovská, Z., et al. (2013). Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. *J. Exp. Bot.* 64, 2805–2815. doi: 10.1093/jxb/ert131

Marchadier, E., and Hetherington, A. M. (2014). Involvement of two-component signalling systems in the regulation of stomatal aperture by light in *Arabidopsis thaliana*. *New Phytol.* 203, 462–468. doi: 10.1111/nph.12813

Maruyama, K., Urano, K., Yoshiwara, K., Morishita, Y., Sakurai, N., Suzuki, H., et al. (2014). Integrated analysis of the effects of cold and dehydration on rice metabolites, phytohormones, and gene transcripts. *Plant Physiol.* 164, 1759–1771. doi: 10.1104/pp.113.231720

Mason, M. G., Jha, D., Salt, D. E., Tester, M., Hill, K., Kieber, J. J., et al. (2010). Type-B response regulators ARR1 and ARR12 regulate expression of AtHKT1;1 and accumulation of sodium in *Arabidopsis* shoots. *Plant J.* 64, 753–763. doi: 10.1111/j.1365-313X.2010.04366.x

McAinsh, M. R., and Hetherington, A. M. (1998). Encoding specificity in Ca2+ signalling systems. *Trends Plant Sci.* 3, 32–36. doi: 10.1016/S1360-1385(97)01150-3

Merchante, C., Brumos, J., Yun, J., Hu, Q., Spencer, K. R., Enríquez, P., et al. (2015). Gene-specific translation regulation mediated by the hormone-signaling molecule EIN2. *Cell* 163, 684–697. doi: 10.1016/j.cell.2015.09.036

Mira-Rodado, V., Sweere, U., Grefen, C., Kunkel, T., Fejes, E., Nagy, F., et al. (2007). Functional cross-talk between two-component and phytochrome B signal transduction in *Arabidopsis*. *J. Exp. Bot.* 58, 2595–2607. doi: 10.1093/jxb/erm087

Mittler, R., Finka, A., and Goloubinoff, P. (2012). How do plants feel the heat? *Trends Biochem. Sci.* 37, 118–125. doi: 10.1016/j.tibs.2011.11.007

Miura, K., and Furumoto, T. (2013). Cold signaling and cold response in plants. *Int. J. Mol. Sci.* 14, 5312–5337. doi: 10.3390/ijms14035312

Müller, B., and Sheen, J. (2007). Arabidopsis cytokinin signaling pathway. *Sci. STKE* 2007:cm5. doi: 10.1126/stke.4072007cm5

Müller, M., and Munné-Bosch, S. (2015). Ethylene response factors: a key regulatory hub in hormone and stress signaling. *Plant Physiol.* 169, 32–41. doi: 10.1104/pp.15.00677

Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S., et al. (2009). Three arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, *SRK*2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol.* 50, 1345–1363. doi: 10.1093/pcp/pcp083

Nakashima, K., and Yamaguchi-Shinozaki, K. (2013). ABA signaling in stress-response and seed development. *Plant Cell Rep.* 32, 959–970. doi: 10.1007/s00299-013-1418-1

Ng, L. M., Melcher, K., Teh, B. T., and Xu, H. E. (2014). Abscisic acid perception and signaling: structural mechanisms and applications. *Acta Pharmacol. Sin.* 35, 567–584. doi: 10.1038/aps.2014.5

Nguyen, K. H., Ha, C., Van, Nishiyama, R., Watanabe, Y., Leyva-González, M. A., et al. (2016). Arabidopsis type B cytokinin response regulators ARR1, ARR10, and ARR12 negatively regulate plant responses to drought. *Proc. Natl. Acad. Sci. U.S.A.* 113, 3090–3095. doi: 10.1073/pnas.1600399113

Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D. T., Kojima, M., Werner, T., et al. (2011). Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. *Plant Cell* 23, 2169–2183. doi: 10.1105/tpc.111.087395

Nitschke, S., Cortleven, A., Iven, T., Feussner, I., Havaux, M., Riefler, M., et al. (2016). Circadian stress regimes affect the circadian clock and cause jasmonic acid-dependent cell death in cytokinin-deficient *Arabidopsis* plants. *Plant Cell* 28, 1616–1639. doi: 10.1105/tpc.16.00016

Nitschke, S., Cortleven, A., and Schmülling, T. (2017). Novel stress in plants by altering the photoperiod. *Trends Plant Sci.* 22, 913–916. doi: 10.1016/j.tplants.2017.09.005

Njimona, I., Yang, R., and Lamparter, T. (2014). Temperature effects on bacterial phytochrome. *PLoS One* 9:e109794. doi: 10.1371/journal.pone.0109794

Nukarinen, E., Ngele, T., Pedrotti, L., Wurzinger, B., Mair, A., Landgraf, R., et al. (2016). Quantitative phosphoproteomics reveals the role of the AMPK plant ortholog SnRK1 as a metabolic master regulator under energy deprivation. *Sci. Rep.* 6:31697. doi: 10.1038/srep31697

O'Brien, J. A., and Benková, E. (2013). Cytokinin cross-talking during biotic and abiotic stress responses. *Front. Plant Sci.* 4:451. doi: 10.3389/fpls.2013.00451

Park, S.-Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., et al. (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324, 1068–1071. doi: 10.1126/science.1173041

Peirats-Llobet, M., Han, S. K., Gonzalez-Guzman, M., Jeong, C. W., Rodriguez, L., Belda-Palazon, B., et al. (2016). A direct link between abscisic acid sensing and the chromatin-remodeling ATPase BRAHMA via Core ABA signaling pathway components. *Mol. Plant* 9, 136–147. doi: 10.1016/j.molp.2015.10.003

Pham, J., and Desikan, R. (2012). Modulation of ROS production and hormone levels by AHK5 during abiotic and biotic stress signaling. *Plant Signal. Behav.* 7, 893–897. doi: 10.4161/psb.20692

Pham, J., Liu, J., Bennett, M. H., Mansfield, J. W., and Desikan, R. (2012). Arabidopsis histidine kinase 5 regulates salt sensitivity and resistance against bacterial and fungal infection. *New Phytol.* 194, 168–180. doi: 10.1111/j.1469-8137.2011.04033.x

Pischke, M. S., Jones, L. G., Otsuga, D., Fernandez, D. E., Drews, G. N., and Sussman, M. R. (2002). An *Arabidopsis* histidine kinase is essential for megagametogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15800–15805. doi: 10.1073/pnas.232580499

Potter, K. C., Wang, J., Schaller, G. E., and Kieber, J. J. (2018). Cytokinin modulates context-dependent chromatin accessibility through the type-B response regulators. *Nat. Plants* 4, 1102–1111. doi: 10.1038/s41477-018-0290-y

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., et al. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F box proteins: EBF1 and EBF2. *Cell* 115, 679–689. doi: 10.1016/S0092-8674(03)00968-1

Qiao, H., Chang, K. N., Yazaki, J., and Ecker, J. R. (2009). Interplay between ethylene, ETP1/ETP2 F-box proteins, and degradation of EIN2 triggers ethylene responses in *Arabidopsis*. *Genes Dev.* 23, 512–521. doi: 10.1101/gad.1765709

Qiao, H., Shen, Z., Huang, S. S. C., Schmitz, R. J., Urich, M. A., Briggs, S. P., et al. (2012). Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. *Science* 338, 390–393. doi: 10.1126/science.1225974

Quail, P. H., Boylan, M. T., Parks, B. M., Short, T. W., Xu, Y., and Wagner, D. (1995). Phytochromes: photosensory perception and signal transduction. *Science* 268, 675–680. doi: 10.1126/science.7732376

Radchuk, R., Radchuk, V., Weschke, W., Borisjuk, L., and Weber, H. (2006). Repressing the expression of the SUCROSE NONFERMENTING-1-RELATED PROTEIN KINASE gene in pea embryo causes pleiotropic defects of maturation similar to an abscisic acid-insensitive phenotype. *Plant Physiol.* 140, 263–278. doi: 10.1104/pp.105.071167

Raines, T., Blakley, I. C., Tsai, Y. C., Worthen, J. M., Franco-Zorrilla, J. M., Solano, R., et al. (2016). Characterization of the cytokinin-responsive transcriptome in rice. *BMC Plant Biol.* 16:260. doi: 10.1186/s12870-016-0932-z

Rashotte, A. M., Mason, M. G., Hutchison, C. E., Ferreira, F. J., Schaller, G. E., and Kieber, J. J. (2006). A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11081–11085. doi: 10.1073/pnas.0602038103

Rockwell, N. C., Su, Y. S., and Lagarias, J. C. (2006). Phytochrome structure and signaling mechanisms. *Annu. Rev. Plant Biol.* 57, 837–858. doi: 10.1146/annurev.arplant.56.032604.144208

Rodríguez-Gacio Mdel, C., Matilla-Vázquez, M. A., and Matilla, A. J. (2009). Seed dormancy and ABA signaling: the breakthrough goes on. *Plant Signal. Behav.* 4, 1035–1049. doi: 10.4161/psb.4.11.9902

Roeber, V. M., Bajaj, I., Rohde, M., Schmülling, T., and Cortleven, A. (2020). Light acts as a stressor and influences abiotic and biotic stress responses in plants. *Plant. Cell Environ.* 1–20. doi: 10.1111/pce.13948

Rusaczonek, A., Czarnocka, W., Kacprzak, S., Witoń, D., Ślesak, I., Szechyńska-Hebda, M., et al. (2015). Role of phytochromes A and B in the regulation of cell death and acclimatory responses to UV stress in *Arabidopsis thaliana*. *J. Exp. Bot.* 6679–6695. doi: 10.1093/jxb/erv375

Sah, S. K., Reddy, K. R., and Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. *Front. Plant Sci.* 7:571. doi: 10.3389/fpls.2016.00571

Salomé, P. A., To, J. P. C., Kieber, J. J., and McClung, C. R. (2006). *Arabidopsis* response regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. *Plant Cell* 18, 55–69. doi: 10.1105/tpc.105.037994

Sami, F., Faizan, M., Faraz, A., Siddiqui, H., Yusuf, M., and Hayat, S. (2018). Nitric oxide-mediated integrative alterations in plant metabolism to confer abiotic stress tolerance, NO crosstalk with phytohormones and NO-mediated post translational modifications in modulating diverse plant stress. *Nitric Oxide Biol. Chem.* 73, 22–38. doi: 10.1016/j.niox.2017.12.005

Samsonová, Z., Kiran, N. S., Novák, O., Spyroglou, I., Skalák, J., Hejátko, J., et al. (2020). Steady-state levels of cytokinins and their derivatives may serve as a unique classifier of arabidopsis ecotypes. *Plants* 9:116. doi: 10.3390/plants9010116

Schaller, G. E., and Bleecker, A. B. (1995). Ethylene-binding sites generated in yeast expressing the *Arabidopsis* ETR1 gene. *Science* 270, 1809–1811. doi: 10.1126/science.270.5243.1809

Scharein, B., Voet-van-Vormizeele, J., Harter, K., and Groth, G. (2008). Ethylene signaling: identification of a putative ETR1-AHP1 phosphorelay complex by fluorescence spectroscopy. *Anal. Biochem.* 377, 72–76. doi: 10.1016/j.ab.2008.03.015

Schepetilnikov, M., and Ryabova, L. A. (2018). Recent discoveries on the role of tor (Target of rapamycin) signaling in translation in plants. *Plant Physiol.* 176, 1095–1105. doi: 10.1104/pp.17.01243

Sharp, R. E., and Lenoble, M. E. (2002). ABA, ethylene and the control of shoot and root growth under water stress. *J. Exp. Bot.* 53, 33–37. doi: 10.1093/jxb/53.366.33

Sheerin, D. J., Menon, C., zur Oven-Krockhaus, S., Enderle, B., Zhu, L., Johnen, P., et al. (2015). Light-activated Phytochrome A and B interact with members of the SPA family to promote photomorphogenesis in *Arabidopsis* by reorganizing the COP1/SPA complex. *Plant Cell* 27, 189–201. doi: 10.1105/tpc.114.134775

Shi, X., Gupta, S., and Rashotte, A. M. (2014). Characterization of two tomato AP2/ERF genes, SlCRF1 and SlCRF2 in hormone and stress responses. *Plant Cell Rep.* 33, 35–45. doi: 10.1007/s00299-013-1510-6

Shi, Y., Tian, S., Hou, L., Huang, X., Zhang, X., Guo, H., et al. (2012). Ethylene signaling negatively regulates freezing tolerance by repressing expression of and Type-A genes in *Arabidopsis*. *Plant Cell* 24, 2578–2595. doi: 10.1105/tpc.112.098640

Shin, D. H., Choi, M., Kim, K., Bang, G., Cho, M., Choi, S.-B., et al. (2013). HY5 regulates anthocyanin biosynthesis by inducing the transcriptional activation of the MYB75/PAP1 transcription factor in *Arabidopsis*. *FEBS Lett.* 587, 1543–1547. doi: 10.1016/j.febslet.2013.03.037

Shin, J., Park, E., and Choi, G. (2007). PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in *Arabidopsis*. *Plant J.* 49, 981–994. doi: 10.1111/j.1365-313X.2006.03021.x

Siegel, R. S., Xue, S., Murata, Y., Yang, Y., Nishimura, N., Wang, A., et al. (2009). Calcium elevation-dependent and attenuated resting calcium-dependent abscisic acid induction of stomatal closure and abscisic acid-induced enhancement of calcium sensitivities of S-type anion and inward-rectifying K+ channels in *Arabidopsis* guard cells. *Plant J.* 59, 207–220. doi: 10.1111/j.1365-313X.2009.03872.x

Skalák, J., Černý, M., Jedelský, P., Dobrá, J., Ge, E., Novák, J., et al. (2016). Stimulation of ipt overexpression as a tool for elucidation of the role of cytokinins in high temperature responses of *Arabidopsis thaliana*. *J. Exp. Bot.* 67, 2861–2873. doi: 10.1093/jxb/erw129

Skirycz, A., Claeys, H., De Bodt, S., Oikawa, A., Shinoda, S., Andriankaja, M., et al. (2011). Pause-and-stop: the effects of osmotic stress on cell proliferation during early leaf development in *Arabidopsis* and a role for ethylene signaling in cell cycle arrest. *Plant Cell* 23, 1876–1888. doi: 10.1105/tpc.111.084160

Soma, F., Takahashi, F., Suzuki, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2020). Plant Raf-like kinases regulate the mRNA population upstream of ABA-unresponsive SnRK2 kinases under drought stress. *Nat. Commun.* 11:1373. doi: 10.1038/s41467-020-15239-3

Soto-Burgos, J., and Bassham, D. C. (2017). SnRK1 activates autophagy via the TOR signaling pathway in *Arabidopsis thaliana*. *PLoS One* 12:e0182591. doi: 10.1371/journal.pone.0182591

Srivastava, A. K., Senapati, D., Srivastava, A. K., Chakraborty, M., Gangappa, S. N., and Chattopadhyay, S. (2015). SHW1 interacts with HY5 and COP1, and promotes COP1-mediated degradation of HY5 during *Arabidopsis* seedling development. *Plant Physiol.* 169, 2922–2934. doi: 10.1104/pp.15.01184

Stavang, J. A., Gallego-Bartolomé, J., Gómez, M. D., Yoshida, S., Asami, T., Olsen, J. E., et al. (2009). Hormonal regulation of temperature-induced growth in *Arabidopsis*. *Plant J.* 60, 589–601. doi: 10.1111/j.1365-313X.2009.03983.x

Street, I. H., Aman, S., Zubo, Y., Ramzan, A., Wang, X., Shakeel, S. N., et al. (2015). Ethylene inhibits cell proliferation of the *Arabidopsis* root meristem. *Plant Physiol.* 169, 338–350. doi: 10.1104/pp.15.00415

Sung, D. Y., Kaplan, F., Lee, K. J., and Guy, C. L. (2003). Acquired tolerance to temperature extremes. *Trends Plant Sci.* 8, 179–187. doi: 10.1016/S1360-1385(03)00047-5

Sweere, U., Eichenberg, K., Lohrmann, J., Mira-Rodado, V., Bäurle, I., Kudla, J., et al. (2001). Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. *Science* 294, 1108–1111. doi: 10.1126/science.1065022

Takatsuka, H., and Umeda, M. (2019). ABA inhibits root cell elongation through repressing the cytokinin signaling. *Plant Signal. Behav.* 14:e1578632. doi: 10.1080/15592324.2019.1578632

Terry, M. J., Linley, P. J., and Kohchi, T. (2002). Making light of it: the role of plant haem oxygenases in phytochrome chromophore synthesis. *Biochem. Soc. Trans.* 30, 604–609. doi: 10.1042/bst0300604

Todaka, D., Zhao, Y., Yoshida, T., Kudo, M., Kidokoro, S., Mizoi, J., et al. (2017). Temporal and spatial changes in gene expression, metabolite accumulation and phytohormone content in rice seedlings grown under drought stress conditions. *Plant J.* 90, 61–78. doi: 10.1111/tpj.13468

Tran, L. S. P., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2010). Role of cytokinin responsive two-component system in ABA and osmotic stress signalings. *Plant Signal. Behav.* 5, 148–150. doi: 10.4161/psb.5.2.10411

Tran, L.-S. P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., et al. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20623–20628. doi: 10.1073/pnas.0706547105

Tun, N. N., Livaja, M., Kieber, J. J., and Scherer, G. F. E. (2008). Zeatin-induced nitric oxide (NO) biosynthesis in *Arabidopsis thaliana* mutants of NO biosynthesis and of two-component signaling genes. *New Phytol.* 178, 515–531. doi: 10.1111/j.1469-8137.2008.02383.x

Urao, T., Miyata, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2000). Possible his to Asp phosphorelay signaling in an *Arabidopsis* two- component system. *FEBS Lett.* 478, 227–232. doi: 10.1016/S0014-5793(00)01860-3

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., et al. (1999). A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell* 11, 1743–1754. doi: 10.1105/tpc.11.9.1743

Vandenbussche, F., Habricot, Y., Condiff, A. S., Maldiney, R., Van Der Straeten, D., and Ahmad, M. (2007). HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. *Plant J.* 49, 428–441. doi: 10.1111/j.1365-313X.2006.02973.x

Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R. K., et al. (2017). Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. *Front. Plant Sci.* 8:161. doi: 10.3389/fpls.2017.00161

Waidyarathne, P., and Samarasinghe, S. (2018). Boolean calcium signalling model predicts calcium role in acceleration and stability of abscisic acid-mediated stomatal closure. *Sci. Rep.* 8:17635. doi: 10.1038/s41598-018-35872-9

Wang, F., Guo, Z., Li, H., Wang, M., Onac, E., Zhou, J., et al. (2016). Phytochrome A and B function antagonistically to regulate cold tolerance via abscisic acid-dependent jasmonate signaling. *Plant Physiol.* 170, 459–471. doi: 10.1104/pp.15.01171

Wang, H. (2015). Phytochrome signaling: time to tighten up the loose ends. *Mol. Plant* 8, 540–551. doi: 10.1016/j.molp.2014.11.021

Wang, P., Zhao, Y., Li, Z., Hsu, C. C., Liu, X., Fu, L., et al. (2018). Reciprocal regulation of the TOR Kinase and ABA receptor balances plant growth and stress response. *Mol. Cell* 69, 100.e10–112.e10. doi: 10.1016/j.molcel.2017.12.002

Wang, Q., Liu, Q., Wang, X., Zuo, Z., Oka, Y., and Lin, C. (2018). New insights into the mechanisms of phytochrome–cryptochrome coaction. *New Phytol.* 217, 547–551. doi: 10.1111/nph.14886

Wang, Y., Li, L., Ye, T., Zhao, S., Liu, Z., Feng, Y. Q., et al. (2011). Cytokinin antagonizes ABA suppression to seed germination of *Arabidopsis* by downregulating ABI5 expression. *Plant J.* 68, 249–261. doi: 10.1111/j.1365-313X.2011.04683.x

Webb, A. A. R., Larman, M. G., Montgomery, L. T., Taylor, J. E., and Hetherington, A. M. (2001). The role of calcium in ABA-induced gene expression and stomatal movements. *Plant J.* 26, 351–362. doi: 10.1046/j.1365-313X.2001.01032.x

Wen, X., Zhang, C., Ji, Y., Zhao, Q., He, W., An, F., et al. (2012). Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus. *Cell Res.* 22, 1613–1616. doi: 10.1038/cr.2012.145

Wilson, R. L., Bakshi, A., and Binder, B. M. (2014a). Loss of the ETR1 ethylene receptor reduces the inhibitory effect of far-red light and darkness on seed germination of *Arabidopsis thaliana*. *Front. Plant Sci.* 5:433. doi: 10.3389/fpls.2014.00433

Wilson, R. L., Kim, H., Bakshi, A., and Binder, B. M. (2014b). The ethylene receptors ethylene response1 and ethylene response2 have contrasting roles in seed germination of arabidopsis during salt stress. *Plant Physiol.* 165, 1353–1366. doi: 10.1104/pp.114.241695

Wright, S. T. C. (1977). The relationship between leaf water potential ψleaf and the levels of abscisic acid and ethylene in excised wheat leaves. *Planta* 134, 183–189. doi: 10.1007/BF00384969

Wulfetange, K., Lomin, S. N., Romanov, G. A., Stolz, A., Heyl, A., and Schmülling, T. (2011). The cytokinin receptors of arabidopsis are located mainly to the endoplasmic reticulum. *Plant Physiol.* 156, 1808–1818. doi: 10.1104/pp.111.180539

Xiong, L., Schumaker, K. S., and Zhu, J.-K. (2002). Cell signaling during cold, drought, and salt stress. *Plant Cell* 14, S165–S183. doi: 10.1105/tpc.000596

Xu, X., Paik, I., Zhu, L., and Huq, E. (2015). Illuminating progress in phytochrome-mediated light signaling pathways. *Trends Plant Sci.* 20, 641–650. doi: 10.1016/j.tplants.2015.06.010

Yaish, M. W., Colasanti, J., and Rothstein, S. J. (2011). The role of epigenetic processes in controlling flowering time in plants exposed to stress. *J. Exp. Bot.* 62, 3727–3735. doi: 10.1093/jxb/err177

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., et al. (2001). The arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol.* 42, 1017–1023. doi: 10.1093/pcp/pce127

Yu, F., Wu, Y., and Xie, Q. (2016). Ubiquitin-proteasome system in ABA signaling: from perception to action. *Mol. Plant* 9, 21–33. doi: 10.1016/j.molp.2015.09.015

Yuan, L., Liu, Z., Song, X., Johnson, C., Yu, X., and Sundaresan, V. (2016). The CKI1 histidine kinase specifies the female gametic precursor of the endosperm. *Dev. Cell* 37, 34–46. doi: 10.1016/j.devcel.2016.03.009

Zdarska, M., Cuyacot, A. R., Tarr, P. T., Yamoune, A., Szmitkowska, A., Hrdinová, V., et al. (2019). ETR1 integrates response to ethylene and cytokinins into a single multistep phosphorelay pathway to control root growth. *Mol. Plant* 12, 1338–1352. doi: 10.1016/j.molp.2019.05.012

Zdarska, M., Dobisová, T., Gelová, Z., Pernisová, M., Dabravolski, S., and Hejátko, J. (2015). Illuminating light, cytokinin, and ethylene signalling crosstalk in plant development. *J. Exp. Bot.* 66, 4913–4931. doi: 10.1093/jxb/erv261

Zhang, F., Wang, L., Qi, B., Zhao, B., Ko, E. E., Riggan, N. D., et al. (2017). EIN2 mediates direct regulation of histone acetylation in the ethylene response. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10274–10279. doi: 10.1073/pnas.1707937114

Zhao, H., Duan, K.-X., Ma, B., Yin, C.-C., Hu, Y., Tao, J.-J., et al. (2020). Histidine kinase MHZ1/OsHK1 interacts with ethylene receptors to regulate root growth in rice. *Nat. Commun.* 11:518. doi: 10.1038/s41467-020-14313-0

Zhao, X. C., and Schaller, G. E. (2004). Effect of salt and osmotic stress upon expression of the ethylene receptor ETR1 in *Arabidopsis thaliana*. *FEBS Lett.* 562, 189–192. doi: 10.1016/S0014-5793(04)00238-8

Zhong, S., Zhao, M., Shi, T., Shi, H., An, F., Zhao, Q., et al. (2009). EIN3/EIL1 cooperate with PIF1 to prevent photo-oxidation and to promote greening of *Arabidopsis* seedlings. *Proc. Natl. Acad. Sci. U.S.A.* 106, 21431–21436. doi: 10.1073/pnas.0907670106

Zhou, W., Lozano-Torres, J. L., Blilou, I., Zhang, X., Zhai, Q., Smant, G., et al. (2019). A jasmonate signaling network activates root stem cells and promotes regeneration. *Cell* 177, 942.e14–956.e14. doi: 10.1016/j.cell.2019.03.006

Zhu, L., Bu, Q., Xu, X., Paik, I., Huang, X., Hoecker, U., et al. (2015). CUL4 forms an E3 ligase with COP1 and SPA to promote light-induced degradation of PIF1. *Nat. Commun.* 6:7245. doi: 10.1038/ncomms8245

Źróbek-Sokolnik, A. (2012). *Temperature stress and responses of plants*, eds P. Ahmad and M. N. V. Prasad (Berlin: Springer).

Zubo, Y. O., Blakley, I. C., Yamburenko, M. V., Worthen, J. M., Street, I. H., Franco-Zorrilla, J. M., et al. (2017). Cytokinin induces genome-wide binding of the type-B response regulator ARR10 to regulate growth and development in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 114, E5995–E6004. doi: 10.1073/pnas.1620749114

Zürcher, E., and Müller, B. (2016). Cytokinin synthesis, signaling, and function-advances and new insights. *Int. Rev. Cell Mol. Biol.* 324, 1–38. doi: 10.1016/bs.ircmb.2016.01.001

Zwack, P. J., De Clercq, I., Howton, T. C., Hallmark, H. T., Hurny, A., Keshishian, E. A., et al. (2016). Cytokinin response factor 6 represses cytokinin-associated genes during oxidative stress. *Plant Physiol.* 172, 1249–1258. doi: 10.1104/pp.16.00415

Zwack, P. J., and Rashotte, A. M. (2015). Interactions between cytokinin signalling and abiotic stress responses. *J. Exp. Bot.* 66, 4863–4871. doi: 10.1093/jxb/erv172

Zwack, P. J., Robinson, B. R., Risley, M. G., and Rashotte, A. M. (2013). Cytokinin response factor 6 negatively regulates leaf senescence and is induced in response to cytokinin and numerous abiotic stresses. *Plant Cell Physiol.* 54, 971–981. doi: 10.1093/pcp/pct049

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 23 February 2021
doi: 10.3389/fpls.2021.613488

The Cytokinin Status of the Epidermis Regulates Aspects of Vegetative and Reproductive Development in *Arabidopsis thaliana*

*Sören Werner[1], Isabel Bartrina[1,2], Ondřej Novák[3], Miroslav Strnad[3], Tomáš Werner[1,2] and Thomas Schmülling[1]**

[1] Institute of Biology/Applied Genetics, Dahlem Centre of Plant Sciences (DCPS), Freie Universität Berlin, Berlin, Germany, [2] Institute of Biology, NAWI Graz, University of Graz, Graz, Austria, [3] Laboratory of Growth Regulators, Faculty of Science, Palacký University & Institute of Experimental Botany, The Czech Academy of Sciences, Olomouc, Czechia

OPEN ACCESS

Edited by:
G. Eric Schaller,
Dartmouth College, United States

Reviewed by:
Stefan de Folter,
UGA-Langebio, Center for Research and Advanced Studies (CINVESTAV), Mexico
Hitoshi Sakakibara,
RIKEN Center for Sustainable Resource Science (CSRS), Japan

***Correspondence:**
Thomas Schmülling
tschmue@zedat.fu-berlin.de

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: 02 October 2020
Accepted: 13 January 2021
Published: 23 February 2021

Citation:
Werner S, Bartrina I, Novák O, Strnad M, Werner T and Schmülling T (2021) The Cytokinin Status of the Epidermis Regulates Aspects of Vegetative and Reproductive Development in Arabidopsis thaliana. Front. Plant Sci. 12:613488. doi: 10.3389/fpls.2021.613488

The epidermal cell layer of plants has important functions in regulating plant growth and development. We have studied the impact of an altered epidermal cytokinin metabolism on *Arabidopsis* shoot development. Increased epidermal cytokinin synthesis or breakdown was achieved through expression of the cytokinin synthesis gene *LOG4* and the cytokinin-degrading *CKX1* gene, respectively, under the control of the epidermis-specific *AtML1* promoter. During vegetative growth, increased epidermal cytokinin production caused an increased size of the shoot apical meristem and promoted earlier flowering. Leaves became larger and the shoots showed an earlier juvenile-to-adult transition. An increased cytokinin breakdown had the opposite effect on these phenotypic traits indicating that epidermal cytokinin metabolism can be a factor regulating these aspects of shoot development. The phenotypic consequences of abbreviated cytokinin signaling in the epidermis achieved through expression of the ARR1-SRDX repressor were generally milder or even absent indicating that the epidermal cytokinin acts, at least in part, cell non-autonomously. Enhanced epidermal cytokinin synthesis delayed cell differentiation during leaf development leading to an increased cell proliferation and leaf growth. Genetic analysis showed that this cytokinin activity was mediated mainly by the AHK3 receptor and the transcription factor ARR1. We also demonstrate that epidermal cytokinin promotes leaf growth in a largely cell-autonomous fashion. Increased cytokinin synthesis in the outer layer of reproductive tissues and in the placenta enhanced ovule formation by the placenta and caused the formation of larger siliques. This led to a higher number of seeds in larger pods resulting in an increased seed yield per plant. Collectively, the results provide evidence that the cytokinin metabolism in the epidermis is a relevant parameter determining vegetative and reproductive plant growth and development.

Keywords: cytokinin, epidermis, *Arabidopsis*, shoot growth, seed yield, developmental transitions

INTRODUCTION

The communication between different cell types is essential for the coordination of growth processes in multicellular organisms (Savaldi-Goldstein and Chory, 2008). In plants, the cells originate from meristems. Meristematic cells are arranged in an organized layered pattern which are maintained by the controlled orientation of cell divisions within layers. All above-ground organs originate from three clonally distinct cell layers in the shoot apical meristem (SAM): the cells of the single-row L1 and L2 layers only divide anticlinally and form the epidermis and subepidermal tissues, respectively, while the multidirectionally dividing cells of the L3 layer produce the SAM corpus and the different cell types of the ground and vasculature tissues (Ha et al., 2010). Coordination of cell division and cell expansion between these different cell layers is important and is achieved by exchange of phytohormones and other endogenous factors as well as external signals (Ingram, 2004).

So far, little is known about the contribution of the three cell layers of the SAM and the autonomous and non-autonomous factors that they produce to regulate development and growth of shoot lateral organs. It is clear however, that the outer cell layer (epidermis) forms not only a protective barrier to the environment (Yeats and Rose, 2013) but that it plays also a role in growth control (Czesnick and Lenhard, 2015). Analysis of periclinal chimeras of tobacco indicated that cell proliferation in the epidermis affects the proliferative activity of the subepidermal tissues (Marcotrigiano, 2010). Several studies have shown that cells in the epidermis both promote and restrict shoot growth by sending signals to the inner layers (Savaldi-Goldstein and Chory, 2008). For example, transgenic expression of a brassinosteroid synthesis gene specifically in the epidermis was sufficient to rescue the dwarf phenotype caused by mutation of the respective gene, which is expressed also in inner tissues. This indicated that the epidermis produces a signal promoting cell division and growth in inner tissues (Savaldi-Goldstein et al., 2007). In contrast, proliferation in inner leaf tissues was repressed by an epidermal signal mediated by very long chain fatty acids (Nobusawa et al., 2013). The main site of ethylene action in controlling leaf growth in *Arabidopsis* is the epidermis (Vaseva et al., 2018). Vaseva et al. (2018) have also demonstrated that ethylene-mediated cell expansion of the epidermis is rate-limiting for leaf growth. Much less is known about the role of the epidermal cell layer in other shoot organs such as the reproductive structures.

Another factor regulating plant growth and morphogenesis is the hormone cytokinin (CK) (Hwang et al., 2012; Kieber and Schaller, 2018; Werner and Schmülling, 2009). In the shoot, CK stimulates mitotic cell divisions and retards cell differentiation. Accordingly, plants with a lowered CK content formed smaller SAMs and displayed a reduced shoot growth (Werner et al., 2001, 2003). On the other hand, plants with an increased CK content or signaling showed opposite effects (Bartrina et al., 2011, 2017; Kiba et al., 2013). During reproductive development of *Arabidopsis*, CK has a profound influence on several processes. It regulates the inflorescence meristem activity and thus the number of flowers and siliques (Bartrina et al., 2011), the size of the gynoecia, and it acts as a positional cue during ovule formation (Bartrina et al., 2011; Zúñiga-Mayo et al., 2018, 2019), determines the duration of flowering (Bartrina et al., 2017) and is involved in seed size regulation (Werner et al., 2003; Riefler et al., 2006; Li et al., 2013). *Arabidopsis* plants defective in certain CK degradation genes have a higher seed yield (Bartrina et al., 2011).

The CK metabolism and signaling genes of *Arabidopsis* are known. Isopentenyltransferases (IPTs) and cytochrome P450 monooxygenases (CYP735A1 and CYP735A2) catalyze the formation of N^6-isopentenyladenine (iP) and *trans*-zeatin (*t*Z) ribotides (Kakimoto, 2001; Takei et al., 2001, 2004; Miyawaki et al., 2006). The corresponding bioactive free bases are released by CK phosphoribohydrolases, belonging to the LONELY GUY (LOG) family (Kurakawa et al., 2007; Kuroha et al., 2009). The levels of active CKs are reduced either via degradation by CK oxidase/dehydrogenases (CKXs) or inactivation by glycoconjugation (Hou et al., 2004; Werner et al., 2006).

The CK signal transduction pathway is a His-Asp phosphorelay constituting a plant two-component signaling system (Kieber and Schaller, 2014). The *Arabidopsis* genome encodes three membrane-bound CK receptors ARABIDOPSIS HISTIDINE KINASE 2 (AHK2), AHK3, and CYTOKININ RESPONSE 1 (CRE1)/AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Yamada et al., 2001). From the receptors, the signal is transmitted via histidine phosphotransfer proteins (AHPs) to transcription factors, type-B response regulators (ARRs), which regulate CK target genes (Sakai et al., 2000, 2001; Hwang and Sheen, 2001; Tanaka et al., 2004; To et al., 2004; Brenner et al., 2012; Bhargava et al., 2013; Zubo et al., 2017). Artificial repression of CK signaling can be achieved by translational fusion of a modified amino acid motif from class II ethylene response factors (ERFs) named SRDX (Ohta et al., 2001; Hiratsu et al., 2003) to type-B ARRs (Heyl et al., 2008).

Not much is known about the putative involvement of epidermal CK metabolism and signaling in growth regulation. In the SAM, CK synthesized in the L1 layer is predicted to form a gradient over several cell layers and position the organizing center, suggesting that it can act as a paracrine signal (Chickarmane et al., 2012; Gruel et al., 2016). Consistently, the epidermal cell layer expresses several CK metabolism and signaling genes (Yadav et al., 2014). CK reporter and marker gene analysis suggested that the entire leaf epidermis has the potential to sense CK and modify CK levels (Vatén et al., 2018). CK regulates the formation of stomata (Vatén et al., 2018) and promotes stomatal closure in defense to pathogens (Arnaud et al., 2017) showing that it has specific functions in the leaf epidermis. It is not known whether epidermal CK has specific functions in reproductive tissues.

We were interested to explore whether an altered CK metabolism or signaling in the epidermis would have an impact on growth and development of the plant shoot. To this end we have generated transgenic *Arabidopsis* plants expressing the *LOG4* or *CKX1* genes under control of an epidermis-specific promoter. In addition, we have expressed the CK repressor gene *ARR1-SRDX* in the epidermis in order to abbreviate CK output in a cell-autonomous fashion. The results showed that perturbations of CK metabolism in the epidermis impact shoot

development consistent with the possibility that the epidermis is a source or sink of the hormone. Abbreviating CK signaling in the epidermis revealed a contribution of epidermal CK to leaf size regulation, presumably through regulating cell division during early leaf development.

RESULTS

Targeted Expression of *LOG4*, *CKX1*, and *ARR1-SRDX* in the Epidermis Alters Shoot Development

In order to investigate a possible role of epidermal CK to regulate shoot organ growth, we expressed *LOG4*, *CKX1*, and *ARR1-SRDX* in the epidermis under the control of the *AtML1* promoter. This promoter provides a highly specific expression limited to the epidermis of the growing shoot (Sessions et al., 1999) and has been used in several studies (Grandjean et al., 2004; Savaldi-Goldstein et al., 2007; Takada and Jürgens, 2007; Takada et al., 2013). The epidermal specificity of the cloned promoter fragment was verified in *AtML1*:*GUS* transgenic lines (**Supplementary Figure S1**). We generated independent transgenic lines ($n > 17$) expressing *AtML1*:*LOG4*, *AtML1*:*CKX1*, and *AtML1*:*ARR1-SRDX* constructs. Two representative lines per construct expressing the transgene and displaying typical phenotypic changes (**Figure 1**) were selected for detailed analysis.

In order to test how the epidermis-specific expression of *LOG4* and *CKX1* affects the CK concentration in the plants, CK metabolites were measured (**Figure 2A**, **Table 1**, and **Supplementary Table S1**). In AtML1:LOG4 seedlings, the total CK content was significantly increased by up to 17% in comparison to the wild type (**Table 1**). The concentrations of biologically active free bases were increased, in particular of *t*Z and *c*Z (**Figure 2A** and **Supplementary Table S1**). In contrast, *AtML1*:*CKX1* expression reduced the total CK content to about one-third compared to the wild type (**Table 1**) affecting most of the analyzed metabolites, including active CK bases (**Figure 2A** and **Supplementary Table S1**). Transcript levels of five selected CK-responsive A-type *ARR* genes, *ARR4*, *ARR5*, *ARR6*, *ARR7*, and *ARR9*, were tested. The steady state mRNA levels of these *ARR* genes were increased in AtML1:LOG4 and reduced in AtML1:CKX1 plants (**Figure 2B**). This suggested that the altered CK levels caused corresponding changes of expression of genes controlled by the hormone. In AtML1:ARR1-SRDX plants, *ARR4* and *ARR7* showed a downregulation, indicating repression of CK signaling (**Figure 2B**).

Phenotypic analysis of the generated transgenic lines revealed a number of quantitative changes during vegetative and reproductive shoot growth. The most obvious changes were a larger rosette and early flowering in AtML1:LOG4 lines and a diminished rosette growth and retarded flowering in AtML1:CKX1 lines as compared to wild type (**Figure 1A**). The shoot growth of AtML1:ARR1-SRDX plants was reduced, however, the changes were less pronounced than in AtML1:CKX1 (**Figure 1B**; see further below).

Altered Epidermal CK Metabolism Influences the Size and Activity of the Shoot Apical Meristem

The CK status substantially influences the size and activity of the SAM as well as the size of the organs originating from it. A higher CK status leads to an increase in SAM size with more cells and delayed differentiation (Bartrina et al., 2011; Niemann et al., 2015), whereas CK deficiency results in the formation of smaller SAMs because of a reduced cell number and earlier differentiation (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Tokunaga et al., 2012). To study if the CK originated from the L1 layer of the meristem controls its size, we compared the morphology of the vegetative and inflorescence meristems in the wild type and transgenic lines with the perturbed CK content. **Figures 3A,C** show that AtML1:LOG4 plants displayed an increase in the size of the vegetative meristem, whereas the SAMs of the AtML1:CKX1 lines were smaller compared to the wild type (**Figures 3A,C**). The extent of changes correlated with the expression level of the transgenes (**Figures 1C,D**). Similar although smaller changes were also detected in the inflorescence meristem (**Figures 3B,D**).

In order to investigate the activity of the vegetative SAM, we determined the plastochrone index, which is defined as the number of days a plant needs to produce one leaf. Given that CK integrates light signals during shoot organ formation (Yoshida et al., 2011), we carried out the analysis under different light regimes. Interestingly, we did not observe strong plastochrone differences under long day (LD) conditions (**Figure 3E**). However, correlating with the reduced meristem size, the leaf production rate was decreased in AtML1:CKX1 plants under short day (SD) conditions (**Figure 3F**).

SAM size is positively correlated with stem diameter (Schnablovà et al., 2017) and corresponding changes were noted in the transgenic lines (**Figure 3G**). The AtML1:LOG4 lines showed an increase in stem diameter of about 9% compared to the wild type and stem thickness was reduced to 68% and 89% of the wild type in the AtML1:CKX1 lines. The stem diameter of AtML1:ARR1-SRDX lines was comparable to the wild type (**Figure 3G**).

Altered Epidermal CK Metabolism Alters Leaf Cell Number and Size

The altered rosette size of transgenic plants (**Figure 1A**) led us to compare leaf development in more detail. In accordance with the changes in rosette size, the leaf blades of AtML1:LOG4 and AtML1:CKX1 plants were significantly bigger and smaller compared to the wild type, respectively (**Figures 4A,B**). The blade surface area of the seventh leaf in AtML1:LOG4 showed up to 45% increase, whereas AtML1:CKX1 leaves reached only 35–40% of the wild-type size. *AtML1*:*ARR1-SRDX* expression caused a significantly weaker, yet reproducible reduction of leaf blade size (**Figure 4B**). Since the differences in leaf size could be due to altered cell proliferation or expansion, we examined cell number and leaf cell size. Epidermal cell size in the AtML1:LOG4 leaves was similar to wild type, indicating that the enlarged leaf blade area resulted from increased cell

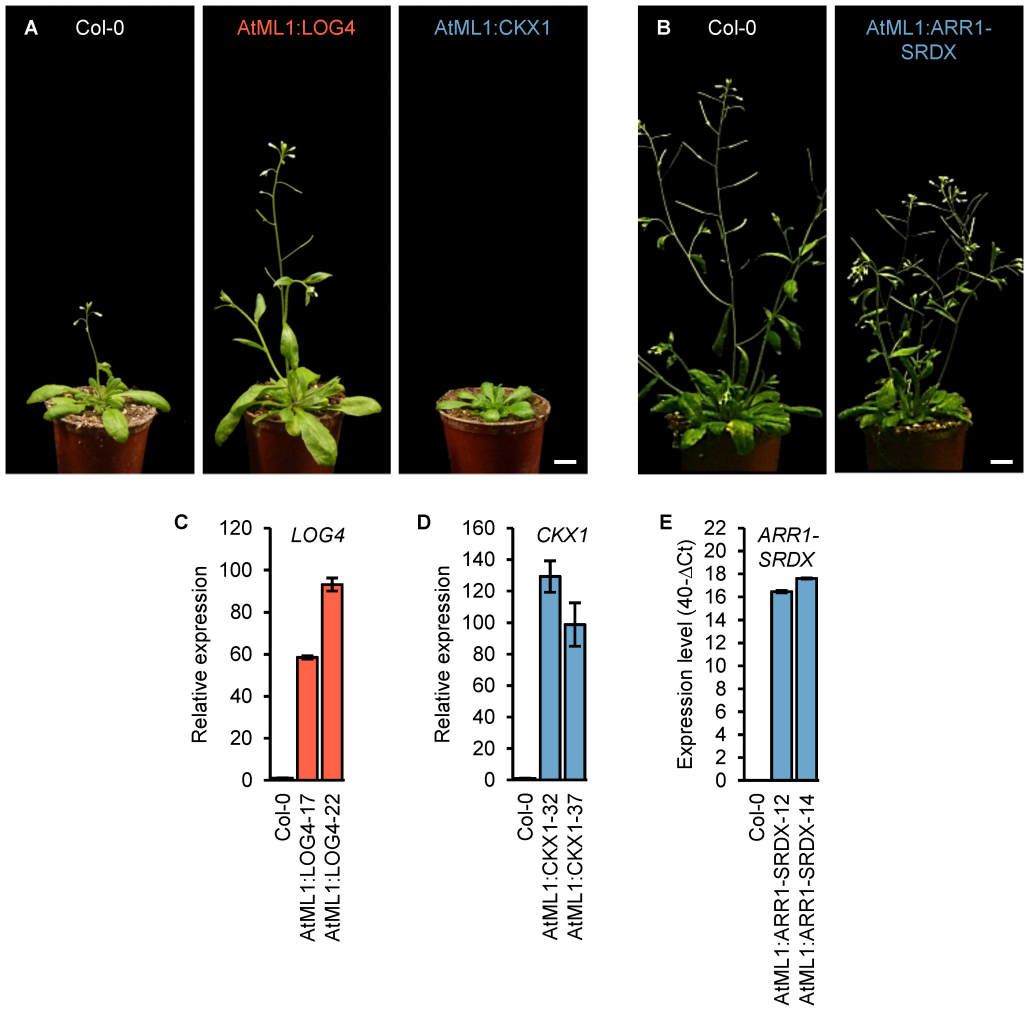


FIGURE 1 | Expression of *ARR1-SRDX*, *CKX1*, and *LOG4* under the control of the *AtML1* promoter influences the development of *Arabidopsis* plants. **(A)** Phenotypes of transgenic AtML1:CKX1 and AtML1:LOG4 plants compared to wild-type plants grown under LD conditions (31 DAG); scale bar = 1 cm. **(B)** Phenotype of transgenic AtML1:ARR1-SRDX plants compared to the wild type grown under LD conditions (36 DAG); scale bar = 1 cm. **(C–E)** Expression level of *LOG4* **(C)**, *CKX1* **(D)**, and *ARR1-SRDX* **(E)** in shoots of 13-day-old plants grown under LD conditions. Transcript levels were determined by qRT-PCR, data were normalized to *PP2AA2* and *TAFII15* **(C,D)** or *PP2AA2* **(E)**, values are mean ± SEM, $n = 4$.

proliferation and a larger total number of leaf cells. In contrast, the AtML1:CKX1 leaves contained only about a quarter of the cells of the wild type (**Figure 4E**), and the reduced cell number was partially compensated by an up to 60% enhancement of cell expansion (**Figures 4C,D**). These results are consistent with the role of CK in sustaining the cell proliferation phase by delaying cell differentiation (Holst et al., 2011; Skalák et al., 2019) and suggest that the epidermis-derived CK is relevant for this regulation. Reduced CK signaling output in the epidermis by the *AtML1:ARR1-SRDX* expression caused only a weak reduction of cell proliferation (**Figures 4B–D**), suggesting that the leaf growth control involves largely a non-cell-autonomous CK activity in the epidermis.

Finally, we took a closer look on the distribution of stomata, which are a specialized cell type in the lower epidermis. The stomatal index, which is defined as the proportion of stomata in the total population of epidermal cells, can provide information about possible influences of the altered CK status on the differentiation of these cells. However, neither the altered CK metabolism in the AtML1:LOG4 and AtML1:CKX1 nor the reduced CK signaling in the AtML1:ARR1-SRDX lines changed the stomatal index (**Figure 4F**) suggesting that this parameter is not under control of CK.

Altered Epidermal CK Metabolism Impacts Developmental Transitions

CK has an influence on the juvenile-to-adult phase transition (Kiba et al., 2013; Sören Werner, Isabel Bartrina, Thomas Schmülling, unpublished result) as well as on flowering time (Michniewicz and Kamieńska, 1965; Werner et al., 2003; Nishimura et al., 2004; Riefler et al., 2006). The epidermis-specific modulation of CK metabolism and signaling impacted these

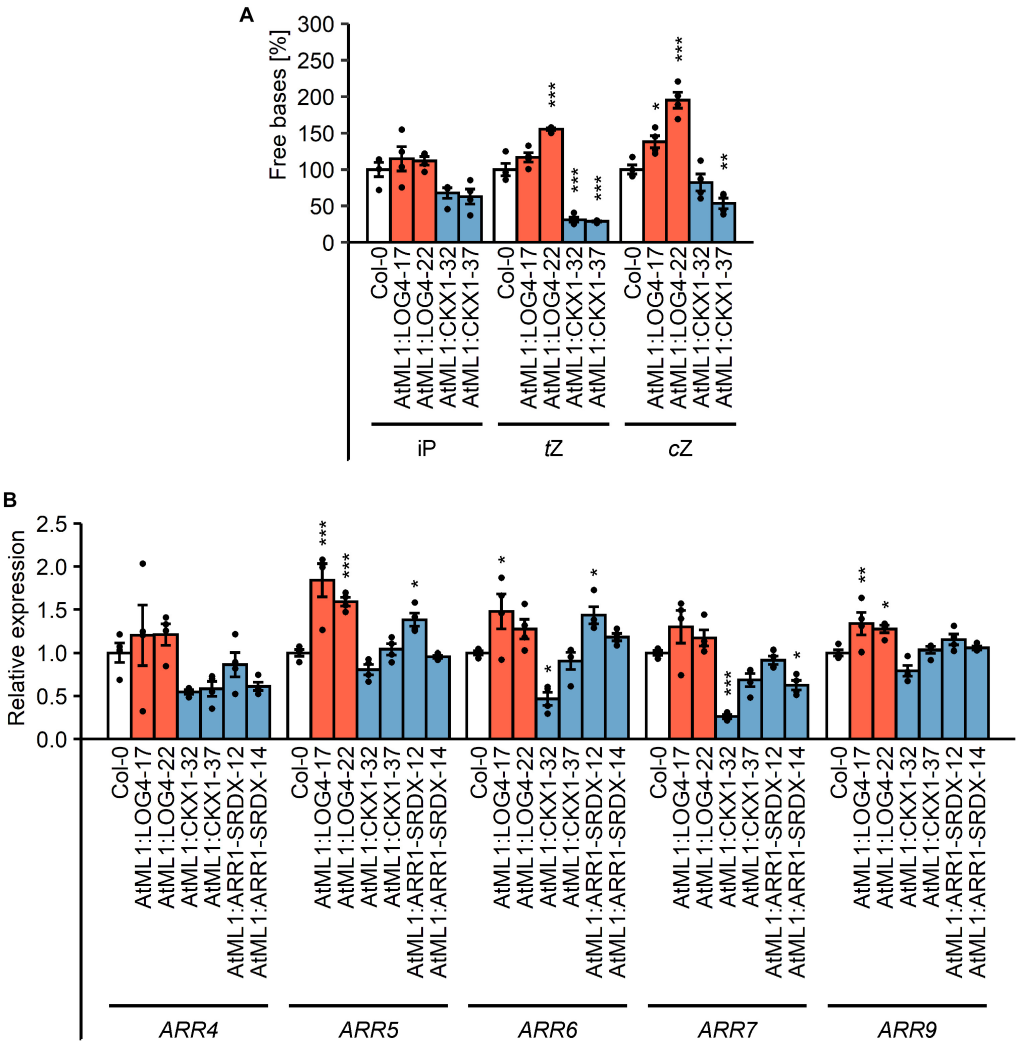


FIGURE 2 | Impact of the expression of *AtML1:LOG4* and *AtML1:CKX1* on the CK status. **(A)** Amount of free CK bases in the transgenic lines relative to the wild type. Twenty milligrams of shoot material of 7-day-old seedlings grown under LD conditions were harvested and pooled for every biological replicate. Values are mean ± SEM ($n = 4$). Asterisks indicate significant differences compared to the wild type, as calculated by Kruskal–Wallis test, *post hoc* Dunn's test (iP) or one-way ANOVA, *post hoc* Dunnett's test (*tZ*, *cZ*) (*$p < 0.05$; **p < 0.01; ***$p < 0.001$). iP, N^6-isopentenyl adenine; *tZ*, *trans*-zeatin; *cZ*, *cis*-zeatin; for a complete list of metabolites see **Supplementary Table S1**. **(B)** Transcript levels of A-type *ARR* genes in shoot material of 13-day-old LD-grown seedlings. Transcript levels were determined by qRT-PCR. Data were normalized to *PP2AA2* and *TAFII15*. Values are mean ± SEM ($n = 4$). Statistical analysis was conducted using one-way ANOVA, *post hoc* Dunnett's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

developmental transitions (**Figure 5**). The transgenic lines were grown under SD and LD conditions and the number of leaves without abaxial trichomes as a measure for juvenile leaf number (Telfer et al., 1997) as well as the time until bolting were examined.

The higher CK status in AtML1:LOG4 lines resulted in an earlier transition from the juvenile to the adult phase under both LD and SD, whereas the reduction of CK content in the AtML1:CKX1 lines led to significant increases in juvenile leaf number (**Figures 5A,B**). Interestingly, the juvenile-to-adult transition was significantly affected by the *AtML1:ARR1-SRDX* transgene (**Figure 5B**), suggesting an at least partly epidermis-autonomous control of this process by CK.

Transition to flowering occurred earlier in AtML1:LOG4 plants than in wild type and was delayed in AtML1:CKX1 under both LD and SD conditions (**Figures 5C,D**). In contrast, the flowering time of AtML1:ARR1-SRDX plants was unchanged in comparison to wild type (**Figures 5C,D**), suggesting that the mobile nature of the signal controlling the developmental transition to flowering is relevant.

Expression of *AtML1:LOG4* and *AtML1:CKX1* Influences Seed Yield

CK has a strong impact on reproductive development of *Arabidopsis* (Bartrina et al., 2011; Werner et al., 2003; Zúñiga-Mayo et al., 2018, 2019). In order to explore the potential

TABLE 1 | Cytokinin concentration in Col-0, AtML1:LOG4, and AtML1:CKX1 plants.

Genotype	iP metabolites	*t*Z metabolites	*c*Z metabolites	Total CKs
Col-0	36.97 ± 2.22	92.44 ± 6.43	7.35 ± 0.49	136.77 ± 8.59
AtML1:LOG4-17	35.80 ± 2.08	98.54 ± 4.02	6.56 ± 0.20	140.90 ± 6.01
AtML1:LOG4-22	41.85 ± 1.67	**111.27 ± 2.15****	6.77 ± 0.15	**159.89 ± 3.71***
AtML1:CKX1-32	**14.61 ± 0.84*****	**22.67 ± 1.82*****	**2.90 ± 0.15****	**40.18 ± 2.77*****
AtML1:CKX1-37	**15.61 ± 0.70*****	**24.51 ± 1.29*****	**3.31 ± 0.14***	**43.42 ± 2.00*****

Twenty milligrams of shoot material of 7-day-old seedlings grown under LD conditions were harvested and pooled for every biological replicate. Values are pmol g FW ± SEM (n = 4). Cytokinin concentrations that are significantly different from the wild type are printed in bold. Asterisks indicate significant differences compared to the wild type, as calculated by one-way ANOVA, post hoc Dunnett's test (iP, tZ, total CKs) or Kruskal–Wallis test, post hoc Dunn's test (cZ) ($p < 0.05$; **$p < 0.01$; ***$p < 0.001$). iP, N^6-isopentenyladenine; tZ, trans-zeatin; cZ, cis-zeatin; for a complete list of metabolites see* ***Supplementary Table S1****.*

relevance of CK of the epidermal layer in regulating reproductive traits we compared various parameters of reproductive growth and development between the transgenic lines and wild type. The flowers and gynoecia of lines with a reduced and increased CK status were smaller and bigger, respectively, than wild type. However, these differences were not in all cases statistically significant at the developmental stage that was evaluated (**Supplementary Figure S2**). The siliques of AtML1:LOG4 and AtML1:CKX1 lines were bigger and smaller, respectively, showing that transgene expression influences growth processes of the reproductive organs (**Figures 6A,B**). The altered silique sizes in these lines correlated with a higher and lower number of seeds developed per silique, respectively (**Figure 6C**), indicating an impact of transgene expression on ovule formation during gynoecia development. The seed yield per plant was increased in the AtML1:LOG4 lines by 9% and decreased in AtML1:CKX1 lines by 41% (**Figure 6D**).

In order to investigate if the impact of transgene expression on seed yield can be attributed to the altered silique length alone, we analyzed other growth parameters contributing to seed yield. AtML1:CKX1 plants produced about 16% bigger seeds as compared to wild type (**Supplementary Figures S3A,B**), nonetheless showing a lower total seed weight than wild type (**Figure 6D**). Among the transgenic lines, only AtML1:CKX1 plants exhibited a reduced number of siliques at the main stem (**Supplementary Figure S3C**), but the average silique number per plant was not altered due to an apparently reduced apical dominance and strongly increased number of axillary branches (**Supplementary Figures S3D,E**). Plant height was affected in AtML1:LOG4 plants, which grew 16% larger than wild type (**Supplementary Figure S3F**), however, the total number of siliques remained similar to wild type (**Supplementary Figures S3C,D**). In AtML1:ARR1-SRDX plants all these parameters were similar to wild type (**Supplementary Figures S3C–F**) indicating that abbreviation of CK signaling output in the epidermal cell layer has no impact on silique number, branching, and plant height. It can be concluded that the impact of *AtML1:LOG4* and *AtML1:CKX1* expression on seed yield is mainly attributable to the altered number of seeds harbored by individual pods.

AHK2, AHK3, and ARR1 Mediate the Effect of Epidermal CK

Next we were interested to investigate through which signaling pathway the epidermis-derived CK signal is transmitted. To this end, the stronger expressing AtML1:LOG4-22 line (**Figure 1C**) was introgressed into three CK receptor double mutants (*ahk2 cre1*, *ahk3 cre1*, and *ahk2 ahk3*) as well as into double mutants of the type-B response regulator genes *ARR1*, *ARR10*, and *ARR12* (*arr1,10*, *arr1,12*, and *arr10,12*). **Supplementary Figure S4** shows that the transgene was expressed to a similar level in the different mutant backgrounds. As a parameter to evaluate the genetic interaction, we chose terminal rosette diameter, which was significantly altered in all transgenic lines. On average, AtML1:LOG4 lines had an 11% increase in rosette size (13.6% in the stronger expressing line AtML1:LOG4-22), AtML1:CKX1 showed a reduction of about 15% and the AtML1:ARR1-SRDX lines exhibited a decrease in rosette diameter of 8% in comparison to the wild type (**Figure 7A**). Corresponding changes in rosette growth rate were detected in the respective transgenic lines (**Figure 7B**).

In the wild-type background, the *ahk2 cre1* double mutant had no impact on rosette diameter, *ahk3 cre1* showed a 22.6% reduction and *ahk2 ahk3* showed with 48.8% reduction the strongest change compared to wild type. Expression of *AtML1:LOG4* caused an increase of the rosette diameter in *ahk2 cre1* indicating that AHK3 alone is sufficient to transmit the CK signal (**Figure 7C**). AtML1:LOG4 *ahk3 cre1* hybrids showed a reduction of rosette diameter as compared to AtML1:LOG4 which was even stronger in the AtML1:LOG4 *ahk2 ahk3* hybrids. AtML1:LOG4 significantly enhanced the rosette diameter of *ahk3 cre1*, whereas no effect was observed in *ahk2 ahk3* mutants (**Figure 7C**). This suggests also a partial contribution of AHK2 to mediate the CK effect while CRE1/AHK4 had apparently no role.

ARR1, *ARR10*, and *ARR12* are among the most highly expressed type-B *ARR* genes in leaves (Mason et al., 2004; Tajima et al., 2004) and they mediate most if not all of the effects of CK on vegetative growth (Argyros et al., 2008; Ishida et al., 2008). To test their involvement in the enhanced rosette growth observed in AtML1:LOG4, we introgressed the *AtML1:LOG4* transgene into all three double mutant combinations. The transgene expression enhanced the rosette size only in the background of *arr10,12* (**Figure 7D**), suggesting an involvement of ARR1 in mediating rosette growth by epidermal CK.

CK Signaling From the Epidermis Promotes Leaf Growth

Increased CK biosynthesis by *AtML1:LOG4* expression enhanced leaf growth. This could be due to a cell-autonomous effect of CK in the epidermis, and/or due to a growth stimulatory effect of epidermally produced CK on the underlying tissues. To address this question experimentally, we suppressed the CK signaling in the epidermis of AtML1:LOG4 plants by crossing

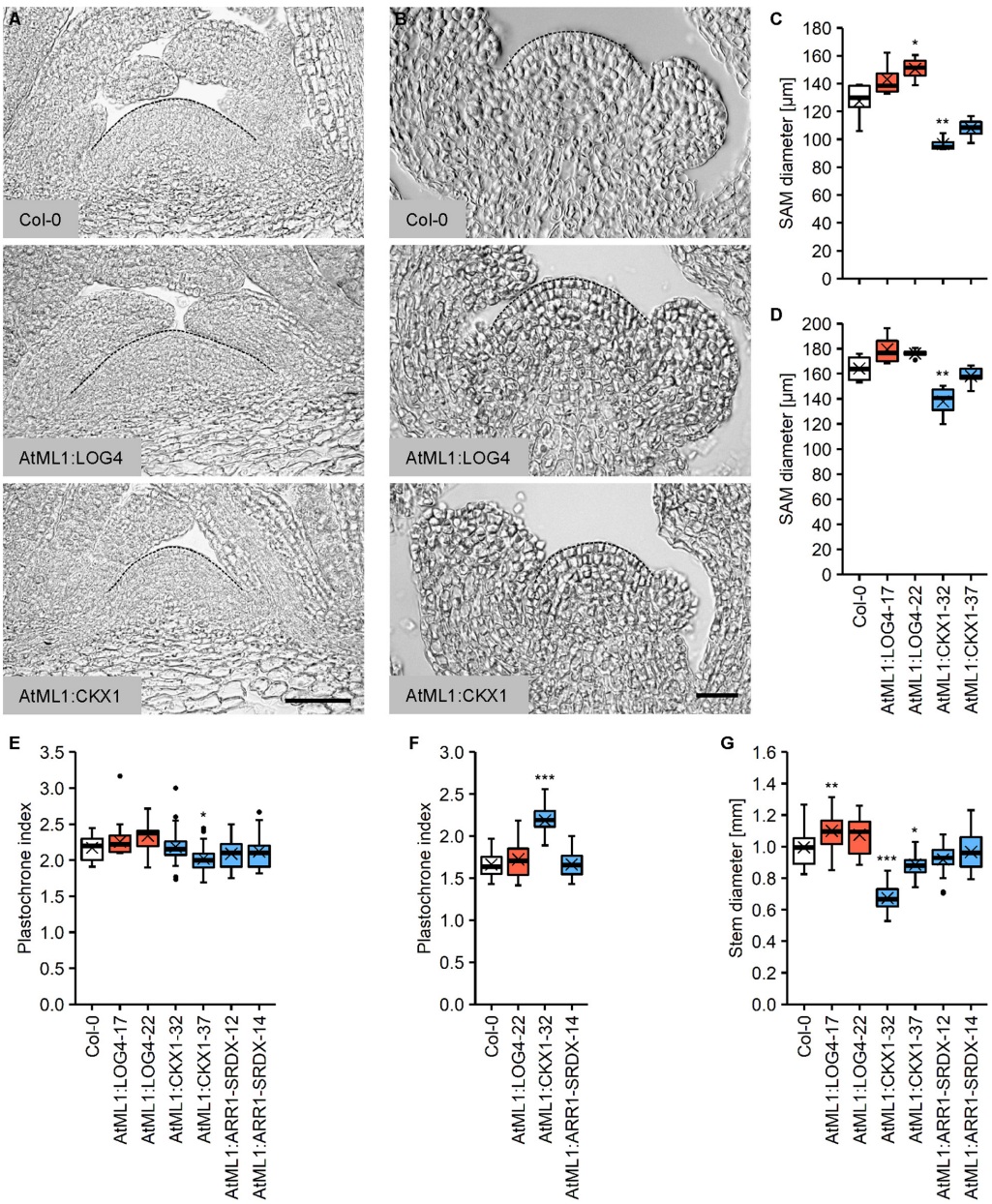


FIGURE 3 | Altered epidermal cytokinin metabolism in AtML1:LOG4 and AtML1:CKX1 influences the size of the shoot apical meristem and the thickness of the stem. **(A)** Median longitudinal sections of the vegetative SAMs of 30-day-old SD-grown wild-type, AtML1:LOG4 and AtML1:CKX1 plants. Scale bar = 50 µm. **(B)** Median longitudinal sections of the inflorescence meristems of LD-grown wild-type, AtML1:LOG4 and AtML1:CKX1 plants. Scale bar = 50 µm. **(C,D)** Diameter of the vegetative (**C**; n = 4–5) and inflorescence meristem (**D**; n = 4–6). **(E,F)** Leaf initiation rate under LD (**C**; n = 28–30) and SD conditions (**D**; n = 17–20). **(G)** Stem diameter (n = 17–24). Asterisks indicate significant differences compared to the wild type, as calculated by one-way ANOVA, *post hoc* Dunnett's test **(C,D,G)** or Kruskal–Wallis test, *post hoc* Dunn's test **(E,F)** (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

to AtML1:ARR1-SRDX. The rosette diameter of a homozygous hybrid line expressing both transgenes (**Supplementary Figure S5**) was analyzed. **Figure 7E** shows that the rosette size of the transgenic hybrid was comparable to wild type, indicating that the growth promoting effect of *AtML1:LOG4* was abolished. Hence, the results suggest that CK signaling in the epidermal layer promotes leaf growth in a, at least partially, cell-autonomous fashion.

DISCUSSION

Enhanced CK biosynthesis or breakdown in the epidermis caused numerous developmental changes of shoot growth that are known from *Arabidopsis* mutants and transgenic plants with an altered CK content or signaling (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Argyros et al., 2008; Ishida et al., 2008; Holst et al., 2011;

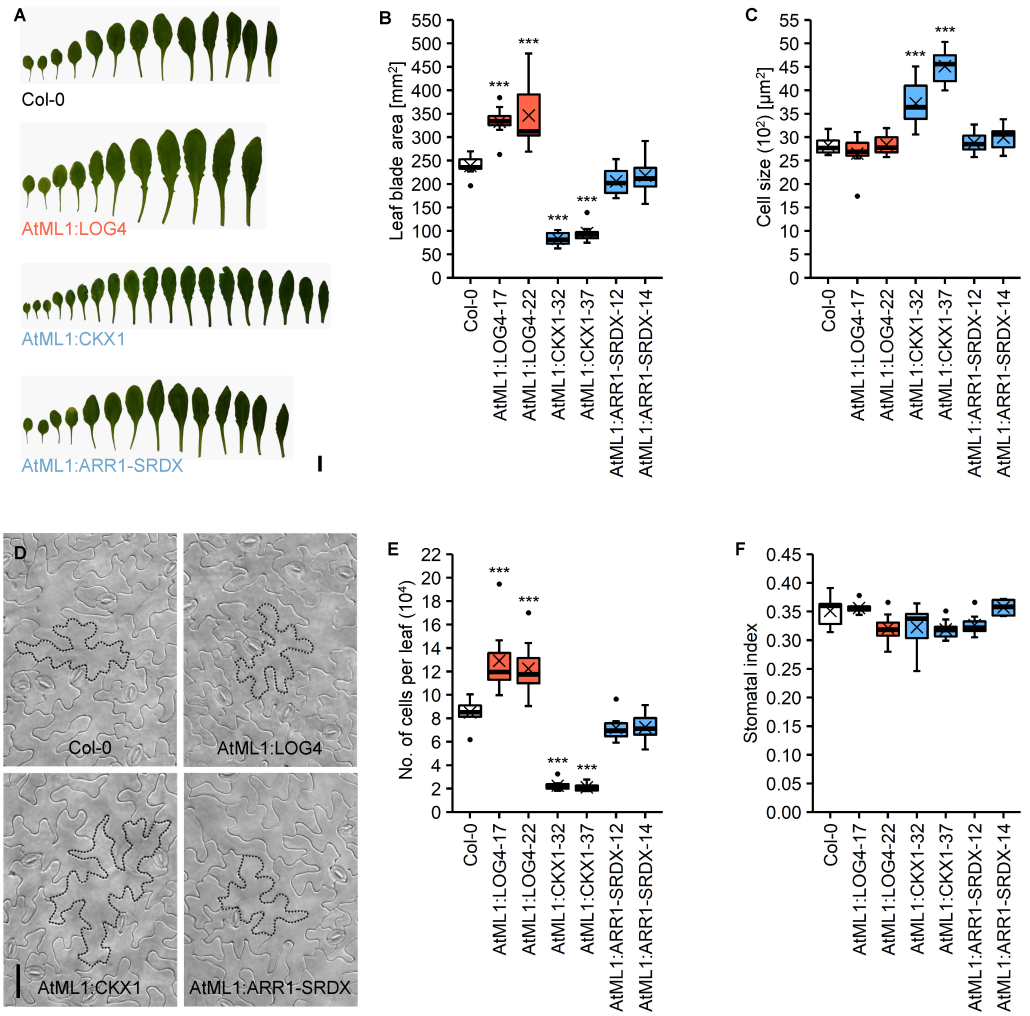


FIGURE 4 | Analysis of the abaxial epidermis of the seventh rosette leaf of LD-grown *AtML1* lines. **(A)** Morphology of full grown leaves of Col-0, AtML1:LOG4-22, AtML1:CKX1-32, and AtML1:ARR1-SRDX-12 plants; scale bar = 1 cm. **(B)** Leaf blade size ($n = 8$). **(C)** Cell size ($n = 8$). **(D)** Abaxial epidermis of Col-0, AtML1:LOG4-22, AtML1:CKX1-32, and AtML1:ARR1-SRDX-12 leaves; exemplary cells are outlined with dotted lines; scale bar = 50 µm. **(E)** Number of cells per leaf ($n = 8$). **(F)** Stomatal index, defined as proportion of stomata within the total population of abaxial epidermal cells ($n = 8$). Asterisks indicate significant differences compared to the wild type, as calculated by one-way ANOVA, *post hoc* Dunnett's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Bartrina et al., 2011, 2017; Niemann et al., 2015; Skalák et al., 2019). These changes included an altered size of the shoot apical meristem, altered leaf and rosette size, altered flowering time, and an impact on seed size and seed yield. This indicates that CK breakdown and CK synthesis in the epidermis can be a factor regulating various aspects of shoot development. The fact that numerous CK metabolism and signaling genes are expressed in the epidermis (Yadav et al., 2014) is consistent with this conclusion.

It needs to be considered that CK is transported and some CK metabolites might also diffuse to surrounding cells or reach them through plasmodesmata. Therefore, it is likely that enhanced degradation of CK in the epidermis weakened its function as source of the hormone and thus indirectly affected tissues adjacent to the epidermis. In an opposite scenario, it cannot be excluded that enhanced CK degradation in the epidermis drained CK from underneath tissues. Similarly, enhanced CK synthesis in the epidermis could act in the epidermis itself or in deeper cell layers. In fact, CK synthesized in the L1 has been proposed to form gradients over several cell layers to regulate *WUS* expression in the organizing center (Chickarmane et al., 2012; Gruel et al., 2016). Further, other work has shown that CK regulating the SAM might come from the young leaf primordia (Holst et al., 2011) or even from roots (Osugi et al., 2017) documenting the mobile nature of the hormone. Notably, a non-cell-autonomous effect of CK was also observed when CK metabolism was experimentally altered during gynoecium development (Marsch-Martínez et al., 2012). Taken together, the phenotypic changes caused by expression of *AtML1:CKX1* and *AtML1:LOG4* must not be or at least not entirely be due to events in the epidermis itself. Consistently, the consequences of *AtML1:ARR1-SRDX*

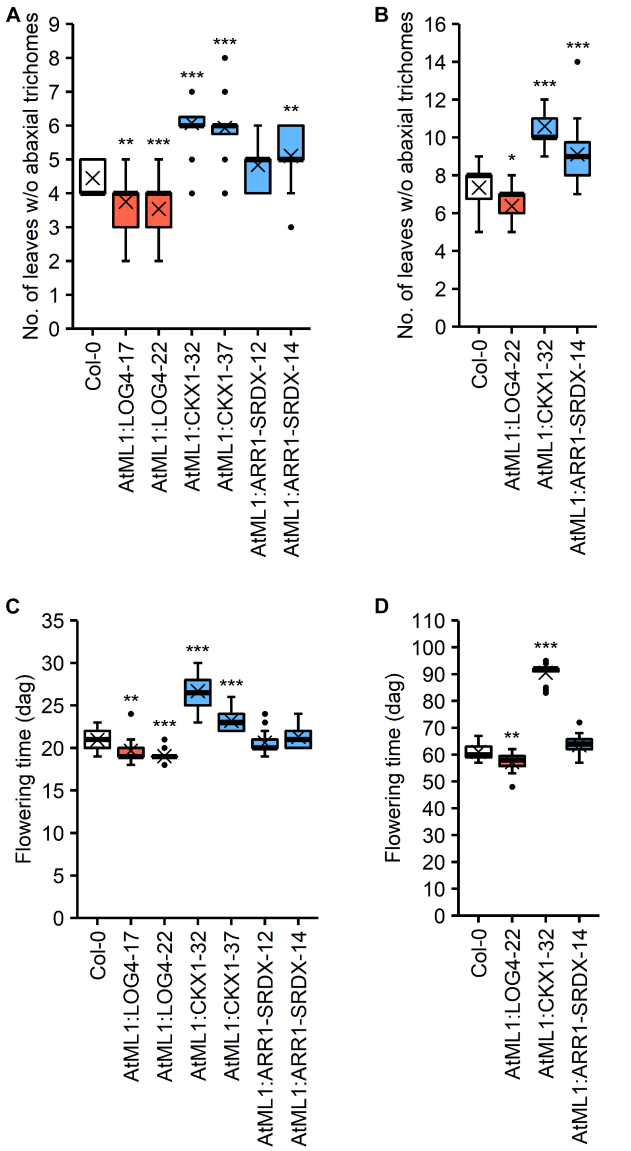


FIGURE 5 | Impact of the expression of *AtML1*:*LOG4, AtML1*:*CKX1*, and *AtML1:ARR1-SRDX* on developmental transitions. **(A,B)** Number of leaves without abaxial trichomes under LD (**A**; n = 28–30) and SD conditions (**B**; n = 17–20). **(C,D)** Flowering time of *AtML1* transgenic lines compared to the wild type under LD (**A**; n = 28–30) and SD conditions (**B**; n = 19–20). Asterisks indicate significant differences compared to the wild type, as calculated by one-way ANOVA, *post hoc* Dunnett's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

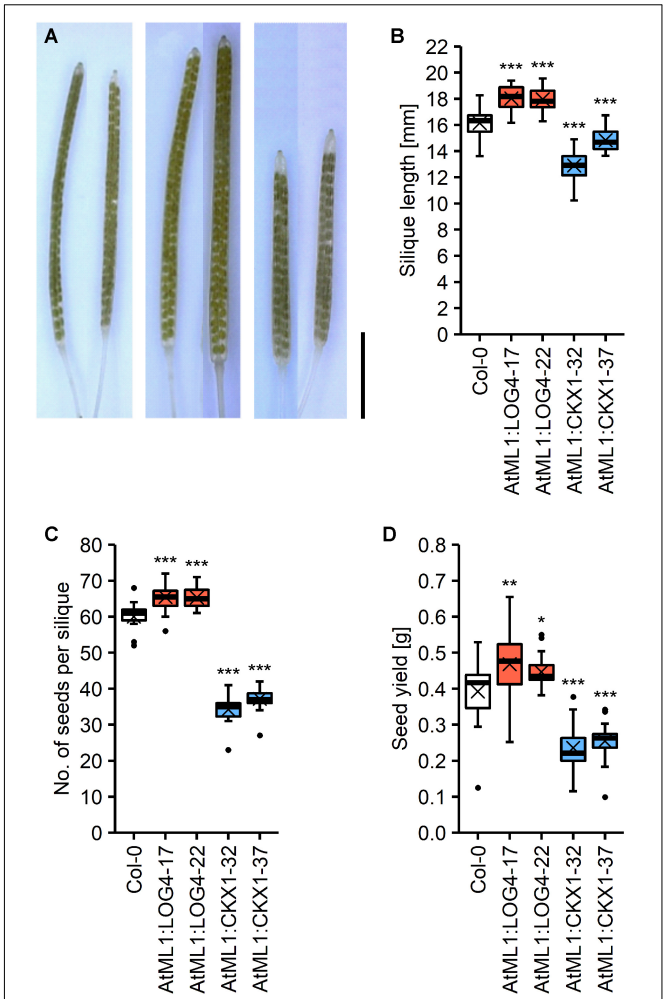


FIGURE 6 | Expression of *AtML1*:*LOG4* and *AtML1*:*CKX1* influences seed yield. **(A)** Cleared siliques; order from left to right: Col-0, AtML1:LOG4-22, AtML1:CKX1-32 (scale bar = 5 mm). **(B)** Silique length (n = 18–30). **(C)** Number of seeds per silique (n = 18–30). **(D)** Seed yield per plant (n = 24–25). Asterisks indicate significant differences compared to the wild type, as calculated by one-way ANOVA, *post hoc* Dunnett's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

expression were generally smaller or even absent as compared to those of *AtML1:CKX1*.

If and to which extent CK acts in the epidermis itself is probably best illustrated by the analysis of leaf and rosette size (reflecting leaf length) of the transgenic lines. CK has numerous functions during leaf development. It regulates cell division during the early phase, the transition of cells from the division to the differentiation phase, the following cell enlargement, and finally termination of leaf development (Werner et al., 2003; Holst et al., 2011; Efroni et al., 2013; Bartrina et al., 2017; Skalák et al., 2019). It has been shown that it is sufficient to alter the CK content during the early cell division phase of leaf development to obtain profound effects on leaf size indicating that its effect on cell division and the inhibition of cell differentiation are most relevant for regulating leaf size (Holst et al., 2011; Skalák et al., 2019).

Both AtML1:CKX1 and AtML1:ARR1-SRDX plants had a reduced leaf and rosette size compared to wild type. The leaf surface in AtML1:CKX1 plants was reduced by about 65% and the rosette diameter by about 15%, while the size reduction was smaller in AtML1:ARR1-SRDX plants reaching on the average 89% of leaf size and 92% of the rosette diameter of wild type. Notably, the size reduction of AtML1:CKX1 leaves was less strong than in leaves of 35S:CKX1 and 35:CKX3 plants (Werner et al.,

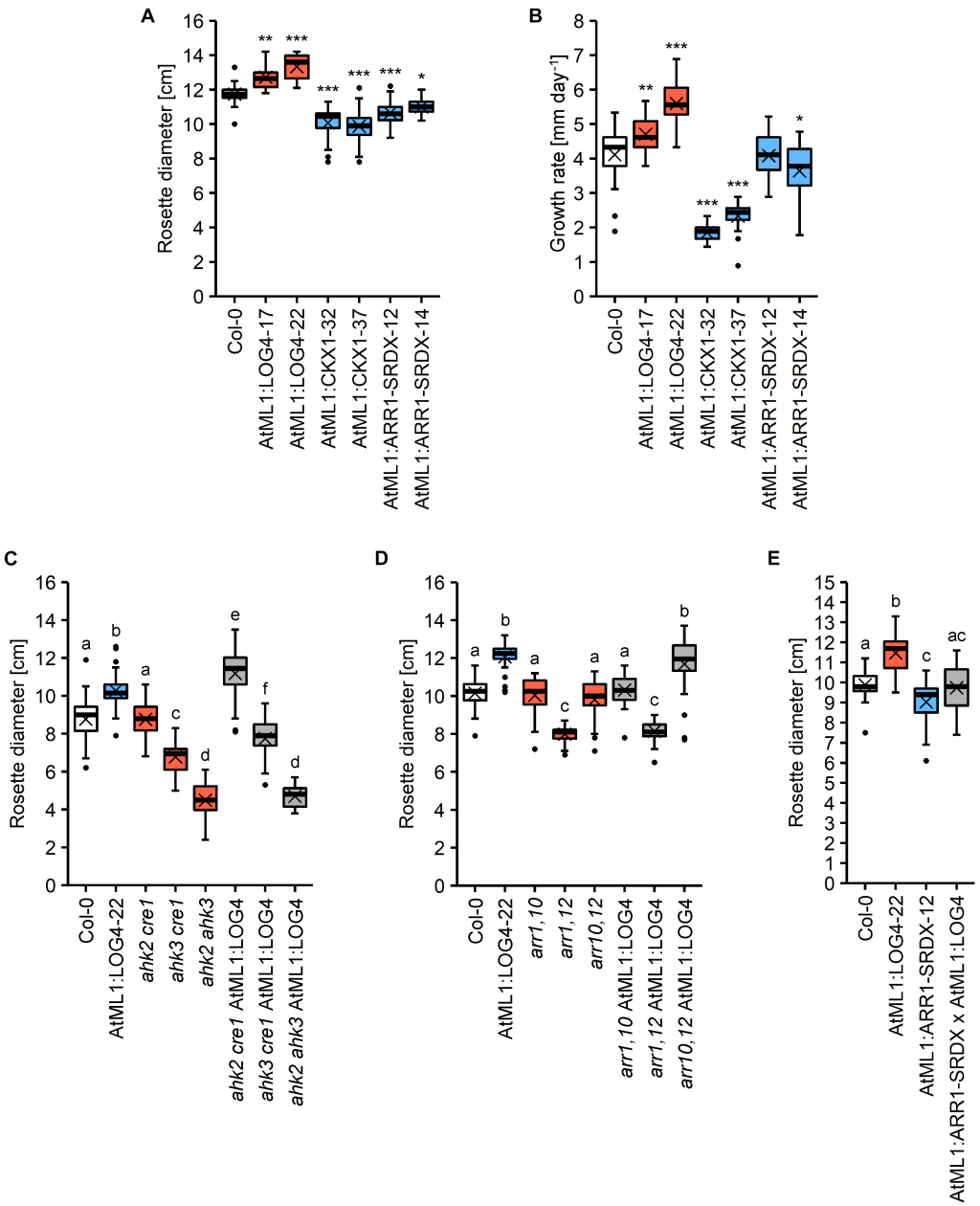


FIGURE 7 | Rosette growth of *AtML1* lines. **(A)** Rosette diameter (n = 18–24) and **(B)** growth rate of the rosette (n = 26–27) of *AtML1* lines. Asterisks indicate significant differences compared to the wild type, as calculated by one-way ANOVA, *post hoc* Dunnett's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). **(C)** Influence of CK receptor mutations on the enhanced rosette growth of AtML1:LOG4 plants grown under LD conditions for 33 days (n = 28). **(D)** Influence of mutations of type-B response regulators on the enhanced rosette growth of AtML1:LOG4 plants grown under LD conditions for 29 days (n = 27–28). Letters indicate significant differences between the genotypes, as calculated by one-way ANOVA, *post hoc* Tukey's test ($p < 0.05$). **(E)** Rosette diameter of LD-grown AtML1:ARR1-SRDX AtML1:LOG4 plants (28 DAG) compared to the parental lines (n = 21–24).

2003; Holst et al., 2011). Similarly, systemically reduced CK signaling in 35S:ARR1-SRDX plants had a much stronger effect on leaf size (Heyl et al., 2008) than epidermal expression of the transcriptional repressor in AtML1:ARR1-SRDX. The stronger consequences of systemic interference with CK metabolism or signaling suggests that the CK status of deeper cell layer is also a relevant factor in regulating leaf growth.

Furthermore, cells of the abaxial epidermis of AtML1:CKX1 plants were 30–60% larger than in wild type indicating that a compensatory effect had been initiated. An increase in cell size to compensate for a lower cell number is known from CK-deficient plants (Werner et al., 2003; Holst et al., 2011; Skalák et al., 2019) as well as from plants with altered cell cycling (reviewed by Horiguchi and Tsukaya, 2011). In

contrast, the size of cells in the epidermis of AtML1:ARR1-SRDX and AtML1:LOG4 leaves was similar to wild type, but leaves of AtML1:LOG4 plants formed about 35% more cells. We conclude that epidermal CK metabolism impacts cell division and the transition to cell differentiation thus regulating leaf cell number and leaf size. Notably, the increase in cell size in AtML1:CKX1 leaves was similar to 35S:CKX3 leaves and larger than in ANT:CKX3 leaves (Holst et al., 2011). This supports the conclusion that CK restricts cell enlargement during leaf expansion growth when the *AtML1* and *35S* promoters are still active but the primordia-specific *ANT* promoter is no longer expressed.

The relevance of epidermal CK acting in the epidermis itself to regulate leaf size might be concluded from the phenotype of AtML1:LOG4/AtML1:ARR1-SRDX hybrid plants. In these plants, the promotive influence of CK synthesis in the epidermis on leaf size was strongly suppressed by abbreviating CK signaling in this cell layer. Principally, the LOG4-driven CK production in the epidermis could still increase CK concentrations in deeper tissues but this had apparently no strong influence on leaf size. Thus, the hybrid phenotype indicated that CK synthesized in the epidermis acted on epidermal cells delaying their differentiation. This example clearly illustrates that CK promotes leaf growth in the epidermal layer in an at least partially layer-autonomous fashion.

Genetic analysis showed that the AHK2 and AHK3 receptors together with the transcription factor ARR1 mediate the CK effect on rosette growth in AtML1:LOG4 plants as mutation of these genes abolished the LOG4-induced growth enhancement. *LOG4* expression in the *ahk2 ahk3* background had no effect on leaf size at all consistent with the known relevance of these receptors in regulating leaf traits (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Cortleven et al., 2014), their strong signaling activity in leaves (Stolz et al., 2011), and their expression in the epidermis (Yadav et al., 2014). Furthermore, AHK2 and AHK3 are prime candidates to perceive the CK signal in the cell internal membrane system (Romanov et al., 2006; Wulfetange et al., 2011; Antoniadi et al., 2020), which may be required for the cell-autonomous activity of CK in the epidermis. Mutation of *ARR1* also blocked completely the response to *AtML1:LOG4* and ARR1 alone was sufficient to mediate the growth enhancement by CK production in the epidermis in the *arr10,12* mutant. This indicates a singular role for this transcription factor in mediating CK activity in the epidermis. The more drastic growth reduction of the rosettes in *ahk2 ahk3 cre1* and *arr1,10,12* triple mutants (Nishimura et al., 2004; Higuchi et al., 2004; Riefler et al., 2006; Argyros et al., 2008; Ishida et al., 2008) suggests that CRE1/AHK4, ARR10, and ARR12 also have functions in regulating leaf growth but probably not in the epidermal cell layer.

CK synthesized in the epidermis of AtML1:LOG4 plants promoted the juvenile-to-adult phase transition while increased CK breakdown or reduced signaling caused a later phase transition (**Figures 5A,B**). Vegetative phase change relies strongly on the balance of miR156 and miR172 and the activity of their respective target genes (Huijser and Schmid, 2011; Poethig, 2013). The abaxial trichomes formed on adult leaves are a commonly used indicator for the transition from the juvenile to the adult phase (Telfer et al., 1997) and they are derived from the epidermis. Thus it could be that the hormone exerts its activity in part directly in the layer where it is produced, which is consistent with the later phase transition of AtML1:ARR1-SRDX. Their weak phenotype compared to AtML1:CKX1 suggests that not the epidermis alone, but rather the whole leaf determines epidermal identity. The regulation of trichome formation from mesophyll depending on the transcription factor TEMPRANILLO shows that there are (also) actions from a distance (Matías-Hernández et al., 2016). Whether CK also affects other leaf traits accompanying vegetative phase change needs to be determined.

Another developmental transition, the onset of flowering showed a different picture. Increased CK synthesis and CK breakdown in the epidermal cell layer caused earlier and later flowering, respectively (**Figures 5C,D**). This is consistent with the known promotive effect of CK on flowering in *Arabidopsis* (Kieber and Schaller, 2018). Abbreviating CK signaling by epidermal expression of *AtML1:ARR1-SRDX* did not alter flowering time suggesting that the CK status of the epidermis itself has no influence on flowering but that altered epidermal CK metabolism influenced the activity of a mobile signal promoting the transition to flowering. Given the long-distance transport of CK in plants, it can be hypothesized that this mobile signal is CK itself.

The higher seed yield of AtML1:LOG4 plants shows that a limited and layer-specific increase in CK content may cause a higher seed yield. This increase was mainly due to the higher number of seeds harbored by individual siliques which were larger than in wild type. Consistently, AtML1:CKX1 plants had smaller siliques with less seeds. Furthermore, the difference in seed yield may be related to the number of ovules that were initiated in the gynoecia. Indeed, CK promotes the activity of the placenta and regulates the ovule number and the distance between ovules (Bartrina et al., 2011; Bencivenga et al., 2012; Cerbantez-Bueno et al., 2020) and *AtML1* is expressed in the placenta (Lu et al., 1996). Interestingly, other yield traits previously shown to be regulated by CK such as the activity of the inflorescence meristem and the duration of flowering (Bartrina et al., 2011, 2017) were not regulated by epidermal CK. In any case, the demonstration that expression of *AtML1:LOG4* as a single dominant gene increases seed yield is of potential biotechnological interest as yield traits are also controlled by CK in oilseed rape (*Brassica napus* L.), which is an important source for vegetable oil and close relative of *Arabidopsis* (Zúñiga-Mayo et al., 2018; Schwarz et al., 2020).

Collectively, the analysis has shown that manipulating the CK metabolism in the epidermis can alter plant growth and development. It is clear from the analysis of leaf size regulation that layer-autonomous activity of CK affects primarily cell division and differentiation. This contrasts with brassinosteroid and ethylene, which both regulate cell expansion of epidermal cells (Savaldi-Goldstein et al., 2007; Vaseva et al., 2018) in a layer-autonomous fashion. Clearly, further experiments are required to resolve the spatial organization of CK action and its potential role

in coordinating cell division and differentiation of the epidermal cells and cells of underlying tissues.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* served as the wild type. All mutants used in this study are listed in **Supplementary Table S2**. All genotypes were confirmed by PCR analysis and the primers used for genotyping are listed in **Supplementary Table S3**. For all experiments conducted on soil, sown *Arabidopsis* seeds were kept at 4°C for 2 days in the dark and then exposed to LD (16 h light/8 h dark cycle) or SD conditions (8 h light/16 h dark cycle), at 22°C and 60% humidity and a light intensity of 100–150 μmol m^{-2} s^{-1}. Individuals of different lines were randomized to minimize positional effects. For *in vitro* experiments, seeds were surface sterilized using a 1.2% (v/v) sodium hypochlorite/0.01% (v/v) Triton X-100 solution. After stratification, seedlings were grown at 22°C on 1/2 × Murashige and Skoog (MS) medium containing 0.22% (w/v) MS basal salt, 0.1% (w/v) sucrose, 0.05% (w/v) MES, and 0.8% (w/v) agar (pH adjusted to 5.7).

Plasmid Constructions

For the generation of *AtML1*:*GFP-GUS*, *AtML1*:*LOG4*, *AtML1*:*CKX1*, and *AtML1*:*ARR1-SRDX* constructs, the Gateway® system (ThermoFisher, Waltham, MA, United States) was used. All primers used for cloning purposes are listed in **Supplementary Table S4**. A 3.9 kb long *AtML1* promoter fragment (−5488 to −1581 bp upstream of the *AtML1* start codon) was amplified from Col-0 genomic DNA using Phusion High-Fidelity DNA-Polymerase (ThermoFisher). The fragment was linked to the Gateway® attB1/attB2 and attB4/attB1R sites by PCR and cloned into pDONR221 and pDONR P4-P1R, respectively, using Gateway BP Clonase Enzyme Mix (ThermoFisher). The pDONR221/*pAtML1* vector was used to generate a transcriptional fusion with the *GUS* gene in pKGWFS7 (Karimi et al., 2002) using Gateway LR Clonase Enzyme Mix (ThermoFisher). The *LOG4* gene coding sequence was amplified from Col-0 cDNA including the stop codon. For *CKX1*, the genomic sequence from Col-0 lacking the stop codon was used for cloning. The protein coding region of *A. thaliana* accession C24 *ARR1* fused to the SRDX peptide (LDLDLELRLGFA) was amplified from pDONR201/ARR1-SRDX (Heyl et al., 2008) without stop codon. All gene fragments were cloned first into pDONR221 and then combined with *AtML1*-attB4/attB1R in pB7m34GW (Karimi et al., 2005) using LR Clonase II Enzyme Mix (ThermoFisher). The third position at the 3′ end in pB7m34GW was filled with a 4xMyc tag, creating a translational fusion in case of *CKX1* and *ARR1-SRDX*. All cloned DNA fragments were fully sequenced to exclude PCR errors. All plasmids were individually transformed into the *Agrobacterium tumefaciens* strain GV3101:pMP90 by electroporation and the resulting strains were subsequently used to transform *Arabidopsis* plants using the floral-dipping method (Clough and Bent, 1998).

RNA Preparation and Quantitative RT-PCR

Approximately 100 mg of plant material was harvested and frozen in liquid nitrogen at the indicated time points. The frozen samples were ground using a Retsch mill in precooled adapters. Total RNA was extracted using TRIsure™ (Bioline) following the manufacturer's instructions. The RNA pellet was resuspended in 40–50 µl of nuclease-free water and treated with DNase I (ThermoFisher), following the manufacturer's instructions. For cDNA synthesis, 1.0–1.5 µg of total RNA was reverse transcribed using SuperScript™ III (ThermoFisher), 4.5 µM of N9 random oligos, and 2.5 µM of oligo-dT_{25} in a 20 µl reaction. Mix 1 containing RNA, 2 mM of dNTP mix and oligos was incubated for 5 min at 65°C and placed on ice afterwards. Mix 2 (first strand buffer, 5 mM DTT, SuperScript™ III) was added and samples were incubated for 30 min at 25°C, 60 min at 50°C, and 15 min at 70°C. The resulting cDNA was diluted 1:5. For qRT-PCR analysis, *PROTEIN PHOSPHATASE 2A SUBUNIT A2 (PP2AA2)* and *TBP-ASSOCIATED FACTOR II 15 (TAFII15)* served as reference genes. All qRT-PCR primers used in this study are listed in **Supplementary Table S5**. qRT-PCR was performed with the 7500 Fast RealTime PCR system (Applied Biosystems) using SYBR Green I technology and universal FAST cycling conditions (15 min at 95°C, 40 cycles of 5 s at 95°C, 15 s at 55°C, and 10 s at 72°C) followed by the generation of a dissociation curve to check for specificity of the amplification. Gene expression data analysis was carried out according to Vandesompele et al. (2002). For analysis of *ARR1-SRDX* transgene expression, the $40-\Delta Ct$ method was used, as described by Morcuende et al. (2007).

Cytokinin Measurements

For the determination of CK metabolites, plants were grown on soil under LD conditions. Approximately 20 mg of shoot material of 7-day-old seedlings were pooled, frozen in liquid nitrogen and stored at −80°C until further analysis. Four independent samples were analyzed for each genotype. The CK content was determined by ultraperformance liquid chromatography-electrospray tandem mass spectrometry (Novák et al., 2008).

Phenotypic Analyses

Plant height and rosette diameter were measured with a ruler. Plant rosette size was determined by measuring the maximal rosette diameter after progression to the reproductive phase. For the analysis of the rosette growth rate, the rosette diameter was measured every 2–3 days. The calculation of the growth rate was based on the linear growth phase. Flowering time was defined as the day the inflorescence stem was 0.5 cm long. Silique, flower and gynoecium length as well as SAM and seed diameter were measured using ImageJ (Abràmoff et al., 2004). To determine the size of the vegetative and inflorescence meristems, the distance at the base of the SAM between the primordia was measured. For the determination of seed size, length and width of every seed were measured and the mean of these values is shown as the average seed diameter.

Histology and Histochemistry

For the determination of cell size, cell number, and stomatal index, the seventh rosette leaves were cleared (Malamy and Benfey, 1997) and analyzed by a stereomicroscope (SZX12; Olympus, Tokyo, Japan) and a microscope (Axioskop 2 plus with AxioCam ICc3 camera; Zeiss, Jena, Germany). The whole leaf blade area was measured using the ImageJ plugin LeafJ (Maloof et al., 2013) and the number of pavement and guard cells per defined area at six different positions of the abaxial epidermis were determined from digital micrographs: at 25%, 50%, and 75% distance between the base and the tip of the leaf blade, in the midst of the leaf margin and the main vein on both sides. From these data, the number of cells per leaf, average cell size, and stomatal index were calculated. To conduct tissue sections, plant material was vacuum-infiltrated with fixative solution containing 10% (v/v) formaldehyde, 5% (v/v) acetic acid, and 50% (v/v) ethanol. After incubation at 4°C for 8–12 h, plant material was dehydrated by subjecting it to 50%, 60%, 70%, 85%, 95%, and 100% ethanol for 45 min each at 4°C. Samples were incubated in ethanol with increasing amounts of Roti®-Histol (Carl Roth, Karlsruhe, Germany) at room temperature. Paraplast X-TRA® (Sigma-Aldrich, Munich, Germany) was added to the vials and incubated at 58°C overnight. Wax was changed several times for 3 days and ultimately was solidified at room temperature. Cross sections were performed at a rotary microtome (RM2255, Leica, Wetzlar, Germany). For the analysis of the stem diameter, stem fragments were taken 2–3 cm above the rosette when the main inflorescence was 12–15 cm high. The stem segments were photographed using the SZX12 stereomicroscope and 14 μm thick cross sections were prepared. After staining with 0.1% toluidine blue, sections were analyzed with the Axioskop microscope. Stem diameter was measured using ImageJ (Abràmoff et al., 2004). GUS staining of *Arabidopsis* seedlings was performed according to Hemerly et al. (1993). The tissue was cleared for the microscopical analysis according to Malamy and Benfey (1997). For the confirmation of the epidermal expression, 8 μm tissue sections of the SAM area were produced and analyzed with the Axioskop microscope. For the analysis of SAM size, 30-day-old SD-grown plants as well as the inflorescences of LD-grown plants were fixed as described and 8 μm tissue sections were generated.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism, version 8 (GraphPad Software, La Jolla, CA, United States). Statistical tests used are indicated in the figure and table legends. A p-value < 0.05 was considered to indicate a significant difference.

Accession Numbers

Sequences of genes and intergenic regions described in this article can be found in The Arabidopsis Information Resource (http://www.arabidopsis.org/) under the following accession numbers: *AHK2* (AT5G35750), *AHK3* (AT1G27320), *CRE1/AHK4* (AT2G01830), *ARR1* (AT3G16857), *ARR4* (AT1G10470), *ARR5* (AT3G48100), *ARR6* (AT5G62920), *ARR7* (AT1G19050), *ARR9* (AT3G57040), *ARR10* (AT4G31920), *ARR12* (AT2G25180), *AtML1* (AT4G21750), *CKX1* (AT2G41510), and *LOG4* (AT3G53450).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SW, IB, TW, and TS developed the project. SW performed experiments. SW, IB, TW, and TS analyzed the data. ON and MS measured cytokinin concentrations. SW and TS wrote the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Susanne Gerber and Gabriele Grüschow for skillful technical assistance and acknowledge support by the Open Access Publication Initiative of Freie Universität Berlin. This work was supported by the grant 18-07563S from the Czech Science Foundation and ERDF Project No. CZ.02.1.01/0.0/0.0/16_019/0000827 (plants as a tool for sustainable global development).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.613488/full#supplementary-material

REFERENCES

Abràmoff, M. D., Magalhães, P. J., and Ram, S. J. (2004). Image processing with ImageJ. *Biophoton. Int.* 11, 36–42.

Antoniadi, I., Novák, O., Gelova, Z., Johnson, A., Plíhal, O., Simerský, R., et al. (2020). Cell-surface receptors enable perception of extracellular cytokinins. *Nat. Commun.* 11:4284. doi: 10.1038/s41467-020-17700-9

Argyros, R. D., Mathews, D. E., Chiang, Y. H., Palmer, C. M., Thibault, D. M., Etheridge, N., et al. (2008). Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. *Plant Cell* 20, 2102–2116. doi: 10.1105/tpc.108.059584

Arnaud, D., Lee, S., Takebayashi, Y., Choi, D., Choi, J., Sakakibara, H., et al. (2017). Cytokinin-mediated regulation of reactive oxygen species homeostasis modulates stomatal immunity in *Arabidopsis*. *Plant Cell* 29, 543–559. doi: 10.1105/tpc.16.00583

Bartrina, I., Jensen, H., Novák, O., Strnad, M., Werner, T., and Schmülling, T. (2017). Gain-of-function mutants of the cytokinin receptors AHK2 and AHK3 regulate plant organ size, flowering time and plant longevity. *Plant Physiol.* 173, 1783–1797. doi: 10.1104/pp.16.01903

Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmülling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. *Plant Cell* 23, 69–80. doi: 10.1105/tpc.110.079079

Bencivenga, S., Simonini, S., Benkova, E., and Colombo, L. (2012). The transcription factors BEL1 and SPL are required for cytokinin and auxin signaling during ovule development in *Arabidopsis*. *Plant Cell* 24, 2886–2897. doi: 10.1105/tpc.112.100164

Bhargava, A., Clabaugh, I., To, J. P., Maxwell, B. B., Chiang, Y. H., Schaller, G. E., et al. (2013). Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-seq in *Arabidopsis*. *Plant Physiol.* 162, 272–294. doi: 10.1104/pp.113.217026

Brenner, W. G., Ramireddy, E., Heyl, A., and Schmülling, T. (2012). Gene regulation by cytokinin in *Arabidopsis*. *Front. Plant Sci.* 3:8. doi: 10.3389/fpls.2012.00008

Cerbantez-Bueno, V. E., Zúñiga-Mayo, V. M., Reyes-Olalde, J. I., Lozano-Sotomayor, P., Herrera-Ubaldo, H., Marsch-Martínez, N., et al. (2020). Redundant and non-redundant functions of the AHK cytokinin receptors during gynoecium development. *Front. Plant Sci.* 11:568277. doi: 10.3389/fpls.2020.568277

Chickarmane, V. S., Gordon, S. P., Tarr, P. T., Heisler, M. G., and Meyerowitz, E. M. (2012). Cytokinin signaling as a positional cue for patterning the apical-basal axis of the growing *Arabidopsis* shoot meristem. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4002–4007. doi: 10.1073/pnas.1200636109

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743. doi: 10.1046/j.1365-313x.1998.00343.x

Cortleven, A., Nitschke, S., Klaumünzer, M., Abdelgawad, H., Asard, H., Grimm, B., et al. (2014). A novel protective function for cytokinin in the light stress response is mediated by the ARABIDOPSIS HISTIDINE KINASE2 and ARABIDOPSIS HISTIDINE KINASE3 receptors. *Plant Physiol.* 164, 1470–1483. doi: 10.1104/pp.113.224667

Czesnick, H., and Lenhard, M. (2015). Size control in plants – lessons from leaves and flowers. *Cold Spring Harb. Perspect. Biol.* 7:a019190. doi: 10.1101/cshperspect.a019190

Efroni, I., Han, S. K., Kim, H. J., Wu, M. F., Steiner, E., Birnbaum, K. D., et al. (2013). Regulation of leaf maturation by chromatin-mediated modulation of cytokinin responses. *Dev. Cell* 24, 438–445. doi: 10.1016/j.devcel.2013.01.019

Grandjean, O., Vernoux, T., Laufs, P., Belcram, K., Mizukami, Y., and Traas, J. (2004). In vivo analysis of cell division, cell growth, and differentiation at the shoot apical meristem in *Arabidopsis*. *Plant Cell* 16, 74–87. doi: 10.1105/tpc.017962

Gruel, J., Landrein, B., Tarr, P., Schuster, C., Refahi, Y., Sampathkumar, A., et al. (2016). An epidermis-driven mechanism positions and scales stem cell niches in plants. *Sci. Adv.* 2:e1500989. doi: 10.1126/sciadv.1500989

Ha, C. M., Jun, J. H., and Fletcher, J. C. (2010). Shoot apical meristem form and function. *Curr. Top. Dev. Biol.* 91, 103–140. doi: 10.1016/S0070-2153(10)91004-1

Hemerly, A. S., Ferreira, P., Engler, J. D., Van Montagu, M., Engler, G., and Inzé, D. (1993). *Cdc2a* expression in *Arabidopsis* is linked with competence for cell division. *Plant Cell* 5, 1711–1723. doi: 10.1105/tpc.5.12.1711

Heyl, A., Ramireddy, E., Brenner, W. G., Riefler, M., Allemeersch, J., and Schmülling, T. (2008). The transcriptional repressor ARR1-SRDX suppresses pleiotropic cytokinin activities in *Arabidopsis*. *Plant Physiol.* 147, 1380–1395. doi: 10.1104/pp.107.115436

Higuchi, M., Pischke, M. S., Mähönen, A. P., Miyawaki, K., Hashimoto, Y., Seki, M., et al. (2004). *In planta* functions of the *Arabidopsis* cytokinin receptor family. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8821–8826. doi: 10.1073/pnas.0402887101

Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. *Plant J.* 34, 733–739. doi: 10.1046/j.1365-313X.2003.01759.x

Holst, K., Schmülling, T., and Werner, T. (2011). Enhanced cytokinin degradation in leaf primordia of transgenic *Arabidopsis* plants reduces leaf size and shoot organ primordia formation. *J. Plant Physiol.* 168, 1328–1334. doi: 10.1016/j.jplph.2011.03.003

Horiguchi, G., and Tsukaya, H. (2011). Organ size regulation in plants: insights from compensation. *Front. Plant Sci.* 2:24. doi: 10.3389/fpls.2011.00024

Hou, B., Lim, E. K., Higgins, G. S., and Bowles, D. J. (2004). N-glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. *J. Biol. Chem.* 279, 47822–47832. doi: 10.1074/jbc.M409569200

Huijser, P., and Schmid, M. (2011). The control of developmental phase transitions in plants. *Development* 138, 4117–4129. doi: 10.1242/dev.063511

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* 413, 383–389. doi: 10.1038/35096500

Hwang, I., Sheen, J., and Müller, B. (2012). Cytokinin signaling networks. *Annu. Rev. Plant Biol.* 63, 353–380. doi: 10.1146/annurev-arplant-042811-105503

Ingram, G. C. (2004). Between the sheets: inter-cell-layer communication in plant development. *Philos. Trans. R. Soc. London [Biol.]* 359, 891–906. doi: 10.1098/rstb.2003.1356

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., et al. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* 409, 1060–1063. doi: 10.1038/35059117

Ishida, K., Yamashino, T., Yokoyama, A., and Mizuno, T. (2008). Three type-B response regulators, ARR1, ARR10 and ARR12, play essential but redundant roles in cytokinin signal transduction throughout the life cycle of *Arabidopsis thaliana*. *Plant Cell Physiol.* 49, 47–57. doi: 10.1093/pcp/pcm165

Kakimoto, T. (2001). Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. *Plant Cell Physiol.* 42, 677–685. doi: 10.1093/pcp/pce112

Karimi, M., De Meyer, B., and Hilson, P. (2005). Modular cloning in plant cells. *Trends Plant Sci.* 10, 103–105. doi: 10.1016/j.tplants.2005.01.008

Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY^{TM} vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* 7, 193–195. doi: 10.1016/s1360-1385(02)02251-3

Kiba, T., Takei, K., Kojima, M., and Sakakibara, H. (2013). Side-chain modification of cytokinins controls shoot growth in *Arabidopsis*. *Dev. Cell* 27, 452–461. doi: 10.1016/j.devcel.2013.10.004

Kieber, J. J., and Schaller, G. E. (2014). Cytokinins. *Arabidopsis Book* 12:e0168. doi: 10.1199/tab.0168

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:dev149344. doi: 10.1242/dev.149344

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., et al. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445, 652–655. doi: 10.1038/nature05504

Kuroha, T., Tokunaga, H., Kojima, M., Ueda, N., Ishida, T., Nagawa, S., et al. (2009). Functional analyses of LONELY GUY cytokinin-activating enzymes reveal the importance of the direct activation pathway in *Arabidopsis*. *Plant Cell* 21, 3152–3169. doi: 10.1105/tpc.109.068676

Li, J., Nie, X., Tan, J. L., and Berger, F. (2013). Integration of epigenetic and genetic controls of seed size by cytokinin in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15479–15484. doi: 10.1073/pnas.1305175110

Lu, P. Z., Porat, R., Nadeau, J. A., and Oneill, S. D. (1996). Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. *Plant Cell* 8, 2155–2168. doi: 10.1105/tpc.8.12.2155

Malamy, J. E., and Benfey, P. N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development* 124, 33–44.

Maloof, J. N., Nozue, K., Mumbach, M. R., and Palmer, C. M. (2013). LeafJ: an ImageJ plugin for semi-automated leaf shape measurement. *J. Vis. Exp.* 21:50028. doi: 10.3791/50028

Marcotrigiano, M. (2010). A role for leaf epidermis in the control of leaf size and the rate and extent of mesophyll cell division. *Am. J. Bot.* 97, 224–233. doi: 10.3732/ajb.0900102

Marsch-Martínez, N., Ramos-Cruz, D., Reyes-Olalde, J. I., Lozano-Sotomayor, P., Zúñiga-Mayo, V. M., and de Folter, S. (2012). The role of cytokinin during *Arabidopsis* gynoecia and fruit morphogenesis and patterning. *Plant J.* 72, 222–234. doi: 10.1111/j.1365-313X.2012.05062

Mason, M. G., Li, J., Mathews, D. E., Kieber, J. J., and Schaller, G. E. (2004). Type-B response regulators display overlapping expression patterns in *Arabidopsis*. *Plant Physiol.* 135, 927–937. doi: 10.1104/pp.103.038109

Matías-Hernández, L., Aguilar-Jaramillo, A. E., Cigliano, R. A., Sanseverino, W., and Pelaz, S. (2016). Flowering and trichome development share hormonal and

transcription factor regulation. *J. Exp. Bot.* 67, 1209–1219. doi: 10.1093/jxb/erv534

Michniewicz, M., and Kamieńska, A. (1965). Flower formation induced by kinetin and vitamin E treatment in long-day plant (*Arabidopsis thaliana*) grown in short day. *Naturwissenschaften* 52:623.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., et al. (2006). Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16598–16603. doi: 10.1073/pnas.0603522103

Morcuende, R., Bari, R., Gibon, Y., Zheng, W. M., Pant, B. D., Bläsing, O., et al. (2007). Genome-wide reprogramming of metabolism and regulatory networks of *Arabidopsis* in response to phosphorus. *Plant Cell Environ.* 30, 85–112. doi: 10.1111/j.1365-3040.2006.01608.x

Niemann, M. C., Bartrina, I., Ashikov, A., Weber, H., Novák, O., Spíchal, L., et al. (2015). *Arabidopsis* ROCK1 transports UDP-GlcNAc/UDP-GalNAc and regulates ER protein quality control and cytokinin activity. *Proc. Natl. Acad. Sci. U.S.A.* 112, 291–296. doi: 10.1073/pnas.1419050112

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. *Plant Cell* 16, 1365–1377. doi: 10.1105/tpc.021477

Nobusawa, T., Okushima, Y., Nagata, N., Kojima, M., Sakakibara, H., and Umeda, M. (2013). Synthesis of very-long-chain fatty acids in the epidermis controls plant organ growth by restricting cell proliferation. *PLoS Biol.* 11:e1001531. doi: 10.1371/journal.pbio.1001531

Novák, O., Hauserová, E., Amakorová, P., Doležal, K., and Strnad, M. (2008). Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry* 69, 2214–2224. doi: 10.1016/j.phytochem.2008.04.022

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* 13, 1959–1968. doi: 10.1105/TPC.010127

Osugi, A., Kojima, M., Takebayashi, Y., Ueda, N., Kiba, T., and Sakakibara, H. (2017). Systemic transport of trans-zeatin and its precursor have differing roles in *Arabidopsis* shoots. *Nat. Plants* 3:17112. doi: 10.1038/nplants.2017.112

Poethig, R. S. (2013). Vegetative phase change and shoot maturation in plants. *Curr. Top. Dev. Biol.* 105, 125–152. doi: 10.1016/B978-0-12-396968-2.00005-1

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18, 40–54. doi: 10.1105/tpc.105.037796

Romanov, G. A., Lomin, S. N., and Schmülling, T. (2006). Biochemical characteristics and ligand-binding properties of *Arabidopsis* cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. *J. Exp. Bot.* 57, 4051–4058. doi: 10.1093/jxb/erl179

Sakai, H., Aoyama, T., and Oka, A. (2000). *Arabidopsis* ARR1 and ARR2 response regulators operate as transcriptional activators. *Plant J.* 24, 703–711. doi: 10.1046/j.1365-313x.2000.00909.x

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., et al. (2001). ARR1, a transcription factor for genes immediately responsive to cytokinins. *Science* 294, 1519–1521. doi: 10.1126/science.1065201

Savaldi-Goldstein, S., and Chory, J. (2008). Growth coordination and the shoot epidermis. *Curr. Opin. Plant Biol.* 11, 42–48. doi: 10.1016/j.pbi.2007.10.009

Savaldi-Goldstein, S., Peto, C., and Chory, J. (2007). The epidermis both drives and restricts plant shoot growth. *Nature* 446, 199–202. doi: 10.1038/nature05618

Schnablovà, R., Herben, T., and Klimešova, J. (2017). Shoot apical meristem and plant body organization: a cross-species comparative study. *Ann. Bot.* 120, 833–843. doi: 10.1093/aob/mcx116

Schwarz, I., Scheirlinck, M. T., Otto, E., Bartrina, I., Schmidt, R. C., and Schmülling, T. (2020). Cytokinin regulates the activity of the inflorescence meristem and components of seed yield in oilseed rape. *J. Exp. Bot.* 71, 7146-7159. doi: 10.1093/jxb/eraa419

Sessions, A., Weigel, D., and Yanofsky, M. F. (1999). The *Arabidopsis thaliana MERISTEM LAYER 1* promoter specifies epidermal expression in meristems and young primordia. *Plant J.* 20, 259–263. doi: 10.1046/j.1365-313x.1999.00594.x

Skalák, J., Vercruyssen, L., Claeys, H., Hradilová, J., Cerny, M., Novák, O., et al. (2019). Multifaceted activity of cytokinin in leaf development shapes its size and structure in *Arabidopsis*. *Plant J.* 97, 805–824. doi: 10.1111/tpj.14285

Stolz, A., Riefler, M., Lomin, S. N., Achazi, K., Romanov, G. A., and Schmülling, T. (2011). The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. *Plant J.* 67, 157–168. doi: 10.1111/j.1365-313X.2011.04584.x

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., and Mizuno, T. (2001). The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinins. *Plant Cell Physiol.* 42, 107–113. doi: 10.1093/pcp/pce037

Tajima, Y., Imamura, A., Kiba, T., Amano, Y., Yamashino, T., and Mizuno, T. (2004). Comparative studies on the type-B response regulators revealing their distinctive properties in the His-to-Asp phosphorelay signal transduction of *Arabidopsis thaliana*. *Plant Cell Physiol.* 45, 28–39. doi: 10.1093/pcp/pcg154

Takada, S., and Jürgens, G. (2007). Transcriptional regulation of epidermal cell fate in the *Arabidopsis* embryo. *Development* 134, 1141–1150. doi: 10.1242/dev.02803

Takada, S., Takada, N., and Yoshida, A. (2013). *ATML1* promotes epidermal cell differentiation in *Arabidopsis* shoots. *Development* 140, 1919–1923. doi: 10.1242/dev.094417

Takei, K., Sakakibara, H., and Sugiyama, T. (2001). Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J. Biol. Chem.* 276, 26405–26410. doi: 10.1074/jbc.M102130200

Takei, K., Yamaya, T., and Sakakibara, H. (2004). *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. *J. Biol. Chem.* 279, 41866–41872. doi: 10.1074/jbc.M406337200

Tanaka, Y., Suzuki, T., Yamashino, T., and Mizuno, T. (2004). Comparative studies of the AHP histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in *Arabidopsis thaliana*. *Biosci. Biotechnol. Biochem.* 68, 462–465. doi: 10.1271/bbb.68.462

Telfer, A., Bollman, K. M., and Poethig, R. S. (1997). Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* 124, 645–654.

To, J. P. C., Haberer, G., Ferreira, F. J., Deruere, J., Mason, M. G., Schaller, G. E., et al. (2004). Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* 16, 658–671. doi: 10.1105/tpc.018978

Tokunaga, H., Kojima, M., Kuroha, T., Ishida, T., Sugimoto, K., Kiba, T., et al. (2012). *Arabidopsis LONELY GUY (LOG)* multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. *Plant J.* 69, 355–365. doi: 10.1111/j.1365-313X.2011.04795.x

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034.1. doi: 10.1186/gb-2002-3-7-research0034

Vaseva, I., Qudeimat, E., Potuschak, T., Du, Y., Genschik, P., Vandenbussche, F., et al. (2018). The plant hormone ethylene restricts *Arabidopsis* growth via the epidermis. *Proc. Natl. Acad. Sci. U.S.A.* 115, E4130–E4139. doi: 10.1073/pnas.1717649115

Vatén, A., Soyars, C. L., Tarr, P. T., Nimchuk, Z. L., and Bergmann, D. C. (2018). Modulation of asymmetric division diversity through cytokinin and SPEECHLESS regulatory interactions in the *Arabidopsis* stomatal lineage. *Dev. Cell* 47:e55. doi: 10.1016/j.devcel.2018.08.007

Werner, T., Köllmer, I., Bartrina, I., Holst, K., and Schmülling, T. (2006). New insights into the biology of cytokinin degradation. *Plant Biol.* 8, 371–381. doi: 10.1055/s-2006-923928

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15, 2532–2550. doi: 10.1105/tpc.014928

Werner, T., Motyka, V., Strnad, M., and Schmülling, T. (2001). Regulation of plant growth by cytokinin. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10487–10492. doi: 10.1073/pnas.171304098

Werner, T., and Schmülling, T. (2009). Cytokinin action in plant development. *Curr. Opin. Plant Biol.* 12, 527–538. doi: 10.1016/j.pbi.2009.07.002

Wulfetange, K., Lomin, S. N., Romanov, G. A., Stolz, A., Heyl, A., and Schmülling, T. (2011). The cytokinin receptors of *Arabidopsis* are located mainly to the

endoplasmic reticulum. *Plant Physiol.* 156, 1808–1818. doi: 10.1104/pp.111.180539

Yadav, R. K., Tavakkoli, M., Xie, M., Girke, T., and Reddy, G. V. (2014). A high-resolution gene expression map of the *Arabidopsis* shoot meristem stem cell niche. *Development* 141, 2735–2744. doi: 10.1242/dev.106104

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., et al. (2001). The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol.* 42, 1017–1023. doi: 10.1093/pcp/pce127

Yeats, T. H., and Rose, J. K. (2013). The formation and function of plant cuticles. *Plant Physiol.* 163, 5–20. doi: 10.1104/pp.113.222737

Yoshida, S., Mandel, T., and Kuhlemeier, C. (2011). Stem cell activation by light guides plant organogenesis. *Genes Dev.* 25, 1439–1450. doi: 10.1101/gad.631211

Zubo, Y. O., Blakley, I. C., Yamburenko, M. V., Worthen, J. M., Street, I. H., Franco-Zorrilla, J. M., et al. (2017). Cytokinin induces genome-wide binding of the type-B response regulator ARR10 to regulate growth and development in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 114, E5995–E6004. doi: 10.1073/pnas.1620749114

Zúñiga-Mayo, V. M., Baños-Bayardo, C. R., Díaz-Ramírez, D., Marsch-Martínez, N., and De Folter, S. (2018). Conserved and novel responses to cytokinin treatments during flower and fruit development in *Brassica napus* and *Arabidopsis thaliana*. *Sci. Rep.* 8:6836. doi: 10.1038/s41598-018-25017-3

Zúñiga-Mayo, V. M., Gómez-Felipe, A., Herrera-Ubaldo, H., and De Folter, S. (2019). Gynoecium development: networks in *Arabidopsis* and beyond. *J. Exp. Bot.* 70, 1447–1460. doi: 10.1093/jxb/erz026

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SF declared a past co-authorship with one of the authors ON to the handling editor.

frontiers in Plant Science

PERSPECTIVE
published: 29 March 2021
doi: 10.3389/fpls.2021.641257

Interpreting Cytokinin Action as Anterograde Signaling and Beyond

Yoshihisa Ikeda[1], David Zalabák[2], Ivona Kubalová[3], Michaela Králová[1], Wolfram G. Brenner[4] and Mitsuhiro Aida[5]*

[1] Centre of the Region Haná for Biotechnological and Agricultural Research, Czech Advanced Technology and Research Institute, Palacký University, Olomouc, Czechia, [2] Laboratory of Growth Regulators, Palacky University and Institute of Experimental Botany AS CR, Olomouc, Czechia, [3] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, [4] General and Applied Botany, Institute of Biology, Universität Leipzig, Leipzig, Germany, [5] International Research Organization for Advanced Science and Technology (IROAST), Kumamoto University, Kumamoto, Japan

Among the major phytohormones, the cytokinin exhibits unique features for its ability to positively affect the developmental status of plastids. Even early on in its research, cytokinins were known to promote plastid differentiation and to reduce the loss of chlorophyll in detached leaves. Since the discovery of the components of cytokinin perception and primary signaling, the genes involved in photosynthesis and plastid differentiation have been identified as those directly targeted by type-B response regulators. Furthermore, cytokinins are known to modulate versatile cellular processes such as promoting the division and differentiation of cells and, in concert with auxin, initiating the *de novo* formation of shoot apical meristem (SAM) in tissue cultures. Yet how cytokinins precisely participate in such diverse cellular phenomena, and how the associated cellular processes are coordinated as a whole, remains unclear. A plausible presumption that would account for the coordinated gene expression is the tight and reciprocal communication between the nucleus and plastid. The fact that cytokinins affect plastid developmental status via gene expression in both the nucleus and plastid is interpreted here to suggest that cytokinin functions as an initiator of anterograde (nucleus-to-plastid) signaling. Based on this viewpoint, we first summarize the physiological relevance of cytokinins to the coordination of plastid differentiation with *de novo* shoot organogenesis in tissue culture systems. Next, the role of endogenous cytokinins in influencing plastid differentiation within the SAM of intact plants is discussed. Finally, a presumed plastid-derived signal in response to cytokinins for coupled nuclear gene expression is proposed.

Keywords: anterograde signaling, cytokinin, chloroplast, organelle communication, retrograde signaling, shoot apical meristem, tissue culture, *WUSCHEL*

OPEN ACCESS

Edited by:
Anna N. Stepanova, North Carolina State University, United States

Reviewed by:
Venugopala Reddy Gonehal, University of California, Riverside, United States
Yuling Jiao, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China
Cristiana T. Argueso, Colorado State University, United States

****Correspondence:***
Yoshihisa Ikeda
yoshihisa.ikeda@upol.cz

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *13 December 2020*
Accepted: *08 March 2021*
Published: *29 March 2021*

Citation:
Ikeda Y, Zalabák D, Kubalová I, Králová M, Brenner WG and Aida M (2021) Interpreting Cytokinin Action as Anterograde Signaling and Beyond. Front. Plant Sci. 12:641257. doi: 10.3389/fpls.2021.641257

INTRODUCTION

Since their discovery over 60 years ago, cytokinins (CKs), in concert with auxin, have been shown to promote cell division and shoots in an *in vitro* tissue culture system (Miller et al., 1955; Skoog and Miller, 1957), while a treatment of kinetin alone mitigated the chlorophyll losses in detached *Xanthium* leaves (Richmond and Lang, 1957). Although dark-grown seedlings treated with CKs

exhibited de-etiolated traits, it appears that CKs and light signaling operate independently or sequentially via partially overlapping pathways (Chory et al., 1994). Myriad studies confirm the positive effects of CKs on plastids' functioning: protecting them from high light-induced damage, and preventing chlorophyll degradation under dark conditions (Cortleven and Schmülling, 2015). Indeed, the expression of genes encoding a subset of enzymes in the chlorophyll biosynthesis pathway and those encoding the multiple subunits of the light-harvesting complex (LHC) were augmented by a CK treatment (Cortleven et al., 2016).

All plastids present in aboveground tissues are derived from those in the central zone (CZ) of the shoot apical meristem (SAM). The developmental status of plastids in the SAM was considered proplastid (Lopez-Juez and Pyke, 2005; Fleming, 2006; Sakamoto et al., 2008) until recently, when Charuvi et al. (2012) revealed that it varied in the shoot apex, depending on the position or layer they were located: plastids in the CZ and peripheral zone (PZ) of the L1 and L3 could partially develop thylakoid membranes. Several studies demonstrated the CZ of L3 (organizing center/OC; also referred to as the rib meristem) is the site for endogenous cytokinin perception (Gordon et al., 2009; Zürcher et al., 2013; Gruel et al., 2016; Snipes et al., 2018). Despite the fact that plastids in endogenous CK-rich domains are poorly developed, a high-resolution gene expression map in the SAM uncovered greater expression of genes encoding the photosystem I (PSI) LHC A and its subunit proteins, as well as photosystem II (PSII) components and its subunit protein in the L3, by >1.5-fold (relative to other domains) (Yadav et al., 2014). These findings suggest the CZ of the shoot apex provides a unique system for investigating the impact of endogenous CKs in the early phase of plastid development.

In this perspective article, we attempt to interpret the action of CK as anterograde signaling. As proposed in prior reviews, the term "anterograde signaling" refers to the process whereby nucleus-derived regulators that transmit information express proteins to coordinate the expression of genes in the nucleus and plastids at multiple levels (Pesaresi et al., 2007; Kleine et al., 2009; Jung and Chory, 2010; Berry et al., 2013). In this way, multiprotein complex machineries, such as photosystems, cytochrome b6/f or ATP synthase, are optimally assembled (Kleine et al., 2009). We begin by focusing on how exogenous and endogenous CKs affect the expression of genes encoding tetrapyrrole biosynthesis, a fundamental metabolic pathway in all living organisms (**Figure 1A**; Tanaka et al., 2011), and then summarizing these effects. Although all genes involved in tetrapyrrole biosynthesis are in the nuclear genome, we scrutinized them because some were found to be CK-responsive (reviewed by Cortleven and Schmülling, 2015). Lastly, based on the results obtained from an *in vitro* tissue culture system (Kubalová et al., 2019), the plausible involvement of retrograde signaling in modulating expression of the nuclear gene *WUSCHEL (WUS)*, a master regulator of stem cell niche, is explored.

INITIATION OF ANTEROGRADE REGULATION BY CKs

An exogenous CK treatment can greatly impact plastids' development and functioning (Cortleven and Schmülling, 2015). Gene expression analyses have corroborated this, by uncovering upregulated plastid-encoded genes upon CK treatment in Arabidopsis and barley (Brenner et al., 2005; Zubo et al., 2008; Danilova et al., 2017a,b; Andreeva et al., 2020). In Arabidopsis, upregulated plastid genes include components of cytochrome b6/f (*petA*) and PSII (*PsbG*, *psbA*, and *psbI*) (Brenner et al., 2005). Both machineries consist of protein complexes encoded in the nucleus and plastids. Additionally, CK indirectly affects plastids' gene expression by upregulating nuclear genes that encode components of their transcription machinery (i.e., phage-type RNA-polymerases, sigma factors, and plastid RNA polymerase-associated proteins; Danilova et al., 2017a; Andreeva et al., 2020). These results imply that CK-coordinated gene expression activity in the nucleus and plastid provides appropriate information for balancing metabolic flows in plastids and cellular functions.

RELATIONSHIP BETWEEN PLASTID STATUS IN THE SAM AND CK SIGNALING IN TISSUE CULTURE

The *in vitro* tissue culture technique has been employed to study pluripotency. In such a system, *de novo* organogenesis is manipulated by a defined cytokinin-to-auxin ratio in cultured medium. As **Figure 1B** (top) illustrates, this widely used method has two sequential steps (Valvekens et al., 1988). The first involves pre-incubation of explants on an auxin-rich callus-inducing medium (CIM), to induce a mass of growing cells, termed a callus. The second step promotes the greening of foci when these induced calli are cultured on shoot-inducing medium (SIM) to which a high cytokinin-to-auxin ratio has been applied. The underlying mechanisms of these processes are detailed in several recent review articles (Shin et al., 2020; Xu and Hu, 2020). The external application of CKs induces *WUS* expression likely via direct activation by type B Arabidopsis Response Regulators (B-ARRs) (Dai et al., 2017; Meng et al., 2017; Zhang et al., 2017; Zubo et al., 2017; Xie et al., 2018); albeit a high CK concentration is required (Gordon et al., 2009). The tight linkage between CK-stimulated *WUS* expression and *de novo* shoot regeneration has been corroborated by genetic studies. Loss-of-function mutations in *WUS* compromised *de novo* shoot regeneration (Gordon et al., 2007; Chatfield et al., 2013; Zhang et al., 2017). Mutants with decreased CK perception were characterized by poor shoot regeneration (Ueguchi et al., 2001; Nishimura et al., 2004; Riefler et al., 2006; Pernisova et al., 2018). By contrast, root explants of CK-hypersensitive mutants—*ckh1* defective in the gene encoding TATA-box binding protein (TBP)-associated factor TAF12-like protein, or *ckh2/pkl (pickle)* defective in gene encoding CHD3 class of SWI/SNF chromatin remodeling factor—promoted tissue greening on CIM culture (Furuta et al., 2011) and developed *de novo* shoots more rapidly

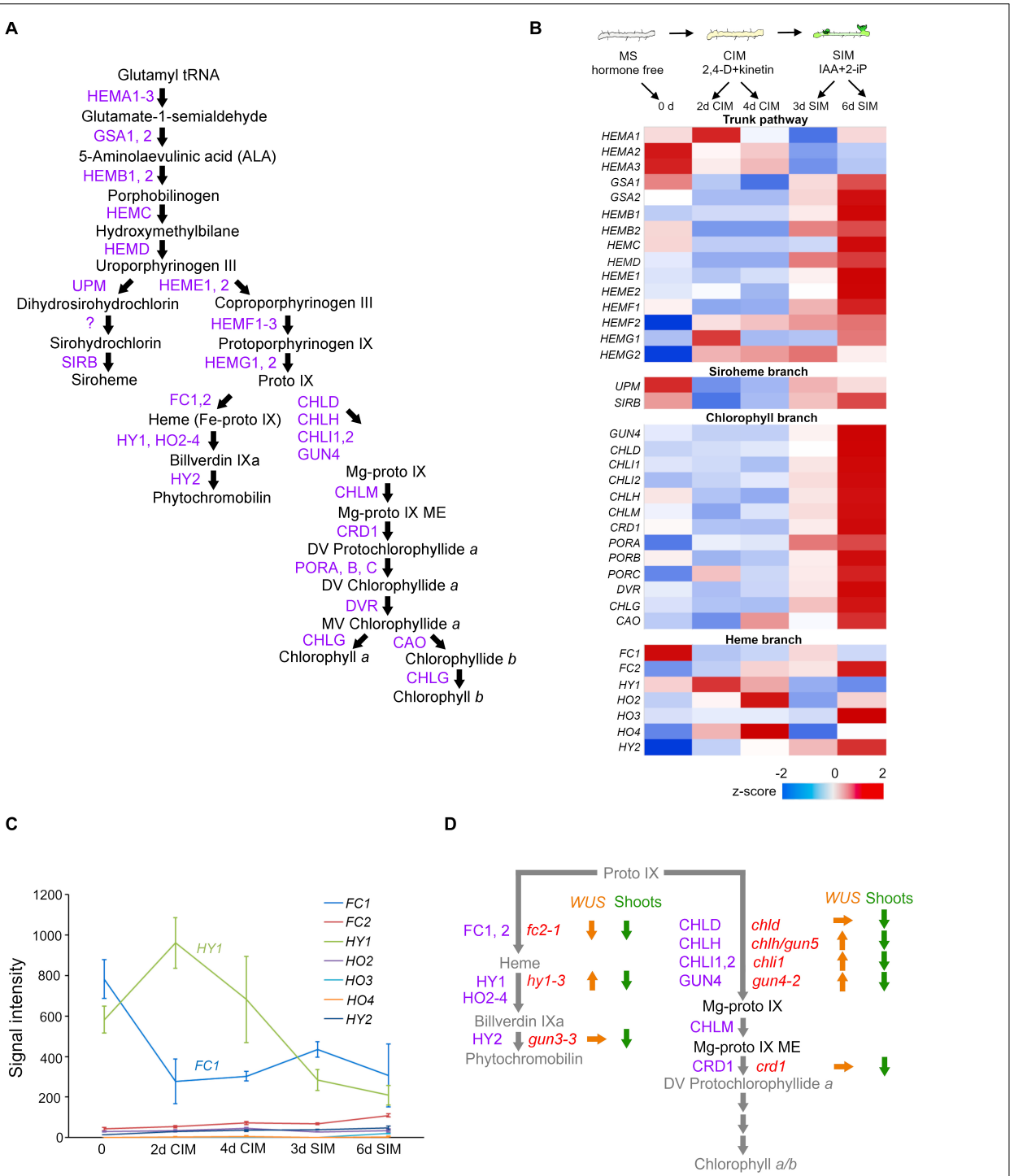


FIGURE 1 | The relationship between the tetrapyrrole biosynthesis pathway and cytokinin-mediated *de novo* shoot apical meristem (SAM) development in tissue culture. **(A)** Tetrapyrrole biosynthesis pathway. Arrows indicate enzymatic reactions. Abbreviations of enzyme names are depicted in purple. **(B)** Schematic morphological representation of excised root explants cultured on MS (Murashige and Skoog medium), CIM (callus-inducing medium), and SIM (shoot-inducing medium) (top), and the expression profiles of genes involved in tetrapyrrole biosynthesis at indicated time points (day) of the indicated culture medium (bottom). Relative expression levels (*z*-scores) are displayed using the color code (blue to red). All the data came from the Arabidopsis eFP browser (http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi?dataSource~=~Regeneration). **(C)** Signal intensity of genes involved in the heme branch. **(D)** Summary of relative expression levels of *WUS* (orange arrows) and shoot regeneration efficiency (green arrows) in root explants of tetrapyrrole mutants cultured on SIM. Up, down, and right arrows indicate an increased *WUS* transcript level, decreased *WUS* transcript level or compromised shoot regeneration, or statistically insignificant changes in the *WUS* transcript level, respectively. The names of enzymes and corresponding mutants are, respectively, depicted in purple and red.

and more frequently during the course of SIM incubation. The shoot regeneration efficiencies in the mutants with altered CK perception coincided well with their *WUS* expression levels (Kubalová and Ikeda, 2017; Kubalová et al., 2019).

CHANGES TO TETRAPYRROLE BIOSYNTHESIS GENE EXPRESSION AT THE ONSET OF SHOOT REGENERATION

As expected from the greening of a tissue phenotype of root explants cultured on SIM (**Figure 1B** top), analysis of a previously reported microarray expression profile (eFP browser)[1] (Che et al., 2002) confirms the increased expression of genes involved in the pathway leading to the chlorophyll *a/b* (trunk pathway and chlorophyll branch) (**Figure 1B**). The exception was the *HEMA* genes, whose products catalyze the initial step in the trunk tetrapyrrole pathway (Kumar and Söll, 2000), their transcripts were substantially reduced when root explants were transferred from CIM onto SIM. This is very intriguing because *HEMA1*, encoding GLUTAMYL-tRNA-REDUCTASE (GluTR), is mainly expressed in photosynthetic tissues, regulated by light through phytochromes (Papenbrock et al., 1999; McCormac et al., 2001), and induced by CK when applied to dark-grown seedlings (Cortleven et al., 2016). In stark contrast to the increased expression of genes involved in the chlorophyll branch, the expression of genes involved in the heme branch did not respond well to SIM incubation. Rather, transcripts of *LONG HYPOCOTYL 1 (HY1)/HEME OXYGENASE 1 (HO1)* declined over time (**Figure 1B**). The expression levels of *HO3* and *HO4* increased, but their contribution seems minor because the signal intensities of their transcripts are negligible when compared to that of *HY1* (**Figure 1C**). Although no quantification results of tetrapyrrole molecules during SIM incubation are currently available, we may reasonably speculate that this reduced *HEMA* expression is due to an insufficient availability of phytochromobilin, the chromophore of phytochromes. It has been repeatedly documented that a wide range of phytochrome-mediated light responses become impaired in *hy1* plants; this suggests light-induced *HEMA1* expression is impaired when the *HY1*'s transcription diminished under the SIM incubation. Notably, HEMA enzymatic activity is attenuated by heme accumulation, in a feedback mechanism (Pontoppidan and Kannangara, 1994), and HEMA proteins are destabilized by heme (Richter et al., 2019). Nevertheless, the *HEMA* transcripts in *hy1* are comparable to those in the wild type (Goslings et al., 2004). Collectively, those findings suggest that, although expression of the rate-limiting *HEMA1* is repressed, chlorophyll *a/b* can readily accumulate during SIM incubation (as evinced by tissue greening). Conversely, the heme branch is likely to be attenuated by decreased expression of *HY1*, implying that heme accumulation occurs when *de novo* SAM is being initiated in the CK-enriched culture. Further analysis to determine the tetrapyrrole molecule contents during cytokinin-stimulated *de novo* SAM emergence is required.

[1]http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi?dataSource=Regeneration

WUS EXPRESSION IS UNCOUPLED FROM *DE NOVO* SAM FORMATION IN MUTANTS DEFECTIVE IN TETRAPYRROLE BIOSYNTHETIC PATHWAY

Microarray results also validate the greater expression of meristem-related genes upon CK-rich SIM incubation (Che et al., 2002). B-ARRs encoding GARP transcription factors directly activated *WUS* expression (Meng et al., 2017; Zhang et al., 2017; Xie et al., 2018). Loss-of-function mutations of tetrapyrrole biosynthesis provide a valuable tool to study the relationship between tissue greening and shoot regeneration. All the mutants defective in the heme branch (*protoporphyrin ix ferrochelatase 2/fc2*, *hy1*, *genome-uncoupled 3/gun3*), and chlorophyll branch (*gun4*, *Mg-chelatase d/chld*, *chlh/gun5*, *chli1*, *copper response deficient 1/crd1*) exhibiting reduced chlorophyll contents resulted in decreased shoot regeneration efficiency (Kubalová et al., 2019); hence, proper chlorophyll contents are required for the acquisition of *de novo* SAM competence or shoot regeneration efficiency. Unexpectedly, of eight tetrapyrrole biosynthesis mutants with substantially compromised shoot regeneration efficiency, in four (*hy1-3*, *gun4-2*, *chlh/gun5*, *chli1*) the expression level of *WUS* significantly increased (Kubalová et al., 2019). This suggests tetrapyrrole intermediates influence nuclear-encoded *WUS* expression. Notably, *WUS* was expressed most in the *hy1-3* mutant despite its shoot regeneration efficiency being among the lowest (Kubalová et al., 2019). In summary, *WUS* expression in *hy1-3*, *gun4-3*, *chlh,* and *chli1* mutants is uncoupled from shoot regeneration efficiency (**Figure 1D**), suggesting that, in response to the altered plastid developmental status stimulated by the high cytokinin-to-auxin ratio in SIM, plastid-to-nucleus communication occurs to fine-tune nuclear *WUS* expression during *de novo* shoot organogenesis.

ALTERED PLASTID DIFFERENTIATION STATUS IN THE SAM

All aboveground tissues arise from the SAM, which contains stem cells in its CZ. The plastids in SAM were once considered proplastids (Lopez-Juez and Pyke, 2005; Fleming, 2006; Sakamoto et al., 2008). Despite that prevailing concept, sophisticated morphological analyses of plastids in Arabidopsis SAM revealed their developmental status was not necessarily null and, depending on their position within the SAM, it had dynamic features: plastids underwent different developmental processes that could lead to either the acquisition or loss of thylakoid membranes (Charuvi et al., 2012). The outermost layer L1 (protoderm) gives rise to the epidermis whose photosynthetic activity is lost once leaves differentiate. Yet, plastids within L1 layer's cells of the SAM (both in CZ and PZ) developed partially

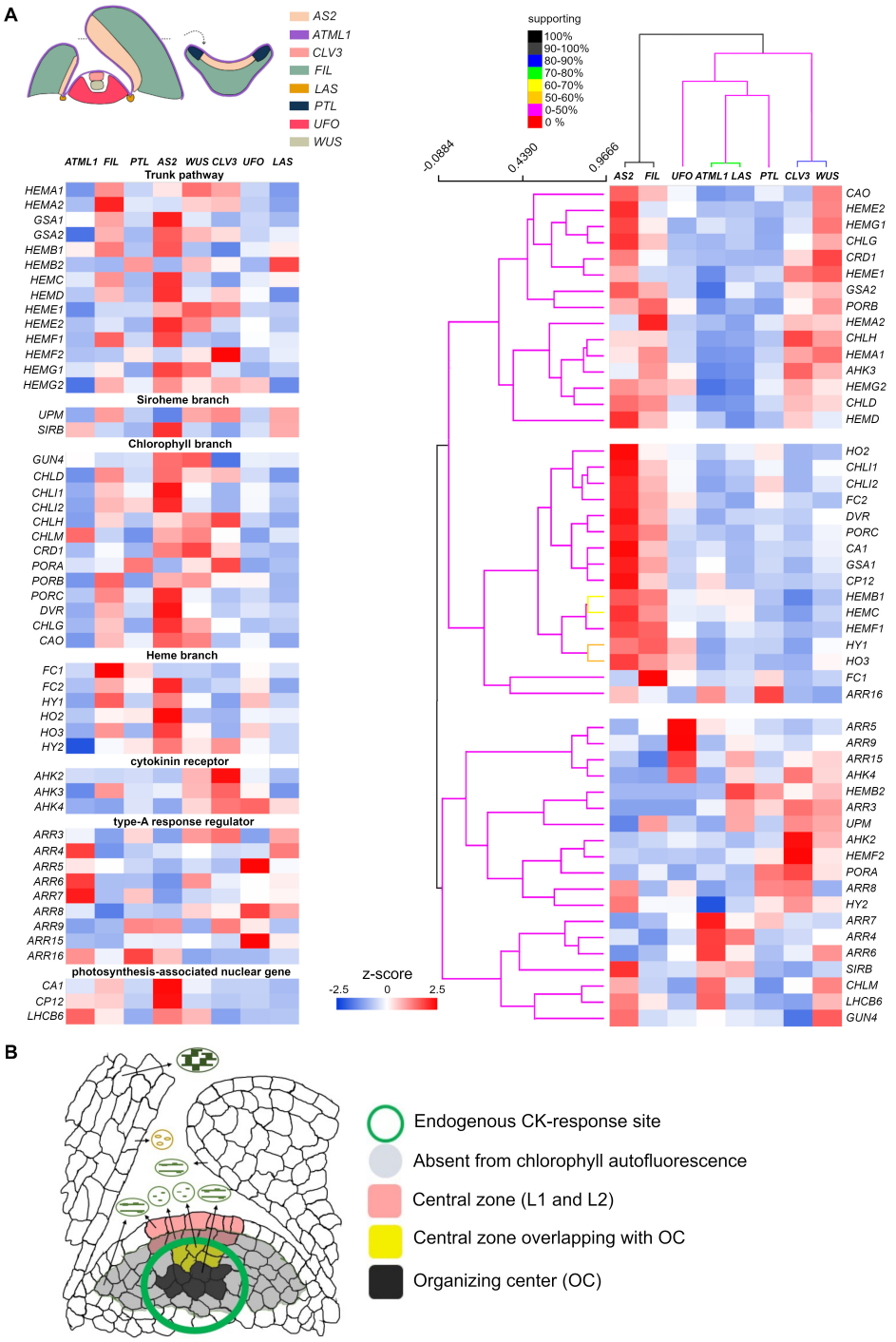


FIGURE 2 | SAM as a unique tissue to study endogenous cytokinin (CK) action on early developmental status of plastids. **(A)** Expression of genes involved in tetrapyrrole biosynthesis, with CK-related and light-responsive genes serving as references for CK or light perception, respectively. The same sets of genes are arranged according to the functional category (left) and to their expression patterns deduced from the clustering analysis (right). Genes and shoot apex domains shown in the left panel were clustered for similarity, in terms of their gene expression pattern, by the Support Tree algorithm implemented in TIGR MeV4 (Saeed et al., 2003), using n = 100 iterations of bootstrapping both genes and shoot apex domains. Relative expression levels determined by the z-scores are color-coded (in blue to red). Percentage of support for the clustering analysis is depicted according to a color intensity gradient [from red (0%) to black (100%)]. All the data came from the Arabidopsis eFP browser (http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi?dataSource~=~Shoot_Apex), except *HEMA3*, *HO4*, and *ARR17* (Tian et al., 2019). **(B)** Summary of plastid differentiation status in cells differentially positioned in the shoot apex. The plastid status in various types of cells is illustrated, with those cells lacking autofluorescence highlighted in gray [adapted from Charuvi et al., 2012)]. Cells able to respond to endogenous CKs (as revealed by *TCS* reporters) are indicated by a green circle (Zürcher et al., 2013). The L1 and L2 cells within the CZ of SAM are highlighted in orange; the cells in L3 overlapping with the organizing center (OC) appear in yellow. Cells comprising the OC are shown in black.

differentiated thylakoid networks. The subepidermal layer L2, located below L1, is the progenitor of outer mesophyll tissue. Despite the fact that the L2 is the major source of photosynthetic tissues, the plastids in the CZ of L2 persist in the most undeveloped state, devoid of thylakoid networks characteristic of proplastids. By contrast, plastids in the PZ cells of L2 develop thylakoid membranes. Beneath them is L3, a multilayer where a small group of cells comprise an OC whose cell fate remains undifferentiated. Inner tissues, such as inner mesophyll and vascular cells, originate from the L3. The differentiation status of plastids in L3's uppermost layer in CZ is similar to that of L2 in CZ (proplastids), whereas plastids in L3's inner layers are closer to maturity, having partially developed thylakoid networks.

So the developmental status of plastids within the SAM seems under internal control, and light signaling does not appear to figure prominently because the plastids can better develop thylakoid networks in the inner L3 than in L2. Expression of a CK-reporter gene, *TCS (Two-component Signaling Sensor)* and its derivative *TCSn*, indicated that the primary CK-signaling response was confined within the CZ of L3 (Gordon et al., 2009; Zürcher et al., 2013; Snipes et al., 2018). The majority of molecules participating in primary CK signaling—ARABIDOPSIS HISTIDINE KINASE/AHK receptors, AHP phosphotransmitters, and B-ARRs—are expressed to some extent in Arabidopsis SAM (Nishimura et al., 2004; Gordon et al., 2009; Gruel et al., 2016). Three CK receptors, *AHK2–AHK4*, are predominantly expressed in L3 cells (Gruel et al., 2016) and *WUS*-expressing cells coincide well with *AHK4*-expressing cells (Gordon et al., 2009). Recently, using several tissue-specific promoters, Tian et al. (2019) determined the differential expression pattern of translating RNAs in differing shoot domains, termed "domain-specific translatome map." To gain insight into CK action's relation to plastid differentiation status in the SAM, we extracted expression profiles of genes involved in tetrapyrrole biosynthesis, whose results were standardized and conveyed here using *z*-scores (**Figure 2A**). Although for three CK receptor genes, their expression is reportedly absent in L1 (Gruel et al., 2016), their abundant expression in domains of *CLV3* (*CLAVATA 3*) and *WUS* agree well with those of high-CK responses revealed by expression of the *TCS* reporters (**Figure 2A**). These results strongly suggest the SAM, especially the CZ of L3, is the active site for the perception of endogenous CKs. Nevertheless, those plastids within L2 and L3 remained the least differentiated; in fact, chlorophyll autofluorescence in the corresponding region was below the detection threshold (**Figure 2B**; Charuvi et al., 2012; Yadav et al., 2019). Unlike for poorly developed plastid conditions in the *CLV3* or *WUS* domain, several tetrapyrrole biosynthesis genes (*HEMA1*, *HEME1*, *HEMF2*, *CHLH*, *CRD1*, and *PORA*) exhibited the highest transcript levels (**Figure 2A**), notwithstanding the fact that the expression of *HEMA1*, *CHLH*, and *CRD1* was predominantly induced by light in photosynthetic tissues (Matsumoto et al., 2004). These results suggest that a handful of genes involved in tetrapyrrole biosynthesis are not only regulated by light but also by intrinsic signal(s). A plausible candidate for this might be CKs or the interplay between them and other hormones. It appears that upregulation of the above genes contributes little or negligibly to chlorophyll biosynthesis in the CZ of L2 and L3, since chlorophyll autofluorescence is undetectable in these layers (**Figure 2B**; Charuvi et al., 2012; Yadav et al., 2019). The expression of genes involved in the heme branch is generally low in both *CLV3* and *WUS* domains, especially that of *HY1* (lowest in the *CLV3* domain; **Figure 2A**), indicative of heme accumulation. To gain further insight into the negative relationship between *HY1*'s expression and CK signaling, those genes involved in CKs' perception and response, as well as light-responsive genes, were analyzed by clustering. These results confirmed that *HY1* fell into a cluster distinct from that harboring the two CK receptors (*AHK2* and *AKH4*) and eight of nine A-ARRs (**Figure 2A**, right panel). Studying the developmental status of plastids in cells in the CZ of L2 and the uppermost layers of L3 in the CZ, by manipulating endogenous CK levels, would provide a new opportunity to reveal as-of-yet-unknown CK functions in early plastid development. In two different systems, for *de novo* organogenesis in tissue culture and the shoot apices of light-grown seedlings, the *HY1* expression levels are negatively correlated with exogenous CK or endogenous CK-rich domains (**Figures 1**, **2A**). Although further analyses are required to uncover a negative correlation between the two mentioned above, it is likely that, in response to CK as an initiator of anterograde signaling, the plastid-to-nucleus communication happens predominantly in meristematic cells where endogenous CK maxima are established.

CONCLUSION AND PERSPECTIVE

Bioactive CKs are molecules perceived by the CHASE domain of CK receptors, for which the primary two-component multistep phosphorelay represents the output module able to regulate gene expression. Given the fact that exogenous CK application to dark-grown plants promotes etioplast-to-chloroplast transition, probably via altered gene expression both in the nucleus and plastids, CKs could positively regulate anterograde signaling to promote plastid differentiation. It appears that the particular developmental and differentiation status affects the sensitivity to CK in plant cells. We propose the CZ of SAM as a promising site to investigate endogenous CK action influencing early plastid development (**Figure 2B**). An alternative motive for this approach is to gain better insight into the coordinated regulation of stem cell determination vis-à-vis the plastids' developmental status. The molecular regulatory mechanism underlying diminished expression of *HY1* by CK is not entirely clear. The lower auxin-to-cytokinin ratio in SIM may be responsible for declines in *HY1* expression because *HY1* is induced by auxin and acts downstream of it (Ma et al., 2014). Besides, an auxin minimum is known to exist in the OC of SAM (Shi et al., 2018). Either way, genetic studies have revealed the overlooked contribution of the heme branch (Kubalová et al., 2019). Although tetrapyrrole biosynthesis is controlled under complex regulation, it is reasonable to argue that substrates accumulate when the catalytic enzymatic step is blocked. Since *WUS* expression in *fc2-1* is evidently opposite to that in *hy1-3*, *gun4-3*, *chlh,* and *chli1* (**Figure 1D**), it is

tempting to speculate that heme, whose synthesis is blocked in *fc2-1* but which accumulates in *hy1-3*, is the plausible candidate molecule modulating *WUS* expression. It goes without saying that the alternative interpretations remain open to account for this. Interestingly, *hy1* mutant seedlings accumulate substantially elevated level of endogenous jasmonates by unknown mechanisms and, as a result, jasmonic acid responses are constitutively active (Zhai et al., 2007). Alternatively, the decreased carbon monoxide, a byproduct of heme oxygenase enzymatic reaction, may come into play (Xuan et al., 2008; Ma et al., 2014).

Beyond its diverse functions to serve as cofactors of hemoproteins in various organelles (Layer et al., 2010), several recent studies pose heme as a retrograde signal that is involved in various physiological regulations (Mense and Zhang, 2006; Von Gromoff et al., 2008). Besides, biochemical evidence corroborates this, i.e., half of the identified heme-binding proteins localize in the nucleus in plants (Shimizu et al., 2020). Measuring or monitoring heme content during *de novo* organogenesis and in the CZ of the SAM of intact plants will provide us with a new insight into the role of the heme branch in nuclear gene expression.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi?dataSource=Regeneration; http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi?dataSource=Shoot_Apex.

AUTHOR CONTRIBUTIONS

YI conceived the study's idea. YI, IK, MK, DZ, WB, and MA wrote the manuscript. WB conducted the clustering analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Czech Science Foundation grants (GACR17-23702S and GACR 18-23972Y) and IROAST research unit on plant stem cells and regeneration.

ACKNOWLEDGMENTS

We acknowledge the Czech Science Foundation grants (GACR17-23702S and GACR 18-23972Y) and IROAST research unit on plant stem cells and regeneration, based at Kumamoto University and Véronique Bergougnoux-Fojtik for critical reading of the manuscript.

REFERENCES

Andreeva, A. A., Vankova, R., Bychkov, I. A., Kudryakova, N. V., Danilova, M. N., Lacek, J., et al. (2020). Cytokinin-regulated expression of *Arabidopsis thaliana* pap genes and its implication for the expression of chloroplast-encoded genes. *Biomolecules* 10, 1–18. doi: 10.3390/biom10121658

Berry, J. O., Yerramsetty, P., Zielinski, A. M., and Mure, C. M. (2013). Photosynthetic gene expression in higher plants. *Photosynth Res.* 117, 91–120. doi: 10.1007/s11120-013-9880-8

Brenner, W. G., Romanov, G. A., Köllmer, I., Bürkle, L., and Schmülling, T. (2005). Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. *Plant J.* 44, 314–333. doi: 10.1111/j.1365-313X.2005.02530.x

Charuvi, D., Kiss, V., Nevo, R., Shimoni, E., Adam, Z., and Reich, Z. (2012). Gain and loss of photosynthetic membranes during plastid differentiation in the shoot apex of *Arabidopsis*. *Plant Cell* 24, 1143–1157. doi: 10.1105/tpc.111.094458

Chatfield, S. P., Capron, R., Severino, A., Penttila, P. A., Alfred, S., Nahal, H., et al. (2013). Incipient stem cell niche conversion in tissue culture: using a systems approach to probe early events in WUSCHEL-dependent conversion of lateral root primordia into shoot meristems. *Plant J.* 73, 798–813. doi: 10.1111/tpj.12085

Che, P., Gingerich, D. J., Lall, S., and Howell, S. H. (2002). Global and hormone-induced gene expression changes during shoot development in *Arabidopsis*. *Plant Cell* 14, 2771–2785. doi: 10.1105/tpc.006668

Chory, J., Reinecke, D., Sim, S., Washburn, T., and Brenner, M. (1994). A role for cytokinins in de-etiolation in *Arabidopsis*. det Mutants have an altered response to cytokinins. *Plant Physiol.* 104, 339–347. doi: 10.1104/pp.104.2.339

Cortleven, A., Marg, I., Yamburenko, M. V., Schlicke, H., Hill, K., Grimm, B., et al. (2016). Cytokinin regulates the etioplast-chloroplast transition through the two-component signaling system and activation of chloroplast-related genes. *Plant Physiol.* 172, 464–478. doi: 10.1104/pp.16.00640

Cortleven, A., and Schmülling, T. (2015). Regulation of chloroplast development and function by cytokinin. *J. Exp. Bot.* 66, 4999–5013. doi: 10.1093/jxb/erv132

Dai, X., Liu, Z., Qiao, M., Li, J., Li, S., and Xiang, F. (2017). ARR12 promotes de novo shoot regeneration in *Arabidopsis thaliana* via activation of WUSCHEL expression. *J. Integr. Plant Biol.* 59, 747–758. doi: 10.1111/jipb.12567

Danilova, M. N., Doroshenko, A. S., Zabrodin, D. A., Kudryakova, N. V., Oelmüller, R., and Kusnetsov, V. V. (2017a). Cytokinin membrane receptors modulate transcript accumulation of plastid encoded genes. *Russ. J. Plant Physiol.* 64, 301–309. doi: 10.1134/S1021443717030062

Danilova, M. N., Kudryakova, N. V., Doroshenko, A. S., Zabrodin, D. A., Rakhmankulova, Z. F., Oelmüller, R., et al. (2017b). Opposite roles of the *Arabidopsis* cytokinin receptors AHK2 and AHK3 in the expression of plastid genes and genes for the plastid transcriptional machinery during senescence. *Plant Mol. Biol.* 93, 533–546. doi: 10.1007/s11103-016-0580-6

Fleming, A. (2006). Metabolic aspects of organogenesis in the shoot apical meristem. *J. Exp. Bot.* 57, 1863–1870. doi: 10.1093/jxb/erj178

Furuta, K., Kubo, M., Sano, K., Demura, T., Fukuda, H., Liu, Y. G., et al. (2011). The CKH2/PKL chromatin remodeling factor negatively regulates cytokinin responses in *Arabidopsis calli*. *Plant Cell Physiol.* 52, 618–628. doi: 10.1093/pcp/pcr022

Gordon, S. P., Chickarmane, V. S., Ohno, C., and Meyerowitz, E. M. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16529–16534. doi: 10.1073/pnas.0908122106

Gordon, S. P., Heisler, M. G., Reddy, G. V., Ohno, C., Das, P., and Meyerowitz, E. M. (2007). Pattern formation during de novo assembly of the *Arabidopsis* shoot meristem. *Development* 134, 3539–3548. doi: 10.1242/dev.010298

Goslings, D., Meskauskiene, R., Kim, C., Lee, K. P., Nater, M., and Apel, K. (2004). Concurrent interactions of heme and FLU with Glu tRNA reductase (HEMA1), the target of metabolic feedback inhibition of tetrapyrrole biosynthesis, in dark-And light-grown *Arabidopsis* plants. *Plant J.* 40, 957–967. doi: 10.1111/j.1365-313X.2004.02262.x

Gruel, J., Landrein, B., Tarr, P., Schuster, C., Refahi, Y., Sampathkumar, A., et al. (2016). An epidermis-driven mechanism positions and scales stem cell niches in plants. *Sci. Adv.* 2:e1500989. doi: 10.1126/sciadv.1500989

Jung, H. S., and Chory, J. (2010). Signaling between chloroplasts and the nucleus: can a systems biology approach bring clarity to a complex and highly regulated pathway? *Plant Physiol.* 152, 453–459. doi: 10.1104/pp.109.149070

Kleine, T., Maier, U. G., and Leister, D. (2009). DNA transfer from organelles to the nucleus: the idiosyncratic genetics of endosymbiosis. *Annu. Rev. Plant Biol.* 60, 115–138. doi: 10.1146/annurev.arplant.043008.092119

Kubalová, I., and Ikeda, Y. (2017). Chlorophyll measurement as a quantitative method for the assessment of cytokinin-induced green foci formation in tissue culture. *J. Plant Growth Regul.* 36, 516–521. doi: 10.1007/s00344-016-9637-7

Kubalová, I., Zalabák, D., Mičúchová, A., and Ikeda, Y. (2019). Mutations in tetrapyrrole biosynthesis pathway uncouple nuclear WUSCHEL expression from de novo shoot development in *Arabidopsis*. *Plant Cell. Tissue Organ Cult.* 139, 395–401. doi: 10.1007/s11240-019-01680-w

Kumar, A. M., and Söll, D. (2000). Antisense HEMA1 RNA expression inhibits heme and chlorophyll biosynthesis in *Arabidopsis*. *Plant Physiol.* 122, 49–56. doi: 10.1104/pp.122.1.49

Layer, G., Reichelt, J., Jahn, D., and Heinz, D. W. (2010). Structure and function of enzymes in heme biosynthesis. *Protein Sci.* 19, 1137–1161. doi: 10.1002/pro.405

Lopez-Juez, E., and Pyke, K. A. (2005). Plastids unleashed: their development and their integration in plant development. *Int. J. Dev. Biol.* 49, 557–577. doi: 10.1387/ijdb.051997el

Ma, F., Wang, L., Li, J., Samma, M. K., Xie, Y., Wang, R., et al. (2014). Interaction between HY1 and H2O2 in auxin-induced lateral root formation in *Arabidopsis*. *Plant Mol. Biol.* 85, 49–61. doi: 10.1007/s11103-013-0168-3

Matsumoto, F., Obayashi, T., Sasaki-Sekimoto, Y., Ohta, H., Takamiya, K., and Masuda, T. (2004). Gene expression profiling of the tetrapyrrole metabolic pathway in *Arabidopsis* with a mini-array system. *Plant Physiol.* 135, 2379–2391. doi: 10.1104/pp.104.042408

McCormac, A. C., Fischer, A., Kumar, A. M., Söll, D., and Terry, M. J. (2001). Regulation of HEMA1 expression by phytochrome and a plastid signal during de-etiolation in *Arabidopsis thaliana*. *Plant J.* 25, 549–561. doi: 10.1046/j.1365-313x.2001.00986.x

Meng, W. J., Cheng, Z. J., Sang, Y. L., Zhang, M. M., Rong, X. F., Wang, Z. W., et al. (2017). Type-B *Arabidopsis* response regulators specify the shoot stem cell niche by dual regulation of WUSCHEL. *Plant Cell* 29, 1357–1372. doi: 10.1105/tpc.16.00640

Mense, S. M., and Zhang, L. (2006). Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases. *Cell Res.* 16, 681–692. doi: 10.1038/sj.cr.7310086

Miller, C. O., Skoog, F., Von Saltza, M. H., and Strong, F. M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. *J. Am. Chem. Soc.* 77:1392. doi: 10.1021/ja01610a105

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. *Plant Cell* 16, 1365–1377. doi: 10.1105/tpc.021477

Papenbrock, J., Mock, H. P., Kruse, E., and Grimm, B. (1999). Expression studies in tetrapyrrole biosynthesis: inverse maxima of magnesium chelatase and ferrochelatase activity during cyclic photoperiods. *Planta* 208, 264–273. doi: 10.1007/s004250050558

Pernisova, M., Grochova, M., Konecny, T., Plackova, L., Harustiakova, D., Kakimoto, T., et al. (2018). Cytokinin signalling regulates organ identity via the AHK4 receptor in *Arabidopsis*. *Development* 145:dev163907. doi: 10.1242/dev.163907

Pesaresi, P., Schneider, A., Kleine, T., and Leister, D. (2007). Interorganellar communication. *Curr. Opin. Plant Biol.* 10, 600–606. doi: 10.1016/j.pbi.2007.07.007

Pontoppidan, B., and Kannangara, C. G. (1994). Purification and partial characterisation of barley glutamyl-tRNA(Glu) reductase, the enzyme that directs glutamate to chlorophyll biosynthesis. *Eur. J. Biochem.* 225, 529–537. doi: 10.1111/j.1432-1033.1994.00529.x

Richmond, A. E., and Lang, A. (1957). Effect of kinetin on protein content and survival of detached xanthium leaves. *Science* 125, 650–651. doi: 10.1126/science.125.3249.650-a

Richter, A. S., Banse, C., and Grimm, B. (2019). The GluTR-binding protein is the heme-binding factor for feedback control of glutamyl-tRNA reductase. *eLife* 8, 1–18. doi: 10.7554/elife.46300

Riefler, M., Novak, O., Strnad, M., and Schmülling, T. (2006). *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18, 40–54. doi: 10.1105/tpc.105.037796

Saeed, A. I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., et al. (2003). TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34, 374–378. doi: 10.2144/03342mt01

Sakamoto, W., Miyagishima, S., and Jarvis, P. (2008). Chloroplast biogenesis: control of plastid development, protein import, division and inheritance. *Arab. B.* 6:e0110. doi: 10.1199/tab.0110

Shi, B., Guo, X., Wang, Y., Xiong, Y., Wang, J., Hayashi, K. I., et al. (2018). Feedback from lateral organs controls shoot apical meristem growth by modulating auxin transport. *Dev. Cell* 44, 204.e6–216.e6. doi: 10.1016/j.devcel.2017.12.021

Shimizu, T., Yasuda, R., Mukai, Y., Tanoue, R., Shimada, T., Imamura, S., et al. (2020). Proteomic analysis of haem-binding protein from *Arabidopsis thaliana* and *Cyanidioschyzon merolae*. *Philos. Trans. R. Soc. B Biol. Sci.* 375:488. doi: 10.1098/rstb.2019.0488

Shin, J., Bae, S., and Seo, P. J. (2020). De novo shoot organogenesis during plant regeneration. *J. Exp. Bot.* 71, 63–72. doi: 10.1093/jxb/erz395

Skoog, F., and Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* 11, 118–130.

Snipes, S. A., Rodriguez, K., DeVries, A. E., Miyawaki, K. N., Perales, M., Xie, M., et al. (2018). Cytokinin stabilizes WUSCHEL by acting on the protein domains required for nuclear enrichment and transcription. *PLoS Genet.* 14:e1007351. doi: 10.1371/journal.pgen.1007351

Tanaka, R., Kobayashi, K., and Masuda, T. (2011). Tetrapyrrole metabolism in *Arabidopsis thaliana*. *Arab. B.* 9:e0145. doi: 10.1199/tab.0145

Tian, C., Wang, Y., Yu, H., He, J., Wang, J., Shi, B., et al. (2019). A gene expression map of shoot domains reveals regulatory mechanisms. *Nat. Commun.* 10, 1–12. doi: 10.1038/s41467-018-08083-z

Ueguchi, C., Sato, S., Kato, T., and Tabata, S. (2001). The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in *Arabidopsis thaliana*. *Plant Cell Physiol.* 42, 751–755. doi: 10.1093/pcp/pce094

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. U.S.A.* 85, 5536–5540. doi: 10.1073/pnas.85.15.5536

Von Gromoff, E. D., Alawady, A., Meinecke, L., Grimm, B., and Beck, C. F. (2008). Heme, a plastid-derived regulator of nuclear gene expression in Chlamydomonas. *Plant Cell* 20, 552–567. doi: 10.1105/tpc.107.054650

Xie, M., Chen, H., Huang, L., O'Neil, R. C., Shokhirev, M. N., and Ecker, J. R. (2018). A B-ARR-mediated cytokinin transcriptional network directs hormone cross-regulation and shoot development. *Nat. Commun.* 9:1604. doi: 10.1038/s41467-018-03921-6

Xu, C., and Hu, Y. (2020). The molecular regulation of cell pluripotency in plants. *aBIOTECH* 1, 169–177. doi: 10.1007/s42994-020-00028-9

Xuan, W., Zhu, F. Y., Xu, S., Huang, B. K., Ling, T. F., Qi, J. Y., et al. (2008). The heme oxygenase/carbon monoxide system is involved in the auxin-induced cucumber adventitious rooting process. *Plant Physiol.* 148, 881–893. doi: 10.1104/pp.108.125567

Yadav, D., Zemach, H., Belausov, E., and Charuvi, D. (2019). Initial proplastid-to-chloroplast differentiation in the developing vegetative shoot apical meristem of *Arabidopsis*. *Biochem. Biophys. Res. Commun.* 519, 391–395. doi: 10.1016/j.bbrc.2019.09.019

Yadav, R. K., Tavakkoli, M., Xie, M., Girke, T., and Venugopala, R. G. (2014). A high-resolution gene expression map of the *Arabidopsis* shoot meristem stem cell niche. *Development* 141, 2735–2744. doi: 10.1242/dev.106104

Zhai, Q., Li, C. B., Zheng, W., Wu, X., Zhao, J., Zhou, G., et al. (2007). Phytochrome chromophore deficiency leads to overproduction of jasmonic acid and elevated expression of jasmonate-responsive genes in *Arabidopsis*. *Plant Cell Physiol.* 48, 1061–1071. doi: 10.1093/pcp/pcm076

Zhang, T.-Q., Lian, H., Zhou, C.-M., Xu, L., Jiao, Y., and Wang, J.-W. (2017). A Two-Step Model for de Novo Activation of WUSCHEL during Plant Shoot Regeneration. *Plant Cell* 29, 1073–1087. doi: 10.1105/tpc.16.00863

Zubo, Y. O., Blakley, I. C., Yamburenko, M. V., Worthen, J. M., Street, I. H., Franco-Zorrilla, J. M., et al. (2017). Cytokinin induces genome-wide binding of the type-B response regulator ARR10 to regulate growth and development in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 114, E5995–E6004. doi: 10.1073/pnas.1620749114

Zubo, Y. O., Yamburenko, M. V., Selivankina, S. Y., Shakirova, F. M., Avalbaev, A. M., Kudryakova, N. V., et al. (2008). Cytokinin stimulates chloroplast transcription in detached barley leaves. *Plant Physiol.* 148, 1082–1093. doi: 10.1104/pp.108.122275

Zürcher, E., Tavor-Deslex, D., Lituiev, D., Enkerli, K., Tarr, P. T., and Müller, B. (2013). A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network in planta. *Plant Physiol.* 161, 1066–1075. doi: 10.1104/pp.112.211763

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 April 2021
doi: 10.3389/fpls.2021.670101

Nitrogen Nutrition Promotes Rhizome Bud Outgrowth via Regulation of Cytokinin Biosynthesis Genes and an *Oryza longistaminata* Ortholog of *FINE CULM 1*

*Kyohei Shibasaki[1], Arika Takebayashi[1], Nobue Makita[1], Mikiko Kojima[1], Yumiko Takebayashi[1], Misato Kawai[2], Takushi Hachiya[2,3] and Hitoshi Sakakibara[1,2]**

[1] RIKEN Center for Sustainable Resource Science, Yokohama, Japan, [2] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan, [3] Department of Molecular and Functional Genomics, Interdisciplinary Center for Science Research, Shimane University, Matsue, Japan

OPEN ACCESS

Edited by:
Jan Hejatko,
Central European Institute of Technology (CEITEC), Czechia

Reviewed by:
Helena G. Carvalho,
Universidade do Porto, Portugal
Weibing Yang,
Institute of Plant Physiology and Ecology,
Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China

***Correspondence:**
Hitoshi Sakakibara
sakaki@agr.nagoya-u.ac.jp

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: 20 February 2021
Accepted: 08 April 2021
Published: 30 April 2021

Citation:
Shibasaki K, Takebayashi A, Makita N, Kojima M, Takebayashi Y, Kawai M, Hachiya T and Sakakibara H (2021) Nitrogen Nutrition Promotes Rhizome Bud Outgrowth via Regulation of Cytokinin Biosynthesis Genes and an Oryza longistaminata Ortholog of FINE CULM 1. Front. Plant Sci. 12:670101. doi: 10.3389/fpls.2021.670101

Oryza longistaminata, a wild rice, can propagate vegetatively via rhizome formation and, thereby, expand its territory through horizontal growth of branched rhizomes. The structural features of rhizomes are similar to those of aerial stems; however, the physiological roles of the two organs are different. Nitrogen nutrition is presumed to be linked to the vegetative propagation activity of rhizomes, but the regulation of rhizome growth in response to nitrogen nutrition and the underlying biological processes have not been well characterized. In this study, we analyzed rhizome axillary bud growth in response to nitrogen nutrition and examined the involvement of cytokinin-mediated regulation in the promotion of bud outgrowth in *O. longistaminata*. Our results showed that nitrogen nutrition sufficiency promoted rhizome bud outgrowth to form secondary rhizomes. In early stages of the response to nitrogen application, glutamine accumulated rapidly, two cytokinin biosynthesis genes, isopentenyltransferase, and *CYP735A,* were up-regulated with accompanying cytokinin accumulation, and expression of an ortholog of *FINE CULM1*, a negative regulator of axillary bud outgrowth, was severely repressed in rhizomes. These results suggest that, despite differences in physiological roles of these organs, the nitrogen-dependent outgrowth of rhizome axillary buds in *O. longistaminata* is regulated by a mechanism similar to that of shoot axillary buds in *O. sativa.* Our findings provide a clue for understanding how branched rhizome growth is regulated to enhance nutrient acquisition strategies.

Keywords: axillary bud outgrowth, cytokinin, nitrogen, *Oryza longistaminata*, rhizome

INTRODUCTION

Seed reproduction and vegetative reproduction are the two major modes of propagation in plants. Some perennial plant species that vegetatively reproduce expand their territories through stolon or rhizome growth (Guo et al., 2020). Rhizomes grow horizontally underground and form branches as secondary and tertiary rhizomes by the outgrowth of axillary buds that develop at rhizome nodes.

Some rhizome tips developmentally transform into aerial green shoots and appear aboveground, becoming new ramets.

Wild rice is known for its substantial ecological diversity. *Oryza longistaminata*, an African wild rice species, is mainly propagated vegetatively via rhizome formation and proliferates vigorously (Vaughan, 1994). Both *O. longistaminata* and *Oryza sativa* contain the AA genome and have a high degree of genome synteny as revealed by genome sequencing (Reuscher et al., 2018; Li et al., 2020). *O. longistaminata* has also been used to study the genetic basis of rhizome development (Hu et al., 2011; Yoshida et al., 2016; Fan et al., 2017; Kyozuka, 2017; Bessho-Uehara et al., 2018; Toriba et al., 2020). The basic structural features of rhizomes are similar to those of aerial stems; rhizomes are composed of phytomer units consisting of a node, an internode, and an axillary bud (Yoshida et al., 2016). Crown roots develop at most of the rhizome nodes, suggesting that the branching growth of rhizomes is a strategy for efficient nutrition acquisition from the soil. Whereas rhizomes and aerial stems have similar anatomical features, the physiological features are different. Rhizomes are photosynthetic sink organs in which the internodes can elongate even during the vegetative stage of the parental ramet, whereas aerial stems are photosynthetic source organs in which internode elongation is tightly linked with the reproductive transition.

Studies of the molecular basis for shoot branching (tillering) in *O. sativa* show that three phytohormones, auxin, cytokinin, and strigolactone play key roles in regulating this developmental process (Beveridge and Kyozuka, 2010; Wang and Li, 2011; Gallavotti, 2013). Apically-derived auxin negatively regulates cytokinin biosynthesis by repressing *IPT4* and positively regulating strigolactone biosynthesis by *D10* (Arite et al., 2007; Minakuchi et al., 2010). Strigolactone induces the expression of *FC1* that negatively regulates axillary bud outgrowth, whereas expression of *FC1* is repressed by cytokinin (Minakuchi et al., 2010). As for nutritional factors that influence shoot branching in *O. sativa*, phosphate-starvation induces *de novo* synthesis of strigolactones that suppress shoot branching (Umehara et al., 2010). In contrast, nitrogen sufficiency up-regulates glutamine-mediated *IPT4* gene expression and plays a role in the promotion of shoot axillary bud outgrowth (Kamada-Nobusada et al., 2013; Ohashi et al., 2017; Sakakibara, 2021). A loss-of-function mutant of *glutamine synthetase1;2* (*GS1;2*) reduced the nitrogen-dependent induction of *IPT4* and the length of axillary buds, a phenotype that was rescued by the application of cytokinins (Ohashi et al., 2017). Nevertheless, how rhizome branching in *O. longistaminata* is regulated by nitrogen nutrition and the biological processes underlying rhizome axillary bud outgrowth remain to be elucidated.

In this study, we analyzed the growth of rhizome axillary buds in response to nitrogen nutrition and examined the involvement of cytokinin-mediated regulation in the promotion of bud outgrowth in *O. longistaminata*. Our results suggest that the nitrogen-dependent outgrowth of rhizome axillary buds in *O. longistaminata* is regulated by a mechanism similar to that for shoot axillary buds in *O. sativa*. This finding provides important insights for understanding the regulation of rhizome branching and growth in nutrient acquisition strategies.

RESULTS

Effect of Nitrogen Nutrition on Rhizome Bud Outgrowth in *O. longistaminata*

To examine the growth response of rhizome axillary buds to an inorganic nitrogen source, young ramets of comparable growth stages (**Figure 1A**) were hydroponically grown in a low-nitrogen (Low-N, 200 μM NH_4NO_3) medium for 2 weeks. The young ramets were further grown under a Low-N or a high-nitrogen (High-N, 2 mM NH_4NO_3) medium for another 2 weeks, and growth of the axillary buds was measured. The resulting growth status of the axillary buds was categorized into one of four patterns: secondary rhizomes whose internodes elongated greater than 1 cm (Rhizome), a bud that grew slightly but the length was less than 1 cm (Bud), a degenerated bud undergoing necrosis (Degenerated), and a developmentally transformed green shoot (Shoot; **Figure 1A**). The outgrowth of rhizome axillary buds was promoted under the High-N treatment with the percentage of secondary rhizomes significantly higher than in the Low-N treatment (**Figure 1B**). By contrast, the percentage of degenerated buds in the Low-N treatment was significantly higher than for the High-N treatment. In both nitrogen treatments, about 30 to 40% of the axillary buds became green shoots (**Figure 1B**). These results suggest that an inorganic nitrogen source promotes rhizome bud outgrowth to form secondary rhizomes.

Phytohormone Profile in Rhizomes in Response to Nitrogen Application

In *O. sativa*, *de novo* cytokinin biosynthesis in roots and shoots is up-regulated by nitrogen sources, and cytokinins are involved in the promotion of axillary bud outgrowth of shoot stems (Kamada-Nobusada et al., 2013; Ohashi et al., 2017). To evaluate the effect of nitrogen application on the levels of phytohormones in rhizomes, we profiled the accumulation levels of the major phytohormones (cytokinins, ABA, and IAA) in crown roots, axillary buds, and nodes at 6 h after 2 mM NH_4NO_3 application. For the cytokinins, levels of *trans*-zeatin (tZ) and its precursors, such as tZ riboside and tZ riboside 5'-phosphates (tZRPs), and N^6-(Δ^2-isopentenyl)adenine riboside 5'-phosphates (iPRPs), precursors of iP, increased in crown roots, buds, and nodes of the NH_4NO_3 -treated rhizomes (**Figure 2** and **Supplementary Table 1**). In contrast, the levels of other cytokinins and their precursors, such as cZ, cZR, and cZRPs, and most of the glucosides decreased after NH_4NO_3 application to the crown roots, but the levels of these compounds did not change in other plant organs (**Figure 2** and **Supplementary Table 1**). Since tZRPs and iPRPs are initial products catalyzed by adenosine phosphate-isopentenyltransferase (IPT) and CYP735A (Sakakibara, 2006), these results suggest that *de novo* synthesis of cytokinins is activated by nitrogen application to the rhizomes. Interestingly, there was no significant difference in the levels of ABA or IAA resulting from supplemental NH_4NO_3 application to crown roots, axillary buds, or nodes (**Supplementary Table 1**).

To confirm a link between cytokinin accumulation and axillary bud outgrowth, we examined the effect of applying exogenous cytokinin on the rhizome. When we treated rhizomes

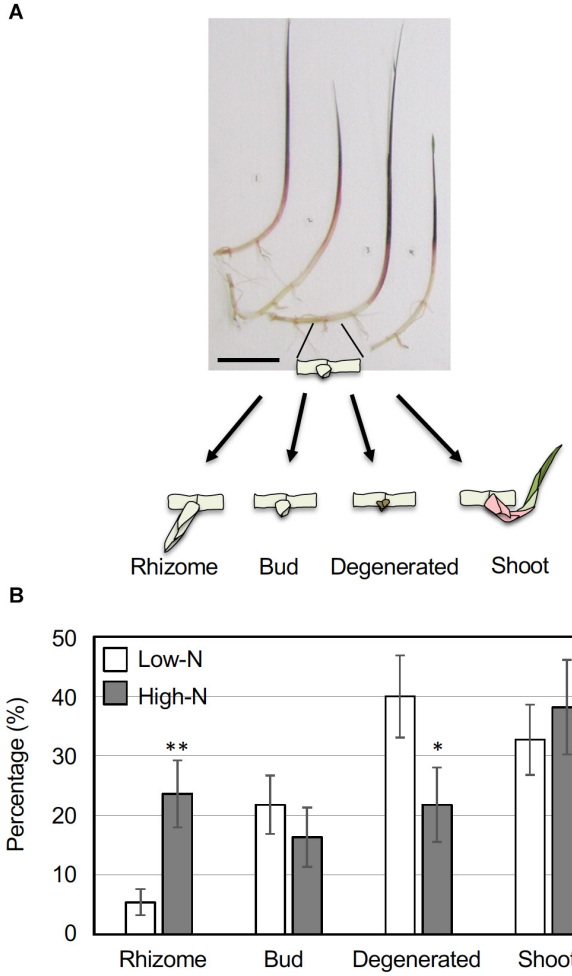


FIGURE 1 | Growth response of *O. longistaminata* rhizome axillary buds to nitrogen nutrition. Young ramets having rhizomes at a similar growth stage were hydroponically grown in a low-N (200 μM NH_4NO_3) medium for 2 weeks. The ramets were subsequently transferred to either a low-N or high-N (2 mM NH_4NO_3) medium and incubated for an additional 2 weeks. Growth of the axillary buds on the rhizome nodes was monitored and classified into one of four categories: Rhizome (length > 10 mm), Buds (length < 10 mm), Degenerated, and Shoot. Detailed category definitions are explained in the text. **(A)** Typical ramets used in this experiment. Scale bar = 10 cm. **(B)** A comparison of rhizome growth in low-N and high-N media. Vertical bars represent the mean ± S.E. [*n* (ramet) = 13 for low-N, *n* = 14 for high-N]. Each ramet rhizome had 2 to 7 nodes, and the total number of nodes was 55 for low-N and 55 for high-N samples. $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t* test) compared to the low-N medium.

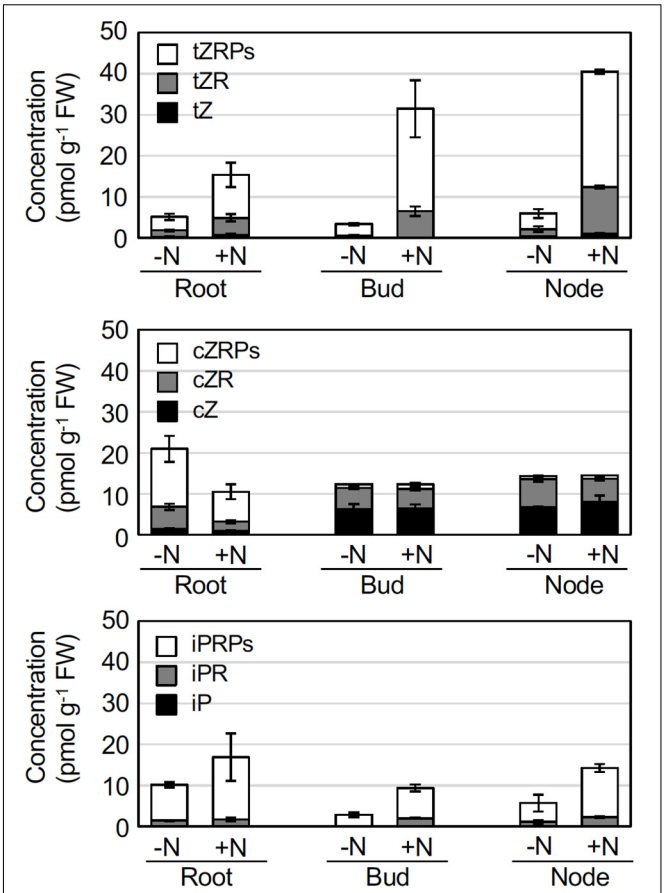


FIGURE 2 | Effects of nitrogen applications on cytokinin concentrations in rhizome roots, buds, and nodes. Young ramets having rhizomes at a similar growth stage were hydroponically grown in a low-N (200 μM NH_4NO_3) medium for 2 weeks. The ramets were subsequently transferred to culture media containing no nitrogen (-N) or 2 mM NH_4NO_3 (+N). 6 h after transfer, rhizome roots, buds, and nodes were separately harvested, and the cytokinin hormone content was analyzed. Data are presented as the mean ± S.E. (*n* = 3). The complete data set is presented in **Supplementary Table 1**. tZ, *trans*-zeatin; tZR, tZ riboside; tZRPs, tZR 5'-phosphates; cZ, *cis*-zeatin; cZR, cZ riboside; cZRPs, cZR 5'-phosphates; iP, N^6-(Δ^2-isopentenyl)adenine; iPR, iP riboside; iPRPs, iPR 5'-phosphates; and FW, fresh weight.

with 1 μM tZ for 2 weeks, axillary bud growth was promoted (**Supplementary Figure 1**). These results support the hypothesis that cytokinin action is involved in nitrogen-dependent rhizome axillary bud outgrowth.

Expression of Cytokinin Biosynthesis Genes in Response to Nitrogen Supply

To understand the underlying events leading to the nitrogen-dependent accumulation of cytokinins, we analyzed the expression of genes involved in cytokinin biosynthesis by reverse transcription-quantitative PCR (RT-qPCR). To specify the responsive genes, we selected orthologs of three cytokinin biosynthesis genes, *IPT, CYP735A*, and *LOG* from the *O. longistaminata* genomic database[1] (**Supplementary Table 2**), and first analyzed the accumulation of the corresponding transcripts in rhizome nodes that include axillary buds at 6 h after the 2 mM NH_4NO_3 application (**Supplementary Figure 2**). Among the analyzed genes, the transcripts for *OlIPT4, OlIPT5, OlIPT8, and OlCYP735A4* accumulated in response to nitrogen application, suggesting that *de novo* cytokinin biosynthesis is up-regulated by nitrogen sources in the rhizome. *NADH-GOGAT1* that encodes a glutamate synthase was used as an indicator for the nitrogen response (Hirose et al., 1997). We further

[1]http://olinfres.nig.ac.jp/

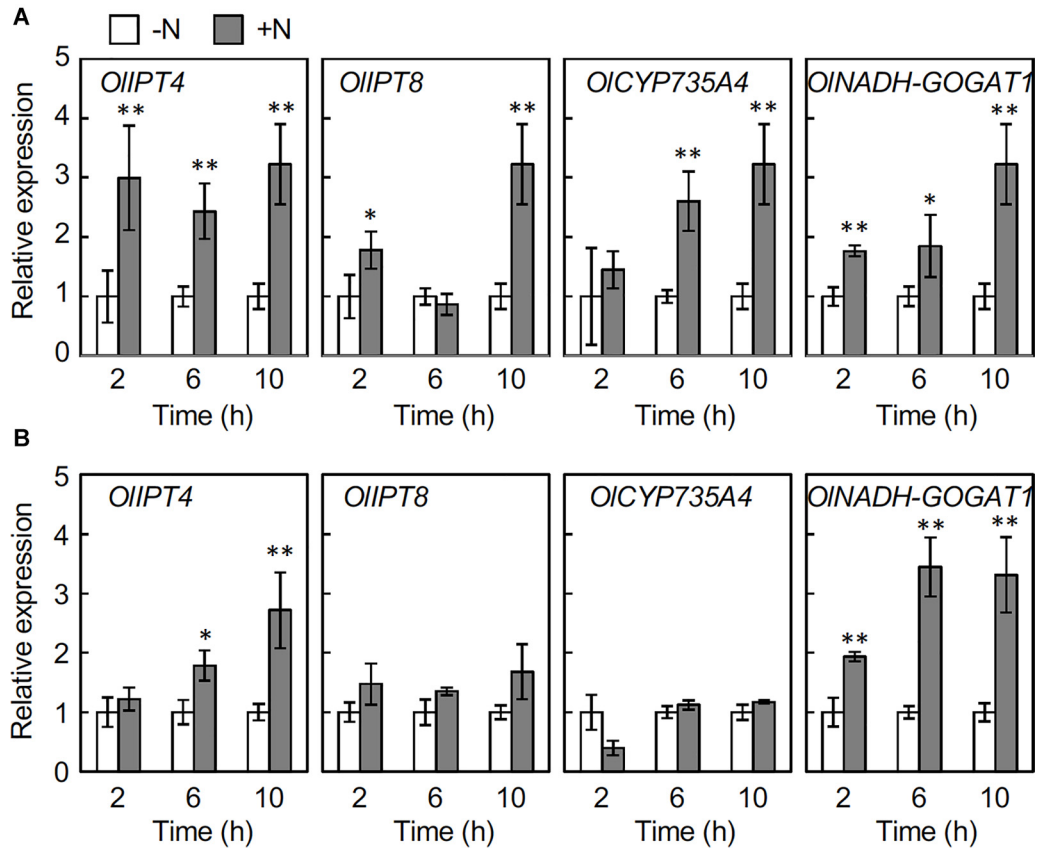


FIGURE 3 | Changes in the expression of cytokinin biosynthesis genes in rhizomes responding to nitrogen application. Young ramets having rhizomes at a similar growth stage were hydroponically grown in a low-N (200 μM NH_4NO_3) medium for 2 weeks. The ramets were subsequently transferred to culture media containing no nitrogen (-N) or 2 mM NH_4NO_3 (+N). After incubation for the indicated times, rhizome roots and nodes with axillary buds were separately harvested. Total RNA prepared from the samples was subjected to RT-qPCR. Expression of the cytokinin biosynthesis genes in nodes including axillary buds **(A)** and roots **(B)**. Transcript abundance was normalized with *OlUBQ1* and expressed as relative values with respect to the value in the -N treatment. Values are means ± SE ($n = 3$). $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t* test) compared to the -N treatment.

analyzed the expression of *OlIPT4, OlIPT8, and OlCYP735A4* in a time-course experiment. In the rhizome node that includes an axillary bud, the *OlIPT4* transcript accumulated within 2 h after 2 mM NH_4NO_3 application (**Figure 3A**). Transcripts for *OlIPT8* and *OlCYP735A4* also accumulated in response to nitrogen application but more slowly. In roots, the *OlIPT4* transcript accumulated gradually, but no clear induction of *OlIPT8* or *OlCYP735A4* was observed, although the expression of the nitrogen-responsive marker gene *OlNADH-GOGAT1* was induced (**Figure 3B**).

Accumulation of Amino Acids in Response to the Nitrogen Supply

In *O. sativa*, a glutamine-related signal is a key indicator of *IPT4* gene induction (Kamada-Nobusada et al., 2013; Ohashi et al., 2017). To obtain insight into the relationship between glutamine and *IPT4* expression in *O. longistaminata*, we analyzed amino acid concentrations in rhizome nodes that include axillary buds and in roots in the presence or absence of supplemental nitrogen. Upon exposure to 2 mM NH_4NO_3, glutamine accumulated significantly in both roots and nodes within 2 h (**Figure 4**). Although the difference was rather smaller, threonine also significantly accumulated in both organs. Aspartate levels in nodes were higher than those in roots, but no substantial differences in the levels of other amino acids were found between organs.

To obtain more direct evidence, we examined the effect of an exogenous application of glutamine on the expression of *OlIPT4*. When we treated rhizomes with 50 mM glutamine, *OlIPT4* expression in rhizome nodes was up-regulated in 6 h (**Supplementary Figure 3**), suggesting that glutamine-related signaling is involved in *OlIPT4* induction.

Downregulation of *OlFC1* Expression in Response to the Nitrogen Supply

To further delineate the underlying processes regulating rhizome axillary bud outgrowth, we analyzed the effect of nitrogen supply on the expression of *OlFC1*, a key regulator of shoot axillary bud growth. Transcript accumulation markedly decreased in rhizome axillary buds in response to nitrogen supply at 6 h,

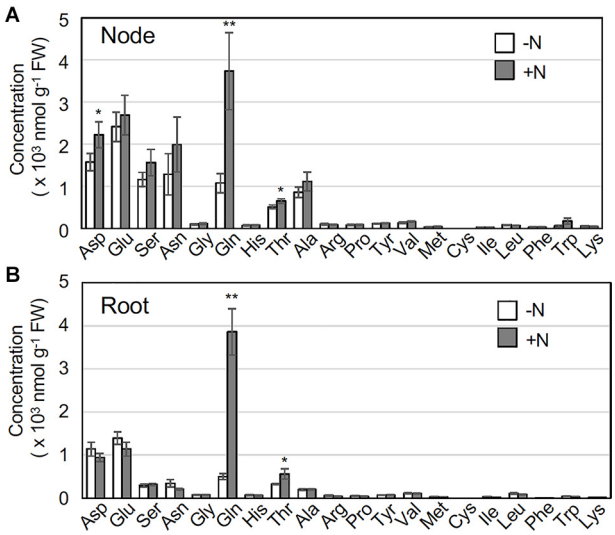


FIGURE 4 | Effects of nitrogen application on amino acid concentrations in rhizome nodes and roots. Young ramets having rhizomes at a similar growth stage were hydroponically grown in a low-N (200 μM NH_4NO_3) medium for 2 weeks. The ramets were subsequently transferred to culture media containing no nitrogen (-N) or 2 mM NH_4NO_3 (+N) and incubated for 2 h. Rhizome nodes and roots were separately harvested and subjected to amino acid analysis. The amino acid concentrations in nodes including axillary buds **(A)** and roots **(B)**. Data are presented as the mean ± S.E. (n = 3). *P < 0.05; **P < 0.01 (Student's t test) compared to the -N treatment. FW, fresh weight.

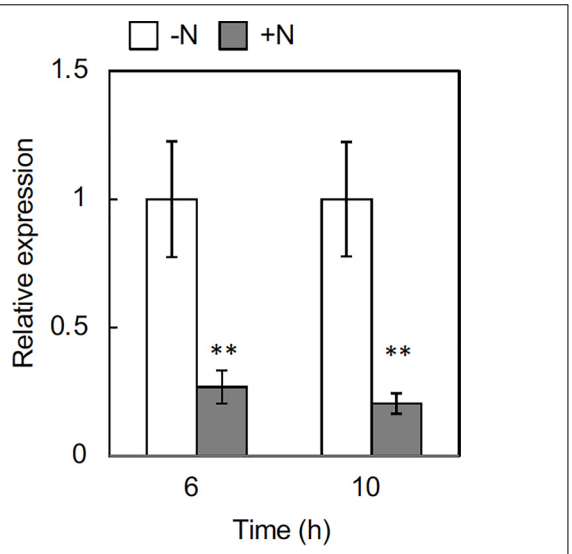


FIGURE 5 | Effects of nitrogen application on the expression of *OlFC1* in rhizome buds. Young ramets having rhizomes at a similar growth stage were hydroponically grown in a low-N (200 μM NH_4NO_3) medium for 2 weeks. The ramets were subsequently transferred to culture media containing no nitrogen (-N) or 2 mM NH_4NO_3 (+N) and incubated for 6 h and 10 h. Total RNA prepared from the samples was subjected to RT-qPCR analysis. Transcript levels were normalized by *OlUBQ1* and expressed as relative values with respect to the value in the -N treatment. Values are the means ± SE (n = 3). **P < 0.01 (Student's t test) compared to the -N treatment.

and the repression continued at 10 h after treatment (**Figure 5**). This result suggests that nitrogen sufficiency down-regulates *OlFC1* expression.

DISCUSSION

In this study, we have shown that nitrogen nutrition promotes rhizome bud outgrowth to secondary rhizomes in *O. longistaminata*. The regulation of secondary rhizome formation in *O. longistaminata* relies on a mechanism similar to that for shoot axillary buds in *O. sativa*. In general, ample nitrogen nutrition promotes shoot growth but suppresses root system growth, with the opposite effect under nitrogen deficient conditions (Hermans et al., 2006; Gruber et al., 2013). Increasing the number of shoots (tillers) in response to nitrogen nutrition leads to larger amounts of aboveground biomass and more photosynthetic assimilation capacity. Also, prioritizing root system growth when nitrogen is deficient is a strategy for efficient nitrogen acquisition. In longer periods of *O. longistaminata* growth in the presence of different nitrogen treatments, growth of the secondary rhizomes was promoted and tertiary rhizomes branched under nitrogen-sufficient conditions (2 mM NH_4NO_3). Conversely, rhizome branching was suppressed and crown root growth was promoted under nitrogen-limiting conditions (200 μM NH_4NO_3; **Supplementary Figure 4**). Thus, the nitrogen-dependent promotion of rhizome elongation contributes to an expansion of the horizontal territory of vegetatively-propagated plants.

In the analysis of axillary bud growth patterns under Low-N and High-N treatments, there was no difference in the rate of differentiation to green shoots (**Figure 1**). Perhaps some of the axillary buds on rhizomes were already primed to become green shoots prior to the nitrogen treatment. Typically, the two basal nodes of ramets develop aerial shoot-type buds (Yoshida et al., 2016). The rate of axillary bud degeneration was higher in the low-N treatment. Deficiencies in the nitrogen supply could abort the growth of rhizome axillary buds in order to efficiently allocate nitrogen resources to a reduced number of rhizomes.

The expression of *OlIPT4* was up-regulated by supplemental nitrogen or exogenously applied glutamine (**Figure 3** and **Supplementary Figure 3**). Also, the expression of *OlFC1* was repressed by an increased nitrogen supply (**Figure 5**). Studies on the regulation of shoot axillary bud outgrowth in *O. sativa* have shown that the expression of *FC1* is repressed by cytokinin (Minakuchi et al., 2010). Our findings in this study suggest a model for the nitrogen-dependent promotion of rhizome axillary bud outgrowth (**Figure 6**) that is similar to how shoot axillary bud outgrowth is regulated in *O. sativa*. A supply of inorganic nitrogen rapidly increases the concentration of glutamine and triggers the induction of *de novo* cytokinin biosynthesis via *OlIPT4* induction. The accumulated cytokinin suppresses the expression of *OlFC1*, thereby promoting outgrowth of the axillary bud.

The degree of nitrogen-dependent induction of *OlIPT4* and *OlCYP735A4* expression in the node was greater than that in

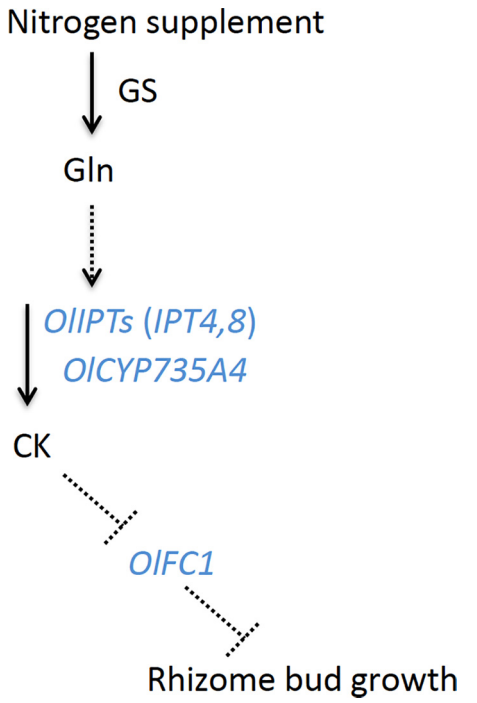


FIGURE 6 | A model of hormonal control of rhizome axillary bud outgrowth in *O. longistaminata*. In this model, the nitrogen supply induces the accumulation of glutamine. Subsequently, a glutamine-related signal activates the expression of cytokinin biosynthesis genes, including *OlIPT4, OlIPT8,* and *OlCYP735A4*. The cytokinins negatively regulate the expression of *OlFC1*, the product of which functions to repress the outgrowth of the rhizome axillary bud. GS, glutamine synthetase; CK, cytokinin. Solid arrows represent the metabolic flow. Dashed arrows and T-end lines represent positive and negative regulation, respectively.

the root (**Figure 3**) and corresponded with the changes in tZ-type cytokinin accumulation (**Figure 2**). The regulation of cytokinin biosynthesis gene expression in response to nitrogen nutrition in *O. sativa* has been primarily analyzed in roots but not in nodes. *IPT4* in *O. sativa* is mainly expressed in the phloem of vascular bundles (Kamada-Nobusada et al., 2013). *O. longistaminata* nodes might be a major organ for *de novo* cytokinin biosynthesis in response to nitrogen because nodes have a complex and dense vascular system (Yamaji and Ma, 2014).

FC1 expression is induced by strigolactones in *O. sativa* (Minakuchi et al., 2010; Xu et al., 2015), whereas strigolactone biosynthesis, important for tiller growth, is induced by phosphorus deficiency (Umehara et al., 2010). The biosynthetic pathways and molecular species of strigolactones are very diverse among plant species. In maize, nitrogen deficiency strongly induces the biosynthesis of zealactone, a strigolactone (Ravazzolo et al., 2019). The regulation strigolactone biosynthesis in *O. longistaminata* has not yet been investigated. The involvement of strigolactones in axillary bud elongation of underground stems in response to nitrogen nutrition and the interplay between cytokinin and strigolactone signalings will be the subjects of future research.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The perennial wild rice species *O. longistaminata* (IRGC10404) was used in this study. Plants were grown in soil in a temperature-controlled greenhouse with supplemental artificial light at 12 h light (30°C)/12 h dark (25°C) photoperiod. Young ramets of comparable growth stages (shoot lengths: 20 to 30 cm, rhizome lengths: 10 to 20 cm) were excised from parental ramets and grown for 2 weeks with aeration in a hydroponic culture solution (Kamachi et al., 1991) in which the NH_4NO_3 concentration was modified to either 200 μM NH_4NO_3 (low-N) or 2 mM NH_4NO_3 (high-N).

Quantification of Phytohormones

Extraction and semi-purification of phytohormones from about 100 mg fresh weight of plant samples were performed as described previously (Kojima et al., 2009; Kojima and Sakakibara, 2012). Cytokinins were quantified using an ultra-performance liquid chromatography (UPLC)-tandem quadrupole mass spectrometer (ACQUITY UPLC™ System/XEVO-TQS; Waters, Milford, MA, United States) with an octadecylsilyl (ODS) column (ACQUITY UPLC HSS T3, 1.8 μm, 2.1 mm × 100 mm, Waters; Kojima et al., 2009). ABA and IAA were quantified using an ultra-high performance liquid chromatography (UHPLC)-quadrupole-orbitrap mass spectrometer (UHPLC/Q-Exactive™; Thermo Fisher Scientific, Waltham, MA, United States) with an ODS column (ACQUITY UPLC HSS T3, 1.8 μm, 2.1 mm × 100 mm; Waters; Kojima and Sakakibara, 2012; Shinozaki et al., 2015).

Gene Cloning

Total RNA was prepared from plant samples using an RNeasy Plant Mini Kit with an RNase-free DNase Set (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized by reverse transcription using a SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific) with oligo$(dT)_{12-18}$ primers. cDNAs were amplified by PCR with specific primers and cloned into the pCR™2.1-TOPO TA vector (Thermo Fisher Scientific).

Quantitative PCR Analysis

Quantitative PCR (qPCR) analysis was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems, Waltham, MA, United States) with a KAPA SYBR® Fast qPCR Master Mix (2×) kit (KAPA Biosystems, London, United Kingdom). Transcript abundance was estimated using the relative quantification method (Livak and Schmittgen, 2001) with a *UBQ1* homolog as the internal standard for normalization. Gene locus IDs and the specific primers are listed in **Supplementary Table 3**.

Determination of Free Amino Acids

Plant samples were powdered in liquid nitrogen and homogenized in 10 volumes of 10 mM HCl with 0.2 mM methionine sulfone as an internal control. The homogenate was centrifuged, and the supernatant was filtered through

Ultrafree® -MC filters (Merck Millipore, Burlington, MA, United States). Derivatization of amino acids was carried out using the Pico-Tag® method (Waters), and the resulting derivatives were analyzed with an HPLC System (Alliance 2695 HPLC system/2475, Waters) using a Pico-Tag column as described in the manufacturer's instruction manual.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KS and HS designed the research project. KS, AT, NM, MKa, MKo, YT, and TH conducted the research. KS, MKo, YT, and HS analyzed the data. KS and HS wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by Advanced Low Carbon Technology Research and Development Program from the Core Research for Evolutional Science and Technology by JST (to HS; no. JPMJCR13B1) and Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to HS; JP17H06473 and JP19H00931).

ACKNOWLEDGMENTS

We are grateful to Drs. Motoyuki Ashikari, and Stephan Reuscher (Nagoya University) for their helpful discussion and providing the genome information of *O. longistaminata*.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.670101/full#supplementary-material

REFERENCES

Arite, T., Iwata, H., Ohshima, K., Maekawa, M., Nakajima, M., Kojima, M., et al. (2007). DWARF10, an RMS1/MAX4/DAD1 ortholog, controls lateral bud outgrowth in rice. *Plant J.* 51, 1019–1029. doi: 10.1111/j.1365-313X.2007.03210.x

Bessho-Uehara, K., Nugroho, J. E., Kondo, H., Angeles-Shim, R. B., and Ashikari, M. (2018). Sucrose affects the developmental transition of rhizomes in *Oryza longistaminata*. *J. Plant Res.* 131, 693–707. doi: 10.1007/s10265-018-1033-x

Beveridge, C. A., and Kyozuka, J. (2010). New genes in the strigolactone-related shoot branching pathway. *Curr. Opin. Plant Biol.* 13, 34–39. doi: 10.1016/j.pbi.2009.10.003

Fan, Z., Cai, Z., Shan, J., and Yang, J. (2017). Bud position and carbohydrate play a more significant role than light condition in the developmental transition between rhizome buds and aerial shoot buds of *Oryza longistaminata*. *Plant Cell Physiol.* 58, 1281–1282. doi: 10.1093/pcp/pcx061

Gallavotti, A. (2013). The role of auxin in shaping shoot architecture. *J. Exp. Bot.* 64, 2593–2608. doi: 10.1093/jxb/ert141

Gruber, B. D., Giehl, R. F. H., Friedel, S., and von Wirén, N. (2013). Plasticity of the *Arabidopsis* root system under nutrient deficiencies. *Plant Physiol.* 163, 161–179. doi: 10.1104/pp.113.218453

Guo, L., Plunkert, M., Luo, X., and Liu, Z. (2020). Developmental regulation of stolon and rhizome. *Curr. Opin. Plant Biol.* 59:101970. doi: 10.1016/j.pbi.2020.10.003

Hermans, C., Hammond, J. P., White, P. J., and Verbruggen, N. (2006). How do plants respond to nutrient shortage by biomass allocation? *Trends Plant Sci.* 11, 610–617. doi: 10.1016/j.tplants.2006.10.007

Hirose, N., Hayakawa, T., and Yamaya, T. (1997). Inducible accumulation of mRNA for NADH-dependent glutamate synthase in rice roots in response to ammonium ions. *Plant Cell Physiol.* 38, 1295–1297. doi: 10.1093/oxfordjournals.pcp.a029120

Hu, F., Wang, D., Zhao, X., Zhang, T., Sun, H., Zhu, L., et al. (2011). Identification of rhizome-specific genes by genome-wide differential expression analysis in *Oryza longistaminata*. *BMC Plant Biol.* 24:11. doi: 10.1186/1471-2229-11-18

Kamachi, K., Yamaya, T., Mae, T., and Ojima, K. (1991). A role for glutamine synthetase in the remobilization of leaf nitrogen during natural senescence in rice Leaves. *Plant Physiol.* 96, 411–417. doi: 10.1104/pp.96.2.411

Kamada-Nobusada, T., Makita, N., Kojima, M., and Sakakibara, H. (2013). Nitrogen-dependent regulation of *de novo* cytokinin biosynthesis in rice: the role of glutamine metabolism as an additional signal. *Plant Cell Physiol.* 54, 1881–1893. doi: 10.1093/pcp/pct127

Kojima, M., Kamada-Nobusada, T., Komatsu, H., Takei, K., Kuroha, T., Mizutani, M., et al. (2009). Highly sensitive and high-throughput analysis of plant hormones using ms-probe modification and liquid chromatographytandem mass spectrometry: an application for hormone profiling in *Oryza sativa*. *Plant Cell Physiol.* 50, 1201–1214. doi: 10.1093/pcp/pcp057

Kojima, M., and Sakakibara, H. (2012). Highly sensitive high-throughput profiling of six phytohormones using MS-probe modification and liquid chromatography-tandem mass spectrometry. *Methods Mol. Biol.* 918, 151–164. doi: 10.1007/978-1-61779-995-2_11

Kyozuka, J. (2017). Analysis of rhizome development in *Oryza longistaminata*, a wild rice species. *Plant Cell Physiol.* 58, 1283. doi: 10.1093/pcp/pcx064

Li, W., Li, K., Zhang, Q. J., Zhu, T., Zhang, Y., Shi, C., et al. (2020). Improved hybrid *de novo* genome assembly and annotation of African wild rice, *Oryza longistaminata*, from Illumina and PacBio sequencing reads. *Plant Genome* 13:e20001. doi: 10.1002/tpg2.20001

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Minakuchi, K., Kameoka, H., Yasuno, N., Umehara, M., Luo, L., Kobayashi, K., et al. (2010). *FINE CULM1* (*FC1*) works downstream of strigolactones to inhibit the outgrowth of axillary buds in rice. *Plant Cell Physiol.* 51, 1127–1135. doi: 10.1093/pcp/pcq083

Ohashi, M., Ishiyama, K., Kojima, S., Kojima, M., Sakakibara, H., Yamaya, T., et al. (2017). Lack of cytosolic glutamine synthetase1;2 activity reduces nitrogen-dependent biosynthesis of cytokinin required for axillary bud outgrowth in rice seedlings. *Plant Cell Physiol.* 58, 679–690. doi: 10.1093/pcp/pcx022

Ravazzolo, L., Trevisan, S., Manoli, A., Boutet-Mercey, S., Perreau, F., and Quaggiotti, S. (2019). The control of zealactone biosynthesis and exudation is involved in the response to nitrogen in maize root. *Plant Cell Physiol.* 60, 2100–2112. doi: 10.1093/pcp/pcz108

Reuscher, S., Furuta, T., Bessho-Uehara, K., Cosi, M., Jena, K. K., Toyoda, A., et al. (2018). Assembling the genome of the African wild rice *Oryza longistaminata* by exploiting synteny in closely related *Oryza* species. *Commun. Biol.* 1:162. doi: 10.1038/s42003-018-0171-y

Sakakibara, H. (2006). Cytokinins: activity, biosynthesis, and translocation. *Annu. Rev. Plant Biol.* 57, 431–449. doi: 10.1146/annurev.arplant.57.032905.105231

Sakakibara, H. (2021). Cytokinin biosynthesis and transport for systemic nitrogen signaling. *Plant J.* 105, 421–430. doi: 10.1111/tpj.15011

Shinozaki, Y., Hao, S., Kojima, M., Sakakibara, H., Ozeki-Iida, Y., Zheng, Y., et al. (2015). Ethylene suppresses tomato (*Solanum lycopersicum*) fruit set through modification of gibberellin metabolism. *Plant J.* 83, 237–251. doi: 10.1111/tpj.12882

Toriba, T., Tokunaga, H., Nagasawa, K., Nie, F., Yoshida, A., and Kyozuka, J. (2020). Suppression of leaf blade development by BLADE-ON-PETIOLE orthologs is a common strategy for underground rhizome growth. *Curr. Biol.* 30, 509.e–516.e. doi: 10.1016/j.cub.2019.11.055

Umehara, M., Hanada, A., Magome, H., Takeda-Kamiya, N., and Yamaguchi, S. (2010). Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. *Plant Cell Physiol.* 51, 1118–1126. doi: 10.1093/pcp/pcq084

Vaughan, D. A. (1994). *The Wild Relatives of Rice: A Genetic Resources Handbook*. Los Banos, CA: IRRI.

Wang, Y., and Li, J. (2011). Branching in rice. *Curr. Opin. Plant Biol.* 14, 94–99. doi: 10.1016/j.pbi.2010.11.002

Xu, J., Zha, M., Li, Y., Ding, Y., Chen, L., Ding, C., et al. (2015). The interaction between nitrogen availability and auxin, cytokinin, and strigolactone in the control of shoot branching in rice (*Oryza sativa* L.). *Plant Cell Rep.* 34, 1647–1662. doi: 10.1007/s00299-015-1815-8

Yamaji, N., and Ma, J. F. (2014). The node, a hub for mineral nutrient distribution in graminaceous plants. *Trends Plant Sci.* 19, 556–563. doi: 10.1016/j.tplants.2014.05.007

Yoshida, A., Terada, Y., Toriba, T., Kose, K., Ashikari, M., and Kyozuka, J. (2016). Analysis of rhizome development in *Oryza longistaminata*, a wild rice species. *Plant Cell Physiol.* 57, 2213–2220. doi: 10.1093/pcp/pcw138

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 24 August 2021
doi: 10.3389/fpls.2021.677585

Cytokinin Regulation of Source-Sink Relationships in Plant-Pathogen Interactions

Kathryn E. McIntyre[1,2], *Daniel R. Bush*[3] *and Cristiana T. Argueso*[1,2]*

[1]Department of Agricultural Biology, Colorado State University, Fort Collins, CO, United States, [2]Graduate Program in Cell and Molecular Biology, Colorado State University, Fort Collins, CO, United States, [3]Department of Biology, Colorado State University, Fort Collins, CO, United States

Cytokinins are plant hormones known for their role in mediating plant growth. First discovered for their ability to promote cell division, this class of hormones is now associated with many other cellular and physiological functions. One of these functions is the regulation of source-sink relationships, a tightly controlled process that is essential for proper plant growth and development. As discovered more recently, cytokinins are also important for the interaction of plants with pathogens, beneficial microbes and insects. Here, we review the importance of cytokinins in source-sink relationships in plants, with relation to both carbohydrates and amino acids, and highlight a possible function for this regulation in the context of plant biotic interactions.

Keywords: cytokinins, source-sink relationships, plant growth, plant yield, plant-pathogen interactions, carbon allocation, amino acid translocation

OPEN ACCESS

Edited by:
Klára Hoyerová, Academy of Sciences of the Czech Republic, Czechia

Reviewed by:
Robert Malinowski, Polish Academy of Sciences, Poland
Karel Dolezal, Academy of Sciences of the Czech Republic, Czechia

***Correspondence:**
Cristiana T. Argueso
cris.argueso@colostate.edu

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *08 March 2021*
Accepted: *12 July 2021*
Published: *24 August 2021*

Citation:
McIntyre KE, Bush DR and Argueso CT (2021) Cytokinin Regulation of Source-Sink Relationships in Plant-Pathogen Interactions. Front. Plant Sci. 12:677585. doi: 10.3389/fpls.2021.677585

INTRODUCTION

Cytokinins are a group of plant hormones derived from adenine, classified by the presence of an isoprenoid or an aromatic chain at the N^6 position of their adenine moieties (Mok and Mok, 2001). Although different compounds with cytokinin activity have been shown to regulate various physiological processes in plants, cytokinins are broadly described as growth promoting plant hormones. The first cytokinin discovered by Miller and Skoog in the 1950s, kinetin, was defined as a plant-derived chemical that could promote cell division (Miller et al., 1956). In a following study, it was demonstrated that kinetin, in combination with auxin, was responsible for promoting cell division and organ development from undifferentiated cells in culture (Skoog and Miller, 1957). While the study of cytokinins began in the middle of the 1900s, they are in fact an ancient hormone, being one of the first four hormones to emerge in photosynthetically capable organisms (Wang et al., 2015). Evolutionary studies indicate that the common ancestor of all land plants, charophytes, contains the genetic sequences of orthologs to known members the cytokinin signaling pathway (Wang et al., 2015). These data suggest that cytokinins had a role in plants as early as 450 million years ago. Today, cytokinins are known for their broad role in plant growth (Kieber and Schaller, 2018), and also roles in preventing senescence, as well regulation of biotic and abiotic stress tolerance (Argueso et al., 2009; Cortleven et al., 2019).

One important physiological response that has been classically associated with cytokinins is the regulation of source-sink relationships and nutrient allocation in plants. Shortly after cytokinins were discovered to have a role in cell division, a study in 1961 in *Nicotiana rustica* demonstrated that exogenous application of kinetin to leaves led to increased accumulation

of the amino acid glycine to the area of hormone application (Mothes and Engelbrecht, 1961). Similarly, kinetin application to leaves of fava bean plants that had been unrooted was also shown to correlate with the movement of the amino acid alanine to the site of hormone application, which the researchers termed "mobilization" (Mothes and Engelbrecht, 1963). These early reports indicated that cytokinins could have a pivotal role in the allocation of amino acids in plants, with important consequences for plant growth.

In this review, we start by providing readers with an overview of the process of source-sink relationships in plants, and then proceed to highlight the evidence for a regulatory role for cytokinins in this important physiological process, starting with their first initial association and finishing with the most recent evidence. We conclude by pointing out some emerging evidence of the importance of this plant hormone as a regulator of nutrient availability in plant biotic interactions during disease susceptibility and promotion of plant immunity.

OVERVIEW OF SOURCE-SINK RELATIONSHIPS IN PLANTS

Photosynthesis leads to the production of reduced carbon products, also known as photoassimilates. Photoassimilates generated in the mesophyll cells, such as sucrose, oligosaccharides, and amino acids, are transported to other parts of the plant to maintain plant growth. Generally, the rate of photosynthetic activity and the accumulation of photoassimilates can be used to classify organs as sinks or sources. Sources are defined as photosynthetically active leaves that export photoassimilates to heterotrophic sink tissues that are dependent on imported sugars and amino acids for growth and development. Fully mature source leaves export as much as 80% of photoassimilate to sink tissues (Kalt-Torres et al., 1987). Sinks are defined as the opposite: an organ that is dependent on sugar and amino acid import to support growth and development. Sinks include young leaves, reproductive organs, and roots. Photosynthetic activity changes during the course of leaf development. Young leaves are sink organs that need to import photoassimilates from mature leaves to support growth and development (Geiger and Sheigh, 1993). As the immature leaf grows, it becomes photosynthetically active and eventually becomes an exporter of photoassimilates, through a process known as the sink-source transition (Turgeon, 1989). The relationship between source and sink organs has been the focus of intensive research because of its impact on plant growth and yield (White et al., 2016) and its potential for using transgenic approaches for modifying yield and/or nutritional quality (Yadav at al., 2015).

Sucrose is the end product of photosynthesis, and the primary sugar transported within plants. In source leaves, sucrose produced, from photosynthesis during the day or starch degradation occurring at night, is loaded into the phloem for transport to sinks (**Figure 1A**). Although this review focuses mostly on sucrose, as it is present in the phloem sap of all plant species, it should be noted that the phloem sap of some plant species also contains sugar alcohols and/or oligosaccharides from the raffinose family (Zimmermann and Ziegler, 1975; Noiraud et al., 2001). Depending on the anatomical connections of the plant species, loading of sucrose into the phloem can be achieved by three different loading mechanisms: symplastic, apoplastic, and polymer trapping (Braun et al., 2014). For the purposes of this review, we will focus on the apoplastic loading pathway, which is the predominant pathway used in most plant species, including the model plant species *Arabidopsis thaliana* (hereafter, Arabidopsis).

Apoplastic phloem loading in the leaf is mediated by a proton-sucrose symporter (Bush, 1993). Sucrose is transported out of mesophyll cells into the intercellular space by sucrose transporters known as SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS (SWEETs) (Chen et al., 2012). Once in the intercellular space, sucrose is then actively loaded into the phloem cells against a significant concentration gradient by proton-sucrose symporters, named SUCROSE TRANSPORTERS/CARRIERS (SUTs/SUCs) (Reinders et al., 2012; Zhang and Turgeon, 2018). Sucrose accumulates to molar levels in the leaf phloem thereby creating a high osmotic potential that draws in water. Since the phloem cells are surrounded by an inelastic cell wall, this creates high hydrostatic pressure that drives mass flow of solution to sink tissues where sucrose is released and used for growth, development or carbohydrate storage (Bush, 2020). There are two main mechanisms by which sucrose is moved into sink cells (Braun et al., 2014): (i) it is released into the intracellular space by SWEETs and then transported into the sink tissue by SUTs/SUCs (Weber et al., 1997) or (ii) it is released into intracellular space by SWEETs and then hydrolyzed into glucose and fructose by extracellular invertases (Ruan et al., 2010) followed by import into sink cells by proton/hexose symporters (HXTs) (Zhang et al., 2006; Hayes et al., 2007). Due to sucrose being the major form of carbon being translocated from source to sink (Fife et al., 1962; Turgeon, 1989), and starch being the main storage form of sucrose, the homeostasis of these two carbohydrates is essential for the regulation of their metabolism and allocation in plants (Smith and Stitt, 2007).

The production, storage, and movement of amino acids can also define organs as sinks or sources (**Figure 1A**) (Bush, 1999). Roots are the site of uptake of inorganic nitrogen (N) from the soil, in the form of nitrate or ammonium, a process regulated by transporters located in root epidermal hairs and root cortical and endodermal cells (Tegeder, 2014). However, some plant species are also able to take up organic N in the form of amino acids, depending on environmental and soil conditions (Nasholm et al., 2009; Tegeder and Rentsch, 2010; Bloom, 2015). The location of N assimilation, or the conversion of inorganic N into amino acids, varies among plant species (Masclaux-Daubresse et al., 2010). Nitrate taken up by the roots is primarily transported to the shoot prior to assimilation, while ammonium, due its toxic nature, is assimilated after uptake in the roots (Tegeder and Masclaux-Daubresse, 2018).

Transport of amino acids from the roots to the above ground areas of the plant occurs through the xylem, while translocation between source and sink organs occurs via the phloem (Tegeder and Masclaux-Daubresse, 2018) (**Figure 1A**). Once formed in

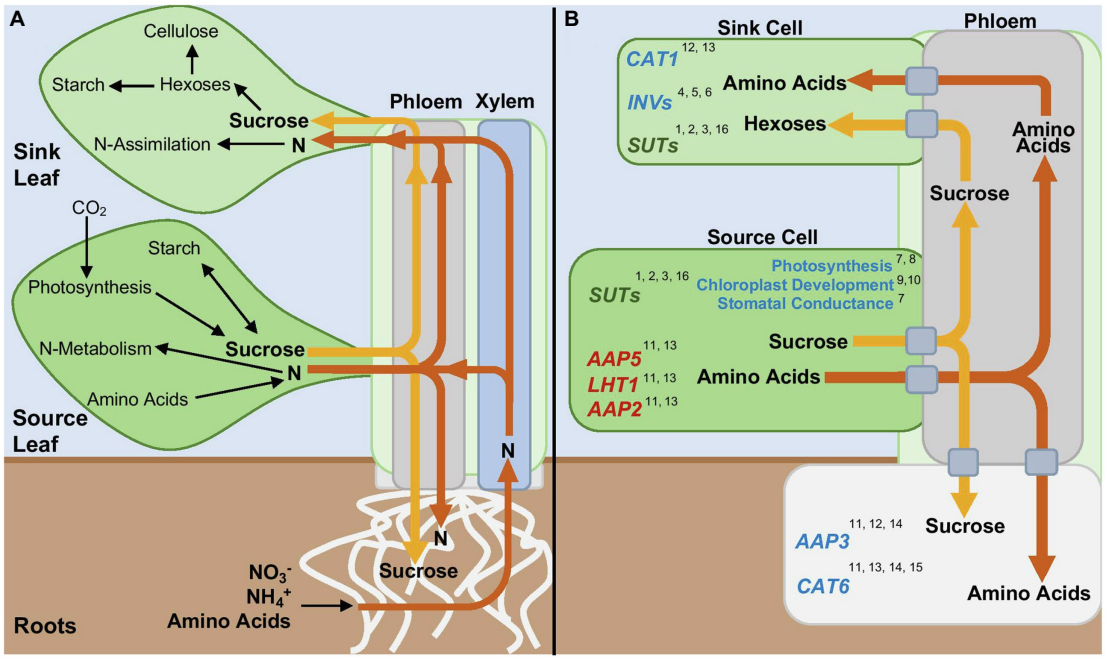


FIGURE 1 | Source-Sink Relationships in Plants. **(A)** Whole plant movement of nitrogen containing compounds (N, orange arrows) and sugars (yellow arrows) between source and sink tissues. **(B)** Regulation by cytokinin of specific enzymes, transporters, and processes involved in source-sink relationships. Blue and red symbolize positive or negative regulation by cytokinin, respectively. Green symbolizes both a positive and negative regulation by cytokinin. Numbers correspond to references. References: 1. Harms et al., 1994; 2. Ninan et al., 2019; 3. Song et al., 2015; 4. Ehness and Roitsch, 1997; 5. Godt and Roitsch, 1997; 6. Yang et al., 2014; 7. Chernyad'ev, 2000; 8. Ahanger et al., 2018; 9. Boasson and Laetsch, 1969; 10. Okazaki et al., 2009; 11. Brenner et al., 2005; 12. Lee et al., 2007; 13. Kiba et al., 2011; 14. Kiba et al., 2005; 15. Yokoyama et al., 2007; and 16. Jian et al., 2016.

source leaves or in roots, amino acids are loaded into the phloem and then unloaded into sinks tissues by amino acid transporters (Okumoto et al., 2004; Tegeder and Hammes, 2018). Also relevant to amino acid allocation is the process of plant senescence. During senescence leaf proteins are degraded, providing a large quantity of amino acids that is used for growth in other organs, a process known as amino acid remobilization. Amino acid remobilization also occurs through the action of amino acid transporters. In both Arabidopsis and *Brassica napus*, it has been shown through ^{15}N tracing that senescing leaves are the primary source of N provided to sink tissues during the late vegetative phase or to flowers and seeds during the reproductive phase (Malagoli et al., 2005; Diaz et al., 2008; Lemaitre et al., 2008).

CYTOKININ REGULATION OF SOURCE-SINK RELATIONSHIPS: EFFECT ON PHOTOSYNTHESES AND SUCROSE TRANSPORT

In many plant species, cytokinins positively affect photosynthetic rates (reviewed in Cherniad'ev, 2000). This effect is associated with increases in stomatal conductance and gas exchange, leading to higher photosynthetic rates and sucrose production (Ahanger et al., 2018, 2020). Cytokinins have also long been associated with an increase in chloroplast number per cell (Boasson and Laetsch, 1969), a process that has been coupled to the anti-senescence activity of this hormone. In Arabidopsis, this increase in chloroplast number per cell is facilitated by the transcriptional regulation of components of the chloroplast division machinery, which is mediated by the cytokinin-regulated transcription factor CYTOKININ RESPONSE FACTOR 2 (CRF2) (Okazaki et al., 2009). Because the number of chloroplasts within a cell can affect overall photosynthetic rates (Austin II and Webber, 2005; Xiong et al., 2017), cytokinin regulation of chloroplast number and their development may have a role in regulating source activity and strength and the availability of photoassimilates.

The photosynthetic activity of source tissues also changes, depending on the demand for photoassimilates by sinks (Paul and Foyer, 2001; Sonnewald and Fernie, 2018). When source leaves are shaded or removed by defoliation, the remaining source leaves display an increase in their rate of photosynthesis, compensating for the removed/shaded source leaves, responding to the rate of utilization of carbohydrates in sinks (Thorne and Koller, 1974; Peet and Kramer, 1980; McCormick et al., 2006). A study in tomato provided evidence that endogenous cytokinin levels could be responsible for altering the response of source leaves following defoliation. After defoliation, the increased photosynthesis levels observed in the remaining source leaves were positively correlated not only to increased levels of the cytokinin *trans*-zeatin riboside, but also to increased

leaf expansion and decreased levels of sugar export. This study suggests that the increased cytokinin concentration in the source leaves caused higher photosynthetic activity, resulting in sugar production that was used for leaf expansion, instead of transport to sinks, which ultimately increased the strength of the source tissue (Glanz-Idan et al., 2020).

As previously mentioned, one method of sugar uptake in sinks following unloading from the phloem is through the activity of extracellular invertases, located within the cell wall. Extracellular invertases catalyze the hydrolysis of sucrose into glucose and fructose (Ruan, 2014), a process that can influence the sink strength (Ho, 1988; Herbers and Sonnewald, 1998; Sturm and Tang, 1999; Lemoine et al., 2013). This process seems to be regulated by cytokinins: Exogenous application of the cytokinin *trans*-zeatin can increase the expression of extracellular invertases (Godt and Roitsch, 1997; Lara et al., 2004) and in a detached leaf assay, cytokinins can prevent senescence and maintain sink strength through regulation of the activity of extracellular invertases (Lara et al., 2004).

Cytokinins may also have a role in sucrose transport from source to sink organs by regulating the expression of *SWEET* and *SUT/SUC* transporters (**Table 1**). In potato, expression of the sucrose transporter *StSUT1* was shown to be induced in mature leaves following exogenous treatment with the cytokinin benzyladenine (BA) (Harms et al., 1994) and in *Brassica napus* expression of *BnSUT*s and *BnSWEET*s was increased after exogenous application of BA to leaves (Jian et al., 2016). Endogenous levels of cytokinins also regulate *SUC/SUT/SWEET* expression. In peas, the content of several cytokinin species in source leaves is correlated with the increase in expression of genes encoding SWEETs and SUTs (Ninan et al., 2019). Developing fruits and seeds are considered major sink tissues. As seeds develop, the walls of siliques in *B. napus* show an increase in expression of *BnSUTs*, which is also correlated with an increased expression in genes responsible for cytokinin biosynthesis (Song et al., 2015). Although the correlation in the expression of sugar transporters and cytokinin content is not necessarily causative, evidence exists for a functional role of this regulation in sugar transport: Application of the cytokinins BA, kinetin, or *trans*-zeatin to *Chenopodium rubrum* suspension cells did not only lead to increased expression of the hexose transporter genes *CST2* and *CST3*, but also to increased uptake of ^{14}C-labeled glucose from the cell suspension media as compared to untreated cells (Ehness and Roitsch, 1997).

TABLE 1 | Transcriptional regulation by cytokinins of genes encoding invertases, sugar, and amino acid transporters in various plant species.

Species	Gene	Gene description	CK regulation
Carbohydrate-related			
Zea mays	*IVR1*	Vacuolar invertase	+
	IVR2	Vacuolar invertase	+
Chenopodium rubrum	*CIN1*	Extracellular invertase	+
Solanum lycopersicum	*LIN6*	Extracellular invertase	+
Sucrose transport			
Arabidopsis thaliana	*CST2*	Hexose transporter	+
	CST3	Hexose transporter	+
Pisum sativum	*PsSW12*	Sucrose transporter	–
	PsSUT1	Sucrose transporter	–
	PsSUT2	Sucrose transporter	–
Solanum tuberosum	*StSUT1*	Sucrose transporter	+
Brassica napus	*BnSUT1*	Sucrose transporter	+
	BnSUT2	Sucrose transporter	+
	BnSUT3	Sucrose transporter	+
	BnSUT4	Sucrose transporter	+
Amino acid transporter			
Arabidopsis thaliana	*AAP2*	Amino acid transporter	–
	AAP3	Amino acid transporter	+
	AAP5	Amino acid transporter	–
	CAT1	Amino acid transporter	+
	CAT6	Amino acid transporter	+
	LHT1	Amino acid transporter	–
Brassica napus	*BnAAP1*	Amino acid transporter	–
	BnAAP2	Amino acid transporter	–
	BnAAP4	Amino acid transporter	–
	BnAAP5	Amino acid transporter	–
	BnAAP6	Amino acid transporter	–
	BnAAP7	Amino acid transporter	–
	BnAAP8	Amino acid transporter	–
Pisum sativum	*PsAAP3*	Amino acid transporter	–
	PsAAP6a	Amino acid transporter	–
	PsAAP7b	Amino acid transporter	–
Oryza sativa	*OsAAP1*	Amino acid transporter	+
	OsLHT1	Amino acid transporter	+

CYTOKININ REGULATION OF SOURCE-SINK RELATIONSHIPS: EFFECT ON AMINO ACID TRANSPORT

The first evidence of a potential role for cytokinins in source-sink relationships came from studies on the movement of amino acids in response to kinetin application to plants. Mothes and Engelbrecht showed that when kinetin was applied to detached leaves of *Nicotiana rustica*, ^{14}C-labeled glycine migrated to the site of kinetin application (Mothes and Engelbrecht, 1961). A similar experiment in unrooted seedlings of fava beans also showed translocation of ^{14}C-labeled alanine to sites of kinetin application. However, if plants were rooted, ^{14}C-labeled alanine migration to sites of cytokinin application was diminished, with more ^{14}C-labeled alanine being mobilized to roots (Mothes and Engelbrecht, 1963). Given that roots are sites of cytokinin biosynthesis (Miyawaki et al., 2004), these experiments showed a direct link between amino acid mobilization and cytokinin content. A similar effect of cytokinin on amino acid mobilization was shown in other plant species, including monocot species, such as oats (Gunning and Barkley, 1963), as well as beans and maize plants (Leopold and Kawase, 1964). Importantly, non-proteinogenic amino acids, such as α-aminoisobutyric acid, are also mobilized to sites of cytokinin application, indicating that the effect of cytokinin is not due to an increased need of amino acids for protein synthesis, but on amino acid translocation *per se* (Mothes and Engelbrecht, 1961).

The relationship between cytokinin and amino transporters has been examined mostly at the level of regulation of gene expression of amino acid transporter genes, such as those from

the family amino acid permeases (AAP), lysine and histidine transporters (LHT), and cationic amino acid transporters (CAT). Application of cytokinin increases the expression of *AAP3* (Brenner et al., 2005; Kiba et al., 2005), *CAT1* (Kiba et al., 2005), and *CAT6* (Brenner et al., 2005; Kiba et al., 2005; Yokoyama et al., 2007), and decreases expression of *AAP2, AAP5,* and *LHT1* (Brenner et al., 2005; Kiba et al., 2011; **Figure 1B** and **Table 1**). Further, transgenic plants with reduced cytokinin signaling display decreased expression of *CAT1* and *AAP3* (Lee et al., 2007).

The different effects that cytokinins have on the levels of expression of genes encoding amino acids transporters are likely explained by the differences in the ability of these transporters in facilitating the movement of specific amino acids, as well as their distinct expression patterns in different tissues within plants. In general, those that are upregulated by cytokinin tend to be expressed in sink organs, such as roots and flowers (Okumoto et al., 2004; Su et al., 2004; Tegeder et al., 2011), and some of them, such as *CAT6,* which is expressed in root tips, have been shown genetically to function in supplying sink cells with amino acids (Hammes et al., 2006). *AAP2, AAP5,* and *LHT1,* on the other hand, are downregulated by cytokinins, and their function and expression patterns seem to be associated with phloem loading in sources. *aap2* mutants display reduced amino acid content in the phloem, thus suggesting a function in phloem loading (Zhang et al., 2010). *AAP5* expression is observed in source leaves, but not sink leaves (Fischer et al., 1995, 2002). Similarly, *LHT1* expression is observed mostly in source organs and is likely involved in the transport of amino acids between mesophyll cells and the xylem (Ehness and Roitsch, 1997; Hirner et al., 2006). Experiments outside of the model plant species Arabidopsis have also provided evidence of the association between cytokinins and regulation of the expression of genes involved in amino acid transport (Song et al., 2015; Ninan et al., 2019; Zhu et al., 2020). In addition to these observed changes in expression of amino acid transporter genes in response to cytokinins, corresponding changes in amino acid translocation are also observed. A study in wheat showed that application of the cytokinin BA to source leaves dramatically decreases the content of amino acids present in the phloem, thus suggesting a function in decreasing phloem loading (Criado et al., 2009).

Finally, amino acid and sugar metabolism are connected in several ways, including through the non-proteinogenic amino acid γ-amino butyric acid (GABA). GABA is synthesized through the GABA shunt pathway, named as such because it bypasses two steps of the tricarboxylic acid (TCA) cycle that is essential to the catabolism of sugars for cellular respiration (Bouche and Fromm, 2004). GABA production through the GABA shunt results from the decarboxylation of the amino acid glutamate, and GABA catabolism leads to the production of succinate that then enters the TCA cycle. Thus, GABA connects amino acid production and sugar utilization. Cytokinins have not been directly associated with GABA production, but plants with increased levels of the cytokinin *trans*-zeatin accumulate GABA at higher levels and that are correlated to increased drought tolerance (Merewitz et al., 2012).

CYTOKININS AND SOURCE-SINK RELATIONSHIPS IN THE OUTCOME OF PLANT BIOTIC INTERACTIONS

Although cytokinins are broadly known as plant hormones involved in the regulation of plant growth, in the last few decades, their involvement in plant-pathogen interactions has become evident (reviewed in Albrecht and Argueso 2017; Akhtar et al., 2020). Similarly, a growing body of evidence has accumulated that indicates an important role for nutrient partitioning in creating metabolic conditions that favor or restrict pathogen growth in plant hosts. In the paragraphs that follow, we highlight a role for source-sink relationships in plant biotic interactions, with emphasis on plant-pathogen interactions, and suggest a function for the plant hormone cytokinin in the regulation of this process.

MAY I OFFER YOU SOMETHING TO EAT? CYTOKININS AND SOURCE-SINK RELATIONSHIPS IN DISEASE SUSCEPTIBILITY

After successful invasion of the host, plant pathogens use effectors (secreted proteins, secondary metabolites, or nucleic acids of pathogen origin) to colonize the host and create host metabolic conditions that are favorable for pathogens, leading to plant susceptibility. Such metabolic conditions include the manipulation of plant metabolism to feed the growing number of pathogens that starts to multiply on the infected plant tissue. While some examples exist of studies on the importance of source-sink relationships in the association of plants with necrotrophic pathogens (Lemonnier et al., 2014; Veillet et al., 2016), which are those that kill plant host cells for their nutrition, the majority of studies has focused on the association of plants with biotrophic pathogens, given the dependency of such pathogens on living plant cells as their source of nutrients.

The role of cytokinins in increasing plant susceptibility to pathogen attack has been well documented. This effect is most commonly seen when lower concentrations of cytokinins are applied to plants prior to pathogen infection (Babosha, 2009; Argueso et al., 2012; Hann et al., 2014). However, in addition to plants, several other organisms can also produce cytokinins or manipulate cytokinin metabolism and/or signaling in plants, including parasitic plants, insects, and plant-associated microbes (reviewed in Spallek et al., 2018). Such microbes include plant pathogenic microbes, able to cause disease on plants, and also beneficial ones, whose association with plants results in enhanced plant growth and protection from disease. By manipulating cytokinin metabolism and/or signaling in plants, such organisms can also potentially regulate plant susceptibility, through manipulation of host physiology.

For the most part, the majority of interactions involving cytokinin production or manipulation by pathogens involves the creation of sink tissues for pathogen nutrition, accompanied by plant developmental changes, such as galls, tumors, and

knots, which are usually noted as disease symptoms. Such developmental changes are associated with one of the primary functions of cytokinins, namely, cell division. However, as a secondary effect, these regions of cell proliferation and growth in fact create new sink tissues and thus alter the balance of source-sink relationships within the plant. A classic example of a plant pathogen that utilizes biosynthesis of cytokinins to create new sink tissues is *Agrobacterium tumefaciens*, the causal agent of crown gall disease. Agrobacterium cells carry a *Tumor-inducing* (*Ti*) plasmid containing the cytokinin biosynthesis gene *trans-zeatin synthesizing* (*tzs*), which is inserted into the plant genome to lead to cytokinin biosynthesis in plant cells (Liu and Kado, 1979; Akiyoshi et al., 1984, 1987; Kutáček and Rovenská, 1991; Lee et al., 2009; Hwang et al., 2010). Along with bacterial-induced auxin biosynthesis, the induction of cytokinin biosynthesis by Agrobacterium results in cell proliferation and the formation of galls. Metabolites needed for gall tumor growth are then rerouted from host plant source leaves to the crown gall tumor, which becomes a strong sink (reviewed in Gohlke and Deeken, 2014).

Another root gall-forming plant pathogen, the obligate biotroph *Plasmodiophora brassicae,* causes clubroot disease in cruciferous plants. The genome of *P. brassicae* contains two cytokinin biosynthesis genes (Schwelm et al., 2015) that were shown to contribute, albeit in a small manner, to the overall cytokinin content in infected tissue (Malinowski et al., 2016). Infection of Arabidopsis by *P. brassicae* alters carbohydrate metabolism of the host, resulting in increased sugar and starch content at the site of infection (Williams et al., 1968; Evans and Scholes, 1995; Brodmann et al., 2002). This carbohydrate mobilization was suggested to be due to high localized concentrations of cytokinins, which create a carbohydrate sink (Dekhuijzen, 1980) mediated by the sugar transporters SWEET11 and SWEET12 (Walerowski et al., 2018). However, decreased disease symptoms were seen after *P. brassicae* infection of the cytokinin biosynthesis mutant ipt1;3;5;7 indicating that the pathogen-derived cytokinins are not sufficient to create a sink (Malinowski et al., 2016). The gall-forming bacteria, *Rhodococcus fascians,* is also known to produce cytokinins as part of its virulence strategy (Stes et al., 2013). Pea plants infected with *R. fascians* show an increase in chlorophyll content, bacterial produced cytokinins, and endogenous plant-derived cytokinins in infected cotyledons (Depuydt et al., 2008; Dhandapani et al., 2017). Moreover, this is accompanied by an increase in expression of *PsCWINV*, *PsSUT*, and *PsSW* (*SWEET*) sugar transporter genes (Dhandapani et al., 2017), suggesting that during infection cytokinins may play a role in creating and maintaining infection sites as sinks tissues. A similar relationship is seen between Arabidopsis and the cyst nematode *Heterodera schachtii*. Upon invading plant roots, this species of nematode induces the formation of specialized structures named syncytia. *H. schachtii* was shown to produce and secrete cytokinins during infection of plant cells, and silencing of the *HsIPT* gene encoding the nematode cytokinin biosynthetic enzyme led to decreased syncytia size and decreased nematode size (Siddique et al., 2015). Given that syncytia are essential sites for juvenile feeding, these results implicate cytokinin as a nematode factor that is necessary to establish nematode feeding sites as sinks, promoting pathogen growth. Further, Arabidopsis amino acid transporters *AAP3* and *AAP6*, which belong to a class of amino acid transporters known to be transcriptionally regulated by cytokinins (Brenner et al., 2005; Kiba et al., 2005; Lee et al., 2007), are necessary for infection of Arabidopsis plants by the root-knot nematode *Meloidogyne incognita,* indicating that successful colonization is dependent on amino acid transport to the sites of infection (Marella et al., 2013), in a process that may be mediated by cytokinins.

Plant-pathogen associations involving cytokinins can also contribute to changes in source-sink relationships without the activation of cell division to create sinks. Such an effect of cytokinins can be seen in the formation of green islands, small areas of live and green leaf tissue surrounded by yellow, senescing tissue, in plants infected with biotrophic fungi (Bushnell, 1967). Green islands have an increased cytokinin content within the green areas (López-Carbonell et al., 1998), which also display increased rates of photosynthesis in comparison with the surrounding senescing tissue (Walters et al., 2008), as well as increased levels of amino acids, sugars, and starch (Raggi, 1974, 1976; Angra and Mandahar, 1991; Angra-Sharma and Mandahar, 1993). These physiological changes in green islands are reminiscent of cytokinin-mediated changes in source-sink relationships mediated by cell wall invertases (Lara et al., 2004), and likely function to maintain these sites as sinks suitable for biotrophic pathogen growth. *Magnaporthe oryzae,* the rice blast fungus, also has the ability to produce cytokinin (Chanclud et al., 2016). *M. oryzae* mutants in the cytokinin biosynthetic gene *CKS1* have reduced virulence and are impaired in their ability to multiply *in planta,* but not *in vitro*, implicating pathogen nutrition through host-derived mechanisms in the reduced virulence phenotype of the mutant (Chanclud et al., 2016). This cytokinin-dependent virulence was associated with the allocation of sugars and amino acids (namely, aspartate and glutamate) to the sites of infection (Chanclud et al., 2016), thus suggesting a function for cytokinin in acting to change source-sink relationships and nutrient allocation in sites of infection, promoting conditions for pathogen multiplication (**Figure 2**).

On a final note, it is important to mention that beneficial microbes also utilize cytokinins in their association with plants to manipulate source-sink relationships and plant growth. In one of the most well-studied examples, cytokinins are essential for nodule formation during the interaction between Rhizobia bacteria and legume plants. In such interactions, plants redirect photoassimilates, mainly in the form of sucrose, to the bacteria, in exchange for organic nitrogen (Kennedy, 1966; Bergersen and Turner, 1967; Kouchi and Yoneyama, 1984). Root nodules can then be classified as sink organs, which require cytokinin for their formation. In *Medicago truncatula*, this requirement for cytokinins is mediated by the ABC transporter ABCG56, which functions as a cytokinin exporter and is required for nodule formation (Jarzyniak et al., 2021). In addition, plant-derived cytokinins are also needed for the activity of certain volatile organic compounds produced by beneficial rhizobacteria, which induce plant growth. This cytokinin-dependent,

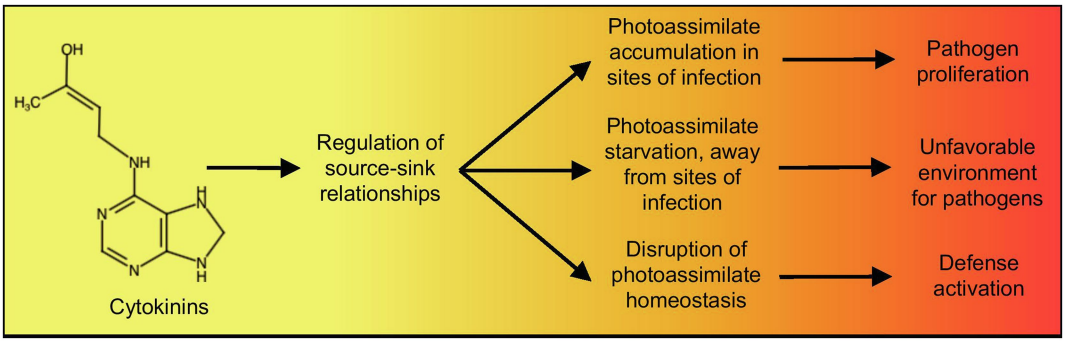


FIGURE 2 | Summary of the proposed effect of cytokinins, through their role in the regulation of source-sink relationships, on the outcome plant-pathogen interactions. Chemical structure created using http://chem-space.com.

rhizobacteria-mediated plant growth is associated with increased photosynthesis and nutrient acquisition, thus linking it to source-sink relationships (Ryu et al., 2003; Zhang et al., 2008; Gutiérez-Luna et al., 2010; Vacheron et al., 2013; Ditengou et al., 2015; Cordovez et al., 2018).

STARVING THE ATTACKER AND SOUNDING THE ALARM: CYTOKININS AND SOURCE-SINK RELATIONSHIPS IN DEFENSE RESPONSES

In addition to a role in creating sink tissues for pathogen nutrition, accumulating evidence also exists for a role of source-sink relationships in defense responses as well. In Arabidopsis, regulation of the sugar transporter STP13 leads to altered susceptibility to pathogens (Yamada et al., 2016). *STP13* is expressed in leaf tissues after infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. Its transport activity was shown to be suppressed via phosphorylation by a protein complex composed of the extracellular immune receptor FLS2 (FLAGELLIN SENSITIVE 2) and its associated kinase BIK1 (BRASSINOSTEROID INSENSITIVE 1). Thus, upon perception of pathogen presence by the FLS2/BIK1 complex, plants diminish STP13 activity, effectively halting sugar transport to the apoplast and preventing pathogen feeding and multiplication (Yamada et al., 2016). STP13 is also important for resistance to the necrotrophic pathogen *Botrytis cinerea*, although it is unknown whether the regulatory mechanisms cited above also apply (Lemonnier et al., 2014).

Other examples of source-sink relationships being modified for defense responses to pathogens, rather than pathogen feeding, include the genes encoding proteinaceous invertase inhibitors. Proteinaceous invertase inhibitors are endogenous plant signals for invertase regulation in plants. In response to *Pseudomonas syringae* pv. *tomato* DC3000 infection, the expression of genes encoding these invertase inhibitors in Arabidopsis is downregulated, a fact that has always been interpreted as manipulation of plant metabolism by the pathogen to increase glucose and fructose availability for pathogen nutrition (Bonfig et al., 2006). However, the activity of these invertase inhibitors has been shown to in fact increase in infected resistant plants, thus functioning as a defense mechanism to prevent the pathogen from cleaving sucrose for its nutritional needs (Bonfig et al., 2010). Finally, the sugar transporters SUT1 and SUT2 in tomato have also been connected to defense responses in plants. *SUT1* and *SUT2* expression are downregulated during the infection of tomato plants with *Candidatus Phytoplasma solani*, an obligate biotrophic bacterial pathogen that inhabits host phloem cells (De Marco et al., 2021). Antisense analyses of *SUT1* and *SUT2* genes in tomato showed that absence of SUT1 and SUT2 function decreases susceptibility of tomato plants to this pathogen, without compromising plant growth, and at the same time increasing the expression of defense genes (De Marco et al., 2021), thus connecting source-sink relationships to defense activation. While a function for cytokinins in the control of source-sink relationships for pathogen nutritional deprivation has not yet been demonstrated, the general importance of this plant hormone in the physiological processes cited above makes it a likely candidate for such regulatory action.

In further agreement with a general role for source-sink relationships in defense is the fact that not only changes in sugar allocation, but also changes in amino acid allocation, lead to altered susceptibility to pathogens. Mutations or overexpression of genes encoding amino acid transporters can also lead to decreased susceptibility to pathogens. This is the case, for example, of the cytokinin-regulated amino acid transporter gene *LHT1*. *lth1* mutants display decreased susceptibility to *Pseudomonas syringae* p.v. *tomato*, as well the hemibiotrophic fungus *Colletotrichum higginsianum* and the biotrophic fungus *Golovinomyces cichoracearum* (Liu et al., 2010). Overexpression of the gene encoding the amino acid transporter *CAT1,* whose expression is also transcriptionally regulated by cytokinins (Kiba et al., 2005), leads to a decrease in susceptibility to *Pseudomonas syringae* p.v. *tomato* (Yang et al., 2014). Most recently, a mutation in the gene encoding the amino acid transporter USUALLY MULTIPLE ACIDS MOVE IN AND OUT 36 (UMAMIT 36) was shown to confer resistance to the oomycete *Phytophthora parasitica* (Pan et al., 2016), and overexpression of *UMAMIT 14* was shown to decrease

susceptibility of Arabidopsis to another oomycete, *Hyaloperonospora arabidopsidis* (Besnard et al., 2021). What is interesting about the examples cited above is that the decreased susceptibility phenotypes of the lines with altered amino acid transporter genes are also accompanied by an increase in the endogenous levels of the defense hormone salicylic acid (SA), and elevated basal levels of the known SA defense marker gene *PATHOGENESIS-RELATED-1* (*PR-1*). Thus, the decrease in pathogen susceptibility is likely not due to altered amino acid transport leading to nutritional deprivation but is in fact due to activation of plant defense pathways and responses.

Amino acids are directly linked to the production of secondary metabolites with important roles in defense, such as glucosinolates and camalexins (derived from tryptophan), SA biosynthesis (derived from phenylalanine), and the biosynthesis of a primer of defense responses, pipecolic acid (derived from lysine). Therefore, it would be reasonable to conclude that these genetic alterations on amino acid transporter genes lead to changes in the cellular amino acid pool, with consequences to the biosynthesis of defense compounds and defense activation. Counterarguments to this amino acid pool hypothesis are several: (i) the fact that the amino acid transporter genes linked to altered pathogen responses are not directly linked to transport of the particular amino acids necessary for the corresponding defense compounds; (ii) that the resistance observed seems to be broad spectrum, and not associated with the effect of a particular defense compound; (iii) that increased levels of GABA, the non-proteinogenic amino acid involved in connecting N and C metabolism, are also associated with abiotic stress tolerance and resistance to pests (Seifikalhor et al., 2019; Tarkowiski et al., 2020); and (iv) and most importantly, that alterations in sugar transport and signaling also seem to activate defense responses in a similar manner to changes in amino acid homeostasis (Gebauer et al., 2017; De Marco et al., 2021). Such counterarguments favor another hypothesis, where cellular metabolic alterations may lead to the activation of defense responses, through a mechanism similar to metabolic priming. Priming is an activated state where plants are able to deploy stronger and faster defenses, resulting in enhanced pathogen protection (reviewed in Mauch-Mani et al., 2017), and the idea of metabolic priming for defense responses has recently been further investigated (Liu et al., 2010; Schwachtje et al., 2018, 2019).

The concept of metabolic priming shares remarkable similarities with the effect of cytokinins on plants. When applied in high concentrations to plants cytokinins can also lead to reduced susceptibility to a broad spectrum of pathogens (reviewed in Akhtar et al., 2020). This is accompanied by the increased production of antimicrobial compounds, such as phytoalexins (Ko et al., 2010; Grosskinsky et al., 2011), and also the production of reactive oxygen species (ROS) and increased defense gene expression, in a manner that is dependent on the defense hormones SA (Choi et al., 2010; Argueso et al., 2012; Naseem et al., 2012) and jasmonic acid (Gupta et al., 2020). Of note, similarly to what happens in defense priming, these responses to cytokinin only happen after pathogen detection. Therefore, cytokinins do not directly activate responses; rather, they trigger physiological conditions that potentiate defense.

The two hypotheses mentioned above, namely, changes in photoassimilate availability altering production of defense compounds or changes in photoassimilate availability altering cellular metabolic stress leading to priming, are not mutually exclusive. Both hypotheses could be parts of an integrated plant defense response involving the regulation of source-sink relationships, coordinated by the plant growth hormone cytokinin (**Figure 2**). In this context, cytokinin levels, through their general effect on source-sink relationships, would serve as a signal for changes in cellular and organismal metabolism that would activate defense. Such a mechanism would likely be beneficial to plants, as it would provide a way to connect defense activation to photoassimilate production, depending on fluctuating environmental conditions.

CONCLUSIONS AND PERSPECTIVES

Because plants are sessile organisms, their ability to effectively respond to environmental change is vital to their survival. To maintain proper growth and development, plants have adapted response mechanisms to regulate photosynthetic ability and photoassimilate partitioning, depending on environmental conditions, such as light intensity, temperature, and water availability. Just like other plant hormones that act on the regulation of cell expansion or cell division, cytokinins have long been associated with promotion of plant growth. In the case of cytokinins, the ability to promote greening and increasing photosynthesis rates is likely also involved in its stimulation of plant growth and yield, as this is centrally linked to the generation of more photoassimilates for plant growth. Further, how these photoassimilates are distributed in the plant are just as important for plant growth and yield, and it is in this aspect that the regulation by cytokinins of source-sink relationships plays a significant role, so much so that genes involved in aspects of cytokinin metabolism and signaling have been a frequent target of crop breeding programs centered on yield improvement (White et al., 2016). Because breeding programs target increased yields in different parts of the plant (seed, fruits, and vegetative organs) depending on the crop, the role of cytokinins in regulating sugar and amino acid transporters with tissue-specific patterns of expression may be of particular interest and importance.

Similarly, response to pathogen attack also requires complex responses by plants. To do so, plants have evolved sophisticated perception and signaling strategies, often mediated by plant hormones, including cytokinins. Timing and degree of defense activation must be tightly controlled, as insufficient defense responses could lead to host death, whereas excessive defense may result in inhibition of plant growth (reviewed in Albrecht and Argueso, 2017). Maintenance of balanced source-sink relationships is therefore vital to sustain growth while ensuring proper defense response against the pathogen. Evidence for the importance of this balanced response comes from the fact that pathogens have developed mechanisms of manipulation of source-sink relationships, in order to obtain nutrients for growth and multiplication. As it is common in the always evolving arms race between plants and pathogens, plants have

also evolved ways to manipulate these source-sink relationships for defense purposes, and there is evidence that both processes may be partly regulated by cytokinins. Given the negative effect of plant pathogens on plant growth and yield, and the importance of photoassimilate partitioning to plant susceptibility and resistance, investigating the role of cytokinin-mediated source-sink relationships in the context of plant-pathogen interactions may provide new avenues not only for yield improvement, but also for pathogen resistance.

AUTHOR CONTRIBUTIONS

KM wrote the manuscript and prepared the figures. CA wrote and edited the manuscript and edited the figures. DB edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the grants COL00781 and 1023089 from the United States Department of Agriculture (USDA) and the National Institute of Food and Agriculture (NIFA).

ACKNOWLEDGMENTS

We thank Dr. Bettina Broeckling for comments on the manuscript and Dr. Karl Ravet for help with figure preparation.

REFERENCES

Ahanger, M. A., Alyemeni, M. N., Wijaya, L., Alamri, S. A., Alam, P., Ashraf, M., et al. (2018). Potential of exogenously sourced kinetin in protecting *Solanum lycopersicum* from NaCl-induced oxidative stress through up-regulation of the antioxidant system, ascorbate-glutathione cycle and glyoxalase system. *PLoS One* 13:9. doi: 10.1371/journal.pone.0202175

Ahanger, M. A., Aziz, U., Sahli, A. A., Alyemeni, M. N., and Ahmad, P. (2020). Combined kinetin and spermidine treatments ameliorate growth and photosynthetic inhibition in *Vigna angularis* by up-regulating antioxidant and nitrogen metabolism under cadmium stress. *Biomol. Ther.* 10:147. doi: 10.3390/biom10010147

Akhtar, S. S., Mekureyaw, M. F., Pandey, C., and Roitsch, T. (2020). Role of cytokinins for interactions of plants with microbial pathogens and pest insects. *Front. Plant Sci.* 10:6. doi: 10.3389/fpls.2019.01777

Akiyoshi, D. E., Klee, H., Amasino, R. M., Nester, E. W., and Gordon, M. P. (1984). T-DNA of *Agrobacterium-tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* 81, 5994–5998. doi: 10.1073/pnas.81.19.5994

Akiyoshi, D. E., Regier, D. A., and Gordon, M. P. (1987). Cytokinin production by *Agrobacterium* and *Pseudomonas* spp. *J. Bacteriol.* 169, 4242–4248. doi: 10.1128/jb.169.9.4242-4248.1987

Albrecht, T., and Argueso, C. T. (2017). Should I fight or should I grow now? The role of cytokinins in plant growth and immunity and in the growth-defence trade-off. *Ann. Bot.* 119, 725–735. doi: 10.1093/aob/mcw211

Angra, R., and Mandahar, C. L. (1991). Pathogenesis of barley leaves by *Helminthosporium teres* I: green island formation and the possible involvement of cytokinins. *Mycopathologia* 114, 21–27. doi: 10.1007/BF00436687

Angra-Sharma, R., and Mandahar, C. L. (1993). Involvement of carbohydrates and cytokinins in pathogenicity of *Helminthosporium carbonum*. *Mycopathologia* 121, 91–99. doi: 10.1007/BF01103576

Argueso, C. T., Ferreira, F. J., Epple, P., To J. PHutchison, C. E., Schaller, G. E., et al. (2012). Two-component elements mediate interactions between cytokinin and salicylic acid in plant immunity. *PLoS Genet.* 8:1. doi: 10.1371/journal.pgen.1002448

Argueso, C. T., Ferreira, F. J., and Kieber, J. J. (2009). Environmental perception avenues: the interaction of cytokinin and environmental response pathways. *Plant Cell Environ.* 32, 1147–1160. doi: 10.1111/j.1365-3040.2009.01940.x

Austin, J. II, and Webber, A. N. (2005). Photosynthesis in *Arabidopsis thaliana* mutants with reduced chloroplast number. *Photosynth. Res.* 85, 373–384. doi: 10.1007/s11120-005-7708-x

Babosha, A. V. (2009). Regulation of resistance and susceptibility in wheat-powdery mildew pathosystem with exogenous cytokinins. *J. Plant Physiol.* 166, 1892–1903. doi: 10.1016/j.jplph.2009.05.014

Bergersen, F. J., and Turner, G. L. (1967). Nitrogen fixation by the bacteriod fraction of breis of soybean root nodules. *Biochim. Biophys. Acta Biochim Biophys Acta.* 141, 507–515. doi: 10.1016/0304-4165(67)90179-1

Besnard, J., Sonawala, U., Maharjan, B., Collakova, E., Finlayson, S. A., Pilot, G., et al. (2021). Increased expression of UMAMIT amino acid transporters results in activation of salicylic acid dependent stress response. *Front. Plant Sci.* 11:606386. doi: 10.3389/fpls.2020.606386

Bloom, A. J. (2015). The increasing importance of distinguishing among plant nitrogen sources. *Curr. Opin. Plant Biol.* 25, 10–16. doi: 10.1016/j.pbi.2015.03.002

Boasson, R., and Laetsch, W. M. (1969). Chloroplast replication and growth in tobacco. *Science* 166, 749–751. doi: 10.1126/science.166.3906.749

Bonfig, K. B., Gabler, A., Simon, U. K., Luschin-Ebengreuth, N., Hatz, M., Berger, S., et al. (2010). Post-translational derepression of invertase activity in source leaves via down-regulation of invertase inhibitor expression is part of the plant defense response. *Mol. Plant* 3, 1037–1048. doi: 10.1093/mp/ssq053

Bonfig, K. B., Schreiber, U., Gabler, A., Roitsch, T., and Berger, S. (2006). Infection with virulent and avirulent *P. syringae* strains differentially affects photosynthesis and sink metabolism in *Arabidopsis* leaves. *Planta* 225, 1–12. doi: 10.1007/s00425-006-0303-3

Bouche, N., and Fromm, H. (2004). GABA in plants: just a metabolite? *Trends Plant Sci.* 9, 100–115. doi: 10.1016/j.tplants.2004.01.006

Braun, D. M., Wang, L., and Ruan, Y. L. (2014). Understanding and manipulating sucrose phloem loading, unloading, metabolism, and signalling to enhance crop yield and food security. *J. Exp. Biol.* 65, 1713–1735. doi: 10.1093/jxb/ert416

Brenner, W. G., Romanov, G. A., Kollmer, I., Burkle, L., and Schmulling, T. (2005). Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. *Plant J.* 44, 314–333. doi: 10.1111/j.1365-313X.2005.02530.x

Brodmann, A., Schuller, A., Ludwig-Miller, J., Aeschbacher, R. A., Wiemken, A., Boller, T., et al. (2002). Induction of trehalase in *Arabidopsis* plants infected with the trehalose-producing pathogen *Plasmodiophora brassicae*. *Mol. Plant-Microbe Interact.* 15, 693–700. doi: 10.1094/MPMI.2002.15.7.693

Bush, D. R. (1993). Proton-coupled sugar and amino acid transporters in plants. *Ann. Rev. Plant. Physiol. Plant. Mol. Biol.* 44, 513–542. doi: 10.1146/annurev.pp.44.060193.002501

Bush, D. R. (1999). "Amino acid transport," in *Plant Amino Acids: Biochemistry and Biotechnology*. ed. B. K. Singh (NY: Marcel Dekker), 305–318.

Bush, D. R. (2020). Identifying the pathways that control resource allocation in higher plants. *Proc. Natl. Acad. Sci. U. S. A.* 117, 8669–8671. doi: 10.1073/pnas.2002581117

Bushnell, W. R. (1967). "The dynamic role of molecular constituents in plant-parasite interactions," in *Symptom Development in Mildewed and Rusted Tissue*. eds. C. J. Mirocha and I. Uritani (St. Paul, MN: Bruce Publishing Company), 21–39.

Chanclud, E., Kisiala, A., Emery, N. R., Chalvon, V., Ducasse, A., Romiti-Michel, C., et al. (2016). Cytokinin production by the rice blast fungus is a pivotal requirement for full virulence. *PLoS Path.* 12:2. doi: 10.1371/journal.ppat.1005457

Chen, L. Q., Qu, X. Q., Hou, B. H., Sosso, D., Osorio, S., Fernie, A. R., et al. (2012). Sucrose efflux by SWEET proteins as a key step for pholem transport. *Science* 335, 207–211. doi: 10.1126/science.1213351

Cherniad'ev, I. (2000). Ontogenetic changes in the photosynthetic apparatus and effects on cytokinins. *Appl. Biochem. Microbiol.* 36, 611–625. doi: 10.1023/A:1026628119243

Choi, J., Huh, S. U., Kojima, M., Sakakibara, H., Paek, K. H., and Hwang, I. (2010). The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in *Arabidopsis*. *Dev. Cell* 19, 284–295. doi: 10.1016/j.devcel.2010.07.011

Cordovez, V., Schop, S., Hordijk, K., Dupré de Boulois, H., Coppens, F., Hanssen, I., et al. (2018). Priming of plant growth promotion by volatiles of root-associated *Microbacterium* spp. *Appl. Environ. Microbiol.* 84:22. doi: 10.1128/AEM.01865-18

Cortleven, A., Leuendorf, J. E., Frank, M., Pezzetta, D., Bolt, S., and Schmulling, T. (2019). Cytokinin action in response to abiotic and biotic stresses in plants. *Plant Cell Environ.* 42, 998–1018. doi: 10.1111/pce.13494

Criado, M. V., Caputo, C., Roberts, I. N., Castro, M. A., and Barneix, A. J. (2009). Cytokinin-induced changes of nitrogen remobilization and chloroplast ultrastructure in wheat (*Triticum aestivum*). *J. Plant Physiol.* 166, 1775–1785. doi: 10.1016/j.jplph.2009.05.007

De Marco, F., Batailler, B., Thorpe, M. R., Razan, F., Le Hir, R., Vilaine, F., et al. (2021). Involvement of SUT1 and SUT2 sugar transporters in the impairment of sugar transport and changes in phloem exudate contents in *Phytoplasma*-infected plants. *Int. J. Mol. Sci.* 22:2. doi: 10.3390/ijms22020745

Dekhuijzen, H. M. (1980). The occurrence of free and bound cytokinins in clubroots and *Plasmodiophora brassicae*. *Physiol. Plant.* 49, 169–176. doi: 10.1111/j.1399-3054.1980.tb02647.x

Depuydt, S., Dolezal, K., Van Lijsebettens, M., Moritz, T., Holsters, M., and Vereecke, D. (2008). Modulation of the hormone setting by *Rhodococcus fascians* results in ectopic KNOX activation in *Arabidopsis*. *Plant Physiol.* 146, 1267–1281. doi: 10.1104/pp.107.113969

Dhandapani, P., Song, J. C., Novak, O., and Jameson, P. E. (2017). Infection by *Rhodococcus fascians* maintains cotyledons as a sink tissue for the pathogen. *Ann. Bot.* 119, 841–852. doi: 10.1093/aob/mcw202

Diaz, C., Lemaitre, T., Christ, A., Azzopardi, M., Kato, Y., Sato, F., et al. (2008). Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in *Arabidopsis* under low nitrogen nutrition. *Plant Physiol.* 147, 1437–1449. doi: 10.1104/pp.108.119040

Ditengou, F. A., Müller, A., Rosenkranz, M., Felten, J., Lasok, H., Miloradovic van Doorn, M., et al. (2015). Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nat. Commun.* 6:6279. doi: 10.1038/ncomms7279

Ehness, R., and Roitsch, T. (1997). Cooordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium rubrum* by cytokinins. *Plant J.* 11, 539–548. doi: 10.1046/j.1365-313x.1997.11030539.x

Evans, J. L., and Scholes, J. D. (1995). How does clubroot alter the regulation of carbon metabolism in its host? *Asp. Appl. Biol.* 42, 125–132.

Fife, J. M., Price, C., and Fife, D. C. (1962). Some properties of phloem exudate collected from root of sugar beet. *Plant Physiol.* 37, 791–792. doi: 10.1104/pp.37.6.791

Fischer, W.-N., Kwart, M., Hummel, S., and Frommer, W. B. (1995). Substrate specificity and expression profile of amino acid transporters (AAPs) in *Arabidopsis*. *J. Biol. Chem.* 270, 16315–16320. doi: 10.1074/jbc.270.27.16315

Fischer, W.-N., Loo, D. D. F., Koch, W., Ludewig, U., Borrer, K. J., Tegeder, M., et al. (2002). Low and high affinity amino acid H+-cotransporters for cellular import of neutral and charged amino acids. *Plant J.* 29, 717–731. doi: 10.1046/j.1365-313X.2002.01248.x

Gebauer, P., Korn, M., Engelsdorf, T., Sonnewald, U., Koch, C., and Voll, L. M. (2017). Sugar accumulation in leaves of *Arabidopsis sweet11/sweet12* double mutants enhances priming of the salicylic acid-mediated defense response. *Front. Plant Sci.* 8:1378. doi: 10.3389/fpls.2017.01378

Geiger, D. R., and Sheigh, W.-J. (1993). Sink strength: learning to measure, measuring to learn. *Plant Cell Environ.* 16, 1017–1018. doi: 10.1111/j.1365-3040.1996.tb02048.x

Glanz-Idan, N., Tarkowski, P., Tureckova, V., and Wolf, S. (2020). Root-shoot communication in tomato plants: cytokinin as a signal molecule modulating leaf photosynthetic activity. *J. Exp. Bot.* 71, 247–257. doi: 10.1093/jxb/erz399

Godt, D. E., and Roitsch, T. (1997). Regulation and tissue-specific distribution of mRNAs for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. *Plant Physiol.* 115, 273–282. doi: 10.1104/pp.115.1.273

Gohlke, J., and Deeken, R. (2014). Plant responses to *Agrobacterium tumefaciens* and crown gall development. *Front. Plant Sci.* 5:155. doi: 10.3389/fpls.2014.00155

Grosskinsky, D. K., Naseem, M., Abdelmohsen, U. R., Plickert, N., Engelke, T., Griebel, T., et al. (2011). Cytokinins mediate resistance against *Pseudomonas syringae* in tobacco through increased antimicrobial phytoalexin synthesis independent of salicylic acid signaling. *Plant Physiol.* 157, 815–830. doi: 10.1104/pp.111.182931

Gunning, B. E. S., and Barkley, W. K. (1963). Kinin-induced directed transport and senescence in detached oat leaves. *Nature* 199, 262–265. doi: 10.1038/199262a0

Gupta, R., Pizarro, L., Leibman-Markus, M., Marash, I., and Bar, M. (2020). Cytokinin response induces immunity and fungal pathogen resistance, and modulates trafficking of the PRR LeEIX2 in tomato. *Mol. Plant Pathol.* 21, 1287–1306. doi: 10.1111/mpp.12978

Gutiérez-Luna, F. M., López-Bucio, J., Altamiramo-Hernández, J., Valencia-Cantero, E., Reyes de la Cruz, H., and Macías-Rodríguez, L. (2010). Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. *Symbiosis* 51, 75–83. doi: 10.1007/s13199-010-0066-2

Hammes, U. Z., Nielsen, E., Honaas, L. A., Taylor, C. G., and Schachtman, D. P. (2006). AtCAT6, a sink-tissue-localized transporter for essential amino acids in *Arabidopsis*. *Plant J.* 48, 414–426. doi: 10.1111/j.1365-313X.2006.02880.x

Hann, D. R., Dominguez-Ferreras, A., Motyka, V., Dobrev, P. I., Schornack, S., Jehle, A., et al. (2014). The *Pseudomonas* type III effector HopQ1 activates cytokinin signaling and interferes with plant innate immunity. *New Phytol.* 201, 585–598. doi: 10.1111/nph.12544

Harms, K. V. W. R., Schulz, B., and Frommer, W. B. (1994). Isolation and characterization of P-type H+-ATPase genes from potato. *Plant Mol. Biol.* 26, 979–988. doi: 10.1007/BF00028864

Hayes, M. A., Davies, C., and Dry, I. B. (2007). Isolation, functional characterization, and expression analysis of grapevine (*Vitis vinifera* L.) hexose transporters: differential roles in sink and source tissues. *J. Exp. Biol.* 58, 1985–1997. doi: 10.1093/jxb/erm061

Herbers, K., and Sonnewald, U. (1998). Molecular determinants of sink strength. *Curr. Opin. Plant Biol.* 1, 207–216. doi: 10.1016/S1369-5266(98)80106-4

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., et al. (2006). *Arabidopsis* LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *Plant Cell* 18, 1931–1946. doi: 10.1105/tpc.106.041012

Ho, L. C. (1988). Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39, 355–378. doi: 10.1146/annurev.pp.39.060188.002035

Hwang, H. H., Wang, M. H., Lee, Y. L., Tsai, Y. L., Li, Y. H., Yang, F. J., et al. (2010). *Agrobacterium*-produced and exogenous cytokinin-modulated *Agrobacterium*-mediated plant transformation. *Mol. Plant Pathol.* 11, 677–690. doi: 10.1111/j.1364-3703.2010.00637.x

Jarzyniak, K., Banasiak, J., Jamruszka, T., Pawela, A., Di Donato, M., Novák, O., et al. (2021). Early stages of legume–rhizobia symbiosis are controlled by ABCG-mediated transport of active cytokinins. *Nat. Plants* 7, 428–436. doi: 10.1038/s41477-021-00873-6

Jian, H., Lu, K., Yang, B., Wang, T., Zhang, L., Zhang, A., et al. (2016). Genome-wide analysis and expression profiling of the SUC and SWEET gene families of sucrose transporters in oilseed rape (*Brassica napus* L.). *Front. Plant Sci.* 7:1464. doi: 10.3389/fpls.2016.01464

Kalt-Torres, W., Kerr, P. S., Usuda, H., and Huber, S. C. (1987). Diurnal changes in maize leaf photosynthesis. *Plant Physiol.* 83, 294–298e. doi: 10.1104/pp.83.2.294

Kennedy, I. R. (1966). Primary products of symbiotic nitrogen fixation II. Pulse-labelling of serradella nodules with 15N2. *Acta. Biochim. Biophys. Acta.* 130, 295–303. doi: 10.1016/0304-4165(66)90224-8

Kiba, T., Kudo, T., Kojima, M., and Sakakibara, H. (2011). Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. *J. Exp. Bot.* 62, 1399–1409. doi: 10.1093/jxb/erq410

Kiba, T., Naitou, T., Koizumi, N., Yamashino, T., Sakakibara, H., and Mizuno, T. (2005). Combinatorial microarray analysis revealing *Arabidopsis* genes implicated in cytokinin responses through the His->Asp phosphorelay circuitry. *Plant Cell Physiol.* 46, 339–355. doi: 10.1093/pcp/pci033

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:dev149344. doi: 10.1242/dev.149344

Ko, K. W., Okada, K., Koga, J., Jikumaru, Y., Nojiri, H., and Yamane, H. (2010). Effects of cytokinin on production of diterpenoid phytoalexins in rice. *J. Pestic. Sci.* 35, 412–418. doi: 10.1584/jpestics.G09-63

Kouchi, H., and Yoneyama, T. (1984). Dynamics of carbon photosynthetically assimilated in nodulated soy bean plants under steady-state conditions. 2. The incorporation of 13C into carbohydrates, organic acids, amino acids, and some storage compounds. *Ann. Bot.* 53, 883–896. doi: 10.1093/oxfordjournals.aob.a086758

Kutáček, M., and Rovenská, J. (1991). Auxin synthesis in *Agrobacterium-tumefaciens* and *A. tumefaciens*-transformed plant-tissue. *Plant Growth Regul.* 10, 313–327. doi: 10.1007/BF00024591

Lara, M. E. B., Garcia, M. C. G., Fatima, T., Ehness, R., Lee, T. K., Proels, R., et al. (2004). Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. *Plant Cell* 16, 1276–1287. doi: 10.1105/tpc.018929

Lee, C. W., Efetova, M., Englemann, J. C., Kramell, R., Wasternack, C., Ludwig-Miller, J., et al. (2009). *Agrobacterium tumefaciens* promotes tumor induction by modulating pathogen defense in *Arabidopsis thaliana*. *Plant Cell* 21, 2948–2962. doi: 10.1105/tpc.108.064576

Lee, D. J., Park, J. Y., Ku, S. J., Ha, Y. M., Kim, S., Kim, M. D., et al. (2007). Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. *Mol. Gen. Genomics.* 277, 115–137. doi: 10.1007/s00438-006-0177-x

Lemaitre, T., Gaufichon, L., Boutet-Mercey, S., Christ, A., and Masclaux-Daubresse, C. (2008). Enzymatic and metabolic diagnostic of nitrogen deficiency in *Arabidopsis thaliana* Wassileskija accession. *Plant Cell Physiol.* 49, 1056–1065. doi: 10.1093/pcp/pcn081

Lemoine, R., La Camera, S., Atanassova, R., Deedaldeechamp, F., Allario, T., Pourtau, N., et al. (2013). Source-to-sink transport of sugar and regulation by environmental factors. *Front. Plant Sci.* 4:272. doi: 10.3389/fpls.2013.00272

Lemonnier, P., Gaillard, C., Veillet, F., Verbeke, J., Lemoine, R., Coutos-Thevenot, P., et al. (2014). Expression of *Arabidopsis* sugar transport protein STP13 differentially affects glucose transport activity and basal resistance to *Botrytis cinerea*. *Plant Mol. Biol.* 85, 473–484. doi: 10.1007/s11103-014-0198-5

Leopold, A. C., and Kawase, M. (1964). Benzladenine effects on bean leaf growth and senscence. *Am. J. Bot.* 51, 294–298. doi: 10.1002/j.1537-2197.1964.tb06633.x

Liu, G., Ji, Y., Bhuiyan, N. H., Pilot, G., Selvaraj, G., Zou, J., et al. (2010). Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in *Arabidopsis*. *Plant Cell* 22, 3845–3863. doi: 10.1105/tpc.110.079392

Liu, S. T., and Kado, C. I. (1979). Indoleacetic acid production: a plasmid function of *Agrobacterium tumefaciens* C58. *Biochem. Biophys. Res. Commun.* 90, 171–178. doi: 10.1016/0006-291X(79)91605-X

López-Carbonell, M. A., and Nadal, M. (1998). Change in cell ultrastructure and zeatin riboside concentrations in *Hedera helix*, *Pelargonium zonale*, *Prunus avium*, and *Rubus ulmifolius* leaves infected by fungi. *Plant Dis.* 82, 914–918. doi: 10.1094/PDIS.1998.82.8.914

Malagoli, P., Laine, P., Rossato, L., and Ourry, A. (2005). Dynamics of nitrogen uptake and mobilization in field-grown winter oilseed rape (*Brassica napus*) from stem extension to harvest. I. Global N flows between vegetative and reproductive tissues in relation to leaf fall and their residual N. *Ann. Bot.* 95, 853–861. doi: 10.1093/aob/mci091

Malinowski, R., Novak, O., Borhan, M. H., Spichal, L., Strnad, M., and Rolfe, S. A. (2016). The role of cytokinins in clubroot disease. *Eur. J. Plant Pathol.* 145, 543–557. doi: 10.1007/s10658-015-0845-y

Marella, H. H., Nielsen, E., Schachtman, D. P., and Taylor, C. G. (2013). The amino acid permeases AAP3 and AAP6 are involved in root-knot nematode parasitism of *Arabidopsis*. *Mol. Plant-Microbe Interact.* 26, 44–54. doi: 10.1094/MPMI-05-12-0123-FI

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L., and Suzuki, A. (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* 105, 1141–1157. doi: 10.1093/aob/mcq028

Mauch-Mani, B., Baccelli, I., Luna, E., and Flors, V. (2017). Defense priming: an adaptive part of induced resistance. *Annu. Rev. Plant Biol.* 68, 485–512. doi: 10.1146/annurev-arplant-042916-041132

McCormick, A. J., Cramer, M. D., and Watt, D. A. (2006). Sink strength regulates photosynthesis in sugarcane. *New Phytol.* 171, 759–770. doi: 10.1111/j.1469-8137.2006.01785.x

Merewitz, E. B., Du, H., Yu, W., Liu, Y., Gianfagna, T., and Huang, B. (2012). Elevated cytokinin content in ipt transgenic creeping bentgrass promotes drought tolerance through regulating metabolite accumulation. *J. Exp. Biol.* 15, 1315–1328. doi: 10.1093/jxb/err372

Miller, C. O., Skoog, F., Okumura, F. S., Von Saltza, M. H., and Strong, F. M. (1956). Isolation, structure and synthesis of kinetin, a substance promoting cell division. *J. Am. Chem. Soc.* 78, 1375–1380. doi: 10.1021/ja01588a032

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant J.* 37, 128–138. doi: 10.1046/j.1365-313X.2003.01945.x

Mok, D. W. S., and Mok, M. C. (2001). Cytokinin Metabolism and Action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 89–118. doi: 10.1146/annurev.arplant.52.1.89

Mothes, K., and Engelbrecht, L. (1961). Kinetin-induced directed transport of substances in excised leaves in the dark. *Phytochemistry* 1, 58–62. doi: 10.1016/S0031-9422(00)82812-5

Mothes, K., and Engelbrecht, L. (1963). On the activity of kinetin-like root factor. *Life Sci.* 2, 852–857. doi: 10.1016/0024-3205(63)90098-5

Naseem, M., Philippi, N., Hussain, A., Wangorsch, G., Ahmed, N., and Dandekar, T. (2012). Integrated systems view on networking by hormones in *Arabidopsis* immunity reveals multiple crosstalk for cytokinin. *Plant Cell* 24, 1793–1814. doi: 10.1105/tpc.112.098335

Nasholm, T., Kielland, K., and Ganeteg, U. (2009). Uptake of organic nitrogen by plants. *New Phytol.* 182, 31–48. doi: 10.1111/j.1469-8137.2008.02751.x

Ninan, A. S., Grant, J., Song, J. C., and Jameson, P. E. (2019). Expression of genes related to sugar and amino acid transport and cytokinin metabolism during leaf development and senescence in *Pisum sativum* L. *Plants* 8:3. doi: 10.3390/plants8030076

Noiraud, N., Maurousset, L., and Lemoine, R. (2001). Transport of polyols in higher plants. *Plant Physiol. Biochem.* 39, 717–728. doi: 10.1016/S0981-9428(01)01292-X

Okazaki, K., Kabeya, Y., Suzuki, K., Mori, T., Ichikawa, T., Matsui, M., et al. (2009). The PLASTID DIVISION1 and 2 components of the chloroplast division machinery determine the rate of chloroplast division in land plant cell differentiation. *Plant Cell* 21, 1769–1780. doi: 10.1105/tpc.109.067785

Okumoto, S., Koch, W., Tegeder, M., Fischer, W. N., Biehl, A., Leister, D., et al. (2004). Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3. *J. Exp. Bot.* 55, 2155–2168. doi: 10.1093/jxb/erh233

Pan, Q. N., Cui, B. M., Deng, F. Y., Quan, J. L., Loake, G. J., and Shan, W. X. (2016). RTP1 encodes a novel endoplasmic reticulum (ER)-localized protein in *Arabidopsis* and negatively regulates resistance against biotrophic pathogens. *New Phytol.* 209, 1641–1654. doi: 10.1111/nph.13707

Paul, M. J., and Foyer, C. H. (2001). Sink regulation of photosynthesis. *J. Exp. Bot.* 52, 1383–1400. doi: 10.1093/jexbot/52.360.1383

Peet, M. M., and Kramer, P. J. (1980). Effects of decreasing source/sink ratio in soybeans on photosynthesis, photorespiration, transpiration, and yield. *Plant Cell Environ.* 2, 201–206. doi: 10.1111/1365-3040.ep11581547

Raggi, V. (1974). Free and protein amino acids in the pustules and surrounding tissues of rusted bean. *Phytopathol. Z.* 81, 289–300. doi: 10.1111/j.1439-0434.1974.tb02804.x

Raggi, V. (1976). Amino acids in mycelium of *Sphaerotheca pannosa* var. *persicae* and in the infected and surrounding tissues of peach leaves. *Phytopathol. Mediterr.* 15, 110–114.

Reinders, A., Sivitz, A. B., and Ward, J. M. (2012). Evolution of plant sucrose uptake transporters. *Front. Plant Sci.* 3:22. doi: 10.3389/fpls.2012.00022

Ruan, Y. L. (2014). Sucrose metabolism: gateway to diverse carbon use and sugar signaling. *Annu. Rev. Plant Biol.* 65, 33–67. doi: 10.1146/annurev-arplant-050213-040251

Ruan, Y. L., Jin, Y., Yang, Y. J., Li, G. J., and Boyer, J. S. (2010). Sugar input, metabolism, and signaling mediated by invertase: roles in development,

yield potential, and response to drought and heat. *Mol. Plant* 3, 942–955. doi: 10.1093/mp/ssq044

Ryu, C.-M., Farag, M. A., Hu, C.-H., Reddy, M. S., Wei, H.-X., Paré, P. W., et al. (2003). Bacterial volatiles promote growth in *Arabidopsis*. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4927–4932. doi: 10.1073/pnas.0730845100

Schwachtje, J., Fischer, A., Erban, A., and Kopka, J. (2018). Primed primary metabolism in systemic leaves: a functional systems analysis. *Sci. Rep.* 8:216. doi: 10.1038/s41598-017-18397-5

Schwachtje, J., Whitcomb, S. J., Firmino, A. A. P., Zuther, E., Hincha, D. K., and Kopka, J. (2019). Induced, imprinted, and primed responses to changing environments: does metabolism store and process information? *Front. Plant Sci.* 10:106. doi: 10.3389/fpls.2019.00106

Schwelm, A., Fogelqvist, J., Knaust, A., Julke, S., Lilja, T., Bonilla-Rosso, G., et al. (2015). The *Plasmodiophora brassicae* genome reveals insights in its life cycle and ancestry of chitin synthases. *Sci. Rep.* 5:11153. doi: 10.1038/srep11153

Seifikalhor, M., Aliniaeifard, S., Hassani, B., Niknam, V., and Lastochkina, O. (2019). Diverse roles of gamma aminobutyric acid in dynamic plant cell responses. *Plant Cell Rep.* 38, 847–867. doi: 10.1007/s00299-019-02396-z

Siddique, S., Radakovic, Z. S., De La Torre, C. M., Chronis, D., Novak, O., Ramireddy, E., et al. (2015). A parasitic nematode releases cytokinin that controls cell division and orchestrates feeding site formation in host plants. *Proc. Natl. Acad. Sci. U. S. A.* 112, 12669–12674. doi: 10.1073/pnas.1503657112

Skoog, F., and Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. Exp. Biol.* 11, 118–130.

Smith, A. M., and Stitt, M. (2007). Coordination of carbon supply and plant growth. *Plant Cell Environ.* 30, 1126–1149. doi: 10.1111/j.1365-3040.2007.01708.x

Song, J., Jiang, L., and Jameson, P. E. (2015). Expression patterns of *Brassica napus* genes implicate IPT, CKX, sucrose transporter, cell wall invertase, and amino acid permease gene family members in leaf, flower, silique, and seed development. *J. Exp. Bot.* 66, 5067–5082. doi: 10.1093/jxb/erv133

Sonnewald, U., and Fernie, A. R. (2018). Next-generation strategies for understanding and influencing source-sink relations in crop plants. *Curr. Opin. Plant Biol.* 43, 63–70. doi:10.1016/j.pbi.2018.01.004, PMID: 29428477

Spallek, T., Gan, P., Kadota, Y., and Shirasu, K. (2018). Same tune, different song - cytokinins as virulence factors in plant-pathogen interactions? *Curr. Opin. Plant Biol.* 44, 82–87. doi: 10.1016/j.pbi.2018.03.002

Stes, E., Francis, I., Petry, I., Dolzblasz, A., Depuydt, S., and Vereechke, D. (2013). The leafy gall syndrome induced by *Rhodococcus fascians*. *FEMS Microbiol. Lett.* 342, 187–195. doi: 10.1111/1574-6968.12119

Sturm, A., and Tang, G.-Q. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends Plant Sci.* 4, 401–407.

Su, Y. H., Frommer, W. B., and Ludewig, U. (2004). Molecular and functional characterization of a family of amino acid transporters from *Arabidopsis*. *Plant Physiol.* 136, 3104–3113. doi: 10.1104/pp.104.045278

Tarkowiski, L., Signorelli, S., and Hofte, M. (2020). Gamma-aminobutyric acid and related amino acids in plant immune responses: emerging mechanisms of action. *Plant Cell Environ.* 43, 1103–1116. doi: 10.1111/pce.13734

Tegeder, M. (2014). Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement. *J. Exp. Biol.* 65, 1865–1878. doi: 10.1093/jxb/eru012

Tegeder, M., and Hammes, U. Z. (2018). The way out and in: phloem loading and unloading of amino acids. *Curr. Opin. Plant Biol.* 43, 16–21. doi: 10.1016/j.pbi.2017.12.002

Tegeder, M., and Masclaux-Daubresse, C. (2018). Source and sink mechanisms of nitrogen transport and use. *New Phytol.* 217, 35–53. doi: 10.1111/nph.14876

Tegeder, M., and Rentsch, D. (2010). Uptake and partitioning of amino acids and peptides. *Mol. Plant* 3, 997–1011. doi: 10.1093/mp/ssq047

Tegeder, M., Rentsch, D., and Patrick, J. W. (2011). "Organic carbon and nitrogen transporters," in *The Plant Plasma Membrane*. eds. A. Murphy, B. Schulz and W. Peer (Berlin, Heidelberg: Springer), 331–352.

Thorne, J. H., and Koller, H. R. (1974). Influence of assimilate demand on photosynthesis, diffusive resistances, translocation, and carbohydrate levels of soybean leaves. *Plant Physiol.* 54, 201–207. doi: 10.1104/pp.54.2.201

Turgeon, R. (1989). The sink-source transition in leaves. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40, 110–138. doi: 10.1146/annurev.pp.40.060189.001003

Vacheron, J., Desbrosses, G., Bouffaud, M.-L., Touraine, B., Moënne-Loccoz, Y., Muller, D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci.* 4:356. doi: 10.3389/fpls.2013.00356

Veillet, F., Gaillard, C., Coutos-Thevenot, P., and La Camera, S. (2016). Targeting the AtCWIN1 gene to explore the role of invertases in sucrose transport in roots and during *Botrytis cinerea* infection. *Front. Plant Sci.* 7:1899. doi: 10.3389/fpls.2016.01899

Walerowski, P., Gündel, A., Yahaya, N., Truman, W., Sobczak, M., Olszak, M., et al. (2018). Clubroot disease stimulates early steps of phloem differentiation and recruits SWEET sucrose transporters within developing galls. *Plant Cell* 30, 3058–3073. doi: 10.1105/tpc.18.00283

Walters, D. R., McRoberts, N., and Fitt, B. D. L. (2008). Are green islands red herrings? Significance of green islands in plant interactions with pathogens and pests. *Biol. Rev.* 83, 79–102. doi: 10.1111/j.1469-185X.2007.00033.x

Wang, C., Liu, Y., Li, S. S., and Han, G. Z. (2015). Insights into the origin and evolution of the plant hormone signaling machinery. *Plant Physiol.* 167, 872–886. doi: 10.1104/pp.114.247403

Weber, H., Borisjuk, L., Heim, U., Sauer, N., and Wobus, U. (1997). A role for sugar transporters during seed development: molecular characterization of a hexose and a sucrose carrier in fava bean seeds. *Plant Cell* 9, 895–908. doi: 10.1105/tpc.9.6.895

White, A. C., Rogers, A., Rees, M., and Osborne, C. P. (2016). How can we make plants grow faster? A source-sink perspective on growth rate. *J. Exp. Bot.* 67, 31–45. doi: 10.1093/jxb/erv447

Williams, P. H., Keen, N. T., Strandberg, J. O., and Mcnabola, S. S. (1968). Metabolite synthesis and degradation during clubroot development in cabbage hypocotyls. *Phytopathology* 58, 921–928.

Xiong, D., Huang, J., Peng, S., and Li, Y. (2017). A few enlarged chloroplasts are less efficient in photosynthesis than a large population of small chloroplasts in *Arabidopsis thaliana*. *Sci. Rep.* 7:5782. doi: 10.1038/s41598-017-06460-0

Yadav, U. P., Ayre, B. G., and Bush, D. R. (2015). Transgenic approaches to altering carbon and nitrogen partitioning in whole plants: assessing the potential to improve crop yields and nutritional quality. *Front. Plant Sci.* 6:275. doi: 10.3389/fpls.2015.00275

Yamada, K., Saijo, Y., Nakagami, H., and Takano, Y. (2016). Regulation of sugar transporter activity for antibacterial defense in *Arabidopsis*. *Science* 354, 1427–1430. doi: 10.1126/science.aah5692

Yang, H., Postel, S., Kemmerling, B., and Ludewig, U. (2014). Altered growth and improved resistance of *Arabidopsis* against *Pseudomonas syringae* by overexpression of the basic amino acid transporter AtCAT1. *Plant Cell Environ.* 37, 1404–1414. doi: 10.1111/pce.12244

Yokoyama, A., Yamashino, T., Amano, Y., Tajima, Y., Imamura, A., Sakakibara, H., et al. (2007). Type-B ARR transcription factors, ARR10 and ARR12, are implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of *Arabidopsis thaliana*. *Plant Cell Physiol.* 48, 84–96. doi: 10.1093/pcp/pcl040

Zhang, L., Tan, Q., Lee, R., Trethewy, A., Lee, Y. H., and Tegeder, M. (2010). Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in *Arabidopsis*. *Plant Cell* 22, 3603–3620. doi: 10.1105/tpc.110.073833

Zhang, C., and Turgeon, R. (2018). Mechanisms of phloem loading. *Curr. Opin. Plant Biol.* 43, 71–75. doi: 10.1016/j.pbi.2018.01.009

Zhang, X. Y., Wang, X. L., Wang, X. F., Xia, G. H., Pan, Q. H., Fan, R. C., et al. (2006). A shift of phloem unloading from symplasmic to apoplasmic pathway is involved in developmental onset of ripening in grape berry. *Plant Physiol.* 142, 220–232. doi: 10.1104/pp.106.081430

Zhang, H., Xie, X., Kim, M.-S., Kornyeyev, D. A., Holaday, S., and Paré, P. W. (2008). Soil bacteria augment *Arabidopsis* photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. *Plant J.* 56, 264–273. doi: 10.1111/j.1365-313X.2008.03593.x

Zhu, K., Zhou, Q., Shen, Y., Yan, J., Xu, Y., Wang, Z., et al. (2020). Agronomic and physiological performance of an indica –japonica rice variety with a high yield and high nitrogen use efficiency. *Crop Sci.* 60, 1556–1568. doi: 10.1002/csc2.20150

Zimmermann, M. H., and Ziegler, H. (1975). Mechanisms of phloem loading. in *Encyclopedia of Plant Physiology.* New series, eds. F.A Loewus and W. Tanner (Berlin, Hiedlberg: Springer), 480–503.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership