RECENT ADVANCES IN UNDERSTANDING PLANT HORMONE TRANSPORTERS

EDITED BY: Markus Geisler

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RECENT ADVANCES IN UNDERSTANDING PLANT HORMONE TRANSPORTERS

Topic Editor:
Markus Geisler, Université de Fribourg, Switzerland

Since the first postulation of auxin function by the Darwins, many other plant hormones have been identified and most of them have been found to be synthesized at different sites from their places of action. Hormone transport and thus the responsible hormone transporters are therefore essential for a precise regulation of plant hormone action, which has been repeatedly supported by severe developmental and physiological phenotypes reported for hormone transporter loss-of-function mutants.

Plant transporters have been shown to be involved in short and long-distance transport of hormones. Short-distance transport between cells seems to be sufficient for a local hormone action in some tissues (such as seeds), which seem to require exporter and importer proteins in adjacent cells as shown for example for abscisic acid. During long distance transport with the transpiration stream or in the phloem, demonstrated for many (but not all) plant hormones (including auxins, abscisic acid, cytokinins, gibberelins, strigolactones, and salicylic acid), transporters are thought to function in loading and unloading processes. Similarly, in cases where long-distance transport is achieved by cell-to-cell transport (such as for auxins), the highly coordinated action of import and export transporters at the contact surfaces of neighboring cells is apparently needed, however, all these processes are far from being understood on the molecular level.

Currently, it appears that many hormones are transported by members of distinct transporter classes, ranging from primary active pumps (that couple hormone translocation to direct ATP hydrolysis), antiporters and symporters (that use the proton motive force to create hormone concentration gradients), and to facilitators. Among those, the ATP-binding cassette (ABC) family and the Nitrate transport1/ Peptide transporter family (NPF) seem to be dominant but currently it is unclear how individual transporters cooperate to achieve a systemic level of transport. Furthermore, in most cases several pairs of importers and exporters are required but how these are correct allocated in order to guarantee the function of a complex hormonal network is unknown.

While remarkable progress has been made on hormone transporter regulation on the transcription and post-transcriptional level for transporters involved in long-distance transport (such as auxin), regulation of transporter trafficking, stability and activity is less understood for other hormones.

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PERSPECTIVE
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Transporter-Mediated Subcellular Distribution in the Metabolism and Signaling of Jasmonates

*Feifei Wang, Guanghui Yu and Pei Liu**

Department of Ecology, College of Resources and Environmental Sciences, China Agricultural University, Beijing, China

Jasmonates (jasmonic acid and its relatives) are a group of oxylipin phytohormones that are implicated in the regulation of a range of developmental processes and responses to environmental stimuli in plants. The biosynthesis of JAs occur sequentially in various subcellular compartments including the chloroplasts, peroxisomes and the cytoplasm. The biologically active jasmonoyl-isoleucine (JA-Ile) activates the core JA signaling in the nucleus by binding with its coreceptor, SCF^{COI1}-JAZ. Five members of a clade of ATP-binding cassette G (ABCG) transporters of *Arabidopsis thaliana* were identified as the candidates of jasmonate transporters (JATs) in yeast cells. Among these JATs, AtJAT1/AtABCG16, has a dual localization in the plasma membrane and nuclear envelop and mediates the efflux of jasmonic acid (JA) across the plasma membrane and influx of JA-Ile into the nucleus. Genetic, cellular and biochemical analyses have demonstrated that AtJAT1/AtABCG16 is crucial for modulating JA-Ile concentration in the nucleus to orchestrate JA signaling. AtJAT1 could also be involved in modulating the biosynthesis of JA-Ile by regulating the distribution of JA and JA-Ile in the cytoplasm and nucleus, which would contribute to the highly dynamic JA signaling. Furthermore, other JAT members are localized in the plasma membrane and possibly in peroxisomes. Characterization of these JATs will provide further insights into a crucial role of transporter-mediated subcellular distribution in the metabolism and signaling of plant hormones, an emerging theme supported by the identification of increasing number of endomembrane-localized transporters.

Keywords: jasmonates, biosynthesis, metabolism, signaling, jasmonate transporters, subcellular distribution, plasma membrane, nuclear envelope

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Markus Geisler,
Université de Fribourg, Switzerland

Reviewed by:
Joelle Sasse Schlaepfer,
Lawrence Berkeley National Laboratory, United States Department of Energy (DOE), United States
Enrique Rojo,
Centro Nacional de Biotecnología (CSIC), Spain

***Correspondence:**
Pei Liu
peiliu@cau.edu.cn

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PARTITION OF THE BIOSYNTHESIS AND SIGNALING OF JASMONATES IN VARIOUS SUBCELLULAR COMPARTMENTS

Jasmonates (jasmonic acid and its relatives, JAs) are a class of oxylipin phytohormones that play important roles in various developmental processes, responses to abiotic and biotic stimuli, in particular immunity against herbivores or pathogen attack (Browse, 2009; Wasternack et al., 2013). JA biosynthesis occurs sequentially in the chloroplasts and peroxisomes, starting from the releasing of α-linolenic acid (18:3) (α-LeA) from galactolipids of chloroplast membranes likely catabolized by a phospholipase1 (PLA_1). The α-LeA is then oxygenated by 13-lipoxygenase (13-LOX) to form (13S)-hydroperoxy-linolenic acid, which is rearranged and cyclized by allene oxide synthase

(AOS) and allene oxide cyclase (AOC) to form 12-oxo-phytodienoic acid (OPDA). OPDA is then translocated to the peroxisomes, where it is converted to JA after reduction by 12-oxophytodienoate reductase (OPR) and three steps of β-oxidation by acyl-CoA-oxidase. JA is then translocated to the cytosol and converted to a number of JA derivatives, among which the biologically active JA conjugate, jasmonoyl-isoleucine (JA-Ile), is formed by jasmonate resistant 1 (JAR1) (Browse, 2009; Fonseca et al., 2009; Gfeller et al., 2010; Song et al., 2011; Wasternack and Hause, 2013). JA-Ile is then bound with its coreceptor SCF^{COI1}-JAZ, targeting the jasmonate ZIM domain (JAZ) transcriptional repressors for ubiquitination and degradation. The degradation of JAZ proteins relieves their repression on the activity of the key transcription factors (e.g., MYC2), activating the core JA signaling pathway (Chini et al., 2007; Thines et al., 2007; Browse, 2009; Sheard et al., 2010). Both the COI1 and JAZ proteins are localized in the nucleus and their nuclear localization was not altered in the mutants of the other interacting partner (Chini et al., 2007; Withers et al., 2012). These findings suggest that the assembling of the SCF^{COI1}-JAZ-JA-Ile receptor-ligand complex occurs in the nucleus, thereby the nuclear entry of JA-Ile is required.

Besides JA-Ile, JA can also be converted to other derivatives by glycosylation, hydroxylation, methyl esterification and sulfonation, which could be involved in modulating the homeostasis of JAs, cross-talking with other signals and/or could act as signaling molecules. JA conjugates such as sugar (e.g., glucose and gentiobiose)-conjugated JAs are likely storage forms of JAs to regulate the activation/deactivation of JA-Ile mediated core JA signaling pathway by modulating the availability of JA in the cytosol. Furthermore, the hydroxylated JA, (-)-12-hydroxyJA (tuberonic acid, TA), (-)-9,10-dihydroJA, and (-)-11,12-dihydroJA (Yoshihara et al., 1989) may also be involved in regulating the availability of cytosolic JA for the biosynthesis of JA-Ile. In addition, oxidation of JA-Ile mediated by wound-inducible cytochromes P450, CYP94C1, and CYP94B3 are involved in the inactivation of JA-Ile (Heitz et al., 2012). Although the cytoplasmic localization of enzymes catabolizing reactions to produce these JA derivatives was only demonstrated experimentally for JAR1, it is generally accepted that these reactions occur in the cytoplasm. JA carboxy methyl transferase (JMT) catalyzing the formation of methyl-JA (MeJA), a volatile involved in plant-plant communication, is also thought to localize in the cytosol (Seo et al., 2001). In addition, the biosynthesis of *cis*-jasmone (CJ) was proposed to occur in the peroxisomes by decarboxylation of JA and act as the disposal pathway of JA due to its high volatility (Koch et al., 1997), though an alternative origin from *iso*-OPDA has been proposed (Dabrowska and Boland, 2007). CJ is critical for indirect defense by attracting the predators/parasites of herbivores (e.g., seven-spot ladybirds as a kairomone) directly as a semiochemical (Birkett et al., 2000) and indirectly as a signaling molecule (Bruce et al., 2008). Through a signaling pathway distinct from JA-Ile, CJ triggers emitting of a mixture of volatile organic compounds (VOCs) critical for beneficial animal attraction (Birkett et al., 2000; Bruce et al., 2008; Matthes et al., 2010). Although more data remain to be furnished, the biosynthesis and signaling of JAs has been shown to partition in distinct subcellular compartments including chloroplasts, peroxisomes, cytosol and nuclei. Therefore, the distribution of JAs in different subcellular compartments would significantly modulate the metabolism and signaling of these JAs.

SUBCELLULAR AND LONG DISTANCE TRANSPORT OF JASMONATES

Information on the intracellular transport of JAs is limited, due to technical difficulties in measuring JAs in different subcellular compartments (Skalicky et al., 2018). The significance of subcellular distribution on the metabolism and signaling of JAs has been revealed by the identification of peroxisomal ATP-binding cassette (ABC) COMATOSE (CTS) transporter. CTS represents the key point for the peroxisomal entry of the substrates of β-oxidation including the key JA precursor, 12-oxophytodienoic acid (OPDA) (Footitt et al., 2007). However, null *cts* alleles do not show the characteristic male sterility of mutants defective in JA biosynthesis or signaling. The *cts* mutants have residual basal levels of JA and can synthesize a modest amount of JA in response to wounding, suggesting that OPDA could also be imported into peroxisomes via passive diffusion or a second importer (Theodoulou et al., 2005). Impaired OPDA import in *cts* mutants leads to elevated levels of this signal molecule in the cytosol, triggering the activation of the distinct OPDA signaling pathway (Dave and Graham, 2012; Maynard et al., 2018). These findings highlight that CTS-mediated distribution of OPDA between the cytosol and peroxisome plays an essential role in regulating the homeostasis and signaling between OPDA and JA.

Besides transport between subcellular compartments, long distance transmission of JAs could play an essential role in triggering wound-induced systemic resistance (WSR) in the distal undamaged leaves. In tomato and tobacco, reciprocal stem-stem grafting between wild type and mutants defective in JA biosynthesis and signaling showed that both the biosynthesis of JA/JA-Ile at the local damaging sites and the capability to perceive JA signal in the distal sites were required, indicating that JAs could act as the mobile wound signal (Li et al., 2002, 2005; Schilmiller and Howe, 2005; Bozorov et al., 2017). Consistently, radiolabeled JAs show that JA (Sato et al., 2009; Bozorov et al., 2017), MeJA (Thorpe et al., 2007), and JA-Ile (Sato et al., 2011) can all translocate from the local damaged to the distal undamaged leaves. Consistent with the observation in the wild tobacco *N. sylvestris* that exogenous radio-labeled JA can translocate from the older leaves to the younger leaves, the long-distance transport of ^{11}C-labeled MeJA was via the phloem pathway in *N. tabacum* (Zhang and Baldwin, 1997). Inconsistent results have been reported on the long-distance transport of radiolabeled JA-Ile (Wang et al., 2008; Sato et al., 2011), however, grafting experiments and biochemical studies indicate that JA, but not JA-Ile, is the most likely candidate of the mobile oxylipin signal (Gasperini et al., 2015; Bozorov et al., 2017). In contrary, biochemical studies show that wound-induced JA/JA-Ile in systemic leaves is predominantly derived

from the *de novo* synthesis, rather than transported from the local damaged leaves (Matsuura et al., 2012) and the mobile signal moves at a faster speed than that of solute transport along the phloem in *Arabidopsis*. These results cast doubts on whether JAs could act as the transmissible wound signals (Farmer et al., 2003; Windt et al., 2006; Glauser et al., 2009; Koo et al., 2009; Mousavi et al., 2013). However, this debate would be reconciled by postulating that an iterative JA auto-amplification process along the phloem network could rapidly transmit the wound signal(s) to the distal leaves (Truman et al., 2007). However, this self-reinforcing mode of JA signal relay requires rapid cell-cell transport of JA (Schilmiller and Howe, 2005; Truman et al., 2007). Therefore, transporters mediating active transport of JAs has been long predicted. Furthermore, inhibitors of membrane transporters inhibited the loading and accordingly the long-distance transport of the radio-labeled MeJA (and the converted ^{11}C-JA) (Thorpe et al., 2007), supporting that transporter(s) may mediate the active loading and long-distance transport of JAs in the phloem.

TRANSPORTERS OF JASMONATES

Similar to other phytohormones including salicylic acid (SA), abscisic acid (ABA), indole acetic acid (IAA), and gibberellic acid (GA), JA is also a weak acid with a pK_a of ∼4.5 that will be trapped (in the form of JA^-) in the relatively alkaline cytosol (pH, 7.2) rather than in the apoplasm (pH, 5.5) (Robert and Friml, 2009; Kang et al., 2010). It has been long postulated that transporters are essential to mediate cellular efflux of these phytohormones into the apoplasm in the source cells. In addition, importers mediating the influx of these molecules from the apoplasm into the cells would facilitate the uptake of the phytohormones in the target cells, thereby promoting cell-cell transport. A number of plasma-membrane localized transporters have been identified for plant hormones, in particular for IAA (Park et al., 2017). The active transport of most of these plant hormones implicates the ATP-binding cassette (ABC), particularly the ABCG family, transporters and Nitrate transporter1/peptide transporters (NPFs). While most of the ABC transporters exhibited relatively higher substrate specificity of transport, the NPF proteins usually have broad substrates (Park et al., 2017).

Several member of NPFs were identified in a yeast-two-hybrid assay that detected JA-Ile-dependent binding of COI1 and JAZ3 as the candidates of jasmonate transporters (JATs). Using LC-MS/MS, a JA-Ile transport activity has been demonstrated for NPF4.1/AIT3 in yeast cells (Chiba et al., 2015). Another NFP member NPF2.10/GTR1 showed activity for the uptake of JA-Ile and JA in Xenopus oocytes (Saito et al., 2015). Their role in JA transport, however, remains to be clarified *in planta*. Furthermore, NPF4.1/AIT3 also showed transport activity for ABA and GAs in yeast cells (Chiba et al., 2015), while NPF2.10/GTR1 showed transport activity for GAs, $NO3^-$ and glucosinolates (Saito et al., 2015). Although the translocation of JA/JA-Ile from local wounded leaves to distal undamaged leaves was impaired in *GTR1* loss-of-function mutant, JA-Ile mediated core JA signaling was increased (Ishimaru et al., 2017) and exogenous GA3, but not JA, recovered male fertility to *gtr1* mutant (Saito et al., 2015). These results indicate that multiple-functional NPF4.1/AIT3 and NPF2.10/GTR1 with JA/JA-Ile transport activity may primarily involve in the interplays between JA and other signals (e.g., GA).

Exogenous JA can inhibit the growth of yeast cells, thus we exploited this system to screen JATs in yeast cells **(Figure 1A)**. Three genes encoding a clade of half-molecule ABCG proteins with 5 members (AtABCG1, AtABCG2, AtABCG6, AtABCG16, and AtABCG20) were induced by exogenous JA treatment in stamens (Mandaokar et al., 2006), and thus are the most likely candidates of JATs **(Figure 1B)**. In addition, AtABCG2, 6, and 20 function redundantly in the synthesis of effective suberin barriers of seed coats and roots and the exine of pollen coat (Yadav et al., 2014). However, the preferential expression of these ABCGs in the vascular tissues indicates that they are unlikely involved directly in the synthesis of suberin. The *atabcg1abcg16abcg20* triple mutant exhibited male sterility (Yadav et al., 2014), characteristics of mutants in JA biosynthesis and signaling, providing genetic evidence to support an involvement of these ABCGs in JA signaling. Yeast cells expressing AtABCG16 exhibited enhanced sensitivity to exogenous JA and increased cellular JA retention, indicating that AtABCG16 is a jasmonate transporter (named as AtJAT1) (Li et al., 2017). More interestingly, an additional localization of AtJAT1/ABCG16-GFP in, besides plasma membrane (PM), the nuclear envelope (NE) in transgenic *Arabidopsis* plant has been revealed. Utilizing cultured suspension cells and isolated nuclei, AtJAT1/AtABCG16 was shown to mediate the cellular export of JA and nuclear import of JA-Ile in a high-affinity mode (Li et al., 2017). The *atjat1* mutant show significantly compromise in JA-Ile-mediated core JA signaling as evident by expression of JA-responsive genes, JA-Ile induced degradation of JAZ1 protein and resistance to the necrotrophic fungal pathogen, *B. cinerea*. Significantly, *atjat1; atjar1* double mutant, but not *atjat1* or *atjar1* mutant, exhibited characteristic male sterility, which was rescued by exogenous JA. A cooperation between AtJAT1 and AtJAR1, which catabolizes the formation of JA-Ile, in regulating JA-Ile-mediated core JA signaling provide robust genetic evidence to support the finding that AtJAT1 mediates nuclear import of JA-Ile.

Semi-quantitative electron microscopic autoradiography using the *atjar1* mutant cells, in which the forming of the biological active JA-Ile is largely depleted (Suza and Staswick, 2008), showed that JA-Ile, but not JA enter the nuclei. Consistent with a role of ATJAT1 in nuclear import of JA-Ile, JA-Ile in the nucleus was remarkably decreased (Li et al., 2017). AtJAT1-mediated nuclear influx of JA-Ile would ensure cells to accumulate a critical nuclear JA-Ile concentration for activating JA signaling rapidly and substantially. In animals, nuclear receptors are activated by binding with their cognate small-molecule hormonal ligands and function as transcription factors to bind directly with the hormone response elements (HREs) in the promoters of their target genes. The binding of nuclear receptors can occur in either the cytoplasm (type I nuclear receptors e.g., steroid receptors) or the nucleus (type II

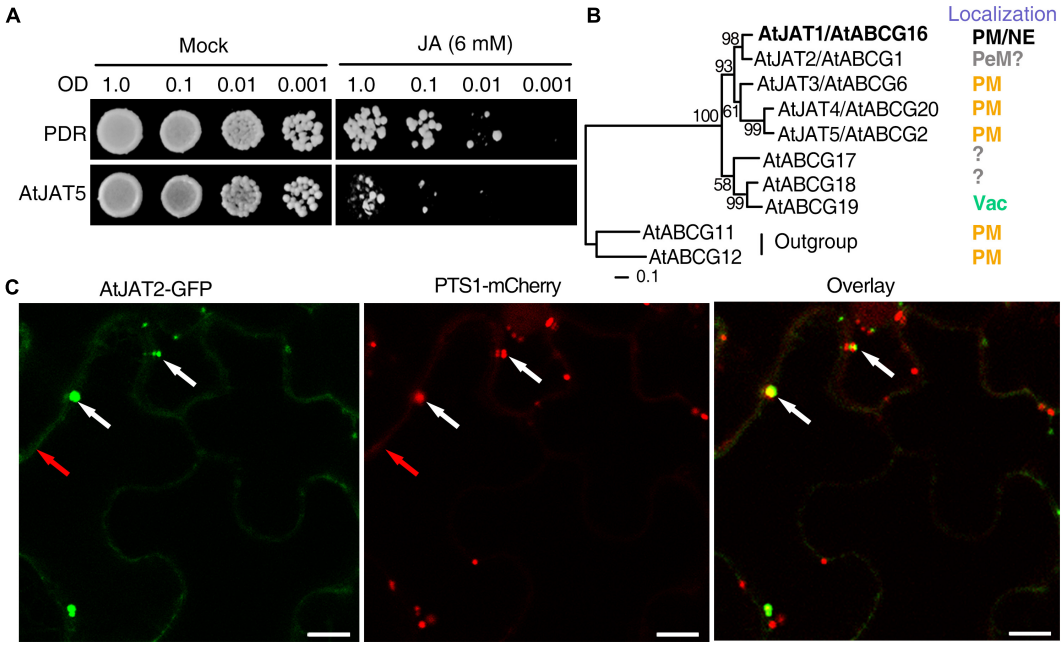

FIGURE 1 | Identification of jasmonate transporters (JATs) in yeast cells and the diverse subcellular localization of JAT family members. **(A)** Exogenous JA (6 mM) inhibits the growth of yeast cells. Yeast cells were diluted (OD_{600} = 1.0, 0.1, 0.01, and 0.001) and grown on SD-URA plate (Mock) or SD-URA supplemented with exogenous JA for 5–7 days. As illustrated for AtJAT5, putative jasmonate transporters (JATs) were identified by altered sensitivity to exogenous JA when the candidates were heterogeneously expressed in yeast cells. **(B)** Phylogenic relationship of 5 ABCG members of JAT family and their subcellular localization (PM, plasma membrane; NE, nuclear envelope; PeM, peroxisomal membrane; Vac, Vacuole). The question marks indicate the subcellular localizations that are tentatively inferred (e.g., AtJAT2) or unkown. AtJAT1 that has been characterized as a jasmonate transporter was indicated by bold letters. **(C)** The AtJAT2-GFP signal was colocalized with PTS1-mCherry/RFP (the peroxisomal marker) signals predominantly in the peroxisomes (indicated by white arrows), although weak signal was also detected in the PM (indicated by red arrows) in the epidermal cells of tobacco. The confocal microscopy analyses were performed 2 days after coinfiltration of *Agrobacterium tumefaciens* strains EHA105 carrying AtJAT2-GFP and PTS1-RFP constructs. Scale bars, 10 μm.

nuclear receptors e.g., triiodothyronine receptors) (Tata, 2002; Puzianowska-Kuznicka et al., 2013). Although plants do not have canonical nuclear hormone receptors, the receptors for auxin, JA, GA, and ABA are nucleus-localized and these receptors are proposed to bind with their hormonal ligands in the nucleus (Santner and Estelle, 2009; Lumba et al., 2010). Contrary to the view that small molecules diffuse passively through the nuclear pore complex (NPC) (Terry et al., 2007; Meier and Brkljacic, 2010), transport across the nuclear envelope (NE) is mediated by Na/Ca exchanger for Ca^{2+} in cultured animal cells (Wu et al., 2009; Galva et al., 2012) and by P-glycoprotein (P-gp) transporter for small-molecule medicines in multiple drug resistant tumor cells (Szaflarski et al., 2013). The identification of AtJAT1 shows that JA-Ile is actively transported into the nucleus rather than diffused passively through NPC. Removal of JA-Ile from the cytosol will consequently effect the equilibrium between JA and JA-Ile (Woldemariam et al., 2012) or hydroxylation of JA-Ile (Koo et al., 2011). Moreover, AtJAT1-mediated cellular efflux of cytosolic JA and nuclear entry of JA-Ile would significantly impact the metabolism of JA occurred in the cytoplasm, in particular the conjugation of JA with Ile (Staswick et al., 2002). Thus, this AtJAT1-modulated distribution of JA-Ile between nucleus and cytoplasm would play a significant role for the dynamics of JA biosynthesis, metabolism and signaling. The mechanism of the high but distinct substrate specificity of AtJAT1 in mediating the nuclear import of JA-Ile (but not JA) and cellular export of JA (but not JA-Ile) remains to be addressed. To execute their functions in substrate transport, half molecule ABCG proteins interact with their self or other ABCG partners to form homodimers and/or heterodimers, which could determine the subcellular localization and substrate specificity of these transporters (McFarlane et al., 2010; Le Hir et al., 2013). Therefore, the substrate-specificity of AtJAT1 might be established by its interacting partners. More remarkably, how AtJAT1 mediates JA-Ile across NE with double-membranes and whether there is basal diffusion of JA/JA-Ile across the PM/NE await to be addressed in future studies.

FUTURE PERSPECTIVES

Due to the PM- and NE-dual localization, AtJAT1 is outstanding among the various phytohormone transporters characterized so far, the majority of which are PM localized (Park et al., 2017). However, dual localizations at the ER and PM of the short PIN (PIN-FORMED) permease-like auxin transporters, which lack the extensive hydrophilic loop separating two transmembrane domains, have also been revealed in *Arabidopsis* epidermal and root hair cells, as well as in tobacco BY-2 cells. These ER-localized PINs presumably regulate auxin homeostasis by

pumping auxin into (PIN5) or out (PIN8) of the ER lumen or hypothetically from the ER lumen into the nucleus (PIN6 and PIN8) (Mravec et al., 2009; Bender et al., 2013; Viaene et al., 2013; Park et al., 2017; Skalicky et al., 2018). The identification of AtJAT1 further supports a common theme that the transporter-modulated subcellular partition plays an essential role in modulating the signaling of plant hormones. Since the receptors of IAA, ABA and GA are also nucleus-localized, it is intriguing to know whether these hormonal ligands enter the nucleus, and if so, whether nuclear importers are involved. Direct evaluation of the substrate specificity of hormonal transporters by the transport of radio-labeled chemicals still remains challenging due to the cost and labor to test diverse radiolabeled potential transport substrates. Besides AtJAT1/AtABCG16, the other 4 members (AtABCG1, AtABCG6, AtABCG20, and ABCG2) in the same monophyletic clade may also mediate the transport of jasmonates. Supporting this notion, yeast cells expressing AtABCG2/AtJAT5 showed increased sensitivity to 6 mM exogenous JA (**Figure 1A**). When transiently expressed in the epidermal cells of tobacco (*N. benthamiana*), AtABCG6/AtJAT3-, AtABCG20/AtJAT4-, and AtABCG2/AtJAT5-GFP signal was previously detected in the PM (Yadav et al., 2014). However, when carefully examined, AtABCG1/AtJAT2-GFP signal was principally detected in the peroxisomes, though weak signal was also observed in the PM when transiently expressed in tobacco epidermal cells (**Figure 1C**). The localization of AtABCG1/AtJAT2-GFP signal in peroxisomes was later confirmed in the root cells of stable transgenic *Arabidopsis* plants (Manuscript in preparation). Although genetic and biochemical data await to be implemented, we propose that the peroxisome-localized AtJAT2/AtABCG1 might mediate the peroxisomal export of JA (**Figure 2**). The three PM-localized JATs (AtJAT3/4/5) are among the most likely candidates of JATs. Further evidence supports that AtJAT3/4 mediate the cellular import of JAs and AtJAT5 mediates the cellular export of JAs (unpublished data). The identification of PM-localized JATs will provide an opportunity to address whether JAs act as the mobile signal(s) in systemic wound response (Nguyen et al., 2017). Furthermore, JAT1 could form heterodimers with one of these three ABCGs in mediating cellular export of JA across the PM. As the only JAT that is present in the NE, AtJAT1 may thus form homodimers in mediating nuclear import of JA-Ile. This speculation provides an explanation for the distinct transport properties (substrate specificity and affinity) of AtJAT1 in the PM and NE. The interactions among these PM-localized AtJATs would be important to reveal the possible mechanism to determine substrate specificity of these transporters. Many chemicals, in particular plant secondary metabolites, use the vacuole as the sites for cellular storage, which play an important role in regulating the homeostasis of these chemicals

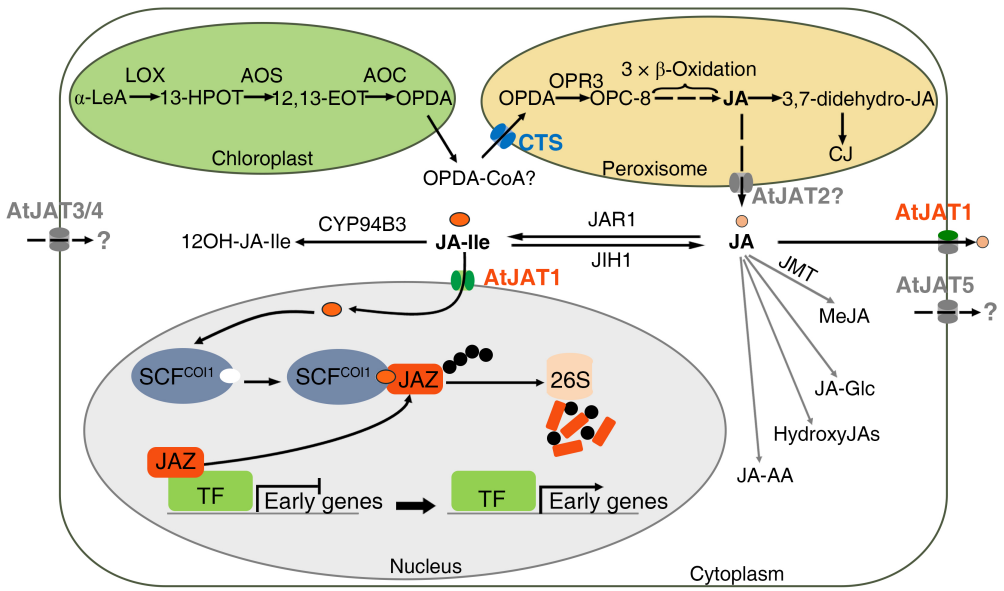

FIGURE 2 | Diverse subcellular localization of JAT family members and their roles in modulating the metabolism and signaling of jasmonates. Schematic model showing transporter-mediated subcellular distribution in modulating the biosynthesis, metabolism and signaling of JAs. The biosynthesis, metabolism and signaling occur sequentially in the chloroplast, peroxisome, cytoplasm and nucleus. CTS is localized in the peroxisome and involved in the peroxisomal entry of the key precursor, OPDA, which is produced in the chloroplast by the oxygenation of α-LeA. The CTS-modulated distribution of OPDA between the cytoplasm and the peroxisome significantly impacts the biosynthesis of JA and accordingly JA and OPDA signaling. AtJAT1 shows dual localizations in the nuclear envelope and plasma membrane and mediates the influx of JA-Ile into the nucleus and efflux of JA from the cytoplasm to the apoplast, accordingly modulating the subcellular distribution of JA-Ile and JA in the nucleus and cytoplasm. Furthermore, AtJAT2 is likely localized in the peroxisomes, which may be involved in the peroxisomal export of JA. While AtJAT3/4/5 are all localized in the PM, AtJAT3/4 may mediate the import of JAs and AtJAT5 may mediate the export of JAs across the PM. The AtJAT2 and AtJAT3/4/5 transporters were indicated in gray since the activity and directionality in jasmonate transport of these transporters remain to be determined. Characterization of these JATs in the future studies will provide further insights in a crucial role of transporter-mediated subcellular distribution in modulating the biosynthesis, metabolism and signaling of JAs.

(de Brito Francisco and Martinoia, 2018). A vacuolar auxin transporter facilitator, WAT1, has been identified in *Arabidopsis* and is required for auxin homeostasis and signaling (Ranocha et al., 2013). In the most closely related clade of the JAT family, there are 3 ABCG members (ABCG17, ABCG18, and AtABCG19) (**Figure 1B**), in which AtABCG19 has been shown to localize in the vacuole and involve in kanamycin resistance (Mentewab and Stewart, 2005). Hence, it is intriguing to know whether these ABCGs also have a transport activity for JAs. In addition, whether there is jasmonate transporter(s) in other transporter family or families still remains an open question. The heterologous system could also be valuable in screening, besides these JATs, such jasmonate transporter(s) in yeast cells. In summary, we propose that the coordinated actions of CTS, AtJAT1 and other uncharacterized JATs play crucial role in modulating the subcellular distribution of OPDA and JAs and accordingly connecting and orchestrating JA biosynthesis, metabolism and signaling partitioned in diverse cellular compartments (**Figure 2**). The identification of JATs localized in PM and endomembrane of various organelles will provide a unique opportunity to explore the roles of transporter-mediated subcellular partition in modulating the biosynthesis, metabolism and signaling of plant hormones.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FW collected information on transporters of plant hormones, prepared the figures. GY was involved in preparing the figure. PL conceived the idea and wrote this perspective and collected all other information on jasmonate transporters. All the authors participated in writing and approved the manuscript for publication.

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REVIEW
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Intra and Extracellular Journey of the Phytohormone Salicylic Acid

Israel Maruri-López[1], Norma Yaniri Aviles-Baltazar[1,2], Antony Buchala[3] and Mario Serrano[1]**

[1] Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Mexico, [2] Instituto de Investigación en Ciencias Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos, Cuernavaca, Mexico, [3] Department of Biology, University of Fribourg, Fribourg, Switzerland

Salicylic acid (SA) is a plant hormone that has been described to play an essential role in the activation and regulation of multiple responses to biotic and to abiotic stresses. In particular, during plant-microbe interactions, as part of the defense mechanisms, SA is initially accumulated at the local infected tissue and then spread all over the plant to induce systemic acquired resistance at non-infected distal parts of the plant. SA can be produced by either the phenylalanine or isochorismate biosynthetic pathways. The first, takes place in the cytosol, while the second occurs in the chloroplasts. Once synthesized, free SA levels are regulated by a number of chemical modifications that produce inactive forms, including glycosylation, methylation and hydroxylation to dihydroxybenzoic acids. Glycosylated SA is stored in the vacuole, until required to activate SA-triggered responses. All this information suggests that SA levels are under a strict control, including its intra and extracellular movement that should be coordinated by the action of transporters. However, our knowledge on this matter is still very limited. In this review, we describe the most significant efforts made to date to identify the molecular mechanisms involved in SA transport throughout the plant. Additionally, we propose new alternatives that might help to understand the journey of this important phytohormone in the future.

Keywords: salicylic acid, transport, phytohormone, defense response, systemic acquired resistance, plant-microbe interactions

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Viktor Zarsky,
Charles University in Prague, Czechia

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Tibor Janda,
Centre for Agricultural Research (MTA), Hungary
Daniel F. Klessig,
Boyce Thompson Institute, United States

****Correspondence:***
Israel Maruri-López
ismaruri@ccg.unam.mx
Mario Serrano
serrano@ccg.unam.mx

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DISCOVERY OF SALICYLIC ACID

Salicylic acid (SA) belongs to a group of molecules collectively named salicylates, which are phenolic compounds synthesized by plants, that possess an aromatic ring and a hydroxyl group. Even before salicylates were chemically identified, for thousands of years, humans used them as pain killers (Klessig et al., 2018). The first known evidence of this use was discovered on the dental plaque from Neanderthals fossils from El Sidrón cave (Weyrich et al., 2017). These fossils contained remains of poplar bark, suggesting that the individuals chewed the plant to relieve the pain of dental abscesses. Use of plants containing salicylates as analgesics, can be found across many ancient cultures from Europe, Asia and America (Vlot et al., 2009). Nevertheless, it was not until 1828 that salicin was purified from willow bark by Johann A. Buchner. Subsequently around 1838, salicin was separated into a sugar and an aromatic compound that could be converted into an acid, named salicylic

acid (2-hydroxybenzoic acid), after the Latin name of the white willow tree *Salix alba* by Raffaele Piria (Vlot et al., 2009; Kumar, 2014). Free SA is poorly soluble in water and very soluble in polar organic solvents, with a pH value of a saturated aqueous solution of 2.4. SA fluoresces at 412 nm when excited at 301 nm and this property has been used to detect it in several plants (Raskin, 1992).

In 1859, Hermann Kolbe and coworkers synthesized SA, leading to an increase in its consumption due to easy availability and decreased cost. Then, years later, in order to avoid side effects induced by SA consumption (irritation and bleeding in the stomach), Felix Hoffmann reported that acetylsalicylic acid, caused less damage to the digestive system, which finally ended in the product that is nowadays used worldwide – aspirin (Vlot et al., 2009; Klessig et al., 2018). Even though the effects and benefits of aspirin in humans to treat fever, pain or swelling and to reduce the risk of heart attack, stroke and certain cancers, have been well described and studied (Klessig et al., 2018), its role as secondary metabolite in plant biology was only characterized in the late 20th century.

THE PLANT HORMONE SA

Plant hormones have been described to play essential biological roles regulating plant growth, development, reproduction and survival; and many of these mechanisms are regulated by cross-communication and signal-transduction pathways, within which plant hormones fulfill central roles (Verhage et al., 2010; De Vleesschauwer et al., 2013).

For many years, SA was considered just one of the thousands of phenolic compounds produced by the plants, another secondary metabolite with a relatively unimportant biological function (Raskin, 1992; Métraux and Raskin, 1993). However, in 1974, SA was described for the first time as a mobile signaling molecule localized in the phloem, that can induce flowering of *Xanthium strumarium* and *Lemna gibba*, suggesting a role as a plant hormone (Cleland, 1974; Cleland and Ajami, 1974). Nevertheless, the evidence that SA was a phytohormone came from the description of its role during the thermogenesis in voodoo lily (*Sauromatum guttatum*) (Raskin et al., 1989). The authors identified a 100-fold increase of endogenous SA during this event that can be also specifically stimulated by exogenous SA or its derivatives, but not by other structurally related compounds. After this initial characterization, multiple reports studied the role of SA as a phytohormone, including its involvement in the resistance and tolerance to many abiotic stresses, including ozone, UV radiation, paraquat, heat, cold, metal, and salinity/osmotic stresses (Horváth et al., 2007; Yuan and Lin, 2008; Rivas-San Vicente and Plasencia, 2011; Dempsey and Klessig, 2017). In addition, there is evidence that application of SA affects multiple aspects of plant growth and development, including seed germination, vegetative growth, flowering, fruit yield, senescence, stomatal closure, thermogenesis, photosynthesis, respiration, changes in the alternative respiratory pathway, glycolysis and the Krebs cycle (Khan et al., 2015; Dempsey and Klessig, 2017; Klessig et al., 2018). Nevertheless, one of the better- characterized SA-induced responses is that involving plant-microbe interactions, described below.

SA, AN ESSENTIAL REGULATOR OF PLANT-MICROBE INTERACTIONS

Plants are constantly interacting with millions of microorganisms, including bacteria, yeast, fungi, and viruses (Lindow and Brandl, 2003; Baldwin et al., 2017). In response, an intimate dialogue is established between plants and microbes, which modifies the growth and development of the pathogens and the induction of the plant defense responses (Aragón et al., 2017). The first line of defense is grouped into the innate immunity, which can be divided into pathogen-associated molecular pattern-triggered immunity (PTI) and effector triggered immunity (ETI). During PTI, accumulation of reactive oxygen species (ROS), mitogen activated protein kinase (MAPK)-dependent signaling cascades and transcriptional reprograming are induced, while during ETI, induction of the above PTI responses is stronger and/or more rapid, which is usually accompanied by programmed cell death or hypersensitive response at the infection site (Boller and Felix, 2009; Zipfel, 2014; Conrath et al., 2015). The effect of ETI and PTI can block the infection of unadapted pathogens, both at the local infected tissue and systemically in uninfected leaves (Craig et al., 2009). After these early events, secondary or late defense responses are triggered, including the activation of hormone-induced signaling pathways. The main hormones involved during the innate immunity are SA, jasmonic acid, and ethylene (Yang et al., 2013). However, abscisic acid, gibberellins, auxins, cytokinins, and brassinosteroids can also function as modulators of the plant immune signaling networks (Pieterse et al., 2009, 2012).

The role of SA during plant-microbe interactions, was recorded for the first time in tobacco and cucumber plants in 1990. Plants with an increased resistance to tobacco mosaic virus (TMV) showed a strong accumulation of SA, while with TMV-susceptible plants, SA levels were significantly reduced (Malamy et al., 1990). Multiple reports have been published showing the effect of SA regulating the interactions between plants and microbes and recent reviews have made detailed description of this regulation (Dempsey and Klessig, 2017; Klessig et al., 2018). Here, we describe some of the most relevant findings.

After the initial observation with tobacco and cucumber plants, several studies found that tobacco and *Arabidopsis thaliana* plants transformed with the bacterial gene *nahG*, encoding a bacterial salicylate hydroxylase that degrades SA to catechol, showed enhanced susceptibility to pathogens (Gaffney et al., 1993; Delaney et al., 1994; Lawton et al., 1995). Through genetic and biochemical analysis, the SA-dependent signaling pathway has been characterized during the past few years (**Figure 1**). Recently, the protein NPR1 was described as the receptor of SA (Wu et al., 2012). Uninfected plants, with low levels of SA, NPR1 forms an oligomeric complex localized in the cytosol (**Figure 1A**), modulating the cross talk between SA and jasmonic acid, but not inducing SA-dependent

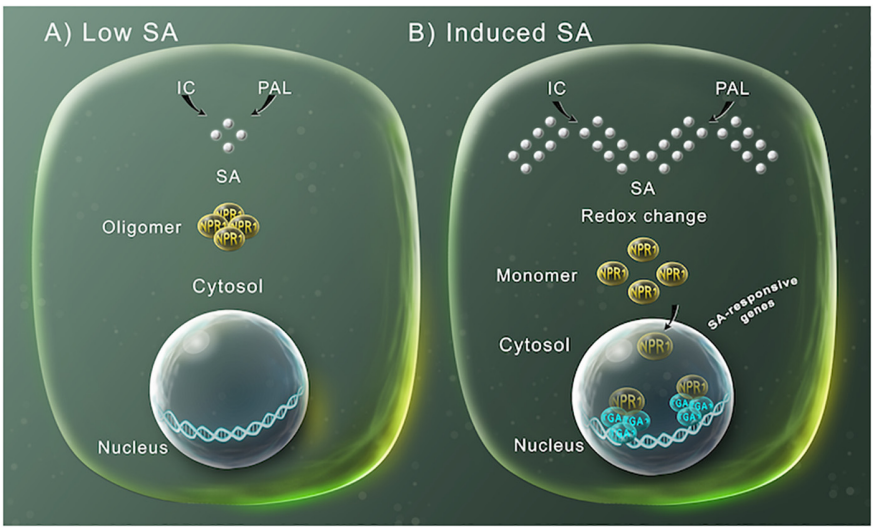

FIGURE 1 | SA-mediated gene expression regulation for plant defense. **(A)** SA (silver spheres) is synthesized by either the phenylalanine ammonia-lyase (PAL) or the isochorismate (IC) pathways. At low SA levels, NPR1 occurs in the cytosol as an oligomer bound by disulfide bridges. **(B)** At high SA intracellular concentration, the increase in SA modifies the cellular reduction potential, which leads to NPR1 structure changes to monomers through the reduction of the intermolecular bridges by a change in redox. This allows NPR1 to enter the nucleus, where it binds to specific TGA transcriptions factors inducing the expression of SA-induced defensive response genes.

defense genes (Spoel et al., 2003). However, upon pathogen infection or treatment with exogenous SA, a change in the cellular redox system occurs, that has been associated with the SA-triggered suppression of JA responses (Holuigue et al., 2016), which induces NPR1-complex dissociation to monomers, by the reduction of disulfide links (**Figure 1B**) (Tada et al., 2008). These monomeric forms can then be translocated into the nucleus, where they interact with basic leucine zipper (bZIP) TGA-type transcription factors, inducing the modification of the transcriptome, including the transcriptional activation of the defense-related genes, such as *PR-1* (**Figure 1B**) (Fu and Dong, 2013; Birkenbihl et al., 2017).

Additionally, NPR1 activity is regulated by proteasome-mediated degradation. This process is carried out by the NPR1 paralogues, NPR3 and NPR4, which are adaptors for the Cullin 3 ubiquitin E3 ligase and mediates the NPR1 degradation in a SA-dependent manner. In uninfected cells, when SA levels are low, NPR4 is proposed to maintain low NPR1 levels. However, after infection, when SA levels increase, NPR4-NPR1 interaction is disrupted, allowing the accumulation of NPR1. Additionally, when the level of SA is extremely high, NPR3 binds NPR1 leading to NPR1 degradation (Spoel et al., 2009; Fu et al., 2012; Dempsey and Klessig, 2017). NPR1 turnover ensures a correct defense activation and is required for full induction of target genes and the establishment of SA-induced responses (Spoel et al., 2009). However, until now only the ubiquitin ligase adapter function has been attributed to NPR3 and NPR4, but they also might be involved in transcriptional regulation of SA-induced defense genes. *Arabidopsis thaliana* plants harboring NPR3 and NPR4 mutant versions, unable to bind SA, showed constitutive repression of SA-induced immune responses and enhanced SA-induced defense gene expression, respectively (Ding, 2018).

After these initial SA-triggered responses in the local infected/treated tissue, plants can induce a long-lasting and broad-spectrum defense syndrome called systemic acquired resistance (SAR), which includes the accumulation of PR proteins and the induction of further biosynthesis of SA (Mishina and Zeier, 2007). SAR promotes a priming of defense mechanisms which is a faster and stronger response to a secondary infection inflicted by virus, bacterial and fungal pathogens (Vlot et al., 2009; Conrath et al., 2015). The chemical derivative of SA (MeSA) was initially described as the mobile signal responsible for inducing SAR (Rasmussen et al., 1991; Park et al., 2007; Vlot et al., 2009). However, while several reports suggest that SA is an important molecule to induce SAR, it is not the only one (Smith et al., 1991; Vernooij et al., 1994). Later work has described that together with SA, other compounds participate as inducers of SAR including: dicarboxylic acid azelaic acid (AzA), glycerol-3-phosphate (G3P), dehydroabietinal (DA), and pipecolic acid (Pip) [reviewed by Singh et al. (2017)]. Nevertheless, these results show the important role of SA during the interactions between plants and pathogens.

SA BIOSYNTHESIS AND STORAGE ARE COMPARTMENTALIZED

Salicylic acid is probably present in all plants, however, its concentration can fluctuate widely between the species and even between members of the same family (Raskin et al., 1990). For example, tobacco leaves can contain <100 ng/g fresh weight (FW) of SA, while in potato can reach 10 μg/g FW (Malamy et al., 1992; Coquoz et al., 1998). In the model plant *Arabidopsis thaliana*, the basal level of SA has been quantified

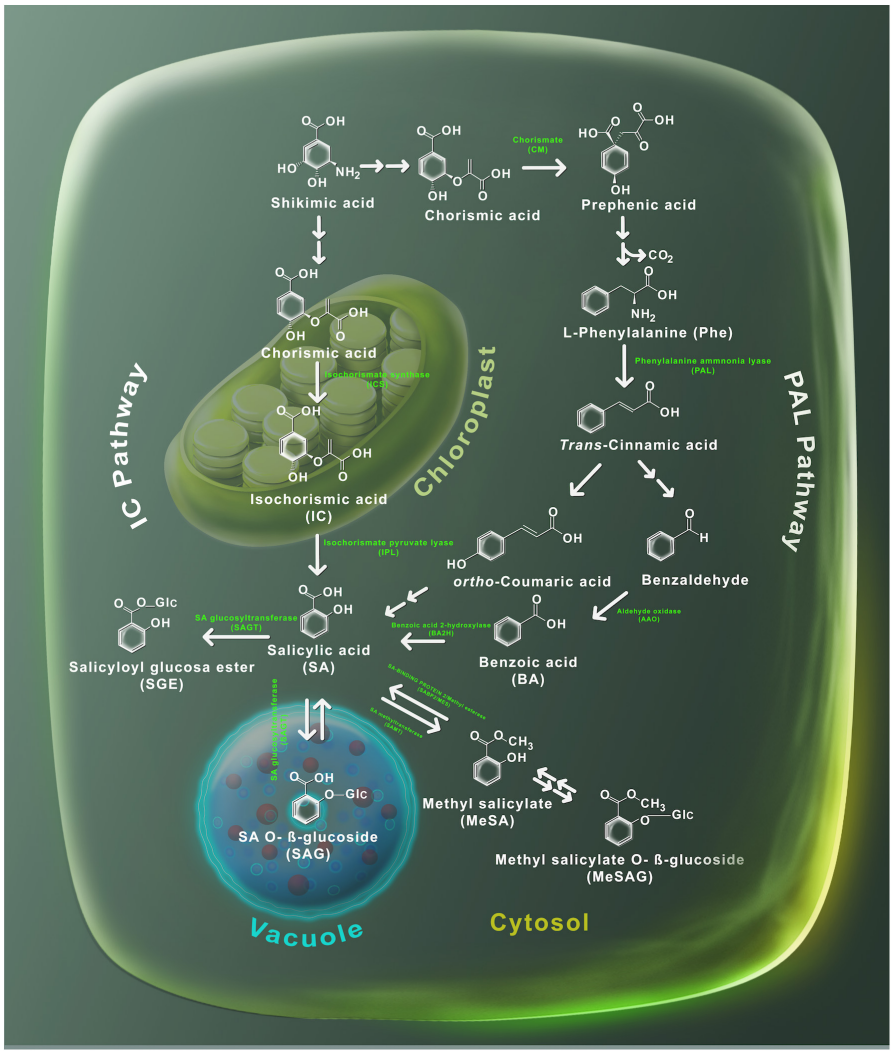

FIGURE 2 | Metabolism of SA. Plants utilize two pathways to produce SA, the phenylalanine ammonia-lyase (PAL) and the isochorismate (IC). Shikimic acid serves as precursor in both routes. The PAL route is carried out in the cytosol, where shikimic acid is transformed into chorismic acid and then into phenylalanine by the action of the chorismic mutase enzyme. Later, the PAL enzyme uses phenylalanine to produce *trans*-cinnamic acid which in turn is converted into both *ortho*-coumaric acid and benzaldehyde. Subsequently, benzaldehyde is converted to benzoic (BA) acid by the aldehyde oxidase (AAO) enzyme. BA is transformed to SA through a reaction catalyzed by the benzoic acid hydroxylase (BA2H) enzyme. The IC pathway takes place at the chloroplast – SA is synthetized by the isochorismate synthase to generate isochorismate, which is later transformed into SA through the action of the isochorismate pyruvate lyase enzymes. Salicylic acid glucosides (SAG and SGE) are produced by glucosyltransferases (SAGT); while, methylation of SA is performed by the methyltransferases enzyme. The chemical structures and its subcellular localization of SA substrates and derivatives are shown.

TABLE 1 | List of plant SA transporter mechanisms.

Plant species	Carrier name	Transporter class	Substrate	Subcellular localization	References
Arabidopsis thaliana	EDS5	Multidrug and toxin extrusion-like (MATE)	SA	Chloroplast envelope	(Serrano et al., 2013)
Glycine max	n.d.	ATP-binding cassette (ABC)	SAG	Vacuole tonoplast	(Dean and Mills, 2004)
Beta vulgaris	n.d.	H^+-antiporter*	SAG	Vacuole tonoplast	(Dean and Mills, 2004)
Nicotiana tabacum	n.d.	H^+-antiporter*	SAG	Vacuole tonoplast	(Dean et al., 2005)
A. thaliana	n.d.	ABC/H^+-antiporter*	SAG	Vacuole tonoplast	(Vaca et al., 2017)
Ricinus communis	n.d.	pH-dependent carrier	SA	Phloem	(Rocher et al., 2006, 2009)
N. tabacum		Diffusion	MeSA	Phloem	(Liu et al., 2011)
A. thaliana		Diffusion	MeSA	Phloem	(Liu et al., 2011)

*n.d., not determined. *likely MATE.*

between 0.250 and 1 μg/g FW (Nawrath and Métraux, 1999; Wildermuth et al., 2001). Several reviews have recently made a detailed description of SA biosynthesis (Dempsey et al., 2011; Dempsey and Klessig, 2017; Klessig et al., 2018). To date, the biosynthesis of SA has not been fully defined, however, genetic and biochemical evidence pointed out that SA can be produced from two independent and compartmentalized pathways: the isochorismate (IC) pathway localized into the plastids and the phenylalanine ammonia-lyase (PAL) pathway that takes place in the cytosol (**Figure 2**). Both pathways begin by the accumulation of chorismic acid, produced by shikimic acid biosynthesis. In the IC pathway, chorismic acid is converted into isochorismic acid (IC) by isochorismate synthase and then through the activity of isochorismate pyruvate lyase (IPL) SA is produced in the chloroplast and afterward exported to the cytosol (Fragnière et al., 2011). On the other hand, in the PAL pathway, prephenic acid is produced from the chorismic acid by chorismate mutase (CM), then reduced to L-phenylalanine (Phe) that is converted into *trans*-cinnamic acid (*t*-CA) by PAL activity. From *t*-CA two metabolites can be produced: *ortho*-courmaric acid (*o*-CA) and benzaldehyde. SA can be synthesized directly from *o*-CA, while benzaldehyde is first transformed into benzoic acid (BA) by aldehyde oxidase (AAO) and then to SA by the activity of benzoic acid 2-hydroxylase (BA2H) (**Figure 2**). Once SA is synthesized, its levels are regulated by a number of chemical modifications, to produce inactive forms, including salicyloyl glucose ester (SGE), SA O-β-glucoside (SAG), methyl salicylate (MeSA), and methyl salicylate O-β-glucoside (MeSAG). These inactive molecules can be stored until required to activate the SA-triggered responses.

In *Arabidopsis thaliana*, glucosylation of SA is performed by the action of UDP-glucosyltransferase enzymes UGT74F1 and UGT74F2 (Lim et al., 2002; Dean and Delaney, 2008). Both genes *UGT74F1* and *UGT74F2* are SA-induced and localized in the cytosol (Dean and Delaney, 2008; Park et al., 2017). The *ugt74f1* mutant accumulates less SAG than *ugt74f2* mutant and wild-type plants, while SGE was not formed in the *ugt74f2* background (Dean and Delaney, 2008). It has been demonstrated that UGT74F1 catalyzes the formation of SAG and UGT74F2 forms primarily SGE, but also synthetizes SAG (Dean and Delaney, 2008; Dempsey et al., 2011). On the other hand, SA produces MeSA by the action of a carboxyl methyltransferase (SAMT) (Ross et al., 1999; Zubieta et al., 2003). In *Arabidopsis thaliana*, *AtBSMT1* encodes for a carboxyl methyltransferase, which may use either BA or SA as substrates to form MeSA (Chen et al., 2003) (**Figure 2**). It was observed that *AtBSMT1* gene expression and hence MeSA production were induced in *Arabidopsis thaliana* leaves by alamethicin treatment, a pore-forming peptide that emulates pathogen damage (Chen et al., 2003). Therefore, it is suggested that AtBSMT1 performs MeSA production mainly during pathogen infection (Liu et al., 2009). Mutants impaired in the expression of *AtBSMT1* did not accumulate MeSA after pathogen attack, while overexpression of *AtBSMT1* led to an incremented accumulation of MeSA at the infection zone (Liu et al., 2009). Interestingly, overexpression and mutants of *AtBSMT1* were not able to accumulate SA or SAG at the distal leaves and they did not establish SAR (Liu et al., 2009). Likely, the overexpression of *AtBSMT1* leads to excessive conversion of free SA to MeSA, which avoids the required accumulation of SA to develop SAR at the systemic leaves. A similar observation was made when *Arabidopsis thaliana* plants overexpress the *OsBSMT1* gene from rice, which accumulated MeSA constitutively and failed to accumulate SA or SAG and were vulnerable to pathogen disease (Koo et al., 2007). Surprisingly, the increase in MeSA levels released by transgenic plants was enough to provoke SAR in nearby wild-type plants in an ICS1-independent process (Koo et al., 2007). Finally, in *Nicotiana benthamiana* virus-infected plants, SAR was impaired when the *SAMT* gene was silenced (Zhu et al., 2014).

Taken together, the fact that SA is produced into the chloroplast and in the cytosol, its inactive form (SAG) is stored in different cellular compartments (**Figure 2**) and afterward spread all over the plant to participate in SAR, suggests an intra and extracellular movement, that should be coordinated by the action of transporters.

THE JOURNEY OF SA

Similarly, to animal hormones, phytohormones are frequently synthesized in a different place to where they develop their function, thus requiring either local or long-distance communication. Plants employ numerous delivery mechanisms that depend on the distance and direction of the transport (Bonnemain et al., 2013; Park et al., 2017). In the next sections, we examine the most exciting advances about intracellular, cell-to-cell, and long-distance transport of SA (**Table 1** and **Figure 3**).

INTRACELLULAR TRANSPORT: FROM SA SYNTHESIS TO STORAGE

The synthesis of SA, induced by biotic and abiotic stress, is localized in the chloroplast and afterward transported to the cytosol (Dean et al., 2005; Garcion et al., 2008; Fragnière et al., 2011). However, the molecular mechanism involved in this active transport was for a long time unknown. At the beginning of 2000's, two *Arabidopsis thaliana* mutants, named SA induction–deficient (*sid1* and *sid2*) were identified (Nawrath and Métraux, 1999). *sid* mutants were described to be impaired in SA biosynthesis and to show enhanced disease susceptibility to bacterial and fungal pathogens (Nawrath and Métraux, 1999). *sid1* was identified to be allelic to *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*), a member of the multidrug and toxin extrusion (MATE) transporter family (Nawrath and Métraux, 1999; Nawrath et al., 2002). *Arabidopsis thaliana* plants overexpressing *EDS5* show enhanced resistance to viruses, validating the role of *EDS5* during the plant defense responses (Ishihara et al., 2008). Additionally, other reports have shown that EDS5 is localized at the chloroplast envelope membrane, suggesting that it might be involved in SA transport (Serrano et al., 2013; Yamasaki et al., 2013). Analyzing the movement of radiolabeled SA in isolated chloroplasts overexpressing *EDS5*

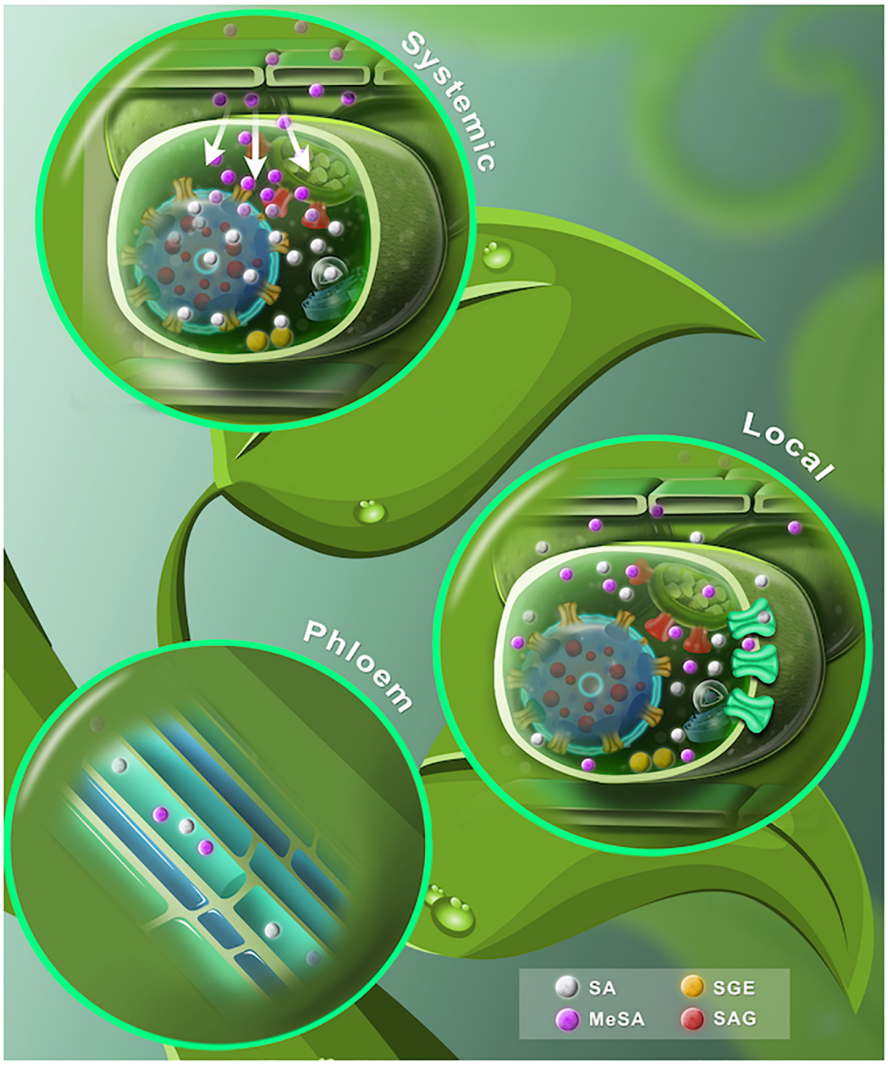

FIGURE 3 | A proposed dynamic model of free and conjugated SA. **(Lower leaf)** The SA synthesized by ICS and PAL pathways is shown in gray spheres. At the chloroplast, SA is translocated to the cytosol through EDS5 transporters (red carrier). Once extruded, SA is conjugated with glucose to form SAG or SGE (red and yellow spheres, respectively). Later, SAG is transported into the vacuole by ABC transporter/H+ -antiporter systems (yellow carriers). SA may spread out to the apoplast by a carrier-mediated system (green carriers). SA is converted to volatile MeSA (purple spheres) by carboxyl methyltransferase enzyme (SAMT). **(Stem)** SA phloem transport may be based on a symplastic outer cell transport, phloem apoplast intake through an ion trap mechanism and an apoplast intake mediated by a carrier system. MeSA also can be found in the phloem. **(Upper leaf)** After a pathogen attack, SA levels rise in the primary infected tissue. SA is converted to MeSA (purple spheres). The accumulating MeSA is translocated to the uninoculated systemic tissue. MeSA is demethylated to form SA and induces *de-novo* synthesis of SA at the distal tissue.

from *Arabidopsis thaliana*, and in a yeast heterologous system, it was confirmed that EDS5 is involved in the export of SA from the chloroplast to the cytosol (Serrano et al., 2013).

Interestingly, the *EDS5* homolog (*EDS5H*) also shows a chloroplastic envelope location, but, however, is induced neither by pathogens nor by application of exogenous SA and its mutant *eds5h* is not impaired in the biosynthesis of SA (Parinthawong et al., 2015). EDS5H most likely transports other phenolic compounds, but not SA, which suggests that the protein-mediated transport system of SA is only performed by specific carriers (Parinthawong et al., 2015). This evidence suggests that EDS5, to our knowledge, is the unique SA plant transporter identified so far involved in its movement from chloroplast to cytosol.

Once synthesized or transported in the cytosol, SA can undergo conjugation with glucose in order to render an inactive form that is water soluble for storage (**Figure 3**). Depending on where glucose is attached, either the hydroxyl group or the carboxyl group, SA can be transformed into SA-glucoside (SAG) or SA glucose ester (SGE), respectively (Lim et al., 2002; Dean et al., 2003, 2005; Dean and Mills, 2004; Park et al., 2007). It is known that glucosides, such as flavonoid glucosides and SAG, are stable forms for storage of other small phenolic compounds, while glucose esters, such as SGE, may act as intermediary

substrates in a biosynthetic pathway (Petrussa et al., 2013; Vaca et al., 2017). Interestingly, reports in tobacco and poplar suggest that the glucose ester of BA and SGE might be precursors for SA formation, indicating that SGE might accumulate in the cytosol to be used as a ready-to-use precursor for SA while SAG is accumulated in the vacuole until the plant needs it (Chong et al., 2001; Ruuhola and Julkunen-Tiitto, 2003).

It has been shown that SAG is transported into the vacuole in various plant species, such as soybean, tobacco, red beet and *Arabidopsis thaliana* (**Table 1**) (Dean and Mills, 2004; Dean et al., 2005; Vaca et al., 2017). For soybean, SAG appears to be exported into the vacuole by ATP-binding cassette (ABC) transporters (Dean and Mills, 2004). In tobacco and red beet, transport is carried out by H^+-antiporters (Dean et al., 2005). While in *Arabidopsis thaliana*, SAG is stored in the vacuole and SGE is only found in the cytoplasm (Vaca et al., 2017). An uptake analysis of radiolabeled SAG, in vacuoles of *Arabidopsis thaliana*, revealed that it is translocated toward the vacuole through a MgATP-dependent process (Vaca et al., 2017). Moreover, vacuolar transport of SAG was blocked by inhibitors of ABC transporters and H^+-antiporter systems, indicating that glycosylated SA transport may be performed by these types of pumps (Vaca et al., 2017).

SA-glucoside is reconverted to the active form of SA when hydrolyzed. In tobacco, SAG is reconverted to SA at the apoplast (Hennig et al., 1993), which makes sense with the extracellular localization of β-glucosidases in dicotyledonous plants and also corresponds to the role played by these enzymes during plant pathogen interactions (Morant et al., 2008). However, it is still unknown if vacuolar SAG is transported to the apoplast and/or if it is released after a pathogen-induced response. Additionally, it cannot be discounted that intracellular enzymes may hydrolyze SAG under special conditions (Dempsey et al., 2011). Taking together, these observations indicate that the intracellular level of SA and its inactive forms are under the control of transporters.

CELL TO CELL TRANSPORT: INFLUX AND EFFLUX OF SA

Once SA is synthesized inside the cells, the next step in the SA journey is its dissemination to neighboring cells (**Figure 3**) (Kawano et al., 2004). Often SA is spread via the apoplast (Lim et al., 2016; Singh et al., 2017). Because of its chemical features, such as weak acid and poor water solubility, SA crosses through animal and plant cell plasma membranes by pH-dependent diffusion and carrier-mediated mechanisms (Chatton and Roch-Ramel, 1992; Takanaga et al., 1994; Chen et al., 2001; Emoto et al., 2002; Bonnemain et al., 2013). In mammals, a monocarboxylate transporter is localized at the cell plasma membrane (Enerson and Drewes, 2003). In plants, the influx of radiolabeled SA has been reported in the aquatic plant *Lemna gibba*, where around 90% of 10 μM SA applied to the medium was taken up in a half hour, however, the mechanisms by which the SA was internalized are unknown (Ben-Tal and Cleland, 1982). Nevertheless, after uptake, SA was localized either in the cytosol or vacuole and no further plasmodesmata transport was observed in *L. gibba* cells (Ben-Tal and Cleland, 1982). It is likely that SA was quickly conjugated with glucose and stored in vacuoles.

Interestingly, SA uptake was faster in a tobacco cell suspension than in *L. gibba* plants, taking up to 200 μM SA in just 5 min (Chen et al., 2001). Nevertheless, after 5 h, over 90% of the radiolabeled SA absorbed was released to the medium. The SA influx could be inhibited by adding a chelating agent (EGTA), but the efflux was restored by addition of Ca^{2+} and moreover, a protein synthesis inhibitor blocked SA excretion (Chen et al., 2001; Kawano et al., 2004). The authors suggest the presence of a SA efflux transporter that may be induced at high SA concentrations and which may involve ROS, Ca^{2+}, *de-novo* protein synthesis and a protein phosphorylation signaling pathway (Chen et al., 2001; Kawano et al., 2004; Bonnemain et al., 2013). However, a constitutive SA efflux carrier involved during low SA concentrations, independent of ROS, $Ca2^+$, and protein kinase cascade signaling has been also proposed (Kawano et al., 2004; Bonnemain et al., 2013).

LONG-DISTANCE TRANSPORT

Along with azelaic acid (AzA), glycerol-3-phosphate (G3P), methyl jasmonate (MeJA) and pipecolic acid (Pip), SA participates as a critical long-distance inducer for SAR (Conrath et al., 2015; Klessig et al., 2018). Rapid translocation of radiolabeled SA, injected to the end of tobacco leaf petioles, was observed in systemic neighboring upper and lower leaves (Ohashi et al., 2004). When ^{14}C-labeled BA was applied to cucumber cotyledons infected with tobacco necrosis virus, labeled SA was subsequently found in the phloem and in the upper uninfected leaf (Mölders et al., 1996). During pathogen infection, SA is accumulated in the phloem, via the apoplast in *Arabidopsis thaliana* and tobacco plants (Yalpani et al., 1991; Zhu et al., 2014; Lim et al., 2016). Additionally, it has been reported that translocation of radiolabeled SA occurs from tobacco virus-inoculated leaves toward uninfected distal zones (Shulaev et al., 1995). SA translocation by the xylem has also been reported in tobacco and *Ricinus communis* seedlings, although the contribution of xylem in SA-mediated transport to develop SAR is not yet clear (Ohashi et al., 2004; Rocher et al., 2006).

In *Ricinus communis* seedlings, the SA phloem transport mechanisms comprise a high specificity pH-dependent carrier system, which may be placed in internal tissues, mainly at the cotyledon veins (**Table 1**) (Rocher et al., 2006, 2009). The authors suggest that SA phloem loading may be based on a symplastic outer cell transport, phloem apoplast intake, through an ion trap mechanism and an apoplast intake mediated by a carrier system (**Figure 3**) (Rocher et al., 2009).

The major barrier to prevent the free diffusion of SA is the plant cuticle (Niederl et al., 1998; Ohashi et al., 2004). Controversially, it is well known that the exogenous application of SA, either by seed priming (soaked seeds before sowing), the addition of SA to a hydroponic solution or spraying plants with SA solution, is favorable for plant growth and helps to protect them against abiotic stresses (Hayat et al., 2010). For instance, seed priming with SA, increased the activities of antioxidant

enzymes in young pea (*Pisum sativum* L.) and leads to the *de-novo* synthesis of SA (Szalai et al., 2011). When sweet basil (*Ocimum baslicum* L.) plants were sprayed with citric and SA, supplemented with a surfactant agent, the root nutrient acquisition pattern changed to assimilating more boron and sulfur, therefore enhancing their uptake to plant shoot, thus increasing fresh biomass and photosynthetic efficiency (Ghazijahani et al., 2014). Moreover, in wheat plants (*Triticum aestivum*) the abiotic stress caused by the insecticide chlorpyrifos was mitigated by spraying exogenous SA; probably due to an activity improvement of antioxidant enzymes. Also, SA was able to avoid the uptake of chlorpyrifos in wheat plants (Wang and Zhang, 2017). However, exogenous applications of SA are often carried out to run-off, with the possibility that could enter via stomata and that soft mechanical damage can induce the defense mechanisms (Benikhlef et al., 2013). Nevertheless, the mechanisms involved in the assimilation and/or transport of SA under these conditions has not been described.

It has been demonstrated that cuticle is hardly permeable to SA, unless it is converted to a volatile form (**Figure 3**). MeSA is a volatile SA derivative and it acts as an airborne defense signal (**Table 1**) (Koo et al., 2007; Park et al., 2007). MeSA is mobilized through the phloem to activate SAR (Yalpani et al., 1991; Kumar and Klessig, 2003). MeSA becomes active when it is reversed to SA by the MeSA esterase activity of SA-binding protein 2 (SABP2) in the systemic tissue (**Figure 2**) (Seskar et al., 1998; Park et al., 2007, 2009; Vlot et al., 2008; Manosalva et al., 2010). In fact, mutations in either *SAMT* or *SABP2*, compromise SAR (Vlot et al., 2008). Altogether, this evidence supports the knowledge that the transport of SA and MeSA serves as a long-distance phloem-mobile signal and that correct homeostasis must be carried out for a successful triggering of SAR. While SA belongs to an orchestrated mechanism that induces SAR, it is not the only one – but it is a fundamental player in the activation of such a significant task of plants when facing the attack of pathogens.

WHAT TO EXPECT ABOUT SA TRANSPORT IN THE FUTURE?

Numerous studies confirm that SA plays a crucial role during plant growth and development and in particular during the plant innate immunity (Klessig et al., 2018). In contrast, discovery and characterization of transport systems and signaling pathways remain largely elusive. While genomic approaches, radiolabeled SA tracking analysis and inhibition of channel gating, have provided insights in SA intracellular and long-distance translocation (Dean and Mills, 2004; Park et al., 2007; Serrano et al., 2013; Zhu et al., 2014; Vaca et al., 2017), there are too many open questions to be answered about the molecular and cellular mechanisms that guide the trafficking of intra- and extra-cellular SA.

We now need to move beyond, to approaches that allow identifying new SA carriers, receptors and targets. For example, the mapping of SA-mediated protein-protein interactions can be a suitable tool for this aim. It is likely that more MATE- and ABC-type transporters will be found to be involved in the transport of SA, since these transporter families have been reported to transport such a diversity of chemicals, including other phytohormones (Zhang et al., 2014; Hwang et al., 2016). Membrane protein-protein interaction and transmission surface plasmon resonance (TSPR) approaches among others, might offer tremendous insight in new transport and receptors of SA (Lertvachirapaiboon et al., 2018). For instance, in the future we shall be capable of monitoring the distribution of intracellular SA pools by biosensor imaging development, using these newly identified SA receptors (Jones, 2016).

Additionally, mathematical modeling has been used to predict phytohormone transport and signaling pathways (Voß et al., 2014) e.g., the case of the cellular and long-distance transport of auxin in tobacco and *Arabidopsis thaliana*, respectively (Hošek et al., 2012; Boot et al., 2016). Also, to predict sequential induction of both auxin efflux and influx carriers in the regulation of lateral root emergence in *Arabidopsis thaliana* (Péret et al., 2013). Modeling predictions are useful to select appropriate experimental systems and generate hypothetical models of SA transport (Otto et al., 2018), which in parallel with experimental observations will be a powerful tool to develop and establish models of the regulation mechanism for SA transport.

AUTHOR CONTRIBUTIONS

IM-L, NYA-B, AB, and MS wrote and revised the manuscript. All the authors approved the final version of the manuscript.

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REVIEW
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Filling the Gap: Functional Clustering of ABC Proteins for the Investigation of Hormonal Transport *in planta*

Lorenzo Borghi, Joohyun Kang and Rita de Brito Francisco*

Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland

Plant hormones regulate a myriad of plant processes, from seed germination to reproduction, from complex organ development to microelement uptake. Much has been discovered on the factors regulating the activity of phytohormones, yet there are gaps in knowledge about their metabolism, signaling as well as transport. In this review we analyze the potential of the characterized phytohormonal transporters belonging to the ATP-Binding Cassette family (ABC proteins), thus to identify new candidate orthologs in model plants and species important for human health and food production. Previous attempts with phylogenetic analyses on transporters belonging to the ABC family suggested that sequence homology *per se* is not a powerful tool for functional characterization. However, we show here that sequence homology might indeed support functional conservation of characterized members of different classes of ABC proteins in several plant species, e.g., in the case of ABC class G transporters of strigolactones and ABC class B transporters of auxinic compounds. Also for the low-affinity, vacuolar abscisic acid (ABA) transporters belonging to the ABCC class we show that localization-, rather than functional-clustering occurs, possibly because of sequence conservation for targeting the tonoplast. The ABC proteins involved in pathogen defense are phylogenetically neighboring despite the different substrate identities, suggesting that sequence conservation might play a role in their activation/induction after pathogen attack. Last but not least, in case of the multiple lipid transporters belong to different ABC classes, we focused on ABC class D proteins, reported to transport/affect the synthesis of hormonal precursors. Based on these results, we propose that phylogenetic approaches followed by transport bioassays and *in vivo* investigations might accelerate the discovery of new hormonal transport routes and allow the designing of transgenic and genome editing approaches, aimed to improve our knowledge on plant development, plant–microbe symbioses, plant nutrient uptake and plant stress resistance.

Keywords: ATP-Binding Cassette, plant hormones, strigolactones, auxins, ABA, vacuolar import, plant-defense, hormonal precursors

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University of Copenhagen, Denmark

****Correspondence:***
Lorenzo Borghi
lorenzo.borghi@uzh.ch

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INTRODUCTION

In plants, hormonal signaling pathways integrate internal and external cues to adapt the growth of these sessile organs to the surrounding, changing environment. Phytohormones tune the regulation of plant morphogenesis, plant development, biotic and abiotic stress resistance and plant–microbe interactions, either individually or in crosstalk network (Santner et al., 2009; Kamiya, 2010). Biosynthesis, perception and catabolism of phytohormones play an important role in the modification of such signaling pathways. As well, cell-to-cell transport, inner allocation and excretion of phytohormones toward the outer environment are important regulators of hormonal action (Park et al., 2017), which might span both long and short distances between the site of biosynthesis and the target of the action. Some years ago scientific works reported the severe growth defects caused by the loss-of-function of phytohormonal transporters (Gälweiler et al., 1998; Noh et al., 2001). This recognized need for hormone transporters during plant development overtook previous hypotheses that considered transporters not as necessary for hormonal distribution. Passive diffusion across the lipid bilayer was considered likely the main drive for hormonal transport (cellular import), because of the protonated form that most of the weak acid phytohormones have in physiological conditions. Instead, we know nowadays that diffusion plays only a partial role, e.g., for auxins (Vanneste and Friml, 2009). Different protein families regulate or facilitate phytohormonal distribution (Abualia et al., 2018) like PIN-FORMED (PIN, Adamowski and Friml, 2015), PIN-like (PILS, Feraru et al., 2012; Grones and Friml, 2015), NITRATE TRANSPORTER (NRT, Fan et al., 2017), MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE), SWEET transporters (Takanashi et al., 2014; Julius et al., 2017) and ATP-BINDING CASSETTE (ABC) transporters, the latter being the focus of this review. ABC proteins are one of the largest superfamily of multifunctional, mostly transmembrane transporters that utilize the ATP hydrolysis energy to carry out the translocation of various substrates across membranes (Kang et al., 2011; Hwang et al., 2016; Do et al., 2018). Depending on sequence and domain organization they have been classified in several subfamilies, from A to I that are mostly present in plants, animals and fungi and share function especially related to detoxification mechanisms (Hwang et al., 2016). Originally isolated for their ability to confer multidrug resistance, nowadays we know that ABC transporters are involved in several aspects of plant growth and adaptation also through the mobilization of plant hormones. Shared-function ABC transporters involved into phytohormonal transport have been characterized both in mono- and di-cotyledon species, thus suggesting a high level of conservation in the plant kingdom. In contrast to their animal, low-selective counterparts, several plant ABC proteins transport specific hormones toward storage organelles, target organs and plant outer surfaces. Until recently, phylogeny was not considered a valuable tool to infer protein function in the ABC protein family. Reported cases of sequence homology between strigolactone and abscisic acid (ABA) transporters (Kretzschmar et al., 2012) or the phylogenetic distance between the several characterized ABA transporters supported that assumption. We show here that the increased characterization of ABC proteins in different plant species (**Figure 1**) that was carried out in the last decade allows now for wider phylogenetic analyses, suggesting the possibility of functional clustering in the ABC family and, based on the previously characterized ABC hormone transporters (**Table 1**), might permit the isolation of putative orthologs in several model and non-model plant species.

Phylogenetic analyses carried on characterized ABC transporters and their sequence homologs from different plant species (see **Supplementary Material and Methods**) shows that proteins belonging to the same class, such as ABC class B proteins for auxinic molecule transport could cluster together. Also, proximity is obtained with ABC proteins sharing the same substrate, like for the SL, ABC class G transporters or for the class D transporters of lipids that are precursors of phytohormones. In some cases, only single ABC proteins have been up-to-date characterized for specific hormonal transport, like AtABCG14 for cytokinins (CKs) (Ko et al., 2014; Zhang K. et al., 2014) and therefore phylogenetic analysis is not very informative. In other cases, ABC proteins share same physiological functions despite transporting different substrates, like for the transporters involved in plant–pathogen defense. Or they have just the subcellular localization in common, e.g., the tonoplast in case of the ABC class C, but not the transported substrate. We are therefore aware that phylogenetic analyses are not sufficient to discover new functions and substrates of ABC transporters, unless coupled with parallel strategies aimed to identify the (possibly specific) substrate(s) and functions via molecular and vesicular transport assays. We finally discuss the basic and applied potentials of such phylogenetic analysis and the plant species and agricultural practices that could benefit from it.

ABCB SUBFAMILY AND TRANSPORT OF AUXINIC MOLECULES

In plants, the second most numerous subfamily of ABC transporters after ABCGs is class B (Geisler, 2014). Several ABCBs were characterized as transporters of the phytohormone auxin in Arabidopsis (ABCB1/PGP1, ABCB19/PGP19/MDR1, ABCB19, ABCB21) but also in rice (*Oryza sativa*, OsABCB14), maize (*Zea mays*, ZmABCB1) and *Lotus japonicus* (LjABCB1) (Xu et al., 2014; Geisler et al., 2017). Still, not all ABCBs are transporters of auxins or auxinic compounds like Indole-3-butyric acid (IBA). For example, in *Coptis japonica* CjABCB1/2 (Shitan et al., 2003, 2013) transport the alkaloid berberine. ABCBs regulating auxin transport are mostly exporters of auxins from biosynthetic active tissues toward the apoplast, therefore they are mainly localized in the plasma membrane of meristematic cells, where auxins are mostly synthetized, and along the stem vasculature where auxins are long-distance transported (Blakeslee et al., 2007). Other proteins like

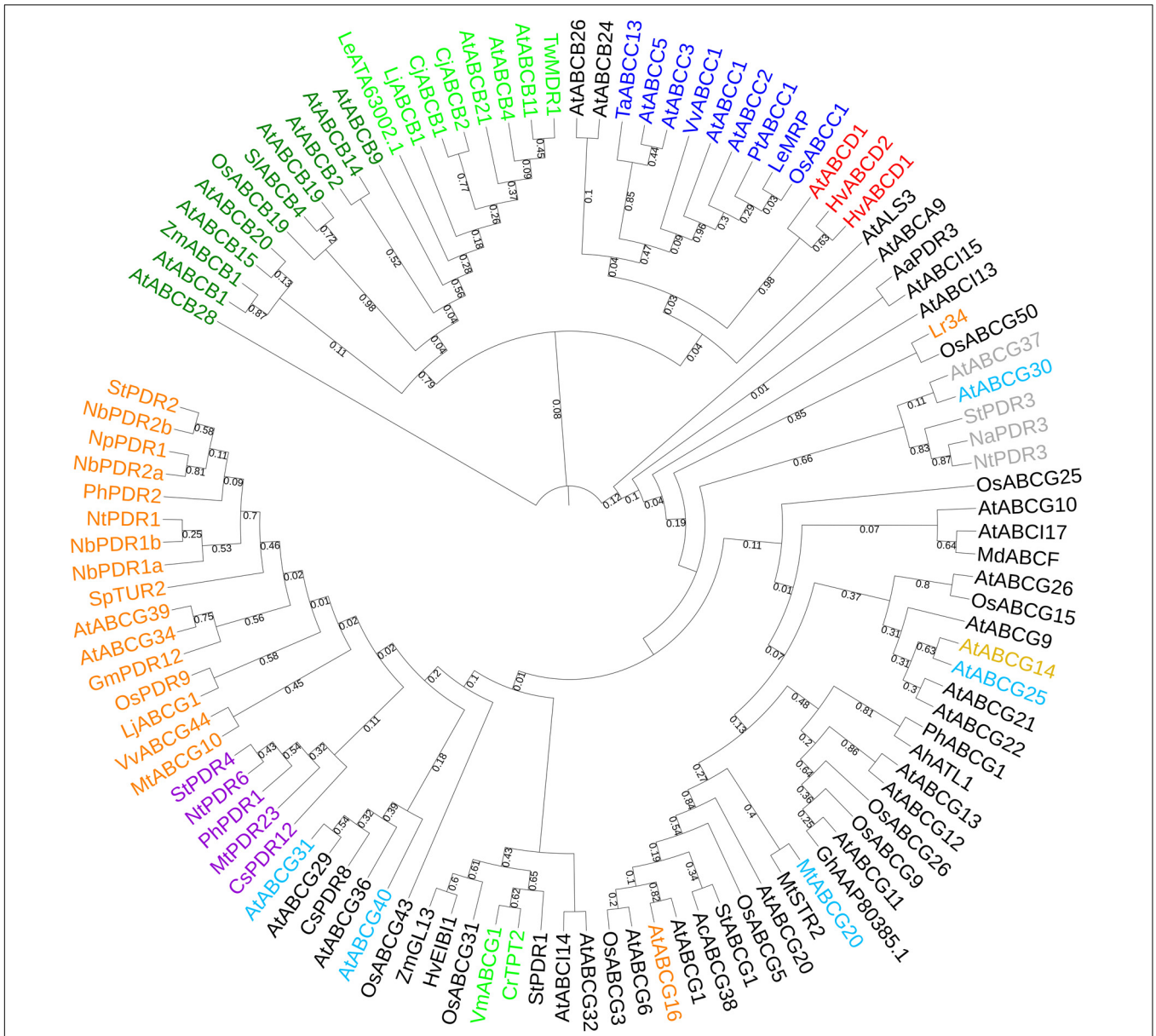

FIGURE 1 | Phylogenetic tree comprising up-to-date characterized ABC proteins (Lefèvre and Boutry, 2018) in plants. Violet clade: strigolactone (SL) transporters and sequence homologs. Orange clade: transporters involved in plant defense. Light green clade: alkaloid transporters. Red clade: lipid, hormonal-precursor transporters. Gray clade: coumarin transporters. Blue clade: ABC class C proteins (tonoplast importers). Dark green clade: auxin transporters. Cyan leaves: ABA transporters. Brown leaf: CKs transporter. Bootstrap n: 100. No branches deleted due to the high variety of sequences included in this analysis. The tree with the highest log likelihood (–4735.73) is shown. The analysis involved 109 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 42 positions in the final dataset.

AtABCB14 and AtABCB15 were not directly demonstrated as auxin transporters in cellular or vesicular systems, still they are expressed mostly along the stem vasculature and reported to affect polar auxin transport in those tissues (Kaneda et al., 2011), supporting that ABCBs can regulate both short and long distance transport of auxins (Cho and Cho, 2013). ABCBs were initially characterized after sequence homology to their mammalian *P*-glycoproteins counterparts (Dudler and Hertig, 1992) and their inducibility by auxin (Noh et al., 2001). Through their sensitivity toward 1-N-Naphthylphthalamic acid (NPA), one of the most popular auxin transport inhibitors, their loss-of-function dwarf phenotypes as well as the strong reduction in polar auxin transport, not only ABCBs but also their partner proteins like TWISTED DWARF1 (TWD1, Geisler et al., 2004) were later characterized. Plant ABCBs are selective for their auxinic substrates, in contrast to their mammalian orthologs, isolated for their multidrug resistance ability (MDR). Similar to other proteins in charge of auxin trafficking but belonging

TABLE 1 | Characterized ABC hormone transporters after (Lefèvre and Boutry, 2018).

Hormone	Transporter	Subfamily
Abscisic acid	AtABCG25	ABCG
Abscisic acid	AtABCG30	ABCG
Abscisic acid	AtABCG40	ABCG
Abscisic acid	MtABCG20 (Pawela et al., 2019)	ABCG
Auxins	AtABCB1	ABCB
Auxins	AtABCB14	ABCB
Auxins	AtABCB15	ABCB
Auxins	AtABCB19	ABCB
Auxins	AtABCB21	ABCB
Auxins	AtABCB4	ABCB
Auxins	AtABCG36	ABCG
Auxins	AtABCG37	ABCG
Auxins	LjABCB1	ABCB
Auxins	OsABCB14	ABCB
Auxins	ZmABCB1	ABCB
Cytokinins	AtABCG14	ABCG
Jasmonic acid	AtABCG16	ABCG
Strigolactones	PaPDR1	ABCG

In this list, only the transporters for in vivo identified substrates are present.

to the PIN family, AtABCB1 and AtABCB19 contain a large hydrophilic, cytosolic loop whose phosphorylation regulates protein activity and localization (Henrichs et al., 2012). Interestingly, some ABCB auxin transporters, like AtABCB4 and AtABCB21 (Santelia et al., 2008; Kamimoto et al., 2012; Kubeš et al., 2012) have the ability to regulate auxin trafficking inwards and outwards. This double directional transport of auxins is likely present to finely tune the homeostasis of intra- and extracellular auxins.

Several investigations reported the presence of stable or transient protein–protein interactions, such as TWD1 with AtABCB1/4/19 (Geisler et al., 2004; Bailly et al., 2008) thus to regulate the activity and directionality of ABCBs involved in auxin transport. TWD1 functions as chaperone protein and regulates ABCB transport from the endoplasmic reticulum (ER) to the plasma membrane, this role shared with its mammalian ortholog FKBP38 (Geisler, 2014). In addition, protein–protein interaction or just shared localization has been shown between ABCBs and PINs, like for PIN1/PIN2 with AtABCB1/AtABCB19 and PIN3 with AtABCB4 (Titapiwatanakun et al., 2009; Geisler et al., 2017). The role and duration of these interactions are not fully clear, yet. Still, both specificity and transport efficiency of auxins are increased by co-expressing these proteins in Arabidopsis or in heterologous systems, suggesting that ABCB-PIN co-expression might synergistically or antagonistically co-ordinate auxin transport. Activity and localization of ABCBs, like reported in PINs, can be regulated by phosphorylation through common AGC kinases, such as PINOID and D6PK (Wang et al., 2012; Armengot et al., 2016). These common factors of PINs and ABCBs suggest the presence of crosstalk regulation between these two families of auxin transporters.

Through the protein sequences of characterized ABCB transporters we carried phylogenetic analyses for isolating ABCB transporter candidates in staple food/economically relevant model plants: soya bean, poplar, manioc, tomato, potato, barrel medic, grapevine, wheat, rice, *L. japonicus*, *Physcomitrella patens*, *Marchantia polymorpha*, barley, *Sorghum bicolor* and maize. The first 100 unique homolog hits (or the ones with sequence identity > 70%) were selected (**Figure 2**). AtABCB19 and SlABCB4 group together, while the other characterized ABCBs from Arabidopsis, rice, maize and lotus are dispersed in the tree, except for AtABCB4 and AtABCB21 forming a separated leaf. Despite their proximity and their common substrate, AtABCB19 and SlABCB4 likely do not share the same function. SlABCB4 is expressed in developing tomato (Ofori et al., 2018a), thus it is supposed to be important during fruit development. AtABCB19 regulates hypocotyl gravitropism and phototropism in Arabidopsis (Nagashima et al., 2008). These two characterized ABCBs cluster together with two ABCB19-like or predicted ABCB19 proteins from manioc (*Manihot esculenta*, Me) and one candidate from potato (*Solanum tuberosum,* St), barrel medic (*Medicago truncatula*, Mt), soya bean (*Glycine max*, Gm), poplar (*Populus trichocarpa*, Pt), and grapevine (*Vitis vinifera*, Vv). The closest leaf stemming from the same node bears the characterized OsABCB19 together with candidates from grasses (sorghum, maize, and rice). OsABCB19 is an auxin transporter involved in the regulation of gravity sensing and cytoplasmic streaming (Okamoto et al., 2015). This targeted search for ABCB homologs in model/crop plants shows several putative orthologs from soya bean and barrel medic proximal to AtABCB1. A close by leaf consists of the characterized ZmABCB1 and its putative ortholog OsABCB1. In maize, ABCB1 is expressed in nodal meristems and regulates auxin transport in mesocotyls and coleoptiles, suggesting that ABCB1 function is conserved in dicots and monocots (Knöller et al., 2010). AtABCB4 together with AtABC21, LjABCB1, AtABCB9, AtABCB24, AtABCB14, and AtABCB15 are present in isolated nodes. AtABCB4 is an auxin efflux transporter that regulates the elongation of root hair cells as well as lateral root development (Cho et al., 2007; Santelia et al., 2008). AtABCB21, together with AtABCB4 are facultative, bi-directional auxin transporter: they are cellular importers at low auxin concentrations and cellular exporters when auxin concentrations are high (Kamimoto et al., 2012). LjABCB1 is homolog and, as expected, proximal to AtABCB4. Its expression pattern is close to rhizobium-nodule infected cells and detected during nodulation, only (Takanashi et al., 2012). Therefore LjABCB1 is suggested to regulate auxin transport toward nodulation tissues, where auxins play an important role for the formation of nodule vasculature (Pacios-Bras et al., 2003). AtABCB9 is possibly an auxin transporter involved in pollen germination (Wang et al., 2008). AtABCB24 is mitochondrial expressed and it is involved in iron homeostasis but not in auxin transport. AtABCB24 is included in this analysis as outsider compared to ABCBs that regulate auxin transport. AtABCB14 was initially characterized as a malate importer involved in the modulation of stomatal response to CO_2 (Lee et al., 2008). Together with AtABCB15,

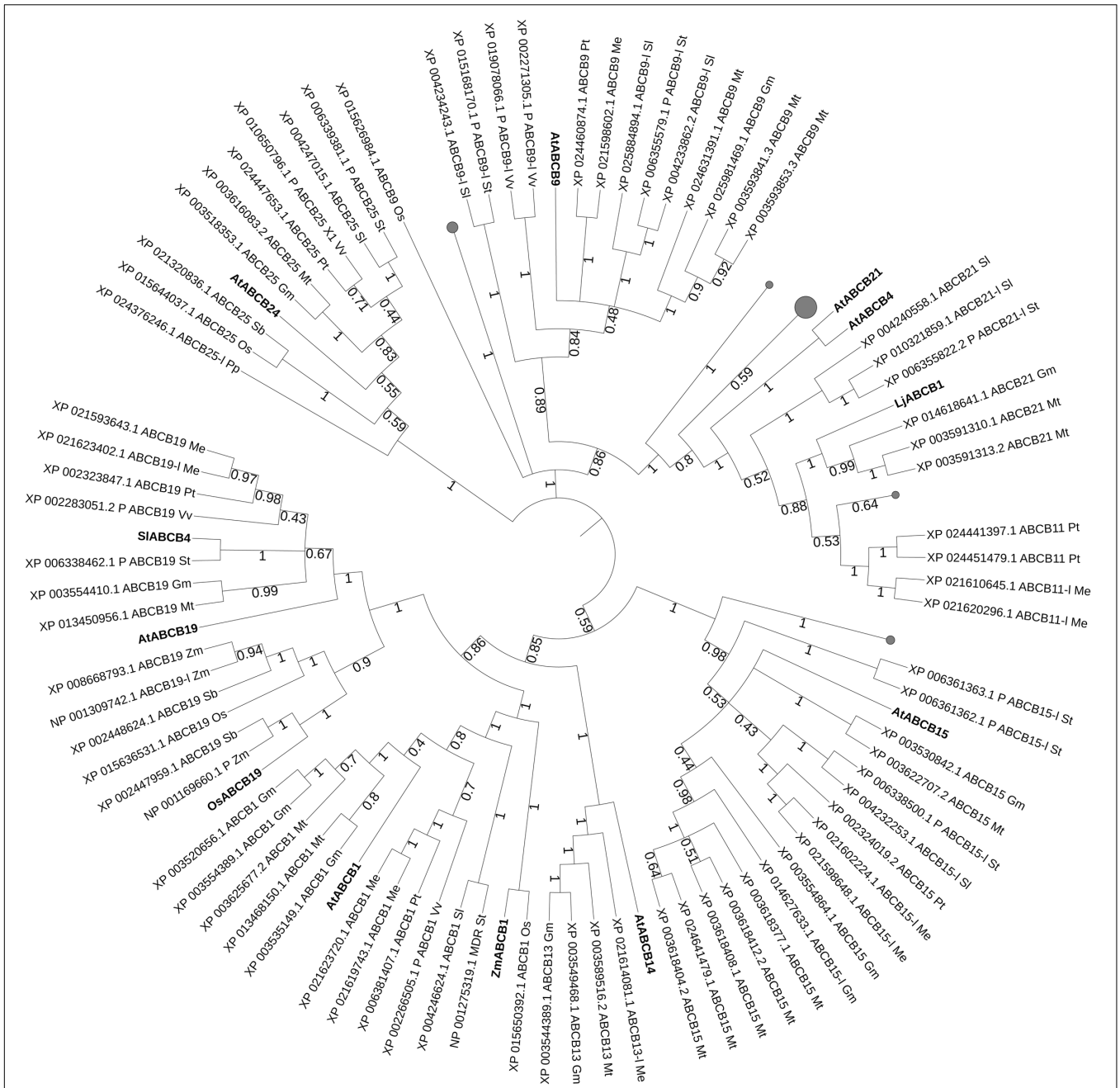

FIGURE 2 | Best hits (sequence identity > 70%) to characterized ABCB proteins. Sequences from *Glycine max* (*Gm*, soya bean), *Populus trichocarpa* (*Pt*, poplar), *Manihot esculenta* (*Me*, manioc), *Solanum lycopersicum* and *tuberosum* (*Sl*, tomato and *St*, potato), *Medicago truncatula* (*Mt*, barrel medic), *Vitis vinifera* (*Vv*, grapevine), *Triticum aestivum* (*Ta*, wheat), *Oryza sativa* (*Os*, rice), *Lotus japonicus* (*Lj*), *Physcomitrella patens* (*Pp*), *Marchantia polymorpha* (*Mp*), *Hordeum vulgare* (*Hv*, barley), *Sorghum bicolor* (*Sb*), and *Zea mays* (*Zm*, maize). P, predicted; -l, -like. Bootstrap n: 100. Branches with bootstrap values < 0.4 are deleted. Maximum 3 isoforms displayed for size restriction. Gray circles (proportionally sized) represent collapsed nodes for size restrictions. The tree with the highest log likelihood (–122794.47) is shown. The analysis involved 136 amino acid sequences. There were a total of 1524 positions in the final dataset.

AtABCB14 is expressed in vasculature tissues of stems and they both participate in auxin transport along the inflorescence (Kaneda et al., 2011).

Interestingly, most of the characterized ABCBs cluster together in a group clearly separated from most of the "land plants" candidates (**Supplementary Figure S1**), showing that the search for orthology through sequence homology was possible and successful in the ABCB family. Out of this cluster, LjABCB1 is part of a leaf comprising several *Fabaceae* candidates. AtABCB21 is proximal to *Arabidopsis lyrata* isoforms and other *Brassicaceae* candidates.

ABCC SUBFAMILY AND HINTS FOR VACUOLAR HORMONAL IMPORT

Plant ABC class C transporters, previously known as multidrug resistant-associated proteins (MRPs), were first described as being involved in barley (*Hordeum vulgare*) xenobiotics detoxification mechanisms (Martinoia et al., 1993). Recently, Lane et al. (2016) investigated the diversity of ABC transporters across plants via the One Thousand Plants Transcriptome Project[1]. Not surprisingly, algae presented a smaller number of unique ABCC genes when compared to other analyzed plant taxa, which may reflect algae life style and cellular organization, as algae in their living environments have direct access to nutrients.

Genome analysis predicts that Arabidopsis contains a total of 15 ABCC genes, *Brassica rapa* 21, *Hevea brasiliensis* 3 (such low number possibly due to the absence of typical central vacuoles), grapevine 26, lotus 17, maize 13, rice 17, tomato 26, and wheat 18 (Sánchez-Fernández et al., 2001; Garcia et al., 2004; Sugiyama et al., 2006; Çakır and Kılıçkaya, 2013; Pang et al., 2013; Bhati et al., 2015; Zhiyi et al., 2015; Chen et al., 2017; Ofori et al., 2018b).

Arabidopsis ABCC transporters are likely tonoplast localized (Jaquinod et al., 2007; Kang et al., 2011) and the same was suggested for ABCCs of grapevine and rice (Francisco et al., 2013; Song et al., 2014). Vacuoles are multifunctional organelles that accumulate chemically diverse compounds and exert many fundamental functions *in planta*, such as detoxification and storage of nutrients. Plants import into the vacuole endogenous compounds and xenobiotics mostly conjugated to polar β-D-glucosides, glutathione and amino acids. Alternatively or additionally, these compounds are excreted to the apoplast but not via ABCC. As recently discussed, vacuoles are also proposed to play a role in plant hormone homeostasis (Martinoia, 2018). Several reports identified hormones and/or hormone conjugates in the vacuolar sap. Gibberellin conjugates have been isolated in cowpea (*Vigna unguiculata*) and barley vacuoles (Garcia-Martinez et al., 1981). In Arabidopsis, indole-3-acetic acid (IAA), IAA conjugates, CKs and CK glucosides were quantified in isolated vacuoles (Ranocha et al., 2013; Jiskrová et al., 2016). Abscisic acid glucosyl ester (ABA-GE) has been described to accumulate in vacuoles of broad bean (*Vicia faba*) (Bray and Zeevaart, 1985) and in cell suspension cultures of tomato (Lehmann and Glund, 1986). The conjugated forms of the phenolic compound salicylic acid (SA), which represent most of the synthesized SA (Rivas-San Vicente and Plasencia, 2011) are stored in tobacco and Arabidopsis vacuoles (Dean and Mills, 2004; Dean et al., 2005; Vaca et al., 2017). In higher plants, hormone conjugates exist in two major forms: amine-linked bound to amino acids or proteins and ester-linked to sugars, (Staswick, 2009; Ludwig-Müller, 2011; Piotrowska and Bajguz, 2011; Korasick et al., 2013). It is still a matter of debate if these hormonal forms should be considered inactive and if the accumulation of these modified forms efficiently contributes to hormonal homeostasis. In some cases, hormone conjugates have been shown to have specific roles in plant development. It was reported (Staswick, 2009) that in Arabidopsis Jasmonic acid-Tryptophan (JA-Trp) and IAA-Trp interfere with root gravitropism. Recently, *VAS2/GH3.17* (IAA-amido synthetase Gretchen Hagen 3), which is predominantly expressed in hypocotyls, was shown to regulate hypocotyl growth. It was observed that *vas2* mutants accumulate higher levels of IAA, low levels of IAA-Glutamate (IAA-Glu) and presented longer hypocotyls under normal growth conditions compared to wild-type (WT) plants (Zheng et al., 2016). Latest studies also revealed a link between auxin-conjugates and ER-localized auxin carriers (Mravec et al., 2009; Barbez et al., 2012; Ding et al., 2012). *PIN5* and *PIN8* encode functional auxin transporters that regulate intracellular auxin homeostasis (Mravec et al., 2009; Ding et al., 2012). The analysis of *pin5* mutant phenotypes showed defects in lateral root initiation as well as in root and hypocotyl growth. Defective growth in root, hypocotyl and not fully expanded, epinastic cotyledons were present in *PIN5* gain-of-function mutants (Mravec et al., 2009). Auxin metabolic profile confirmed that free IAA levels decreased whereas the capacity to produce amino acid conjugates such as IAA–Aspartate (IAA-Asp) or IAA-Glu upon PIN5 induction increased (Mravec et al., 2009). PIN8 is expressed in the Arabidopsis male gametophyte and has a role in pollen development and functionality. Decreased transmission ability through the male gametophyte was observed in *pin8* mutants compared to wild-type pollen (Ding et al., 2012). In *pin8* mutants no significant alteration of free IAA was observed. However, in *PIN8* overexpressor lines free IAA levels increased whereas IAA-Asp and IAA-Glu decreased. Through results of complementation assays the authors suggested antagonistic roles of PIN5 and PIN8 (Ding et al., 2012). Similarly to PIN5, PILS2 and PILS5 activity was shown to increase free IAA levels and reduce IAA-Asp and IAA-Glu, presumably by regulating auxin compartmentalization into the ER lumen (Barbez et al., 2012). How the activity of IAA-amino synthetases, which biosynthesize the amino-IAA conjugates, and the above PINs and PILS activity is integrated is, however, still poorly understood.

The ABCCs have been long proposed to be involved in the vacuolar import of hormone conjugates, e.g., in case of ABA. ABA-GE was reported to be transported into mesophyll vacuoles via ABC protein-type dependent mechanism (Burla et al., 2013). The vacuolar conjugate ABA-GE was shown to be an important source of free ABA when plants experience abiotic stresses, via the activation of vacuolar β-glucosidase (BG2) under osmotic stress that induces the consequent hydrolyses of ABA-GE and release of free ABA (Xu et al., 2012). Arabidopsis AtABCC1 and especially AtABCC2, when heterologously expressed in yeast exhibit ABA-GE transport activity (Burla et al., 2013). However, since no apparent ABA-related phenotype could be observed either in the single mutant *atabcc1* and *atabcc2* or in *atabcc1 atabcc2* double mutants the authors proposed that multi-specific transporters might be involved in the vacuolar sequestration of conjugated hormonal metabolites (Burla et al., 2013). Interestingly, the vacuolar ABA-GE import was not exclusively performed by an ABC-type mechanism since it was shown that also antiporters are involved in such process. This was also the case for SA

[1] 1KP, https://sites.google.com/a/ualberta.ca/onekp/

conjugates, namely SA 2-O-beta-d-glucose (SAG). SAG uptake by vacuolar membrane-enriched vesicles was stimulated by Mg-ATP and inhibited by vanadate and bafilomycin A1, an ABC transporter inhibitor and a vacuolar H^+-ATPase inhibitor, respectively (Vaca et al., 2017).

For what concerns auxin conjugates, the function of such compounds in the vacuole is still unclear. Although certain auxin conjugates can be enzymatically hydrolyzed to produce free auxin (Ludwig-Müller, 2011; Piotrowska and Bajguz, 2011), this unlikely occurs in the vacuole, at least for IAA-amino-conjugates, as most of Arabidopsis auxin amino-hydrolases are predicted to be targeted to the ER (LeClere et al., 2002; Campanella et al., 2003). Up to now the identification of transporters regulating the import of auxin conjugates into the vacuole has not been addressed. So far the only functionally characterized tonoplastic transporter is involved in auxin metabolism. WALLS ARE THIN1 (WAT1) is an H^+-IAA-symporter and it was shown to mediate the export of auxin from vacuoles (Ranocha et al., 2013). *wat1* mutants showed a decrease in stem fiber cell wall thickness that could be restored by exogenous auxin application to stems (Ranocha et al., 2010, 2013).

The maintenance of endogenous CKs levels is mediated by CK dehydrogenases/oxidases, which are the main enzymes mediating CKs degradation. Some members were shown to be localized in the vacuole (Werner et al., 2003; Skalický et al., 2018). Also, very little is known about the role of vacuolar-localized glycosylated-CK; for example, if they can be a source for free CKs as it was demonstrated for ABA-GE (Schroeder and Nambara, 2006). No candidate has yet been proposed for their import into the vacuole.

Although the evidence supports that the vacuole is involved in hormone homeostasis, in general not much is known about the transport mechanisms involved in such processes. According to the recent review by Lefèvre and Boutry (2018) on the substrates of ABC transporters, so far no transporter was univocally functionally characterized to import hormone conjugates into the vacuole.

In the phylogenetic tree with all characterized ABC proteins in plants (**Figure 1**), ABCCs form a cluster with two separated clades. The first comprises the transporters described as being involved in xenobiotics detoxification (AtABCC1, AtABCC2, PtABCC1, and OsABCC1), in naphthoquinone shikonin transport (LeMRP) and anthocyanin import (VvABCC1). The second clade is composed of transporters that are involved in xenobiotics detoxification (AtABCC3), and organic acids/phytate (AtABCC5; TaABCC13). In the attempt to expand our analysis to yet non-functional characterized ABCC that might putatively be involved in hormonal transport in plant models and crops, we performed a phylogenetic analysis excluding the characterized proteins of **Figure 1** and including instead Arabidopsis, less characterized ABCCs (**Figure 3**), where function or substrate specificity are not yet fully understood.

Several clusters could be identified that could help a future characterization of ABCC protein in several plant species. AtABCC4 and AtABCC14 cluster with predicted ABCC4 proteins from manioc, one candidate from soya bean and one from grapevine. Although it was initially hypothesized that AtABCC14 could be involved in ABA-GE transport, membrane vesicles isolated from yeast heterologously expressing AtABCC14 did not exhibit detectable ABA-GE transport activity (Burla et al., 2013). Up-to-date no ABCC transporter was reported to be exclusively/directly involved in the vacuolar uptake of hormone conjugates possibly due to functional redundancy of several elements of this family, as also highlighted in the phylogeny analysis (**Figure 3**). In addition, other classes of vacuolar transporters, e.g., MATES could also participate in this process (Burla et al., 2013; Vaca et al., 2017). Strategies that could allow us a wider identification of novel functions of ABCC proteins could include simultaneous knockouts against redundancy, and overexpressing transgenic lines of multiple ABCC transporters. Genome editing technologies, such as CRISPR/Cas9 could be a powerful tool to accelerate our progresses in this field.

ABCG SUBFAMILY AND STRIGOLACTONE TRANSPORT

Research on hormones belonging to the strigolactone (SL) family began in 1960s, when plant root exudates triggering the germination of parasitic weeds of the Striga family were discovered and named *Strigol* (Cook et al., 1966). Only in 2005 SLs were reported as inducer of hyphal branching in symbiotic arbuscular mycorrhizal fungi belonging to the ancient phylum *Glomeromycota* (Akiyama et al., 2005). In 2008 SLs were further characterized as regulators of plant shoot lateral branching (Gomez-Roldan et al., 2008) and later as phytohormones shaping above and below ground plant architecture (Al-Babili and Bouwmeester, 2015).

In recent years, SL research groups discovered new functions of the SL signaling pathway in different plant species (Goldwasser et al., 2008; Lopez-Raez et al., 2008; Liang et al., 2010; Proust et al., 2011; Delaux et al., 2012; Hamiaux et al., 2012; Kretzschmar et al., 2012; Rasmussen et al., 2012; An et al., 2016; Charnikhova et al., 2017; Rozpądek et al., 2018), thus showing the conservation of the SL signaling pathway in the plant kingdom from green algae to land plants. SL integrates plant development with environmental stimuli such as nutrient abundance, plant-rhizosphere symbioses and light quality (Nagata et al., 2016). Nowadays the scientific community holds a strong knowledge on SL biosynthesis, SL signaling (Lopez-Obando et al., 2015) and moved several steps toward the discovery of SL functions in mono- and di-cotyledon species, e.g., Arabidopsis (Sorefan et al., 2003; Booker et al., 2004, 2005; Bennett and Leyser, 2014; Brewer et al., 2016), rice (Song et al., 2017), *Fabaceae* (Foo et al., 2001; Yoneyama et al., 2008; Dun et al., 2013) and *Solanaceae* (Lopez-Raez et al., 2008; Drummond et al., 2011, 2015; Kretzschmar et al., 2012; Sasse et al., 2015; Xie et al., 2015). Several SL-driven developmental modules have been shown to be highly conserved in the plant kingdom, like shoot lateral branching, root hair elongation, leaf aging and crosstalk with most phytohormones. The recent development of SL-based strategies seems endless, going from approaches targeted

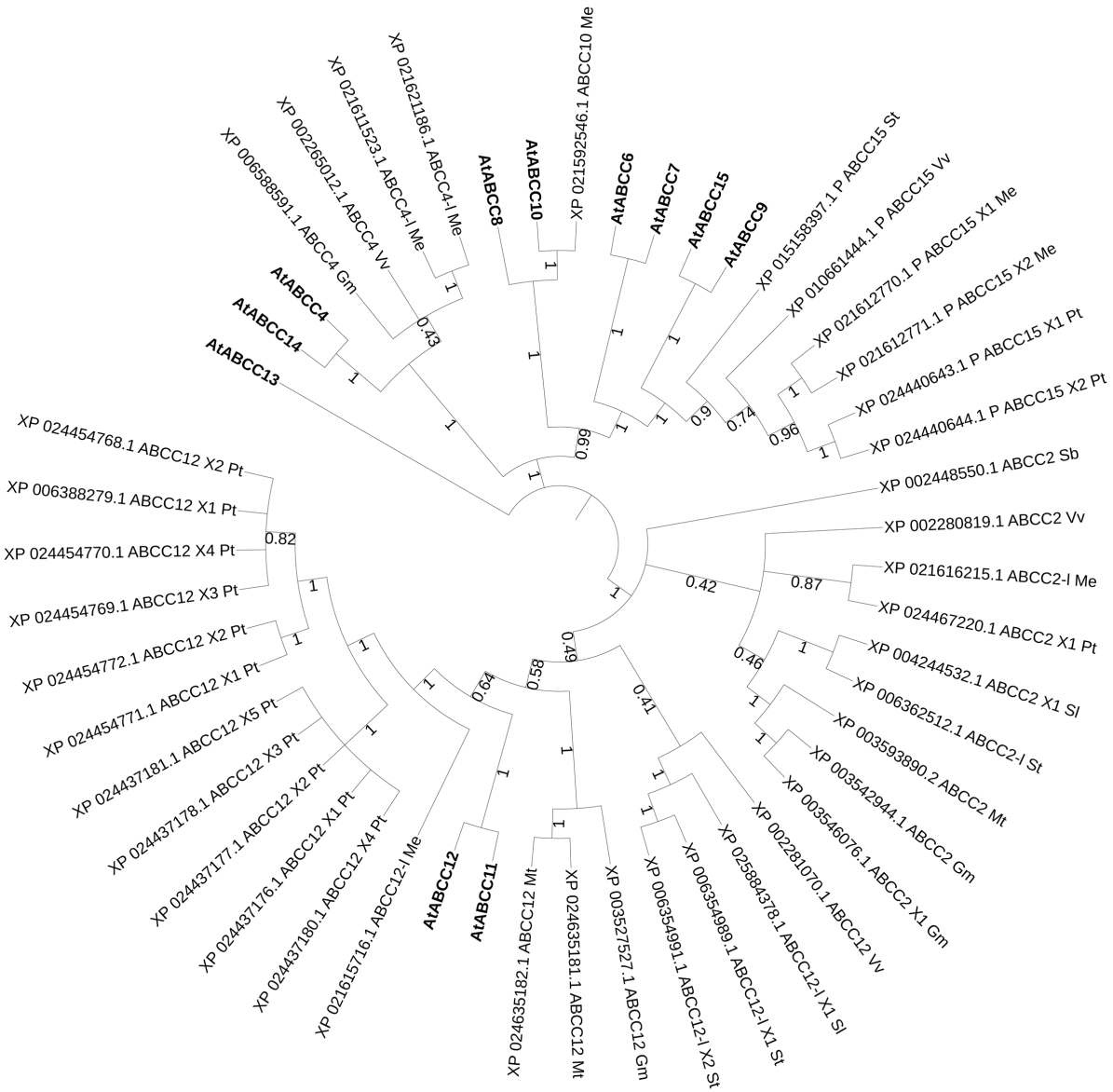

FIGURE 3 | Best hits (sequence identity > 70%) homolog to poorly-characterized ABCC transporters in Arabidopsis. Sequences from *Glycine max* (*Gm*, soya bean), *Populus trichocarpa* (*Pt*, poplar), *Manihot esculenta* (*Me*, manioc), *Solanum lycopersicum* and t*uberosum* (*Sl*, tomato and *St*, potato), *Medicago truncatula* (*Mt*, barrel medic), *Vitis vinifera* (*Vv*, grapevine), *Triticum aestivum* (*Ta*, wheat), *Oryza sativa* (*Os*, rice), *Lotus japonicus* (*Lj*), *Physcomitrella patens* (*Pp*), *Marchantia polymorpha* (*Mp*), *Hordeum vulgare* (*Hv*, barley), *Sorghum bicolor* (*Sb*) and *Zea mays* (*Zm*, maize). P, predicted; -l, -like. Bootstrap n: 100. Branches with bootstrap values < 0.4 are deleted. Maximum 5 isoforms displayed for size restriction. Gray circles (proportionally sized) represent: collapsed nodes for size restriction. The tree with the highest log likelihood (–31375.71) is shown. The analysis involved 50 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 1014 positions in the final dataset.

to the improvement of good agricultural practices (Pavan et al., 2016; Cochetel et al., 2018; Liu et al., 2018a; Mohemed et al., 2018) to the amelioration of human health through the recently revealed apoptotic function of SL mimics in different kinds of cancers and diseases (Modi et al., 2017, 2018; Hasan et al., 2018).

Several factors affect SL activity in plant tissues and in the rhizosphere. First, in contrast to other hormone-receptor complexes, SLs are reported to permanently bind their heterodimeric receptor MORE AXILLARY GROWTH2/DWARF14, which after activation is targeted to degradation (Shabek et al., 2018; Yao et al., 2018). This means that the receptor-ligand complex might negatively regulate the amount of free SLs in the target tissue. Developmental and environmental signals adjust the amount of SL amounts in plants and rhizosphere, too: SL is highly synthetized in roots and its synthesis is induced, e.g., by high red/far-red ratios or low nutrient (mainly phosphate or nitrogen) conditions

(Breuillin et al., 2010; Foo et al., 2013; Nagata et al., 2015; Kameoka and Kyozuka, 2018). Another factor that was shown to regulate the availability of SL in and out plants is the SL transporter PLEIOTROPIC DRUG RESISTANCE 1 from *Petunia hybrida* (PhPDR1). PhPDR1 is an ABC class G protein, originally isolated in petunia (Kretzschmar et al., 2012). PhPDR1 is expressed in root tips and in specialized root cortex cells, named hypodermal passage cells (HPCs) that are unsuberized gates for mycorrhizal hyphae and exudation points for SLs. HPC abundance is regulated by environmental signaling and plant age (Sharda and Koide, 2008). PhPDR1 is also expressed in the shoot lateral axils close to dormant lateral buds, and it is suggested to transport SLs into the buds thus contributing to the inhibition of lateral bud outgrowth. At support of this hypothesis, mutants for *pdr1* exude little SL to the rhizosphere, their roots are scarcely mycorrhized, shoots are heavily branched and *pdr1* seedlings accumulate SLs in biosynthetically active tissues such as the root tip. PhPDR1 is asymmetrically localized in the plasma membrane of cells that transport SLs (Sasse et al., 2015), supporting the hypothesis that PhPDR1 can directionally export SLs from SL biosynthetic-tissues and additionally release a SL-driven negative feedback on the expression of its biosynthetic enzyme *CAROTENOID CLEAVAGE DIOXYGENASE1/DECREASED APICAL DOMINANCE1* (*CCD8/DAD1*). The presence of this feedback regulation between SL transport and synthesis was also assayed by altering PhPDR1 levels with a *PhPDR1* overexpressor based strategy (PDR1 OE) (Liu et al., 2018b). PDR1 OE transgenic plants contained low SL amounts in leaves and a stay-green phenotype was induced, suggesting that PDR1 OE could counteract the reported SL-driven leaf senescence (Yamada and Umehara, 2015). Also, PDR1 OE plants exuded higher than WT amounts of SLs to the rhizosphere and could quickly induce mycorrhization and parasitic weed germination at higher levels than the WT. Last but not least, PDR1 OE plants increased their root and shoot biomass by inducing lateral root development, stem secondary growth and increased plant nutrient uptake (phosphate) on poor soil conditions. These positive effects of PDR1 OE on plant biomass production were also present in simulated-microgravity conditions (Liu et al., 2018a) that could negatively influence the development/branching of mycorrhizal fungi. These several changes in plant development and plant-fungal interactions caused by different expression levels of PhPDR1 show that transport and allocation of SL operated by PhPDR1 play a prominent role in the efficiency of the SL signaling pathway and support the hypothesis that phytohormonal transport is key to regulation of plant development and plant-environment interactions.

What regulates the expression level, cellular and sub-cellular localization of PhPDR1, so that SL transport can be finely tuned in space and time? Does SL transport require an ABCG protein only in *P. hybrida*? Until now, these points were difficult to address. The fact that in Arabidopsis the sequence homolog of PhPDR1 is the ABA transporter ABCG40 (Kang et al., 2010) and that PhPDR1 does not transport ABA (Kretzschmar et al., 2012) did not speed up the investigation of SL transport out of petunia, as the rich molecular too-box, small size and fast developmental speed of Arabidopsis could not be exploited, not considering that Arabidopsis is seen as non-host to arbuscular mycorrhizal fungi, despite that it was shown to have the potential to be colonized (Veiga et al., 2013). A likely PhPDR1 ortholog in *Nicotiana tabacum*, NtPDR6, was recently reported (Xie et al., 2015), but no bioassays or transport experiments were shown yet at proof that NtPDR6 is a SL transporter. An ongoing research line in *M. truncatula* strongly suggests that MtPDR23 (Banasiak and Jasiński, 2014) is the ortholog of PhPDR1. Confirmation of this hypothesis will be important to show that active SL transport is also required out of *Solanaceae* and even in plants without HPCs. We report here that the PDR1/-like transporters above mentioned share a high sequence similarity, higher than with AtABCG40 or its best scoring sequence homologs. More in detail, BLAST hits similar to NtPDR6 and PhPDR1 from a subset of land plant accessions (**Supplementary Figure S2**) comprise sequence homologs from *Capsicum annuum* (*Can*), *S. tuberosum* (*St*)/*S. lycopersicum* (*Sl*)/*Solanum pennellii* (*Sp*), *N. sylvestris* (*Ns*)/*N. tabacum* (*Nt*)/*Nicotiana attenuata* (*Na*) and *Nicotiana tomentosiformis* (*Nto*). NtPDR6 and PhPDR1 are proximal to PDR1-like candidates from *Ipomoea nil* (*In*, Japanese morning glory). MtPDR23 is separated and clusters with a candidate from *Cicer arietinum* (*Ca*, chickpea) and it is a subgroup of a clade containing MtPDR23-like candidates from *Cajanus cajan* (*Ccaj*), *Glycine max* (*Gm*), *Phaseolus vulgaris* (*Pv*), and *Vigna radiata* (*Vr*). AtABCG40 forms a clade unrelated to SL characterized transporters together with ABCG40/PDR12 candidates from *A. thaliana*/*A. lyrata* (*Al*), the related *Capsella rubella* (*Cr*), and proximal to ABCG40 candidate from *Camelina sativa* (*Cs*), *Brassica oleracea* (*Bo*)/*Brassica napus* (*Bn*)/*B. rapa* (*Br*), *Raphanus sativus* (*Rs*) and *Eutrema salsugineum* (*Es*).

We then took advantage of the three published SL transporters to run phylogenetic analyses for isolating SL transporter candidates in staple food/economically relevant model plants: soya bean, poplar, manioc, tomato, potato, barrel medic, grapevine, wheat, rice, *L. japonicus*, *P. patens*, *M. polymorpha*, barley, *S. bicolor* and maize. The first 100 unique homolog hits (or the ones with sequence identity > 70%) to MtPDR23, PhPDR1 and NtPDR6 were selected. No grass sequence homologs clustered together with the known SL transporters (**Figure 4**). Predicted PDR1-like hits from tomato and from potato clustered with PhPDR1 and NtPDR6. Also, several not characterized PDR1 and isoforms from *Glycine max* grouped with MtPDR23, as well as from *P. trichocarpa*, *M. esculenta*, and *V. vinifera*. Some, but not all ABC transporters are reported to be induced by their substrate (Lefèvre and Boutry, 2018). We previously showed that PhPDR1 but not its closest sequence homolog in Petunia (PhPDR4) is induced by GR24, a SL mimic (Borghi et al., 2016). We suggest therefore that the sensitivity to GR24 or to a SL mimic might help screening for PDR1 putative candidates out of the results from this phylogenetic analysis. From the additional phylogenetic analysis based on all characterized ABC transporters in plants up-to-date,

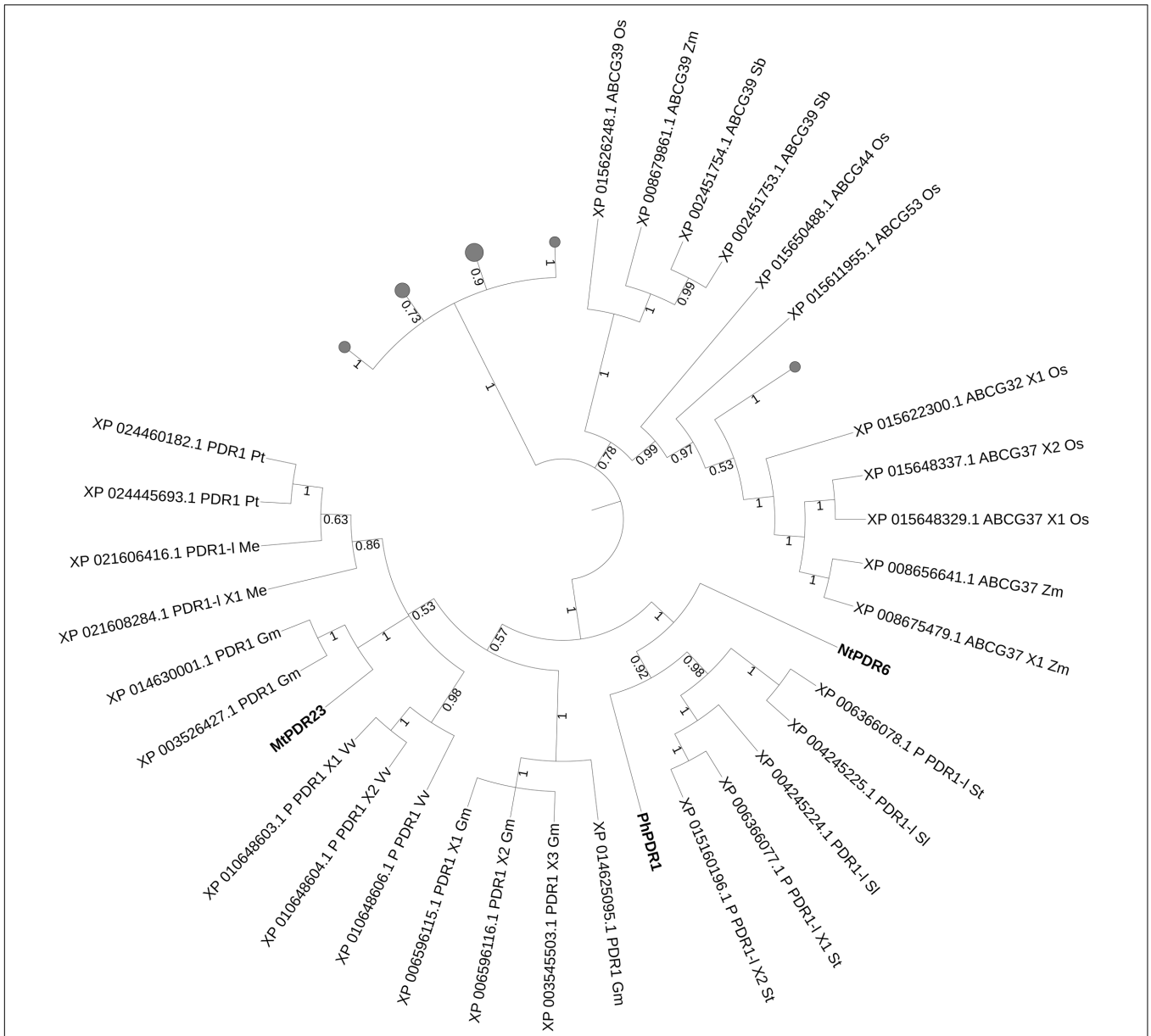

FIGURE 4 | Best hits (sequence identity > 70%) to published SL transporters MtPDR23, PhPDR1, and NtPDR6. Sequences from *Glycine max* (*Gm*, soya bean), *Populus trichocarpa* (*Pt*, poplar), *Manihot esculenta* (*Me*, manioc), *Solanum lycopersicum* and t*uberosum* (*Sl*, tomato and *St*, potato), *Medicago truncatula* (*Mt*, barrel medic), *Vitis vinifera* (*Vv*, grapevine), *Triticum aestivum* (*Ta*, wheat), *Oryza sativa* (*Os*, rice), *Lotus japonicus* (*Lj*), *Physcomitrella patens* (*Pp*), *Marchantia polymorpha* (*Mp*), *Hordeum vulgare* (*Hv*, barley), *Sorghum bicolor* (*Sb*) and *Zea mays* (*Zm*, maize). P, predicted; -l, -like. Bootstrap n: 100. Branches with bootstrap values < 0.4 are deleted. Maximum 3 isoforms displayed for size restriction. Gray circles (proportionally sized) represent collapsed nodes for size restriction. The tree with the highest log likelihood (–77409.74) is shown. The analysis involved 112 amino acid sequences. There were a total of 1755 positions in the final dataset.

NtPDR6, MtPDR23, and PhPDR1 (violet clade) form a well-separated group with StPDR4 and CsPDR12 (**Figure 1**). *StPDR4* (Ruocco et al., 2011) expression levels are induced by heavy metals; *CsPDR12* by phytohormones (Migocka et al., 2012). The authors who characterized CsPDR12 speculate these ABC proteins might be involved in the transport of ABA or auxinic precursors. Based on this analysis, we suggest that CsPDR12 and StPDR4 could be further tested for a possible role in SL transport.

SL IMPORTANCE FOR CROP DESIGN

Strategies focused on the alteration of SL synthesis and/or allocation are promising for the promotion of plant biomass production on nutrient scarce soils. Previous projects based on low SL exuding varieties such as NERICA *New Rice for Africa* (Cissoko et al., 2011) or *S. bicolor* (Mohemed et al., 2018) proved to be successful by reducing the germination of parasitic weeds in arable lands of subtropical areas. In farm lands with no

parasitic weeds, enhanced SL exudation could on the contrary improve mycorrhization and likely plant nutrient uptake. Or, alternatively, high SL exuding plants could be included in push-pull strategies, where parasitic-weed infected plants are physically removed from the fields before planting the crop of interest (Parker, 2012). As well, high SL exuding non-host plants to parasitic-weeds could drive suicidal germination (Kgosi et al., 2012) and help clearing agriculture fields before usage, or high mycorrhization levels could induce protection against parasitic weed as shown with *Z. mays* (Othira et al., 2012). Low SL biosynthesis obtained either by knock out or breeding equally affects root and shoot architectures with effects not always desirable for agricultural practices. For example, the loss of control on lateral bud outgrowth obtained in SL biosynthesis mutants decreases the germination of parasitic weeds but increases lateral branching/foliage also when not needed and reduces internode elongation. In contrast, plants with high SL exudation and/or production are advantaged on nutrient scarce soils but also show apical dominance, detrimental in case fruits/seeds are produced on lateral branches. As well, such plants would promote higher germination of parasitic weed seeds if present in the soil. The development of new techniques affecting SL transport/synthesis only above or belowground might be a further step to select/design breeds with enhanced plant–microbe symbioses but with no bushy/dwarf shoot architectures or plants with regulated shoot radial/lateral growth without the collateral germination of detrimental weeds.

ABC CLASS G TRANSPORTERS AND RESISTANCE TO PLANT–PATHOGENS

Latterly, most studies on the role of hormones in plant–pathogen defense focused on classical defense hormones: jasmonic acid (JA), ethylene (ET) and SA (Glazebrook, 2005). New, ongoing progress has revealed more complex hormonal crosstalk with additional hormones such as auxins (Kazan and Manners, 2009), ABA (Cao et al., 2011), CKs (Choi et al., 2014), and brassinosteroids (Belkhadir et al., 2012). These interactions allow fast defense reactions against plant–pathogens (Berens et al., 2017). We highlight here the up-to-date knowledge on ABC transporters which regulate the distribution of defense hormones. AtJAT1/AtABCG16 modulates the intracellular distribution of JA by mediating both cellular efflux of JA and nuclear influx of jasmonoyl-isoleucine (JA-Ile) (Li et al., 2017), the latter critical to activate JA signaling in the nucleus. Interestingly, AtABCG16 clusters with several ABC class G transporters (**Figure 1**), which are reported as lipid/lipid-related molecules transporters. This phylogenetic proximity of AtABCG1 and AtABCG16 is supported by their shared function as transporters of hydrophobic molecules for pollen coat formation (Yadav et al., 2014; Yim et al., 2016).

Apart from AtABCG16, several ABC class G transporters regulate plant–pathogen defense by orchestrating SA and JA signaling pathways and/or actively exuding compounds with herbivore/fungal/bacterial toxicity toward the plant surfaces (Hwang et al., 2016; Khare et al., 2017). Phylogenetic analyses with up-to-date characterized ABC transporters in plants (**Figure 1**) show the presence of a large cluster for plant–pathogen defense. This cluster contains mainly players characterized for biotic stress resistance in grapevine, lotus and several *Solanaceae*. The expression level of some of these transporters is induced by the presence of Pathogen-Associated Molecular Pattern (PAMP)-triggers and/or with the activation of a pathogen-defense, hormonal-signaling pathway. Like in the case of elicitin INF1, an elicitor protein secreted by the potato late blight pathogen *Phytophthora infestans* for NbPDR1a/b and NbPDR2a/b (Pierman et al., 2017), or *Phytophthora medicaginis* cell-wall oligosaccharides for MtABCG10 (Jasinski et al., 2009) or MeJA for NpPDR1, NtPDR1, and LjABCG1 (Grec et al., 2003; Crouzet et al., 2013; Sugiyama et al., 2015). GmPDR12 from soya bean is also induced by MeJa and SA (Eichhorn et al., 2006).

Major substrates of ABC transporters belonging to this cluster are secondary metabolites: terpenoids, alkaloids and flavonoids. The *Solanaceae* NtPDR1, NpPDR1, NbPDR1, and NbPDR2 support the transport of terpenes (Stukkens et al., 2005; Shibata et al., 2016; Pierman et al., 2017; Rin et al., 2017). AtABCG34 secretes to the leaf surface camalexin that is a major phytoalexin in Arabidopsis and thereby fights *Alternaria brassicicola* infection (Khare et al., 2017). The mutant of *atabcg34* was screened out of altered sensitivity to sclareol, a natural diterpene known to act as an antimicrobial and defense-related molecule, whereas the mutant for *atabcg39*, the closest homolog of *AtABCG34* has no altered sensitivity to sclareol (Khare et al., 2017). AtABCG39 plays a role in the cellular import of non-selective paraquat (Xi et al., 2012). MtABCG10 might modulate isoflavonoids amounts related to phytoalexin production (Banasiak et al., 2013). VvABCG44 is suggested as promising transporter of phenolic compounds, such as resveratrol (Suzuki et al., 2014). Last but not least, PhPDR2 is involved in the accumulation of steroidal compounds in leaves and trichomes (petuniasterone precursors) (Sasse et al., 2016).

Interestingly, *Lr34/Yr18/Pm38* multi-pathogen resistance gene exists as solo player and it is not included in the above cluster (**Figure 1**). The wheat Lr34-resistance (Lr34res) allele increases durable disease resistance against multiple fungal pathogens in different crop species (Krattinger et al., 2009, 2013). Its mechanism of action and substrate are yet unknown. OsABCG50 shares 85% of identity with Lr34 but cannot confer rice blast resistance (Krattinger et al., 2013). Despite being described as ortholog, OsABCG50 does not share a common function with Lr34res as it has the same SNP with Lr34-susceptible allele and not with Lr34res (Krattinger et al., 2013).

Clades with candidates for plant–pathogen defense in crop/model plants are present after phylogenetic analyses with characterized plant-defense ABCs (**Figure 5**). Sequence homologs from selected crop/model plants to the characterized defense-involved transporters were isolated in manioc, barrel medic, grapevine, tomato, potato, soya and sorghum. Several candidates from tomato and potato group together with PhPDR2, StPDR2 and *Nicotiana* spp. PDR1/2. VvABCG44 clusters with and is proximal to multiple grapevine candidates, suggesting that the presence of this compound is mainly, if not only, in grapevine among the analyzed species. A small leaf comprising

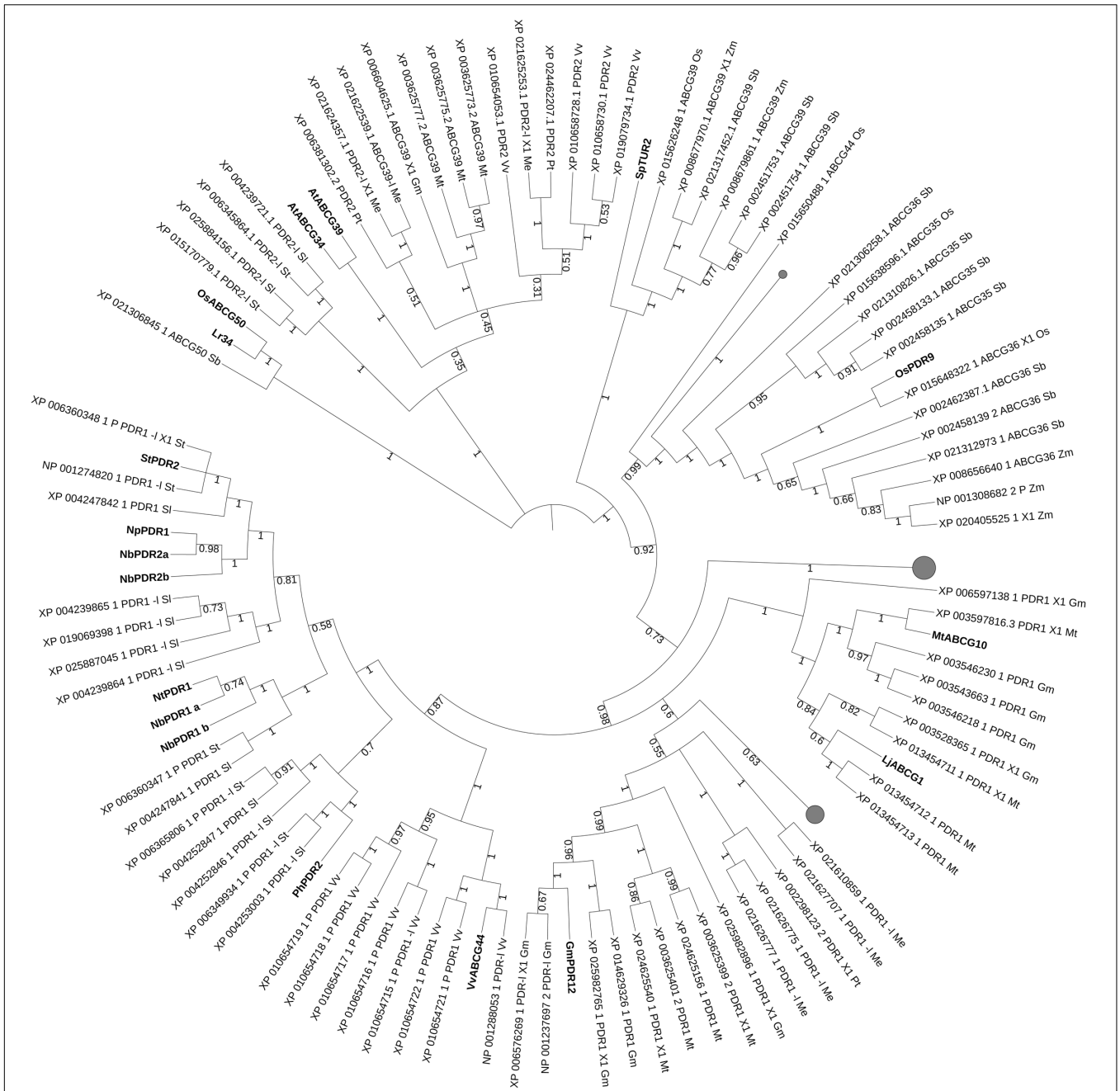

FIGURE 5 | Best hits (sequence identity > 70%) to ABC transporters involved into response to pathogen attack. Sequences from *Glycine max* (*Gm*, soya bean), *Populus trichocarpa* (*Pt*, poplar), *Manihot esculenta* (*Me*, manioc), *Solanum lycopersicum* and t*uberosum* (*Sl*, tomato and *St*, potato), *Medicago truncatula* (*Mt*, barrel medic), *Vitis vinifera* (*Vv*, grapevine), *Triticum aestivum* (*Ta*, wheat), *Oryza sativa* (*Os*, rice), *Lotus japonicus* (*Lj*), *Physcomitrella patens* (*Pp*), *Marchantia polymorpha* (*Mp*), *Hordeum vulgare* (*Hv*, barley), *Sorghum bicolor* (*Sb*) and *Zea mays* (*Zm*, maize). P, predicted; -l, -like. Bootstrap n: 100. No branches deleted as bootstrap values in branches of interest > 0.4. Maximum 1 isoform displayed for size restriction. Gray circles (proportionally sized) represent collapsed nodes for size restriction. The tree with the highest log likelihood (–66697.92) is shown. The analysis involved 130 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 1097 positions in the final dataset.

Lr34 and OsABCG50 shows the presence of a homolog candidate in sorghum. MtABCG10, LjABCG1 and GmPDR12 are proximal to soya and barrel medic transporters, which might be candidates for a role in plant defense. Loss of function of lipid transporters regulating the presence of hydrophobic barriers aimed to protect against harmful environments (Burghardt and Riederer, 2007) can also lead to weaknesses against pathogen attacks. These barriers consist of different types of hydrophobic polymers depending on the organ tissue and several ABC class G transporters regulate the export of each hydrophobic monomers

to the right layer (Do et al., 2018). OsABCG31, from rice and HvABCG31/HvEIBI1, from barley also contribute to cuticle formation (Chen et al., 2011a,b). The cuticular alterations in *osabcg31* mutant plants result in reduction of infection structures of the rice blast fungus *Magnaporthe oryzae* in the plant, as well as in the constitutive up-regulation of genes involved in pathogen resistance (Garroum et al., 2016). The abnormal cuticle formation and the consequent lack of surface lipid molecules/transporters may affect the signaling for plant–pathogen defense.

ABCD SUBFAMILY AND TRANSPORT OF HORMONAL PRECURSORS

Particular classes of multiple ABC transporters participate to the transport of different lipids, including fatty acids, waxes, and sterols. It is a very remarkable feature of ABC proteins in all phyla. In case of human, almost half of ABC proteins participate in the translocation of lipids or lipid-related molecules (Tarling et al., 2013). Numerous ABC transporters *in planta* also contribute to lipid transport and are classified on the three main transported substrates: hydrophobic monomers for cell surface lipid barriers, fatty acid precursors for hormonal biosynthesis and components of membrane lipids (Kang et al., 2011). Deeper reporting on ABC proteins involved in the transport of lipid/lipid related molecules is out of the scope of this review. In spite of that the lipid transporters have reason to be mentioned, because they comprise fatty acid transporters for hormone precursors (Theodoulou et al., 2005). In addition, several ABC class G proteins which transport hydrophobic monomers as components for surface lipid barrier assist the role of SA/JA transporters in plant–pathogen defense function (Aragón et al., 2017; Ziv et al., 2018).

Peroxisomal ABC proteins (ABC class D proteins) contribute to the process of phytohormonal biosynthesis by transporting fatty acids that are hormonal precursors. HvABCD1/2 and AtABCD1 are ABC peroxisomal transporters that can complement yeast mutants for fatty acid beta-oxidation (Mendiondo et al., 2014). AtABCD1/CTS has been implicated in the transport of β-oxidation substrates related to hormone precursors: 12-oxo-phpytodienoic acid (OPDA); JA precursor (Theodoulou et al., 2005; Dave et al., 2011), 2, 4-dichlorophenoxybutric acid (2, 4-DB) and IBA; auxin precursors (Zolman et al., 2001; Hayashi et al., 2002). Blasting these characterized ABCD transporters on a plant-specific, narrow multiple-species database resulted in a number of sequence homology-based, annotated candidates for transport of fatty acids (**Supplementary Figure S3**).

THE DISPERSED ABA TRANSPORTERS

Abscisic acid rapidly accumulates in its target sites in response to a variety of biotic and abiotic stresses, such as pathogen, wounding, drought, radical temperature change or salinity. ABA plays a critical role in plants for overcoming these environmental stresses (Ton et al., 2009; Cutler et al., 2010). ABA research has made great progress during the last century and many different aspects of signal transduction, biosynthesis/catabolism and molecular mechanisms of action have been revealed (Nambara and Marion-Poll, 2005; Cutler et al., 2010; Raghavendra et al., 2010). However, ABA transport was not studied much, partly because ABA was regarded as able to cross the lipid bilayer and enter cells as protonated form and therefore a transporter was considered not necessary (Heilmann et al., 1980). However, results on the kinetics of ABA uptake disprove the mere action of passive diffusion (Windsor et al., 1992; Wilkinson and Davies, 1997). Finally, two different ABC class G ABC proteins were identified as ABA transporters until 2010: AtABCG40, an ABA importer in guard cell plasma-membrane, and AtABCG25, an ABA exporter in vascular tissue where ABA biosynthesis occurs (Kang et al., 2010; Kuromori et al., 2010). Recently, multiple ABA transporters are reported to regulate each cell-, tissue type specific ABA transport also by establishing a complex feedback network with ABA biosynthesis (Kuromori et al., 2018).

When plants suffer water deficit, ABA has to be immediately synthesized and transported from specific biosynthetic sites to the action site thus to cope with drought stress. AtABCG25 pumps *de novo* biosynthetic ABA out of vascular cells into the apoplast (Kuromori et al., 2010). Apoplastic ABA is then rapidly loaded into guard cells via the ABA importer AtABCG40, which is expressed in guard cells to positively regulate stomata closing (Kang et al., 2010). ABA regulates additional developmental processes in plants. *De novo* ABA biosynthesis continuously occurs in the endosperm, but not in embryo, thus to maintain embryo dormancy (Ali-Rachedi et al., 2004; Penfield et al., 2006; Barrero et al., 2010). The endosperm, a single layer tissue surrounding the embryo releases ABA to repress embryonic development and consequent germination (Lee et al., 2010). The cooperation of four different ABA transporters from Arabidopsis ABC class G proteins is responsible for the relocation of ABA to control seed dormancy. AtABCG31 and AtABCG25 are expressed in the endosperm and secrete ABA from the endosperm to the embryo. AtABCG30 and AtABCG40, located in the embryo participate to ABA accumulation in the embryo (Kang et al., 2015). Recently, MtABCG20 was characterized as ABA exporter from *M. truncatula*. MtABCG20 is localized in the plasma membrane of roots and germinating seeds. It secretes ABA from ABA biosynthetic sites in a homo-dimer form and thereby positively affects lateral root primordium formation, inhibits the development of nodule primordia and promotes the embryonic germination in Medicago (Pawela et al., 2019).

The up-to-date characterized ABC proteins involved into ABA transport do not form remarkable clusters. Instead, they are dispersed in different small branches, suggesting that any eventual sequence/domain similarity is not large or not conserved enough in transporters for ABA, or that ABA transporters do not share significant similarities at all.

Already reported ABA transporters in Arabidopsis belong also to different protein families: ABC protein superfamily, nitrate transporter 1/Peptide transporter family (NPF; NPF4.6, used to be called AIT1) (Kanno et al., 2012), and detoxification efflux carrier like DTX50, which is a member of the MATE family (Zhang H. et al., 2014).

AtABCG31, an ABA exporter from the endosperm to the embryo thus regulating seed dormancy (Kang et al., 2015) makes a small group with AtABCG40, ABA importer into guard cells. This cluster is composed by full size ABC class G transporters, called PDR: the multi-functional ABC transporter AtABCG36 (Stein et al., 2006; Kim et al., 2007; Strader and Bartel, 2009) and its ortholog CsPDR8 from cucumber (Migocka et al., 2012), and the monolignol transporter AtABCG29 (Alejandro et al., 2012). AtABCG25 exports ABA from its biosynthetic site to the apoplast (Kuromori et al., 2010) and groups with AtABCG21 and AtABCG22, which are putative ABA importers/related to ABA (Kuromori et al., 2011, 2017). This cluster also includes the CKs transporter AtABCG14 (Ko et al., 2014).

AtABCG30, MtABCG20, and AhATL1 exist as solo players (**Figure 1**). AtABCG30, another embryonic ABA importer regulating seed dormancy together with AtABCG31 (Kang et al., 2015) clusters with a coumarin/IBA transporter from Arabidopsis, AtABCG37 (Ruzicka et al., 2010; Ziegler et al., 2017). MtABCG20, an ABA exporter regulating root morphology and seed germination is close to MtSTR2 that is required for mycorrhizal arbuscule development in Medicago (Zhang et al., 2010). The potential ABA transporter, ABA transporter-like 1(AhATL1; Ah_AQW44869.1) from *Arachis hypogaea* (peanut) belongs to the ABC class G of ABC proteins. AhATL1-overexpressor was shown to reduce drought resistance by inhibiting the drought induced *AtABCG40* expression in guard cells of Arabidopsis (Ge et al., 2017). AhATL1 groups with the floral volatile organic compound transporter PhABCG1 (Adebesin et al., 2017).

CONCLUSION AND OUTLOOK

These phylogenetic analyses on characterized ABC proteins involved in phytohormonal transport and its regulation generated different amounts of potential functional homologs for each ABC subfamily. Still, as long as no bioassays are carried on for testing the hormonal substrate specificity (if any) of such transporters, these results are only hints toward candidate orthologs. Characterized proteins belonging to the ABCB class and their sequence homologs from selected crop and model plants generally did not group into common clades (**Figure 2**). Exceptions were SlABCB4 and AtABCB19, auxin transporters with reported different function during fruit and vegetative development, respectively. The proximal leaves from potato, barrel medic, soya, grapevine, and poplar are candidates for auxin transport in these plant species. The clustering of SlABCB4 and AtABCB19 is conserved after the phylogenetic tree was built on the larger "land plants" group sequence homologs (**Supplementary Figure S1**), with the additional proximity of OsABCB19. Another clade containing characterized ABCB transporters from different plant species consists of ZmABCB1 and AtABCB1. The other characterized candidates are again clearly separated and the sequence homologs present in this setup are mostly from *Brassicaceae*, except for the cluster containing lotus ABCB1 and six *Fabaceae* candidates.

Following the phylogenetic analyses based on SL transport candidates, AtABCG40 and its clade resulted to be no close homologs to known SL transporters (**Figure 1** and **Supplementary Figure S2**). NtPDR6 and PhPDR1 defined a cluster where single candidates from *N. tomentiformis*, *N. sylvestris*, and *N. attenuata* are present. The characterization of these candidates might open the possibility for improving plant nutrient uptake and biomass production in *Nicotiana* species. Pepper and tomato spp. likely underwent duplication of PhPDR1 homologs as two candidates are present in each. An efficient strategy for investigating SL transport in these species, as well as in the tetraploid potato and tobacco might be therefore testing the involvement of these candidates in SL transport with (i) molecular and vesicular approaches and (ii) by targeting the candidates with a CRISPR/Cas9-based strategy thus to (iii) study the effect of eventual SL re-distribution directly *in planta*. MtPDR23 is a promising runner for SL transport in *Fabaceae*. The discovery of additional PhPDR1 orthologs in beans might allow new investigations in this clade, where only single candidates from pea, chickpea and mung bean are present. However, the unicity of these candidates might be as well due to the incomplete coverage of their genomes, e.g., 74% in chickpea (Varshney et al., 2013) and 82% in mung bean (Kang et al., 2014). Grasses were introduced into the phylogenetic analysis (**Figure 4**) together with known SL transporters and a clear separation was obtained from non-grasses candidates. This division suggests that no ABCG protein is involved into the regulation of SL transport in grasses, and that possibly other transporter families regulate SL transport and exudation in crops. Two branches close to MtPDR23 suggest that SL transporters belonging to the PhPDR1 family might be present also in grapevine, manioc and poplar. Despite the low amounts of sequence homologs to ABCD proteins regulating the transport of hormonal precursors (**Supplementary Figure S3**), the two barley homologs clearly indicate two rice proteins as putative candidates for functional conservation.

None of the characterized ABC class C proteins is a high affinity transporter for hormones, although hormones and their conjugate forms were identified in the vacuole. This is the reason why in this review a phylogenetic analysis was run on ABCC candidates with yet weakly characterized/unknown function (**Figure 3**). A CRISPR/Cas9 approach could be set to downregulate the expression of redundant homologs to these Arabidopsis ABCCs, thus to test with *in vitro*, vacuolar transport assays their affinity for hormonal substrates.

The phylogenetic isolation of GmPDR12 and Lr34 does not allow speculating about ortholog candidates for plant defense in other plant species, apart for suggesting a sorghum, yet uncharacterized protein. As well, the *Fabaceae* cluster comprising LjABCG1 and MtABCG10 is not informative on the

function of barrel medic and soya proteins, as no substrates were isolated, yet.

Last but not least, although plant ABC transporters are considered being more specific than their human homologs, at least for their high abundance and wide diversification that occurred during evolution (Hwang et al., 2016), also in plants there are cases of strong substrate ambiguity. In addition to the already mentioned ABCC subfamily, AtABCG37 and ABA transporters, AtABCG36/PDR8/PEN3 is a robust example of absent substrate specificity. AtABCG36 has been reported involved in non-host resistance (Stein et al., 2006), sensitivity to IBA (Strader and Bartel, 2009), heavy metal toxicity resistance (Kim et al., 2007) and its overexpression has been shown to improve drought stress resistance by reducing sodium content in plants (Kim et al., 2010). Likely, rather than substrate specificity, AtABCG36 expression patterns, the factors regulating its activation and transcription levels and its possible recruitment for fast-reactive signaling pathways such as pathogen-resistance might have modeled its functions during evolution. As pointed out in Lefèvre and Boutry (2018), substrates of ABC transporters have been often suggested based on physiological data rather than on direct transport assays. Complementary approaches are needed to confirm supposed substrate specificities and to assay new ones, such as combinations of *in vitro* transport assays with monitoring of ATP hydrolysis in the presence of the ABC transporter of interest and the candidate substrate. In a near future, we foresee that automatized assays aimed to identify ABC transporter substrates, coupled to new knowledge on transcriptional and post-translational regulation of ABC proteins will shed new lights on ABC substrate specificity and ABC functions in plants.

AUTHOR CONTRIBUTIONS

JK and RdBF contributed significant parts of the manuscript. LB was the main coordinator and wrote large part of the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Cytokinin Transporters: Multisite Players in Cytokinin Homeostasis and Signal Distribution

Chang-Jun Liu[1], Yunjun Zhao[1] and Kewei Zhang[2]*

[1] Department of Biology, Brookhaven National Laboratory, Upton, NY, United States, [2] Department of Biology, College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua, China

Cytokinins (CKs) are a group of mobile adenine derivatives that act as chemical signals regulating a variety of biological processes implicated in plant development and stress responses. Their synthesis, homeostasis, and signaling perception evoke complicated intracellular traffic, intercellular movement, and in short- and long-distance translocation. Over nearly two decades, subsets of membrane transporters have been recognized and implicated in the transport of CKs as well as the related adenylates. In this review, we aim to recapitulate the key progresses in exploration of the transporter proteins involved in cytokinin traffic and translocation, discuss their functional implications in the cytokinin-mediated paracrine and long-distance communication, and highlight some knowledge gaps and open issues toward comprehensively understanding the molecular mechanism of membrane transporters in controlling spatiotemporal distribution of cytokinin species.

Keywords: cytokinin, ATP-binding cassette transporter, purine permease, equilibrative nucleoside transporter, isopentenyl adenine, *trans*-zeatin

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****Correspondence:***
Chang-Jun Liu
cliu@bnl.gov

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INTRODUCTION

Cytokinins (CKs) are the mobile adenine derivatives that carry N^6-linked isopentenyl or aromatic side chains. They serve as hormonal signals functioning in a myriad of biological processes, such as cell division and differentiation, seed germination, apical dominance, leaf senescence, root growth, branching and nodulation, nutrient homeostasis, and stress responses (Mok and Mok, 2001; Sakakibara, 2006; Werner and Schmulling, 2009; Kieber and Schaller, 2018). The majority of naturally occurring CKs *in planta* are the N^6-isopentenyl conjugated adenine derivatives, in addition to a small amount of N^6-aromatic CK species. The isoprenoid CKs chemically differ in their side chain structures, including the hydroxylation at the side chain terminus, the stereo-isomeric position, and the hydrocarbon chain saturation status. Correspondingly, they are classified into four main categories: N^6-(Δ^2-isopentenyl) adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DHZ) (Sakakibara, 2006). Structural variations of the side chain may confer substantial differences in chemical properties of CK species, especially for their lipophilicity/hydrophobicity. Predictably, the iP-type CKs that lack hydroxyl moiety at their N^6-linked side chains would possess the highest hydrophobicity compared to the others.

The isoprenoid CKs are generated through *N*-prenylation of adenosine 5′-phosphates (AMP, ADP, or ATP) or tRNAs, which is catalyzed by adenosine 5′-phosphate isopentenyltransferase (IPT) (Kakimoto, 2001; Takei et al., 2001, 2004a; Miyawaki et al., 2006). The resulting product

isopentenyl adenosine 5′-phosphates, i.e., iP nucleotides, are then converted to tZ derivatives by *trans*-hydroxylases, the cytochrome P450 enzymes CYP735A1 and CYP735A2 (Takei et al., 2004b). Both iP- and tZ- nucleotides can be activated by phosphoribohydrolase LONELY GUY (LOG), yielding free CK bases (Kurakawa et al., 2007; Ueda et al., 2007). Alternatively, the formation of free CK bases may potentially undergo a two-step sequential dephosphorylation and deribosylation process. However, only a nucleoside *N*-ribohydrolase that mediates deribosylation reaction has been recognized so far (Jung et al., 2009; Kopecna et al., 2013). The enzymes involved in the two-step conversion *in planta* remain to be further identified.

Cytokinins are synthesized in a number of different cell types in both roots and shoots, and cross-talk with other phytohormones, particularly auxins, to regulate plant growth and development (Sakakibara, 2006). As chemical signals, CKs mediate both local and long-distance communications, and are transported either in short distance among neighboring cells or as acropetal and basipetal messengers translocated in long distance between roots and shoots. In Arabidopsis, tZ- and iP-type of CK species are confirmed as the active forms in respect to their specific recognition by three sensor histidine kinases, AHK2, AHK3, and CRE1/AHK4 (Inoue et al., 2001; Kakimoto, 2003; Romanov et al., 2006; Lomin et al., 2015). The tZ-type CKs are mainly synthesized in roots and transported apoplastically to shoots, which promote the growth of the above-ground parts of the plant (Beveridge et al., 1997; Hirose et al., 2008). In contrast, the iP- and cZ type CKs are the major forms found in phloem and are translocated rootward to transmit messages from shoots to roots (Corbesier et al., 2003; Hirose et al., 2008). The shoot-borne iP-type CKs have been suggested to serve as a signal of nitrogen satiety, regulating root architecture, suppressing nitrogen uptake in the root, and/or modulating nodulation (Sasaki et al., 2014). In addition to nutritional signaling, the shoot-derived iP-type CKs also regulate root development by modulating polar auxin transport and vascular patterning in the root meristem (Bishopp et al., 2011). Interestingly, although the iP-type CKs are considered to be synthesized throughout the whole plant body of Arabidopsis (Miyawaki et al., 2004, 2006; Takei et al., 2004a), a couple of recent studies reveal that iP ribosides can be predominantly synthesized in the roots of young tobacco and Arabidopsis seedlings (Zd'arska et al., 2013; Gelova et al., 2018), which implicate that a shootward transport of iPR via phloem could also be possible, although its underlying mechanism and related physiological functions remain to be explored.

As mobile signals, CKs biosynthesis, metabolism, distribution and perception evoke considerable intra- and inter-cellular movement and translocation. However, compared with the knowledge on transport and distribution of other plant growth and development related phytohormones, such as auxin, our understanding on molecular mechanisms of CK transport is just emerging. During the last two decades, three types of membrane transporters have been recognized and implicated in the CK transmembrane transport and intercellular translocation (**Figure 1** and **Table 1**). These proteins include the subsets of purine permeases (PUPs) and equilibrative nucleoside transporters (ENTs), which act as influx carriers and are implicated in the transport of CK nucleobases or nucleosides, respectively (**Figure 1** and **Table 1**), and the ATP-binding cassette transporter G subfamily member, ABCG14 in Arabidopsis, that acts as an efflux pump involved in long-distance acropetal translocation of the root-born CKs (**Figure 1** and **Table 1**). Over the years, several excellent review articles have summarized the progress in understanding CK transport and in characterizing the related transporters (Hirose et al., 2008; Kudo et al., 2010; Borghi et al., 2015; Lacombe and Achard, 2016; Duran-Medina et al., 2017; Kang et al., 2017; Ko and Helariutta, 2017; Park et al., 2017). In this article, we aim to recapitulate the advances in identifying and characterizing the membrane transporters involved in short- and long-distance translocation of CKs as well as the related metabolites, to discuss their implicated functions in CK biosynthesis, homeostasis, and signal perception, and to tentatively point out some gaps and open questions toward fully understanding molecular mechanism of CK transport.

TRANSPORT PROTEINS POTENTIALLY INVOLVED IN INTRACELLULAR TRAFFIC OF CYTOKININ

Subcellular compartmentation is a key feature of eukaryotic cells to effectively organize their metabolic and signaling processes (Skalicky et al., 2018). At the site of synthesis, the formation, conversion and storage of CKs take place in different compartments of the cell. Correspondingly the key enzymes/proteins involved in the processes are located within different places (Skalicky et al., 2018). IPTs catalyze *N*-prenylation of adenine ribotides, which diverts both the plastidial and cytosolic sources of terpenoid building blocks, the five carbon units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to CK synthesis (Sakakibara, 2006). Among several IPTs identified in Arabidopsis, AtIPT1, 3, and 5 were proved the major players for iP- and tZ-type CKs formation. Their fluorescently tagged proteins were found to primarily localize to the chloroplasts of mesophyll cells (Kasahara et al., 2004). Interestingly, besides its plastidial localization, AtIPT3 can be farnesylated, which directs it to the nucleus. The subcellular localization of farnesylated and non-farnesylated protein is closely correlated with either iP-type or tZ-type CK biosynthesis (Galichet et al., 2008). By contrast, AtIPT2 and 9 catalyze isopentenylation of tRNA to provide a source for cZ-type CKs (Miyawaki et al., 2006). The AtIPT2-GFP fusion appeared within cytoplasm, indicating that AtIPT2 utilizes isoprenoid precursors synthetized via the mevalonate pathway in cytosol for cZ-type CK synthesis. Additionally, AtIPT7-GFP was observed in mitochondria (Kasahara et al., 2004). Different subcellular localization of IPTs implicates multiple sites occurrence of CK species.

After formation of iP nucleotides, their subsequent hydroxylation is catalyzed by cytochrome P450 monooxygenases CYP735A1 and CYP735A2, which yields tZ-type CKs (Takei et al., 2004b). Cytochrome P450s are the integral endoplasmic reticulum (ER)-resident proteins. Therefore, the hydroxylation

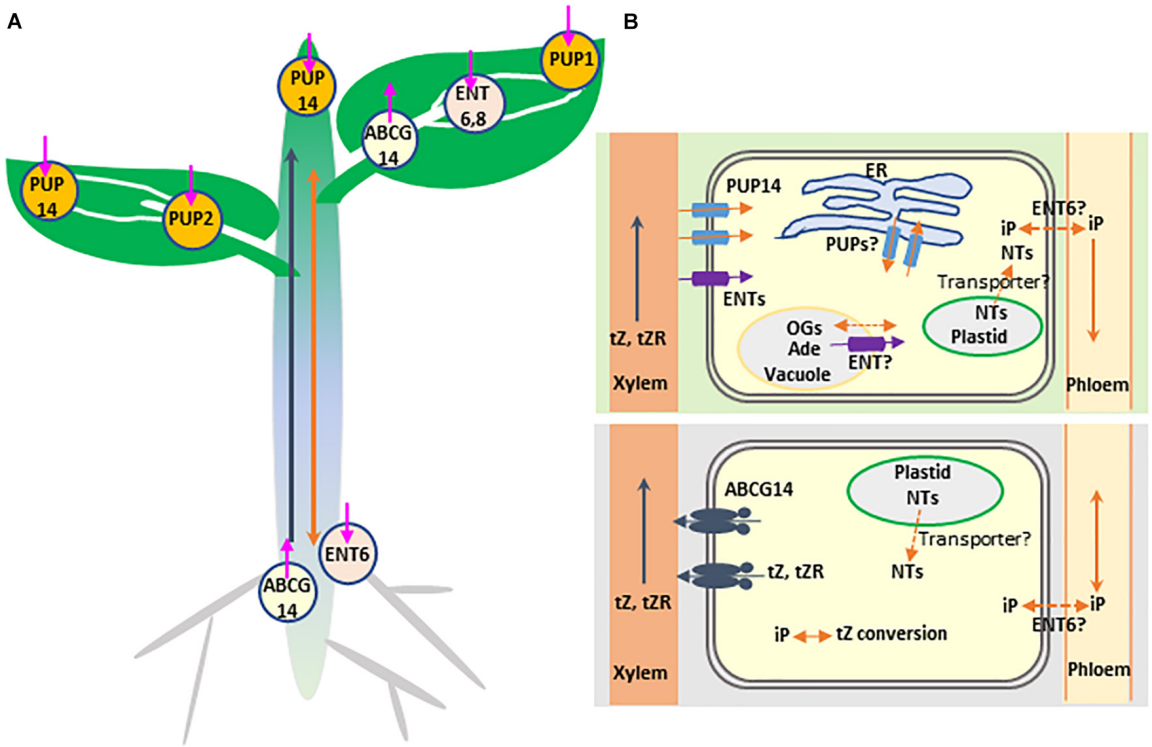

FIGURE 1 | Schematic representation of transporter-mediated long- and short-distance movement of cytokinins in *Arabidopsis thaliana*. **(A)** Involvement of transporters in cytokinin distribution. The determined or the speculated functional sites of cytokinin transporters, based on their expression patterns (see **Table 1** for further details), are mapped on the different tissues. The three types of transporters are indicated with distinctly colored circles. The orientation of the transport is indicated by an arrow pointing inside (influx) or outside (efflux) the circle symbol. Long-distance transport of tZ-type cytokinins from roots to photosynthetic tissues through xylem and the rootward and the hypothetic shootward translocation of iP-type cytokinins through phloem are indicated with blue and orange arrows, respectively. **(B)** Involvement of transporters in the inter-and intra-cellular movements of cytokinins in root (bottom) and shoot (top) cells. The plastid-synthesized iP nucleotides (NTs) may be delivered to the cytoplasm, nucleus, or even endoplasmic reticulum (ER) via transporter activity. ABCG14 as an efflux pump transports tZ-type cytokinins from cytoplasm to apoplast (xylem vessel). PUP14 and ENTs act as importers to take up apoplastic free bases or nucleosides of cytokinins, respectively, into cytoplasm. The ER localized PUP members may involve in sequestering cytokinin species to or out ER lumen. ENT transporter perhaps delivers vacuolar adenosine (Ade) to cytosol; meanwhile, the unidentified transporters might be responsible for the vacuolar sequestration of reversible cytokinin glucosides (OGs). Finally, transport of iP-type cytokinin species in or out of the cells of their synthesis and the signaling target sites as well as phloem may also require transporter proteins; ENT6 was considered as one of the candidates.

reaction of CKs must take place on the surface of ER. In addition, the resulting CK nucleotides need to be dephosphoribosylated to become active free-base forms (Kurakawa et al., 2007). The responsible enzyme LOGs have been demonstrated to predominantly present in the nucleus and cytosol (Kuroha et al., 2009). Therefore, the translocation of iP nucleotides from plastid to cytoplasm, to nucleus, or even to ER is conceivable (**Figure 1**). However, until now there is no specific study elucidating how the CK biosynthetic intermediates are intracellularly sequestrated, and whether the specific transporters are required. An Arabidopsis plastidial membrane-localized nucleotide uniport carrier, Brittle 1, has been found to transport adenylates such as AMP, ADP, and ATP into the cytosol (Kirchberger et al., 2008). The gene encoding Brittle 1 primarily expressed in the root tips and the maturating or germinating pollens. Down-regulation of *Brittle 1* gene expression substantially retarded plant growth; but feeding adenosine complemented the growth defect of the mutant (Kirchberger et al., 2008). Giving the structural similarity of prenylated adenosine-5′-phosphate to the reported substrates of Brittle 1, it remains interesting to examine whether such a plastidial purine nucleotide exporter would have overlapped functions in delivering CK biosynthetic intermediates from plastid to cytoplasm.

The free CK bases within the cell can be reversibly glycosylated at their hydroxyl moieties of the N^6-side chains (for tZ, cZ, and DHZ), or irreversibly glycosylated at N^7- or N^9- positions by cytosolic localized glycosyltransferases and sequestered into the vacuole for storage. The vacuolar stored CK glucosides can also be reconverted back to the active CK species via β-glucosidases (Brzobohaty et al., 1993) and released to cytosol. In addition, the free CK bases can also be reversibly inactivated into their nucleotide forms by enzymes in common with purine metabolism, such as adenine phosphoribosyltransferases, which appear to be cytosolic and act antagonistically to LOGs (Moffatt et al., 1991; Zhang et al., 2013). Such a complexity in subcellular compartmentation of CK *de novo* synthesis, bioconversion, storage and inactivation implicates the presence of a sophisticated intracellular CK transport network. Members

TABLE 1 | The identified and/or putative cytokinin transporters*.

Transporter family	Name	Substrates (organism for detection)	Physiological functions in plant	Subcellular localization	Expression pattern	References
ENT	AtENT1	Adenosine in yeast	Unknown	Plasma membrane or vacuolar membrane		Li and Wang, 2000; Sun et al., 2005; Jaquinod et al., 2007
	AtENT8/SOI33	iPR in seedlings	Reduced sensitivity of *soi33-1* to iPR; Overexpression increases sensitivity to iPR	Unknown	Seedling, hypocotyls, flowers, midribs, abscission zones, siliques	Sun et al., 2005
	AtENT3	iPR in seedlings; adenosine in yeast; nucleoside in Xenopus oocytes	Reduced uptake efficiency of nucleoside-type CK (iPR), seedlings	Unknown	Unknown	Li et al., 2003; Sun et al., 2005
	AtENT6	iPR, tZR and adenosine in yeast	Unknown	Plasma membrane	Vascular tissues of root, leaf and flower, and cotyledons, stomata	Wormit et al., 2004; Hirose et al., 2008
	OsENT2	iPR, tZR, adenosine and uridine in yeast	Unknown	Unknown	Vascular tissues of leaf and root	Hirose et al., 2005
PUP	AtPUP1	Hypoxanthine, zeatin, kinetin, caffeine in yeast (proton-gradient dependent symporter)	Unknown	Unknown	Leaf mesophyll cells, stems, flowers, siliques, and hydathodes, stigma	Gillissen et al., 2000; Bürkle et al., 2003
	AtPUP2	Adenine, tZ, cZ, iPA, kinetin, BAP and tZR in yeast (proton-gradient dependent symporter)	Unknown	Unknown	Vascular tissues of leaf, stem, particularly in phloem, pollen	Bürkle et al., 2003
	AtPUP14	tZ, iPA, BA in protoplast and microsomes of plant. (ATP-dependent)	CK signaling, morphogenesis in embryos, roots and the shoot apical meristem	Plasma membrane	Various organ and tissues, including seedlings, embryos and mesophyll cells	Zürcher et al., 2016
	OsPUP7	Caffeine in yeast	Plant height, seed size and flowering time. Higher iP and iPR levels in mutant plant	Endoplasmic reticulum	Vascular bundle of roots, culms and leaves, hull vein and flower, stigma, style, stamens	Qi and Xiong, 2013; Kirschner et al., 2018; Xiao et al., 2018
	OsPUP4/BG3	Unknown	Seed size	Plasma membrane	Parenchyma cells near vascular tissue	Xiao et al., 2018
ABCG	AtABCG14	tZ-type CKs	Reduced root-to-shoot translocation in mutant. Shoot and root growth, nitrogen signaling	Plasma membrane	Vascular tissue in root (mainly) and shoot, leaf midribs, veins, mature anthers	Ko et al., 2014; Zhang et al., 2014; Poitout et al., 2018

**iPA or iP, isopentenyladenine; iPR, isopentenyladenosine; BAP or BA, benzylaminopurine or benzyladenine; tZ, trans-zeatin; tZR, trans-zeatin riboside.*

of equilibrative nucleoside transporter (ENT) family have been demonstrated with broad substrate specificities, transporting both purine and pyrimidine nucleosides (Mohlmann et al., 2001; Wormit et al., 2004; **Table 1**). Eight members (AtENT1-8) are found in Arabidopsis and four (OsENT1-4) in rice (Li et al., 2003; Hirose et al., 2004, 2005). Among them AtENT1 was the first member with demonstrated adenosine transport activity by a complementation assay with Arabidopsis cDNAs in yeast cells deficient in synthesizing adenine (Mohlmann et al., 2001). Instead of the previously reported plasma membrane localization (Li and Wang, 2000), AtENT1 was re-verified to present on vacuolar membrane (Jaquinod et al., 2007; Bernard et al., 2011; Schulze et al., 2012). Overexpression of *AtENT1* reduced vacuolar contents of adenosine and 2',3'-adenosine monophosphate, the RNA degradation products (Bernard et al., 2011). Conversely, down-regulation of *AtENT1* by RNAi

resulted in a significant increase in vacuolar accumulation of both metabolites. Furthermore, upregulating AtENT1-mediated vacuolar export activity concurred with the increase of the cytosolic adenosine kinase activity (Bernard et al., 2011). These observations suggest that AtENT1 might participate in the export of vacuolar nucleosides and/or RNA breakdown products to cytosol, and the exported nucleosides may re-enter the salvage pathways of adenylates with actions of nucleoside kinases, hydrolases, and/or phosphoribosyltransferases (Bernard et al., 2011). Therefore, expectedly AtENT1 activity might connect to the cytosolic CK biosynthesis and CK homeostasis. Although it was reported that the null mutant line of *AtENT1* exhibited no substantial effect on the CK response (Sun et al., 2005), it is worthy to re-examine the functions of this transporter in CK metabolism and homeostasis by using more sensitive CK signaling read-out reporters.

TRANSPORTERS RESPONSIBLE FOR CYTOKININ LONG DISTANCE TRANSLOCATION

After synthesis, CKs undergo intercellular movement and long-distance translocation from the sites of their synthesis to the target cells. Consistently, CKs are detected in the transduction systems phloem and xylem sap. In xylem sap, tZ, and tZR account for about 15 and 80% of CK species, respectively (Beveridge et al., 1997; Hirose et al., 2008; Kuroha et al., 2009; Osugi et al., 2017); whereas in phloem sap, the major forms are the iP- and cZ-type CKs (Corbesier et al., 2003; Hirose et al., 2008). The compartmentation of tZ- and iP-type of CKs in distinct vascular tissues suggest that plant has elaborated sophisticated selective transport mechanisms for distributing different types of CKs.

Translocation of the root-born tZ-type of CKs from roots to shoots requires an ABC transporter (**Figure 1**). A few years ago, we adopted a reverse genetics approach to systematically characterize the functions of ABCG subfamily proteins. We noticed that disruption of ABCG14 in Arabidopsis caused obvious morphological alterations, such as smaller inflorescences, smaller rosettes, slender stems with reduced number of vascular bundles, severely retarded primary root growth, and differential responses to the exogenously supplied phytohormones. Such developmental defects resembled those of the cytokinin biosynthetic mutants defective in CK signaling (Zhang et al., 2014). AtABCG14 expressed primarily in the pericycle and stele of roots. Profiling CKs in both roots and shoots of Arabidopsis seedling deficient in *abcg14* revealed a clear overaccumulation of the root-derived tZ- and DHZ-type CKs in the roots and reduced content in the shoots, indicating that disruption of AtABCG14 substantially inhibits the translocation and distribution of the root-synthesized CKs to the shoots. Interestingly, in contrast to the decline of both tZ- and DHZ-type CKs in the shoots, the iP-type CKs levels were slightly increased in both the shoots and roots of *abcg14*, so was the cZ-type CKs (Zhang et al., 2014). These data point out that (1) AtABCG14 participates in the long-distance translocation of the root-derived CKs. (2) AtABCG14 possesses profound substrate specificity and is able to discriminate the subtle structural variation of the isopentenyl side chain of CK species. (3) An intrinsic CK homeostatic mechanism occurs in plant cells that compensates the perturbation of CK allocation. Furthermore, by employing radiolabeled tZ in an *in planta* feeding and in the detached leaf assay with *ABCG14* overexpressing plants, we demonstrated that ABCG14, as a plasma membrane-localized protein, functioned as an efflux pump (Zhang et al., 2014). The data affirm that ABCG14 is a CK exporter responsible for loading the root-born tZ- and DHZ-type CKs into xylem vessels from xylary parenchyma cells. Coincidently, a couple of months later, Ko et al. (2014) reported a very similar study and finding, with additional reciprocal grafting experiments confirming the roles of AtABCG14 in the long-distance translocation of the root-synthesized CKs to the shoots (Ko et al., 2014). ABC transporter is an ATP-dependent primary transporter. Its activity consumes ATP yielding AMP, which directly links to the purine nucleotide metabolism. Therefore, it might have metabolic and regulatory advantages for plant to adopt this type of transporter to deliver CKs species in terms of the potential co-sharing and recycling of purine nucleotides, and more effective coordination and organization of energy flow, CKs synthesis and transport processes.

When *AtABCG14::GUS* was employed to examine gene expression, we noticed that in adult plant, *AtABCG14* was not only expressed in the roots but also in young rosette leaves, especially in the leaf midribs, veins and the nearby leaf cells. In addition, *AtABCG14* was also detected in mature anthers, and in siliques (Zhang et al., 2014; **Table 1**). The broad expression pattern of *AtABCG14* hints its possibility of more comprehensive functions than the involvement in xylem loading of root CKs. Speculatively, AtABCG14 may also reside on the cell membrane of the lignifying yet alive vascular cells near midribs or veins of young aerial tissues, potentially as an efflux pump responsible for the redistribution of CK species that are accumulated in those alive xylary cells (**Figure 1**), although currently it remains unclear how the CK species from xylem sap enrich in those cells. Alternatively, when AtABCG14 expresses in the leaf vein neighboring cells, it may also be possible to switch its transport direction to function as an importer taking up the xylem sap CKs to the cells. Although it is quite a rare case and mechanistically unclear, some ABC transporters seem pretty versatile in their flux pump direction. For example, AtABCB4, the transporter involved in auxin distribution, exhibited a substrate concentration-dependent switch from auxin influx to efflux when it was heterologously expressed in tobacco BY-2 cells, yeast, or HeLa cells (Terasaka et al., 2005; Yang and Murphy, 2009; Kubes et al., 2012). It is unknown whether such a unique behavior occurs *in planta*. Clearly, more refined experimental evidences are required for corroborating the postulated dual roles of AtABCG14 in CK translocation and cellular distribution.

While ABCG14 mediates acropetal transport of the root-born tZs to the shoots, systemic transport of the shoot-derived CKs (primarily iP- and cZ-types) takes place in phloem and likely involves cell to cell translocation through symplastic connection (**Figure 1**). Plasmodesmata connects neighboring cells and forms a highway for the movement of endogenous CKs as well as other photosynthetic assimilates from the site of synthesis to

phloem and from phloem to the target cells (Romanov et al., 2018). This symplastic process appears to have no requirement for the involvement of particular transporters. However, previous studies revealed that AtENT6 in Arabidopsis and OsENT2 in rice were likely involved in mediating the transport of adenosine and other nucleosides, including iP riboside (iPR) (Hirose et al., 2005, 2008; **Table 1**). Comparably, the affinity of ENTs to iPR was much higher than that to tZR (Hirose et al., 2005, 2008). Moreover, AtENT6 predominantly expressed in the vascular tissues of rosette leaves, flowers, cotyledons and roots (Hirose et al., 2008), while OsENT2 expressed primarily in rice leaf vascular bundles and phloem tissues (Hirose et al., 2005; **Table 1**). These data led to the speculation that ENTs may be responsible at least in part for the selective transport of iP nucleosides in vascular tissues (Sakakibara, 2006). Considering the hydrophobic characteristics of iP species, another possibility is that the concentration-dependent free diffusion might also contribute to iPs' spatial distribution and movement. This notion seems to be supported with the observation that the iP-type CKs abundantly presented in xylem sap when both *CYP735A1* and *CYP735A2* genes were knocked-out (Ko et al., 2014). Presumably disruption of two *CYP735A* genes depleted conversion of iP to tZ, consequently the overaccumulation of iP species in the xylem parenchyma cells resulted in their passive release into apoplastic space (xylem vessel). Alternatively, it is also possible for the existence of a low affinity exporter for iP-type species in root xylary cells. When high concentration of iPs accumulate, the transporter turns on. Molecular mechanism for iP systemic translocation needs to be further clarified.

TRANSPORTERS INVOLVED IN CK SIGNAL PERCEPTION

The transported CKs, when reaching the target cells, will eventually meet their receptors either at the cell surface or inside the cells to trigger signaling cascade. The activity of CK transporters guides cellular and subcellular localization of CKs and affects ligand-receptor interactions. Fragmentary evidences indicate that both PUP and ENT family transporters participate in CK uptake, therefore, directly or indirectly affect CK cellular or subcellular distribution and signal perception. PUPs are a class of small, integral membrane proteins that exhibit influx activity to a broad range of nucleobases (Girke et al., 2014; **Table 1**). Similar to the discovery of ENTs, the recognition of PUPs also came from complementation assay by adopting a purine transport-deficient yeast mutant transformed with an Arabidopsis cDNA library. In the subsequent competition assays, besides adenine and other nucleobases, it was found that AtPUP1 also took up CKs kinetin and zeatin, meanwhile showed a lower activity to their ribosides (Gillissen et al., 2000). This discovery eventually led to the recognition of 23 PUP family members in Arabidopsis (Schwacke et al., 2003; Zürcher et al., 2016). So far AtPUP1, AtPUP2, and AtPUP14 have been demonstrated with direct CK uptake activity in yeast expression system, Arabidopsis mesophyll protoplasts, and/or microsomes derived from *Nicotiana benthamiana* transfected with *PUP* genes (Bürkle et al., 2003; Zürcher et al., 2016; **Table 1**). Interestingly, AtPUP1 and AtPUP2 behaved as the proton-gradient dependent symporter. Protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and H^+-ATPase inhibitor significantly depleted their uptake activity to the radiolabeled tZ (Bürkle et al., 2003; Cedzich et al., 2008); whereas the activity of AtPUP14 is seemingly independent of proton-gradient potential but requests the presence of ATP (Zürcher et al., 2016). This phenomenon suggests that PUP members differ substantially in their biochemical properties. Moreover, *PUP* members *in planta* exhibit substantially different gene expression patterns, which infer their potential multifaceted physiological roles. For instance, *AtPUP1* is mainly expressed in the epithem of hydathodes and at the stigma surface of siliques (**Figure 1**), implicating its potential role in the retrieval of nucleobase derivatives in its expressing tissues (Bürkle et al., 2003). *AtPUP14* prevailed in all the examined tissues including seedlings, embryos and mesophyll protoplasts, where it displays the highest expression level among all other family members (Zürcher et al., 2016). In heart stage embryos, the expression of *AtPUP14* was inversely correlated to the cytokinin signaling readout. The reciprocal correlation led to a conclusion that AtPUP14, as a principal plasma membrane-resident protein, is able to import the active CK nucleobases into the cells consequently depleting apoplastic CK pools and inhibiting perception of CK signals by plasma membrane-localized receptors, by which, AtPUP14 modulates spatiotemporal CK sink patterns and influences plant morphogenesis (Zürcher and Muller, 2016; Zürcher et al., 2016). This hypothesis, however, evoked substantial argument in respect to whether the CK signaling initiation site occurs at plasma membrane or ER, since so far the experimentally defined CK receptors in different plant species appeared as the ER-localization (Romanov et al., 2006; Lomin et al., 2011, 2018). In addition, there are also some concerns about the binding affinity of PUPs versus the CK receptors to the ligands. Even if CK sensors do localize on the plant membrane, unless they have substantially lower ligand binding affinity than PUPs, it is not easy to explain the complete inhibition of CK signaling by the activity of AtPUP14 (Duran-Medina et al., 2017; Kang et al., 2017; Romanov et al., 2018). With these concerns, Romanov et al. (2018) argued that AtPUP14 or other PUP family members, instead of as the PM-localized proteins, might function at the ER membrane, where they pump CKs from ER lumen to the cytosol, thereby modulating CK signaling. Recently a rice PUP member OsPUP7 has been proved to localize at the ER and be involved in CK transport, although its flux pump direction remains ambiguous (Xiao et al., 2018), which seemingly aligns with Romanov et al's argument.

In favor of CK signal perception primarily at the ER, Romanov et al proposed a model of multiple sites cellular CK signaling initiation and perception (Romanov et al., 2018), which integrates the importer activities of both PUP and ENT family proteins and differentiates the fate of tZ and tZR. The root-derived tZ that is transported through xylem to the particular region or cell types of the shoot directly interacts with the hypothetic plasma membrane-localized CK sensors, thereby, triggering hormonal signaling at the surface of cells. The established apoplastic CK

pool can also be either degraded by the extracellular cytokinin oxidases, or transformed into the corresponding riboside by extracellular purine nucleoside phosphorylases, or imported into the cytoplasm by PUP transporters. The imported CK bases then could be either degraded or transformed into the inactive tZRP, and re-enter into the intracellular CK pool. On the other hand, the xylem-delivered tZR is taken up into the cells of the shoot via nucleoside importer ENTs. The imported tZRs in the cytosol could further undergo metabolic conversion by tZR kinase into tZRP, and by the activity of LOG enzymes release the biologically active form, free base of tZ.

While this multiple-site CK signal perception model integrates well with the experimental evidences on the ER-localization of CK sensors, the proposed transporter components call for more solid experimental confirmation. Primarily, the transport efficiency and specificity of ENTs to tZR is a matter of concern. The ENTs identified from Arabidopsis and rice exhibit quite broad substrate specificity and are apparently not optimal for the transport of tZR, since the affinity of the transporters to tZR (K_m = 630∼660 μM) is substantially lower than that to iPR (K_m = 17∼32 μM) or adenosine (K_m = 3∼91 μM) (Hirose et al., 2005, 2008). Although it was reported that mutation of AtENT8 has suppressed the phenotypes of a plant with overproduction of CKs, and a loss-of-function of AtENT3 presented reduced CK uptake (Sun et al., 2005), compelling biological evidence for ENTs' involvement in the tZR uptake is still missing.

FUTURE PERSPECTIVES

Short- and/or long-distance translocation of CKs are important components in CK biosynthesis, salvage, homeostasis, and signaling processes. Elucidating molecular mechanism of CK transport and identifying the related molecular factors of CK translocation have substantially deepened our understandings of the physiological roles that CKs play with in plant growth and development, and in plant-environmental interactions. Grafting experiments between *abcg14* mutant and WT have revealed that root-to-shoot transport of CKs is crucially required for the regulation of shoot growth and development (Ko et al., 2014). A recent study through disturbing root CK synthesis and translocation discovered that both tZ and tZR act as systemic signals traveling from root to shoot through xylem, differentially influencing leaf size and/or vegetative meristem activity (Osugi et al., 2017). Moreover, the long-distance translocated CKs also function as second messengers to signal the level of nutrient availability, particularly the soil nitrate level, to the shoot apical meristem, thereby mediating the organogenesis activity of shoot apical meristem in respect to the availability of soil mineral nutrients (Landrein et al., 2018). Aided with split-root system to uncouple local and systemic signaling, analysis of mutants *ipt3,5,7* and *abcg14* deficient in the root CK synthesis and translocation revealed that accumulation of the root-born tZ in shoots is an integral component of systemic signaling network of the root-shoot-root, which mediates the molecular and physiological responses of plants to nitrate heterogeneity in their roots, and controls gene expression in roots and shoots (Poitout et al., 2018). These studies exemplify the importance of unveiling CKs transport in understanding the physiological functions of this group of mobile hormonal molecules.

On the other hand, although a few transporter proteins have been implicated or partially evidenced in CK signal transport and distribution, the biological relevance of many putative CK transporter members require more detail and systematic exploration. PUP as well as ENT proteins are encoded by a multigene family *in planta*. The potential functional redundancy and their substrate promiscuity pose significant challenges for pinpointing their biochemical and biological functions in CK synthesis, intracellular traffic, cellular distribution and signal perception. To unequivocally elucidate the functions of ENTs and PUPs, it requires the integrated biochemical and biophysical approaches to precisely determine their influx or efflux carrier properties, substrate preferences, and subcellular localizations. Meanwhile, establishing higher order of mutant lines via conventional genetic cross and/or by CRISPR-Cas9 gene editing, together with the employment of high sensitive CK signal reporter system such as *TCSn::GFP* (Zürcher et al., 2013; Kirschner et al., 2018) could facilitate the determination and discrimination of biological roles of both PUP and ENT members in CK metabolism and signaling.

Recent study revealed that tZ and tZR transported through xylem flow exert distinct physiological roles for leaf development and apical meristem activity. The ratio of tZ and tZR in xylem flow and their delivery rate are modulated in response to the environmental conditions, which render different physiological consequence of shoot growth (Osugi et al., 2017). Currently it remains unclear if transporter proteins are involved in determining the ratio and delivery rate of root-born CK species in xylem flow. In the future, besides further tackling down additional physiological roles of ABCG14 in aerial tissues pertaining to the CK distribution, it is also intriguing to explore whether ABCG14, the primary transporter for xylem loading of tZ and tZR in root, coordinates with particular PUPs and/or ENTs in shoot to fine-tune the status and flow rate of different CK species in xylem sap and their differential cellular distribution, in response to plant growth and development cues and environmental stimuli.

AUTHOR CONTRIBUTIONS

C-JL wrote the manuscript. C-JL, YZ, and KZ prepared the data. All authors have read, edited, and agreed to the content.

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The Arabidopsis ATP-BINDING CASSETTE Transporter ABCB21 Regulates Auxin Levels in Cotyledons, the Root Pericycle, and Leaves

Mark K. Jenness[1], *Nicola Carraro*[2], *Candace A. Pritchard*[1] *and Angus S. Murphy*[1,2]*

[1] Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD, United States,
[2] Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN, United States

The phytohormone auxin plays significant roles in regulating plant growth and development. In Arabidopsis, a subset of ATP-BINDING CASSETTE subfamily B (ABCB) transporters participate in polar movement of auxin by exclusion from and prevention of reuptake at the plasma membrane. A previous analysis identified ABCB21 as a conditional auxin uptake/efflux transporter that regulates cellular auxin levels, but clear physiological roles for ABCB21 *in planta* remain unknown. Here we show that ABCB21 maintains the acropetal auxin transport stream by regulating auxin levels in the pericycle. Loss of ABCB21 reduces rootward auxin transport and delays lateral root emergence. In seedling shoots, ABCB21 regulates mobilization of auxin from the photosynthetic cotyledons that is important for phototropic bending. In rosette leaves ABCB21 contributes to lateral auxin distribution. These results support a primary role for ABCB21 in regulating auxin distribution supplementary to the primary ABCB auxin transporters ABCB1 and 19.

Keywords: ABCB transporter, *Arabidopsis thaliana*, auxin, development, seedling

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Faculty of Science, Charles University, Czechia
Eilon Shani,
Tel Aviv University, Israel

***Correspondence:**
Angus S. Murphy
asmurphy@umd.edu

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INTRODUCTION

Optimization of light capture from sunlight and accumulation of water and nutrients from the soil during seedling establishment are major determinants of plant fitness. In Arabidopsis seedlings, light promotes synthesis of the phytohormone auxin (indole-3-acetic acid, IAA) in the cotyledons and young developing leaves (Bhalerao et al., 2002). During early post-photomorphogenic seedling growth auxin regulates expansion of the photosynthetically active cotyledons (Ni et al., 2001; Lewis et al., 2009) and tropic bending toward the light source (Christie et al., 2011). During later stages of seedling growth auxin regulates lateral root development in a sequence of events that can be grouped into two distinct major phases: initiation and elongation/emergence (Péret et al., 2009). Auxin originating from or redirected at the root cap controls transcriptional oscillations that initiate lateral root primordia (Van Norman, 2015; Xuan et al., 2015) within the xylem pole pericycle which surrounds the central vascular cylinder (Dolan et al., 1993; Dubrovsky et al., 2000; Casimiro et al., 2001; Van Norman et al., 2013; Kircher and Schopfer, 2016). Excision and labeling experiments in Arabidopsis demonstrate that auxin involved in both lateral root initiation and emergence is initially shoot derived and transported in a rootward stream between 5 and 7 d after

germination (Busse and Evert, 1999; Bhalerao et al., 2002; Lewis et al., 2007; Swarup et al., 2008; Peer et al., 2014). The requirement for auxin synthesis at the root apex as seedlings mature was more definitively demonstrated when root growth of quadruple *yucca* auxin biosynthesis mutants was rescued by exogenous auxin in the media, but not by auxin overproduction in the shoot (Chen et al., 2014). Further, although shoot-derived auxin induces lateral root emergence, local auxin biosynthesis in the root tip is also required for root meristem maintenance (Brumos et al., 2018). At 10 days post-germination the root apex increases competence to synthesize auxin and, subsequently, root-derived auxin maintains primary root growth (Bhalerao et al., 2002; Brumos et al., 2018).

The rootward polar auxin stream in seedlings is primarily attributed to a cellular transport process that involves gradient-driven, directed release to the apoplast of auxin from one cell followed by uptake into an adjoining cell. Bulk auxin movement in phloem transport makes an additional contribution to movement as seedlings mature (Swarup et al., 2001; Marchant et al., 2002). At the cellular level, isotropic auxin (IAA) uptake occurs via lipophilic diffusion of the protonated acid or H^+ symport of the prevalent anionic form via AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) permeases. AUX1/LAX proteins play a primary role in auxin redirection at the root apex and uptake into cortical cells during lateral root emergence (Bennett et al., 1996; Swarup and Péret, 2012). Polarized PIN-FORMED (PIN) proteins facilitate directional cellular efflux vectors to amplify overall polar streams (reviewed in Adamowski and Friml, 2015), while the activity of ATP-BINDING CASSETTE subfamily B (ABCB) efflux transporters limits auxin reuptake at efflux sites (Blakeslee et al., 2007; Aller et al., 2009; Bailly et al., 2011).

Observations of cellularly-polarized PIN proteins that function in organogenic growth by amplifying vectoral auxin streams (Benková et al., 2003; Friml et al., 2003) harmonize well with predictions of early polar auxin transport models (Rubery and Sheldrake, 1974; Raven, 1975; Goldsmith, 1977). Polar transport defects evident in mutants where transport sinks generated by AUX1/LAX uptake are absent (Bennett et al., 1996; Marchant et al., 1999; Swarup et al., 2001; Péret et al., 2012) are consistent with a requirement for uptake sinks included in more robust models (Lomax et al., 1995; Kramer and Bennett, 2006). Finally, alterations in plant stature, changes in leaf morphology, and reductions in long distance polar auxin streams associated with loss of ABCB function (Noh et al., 2001; Multani et al., 2003; Geisler et al., 2005; Santelia et al., 2005; Terasaka et al., 2005; Blakeslee et al., 2007; Knöller et al., 2010) are consistent with cellular efflux models that include cellular exclusion at the PM interface (Bailly et al., 2011; Jenness and Murphy, 2014). These later models factor in membrane partitioning of auxin (Gutknecht and Walter, 1980) and direct binding of ABCB transporters with the auxin efflux inhibitor 1-naphthylphthalamic acid (NPA) (Noh et al., 2001; Murphy et al., 2002; Geisler et al., 2003; Bernasconi et al., 2016), as well as experimentally-determined losses of rootward auxin transport (60–75% in Arabidopsis *abcb1 abcb19* seedlings (Blakeslee et al., 2007).

Except during cell division, ABCB proteins exhibit nonpolar distributions on the plasma membrane (PM) (Geisler et al., 2005; Blakeslee et al., 2007; Wu et al., 2007; Mravec et al., 2008; Kubeš et al., 2012). Accordingly, *ABCB* mutants are competent in embryo- and organogenesis, but exhibit vegetative phenotypes indicative of reduced and irregular cell elongation/expansion (Noh et al., 2001; Wu et al., 2007). In almost all plant species studied, a highly similar pair of ABCB proteins (ABCB1 and 19 in Arabidopsis) are primary contributors to rootward auxin transport (Knöller et al., 2010). In maize and other grasses, ABCB1/Brachytic2/Dwarf3 is a primary regulator of rootward auxin transport (Multani et al., 2003; Cassani et al., 2010; Knöller et al., 2010; McLamore et al., 2010; Balzan et al., 2018; Wei et al., 2018). In Arabidopsis and other dicots, ABCB19 is the more distinguishable isoform, and loss of ABCB19 results in enhanced phototropic bending (Noh et al., 2003; Christie et al., 2011), reduced plant stature (Noh et al., 2001), decreased auxin reporter activity in early stage lateral roots, and reduced lateral root outgrowth (Wu et al., 2007). The additional contribution of ABCB1 to rootward streams is best visualized in *abcb1 abcb19* double mutants (Lin and Wang, 2005; Blakeslee et al., 2007; Wu et al., 2007).

However, the Arabidopsis genome encodes 22 full-length ABCB transporters, including the pseudogene *ABCB8* (Verrier et al., 2008). Some of these isoforms appear to function in localized maintenance of rootward auxin transport streams, as treatment of Arabidopsis seedlings with the ABCB-associated auxin transport inhibitors NPA, Gravacin, and BUM (2-[4-(diethylamino)-2-hydroxybenzoyl benzoic acid) causes delayed lateral root formation and emergence to a greater extent than is observed in *abcb1 abcb19* mutants alone (Casimiro et al., 2001; Rojas-Pierce et al., 2007; Kim et al., 2010). Recently, the ABCB6 and ABCB20 auxin transporters were shown to contribute to rootward auxin streams in inflorescences (Zhang et al., 2018), and the biochemically uncharacterized ABCB11/12 pair, guard cell malate/citrate transporter ABCB14 (Lee et al., 2008), and ABCB15 in the Arabidopsis inflorescence have also been implicated in maintenance of rootward auxin streams (Kaneda et al., 2011).

A contribution of ABCB21 to acropetal auxin streams in the root has also been inferred by localization of *proABCB21:GUS* signals to the root vasculature and biochemical characterizations of conditional auxin transport in protoplasts and yeast exhibiting attributes that are highly similar to the root epidermal/cortical ABCB4 transporter (Kamimoto et al., 2012). This suggests that ABCB21 functions in the vascular cylinder of the root maturation zone and above to provide a regulated lateral boundary for the rootward auxin transport stream. Such function would require a conditional auxin uptake/efflux transport activity in the pericycle of the maturation zone. This function is hypothesized to be similar to ABCB4 modulation of constitutive shootward auxin transport from the root apex mediated by AUX1 and PIN2 in epidermal cells near the root elongation zone (Santelia et al., 2005; Terasaka et al., 2005; Cho et al., 2007; Yang and Murphy, 2009; Kubeš et al., 2012). Additionally, ABCB21 expression in young leaves suggests an analogous function in those organs (Kamimoto et al., 2012).

Here we show that ABCB21 maintains the acropetal auxin transport stream by regulating auxin levels in the pericycle and functions in the distribution of auxin in cotyledons and young leaves. Loss of ABCB21 results in reduced rootward auxin transport and defects in lateral root outgrowth. In aerial tissues, *abcb21* exhibits reduced cotyledon-hypocotyl auxin transport, defects in phototropic bending, and alterations in lateral auxin movement in leaves. While the exclusionary role of ABCB21 supplements the activity of ABCB19, the conditional uptake/efflux activity provides an additional and unique level of auxin transport regulation. Sequence similarity implies functional redundancy between the ABCB4/21 pair, as is observed with ABCB1/19 and ABCB6/20 (Noh et al., 2001; Zhang et al., 2018). Unlike these pairs, ABCB4 and ABCB21 function in discrete domains, indicating distinct spatio-temporal roles during growth and development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used for all experiments. Lines used are listed in **Supplementary Table 3**. Seeds were surface sterilized and sown on ¼ MS medium (pH 5.6; Caisson Labs, Smithfield, UT, USA) containing 1 g L^{-1} MES, 0.5% sucrose, and 0.8% agar, pH 5.5. For seedling assays, seeds were stratified 4°C for 2 d, then grown vertically under continuous 100 μmol m^{-2} s^{-1} light at 22°C for the times indicated. For mature plants, seeds were sown on soil, stratified 4°C for 2 d, then grown in growth chambers under 100 μmol m^{-2} s^{-1} light (16 h photoperiod) at 22°C for the times indicated.

Yeast Transport Assays

Yeast assays were conducted as described (Yang and Murphy, 2009). The ABCB21 expression construct was created by amplifying the *ABCB21* coding sequence with Gateway BP primers (**Supplementary Table 4**) and recombining the product into pDONR/Zeo by BP reaction (Thermo Fisher Scientific). *ABCB21* was then transferred into pREP41GW by LR reaction (Thermo Fisher Scientific). Expression vectors were transformed into *S. pombe* by electroporation. Assays were performed using 40 nM [^{3}H]IAA, which is within the physiological range for Arabidopsis (Novák et al., 2012).

Histochemical Staining

The 0.625 kb promoter fragment of *ABCB21* upstream of the start codon was cloned into pENTR/D-TOPO (Thermo Fisher Scientific) then transferred into the Gateway compatible vector pGWB3 (Nakagawa et al., 2007) by LR reaction (Thermo Fisher Scientific). Constructs were transformed into Col-0 via floral dip (Clough and Bent, 1998). For GUS staining, tissues were incubated in 90% acetone for 20 min on ice, then immersed in staining solution (50 mM sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 1 mM X-gluc) and incubated in the dark at 37°C for 5 h, unless otherwise noted. Stained samples were cleared with 70% ethanol before imaging. For sectioning tissue was dehydrated in a series of tert-butanol (TBA) and embedded in Paraplast Plus. Twenty micrometer sections were prepared using a Leica Reichert-Jung 2030 rotary microtome.

Seedling Transport Assays

Rootward seedling transport assays were conducted as described (Christie et al., 2011), except that 6% agarose beads (Colloidal Science Solutions; AMB-0601-0010) were substituted for polystyrene beads. For cotyledon-hypocotyl transport assays 5 d seedlings were placed on filter paper (Whatman 3MM) saturated with ¼ MS with the hypocotyl and cotyledons not touching any surface. Seedlings were allowed to equilibrate vertically in light for 1 h. A 6% agarose bead incubated in solution containing 2 μM IAA (1:1 cold IAA:[^{3}H]IAA; 25 Ci $mmol^{-1}$, American Radiolabeled Chemicals) was placed in the middle of one cotyledon per seedling. After 2 h, both cotyledons were removed by cutting just below the cotyledonary node using a surgical blade. [^{3}H]IAA transported from the cotyledons to the hypocotyl and root was measured by liquid scintillation counting.

Leaf Transport Assays

Agarose beads coated in [^{3}H]IAA were placed on equal size rosette leaves of 35 d plants at the positions indicated. After bead placement plants were incubated under 20 μmol m^{-2} s^{-1} yellow light and 55% relative humidity for 3 h. 0.5 mm punches were collected at positions indicated and measured for radioactivity.

IAA Quantifications

Free IAA quantifications were conducted as described (Novák et al., 2012) with minor modifications. Briefly, 10–16 mg Arabidopsis tissue was collected and frozen in liquid nitrogen before storing in −80°C until use. Samples were ground in liquid nitrogen, and 1 mL cold 50 mM sodium phosphate buffer (pH 7.0) containing 1% diethyldithiocarbamic acid. Indole proprionic acid was added as an internal standard. Samples were vortexed and extracted for 20 min at 4°C, then centrifuged at 12,000 × g for 15 min at 4°C. The pH value of supernatant was adjusted to 3 using 1 N HCl, and the supernatants were purified using an HLB Column. The column was conditioned with 1 mL methanol (Fisher Scientific, LC-MS/MS grade, A456-1) followed with 1 mL water and 0.5 mL 50 mM sodium phosphate buffer, pH 2.7. After loading the sample, the column was washed with 2 mL 5% (vol/vol) methanol. Finally, analytes were eluted with 2 mL 80% (vol/vol) methanol. The eluted samples were dried under nitrogen gas, dissolved with 500 μL methanol, and filtered through 0.2-μm PTFE Filters (Fisher Scientific, 03–391-4E); 1 μL of each sample was injected for LC-MS/MS analyses. Compounds were quantified in positive ion mode, and MS/MS settings were as described (Novák et al., 2012) and conducted by Agilent 6460 triple quadrupole LC-MS/MS.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR total RNA was extracted using ZR Plant RNA Mini Prep kit (Zymo Research) followed by treatment with DNaseI (New England Biolabs). Total RNA (1.5 μg) was used for first-strand synthesis using SuperScript III reverse transcriptase (Thermo Fisher Scientific). qRT-PCR was performed on a CFX

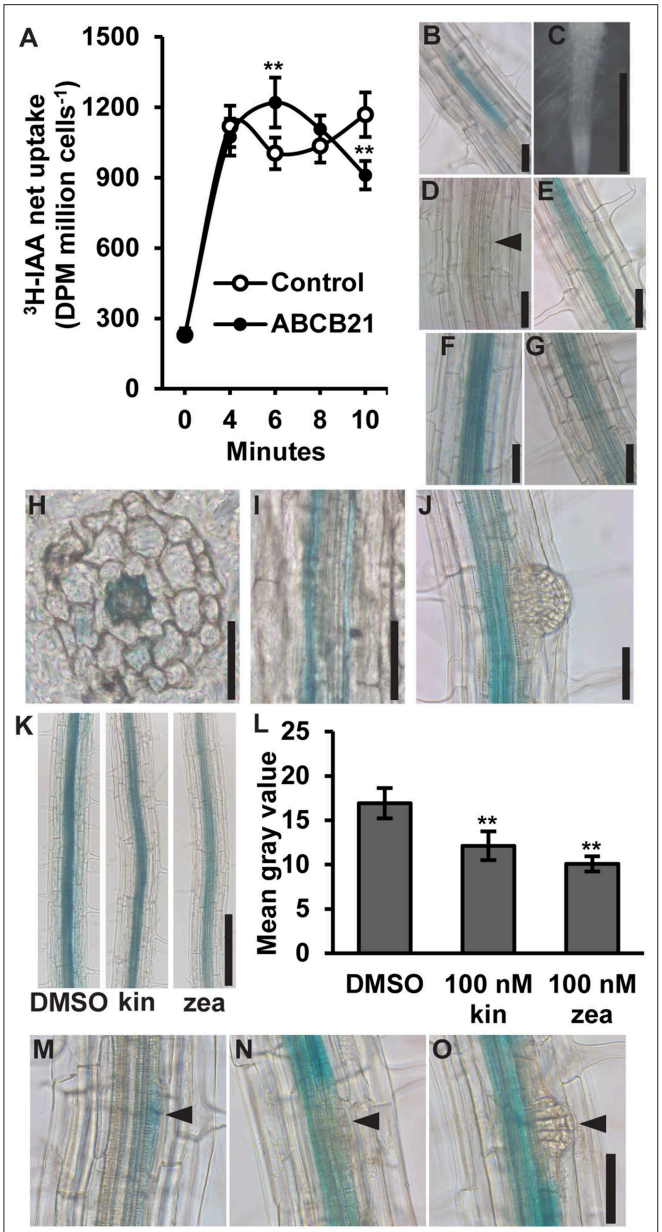

FIGURE 1 | Auxin transport activity and expression of *ABCB21*. **(A)** ABCB21 exhibits conditional auxin uptake/efflux in *S. pombe*. Data shown are means ± SD ($n = 6$–8 from two experiments with 3- independent transformants). **(B)** *proABCB21:GUS* expression in 4 d etiolated seedling roots. **(C–G)** *proABCB21:GUS* expression in **(C)** 1 d **(D)** 3 d, **(E)** 5 d, **(F)** 7 d, and **(G)** 10 d light-grown seedling roots. No GUS signal is observed in 1 d roots. Arrow indicates area of low level expression in 3 d roots. **(H)** Cross and **(I)** longitudinal sectioning confirms expression is primarily associated with the root pericycle. **(J)** *proABCB21:GUS* is absent in emerging lateral roots. **(K)** Treatment with cytokinins reduces *proABCB21:GUS* expression in the pericycle of 7 d roots. Representative images of *proABCB21:GUS* expression after 3 h treatment with 100 nM kinetin (kin) or *trans*-zeatin (zea) prior to GUS staining. **(L)** Quantification of *proABCB21:GUS* signal in **(K)**. Images were taken between the lowest two emerged lateral roots. Data shown are means ± SD for the five most heavily stained roots. **(M–O)** *proABCB21:GUS* expression during early lateral root development. Arrows indicate lateral root primordia. Asterisks indicate statistical difference from Col-0 by Student's *t*-test for ** $P < 0.01$. Scale bars: **(B–J,M–O)** 50 μm; **(K)** 200 μm.

Connect (Bio-Rad Laboratories) using EvaGreen qPCR master mix (Biotium) according to manufacturer's instructions. Primers used are listed in **Supplementary Table 4**. Transcript levels normalized against *PP2A* (AT1G69960) or *ACT2* (AT3G18780) produced similar results.

Phototropism Assays

Surface sterilized seeds were sown on MS, 0.5% sucrose, and 0.8% agar plates then stratified at 4°C for 2–4 d in the dark. To induce germination seeds were placed in light for 12 h. For etiolated bending assays seeds were removed from light and placed in dark for 3 d at 22ÂC until hypocotyls reached 7–8 mm in height. For de-etiolated bending assays seeds were placed in light ∼36 h to undergo photomorphogenesis, then place in dark to induce hypocotyl elongation to 7–8 mm in height. 3 d seedlings were transferred from vertical plates to 60 mm petri dishes filled with silicon dioxide (Sigma, St. Louis, MO; Cat #274739) and water. After a 30 min acclimation, seedlings were exposed to 0.4–0.8 μmol m^{-2} s^{-1} unilateral LED blue light illumination. Images were captured every 10 min with a USB3 uEYE CP camera (IDS Imaging Development Systems, Woburn, MA, USA) and processed for clarity with Photoshop (Adobe Systems Inc., Cupertino CA). All phototropic bending and hypocotyl elongation measurements were made using FIJI software (Schindelin et al., 2012; Schneider et al., 2012). Bending angle measurements are described in **Supplementary Figure 6**.

Leaf Petiole Angle Assays

Assays were conducted as described (de Carbonnel et al., 2010). Briefly, soil was placed in 90 × 15 mm petri dishes with holes punched in the bottom. Dishes were then placed in trays and watered by bottom infiltration. Seeds were sown onto the soil and stratified for 48 h. Seedlings were grown in a growth chamber under 80 μmol m^{-2} s^{-1} white light, 16 h photoperiod. Upon reaching stage 1.01, seedlings were transferred to continuous 50 μmol m^{-2} s^{-1} white light or 50 μmol m^{-2} s^{-1} red light for an additional 72 h. For true leaf petiole angle measurements plants were photographed from the side. Angles were determined by measuring the angle formed between the hypocotyl and the petiole minus 90°.

Petal Break-Strength Assays

Plants that were grown 35 d at 21°C temp with 16 h 100 μmol m^{-2} s^{-1} light/8 h dark. Detached flowers were suspended on a force transducer (Aurora Scientific, Inc.: Model 404A: range, 0–100 mN; sensitivity; 10.0 mN; resolution, 2000 nN) with an alligator clip lined with soft rubber (**Supplementary Figure 7A**). Single flower petals were then attached to flat end forceps mounted on a computer controlled translation stage (Thor Labs OptoDC Servo Motor) programmed to move 0.05 mm s^{-1}. The vertical displacement of the stage resulted in reproducible detachment of the petal at the receptacle (**Supplementary Figure 7B**). The maximum voltage value from the acquisition during petal pulling was used to calculate the petal break-strength. The measurements were converted to gram equivalents according to a linear standard curve (voltage as a function of weight) corrected for the weight of

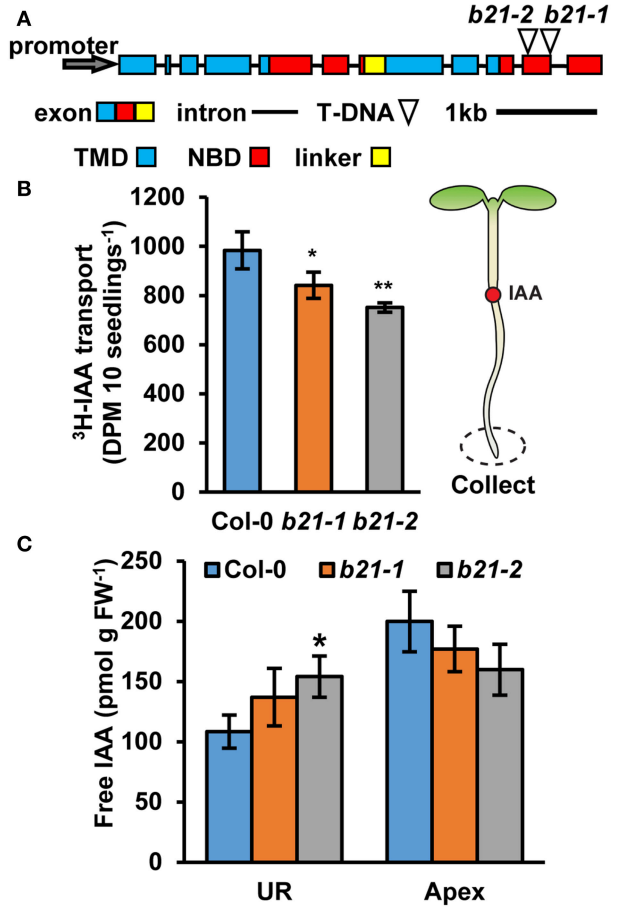

FIGURE 2 | Auxin transport and levels in *abcb21* roots. *abcb21* mutants exhibit reduced acropetal auxin transport in roots. **(A)** *ABCB21* gene model indicating T-DNA insertion positions for *abcb21-1* (WiscDsLox1C2) and *abcb21-2* (Gabi_954H06). Boxes correspond to exons with approximate positions of transmembrane domains (TMD; blue), nucleotide-binding domains (NBD; red), and linker region (yellow). **(B)** [^{3}H]IAA transport from the root-shoot transition zone to the root tip in 5.5 d seedlings. Data shown are means ± SD ($n = 3$ pools of 10). **(C)** Quantification of free IAA levels in 5.5 d light grown seedlings. UR, upper root; Apex, apical 2 mm root section. Data shown are means ± SD ($n = 3$ pools of 10). Asterisks indicate statistical difference from Col-0 by Student's *t*-test for * $P < 0.05$ and ** $P < 0.01$.

the clip (**Supplementary Figure 7C**). Flowers 2, 4, and 6 were measured with position 1 considered to be the first flower with visible petals.

Lignin Content and *p*-coumaryl Alcohol Growth Assays

Analysis of lignin thioacidolysis products and *p*-coumaryl alcohol growth assays were conducted as described (Alejandro et al., 2012).

Statistical Analysis

All statistical analyses were performed using JMP PRO 13.

RESULTS

ABCB21 Exhibits Conditional Auxin Uptake/Efflux Activity

ABCB21 was previously shown to exhibit conditional uptake/efflux activity using *RNAi* knockdown in mesophyll protoplasts and expression in *Saccharomyces cerevisiae* (Kamimoto et al., 2012). This activity was validated by expressing ABCB21 in *Schizosaccharomyces pombe* (**Figure 1A**). Cells expressing ABCB21 accumulated ∼25% more [^{3}H]IAA after 6 min than control lines. However, after 10 min cells expressing ABCB21 accumulated ∼23% less [^{3}H]IAA than the controls. The decrease in IAA accumulation in control lines between 4 and 6 min is indicative of efflux by endogenous low affinity IAA transporters which is not observed until higher cellular IAA levels are reached (Yang and Murphy, 2009). Short timeframes is one of the limitations with using this assay system. After ∼10 min the results become more variable and difficult to interpret due to endogenous transport activities and reduced integrity of the yeast cells. These results suggest that, like ABCB4, ABCB21 exhibits initial IAA uptake activity and efflux is activated by reaching a threshold intracellular IAA concentration. The timing of the switch between uptake and efflux is very similar between ABCB21 and those observed for ABCB4 (Yang and Murphy, 2009), suggesting they may share similar transport and/or regulatory mechanisms.

ABCB21 Expression in Seedlings Is Associated With Auxin Conducting Tissues

ABCB21 expression was previously analyzed using a promoter sequence 0.75 kb upstream of the *ABCB21* start codon fused to the *β-glucuronidase* (*GUS*) reporter (Kamimoto et al., 2012). This promoter, however, included 122 nucleotides of the 3′ UTR of the upstream gene (At3g62160). To see if this fragment had any effect on expression, a shorter 0.625 kb promoter was fused to *GUS* and transformed into Col-0. Overall, there were no observable expression differences between the two promoters. In the root, *proABCB21:GUS* is primarily expressed in the root vasculature of 5–10 d seedlings (**Figures 1B–G**). Before 5 d and after 10 d, expression in the pericycle is mostly absent and the signal that is present is highly variable and discontinuous (**Figures 1B–D,G**). Cross and longitudinal sections of roots show that expression is primarily associated with the pericycle (**Figures 1H,I**). Expression in the pericycle is continuous throughout the top two-thirds of the root, but absent in lateral root primordia and emerging lateral roots (**Figure 1J**). Negative regulation of *ABCB21* expression by cytokinin has been reported in transcriptomic studies (Winter et al., 2007) and is consistent with the observed lack of expression in the root tip where cellular cytokinin levels are high (Antoniadi et al., 2015). Treatment with 100 nM kinetin or *trans*-zeatin (similar to levels reported in the root tip) reduced *proABCB21:GUS* expression in root pericycle cells situated between emerged lateral roots (**Figures 1K,L**). Negative regulation of *proABCB21:GUS* expression in these tissues is consistent with previously reported regions of increased cytokinin signaling visualized with the *TCS:GFP* cytokinin reporter, particularly with exogenous

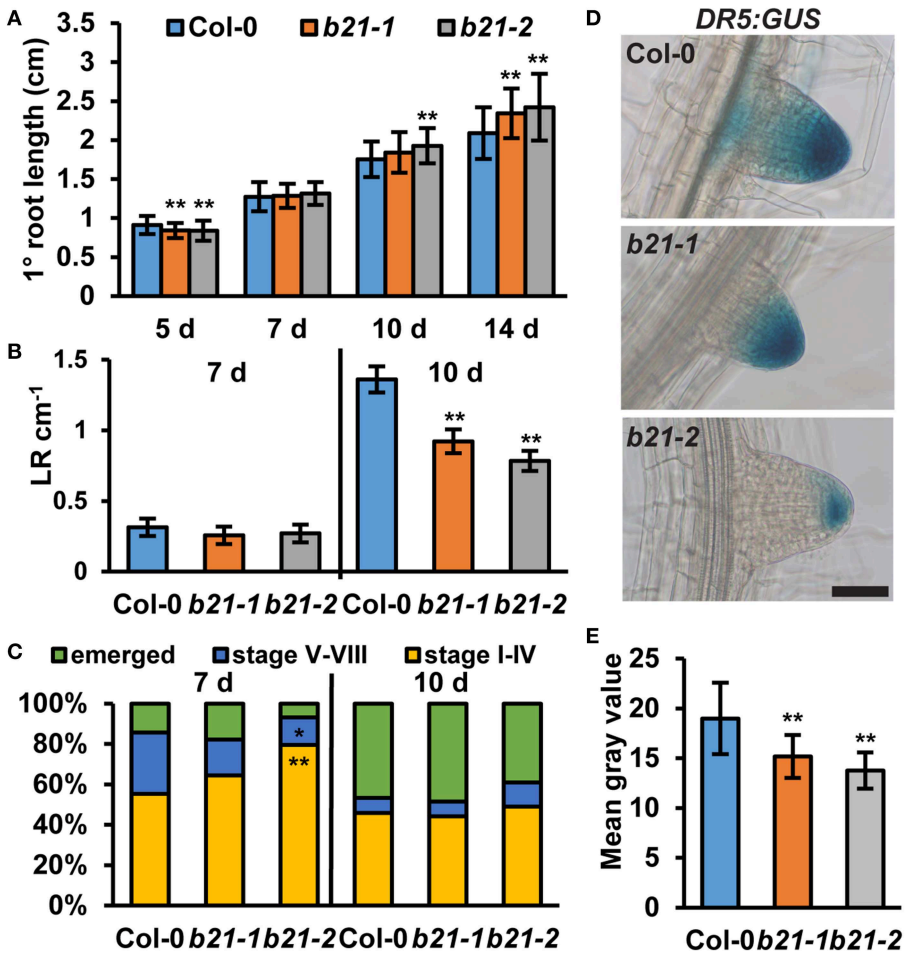

FIGURE 3 | Root phenotypes in *abcb21*. *abcb21* mutants exhibit altered primary root elongation and lateral root development. **(A)** Primary root length at 5, 7, 10, and 14 d. Data shown are means ± SD ($n = 40$–50). **(B)** Emerged lateral root density in 7 and 10 d seedlings. Data shown are means ± SE ($n = 40$–50). **(C)** Distribution of stage I–IV, stage V–VI, and emerged lateral roots in 7 and 10 d seedlings. **(D)** *DR5:GUS* expression is reduced in emerging and newly emerged *abcb21* lateral root tips. **(E)** Quantification of GUS signal in **(D)**. Data shown are means ± SD ($n \geq 24$ from two independent experiments). Asterisks indicate statistical difference from Col-0 by Student's *t*-test for * $P < 0.05$ and ** $P < 0.01$. Scale bar: 50 μm.

cytokinin treatment (Bielach et al., 2012). This suggests that cytokinin negatively regulates *ABCB21* in immature root tissues, and may also contribute to negative regulation of *ABCB21* during lateral root formation and emergence (Laplaze et al., 2007; Bielach et al., 2012). Since *proABCB21:GUS* expression levels are low in regions of lateral root primordia initiation, visualization of changes following cytokinin treatment were difficult to interpret. However, loss of *proABCB21:GUS* signal is observed following initial cell divisions during primordia initiation (**Figures 1M–O**), which correlates with the timing of published cytokinin signaling increases during lateral root development (Bielach et al., 2012).

The previously described *abcb21-1* (WiscDsLox1C2) allele forms a partial transcript (Kamimoto et al., 2012). Therefore, a new allele, *abcb21-2* (Gabi_954H06) was obtained. Mutants were backcrossed to Col-0 three times and genotyped before subsequent analysis (**Supplementary Figure 1A**). Reverse transcription PCR (RT-PCR) indicates *abcb21-2* also forms a transcript that corresponds to the coding region upstream of the T-DNA insertion (**Supplementary Figure 1B**). Additionally, expression levels determined by quantitative real-time PCR (qRT-PCR) are not different from Col-0 (**Supplementary Figure 1C**). However, since the T-DNA insertion in *abcb21-2* is farther upstream compared to *abcb21-1* it was hypothesized that it would represent a stronger allele (**Figure 2A**). Due to the overlap in expression of *ABCB21* with *ABCB19* surrounding the vasculature, *abcb21-2* was tested for compensation by *ABCB19*. While loss of *abcb1* results in ∼5X increase in *ABCB19* transcript, no change is observed in the *abcb21-2* mutant (**Supplementary Figure 1D**).

As *ABCB19* and *ABCB21* expression domains overlap in the root, it was hypothesized ABCB21 may function in restriction of auxin to the root vasculature. When [^{3}H]IAA was placed

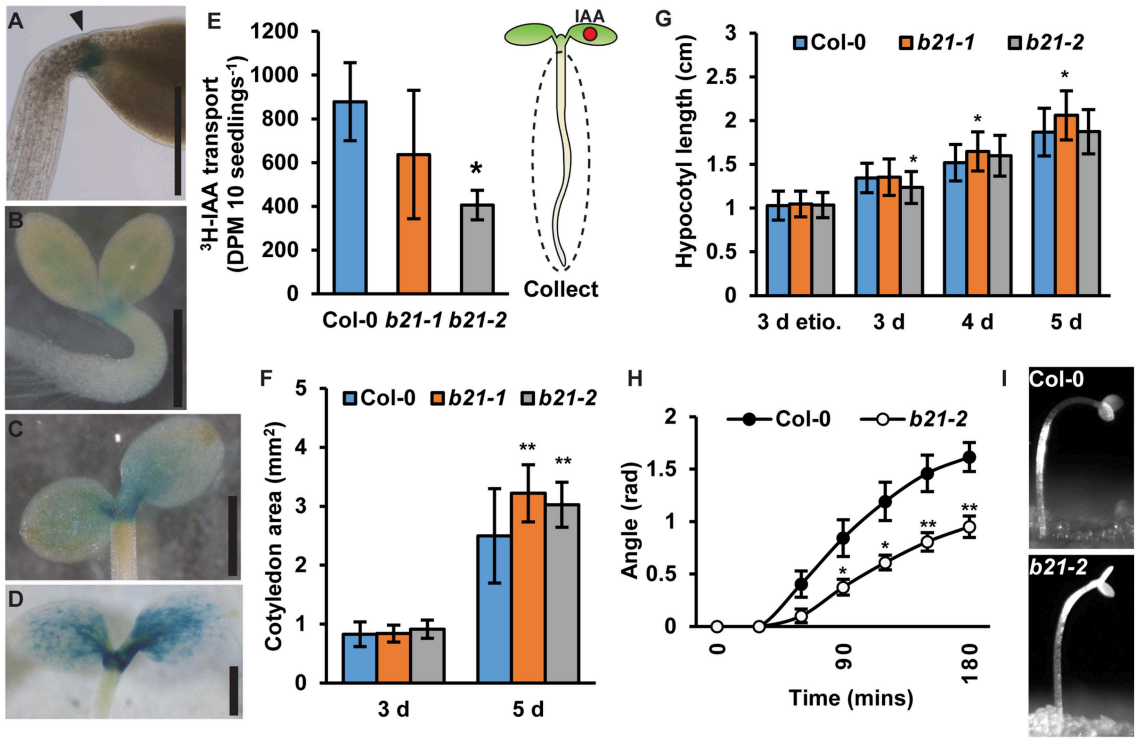

FIGURE 4 | ABCB21 mediates cotyledon-hypocotyl auxin transport. **(A)** *proABCB21:GUS* expression at the base of petioles in 4 d etiolated seedlings. **(B–D)** *proABCB21:GUS* expression in the petioles and cotyledonary node of **(B)** 1 d, **(C)** 3 d, and **(D)** 5 d light-grown seedlings. **(E)** Cotyledon-hypocotyl [^{3}H]IAA transport in 5.5 d seedlings. Data shown are means ± SD ($n = 3$ pools of 12). **(F)** Cotyledon areas of 3 and 5 d light-grown seedlings. Data shown are means ± SD ($25 \leq n \leq 32$). **(G)** Hypocotyl length in 3 d etiolated (etio.), and 3–5 d light-grown *abcb21* seedlings. Data shown are means ± SD ($n > 45$ from 3 replicates). **(H)** Phototropic curvature in light-treated Col-0 and *abcb21-2* seedlings. Data shown are means ± SE ($n = 8$ from 2 replicates). **(I)** Representative images of Col-0 and *abcb21-2* after phototropic bending for 3 h. Asterisks indicate statistical difference from Col-0 by Student's *t*-test for * $P < 0.05$ and ** $P < 0.01$. Scale bars: **(A–D)** 500 μm.

at the root-shoot transition zone (RSTZ) transport to the root tip was reduced by ∼14% and ∼24% in *abcb21-1* and *abcb21-2*, respectively (**Figure 2B**). Quantification of free IAA levels in *abcb21* indicate auxin levels are increased in the upper root and reduced in the root apex (**Figure 2C**). This suggests the reduction in rootward auxin transport in *abcb21* causes auxin to back up and pool in the upper root. This pooling, however, was not enough to activate the *DR5:GUS* auxin reporter in either *abcb21* background. Phenotypic analysis revealed *abcb21-2* 5 d primary roots are shorter than Col-0, but slightly longer in 10 and 14 d seedlings (**Figure 3A**). 10 d *abcb21-2* mutants exhibit reduced lateral root density (**Figure 3B**), but no difference in the distribution among developmental stages was observed (**Figure 3C**). Seven days lateral root density is not different between Col-0 and *abcb21* mutants (**Figure 3B**). However, the proportion of stage I-IV in *abcb21-2* mutants is increased and the proportion of stage V-VIII and emerged lateral roots is reduced in *abcb21-2* (**Figure 3C**). *abcb21-1* exhibits intermediate primary root and lateral root phenotypes which are consistent with it representing a weak allele compared to *abcb21-2*. *DR5:GUS* signal is reduced in emerging and newly emerged lateral roots indicating the defect in lateral root outgrowth is due to reduced auxin levels (**Figures 3D,E**).

ABCB21 Mobilizes Phototropic Auxin Supply From the Cotyledons

In seedlings, *proABCB21:GUS* expression is high at the base of the cotyledons, the petioles, and the cotyledonary node (**Figures 4A–D**) suggesting ABCB21 may function in mobilizing auxin from these tissues. To compare auxin transport in *abcb21* to wild type, [^{3}H]IAA was placed at the center of one cotyledon, then the hypocotyl and roots were collected after 2 h (**Figure 4E**). [^{3}H]IAA transport in *abcb21-2* is reduced by >50%. Transport in *abcb21-1* was also reduced, but was highly variable. Auxin transport from the shoot apex to the RSTZ was not different in either *abcb21* mutant (data not shown), which is consistent with the lack of *proABCB21:GUS* expression in the hypocotyl. The defects in mobilization of auxin from the cotyledons leads to a significant increase in cotyledon expansion in 5 d seedlings (**Figure 4F**), but only small differences in hypocotyl elongation (**Figure 4G**). Removal of the cotyledons in post-photomorphogenic seedlings reduces phototropic bending, suggesting cotyledon-derived auxin contributes to phototropic bending (Preuten et al., 2013). Similarly, phototropic bending was severely reduced in post-photomorphogenic *abcb21-2* seedlings (**Figures 4H,I**; **Supplementary Movie 1**). No difference was observed in etiolated seedlings.

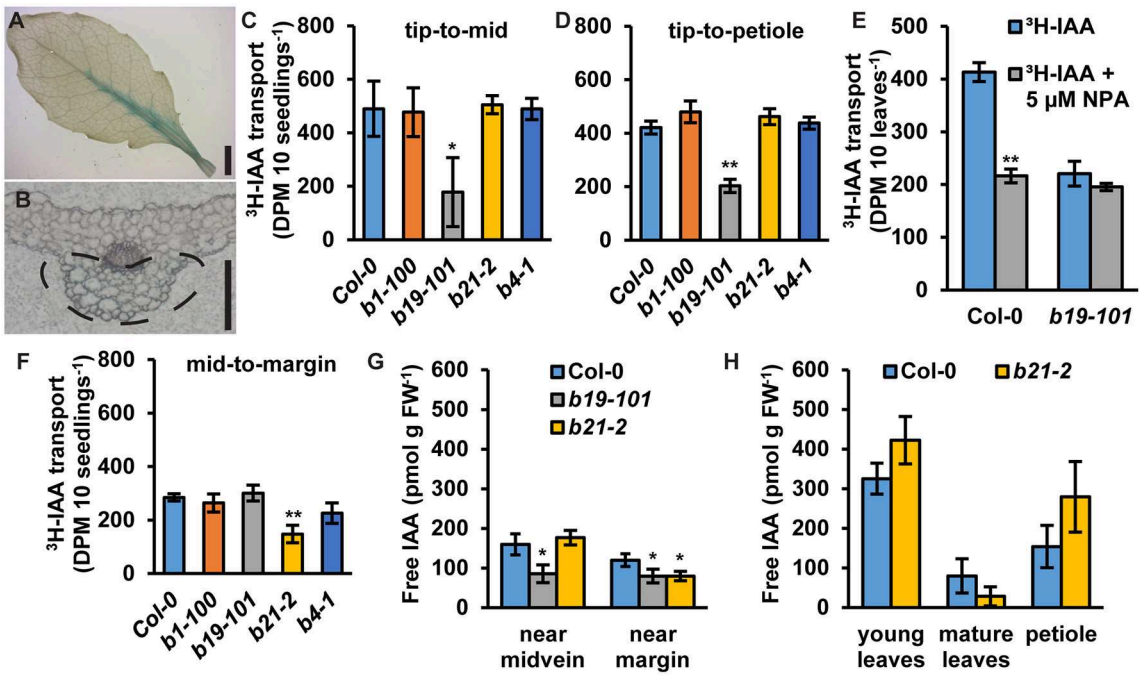

FIGURE 5 | Expression of *ABCB21* and auxin transport activity in leaves. **(A)** *proABCB21:GUS* is expressed in rosette leaves associated with the midvein. **(B)** Cross section showing *proABCB21:GUS* expression is primarily in the bundle sheath and collenchyma on the abaxial side of the leaf. Dotted line indicates area of GUS staining. **(C,D)** Transport of [^{3}H]IAA from the leaf tip to the **(C)** leaf midpoint or **(D)** petiole. **(C,D)** share the same scale/units. Data shown are means ± SD ($n = 3$ pools of 10). **(E)** Transport of ^{3}H-IAA from the leaf tip to the petiole with NPA treatment. Data shown are means ± SD ($n = 3$ pools of 10). **(F)** Transport of [^{3}H]IAA from the leaf midvein to the margin. Data shown are means ± SD ($n = 3$ pools of 10). **(G)** Free IAA levels in rosette leaves near the midvein and near the margin. **(H)** Free IAA levels in young leaves, mature leaves, and petioles. Data shown are means ± SD ($n = 3$ pools of 10). Asterisks indicate statistical difference from Col-0 by Student's *t*-test for * $P < 0.05$ and ** $P < 0.01$. Scale bars: **(A)** 2 mm; **(B)** 200 µm.

ABCB21 Contributes to Lateral Auxin Distribution in Rosette Leaves

In rosette leaves, *proABCB21:GUS* expression is observed near the leaf midvein (**Figure 5A**). Cross sections revealed that expression is primarily associated with the collenchyma and bundle sheath cells on the abaxial side of the leaf and not within the midvein (**Figure 5B**). To see if ABCB21 function in the leaf might resemble its function in the root *abcb21-2* mutants were analyzed for defects in auxin transport using intact rosette leaves. *abcb1*, *abcb19*, and *abcb4* were also included. For transport along the tip-petiole axis, [^{3}H]IAA-soaked agarose beads were placed on leaf tips. After 3 h, petioles or 0.5 mm mid-leaf punches were collected and measured for radioactivity. For centro-lateral transport, [^{3}H]IAA-soaked agarose beads were placed on the leaf midvein. After 3 h, 0.5 mm punches were collected from the leaf margin and measured for radioactivity. Transport of [^{3}H]IAA from the tip to the mid-leaf and petiole was significantly reduced in *abcb19* (**Figures 5C,D**). In Col-0, treatment with 5 µM NPA reduced transport from the leaf tip to the petiole ∼50%, which is equal to the transport in *abcb19* (**Figure 5E**). Treatment of *abcb19* with 5 µM NPA did not cause any further reduction, suggesting ABCB19 is a primary target for NPA inhibition at this concentration (**Figure 5E**). No additional reduction in auxin transport from the leaf tip to the midpoint or petiole was observed in Col-0 using 10 µM NPA (**Supplementary Figures 2A,B**). However, treatment with 20 µM NPA resulted in additional inhibition of transport which is likely due to blocking of other ABCBs and/or PINs. In contrast to *abcb19*, *abcb21* showed a significant decrease in transport of [^{3}H]IAA from the midvein to the margin (**Figure 5F**). For lateral auxin transport 10 µM NPA inhibited auxin transport in Col-0 to *abcb21* levels (**Supplementary Figure 2C**). No additional effect was observed using 20 µM NPA (**Supplementary Figure 2C**) suggesting ABCB21 is the primary target for NPA inhibition of the measured lateral auxin movement. Consistent with these results, endogenous IAA levels are significantly reduced near the midvein of *abcb19* (**Figure 5G**). IAA levels along the margin are reduced by ∼30% in *abcb19* and *abcb21* (**Figure 5G**). Overall auxin levels in young leaves, mature leaves, and petioles were not statistically different from Col-0 (**Figure 5H**). Despite the auxin transport defects in leaves, *abcb21* mutants do not exhibit any observable differences from Col-0 in rosette leaf morphology or phyllotaxis under our standard growth conditions (**Supplementary Figures 3A,B**). It was noted, however, that *abcb21* occasionally exhibited larger variation in rosette leaf size in the greenhouse when light and temperature were more inconsistent.

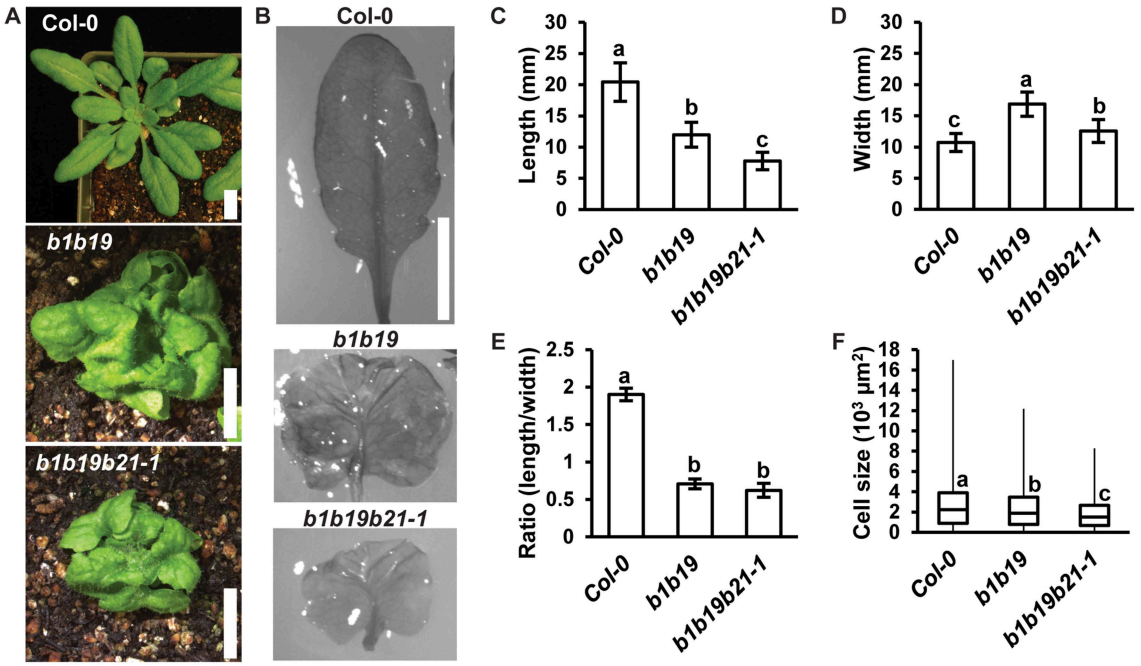

FIGURE 6 | *abcb21* triple mutants exhibit enhanced morphological defects in leaves. **(A)** Representative images of 4 weeks rosettes grown under 100 µmol m^{-2} s^{-1} white light. **(B)** 5th rosette leaf removed from A. Leaves were soaked in ethanol to allow curled leaves to lay flat. **(C–E)** Measurement of **(C)** length, **(D)** width, and **(E)** length/width ratio in leaves from **(B)**. Data shown are means ± SD ($n \geq 10$). Letters indicate statistical difference by ANOVA $p < 0.001$, Tukey's *post-hoc* $p < 0.05$. **(F)** Boxplot of showing 5th leaf cell size ($n \geq 219$ from at least 3 leaves per line). Abaxial epidermal cells were measured at a midpoint from the leaf tip to the petiole and half way from the leaf margin. Letters indicate statistical difference by ANOVA $P < 0.001$, Tukey's *post-hoc* $P < 0.05$. Scale bars: 1 cm.

Loss of *abcb1* and *abcb19* results in compact and severely curled rosette leaves (Noh et al., 2001; Geisler et al., 2003; Blakeslee et al., 2007). It was hypothesized that addition of *abcb21* would result in enhancement of these leaf morphology defects. Careful examination of leaf development revealed no defects in basic abaxial/adaxial definition, veination, or leaf margin development in *abcb1 abcb19* or *abcb1 abcb19 abcb21* (**Figures 6A,B**). Although the weak *abcb21-1* allele was used, enhancement of defects in leaf morphology were observed. Measurement of leaf length and width revealed *abcb1 abcb19 abcb21* leaves were significantly shorter than Col-0 and *abcb1 abcb19* (**Figure 6C**) and wider than Col-0, but not *abcb1 abcb19* (**Figure 6D**). While triple mutants developed smaller leaves compared to the double mutant, their overall shapes were not notably different (**Figures 6B,E**). These alterations in morphology appear to be due to reduced abaxial pavement cell expansion, as decreasing cell size compared to Col-0 is observed in *abcb1 abcb19* and *abcb1 abcb19 abcb21*, respectively (**Figure 6F**).

At flowering stage single *abcb21* mutants are slightly taller than Col-0 plants and have increased secondary inflorescence number (**Supplementary Figures 4A–C**). No difference in primary branch number or internode length were observed (**Supplementary Figures 4D,E**). Compensation and functional redundancy lead to enhanced phenotypes in double *abcb1 abcb19* and *abcb6 abcb20* mutants (Noh et al., 2001; Geisler et al., 2003; Blakeslee et al., 2007; Zhang et al., 2018). Although no compensation in *ABCB4* expression was detected in *abcb21* knockdown lines (Kamimoto et al., 2012) and their expression domains do not overlap, *abcb4 abcb21* double mutants were examined for morphological defects not observed in the single mutants. This, however, did not result in any synergistic phenotypes (**Supplementary Figures 3A,B, 4A–E**).

ABCB21 Expression Is Rapidly Induced During Wounding

As reported previously (Kamimoto et al., 2012), *proABCB21:GUS* expression in late stage mature tissues is restricted to the abscission zones of flowers, as well as rosette and cauline leaves (**Figures 7A–E**). Auxin regulation of leaf positioning (Peeters et al., 2009; de Carbonnel et al., 2010) and floral organ shedding/abscission (Tang et al., 2013) suggests a possible role for ABCB21 in regulation of localized auxin accumulations in these tissues. However, no differences in light-mediated leaf positioning were observed in *abcb21* mutants when responses under continuous 50 µmol m^{-2} s^{-1} red plus far red light, 50 µmol m^{-2} s^{-1} red light, or 50 µmol m^{-2} s^{-1} white light were examined (**Figure 7F**), and measurements of petal break-strength was not different between Col-0 and *abcb21-2* (**Figure 7G**). It is unclear whether *ABCB21* expression at these junction sites

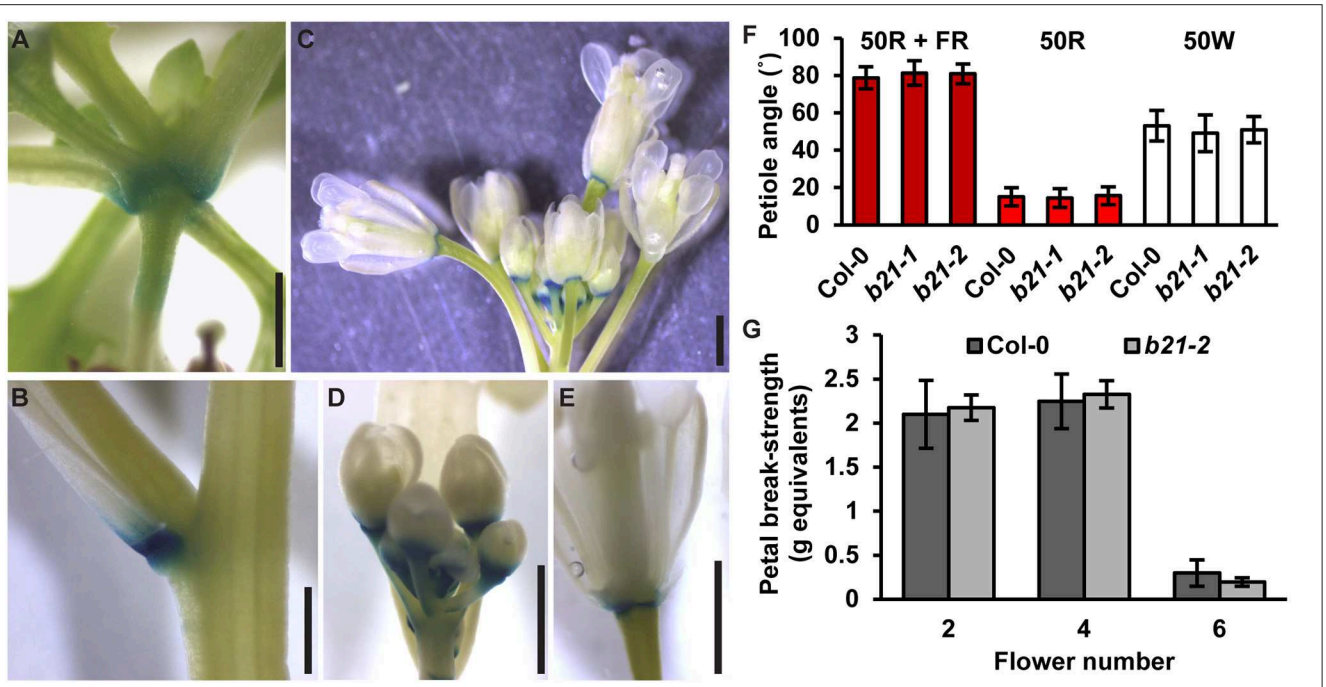

FIGURE 7 | Expression of *ABCB21* in abscission zones. *proABCB21:GUS* is expressed in the abscission zones of **(A)** rosette leaves, **(B)** cauline leaves, and **(C)** floral organs. **(D,E)** *proABCB21:GUS* expression domain is expanded in **(D)** young flowers and **(E)** restricted to the abscission zone in mature flowers. Plants were GUS stained for 16 h. **(F)** True leaf petiole angles in *abcb21*. Plants were grown on soil under 80 μmol m^{-2} s^{-1} white light, 16 h photoperiod. When plants reached stage 1.01 they were transferred to continuous 50 μmol m^{-2} s^{-1} red plus far-red light (50R + FR; burgundy bars), 50 μmol m^{-2} s^{-1} red light (50R; red bars), or 50 m^{-2} s^{-1} white light (50W; white bars) and allowed to grow an additional 3 d. Angle was determined by measuring the angle formed between the hypocotyl and the two first true leaf petioles minus 90°. Data shown are means ± SD ($n = 60$). **(G)** Flower petal break-strength in *abcb21*. Flower 1 was designated as the first flower with visible flower petals. Methods are detailed the methods section and **Supplementary Figure 7**. Data shown are means ± SD ($n = 15$). Scale bars: 1 mm.

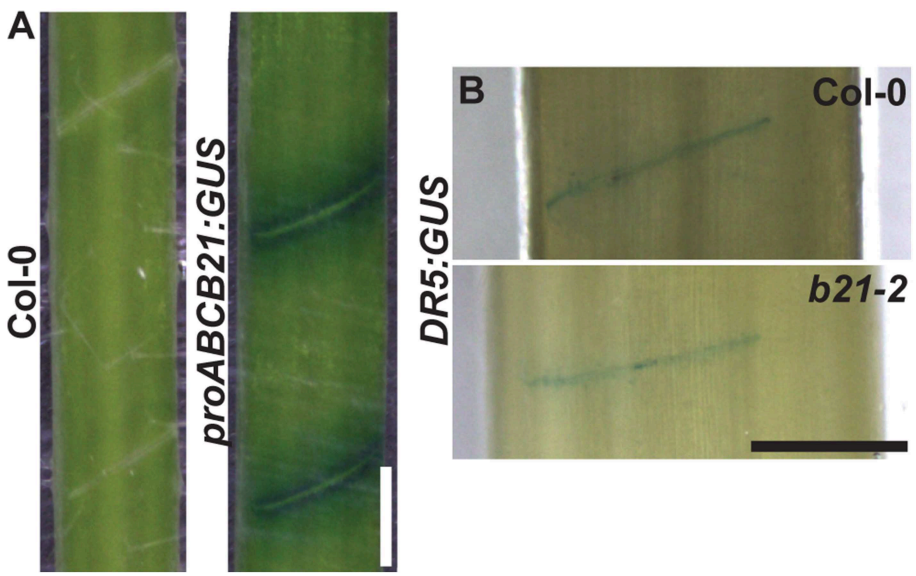

FIGURE 8 | *proABCB21:GUS* and *DR5:GUS* expression after wounding. **(A)** *proABCB21:GUS* expression is induced after manual wounding. Mature inflorescence stems were cut with a razor blade at the bottom internode. Attached stems were left for 2 h prior to GUS staining. **(B)** Initial *DR5:GUS* expression is not different between Col-0 and *abcb21-2*. Plants were treated the same as in **(A)** except cleared with Visikol prior to imaging to enhance visualization. Scale bars: 0.5 mm.

is responsive or causal. However, wounding increases *ABCB21* expression ∼1.7X between 30 and 60 min before returning to pre-wound levels or below (Kilian et al., 2007). Rapid induction of *proABCB21:GUS* expression is observed in stem tissues after wounding (**Figure 8A**). No GUS staining was observed in Col-0 indicating staining was not due to non-specific

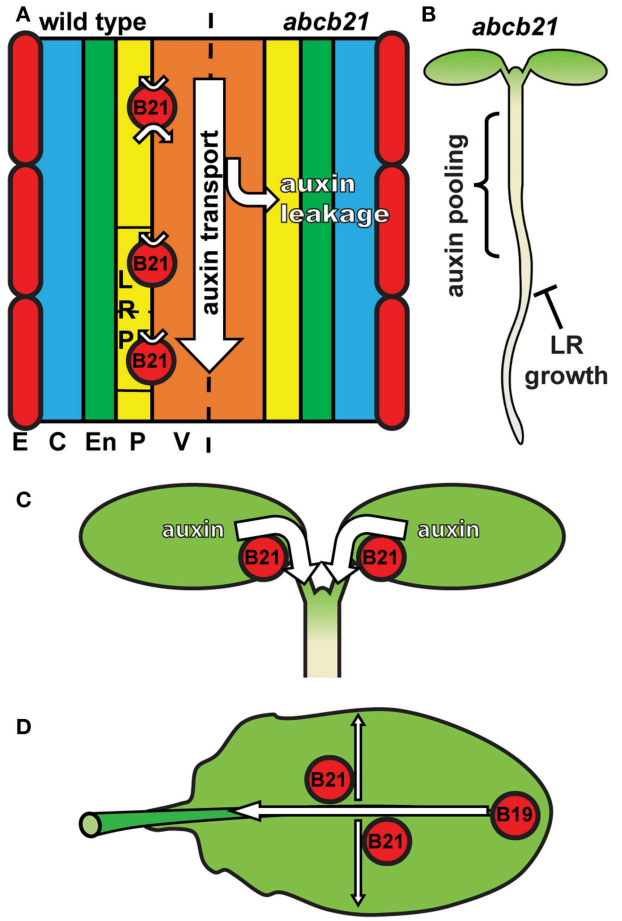

FIGURE 9 | Model for ABCB21 function in seedlings and leaves. **(A)** ABCB21 functions primarily in restricting auxin to within the rootward auxin transport stream. Loss of ABCB21 leads to leakage of auxin from the central cylinder, resulting in reduced rootward auxin transport. E, epidermis; C, cortex; En, endodermis; P, pericycle; V, vasculature. **(B)** The reduction in auxin transport in the root causes pooling of auxin in the upper root and lower hypocotyl, reducing the supply needed for lateral root outgrowth. **(C)** ABCB21 mediates cotyledon-hypocotyl auxin transport to load the rootward auxin transport stream. **(D)** In leaves ABCB21 contributes to lateral auxin transport, while ABCB19 primarily mediates transport from the leaf tip to the petiole.

enzymatic activity. However, similar discrete *DR5:GUS* signals are initially observed in both Col-0 and *abcb21-2* suggesting initial auxin accumulations are not affected (**Figure 8B**). A downstream role in wound-induced vascularization is possible, but does not appear to involve monolignol transport, as is observed with ABCG29 (Alejandro et al., 2012). No differences in seedling root growth on *p*-coumaryl alcohol were observed in *abcb21* under conditions where *abcg29* root growth is more inhibited than Col-0 (**Supplementary Table 1**), and no differences in lignin content or speciation were detected in seedling roots (**Supplementary Table 2**). A more localized impact on auxin-dependent vascularization is possible, but could not be reproducibly verified.

DISCUSSION

The results presented herein are consistent with a role for ABCB21 in regulating cellular auxin levels in a manner similar to ABCB4 in the root epidermis. In roots, *ABCB21* expression in the pericycle up to 7 d coincides with the rootward pulse of shoot derived auxin that triggers lateral root outgrowth (Bhalerao et al., 2002). *abcb21* mutants exhibit reduced rootward auxin transport and delays in lateral root emergence, which is consistent with the activity of ABCB19 (Wu et al., 2007). This suggests that in the root pericycle not associated with lateral roots ABCB21 primarily plays a supplementary role to ABCB19 in excluding auxin from the pericycle and maintaining it within the central cylinder (**Figure 9A**). Although ABCB21 exhibits conditional uptake/efflux activity, the endogenous auxin levels in the seedling root suggests ABCB21 is primarily acting in efflux in these tissues. The defects in lateral root emergence are likely due primarily to the defects in rootward auxin transport (**Figure 9B**). However, the loss of *ABCB21* expression in developing and emerging lateral roots suggests ABCB21 may also play a more localized role during lateral root development. Since ABCB21 exhibits auxin uptake activity at low intracellular concentrations, ABCB21 may help regulate initial auxin accumulations during the early stages of lateral root development. Expression of *ABCB21* during the first cell divisions during early lateral root development is consistent with this function. Additionally, treatment of lateral root primordia with cytokinins delays the establishment of auxin maxima and subsequent lateral root outgrowth (Bielach et al., 2012). These delays are also observed in *abcb21* suggesting cytokinins may negatively regulate *ABCB21* to allow for development of auxin maxima and progression of lateral root development after initiation. Loss of *ABCB21* expression during late stage development and emergence could be expected, as preventing reloading of auxin back into the rootward stream seems necessary for the establishment of auxin maxima at the primordia tip (Benková et al., 2003).

In cotyledons *ABCB21* expression coincides with the timing of shoot-derived auxin production (Bhalerao et al., 2002). *abcb21* mutants exhibit reduced cotyledon-hypocotyl auxin transport, increased cotyledon expansion, and decreased phototropic bending. These results reflect a role for ABCB21 in mobilizing auxin from the cotyledons to load the rootward auxin transport stream during the 5-10 d window when the cotyledons supply auxin to the root (Bhalerao et al., 2002) (**Figure 9C**). It was hypothesized that that loss of *ABCB21* would result in decreased transport from the leaf tip to the petiole. However, this resulted in reduced lateral auxin transport, not reduced transport from the tip to the petiole. While the precise cellular transport mechanisms remain to be determined, these results support a role for ABCB21 in contributing to auxin lateral distribution within the leaf, while ABCB19 primarily contributes to transport from the leaf tip to the petiole (**Figure 9D**). This is further supported by the observed alterations in leaf morphology and reduced epidermal cell size in *abcb1 abcb19 abcb21* triple mutants compared to *abcb1 abcb19* double mutants.

The role of ABCB21 in rosette leaf and floral organ abscission zones remains unclear. Treatment with auxin, salicylic acid, or methyl-jasmonate does not induce *ABCB21* expression (Kamimoto et al., 2012). However, H_2O_2 and UV-B, presumably by UV-induced reactive oxygen species (ROS), increases *ABCB21* expression ∼1.9X and ∼6X, respectively (Kilian et al., 2007; Gutiérrez et al., 2014). During wounding, increased ROS levels at the wound site within minutes (L'Haridon et al., 2011; Beneloujaephajri et al., 2013) correlates with the rapid induction of *ABCB21* expression. However, no difference in auxin accumulations or auxin related phenotypes are observed in these tissues, suggesting a role for ABCB21 in the transport of other substrates besides auxin.

ABCB4 and ABCB21 share high protein sequence similarity (83.8% identity/92.4% similarity) (**Supplementary Figure 5**). Previous analysis of protein sequence and structure identified an N-terminal coiled-coil domain that is present in ABCB4 and ABCB21, but not ABCB1 or ABCB19 (Yang and Murphy, 2009). The function of this domain remains unknown, but appears to be unique to ABCBs associated with substrate uptake in addition to efflux (Shitan et al., 2003; Santelia et al., 2005; Terasaka et al., 2005; Lee et al., 2008; Yang and Murphy, 2009; Kubeš et al., 2012). Although ABCB4 and ABCB21 function in a similar manner at the cellular level, compensatory activity that is observed with ABCB1/19 and ABCB6/20 (Noh et al., 2001; Zhang et al., 2018) is not present. This lack of functional redundancy is explained by the non-overlapping expression domains and points to involvement in distinct developmental processes. ABCB21 represents the final complement to the primary ABCB auxin transporter pairs. Therefore, ABCB1/19, ABCB6/20, and ABCB4/21 appear to represent the major ABCB auxin transporters in Arabidopsis.

DATA AVAILABILITY

This manuscript contains previously unpublished data. The name of the repository and accession number are not available.

AUTHOR CONTRIBUTIONS

MJ, NC, and AM designed the research. MJ, NC, CP, and AM performed the experiments and analyzed the data. MJ and AM wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00806/full#supplementary-material

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REVIEW
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Indole 3-Butyric Acid Metabolism and Transport in *Arabidopsis thaliana*

Suresh Damodaran[1] *and Lucia C. Strader*[1,2,3]*

[1]Department of Biology, Washington University in St. Louis, St. Louis, MO, United States, [2]Center for Science and Engineering Living Systems, Washington University in St. Louis, St. Louis, MO, United States, [3]Center for Engineering MechanoBiology, Washington University in St. Louis, St. Louis, MO, United States

Auxin is a crucial phytohormone involved in multiple plant developmental processes. Spatiotemporal regulation of auxin levels is necessary to achieve development of organs in the proper place and at the proper time. These levels can be regulated by conversion of auxin [indole 3-acetic acid (IAA)] from its conjugated forms and its precursors. Indole 3-butyric acid (IBA) is an auxin precursor that is converted to IAA in a peroxisomal β-oxidation process. In Arabidopsis, altered IBA-to-IAA conversion leads to multiple plant defects, indicating that IBA contributes to auxin homeostasis in critical ways. Like IAA, IBA and its conjugates can be transported in plants, yet many IBA carriers still need to be identified. In this review, we discuss IBA transporters identified in Arabidopsis thus far, including the pleiotropic drug resistance (PDR) members of the G subfamily of ATP-binding cassette transporter (ABCG) family, the TRANSPORTER OF IBA1 (TOB1) member of the major facilitator superfamily (MFS) family and hypothesize other potential IBA carriers involved in plant development.

Keywords: auxin, indole-3-butyric acid, phytohormone, ATP-binding cassette transporter, TRANSPORTER OF IBA1, transporters

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***Correspondence:**
Lucia C.Strader
strader@wustl.edu

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INTRODUCTION

Multiple pathways coordinate plant development; many of which require auxin for their effect on development. Auxin is a well-studied plant hormone that is important for multiple plant developmental processes with major roles in cell division, differentiation, and elongation (Ljung, 2013; reviewed in Zhao, 2010). Endogenous active auxins include indole acetic acid (IAA), phenyl acetic acid (PAA), and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Cook, 2019; reviewed in Korasick et al., 2013). IAA is widely considered to be the predominant form of active auxin within the plant and likely contributes to the majority of auxin activity in many plants.

Attaining auxin maxima in specific tissues is essential for organogenesis and for environmental responses. Mechanisms that contribute to the regulated distribution of auxin include polar auxin transport and IAA metabolism. The polarized localization of IAA transporters regulates auxin movement in specific directions, whereas local auxin metabolism provides the IAA for this transport and is also critical for establishing auxin maxima. These metabolism and transport mechanisms for IAA likely act in concert (Brumos et al., 2018; Morffy and Strader, 2018).

Auxin is primarily synthesized through a two-step tryptophan-dependent auxin biosynthesis pathway catalyzed by the tryptophan aminotransferase of Arabidopsis (TAA) and YUCCA (flavin monooxygenase enzyme) families of enzymes (reviewed in Zhao, 2012). TAA family enzymes convert the aromatic amino acid tryptophan into indole-3-pyruvic acid (IPyA);

this is subsequently converted into IAA by YUCCA family members. Higher order *taa* and higher order *yucca* mutants display drastic developmental phenotypic defects (Zhao et al., 2001; Cheng et al., 2006, 2007; Stepanova et al., 2008; Tao et al., 2008), suggesting functional redundancy within these families and also revealing critical roles for *de novo* auxin biosynthesis in plant development.

In addition to *de novo* auxin biosynthesis, IAA can be released from conjugates with sugars, amino acids, and the chain-lengthened precursor indole-3-butyric acid (IBA) (reviewed in Korasick et al., 2013; Frick and Strader, 2018). Several amide-linked IAA conjugates have been identified in plants, including IAA-Ala and IAA-Leu, which can be hydrolyzed to release free IAA in Arabidopsis, maize, and several other plant species (Bartel and Fink, 1995; LeClere et al., 2002; Campanella et al., 2003a,b, 2008; Rampey et al., 2004). IAA stored in the form of an ester-linked IAA-sugar conjugate is hydrolyzed to release active IAA in both monocots and dicots (Jakubowska et al., 1993; Jakubowska and Kowalczyk, 2005; Campanella et al., 2008). The auxin precursor IBA is converted in to active IAA through a β-oxidation process in the peroxisome (Strader et al., 2011).

Metabolism of Indole 3-Butyric Acid and Its Role as an Auxin Precursor

IBA was initially assumed to be a synthetic auxinic compound that was primarily used as a rooting media agent. In marigold, tomato, buckwheat, pea, bean, sunflower, and few other plant species, exogenous IBA induces root elongation, leaf epinasty, and stem bending (Zimmerman and Wilcoxon, 1935). Although IBA was once thought to be a synthetic auxin, it was later detected as an endogenous compound in potato peelings using paper chromatography (Blommaert, 1954). Endogenous IBA has been detected in multiple plant species, including Arabidopsis, tobacco, pea, and maize (reviewed in Korasick et al., 2013). This widespread occurrence of IBA suggests that this molecule may play a conserved role across species.

Although IBA has been identified as an endogenous compound in multiple plant species, it is often present at low levels and is difficult to detect. Indeed, the presence of IBA as an endogenous molecule has been questioned in one study, due to an inability to detect it using gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry in Arabidopsis, poplar, and wheat (Novak et al., 2012). In studies in which endogenous IBA has been detected, its levels are often found at a lower level than IAA (Sutter and Cohen, 1992; Ludwig-Müller et al., 1993). Among these studies, differences in IBA detection could reflect (1) differences in metabolite extraction techniques, (2) detection limits of the technology being used, and/or (3) differences in IBA accumulation in plants grown under distinct laboratory conditions. IBA contribution to overall auxin level varies among species and even among accessions within a single species, suggesting differential IBA metabolism in different plants (Ludwig-Müller and Epstein, 1991; Ludwig-Müller et al., 1993). Resolving these differences in the future will be key to understanding the prevalence of IBA contributions to the auxin pool.

IBA is structurally similar to IAA except for the side chain, in which IAA has two carbons and IBA has a four-carbon side chain (**Figure 1**). IAA binds to the TIR1/AFB-Aux/IAA (TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN-Auxin/INDOLE-3-ACETIC ACID) co-receptor complex to initiate downstream auxin-responsive gene expression (reviewed in Lavy and Estelle, 2016). Because of its lengthened side chain, IBA is unable to stimulate formation of the auxin co-receptor complex (Uzunova et al., 2016). Thus, physiological effects of IBA treatment are likely caused by IBA-derived IAA and not by the IBA molecule itself.

The major mechanism by which IBA influences plant development is through the reduction of its carbon side chain to convert in to IAA (reviewed in Strader and Bartel, 2011). IBA-to-IAA conversion occurs through a fatty acid β-oxidation process housed in the peroxisome (Zolman et al., 2000; Strader et al., 2010). Enzymes specifically involved in catalyzing IBA conversion include the short-chain dehydrogenase/reductase indole-3-butyric acid response 1 (IBR1; Zolman et al., 2007), the acyl-coA dehydrogenase/oxidase-like IBR3 (Zolman et al., 2007), the predicted enoyl coA hydratase IBR10 (Zolman et al., 2008), and enoyl-COA hydratase2 (ECH2; Strader et al., 2011). In addition, generalist enzymes such as PED1 (3-ketoacyl COA thiolase) (Zolman et al., 2000) and ACX enzymes (Adham et al., 2005) may also act in the conversion of IBA to IAA. Peroxisomal fatty acid β-oxidation of oil bodies is required in Arabidopsis to fuel growth prior to photosynthesis. Thus, dark-grown seedlings defective in fatty acid β-oxidation display reduced growth unless the media is supplemented with an exogenous carbon source such as sucrose (Zolman et al., 2000). Because mutants defective in *IBR1*, *IBR3*, *IBR10*, or *ECH2* grow normally in the dark in the absence of sucrose for growth (Zolman et al., 2007, 2008; Strader et al., 2011), these enzymes are unlikely to be involved in fatty acid β-oxidation; however, it is possible they are involved in β-oxidation of substrates in addition to IBA. Conversely, mutants defective in PED1 or ACX enzymes are resistant to IBA and also require an exogenous carbon source to fuel dark-grown growth (Zolman et al., 2000; Adham et al., 2005), suggesting that these are in involved in both IBA and fatty acid β-oxidation.

Mutants defective in IBA-to-IAA conversion enzymes display multiple plant developmental defects, including reduced cotyledon expansion, reduced apical hook curvature, reduced lateral root formation, and smaller root apical meristems, along with decreased levels of free IAA (Zolman et al., 2008; Strader et al., 2010, 2011). IBA treatment fails to stimulate lateral root organogenesis in mutants defective in IBA conversion enzymes (Strader et al., 2011), suggesting that IBA cannot function directly to stimulate lateral root production but rather acts through its conversion to IAA (Strader et al., 2011). Further, the chemical naxillin stimulates lateral root production through stimulation of IBA-to-IAA conversion in Arabidopsis (De Rybel et al., 2012), confirming strong roles for IBA contribution to the pool of active auxin to regulate production of lateral roots.

Similar to IAA conjugates, both amide- and ester-linked conjugates of IBA have been detected in plants (Ludwig-Müller et al., 1993; Tognetti et al., 2010; Liu et al., 2012; Sherp et al., 2018). Overexpression of *UGT74E2*, encoding a glucosyltransferase, results in reduced IBA levels and elevated IBA-glucose conjugate

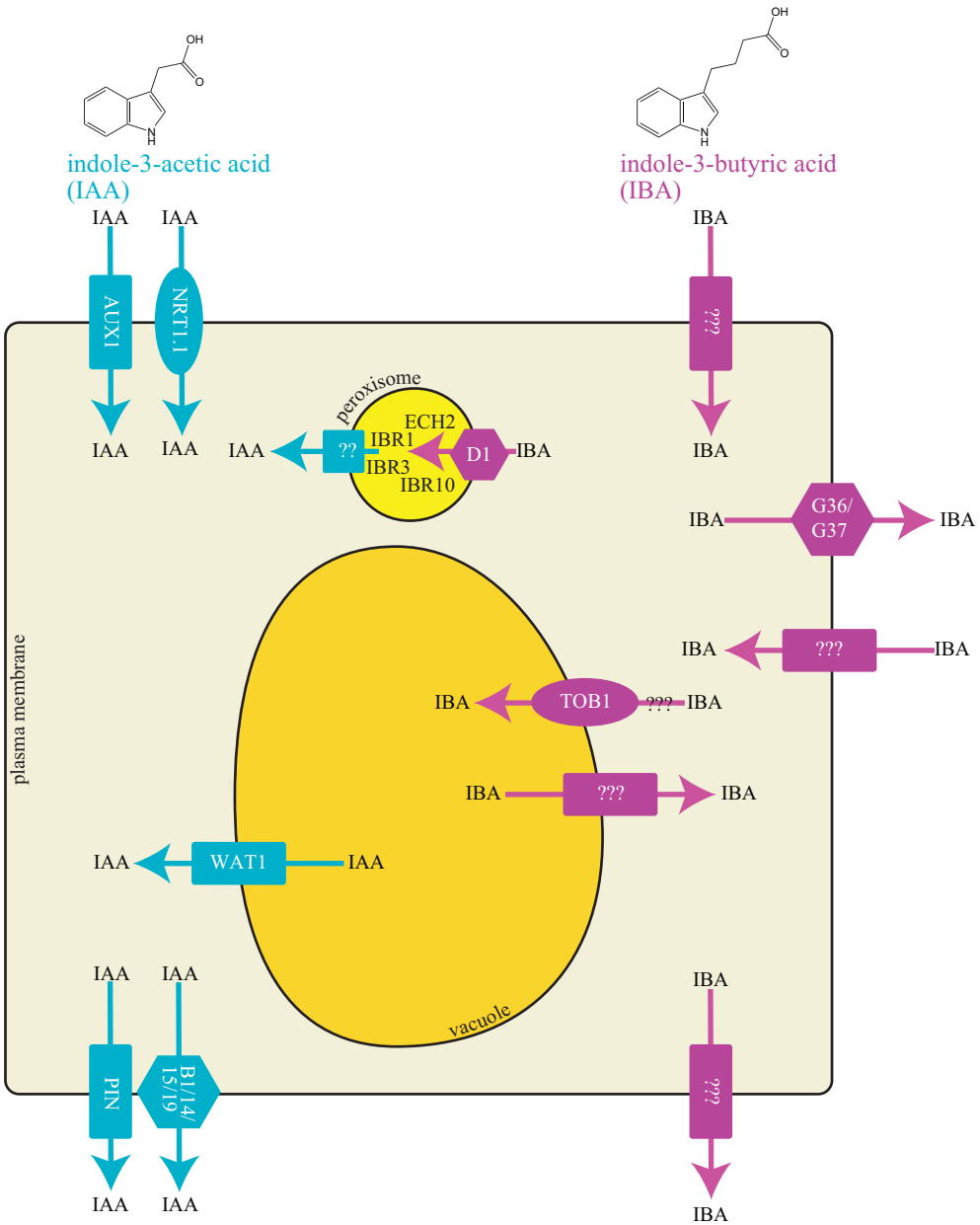

FIGURE 1 | Cellular model of IAA and IBA transporters. IAA and IBA are indolic compounds with two and four carbon side chains, respectively. Graphical representation of a cell with different transport proteins involved in transport of IAA and IBA.

levels in Arabidopsis (Tognetti et al., 2010), suggesting that UGT74E2 catalyzes conjugation of IBA to glucose. Further, Arabidopsis plants overexpressing *UGT74E2* display increased shoot branching and improved abiotic stress tolerance (Tognetti et al., 2010), consistent with the possibility that altered IBA homeostasis affects plant growth and stress response. In these experiments, it is unclear whether depletion of the IBA molecule itself or depletion of IBA-derived IAA affects growth and stress responses when *UGT74E2* is overexpressed. In addition to conjugation to glucose, IBA is also conjugated to amino acids (reviewed in Korasick et al., 2013). The Arabidopsis GH3-15 acyl acid amido synthetase enzyme specifically conjugates IBA to aspartate; however, whether this conjugated form is for IBA storage or for degradation is unknown (Sherp et al., 2018). Overall, these data suggest that homeostasis of IBA and its conjugates play important roles in maintaining IAA levels.

Indole 3-Butyric Acid Transport

Polar auxin transport regulates IAA distribution *via* specific cellular carriers. Similar to IAA, IBA is moved by transporters (reviewed in Strader and Bartel, 2011; Michniewicz et al., 2014). Because IBA is structurally similar to IAA (**Figure 1**), the question arises whether IAA transporters can also transport IBA. IAA is transported directionally with the aid of

tissue-specific influx and efflux proteins (reviewed in Vieten et al., 2007; Peer et al., 2011). Transporters involved in polar IAA transport include both uptake and efflux carriers. IAA uptake is carried out by members of the amino acid permease-like AUXIN RESISTANT1 (AUX1) family. Two transporter families mediate cellular IAA efflux: the family of PIN-FORMED (PIN) proteins and the MULTIDRUG RESISTANCE/GLYCOPROTEIN (PGP) class of ATP-binding cassette (ABC) transporters (reviewed in Vieten et al., 2007; Petrášek and Friml, 2009). No examined IAA transporter appears to mediate IBA transport (see sections below) suggesting the presence of IBA specific transporters in Arabidopsis.

Long-distance IBA transport was first suggested based on evidence that localized IBA treatment affected distal developmental events in marigold, tomato, sweet pea, and few other species (Zimmerman and Wilcoxon, 1935). Long-distance transport of radiolabeled IBA allowed tracking of IBA movement in cleopatra mandarin (Epstein and Sagee, 1992) and Arabidopsis (**Figure 1**; Ludwig-Müller et al., 1995; Rashotte et al., 2003). However, a major caveat to these tracking experiments is that the identity of the tracked molecule is unknown; thus, movement of [^{3}H]IBA is indistinguishable from movement of [^{3}H]IBA-conjugates or [^{3}H]IAA derived from [^{3}H]IBA. To overcome this limitation, analytical methods have been used to determine the identity of IBA-derived molecules in transport assays (Ruzicka et al., 2010; Liu et al., 2012). Application of [^{3}H]-IBA to Arabidopsis root columella cells results in high-performance liquid chromatography-based detection of [^{3}H]-IAA in root tissue 4 mm above the application site after a 2-h incubation (Ruzicka et al., 2010), suggesting that most of the transported molecule in this assay was IBA-derived IAA. Further, gas chromatography-mass spectrometry (GC/MS) methods were used to determine transported molecules across the Arabidopsis hypocotyl and inflorescence stem after heavy IBA application; multiple IBA-derived molecules were transported through these tissues, including IAA and ester-linked IBA (Liu et al., 2012). By comparison, movement of heavy IBA through these tissues occurred at lower levels, suggesting that, in Arabidopsis, IBA metabolites are transported long distances more efficiently than the IBA molecule itself (Liu et al., 2012). Thus, it seems that much of the long-distance transport of "IBA" may be of IBA conjugates, rather than the IBA molecule itself. Further, no identified IBA transporter has been shown to have roles in long-distance IBA transport.

IBA uptake is a saturable process (Ludwig-Müller et al., 1995; Rashotte et al., 2003), suggesting that IBA uptake is mediated by carriers rather than by simple diffusion. IBA transport is unaltered in the *aux1* mutant in both long-distance (Rashotte et al., 2003) and root tip (Strader and Bartel, 2009) transport assays. Thus, IBA is likely not a substrate for AUX1; however, it remains possible that it may be a substrate for other members of the AUX1 family. In particular, LIKE AUX1 (LAX3) appears to display some affinity toward IBA when heterologously expressed in *Xenopus* oocytes (Swarup et al., 2008), suggesting that this transporter may use IBA, in addition to IAA, as a substrate.

IBA efflux appears to be mediated by carriers distinct from IAA efflux carriers. Application of the polar auxin transport inhibitors 1-N-napthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) fail to block IBA efflux, whereas they have a dramatic effect on IAA transport (Rashotte et al., 2003; Liu et al., 2012), suggesting independent efflux mechanisms for these two molecules. Further, heterologously expressed PIN2, PIN7, ABCB1, and ABCB19 display no IBA efflux activity (Ruzicka et al., 2010). Thus, plants seem to use distinct efflux carriers for IBA and IAA. Indeed, several transporters that use IBA, but not IAA, as a substrate have been described. Using genetic and molecular approaches in Arabidopsis, IBA transporters including PXA1/ABCD1, ABCG36, ABCG37, and TOB1 have been identified, and their roles are elaborated in the following text.

Peroxisomal Indole 3-Butyric Acid Transporter-PXA1

IBA to IAA conversion occurs in the peroxisome; thus, an intracellular carrier is necessary for movement of IBA into peroxisomes. The peroxisomal ABC transporter PEROXISOMAL ABC TRANSPORTER1/ABCD1/PXA1 appears to be the influx carrier involved in transporting IBA for IAA conversion through the β-oxidation process (**Figure 1**; Zolman et al., 2001b). The loss-of-function *pxa1* mutant displays resistance to the long-chain auxin precursor IBA, but wild-type sensitivity to the active auxin IAA (Zolman et al., 2001b), due to a reduction in IBA-to-IAA conversion (Strader et al., 2010). PXA1 likely transports compounds in addition to IBA for peroxisomal β-oxidation, such as fatty acid and jasmonic acid (Zolman et al., 2001a; Linka et al., 2008; Kunz et al., 2009). The peroxisomal transporter required for efflux of IBA-derived IAA from the peroxisome is unknown.

Pleiotropic Drug Resistance Proteins Functions as Indole 3-Butyric Acid Efflux Transporters

The ABCG36 and ABCG37 members of the pleiotropic drug resistance (PDR) subclade of the ABCG family of ATP-binding cassette transporters (Strader et al., 2008; Strader and Bartel, 2009; Ruzicka et al., 2010) are required for efflux of IBA from the root. Mutants defective in ABCG36 were found in a screen for altered IBA sensitivity (Strader and Bartel, 2009). Mutants defective in ABCG37 were identified in screens for altered sensitivity to 2,4-dichlorophenoxyacetic acid (2,4-D) (Ito and Gray, 2006), for altered sensitivity to polar auxin transport inhibitors (Ruzicka et al., 2010), or for altered sensitivity to IBA (Strader et al., 2008). Excised root tips from *abcg36* and *abcg37* loss-of-function mutants hyperaccumulate [^{3}H]-IBA, but not [^{3}H]-IAA, suggesting that the ABCG36 and ABCG37 transporters act to efflux IBA (**Figure 1**; Strader et al., 2008; Strader and Bartel, 2009; Ruzicka et al., 2010). The hyperaccumulation of [^{3}H]-IBA, combined with the localization of ABCG36 and ABCG37 to the outer polar domain of root epidermal and lateral root cap cells (Stein et al., 2006; Strader and Bartel, 2009; Ruzicka et al., 2010), suggest that these transporters move IBA out of the root.

To determine whether ABCG37 directly transports IBA, it was expressed in *Schizosaccharomyces pombe* (see **Table 1**), in which ABCG37-expressing cells accumulated less [^{3}H]-IBA compared to control cells, with no change in [^{3}H]-IAA accumulation (Ruzicka et al., 2010), suggesting that ABCG37 directly transports IBA. Although ABCG36 has not been evaluated in a heterologous system for efflux activity, the IBA hyperaccumulation in the *abcg36* mutant (Strader and Bartel, 2009) suggests that ABCG36 could be functionally similar to ABCG37 in IBA transport. Further, the *abcg36 abcg37* double mutant displays additive IBA hypersensitivity and [^{3}H]-IBA hyperaccumulation in comparison to either the *abcg36* or *abcg37* single mutants, suggesting overlapping functions (Ruzicka et al., 2010).

ABCG37 and ABCG36 appear to transport substrates in addition to IBA. PDR proteins typically have broad range of substrates (reviewed in van den Brule and Smart, 2002; Verrier et al., 2008). ABCG37 displays broad substrate specificity, including several auxinic compounds such as the synthetic auxin 2,4-D (Ito and Gray, 2006; Strader et al., 2008), 2,4-dichlorophenoxy butyric acid (2,4-DB) (Strader et al., 2008; Ruzicka et al., 2010), and the auxin transport inhibitor (NPA) (Ito and Gray, 2006). ABCG36 likely transports cadmium (Kim et al., 2007) and the indolic defense compound 4-methoxyindol-3-yl-methanol (Matern et al., 2019).

ABCG36 and ABCG37 play roles in distinct areas of plant development. In particular, the *abcg36* mutant displays an increased number of lateral roots, larger cotyledons, and increased root hair lengths (Strader and Bartel, 2009), consistent with elevated auxin levels in these tissues. ABCG36 and ABCG37 localize to the outward face of epidermal cells in leaves (ABCG36) and roots (ABCG36 and 37) (Strader and Bartel, 2009; Langowski et al., 2010; Ruzicka et al., 2010), suggesting that these transporters exude IBA into the environment. This activity may be crucial for maintaining IBA levels and auxin homeostasis, as *abcg36* and *abcg37* mutant display phenotypes consistent with elevated auxin accumulation. Further, blocking IBA-to-IAA conversion in the *abcg36* mutant suppresses these phenotypes (Strader et al., 2011), suggesting that IBA conversion is necessary for the developmental defects in *abcg36*. In roots, ABCG36 and ABCG37 are polarly localized to the outward-facing plasma membrane of root epidermal and lateral root cap cells (Strader and Bartel, 2009; Langowski et al., 2010; Ruzicka et al., 2010) and may exude IBA into the rhizosphere, which could have potential implications on the microbial diversity.

TRANSPORTER OF IBA1 Transports Indole 3-Butyric Acid

TRANSPORTER OF IBA1 (TOB1) was identified in a forward genetics screen for suppression of *abcg36* IBA hypersensitivity (Michniewicz et al., 2019). TOB1 belongs to the NTR1 PTR FAMILY (NPF), a part of the larger group of major facilitator superfamily (MFS) transporters (Leran et al., 2014). The *tob1* loss-of-function mutant displays mild resistance to 2,4-DB and IBA and wild-type sensitivity to IAA and 2,4-D in root elongation assays (Michniewicz et al., 2019). Indeed, transport assays using excised Arabidopsis root tips reveal reduced accumulation of [^{3}H]-IBA and no difference in [^{3}H]-IAA accumulation in *tob1* compared to wild type root tips (Michniewicz et al., 2019). Further, TOB1 directly transports IBA in heterologous systems (see **Table 1**) such as yeast and *Xenopus* oocytes (Michniewicz et al., 2019). In addition, TOB1 transports nitrate (He et al., 2017; Michniewicz et al., 2019). In oocytes, nitrate serves as a better substrate than

TABLE 1 | Heterologous expression systems for transport assays.

Heterologous system	Pros and cons	Auxin-related transporters characterized
Yeast–*Saccharomyces cervisiae* and *Schizosaccharomyces pombe*	1. Easy to manipulate and cultivate in standard lab conditions. 2. Allows proper folding and translocation of transmembrane proteins. 3. Overexpression can lead to aggregates of misfolded protein.	ABCB, AUX, and PIN family proteins (Yang and Murphy, 2009). TOB1 (Michniewicz et al., 2019).
Xenopus oocyte–Oocytes harvested from the South African clawed frog *Xenopus laevis*	1. Electrophysiological measurements using the two-electrode voltage clamp technique. 2. Large oocyte size facilitates handling and microinjection. 3. Exogenous transporters post-translationally modified before plasma membrane localization.	PIN (Zourelidou et al., 2014), TOB1 (Michniewicz et al., 2019).
Insect cell–Baculovirus/*Spodoptera frugiperda* (Sf9) insect cells	1. Ability to express large quantities of eukaryotic transmembrane protein. 2. Media is expensive and protein yield is minimal. 3. Need to infect cells with virus for protein expression each time; stable lines difficult to maintain. 4. Tissue culture conditions needed.	AUX1 (Carrier et al., 2008), ABCG25 (Kuromori et al., 2010)
Mammalian cell lines such as HeLa	1. Standardized protocols available for transfection and stable expression of transmembrane protein 2. Media is expensive and protein yield is minimal. 3. Tissue culture conditions needed.	ABCG37 (Ruzicka et al., 2010)
Plant protoplast tobacco BY-2 cells	1. Transformation protocols are standardized. 2. Environment similar to native environment of transporter. 3. Possible interference by endogenous transmembrane proteins. 4. Unclear whether transport activity is due to direct transport or by modulation of existing plant transporters.	

IBA, although IBA can compete with nitrate uptake in *TOB1*-expressing oocytes (Michniewicz et al., 2019).

TOB1 localizes to the vacuolar membrane and is primarily expressed in cells adjacent to the lateral root primordia and in the lateral root cap cells of the primary root (Michniewicz et al., 2019). The *tob1* loss-of-function mutants display increased numbers of lateral roots and altered root system architecture (Michniewicz et al., 2019), suggesting that TOB1 normally limits production of lateral roots, possibly by sequestering IBA to the vacuole to limit its contributions to the pool of active auxin in these tissues. *TOB1* is a direct target of the cytokinin reponse regulator ARR10 (Zubo et al., 2017) and *TOB1* expression is induced by cytokinin treatment (Michniewicz et al., 2019). Further, the *tob1* mutant is resistant to the inhibitory effects of cytokinin on lateral root production (Michniewicz et al., 2019), suggesting that cytokinin's inhibitory effects on lateral root production are at least partially mediated by moving IBA into the vacuole. These data suggest a model in which cytokinin regulates *TOB1* expression to regulate IBA contributions to auxin homeostasis during lateral root development.

Thus far, four proteins, PXA1 (Zolman et al., 2001b), ABCG36 (Strader and Bartel, 2009), ABCG37 (Strader et al., 2008; Ruzicka et al., 2010), and TOB1 (Michniewicz et al., 2019) have been implicated in IBA transport. Identification of these transporters has demonstrated the significance of IBA transporters in regulating aspects of development. Although progress has been made in identification of IBA transporters and IBA metabolic enzymes, much remains to be uncovered. Identifying additional transporters and determining their substrates (either IBA or IBA conjugates), combined with a detailed knowledge of IBA metabolism, will shine more light into understanding the plant developmental pathways.

Remaining questions on IBA transport:

- *How and where is IBA made and metabolized?* IBA may be derived from IAA in maize (Ludwig-Müller and Epstein, 1991; Ludwig-Müller et al., 1995a) and Arabidopsis (Ludwig-Muller, 2007). Yet, enzymes involved in IBA biosynthesis remain unidentified. IBA-derived auxin appears to be important in very specific aspects of plant development; it seems an inefficient system to create IBA from IAA, just to metabolize it to IAA again. Because IBA is important for early seedling growth, a stage in which peroxisomal activity is high in metabolizing storage oils, and because IBA β-oxidation releases not only free IAA but also acetyl-CoA, it may be possible that IBA is used in this scenario to not only provide auxin, but also energy, to drive growth. In addition, much of the transported IBA is in the form of IBA ester-linked conjugates (likely conjugated to sugars) (Liu et al., 2012). Whether IBA conjugates are made in one tissue; then transported to another for metabolism remains an open question. A greater understanding of tissue-type expression of IBA conversion enzymes and IBA transporters will be beneficial in understanding this system.
- *Roles for additional ABCG or TOB proteins in IBA transport.* Known IBA transporters belong to the PDR clade of the ATP-binding cassette (ABC) family and of the NPF clade of the major facilitator superfamily. Additional members of these clades may be involved in mediating IBA transport. In particular, mutants defective in ABCG29/PDR1 and ABCG33/PDR5 display hypersensitivity to the synthetic IBA mimic 2,4-DB (Michniewicz et al., 2014), consistent with the possibility that, similar to ABCG36 and ABCG37 family members, ABCG29 and ABCG33 transport IBA. In addition, TOB1 also has six closely related paralogs, which could function, similar to TOB1, in transporting IBA (Michniewicz et al., 2019). Functional characterization of these close paralogs could reveal potential IBA carriers. To determine whether these are *bona fide* IBA transporters, IBA transport activity must be demonstrated in a heterologous system (**Table 1**) such as yeast, *Xenopus* oocytes, insect cells, or mammalian cells (Haferkamp and Linka, 2012). There is a possibility that TOB1 paralogs (Michniewicz et al., 2019) could transport IBA in distinct tissues of Arabidopsis and promote the development of different tissues. Characterization of these members in addition to other potential transporters would aid in understanding the role of IBA transporters function at spatial levels to indirectly regulate IAA levels.
- *IBA uptake carriers.* IBA uptake is a rate-limited process (Rashotte et al., 2003), suggesting that its import into the cell is mediated by a carrier, rather than the effect of diffusion. Thus far, no IBA uptake carriers have been reported. A potential candidate for an uptake carrier is RESISTANT TO IBA1 (RIB1). The semi-dominant mutant *rib1* displays IBA resistance (Poupart and Waddell, 2000) and altered IBA transport (Poupart et al., 2005), consistent with the possibility that RIB1 mediates IBA uptake. Identification of the underlying mutation and molecular characterization of the gene product will allow for understanding the role of RIB1 in IBA transport.
- *Directionality of TOB1 transport.* The reduced 3H-IBA accumulation in *tob1* mutants combined with developmental phenotypes consistent with increased IBA contributions to the auxin pool suggests that TOB1 sequesters IBA in to vacuole (Michniewicz et al., 2019). Conversely, *tob1* resistance to the inhibitory effects of exogenous IBA on root elongation and electrophysiology assays are consistent with the possibility that TOB1 moves IBA out of the vacuole (Michniewicz et al., 2019). These contradictory pieces of data may reflect concentration-dependent differences in TOB1 transport direction. Thus, the direction of IBA movement by TOB1 remains unclear (**Figure 1**).
- *Identity of transported molecules.* Although it is clear that PDR9 and TOB1 directly transport IBA based on heterologous transport assays, it is not clear whether IBA itself or IBA-derived molecules are being tracked in other assays. Liu et al. (2012) elegantly used heavy IBA and mass spec analysis to determine that, in Arabidopsis hypocotyl tissues, most of the transported material was IBA-derived IAA or IBA conjugates. Likewise, in Ruzicka et al. (2010), [^{3}H]-IBA applied to the columella was IBA-derived [^{3}H]-IAA when sampled 4 mm from the root tip. Clearly, analytical methods need to be combined with transport assays for both IBA and IAA.

Indeed, transport assays in heterologous systems, such as *Xenopus* oocytes, can be done on a time scale for which metabolism of molecules is not a factor.

- *IAA efflux carrier in peroxisome*. IBA-to-IAA conversion occurs in the peroxisome (reviewed in **Figure 1**; Strader and Bartel, 2011). However, the transporter involved in movement of IBA-derived IAA from the peroxisome into the cytoplasm is unidentified.
- *Long distance IBA efflux carriers.* Although long-distance transport has been speculated to exist based on radiotracer experiments, it seems that much of this tracked material was IBA metabolites, rather than free IBA. Certainly, none of the identified IBA transporters are involved in long-distance IBA transport. At this point, transporters involved in the long-distance transport of IBA or IBA conjugates remain elusive. Further, developmental roles for transport of IBA or IBA remain unknown; thus, it is possible that this mechanism is not a contributor to physiologically relevant processes.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Plant Science

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ABCG36/PEN3/PDR8 Is an Exporter of the Auxin Precursor, Indole-3-Butyric Acid, and Involved in Auxin-Controlled Development

*Bibek Aryal[1], John Huynh[1], Jerôme Schneuwly[1], Alexandra Siffert[1], Jie Liu[1], Santiago Alejandro[2], Jutta Ludwig-Müller[2], Enrico Martinoia[3] and Markus Geisler[1]**

[1] Department of Biology, University of Fribourg, Fribourg, Switzerland, [2] Institut für Botanik, Technische Universität Dresden, Dresden, Germany, [3] Institute for Plant and Microbial Biology, Zurich, Switzerland

The PDR-type ABCG transporter, ABCG36/PDR8/PEN3, is thought to be implicated in the export of a few structurally unrelated substrates, including the auxin precursor, indole-3-butyric acid (IBA), although a clear-cut proof of transport is lacking. An outward facing, lateral root (LR) location for ABCG36 fuelled speculations that it might secrete IBA into the rhizosphere. Here, we provide strong evidence that ABCG36 catalyzes the export of IBA – but not of indole-3-acetic acid – through the plasma membrane. ABCG36 seems to function redundantly with the closely related isoform ABCG37/PDR9/PIS1 in a negative control of rootward IBA transport in roots, which might be dampened by concerted, lateral IBA export. Analyses of single and double mutant phenotypes suggest that both ABCG36 and ABCG37 function cooperatively in auxin-controlled plant development. Both seem to possess a dual function in the control of auxin homeostasis in the root tip and long-range transport in the mature root correlating with non-polar and polar expression profiles in the LR cap and epidermis, respectively.

Keywords: IBA, IAA, ABCG, ABC transporter, PDR, PEN3, auxin, plant development

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Gloria Muday, Wake Forest University, United States

****Correspondence:***
Markus Geisler
markus.geisler@unifr.ch

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INTRODUCTION

Initially, IBA was described as a synthetic auxin that elicited auxin-like effects such as root initiation and was thus used for plant propagation in a process called rooting (reviewed in Frick and Strader, 2018). It has now been established that IBA is an endogenous compound in a variety of plant species examined (Frick and Strader, 2018) Its existence, however, in the model plant *Arabidopsis thaliana* has been questioned (Novak et al., 2012). Currently, it appears that this might simply be a question of extraction and detection methods (Frick and Strader, 2018; Matern et al., 2019). However, until now, it is unclear if IBA acts as an auxin by itself or if it acts strictly via IAA, the major auxin, for which it functions as a precursor (Ludwig-Muller, 2007; Strader and Bartel, 2008; Schlicht et al., 2013). Conversion of IBA to IAA is a peroxisome-dependent

Abbreviations: ABCB, ATP-binding cassette protein subfamily B; ABCG, ATP-binding cassette protein subfamily G; dag, day(s) after germination; dai, day(s) after infection; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NPA, 1-*N*-naphthylphthalamic acid; PAT, polar auxin transport; PDR, pleiotropic drug resistance; PIN, pin-formed; WT, wild-type.

reaction (Zolman et al., 2008) and peroxisomal import is thought to occur by PEROXISOMAL TRANSPORTER1/COMATOSE/ABCD1 (PXA1/CTS/ABCD1) belonging to the ABCD family of ABC transporters (Zolman et al., 2001; Footitt et al., 2002; Hooks et al., 2007).

Indole-3-butyric acid uptake is a saturable process (Rashotte et al., 2003), suggesting that IBA uptake into plant cells is mediated by unidentified IBA uptake carriers (Frick and Strader, 2018; Matern et al., 2019). IBA export is thought to be catalyzed by members of the PDR transporters belonging to the ABCG family of ABC transporters that is limited to plants and fungi (Strader and Bartel, 2009; Kang et al., 2010; Kretzschmar et al., 2012). Interestingly, members of the PIN and ABCB families of IAA transporters do not appear to transport IBA (Ruzicka et al., 2010; Matern et al., 2019) indicating an independent evolution of auxin substrate specificities for distinct transporter classes. Plant PDRs have been assigned to the transport of substrates that are involved in several biotic and abiotic responses: for example, tobacco PDR1 was identified as an exporter of the antifungal terpenoid, sclareol (Jasinski et al., 2001), while PDR5 is induced by the defense hormone, jasmonic acid, and wounding and plays a role in herbicide resistance (Bienert et al., 2012). The PDR, Leaf Rust Resistance34 (Lr34), confers durable, race-specific resistance to multiple fungal pathogens in wheat (Krattinger et al., 2009), while *Petunia* PDR1 has been shown to export the hormone strigolactone in the context of arbuscular mycorrhizal establishment (Kretzschmar et al., 2012).

Mutant alleles of *PDR8/PEN3/ABCG36* (hereafter referred to as ABCG36) were first shown to display altered responses to diverse pathogens (Kobae et al., 2006; Stein et al., 2006), decreased extracellular accumulation of flagellin22-induced callose (Clay et al., 2009) and hyper-accumulation of flagellin22- or pathogen-elicited indole glucosinolate derivatives of the PEN2 pathway (Bednarek et al., 2009; Clay et al., 2009). A striking feature of ABCG36 is its focal accumulation at the site of leaf pathogen entry, where it is thought to export as yet unidentified defense compounds through the plasma membrane (PM) (Stein et al., 2006; Xin et al., 2013). Recently, the indole, 4-methoxyindol-3-yl methanol, was identified as a substrate for ABCG36 functioning in the induced deposition of callose by flagellin22 (Matern et al., 2019).

On the other hand, *abcg36* alleles were found to independently hyper-accumulate and to be hypersensitive to IBA (Strader and Bartel, 2009; Lu et al., 2015). In the root, ABCG36 is predominantly laterally localized at the outermost root PM domains (Langowski et al., 2010; Ruzicka et al., 2010). In these lateral domains, which were defined as the root-soil interface (Langowski et al., 2010; Ruzicka et al., 2010), ABCG36 was shown to co-localize widely with ABCG37 and was thus suggested to act redundantly in mediating root auxin homeostasis (Ruzicka et al., 2010). In agreement, both *ABCG36* and *ABCG37* were identified in chemical genetic screens for hypersensitivity toward auxinic compounds and auxin transport inhibitors, including IBA (Strader and Bartel, 2009; Ruzicka et al., 2010; Frick and Strader, 2018). Accumulation and reduced efflux from entire root tips were found for different *abcg36* alleles (Strader and Bartel, 2009). Finally, by heterologous expression, ABCG37/PDR9/PIS1 was shown to function as a transporter of IBA and 2.4-D but not of IAA (Ruzicka et al., 2010). In summary, genetic and biochemical approaches in Arabidopsis support the idea that IBA efflux from root cells is catalyzed by at least two PDRs of the ABCG family of ABC transporters, ABCG36/PDR8/PEN3 (Strader and Bartel, 2009; Lu et al., 2015) and ABCG37/PDR9/PIS1 (Strader et al., 2008; Ruzicka et al., 2010). IBA hypersensitivity phenotypes of mutants defective in these transporters suggest that IBA is a common substrate exported by both ABCG36 and ABCG37 (Strader and Bartel, 2009) but clear IBA transport data have so far only been provided for ABCG37 (Ruzicka et al., 2010).

Here we provide strong evidence that ABCG36 functions as a PM exporter of IBA that dampens polar (rootward) IBA transport in the root. Further, our data support a functional interplay with ABCG37 in auxin-controlled plant physiology.

MATERIALS AND METHODS

Plant Material and Phenotypic Analyses

The following *Arabidopsis thaliana* lines in ecotype Columbia (Col Wt) were used: *abcg36-4 (pen3-4)* (Stein et al., 2006), *abcg37-2 (pdr9-2)* (Ito and Gray, 2006), *abcg36-4 abcg37-2 (pen3-4 pdr9-2)* (Ruzicka et al., 2010), *35S:ABCG37-GFP (35S:PIS1-GFP)* (Ruzicka et al., 2010), *ABCG36:ABCG36-GFP (PEN3:PEN3-GFP)* (Stein et al., 2006). *DR5:GFP* (Ottenschlager et al., 2003), was crossed into *abcg36-4 and abcg37-2* and isogenic, homozygous lines for the transgene in the F3 generations were used for further analyses. *35S:ABCG36* Arabidopsis lines were constructed by transforming *35S:PDR8* (Kim et al., 2007) into the *abcg36-4*/*pen3-4* mutant.

Seedlings were generally grown on vertical plates containing 0.5 Murashige and Skoog media, 1% sucrose, and 0.75% phytoagar in the dark or at 16 h (long day) light per day. Developmental parameters, such as primary root lengths, lateral root (LR) number and root gravitropism, primary root and hypocotyl elongation were quantified from scanned plates using the Fiji plugin[1] for ImageJ[2]. All experiments were performed at least in triplicate with 30 to 40 seedlings per experiment.

Yeast Work

cDNA of ABCG36 was PCR-amplified and inserted into BamHI/XhoI sites of pYES2NT/C-ABCG36. ABCG36 and ABCG37 were expressed from shuttle vectors pYES2NT/C-ABCG36, pNEV-ABCG37-HA (Ruzicka et al., 2010) in Wt strain JK93da (Hemenway and Heitman, 1996). Yeast IBA/2.4-D transport was performed as described (Kim et al., 2010).

Confocal Laser Scanning Imaging

For confocal laser scanning microscopy work, a SP5 confocal laser microscope was used. Confocal settings were set to record the emission of GFP (excitation 488 nm, emission 500–550 nm) and FM4-64 (excitation 561 nm, emission 600–680 nm).

[1]https://imagej.net/Fiji

[2]https://imagej.net

Plant Auxin Transport

Simultaneous export of [ring-^{3}H]-IBA (specific activity 25 Ci mmol^{-1}; American Radiolabeled Chemicals, ART1112) with either [carboxyl-^{14}C]-2.4-D (specific activity 50 mCi mmol^{-1}; American Radiolabeled Chemicals, ARC0722) or [1-^{14}C]-IAA (specific activity 55 mCi mmol^{-1}; American Radiolabeled Chemicals, ARC1060) from Arabidopsis and *Nicotiana benthamiana* mesophyll protoplasts was analyzed as described (Henrichs et al., 2012). *N. benthamiana* mesophyll protoplasts were measured 4 days after *Agrobacterium*-mediated transfection of *35S:ABCG36/35S:PDR8* (Kim et al., 2007) or *35S:ABCG37-GFP* (Ruzicka et al., 2010). In short, after loading, external radioactivity was removed by separating protoplasts in a 50-30-5% Percoll gradient. Transport was initiated by incubation at 25°C and halted by silicon oil centrifugation. Exported radioactivity was determined by scintillation counting of aqueous phases and is presented as the relative efflux of the initial efflux (efflux prior to temperature incubation), which was set to zero. Relative export from protoplasts was calculated from exported radioactivity into the supernatant as follows: (radioactivity in the supernatant at time t = x min) – (radioactivity in the supernatant at time t = 0) * (100%)/(radioactivity in the supernatant at t = 0 min); mean values are presented from four independent experiments. Rootward (acropetal) and shootward (basipetal) PAT in roots was measured as described in Lewis and Muday (2009).

In planta Analysis of Auxin Contents and Responses

Endogenous free IBA and IAA were quantified from shoot and root segments of 9-days-after-germination (dag) light-grown (16 h) Arabidopsis seedlings by using gas chromatography-mass spectrometry (GC–MS) as described in Leljak-Levanic et al. (2010). Methylation was performed by adding equal sample amounts of a 1:10 diluted solution (in diethylether) of trimethylsilyldiazomethane solution (Sigma-Aldrich) for 30 min at room temperature. The mixture was then evaporated and resuspended in 50 μl of ethyl acetate for GC–MS analysis. Data are means of four independent lots of 30–50 seedlings each, and equivalent to ca. 100 mg root shoot material, respectively.

Homozygous generations of Arabidopsis *abcg36-4* and *abcg36-4 abcg37-2* expressing DR5:GFP were obtained by crossing with DR5:GFP lines (Ottenschlager et al., 2003). Seedlings were grown vertically for 5 (dag) and then for 4 h on 5 (M IBA plates and analyzed by confocal laser-scanning microscopy. DR5:GFP signals in the very root tip were analyzsed using the Fiji software[3].

Data Analysis

Data were statistically analyzed using Prism 7.0a (GraphPad Software, San Diego, CA, United States) and the R software package of the Comprehensive R Archive Network (CRAN)[4].

[3]https://imagej.net/Fiji

[4]http://cran.r-project.org

RESULTS

ABCG36 Functions as an Exporter of IBA

Recently, by heterologous expression in yeast and HeLa cells, ABCG37/PDR9/PIS1 was shown to function as a transporter of IBA, 2.4-D and NPA but not of IAA (Ruzicka et al., 2010). For different *abcg36* alleles, increased accumulation and reduced efflux from entire root tips were measured (Strader and Bartel, 2009) but no direct transport data, especially in the absence of plant-specific factors, was provided.

In order to demonstrate such a transport activity, we functionally expressed *ABCG36* in the heterologous, non-plant and plant systems, baker's yeast and tobacco (*N. benthamiana*), respectively. ABCG36- and ABCG37-expressing yeast (the latter was used here as a positive control) revealed significantly reduced retention of IBA and 2.4-D, assayed in parallel by double isotope labeling (**Figure 1A**). Reduced retention argues for an export activity. *N. benthamiana* transfection resulted in expression of ABCG36 at the PM of epidermal cells as was shown by co-localization with FM-4-64 after short-term incubation of transfected leaves (**Figure 1C**). Confocal imaging was verified biochemically by demonstrating co-sedimentation of ABCG36-positive microsomal fractions with the PM marker, PIP2;1, using Western blotting after fractionation on a linear sucrose gradient. ABCG36 expression on the PM greatly enhanced IBA and 2.4-D export from prepared tobacco protoplasts (**Figure 1B**) as was shown before for ABCG37 (Ruzicka et al., 2010).

Next, we quantified IBA/2.4-D export from stable Arabidopsis lines over-expressing ABCG36 (Kim et al., 2007) and ABCG37-GFP (Ruzicka et al., 2010). Both lines exported significantly more IBA and 2.4-D (**Figure 2A**) but not IAA (**Supplementary Figure 1**) compared to the corresponding Wt. In agreement, T-DNA insertion, null alleles of *ABCG36*, *abcg36-3*, *abcg36-4*, *abcg36-7*, previously shown to express no ABCG36 protein (Lu et al., 2015), revealed significantly reduced IBA and 2.4-D export (**Figure 2B**). In summary, these data verify a PM export activity of ABCG36 for IBA but additionally indicate a high degree of substrate specificity as was already found for ABCG37 (Ruzicka et al., 2010).

ABCG36 Functions in the Polar Distribution of IBA

Previous work has established that in roots both IBA and IAA both move with distinct polarities at similar transport rates (Rashotte et al., 2003). Inspired by the striking polar expression of ABCG36 and ABCG37 in the root epidermis (Ruzicka et al., 2010; Mao et al., 2016; see **Figure 3C**) and in order to demonstrate an involvement of ABCG36 in this process, we quantified polar IBA and IAA transport in 5 mm segments from the source. Both radiotracers were applied to root-shoot junctions and root tips by diffusion into plant tissues from agar beads functioning as source (Lewis and Muday, 2009). Results revealed to our surprise that rootward (acropetal) transport is enhanced in *abcg36, abcg37* and *abcg36 abcg37* mutant roots, although significantly differences were only found

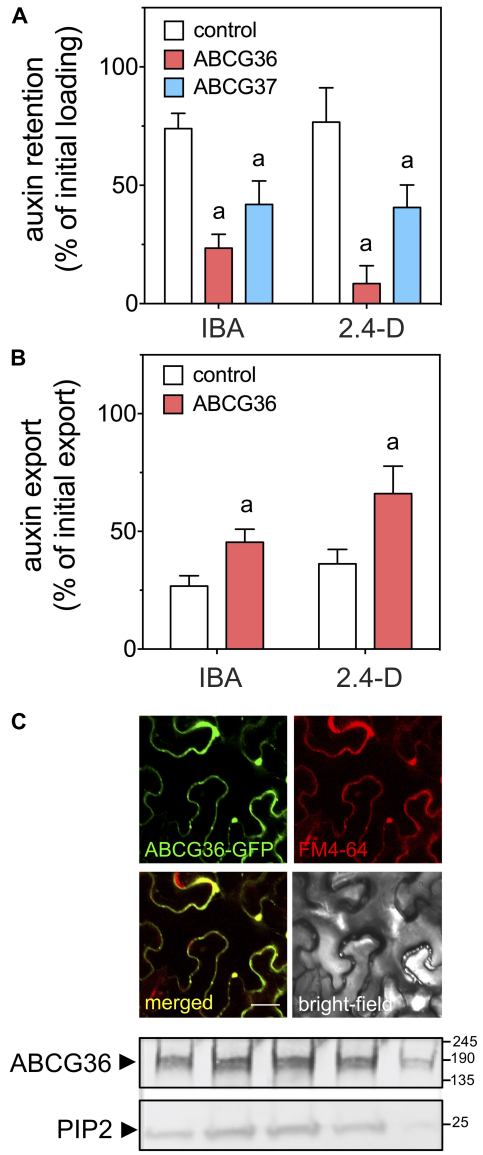

FIGURE 1 | Heterologous expression of ABCG36 results in enhanced plasma membrane export of the native and synthetic auxins, IBA and 2.4-D. **(A)** Yeast expressing ABCG36 or ABCG37 retain significantly less IBA and 2.4-D indicating higher export. **(B)** Enhanced IBA and 2.4-D export from *Nicotiana benthamiana* protoplasts prepared after *Agrobacterium*-mediated leaf transfection with 35S:*ABCG36*. Significant differences (unpaired *t*-test with Welch's correction, $p < 0.05$) to vector control are indicated by an 'a' (mean ± SE; $n \geq 4$ transport experiments generated from independent yeast transformations or tobacco transfections). **(C)** ABCG36 is expressed on the plasma membrane revealed by confocal imaging of ABCG36-GFP (upper panel) and Western analyses of sucrose gradient fractions prepared from tobacco leaves transfected with *ABCG36:ABCG36-GFP*. Microsomes were separated by a linear 10–50% sucrose gradient and plasma membrane (PIP2)-positive fractions 9–13 (corresponding to 37–50% sucrose) were probed with anti-GFP (lower panel).

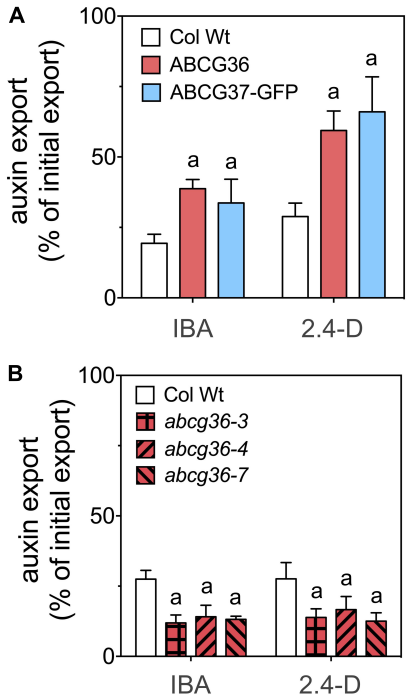

FIGURE 2 | ABCG36 transports the native and synthetic auxins, IBA and 2.4-D, in Arabidopsis. **(A)** Enhanced IBA and 2.4-D efflux from leaf protoplasts prepared from *ABCG36* (*35S:ABCG36*) and *ABCG37* (*35S:ABCG37-GFP*) gain-of-function plants. **(B)** Reduced IBA and 2.4-D efflux from leaf protoplasts prepared from indicated *ABCG36* loss-of-function plants. Significant differences (unpaired *t*-test with Welch's correction, $p < 0.05$) to Wt (Col Wt) are indicated by an 'a' (mean ± SE; $n \geq 4$ transport experiments generated from independent protoplast preparations).

with the double mutant (**Figure 3A**). This effect is specific for IBA as it was not found with IAA assayed simultaneously. Shootward (basipetal) transport rates were likewise significantly enhanced in *abcg36* and *abcbg36 abcbg37* but not in *abcg37* roots, which might indicate a selective contribution of ABCG37 in this transport stream. Slightly reduced, although not significant, shootward transport in *abcg37* roots is in agreement with a recent report (Ruzicka et al., 2010), however, it is important to mention that application methods employed differ. While we here applied radiolabeled IAA by placing agar beads containing low amount of radiotracers next to the plant tissue (Lewis and Muday, 2009), Ruzicka et al. (2010) used direct nano-droplet application to the plant tissues, which might result in external passage of auxin-containing microfluids by capillary forces.

Enhanced rootward and shootward transport rates for *ABCG36/37* transporter loss-of-function mutants might seem counter-intuitive at first sight. In light of the strict lateral, outward-facing distribution for ABCG36 (Strader and Bartel, 2009, see **Figure 3C**) and ABCG37 (Ruzicka et al., 2010) in the region that is relevant for this transport measurement, a plausible explanation is that both transporters might function in lateral exclusion of IBA from the apical-basal transport stream. Loss-of-function would thus result in enhanced polar IBA transport (see **Figure 3D**). Altogether, this dataset further substantiates that ABCG36 like ABCG37 acts as an IBA exporter and is involved in the regulation of its polar

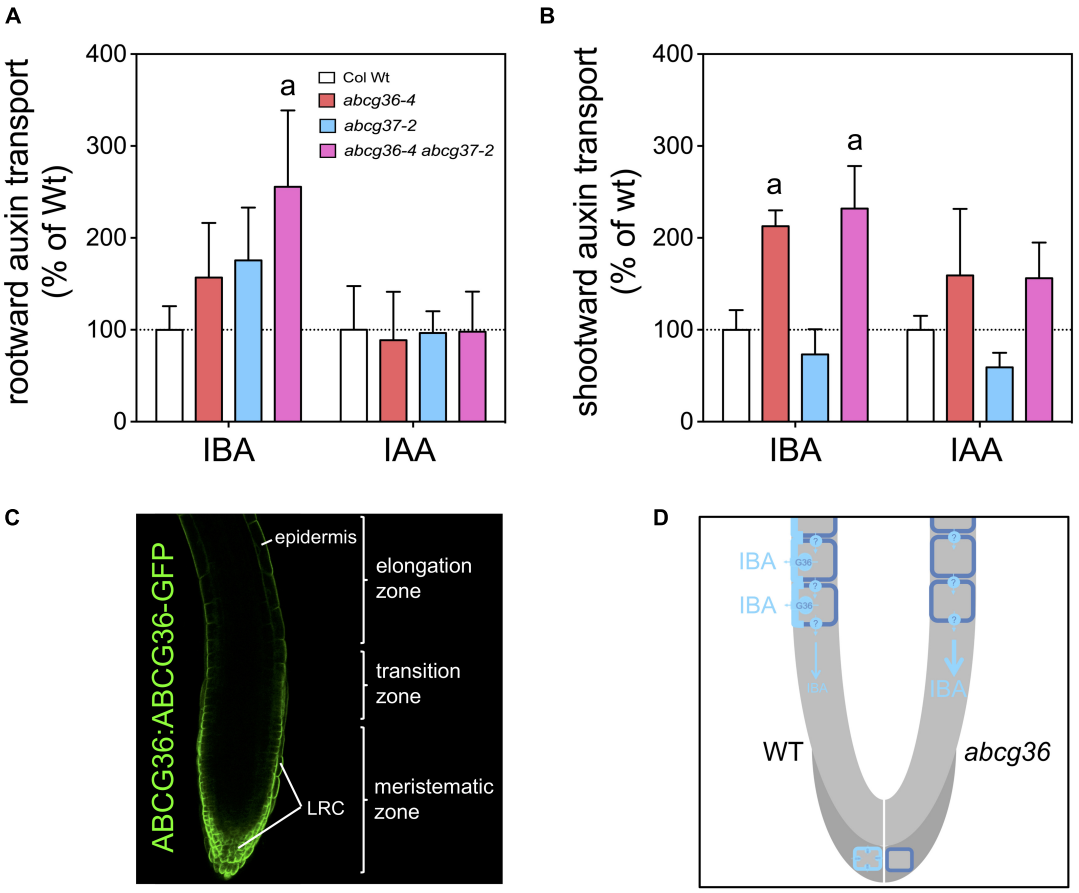

FIGURE 3 | ABCG36 functions in rootward IBA transport in the Arabidopsis root. Rootward (**A**, acropteal) and shootward (**B**, basipetal) root transport of ^{3}H-IBA and ^{14}C-IAA assayed in parallel. Significant differences (unpaired *t*-test with Welch's correction, $p < 0.05$) between WT and mutant alleles are indicated by an 'a' (mean ± SE; $n \geq 4$ transport experiments). Both radiotracers were applied to root-shoot junctions and root tips by diffusion into plant tissues from agar beads functioning as source (Lewis and Muday, 2009); 5 mm segments from the source were used for quantification. **(C)** ABCG36-GFP is localized to the periphery of epidermal cells but reveals a non-polar expression in cells of the lateral root cap (LRC). Confocal image of a 5 dag root tip carrying *ABCG36:ABCG36* (*PEN3:PEN3-GFP*) in *abcg36-3/pen3-3* (Underwood and Somerville, 2017). **(D)** Putative model on the role of ABCG36 in polar transport of IBA. ABCG36 contributes to the rootward (acropetal) transport of IBA, which based on **(A,B)** seems to be shared by ABCG37 (not shown here). Lateral, outward-facing PM expression in the WT root epidermis (left panel) suggests lateral IBA excretion into the rhizosphere, taking IBA out of the polar IBA stream provided by so far uncharacterized apical-basal IBA transporters. Deletion of ABCG36 in the mutant (right panel) would enhance polar IBA transport, which is in line with our data. Additionally, ABCG36 seems to contribute to local auxin homeostasis in the very root tip correlating with a predominant non-polar expression in the LRC (dark gray). Absence of *ABCG36* might thus abolish IBA export resulting in DR5-GFP activation (**Figure 4**) and root hypersensitivity on IBA (Strader and Bartel, 2009).

distribution by a yet unidentified apical-basal transport machinery (**Figure 3D**).

ABCG36 and ABCG37 Have an Impact on Auxin Signaling and Accumulation

Recently, hyper-sensitivity of *abcg37-1/pis1-1* roots to IBA was correlated with increased induction of the auxin (IAA) signaling reporter, DR5:GFP, known to be not activated by IBA itself (Ruzicka et al., 2010). Based on quantification of DR5:GFP expression in the root tip, auxin signaling was not found to be different in *abcg36-4* and *abcg36-4 abcg37-2* compared to WT on control media (**Figures 4A,B**), as shown for *abcg37-1/pis1-1* (Ruzicka et al., 2010). However, like *abcg37-1* (Ruzicka et al., 2010), application of IBA led to significantly higher reporter activation in the *abcg36-4* columella cells compared to the WT, and results in additional signals in the stele (**Figures 4A,B**). Interestingly, this effect was reverted in the *abcb36-4 abcg37-2* double mutant to Wt level arguing for compensating roles of both transporters in the columella cells (see section "Discussion").

In order to provide more direct evidence for an involvement of ABCG36 (and ABCG37) in IBA/IAA homeostasis and to dissect IBA and IAA – but also ABCG36,37 – functionalities, we analyzed free IBA and IAA levels from entire root and shoot segments of Arabidopsis seedlings by GC–MS. Both roots and shoots of *abcb36* and *abcg37* single and double mutants contained significantly less IBA than the WT most likely caused by defects in apical-basal IBA distribution (**Figure 4C** and **Supplementary Figure 2**). Interestingly, IAA levels in *abcb36-4* and *abcb36-4 abcg37-2* roots – but not in those of *abcg37-2* – were also reduced,

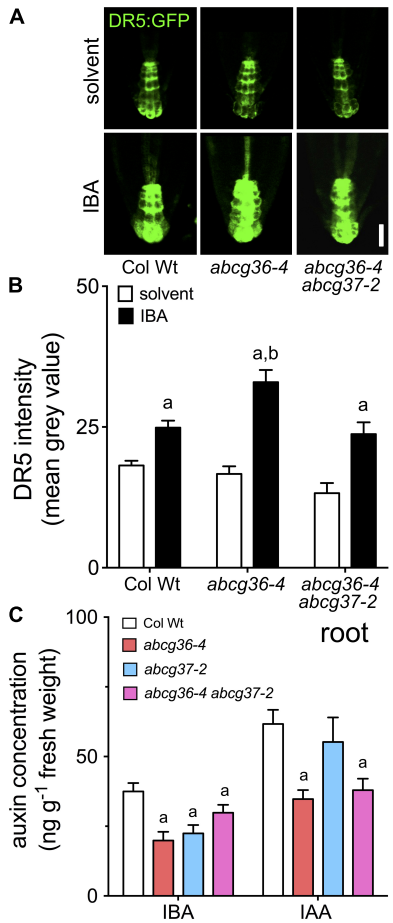

FIGURE 4 | Auxin responses and quantification of free IBA and IAA in *abcg36*. Root auxin responses visualized by the auxin-responsive DR5 reporter in the root tip of 5 dag seedlings **(A)** grown on solvent (DMSO) or 5 μM IBA (**B,** quantification of fluorescence by image analysis of confocal sections). Significant differences (unpaired *t*-test with Welch's correction, $p < 0.05$) to corresponding solvent controls are indicated by an 'a,' while differences between IBA-treated WT and mutant alleles are marked by a 'b' (mean ± SE; $n \geq 20$ images). Bar: 20 μm. **(C)** Free IBA quantified by GC–MS is reduced in *abcg36/37* roots. Significant differences (unpaired *t*-test with Welch's correction, $p < 0.05$) between WT and mutant alleles are indicated by 'a' (mean ± SE; $n = 4$). Shoot quantification is shown in **Supplementary Figure 2**.

which is in line with enhanced polar transport rates for IBA (**Figure 3**). In summary, this data set uncovers that ABCG36 and ABCG37 have both an impact on root and shoot IBA and IAA homeostasis but that their individual impact is tissue-specific.

ABCG36 Is Involved in Some – But Not All – Auxin-Controlled Developmental Programs in an Action That Is Partly Shared With ABCG37

Originally, the *ABCG36* allele, *abcg36-6/pdr8-115*, was isolated in a screen for mutants that were able to restore IBA but not IAA responsiveness to auxin signaling mutants (Strader and Bartel, 2009). In the following, individual mutant alleles of *ABCG36* were tested for their sensitivity toward IBA using root growth as read-out but different technical setups revealed mixed reports: while *abcg36-1/pen3-1*, *abcg36-2/pen3-2*, *abcg36-3-pen3-3*, *abcg36-4/pen3-4,* and *abcg36-6* were found to be hypersensitive (Ruzicka et al., 2010; Strader and Bartel, 2011; Lu et al., 2015), the point mutation allele, *abcg36-5*, was not (Lu et al., 2015). For the *abcg37-2/pdr9-2* allele, hypersensitivity toward IBA as well as a redundant function with ABCG36 were reported (Ruzicka et al., 2010).

We therefore re-analyzed the performance of an established set of *ABCG36* and *ABCG37* single and double loss-of-function lines (Ruzicka et al., 2010) in respect to well-characterized, auxin-controlled growth programs. In order to be able to potentially dissect the role of individual ABCG-type transporters in IBA homeostasis, we also quantified seedling phenotypes after transfer to IBA plates. While hypocotyls at standard conditions (22°C) had no obvious phenotypes, growth at 28°C – known to cause a shift to thermomorphogenesis characterized by elongated hypocotyls most likely triggered by enhanced IAA (Gray et al., 1998; Gil and Park, 2019) – greatly reduced hypocotyl elongation in the order *abcg36* < *abcg37* < *abcg36 abcg37* in comparison to WT (**Figure 5A**). IBA enhanced this phenotype at 28°C but not at 22°C suggesting that increased conversion of IBA to IAA might be responsible for this effect. Analysis of hook opening in etiolated seedlings, another hallmark of auxin-regulated shoot development (Abbas et al., 2013), revealed that single *ABCG36* or *ABCG37* mutation did not significantly affect hook opening angles, while the *abcg36-4 abcg37-2* double mutant revealed an impaired hook opening angle (**Figure 5B**). IBA treatment generally inhibited hook opening compared to the solvent control but also ameliorated the differences between the mutant alleles.

Results for root elongation indicated that *abcg36-4* and *abcg37-2* roots elongate faster on solvent plates as was previously found (Strader and Bartel, 2009; Ruzicka et al., 2010), while this phenotype is lost in *abcg36-4 abcg37-2* (**Figure 5C**). As found for hook opening, IBA inhibited root elongation overall. As shown before *abcg36-4 abcg37-2* was hypersensitive in comparison to Wt (Ruzicka et al., 2010), while *abcg36-4* and *abcg37-2/pdr9-2* was not. The latter is in agreement with a former report (Ito and Gray, 2006) who likewise employed a transfer of seedlings onto IBA medium, while apparently, growth on IBA produces different results (Ruzicka et al., 2010). As a second developmental root parameter, we measured the number of emerged LRs. Again, it was found that *abcg36-4* roots produce slightly more LRs (cf Strader and Bartel, 2009). According to its known rooting potential, IBA increased LR numbers for all accessions tested, and the *abcg36-4* allele was clearly hyper-responsive toward IBA (Strader and Bartel, 2009), while *ABCG37* mutant combinations were not (**Figure 5D**). As a last physiological constraint, we measured root gravitropism for the same mutant set, which to our knowledge has not been analyzed in this respect. In contrast to most measured read-outs, all *ABCG36* and *ABCG37* loss-of-function alleles revealed Wt-like root bending (**Figure 5E**). Also, all mutant lines showed similar sensitivities toward the non-competitive auxin transport inhibitor, *N*-1-naphthylphtalamic acid (NPA; see **Figure 5F**), that efficiently blocks IAA-controlled

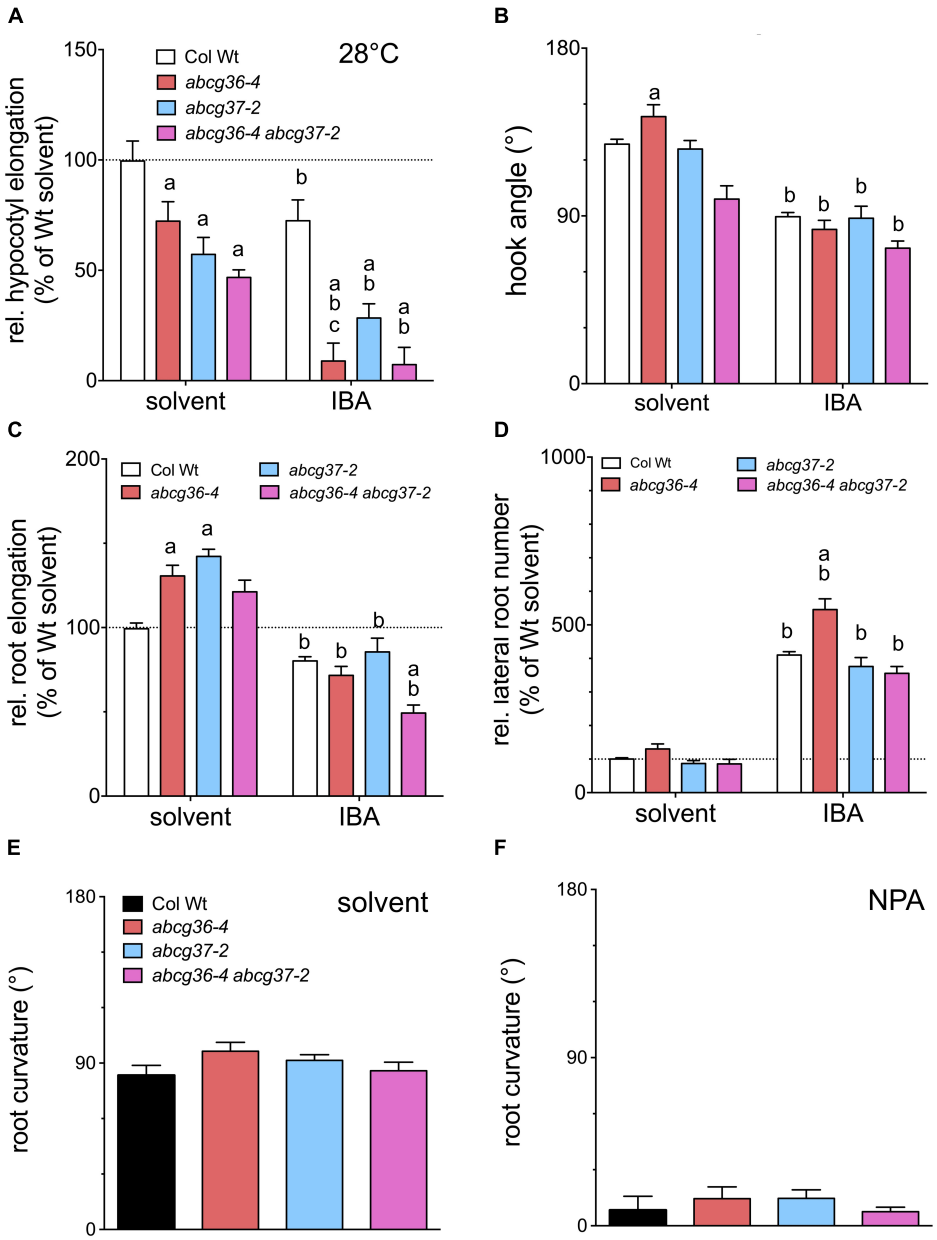

FIGURE 5 | ABCG36 contributes to auxin-controlled plant development. **(A)** Hypocotyl elongation of 5 dag seedlings after transfer onto solvent control or IBA (5 μM) plates after 3 days at 28°C. 22°C control is shown in **Supplementary Figure 3**. **(B)** Hook opening angle of etiolated seedlings grown for 3 days in the dark and transferred onto solvent control or IBA (5 μM) plates in the dark after 4 days. **(C)** Root elongation of 5 dag seedlings after transfer onto solvent control or IBA (5 μM) plates after 24 h. **(D)** Quantification of emerged lateral roots of 5 dag seedlings after transfer onto solvent control or IBA (5 μM) plates after 7 days. Significant differences (unpaired *t*-test with Welch's correction, $p < 0.05$) between corresponding WT and mutant alleles are marked with an 'a,' differences to corresponding WT solvent control with a 'b' (means ± SE; $n = 4$ sets of > 20 seedlings each). For root bending assays, 5 dag seedlings were transferred to solvent control **(E)** or NPA (10 μM) plates **(F)**, and root bending angles were judged after 12 h in the dark.

root gravitropism (Bailly et al., 2008). The finding that *abcg* mutant roots bend like Wt on NPA might indicate that both transporters are not sensitive to NPA as was suggested before for IBA transport in general (Liu et al., 2012).

In summary, these data support an involvement of ABCG36 in most of the auxin-controlled developmental programs tested, such as hypocotyl and root elongation and hook opening. This action of ABCG36 is partly shared with ABCG37, as illustrated by hypocotyl elongation and hook opening. However, root gravitropism data suggest that ABCG36 (and ABCG37) is not involved in all auxin-controlled processes. Our analyses also revealed that IBA altered all four of the developmental read-outs tested. Amongst those, hypersensitivity of *ABCG36/37* mutant alleles to IBA (as for hypocotyl elongation, root elongation and

LR development; **Figure 5**) further supports the involvement of ABCG36/37 in auxin-controlled development.

DISCUSSION

ABCG36 Is an IBA Exporter Negatively Regulating Rootward IBA Transport

The current work enhances the portfolio of ABCG36 substrates by the auxin precursor, IBA. This has been established by heterologous expression in non-plant (yeast) and plant systems (tobacco; **Figure 1**). Recently, expression of ABCG37 in yeast resulted in mislocation to the ER leading to enhanced yeast loading (Ruzicka et al., 2010). Using an identical approach and even the same construct for ABCG37, we here report PM export for ABCG36 and ABCG37; discrepancies in localizations for ABCG37 in yeast between Ruzicka et al. (2010) and this study are currently not known. However, PM exports of IBA (and 2.4-D) were verified in tobacco and *in vivo* by analyses of Arabidopsis *ABCG36* gain- and loss-of-function mutant lines (**Figures 1**, **2**).

It appears that ABCG36 and ABCG37 apparently possess a wider, but clearly delimited, specificity for a few overlapping but structurally unrelated substrates found to be typical for PDR-type ABC transporters (Kang et al., 2010; Borghi et al., 2015). However, it should be mentioned that the substrate sets described are not identical: ABCG37 but not ABCG36 was found to transport phenolic compounds, including coumarin (Fourcroy et al., 2014, 2016; Ziegler et al., 2017). Interestingly, in this substrate category, ABCG36 (like ABCG37) seems to be specific for a few auxinic compounds, such as IBA and 2.4-D, but both do not, for example, transport the major native auxin, IAA (**Supplementary Figure 1**), which only differs from IBA by a two-carbon chain. A future venue for this interesting finding might be offered by the *abcg36-5/pen3-5* allele that was reported to uncouple ABCG36 function in IBA-stimulated root growth, callose and a pathogen-inducible accumulation of salicylic acid from ABCG36 activity in extracellular defense (Lu et al., 2015).

Previous reports left us with a certain degree of uncertainty if IBA is indeed transported in a polar fashion (Rashotte et al., 2003; Ruzicka et al., 2010; Liu et al., 2012). While Rashotte et al. (2003) provided evidence for polar IBA transport in the shoot and roots without analyzing the radiotracer, Liu et al. (2012) clearly demonstrated polar IBA movement, distinct from that of IAA in the hypocotyl. In contrast, Ruzicka et al. (2010) demonstrated that already after 2 h most of the IBA applied to roots is metabolized into IAA. Our careful analysis of PAT in Arabidopsis roots revealed that ABCG36 functions redundantly with ABCG37 in rootward IBA (but not IAA) transport (**Figure 3A**). As discussed above, their role seems to lie in diminishing rootward IAA streams provided by so far unknown IBA transporters, by concerted, lateral IBA export out of the root (**Figure 3D**). Enhanced rootward, long-range IBA transport leads apparently to reduced concentrations of free IBA in *abcg36-4* and *abcg37-2,* measured here over entire roots. Although a final proof of lateral, epidermal ABCG36/37-mediated IBA export is lacking due to technical limitations, the overall concept is in agreement with increased accumulation and reduced efflux from *abcg36* root tips (Strader and Bartel, 2009).

At first view, ABCG36 (but not ABCG37) also seems to have a negative impact on shootward transport of IBA in the root (**Figure 3B**). However, shootward IAA transport profiles remarkably resemble those of IBA, although both ABCG transporters were shown not to transport IAA (**Figure 3B**; Ruzicka et al., 2010). Therefore, we cannot exclude that differences in shootward IAA transport might be caused by peroxisomal conversion of ring-labeled IBA to IAA during the elevated time-frame that is technically necessary for measuring this slow directionality (18 h for shootward vs. 3 h for rootward transport). Although the impact of IBA-to-IAA conversion in Arabidopsis roots has been discussed controversially (Ruzicka et al., 2010; Liu et al., 2012), we believe that polar root transport of IBA using radiotracers can only be safely quantified in a rootward direction.

In contrast to direct IBA/IAA quantifications of entire roots, signals of the columella, maximum IAA reporter, DR5:GFP, were unchanged in *abcg36-4* and *abcg36-4 abcg37-2* on solvent control, as found for *abcg37-2* (Ruzicka et al., 2010). In analogy, IBA significantly enhanced reporter expression compared to Wt in *abcg36-4* (**Figures 4A,B**), most likely by IBA to IAA conversion caused by lack of LRC export. In summary, these data support a dual function of ABCG36 in the polar (rootward) distribution of IBA in the root and local IBA/IAA homeostasis in the very root tip. These features correlate with the mainly polar (lateral) expression pattern in the mature root and a widely non-polar expression in the LR cap cells (see **Figure 3D**; Strader and Bartel, 2009; Ruzicka et al., 2010; Mao et al., 2016, 2017).

ABCG36 and ABCG37 Are Partly Redundant

Widely overlapping substrate profiles and PM localizations between ABCG36 and ABCG37 imply but do not directly prove functional redundancy. A meta-analysis of our data supports the concept that ABCG36 and ABCG37 indeed function at least partly redundantly. This is confirmed by analyses of hook opening, of hypocotyl elongation at 28°C and of root elongation in the presence of exogenous IBA (**Figure 5**). Leaving uncertain or indirect observations, like shootward PAT of IBA or DR5-GFP reporter activation, aside, a few other read-outs do not support a common function in the same pathway. For example, LR development that is enhanced with IBA in *abcb36-4* (**Figure 5D**; Strader and Bartel, 2009) seems to be independent of ABCG37. This is surprising because LRs are thought to be initiated by a shoot-derived auxin maximum (Casimiro et al., 2003; Schlicht et al., 2013), and based on our data, shootward IBA root transport is apparently provided by both ABCG36 and ABCG37 (**Figures 4**, **5**).

Interestingly, quantification of auxin responses, free IBA and root elongation in *abcg36 abcg37* roots (**Figure 2**), revealed a certain degree of compensation of individual single-mutant phenotypes. In light of an overall redundant function in IBA transport this is not easy to understand. An important finding in this respect might be that ABCG37 was found to be a frequent

interactor of ABCG36 (Aryal and Geisler, unpublished). An appealing scenario is therefore that ABCG36 and ABCG37 itself functionally interact, which might explain both the discrepancies found during compensatory and redundant function. The impact of full-size ABC-ABC transporter interaction is currently still unclear but has been recently supported by uncovering the ABC transporter interactome in yeast (Snider et al., 2013).

Analyses of *abcg36/37* Mutants Allowed to Partially Dissect the Roles of IBA and IAA Transport Pathways

The analyses presented here for *abcg36/37* mutants in respect to auxin-controlled growth processes allowed us to partially dissect the roles of IBA and IAA transport pathways and their involvement in either IBA or IAA-controlled plant development. As pointed out above, all of the growth parameters investigated were affected by IBA (**Figure 5**). More interestingly, *abcg36/37* mutants were hypersensitive to IBA in all four of the tested developmental read-outs, although to different degrees (**Figure 5**): while this effect seems to be specific for ABCG36 during hypocotyl elongation and LR initiation (**Figures 5A,D**), this functionality seems to be shared with ABCG37 during root elongation. A concerted action of ABCG36 with ABCG37 contributes to the polar (rootward) distribution of IBA in the plant root and is responsible for auxin-controlled development.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

MG and BA designed the research. BA, JS, JH, AS, JL-M, and JL performed the research. SA and EM provided unpublished material. BA, JS, JH, JL-M, and MG analyzed the data. MG wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00899/full#supplementary-material

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frontiers in Plant Science

REVIEW
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The Nuts and Bolts of PIN Auxin Efflux Carriers

*Marta Zwiewka, Veronika Bilanovičová, Yewubnesh Wendimu Seifu and Tomasz Nodzyński**

Mendel Centre for Plant Genomics and Proteomics, Central European Institute of Technology (CEITEC), Masaryk University, Brno, Czechia

The plant-specific proteins named PIN-FORMED (PIN) efflux carriers facilitate the direction of auxin flow and thus play a vital role in the establishment of local auxin maxima within plant tissues that subsequently guide plant ontogenesis. They are membrane integral proteins with two hydrophobic regions consisting of alpha-helices linked with a hydrophilic loop, which is usually longer for the plasma membrane-localized PINs. The hydrophilic loop harbors molecular cues important for the subcellular localization and thus auxin efflux function of those transporters. The three-dimensional structure of PIN has not been solved yet. However, there are scattered but substantial data concerning the functional characterization of amino acid strings that constitute these carriers. These sequences include motifs vital for vesicular trafficking, residues regulating membrane diffusion, cellular polar localization, and activity of PINs. Here, we summarize those bits of information striving to provide a reference to structural motifs that have been investigated experimentally hoping to stimulate the efforts toward unraveling of PIN structure-function connections.

Keywords: PIN efflux carriers, protein domains, sequence motifs, auxin transport, subcellular trafficking

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***Correspondence:**
Tomasz Nodzyński
tomasz.nodzynski@ceitec.muni.cz

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INTRODUCTION

Auxin is an important regulator of plant development. This signaling molecule provides instructive cues through its accumulation patterns guiding the development from early embryogenesis (Robert et al., 2018) and throughout the entire plant ontogenesis (Vanneste and Friml, 2009), orchestrating the establishment of embryonic apical-basal polarity (Friml et al., 2003), root patterning (Friml et al., 2002a; Ditengou et al., 2008), root system architecture (Lavenus et al., 2016), organogenesis and organ positioning (Heisler et al., 2005; Pahari et al., 2014), and cell differentiation (Marhava et al., 2018). Directional transport of this phytohormone is also involved in responses of plants to critically important environmental stimuli such as gravity (Chen et al., 1998; Luschnig et al., 1998; Kleine-Vehn et al., 2008; Rahman et al., 2010; Rakusová et al., 2011, 2016; Leitner et al., 2012b) and light (Ding et al., 2011; Haga and Sakai, 2012; Zhang et al., 2013).

It has been well established that auxin (with indole-3-acetic acid – IAA – being the most abundant form) exerts its action through the de-repression of numerous auxin-responsive genes (Guilfoyle et al., 1998; Schenck et al., 2010). The main components of auxin signaling machinery encompass proteins from three families: the F-box TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) auxin co-receptors, the Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors, and the AUXIN RESPONSE FACTOR (ARF)

transcription factors. Auxin enhances the interaction between TIR1/AFB and Aux/IAA proteins, leading to a proteasome-dependent degradation of Aux/IAAs and a subsequent release of ARF repression (Salehin et al., 2015; Lavy and Estelle, 2016). Auxin is synthesized in cotyledons, young leaves, and other growing tissues, where development coordination is needed, including expanding leaves (Ljung et al., 2001), roots (Ljung et al., 2005), and reproductive organs (Robert et al., 2015; Lv et al., 2019). IAA is structurally related to the amino acid tryptophan and is synthesized through both enzymatic tryptophan-dependent and independent pathways (Ruiz Rosquete et al., 2011; Kasahara, 2016).

Beside localized synthesis or enzymatic modification, the abundance of active IAA is regulated by auxin carriers. Developmentally crucial polar auxin transport (PAT) is established and maintained by the combined action of auxin transporters from at least three families: AUXIN1-RESISTANT1 (AUX1)/LIKE AUX1 (hereafter AUX1/LAX), PIN-FORMED proteins (hereafter PINs), and members of the B subfamily of ATP binding cassette (ABC) transporters (hereafter ABCBs) (Geisler et al., 2014).

The AUX1/LAXs are part of the amino acid permease superfamily (Bennett et al., 1996) and function as auxin-proton co-transporters (Singh et al., 2018). The variable N- and C-termini of AUX1 are positioned in the cytoplasm and apoplast, respectively and the experiments indicate that the protein has 11 transmembrane domains (Swarup et al., 2004). Similar to PINs, sequence identity to a protein for which AUX1 structure has been solved is so low that homology modeling is unrealistic at present. Nevertheless, the topology predictions allow some comparison to mammalian co-transporter proteins (Fowler et al., 2015). The membrane-spanning helices are likely to drive an alternating-access mechanism (Jardetzky, 1996). In the proposed mode of action, when IAA and protons bind to an outward-open conformation, there is a change in helix arrangement giving rise to an inward-open conformation from which the bound substrates dissociate as the protons dissipate in the proton-poor cytoplasm. However, such predictions require crystallographic validation for AUX1/LAXs (Singh et al., 2018).

Unlike PINs and AUX1/LAX proteins, a subgroup of ABCBs act as primary active auxin pumps that is able to transport against steep auxin gradients (Geisler et al., 2016). Those ATP-binding cassette transporters share a common architecture consisting of two transmembrane domains (TMDs) and two cytosolic nucleotide-binding domains (NBDs). Models derived from high-resolution crystal structures of bacterial ABC transporters have provided important insights into the structure of plant ABCs (Bailly et al., 2012). In the commonly accepted ABC transport mechanism, helices from each TMD participate in substrate binding and form the translocation pathway for it, while both NBDs transmit the necessary ATP-dependent energy to perform a complete transport cycle (Lefèvre and Boutry, 2018). Predictions suggest that substrate specificity in plant ABCBs is determined primarily by the TMDs. The putative IAA-binding sites and translocation surfaces of the plant ABCBs are relatively conserved, although they lack a high degree of sequence identity (Bailly et al., 2012).

Members of PIN-LIKES (PILS) family transporters were shown to function on endomembrane [mainly endoplasmic reticulum (ER)] structures. Identification of this novel putative auxin carrier family was based on the predicted topology of PIN proteins (Barbez et al., 2012). Interestingly, PILS and PIN proteins share only 10–18% sequence identity and belong to distinct protein families. PILS proteins are presumably characterized by two hydrophobic transmembrane regions found at N- and C-termini. The two transmembrane regions flank a relatively short hydrophilic region (loop) with a presumable cytosolic orientation. The loop is less conserved and the most divergent part of the PILS sequences (Feraru et al., 2012). The involvement of PILS in PAT has not been fully clarified but members of this family (PILS6, PILS2, PILS3 and PILS5) have been shown to repress auxin signaling, restrict the nuclear availability and perception of auxin (Barbez et al., 2012). Recently, the ER-localized PILS6 has been show to gate nuclear auxin levels and perception in response to high temperature (Feraru et al., 2019).

The proteins mentioned above do not exhaust the list of membrane localized players that participate in auxin regulation. The tonoplast importer, WALLS ARE THIN 1 (WAT1), was shown to retrieve auxin from vacuoles, suggesting that they also might contribute to auxin homeostasis (Ranocha et al., 2013). In addition, the PM-localized NITRATE TRANSPORTER 1.1 (NRT1.1) is importing IAA during nitrate absence (Krouk et al., 2010).

Last but not least, in many instances, the developmentally crucial auxin maxima are generated *via* the directional auxin efflux facilitated by PINs, in particular, those localized asymmetrically on the PM. This distinct localization is achieved and regulated by vesicular trafficking machinery that mediates directional exocytosis (Łangowski et al., 2016), endocytic recycling, and vacuolar degradation (Kleine-Vehn et al., 2008; Zwiewka et al., 2011; Nodzyński et al., 2013) of those carriers. Vesicles carrying PIN proteins and other PM cargos are formed on donor membranes by the coordinated action of several groups of proteins: small GTPases of ARF/SAR1 family, their guanine-nucleotide exchange factors (ARF-GEFs), GTPase-activating proteins (ARF-GAPs) and coat proteins (Singh and Jürgens, 2018). Among ARF-GEFs, GNOM is often reported as a general regulator of protein secretion since a fungal toxin Brefeldin A (BFA) impairs GNOM function and leads to the formation of intracellular structures composed of aggregated early secretory compartments containing cargoes (Lam et al., 2009), which would normally be recycled back to the PM by exocytosis (Geldner et al., 2003). PINs also aggregate after BFA treatment. However, it is worth highlighting that GNOM plays a role in PIN recycling to the basal but not the apical side of cells (Kleine-Vehn et al., 2008).

The importance of subcellular dynamics facilitating PIN polar localization on the PM with its downstream connection to establishment of auxin accumulation patterns guiding development has been abundantly reported (Nodzyński et al., 2012; Luschnig and Vert, 2014; Naramoto, 2017). Therefore, in this review, we would like to focus on the structure-function connections in PINs that are still insufficiently characterized, primarily discussing amino acid motifs from *Arabidopsis* carriers for which most extensive work has been done.

PINs ARE MEMBRANE PROTEINS WITH TWO HELICAL REGIONS LINKED BY A LOOP

IAA, as well as its synthetic analogs, are weak acids and therefore dissociate in relation to the pH. Based on this characteristic, a chemiosmotic polar diffusion model was proposed (Goldsmith, 1977) postulating that IAA in the low pH of the apoplast (approx. 5.5) is undissociated (IAAH) and able to permeate the PM without any transporters. However, once the molecules have passively diffused into the higher pH (approx. 7.0) of the cytoplasm, they dissociate forming IAA anions that cannot escape the cell unless *via* protein efflux carriers.

It was discovered that PIN proteins fit very well to the chemiosmotic hypothesis since some of them are asymmetrically localized in cells (Gälweiler et al., 1998; Friml et al., 2002b). Later, the capacity of PINs to transport auxin was tested in *Arabidopsis* and also in different heterologous systems (Petrášek et al., 2006; Yang and Murphy, 2009; Zourelidou et al., 2014). The developmental significance of auxin transport is reflected in *Arabidopsis pin1* loss-of-function mutants, which fail to develop floral organs properly and instead generate characteristic naked, pin-like inflorescences, which gave the name PIN-FORMED (often abbreviated as PIN) to this entire protein family (Okada et al., 1991; Gälweiler et al., 1998). In *Arabidopsis*, eight PINs have been identified and they can be divided into two groups, PIN1, 2, 3, 4, and 7, which localize asymmetrically at the plasma membrane of cells (Adamowski and Friml, 2015), and the remaining PIN5, PIN6, and PIN8 that also show localization on the endoplasmic reticulum membrane. At the ER, they mediate auxin exchange between the cytosol and the ER lumen (Barbez and Kleine-Vehn, 2013).

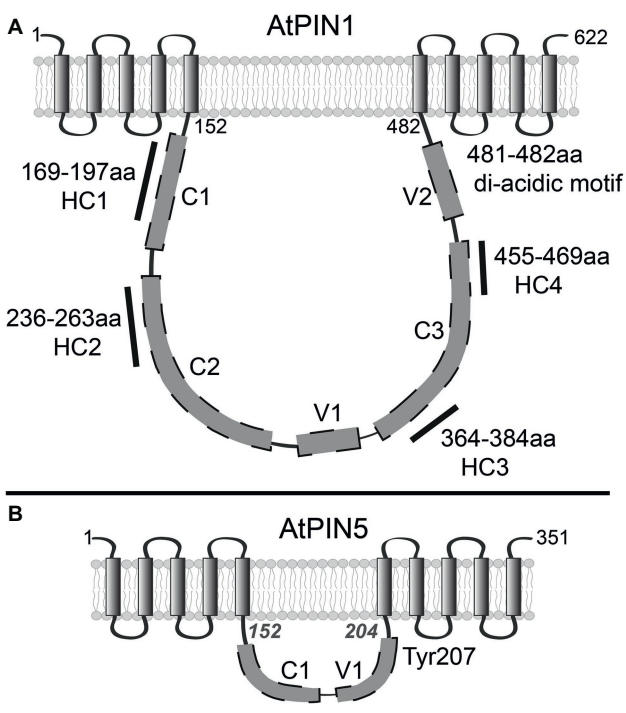

FIGURE 1 | Schematic representation of *Arabidopsis thaliana* long and short PINs. **(A)** Topology of PIN1. Parts of hydrophilic loop marked with gray boxes indicate approximate arrangement of predicted conserved – C and variable – V regions. The positions of highly conserved, canonical motifs HC1–HC4 are indicated with solid lines and amino acid range indications. **(B)** Experimentally not verified topology model of PIN5 with 10 transmembrane domains separated by short hydrophilic region. Amino acid positions in italics indicate the predicted beginning and end of short HL. The tyrosine (Tyr) aligning within the putative tyrosine motif is indicated at position 207.

PINs are integral membrane proteins and it has been experimentally verified that the *Arabidopsis* PINs 1–4 have 10 TMDs grouped in two regions of five alpha helices separated with a large hydrophilic loop (HL) localized in the cytoplasm (**Figure 1A**; Nodzyński et al., 2016). Most of the experiments in those topology studies utilized PIN1 due to the availability of three versions of the protein with GFP inserted in different positions of the HL (GFP-1, 2, and 3). Fluorescence of those reporters was not affected by lowering of the apoplastic pH indicating their cytoplasmic localization along with the HL into which they were inserted. Those results were corroborated by immunolocalization of the HL and the C-terminus in membrane permeable and non-permeable conditions. When PM permeability for the antibodies was reduced, the HA tagged C-terminus was preferentially decorated, while HL showed significantly lower labelling, confirming the cytoplasmic versus apoplastic localization of the HL and C-terminus respectively. Those empirical data limited the amount of valid topological predictions, supporting a 10-TMD PIN topological model. However, the exact position or length of each TMD was not investigated in detail. To gain this information more constructs with tag insertions in the minor loops, linking the helices, would need to be tested. It is unlikely, but not impossible that the total number of TMD may not be 10, just as long as particular PIN parts are positioned accordingly with the experimental data. One such case was published by Wang et al. (2014) depicting a 9-TMD PIN model with the HL and C-terminus positioned on opposite sides of the PM. Such topology was possible when one TMD from the N-terminal helical region was omitted. Not most, but indeed several topology prediction programs indicate such scenario although, usually they depict the N-terminus and the HL as both facing outside the cell which does not correspond with experiments. Also, the topology prediction reliability of the TMDs 1–5 was above 90% (Nodzyński et al., 2016). Still, experimental verification of the N-terminuses position was not done. We were unable to perform it since in our hands N-terminal GFP tagging of PINs was not yielding functional proteins, presumably due to disruption of the PM retention signal. It is also worth to mention that, in the topology studies (Nodzyński et al., 2016), the transmembrane regions were, rather casually, called "bundles" in the sense that there are two groups of five helices. The term "bundles" was not aiming to make deeper structural inferences for which more detailed studies would be required, if one would like to assess which alpha helices bundle together or interact with each other. The same can be stated for the ER-localized carriers for which the topological predictions are even more variable, and their topology has not been verified

experimentally thus far. The PIN5 and PIN8 harbor a relatively short stretch of hydrophilic amino acids between their transmembrane domains (**Figure 1A**; Viaene et al., 2013). Notably, the hydrophilic loop of PIN6 is of intermediate length in comparison to the so-called "long-loop" PINs (PIN1–4 and PIN7) vs. the ER "short-loop" PINs and exhibits a dual ER and PM localization (Simon et al., 2016). The predicted structure of the "long" PINs is similar to the structures of secondary transporters that use an electrochemical gradient across the membrane to facilitate transport (Křeček et al., 2009) sharing also a limited sequence similarity with some prokaryotic (Kerr and Bennett, 2007) and eukaryotic transporters (Gälweiler et al., 1998; Palme and Gälweiler, 1999). Consequently, no ATP binding cassettes have been identified in PIN proteins. There is also experimental data implicating the proton gradient across as the PM as important for auxin transport (Rubery and Sheldrake, 1974; Hohm et al., 2013). However, there are no biochemical studies unequivocally clarifying that the H^+ are the driving force for PIN-mediated auxin efflux.

PIN TRANSMEMBRANE DOMAINS SHOW HIGH SEQUENCE CONSERVATION

Transmembrane domain regions of PINs in *Arabidopsis,* as well as in *Plantae,* are much more conserved than the hydrophilic loop linking them (Křeček et al., 2009; Bennett et al., 2014). Alignment of *Arabidopsis* PINs reveals greater conservation of amino acids in the first (N-terminal) alpha helical region when ER-localized PIN5 and 8 are aligned to the PM-localized PINs 1–4 and 7. However, both the PM PINs (Petrášek et al., 2006) and the ER ones (Mravec et al., 2009; Ding et al., 2012) seem to have the capacity to transport auxin, suggesting that the auxin translocation activity is encoded in the transmembrane domains and not in the hydrophilic loop, which is prominently shortened for the ER PINs. Notably, the transport assays for ER-localized PINs were based on auxin metabolic profiling, showing increased conjugation of IAA in case of PIN5 over-expression (Mravec et al., 2009) while the converse was reported when PIN8 was over-expressed (Ding et al., 2012). Activity assessment of membrane proteins requires their incorporation in a lipid bilayer across which transport can commence. Establishment of such setup is more challenging in comparison to an activity assay of soluble enzymes. However, it would be interesting to reconstitute the ER PINs in liposomes (Nimigean, 2006) to test their activity. Such, more biochemically pure setup would further support the conjecture that PINs are independent efflux carriers and enable to evaluate more precisely their transport specificity.

The length of the TMDs was also analyzed by aligning so-called "canonical" PIN sequences from multiple plant species. Thus, the predicted length of the N-terminal TMD region was very consistent, ranging from 155 to 176 amino acids. The longer sequences, originating mostly from the *Poaceae* family, were usually extended by insertion of up to 18 amino acids between positions 97 and 98 falling in between third and fourth alpha helix of the generalized PIN model. Similarly, predicted C-terminal TMDs were even more consistent with the length of 154 amino acids (Bennett et al., 2014). Calculated frequency of the most common amino acids at each core position in the TMDs, in all canonical PINs, indicated 106 invariant or near-invariant (>99% amino acid identity) positions, and 87 amino acids with more than 90% identity. The predicted TMDs positions did vary but they were still the most highly conserved parts of the whole transmembrane domain region with only helix 3 showing less conservation in the generalized PIN model. The conservation of TMDs likely was evolutionarily selected in relation to the stabilization of the protein within the lipid bilayer or its auxin translocation activity (Bennett et al., 2014).

It is worth noting that the eight very small loops linking the alpha helices, while being more variable, still contain several highly conserved residues which might play important structural or regulatory roles. In congruence with this assumption, recently two evolutionarily-conserved cysteine residues (C39 and C560; **Figure 2A**) have been implicated in regulating PIN2 endocytosis and distribution on the PM. PIN2 mutant version with cysteine-to-alanine mutations shows more cytoplasmic fluorescent signals and more variability in abundance of the efflux carrier on the PM. The mutation also caused more wavy root while the overall gravitropism was not altered. A detailed microscopic analysis of *pin2*^{C39,560A}*:Venus* double mutant revealed modified PIN2 distribution within plasma membrane microdomains (clusters), indicating overall changes in mobility of the protein (Retzer et al., 2017). Indeed in comparison with different apolar cargoes, the lower PM diffusion of PINs, together with their targeted super-polar exocytosis and the endocytosis collecting laterally diffusing auxin carriers, have been proposed as mechanisms contributing to PIN polarity maintenance (**Figure 2B**; Kleine-Vehn et al., 2011). In addition, it has been shown that PIN5 fused with PIN2-HL is able to localize ectopically on the PM, but does not exhibit PIN2-like, polar localization (Ganguly et al., 2014). All those reports seem to indicate that functional elements could be encoded in the small loops linking the alpha helices or in the TMDs themselves, together participating in PIN polarity establishment. This process might also involve interactions between PINs and the cell wall, as protoplasting of epidermal cells has resulted in PIN2 polarity disappearance (Feraru et al., 2011). Therefore, more detailed studies of PIN structure would be instructive in finding out, what motifs play a role in PIN polarity maintenance.

THE HYDROPHILIC LOOP SEQUENCE IS LESS CONSERVED YET WITH IDENTIFIABLE MOTIFS

Based on the size of the central hydrophilic loop, PINs can be tentatively divided into two major subgroups, the "long" and "short" ones (Viaene et al., 2013). The HL sequence is much more divergent than the TMD regions (Křeček et al., 2009), but already one of the initial multi-species alignments revealed conserved domains (C) and variable regions (V) in the HL (**Figure 1A**; Zažímalová et al., 2007). The authors postulated

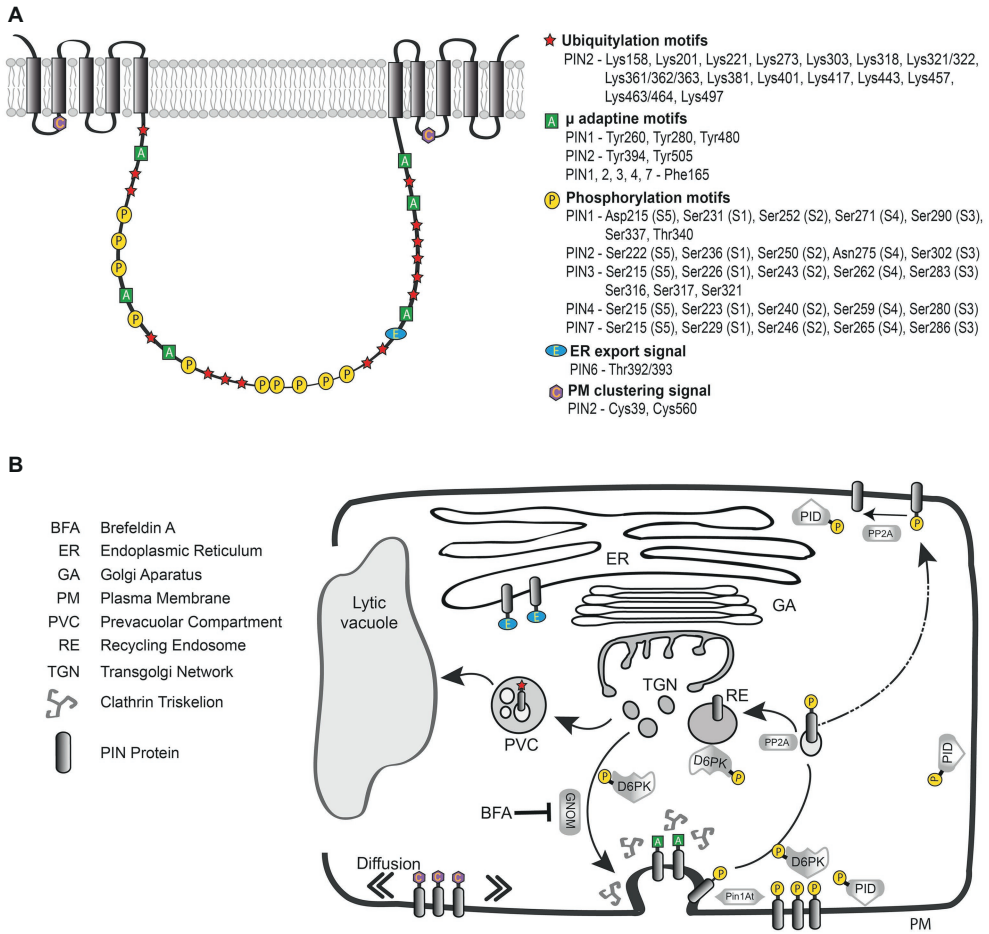

FIGURE 2 | Schematic designation of known sequences and residues in a generalized model of *Arabidopsis thaliana* long PINs and their role during intracellular trafficking. **(A)** Symbols indicate the functional elements and their approximate location in the canonical PIN model, precise positions of those motifs for particular PINs are listed on the right. **(B)** Simplified illustration of PIN vesicular trafficking with the functional motifs indicated at those steps of the intracellular pathway in which they play most prominent roles. The ER export signal present in the long hydrophilic loop of the PIN protein is oriented into the cytoplasm and serves as a signal for further secretion. GNOM is a regulator of protein recycling from early endosomal compartments like TGN and its inhibition by BFA impairs exocytosis. The μ-adaptin motifs in the HL participate in interaction with clathrin machinery. PIN phosphorylation status controlled by kinases (PID, D6PK) and phosphatase (PP2A) is crucial for cycling, PM localization and activity of the auxin efflux carrier. Pin1At-facilitated isomerization of proline residues in the vicinity of phosphorylation sites affects PIN1 polar localization. Cysteines present in small cytosolic loops linking the helices play a role in PIN PM diffusion and trafficking. Ubiquitylation serves as a signal for vacuolar degradation of the carrier.

that long HL includes conserved domains C1, C2, and C3 and variable regions V1 and V2 depicting their schematic, approximate distribution within the HL and already noting that the motifs appear in a particular order. In this report, the short HL was characterized by the presence of only the domain C1 and region V1 (**Figure 1B**). The variable regions were homologous within the group of long HL PINs but, they differed significantly when compared between short and long looped PINs.

Latter more detailed studies were published. Wang et al. (2014) identified 20 motifs after aligning PIN1 proteins sequence from multiple species. The two longest motifs (M) were aligning mostly in the TMD regions, M1 in the N-terminal and M2 in the C-terminal part of the PIN1 protein, again indicating strong conservation of the helices across species. Several shorter motifs were identified in the HL showing that also this region has cross-species conservation. Notably, some of the shorter conserved sequences (M4, M13, M9) encompassed the phosphorylation sites S1–S4 (Barbosa et al., 2018) present in the *Arabidopsis* PM PINs as well as the S337, T340 residues (M11) shown to control polar sorting of PIN1 (**Figure 2A**; Zhang et al., 2010). In the same year, another study focused on the HL region of PINs in multiple plant species. This alignment of PIN sequences has identified four highly conserved (HC) motifs HC1–HC4 in the central HL (**Figures 2A,B**; Bennett et al., 2014). Authors reported that long loop usually harbors all four conserved motifs, always appearing in succession. Notably, the HC4 region was also partially present in the short HL of PIN5 and PIN8 within angiosperm kingdom. Authors of this study designated particular PIN proteins as "canonical" if they matched the

consensus sequence of all four HC motifs with at least 50% identity or 70% similarity. Auxin efflux carriers not fulfilling these criteria were named "noncanonical" (Bennett et al., 2014). With this classification, authors strove to describe more accurately the structural features of PIN proteins, not referring only to the length of the HL, but rather to the content of its sequence. Still, most of the canonical PINs did have long HLs. Using this nomenclature PIN5 and 8 would be noncanonical, while PIN6 might be classified as "semi-canonical".

Authors also stipulated that noncanonical PINs evolved repeatedly by sequence divergence from canonical precursors. They pointed out the unique structural features of *Arabidopsis* PIN5 and PIN8 indicating that they cannot be classified as ancestral precursors of canonical PINs, as surmised prior (Viaene et al., 2013). Indeed, the *Arabidopsis* ER-localized PINs have a more divergent composition of the TMD than the PM-localized ones. Also, in case of *Arabidopsis* PIN5 and PIN8 the topology predictions are much more variable than for PM PINs 1–4 and 7. As mentioned above, a chimeric version of PIN5 harboring the HL of PIN2 was able to more efficiently reach the PM, but without exhibiting the characteristic polar localization (Ganguly et al., 2014) hinting at the functional distinction of ER PINs from the PM ones, supporting their divergent evolution.

THE HYDROPHILIC LOOP HARBORS REGULATORY CUES FOR PIN TRAFFICKING AND ACTIVITY

Numerous studies indicated the presence of information necessary for PIN trafficking and polar targeting within the HL amino acid sequence (**Figure 2B**; Michniewicz et al., 2007; Huang et al., 2010; Zhang et al., 2010; Ganguly et al., 2014). Congruently, the short looped PIN5 and 8 show predominantly ER localization, thus it is presumed, that they lack the molecular cues for PM trafficking (Ganguly et al., 2014). The experimentally verified cytosolic orientation of the HL is also sensible from the functional point of view as it makes the loop accessible for the cytoplasmic, subcellular trafficking machinery. Motifs in the HL are involved in clathrin-mediated endocytosis (Kleine-Vehn et al., 2011; Nodzyński et al., 2016; Sancho-Andrés et al., 2016) and being a target of phosphorylation by cytosolic kinases (Dhonukshe et al., 2010; Huang et al., 2010) as well as ubiquitylation (Abas et al., 2006; Leitner et al., 2012a), which collectively modulate the intracellular trafficking, PM stability and polar delivery of long *Arabidopsis* PINs. Those processes contribute to the regulation of PIN-mediated directional auxin translocation, which can take place when those efflux carriers are present at the PM, distributed in an asymmetrical fashion and active (**Figure 2B**; Wiśniewska et al., 2006; Zourelidou et al., 2014). Below we will discuss some of the amino acid motifs contained in the HLs and their roles uncovered so far.

Tyrosine Sorting Motif Is Involved in PIN Trafficking

Plasma membrane resident PIN proteins undergo clathrin-dependent endocytosis (Dhonukshe et al., 2007) regulating their PM abundance (Adamowski et al., 2018) and maintaining their polar localization (Kitakura et al., 2011; Kleine-Vehn et al., 2011). Cargo sorting into clathrin-coated vesicles is mediated by adaptor protein (AP) complexes, which recognize cytosolic sorting signals in membrane proteins *via* their medium (μ) subunits (Robinson, 2015). The *Arabidopsis* genome encodes adaptin subunits of 4 types of putative AP complexes (AP-1 to AP-4) including five medium subunits, named μA (μ2), μB1 (μ1–1), μB2 (μ1–2), μC (μ4), and μD (μ3) (Boehm et al., 2001; Park et al., 2013). One of the best- characterized sorting signals, which is recognized by the medium (μ) subunit of adaptor complexes, is the YxxΦ motif, where Y denotes the single letter abbreviation for tyrosine, "x" is any amino acid, and Φ is a bulky hydrophobic residue leucine, isoleucine, phenylalanine, methionine or valine (Canagarajah et al., 2013). In a different type of tyrosine-based motif (NPxxY) (Traub, 2009), Tyrosine 480 (Y-480) was proposed to be important for PIN1 localization, since mutagenesis of the amino acid stretch containing this tyrosine (NPNSY to NSLSL), caused PIN1 retention at the ER membrane (Mravec et al., 2009). This tyrosine residue and the motif itself is found in all long HL PINs and seems to be important for PIN1 trafficking and localization (Sancho-Andrés et al., 2016). Interestingly, similar tyrosine containing conserved region was also identified in the short hydrophilic loop of PIN5 and PIN8 (**Figure 1A**; Mravec et al., 2009).

Similarly, the clathrin-dependent PIN internalization was selectively affected by mutating a conserved tyrosine residue of PIN2 required for cargo-specific sorting into clathrin-coated pits (**Figures 2A,B**). While the majority of *PIN2*^{Y505A}*-YFP* was localized to the apical cell side in root epidermal cells, the mutation strongly enhanced PIN2 lateral localization. Notably, *pPIN2:PIN2*^{Y505A}*-YFP* showed reduced PIN internalization and did not fully rescue the *pin2* mutant phenotype (Kleine-Vehn et al., 2011).

Further search for putative sorting signals in HL of PIN1, identified a phenylalanine (F)165 and three tyrosines in positions 260, 328, and 394 participating in the interaction with several μ-adaptins, thus possibly being involved in PIN1 trafficking and polar localization (**Figures 2A,B**). However, only F165, present in all canonical PINs, which has the ability to interact with μA- and μD-adaptins *in vitro*, appeared to be essential for the routing and localization of the efflux carrier *in vivo*, since the PIN1:GFP-F165 mutant showed reduced endocytosis, but also accumulated in intracellular structures (Sancho-Andrés et al., 2016). Nevertheless, PIN1:GFP-F165A mutant was still internalized, therefore other residues in the HL may also participate in PIN1 endocytosis. Even though, precise function of AP-3 in PIN1 trafficking needs further research, the PIN1-GFP accumulation in big intracellular structures present in a μ3-adaptin mutant (Sancho-Andrés et al., 2016) and in mutants of other subunits of the AP-3 complex (Feraru et al., 2010; Zwiewka et al., 2011), solidify the role this complex in PIN1 trafficking and localization.

ER Exit

Membrane-localized proteins are synthesized on the rough ER and remain integrated into the lipid vesicles as they traffic to their final destinations along the secretory pathway.

A starting point of unraveling which protein motifs retain PINs at the ER membrane and which are necessary for ER exit, was provided during the investigation of PIN5 localization. Alignment of short-ER PINs and long-PM ones revealed the presence of a conserved amino acid stretch, named the di-acidic motif presumably, important for trafficking of proteins from the ER (Mravec et al., 2009). In PIN1 HL this sequence is abstracted as NPxxYxxΦ, where "x" represents any amino acid and Φ denotes a residue with a bulky hydrophobic side chain, the other letters designate specific amino acids. The di-acidic motif is composed from the tyrosine-480 motif NPNSY followed by SSL sequence (**Figure 2A**). Alignment of the region spanning the tyrosine motif of all *Arabidopsis* PIN proteins revealed a conserved sequence NPN(S/T)YSSL (where S is found in PIN1 sequence and T is present in other long PINs) in the HL of canonical PINs, but the last three SSL amino acids are missing in the short-looped PIN5 and PIN8. The attempt to mutagenize the di-acidic motif in PIN1 resulted in significant accumulation of the protein at the endoplasmic reticulum (Mravec et al., 2009), supporting its role in ER exit. It is important to highlight however, that the mutagenesis targeted also the conserved tyrosine residue (NPNSY mutated to NSLSL) which could interfere with clathrin mediated endocytosis in general (Ohno et al., 1995; Bouley et al., 2003). On the other hand, manipulations of PIN1 HL residues, especially the F-165 mutation, interacting with the μ-adaptin endocytic machinery resulted in accumulation of the carrier in round, condensed structures (Sancho-Andrés et al., 2016). Whereas, in PIN1-GFP-tyr (NSLSL) line, PIN protein was clearly localized at the ER (Mravec et al., 2009). Nevertheless, it cannot be ruled out that mutagenesis of this specific sequence would result in improper folding and protein retention at the ER.

Interestingly, phosphorylation-dependent ER exit has been recently reported for PIN6 protein. Functional analysis of threonine residues (T) 392 and 393 (**Figure 2A**; Benschop et al., 2007), phosphorylated by mitogen-activated protein kinase (MAPK) MPK4 and MPK6 *in vitro* (although T393 is not phosphorylated by these kinases) revealed a key role of indicated residues in PIN6 ER exit. Those phosphorylation sites also play a role in main root and root hair growth regulation, as well as development of the inflorescence stem at the appropriate, possibly environmentally determined, time (Ditengou et al., 2018). This demonstrates yet another facet of phosphorylation controlling PIN subcellular trafficking beside the earlier discovered control of their PM polar localization.

PIN POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications (PTM) play an important role in regulating protein folding, activity by modifying the targeting to specific subcellular compartments, and interaction with ligands or other proteins (Spoel, 2018). The early steps of processing and sorting of *de novo* synthesized PIN proteins are still largely unknown. PIN post-translational modifications such as glycosylation, an indicator of protein maturation, or proteolytic processing, including signal peptide recognition and cleavage, have not been extensively experimentally verified (Luschnig and Vert, 2014). It is worth noting that online bioinformatic tools and databases can be instructive in search for known and potential sites of post-translational protein modification. Some of recently launched user-friendly bioinformatics online resources: iPTMnet (Huang et al., 2018) and Plant PTM Viewer (Willems et al., 2018) integrate PTM information from text mining, curated databases and ontologies providing visualization tools for exploring PTM networks, conservation across species or even crosstalk. One of the most common PTMs is protein phosphorylation. The database of Phopsho-sites in PlanTs (dbPPT) (Cheng et al., 2014), containing experimentally identified sites in plant proteins, indicates 77.99, 17.81, and 4.20% of phosphorylated serine, threonine and tyrosine residues respectively. Consequently, also for PINs, many phosphorylation sites have been discovered and will be also discussed below.

Extensive PIN Glycosylation Has Not Been Reported

An early review of multiple PIN sequences with the Prosite database (Hulo et al., 2006) suggested two clusters of sequences in the HL region containing potential glycosylation motifs (Zažímalová et al., 2007). They are however positioned in the HL region, which we now know, is in the cytosol (Nodzyński et al., 2016), and it is more likely that parts of the protein facing the lumen of ER and Golgi would be glycosylated and not the cytoplasmic ones. Congruently, the theoretical size of PIN2 (~70 kDa) matched with the observed band on the SDS PAGE gel, indicating no extensive glycosylation of the protein (Müller et al., 1998).

Several Residues in the Hydrophilic Loop of PINs Are Phosphorylated

Protein phosphorylation is an enzymatic reaction involving the addition of a phosphate group $(-PO_4)^{3-}$ to the polar residue -R of various amino acids (usually serine, threonine, and tyrosine, or histidine). This covalent modification is often associated with protein activity regulation related to its conformational change, allowing to interact with other molecules, proteins, and even assemble or uncouple protein complexes (Sacco et al., 2012; Ardito et al., 2017). Kinases (phosphotransferases) reversibly attach a phosphate group while phosphatases do the opposite by hydrolysis.

Three Ser/Thr protein kinase families were reported to be involved in phosphorylation of PINs: AGC kinases (serine/threonine kinases homologous to mammalian protein kinase A, cGMP-dependent kinase, and protein kinase C) (Bögre et al., 2003) belonging to the AGCVIII subfamily (Galván-Ampudia and Offringa, 2007), MITOGEN-ACTIVATED PROTEIN (MAP) KINASES (MPKs/MAPKs) (Jia et al., 2016; Dory et al., 2017), and Ca^{2+}/calmodulin-dependent protein kinase-(CDPK)-related kinases (CRKs) (Rigó et al., 2013). Among the AGCVIII subfamily, two subgroups have been directly implicated in PIN-mediated auxin transport: PINOID (PID) together with

its presumed functional paralogs WAG1, WAG2 (named after *wag* mutants that showed root waving) (Santner and Watson, 2006) as well as D6 PROTEIN KINASE (D6PK) with the three candidate paralogs D6PK-LIKE (D6PKL) 1–3 (reviewed in Willige and Chory, 2015).

Like *pin1* plants (Okada et al., 1991; Gälweiler et al., 1998), PID mutants exhibit the pin-shaped inflorescence (Bennett et al., 1995; Christensen et al., 2000; Benjamins et al., 2001). PID and the closely related WAGs also play roles in hypocotyl phototropism (Ding et al., 2011; Haga et al., 2014), gravitropism (Grones et al., 2018) as well as root gravitropism (Ganguly et al., 2012) and, apical hook opening (Willige et al., 2012). In all those processes PIN polarity has to be modified to redirect auxin flux, facilitating differential growth. The polarity related action of AGCVIII kinases is in *Arabidopsis* counteracted by the protein phosphatase 2A (PP2A) complex, and defects in the regulatory subunits - PP2AA1, PP2AA2, and PP2AA3 result in similar phenotypes as the PID overexpression (Garbers et al., 1996; Michniewicz et al., 2007; Xi et al., 2016). Within the *Arabidopsis* AGCVIII subfamily, the D6PK and related D6PKL1–D6PKL3 (D6PKs) are also required for auxin transport-dependent processes, such as hypocotyl phototropism, negative gravitropism, shade avoidance as well as lateral root and shoot differentiation (Zourelidou et al., 2009; Willige et al., 2013; Kohnen et al., 2016). Interestingly, their phosphorylation function affects PIN-mediated auxin efflux activity, rather than the PM polarity of those carriers (Zourelidou et al., 2009, 2014).

It has been reported that PID action can be regulated by interaction with different Ca^{2+} binding proteins (Benjamins et al., 2003) suggesting a possible role of Ca^{2+} signals in PIN polarization. What is more, mutations or chemical treatments which elevated Ca^{2+} levels, were associated with PID-related polarity shifts of PIN1 in the stele and PIN2 in the cortex cells, but not in the epidermis (Zhang et al., 2011). Consequently, Ca^{2+} was implicated in endomembrane trafficking in plants (Himschoot et al., 2017), but how those signals translate into PIN polarity is not fully understood. In the case of PIN2, the calcium-dependent CRK5 may be a part of the machinery translating Ca^{2+} levels into PIN polar targeting (Rigó et al., 2013). *In vitro* experiments confirmed PIN2 phosphorylation by CRK5, but the respective phosphosites have not been mapped. In *crk5* mutants, PIN2 protein was partially depleted from the apical plasma membrane in epidermis cells and showed polarity defects in the cortex displaying apolar or outer-lateral PIN2 localization, but for other PINs the effect was not observed (Rigó et al., 2013). Upon treatment of the *crk5* mutants with BFA, which is blocking exocytosis of PM cargos, PIN2 showed accelerated accumulation in BFA compartments (Rigó et al., 2013). Moreover, in *crk5*, inhibition of primary root elongation and delay of gravitropic bending of root and shoot were observed. Those data are suggesting that the CRK5 function is ether to inhibit PIN2 endocytosis or to activate its recycling (Rigó et al., 2013).

PIN Phosphorylation as the Hypothetical Down-to-Up-Polarity Switch

The most evident phenotypical aberrations are those related to PIN1 function and the phosphorylation sites in the HL of this carrier are probably most comprehensively experimentally verified. The kinases PID/WAGs and PP2A phosphatase were shown to regulate the phospho-status of the evolutionary conserved S1-S3 residues in PIN1 HL (TPRxS where "x" is S or N, see **Figure 2A**). Phosphorylation of those sites resulted in PIN apicalization (shootward localization) on the PM of cells while de-phosphorylation caused a predominantly basal (rootward) localization of the carrier (**Figure 2B**; Friml et al., 2004; Michniewicz et al., 2007; Kleine-Vehn and Friml, 2008). In connection, it has been observed that in *pid* mutants, PIN1 does not relocalize to the apical plasma membrane to redirect auxin distribution during shoot differentiation (Friml et al., 2004). Consequently, S1–S3 phosphosite mutations interfere with the ability of PIN1 to rescue *pin1* inflorescence phenotype (Dhonukshe et al., 2010). In the wild type root epidermal cells, PIN2 is apical, but in *pid*, *wag1*, *wag2* mutants it is basally localized (Dhonukshe et al., 2010). What is more, in *Arabidopsis* root, PID/WAG overexpression results in a basal to apical PIN polarity shift, which correlates with root meristem collapse due to loss of the root tip auxin maximum (Michniewicz et al., 2007; Dhonukshe, 2011; Weller et al., 2017). S1–S3 phosphosite mutations abrogate the effects of PID overexpression on PIN1 polarity, and phosphorylation-mimicking mutations result in constitutive apicalization of the PIN protein (Dhonukshe et al., 2010; Huang et al., 2010). Importantly, phosphomimetic mutations of PIN3 HL in the previously described conserved TPRxS motif resulted in defective root and hypocotyl gravitropic growth as well as inhibition of gravity-induced PIN3 relocation similar to the effects observed in PID overexpression lines (Grones et al., 2018). Based on the results described above, mostly concerning PIN1, a quite long-standing hypothesis of a binary polarity switch mechanism was proposed in which more phosphorylation resulted in apical PIN trafficking while less phosphorylation made the carrier remain on the basal cell site. However, during those studies there were no tools available to probe PIN phospho-status directly at a given subcellular localization. This knowledge gap was filled more recently and is discussed below.

D6PKs and Phosphosite Antibodies – Updating the PIN Polarity Model

Similarly to PID/WAGs, D6PK phosphorylates PIN proteins at serines S1–S3 as well as at two additional serine residues, S4 and S5 (**Figure 2A**; Zourelidou et al., 2009, 2014; Willige et al., 2013). The S4 and S5 are conserved in PIN3, PIN4, and PIN7, but S5 is absent from PIN1 while PIN2 lacks both S4 and S5 (**Figure 2A**; Zourelidou et al., 2014). Like PID, D6PK activates PIN-mediated auxin efflux (PIN1, PIN3), although with a slightly different PIN phosphosite preference, D6PK phosphorylating the S2 site less (Zourelidou et al., 2014). Unlike in the case of PID or WAG, D6PK overexpression does not result in PIN polarity changes that has been explained by different phosphosite preferences between D6PK and PID/WAGs (Zourelidou et al., 2009, 2014; Barbosa et al., 2014). Furthermore, recent investigations utilizing phosphosite-specific antibodies directed against PIN1 S1–S4 sites, immunodetected

phosphorylated PIN1 at the basal as well as at the apical plasma membranes of root cells. The phosphorylation at the basal PM was strongly sensitive to BFA, rather indicative of D6PKs that is predominantly basally localized but not of the BFA insensitive PID/WAGs (Kleine-Vehn et al., 2009; Weller et al., 2017). Interestingly, when PIN1 was targeted to the apical plasma membrane using a GFP fusion impairing PIN1 trafficking (PIN1::PIN1:GFP-3), the efflux carrier was phosphorylated and the immunodetected P-signal was present even after BFA treatment (Weller et al., 2017). Those results are in agreement with the model of phosphorylation controlling PIN localization but it seems that the basal-to-apical polarity switch does not depend only on increased phosphorylation since apically localized PIN1-GFP-3 is also phosphorylated (Weller et al., 2017). Thus, phosphorylation might be only one of the steps that initiate PIN polarity shifts and it is conceivable that also other proteins participate in the sequence of events leading to PIN polar targeting. Since the HL constitutes almost half of the total size of PM PINs it may serve as a hub for multiple interactions that together with phosphorylation co-regulate PIN polar delivery also depending on the cellular context (**Figures 2A,B**; Wiśniewska et al., 2006).

Pin1At Isomerase – Phosphorylation and Hydrophilic Loop Conformation Impacting PIN Polarity

Phosphorylation has been also linked to protein conformational changes (Humphrey et al., 2015). Interestingly, peptide bond *cis/trans* isomerization of prolines following phosphorylated serine or threonine (S or T–P) by Pin1At (Peptidyl-prolyl cis-trans isomerase NIMA-interacting), was connected to regulation of flowering time in *Arabidopsis* (Wang et al., 2010). The Pin1At accelerates the *cis/trans* conformational change of the phosphorylated Ser/Thr-Pro motifs in the central PIN1 hydrophilic loop and affects polar localization of PIN1 (**Figure 2B**). In other organisms, this isomerase rearranges only phospho-Serine/Threonine-Proline motifs and has been reported to bind to a subset of proteins, thus mediating a post phosphorylation regulatory function (Ping Lu et al., 1996; Abrahamsen et al., 2012; Brichkina et al., 2016).

In *Arabidopsis* Pin1At mediates the antagonistic effects of PID and phosphatases on PIN1 polarity. Overexpression of Pin1At enhances the agravitropic phenotype of *pp2aa1–6*, generating coiled roots, while downregulation of the isomerase in AmiR-Pin1At line suppresses root agravitropism of 35S:PID (Xi et al., 2016). Pin1At most effectively isomerizes the S337, found in the last motif in PIN1-HL out of 4 tested by Nuclear Magnetic Resonance (NMR) (Xi et al., 2016). This serine is targeted by the cascade of MAP Kinase activating the Mitogen-Activated Protein Kinase (MKK7–MPK) 3/6-targeted (Jia et al., 2016). Pin1At, to a lesser extent, also isomerizes the phosphorylated T227, T248, and T286, reported to be targets of MPK4 and MPK6 (Dory et al., 2017). Congruently with previously published data (Zhang et al., 2010), the downregulation of Pin1At suppresses the phosphomimetic mutations (serine to aspartic acid change) S337 and T340 in root stele cells, which are inducing a basal-to-apical shift of PIN1 (Xi et al., 2016). If this role is directly linked to PID remains to be shown because PID could not phosphorylate PIN1 S337 (Zhang et al., 2010). It is worth mentioning, the effects of the isomerase on the polarity of GFP tagged PIN2, 3, 7 were not observed (Xi et al., 2016). This leaves an open possibility for other mechanisms to control the polarity of different PINs. It is not unlikely that the regulatory elements are encoded in the sequence context, extending beyond the phosphorylation sites and into the structural elements of HLs that have not been proven or experimentally investigated yet.

PIN Ubiquitylation

After internalization from cell surface, proteins can either be recycled back from Trans-Golgi Network (TGN) to PM, or sorted to Multivesicular bodies (MVB) and further routed towards the vacuolar proteolysis (**Figure 2B**). In plant cells, the ubiquitylation status seems to signal which membrane cargo will traffic back to PM and which will be degraded (Tian and Xie, 2013). A single molecule of ubiquitin (Ub), or a poly-Ub string, can be attached to a lysine side chain of the targeted protein *via* a C-terminal carboxylate group of ubiquitin (Ub). As mentioned, the Ubs can also be chained together *via* an isopeptide bond linking one of seven lysines (K) of the already bound Ub with the C-terminal glycine of second Ub molecule. Several lysine residues of the cargo protein can be ubiquitinated. The K63-linked ubiquitin chains are generally considered to be critical signals for endosomal-mediated sorting and vacuolar degradation of PM proteins (Reyes et al., 2011; Isono and Kalinowska, 2017).

One of the first immunoprecipitation experiments demonstrated ubiquitylation of a considerable range of PM proteins, including PIN2, but no clear link to PIN protein fate has been established at that point (Abas et al., 2006). Thus far, only one report systematically investigated possible lysine residues targeted by K63-linked polyubiquitylation in PIN2 protein (Leitner et al., 2012a). In this study, authors prepared a collection of PIN2 constructs having increasing numbers of mutated lysines and tested their capacity to rescue the agravitropic *pin2/eir1–4 (pin-formed/ethylene insensitive root 1)* mutant phenotype. Importantly, it was shown that single K mutations and combinations of only a few lysine-to-arginine (R) exchanges did not visibly interfere with PIN degradation. However, combining multiple K-to-R point mutations in the hydrophilic loop did elevate the PM stability of the modified PIN2 to the point that it failed to fully complement the agravitropic *eir1–4* mutant. Consistently, the more lysines in the loop were replaced, the more apparent this effect became. Two constructs that were least effective in rescuing the *eir1–4* had 12 and 17 lysines mutated in amino acid positions ($pin2^{12K\text{-}R}$) *158*, *201*, *318*, *321*, *322*, *361*, *362*, *363*, *381*, *497*, *556*, *614* and ($pin2^{17K\text{-}R}$) *221*, *273*, *303*, *318*, *321*, *322*, *361*, *362*, *363*, *381*, *401*, *417*, *429*, *443*, *457*, *463*, *464* (**Figure 2A**). Overall, these findings imply that K63-linked ubiquitin chain modification, of multiple lysines situated in the hydrophilic loop, redundantly control PIN2 degradation and root gravitropism (Leitner et al., 2012a).

This is consistent with the previously reported vacuolar trafficking of PIN2 in epidermal cells of the convex side of the root during root gravitropic bending, decreasing auxin flux and causing elongation of those cells (Kleine-Vehn et al., 2008). Interestingly, five of above-mentioned lysines (*PIN2: 158, 201, 363, 497, 614*) are conserved among *Arabidopsis* PIN1–4 and 7, hence could be investigated in other PINs.

PIN – ABCB INTERACTIONS

Besides PINs, ABC transporter B subfamily proteins (ABCBs, previously named PGPs - p-glycoprotein subfamily) also participate in auxin efflux from plant cells. ABCBs are primary transporters using adenosine triphosphate (ATP) to power auxin translocation. What is more, physical interactions of those auxin carriers were demonstrated by Y2H and co-immunoprecipitation analyses using C-terminal domains of ABCB1 and ABCB19 pairing with the hydrophilic loops of PIN1 and PIN2, respectively (Blakeslee et al., 2007; Rojas-Pierce et al., 2007). Although PINs are able to efflux auxin on their own (Petrášek et al., 2006), when co-expressed as PIN1-ABCB1 and PIN1-ABCB19 pairs, elevated export rate and increased substrate specificity was reported for PIN1 in comparison with the single transporter assays. While, co-expression of ABCB1 and 19 enhanced only the substrate specificity of PIN2 (Blakeslee et al., 2007), expressing ABCB4 with PINs had an influence on transport directionality. However, the interaction has not been proven biochemically thus far (Bandyopadhyay et al., 2007; Blakeslee et al., 2007). Additional evidence for PIN and ABCB functional interactions came from microscopy-based cell biological studies showing partial co-localization of ABCB1 with PIN1 in the stele and with PIN2 in cortical and epidermal cells of the *Arabidopsis* seedling roots (Blakeslee et al., 2007). Also, genetic analyses were conducted, utilizing mutant combinations, to confirm the ABCB-PIN interactions however, those efforts unraveled yet another layer of complexity (Geisler et al., 2017). Shoot and leaf defects observed in *abcb19 pin1* double mutant were stronger than in *pin1* alone. Notably, they were partially restored in the triple *pin1 abcb1 abcb19* mutant, presumably as a result of ectopic auxin accumulation in the shoot apical meristem (Blakeslee et al., 2007). The *pin2 abcb1 abcb19* triple mutant showed enhanced gravitropism defects in comparison with *pin2* single. Synergistic action could be observed in *pin1 abcb1 abcb19* triple mutants cotyledon patterning showing more severe defects than *pin1* single or *abcb1 abcb19* double combinations (Mravec et al., 2008).

The discussion of PIN-ABCB interactions invokes mentioning NPA (N-1-Naphthylphthalamic Acid) as one of the most often used polar auxin transport inhibitors. The link between the inhibitor, PINs and ABCBs was noticed during PIN1–ABCB1 and PIN1–ABCB19 co-expression studies, demonstrating a higher degree of NPA sensitivity for those carrier combinations (Blakeslee et al., 2007). Indeed, NPA treatment results in pin-like inflorescences (Gälweiler et al., 1998; Kim et al., 2010), but the direct connection between NPA and PINs has not been demonstrated so far. In this context, it is also worth mentioning, that transport activity of PINs has been shown in a heterologous systems such as yeast and *Xenopus* oocytes (Petrášek et al., 2006; Yang and Murphy, 2009; Zourelidou et al., 2014), that are devoid of ABCBs. This depicts PINs as independent transporters and begs for final clarification of the NPA-PIN-ABCB connection.

CONCLUDING REMARKS

Although PINs play very important roles in plant development, their topology has been only recently experimentally assessed and we still have to wait for NMR or crystallographic data resolving the structure of those carriers in detail. Crystallizing the PIN will be a major effort and a landmark achievement when successful. Yet, before the crystals will be grown and analyzed, we should not lay down our arms and exploit all other possibilities to further structurally and biochemically characterize those important auxin efflux carriers. Over the years substantial knowledge has accumulated on the motifs and amino acids being essential in proper PIN function. Mapping those, and assessing their significance, can be very helpful in the preparation of new PIN constructs, when one knows where to insert and where not to insert a particular tag, which flexible domain to stabilize and which one permits sequence changes. Even partial structural characterization of fragments of the protein will enrich our understanding of the PINs, and support the future expression, purification and structure determination strategies, in which we want to participate, to which we invite and encourage.

AUTHOR CONTRIBUTIONS

TN and MZ designed the manuscript outline. TN, MZ, and VB wrote the article. VB and YS prepared the figures. All authors revised the article.

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Identification of Lysine Histidine Transporter 2 as an 1-Aminocyclopropane Carboxylic Acid Transporter in *Arabidopsis thaliana* by Transgenic Complementation Approach

Jungki Choi[1], Sanung Eom[2], Kihye Shin[1], Rin-A Lee[1], Soobin Choi[1], Jun-Ho Lee[2], Sumin Lee[1] and Moon-Soo Soh[1]**

[1] Division of Integrative Bioscience and Biotechnology, College of Life Science, Sejong University, Seoul, South Korea,
[2] Departments of Biotechnology, Chonnam National University, Gwangju, South Korea

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***Correspondence:**
Sumin Lee
smlee55@sejong.ac.kr
Moon-Soo Soh
soh@sejong.ac.kr

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1-Aminocyclopropane-1-carboxylic acid (ACC), a biosynthetic precursor of ethylene, has long been proposed to act as a mobile messenger in higher plants. However, little is known about the transport system of ACC. Recently, our genetic characterization of an ACC-resistant mutant with normal ethylene sensitivity revealed that lysine histidine transporter 1 (LHT1) functions as a transporter of ACC. As amino acid transporters might have broad substrate specificity, we hypothesized that other amino acid transporters including *LHT1* paralogs might have the ACC-transporter activity. Here, we took a gain-of-function approach by transgenic complementation of *lht1* mutant with a selected set of amino acid transporters. When we introduced transgene into the *lht1* mutant, the transgenic expression of *LHT2*, but not of *LHT3* or *amino acid permease 5 (AAP5)*, restored the ACC resistance phenotype of the *lht1* mutant. The result provides genetic evidence that some, if not all, amino acid transporters in Arabidopsis can function as ACC transporters. In support, when expressed in *Xenopus laevis* oocytes, both *LHT1* and *LHT2* exhibited ACC-transporting activity, inducing inward current upon addition of ACC. Interestingly, the transgenic expression of *LHT2*, but not of *LHT3* or *AAP5*, could also suppress the early senescence phenotypes of the *lht1* mutant. Taking together, we propose that plants have evolved a multitude of ACC transporters based on amino acid transporters, which would contribute to the differential distribution of ACC under various spatiotemporal contexts.

Keywords: 1-aminocyclopropane carboxylic acid, amino acid transporter, triple response, LHT1, LHT2, transgenic complementation, *Arabidopsis thaliana*

INTRODUCTION

Transporters of plant hormones play a pivotal role in cell-cell communication, underpinning tissue-specific differentiation and environmental adaptation of higher plants, as exemplified by various defects of hormone-transporter mutants throughout the life cycle (Park et al., 2017; Do et al., 2018). For the past three decades, the molecular genetic analysis combined with transport assay has uncovered various components of plant transporter systems,

including principal transporters of each hormone, except brassinosteroid (Abualia et al., 2018). An emerging theme points to the existence of multiple routes for plant hormones transportation. For example, many transporters implicated in the uptake of metabolites/nutrients take part in hormonal transport (Tsay et al., 1993; Huang et al., 1999; Krouk et al., 2010; Kanno et al., 2012). Despite such progress, we are still far from a comprehensive understanding of the components of hormone transport systems and their regulatory mechanisms (Park et al., 2017).

Ethylene, a gaseous plant hormone, controls diverse aspects of the developmental processes including germination, fruit ripening, leaf and flower senescence, and abscission, as well as the environmental adaptation of higher plants (Lin et al., 2009; Bakshi et al., 2015; Dubois et al., 2018). The triple response of ethylene-treated etiolated seedlings, including apical hook exaggeration, inhibition of root growth, and shortened hypocotyl, represents one of the best characterized ethylene responses. It has been instrumentally exploited for genetic identification of key components of ethylene biosynthesis and signaling (Guzman and Ecker, 1990; Ju and Chang, 2015; Merchante and Stepanova, 2017). Since the first identification of ethylene-insensitive *etr1-1* mutant (Bleecker et al., 1988), molecular genetic analyses in *Arabidopsis* have well established how plant perceive and respond to ethylene *via* core signaling module, which comprised five components, including ethylene receptors (ETR1 and its paralogs)-constitutive triple response1-ethylene insensitive 2-EIN3 binding F-Box 1 (EBF1)/EBF2-ethylene insensitive 3 (EIN3)/EIN3-like (Ju and Chang, 2015; Merchante and Stepanova, 2017).

It has been well characterized how plants control the ethylene level. In higher plants, ethylene is biosynthesized from a nonprotein amino acid, 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC oxidase. ACC is synthesized by ACC synthase (ACS) using S-adenosyl methionine, supplied *via* Yang cycle reaction (Boller et al., 1979; Yang and Hoffman, 1984; John et al., 1985). Synthesis of ACC by ACS is the major rate-limiting step of ethylene biosynthesis (Yang and Hoffman, 1984), while under certain circumstances, e.g., fruit ripening, ACC oxidase also contributes to the increase in ethylene level (Nakatsuka et al., 1998; De Paepe et al., 2004; Rudus et al., 2013). As such, ACS activities are under extensive transcriptional and posttranscriptional regulations (Argueso et al., 2007). Besides its biosynthetic regulation, the amount of ACC is also controlled by conjugation and deamination (Van de Poel and Van Der Straeten, 2014; Vanderstraeten and Van Der Straeten, 2017).

In addition to ACC metabolism, short- or long-distance transport of ACC can also contribute to spatiotemporal distribution of ethylene. The long-distance transport of ACC has been suggested by its presence in the vascular system. (Bradford and Yang, 1980; Finlayson et al., 1991; Else et al., 1995; Morris and Larcombe, 1995). Besides long-distance transport, intracellular transport of ACC was also observed in the vacuole (Tophof et al., 1989) and maize mesophyll vacuole (Saftner and Martin, 1993). While the amino acid transporter system has long been implicated to take part in the transport system of ACC (Lurssen, 1981; Saftner and Baker, 1987), the molecular identity of an ACC transporter has been revealed only very recently (Shin et al., 2015). The loss-of-function mutant of *lysine histidine transporter 1* (*LHT1*) in *Arabidopsis* exhibited dose-dependent resistance to exogenous ACC, but not to gaseous ethylene. In the agreement, the mesophyll protoplast of the *lht1* mutants was impaired in the uptake of ^{14}C-ACC. The findings that the *lht1* mutant showed a normal ethylene response in the presence of higher concentrations of ACC and it retains, although partial, uptake activity of ^{14}C-ACC implicated the presence of additional transporters. Since *LHT1* belongs to a large gene family encoding amino acid transporters, which comprised more than 100 members in *Arabidopsis* (Pratelli and Pilot, 2014; Tegeder and Masclaux-Daubresse, 2018), it is conceivable that transporters other than LHT1 take part in the uptake of ACC. However, there has been no report available about genetic identification of ACC transporters except *LHT1*. Presumably, genetic redundancy, as well as distinct spatiotemporal expression patterns among amino acid transporters, might have hampered the genetic identification of mutants impaired in the uptake of ACC.

To identify additional amino acid transporters that take part in the uptake of ACC, here we undertook a gain-of-function approach making use of the *lht1* mutant, which is defective in the triple response in the presence of 1 μM ACC. After introducing the overexpressing construct of amino acid transporter gene into the *lht1* mutant, we examined whether the defective triple response of *lht1* mutant is restored or not. As a proof of concept, our pilot study with three amino acid transporter genes, namely *LHT2*, *LHT3*, and *AAP5*, revealed that only *LHT2*, but not *LHT3* and *AAP5*, could restore the ACC-induced triple response of the *lht1* mutant. Further, we found that the ectopic expression of *LHT2* could also suppress the early senescence phenotype of the *lht1* mutant. Supporting the hypothesis that LHT2 mediates uptake of ACC, LHT2 exhibited ACC-transporting activity when expressed in *Xenopus* oocytes, as LHT1 did. Together with findings on distinct spatiotemporal expression and substrate specificities of amino acid transporters, we discuss the hypothesis that a multitude of ACC transporting amino acid transporters might have diverse roles during development and environmental adaptation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All of the *Arabidopsis thaliana* plant materials used in this study had the "Col-0" ecotype background. The mutants of *lht1-101* (allelic designation was changed hereafter, substituting the original ones, *are2* or *lht1^{are2}*), *lht2-1* (SAIL_222_C12), and *lht1-101LHT1ox-1* transgenic plants have been previously described (Shin et al., 2015). The *lht1-101lht2-1* double mutant was obtained by genetic crossing. After crossing *lht1-101* with the *lht2-1* mutant, the resulting F2 population was subject to genotyping PCR to select double mutant plants using the genotyping primers as described (Shin et al., 2015). Unless

otherwise stated, seeds were surface sterilized, kept at 4°C for 3 days, and plated on the half strength of MS medium (Duchefa, Haarlem, the Netherlands) supplemented with 0.5% sucrose and 0.7% phytoagar. The seedlings were grown under continuous light. After 7 days, they were transferred to soil (Sunshine Professional Growing Mix #5) for further growth under long days (16 h light/8 h dark) at 22°C. Leaf senescence phenotyping, including leaf yellowing and trypan blue staining, was performed as described (Shin et al., 2015).

Transgenic Complementation Tests

To examine transgenic complementation of the *lht1-101* mutant, we amplified the full-length cDNA of *LHT2*, *LHT3*, or *AAP5 via* reverse transcriptase–polymerase chain reaction (RT-PCR), using primers listed in **Supplementary Table S1**. The sequence-verified amino acid transporter DNA was subcloned into pENTR1A (Invitrogen, Carlsbad, CA, USA) or pENT-D-TOPO (Invitrogen). The resulting entry clone was recombined into a gateway-compatible plant expression vector, pH2GW7 (Karimi et al., 2002), using LR clonase according to the manufacturer's instructions (Invitrogen). The pH2GW7/*LHT2*, *LHT3*, or *AAP5* clones were introduced into *Agrobacterium tumefaciens* GV3101 for transformation into the *lht1-101*mutant plants. Putative transgenic plants were selected based on their hygromycin resistance. For phenotypic analysis, homozygous lines harboring a single T-DNA insertion were selected in the T_2 and T_3 generations.

Assays for Seedling Response to ACC or D-Amino Acids

To test the response of seedlings to ACC or D-amino acid, we sowed stratified seeds on plates of MS media containing various concentrations of ACC (Sigma-Aldrich, St. Louis, MO, USA) or of D-amino acids (Sigma-Aldrich). The seedlings were then further grown under continuous light or dark after irradiation for 12 h. For amino acid competition assay with ACC, seedlings were grown on MS media containing 1 μM of ACC with mock or 100 μM of amino acid for 3.5 days in the dark after irradiation of white light for 12 h. The length of hypocotyls and roots was measured using ImageJ software (http://rsbweb.nih.gov/ij). Seedling images were scanned with an Epson scanner (Epson V700 Photo, Epson, Suwa, Nagoya, Japan).

Expression Analysis

Quantitative real-time PCR (qRT-PCR) analysis was performed with total RNA. After extraction, DNase I–treated total RNA was reverse transcribed by using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Waltham, MA, USA). The 10-fold diluted cDNA was subject to qRT-PCR analysis using specific primers listed in **Supplementary Table S1**. The qRT-PCR analysis was performed with an Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA) using an EvaGreen® qPCR Supermix (Solis Biodyne, Tartu, Estonia). The reactions were performed in technical triplicate for each gene. The comparative ΔCt method was used to evaluate the relative quantities of each amplified product in the samples, as described (Livak and Schmittgen, 2001). Relative expression levels were normalized according to Ct values for *PP2A* (At1g13320), a reference gene (Czechowski et al., 2005).

In Vitro Transcription for cRNA Preparation and Microinjection

The cDNAs encoding *LHT1* or *LHT2* were subcloned into the pGEM-HE vector to be expressed in oocytes using Nde1 restriction enzyme site. After sequence verification, the cRNAs were transcribed from the linearized cDNAs using transcription kit (mMessage mMachine; Ambion, TX, USA) with T7 RNA polymerases. The RNA was dissolved in nucleotide-free water and diluted at a final concentration of 1 μg/μL and then aliquoted and stored at −80°C until use. The nanoinjection of cRNAs (40 ng) into the vegetal or animal pole of each oocyte was carried out using a microinjector (VWR Scientific, Mississauga, Ontario, Canada). The nanoinjector pipettes were pulled out by the glass capillary tubes that were used for the electrodes and polished to a ~20-μm outer diameter.

Xenopus Oocyte Electrophysiology

The handling of *Xenopus laevis* oocytes and preparation of single cells were described in the previous study (Bai et al., 2019). Briefly, frogs caring procedures followed the Chonnam National University animal caring institution guidelines (CNU IACUC-YB-2016-07, July 2016). The removed oocytes from *X. laevis* were collagenized with shaking for 2 h in Ringer solution (96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, and 20 mM HEPES at pH 7.5). The matured oocytes were selected and incubated in ND96 containing: 96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 1.8 mM $CaCl_2$, and 20 mM HEPES at pH 5.6 with 1% penicillin and streptomycin (Sigma). Two electrode voltage clamp experiments were carried out after 48 h for each of the RNA-injected oocytes (Naik, 2019). The oocyte was put in a perfusion chamber (Warner Instrument, Holliston, MA, USA) and flowed with ND96 medium at 1 mL/min. Each oocyte was penetrated with microelectrodes filled up with electrolyte solution. The microelectrodes resistance was from 0.5 to 0.8 MΩ. The electrophysiological experiment was performed at room temperature with oocyte clamp amplifier (OC-726C; Warner Instruments) and acquisition of data was performed using Digidata 1320 and pClamp 9 (Molecular Devices, San Jose, CA, USA).

RESULTS

Previously we showed that LHT1 takes part in the uptake of ACC in *Arabidopsis* (Shin et al., 2015). However, the null alleles of *LHT1* retained, despite reduced, ACC uptake activity, suggesting that LHT1 may not be the sole ACC transporter in *Arabidopsis*. In line with those findings, when applied at high concentration, various amino acids reduced the ACC-induced triple response (**Figure 1**). We examined the triple response of etiolated seedlings that were grown in the presence of 1 μM ACC plus mock solution or 100 μM of each amino acid. The result showed that 8 of 20 amino acids, including alanine, phenylalanine, glycine, isoleucine, leucine, methionine, glutamine, and proline, alleviated the ACC-induced triple response in dark-grown wild-type seedlings.

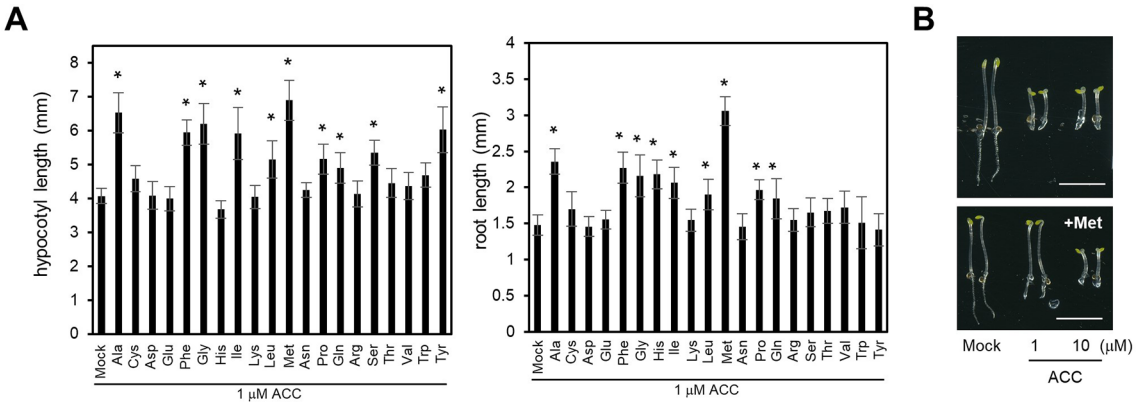

FIGURE 1 | Effect of supplemented amino acids on ACC-induced inhibition of hypocotyl and root growth. **(A)** Hypocotyl (left) and root length (right). The wild-type seedlings were grown under dark for 4 days on MS-sucrose media containing 1 μM of ACC supplemented with each of 20 types of 100 μM amino acid. Values are mean ± SD (n = 15). The significant difference of the length between seedlings grown on the media containing ACC only and ACC with amino acid is indicated with asterisks (*t* test, $^*p < 0.01$). **(B)** Representative wild-type seedlings grown on MS-sucrose media containing either 1 or 10 μM of ACC supplemented without (upper) or with 100 μM of methionine (Met) (lower). Scale bar, 5 mm.

As shown in **Figure 1A**, the hypocotyl length or root length of seedlings that were grown in the presence of ACC plus above stated amino acids was longer than that of seedling grown in the presence of ACC alone. Some amino acids affected only either hypocotyl growth or root growth. For example, the basic amino acid, histidine, reduced the ACC-induced root growth inhibition, but not hypocotyl growth. In the case of serine and tyrosine, these affected only hypocotyl growth (**Figure 1A**). On the contrary, higher concentration of ACC (10 μM) could overcome the inhibitory effect of the amino acid, e.g., methionine, inducing the characteristic triple response: inhibition of hypocotyl elongation, shortened root growth, and exaggerated apical hook formation (**Figure 1B**). These results implied that some, if not all, amino acids may compete with ACC for uptake. Further, the diverse profile of amino acids with inhibitory activity for ACC-induced triple response suggested the presence of a multitude of amino acid transporters with ACC-transporting activity in *Arabidopsis*.

Although *LHT1* was identified as ACC transporter using the loss-of-function approach, all knockout mutants of *LHT* family gene except *lht1* did not show ACC resistance in seedlings (Shin et al., 2015). Because the phenotype of knockout mutants is highly dependent on the developmental stage due to the specific spatiotemporal expression of the genes (**Supplementary Figure 1**) and can be masked by other gene activity with overlapping function, the possibility that other LHTs are involved in ACC transport cannot be ruled out. To overcome the drawbacks of the loss-of-function approach, we adopted a transgenic gain-of-function approach to identify ACC-transporting amino acid transporters. After introducing transgene that overexpresses a gene encoding amino acid transporter into the *lht1-101* mutant, a null allele, we tested if it restored the defect in the ACC-induced triple response of the *lht1* mutant. As a proof of concept, we selected three amino acid transporters (LHT2, LHT3, and AAP5) in two amino acid transporter family, e.g., LHT and AAP family. When the transgene was introduced into the *lht1* mutant, the resulting 17 of 19 T2 lines with *35S*::LHT2 showed normal triple response to ACC as wild type did. In contrast, none of more than 20 T2 lines with *35S*::LHT3 or *35S:*AAP5 exhibited defective triple response to ACC as the *lht1-101* mutant did. After confirmation of the elevated transcript level of each amino acid transporter (**Supplementary Figure 2**), we examined the ACC-induced triple response in detail with two independent homozygous lines that overexpressed each amino acid transporter (**Figure 2**). In the presence of 1 μM of ACC, wild type showed typical triple response including apical hook exaggeration, inhibition of hypocotyl growth, and shortened root growth, whereas *lht1-101* mutant did not (**Figure 2A**). As shown in **Figures 2B**, **C** overexpression of *LHT2*, but not *LHT3* or *AAP5*, could restore the ACC-induced triple response of the *lht1-101* mutant. Next, we analyzed the concentration-dependent ACC response in the *lht1-101* mutant overexpressing *LHT1* or *LHT2*. *LHT1* overexpression in the *lht1-101* mutant restored the ACC response of root growth inhibition completely as wild type did at all concentrations of ACC tested, whereas in transgenic *lht1-101* seedlings overexpressing *LHT2*, the ACC induced root growth inhibition was observed at high concentration of ACC (**Figure 3**). Despite the lack of any visible effect of the *lht2-1* mutation on the ACC-induced triple response (**Supplementary Figure 3**), our results of transgenic complementation demonstrated the role of LHT2 as a potential ACC transporter, functioning likewise LHT1.

LHT1 mediates uptake of broad substrates, including several D-amino acids, illustrated by the strong resistant phenotype of the *lht1* mutant in the presence of a high concentration of D-amino acids (Svennerstam et al., 2007; Gordes et al., 2011). With the assumption that other amino acid transporters take part in the uptake of the D-amino acids, substrates of LHT1, we investigated whether D-amino acid resistance of the *lht1* mutant could be restored by transgenic overexpression of *LHT2*, *LHT3*,

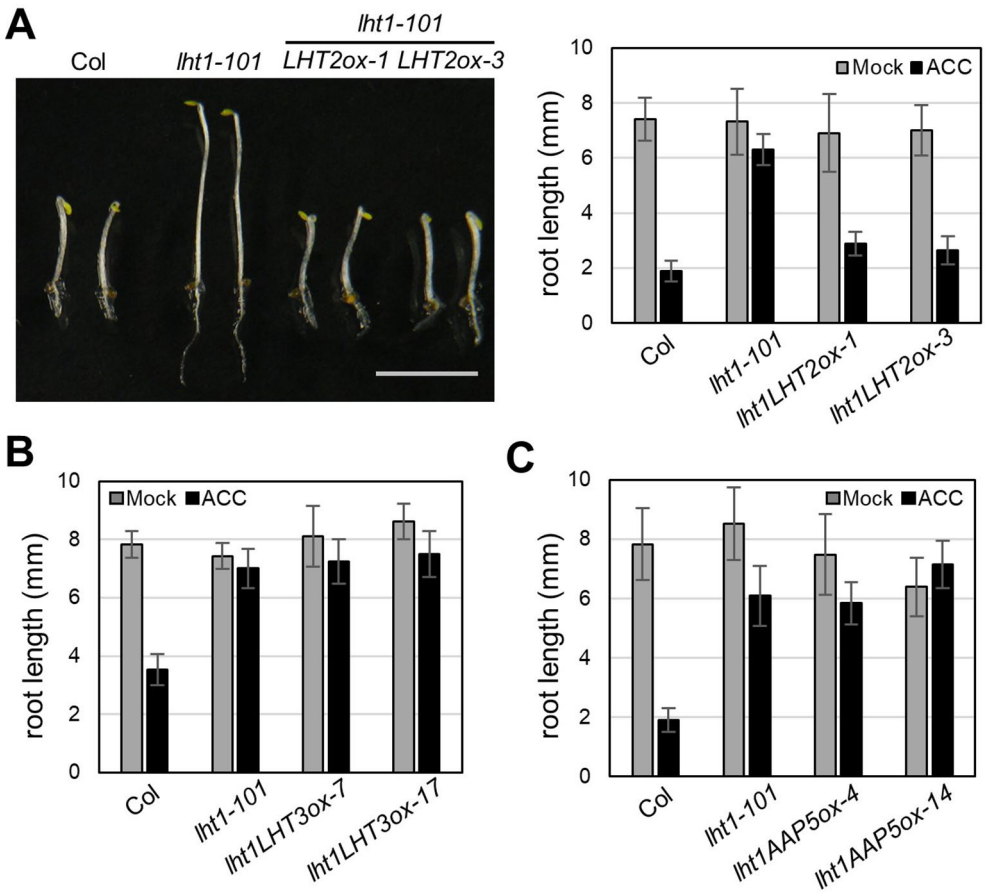

FIGURE 2 | Transgenic complementation of *lht1-101* by overexpression of *LHT2*, *LHT3*, or *AAP5*. **(A)** Restored ACC response of *lht1-101* mutant by overexpression of *LHT2*. Left, Morphology of representative 4-day-old seedlings grown on MS-sucrose under dark media in the presence of 1 μM ACC. Note that the triple response of *lht1-101* mutant was restored in the two independent *LHT2*-overexpressing transgenic lines (*LHT2ox1* and *LHT2ox3*). Scale bar, 5 mm. Right, Root length of the transgenic *lht1-101* seedlings. The seedlings were grown on MS-sucrose media supplemented with either Mock or 1 μM of ACC for 4 days under dark. Values are mean ± SD (n = 15). **(B)** Root length of *LHT3* overexpressing transgenic *lht1-101* seedlings. The seedlings were grown as described in **(A)**. Values are mean ± SD (n = 15). **(C)** Root length of *AAP5* overexpressing transgenic *lht1-101* seedlings. Values are mean ± SD (n = 15). The seedlings were grown as described in **(A)**.

or *AAP5*. The results showed that overexpression of *LHT2*, or *LHT3*, but not of *AAP5*, restored the sensitivity to D-amino acids, including D-Ala, D-Phe, and D-Met in *lht1-101* (**Figure 4**). Together with the finding that *LHT3* could not restore ACC resistance of the *lht1* mutant (**Figure 2**), these results implied that LHT3 might have similar but distinct substrate specificity with LHT1 and LHT2.

Early senescence/cell death syndrome is a characteristic developmental phenotype of the *lht1* mutants (Liu et al., 2010; Shin et al., 2015). We investigated whether the early senescence phenotype of *lht1-101* could be altered in the transgenic plants overexpressing the amino acid transporters. Interestingly, overexpression of *LHT2* restored the accelerated leaf yellowing phenotype, an early leaf senescence syndrome of *lht1* mutants, while overexpression of *LHT3* or *AAP5* did not (**Figure 5A**). When we performed a closer examination of *LHT2* overexpressing *lht1-101* plants, we found that *LHT2* overexpression suppressed the aberrant cell-death phenotype of the *lht1-101* mutant, manifested by trypan blue staining (**Figures 5B**, **C**). As the early senescence phenotype of the *lht1* mutant is accompanied by the massive changes of gene expression (Liu et al., 2010), further we tested whether overexpression of *LHT2* can restore the elevated expression of a set of senescence-associated genes, including *pathogenesis related 1* (*PR1*), *senescence related gene 1* (*SRG1*), *senescence associated gene 12* (*SAG12*), *phytoalexin deficient 4* (*PAD4*), *isochorismate synthase 1* (*ICS1*), *NDR1/HIN1-like 25* (*NHL25*) in the *lht1-101* mutant (**Figure 5D**). The expression of senescence-associated genes in the *lht1-101* was shown to be suppressed by transgenic *LHT2* overexpression. Collectively, these results implied that the ectopically expressed *LHT2* can substitute the role of *LHT1* for the control of leaf senescence.

Given the genetic evidence that LHT1 and LHT2 play similar roles as an ACC/amino acid transporter, we assessed the biochemical activity of LHT1 and LHT2. Taking an electrophysiological approach based on *Xenopus* oocyte system,

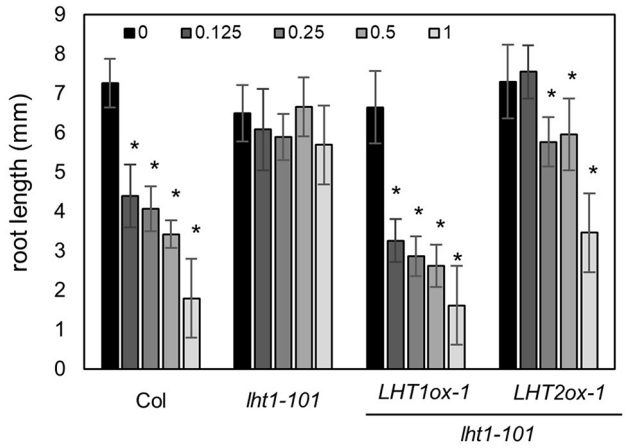

FIGURE 3 | ACC dose-response analysis of transgenic *lht1-101* seedlings overexpressing *LHT2*. Wild-type, *lht1-101*, the transgenic *lht1-101* seeds overexpressing *LHT1* or *LHT2* were grown on MS-sucrose media containing indicated concentrations (μM) of ACC. The seedlings were grown for 4 days in the dark. Values are mean ± SD (n = 15). A significant difference between ACC treated and mock-treated (0 ACC) seedlings was indicated with asterisks (*t* test, $p < 0.01$).

we conducted a two-electrode voltage-clamp electrophysiological recording. After injection into *Xenopus* oocytes with mRNA of *LHT1* or *LHT2*, we measured inward currents elicited after the addition of the substrate, ACC or amino acids. The applied ACC or amino acids (100 mM) did not induce any current in the control oocytes (**Supplementary Figure 4**). In contrast, the treatment of a series of amino acids induced inward currents with differential activity in both *LHT1*- and *LHT2*-expressing oocytes (**Figure 6**). Notably, LHT1 and LHT2 exhibited largely similar, if not identical, substrate selectivity. The results implied that LHT1 and LHT2 transport overlapping set of amino acid substrates, but with differential preference. Under the same condition, exogenously applied ACC elicited the inward currents on both *LHT1*- and *LHT2*-expressing oocytes, confirming their roles as ACC transporters (**Figures 6** and **7**). Further, we evaluated the ACC dose dependency in the *LHT1*- or *LHT2*-expressing oocytes. With the dose-response analysis, we calculated $K_{0.5}$ for ACC with nonlinear regression using Michaelis-Menten's equation. The $K_{0.5}$ values of ACC are 60.9 ± 8.2 mM and 59.1 ± 6.6 mM for *LHT1*- and *LHT2*-expressing oocytes, respectively, suggesting that LHT1 and LHT2 can function as ACC transporter with comparable activity in *Xenopus* oocytes.

DISCUSSION

As a precursor of ethylene, ACC has long been recognized as a mobile molecule in higher plants (Bradford and Yang, 1980; Amrhein et al., 1982; Métraux and Kende, 1983; Else et al., 1995; English et al., 1995; Kende et al., 1998; Vriezen et al., 2003). In accordance, intercellular ACC transport has been suggested to contribute to the ethylene-mediated stress adaptation and development (Woltering, 1990; Jones and Woodson, 1997; Jones and Woodson, 1999; Dugardeyn et al., 2008; Vanderstraeten and Van Der Straeten, 2017). However, the molecular nature of ACC-transporting system has remained elusive until recent genetic identification of *lht1-101*, as an ACC-resistant mutant (Shin et al., 2015). In line with previous biochemical studies (Lurssen, 1981; Saftner and Baker, 1987), we found that a series of amino acids could inhibit the ACC-induced triple response, suggestive of the presence of a multitude of ACC transporters (**Figure 1**). However, it should be taken into account that the inhibitory effects of amino acids may not necessarily involve their competitive effect for ACC uptake, considering that certain amino acids may alter hormonal biosynthesis or cellular signaling components (Qi et al., 2006; Luna et al., 2014; Kong et al., 2016; Smith et al., 2017). Other ACC transporters than LHT1 have also been inferred by the physiological features of the *lht1* mutant, including its partial ACC resistance as well as a partial defect in ACC uptake activity. Still, it is challenging to identify the ACC-transporting amino acid transporters among more than 100 putative amino acid transporters in *Arabidopsis* (Tegeder and Masclaux-Daubresse, 2018).

In this study, we presented that a gain-of-function approach, transgenic complementation of the *lht1* mutant, is a feasible way to identify ACC transporters *in planta*. As a proof of concept, our pilot study revealed that LHT2 can function as an ACC transporter. When overexpressed in the *lht1* mutant background, *LHT2* could restore all of the physiological defects of the *lht1* mutant, including defective ACC-induced triple response, D-amino acid resistance, and early-senescence syndromes (**Figures 2**–**5**). Although we cannot rule out the possibility that the overexpression of *LHT2* may trigger the expression other ACC-transporting amino acid transporters to complement the *lht1* mutant phenotype, we found that the oocytes expressing *LHT1* or *LHT2* were responsive to exogenous ACC, producing inward currents with comparable efficacy (**Figure 7**). The results demonstrate that LHT1/LHT2 act as an ACC transporter in *Xenopus* oocytes at millimolar concentrations of ACC. It is noteworthy that the $K_{0.5}$ values of ACC for LHT1 or LHT2 were higher than expected. Considering that 1 μM of ACC is high enough to elicit the full range of the triple response (**Figure 2**), it seems that our experimental conditions may not be optimal for the transport assay of LHT1 or LHT2 proteins. Alternatively, but not mutually exclusively, LHT1 or LHT2 proteins may require additional plant protein(s) or posttranslational modification for their full activity. Thus, it remains to be proven that LHT2 functions as an ACC transporter at physiologically relevant concentrations *in planta*. While both LHT1 and LHT2 could rescue the ACC-resistant phenotype of *lht1* mutant, we observed slight difference in the ACC dose-response phenotypes among the wild-type and transgenic lines of LHT1 and LHT2 (**Figure 3**). Compared to *LHT1*-overexpressing line, the plants with overexpression of *LHT2* were less sensitive to exogenously applied ACC. It may be due to relatively lower transporter activity of LHT2 compared to LHT1 *in planta*. However, as we did not assess the protein level of LHT1 and LHT2, it cannot be ruled out that protein expression/stability underlies the differential dose-response phenotypes.

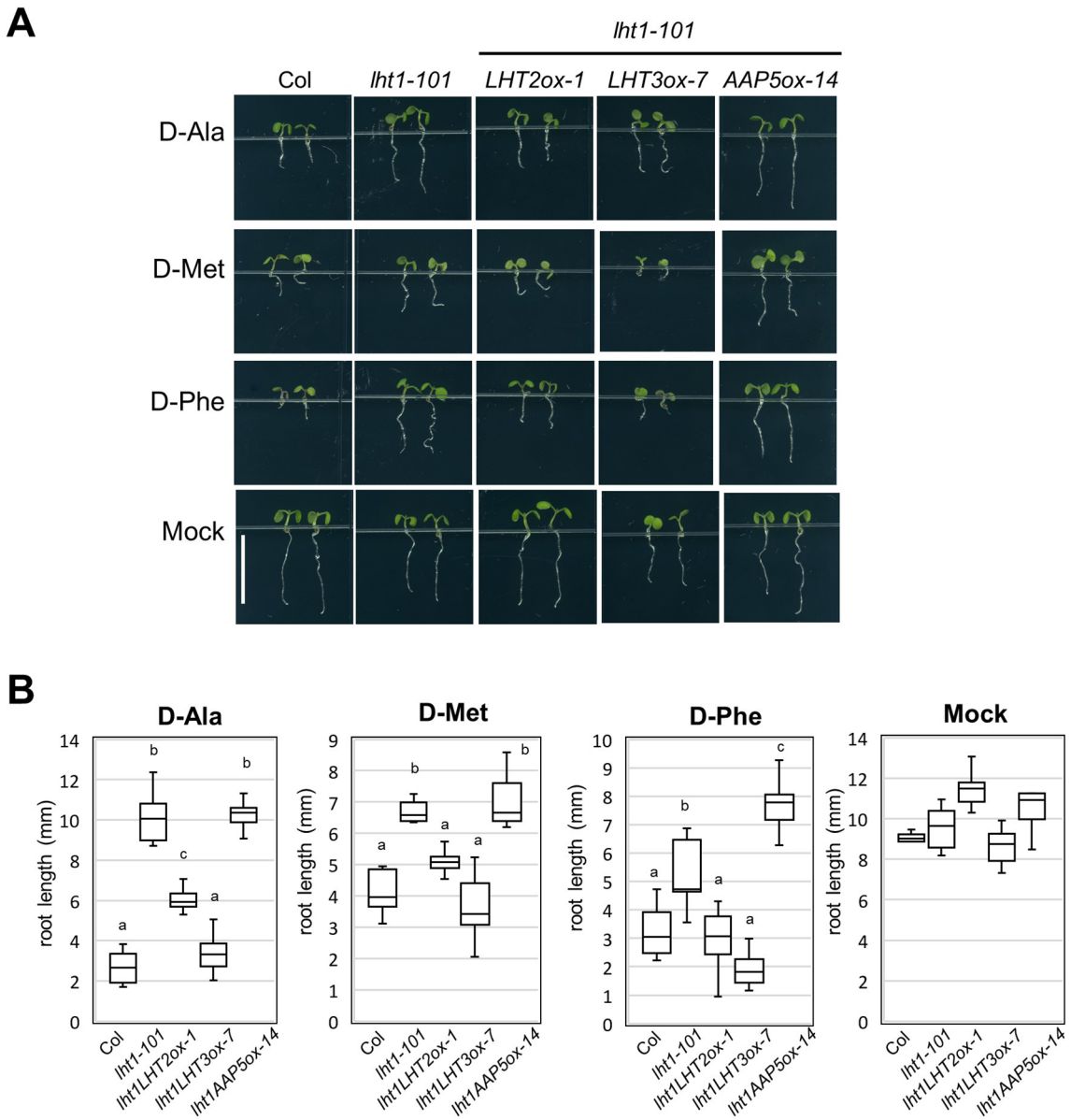

FIGURE 4 | D-Amino acid response of transgenic *lht1-101* seedlings overexpressing amino acid transporters. **(A)** Morphology of representative seedlings that have grown on MS-sucrose media containing mock, 1 mM of D-alanine (D-Ala), 3 mM of D-methionine (D-Met), or 3 mM of D-phenylalanine (D-Phe). Wild-type, *lht1-101*, and the transgenic *lht1-101* plants overexpressing the amino acid transporter, e.g., *LHT2*, *LHT3*, or *AAP5*, were grown for 5 days under continuous light. Scale bar, 5 mm. **(B)** The root length of the seedlings grown as described in **(A)**. The data are presented with box-and-whisker plot (n = 6-11). Different letters indicate significant differences at $p < 0.01$ according to one-way analysis of variance with Tukey honestly significant difference test. Evidence for normality of distribution and homoscedasticity is presented in **Supplementary Table S2.**

On the contrary, *LHT3* and *AAP5* were not effective in the complementation activity of the *lht1* mutant (**Figures 2**–**5**), suggesting that these amino acid transporters have marginal, if any, ACC-transporting activity. In agreement, compared to LHT1 or LHT2 (**Figure 6**), AAP5 has been characterized to have differential substrate selectivity, mediating uptake of basic amino acids (Boorer and Fischer, 1997; Fischer et al., 2002), while the substrate of LHT3 has not been reported yet. It is intriguing that transgenic expression of *LHT3* could restore the resistance of the *lht1* mutant to D-Ala, D-Met, and D-Phe, implicating that LHT3 might have overlapping activity with LHT1 toward a subset of LHT1 substrates, except ACC. Notably, the ACC-transporting LHT1 and LHT2 exhibited similar, but not identical, amino acid selectivity in the oocyte system (**Figure 6**). These results imply that amino acid transporter have delicate transporting channels, selecting specific substrates. It is noteworthy that methionine was not the preferred substrate for LHT1 or LHT2 in the oocyte system

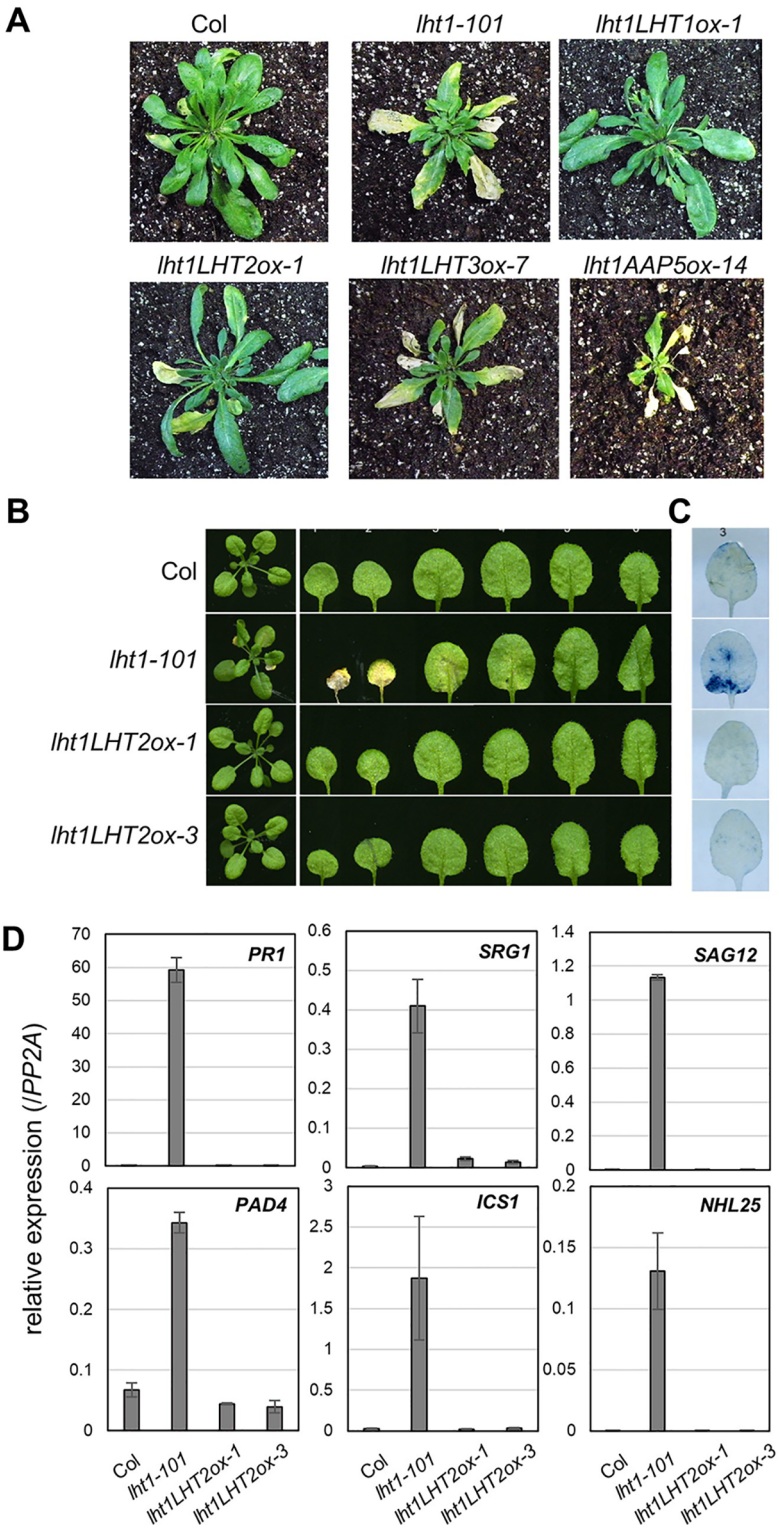

FIGURE 5 | The early senescence syndrome of the transgenic *lht1-101* mutant overexpressing *LHT2*. **(A)** Representative morphology of wild-type, *lht1-101*, and the transgenic plants overexpressing *LHT2*, *LHT3*, or *AAP5* at 38 DAG (days after growth). For clarity, the inflorescence of each plant was removed. **(B)** Representative leaf morphology of the transgenic *lht1-101* plants overexpressing *LHT2* at 27 DAG. **(C)** Trypan blue staining. Cell death in the third leaf of each plant pictured in **(B)** was assayed with trypan blue. **(D)** Expression analysis of senescence-associated genes. The fourth leaves of the plants at 27 DAG were sampled for extraction of total RNA, which was subjected to qRT-PCR analysis. For each gene indicated, the relative expression was presented after normalization with the level of *PP2A*. Values are mean ± SD (n = 3, technical triplicate).

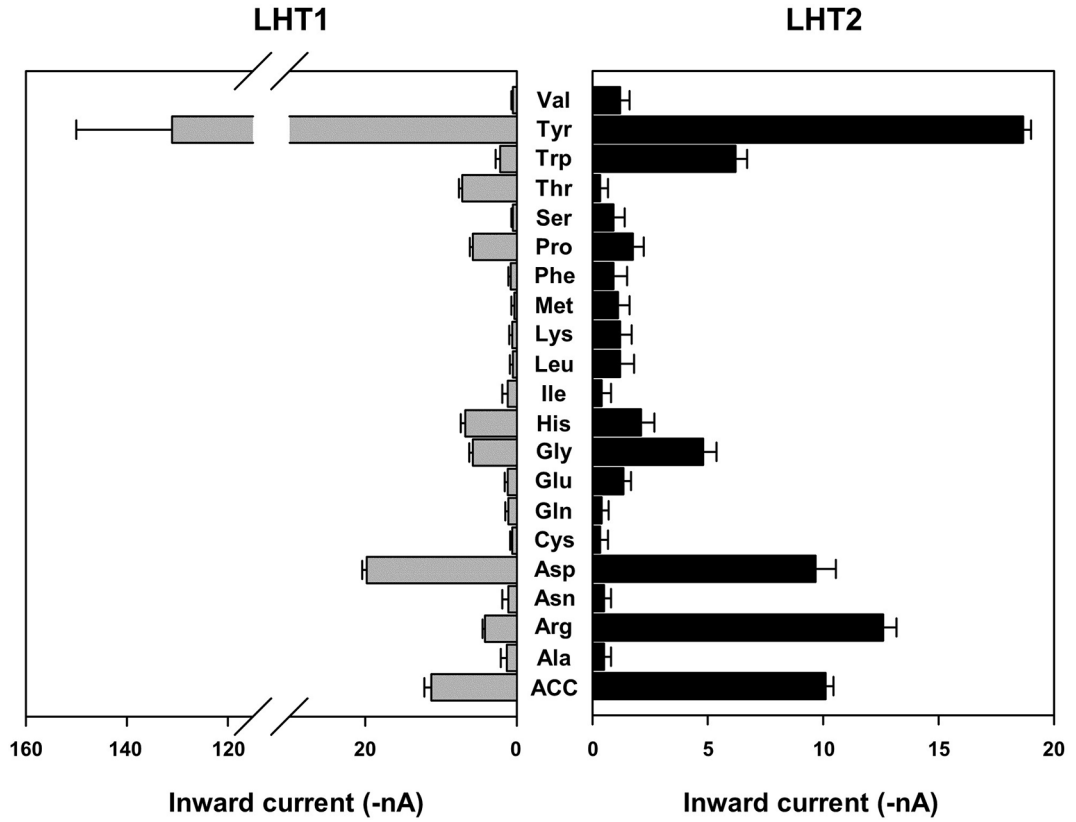

FIGURE 6 | Amino acid selectivity of LHT1 and LHT2 in *Xenopus* oocytes. After perfusion with Ringer solution at pH 5.6, oocytes expressing *LHT1* or *LHT2* were treated with each amino acid (100 mM) or ACC (100 mM). For tyrosine, the currents were measured at 2.5 mM (limit of solubility). The resulting inward currents were recorded at −80 mV. Data represent the means ± SEM (n = 6–8 oocytes/four different frogs).

(**Figure 6**), although it is one of the most effective amino acids in the ACC-competition assay (**Figure 1**; Lurssen, 1981). Taken together, it is plausible that other class of amino acid transporters with differential substrate selectivity may also take part in the uptake of ACC, along with LHT1/LHT2.

LHT2 was previously reported to be expressed preferentially in floral organs, mainly in tapetum tissues and pollens (**Supplementary Figure 1**) (Lee and Tegeder, 2004). The tissue-preferential expression of *LHT2* may account for the lack of defect of *lht2-1* mutant seedlings in regard to the ACC-induced triple response (**Supplementary Figure 3**). For the present, it is not clear whether the ACC-transporting activity of LHT2 has any physiological relevance during reproduction. It is noteworthy that high-order, octuple, *ACS* mutants of *Arabidopsis* exhibited pleiotropic defects unlike mutants with impaired ethylene signaling, including reduced fertility (Tsuchisaka et al., 2009). As we did not observe any reproductive defects in the *lht2-1* mutant and several *LHT* genes are also expressed in pollens (**Supplementary Figure 1**), it is possible that there might be other pollen-expressed, ACC-transporting amino acid transporters, functioning redundantly with LHT2. It will be interesting to examine whether the pollen-expressed *LHT*s have similar substrate selectivity and whether multiple loss-of-function *lht* mutants bear any defects in reproductive processes.

It is intriguing that *LHT2*, but not *LHT3* or *AAP5*, could complement the early senescence syndrome of the *lht1* mutant, which entails transcriptional alteration of several defense/cell death–related genes (**Figure 5**). Although it has not been resolved how *lht1* mutation impacts on leaf senescence and cell death syndrome (Liu et al., 2010; Shin et al., 2015), it is tempting to speculate that ACC or possibly other shared substrates of LHT1 and LHT2 play signaling functions in these processes. Together with accumulating evidence that proteogenic and nonproteogenic amino acids have diverse regulatory roles (Qi et al., 2006; Luna et al., 2014; Ramesh et al., 2015; Kong et al., 2016; Dinkeloo et al., 2018; Shi et al., 2018), future studies about how those amino acid transporters modulate leaf senescence would shed light on novel functions of ACC or amino acids during leaf senescence/cell death syndrome.

Together with electrophysiological analysis, the transgenic complementation analysis of *lht1* mutant would facilitate further characterization of amino acid transporter genes, expanding the repertoire of ACC-transporting amino acid transporters. As ACC has been recently appreciated as a signaling molecule (Xu et al., 2008; Tsuchisaka et al., 2009; Tsang et al., 2011; Yoon and Kieber, 2013), the identification of ACC-transporting amino acid transporters along with their spatiotemporal expression specificity would help to elucidate the role of ACC and amino

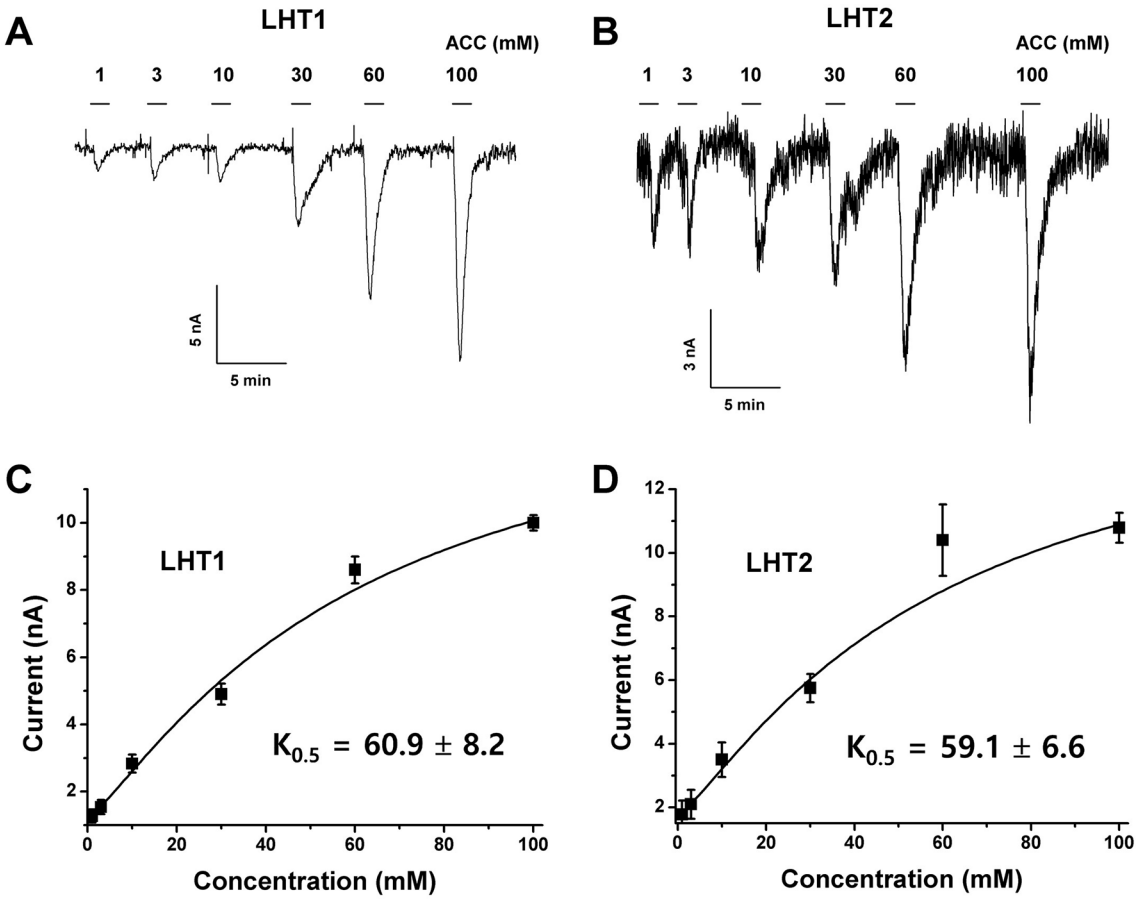

FIGURE 7 | Concentration dependency and kinetic analysis of ACC-induced inward currents in *LHT*-expressing *Xenopus* oocytes. **(A** and **B)** The representative traces of *LHT1*- or *LHT2*-expressing oocytes after addition of various concentration of ACC. Exogenously applied ACC produced a reversible inward current. The holding potential was –80 mV. ACC induced the inward currents with the concentration-dependent manner in each *LHT*-expressing oocyte, respectively. **(C** and **D)** Kinetic analysis of ACC-induced inward currents. $K_{0.5}$ value was calculated with Michaelis-Menten's equation. Data represent the means ± SE.M (n = 6–8 oocytes/four different frogs).

acid transporters during development and environmental adaptation in higher plants.

DATA AVAILABILITY

All datasets [Expression Analysis] for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

M-SS and SL designed the research. JC, SE, KS, RL, SC, and SL performed the research. KS, JL, SL, and M-SS analyzed the data. SL, JL and M-SS wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01092/full#supplementary-material

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An Optimized Screen Reduces the Number of GA Transporters and Provides Insights Into Nitrate Transporter 1/Peptide Transporter Family Substrate Determinants

*Nikolai Wulff[1], Heidi Asschenfeldt Ernst[2], Morten Egevang Jørgensen[1,3,4], Sophie Lambertz[1], Tobias Maierhofer[3], Zeinu Mussa Belew[1], Christoph Crocoll[1], Mohammed Saddik Motawia[5], Dietmar Geiger[3], Flemming Steen Jørgensen[2], Osman Mirza[2] and Hussam Hassan Nour-Eldin[1]**

[1] DynaMo Center, Copenhagen Plant Science Centre, Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark, [2] Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark, [3] Julius-von-Sachs-Institute, Molecular Plant Physiology and Biophysics, University Würzburg, Würzburg, Germany, [4] Carlsberg Research Laboratory, Copenhagen, Denmark, [5] Center for Plant Plasticity, Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark

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Markus Geisler,
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Shenyang Agricultural University,
China

***Correspondence:**
Hussam Hassan Nour-Eldin
Huha@plen.ku.dk

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Based on recent *in vitro* data, a relatively large number of the plant nitrate transporter 1/peptide transporter family (NPF) proteins have been suggested to function as gibberellic acid (GA) transporters. Most GA transporting NPF proteins also appear to transport other structurally unrelated phytohormones or metabolites. Several of the GAs used in previous *in vitro* assays are membrane permeable weak organic acids whose movement across membranes are influenced by the pH-sensitive ion-trap mechanism. Moreover, a large proportion of *in vitro* GA transport activities have been demonstrated indirectly *via* long-term yeast-based GA-dependent growth assays that are limited to detecting transport of bioactive GAs. Thus, there is a need for an optimized transport assay for identifying and characterizing GA transport. Here, we develop an improved transport assay in *Xenopus laevis* oocytes, wherein we directly measure movement of six different GAs across oocyte membranes over short time. We show that membrane permeability of GAs in oocytes can be predicted based on number of oxygen atoms and that several GAs do not diffuse over membranes regardless of changes in pH values. In addition, we show that small changes in internal cellular pH can result in strongly altered distribution of membrane permeable phytohormones. This prompts caution when interpreting heterologous transport activities. We use our transport assay to screen all *Arabidopsis thaliana* NPF proteins for transport activity towards six GAs (two membrane permeable and four non-permeable). The results presented here, significantly reduce the number of *bona fide* NPF GA transporters in *Arabidopsis* and narrow the activity to fewer subclades within the family. Furthermore, to gain first insight into the molecular determinants of substrate specificities toward organic molecules transported in the NPF, we charted all surface exposed amino acid residues in the substrate-binding cavity and correlated them to GA transport. This analysis

suggests distinct residues within the substrate-binding cavity that are shared between GA transporting NPF proteins; the potential roles of these residues in determining substrate specificity are discussed.

Keywords: gibberellic acid, transport, nitrate transporter 1/peptide transporter family, membrane permeable, phytohormones, *in vitro*, pH

INTRODUCTION

Gibberellic acids (GAs) were detected in phloem sap more than 50 years ago suggesting that GAs are mobile phytohormones (Hoad and Bowen, 1968). This has since been supported by multiple studies indicating that GAs can move long distances *in planta* and that transport processes generate local concentration maxima that may be essential for the regulatory roles of GA (Regnault et al., 2015; Regnault et al., 2016; Binenbaum et al., 2018). However, due to lack of molecular knowledge on GA transport, the physiological role of GA mobility remains unclear.

Within recent years, novel *in vitro* and *in vivo* approaches led to the identification of a large number of putative GA transporters (>25 different genes in *Arabidopsis* summarized in (**Supplementary Table 1**). The majority of these genes belong to the nitrate transporter 1/peptide transporter family (NPF) (Kanno et al., 2012; Chiba et al., 2015; Saito et al., 2015; Tal et al., 2016). The physiological roles of two NPF-GA transporters have been investigated *in planta*, namely, NPF3.1 and NPF2.10 (Saito et al., 2015; David et al., 2016; Tal et al., 2016). The GA related phenotypes in plants mutated in these genes are limited compared with those observed in GA-deficient or GA signaling mutants (Sun, 2008; Plackett et al., 2011). The relatively large number of potential NPF-GA transporters implies that there may be widespread functional redundancy among these transporters. Accordingly, experiments in which expression of multiple GA transporting NPF members are inhibited or knocked-out simultaneously may be needed to unveil their distinct roles (Binenbaum et al., 2018).

The NPF proteins are plant specific subfamily members of the proton-coupled oligopeptide transporter (POT) family, which exists in all kingdoms of life and whose members are important for transport of di- and tripeptides across membranes in symport with at least one proton. In humans, there are four POT family members, two of which are prime targets for drug delivery owing to their central importance for delivery of peptidomimetic drugs to intestinal epithelial cells (Daniel and Kottra, 2004). Crystal structures of bacterial POT family members have identified key residues in the POT substrate-binding cavity which interact with the peptidomimetic substrates and are located in a large cavity able to accommodate nearly limitless variations in amino acid side chains and conjugated species (Biegel et al., 2006; Doki et al., 2013; Guettou et al., 2014; Lyons et al., 2014; Newstead, 2015; Newstead, 2017). Moreover, a conserved ExxE[K/R] motif plays an essential role in intra-transporter salt bridge formations that enable coupling between proton and substrate transport to ensure active transport (Solcan et al., 2012; Aduri et al., 2015).

In *Arabidopsis*, 53 NPF members exist, which are divided into eight distinct subclades (Léran et al., 2014). Low affinity nitrate transport activities have been demonstrated in members from subclades 1, 2, 3, 4, 5, 6, and 7; peptide transport in subclades 5 and 8; glucosinolate transport in subclade 2; and transport of the phytohormones abscisic acid (ABA), GA, and jasmonoyl-isoleucine (JA-Ile) transport activities have been demonstrated for members from subclades 1, 2, 3, 4, 5, and 8, as recently reviewed (Corratge-Faillie and Lacombe, 2017; Wang et al., 2018). Hence, for some NPF substrates, transport activity appears relatively confined to distinct subclades with high degree of amino acid identity (e.g. the glucosinolate transporters GTR1–3 (NPF2.9-NPF2.11) with >60% identity and the peptide transporters (NPF8.1–8.3) with >55% identity—excluding PTR3 (NPF5.2). In contrast, for other substrates, such as the 25 putative NPF-GA transporters and the many low-affinity nitrate transporters from *Arabidopsis* (**Supplementary Table 1**); there is no apparent phylogenetic clustering within distinct subclades. This discrepancy could be due to features other than determinants of GA substrate specificity weighing higher towards defining phylogenetic differentiation. Alternatively, it is conceivable that the current number of putative GA transporters may be overestimated. The ExxE[K/R] motif plays a role in coupling proton and substrate transport in the NPF proteins but otherwise our knowledge on the transport mechanism of NPF members remains limited (Jørgensen et al., 2015). Analysis of the recent crystal structures of *Arabidopsis* NPF6.3 suggested that its enigmatic dual affinity transport mode is controlled by Thr101 phosphorylation-dependent dimerization (Liu and Tsay, 2003) and that nitrate binds to His356 that is only conserved in one out of the ~20 other suggested NPF nitrate transporters (Parker and Newstead, 2014; Sun et al., 2014; He et al., 2017; Wang et al., 2018). The structure of NPF6.3 provides a welcomed basis for inferring structure-function relationships for the NPF proteins but has so far not been used to explain the molecular determinants of substrate specificities of any NPF transporter towards organic molecules.

The knowledge gap in our understanding of the relationship between structure and function of NPF transporters complicates the identification NPF transporter substrates based on phylogenetic relationships. Hence, understanding the intricate details of substrate selectivity among plant NPF members will be crucial for predicting and elucidating physiological functions.

Here, we aim to increase our understanding of the relationship between structure and function of plant NPF-GA transporters by searching for distinct features among potential substrate-interacting residues within the substrate-binding cavity of GA

Abbreviations: ABA, Abscisic acid; GA, gibberellic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; MFS, Major Facilitator Superfamily; NPF, nitrate transporter 1/peptide transporter family; OPDA, oxo-phytodienoic acid; PCA, principal component analysis; POT, proton-coupled oligopeptide transporter.

transporting NPF proteins. In this context, it is paramount to ascertain the *in vitro* data assigning GA transport activity to individual NPF members. To achieve this, we develop and optimize a short-term quantitative GA transport assay in *Xenopus* oocytes that in theory is capable of identifying both GA importers and exporters and simultaneously detects transport of intermediate, bioactive, and catabolic GA species. We use the optimized transport assay to identify candidate *Arabidopsis* NPF-GA transporters by characterizing and critically assessing the GA substrate preference map among all *Arabidopsis* NPF members. A surprising result from our study is that some NPF proteins are capable of profoundly altering intracellular pH in *Xenopus* oocytes. In a case study, we show that such alterations may lead to changed accumulation equilibria of membrane permeable phytohormones. In addition, we generate a map of all amino acid residues within the substrate-binding cavity of NPF proteins that may play a role in determining substrate specificity and use the GA substrate preference map to correlate GA transport activity to structural features of NPF proteins. Our findings provide a set of critical considerations that will help in guiding physiological investigations.

MATERIALS AND METHODS

Cloning the Entire NPF and cRNA Generation

Coding DNA sequences of the entire *Arabidopsis* NPF were cloned into the *Xenopus* oocyte expression vector, pNB1u as indicated in (**Supplementary Table 2**) (Nour-Eldin et al., 2006). Seventeen of the CDS were obtained as uracil-containing non-clonal DNA fragments, codon optimized for *Xenopus* oocyte expression from Thermo Fisher Scientific Geneart wherein a uracil had been placed at either end to facilitate direct insertion into pNB1u *via* USER cloning (Jørgensen et al., 2017a). Nine of the CDS were amplified *via* PCR from *Arabidopsis* cDNA. Fifteen of the CDS were amplified from vectors obtained from Pro. Wolf B. Frommer or Dr. Eilon Shani. Nine of the CDS were amplified from vectors obtained from RIKEN Bioresource Center (BRC) (Seki et al., 1998; Seki et al., 2002). The remaining three CDS had been cloned into pNB1u in a previous study (Nour-Eldin et al., 2012). Primers used for PCR amplification are indicated in (**Supplementary Table 2**). All PCR amplified fragments had a uracil incorporated at either end *via* the primers and were cloned into pNB1u *via* USER cloning as described previously (Nour-Eldin et al., 2006). We used the X7 polymerase (Nørholm, 2010) in the PCR reactions. Linearized DNA templates for RNA synthesis were obtained by PCR amplifying the coding sequences surrounded by *Xenopus* β-Globin 5′- and 3′-UTRs from pNB1u using forward primer (5′-AATTAACCCTC ACTAAAGGGTTGTAATACGACTCACTATAGGG-3′) and reverse primer (5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTATACTCAAGCTAGCCTCGAG-3′) PCR products were purified using E.Z.N.A Gel extraction kit (Omega Bio-tek) using the manufacturer's instructions. PCR products were *in vitro* transcribed using the mMessage mMachine T7 transcription kit (InVitrogen) using the manufacturer's instructions.

Synthesis of JA-Ile

JA-Ile conjugate was chemically synthesized as described in Kramell et al. (1988).

Xenopus Oocyte Transport Assays

Defolliculated *Xenopus laevis* oocytes (stage V-VI) were purchased from Ecocyte Biosciences and were injected with 25 ng cRNA in 50.6 nl (500 ng/μl) using a Drummond Nanoject II and incubated for 2 to 4 days at 16°C in HEPES-based kulori (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES pH 7.4) before use. Expressing oocytes were pre-incubated in MES-based kulori (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM MES pH 5) for 4 min, before being transferred to phytohormone-containing MES-based kulori for 60 min. Afterwards, oocytes were washed three times in 25 ml HEPES-based kulori followed by one wash in 25 ml deionized water, homogenized in 50% methanol and stored for >30 min at −20°C. Following centrifugation (25,000*g* for 10 min 4°C), the supernatant was mixed with deionized water to a final methanol concentration of 20% and filtered through 0.22 μm (MSGVN2250, Merck Millipore) before analytical LC-MS/MS as described below.

pH Stabilization

Expressing oocytes were injected with 50.6 nl 0.5 M MES 50 mM EGTA adjusted to pH 5.7 with 0.5 M TRIS for stabilizing internal oocyte pH to ~6.25 or 0.5 M TRIS 50 mM EGTA adjusted to 7.7 with 0.5 M MES for stabilizing internal oocyte pH to ~7.5. Assays were carried out as described above, with the exceptions that sorbitol was included in the MES and the HEPES-based kulori solutions to a final concentration of 50 mM to avoid oocyte swelling, and the timeframe between injection of pH stabilizing buffer to assay was terminated was held to a maximum 30 min.

Metabolite Quantification by LC-MS/MS

Compounds in the diluted oocyte extracts were directly analyzed by LC-MS/MS. The analysis was performed with modifications from the method described in Tal et al. (2016). In brief, chromatography was performed on an Advance UHPLC system (Bruker, Bremen, Germany). Separation was achieved on a Kinetex 1.7u XB-C18 column (100 x 2.1 mm, 1.7 μm, 100 Å, Phenomenex, Torrance, CA, USA) with 0.05% v/v formic acid in water and acetonitrile (with 0.05% formic acid, v/v) as mobile phases A and B, respectively. The elution profiles for GAs, glucosinolates, and glycylsarcosine are as follows: 0–0.5 min, 2% B; 0.5–1.3 min, 2–30% B; 1.3–2.2 min, 30–100% B, 2.2–2.8 min, 100%; 2.8–2.9 min, 100–2% B; and 2.9–4.0 min, 2% B. The elution profiles for ABA, jasmonic acid (JA), Ja-Ile, and oxo-phytodienoic acid (OPDA) are as follows: 0–0.5 min, 2% B; 0.5–1.2 min, 2–30% B; 1.2–2.0 min, 30–100% B, 2.0–2.5 min, 100%; 2.5–2.6 min, 100–2% B; and 2.6–4.0 min, 2% B. The mobile phase flow rate was 400 μl·min^{-1} and column temperature was maintained at 40°C. The liquid chromatography was coupled to an EVOQ Elite triple quadrupole mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ion source operated in positive and

negative ionization mode. Instrument parameters were optimized by infusion experiments with pure standards. For analysis of GAs, glucosinolates, and glycylsarcosine the ion spray voltage was maintained at +4000 V and −4000 V in positive and negative ionization mode, respectively, and heated probe temperature was set to 200°C with probe gas flow at 50 psi. For ABA, JA, JA-Ile, and OPDA the ion spray voltage was maintained at −3300 V in negative ionization mode and heated probe temperature was set to 120°C with probe gas flow at 40 psi. Remaining settings were identical for all analytical methods with cone temperature set to 350°C and cone gas to 20 psi. Nebulizing gas was set to 60 psi and collision gas to 1.6 mTorr. Nitrogen was used as probe and nebulizing gas and argon as collision gas. Active exhaust was constantly on. Multiple reaction monitoring was used to monitor analyte parent ion → product ion transitions for all analytes: Multiple reaction monitoring transitions and collision energies were optimized by direct infusion experiments. Detailed values for mass transitions can be found in (**Supplemental Table 3**). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Bruker MS Workstation software (Version 8.2.1, Bruker, Bremen, Germany) was used for data acquisition and processing. Linearity in ionization efficiencies were verified by analyzing dilution series of standard mixtures. Sinigrin glucosinolate was used as internal standard for normalization but not for quantification. Quantification of all compounds was achieved by external standard curves diluted with the same matrix as the actual samples. All GAs were analyzed together in a single method apart from GA12 which suffered from severe ion suppression when combined with the other GAs. Samples with GA12 were, therefore, analyzed separately and with separate dilution series for quantification. Similarly, other hormones than GAs (ABA, JA, JA-Ile, and OPDA) were analyzed in separate analytical runs. Glycylsarcosine and 4-methylthio-3- butenyl were analyzed in a combined analytical run.

pH Measurements of Oocyte Lumen

pH-electrodes were pulled from borosilicate glass capillaries (KWIK-FIL TW F120-3 with filament) on a vertical puller (Narishige Scientific Instrument Lab), baked for 120 min at 220°C, and silanized for 60 min with dimethyldichlorosilane (Silanization Solution I, Sigma Aldrich). Electrodes were backfilled with a buffer containing 40 mM KH_2PO_4, 23 mM NaOH, and 150 mM NaCl (pH 7.5). The electrode tip was filled with a proton-selective ionophore cocktail (hydrogen ionophore I cocktail A, Sigma-Aldrich) by dipping the tip into the cocktail. Oocytes, as described above, were placed in freshly made HEPES-based ekulori (2 mM $LaCl_3$, 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES pH 7.4) for at least 30 min prior to three-electrode voltage clamp experiments. Before each oocyte a pH calibration curve was made for each oocyte using 100 mM KCl pH 5.5, 100 mM KCl pH 6.5, and 100 mM KCl pH 7.5. Oocytes were clamped at 0 mV and perfused with HEPES-based ekulori pH 7.4, followed by MES-based ekulori (2 mM $LaCl_3$, 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM MES pH 5) and internal pH response was measured continuously as a function of external pH change.

Multiple Sequence Alignment and Structure Guided Identification of Cavity Lining Residues

To bring the GA transport function into a structural context, NPF protein sequences from the 31 plant genomes and two outgroups as performed by (Léran et al., 2014) were retrieved from Phytozome version 9.1(Goodstein et al., 2012) and annotated with their NPF IDs (Léran et al., 2014). Guided by blastp (Altschul et al., 1990), long sequences comprising either multiple NPF modules, or fusions with other proteins, were trimmed to the size of single NPF proteins. Initially, the eight NPF subclades were treated separately, for practical reasons and to facilitate robust multiple sequence alignments produced by MUSCLE (Edgar, 2004). For each group, the *Arabidopsis* NPF6.3 sequence was included as a structural reference (PDB: 4OH3) (Sun et al., 2014). Sequences that due to inserts or gaps were not consistent with an intact Major Facilitator Superfamily (MFS) fold were discarded. In the end, the trimmed multiple sequence alignments for all eight NPF subclades were combined and re-aligned using MAFFT (Katoh and Standley, 2013) followed by manual adjustments. The final alignment comprises 1585 NPF sequences.

To embrace structural variability in the substrate binding site, the selection of a subset of amino acid positions that defines the substrate-binding site was guided using four crystal structures of bacterial POT family members, including two complexes with the peptidomimetic drug alafosfalin with different binding modes, as well as homology models constructed to represent the outward-facing conformation of the transporters. The four bacterial POT structures (PDB: 4IKZ, 4LEP, 2XUT, and 4APS) (Newstead et al., 2011; Solcan et al., 2012; Doki et al., 2013; Guettou et al., 2013), all in inward-facing conformations, were superimposed and surface-exposed residues within 8 Å from the alafosfalin in either of the structures were extracted and included in the subset of amino acids that makes up the binding site residues.

Importantly, all known POT structures represent inward-open conformations of the transporter, whereas generally substrate recognition from uptake, will occur when the transporter is in the outward-open orientation. In an attempt to identify residues that line the substrate-binding cavity in the outward-facing conformation, homology models were constructed based on outward-open structures of members of the MFS. The two different outward-open structures of FucP and YajR (PDB: 3O7Q and 3WDO) (Dang et al., 2010; Jiang et al., 2013) were used as templates. Sequence alignments of GkPOT (PDB: 4IKZ) (Doki et al., 2013), FucP and YajR sequences were made with PROMALS3D (Pei et al., 2008) and further refined by hand. Outward-facing homology models of GkPOT were made using MODELLER v9.12 (Sali and Blundell, 1993). Finally, surface-exposed residues within 12 Å from the central cavity (as measured from R36-NH1 and W306-CZ2, respectively) were extracted and included in the binding site residue subset. Our final binding site residue subset, defining the substrate-binding cavity, comprises 51 positions. The sequence logos of GA transporters and non-GA transporters were prepared by the WebLogo program (Crooks et al., 2004).

Principal Component Analysis

The principal component analysis (PCA) was performed with KNIME using the PCA Compute node (Berthold et al., 2008).

RESULTS

Xenopus Oocyte Membrane Permeability Towards GAs at Different External pH

GAs are weak organic acids with lipophilic properties. Consequently, when heterologous transport assays utilize external media with an acidic pH, membrane permeable GAs will be subject to the so-called ion-trap mechanism (Rubery and Sheldrake, 1973). Whereas import can occur by simple diffusion, export likely necessitates the activity of a transport protein (Kramer, 2006; Binenbaum et al., 2018). Of the more than 130 different GAs existing in nature (Macmillan, 2001) a handful has been tested in various *in vitro* transport assays and shown varying membrane permeation (Saito et al., 2015; David et al., 2016; Kanno et al., 2016; Tal et al., 2016). In particular, products of the C13-hydroxylation pathway appear less membrane permeable than their non C13-hydroxylated counterparts (Binenbaum et al., 2018). To investigate this in more detail, we exposed water injected control oocytes to a 50 µM equimolar mixture of eight different GAs (GA1, GA3, GA4, GA7, GA8, GA9, GA19, and GA24) at three different external pH values (pH 4.7, pH 5.3, and pH 6.0) for 60 min and subsequently quantified internalized GA by LC-MS/MS analysis of oocytes homogenates (**Figure 1A**). The eight GAs represent intermediate, bioactive, and catabolic GA species and characteristically contain a varying number of oxygen atoms (**Figure 1B**). Four of the GAs (GA4, GA7, GA9, and GA24) appeared to permeate the membrane as they were detected in oocytes at all external pH values whereas the remaining four GAs (GA1, GA3, GA8, and GA19) were not detected in oocytes regardless of the external pH. Increasing the external pH lowered the accumulation of membrane permeable GAs. Based on the quantification, we categorized three of the permeating GAs (GA4, GA7, and GA24) as moderately permeating (oocyte GA concentration ≤ external concentration at pH 5.3 and 6.0, respectively) and one (GA9) as highly permeating (internal concentration 20- to 80-fold over external concentration at pH 6 to 4.7). From this dataset, we draw a simple correlation between number of oxygen atoms and membrane permeability (**Figure 1C**). Irrespective of oxygen positions on the GA backbone or the functional group in which they participate, GAs with six or more oxygens (GA1, GA3, GA8, and GA19), did not permeate the *Xenopus* oocyte membrane at any pH. In comparison, GAs with five oxygens (GA4, GA7, and GA24) permeate moderately and GAs with only four oxygens (GA9) permeate to a high degree. Thus, background accumulation

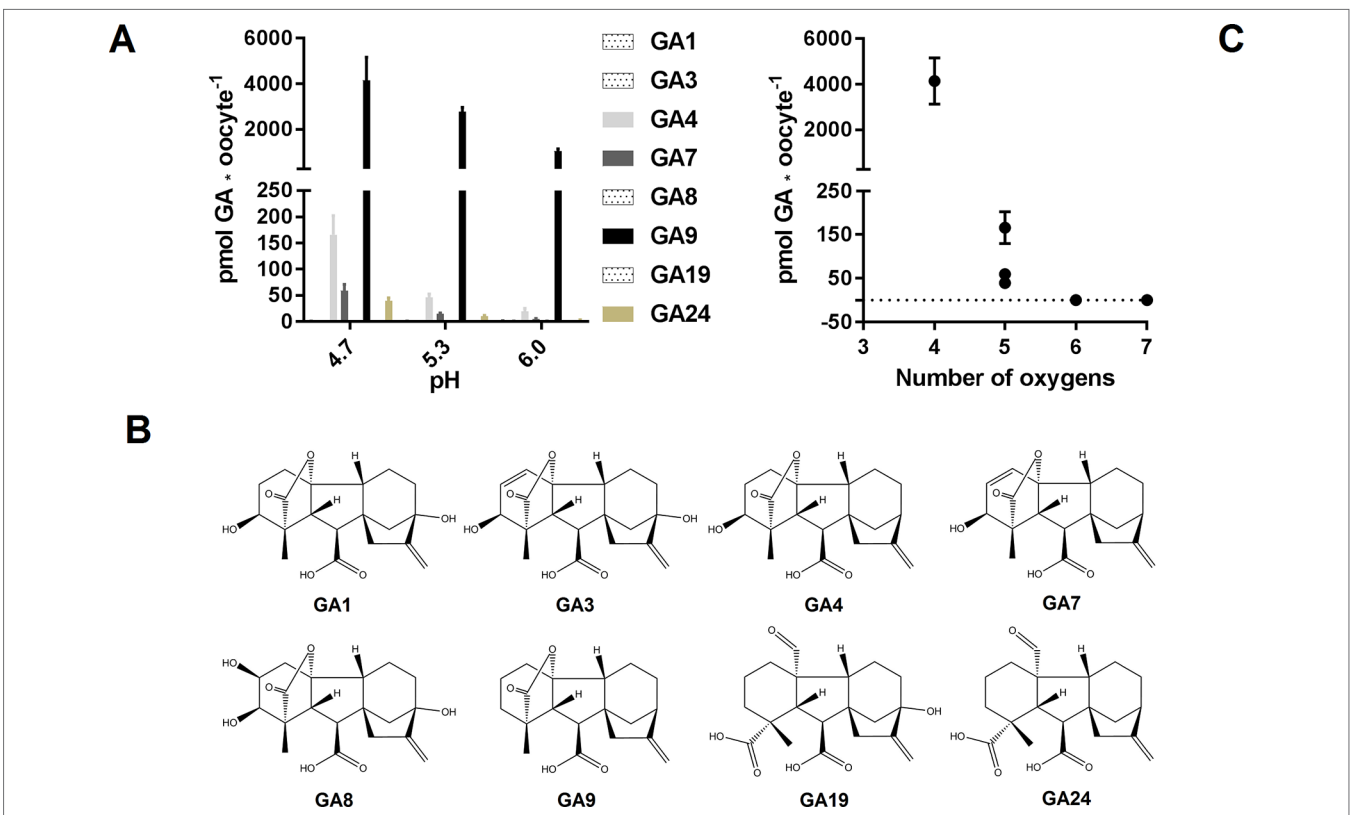


FIGURE 1 | GA membrane permeability is a function of pH and oxygen content. **(A)**. Mock expressing oocytes (n = 7–9) were exposed to a mix of 50 µM GA1, 50 µM GA3, 50 µM GA4, 50 µM GA7, 50 µM GA8, 50 µM GA9, 50 µM GA19, and 50 µM GA24 in pH 4.7, 5.3, and 6.0 for 60 min and GA content was quantified by LC-MS/MS. **(B)**. Chemical structures of the tested GAs. **(C)**. GA content of mock injected oocytes at pH 4.7 as a function of GA oxygen content.

due to diffusion of protonated membrane permeating GAs can—not surprisingly—be minimized by increasing external pH in transport assays. It should be emphasized here that we only investigated membrane permeability toward various GAs in *Xenopus* oocytes and that similar permeability/background uptake cannot necessarily be inferred for other heterologous hosts, such as yeast. For example, GA3, which does not permeate oocytes appears to accumulate weakly in yeast (Saito et al., 2015; David et al., 2016; Kanno et al., 2016; Tal et al., 2016).

Determining Optimal pH for GA Transport Assays in *Xenopus* Oocytes Expressing NPF Proteins

To determine a suitable pH for GA transport assays in *Xenopus* oocytes, we used NPF3.1 as a study case. This transporter imports a wide range of GA species when expressed in *Xenopus* oocytes and assayed at pH 5 (Tal et al., 2016). However, no difference to mock can be seen when exposed to highly permeating GAs, such as GA9 or GA12. One explanation could be that the strong diffusion of GA9 into oocytes may mask any transport activity (**Figure 1A**). We have previously shown that transport activity of NPF3.1 is undetectable at pH 7 (Tal et al., 2016). Here, we test the activity of NPF3.1, in a 180 min time-course uptake assay towards 50 μM of the non-permeating GA3 at pH 5 and 6 (**Figure 2A**). At pH 5, NPF3.1-mediated accumulation of GA3 reached equilibrium after approximately 120 min incubation (~1.5 fold x external medium concentrations). At pH 6, NPF3.1-mediated GA3 uptake also reached equilibrium after 2 h incubation albeit the equilibrium level was approximately 75% lower than at pH 5. For comparison, we tested the membrane permeability of GA9 in a 180 min time-course assay in water injected control oocytes (**Figure 2B**). Accordingly, seeking a compromise between reduced diffusion and reduced transport activity, we retested whether NPF3.1-mediated transport of GA9 into oocytes could be detected at pH 5.5 and pH 6. No difference could be seen to mock oocytes when assayed at pH 5.5 (i.e. both accumulated GA9 to equally high levels). In comparison, when assayed at pH 6, NPF3.1 expressing oocytes accumulate higher amounts of GA9 compared to water injected control oocytes (**Figure 2C**). Thus, for characterizing NPF protein mediated transport of highly permeating GAs in *Xenopus* oocytes, it can be advisable to adjust external media to pH values >5.5 to reduce diffusion to an extent where the contribution of transport activity is distinguishable. As the transport activity is dramatically reduced at pH 6 (**Figure 2A**) we opted for exploring NPF3.1 transport activity towards our selection of non-permeating GAs at pH 5, moderately permeating GAs at pH 5.5 and highly permeating GAs (including GA12) at pH 6. We detected significantly higher accumulation compared to water injected control oocytes for all non-permeating, moderately permeating, and highly permeating GAs at pH 5, pH 5.5, and pH 6, respectively (**Supplementary Figure 1**). Thus, we established optimized conditions for quantitative GA transport assays capable of detecting NPF3.1-mediated accumulation of eight different GAs including the highly membrane permeating species.

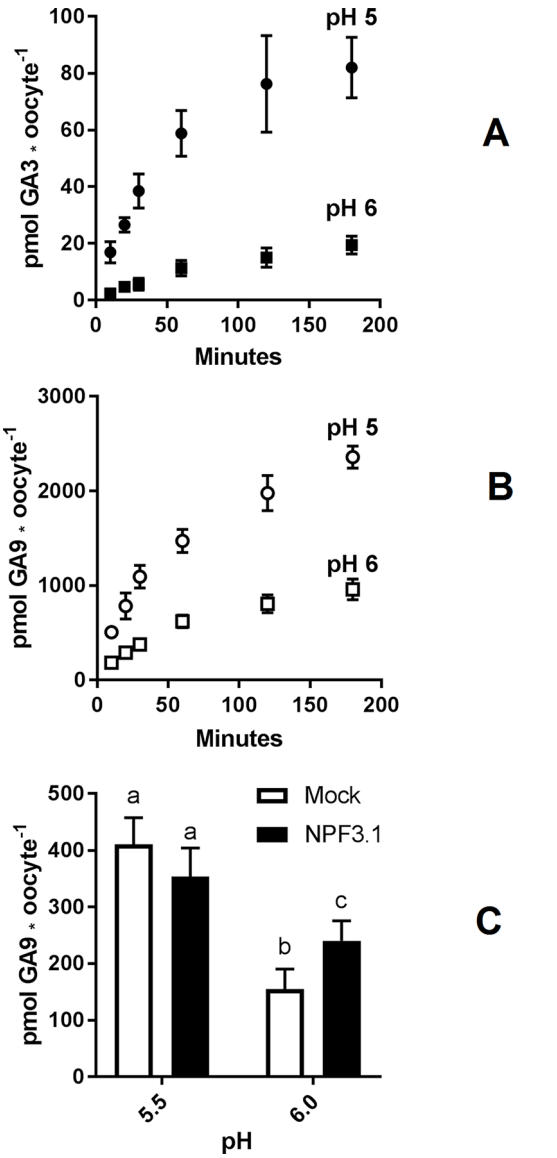
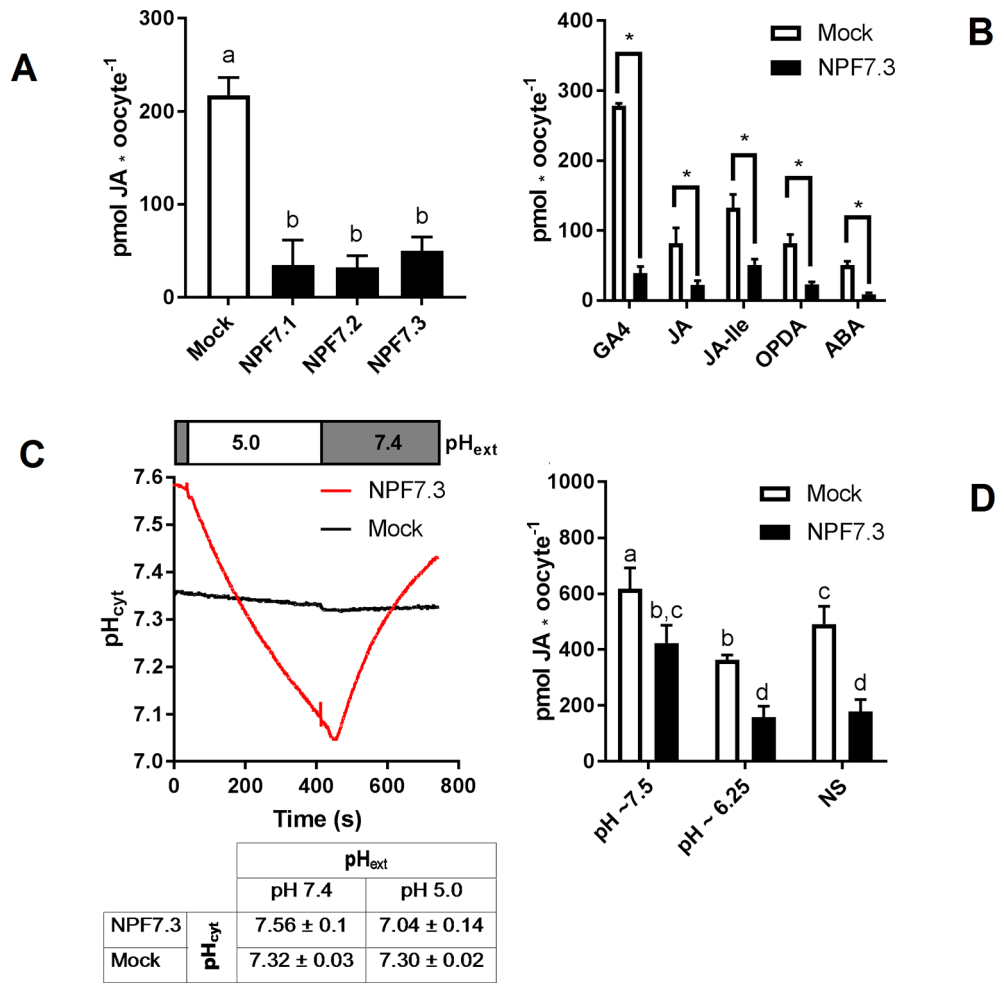


FIGURE 2 | Assessment of GA permeability and NPF transporter function. **(A)**. Time course of NPF3.1 mediate GA3 transport over 180 min. NPF3.1 expressing oocytes were exposed to 50 μM membrane non-permeable GA3 in kulori pH 5 or 6. At each time point, oocytes (n = 6) were taken out for analysis. **(B)**. Time course of GA9 membrane penetration over 180 min. Mock expressing oocytes were exposed to 50 μM GA9 in kulori pH 5 or 6. At each time point, oocytes (n = 6) were taken out for analysis. **(C)**. NPF3.1 mediated GA9 transport at pH 5.5 and 6. Oocytes (n = 6) were exposed to 50 μM GA9 in kulori (Materials and Methods) pH 5.5 or 6 for 60 min. Oocyte GA content was analyzed using LC-MS/MS. Statistical assessment was performed with Holm Sidak two-way ANOVA p = 0.01.

The Proton Potassium Antiport Activity Portraits an Efflux Transporter Artefact

In theory, passive diffusion of moderately permeating GA into oocytes allows screening for exporting transport proteins. For example, if an oocyte expressing a candidate exporter accumulates less GA4 compared to mock oocytes, it could indicate that a portion of the GA4 molecules that have diffused

into the transporter expressing oocytes can move out of the oocyte through the transporter. In our preparatory phases we exposed parts of our NPF library to a mixture of phytohormones at external pH 5. The mixture included JA, which highly permeates oocyte membranes at acidic pH. Strikingly, three related transporters, NPF7.1–7.3 all accumulated 75% to 85% less JA compared to water injected control oocytes (**Figure 3A**). Our excitement about having identified potential JA exporters was, however, curbed by several factors. First, in an attempt to determine the transporters' substrate preference, we exposed NPF7.3 expressing oocytes to five different phytohormones with lipophilic weak acid properties (GA4, ABA, JA, JA-Ile, and OPDA). Despite the diversity in chemical structures, NPF7.3 expressing oocytes accumulated significantly less of all five phytohormones compared to water injected control oocytes (**Figure 3v**). Thus, either this putative exporter appeared to possess the same enigmatic multi-substrate specificity towards phytohormones as described for other NPF proteins (Kanno et al., 2012; Chiba et al., 2015; Tal et al., 2016) or the reduced accumulation within oocytes was possibly due to indirect effects. In the context of *in vitro* transport assays, the accumulation equilibrium generated by the ion-trap mechanism is expectedly sensitive to small pH changes on either side of the membrane (Rubery and Sheldrake, 1973). Recently, NPF7.3 was



		pH_{ext}	
		pH 7.4	pH 5.0
NPF7.3	pH_{cyt}	7.56 ± 0.1	7.04 ± 0.14
Mock	pH_{cyt}	7.32 ± 0.03	7.30 ± 0.02

FIGURE 3 | The proton potassium antiport function of NPF7.3 influences the ion-trap mechanism of membrane permeable weak acids. **(A)**. Oocytes were exposed to 100 μM JA in kulori pH 5 for 60 min and analyzed in three technical replicates of five oocytes. Statistical assessment was performed with Holm Sidak one-way ANOVA p = 0.01. **(B)**. Lower accumulation of membrane permeable phytohormones in NPF7.3 expressing oocytes compared to mock. Oocytes were exposed to 100 μM phytohormone in kulori pH 5 for 60 min (JA, JA-Ile, OPDA, ABA) or 90 min (GA4) and analyzed in three to four technical reps of four to five oocytes. Statistical assessment was performed with two-tailed t-tests p = 0.05 **(C)**. Upper panel: Internal oocyte pH measured using three-electrode voltage-clamp of mock vs NPF7.3 expressing oocytes. Starting in ekulori pH 7.4, the external buffer was changed to ekulori pH 5.0 for 400 s followed by ekulori pH 7.4 for another 400 s. pH was measured continuously at a membrane potential of 0 mV. Representative measurements are shown. Lower panel: Mean values of cytosolic pH in mock or NPF7.3 expressing oocytes in standard ekulori buffer pH 7.4 or 400 s after incubation in ekulori pH 5.0 (n ≥ 3, mean ± SD). **(D)**. JA content at defined cytosolic pH. Oocytes injected with 0.5 M TRIS 50 mM EGTA adjusted to pH 7.7 with 0.5 M MES (pH stabilized at ~7.5), 0.5 M MES 50 mM EGTA adjusted to 5.7 with 0.5 M TRIS (pH stabilized at ~6.25) or water (not stabilized: NS) was exposed to 100 μM JA in kulori pH 5 for 20 min and analyzed in 2 × 3 technical reps of four to five oocytes. Oocyte phytohormone content was analyzed using LC-MS/MS. Statistical assessment was performed with Holm Sidak two-way ANOVA p = 0.01. *significance is p = 0.01 in **(A)**, p = 0.005 in **(B)**, and p = 0.01 in **(C)**.

shown to exhibit a non-electrogenic proton/potassium antiport activity when expressed in oocytes. However, the effect of the imported protons on internal oocyte pH was not investigated (Li et al., 2017). This prompted us to measure intracellular pH in oocytes expressing NPF7.3 using a proton-selective electrode in a three-electrode voltage clamp setup. Oocytes were clamped at a membrane potential of 0 mV and perfused with kulori at pH 7.4. This was followed by 400 s perfusion with ekulori buffer pH 5.0 and then back to pH 7.4 for additional 400 s, while the intracellular pH was monitored continuously. Compared to water injected control oocytes, NPF7.3 expressing oocytes displayed a significant 0.24 pH-units higher cytosolic pH in buffers with pH 7.4. However, within 400 s intracellular pH markedly dropped by 0.5 pH units when external pH was lowered from 7.4 to 5.0, reaching a cytosolic pH 0.26 units lower than in water injected control oocytes (**Figure 3C**). During prolonged exposure (60 min) to ekulori buffer pH 5.0 intracellular pH even decreased to a stable pH of approximately 6.3, that completely reversed when re-subjected to pH 7.4 (**Supplementary Figure 2**). In comparison, oocytes individually expressing a selection of other NPF members; NPF2.5, NPF2.13, NPF3.1, NPF4.1, NPF4.3, and NPF4.6 (**Figure 4**) did not display a drop in intracellular pH that was significantly different than water injected control oocytes. Next we tested whether a decrease in intracellular pH of this magnitude affects the accumulation of membrane permeating phytohormones. Due to its rapid diffusion into oocytes, we used JA as a representative example of diffusing phytohormones. First, we investigated JA accumulation in water injected control oocytes wherein pH was lowered by concentrated MES/TRIS buffer (pH 5.7) injection resulting in an oocyte intracellular pH of ~6.25 that was stable for approximately 30 min (**Supplementary Figure 3**). This procedure mimicked the pH lowering effect of NPF7.3. Next we exposed these oocytes to 100 μM JA for 20 min. In comparison to oocytes that were not injected with the pH-lowering buffer, oocytes with the buffer-controlled intracellular pH of ~6.25 accumulated significantly less JA (**Figure 3D**). In contrast, when we stabilized intracellular pH of NPF7.3 expressing oocytes to ~7.5 *via* buffer injection, these oocytes accumulated JA to the same extent as in mock oocytes with no buffer injection (**Figure 3D**). These results strongly suggest that altered accumulation of phytohormones in NPF7.3 expressing oocytes is an indirect effect of the proton influx and concomitant lowering of intracellular pH.

A Quantitative Screen of the *Arabidopsis* NPF Proteins

To ascertain the *in vitro* based functional annotation of NPF GA transport activity, we used the optimized GA transport conditions to quantitatively screen for GA transport activity toward non-permeating and moderately permeating GAs in oocytes. Therefore, cRNA for translation of all 53 *Arabidopsis* NPF members were injected individually into *Xenopus* oocytes and after 3 days of expression oocytes were exposed for 60 min at pH 5.5 to mixtures of bioactive GAs (50 μM GA1 and 100 μM GA4) (Yamaguchi, 2008), a product of catabolism (50 μM GA8) (Yamaguchi, 2008), a biosynthesis intermediate (50 μM GA19) (Yamaguchi, 2008), phloem transported (50 μM GA24) (Regnault et al., 2015) and the seed specific (50 μM GA3) (Derkx et al., 1994). The data is displayed in two figures (**Figure 5** and **6**). **Figure 5** displays transport activities for non-permeating GAs (GA1, GA3, GA8, and GA19), whereas **Figure 6** displays transport activities of the moderately permeating GAs (GA4 and GA24). Un-normalized data is included in (**Supplementary Figure 4**). From subclade NPF4, only NPF4.1 and NPF4.6 transported non-permeating GAs, where NPF4.6 accumulated only ~15% of GA compared to NPF4.1 levels. The only other transporters displaying uptake activities of similar magnitude as NPF4.1 were

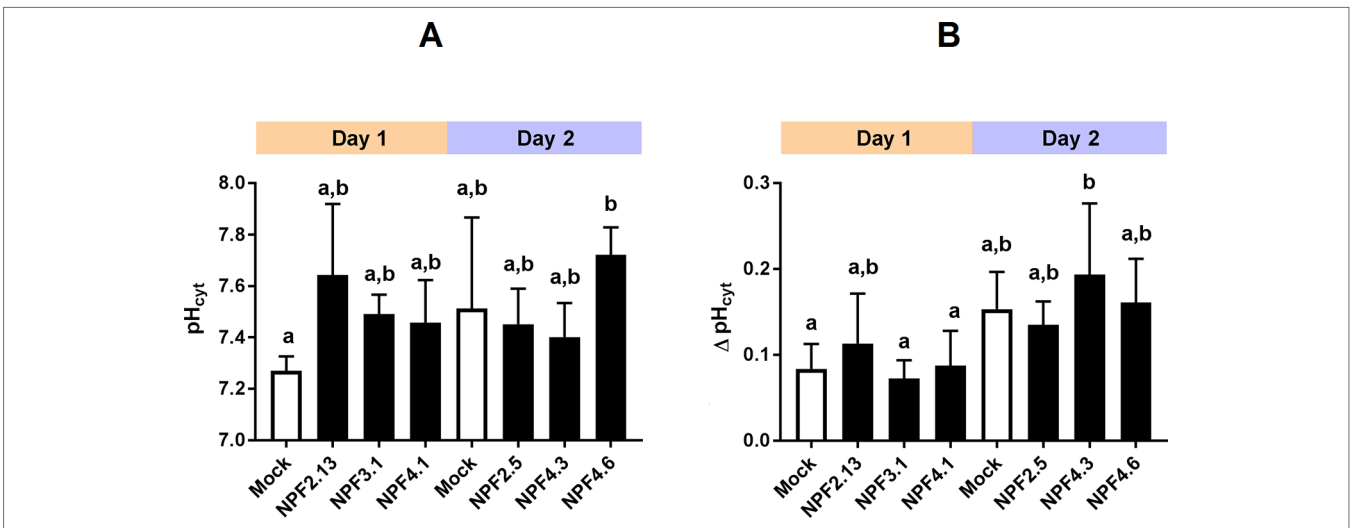


FIGURE 4 | Transporter expression and oocyte maturity influence internal oocyte pH. Internal oocyte pH measured using three-electrode voltage-clamp of mock versus expressing oocytes. **(A)**. after 30 min resting in ekulori pH 7.4 and additional 5 min clamped at 0 mV with pH 7.4 ekulori perfusion. **(B)**. Change in internal pH after additional 5 min perfusion with pH 5 ekulori. Statistical assessment was performed with Holm Sidak two-way ANOVA p = 0.05. a and b indicates statistical indifference.

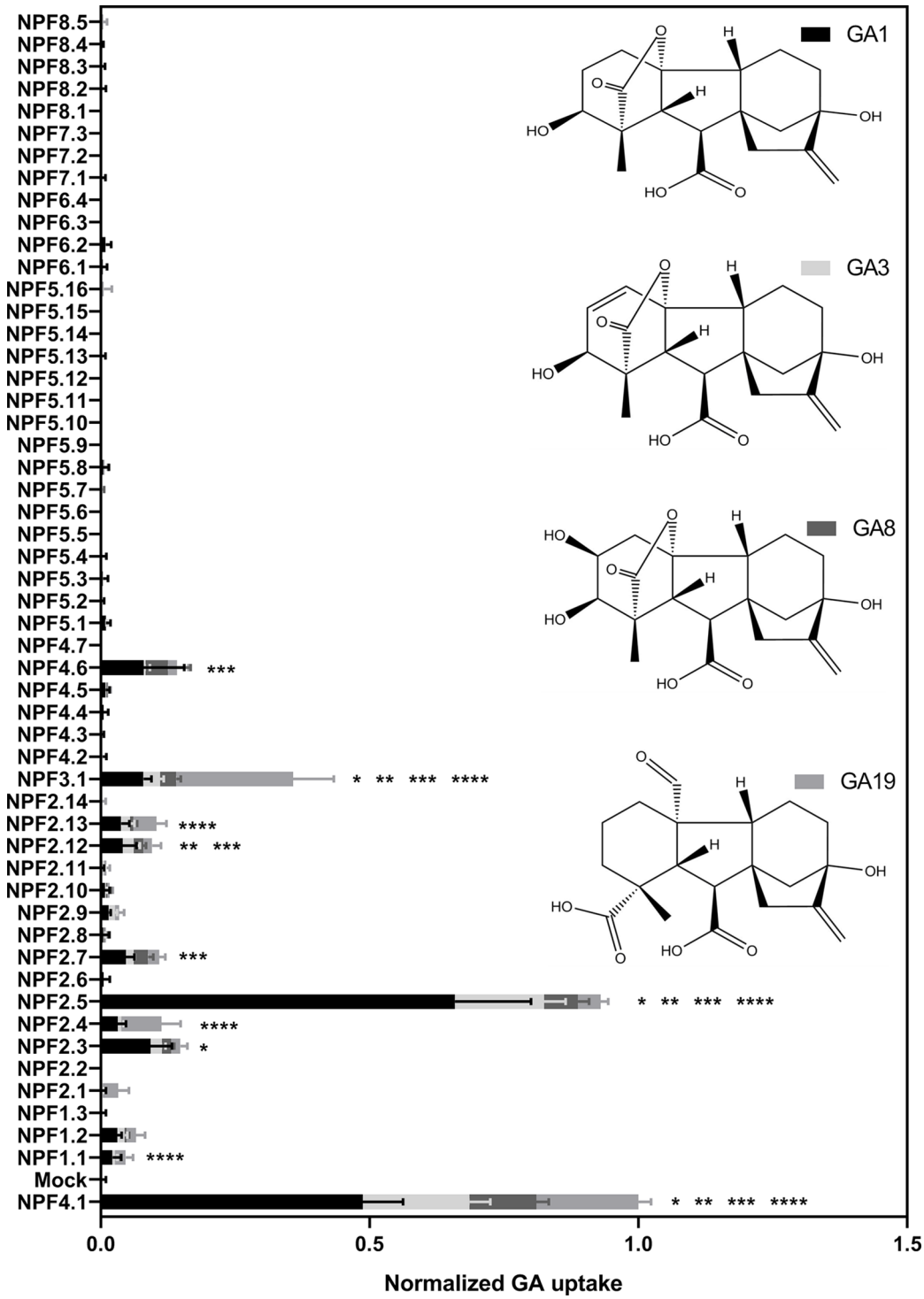


FIGURE 5 | Quantitative screen of the NPFs for GA transport of not membrane non-permeable GAs. Due to logistic considerations, the 53 NPF members were screened in two portions on the same day and normalized to the transport of NPF4.1 of membrane non-permeable GAs. NPF4.1 was chosen for normalization as it is a well characterized GA transporter (Kanno et al., 2012; Saito et al., 2015; Tal et al., 2016). For each transporter the proportion of each GA imported into oocytes is given in proportion to total imported amount of GAs. Statistical significant transport (Holm Sidak one-way ANOVA p = 0.05) is indicated with one asterisk for GA1, two asterisks for GA3, three asterisks for GA8, and four asterisks for GA19. Two assays of 28 and 29 genes, respectively, were performed on same day on the same oocyte batch. Both assays included NPF3.1, NPF4.1, and Mock to normalize. Oocytes (n = 5–6) were exposed to a mix of 50 μM GA1, 50 μM GA3, 100 μM GA4, 50 μM GA8, 50 μM GA19, and 50 μM GA24 in pH kulori 5.5 (Materials and Methods) for 60 min. Oocyte GA content was analyzed using LC-MS/MS.

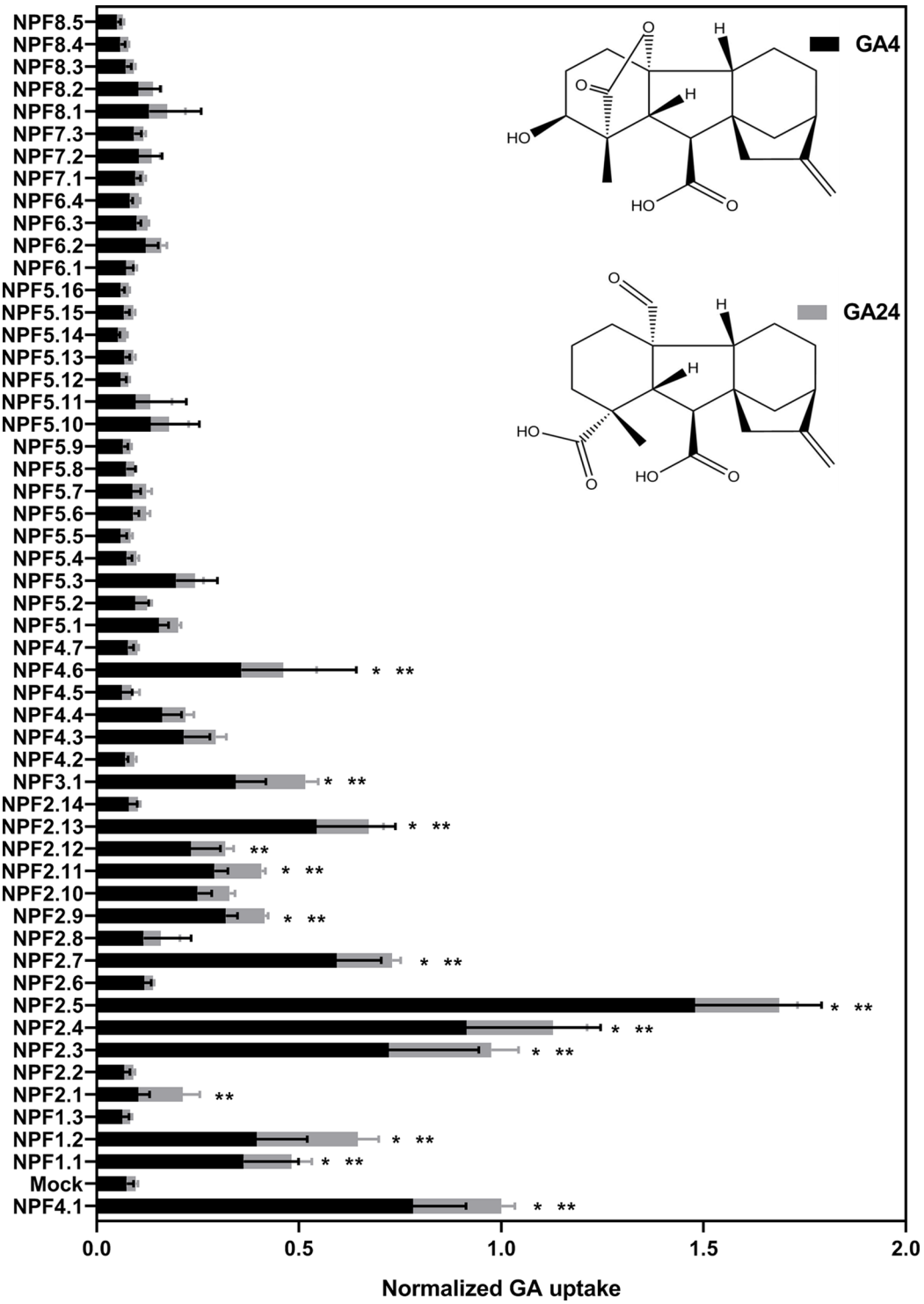


FIGURE 6 | Quantitative screen of the NPF proteins for GA transport of membrane permeable GAs. Due to logistic considerations, the 53 NPF members were screened in two portions on the same day and normalized to the transport of NPF4.1 of membrane permeable GAs. NPF4.1 was chosen for normalization as it is a well characterized GA transporter (Kanno et al., 2012; Saito et al., 2015; Tal et al., 2016). For each transporter the proportion of each GA imported into oocytes is given in proportion to total imported amount of GAs. Statistical significant transport (Holm Sidak one-way ANOVA $p = 0.05$) is indicated with one asterisk for GA4 and two asterisks for GA24. Two assays of 28 and 29 genes, respectively, were performed on same day on the same oocyte batch. Both assays included NPF3.1, NPF4.1, and Mock to normalize. Oocytes (n = 5–6) were exposed to a mix of 50 μM GA1, 50 μM GA3, 100 μM GA4, 50 μM GA8, 50 μM GA19, and 50 μM GA24 in pH kulori 5.5 (Materials and Methods) for 60 min. Oocyte GA content was analyzed using LC-MS/MS.

NPF2.5 and NPF3.1. NPF3.1 imported approximately 40% of NPF4.1 levels. In comparison, NPF1.1, NPF2.3, NPF2.4, NPF2.7, NPF2.12, and NPF2.13 imported GAs to approximately 10% to 15% of NPF4.1 levels. Thus, no significant uptake was detected in oocytes expressing any NPF member from subclades 5 to 8, indicating that GA transporters cluster within subclades 1 to 4.

Interestingly, our screen indicates different substrate preferences among the identified GA transporters. However, due to a necessity for more rigorous characterization we did not score differences in preference statistically. However, some clear potential preferences can be seen for a few genes. For example, NPF3.1 appears to prefer the biosynthesis intermediate GA19, whereas NPF2.5 shows a strong preference for the bioactive GA1.

Transport data on the moderately permeating GAs identifies significant transport activity for more putative GA transporters than uptake on non-permeating GAs. Significant transport is still confined to subclades 1 to 4 but with more members of subclades 1 and 2 identified as potential GA transporters, namely (NPF1.2, NPF2.1, NPF2.9, and NPF2.11).

Delineating Potential Substrate Binding Residues

The uptake results presented above (**Figure 5** and **6**) shows that the apparent GA transport activities are confined to a subset of NPF proteins from subclades 1 to 4. If we assume that GA-transporting NPF proteins must have residues that confer GA selectivity compared to non-transporting NPF proteins; a pertinent question is whether the identified NPF members share these residues to form identifiable GA specific motifs.

The canonical MFS transporter structure consists of two six-helix bundles connected by a long cytosolic loop; the interface between these two bundles contains the residues that interact with the transported substrate and, thus, define the substrate specificity. The crystal structure of NPF6.3 is an excellent example of an MFS structure (Parker and Newstead, 2014; Sun et al., 2014). During its transport cycle the transporter will alternate between conformations with the substrate-binding site open to the extracellular side, be occluded, or open to the cytoplasm (Jardetzky, 1966; Abramson et al., 2003; Huang et al., 2003). POT family members and NPF members are closely related (Fei et al., 1994; Léran et al., 2014; Jørgensen et al., 2015). Several POT crystal structures have been co-crystallized with a number of different peptides or peptidomimetic drugs (Doki et al., 2013; Guettou et al., 2014; Lyons et al., 2014; Newstead, 2015; Newstead, 2017). All peptides bind at relatively equivalent positions between the two six-helix bundles. To identify potential specificity determining residues of the NPF proteins we utilized POT structures crystallized in complex with the peptidomimetic drug alafosfalin (PDB: 4IKZ and 4LEP) (Doki et al., 2013; Guettou et al., 2013). Surface exposed residues within an 8 Å sphere around alafosfalin were selected; additionally, residues predicted to be surface exposed in an outward-facing conformation, as judged from comparisons with the FucP and YajR structures (PDB: 3O7Q and 3WDO) (Dang et al., 2010; Jiang et al., 2013) were also selected; yielding a total of 51 residues (**Figure 7**, **Supplementary Video 1**. From this set of residues, we created sequence logos for the non-membrane permeating GA (GA1, GA3, GA7, GA19) transporting- versus non–GA-transporting *Arabidopsis* NPF proteins (GA(+) and GA(–), respectively, in **Figure 7**) to highlight any preferred positions (Crooks et al., 2004). Only a few distinct positions were observed; hereunder arginine in position 16, hereafter denoted Arg^{Pos16} (Lys164 of NPF6.3), Ser^{Pos28} (Trp353 of NPF6.3) and Gln^{Pos31} (Leu359 of NPF6.3) (**Figure 7**).

For assessing the physiochemical environment in the substrate binding cavity in NPF transporters the same set of residues were converted to the position-dependent numerical descriptors, z-scales, developed by (Hellberg et al., 1987). The original z-scales descriptors have been derived by a PCA of 29 physicochemical variables describing the properties of the 20 natural amino acids, and represent the hydrophilicity (z_1), steric properties (z_2) and polarity of the amino acid (z_3). The z-scales have successfully been used in several proteochemometric studies, for instance to model HIV protease resistance or to alignment-independently classify G-coupled receptors and more (Lapinsh et al., 2002; Van

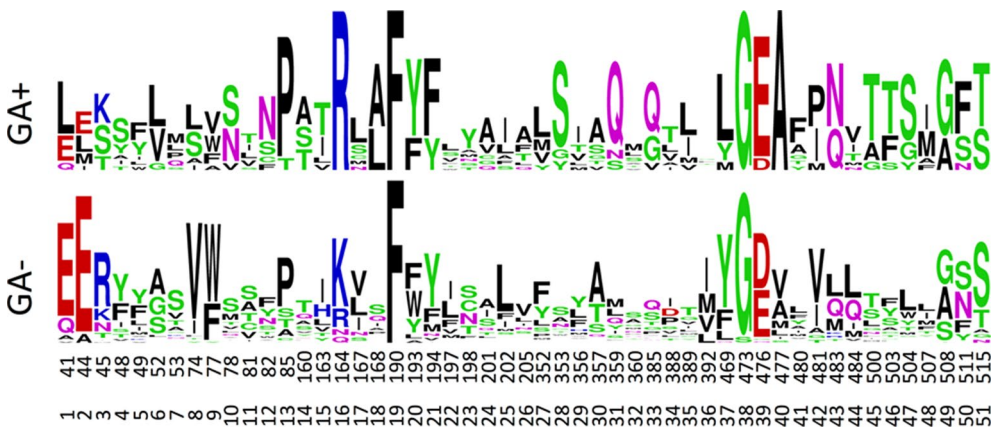


FIGURE 7 | Sequence logos for the GA transporting (GA+) versus GA non-transporting (GA-) NPF proteins. Numbers correspond to the amino acid position of *Arabidopsis thaliana* NPF6.3 and the relative position of the binding site residues. The figure was made in the WebLogo program (Crooks et al., 2004).

Westen et al., 2013; Cortés-Ciriano et al., 2015). The 51 sequences times 51 residues matrix was converted to a 51 sequences times 153 z-scales matrix, which was subjected to a PCA.

The PCA only shows four major clusters indicating shared physicochemical properties in the substrate binding cavity in genes across phylogenetic subclades. Three of the clusters are clearly defined whereas the fourth is larger and more diffuse. Cluster I, contains six NPF2 subclade proteins that include four GA(+) transporters and two GA(−) transporters. Cluster II contains 11 NPF proteins, four GA(+) and seven GA(−) transporters, from the NPF1, NPF2, and NPF3 subclades. Cluster III, contains solely GA(−) transporters from the NPF5 subclade. Finally, cluster IV contains the remaining NPF proteins including the two GA(+) transporters, NPF4.1 and NPF4.6 (**Figure 8**). Glucosinolate transporting NPF proteins (NPF2.9–2.13 and NPF3.1) (Nour-Eldin et al., 2012; Jørgensen et al., 2017b) and peptide transporting NPF proteins (NPF5.2 and NPF8.1-NPF8.3) (**Supplementary Figure 5**) (Frommer et al., 1994; Dietrich et al., 2004; Hammes et al., 2010) group in two tight groups in distinct clusters in the PCA plot (**Figure 8**), and both clusters contain additional genes (clusters II and IV, respectively). Similar to GA(+) transporters, nitrate transporters are scattered in more clusters (**Supplementary Figure 6**).

Cluster I is composed exclusively of genes belonging to the NAXT subclade whose members all lack the ExxE[K/R] motif, i.e. the negatively charged Glu^{Pos1} (Residue 41 of NPF6.3), Glu^{Pos2} (Residue 44 of NPF6.3) and the positively charged Arg/Lys^{Pos3} (Residue 45 of NPF6.3). This motif is otherwise conserved in the rest of the family except in the NPF7 clade (Segonzac et al., 2007; Jørgensen et al., 2015). To exclude bias by the presence/absence of the ExxE[K/R] motif, the entire PCA was repeated for only 48 residues (i.e. omitting positions 1–3). The 48 residue PCA yielded four clusters containing the same genes and a similar clustering as the PCA on the original 51 residues (**Supplementary Figure 7**). Thus, cluster I is not defined based on the absence of the ExxE[K/R] motif.

To identify characteristic properties for each of the four clusters, we created sequence logos for the individual clusters (**Figure 9**). Besides uniquely lacking the ExxE[K/R] motif, cluster I is the only cluster that includes Ala^{Pos18} (Ser168 of NPF6.3), Asn^{Pos43} (Gln483 of NPF6.3), Thr^{Pos46} (Leu503 of NPF6.3), Ser^{Pos47} (Leu504 of NPF6.3) and Phe^{Pos50} (Phe511 of NPF6.3) (**Figure 9**, red arrows; **Figure 10**, green residues). In comparison, only one residue is unique to cluster II, namely, Asn^{Pos12} (Residue 82 of NPF6.3) (**Figure 9**, blue arrow; **Figure 10**, blue residue). It is interesting to compare the differences between cluster I/II versus cluster III/IV, since the majority of the GA transporters are found in clusters I and II. The sequence logos indicate residues that are uniquely shared between clusters I and II. For example, position 16 (Residue 164 in NPF6.3), is conserved as an Arg in

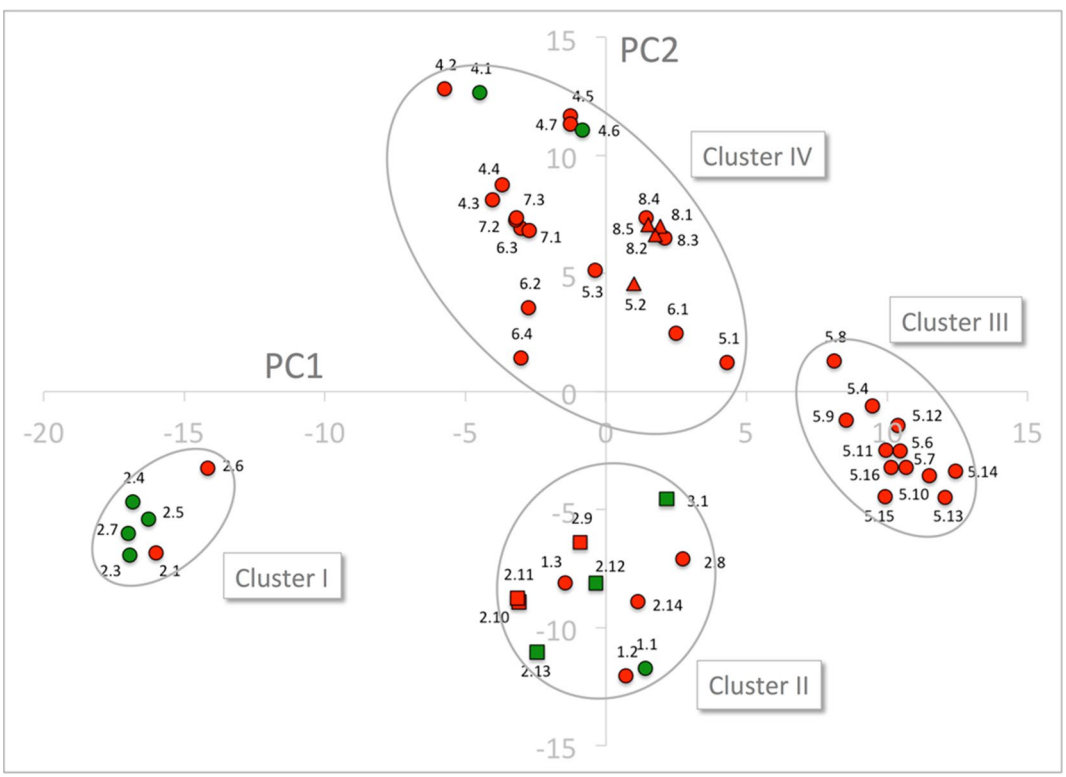


FIGURE 8 | PCA of the 51 NPF sequences expressed by z-scales. GA transporting transporters are shown as green dots and squares and GA non-transporting transporters as red dots, triangles, and squares. Glucosinolate transporting genes and peptide transporting genes are shown as squares and triangles, respectively. The four clusters are marked by ellipses. PC1 and PC2 refer to the first and second principal components, respectively.

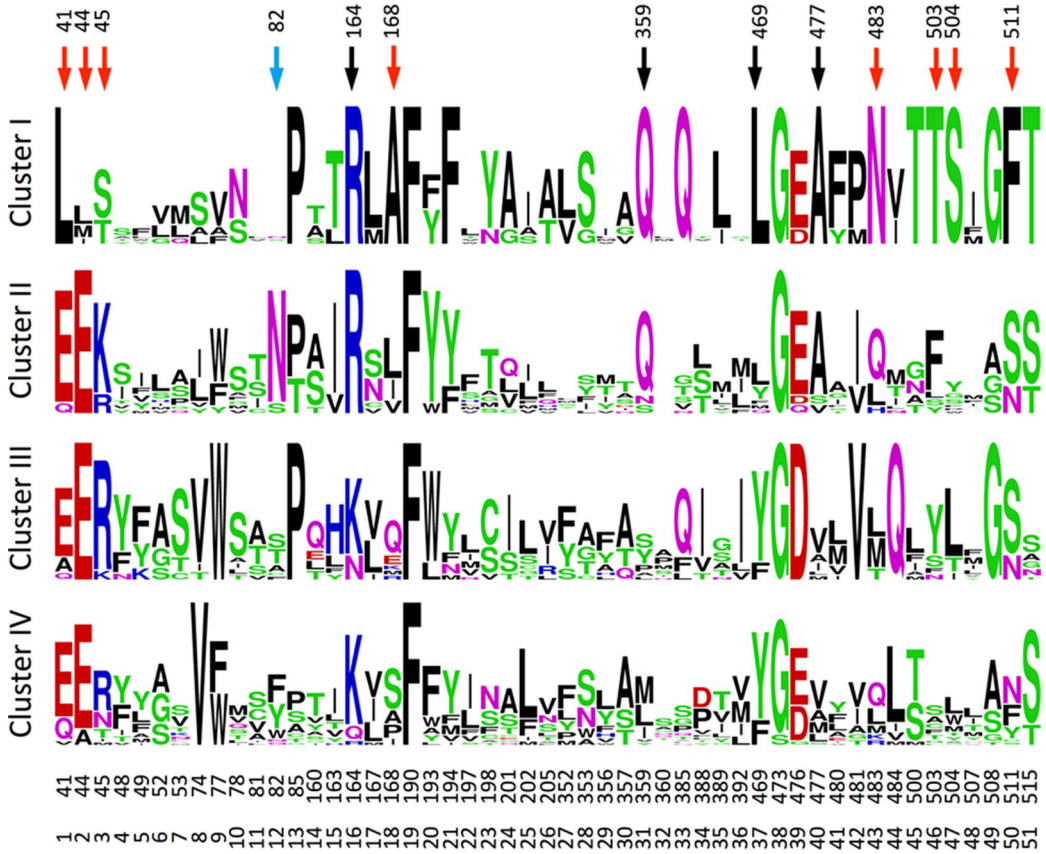


FIGURE 9 | Sequence logos for the transporters in the four clusters. The figure was made in the WebLogo program (Crooks et al., 2004). Red, blue, and black arrows mark positions unique for cluster I, cluster II, and for clusters I and II, respectively.

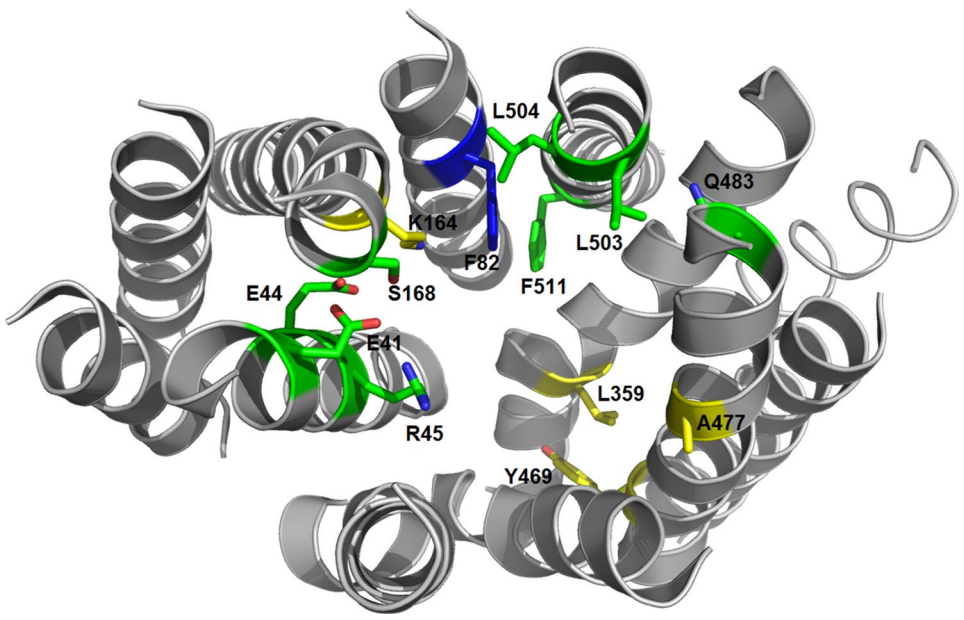


FIGURE 10 | 3D structure of the NPF6.3 [PDB: 4OH3 (Sun et al., 2014)]. Residues unique for cluster I are shown as green sticks, the residue unique for cluster II is shown as a blue stick and residues unique for clusters I and II are shown as yellow sticks.

clusters I and II and Lys in clusters III and IV. In addition, Gln^{Pos31} (Residue 359 of NPF6.3), Leu^{Pos37} (Residue 469 of NPF6.3) and Ala^{Pos40} (Residue 477 of NPF6.3) are characteristic for cluster I/II compared to cluster III/IV (**Figure 9**, black arrows; **Figure 10**, yellow residues).

DISCUSSION

Perhaps, one of the most intriguing features of the NPF phytohormone transporters is the apparent multi-specificity toward phytohormones and other metabolites with distinct chemical structure (Corratge-Faillie and Lacombe, 2017; Wang et al., 2018). This phenomenon has previously prompted speculations in the NPF, providing a basis for the integration of environmental and physiological information linked to the relative availability of the different nutrients (Corratge-Faillie and Lacombe, 2017). However, to delve into this interesting notion of multi-substrate specificity it is necessary to discuss how to distinguish between "real" and "non-" substrates for NPF transporters. In this context, our data provide some novel points for discussion.

First, there appears to be indirect ways that NPF transporters can alter the distribution pattern of membrane permeating substrates with acid/base properties across membranes. For example, our characterization of NPF7.3 provides an alternative explanation to what we first perceived as multi-specificity towards different phytohormones. As NPF7.3 causes a profound reduction in internal oocyte pH when external pH is reduced from 7.4 to 5 (**Figure 3C**), the strength of the ion-trap mechanism is altered, and this affects the diffusion-based accumulation pattern of lipophilic phytohormones across the membrane regardless of their chemical structure. In this context, it is concerning that current *in vitro* data points to multi-substrate specificity for several NPF phytohormone importers (**Supplementary Table 1**). It remains to be investigated whether expression of NPF members in heterologous systems can cause increased internal pH that would lead to an increased ion-trap strength and thereby indirect uptake of many structurally unrelated membrane-permeating phytohormones. This could, for example, happen by the outward movement of protons or potentially by transport of buffer molecules through the transporters. Interestingly, *Streptococcus thermophilus* PepT1, a POT homolog of NPF transporters was recently co-crystallized together with the buffering reagent HEPES—binding within the cavity (Martinez Molledo et al., 2018). The efflux of protons has not yet been reported for any NPF protein nor has transport of HEPES to the best of our knowledge been investigated. We measured pH in oocytes expressing six different NPF proteins with GA import activity and could not detect altered pH in any of them (**Figure 4**). However, we cannot exclude that discrete and transient pH alterations may have eluded detection. In conclusion, when considering the results of transport assays involving membrane permeating lipophilic compounds with weak acid/base properties (such as most phytohormones), it is important to consider and exclude indirect factors that may cause changed distribution across the membrane. For example, if using *Xenopus* oocytes it is possible to inject a buffer with enough buffer capacity to maintain internal pH at neutral (**Figure 3D**).

Second, NPF2.10 and NPF2.11 (GTR1 and GTR2) transport a wide range of glucosinolates that all share the common glucosinolate core structure but with varying amino acid side chains (Nour-Eldin et al., 2012; Andersen et al., 2013; Jørgensen et al., 2017b). Similarly, hPepT1 and hPepT2, two POT transporters involved in dietary peptide uptake in intestines, transport an immense range of peptidomimetics, indicating that a substrate binding cavity accommodating large variations over a common chemical core structure may be a common feature in the NPF (Biegel et al., 2006; Brandsch et al., 2008). Given this apparent plasticity in the substrate-binding cavity, it is conceivable that some NPF members (such as NPF2.10) may accommodate adventitious transport of a wide selection of metabolites (such as JA, ABA, and GAs) at low levels in heterologous systems. Discerning whether such transport activities do represent "real" substrates ultimately, requires deeper insights into the relationship between structure and function of NPF.

Here, we charted the GA substrate preference map of the *Arabidopsis* NPF proteins to elucidate whether it links to distinct structural features within the substrate-binding cavity. Such insights would represent first steps towards understanding the molecular basis of the selectivity of NPF transporters towards organic molecules and lead to an improved ability to predict substrate preference based on sequence information and 3D structure.

To date, almost all NPF-GA transporters have been identified using a two-component GA receptor-based yeast-two-hybrid system, which is qualitative and very sensitive to small influxes of substrate (Chiba et al., 2015). In addition, an *in vivo* approach monitored altered accumulation patterns of a fed bioactive fluorescein-conjugated GA3 in *Arabidopsis* transporter mutants. This approach identified NPF3.1 as a *bona fide* GA transporter with a role in accumulating GA in the endodermis (Shani et al., 2013; Tal et al., 2016). Despite the elegance of these approaches, their qualitative nature offers little distinction between transporters with varying transport activity and are, in their current versions, unable to detect transport of non-bioactive GA shown to undergo long distance transport *in planta* (Regnault et al., 2015; Binenbaum et al., 2018). Given its importance for downstream physiological and structure-function investigations, we found a need for critically re-assessing the *in vitro* data for GA substrate specificity among NPF transporters.

As a first step, we developed an optimized approach to screen for GA transporters among the *Arabidopsis* NPF members. Unlike previous approaches, the one presented here is quantitative, short-term, not limited to bioactive GAs, and gives preliminary insights into GA substrate specificity (Chiba et al., 2015). This is relevant as only some GAs species have been suggested to be transported long-distances in plants (Regnault et al., 2015). Additionally, compartmentalization has been suggested to play a regulatory role in GA signaling, thus, transporters specific for either anabolic or catabolic GA species may be of interest (and can be identified in our screen) (Olszewski et al., 2002). In principle, by screening NPF transporters using a mixture of GAs that include moderately permeating GAs allowed us to screen for both import (increased

accumulation) and export (reduced accumulation of permeating GAs) activity. However, due to the potential ambiguity associated with interpreting accumulation of diffusing GA (described above), we focus on reporting and analyzing import activity only of non-diffusing GAs. In summary, our screen identified 10 *bona fide Arabidopsis* NPF GA transporters belonging to subclades 1 to 4 that transported non-diffusing GAs in oocytes (**Figure 5**).

In parallel, it is equally important to consider which genes our screen did not identify as GA transporters. In contrast to previous *in vitro* data, no members from subclades 5 to 8 were identified in our screen (Chiba et al., 2015). However, lack of GA transport could be attributed to lack of expression or localization to the plasma membrane.

In a study aimed at elucidating the evolutionary origin of glucosinolate transporters, we used YFP tagging to show that >20 NPF proteins from various plant species without exception were successfully expressed and localized to the plasma membrane of *Xenopus* oocytes (Jørgensen et al., 2017b). In other examples, we and others have shown that tonoplast NPF members from Arabidopsis, *Catharanthus roseus* and tomato localize to the plasma membrane of *Xenopus* oocytes (Chiang et al., 2004; Payne et al., 2017) (unpublished data). Thus, from our experience, *Xenopus* oocytes appear to be very well suited for expressing and localizing plant NPFs to the plasma membrane. To a large extent, this property underlies our choice of *Xenopus* oocytes as expression host in this study.

Nevertheless, of the remaining 39 transporters not displaying significant GA uptake in our screen, we note that 15 of these 39 transporters have previously been expressed functionally in *Xenopus* oocytes; NPF2.8, NPF2.10, NPF2.14, NPF5.5, NPF5.10, NPF5.11, NPF5.12, NPF5.16, NPF6.2, NPF6.3, NPF7.2, NPF7.3 NPF8.1, NPF8.2, and NPF8.3 (Tsay et al., 1993; Chiang et al., 2004; Chiu et al., 2004; Dietrich et al., 2004; Komarova et al., 2008; Li et al., 2010; Nour-Eldin et al., 2012; Léran et al., 2015; He et al., 2017; Jørgensen et al., 2017b; Li et al., 2017). Of these, we confirmed activity for NPF2.10 toward glucosinolates and for NPF8.1 towards dipeptides in this study (**Supplementary Figure 5).** Importantly, NPF2.10 shows high uptake activity of glucosinolates, which argues that the low GA uptake by NPF2.10 is not due to low expression of NPF2.10. Based on these observations, we find it highly unlikely that the lack of/low GA transport by these 15 transporters should be due to lack of/or low expression in *Xenopus* oocytes.

We noted that NPF1.3 lacks the large loop between TM6 and TM7 and NPF2.2 lacks a large region equal to parts of TM10 and TM11 of NPF6.3, thus, it is likely that these two transporters are non-functional (data not shown).

Lastly, from (**Figures 5** and **6**) it is evident that a larger number of transporters cause higher accumulation of diffusing GAs. This indicates that these transporters are expressed in the oocytes. However, due to the concerns discussed earlier about inferring activity from diffusing compounds we choose not include these transporters in our list of only 10 *bona fide* NPF GA transporters that are capable of transporting non-diffusing GAs. In conclusion, the quantitative short-term uptake screen presented here significantly reduces the number of potential *Arabidopsis* GA transporters from 25 to 10 and confines them to NPF subclades 1 to 4.

As a second step, we investigated whether molecular determinants of GA substrate specificity could be found among the transporter cavity exposed residues around the substrate binding sites. To identify these residues, we generated a map of all cavity exposed amino acids within an 8 Å sphere to the substrate binding site in all three conformations (outward open, occluded, inward open). This identified 51 positions in the plant NPF structures that we suggest as a general foundation for structure-function elucidation.

PCA analysis of physicochemical properties of the 51 residue subset and corresponding sequence logos did not reveal a clear set of features in the substrate-binding cavity that associated directly with all GA transporting NPFs. However, from our analyses a few residues did emerge that associated with a large portion of GA transporters. Namely, Arg^{Pos16}, Gln^{Pos31}, Leu^{Pos37}, and Ala^{Pos40} are either conserved differently in cluster I and cluster II in the PCA analysis of physicochemical properties compared to clusters III and IV or appear as distinct features in the family wide sequence logo analysis. As clusters I and II contains the majority of GA transporters it is possible that some of these residues are important for GA recognition. Incidentally, an investigation of YePepT from *Yersinia enterocolitica* identified Lys314 as a determinant of specificity towards negatively charged dipeptides (Boggavarapu et al., 2015). Lys314 of YePepT corresponds to position 31 (Leu359 of NPF6.3) in our alignment, which is highly and uniquely conserved as Gln^{Pos31} among the GA transporting NPF members of clusters I and II. Lastly, position 40 (Ala477 of NPF6.3), conserved as an Ala in clusters I and II, is located adjacent to a Glu which is highly conserved in all POTs where it plays a role in binding the N-terminus of peptides (Meredith et al., 2000; Jensen et al., 2012; Solcan et al., 2012). However, in summary as GA transporters NPF4.1 and NPF4.6 are located in cluster IV and that other specificities are known for members of clusters I and II, our analyses indicate that GA may be recognized differently by different NPF GA transporters.

It is intriguing that many of the *Arabidopsis* NPF proteins are able to transport nitrate, without any apparent binding site features substantiating these observations; nitrate is a small molecule, and to some extent, it might be comprehensible that it would be able to trigger the transport mechanism. Our assay results show that GA, a molecule much larger than nitrate, is also able trigger transport mechanism without any apparent conserved binding site (**Figure 8**, **Supplementary Figure 6**).

This apparent promiscuity is concerning as it appears to be accompanied by multi-specificity towards compounds with very different structures. For example, prior to the identification of NPF2.9–2.11 as high affinity glucosinolate transporters (Nour-Eldin et al., 2012; Jørgensen et al., 2017b), NPF2.9 was identified as a low affinity nitrate transporter (Wang and Tsay, 2011). Thus, despite our considerations of technical artifacts we cannot exclude that additional substrates await to be identified for a number of the GA transporters in our list. In fact, we estimate that our knowledge on the substrates transported by members of the plant NPF is still in its infancy. This lack of knowledge confounds investigations on physiological roles and complicates efforts attempting to correlate structure to function. We envision that the development of untargeted approaches to reveal the breath of substrates transported

by each member of the NPF would represent a timely necessity that is required to improve our understanding of this enigmatic family.

AUTHOR CONTRIBUTIONS

NW optimized GA assays, performed the screen, participated in pH measurements, analyzed LC-MS/MS data, contributed to the study design, and wrote the paper based on the draft. MJ did the initial GA experiments including 4-methylthio-3-butenyl and glycylsarcosine screen and contributed to pH measurements in *Xenopus* oocytes, NPF7.3 characterization, data analysis, and the study design. HE made the multiple sequence alignment and defined the binding site residues. SL characterized NPF7.3 mediated phytohormone transport supported by ZB. TM participated in cytosolic pH measurements in *Xenopus* oocytes and data analysis. CC performed the LC-MS/MS analysis. MM synthesized the JA-Ile. DG participated in defining the NPF7.3 effect on the ion-trap mechanism. FJ performed PCA and clustering analysis. OM and HN-E advised and contributed to the study design. HN-E wrote the paper based on the draft. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01106/full#supplementary-material

SUPPLEMENTARY VIDEO 1 | The 51 surface-exposed cavity residues colored green on NPF6.3 (PDB: 4OH3).

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REVIEW
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Developmental Roles of AUX1/LAX Auxin Influx Carriers in Plants

Ranjan Swarup[1,2]* *and Rahul Bhosale*[1,2]

[1] Plant and Crop Sciences, School of Biosciences, University of Nottingham, Nottingham, United Kingdom, [2] Center for Plant Integrative Biology (CPIB), University of Nottingham, Nottingham, United Kingdom

Plant hormone auxin regulates several aspects of plant growth and development. Auxin is predominantly synthesized in the shoot apex and developing leaf primordia and from there it is transported to the target tissues e.g. roots. Auxin transport is polar in nature and is carrier-mediated. AUXIN1/LIKE-AUX1 (AUX1/LAX) family members are the major auxin influx carriers whereas PIN-FORMED (PIN) family and some members of the P-GLYCOPROTEIN/ATP-BINDING CASSETTE B4 (PGP/ABCB) family are major auxin efflux carriers. AUX1/LAX auxin influx carriers are multi-membrane spanning transmembrane proteins sharing similarity to amino acid permeases. Mutations in *AUX1/LAX* genes result in auxin related developmental defects and have been implicated in regulating key plant processes including root and lateral root development, root gravitropism, root hair development, vascular patterning, seed germination, apical hook formation, leaf morphogenesis, phyllotactic patterning, female gametophyte development and embryo development. Recently AUX1 has also been implicated in regulating plant responses to abiotic stresses. This review summarizes our current understanding of the developmental roles of *AUX1/LAX* gene family and will also briefly discuss the modelling approaches that are providing new insight into the role of auxin transport in plant development.

Keywords: auxin, AUX1/LAX, influx carriers, plant, development

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***Correspondence:**
Ranjan Swarup
Ranjan.Swarup@nottingham.ac.uk

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INTRODUCTION

Auxin is a key plant hormone that regulates several aspects of plant growth and development including plant tropic responses, embryo development, root development, shoot development, leaf development and phylotactic patterning and there are several good reviews highlighting the role of auxin in plant development (Reinhardt, 2003; Leyser, 2006; Sieburth and Deyholos, 2006; Vanneste and Friml, 2009; Wang and Estelle, 2014; Lavy and Estelle, 2016).

It is known for a long time that auxin transport is polar in nature. Following its synthesis in the leaf primordia and shoot apical meristem, auxin is transported downwards to its target tissues using either through the bulk flow in the phloem or through the polar auxin transport stream (Swarup et al., 2001; Vanneste and Friml, 2009; Swarup and Péret, 2012; Swarup and Bennett, 2014).

Auxin was the first plant hormone to be discovered (Went, 1927). Indole-3-acetic acid (IAA), the major form of auxin in higher plants, is a weak acid (pKa = 4.85) and, at the intracellular pH, exists in its membrane impermeable (IAA-) form. However, in the extracellular apoplast, where the pH is slightly acidic (pH ~5.5), IAA exists both as membrane permeable IAAH form and membrane impermeable (IAA-) form (Zazímalová et al., 2010; Swarup and Péret, 2012; Swarup and Bennett, 2014). Zazímalová et al. (2010) highlighted the importance of auxin influx carriers when they calculated that at apoplastic pH, 83% of IAA is in its membrane impermeable IAA- form

and would need a carrier for its import in the cell. Interestingly, even before any of the auxin carriers were discovered, as part of the chemiosmotic hypothesis, Rubery and Sheldrake (1974) and Raven (1975) independently proposed that auxin transport is carrier-mediated and the polarity of auxin movement is likely to be provided by asymmetric localization of auxin transporters (Swarup and Bennett, 2014; Mohanta et al., 2018). It is now well established that auxin transport is carrier-mediated and is facilitated by auxin influx carriers and efflux carriers. Auxin influx carriers mediate the uptake of auxin inside the cells whereas auxin efflux carriers are required for the export of auxin out of the plant cells. In *Arabidopsis*, the AUX1/LAX family of auxin transporters represent the major influx carriers (Bennett et al., 1996; Swarup et al., 2001; Swarup et al., 2008; Péret et al., 2012) whereas PIN (Chen et al., 1998; Gälweiler et al., 1998; Luschnig et al., 1998; Steinmann et al., 1999; Friml et al., 2002; Friml et al., 2003; Blilou et al., 2005; Wisniewska et al., 2006; Rahman et al., 2010; Bosco et al., 2012) and PGP/ABCB (Noh et al., 2003; Terasaka et al., 2005; Blakeslee et al., 2007; Cho et al., 2007; Zhang et al., 2018) family members encode the major auxin efflux carriers. Using an *in-silico* approach, Barbez et al. (2012) identified *PILS* (*PIN LIKES*) gene family that also appears to be regulating auxin homeostasis. In addition, it has been suggested that nitrate transporter NRT1.1 can also be involved in auxin transport (Krouk et al., 2010) and can regulate lateral root formation depending on the nitrogen status of the plant (Krouk et al., 2010). This may provide a direct mechanism for soil nutrient status mediated auxin dependent regulation of lateral root development.

This review will focus on our current understanding of the roles of AUX1/LAX proteins in regulating auxin transport during plant development. There have been a few comprehensive reviews covering the role of auxin influx carriers in plant development in general (Swarup and Péret, 2012; Swarup and Bennett, 2014) or AUX1 in particular (Singh et al., 2018) and so this review will only briefly discuss the topics covered in those reviews. Here, we focus primarily on new understanding of AUX1/LAX auxin influx carriers and their roles in plant development.

AUX1/LAX GENE FAMILY IN *ARABIDOPSIS*

In the chemiosmotic hypothesis, both Rubery and Sheldrake (1974) and Raven (1975) independently proposed that auxin transport is carrier-mediated but it was not until 1985 when Lomax et al. (1985) showed that IAA uptake is an active process and is driven by proton motive force.

Working on suspension-cultured tobacco cells, Delbarre et al. (1996) revealed that IAA and 2,4-D (2,4-Dichlorophenoxyacetic acid-a synthetic auxin) uptake in the cells is carrier-mediated but in contrast, lipophilic auxin 1-naphthalene acetic acid (1-NAA) enters the cells through passive diffusion. This indeed turned out to be true when the first auxin influx carrier was cloned and characterized (Bennett et al., 1996; Marchant et al., 1999). Cloning of *AUX1* gene revealed similarity to amino acid permeases. Considering IAA is structurally similar to tryptophan, explains the evolution of these plant specific sub class of the amino acid permease superfamily that now is known as auxin amino acid permease superfamily. In *Arabidopsis*, the *AUX1/LAX* gene family is comprised of four members *AUX1, LAX1, LAX2*, and *LAX3* sharing 75–80% similarity at protein level (Swarup and Péret, 2012; Swarup and Bennett, 2014). These genes encode multi-transmembrane (TM) spanning proteins and share similarity to amino acid transporters (Young et al., 1999; Péret et al., 2012). *Arabidopsis* AUX1/LAX proteins have been shown to take up auxin in heterologous expression systems (Yang et al., 2006; Swarup et al., 2008; Péret et al., 2012) and mutations in *AUX1/LAX* genes result in auxin related developmental defects (Bennett et al., 1996; Swarup et al., 2001; Swarup et al., 2004; Swarup et al., 2005; Swarup et al., 2007; Swarup et al., 2008; Bainbridge et al., 2008; Péret et al., 2012). The founding member of this family, AUX1 has been well studied and shown to regulate root gravitropism (Bennett et al., 1996; Swarup et al., 2001; Swarup et al., 2004; Swarup et al., 2005), whereas AUX1 and LAX3 both shown to regulate lateral root development (Swarup et al., 2008). LAX2 has been shown to facilitate vascular development (Péret et al., 2012) and AUX1, LAX1 and LAX2 have been shown to act in a redundant manner to regulate phyllotactic patterning (Bainbridge et al., 2008). These studies highlight the functional importance of AUX1/LAX proteins in auxin transport.

In the following sections, we will review our understanding of the role of AUX1/LAX auxin influx carriers in regulating plant development in *Arabidopsis* and highlight their importance in several biological processes from seed germination to root, shoot, and flower development and embryogenesis (**Figure 1**).

Root Gravitropism

Gravity in the roots is perceived in the columella cells. Upon gravity perception, differential movement of auxin from the site of gravity perception to the gravity-responsive tissues of the elongation zone results in root bending as higher auxin levels on the lower side of the root inhibits cell elongation whereas cells on the upper side are still elongating (Ottenschläger et al., 2003; Swarup et al., 2013; Sato et al., 2015; Muller et al., 2018). Differential auxin gradient is facilitated by auxin efflux carrier PIN3 that relocalizes to the new bottom of the cell within minutes after the gravity stimulus (Friml et al., 2002). In addition, PIN7 that has been reported to regulate root bending in detached lateral roots (Ruiz Rosquete et al., 2018), appears to be involved in some kind of compensation mechanism in primary roots as PIN7-GFP is expanded into the PIN3 expression domain in the *pin3* mutants (Kleine-Vehn et al., 2010). Using a modelling approach, Band et al. (2012), revealed that auxin asymmetry is transient and lasts only about 100 min and after which the auxin distribution becomes symmetric. They proposed a "tipping point mechanism" that reverses the auxin flow as the root tips reach an angle of 40°.

Among the AUX1/LAX family members in *Arabidopsis*, only AUX1 has been implicated in regulating root gravitropic response. A recent report suggests that AUX1 acts upstream of PIN2 in regulating root gravitropism (Liu et al., 2018) but we believe that they act in concert. Mutation in *aux1* results in agravitropic roots. *AUX1* is expressed in tissues involved in gravity perception (columella), gravity signal transmission (lateral root cap) and

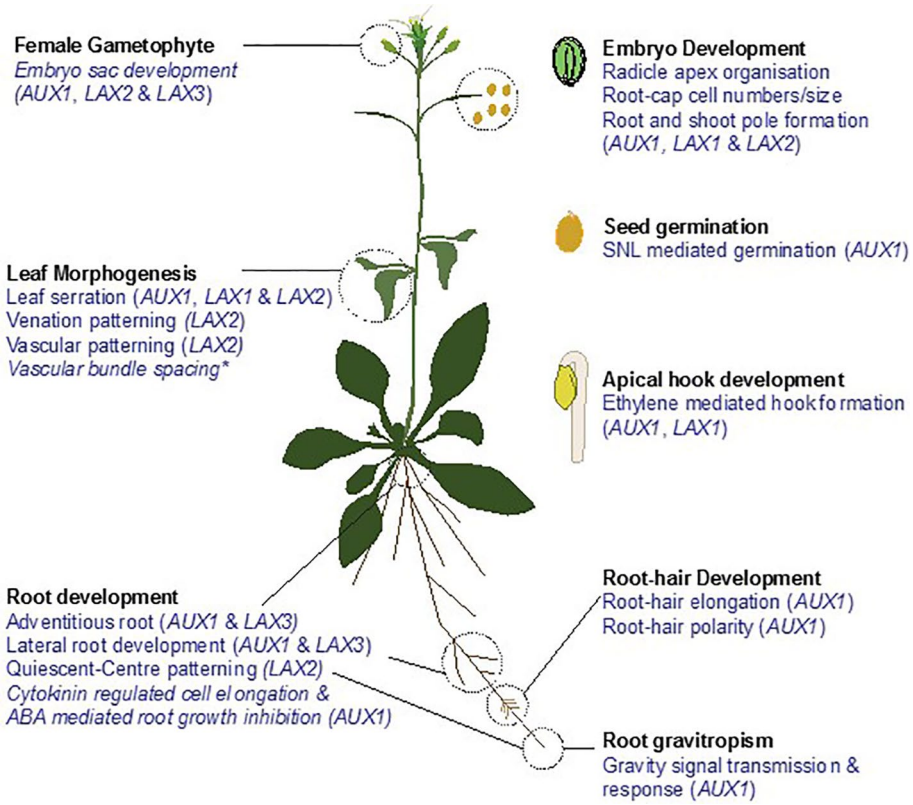

FIGURE 1 | Roles of AUX1/LAX family in development of *Arabidopsis thaliana*. Auxin influx carrier *AUX1* and its homologs *LAX1*, *LAX2*, and *LAX3* have been implicated in regulating root gravitropism; root, lateral & adventitious root development; root-hair development, leaf morphogenesis; female gametophyte development; embryo development; seed germination and apical hook development. Names in parenthesis indicate the genes controlling the respective role. *indicates the role of AUX1/LAX family deciphered from modelling studies and quadruple mutant analysis. Aradidopsis plant cartoon template adapted from (Bhosale et al., 2019).

gravity response (epidermis) (Swarup et al., 2001; Swarup et al., 2004; Swarup et al., 2005). Using a transactivation-based approach, Swarup et al. (2005) probed which AUX1 expression domains are required for root gravitropism and revealed that *AUX1* expression in lateral root cap and epidermal cells was sufficient to rescue root gravitropic defect of *aux1* mutants. Interestingly, *AUX1* columella expression was not required for restoration of root agravitropic defect of *aux1* mutants. Genetic redundancy could be a possible explanation as both *LAX2* and *LAX3* are known to express in the columella cells.

Recently, Pernisova et al. (2016) proposed that AUX1 mediated auxin influx is subject to regulation by cytokinins. They found that multiple cytokinin receptor mutants show altered root angles in gravi-stimulated roots and *35S:CKX* lines over expressing cytokinin degradation enzyme cytokinin dehydrogenase/oxidase show delayed root gravitropism. Interestingly, treatment with exogenous cytokinins does not affect root gravitropic response and so the authors suggest that proper concentration of endogenous cytokinins is crucial for gravity induced auxin redistribution. They also show that AUX1-YFP signal is reduced in these cytokinin depleted lines but the relocalization kinetics of PIN3 and PIN7 was comparable to wild type. It will be interesting to check if treatment with exogenous cytokinins can restore AUX1 signal in the cytokinin depleted lines to further understand the role of cytokinin in regulating auxin influx and root gravitropism.

Vascular Development

Auxin is known to regulate vascular development. Direct treatment with auxin promotes vascular development and several auxin transport and response mutants have defects in vascular patterning (Reinhardt, 2003; Sieburth and Deyholos, 2006; Péret et al., 2009a, Péret et al., 2009b; Petrásek and Friml, 2009; Yang and Wang, 2016). Despite, the role of auxin influx carriers has not been very clear until recently. Now, there is some evidence for the role of LAX2 in regulating vascular patterning in the cotyledons as *lax2* mutants have higher propensity of discontinuity in vascular strands in the cotyledon (Péret et al., 2012). More recently, Fàbregas et al. (2015) used a computational, modelling and experimental approaches to investigate the role of influx carriers in vascular development. Their theoretical approach predicted that the auxin influx carriers increase the periodicity of auxin maxima. They tested these predictions using *aux1/lax* quadruple mutants and showed that these mutants have fewer and more spaced vascular bundles. Additionally, they showed

that AUX1/LAX proteins also regulate xylem differentiation in both the shoot and the root.

Seed Germination

Plant hormones such as Gibberellic Acid (GA) and Abscisic Acid (ABA) are known to regulate seed germination (Koornneef et al., 2002; Finkelstein et al., 2008). GA promotes germination and ABA inhibits germination. Auxin has also been implicated in regulating germination (Holdsworth et al., 2008). Low concentration of auxin promotes seed germination and higher concentrations inhibit germination (Hsueh and Lou, 1947; Brady et al., 2003). Auxin appears to affect seed germination through GA/ABA signaling pathways as auxin response factors ARF10 and ARF16 can modulate expression of *ABSCISIC ACID INSENSITIVE 3 (ABI3)* and imbibed seeds have increased expression of auxin influx and efflux carriers compared to dormant seeds (Carrera et al., 2007; Liu et al., 2013).

It is emerging now that histone modification may play a crucial role in regulating seed germination by regulating the expression of several auxin related genes. Recently, Wang et al. (2017) showed that mutations in histone deacetylase-binding factor genes, *SNL1* (*SWI-INDEPENDENT3 (SIN3)-LIKE1)* and *SNL2* result in increased speed of seed germination and faster radicle protrusion. They also showed that expression of several auxin synthesis, transport and responses genes were up regulated in *snl1snl2* double mutants and these mutants accumulated significantly more IAA than wild type plants. This led Wang et al. (2017) to investigate the seed germination in auxin related mutants. Though many auxin related genes were upregulated in *snl1snl2* double mutant, many of their mutants had no obvious seed germination defects. However, they did find that *aux1* mutants had weakly decreased germination of fresh seeds compared to controls. Further investigation showed that AUX1 levels were higher in the radicle tip of the *snl1snl2* double mutants and AUX1 was a target for SNL mediated H3K9/18 histone deacetylation. Moreover, they also showed that the cell cycle genes *CYCD1;1* and *CYCD4;1* were upregulated in *snl1snl2* double mutants, which is also in agreement with increased cell cycle activity in these mutants. In addition, auxin also can induce the expression of *CYCD1;1* and *CYCD4;1*. Taken together, they showed that SNLs negatively regulate radicle protrusion and AUX1 plays a crucial role in SNL mediated seed germination. They further proposed a model, where during embryo development and seed maturation high SNL levels cause deacetylation of ABA hydrolysis genes causing high ABA levels inhibiting seed germination. During imbibition, the SNL levels go down that promotes expression of auxin synthesis and transport genes including *AUX1*. Increased auxin levels, in turn, switch on cell cycle genes that promote radicle growth.

Apical Hook Development

Apical hook protects the shoot apical meristem during seedling emergence from the soil (Abbas et al., 2013). Light and plant hormones such as auxin, ethylene, gibberellins, and brassinosteroids are key signals regulating apical hook development (Abbas et al., 2013; Smet et al., 2014). Vandenbussche et al. (2010) showed that apical hook formation requires fresh auxin synthesis on the inner side of the apical hook in an ethylene dependent fashion. This results in creation of an auxin gradient and the inhibition of cell elongation on the inner side, in turn, resulting in differential growth and apical hook formation.

Among the auxin influx carriers, AUX1 and LAX3 have been implicated in regulating apical hook development in *Arabidopsis* (Roman et al., 1995; Vandenbussche et al., 2010). Vandenbussche et al. (2010) showed that *aux1* and *lax3* mutants show less exaggerated apical hook upon ethylene treatment. *AUX1* is expressed in the hook region and it is localized in the epidermal cell membranes (Vandenbussche et al., 2010). In contrast, *LAX3* is localized to the plasma membrane in the outer tissues on the basal parts (but not in the apical hook region) of hypocotyl. Vandenbussche et al. (2010) proposed that LAX3 is the major auxin influx carrier in hook development with AUX1 facilitating movement of auxin from the cotyledons and shoot apical meristem to the apical hook whereas LAX3 draining auxin out of the hook.

Though both AUX1 and LAX3 (along with auxin efflux carrier PIN3— Zádníková et al., 2010) regulate apical hook formation, their localization to the plasma membrane appears to be under distinct genetic regulation. Working in the *echidna* mutants, Boutté et al. (2013) showed that *echidna* mutants have defects in apical hook formation. They also have defects in differential auxin gradient formation in the apical hook. Boutté et al. (2013) showed that ECHIDNA is predominantly localized to the secretory vesicles at the trans-Golgi network (TGN) and in *echidna* mutants AUX1 trafficking to the plasma membrane is disrupted. In contrast, LAX3 or PIN3 trafficking is only marginally altered in *echidna* mutants. These findings suggest that not only there are distinct mechanisms for the trafficking of auxin influx and efflux carrier proteins to the plasma membrane, even the closely related AUX1 and LAX3 follow distinct trafficking pathways from the trans-Golgi network (TGN) to the plasma membrane in the hypocotyl.

Kleine-Vehn et al. (2006) had shown previously that polar AUX1 and PIN1 trafficking to the plasma membrane in the root protophloem cells follow distinct pathways with PIN1 but not AUX1 trafficking being GNOM dependent. *GNOM* encodes membrane-associated guanine–nucleotide exchange factor on ADP-ribosylation factor G protein (ARF-GEF) (Steinmann et al., 1999). Guanine nucleotide exchange factors are involved in the activation of small GTPases which act as molecular switches in the intracellular signaling pathways (Casanova, 2007; Richter et al., 2014). In *Arabidopsis*, there are eight ARF-GEFs which are grouped into two classes based on their similarity to human big ARF-GEFs, GBF1, and BIG1 (not to be confused with calossin-like protein BIG/DOC/CRM—Gil et al., 2001). There are three GBF1 related members (GNOM, GLN1, GLN2) and five BIG1 related members (BIG1 to BIG5) (Richter et al., 2014). BIG1-4 have been implicated in post Golgi trafficking.

Recently, Jonsson et al. (2017) showed that in hook development, AUX1 trafficking is mediated by ECHIDNA, ARF1, and BIG proteins. They showed that ARF1 and BIG4 colocalize with ECHIDNA in the TGN and *arf1* and *big* mutants have defects in AUX1 trafficking and show hook developmental defects similar to *aux1* mutants. In *echidna* mutants, ARF1

and BIG cannot localize to the TGN whereas in *arf1* and *big* mutants, ECHIDNA localization is perturbed. They proposed that ECHIDNA and BIG facilitate recruitment of ARF1 to the TGN that then facilitates vesicle formation for AUX1 delivery to the plasma membrane, which is a prerequisite for the ethylene mediated hook development.

Root Development

During root development, cells arise linearly from a group of stem cells surrounding the quiescent center (QC). Stem cells divide asymmetrically to give rise to daughter cells that move away from the stem cell niche and differentiate (Scheres, 2007). Auxin is crucial for specification of the QC and QC is crucial for the maintenance of the stem cell fate. Cytokinin is important for promoting cell differentiation and auxin and cytokinin act antagonistically to regulate root development (Aloni et al., 2006).

Zhang et al. (2013) showed that auxin influx carrier LAX2 plays a role in regulating QC patterning in the roots *via* an auxin–cytokinin module. In *Arabidopsis*, cytokinin signaling is mediated by a multi-step phosphorelay mechanism involving cytokinin receptors AHKs (*Arabidopsis* Histidine Kinases) AHPs (*Arabidopsis* histidine phosphotransfer proteins) and ARRs (*Arabidopsis* response regulators). Upon cytokinins perception, AHKs relay the signal to ARRs *via* AHPs. ARRs act as transcriptional regulators to regulate gene expression (Keshishian and Rashotte, 2015). Zhang et al. (2013) show that cytokinin promotes cell division in the QC and downregulate the expression of several genes including *LAX2* in ARR1 and ARR12 dependent fashion and ARR1 directly binds to *LAX2* gene. Moreover, *lax2* mutants have increased cell division in the QC thus phenocopying cytokinin treatment. Taken together, Zhang et al. (2013) proposed that cytokinin suppresses *lax2* expression to regulate auxin distribution in the root apical meristem.

Cytokinins inhibit cell elongation and cell proliferation. Street et al. (2016) recently showed that cytokinin inhibits root cell elongation *but not cell proliferation* in an AUX1 dependent fashion. Using an innovative screen in *arr1* and *arr12* background to identify novel regulators of cytokinin mediated root development. Two of the enhancers (termed *enhancers of response regulators* or *err*) turned out to have mutations in the *AUX1* gene (Pro371Leu and Gly374Ser) in the extracellular loop between 9th and 10th transmembrane regions suggesting the role of AUX1 in cytokinin mediated root development. Indeed, *aux1* mutants showed reduced root growth inhibition to cytokinin treatment but not *lax1, lax2 or lax3*. They further observed that the expression of the type-B response regulator *ARR10* is auxin and AUX1 dependent and led them to propose that cytokinin and auxin regulate expression of *ARR10* and *AUX1* as part of an auto regulatory circuit.

AUX1 has also been implicated in ABA mediated inhibition of root growth. ABA is known to inhibit root growth at high concentration and recently, Li et al. (2017) showed that this is mediated *via* AUX1 in an ethylene dependent pathway.

Lateral Root Development

Lateral roots originate from the xylem pole pericycle cells that undergo a series of division to create the lateral root primordia (Dubrovsky et al., 2000; Dubrovsky et al., 2001; Péret et al., 2009a; Lavenus et al., 2013; Du and Scheres, 2018). This new primordium has to penetrate several cell layers before emergence (Swarup et al., 2008; Péret et al., 2009b; Swarup and Péret, 2012). Auxin is one of the key signals regulating lateral root development (Benková et al., 2003; Swarup et al., 2008; Péret et al., 2009a; Swarup and Péret, 2012; Du and Scheres, 2018). Mutations in auxin influx carriers *AUX1* result in about 50% reduction in lateral root numbers (Hobbie and Estelle, 1995; Marchant et al., 2002). Swarup et al. (2008) showed that *lax3* mutants also have 50% reduction in lateral root numbers and *aux1lax3* double mutants have severe reduction in lateral root emergence and show almost no emerged lateral root primordia up to day 14. Both *AUX1* and *LAX3* show contrasting and non-overlapping expression patterns with *AUX1* being expressed in the primordia whereas *LAX3* is completely excluded from the primordia and is expressed in the cortical and epidermal cells facing the primordia (Marchant et al., 2002; Swarup et al., 2008). To explain lateral root emergence, Swarup et al. (2008) proposed an elegant model based on the facts that the *LAX3* gene is auxin inducible; auxin maxima is localized at the tip of the developing primordia and several cell wall modelling enzymes (Cosgrove, 2000; Cosgrove, 2005) are auxin inducible in a LAX3 dependent manner. They proposed that auxin from the developing primordia acts as a signal to induce *LAX3* in the cortex. In a positive feedback loop, *LAX3* induction results in build-up of auxin in the cortex cells that then results in the induction of cell wall remodeling enzymes to facilitate smooth passage of the primordia through the cortex. Similar mechanism then facilitates primordia emergence through the epidermis. Porco et al. (2016a) provided further mechanistic insight into the regulation of lateral root emergence. They showed that induction of *LAX3* by auxin is mediated by *LBD16* which acts upstream of LAX3. Additionally, Orman-Ligeza et al. (2016) showed that reactive oxygen species (ROS) also involved in lateral root emergence by cell wall remodeling of overlaying tissues.

Light has been known to regulate root system architecture, but the mechanism has not been well understood. Recently, van Gelderen et al. (2018) revealed that light regulate lateral root development through regulating auxin transport. They showed that low R:FR perception in the shoot inhibits lateral root emergence. This is achieved *via* HY5 (ELONGATED HYPOCOTYL5) that accumulates in the lateral root primordia in phytochrome dependent fashion and regulates the plasma membrane abundance of LAX3 and PIN3 to reduce auxin levels in the overlaying cortex cells to reduce lateral root outgrowth.

Adventitious Root Development

Adventitious roots are post embryonic roots and auxin has been known to regulate their formation (Veloccia et al., 2016; Fattorini et al., 2017). Recently, two independent reports suggest a possible role of AUX1 and LAX3 in this process. Veloccia et al. (2016) showed that both ethylene and auxin regulate adventitious root formation in *Arabidopsis*. Treatment of *Arabidopsis* seedlings with ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) resulted in increased number of adventitious roots in AUX1/LAX3 dependent fashion. It appears that AUX1/LAX3 mediated adventitious root formation is regulated by ARF7/

ARF19-LBD16/LBD18 module as *aux1*, *lax3*, *lbd16*, *lbd18*, *arf7*, and *arf19* single mutants have reduced number of adventitious roots whereas *aux1lax3lbd16lbd18* quadruple mutants lack adventitious roots (Lee et al., 2019).

Root Hair Development

Auxin plays a critical role in root hair development (Knox et al., 2003; Fischer et al., 2006; Salazar-Henao et al., 2016). Auxin treatment promotes root hair elongation and mutations in *AUX1* gene results in shorter root hairs that can be restored to wild type levels by exogenous auxin. Interestingly, *AUX1* is expressed in non-hair epidermal cells but not in the hair cells (Jones et al., 2009). Computer simulation showed that expression of *AUX1* in the non-hair cells can still result in over 10-fold accumulation of auxin in hair cells and thus Jones et al. (2009) concluded that non-hair cells affect auxin abundance in hair cells. PIN2 which is expressed in both root hair and non-hair cells can facilitate auxin efflux out of the non-hair cells and into the apoplast and despite no AUX1 in the hair cells, these root hair cells can still maintain high auxin concentration.

Root hair cell polarity has also been shown to be regulated by auxin (Grebe et al., 2002). Root hairs are formed on the basal side of the hair cells and auxin treatment results in more basal position of root hairs (Grebe et al., 2002). AUX1 has been implicated in regulating this root hair polarity by auxin as in *aux1* mutants root hairs are formed not only on more apical side but they also have 30% increased frequency of double root hairs (Grebe et al., 2002).

Recently, Dindas et al. (2018) showed that AUX1 also mediates proton-coupled auxin transport in root hairs. Membrane depolarization is one of the earliest auxin responses in a cell. Using an electrophysiological approach and measuring membrane potential using intracellular mini-electrodes, Dindas et al. (2018) showed that IAA alters membrane potential in a pH and concentration dependent manner and *aux1* mutants are severely impaired in IAA mediated membrane depolarization. They also showed that IAA influx is coupled with changes in cytosolic calcium and calcium influx is impaired in *aux1* mutants. Interestingly, they find that auxin receptors TIR1/AFB are also involved in IAA mediated membrane depolarization and calcium influx. This suggests that early events in auxin signaling are non-genomic. This led Dindas et al. (2018) to propose a very short auxin signaling module, where a cytosolic component of $SCF^{TIR/AFB}$ binds to IAA that then regulates opening of calcium channels and hence an increase in intracellular Ca^{++} levels.

Auxin mediated root hair elongation is a key adaptive response to low P (Bates and Lynch, 1996; Lynch, 2011). Recently, Giri et al. (2018) and Bhosale et al. (2018) showed that root hair elongation under low P is mediated by AUX1. Bhosale et al. (2018) provided a mechanistic frame work for low P mediated root hair elongation in *Arabidopsis*. They showed that auxin homeostasis (Porco et al., 2016b) is crucial for root hair elongation under low P. They also showed that under low P, there is accumulation of auxin in the root apex through induction of *TAA1*, a key enzyme in auxin biosynthesis. AUX1 then facilitates the movement of this auxin in a shootward direction into the root hair zone where it facilitates root hair elongation. In this study, they not only showed that both *taa1* and *aux1* mutants have defects in root hair elongation under low P but also mapped the tissues required for root hair elongation under low P. They showed that expression of *AUX1* in lateral root cap and epidermal cells files is sufficient to rescue low P mediated root hair elongation defect in *aux1* mutants. But what happens when auxin has reached the root hair zone? What are the down-stream components mediating this low P mediated root hair elongation response? To investigate this, Bhosale et al. (2018) used a global gene expression profiling approach and showed that auxin response factor *ARF19* is induced under low P and genetic analysis confirmed the role of ARF19 in regulating root hair elongation response under low P as *arf19* mutants were defective in root hair elongation under low P. Next, they asked the question, what are the targets for ARF19? bHLH transcription factor RSL4 is a known regulator of root hair elongation and the transcriptome as well as reporter studies suggested that *RSL4* and its close homolog *RSL2* both are induced in the root apex under low P and both *rsl4* and *rsl2* mutants had defects in low P mediated root hair elongation. A close examination of the *RSL2* and *RSL4* promoters revealed several auxin response elements suggesting a possible mechanism for auxin mediated root hair elongation. Based on these results, Bhosale et al. (2018) proposed that low P results in increased auxin accumulation under low P through TAA1. This auxin is then moved to the root hair zone through AUX1 where it induces the expression of auxin inducible ARF19 that then induces the expression of bHLH transcription factors RSL2 and RSL4 that facilitate root hair elongation.

Leaf Morphogenesis

PIN based auxin efflux has been previously shown to define regions of fast and slow growing areas in leaf margins regulated by CUC (CUP-SHAPED COTYLEDON) transcription factors, that are known to regulate organ boundaries in plants (Nikovics et al., 2006). Recently, Kasprzewska et al. (2015) have shown that auxin influx is also required for leaf serration in *Arabidopsis*. *AUX1*, *LAX1*, and *LAX2* are all expressed in the leaves and show non-overlapping and dynamic expression patterns. *AUX1* expression is more confined to the leaf margins. In contrast, *LAX2* expression is excluded from the margins and is localized more towards the center of the leaf primordia and gradually gets confined to the leaf vasculature. *LAX1* expression appears to be most dynamic and is mainly seen in the leaf tip and the flanks in the young leaf primordia. Later, new *LAX1* expression sites are seen at the leaf margins proximal to the original sites which Kasprzewska et al. (2015) argued could be "presumptive sites of serration". Despite non-overlapping expression patterns, single or double *AUX1/LAX* mutants don't show any leaf serration defects. However, *aux1/lax* quadruple mutants and *aux1lax1lax2* triple mutants have reduced leaf serrations. Kasprzewska et al. (2015) argued that this cannot be explained by simple genetic redundancy and so they used a modelling approach and proposed a "*margin-patterning*" model in which AUX1/LAX1/LAX2 auxin import module regulates extent of leaf serration.

More recently, Moreno-Piovano et al. (2017) also showed the involvement of LAX2 in leaf venation patterning and normal xylem development. They showed that *lax2* mutants have

increased xylem length and number of xylem cell rows which can be restored by expression of *LAX2* suggesting that auxin homeostasis regulates leaf venation patterning.

Female Gametophyte Development

Female gametophyte (megagametophyte) development begins with mega spore mother cell undergoing meiotic division and giving rise to four haploid cells. One of the haploid cell becomes a functional megaspore and undergoes three rounds of mitosis to produce seven-celled eight-nucleate highly polarized megagametophyte comprised of two synergid cells, one egg cell, one central cell and three antipodal cells. Auxin efflux carrier PIN1 was previously shown to play a role in regulating female gametophyte development (Ceccato et al., 2013). Recently, Panoli et al. (2015) showed that auxin influx and local auxin biosynthesis are also crucial in this process. They revealed that while AUX1 is primarily localized in the synergids and egg cell membranes, LAX1 is seen localized in the sporophytic tissues of nucellus surrounding the micropylar pole of embryo sac. Single, double and triple and quadruple mutants' analysis of *AUX1/LAX* family members revealed that *aux1lax1lax2* triple mutant and *aux1lax1lax2lax3* quadruple mutant had multiple gametophyte defects with about 29% ovules showing aberrant embryo sacs. Panoli et al. (2015) further provided evidence that besides the auxin import, local auxin biosynthesis through YUCCA/TAA pathway also mediate mitotic cell division and cell specification during female gametophyte development.

Embryo Development

Genetic and pharmacological studies show that auxin is crucial for embryo development (Hardtke and Berleth, 1998; Bhatia et al., 2016). For example, mutations in one of the key auxin signaling genes *MONOPTEROS/AUXIN RESPONSE FACTOR5* result in severe embryonic defects (Hardtke and Berleth, 1998; Bhatia et al., 2016). In addition, previous studies have shown the importance of auxin transport in embryo development as *AUX1/LAX* quadruple mutant are reported to have disorganized radicle apex and an increase in the root-cap cell numbers and/or cell size (Ugartechea-Chirino et al., 2010).

More recently, Robert et al. (2015) showed that auxin influx carriers AUX1, LAX1, and LAX2 are required for embryonic root and shoot pole formation. Of the four *AUX1/LAX* genes, *AUX1*, *LAX1*, and *LAX2* are expressed in the embryo. They showed that *AUX1* is specifically expressed in the 32-cell embryo stage and later in the provascular cells. *LAX2* is also expressed in the perivascular cells from 32-cell embryo stage onward and is also expressed in the hypophysis and the uppermost suspensor cell. In contrast, *LAX1* is expressed very early on from the one-cell stage in the apical cell and from 32-cell stage, its expression is more pronounced in the upper tier cells and by heart stage embryo, *LAX1* expression is confined to the cotyledon tips. No *LAX3* expression is reported in the embryo.

To get a better understanding of the role of auxin influx carriers in embryo development, Robert et al. (2015) specifically used *AUX1/LAX* double and triple mutant combinations as previous studies had reported no embryo related defects in *aux1*, *lax1*, and *lax2* single mutants (Bainbridge et al., 2008). They uncovered patterning defects in the upper pole in the *aux1lax1* double mutants at a low frequency and this was considerably more pronounced both in the severity and the frequency in the *aux1lax1lax2* triple mutants. The embryo defects were manifested later in the seedlings as about one quarter of the seedlings showing mono cotyledon and/or stubby roots. These defects resembled *monopteros* (*mp*) and *bodenlos* (*bdl*) mutants (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Hamann et al., 1999). Furthermore, they observed that expression of *AUX1* and *LAX2* but not *LAX1* was reduced or absent in the strong MP allele *mpB4149*. This led Robert et al. (2015) to conclude that MP/BDL signaling module regulates AUX1/LAX mediated auxin import into the cell.

More recently, Liu et al. (2017) showed that ROPGEF1 regulates AUX1 polar localization in the embryo and the roots. ROPGEFs are guanine nucleotide exchange factors that are known to activate Rho GTPases of plants. AUX1 is localized to the apical face of the cell in the embryo central vascular cells and the protophloem cells. Liu et al. (2017) showed that in *ropgef1* mutant apical AUX1 localization has shifted from apical to basal position in the embryo and also in the root protophloem cells. They also showed that *ropgef1* mutants also have altered accumulation of PIN2 and PIN7 and cannot establish asymmetric auxin gradient in gravistimulated roots and have embryo defects as well as cotyledon vein breaks and altered root gravitropic response.

AUX1/LAX GENE FAMILY AND THEIR ROLES ACROSS PLANT SPECIES

With advances in genome sequencing, *AUX1/LAX* homologs have been identified in several plant species (**Figure 2**) including rice, maize, wheat, barley, *Setaria, Medicago*, and *Brachypodium*. (Hochholdinger et al., 2000; Zhao et al., 2015;Huang et al., 2017; van der Schuren et al., 2018). They all show high similarity to AtAUX1 at protein level (71–90%). In this section, we will briefly review our current understanding of the role of *AUX1/LAX* gene family across plant species. For sake of brevity, we will only focus on model plants where there are supporting functional and/or genetic evidence for the role of *AUX1/LAX* genes in regulating plant development (**Figure 3**).

Inflorescence Architecture in *Setaria* and Maize

Recently, Huang et al. (2017) demonstrated that *AUX1* homologs in *Setaria viridis* (*SvAUX1)* and maize (*ZmAUX1)* are involved in inflorescence development and root gravitropism. Inflorescence architecture is an important agronomic trait as it influences grain yield. Using a forward genetic approach, Huang et al. (2017) showed that in *S. viridis,* mutations in *SvAUX1,* result in major defects in inflorescence branch development leading to sparse panicle (spp) phenotypes. These mutants (*spp1-1* and *spp1-3)* also show decreased plant height, reduced inflorescence branching and spikelet numbers and increased panicle length compared to the control plants.

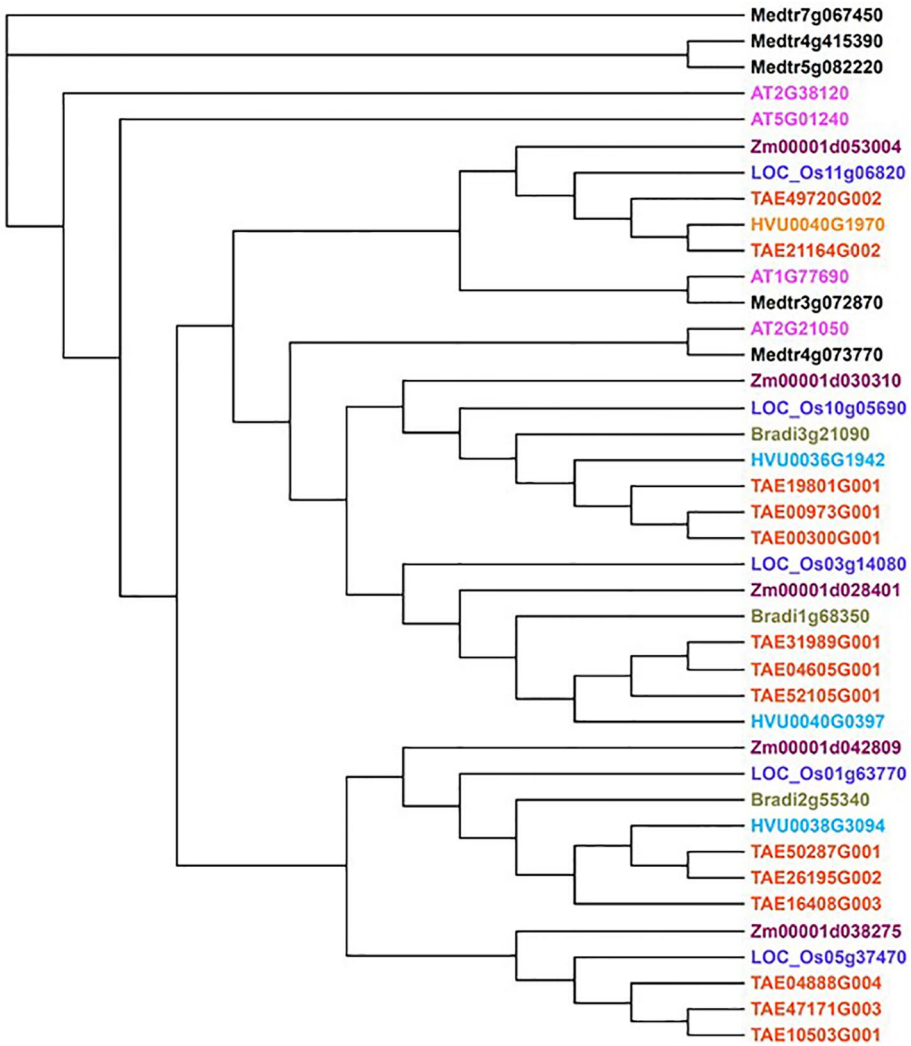

FIGURE 2 | The phylogeny of *AUX1* homologs of selected plant species. This tree was generated using interactive phylogenetic module from Plaza 4.0 (Van Bel et al., 2017). *AtAUX1* was used as seed gene to retrieve homologs of selected species [Arabisdopsis thaliana (AT), Zea maize (Zm), Oryza sativa ssp. Japonica (LOC), Triticum aestivum (TAE), Hordeum vulgare (HVU), Brachypodium distachyon (Bradi) and Medicago truncatula (Medtr); coloured differently] and homologs with >70% protein identity were retained for generating the tree. Some of these homologs have been previously characterised (discussed in the main text).

In maize, auxin synthesis, transport and signaling have been previously linked to inflorescence architecture variation, including branching pattern changes (Gallavotti et al., 2008; Skirpan et al., 2009; Phillips et al., 2011). Auxin efflux carrier *ZmPIN1* has been also implicated in maize inflorescence development (Skirpan et al., 2009). Now Huang et al. (2017) showed that a loss-of-function allele of *ZmAUX1* termed *Zmaux1-0* has reduced inflorescence branching and fewer spikelets in the central spike. Interestingly, Huang et al. (2017) observed that these defects in inflorescence development in *Zmaux1-0* are less severe than previously studied auxin synthesis mutants *sparse inflorescence1* and *vanishing tassel1* and auxin transport mutant *bif2* suggesting potential redundancy among *ZmAUX1* and its other three homologues that show overlapping expression in immature inflorescence.

Additionally, Huang et al. (2017) observed that *spp-1*, *spp1-3*, and *Zmaux1-0* mutants have root agravitropic defects similar to *ataux1*. But unlike *ataux1*, *spp-1* and *spp1-3* mutants have no lateral root defects.

Root and Shoot Development in Rice

Like *Arabidopsis*, *OsAUX1* has been shown to regulate root gravitropism and lateral root development (Zhao et al., 2015) and low P mediated root hair elongation (Giri et al., 2018). Zhao et al. (2015) observed that *OsAUX1* is highly expressed in lateral roots and lateral root primordia. Mutations in *OsAUX1* result in reduced lateral root initiation events whereas *OsAUX1* overexpression plants exhibit increased lateral root initiation events. Transcript levels of several auxin signaling and cell cycle genes are significantly downregulated in *osaux1*, further

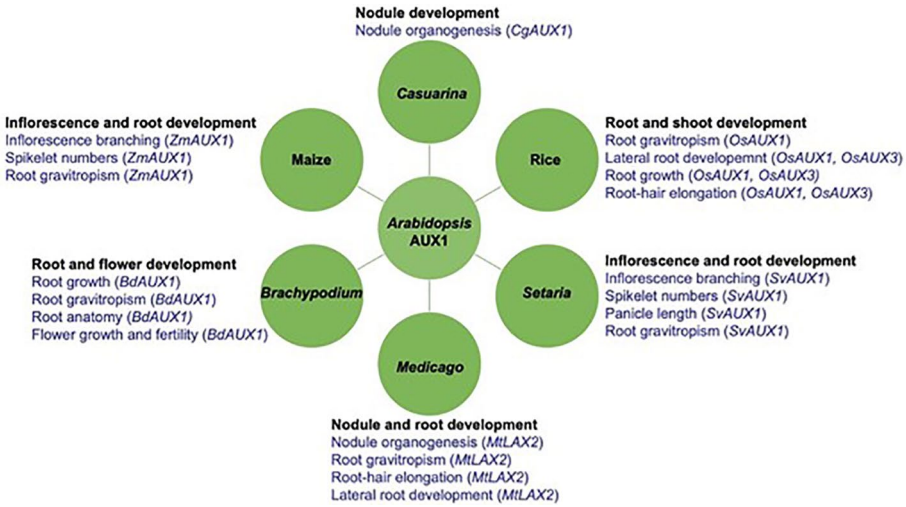

FIGURE 3 | *Arabidopsis AUX1* homoliogs play crucial roles in plant development across severals species. Arabidopsis auxin influx carrier *AUX1* homologs regulate aspects of plant development such as root gravitropism; root architecture (e.g. root/lateral root development; root hair development); root natomy (e.g. cortical cell number and size); inflorescence architecture (e.g. branching; spikelet numbers; panicle lenght); nodule organogenesis and flower development in several plants including the one represented here: Rice, Maize, *Setaria*, *Brachypodium, Medicago,* and *Casuarina*. Names in parenthesis indicate the genes controlling the respective role.

highlighting the importance of OsAUX1 in regulating lateral root development in rice.

OsAUX1 has also been implicated in Cadmium (Cd) stress response. Cd stress induces the production of reactive oxygen species, which trigger cell death in plants. Auxin signaling is known to be involved in activating Cd-induced morphogenic defense responses in wheat, barley and *Arabidopsis* (Tamas et al., 2015; Agami and Mohamed, 2013; Zhu et al., 2013). Yu et al. (2015) showed that *OsAUX1/LAX* genes (*OsAUX1-5*) are induced by Cd stress. Reporter analysis showed that *OsAUX1* is distinctly induced under Cd stress in primary roots, lateral roots and root hairs and *osaux1* mutants are more sensitive to Cd stress. Cd contents in the *osaux1* mutant were not altered, but reactive oxygen species-mediated damage was enhanced, further increasing the sensitivity of the mutant to Cd stress. Taken together, their results indicated that OsAUX1 plays an important role in mediating plant responses to Cd stress. Yu et al. (2015) also showed that in contrast to *Arabidopsis, OsAUX1* is specifically expressed in root hair cell files, suggesting functional differences between monocots and dicots in regulating RH development.

Giri et al. (2018) showed that *OsAUX1* also regulates root hair elongation under low P as *osaux1* mutants are defective in low P mediated root hair elongation. Using direct auxin quantification by mass spectrometry as well as auxin reporter-based approaches, they showed that low P results in increased auxin accumulation in the root apex in OsAUX1 dependent fashion. Although mechanistic details are not yet fully understood in rice, it is tempting to speculate that it is similar to *Arabidopsis* (Bhosale et al., 2018) and may even be conserved in other land plants as well. Further, using an elegant split root experiments, by exposing half of the crown roots from the same plants to low P and the other half to high P, Giri et al. (2018) also showed that low P mediated root hair elongation is a local response irrespective of the plant P status.

More recently, Wang et al. (2019) characterized another AUX1 homolog in rice *OsAUX3*. *OsAUX3* is expressed in primary roots, lateral roots and in the root hairs. Mutations in *OsAUX3* result in shorter primary roots, decreased lateral root density, and longer root hairs compared to control. In addition, it appears that OsAUX3 is also involved in mediating Aluminum (Al) induced inhibition of root growth. *OsAUX3* expression is up-regulated in the root apex under Al stress and one of the *OsAUX3* mutants (*osaux3-2)* is insensitive to Al treatments and Al-induced ROS mediated damage.

Root and Flower Development in *Brachypodium*

Recently, van der Schuren et al. (2018) showed that similar to *AtAUX1, BdAUX1* is expressed in roots displaying expression patterns in root protophloem, epidermis and columella. Additionally, authors observed that unlike *AtAUX1*, *BdAUX1* is also expressed throughout the stele and in the outer cortex layers, encompassing the combined expression domains of *AtAUX1*, *AtLAX2*, and *AtLAX3*. Thus, possibly *BdAUX1* have the combined functions in these tissues. *Bdaux1* mutant roots are agravitropic and show longer root phenotypes due to increased mature cell lengths possibly due to higher free auxin content. Additionally, *Bdaux1* mutants are also significantly thinner due to reduced cell file numbers in every tissue except xylem and phloem and due to smaller cell sizes radially. *Bdaux1* mutants also show reduced root hair length and density. Unlike *AtAUX1*, *Bdaux1* loss-of-function mutants are dwarfs with aberrant flower development,

and consequently infertile suggesting a more crucial role for BdAUX1 in flower development.

Nodule Organogenesis in *Medicago* and *Casuarina*

Péret et al. (2007) have previously shown that nodule organogenesis in *Casuarina glauca* is facilitated by CgAUX1. More recently, Roy et al. (2017) showed that nodule organogenesis in *Medicago* is mediated by MtLAX2. They showed that *MtLAX2* is auxin inducible and is expressed in the nodule primordia, vasculature of developing nodules and at the apex of matured nodules. Upon Rhizobium infection, *mtlax2* mutants have fewer nodules and reduced DR5 activity at the infection sites clearly implicating the role of *MtLAX2* in nodule development.

In addition, Péret et al. (2007) also showed the importance of MtLAX2 in root development. Mutation in *MtLAX2* results in defects in root gravitropism, fewer lateral roots and shorter root hairs suggesting this to be a functional analog of *AtAUX1* (Roy et al., 2017). Interestingly, *MtLAX2* cannot rescue root gravitropic defects of Arabidopsis *aux1* mutant when expressed under *AtAUX1* promoter. This led Roy et al. (2017) to conclude that these genes may have diverged to an extent that they encode biochemically distinct proteins. It is possible that MtLAX2 also cannot get correctly localized in AUX1 expression domain as has been the case for AtLAX2 and AtLAX3 (Péret et al., 2012). Modeling studies on auxin transport.

MODELING STUDIES ON AUXIN TRANSPORT

Computer simulations and modelling approaches in the past decade have proven very useful in getting better understanding of the role of auxin transport in regulating auxin mediated developmental processes in *Arabidopsis* and how auxin fluxes are established and maintained (Swarup et al., 2005; Kramer and Bennett, 2006; Grieneisen et al., 2007; Laskowski et al., 2008; Jones et al., 2009; Prusinkiewicz et al., 2009; Mironova et al., 2010; Szymanowska-Pułka and Nakielski, 2010; Vernoux et al., 2011; Bridge et al., 2012; Steinacher et al., 2012; Novoselova et al., 2013). Increased understanding of the auxin transport proteins and their sub-cellular localization have helped refine previous auxin-transport models and improved our understanding of how changes at cellular level regulate organ-scale auxin patterns. For example, Band et al. (2014) showed the importance of AUX1/LAX proteins in pattern formation at the root tip by taking into consideration the localization of auxin transport proteins as well as cell geometries and further validated their model predictions using DII VENUS auxin sensor (Brunoud et al., 2012). Authors found that, while polar localized auxin efflux carriers provide polarity of the auxin movement, nonpolar AUX1/LAX influx carriers are crucial in determining which tissues have high auxin levels. They concluded that both auxin influx and efflux carriers are required to create a pattern of auxin distribution in the root tip.

More recently, Moore et al. (2017) developed a mechanistic model that also reinforced that auxin pattern formation requires co-ordination between influx and efflux carriers. Their model predicts that the localization of influx carriers can either get more polar when auxin efflux carrier levels are changed or modulate efflux carrier level and polarity to maintain the auxin patterns.

CONCLUSION AND PERSPECTIVES

In the past two decades, there has been a significant increase in our understanding of molecular basis of auxin transport and roles of auxin transporters in plant development. Particularity, there has been a better understanding of auxin influx carriers and how they play crucial roles in almost all aspects of plant growth and development. More advanced computer models and high-resolution imaging and segmentation approaches have proved crucial in providing better understanding of auxin influx carriers in pattern formation especially how changes at the cellular scale affect organ-scale auxin patterns.

Root development is very plastic and respond to their environment. Recently it has been shown that chromium inhibits primary root growth by regulating cell cycle genes. Chromium toxicity can cause major damage to crop yield. Genetic and physiological studies show a role for AUX1 in chromium mediated inhibition of root growth (Wakeel et al., 2018). Similarly, as stated above, AUX1 has also been implicated in Al and Cd mediated inhibition of root growth. Similarly, low P mediated root hair elongation response is mediated *via* AUX1 but early events are not well understood as to how low P status is sensed by the plants. Further understanding of the early events will be crucial for our understanding of the root growth and development in changing environment and may help develop predictive models for future crop improvement programmes.

Alternative splicing is another key area that has not been explored much in the plants but may be crucial for better understanding of the role of alternatively spliced transcripts in regulating and shaping plant development. It appears that alternative splicing in plants is more common than previously appreciated (Li et al., 2016; Swarup et al., 2016). With the advancement in sequencing technology, longer reads and single cell transcriptome, it is possible now to get a much better view of the cellular transcriptome and what if any is the role of alternative spliced transcripts in regulating auxin transport.

AUTHOR CONTRIBUTIONS

RS and RB did the review of literature, prepared figures and wrote the manuscript.

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The Full-Size ABCG Transporter of *Medicago truncatula* Is Involved in Strigolactone Secretion, Affecting Arbuscular Mycorrhiza

Joanna Banasiak[1]*, *Lorenzo Borghi*[2], *Natalia Stec*[1], *Enrico Martinoia*[2] and *Michał Jasiński*[1,3]

[1] Department of Plant Molecular Physiology, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland, [2] Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland, [3] Department of Biochemistry and Biotechnology, Poznan University of Life Sciences, Poznan, Poland

Strigolactones (SLs) are plant-derived signaling molecules that stimulate the hyphal branching of arbuscular mycorrhizal fungi (AMF), and consequently promote symbiotic interaction between the fungus and the plant. Currently, our knowledge on the molecular mechanism of SL transport is restricted to the *Solanaceae* family. In the *Solanaceae* family, SL translocation toward the rhizosphere occurs through the exodermis *via* hypodermal passage cells and involves a member of the G subfamily, of the ATP-binding cassette (ABC) membrane transporters. Most *Fabaceae* species, including those that are agriculturally important, have a different root anatomy compared to most angiosperm plants (i.e., lacking an exodermis). Thus, we have investigated how SL transport occurs in the model legume *Medicago truncatula*. Here, we show that overexpression of a SL transporter from petunia (PaPDR1) enhances AMF colonization rates in *M. truncatula*. This result demonstrates the importance of ABCG proteins for the translocation of orobanchol-type molecules to facilitate arbuscular mycorrhiza, regardless of root anatomy and phylogenetic relationships. Moreover, our research has led to the identification of Medicago ABCG59, a close homologue of Petunia PDR1, that exhibits root specific expression and is up-regulated by phosphate starvation as well as in the presence of *rac*-GR24, a synthetic SL. Its promoter is active in cortical cells, root tips, and the meristematic zone of nodules. The *mtabcg59* loss-of-function mutant displayed a reduced level of mycorrhization compared to the WT plants but had no impact on the number of nodules after *Sinorhizobium meliloti* inoculation. The reduced mycorrhization indicates that less SLs are secreted by the mutant plants, which is in line with the observation that *mtabcg59* exudates exhibit a reduced stimulatory effect on the germination of the parasitic plant *Phelipanche ramosa* compared to the corresponding wild type.

Keywords: ABC transporters, arbuscular mycorrhiza, exodermis, *Medicago truncatula*, strigolactones, symbioses

Abbreviations: AM, arbuscular mycorrhiza; HPC, hypodermal passage cell; LRS, legume-rhizobium symbiosis; SL, strigolactone.

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***Correspondence:**
Joanna Banasiak
joaban@ibch.poznan.pl

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INTRODUCTION

Arbuscular mycorrhiza (AM) represents an ancient and widespread beneficial association, established between most terrestrial plants and filamentous fungi from the subphylum *Glomeromycotina* (Parniske, 2008; Spatafora et al., 2016). Arbuscular mycorrhizal fungi (AMF) improve plant nutrient acquisition, especially phosphate, depletion of which is a major factor limiting plant growth (Karandashov and Bucher, 2005). In return, plants supply the fungi with sugars (Doidy et al., 2012) and lipids (Jiang et al., 2017; Luginbuehl et al., 2017; Rich et al., 2017). This nutrient transfer between symbionts occurs *via* highly branched, fungus-derived structures called arbuscules, formed inside root cortical cells (Parniske, 2008; Luginbuehl and Oldroyd, 2017).

The establishment of AM is preceded by a signal exchange between partners that mediates reciprocal recognition, prior to the establishment of direct contact. One of the breakthroughs in the field of AM research was the discovery of strigolactones (SLs) as host-derived precolonization signals (Akiyama et al., 2005). SLs, released into the rhizosphere upon phosphate deficiency, stimulate fungal mitochondrial metabolism, and promote hyphal branching (Akiyama et al., 2005; Besserer et al., 2006). These signaling molecules also contribute to the transcriptional reprogramming of AMF (Tsuzuki et al., 2016) and induce the extrusion of chitin oligosaccharides, the so-called "myc factors", responsible for the activation of nuclear Ca^{2+} spikes in the host root epidermis (Genre et al., 2013). In the rhizosphere, SLs are active at very low concentrations and are chemically instable. Thus, it has been postulated that SLs form a steep concentration gradient in the soil, indicating the proximity of a plant host and guide fungal hyphae to the root (Akiyama and Hayashi, 2006; Boyer et al., 2012; Nadal and Paszkowski, 2013; Oldroyd, 2013). Moreover, elevated concentrations of SLs near to the root surface may affect the initiation of hyphopodium formation. It has recently been shown that in rice this process is severely attenuated in the SL biosynthetic mutants (Kobae et al., 2018).

Following physical contact and fungal hyphopodium formation on the epidermis, internal root colonization begins. Having penetrated the epidermal cells, the hyphae encounters the hypodermis, the outermost cortical layer. In most angiosperm plants, cell walls within the hypodermis might be modified and form an apoplastic barrier called an exodermis, comprising cells with Casparian bands and suberin lamellae, as well as unsuberized ones. The suberized cells become impenetrable to fungi, therefore deeper cortex colonization occurs only through the suberin-free cells called hypodermal passage cells (HPCs) (Sharda and Koide, 2008). Interestingly, *Petunia hybrida* pleiotropic drug resistance 1 (PhPDR1), the first characterized SL exporter, is specifically expressed in HPCs. Loss-of-function mutations of this gene negatively affects the formation of arbuscular mycorrhiza (Kretzschmar et al., 2012). An orthologue of PhPDR1 from *Petunia axillaris* exhibits a cell-type-specific polar localization in HPCs. PaPDR1 is localized in the outer-lateral plasma membrane of HPCs, in accordance with its proposed function in releasing SL toward the rhizosphere and in creating SL gradients guiding AM fungi to preferred access sites (Sasse et al., 2015). PhPDR1 and PaPDR1 are members of the ancient and ubiquitous ATP-binding cassette (ABC) protein family, and belong to the ABCG subfamily, which is the most abundant in plants and appears to have a great impact on plant adaptations to terrestrial life (Kang et al., 2011; Hwang et al., 2016).

Currently, our knowledge about the molecular mechanisms of SL transport is restricted to the *Solanaceae* family (Kretzschmar et al., 2012; Xie et al., 2015). This is a surprising constraint as SLs influence other types of plant-rhizosphere interactions, both negative and positive. Their presence in the rhizosphere promotes the germination of seeds of parasitic plants of the genera *Striga* and *Orobanche* (Matusova et al., 2005). Recently, a role of SLs in the legume - rhizobium symbiosis (LRS) has also been described. While the function of SL in AM symbiosis is well established, their role in LRS is still emerging. It has been shown, that mutations in the SL biosynthetic genes (*CCD7* and *CDD8*) lead to a decrease in the number of nodules in *Pisum sativum* (pea) and *Lotus japonicus* (Foo and Davies, 2011; Liu et al., 2013). Further analysis in pea revealed that SLs promote the formation of infection threads by influencing the bacterial partner (McAdam et al., 2017). The question about SL transporters seems to be especially intriguing in the case of the leguminous plants (*Fabaceae*), since it was reported that unlike most other angiosperm plants, they do not form an exodermis (Hose et al., 2001; Seago and Fernando, 2013). Moreover, *Fabaceae* such as *Medicago sativa* (alfalfa) and *Glycine max* (soybean) are crops exhibiting an important impact on agriculture and they have evolved several strategies to survive in low nutrient soils (Stagnari et al., 2017). Nitrogen and phosphorus are limiting nutrients in most natural soils. A large part of phosphorus in the soil is not available to plants due to its tendency to interact with cations and it is often present as the sparingly soluble rock phosphate. This non-renewable resource is being mined at an increasing rate to meet the demand for artificial fertilizers (Cordell et al., 2009). In the case of phosphorus, in many ecosystems and, in particular in acidic soils, the response of plants to sparingly available phosphate results mainly in the association with mycorrhizal fungi (Kluber et al., 2012). In this regard it is also worth considering how the root anatomical traits in leguminous plants may influence SL dispersal as well as affect SL coordinated symbiotic processes.

In this study, by functional overexpression of the petunia's PaPDR1 in *Medicago truncatula*, we have shown that SL transport mechanism, based on ABCG transporters, is conserved between species. Additionally, by analyzing transcriptomic and phylogenetic data, we have identified a full-size ATP-binding cassette (ABC) transporter, namely MtABCG59. This protein is important for establishing mycorrhizal symbiosis in Medicago by acting as an SL exporter localized in the root cortex.

MATERIALS AND METHODS

Plant Materials, Treatments, and Growth Conditions

Medicago truncatula seeds were acquired from A. Kondorosi of CNRS, Gif-sur-Yvette, France. Seeds of the *mtabcg59 Tnt1*

retrotransposon insertion mutant lines (NF15758 and NF12356) were obtained from the Noble Research Institute. The presence of Tnt1 was confirmed using gene-specific primers and primers annealing to the Tnt1 border (**Supplementary Table 1**).

The Medicago seeds were scarified with 96% sulfuric acid (10 min), stratified (4°C in the dark for 3 days), and were then germinated on solid, half-strength Murashige and Skoog medium (M5524, Sigma-Aldrich). For all experiments plants were grown in a greenhouse (one plant per 0.5 L pot) or growth chamber (*in vitro* experiments) under a 16 h light/8 h dark (24/22°C) regime, at 50%–60% relative humidity.

For the mycorrhization trials the Medicago plants (R-108 and *mtabcg59* mutants) were grown for three weeks (one plant per 0.5 L pot) in clay granules (Oil Dri US-Special, Damolin) with mycorrhizal inoculum (2 g per pot) (Swiss Collection of Arbuscular Mycorrhizal Fungi (SAF)). Clay granules were supplemented once a week with half-strength Hoagland solution. Afterwards the mycorrhizal roots were collected for quantification of AM colonization or AM-marker genes (*MtBCP1* and *MtPT4)* expression analyses. For the experiments five and four biological replicates were performed respectively, 3 independent plants were pooled per replicate.

For the *rac*-GR24 treatments, seven-day-old *M. truncatula* seedlings were transferred onto fresh half-strength MS medium supplemented with 10 µM *rac*-GR24 (Chiralix) dissolved in acetone or the equal volume of acetone (mock). Roots were collected 24 h after transfer and immediately frozen. Roots from three plants were pooled for each condition and four independent replicates were performed. The collected material was used for qRT-PCR analyses.

For the phosphorous starvation experiments seven-day-old *M. truncatula* seedlings were planted in a mixture of perlite and vermiculite (2:5). Plants were watered twice a week with water and custom prepared fertilizer solution optimized for legumes (**Supplementary Table 2**) or fertilizer without KH_2PO_4 and $NH_4H_2PO_4$. Roots were collected four weeks after they were planted and immediately frozen. Roots from two plants were pooled for each condition and three independent replicates were performed. The collected material was used for qRT-PCR analyses.

Quantification of Mycorrhization

To determine mycorrhizal colonization, a modified gridline intersect method (Mcgonigle et al., 1990) was applied. Mycorrhizal roots were washed in tap water and boiled for 10 min in 10% KOH. Then the roots were rinsed with ddH_2O, boiled for 10 min in a solution containing 5% black ink and 5% acetic acid. After washing and de-staining the roots were stored in 5% acetic acid at 4°C. The roots were then spread evenly on a petri dish with 4 mm^2 grid and the percentage of positive events (roots with intra-radical structures) were calculated from the total amount of segments analyzed. Five independent biological experiments (i.e., 5 pools of 3 plants each) were performed.

Phelipanche ramosa Germination Assay

Rhizotron chambers for assessing the germination of *P. ramosa* seeds were assembled from square petri dishes. Three and one holes were respectively carved on the bottom and on the top of the petri dish to allow for water uptake and plant growth. Ten days after germination on half-strength MS agarose medium, the WT and *mtabcg59* Medicago seedlings were moved onto a round wet filter paper placed in the rhizotron. Then, 150 seeds per filter paper were positioned on the roots and off the roots (seeds placed 1 to 3 mm aside). Another wet filter paper was positioned on the top, creating a paper/roots + seeds/paper sandwich. The rhizotron chambers were then filled up with clay (Oil-Dri), sealed with Micropore Tape (3M) and positioned in a try with water on its bottom to keep the rhizotron moist but not water-logged. Five days later, the rhizotron chambers were opened and germination of *P. ramosa* was quantified. At this stage of development, no significant differences were present for the biomass and surface of seedling roots between the two genotypes (R-108 and *mtabcg59*). No germination was detected without the presence of a plant root. Around 4,000 micro-pilar openings and the eventual presence of radicle were quantified with the help of a binocular microscope.

Nodulation Assay

For *in vitro* nodulation experiments *M. truncatula* seedlings (R-108, *mtabcg59*) were grown vertically on the surface of the modified solid Fahraeus (-N) medium, pH 6.5. *Sinorhizobium meliloti* strain 1021 was grown overnight at 28°C in liquid Bergensen's Modified Medium (BMM) containing streptomycin (500 µg/L), and then diluted with sterile BMM to an OD_{600} = 0.1. Six-day-old *M. truncatula* seedling roots were flood-inoculated with 200 µl of bacterial culture spread along the root. Nodule numbers were counted 14 and 21 days after inoculation. There were five replicates (N = 5) with nine plants (n = 9) each, for each genotype.

Quantitative RT-PCR Analyses

Total RNA of the collected samples were extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The cDNA was then synthesized with an Omniscript Reverse Transcription (RT) Kit (Qiagen) or Moloney Murine Leukemia Virus Reverse Transcriptase Reverse Transcriptase (M-MLV RT) (Promega, Madison, Wisconsin, USA). Quantitative PCR analyses for *MtABCG43*, *MtABCG44*, *MtABCG59, MtCCD7, and MtCCD8* were performed in a CFX Connect Real-Time System machine (BioRad, Hercules, CA, USA) using iTaq Universal SYBR Green supermix (BioRad) with at least three biological replicates each with three technical repeats. Quantitative PCR analyses for the AM-marker genes (*MtBCP1* and *MtPT4*) were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA) using SYBR Green PCR Master Mix (Applied Biosystems) with four biological replicates each with two technical repeats. Expression levels were normalized to *Mtactin* and calculated with the ΔΔCt method. For primer sequences, see **Supplementary Table 1**.

Genetic Constructs and Plant Transformation

A genomic DNA fragment (1,752 bp) corresponding to the promoter region of *MtABCG59* was amplified with KOD Hot

Start DNA Polymerase (Novagen, Madison, Wisconsin, USA), and cloned into the following vectors: (i) pPR97, carrying the β-glucuronidase (gusA) reporter gene (Szabados et al., 1995), by restriction-ligation using the restriction sites for *EcoR*I and *Hind*III; and (ii) pPLV04_v2, carrying a GFP reporter gene tagged with a nuclear localization signal (NLS), by ligation-independent cloning (De Rybel et al., 2011). The genomic DNA fragment (7,500 bp) of *MtABCG59* was used for subcellular localization and was amplified with KOD Hot Start DNA Polymerase (Novagen) and cloned into the Gateway entry vectors pENTR4 (Invitrogen, Carlsbed, CA, USA) by restriction-ligation, using restriction sites for *Not*I and *Sal*I. The resulting entry clone was used for recombination with a destination vector pMDC43 (Curtis and Grossniklaus, 2003) using Gateway LR Clonase II Enzyme Mix (Invitrogen). The gPaPDR1 cloning procedure was described in (Kretzschmar et al., 2012). For primers, see **Supplementary Table 1**.

Plant Transformation

Transgenic roots carrying *ProMtABCG59:GUS* or *ProMtABCG59:NLS-GFP* constructs were obtained from *M. truncatula* after inoculation of a severed radicle with *Agrobacterium rhizogenes* Arqua1 (at least 30 composite plants for each construct). Stably transformed *M. truncatula* plants carrying *ProMtABCG59:GUS* (6 independent F0 lines) or *Pro35S:GFP-PaPDR1* (3 independent F0 lines) were obtained by *Agrobacterium tumefaciens* AGL1-mediated transformation using leaf explants and regeneration *via* somatic embryogenesis. The *M. truncatula* transformation was performed according to the protocol described in the Medicago handbook (http://www.noble.org/medicagohandbook). Arabidopsis protoplasts that transiently expressed *Pro35S:GFP-MtABCG59* or free GFP were obtained using PEG-mediated direct gene transfer, as described previously (Smolarkiewicz et al., 2014).

Microscopic Observation and Staining Procedures

Transgenic hairy roots and stably transformed *M. truncatula* (plants of the F1 generation) carrying GUS reporter constructs were stained using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, as previously described (Gallagher, 1992) and visualized by light microscopy.

Microscopic observations of the transgenic roots carrying the *ProMtABCG59:NLS-GFP* reporter construct and Arabidopsis protoplasts transiently expressing *Pro35S:GFP-MtABCG59* or free GFP were performed using laser scanning confocal microscopy (Leica TCS SP5).

For suberin staining, 6-week-old *M. truncatula* and *Nicotiana tabacum* roots (10 of each species) were incubated in 0.01% solution of Fluorol Yellow 088 (Santa Cruz Biotechnology, Dallas, Texas, USA) at 70°C for 30 min. Free-hand root sections (from the regions located at least 3 cm above the root tip) were observed under UV-light with fluorescence microscopy (Leica DMI 4000B, Wetzlar, Germany).

For arbuscules visualization, mycorrhizal roots were fixed in 50% ethanol for 3 h, cleared in 20% (w/v) KOH for 3 days at room temperature (21°C–23°C), followed by 0.1 M HCl for 1 h. Then roots were washed in PBS and incubated in WGA-AlexaFluor 488 staining solution (1 μg/ml in PBS and 0.01% v/v Tween 20) in the dark for 16 h. The roots were imaged with laser scanning confocal microscopy (Leica TCS SP5).

RESULTS

Medicago truncatula Root Anatomy

It has been shown that the secretion of SLs in petunia, belonging to the *Solanaceae* family, occurs through the hypodermal passage cells (HPCs), located within the exodermis (Kretzschmar et al., 2012). Notably, most *Fabaceae* species have a different root anatomy and do not form an exodermis (Perumalla et al., 1990; Seago and Fernando, 2013). This is also true for the model legume *Medicago truncatula*, as shown by the Fluorol Yellow 088 fluorescent staining of the suberin lamella in cross-sections of its roots. In contrast to a plant such as *Nicotiana tabacum*, whose roots possess two layers, an exo- and endodermis, which are stained by this dye, Medicago possesses only one layer corresponding to the endodermis (**Figure 1**). The lack of exodermis in Medicago roots has been observed regardless of the plant age or the region of the root examined (**Supplementary Figure 1**).

In petunia, SLs have been shown to be translocated and secreted into the rhizosphere by the membrane transporter PDR1, which belongs to the full-size ABCG (PDR) subfamily of ABC transporters (Kretzschmar et al., 2012). Interestingly, overexpression of the PaPDR1 (the only up to date cloned SL transporter), in Medicago enhanced AMF colonization rates, and did not affect the arbuscule structures in Medicago (**Supplementary Figure 2**). This observation underlines the importance of ABCG proteins for the translocation of orobanchol-type molecules to facilitate AM symbiotic interactions, despite the differences in the anatomical features of the roots between the plant families. The *M. truncatula* transgenic plants overexpressing *PaPDR1* also displayed several SL-related phenotypes in the shoot, demonstrating the importance of SL redistribution in Medicago aboveground organs by this transporter. PaPDR1 OE plants possessed: (i) modified leaf shape with deeper margin serrations, (ii) enhanced internode elongation, and iii) petioles of a reduced length (**Supplementary Figure 3**).

Identification of a Potential Strigolactone Transporter in *Medicago truncatula*

We have previously identified several ABCG transporters in the *M. truncatula* genome (Banasiak and Jasinski, 2014). The phylogenetic analysis showed that three ABCG proteins: MtABCG59 (MtPDR23), MtABCG44 (MtPDR8), and MtABCG43 (MtPDR7), clustered with the previously characterized SL transporter PaPDR1 from *P. axillaris* (**Figure 2**, **Supplementary Table 3**). They share 74%, 71%, and 68% amino acid sequence identity with PaPDR1, respectively. Since nutrient starvation stimulates AM

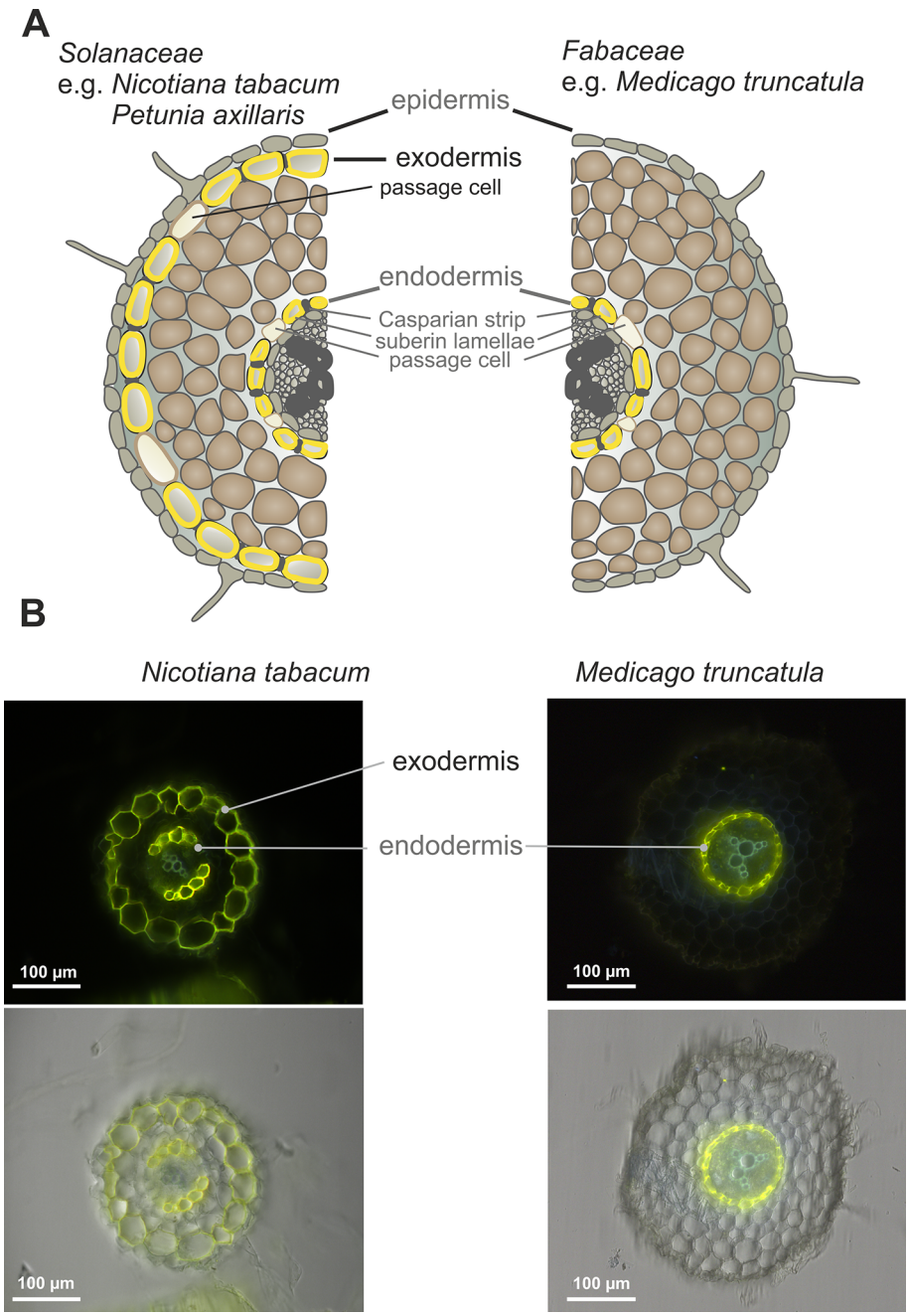

FIGURE 1 | Root anatomical structure. **(A)** Scheme of *Nicotiana tabacum* (*Solanaceae*) and *Medicago truncatula* (*Fabaceae*) root layers. **(B)** Deposition of suberin lamellae in roots of 6-week-old *N. tabacum* and *M. truncatula* (representative images from 10 roots). The cross-sections of the roots comes from the regions located 3 cm above the root tip. Fluoral yellow 088 fluorescence (upper panel), fluorescence images overlayed with corresponding bright-field images (lower panel).

formation as well as biosynthesis and secretion of SLs in Petunia (Liu et al., 2018b), we have tested the response of those three genes to phosphorous deficiency. The highest level of gene induction was observed for *MtABCG59* and *MtABCG43* exhibiting 18 and 17-fold changes, respectively (**Figure 3A**). Furthermore, exogenous applications of a synthetic SLs analogue – *rac*-GR24 onto the roots, increased the mRNA accumulation of *MtABCG59* (9-fold) and again at a lower extent *MtABCG43* (3-fold change) (**Figure 3B**). Previously it was reported that the expression of several ABCG transporters could be substrate inducible (Jasinski et al., 2001; Biala et al., 2017; Pawela et al., 2019). The *MtABCG44* was only slightly

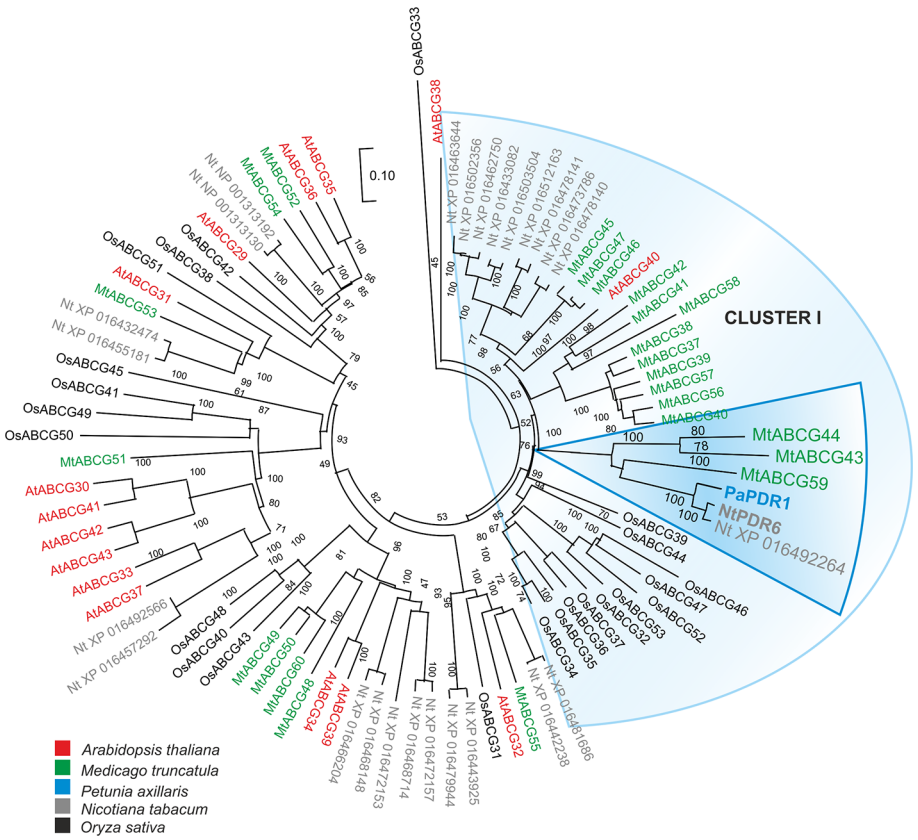

FIGURE 2 | Phylogenetic analysis of full-size ABCG (Pleiotropic drug resistance - PDR) proteins in various plants. Neighbor-joining phylogenetic tree (bootstraps: 1000) was conducted using MEGA X software (Kumar et al., 2018) based on the amino acid sequences generated after multiple sequence alignments with MUSCLE. The highlighted PaPDR1 and NtPDR6, as well as their homologues from *Medicago truncatula* belong to Cluster I (Crouzet et al., 2013).

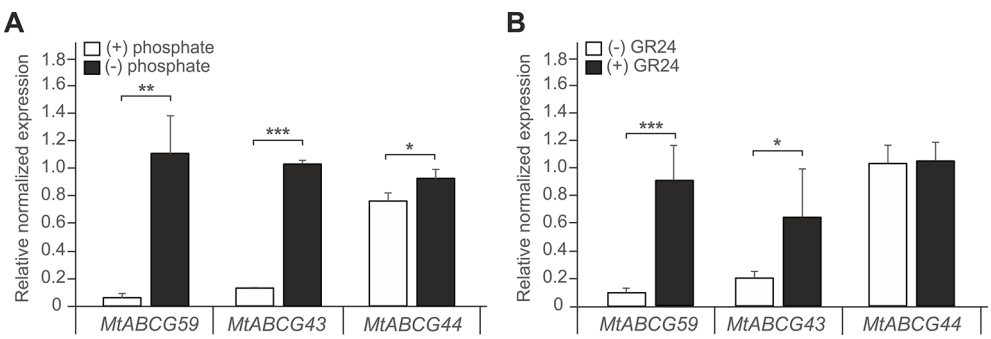

FIGURE 3 | Expression profiles of *Medicago truncatula ABCG59*, *ABCG43*, and *ABCG44*. **(A, B)** Quantitative Real-Time PCR expression analysis of *MtABCG59*, *MtABCG43*, and *MtABCG44* in roots under phosphate-limiting conditions **(A)** or treated with the synthetic strigolactone analogue *rac*-GR24 **(B)**. The transcript levels were normalized to the *actin* gene from *Medicago truncatula*. Data represent the mean ± SD of three and four independent biological experiments, respectively, and three technical repeats. Significant differences from the control plants determined by Student's t-test are indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

induced (1.2-fold change) under phosphate limiting condition, and *rac*-GR24 had no impact on its expression (**Figure 3**). Based on this expression profile, and phylogenetic relationships, we selected MtABCG59 for further and more comprehensive functional analyses.

Gene Expression Pattern of *MtABCG59* in *M. truncatula* and Subcellular Localization of the Corresponding Protein

The qPCR analyses revealed that *MtABCG59* mRNA accumulates in roots and root-derived nodules (**Figure 4A**). To

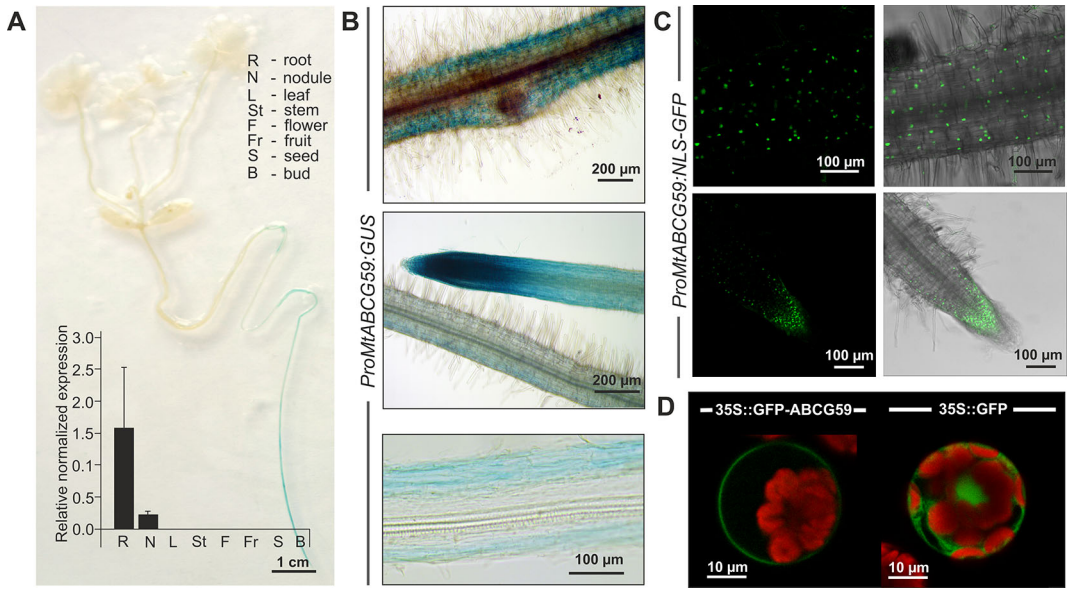

FIGURE 4 | Expression pattern and subcellular localization of MtABCG59. **(A)** GUS staining of 2-week-old transgenic *Medicago truncatula* expressing *ProMtABCG59:GUS* and quantitative PCR expression analysis of *MtABCG59* in different *M. truncatula* organs (three-month-old plants) revealed the *MtABCG59* transcript accumulation exclusively in the roots and nodules. The transcript levels were normalized to the *actin* gene from *M. truncatula*. Data represent the mean ± SD of three independent biological experiments and three technical repeats. **(B)** X-Gluc staining of *ProMtABCG59:GUS* reporter line in root cortex (upper panel) and in root meristem (middle panel), X-Gluc staining of root cross-sections (bottom panel). **(C)** Fluorescence signal of *ProMtABCG59:NLS-GFP* reporter line in root cortex (upper panel) and in root meristem (bottom panel). **(D)** Arabidopsis mesophyll protoplast expressing the fusion gene *Pro35S:GFP-MtABCG59*. The GFP signal was observed in the plasma membrane (left panel). Control Arabidopsis protoplast expressing free cytoplasmic GFP (right panel). The red color represents chlorophyll autofluorescence.

further explore the spatial expression patterns of *MtABCG59*, we generated *M. truncatula* transgenic plants expressing the β-glucuronidase (GUS) reporter gene under the control of the native *MtABCG59* promoter (*ProMtABCG59:GUS*). Histochemical staining of the 2-week-old transgenic seedlings, as well as qPCR, showed that *MtABCG59* expression is root specific (**Figure 4A**). Moreover, microscopic observations of transgenic Medicago hairy roots expressing *ProMtABCG59:GUS* and *ProMtABCG59* fused with a nuclear-localized version of green fluorescent protein (*ProMtABCG59:NLS-GFP*) revealed that the root tip, where SLs are biosynthesized (van Zeijl et al., 2015a), and the cortical cells, are the main sites of *MtABCG59* promoter activity (**Figures 4B, C**).

To determine the subcellular localization of MtABCG59, we fused GFP to the C-terminal end of MtABCG59 and transiently expressed the *Pro35S:GFP-MtABCG59* recombinant gene in Arabidopsis leaf mesophyll protoplasts. Visualization of GFP fluorescence was performed using confocal microscopy. The GFP signal from the fusion protein was detected in the plasma membrane. In control protoplasts, transformed with *Pro35S:GFP*, the fluorescence signal was observed in the cytoplasm (**Figure 4D**).

Mycorrhizal Phenotype of *mtabcg59* Mutant Lines

To test the hypothesis that MtABCG59 can influence the AM symbiosis we identified the tobacco retrotransposon (Tnt1) inserted line (NF15758) for *MtABCG59* and assessed with the grid-line intersect method (see M&M) the colonization rate of AMF in the roots derived from the WT and *mtabcg59-1*. The percent of root length colonization by *Rhizophagus irregularis* was estimated 3 and 5 weeks after inoculation with the fungal spores. The experiments showed that the *mtabcg59-1* mutant exhibits 70% and 11% lower levels of colonization than the WT, respectively (**Figure 5A**). The arbuscule structures appeared unaffected in *mtabcg59-1*, suggesting that MtABCG59 is required for initiation of AM, rather than proper development of arbuscules (**Figure 5B**). The reduction of AM formation in *mtabcg59-1* as well as in the second mutant line (*mtabcg59-2*, NF12356) 3wpi was further demonstrated with the usage of AM-related marker genes *MtBCP1* and *MtPT4* (**Figure 5C**). *MtBCP1*, encoding a blue copper protein, is expressed in the epidermal and cortical cells containing fungal structures and in adjacent noncolonized cortical cells (Hohnjec et al., 2005; Hogekamp and Kuster, 2013; Zhang et al., 2015), while *MtPT4* encodes an arbuscule-specific phosphate transporter (Javot et al., 2007; Hogekamp and Kuster, 2013). Real-time PCR analyses revealed that AM marker genes had lower expression levels in the *mtabcg59-1* and *mtabcg59-2*, reflecting the lower mycorrhization rate in these plants compared to the WT (**Figure 5C**).

Taking into the consideration the possible functional redundancy, we have investigated if MtABCG59 paralogues, namely MtABCG43 and MtABCG44 might take over the

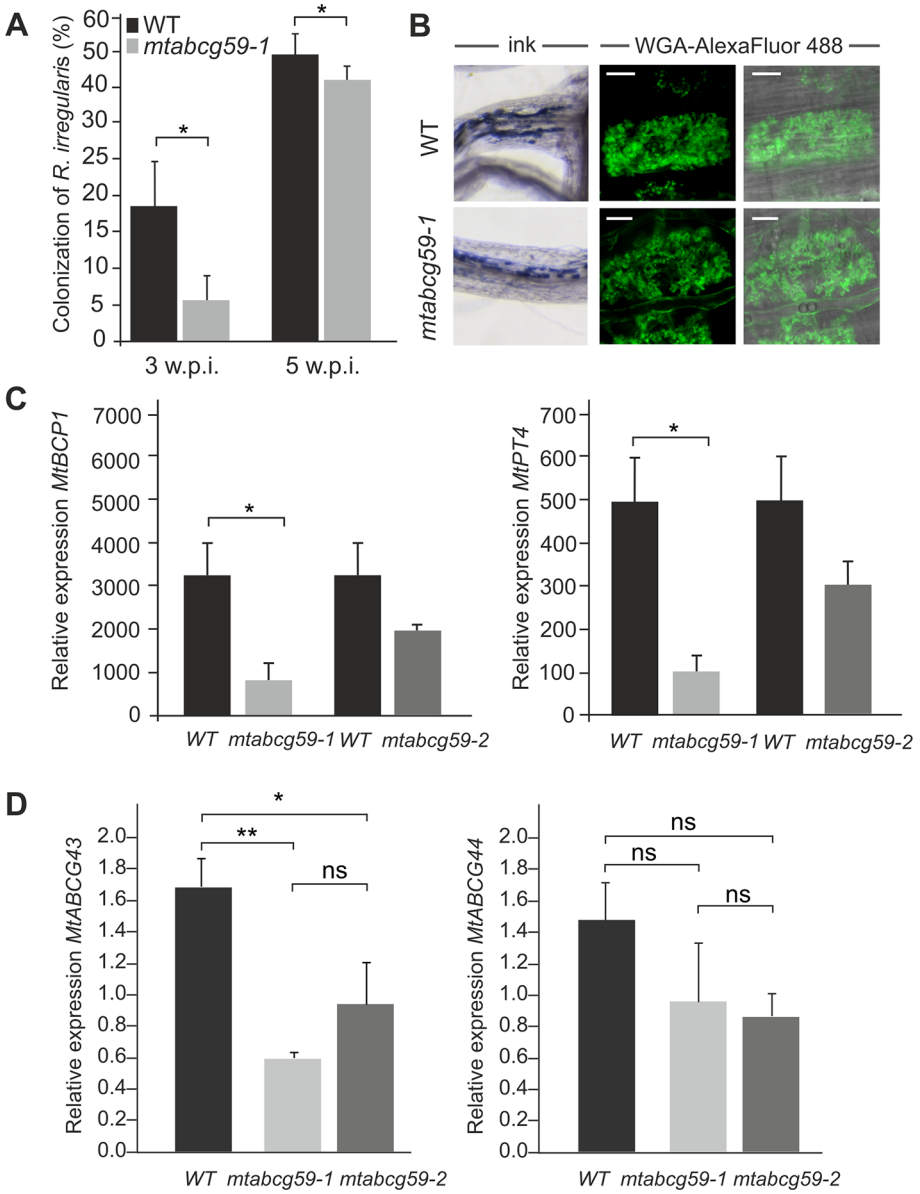

FIGURE 5 | Phenotypic characterization of the *mtabcg59* mutant in mycorrhization contexts. **(A)** Mycorrhizal colonization of *Medicago truncatula* control (WT) and *mtabcg59-1* mutant roots 3 and 5 weeks after inoculation with *Rhizophagus irregularis*. The percentage of the root length colonized by the mycorrhizal fungi is shown. Data represent the means ± SE of five independent biological experiments (i.e. 5 pools of 3 plants each). Significant differences from the control plants were determined by Student's t-test and are indicated by: *P < 0.05. **(B)** Arbuscules formed in the WT and *mtabcg59-1* mutant were morphologically similar. Ink-staining of fungal structures (left panel), WGA-AlexaFluor 488 staining of arbuscules (right panel), scale bar, 10 µm. **(C)** Transcript accumulation of AM-related marker genes *MtBCP1* (left panel) and *MtPT4* (right panel) in *mtabcg59* mutants and control (WT) roots measured by quantitative real-time PCR. The data represents the mean ± SD of four independent biological experiments and two technical repeats. Significant differences from the control plants were determined by Student's t-test and are indicated by: * P < 0.05. **(D)** Relative expression of *MtABCG43* and *MtABCG44* in WT and *mtabcg59* mutant plants. Values represent the mean of three biological replicates ± SD. Significant differences in genes expression between WT and *mtabcg59* mutants were determined by an ANOVA test and Tukey's multiple comparison test, and are as follows: ns, not significant; *P < 0.05, **P < 0.01.

function of MtACG59. Interestingly, we have observed that upon phosphate deficient condition *MtABCG43* is downregulated in both *mtabcg59* mutants background. Concomitantly, the expression of *MtABCG44* did not show significant differences (**Figure 5D**).

Root Exudates From *mtabcg59* Exhibit a Reduced Ability to Promote Germination of *Phelipanche ramosa* Seeds

To investigate whether MtABCG59 is involved in SL secretion, *mtabcg59-1* root exudates were assessed for their ability to

stimulate parasitic weed *Phelipanche ramosa* seed germination. Compared to the WT, a significant decrease in germination capacity was observed for *P. ramosa* seeds exposed to root exudates from *mtabcg59-1*. Interestingly, this difference was present only with seeds placed 1 to 3 mm aside, no difference in germination rates were observed with seeds placed directly on the root surface (**Figure 6**, **Supplementary Figure 4**).

Lack of MtABCG59 Disturbs Strigolactone Biosynthesis

The presence of *MtABCG59* transcripts in root tips suggests that MtABCG59 might be involved in the export of SL from the site of biosynthesis. Since SL content can influence the transcript level of SL biosynthetic genes, *CCD7* and *CCD8* (Foo et al., 2005; Hayward et al., 2009) we have examined their expression in WT and *mtabcg59* mutant lines. We have noticed a decrease in mRNA accumulation of *CCD7* and *CCD8* in both *mtabcg59* mutants background. The observed effect might be due to excessive accumulation of SL in the cells that can trigger a negative feedback regulation mechanism in SL biosynthesis (**Figure 7**).

Lack of MtABCG59 Does Not Affect Nodulation

Recent studies have found that SLs positively affect interactions between legumes and nitrogen-fixing bacteria (McAdam et al., 2017). Moreover, we have shown that the expression pattern of *MtABCG59* in nodule primordia as well as in the meristematic zone of developing and mature nodules (**Figure 8A**) is similar to that reported for genes encoding SL biosynthetic enzymes (van Zeijl et al., 2015a). Despite this, *mtabcg59* plants inoculated with *Sinorhizobium meliloti* had a comparable number of root nodules to the WT, and did not differ morphologically (**Figures 8B, C**), indicating that MtABCG59 is either not required for nodulation or that a redundant, yet unknown protein compensates its loss of function.

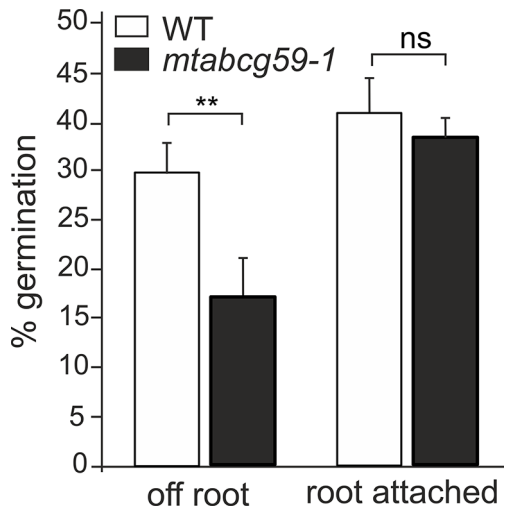

FIGURE 6 | Germination rates of *Phelipanche ramosa* seeds exposed to WT and *mtabcg59-1* exudates. Root attached (seeds placed directly in root surface); off root (seeds placed 1 to 3 mm aside). The data represent the mean ± SE of six to seven independent biological experiments (approx. 150 seeds screened for each biological replicate). Significant differences from the control plants were determined by Student's t-test and are indicated by: ns, not significant; **P < 0.005.

DISCUSSION

The only SL transporter characterized in detail is the PaPDR1 from *Petunia axillaris*, and it operates in a highly cell specific

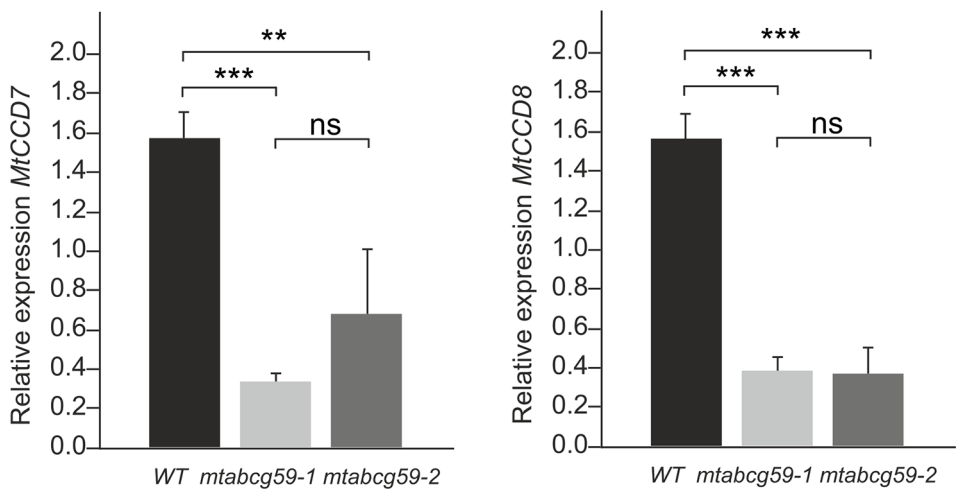

FIGURE 7 | MtABCG59 affects the SL-biosynthetic pathway. Relative expression of SL-biosynthesis gene (*MtCCD7* and *MtCCD8*) in WT and *mtabcg59* mutant plants. Values represent the mean of three biological replicates ± SD. Significant differences in genes expression between WT and *mtabcg59* mutants were determined by an ANOVA test and Tukey's multiple comparison test, and are as follows: ns, not significant; **P < 0.01, ***P < 0.001.

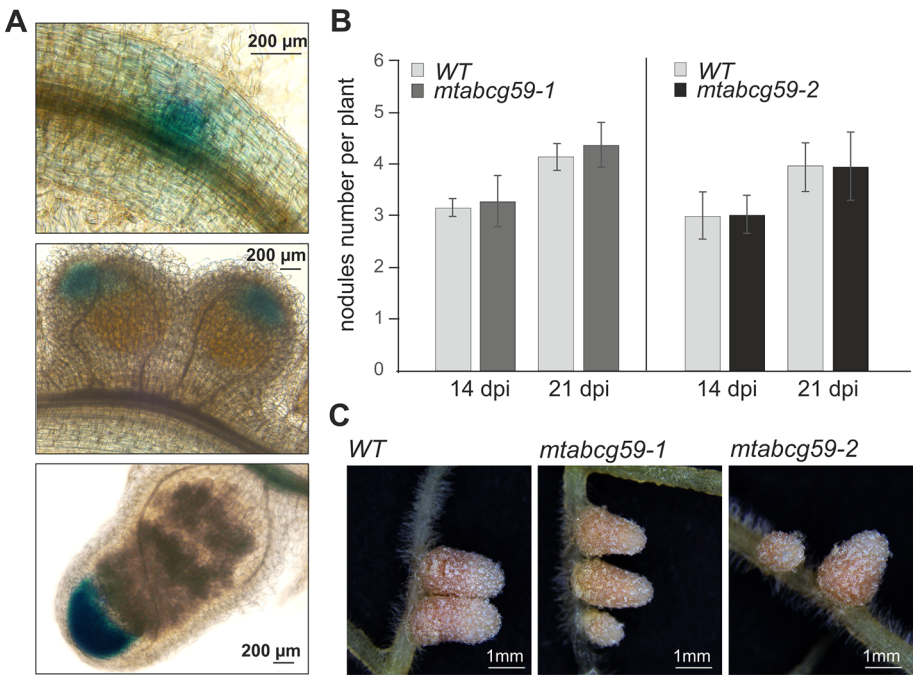

FIGURE 8 | Phenotypic characterization of *mtabcg59* in nodulation context. **(A)** Expression of *ProMtABCG59:GUS* in nodules. **(B)** Average nodule number per plant in WT and *mtabcg59* plants. Six-day-old seedlings were inoculated with *Sinorhizobium meliloti* and grown on modified Fahraeus (-N) medium. At 14- and 21-days post-inoculation (dpi), the nodules were quantified. The data represent the mean ± SD of (N = 5, n = 9), per line. **(C)** WT and *mtabcg59* nodules morphology.

manner, being active *inter alia* in the hypodermal passage cells (HPCs) located within exoderemis (Sasse et al., 2015). We have shown that the roots of the model legume plant *Medicago truncatula*, related to the important forage crop *Medicago sativa,* do not possess an exodermis and HPCs (**Figure 1**). Overexpression of *PaPDR1* in Medicago revealed that despite these anatomical differences, this transporter is functional in Medicago and its presence promotes AM. The increased mycorrhization in Medicago overexpressing *PaPDR1*, indicates that SL transport mechanisms based on ABCG transporters, exhibit similar properties between species. Notably, canonical orobanchol-type molecules are the most abundant SLs exuded into the rhizosphere by both petunia and *M. truncatula* (Kretzschmar et al., 2012; Tokunaga et al., 2015). Thus, the PaPDR1 OE strategy appears to be useful for ameliorating mycorrhization rate in *Fabaceae.* It could improve phosphate acquisition and plant biomass production as it was reported for petunia (Liu et al., 2018a; Liu et al., 2018b).

It has been demonstrated that AMF hyphal branching can be initiated by the SL present in the root exudates of legume plants (Akiyama et al., 2005; Steinkellner et al., 2007). However, transporters responsible for SL release into the rhizosphere from this plant family have not been discovered. To identify a putative SL transporter in *M. truncatula,* we combined the phylogenetic information with expression profile analyses. Among the tested genes, *MtABCG59*, besides sharing the highest level of sequence identity with the previously described SL exporter PaPDR1 from petunia (**Figure 2**), it had the highest induction rates in roots in phosphate limited conditions and after *rac*-GR24 treatment (**Figure 3**). We have shown that in Medicago roots, the expression of the putative SL transporter MtABCG59 occurs mainly in the outer cortical cells (**Figure 4B**). The observed expression pattern is compatible with the proposed function of MtABCG59 in SL secretion from the roots toward the soil to induce hyphal branching of AM fungi and guide them to host roots. As *MtABCG59* is also expressed in Medicago colonized roots (**Supplementary Figure 5**), it cannot be excluded that it also participates in the SL translocation to the apoplast, guiding intraradical hyphal branching within the root cortex. Additionally, the presence of *MtABCG59* transcripts in root tips suggests that MtABCG59 might be involved in the export of SL from the site of biosynthesis towards the upper root, as has been previously revealed for PaPDR1 (Sasse et al., 2015). This hypothesis can be supported by the observation that SL-biosynthesis genes *MtCCD7* and *MtCCD8* are downregulated in *mtabcg59* mutant lines compared to WT (**Figure 7**, **Supplementary Figure 6**). Previously, it was proposed that excessive accumulation of SL in the cells can trigger a negative feedback regulation mechanism in SL biosynthesis and affect mRNA accumulation of *CCD7* and *CCD8* (Foo et al., 2005; Hayward et al., 2009; Liu et al., 2018b).

Phenotypic characterization of the *mtabcg59-1* mutant showed that its loss of function negatively influences AM formation, especially, at the early stage of interaction. Previously, it was proposed that altered presymbiotic AM fungal growth could result in delayed colonization, which is apparent at early time points (Montero et al., 2019). That is also

what we observed for *mtabcg59* mutant (**Figure 5**). Likewise, as in other reported SL-related mutants (e.g., biosynthetic, perception, and transport) (Kretzschmar et al., 2012; Lanfranco et al., 2018), the arbuscular structure in colonized root cells was not altered in *mtabcg59* plants (**Figure 5**). This observation supports the notion that SLs influence the fungal morphology mainly in the rhizosphere but do not affect its intercellular accommodation and development. SLs *ex planta* stimulate the seed germination of parasitic weeds such as witchweeds (Striga spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) (Cook et al., 1966; Yoneyama et al., 2018). To verify whether the decrease of AM formation in *mtabcg59-1* is indeed caused by a reduction in the secretion of SLs, we applied a parasitic seed germination bioassay. This approach allows for the indirect but sensitive determination of the relative amounts of SLs exuded by roots (Guillotin et al., 2017). The SL levels secreted by the *mtabcg59-1* were assessed by the germination of *Phelipanche ramosa* seeds that exhibit a broad host range, including *M. truncatula* (Fernandez-Aparicio et al., 2009). Our experiments revealed that root exudates derived from *mtabcg59-1* possess lower germination-inducing activity than exudates from the WT. Interestingly, the effect observed concerned only seeds which were not directly in touch with the roots. Further studies are needed to test whether this lack of difference in seed germination in seeds that are in touch with the root is due to a second, less active transporter or due to diffusion of the hydrophobic SLs. In Medicago apart from *MtABCG59*, expression of *MtABCG43* is induced by phosphorous deficiency and *rac*-GR24 (**Figure 3**). Further investigation is required to demonstrate that MtABCG43 participates in SL secretion, however, functional redundancy is possible. Nevertheless, since the expression of *MtABCG43* decreased in *mtabcg59* mutants compared to WT (**Figure 5D**), the diffusion of SL through the exodermis-free cortex appears to be more likely. In the case of *mtabcg59* mutant, the concentration of diffusion-derived SL on the root surface is still sufficient for parasitic seed germination. Taken together, our findings suggest that MtABCG59 is involved in SL extrusion to the soil to maintain an SL concentration sufficiently high in the proximity of the roots, allowing to induce hyphal branching and guiding the fungi towards the host root (**Figure 9**).

In addition to arbuscular mycorrhiza, SLs are implicated in the legume-rhizobium symbiosis (LRS) (Lopez-Raez et al., 2017). A characteristic feature of this type of interaction is the formation of root nodules, which are the place of nitrogen fixation and nutrient exchange between partners. Phenotypic analyses of SL biosynthetic mutants of *Pisum sativum*, *Lotus japonicus*, and *Glycine max* demonstrated the positive role of SLs in LRS establishment (Foo and Davies, 2011; Liu et al., 2013; Rehman et al., 2018). In the case of *M. truncatula*, exogenous application of *rac*-GR24 had either a positive or negative effect on this process, depending on the SL concentrations used (De Cuyper et al., 2015). We could not detect a significant difference

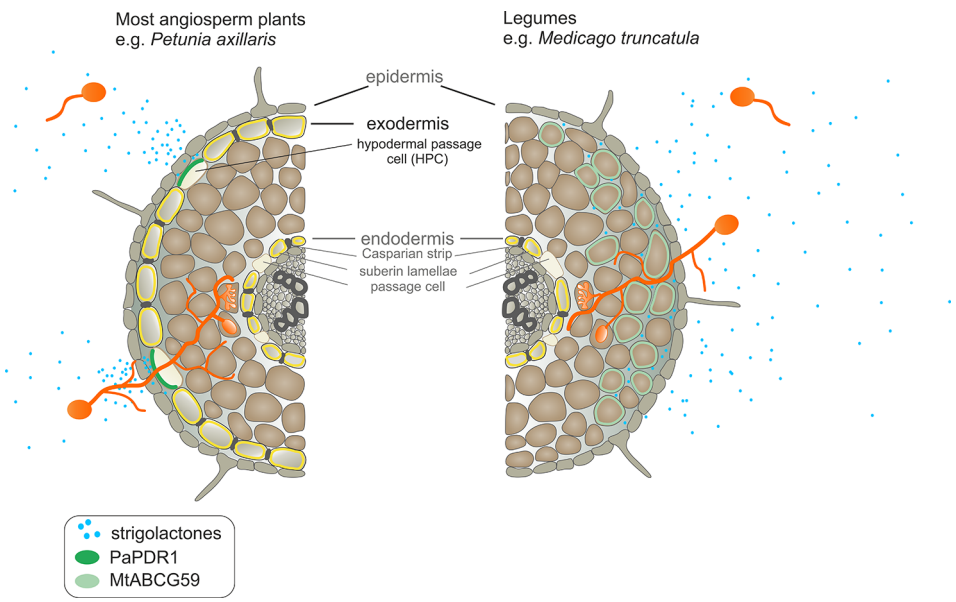

FIGURE 9 | Comparison of strigolactone (SL) exudation into the soil and SL-dependent guidance of arbuscular mycorrhizal fungi to the host root between *Petunia axillaris* and *Medicago truncatula*. In *P. axillaris*, SLs are secreted into the rhizosphere by PaPDR1 that is localized in the hypodermal passage cells (HPCs) within the exodermis. A steep concentration gradient of SLs, created by PaPDR1, guides arbuscular mycorrhizal fungi (AMF) to access penetration sites – i.e. unsuberized HPCs. In the *M. truncatula,* due to a lack of exodermis, SLs could passively enter the rhizosphere because they do not encounter the apoplastic diffusion barrier. However, we suggest that to achieve the full extent of AMF colonization, the active export of SLs, mediated by MtABCG59, is required. Its action ensures sufficiently high concentrations of SLs around the roots to attract AM fungi, which together with the characteristic root anatomical traits in *M. truncatula,* enables the colonization of the whole root surface.

in the root nodule structures or the number of root nodules between the *mtabcg59* and WT despite the fact that *MtABCG59* is expressed in the nodule meristem (**Figure 8**) and co-expressed with the SL biosynthetic genes (*MtD27*, *MtCCD7*, and *MtCDD8*) (van Zeijl et al., 2015a). Although SLs are biosynthesized in mature nodules, their role in this organ remains currently unknown. Pea SL-deficient mutants produced less nodules, but their size and ability to fix atmospheric nitrogen were comparable to those of WT plants. Therefore, it was postulated that SLs affect the LRS mainly in the early stages of the interaction. Recently, it has been shown that SLs promote infection thread formation influencing the bacterial partner (McAdam et al., 2017). Nevertheless, the function of the SL transporter in the nodule, like in the root, could be the removal of SLs from the site of biosynthesis to avoid SL accumulation in the meristem and/or deliver them to the place of perception. It cannot be excluded that a redundant transporter can take over the SL transport under LRS. Analysis of publicly available transcriptomic data showed that in Medicago expression of another *PaPDR1* homologue, *MtABCG43*, is induced after Nod factor treatment (van Zeijl et al., 2015b; Jardinaud et al., 2016) and its mRNA was found to accumulate at the highest level in nodules (**Supplementary Figure 7**). It is also possible that in *M. truncatula* SLs do not play an essential role in determining the nodule number. This assumption can be supported by the observation that the silencing of SL biosynthetic gene *MtD27* did not affect the efficiency of nodulation in Medicago (van Zeijl et al., 2015a).

SL acts not only as a signaling molecule in the rhizosphere but also as a phytohormone that adjusts the overall plant architecture to nutrient availability. They control *inter alia* internode elongation (de Saint Germain et al., 2013) and shoot branching by inhibiting axillary bud outgrowth (Gomez-Roldan et al., 2008; Umehara et al., 2008) as well as increasing root surface, and thus, influencing the lateral root and root hair development (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). It is worth noting that *PhPDR1* and *NtPDR6*, in addition to the roots, are expressed in aerial tissues, especially in nodes. Their mutation and/or silencing enhance shoot branching, giving plants a bushy phenotype (Kretzschmar et al., 2012; Xie et al., 2015). Notably, the *PaPDR*-overexpressing Medicago showed several SL-related phenotypes in the shoot, such as deeper leaf margin serrations and increased internode length. Analogous phenotypes were previously reported for Medicago after the exogenous application of *rac*-GR24 at the primary shoot apex. Conversely, SL deficient (*ccd7*, *ccd8*) and insensitive (*d14*) mutants displayed shallower serrations as well as reduced shoot elongation than the WT (Lauressergues et al., 2015). We did not detect expression of *MtABCG59* in aboveground tissues (**Figure 4A**) or observe alterations in the shoot architecture of the *mtabcg59* mutant (**Supplementary Figure 8**). Hence, we propose that in Medicago one or more additional SL transporter (s) can operate to control aerial developmental traits. One of the candidates might be MtABCG44 (71% amino acid identity to MtABCG59) that exhibits a broad expression pattern (**Supplementary Figure 7**).

CONCLUSION

In conclusion, in the present study we have identified and characterized MtABCG59, an ABC class G transporter involved in SL export towards the soil that positively affects arbuscular mycorrhiza formation in *Medicago truncatula*. We propose that its primary function is releasing SLs into the rhizosphere to attract AMF during the presymbiotic stage of interactions. The root specific expression of *MtABCG59* and the lack of *mtabcg59* aboveground phenotypes suggests that in *M. truncatula*, SL distribution can be mediated by more than one transporter. Moreover, with the overexpression of the petunia SL transporter PaPDR1 in *M. truncatula,* we have demonstrated the importance of ABCG proteins for the translocation of orobanchol-type molecules, regardless of root anatomy and phylogenetic relationships.

DATA AVAILABILITY STATEMENT

Sequence data from this article can be found in the GenBank database under the following accession numbers: MTR_3g107870 for MtABCG59, MTR_1g011640 for MtABCG43, MTR_1g011650 for MtABCG44, MTR_3g095530 for Mtactin, AFA43815 for PaPDR1, MTR_1g028600 for MtPT4, JQ292812 for PaPDR1.

AUTHOR CONTRIBUTIONS

JB and LB conceived and designed the study. JB, LB, and NS performed the experiments. JB and EM secured funding for the project. JB, MJ, LB, and EM analyzed and interpreted the data. JB wrote the manuscript (initial draft preparation). MJ, LB, and EM wrote the manuscript (critical editing and review of the manuscript).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00018/full#supplementary-material

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Functional Characterization of the Arabidopsis Abscisic Acid Transporters NPF4.5 and NPF4.6 in Xenopus Oocytes

Sophie Léran[†‡], *Mélanie Noguero*[‡], *Claire Corratgé-Faillie*, *Yann Boursiac*, *Chantal Brachet and Benoit Lacombe*[*]

BPMP, Univ Montpellier, CNRS, INRAE, Montpellier SupAgro, Montpellier, France

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***Correspondence:**
Benoit Lacombe
benoit.lacombe@supagro.fr

[†]Present address:
Sophie Léran, IPME, Univ Montpellier, CIRAD, IRD, Montpellier, France

[‡]*These authors have contributed equally to this work*

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Few proteins have been characterized as abscisic acid transporters. Several of them are NRT1/PRT Family (NPF) transporters which have been characterized in yeast using reporter systems. Because several members of the NPF4 subfamily members were identified in yeast as ABA transporters, here, we screened for ABA transport activity the seven members of the NPF4 subfamily in Xenopus oocytes using cRNA injection and ^{3}H-ABA accumulation. The ABA transport capacities of NPF4.2, NPF4.5, NPF4.6, and NPF4.7 were confirmed. The transport properties of NPF4.5 and NPF4.6 were studied in more detail. Both ABA transporter activities are pH-dependent and slightly pH-dependent apparent Km around 500 μM. There is no competitive inhibition of the ABA-analogs pyrabactin and quinabactin on ABA accumulation demonstrating a different selectivity compared to the ABA receptors. Functional expression of these ABA transporters in Xenopus oocyte is an opportunity to start structure–function studies and also to identify partner proteins of these hormone transporters.

Keywords: abscisic acid, transport, hormone, Km, pH

INTRODUCTION

The weak acid sesquiterpene abscisic acid (ABA) was identified in plant in the sixties (Zhang, 2014). It is widely described as the stress hormone because it is involved in the plant responses to many biotic and abiotic environmental signals (Cutler et al., 2010). From its discovery to 2010, most of the work was dedicated to the identification of biosynthetic and catabolic pathways, and several enzymes involved in these processes have been identified (Nambara and Marion-Poll, 2005). In 2009, the perception and signaling pathways came back to light with the identification of the ABA receptors from the PYR/PYL/RCAR family (Ma et al., 2009; Park et al., 2009).

While long-distance ABA transport within the plant was characterized years ago, the firsts ABA transmembrane transporters were only identified in 2010 (Boursiac et al., 2013). What are the ABA transporters identified so far?

The first family of protein which have ABA transporters is the ABCG subfamily of ABC (ATP BINDING CASSETTE) transporters, with four of them related to ABA transport: ABCG25, 30, 31, and 40. ABCG25 exports ABA from the vascular parenchyma cells and AtABCG40/PDR12

mediates guard cells ABA uptake to trigger stomatal closure (Kang et al., 2010; Kuromori et al., 2010; Kuromori and Shinozaki, 2010; Kuromori et al., 2016; Kuromori et al., 2018). These transporters, together with AtABCG30 and AtABCG31, are involved in seed dormancy. AtABCG25 and AtABCG31 export ABA from the endosperm whereas AtABCG30 and AtABCG40 import ABA in the embryo to suppress seed germination (Kang et al., 2015). The Medicago MtABCG20 is an ABA exporter involved in root development and seed germination (Pawela et al., 2019).

The second family of ABA transporters is the NPF (NRT1/PTR FAMILY) (Corratgé-Faillie and Lacombe, 2017). An elegant functional screen of ABA transport in yeast was used to identify NPF4.6/AIT1/NRT1.2, NPF4.5/AIT2, NPF4.1/AIT3, and NPF4.2/AIT4 (Kanno et al., 2012). Their ABA transporting activities were tested and confirmed by ABA accumulation studies in either yeast cells or *Sf*9 cells (Kanno et al., 2012). NPF4.6 is expressed around vascular tissues, and mutants defective in AtNPF4.6 have lower surface temperatures than the wild-type, supporting a role as an ABA transporter *in planta* (Kanno et al., 2012). NPF4.6 is also a nitrate transporter (Huang et al., 1999), so the effect of nitrate on ABA accumulation has been tested (Kanno et al., 2013), but an interaction between the two substrates has not been demonstrated. Using the same ABA-dependent two-hybrid system and screening 45 out of the 53 Arabidopsis NPF members, Chiba and coworkers (Chiba et al., 2015) confirmed that NPF4.6, NPF4.1, and NPF4.5—and also additional NPF members such as NPF1.1, NPF2.5, NPF5.1, NPF5.2, NPF5.3, NPF5.7, and NPF8.2—are ABA influx transporters. More recently, Tal et al. (2016) have demonstrated the ability of NPF3.1-expressing oocytes to accumulate ABA. The Medicago MtNPF6.8 is an ABA influx transporter when expressed in Xenopus oocytes (Pellizzaro et al., 2014).

Two other proteins behave as ABA transporters. A DTX/MATE (Detoxification efflux carrier/multidrug and toxic compound extrusion), AtDTX50 is an Arabidopsis efflux transporter involved in ABA sensitivity and drought tolerance (Zhang et al., 2014). In rice, an AWPM-19-family member (OsPM1, PLASMA MEMBRANE PROTEIN1) is an ABA influx transporter involved in drought response (Yao et al., 2018).

Despite the number and the diversity of the ABA transporters, the detailed transport properties of these proteins are largely unknown. The aims of our work were: (i) to identify functional ABA transporters within the 7 NPF4 proteins, using heterologous expression and ^{3}H-ABA and (ii) to perform a detailed characterization of the functional properties of NPF4.5 and NPF4.6. Besides its numerous advantages for membrane transport characterization, the use of Xenopus oocytes also gives the opportunity to determine the transport parameters in other systems.

MATERIALS AND METHODS

Plasmids and cRNA Synthesis

NPF coding sequences (CDS) were either obtained from ABRC (cloned in pENTR223 for NPF4.3, 4.5) or cloned in pENTR/D/TOPO (for clones NPF4.1, 4.2, 4.4, 4.7), and pDONR207 (for clones NPF4.1, 4.6). Each clone was sequenced and compared to Col-0 genomic sequence. LR reaction was performed according to the manufacturer's instructions (Life Technologies), to clone the CDS into the Xenopus oocyte expression vector [pGEM-GWC, (Leran et al., 2015)].

Oocytes Expression

NPFx-pGEM-GWC vectors were linearized and *in vitro* transcribed with mMessage mMachine T7 Ultra Kit following manufacturer protocol (Life Technologies). Xenopus oocytes were purchased from the Centre de Recherche en Biochimie Macromoléculaire (CNRS, Montpellier, France). Oocytes were obtained and injected as previously described (Lacombe and Thibaud, 1998).

ABA Uptake Experiments and ^{3}H-ABA Quantification

For ABA uptake, oocytes were incubated for 20 min in 1 ml of ND96 solution (pH indicated in the figure legends) containing the indicated concentration of ABA (10% of the labeled ^{3}H-ABA, American Radiolabelled Chemicals and 90% of cold ABA, Sigma). They were then washed 4 times in 15 ml of ND96 solution (4°C) containing 5 µM of cold-ABA. Each oocyte was then dissolved in 100 µl of 2% Sodium Dodecyl Sulfate (SDS). Lysis solution was then mixed to 3 ml of scintillating solution (ULTIMAGOLD, PerkinElmer). Incorporated radioactivity was measured by Liquid-Scintillation analyzer (Tri-Carb 2100 TR, Perkin Elmer).

Fitting Procedure

Least squares fit using SIGMAPLOT (11.0, Systat Software Inc.) has been used. The ABA concentration range was between 0 and 5 µM ^{3}H-ABA. Data were fitted by a Michaelis–Menten equation: A = (Amax * [ABA])/(Km + [ABA]), where A is the intracellular ABA accumulation, Amax is the maximum intracellular accumulation, (ABA) is the external ABA concentration and Km is the apparent affinity.

RESULTS

Expression of the Seven AtNPF4 in Xenopus Oocytes

Xenopus oocytes are used to express the seven Arabidopsis NPF4 proteins after injection of *in vitro* transcribed cRNA. Noninjected oocytes were used as negative controls. We used ^{3}H-labeled ABA as a tracer for ABA accumulation into oocytes. After 20 min incubation in ^{3}H-ABA containing ND96 solutions, ^{3}H was quantified into oocytes (**Figure 1**). Control oocytes accumulate low levels of ^{3}H, this could be explained by the membrane diffusion of protonated form of ABA (ABA-H). Whereas in yeast NPF4.1 is an ABA influx transporter (Kanno et al., 2012; Kanno et al., 2013; Chiba et al., 2015), NPF4.1-expressing oocytes accumulate ^{3}H at the same level as the control. NPF4.2 and NPF4.7-expressing oocytes accumulate more than 2.5-fold ^{3}H compared to control oocytes suggesting that ABA is a substrate for these transporters. NPF4.3 and

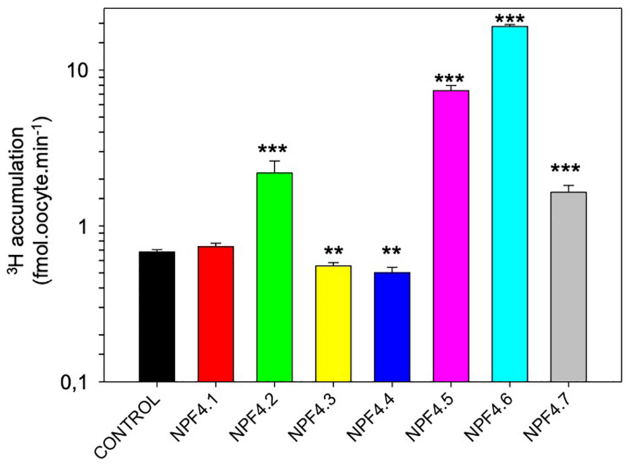

FIGURE 1 | Screen for ABA transport activity of Arabidopsis NPF4 subfamily members in Xenopus oocytes. Control (noninjected) and NPF4-injected oocytes were bathed in 1 µM ^{3}H-ABA (pH = 6.0), and ^{3}H accumulation in oocytes was quantified after 20 min. Values are mean +/– SEM (n = 5–12 oocytes, biological replicates). ***P < 0.001, **0.001 < P < 0.005, two-sided t-test after comparison with control oocytes.

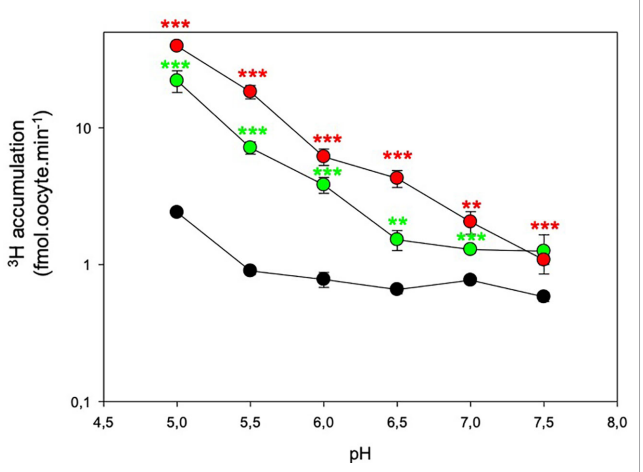

FIGURE 2 | pH-dependent ^{3}H accumulation in NPF4.5 and NPF4.6 in Xenopus oocytes. Control (noninjected, black circles), NPF4.5 (green circles) and NPF4.6 (red circles) injected oocytes were bathed in 1 µM ^{3}H-ABA (pH = 5.0 to 7.5), and ^{3}H accumulation in oocytes was quantified after 20 min. Values are mean +/– SEM (n = 6–20 oocytes, biological replicates). ***P < 0.001, **0.001 < P < 0.005, two-sided *t*-test after comparison with control oocytes.

NPF4.4-expressing oocytes accumulate less ^{3}H; this suggests that they behave as ABA efflux transporters. However, this should be confirmed by performing an experiment specifically designed to identify efflux transporter by injecting ABA into the oocytes. The highest accumulation was obtained in oocytes expressing NPF4.5 and NPF4.6 (**Figure 1**).

Since NPF4.5 and 4.6 showed high ABA accumulation, we focused on these two transporters for further characterization.

Effect of External pH on ABA Accumulation

Most of the NPFs and their animal and bacterial counterparts are proton-coupled transporters. So, we quantified ^{3}H accumulation at different external pH, ranging from 5.0 to 7.5 (**Figure 2**). In control oocytes, ^{3}H accumulation is not affected in the 5.5–7.5 range and slightly increases at pH 5.0. This is probably due to an increase in the concentration of the protonated form of ABA at acidic pH which increases the membrane diffusion of this form. The external pH sensitivity of NPF4.5 and NPF4.6 is equivalent. ^{3}H accumulation is enhanced by acidic pH and NPF-dependent ^{3}H accumulation is very low at pH above 7.0.

ABA Dose Response

An important property of a transporter is its affinity towards its substrate. We have assessed the apparent affinity (Km) of NPF4.5 and NPF4.6 towards ABA by quantifying ^{3}H accumulation into the oocytes at different external ABA concentrations in the 0–5 µM range (**Figure 3**) and at different pH (5.0, 5.5, 6.0, 6.5, 7.0). Data were fitted by a Michaelis–Menten equation: A = (Amax * [ABA])/(Km + [ABA]). This fitting procedure allows determining the apparent affinity of ABA for the transporters (Km). The calculated Km is slightly dependent on the external pH: the Km increases with increase in external pH. At the four tested pH, the Km for both transporters are around 500 nM (**Table 1**).

Effect of Quinabactin and Pyrabactin on ABA Accumulation

Several ABA-analogs have been identified and characterized (Cao et al., 2017). Within these analogs, pyrabactin and quinabactin induce physiological responses, similar to ABA, through their direct binding to the ABA-receptors from the PYR/PYL/RCAR family. However, nothing is known about their effect on ABA transporters. Direct transport of these ABA-analogs was not possible because there is a no labeled-form of these molecules; so we tested their effect on ABA transport (^{3}H accumulation). To test the competition, two concentrations of ABA-analogs were tested at 0.5 and 5 µM in the presence of 1 µM ABA. Neither quinabactin (Okamoto et al., 2013) nor pyrabactin (Park et al., 2009; Kanno et al., 2012) was able to decrease the ^{3}H-ABA accumulation into the oocytes, suggesting that they are not transported by, nor bound to NPF4.5 and NPF4.6.

DISCUSSION

Within the different families of membrane transporters, NPF can transport structurally different substrates (Corratgé-Faillie and Lacombe, 2017). In this family, transporters for different hormones have been identified: auxin, GA, Jasmonate, and ABA. To date, the structure–function relationships are not well defined (Jørgensen et al., 2015; Jørgensen et al., 2017; Longo et al., 2018) and it is not

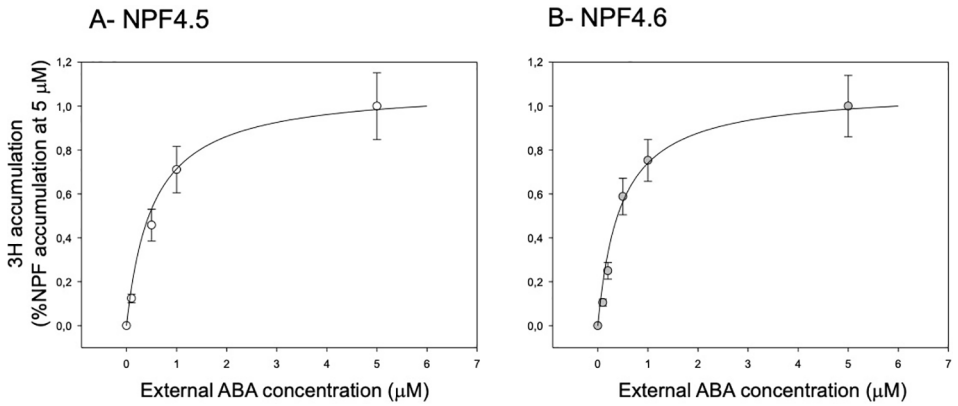

FIGURE 3 | Effect of external ABA concentration on ^{3}H accumulation in NPF4.5 and NPF4.6-expressing oocytes. ^{3}H-accumulation in NPF4.5 **(A)** and NPF4.6 **(B)** expressing oocytes depending on the wide external ABA concentrations range (0–5 µM ^{3}H-ABA). The solid lines are least-squares Michaelian fits. Data are mean +/– SE (n = 6–10 oocytes, biological replicates).

TABLE 1 | Effect of external pH on ABA affinity. ABA accumulations have been determined in different external ABA concentrations (0–5 µM ^{3}H-ABA) at 4 different external pH levels: 5.0, 5.5, 6.0, and 6.5.

pH	5.0		5.5		6.0		6.5	
NPF	**NPF4.5**	**NPF4.6**	**NPF4.5**	**NPF4.6**	**NPF4.5**	**NPF4.6**	**NPF4.5**	**NPF4.6**
Km (nM)	462	395	485	423	522	452	545	472
± SEM	± 35	± 25	± 47	± 33	± 41	± 25	± 55	± 62

*At each pH, data were fitted by a Michaelis–Menten equation: A = (Amax * [ABA])/[Km + [ABA]) to determine the Km. Values are mean +/– SEM (n = 5–12 oocytes, biological replicates).*

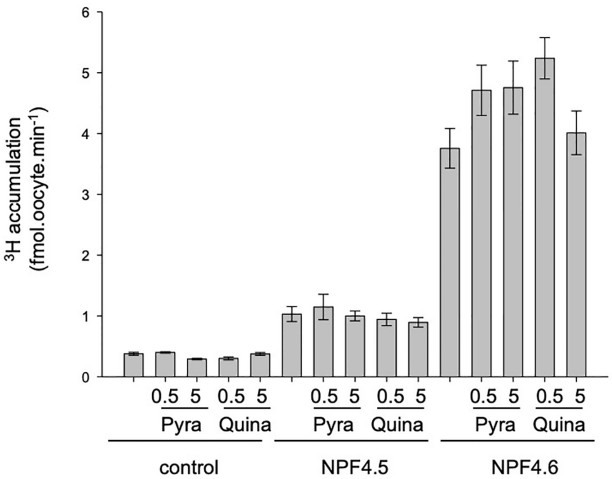

FIGURE 4 | Effect of pyrabactin and quinabactin on ^{3}H accumulation in NPF4.5 and NPF4.6 in Xenopus oocytes. Control (noninjected), NPF4.5 and NPF4.6 injected oocytes were bathed in 1 µM ^{3}H-ABA (pH = 6.0), and ^{3}H accumulation in oocytes was quantified after 20 min in the presence or in the absence of 0.5 or 5 µM of pyrabactin or quinabactin. Values are mean +/– SEM (n = 9–11 oocytes, biological replicates). The pyrabactin and quinabactin treatment has no significant effect on ^{3}H accumulation (two-sided *t*-test).

possible to predict the substrate from the sequence. NPF ABA transporters have been characterized in yeast and Sf9 insect cells (Kanno et al., 2012; Kanno et al., 2013; Chiba et al., 2015). These researches identified the NPF4 as a subfamily with several ABA transporters. This work unveils the transport properties of two of these expressed in Xenopus oocytes, AtNPF4.5 and NPF4.6. These data give new insights into the transmembrane transport of ABA influxer. Furthermore, we present our screen of ABA accumulation in oocytes expressing each member of the Arabidopsis NPF4 subfamily. This demonstrates that Xenopus oocytes combined with ^{3}H-ABA quantification can be used to study plant ABA transporters.

Our screen confirms that ABA is a substrate for NPF4.5/AIT2, NPF4.6/AIT1/NRT1.2 and NPF4.2/AIT4 (**Figure 1**, Chiba et al., 2015). In our experimental conditions, we were not able to demonstrate an ABA transport activity of NPF4.1/AIT3, further experiments in different conditions should be performed to understand the different results obtained in yeast (Kanno et al., 2012; Kanno et al., 2013; Chiba et al., 2015). The data obtained with NPF4.3 and NPF4.4 should also be studied in more detail. Indeed, in all experiments performed, oocytes expressing these transporters always accumulated less ^{3}H (ABA) than control oocytes (**Figure 1**). This is an indication of a putative role in ABA efflux. This could explain the negative results obtained with these transporters expressed in yeast (Kanno et al., 2012; Kanno et al., 2013; Chiba et al., 2015). Finally, NPF4.7 displays ABA transport activity in Xenopus oocytes unlike in yeast (Kanno et al., 2012; Kanno et al., 2013; Chiba et al., 2015). This demonstrates that the use of different heterologous expression systems is a prerequisite to a definitive conclusion about the substrate selectivity of a specific transporter. The functional properties of different

plant transporters have been determined in several expression systems and are known to be affected by the expression host [*e.g.* (Dreyer et al., 1999)]. Several explanations have been proposed and it is not possible from our results to discriminate between them: membrane lipid composition, membrane potentials, expression of endogenous regulators (kinases, phosphatases, …), different cytosolic compositions (pH, calcium). In a previous screen (Leran et al., 2015), the nitrate uptake capacity of NPF4.3, 4.3, 4.5, 4.6 was tested, and none of these proteins displayed nitrate transport properties.

We have identified a strong positive effect of external acidification (**Figure 2**) with small effect on the Km (**Table 1**). This could be indicative of an increase in the diffusion of the protonated membrane-permeable form of ABA (ABA-H) because acidification increases its concentration. ABA is a weak acid in equilibrium between the anionic (ABA^-) form and the protonated (ABA-H) form. The pKa (4.7 for ABA) is the pH at which both forms are at the same concentration (at pH 4.7, 50% of abscisic acid is ABA^- and 50% is ABA-H form). At a more acidic pH, ABA-H is the dominant form; whereas at a basic pH $[ABA^-] > [ABA\text{-}H]$. For example, at pH 7.7, $[ABA^-] = 1000 \times [ABA\text{-}H]$. The protonated form (ABA-H) is uncharged, and therefore, is able to diffuse freely through the membrane lipid bilayer. This phenomenon did not significantly affect ABA accumulation in the control oocyte which is very slightly pH dependent (**Figure 2**). The three other explanations for this are: (i) as most of the NPF characterized so far, NPF4.5 and NPF4.6 are proton coupled transporters, (ii) acidification induces protonation of some amino-acids which induce a modification of the transport properties, and (iii) the transported form of ABA is ABA-H and not the negatively charged ABA^-. It is not yet possible to determine which one of these explanations is the right one. It could even be a combination of two or three of these hypotheses. Further studies using site-directed mutagenesis will give the opportunity to test these hypotheses.

The dose response curve of ABA transport activity *versus* the external ABA concentration follows a Michaelis–Menten behavior (**Figure 3**). Fitting the data allows determining the affinity: the Km is *ca.* 500 nM for both transporters and is only slightly modified by external pH (**Table 1**). This is 10-fold lower than what has been previously determined by NPF4.6-expression in yeast [5 μM, (Kanno et al., 2012)]. These experiments in yeast have been performed at pH 7.5, whereas our experiments were done at pH 6.0. We cannot test this in oocytes because, at pH 7.5, there is no NPF-dependent ^3H accumulation. But the small change in Km in the 5.0–6.5 range (**Table 1**) does not support the fact that the difference in Km observed in yeast and in oocyte is explained by a different external pH.

The selectivity and affinity of ABA receptors have been studied, and several ABA analogs with higher affinity for the receptors have been identified, as pyrabactin and quinabactin **(Figure 4)** (Park et al., 2009; Okamoto et al., 2013). The effect of these molecules on ABA transport has been tested. They have no effect on ^3H accumulation, suggesting that (i) they are not competitive inhibitors of NPF-dependent ABA transport and (ii) they are not transported. However, the development of labeled forms of these molecules is necessary to confirm the absence of transport.

Xenopus oocytes have been used to characterize plant transporters from different transporter families (Larsen et al., 2017). The possibility to use this convenient system for most plant hormones is now established (Wulff et al., 2019), and specific drawbacks have been recently identified (Wulff et al., 2019). This system can be used to characterize ABA transport from the NPF family and will be used to perform a structure–function analysis to identify the amino-acids involved in the ABA selectivity of these transporters. It will be also interesting to study the properties of the ABA transporter from the ABCG family expressed in Xenopus oocytes. Some more data should also be obtained *in planta* to have a better understanding of the transport properties in different tissues (Boursiac et al., 2013).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SL, MN, CC-F, YB, CB, and BL performed the research and analyzed the data. BL conceived the work and wrote the manuscript.

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