AN UPDATE ON BRASSINOSTEROIDS: HOMEOSTASIS, CROSSTALK, AND ADAPTATION TO ENVIRONMENTAL STRESS

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AN UPDATE ON BRASSINOSTEROIDS: HOMEOSTASIS, CROSSTALK, AND ADAPTATION TO ENVIRONMENTAL STRESS

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EDITORIAL
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Editorial: An Update on Brassinosteroids: Homeostasis, Crosstalk, and Adaptation to Environmental Stress

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Editorial on the Research Topic

An Update on Brassinosteroids: Homeostasis, Crosstalk, and Adaptation to Environmental Stress

Over the last three decades, there have been significant advances in the understanding of brassinosteroid (BR) biosynthesis and signaling, particularly in the model plant species *Arabidopsis thaliana*. BRs regulate a variety of morphogenetic and physiological processes throughout plant life. Notably, BR biosynthesis and signaling are interconnected with the signaling pathways of other phytohormones and environmental stresses. Gathering knowledge about these aspects in monocot and dicot crops is of particular importance as it may allow modulation of these processes and enable the development cultivars better adapted to ongoing climate change. This Research Topic, providing *An Update on Brassinosteroids: Homeostasis, Crosstalk, and Adaptation to Environmental Stress* is aimed at introducing the latest findings in the regulation of BR metabolism, the interconnection of the BR signalosome with phytohormonal and stress signaling pathways, and the BR-mediated adaptation of plants to environmental conditions. The Research Topic includes five reviews and one original research article.

As far as the BR biosynthesis pathway is concerned, Bajguz et al. provided a comprehensive review of the complicated process. The authors presented a description of the BR biosynthesis and their sterol precursors. BR biosynthesis leads to the production of the C_{27}-, C_{28}-, or C_{29}-type BRs. Thus, the authors described the early steps of the process that are common for each type and later presented the steps which differentiate the BR biosynthesis pathways. The interconnections between the distinct BR biosynthesis pathways were also described. An important part of this review is focused on the BR biosynthesis inhibitors because they are useful tools for investigating the biosynthetic pathways and for manipulating the BR content in crops.

Another review article by Wei and Li presented the recent advance in elucidating mechanisms that control the BR homeostasis in reaction to both internal and external cues. Understanding this process is of particular importance, as BRs control plant growth and development in a dose-dependent manner, and a fine-tuning of BR homeostasis is critical for optimal BR functions. This article converges with the above-mentioned review and places aspects of BR biosynthesis in a

broader, regulatory context. The review presented the regulation of BR biosynthesis and catabolism by hormonal and environmental cues in Arabidopsis but also in rice and other crop species. It is of significant importance, as elucidating the mechanisms regulating the BR homeostasis may allow the development of new, high yield crops *via* manipulating BR contents.

A series of articles in this Research Topic described the role of BRs in the regulation of plant reaction to environmental stresses. The first article of this Research Topic by Ramirez and Poppenberger discusses the role of BR in the regulation of cold stress tolerance. Interestingly, in comparison with gibberellins, BRs improve cold stress tolerance with fewer trade-offs in terms of growth and yield. The authors indicated that in addition to improving basal tolerance, BRs contribute to acquired freezing tolerance in Arabidopsis. It involves the molecular and biochemical changes induced by low, but non-freezing temperatures. This adaptation may result from the fact that BRs are involved in cell wall remodeling mechanisms. It was also reported that BRs can increase lignin accumulation. The article comprehensively reviewed biochemical and cellular adaptations during plant reaction to the cold stress, with an emphasis on the BR influence on the cold stress perception and signaling.

Another review article by Kour et al. described the role of BRs in the regulation of plant reaction to heavy metal stress. BRs reduce the uptake of heavy metals by altering cell membrane permeability. They also induce antioxidant enzymes which scavenge the reactive oxygen species that accumulate within the cell in reaction to the stress. BRs alleviate heavy metal toxicity by increasing the concentration of potassium and sodium ions, proline, antioxidants, and osmolytes. The article includes a review of various experiments in which crop plants were exposed to heavy metal stress and were simultaneously treated with exogenous BRs. The article also presented an emerging view of interhormonal crosstalk (mostly involving BR, auxin, and cytokinin) and the interplay of BR-polyamines, which regulate reaction to stress.

The molecular mechanisms underlying the regulatory role of BRs in the modulation of plant growth and stress responses are described in a review article by Kono and Yin. The article described the functions and regulation of the BES1/BZR1 transcription factors which are key modulators of the BR-dependent gene expression. Moreover, they constitute hubs interconnecting the BR signaling with the response pathways of other phytohormones and environmental cues. Interestingly, SUMOylation appears to have an opposite effects on the functions of the two (highly similar) transcription factors. It is postulated that the opposite effects result from distinct SUMOylation target sites in each transcription factor. Moreover, the SUMOylation of BZR1 is regulated by salt stress, and the BR and salt-stress signaling pathways interact at multiple targets. The review also described the involvement of the BES1/BZR1 transcription factors in the crosstalk between the BR, jasmonic acid, auxin, and UV-B signaling pathways during plant morphogenesis and reaction to environmental stresses.

The interconnection between BR signaling and salt-stress response is also explored in the original research article by Dong et al. The article describes the development of the mutant alleles of *SERK2* (involved in the BR signaling) by the CRISPR/Cas9 method. The knockout of *SERK2* resulted in a compact plant stature but increased grain size. The *serk2* mutants showed an increased salt sensitivity which seemed to be independent of ABA. On the other hand, overexpression of the *SERK2* gene enhanced salt tolerance without a negative effect on plant architecture. Interestingly, *SERK2* positively regulates salt tolerance, however, it is suppressed by salinity at the transcription level. On the contrary, the SERK2 protein accumulation is greatly induced by stress. Generally, *SERK2* could be a valuable target for engineering plant architecture and salt-stress tolerance.

This Research Topic provides important updates on BR homeostasis and functions. However, several aspects such as the involvement of BRs in the epigenetic and small RNA-mediated regulation of gene expression require further research and elucidation.

AUTHOR CONTRIBUTIONS

DG wrote the manuscript. All authors revised the manuscript and gave final approval for publication.

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MINI REVIEW
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Comprehensive Overview of the Brassinosteroid Biosynthesis Pathways: Substrates, Products, Inhibitors, and Connections

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Brassinosteroids (BRs) as a class of steroid plant hormones participate in the regulation of numerous developmental processes, including root and shoot growth, vascular differentiation, fertility, flowering, and seed germination, as well as in responding to environmental stresses. During four decades of research, the BR biosynthetic pathways have been well studied with forward- and reverse genetics approaches. The free BRs contain 27, 28, and 29 carbons within their skeletal structure: (1): 5α-cholestane or 26-nor-24α-methyl-5α-cholestane for C_{27}-BRs; (2) 24α-methyl-5α-cholestane, 24β-methyl-5α-cholestane or 24-methylene-5α-cholestane for C_{28}-BRs; (3) 24α-ethyl-5α-cholestane, 24(*Z*)-ethylidene-5α-cholestane, 25-methyl-5α-campestane or 24-methylene-25-methyl-5α-cholestane for C_{29}-BRs, as well as different kinds and orientations of oxygenated functions in A- and B-ring. These alkyl substituents are also common structural features of sterols. BRs are derived from sterols carrying the same side chain. The C_{27}-BRs without substituent at C-24 are biosynthesized from cholesterol. The C_{28}-BRs carrying either an α-methyl, β-methyl, or methylene group are derived from campesterol, 24-epicampesterol or 24-methylenecholesterol, respectively. The C_{29}-BRs with an α-ethyl group are produced from sitosterol. Furthermore, the C_{29} BRs carrying methylene at C-24 and an additional methyl group at C-25 are derived from 24-methylene-25-methylcholesterol. Generally, BRs are biosynthesized *via* cycloartenol and cycloartanol dependent pathways. Till now, more than 17 compounds were characterized as inhibitors of the BR biosynthesis. For nine of the inhibitors (e.g., brassinazole and YCZ-18) a specific target reaction within the BR biosynthetic pathway has been identified. Therefore, the review highlights comprehensively recent advances in our understanding of the BR biosynthesis, sterol precursors, and dependencies between the C_{27}-C_{28} and C_{28}-C_{29} pathways.

Keywords: brassinazole, brassinolide, castasterone, inhibitors, mevalonate and nonmevalonate pathways, sterols

INTRODUCTION

Brassinosteroids (BRs) represent the sixth class of plant hormones. Since the discovery of brassinolide (BL) in 1979, about 70 naturally occurring compounds from this group have been reported as free molecules or conjugates with glucose and fatty acids. BRs are structurally very similar to androgens, estrogens, corticoids, and ecdysteroids. Their presence was reported both in lower and higher plants, especially in angiosperms; and also in all plant organs, including roots, stems, leaves, flowers, anthers, pollen, seeds, and grain (Bajguz and Tretyn, 2003; Yokota et al., 2017; Bajguz, 2019; Zullo and Bajguz, 2019). BRs play an essential role in the development and growth of plants. They elicit a broad spectrum of morphological and physiological responses as well as a tolerance against abiotic and biotic stress (Bajguz and Hayat, 2009; Bajguz and Piotrowska-Niczyporuk, 2014; Wei and Li, 2016; Wendeborn et al., 2017; Ahanger et al., 2018; Siddiqui et al., 2018; Nolan et al., 2020).

CHEMICAL STRUCTURE OF BRs

Based on the total number of carbons, BRs are divided into C_{27}, C_{28}, and C_{29}-type. The basic structure of C_{27}-BRs is a 5α-cholestane skeleton, C_{28}-BRs: 5α-ergostane, and C_{29}-BRs: 5α-stigmastane (**Figure S1**). Differences in the structure of these hormones are due to the type and orientation of oxygenated functions in the A- and B-ring, as well as the number and position of functional groups in the side chain of the molecule. These modifications arise during oxidation and reduction reactions. Based on the cholesterol (CR) side chain, BRs are divided by different substituents into C-23, C-24, C-25, 23-oxo, 24*S*-methyl, 24*R*-methyl, 24-methylene, 24*S*-ethyl, 24-ethylidene, 24-methylene-25-methyl, 24-methyl-25-methyl; without substituent at C-23, without substituent at C-24, and without substituents at C-23, C-24. BRs can also conjugate with glucose and fatty acids (Fujioka and Yokota, 2003; Bajguz, 2007; Piotrowska and Bajguz, 2011; Wendeborn et al., 2017; Zullo and Bajguz, 2019).

BRs BIOSYNTHETIC PATHWAYS

Three pathways of the BR biosynthesis leading to the production of C_{27}-, C_{28}-, or C_{29}-type of BRs are currently known (**Figure 1**; **Figure S1**). Early steps of their synthesis are common for each type and may occur *via* mevalonate (MVA) or non-MVA pathway, while later steps differentiate the BR biosynthesis pathways (cycloartenol- and cycloartanol-dependent). So far, most of the reactions, enzymes, and genes were discovered and characterized by the C_{28}-BR biosynthesis pathway (mostly in *Arabidopsis thaliana*, from which the majority of genes in this pathway were isolated). Their biosynthesis includes two major stages: biosynthesis of campesterol and 22α-hydroxycampesterol. The direct substrate of C_{27}-BRs viz. cholesterol (CR) is finally converted to 28-norBL, whereas the biosynthesis of C_{29}-BRs is initiated from β-sitosterol and leads to 28-homoBL. However, not all indirect compounds of these two pathways have been identified (**Figure 1**; **Figure S1**) (Fujioka et al., 2002; Kwon and Choe, 2005; Fujita et al., 2006; Ohnishi et al., 2006b; Chung and Choe, 2013; Roh et al., 2017; Kim et al., 2018; Rozhon et al., 2019).

Early Steps of BRs Biosynthesis

Biosynthesis of isopentenyl pyrophosphate (IPP), which is an indirect compound in the CR synthesis pathway, can occur *via* two pathways: non-MVA in lower plants and MVA in the most of higher plants. The initial compounds in non-MVA pathway are D-glyceraldehyde 3-phosphate and pyruvate, which are transformed into the 1-deoxy-D-xylulose 5-phosphate (DOXP) by the DOXP synthase. Then, DOXP is converted to 2-C-methyl-D-erythritol 4-phosphate (MEP) by the DOXP reductoisomerase. The next step leads to the formation of 4-(cytidine 5'-diphospho)-2-C methyl-D-erythritol (CDP-ME) from MEP. The reaction is catalyzed by the CDP-ME synthase. Then, CDP-ME is converted to CDP-methyl-D-erythritol-2-phosphate by the CDP-ME kinase, and the obtained compound is transformed to 2-C-methyl-D-erythitol-2,4-cyclodiphosphate by the ME cyclodiphosphate synthase. Finally, with the action of reductases and dehydratases, the IPP is synthesized (**Figure 1**; **Figure S1**) (Lichtenthaler, 2000). In the MVA pathway, three molecules of acetyl-CoA are combined to produce 3-hydroxy-3-methyloglutaryl-CoA by the HMG-CoA synthase. The obtained compound is reduced to MVA by the HMG-CoA reductase. IPP is synthesized from MVA through the two indirect phosphorylation intermediates, such as MVA-phosphate and MVA-pyrophosphate (MVA-PP) (Miziorko, 2011). Enzymes involved in these reactions are the MVA kinase, phospho-MVA kinase, and MVA-PP decarboxylase, respectively (**Figure 1**; **Figure S1**) (Wang et al., 2017).

Biochemical changes of IPP *via* geranyl pyrophosphate and farnesyl pyrophosphate lead to the synthesis of squalene, which is oxidized to squalene-2,3-oxide *via* squalene epoxidase and the latter is converted to cycloartenol by the cycloartenol synthase. Cycloartenol is a key compound for the BR biosynthesis because it constitutes a substrate for multistep reactions in few pathways, which lead to the synthesis of C_{27}-, C_{28}-, and C_{29}-BRs. Conversion of cycloartenol *via* cycloartanol and in a series of subsequent reactions to cholesterol/cholestanol and/or 6-oxocholestanol leads to the synthesis of C_{27}-BRs (**Figure 1**; **Figure S1**) (Wang et al., 2017).

Cycloartenol may also be a substrate of the C-24 methylation reaction, which is catalyzed by the sterol C-24 methyltransferase (SMT1), and leads to 24-methylenecycloartanol. The next few reactions are catalyzed by C-4 sterol methyl oxidase (SMO1), cycloprophylsterol isomerase, obtusifoliol 14α-demethylase (CYP51), and sterol C-14 reductase, leading to the synthesis of 4α-methylergostatrienol. Indirect products of these reactions are cycloeucalenol and obtusifoliol. In next step, the sterol C-14 reductase which is encoded by the *FACKEL*/*HYDRA2* gene catalyzes the reduction of 4α-methylergostatrienol to 4α-methylergostadienol, which is converted in the subsequent reaction to 24-methylenelophenol by the sterol 8,7 isomerase

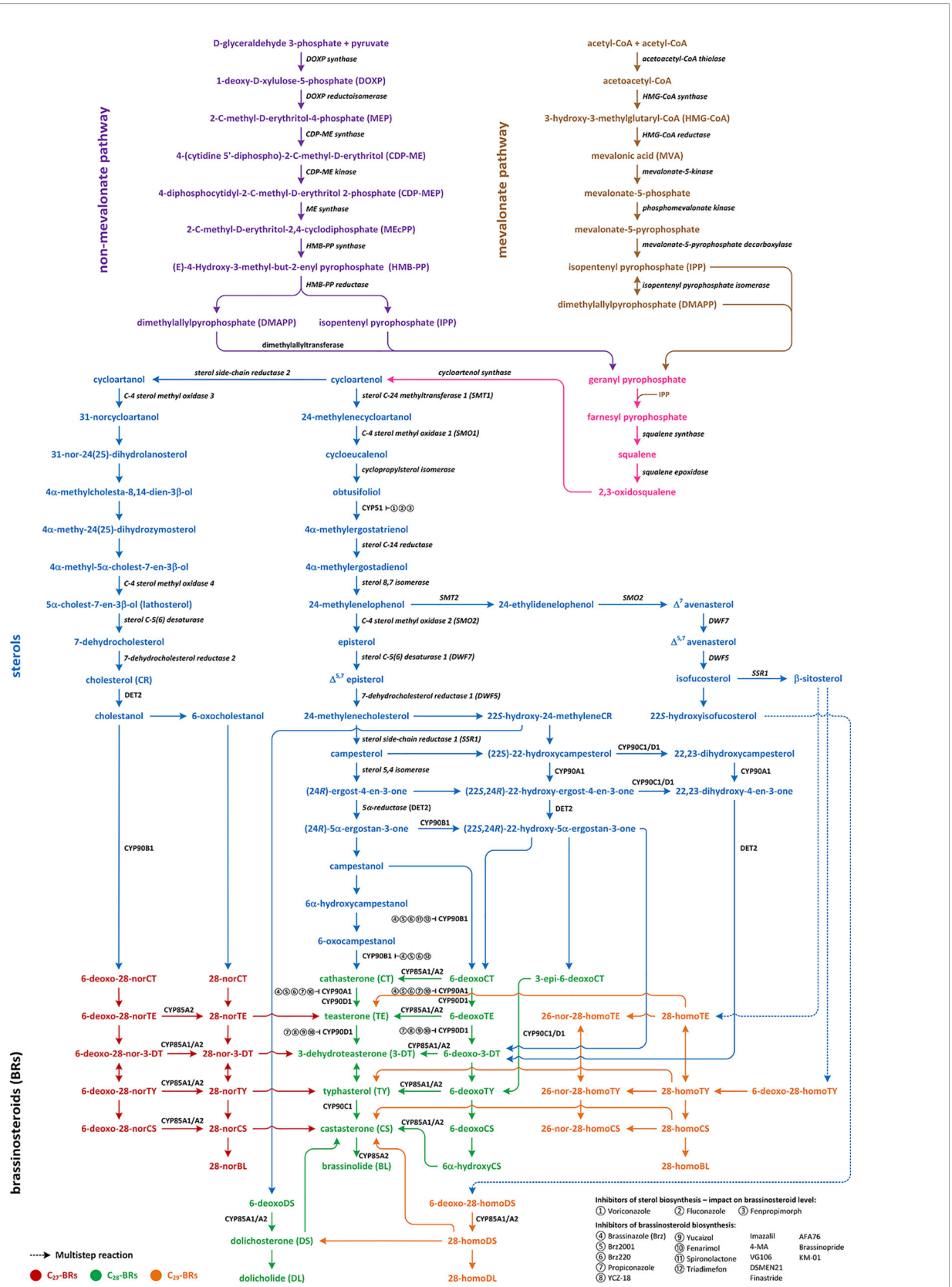

FIGURE 1 | Multistep reactions of brassinosteroids biosynthesis and their sterol biosynthetic precursors.

(Kushiro et al., 2001; Schneider, 2002; Sonawane et al., 2016; Wang et al., 2017).

24-methylenelophenol is a substrate of two independent pathways of sterol biosynthesis. The first leads to the biosynthesis of isofucosterol/β-sitosterol that are precursors of the C_{29}-BR biosynthesis (Xin et al., 2016); the second pathway, 24-converts methylenelophenol to campesterol, which is a substrate of the C_{28}-BR biosynthesis (**Figure 1**; **Figure S1**) (Choe, 2006; Sonawane et al., 2016).

Biosynthesis of C_{27}-BRs

The C_{27}-BR biosynthesis pathway starts from the conversion of cycloartenol to cycloartanol (**Figure 1**; **Figure S1**) by the sterol side chain reductase 2 (SSR2) and proceeds through a synthesis of 31-norcycloartanol from cycloartanol by the C4-sterol methyloxidase3 (SMO3), and further biochemical changes of 31-norcycloartanol up to 31-nor-24(25)-dihydrolanosterol, 4α-methylcholestadienol, 4α-methyl-24(25)-dihydrozymosterol, 4α-methylcholestenol, 5α-cholest-7-en-3β-ol, 7-dehydroCR and finally CR, respectively. The reaction of 5α-cholest-7-en-3β-ol synthesis is catalyzed by the C4-sterol methyloxidase 4 (SMO4), while sterol C5 (6) desaturase catalyzes synthesis of 7-dehydrocholesterol. Cholesterol is synthesized from 7-dehydrocholesterol by the 7-dehydrocholesterol reductase 2 (Sonawane et al., 2016). The biosynthesis of C_{27}-BRs might occur through the late C6 oxidation pathway. Firstly, cholesterol is converted to cholestanol (a C_{27}-BR biosynthesis precursor) by the 5α-reductase encoded by the *DET2* gene. The 5α-reductase DET2 belongs to enzymes that have broad substrate specificity; therefore, it catalyzes reaction (**Figure 1**; **Figure S1**). In the next steps of the C_{27}-BR biosynthesis pathway 6-deoxo-28-norcathasterone, 6-deoxo-28-norteasterone, 6-deoxo-28-nor-3-dehydroteasterone, 6-deoxo-28-nortyphasterol and 6-deoxo-28-norcastasterone are synthesized in the consecutive reactions. Furthermore, the early C6 oxidation pathway is initiated through oxidation of cholestanol into 6-oxocholestanol, which is then followed by a synthesis of 28-norcathasterone, 28-norteasterone, 28-nor-3-dehydroteasterone, 28-nortyphasterol, 28-norcastasterone, and 28-norbrassinolide. Enzymatic conversions of compounds from the late C6 oxidation pathway to the early C6 oxidation counterparts have been evidenced, e.g. 6-deoxo-28-norteasterone to 28-norteasterone, 6-deoxo-28-nortyphasterol to 28-nortyphasterol, and 6-deoxo-28-norcastasterone to 28-norcastasterone. It is known that oxidation/hydroxylation steps in the all BR biosynthetic pathways are catalyzed by cytochrome P450 enzymes. The CYP85A1 and CYP85A2 oxidases, similarly to the 5α-reductase DET2, belong to enzymes of broad substrate specificity. They catalyze the oxidation reactions connecting the late and early counterparts of the C_{27}-BR biosynthesis pathway, and also catalyze corresponding reactions during the C_{28}-BR pathway (**Figure 1**; **Figure S1**) (Kim et al., 2005; Fujita et al., 2006; Joo et al., 2012; Zhao and Li, 2012; Joo et al., 2015).

Biosynthesis of C_{28}-BRs

24-methylenelophenol may also be converted to episterol, which is the first characteristic metabolite in the C_{28}-BR biosynthesis. The reaction is catalyzed by the C-4 sterol methyl oxidase 2 (SMO2) (**Figure 1**; **Figure S1**). Then, episterol is converted to 5-dehydroepisterol by the sterol C-5(6) desaturase encoded by the *DWF7* gene (also known as *STE1*), which is then converted to 24-methyleneCR (catalyzed by 7-dehydrocholesterol reductase encoded by the *DWF5* gene) (Choe, 2006; Ohnishi, 2018). Further stages of the C_{28}-BR biosynthesis may proceed through two parallel pathways, called the late and early C-22 oxidation pathway. Reduction of 24-methyleneCR to campesterol initiates the late C-22 oxidation pathway and is catalyzed by the C-24(25)-sterol reductase in a two-step reaction in which 24-methyl-desmosterol is an intermediate (Dockter et al., 2014). The enzyme (also known as sterol side-chain reductase 1), which catalyzes the production of campesterol, is encoded by the *DWF1* gene. Campesterol is then transformed in the 5,4 isomerization reaction to (24*R*)-ergostan-4-en-3β-one. The latter is then converted through the DET2-mediated 5α-reduction to (24*R*)-5α-ergostan-3-one, which is transformed into campestanol (CN). In the parallel, early C-22 oxidation pathway, C-22α hydroxylation of 24-methyleneCR leads to the synthesis of 22-hydroxy-24-methyleneCR. The reaction of C-22α hydroxylation is catalyzed by the C-22α hydroxylase, which is encoded by the *DWF4* gene. The enzyme belongs to the P450 cytochrome family (Fujiyama et al., 2019). The next reactions are analogous to the late C-22 oxidation pathway and result in the synthesis of 22-hydroxy forms of the corresponding compounds. However, an essential difference between the C-22 oxidation sub-pathways is the synthesis of 6-deoxocathasterone (6-deoxoCT) from (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one, without synthesis of campestanol (CN) (CN-independent pathway of BRs biosynthesis) as a result of the early C-22 oxidation. On the other hand, in each stage of the late C-22 oxidation pathway, the compound can be hydroxylated by the C-22α hydroxylase into hydroxygenated forms of early C-22 pathway (Choe, 2004; Ohnishi, 2018). Moreover, biochemical changes of 22-hydroxymethyleneCR can lead to the synthesis of 6-deoxodolichosterone, which may be further converted into dolichosterone (DS), and dolicholide (DL), as well as to castasterone (CS) and BL (Roh et al., 2017).

Campestanol may be a substrate of the BR biosynthesis in a parallel manner, both in late C6 oxidation pathway (when hydroxylation of carbon atoms in the A-ring and both C-22 and C-23 positions of the side chain occurs before oxidation of C6) and early C-6 oxidation pathway (when hydroxylation takes place after oxidation of C6) (**Figure 1**; **Figure S1**) (Shimada et al., 2001). In *A. thaliana* both the early and late C6 oxidation pathways are functional (Fujioka et al., 2000); however, the late C6 oxidation pathway plays a prominent role during photomorphogenesis, whereas the parallel early C6 oxidation dominates during skotomorphogenesis (Noguchi et al., 2000). Generally, the late C6 pathway is more prominent in plants (e.g., in potato, it is the only type of the C_{28}-BR biosynthesis). The late C6 pathway begins with hydroxylation of CN into the 6-deoxoCT by the 22α-hydroxylase. 6-deoxoCT may also be synthesized directly from 22-hydroxy5α-ergostan-3-one (the CN-independent pathway). Then, 6-deoxoCT is hydroxylated through the C-23 hydroxylase

(encoded by the *CPD* gene) to the 6-deoxoteasterone, which is then C-3 oxidized into the 3-dehydro-6-deoxoteasterone (6-deoxo-3-DT) through the *CYP90D* C3-oxidase. In the next step, 6-deoxo-3-DT is converted to 6-deoxoTY. This reaction is catalyzed by the *D11* CYP724B1 enzyme. Then, 6-deoxoTY is hydroxylated to 6-deoxoCS by the 2α-hydroxylase encoded by the *DDWF1* gene. 6-deoxoCS is converted to castasterone (CS) (BR-6-oxidase1 and BR-6-oxidase2 catalyze the reaction). Then, CS is converted to BL *via* Baeyer-Villiger oxidation by the BR-6-oxidase2 (CYP85A2) (Choe, 2004; Choe, 2006; Vriet et al., 2013; Nakano and Asami, 2014; Ohnishi, 2018).

The early C6 oxidation pathway begins from hydroxylation of CN to 6α-hydroxyCN and its subsequent oxidation to 6-oxo-CN. The latter is transformed to CT by the 22α-hydroxylase. Cathasterone (CT) is converted in the consecutive reactions to teasterone (TE), 3-dehydroteasterone (3-DT), typhasterol (TY), CS, and BL, respectively (Shimada et al., 2001; Fujioka et al., 2002; Kwon and Choe, 2005; Ohnishi et al., 2006a; Ohnishi et al., 2006b; Lee et al., 2010; Lee et al., 2011; Zhao and Li, 2012; Chung and Choe, 2013; Joo et al., 2015; Kim et al., 2018; Ohnishi, 2018; Roh et al., 2020).

Biosynthesis of C_{29}-BRs

The C_{29}-BR biosynthesis is the least known and described route of the BR biosynthesis (**Figure 1**; **Figure S1**). In this pathway 24-methylenelophenol is converted by sterol methyltransferase 2 (SMT2) into 24-ethylidenelophenol that is transformed into avenasterol by the sterol methyl oxidase 2. Then, isofucosterol and β-sitosterol are produced from avenasterol in a series of reactions catalyzed by the DWF7, DWF5, and SSR1 (DWF1) enzymes. β-sitosterol, as a precursor of the C_{29}-BRs, is hydroxylated into 6-deoxo-28-homoTY and oxygenated into 28-homoTY by the *CYP724B2* and *CYP90B3* C-22 hydroxylase, respectively (Ohnishi et al., 2006a). 28-homoTY can be also formed from 28-homoTE, but intermediates of this reaction have not been identified yet. 28-homoTY is converted to 28-homoCS and 28-homoBL *via* the CYP85A1/A2 oxidases. The recent report suggests the way of 28-homodolicholide and CS synthesis from isofucosterol *via* 22-hydroxyisofucosterol, 6-deoxo-28-homoDS, and 28-homoDS, respectively. Moreover, CS can be converted from β-sitosterol, through the 22-homositosterol, 6-deoxohomositosterol, and 28-homoCS. It was found that 28-homoTE, 28-homoTY and 28-homoCS can be converted into 26-nor-28-homoTE, 26-nor-28-homoTY, and 26-nor-28-homoCS, respectively. C-26 demethylation might also serve to a deactivation of the C_{29}-BRs (Lee et al., 2011; Joo et al., 2015; Roh et al., 2017; Kim et al., 2018).

Links Between C_{27}-C_{28} and C_{28}-C_{29} Pathways

The C_{27}-BRs biosynthetic pathway links with the C_{28} pathway through the following reactions: 28-norTE → TE, 28-nor-3-DT → 3-DT, 28-norTY → TY, and 28-norCS → CS. On the other hand, C_{29}-BRs conversion to C_{28}-BRs occurs through the following reactions: 28-homoTE → TE, 28-homoTY → TY, 28-homoCS → CS, 28-homoDS → CS, and 28-homoDS → DS → CS. Therefore, it is suggested that the biosynthetic connection of C_{27}- and C_{29}-BRs with C_{28}-BRs occurs mainly between the end products of the pathways. Five pathways are biosynthetically connected to produce CS, an active BR, in plants (**Figure 1**; **Figure S1**). Direct substrates to synthesize CS are: 28-norCS, DS, 6-deoxoCS by 6α-hydroxyCS, 28-homoDS, and 28-homoCS. Thus, it is most conceivable that all the biosynthetic pathways of BRs in plants are funneled into CS to carry out the relevant biological activities. It is known that the early C-6 oxidation pathways of C_{27}-, C_{28}-, and C_{29}-BRs are commonly interrupted in plant tissues (Fujita et al., 2006; Joo et al., 2012; Joo et al., 2015; Kim et al., 2018). A recent study of barley (*Hordeum vulgare*) BR mutants indicated that the accumulation of 28-homoCS is inversely correlated with the accumulation of CS: mutants deficient in the biosynthesis of CS accumulate the highest concentrations of 28-homoCS, on the other hand, the BR-insensitive line, in which the highest concentration of CS was observed, accumulates the lowest concentration of 28-homoCS (Gruszka et al., 2016).

INHIBITORS OF BR BIOSYNTHESIS

Inhibitors are tools useful not only for investigating biosynthetic pathways, but also for manipulating the BR level in crop plants. Till now, 17 inhibitors (KM-01, brassinozole (Brz), Brz2001, Brz220, propiconazole, YCZ-18, yucaizol, fenarimol, spironolactone, triadimefon, imazalil, 4-MA, VG106, DSMEM21, finastride, AFA76, and brassinopride) have been discovered (**Figure 2**), however, the site of action of only nine compounds is known. The sites of action of inhibitors are as follows:

- campestanol → 6-deoxoCT for brassinazole, Brz2001, Brz220, triadimefon, and spironolactone;
- 6-deoxoCT → 6-deoxoTE for brassinazole, Brz2001, Brz220, propiconazole, and fenarimol;
- 6-deoxoTE → 6-deoxo-3DT for YCZ-18, yucaizol, propiconazole, and fenarimol;
- 6-oxocampestanol → CT for brassinazole, Brz2001, Brz220, and triadimefon;
- CT → TE for brassinazole, Brz2001, Brz220, propiconazole, and fenarimol;
- TE → 3DT for YCZ-18, yucaizol, propiconazole, and fenarimol (Rozhon et al., 2019) (**Figure 1**; **Figure S1**).

The first reported BR inhibitor, i.e., KM-01 was isolated from a microbial culture medium. It inhibited BR activity in a rice lamina inclination test. Despite the unclear site of action, KM-01 exhibits highly potent activity (Kim et al., 1994; Kim et al., 1995; Kim et al., 1998). However, brassinazole (Brz) represents the first specific BR biosynthesis inhibitor, which blocks the conversion of campestanol to 6-deoxoCT, 6-deoxoCT to 6-deoxoTE, 6-oxocampestanol to CT, and CT to TE in the BR biosynthetic pathways. Brz2001 is a modified form of Brz containing an allyl moiety instead of the methyl group. Both inhibitors block the

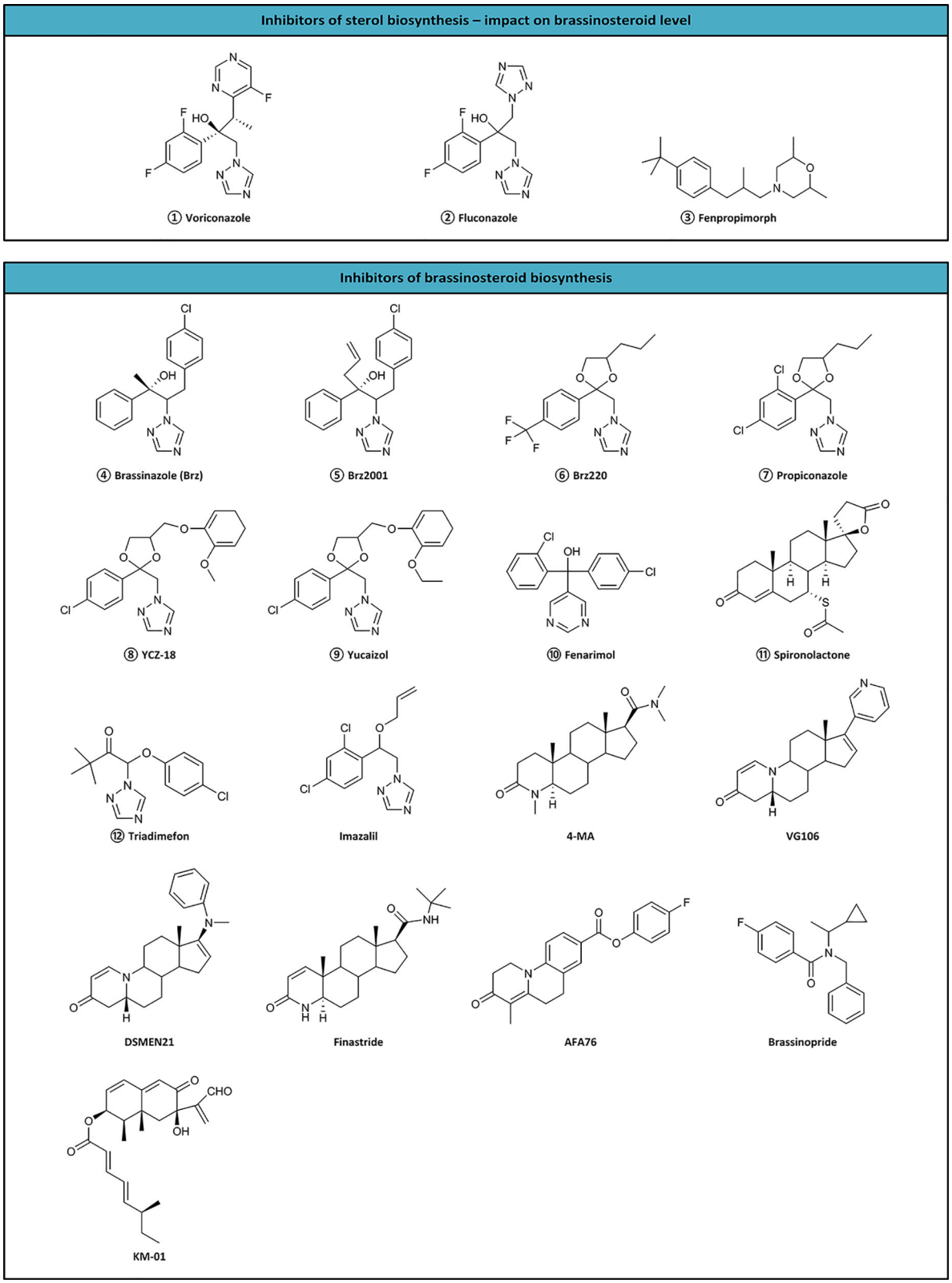

FIGURE 2 | Inhibitors of sterol and brassinosteroid biosynthesis. Numbered inhibitors have a known site of action presented in **Figure 1**.

same reactions (Asami and Yoshida, 1999; Asami et al., 2000; Asami et al., 2001; Asami et al., 2003b). Brz and Brz2001 can induce morphological changes, including dwarfism, altered leaf color, and curling in de-etiolated barley (Sekimata et al., 2001). Brz decreased the level of BRs in the barley leaves, but not in roots. The inhibition effect of Brz on plant growth is reversed by exogenous BR (Bajguz and Asami, 2004; Bajguz and Asami, 2005; Bajguz et al., 2019).

Propiconazole, a triazole compound, also affects similar to Brz (Hartwig et al., 2012). Another triazole-type BR biosynthesis inhibitors, YCZ-18, and yucaizol, bind to the CYP90D1 enzyme and inhibit the BR-induced cell elongation. However, only BL negates the inhibitory effect of YCZ-18 or yucaizol. Therefore, it was suggested that they function differently from Brz (Oh et al., 2015a; Oh et al., 2015b). Fenarimol is known for inhibiting cytochrome P450 monoxides involved in 14α-demethylation during the biosynthesis of ergosterol. Simultaneously, it inhibits the conversion of CT to TE, and evokes the phenotype of BR-deficient mutants with short hypocotyls, de-etiolate dark-grown seedlings, and dark green downward curled leaves of light-grown *A. thaliana* (Wang et al., 2001; Oh et al., 2015a). Plants treated with triadimefon show reduced elongation of stems and petioles, dark green and thicker leaves, delayed senescence, and increased expression levels of the *CPD* gene. The phenotypes could be recovered with CT, TE, TY, CS, and BL (Asami et al., 2003a). On the other hand, imazalil causes severe hypocotyl shortening in *A. thaliana*, which could be reversed by the application of 24-epibrassinolide (Werbrouck et al., 2003). Seedlings of *A. thaliana* treated with spironolactone showed dark, downward curled leaves, and shortened hypocotyls, which could be reversed by BL application (Asami et al., 2004). Although *A. thaliana* mutants viz. *cpd*, *det2-1*, or *cbb1* treated with brassinopride enhanced apical hook formation, the normal phenotype was recovered by BL (Gendron et al., 2008). Voriconazole, fluconazole, and fenproprimorph (**Figure 2**) inhibit cycloeucalenol-obtusifoliol isomerase and have a reductive impact on BRs level (Rozhon et al., 2013; Rozhon et al., 2019).

AUTHOR CONTRIBUTIONS

AB and MC prepared a draft of figures and text. AB and DG corrected and finalized the review.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01034/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Multistep reactions of brassinosteroids biosynthesis and their sterol biosynthetic precursors.

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REVIEW
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Regulation of Brassinosteroid Homeostasis in Higher Plants

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Brassinosteroids (BRs) are known as one of the major classes of phytohormones essential for various processes during normal plant growth, development, and adaptations to biotic and abiotic stresses. Significant progress has been achieved on revealing mechanisms regulating BR biosynthesis, catabolism, and signaling in many crops and in model plant *Arabidopsis*. It is known that BRs control plant growth and development in a dosage-dependent manner. Maintenance of BR homeostasis is therefore critical for optimal functions of BRs. In this review, updated discoveries on mechanisms controlling BR homeostasis in higher plants in response to internal and external cues are discussed.

Keywords: brassinosteroids, phytohormones, homeostasis, cytochrome P450, transcriptional regulation

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INTRODUCTION

Brassinosteroids (BRs) are a group of naturally occurring and polyhydroxylated phytosterols, carrying at least one oxygen moiety at the C3 position and additional ones at one or more of C2, C6, C22, and C23 carbon atoms (Bishop and Yokota, 2001). Since brassinolide (BL), the most active BR compound, was first isolated from *Brassica napus* pollen grains in 1970s, more than 70 BR compounds have been identified and they are ubiquitously presented in the plant kingdom (Mitchell et al., 1970; Grove et al., 1979; Bajguz and Tretyn, 2003). It is widely known that BRs regulate multiple processes during plant growth, development and environmental adaptations, especially controlling many important agronomic traits such as plant architecture, flowering time, seed yield, and stress tolerance (Clouse and Sasse, 1998; Tong and Chu, 2018). Therefore, genetic control of endogenous BR levels or signaling offers a novel approach for crop improvement.

Although application of limited amounts of BRs can significantly enhance growth, excessive BRs are usually harmful to plant growth and development (Clouse et al., 1996). Maintenance and regulation of endogenous BR levels are therefore essential for optimal plant growth and development. Considering that BRs cannot undergo long distance transport, BR biosynthesis and catabolism are two critical antagonistic processes for maintaining BR homeostasis in plants (Symons and Reid, 2004; Ye et al., 2011; Zhao and Li, 2012). In the past decades, extensive researches have been conducted to elucidate the BR biosynthesis pathway in many plant species. Various enzymes catabolizing bioactive BRs through acylation, sulfonation, glycosylation, or other manners in these plants have also been identified. This review focuses on the recent advances in our understanding of the dynamic regulation of BR homeostasis in higher plants in response to various internal and external factors. These pieces of information can be used to facilitate BR application in molecular design for modern agriculture.

BR BIOSYNTHESIS PATHWAYS

BRs are classified as C27, C28, and C29 steroids based on the structure of their C24 alkyl groups (Fujioka and Yokota, 2003). C28 BRs, such as castasterone (CS) and BL, are the most abundant and ubiquitous BRs in plants. Synthesis of CS and BL from campesterol, one of the plant sterols, has been clearly elucidated and is discussed in detail. C27 and C29 BRs use two other compounds, cholesterol, and sitosterol, as their corresponding precursors, and may go through pathways similar to those of C28 BRs (Sakurai, 1999; Fujioka and Yokota, 2003).

Sterol Biosynthesis From Cycloartenol to Campesterol and Sitosterol

The Common Steps

Plant sterols are synthesized from cycloartenol, a plant-specific C30 sterol derived from squalene (**Figure 1**). Most of the enzymes involved in the phytosterol biosynthetic pathway have been characterized in different plant species (**Table 1**). (1) Squalene epoxidase (SQE) catalyzes the oxidation of squalene to squalene-2,3-oxide (Rasbery et al., 2007; Pose et al., 2009; Unland et al., 2018; Liu et al., 2020a). (2) Conversion of squalene-2,3-oxide into cycloartenol is catalyzed by a cycloartenol synthase (Corey et al., 1993; Babiychuk et al., 2008; Gas-Pascual et al., 2014). (3) The first C24 methylation reaction converts cycloartenol into 24-methylene cycloartenol (Shi et al., 1996; Diener et al., 2000; Holmberg et al., 2002; Schrick et al., 2002; Willemsen et al., 2003; Guan et al., 2017). This rate-limiting methylation step leads to subsequent synthesis of 24-methyl (campesterol) or 24-ethyl (sitosterol) instead of 24-desmethyl sterol (cholesterol). The second C24 methylation reaction after several steps determines the formation of 24-ethyl sterols instead of 24-methyl sterols. (4) Cycloeucalenol is produced from 24-methylene cycloartenol through demethylation at C4 position, which is performed with the sequential participation of three enzymes, a sterol 4α-methyl oxidase (SMO), a 4α-carboxysterol-C3-dehydrogenase/C4-decarboxylase (CSD), and a sterone ketoreductase (Darnet et al., 2001; Darnet and Rahier, 2004; Rahier, 2011; Song et al., 2019). Removal of the two methyl groups at C4 position is essential for sterols to be functional. The two separate C4 demethylation reactions in higher plants are catalyzed by two distinctive families of SMO enzymes, whereas the two consecutive C-4 demethylation reactions are catalyzed by the same enzymes in animals and fungi (Rahier, 2011). (5) Cycloeucalenol is then isomerized by cyclopropyl sterol isomerase. As a result, obtusifoliol is produced (Lovato et al., 2000; Men et al., 2008). (6) Subsequently, CYP51, one of the most ancient and conserved cytochrome P450s across the kingdoms, demethylates obtusifoliol at C14 to form 4α-methyl ergostatrienol (Kahn et al., 1996; Bak et al., 1997; Cabello-Hurtado et al., 1999; Kushiro et al., 2001; Burger et al., 2003; Kim et al., 2005a). (7) C14 reduction of 4α-methyl ergostatrienol is catalyzed by FACKEL/HYDRA2/EXTRA-LONG-LIFESPAN 1 (FK/HYD2/ELL1), three alleles from *Arabidopsis* isolated by independent research groups, leading to formation of 4α-methyl fecosterol (Jang et al., 2000; Schrick et al., 2000; Souter et al., 2002). (8) Isomerization of 4α-

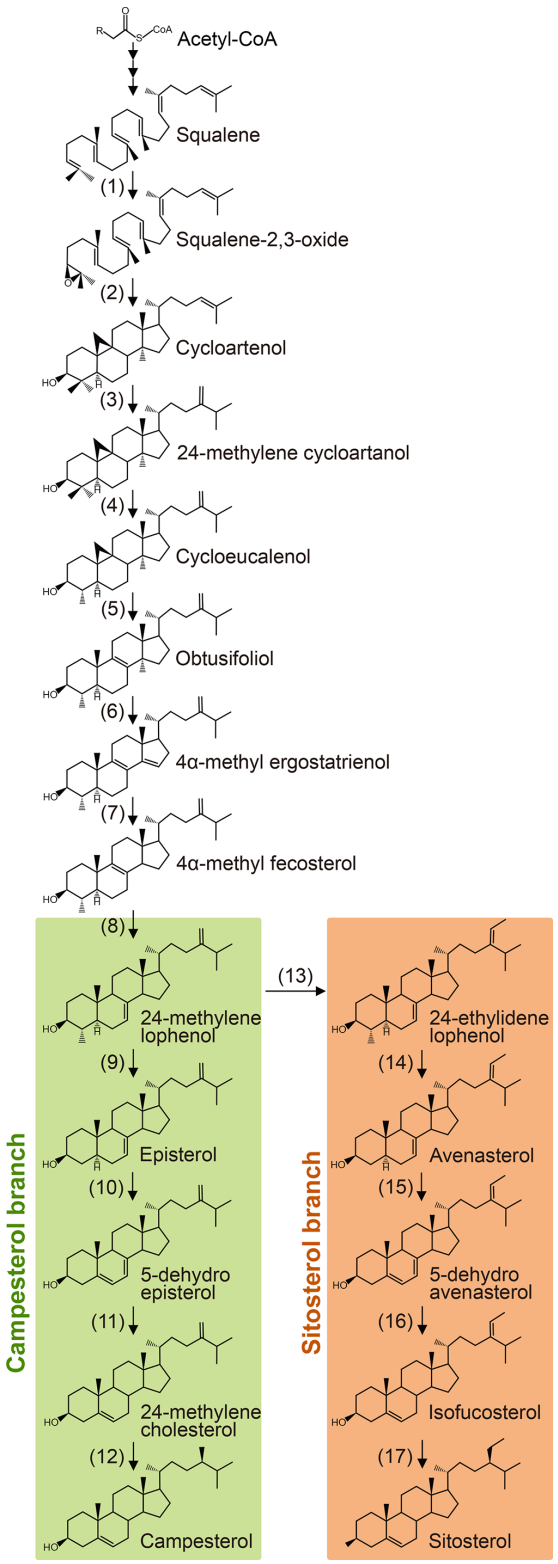

FIGURE 1 | Biosynthesis of campesterol and sitosterol from squalene. Numbers correspond to the description in the text and **Table 1**. Enzymes identified from higher plants are listed in **Table 1**.

TABLE 1 | Sterol biosynthesis enzymes identified in different plant species.

Function	Species	Name	Steps*	Reference
Squalene epoxidase	*Arabidopsis thaliana*	SQE1	1	Rasbery et al., 2007; Pose et al., 2009
	Tripterygium wilfordii	TwSQE6/7	1	Liu et al., 2020a
	Taraxacum koksaghyz	TkSQE1	1	Unland et al., 2018
Cycloartenol synthase	*Arabidopsis thaliana*	CAS1	2	Corey et al., 1993; Babiychuk et al., 2008
	Nicotiana tabacum	NtCAS1	2	Gas-Pascual et al., 2014
Sterol methyltransferase	*Arabidopsis thaliana*	AtSMT1/CPH/ORC	3	Diener et al., 2000; Schrick et al., 2002; Willemsen et al., 2003
		CVP1/SMT2/3	13	Husselstein et al., 1996; Bouvier-Navé et al., 1997; Schaller et al., 1998; Carland et al., 1999; Schaeffer et al., 2001; Carland et al., 2002; Carland et al., 2010
	Glycine max	SMT1	3	Shi et al., 1996
	Nicotiana tabacum	NtSMT1	3	Holmberg et al., 2002
	Tripterygium wilfordii	TwSMT1	3	Guan et al., 2017
	Gossypium hirsuturm	GhSMT2-1/2-2	3	Luo et al., 2008
Sterol 4α-methyl oxidase	*Arabidopsis thaliana*	SMO1	4	Darnet et al., 2001; Darnet and Rahier, 2004; Song et al., 2019
		SMO2	9/14	Zhang et al., 2016
Cyclopropyl sterol isomerase	*Arabidopsis thaliana*	CPI1	5	Lovato et al., 2000; Men et al., 2008
14α-demethylase	*Arabidopsis thaliana*	CYP51A2	6	Kushiro et al., 2001; Kim et al., 2005a
	Oryza sativa	OsCYP51G3	6	Xia et al., 2015
	Sorghum bicolor	CYP51	6	Kahn et al., 1996; Bak et al., 1997
	Triticum aestivum	CYP51	6	Cabello-Hurtado et al., 1999
	Nicotiana benthamiana	CYP51	6	Burger et al., 2003
C14 reductase	*Arabidopsis thaliana*	FK/HYD2/ELL1	7	Jang et al., 2000; Schrick et al., 2000; Souter et al., 2002
Δ8-Δ7 sterol isomerase	*Arabidopsis thaliana*	HYD1	8	Souter et al., 2002
C5 desaturase	*Arabidopsis thaliana*	DWF7/STE1/BUL1	10/15	Gachotte et al., 1995; Choe et al., 1999b; Catterou et al., 2001a; Catterou et al., 2001b
C7 reductase	*Arabidopsis thaliana*	DWF5	11/16	Choe et al., 2000
Δ24 isomerase/reductase	*Arabidopsis thaliana*	DWF1/CBB1/DIM	12/17	Takahashi et al., 1995; Kauschmann et al., 1996; Klahre et al., 1998; Choe et al., 1999a
	Oryza sativa	BRD2/LTBSG1/LHDD10	12/17	Hong et al., 2005; Liu et al., 2016; Qin et al., 2018
	Pyrus ussuriensis	PcDWF1	12/17	Zheng et al., 2020
	Hordeum vulgare	HvDIM	12/17	Dockter et al., 2014
	Zea mays	NA2	12/17	Best et al., 2016

*Step numbers correspond to the description in the text and ***Figure 1***.

methyl fecosterol into 24-methylene lophenol is catalyzed by a Δ^8-Δ^7 sterol isomerase, identified as HYDRA1 (HYD1) in *Arabidopsis* (Souter et al., 2002).

The Campesterol Branch

(9) Removal of the second methyl group at C4 converts 24-methylene lophenol into episterol, which involves a family of SMO enzymes distinctive from the first C4 demethylation reaction (Zhang et al., 2016). (10) Episterol is subsequently converted into 5-dehydro episterol by a C5 desaturase, named DWARF7/STE1/BOULE1 (DWF7/STE1/BUL1) in *Arabidopsis* (Gachotte et al., 1995; Choe et al., 1999b; Catterou et al., 2001b; Catterou et al., 2001a). (11) C7 reductase, also designated as DWARF5 (DWF5) in *Arabidopsis*, reduces 5-dehydro episterol to yield 24-methylene cholesterol (Choe et al., 2000). (12) The $\Delta^{24(28)}$ bond of 24-methylene cholesterol is isomerized into a $\Delta^{24(25)}$ bond, and then the double bond is reduced to produce campesterol, the specific precursor of BR biosynthesis. Both the isomerization and reduction are catalyzed by a single enzyme, named as DWF1/CBB1/DIM and BRD2/LTBSG1/LHDD10 in *Arabidopsis* and rice, respectively (Takahashi et al., 1995; Kauschmann et al., 1996; Klahre et al., 1998; Choe et al., 1999a; Hong et al., 2005; Best et al., 2016; Liu et al., 2016; Qin et al., 2018; Zheng et al., 2020).

The Sitosterol Branch

(13) The first step of sitosterol branch is the second C24 methylation reaction in the plant sterol biosynthesis pathway that converts 24-methylene lophenol into 24-ethylidene lophenol, the fundamental member of 24-ethyl sterols (Husselstein et al., 1996; Bouvier-Navé et al., 1997; Schaller et al., 1998; Carland et al., 1999; Schaeffer et al., 2001; Carland et al., 2002; Luo et al., 2008; Carland et al., 2010). (14-17) Subsequent four consecutive steps, including C4 demethylation, C5 desaturation, C7 reduction, and C24 isomerization/reduction, leads to the final biosynthesis of sitosterol. These four steps are catalyzed by the same enzymes functioning in the parallel campesterol branch (Klahre et al., 1998; Choe et al., 1999b; Choe et al., 2000; Zhang et al., 2016).

Specific Biosynthesis of BL From Campesterol

BR biosynthesis involves parallel and highly networked pathways (**Figure 2**). Campesterol can be first converted into campestanol

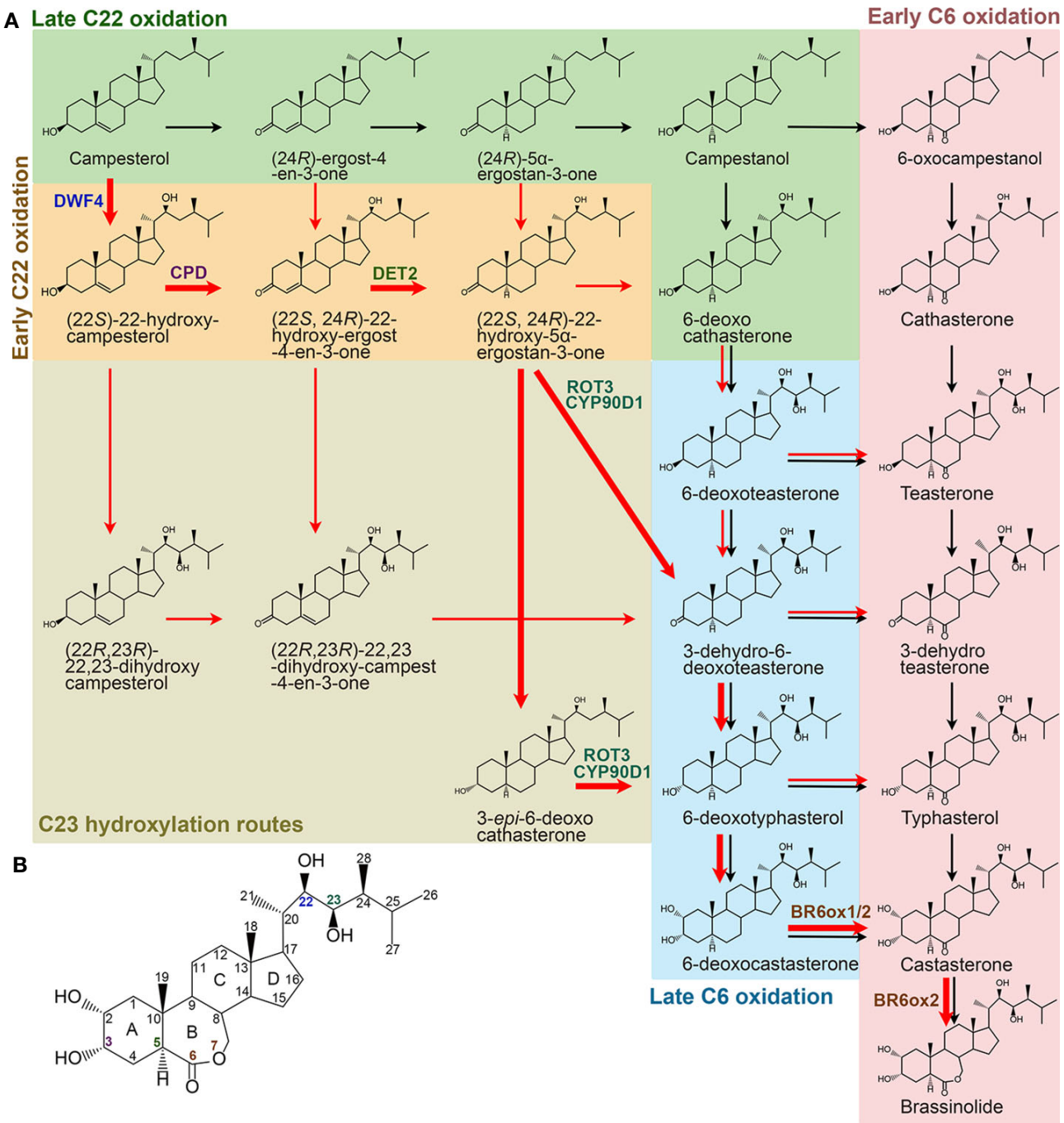

FIGURE 2 | Specific BR biosynthetic pathways from campesterol in higher plants. **(A)** Black and red arrows represent CN-dependent and -independent pathways, respectively. Bold red arrows represent the most dominant and efficient flow of the BR intermediates. Enzymes identified from Arabidopsis are only shown in the dominant routes. **(B)** Molecular structure of BL. The carbons are numbered and the rings are labelled by letters. Colors of the number correspond to the enzymes shown in **(A)**.

(CN) through a late C22 oxidation pathway. CN in turn is converted to CS either *via* an early C6 oxidation or a late C6 oxidation pathway, which is also called a CN-dependent pathway (Fujioka and Yokota, 2003). On the other hand, campesterol can be first converted into 6-deoxocathasterone through an early C22 oxidation pathway, either flowing straightly or *via* a C23 hydroxylation reaction step then going into the C6 oxidation pathways to synthesize CS, which is also designated as a CN-independent pathway (Fujioka et al., 2002; Fujita et al., 2006; Ohnishi et al., 2006b). CS is the end and most bioactive BR

compound in graminaceous plants, such as rice (Kim et al., 2008). Whereas, CS can further be converted into BL in most dicotyledonous plants due to the duplication of a C6 oxidase gene, one of their encoded C6 oxidases developed a BL synthase function (Kim et al., 2005b; Nomura et al., 2005). Compared with their parallel branches, the early C22 oxidation pathway and the late C6 oxidation pathway appear to be the predominant route in many plant species, including *Arabidopsis*, tomato, and pea (Nomura et al., 2001; Fujioka et al., 2002). Furthermore, the C23 hydroxylases prefer to use (22*S*, 24*R*)-22-hydroxy-5α-ergostan-3-one and 3-*epi*-6-deoxocathasterone as their substrates (Ohnishi et al., 2006b). Thus, the most dominant and efficient flow of BR intermediates, campesterol → (22*S*)-22-hydroxy-campesterol → (22*S*, 24*R*)-22-hydroxy-ergost-4-en-3-one → (22*S*, 24*R*)-22-hydroxy-5α-ergostan-3-one → 3-*epi*-6-deoxocathasterone/3-dehydro-6-deoxoteasterone → 6-deoxotyphasterol → 6-deoxocastasterone → CS → BL, is established (Ohnishi et al., 2012). Although there are two more steps in other biosynthetic routes compared with this dominant CN-independent pathway, all the BR biosynthesis routes involve common reaction steps, including hydroxylation at C22, C23, and C2, oxidation and reduction at C3, reduction at C5, and oxidation at C6, and an additional Baeyer-Villiger oxidation in most dicotyledonous plants. Most of the enzymes involved in the reactions were identified in different plant species (**Table 2**). Loss of function of these enzymes leads to similar defective phenotypes, including dwarf and compact plant architecture, short roots, delayed flowering time, reduced biomass and seed yield.

Hydroxylation at C22, C23, and C2

There are at least five C22 hydroxylation reactions in the BR biosynthesis pathway, including campesterol to (22*S*)-22-hydroxy-campesterol, (24*R*)-ergost-4-en-3-one to (22*S*, 24*R*)-22-hydroxy-ergost-4-en-3-one, (24*R*)-5α-ergostan-3-one to (22*S*, 24*R*)-22-hydroxy-5α-ergostan-3-one, CN to 6-deoxocathasterone, and 6-oxocampestanol to cathasterone (Choe et al., 1998; Fujita et al., 2006; Ohnishi et al., 2006c). Although all these C22 hydroxylation reactions are catalyzed by the same cytochrome P450 monooxygenases in different plants, they prefer to take campesterol rather than others as a substrate (Choe et al., 1998; Fujita et al., 2006; Ohnishi et al., 2006c). In *Arabidopsis*, CYP90B1, a cytochrome P450 monooxygenase also known as DWARF4 (DWF4), is mainly responsible for these

TABLE 2 | Specific BR biosynthesis enzymes identified in different plant species.

Function	Species	Name	Reference
C22 hydroxylase	*Arabidopsis thaliana*	DWF4/CYP90B1	Choe et al., 1998; Fujita et al., 2006
		CYP724A1	Zhang et al., 2012
	Oryza sativa	CYP90B2/OsDWF4	Sakamoto et al., 2006
		CYP724B1/D11*	Sakamoto et al., 2006
	Lycopersicon esculentum	CYP90B3	Ohnishi et al., 2006c
		CYP724B2	Ohnishi et al., 2006c
	Zea mays	CYP90B2/ZmDWF4	Liu et al., 2007
	Solanum tuberosum	StDWF4	Zhou et al., 2018
	Populus tomentosa	PtoDWF4	Shen et al., 2018
C23 hydroxylase	*Arabidopsis thaliana*	CYP90C1/ROT3	Ohnishi et al., 2006b
		CYP90D1	
	Oryza sativa	CYP90D2/OsD2*	Sakamoto et al., 2012
		CYP90D3	
	Lycopersicon esculentum	DPY	Koka et al., 2000
C2 hydroxylase	*Pisum sativum*	CYP92A6/DDWF1	Kang et al., 2001
C3 oxidase	*Arabidopsis thaliana*	CYP90A1/CPD	Szekeres et al., 1996; Ohnishi et al., 2012
	Oryza sativa	CYP90A3/4(OsCPD1/2)	Sakamoto and Matsuoka, 2006
		CYP90D2/OsD2*	Hong et al., 2003; Li et al., 2013
	Hordeum vulgare	HvCPD	Dockter et al., 2014
C3 reductase	*Oryza sativa*	CYP724B1/OsD11*	Tanabe et al., 2005
C5 reductase	*Arabidopsis thaliana*	DET2	Chory et al., 1991; Fujioka et al., 1997; Noguchi et al., 1999
	Glycine max	GmDET2a/b	Huo et al., 2018
	Gossypium hirsuturm	GhDET2	Luo et al., 2007
	Cucumis sativus	CsDET2	Hou et al., 2017
	Pisum sativum	LK	Nomura et al., 2004
	Pharbitis nil	PnDET2	Suzuki et al., 2003
C6 oxidase	*Arabidopsis thaliana*	CYP85A1/2 (BR6ox1/2)	Shimada et al., 2001
	Lycopersicon esculentum	CYP85A1(DWARF)/A3	Bishop et al., 1999; Shimada et al., 2001
	Pisum sativum	PsCYP85A1/6	Jager et al., 2007
	Oryza sativa	OsDWARF/BRD1	Hong et al., 2002; Mori et al., 2002
	Hordeum vulgare	HvBRD	Dockter et al., 2014
	Cucumis sativus	SCP1/CsCYP85A1	Wang et al., 2017
	Brachypodium distachyon	BdBRD1	Xu et al., 2015
	Zea mays	ZmBRD1	Makarevitch et al., 2012
	Populus trichocarpa	PtCYP85A3	Jin et al., 2017

Enzymes marked by an asterisk are those with controversial functions.

reactions (Choe et al., 1998; Fujita et al., 2006). CYP724A1 function at least partially as a C22 hydroxylase, since its overexpression can restore the deficiency caused by *dwf4* mutation (Zhang et al., 2012). Homologs of CYP90B1/DWF4 and CYP724A1 in different plant species, such as CYP90B2/OsDWF4 and CYP724B1/OsD11 in rice, CYP90B3 and CYP724B2 in tomato, CYP90B2/ZmDWF4 in maize, StDWF4 in potato (*Solanum tuberosum* L.), and PtoDWF4 in *Populus tomentosa*, were also found to possess similar biological functions (Ohnishi et al., 2006c; Sakamoto et al., 2006; Liu et al., 2007; Shen et al., 2018; Zhou et al., 2018). The C22 hydroxylation is considered as a rate-limiting step in the BR biosynthesis pathway possibly due to a low *DWF4* expression level that cannot effectively catalyze the reaction (Choe et al., 1998). This makes DWF4 an ideal target for manipulating BR biosynthesis to regulate growth and stress adaptation in modern agriculture (Choe et al., 2001; Kim et al., 2006; Sakamoto et al., 2006; Sahni et al., 2016; Li et al., 2018; Zhou et al., 2018).

Six C23 hydroxylation reactions, including (22*S*)-22-hydroxy-campesterol to (22*R*, 23*R*)-22, 23-dihydroxy-campesterol, (22*S*, 24*R*)-22-hydroxy-ergost-4-en-3-one to (22*R*, 23*R*)-22, 23-dihydroxy-campest-4-en-3-one, (22*S*, 24*R*)-22-hydroxy-5α-ergostan-3-one to 3-dehydro-6-deoxoteasterone, 3-*epi*-6-deoxocathasterone to 6-deoxotyphasterol, 6-deoxocathasterone to 6-deoxoteasterone, and cathasterone to teasterone, were identified in the BR biosynthesis pathway (Ohnishi et al., 2006b; Sakamoto et al., 2012). (22*S*, 24*R*)-22-hydroxy-5α-ergostan-3-one and 3-*epi*-6-deoxocathasterone are two favorable substrates for the C23 hydroxylases in plants, leading to a shortcut with two steps less than other biosynthetic routes (Ohnishi et al., 2006b; Sakamoto et al., 2012). The C23 hydroxylases are also members of cytochrome P450 monooxygenases, such as CYP90C1/ROTUNDIFOLIA3 (ROT3) and CYP90D1 in *Arabidopsis*, CYP90D2/OsD2 and CYP90D3 in rice, and CYP90C2/DUMPY (DPY) in tomato (Koka et al., 2000; Ohnishi et al., 2006b; Sakamoto et al., 2012).

The C2 hydroxylation steps, converting 6-deoxotyphasterol to 6-deoxocastasterone and typhasterol to castasterone located in the late and the early C6 oxidation pathways respectively, have only been elucidated in pea (Kang et al., 2001). A dark-induced cytochrome P450, named as DARK-INDUCED DWF-LIKE PROTEIN 1 (DDWF1), is activated by a small G protein PRA2 and then to catalyze the C2 hydroxylation reactions in the BR biosynthetic pathway (Kang et al., 2001).

Oxidation and Reduction at C3

At least twice redox reactions at C3 position were found in the BR biosynthetic pathway. The big difference between the dominant CN-independent route from others is that it contains one less C3 redox reaction (Ohnishi et al., 2012). The first time of C3 oxidation reactions include campesterol to (24*R*)-ergost-4-en-3-one, (22*S*)-22-hydroxy-campesterol to (22*S*, 24*R*)-22-hydroxy-ergost-4-en-3-one, and (22*R*, 23*R*)-22, 23-dihydroxycampesterol to (22*R*, 23*R*)-22, 23-dihydroxy-campest-4-en-3-one. Conversions from 6-deoxoteasterone to 3-dehydro-6-deoxoteasterone and from teasterone to 3-dehydroteasterone in the late and the early C6 oxidation pathways, respectively, are the second C3 oxidation reactions. In *Arabidopsis*, CYP90A1/CPD is responsible for the C3 oxidation and has a broad substrate specificity. Three of the five intermediates, (22*S*)-22-hydroxy-campesterol, (22*R*, 23*R*)-22, 23-dihydroxycampesterol, and 6-deoxoteasterone, can be converted to their respective 3-dehydro derivatives by CYP90A1/CPD, whereas, its preferred substrate is (22*S*)-22-hydroxy-campesterol (Szekeres et al., 1996; Ohnishi et al., 2012). Rice CYP90A3/OsCPD1 and CYP90A4/OsCPD2 were predicted to perform the similar function as *Arabidopsis* CYP90A1/CPD based on their high sequence similarity (Sakamoto and Matsuoka, 2006). However, rice CYP90D2/OsD2 is considered as the C3 oxidase for 6-deoxoteasterone and teasterone by two research groups, while another research group demonstrated that it functions redundantly with CYP90D3 as a C23 hydrolase (Hong et al., 2003; Sakamoto et al., 2012; Li et al., 2013).

C3 reductions include conversions from (24*R*)-5α-ergostan-3-one to campestanol, (22*S*, 24*R*)-22-hydroxy-5α-ergostan-3-one to 6-deoxocathasterone or 3-*epi*-6-deoxocathasterone, 3-dehydro-6-deoxoteasterone to 6-deoxotyphasterol, and 3-dehydroteasterone to typhasterol. In rice, CYP724B1/OsD11 is originally reported as the C3 reductase to produce 6-deoxotyphasterol and typhasterol (Tanabe et al., 2005). However, a different research group declared that it catalyzes the C22 hydroxylation together with CYP90B2/OsDWF4 (Sakamoto et al., 2006). The BR C3 reductase in *Arabidopsis* model plant is yet to be identified in the future.

C5 Reduction

C5 reduction is an early reaction step in the BR biosynthesis pathway, leading to the formation of (24*R*)-5α-ergostan-3-one, (22*S*, 24*R*)-22-hydroxy-5α-ergostan-3-one, and 3-dehydro-6-deoxoteasterone from (24*R*)-ergost-4-en-3-one, (22*S*, 24*R*)-22-hydroxy-ergost-4-en-3-one, and (22*R*, 23*R*)-22, 23-dihydroxy-campest-4-en-3-one, respectively. A steroid 5α-reductase, named as DEETIOLATION 2 (DET2), is responsible for the C5 reduction in *Arabidopsis* (Chory et al., 1991; Li et al., 1996; Fujioka et al., 1997; Li et al., 1997; Noguchi et al., 1999). Paralogs of DET2 in different plant species have also been identified, such as in soybean, cotton, cucumber, pea, and morning glory (Suzuki et al., 2003; Nomura et al., 2004; Luo et al., 2007; Hou et al., 2017; Huo et al., 2018).

C6 Oxidation and Baeyer-Villiger Oxidation

C6 oxidation converts the 6-deoxo BR intermediates in the late C6 oxidation pathway to corresponding 6-oxo compounds in the early C6 oxidation pathway. Although several pairs of substates and products seem to occur naturally in a number of plant species, only the conversion from 6-deoxotyphasterol to typhasterol and from 6-deoxocastasterone to castasterone have been verified in *Arabidopsis* and rice (Shimada et al., 2001; Mori et al., 2002). Conversions from 6-deoxoteasterone to teasterone and from 3-dehydro-6-deoxoteasterone to 3-dehydroteasterone were also thought to occurr but remain tentative in *Arabidopsis* and possibly other plants (Shimada et al., 2001). Whereas, in tomato, conversion from 6-deoxocastasterone to castasterone seems to be the only major C6 oxidation pathway (Bishop et al., 1999; Shimada et al., 2001). The C6 oxidases encoded by cytochrome P450s have been

identified in different plant species, such as CYP85A1/2 (also name as BR6ox1/2) in *Arabidopsis*, DWARF/CYP85A1 and CYP85A3 in tomato, PsCYP85A1 and PsCYP85A6/LKE in pea, OsDWARF/BRD1 in rice, SCP1/CsCYP85A1 in cucumber, HvBRD in barley, BdBRD1 in *Brachypodium distachyon*, ZmBRD1 in maize, PtCYP85A3 in *Populus trichocarpa*, and so on (Bishop et al., 1999; Shimada et al., 2001; Hong et al., 2002; Mori et al., 2002; Jager et al., 2007; Makarevitch et al., 2012; Dockter et al., 2014; Xu et al., 2015; Jin et al., 2017; Wang et al., 2017). It should be noted that the C6 oxidation is also a rate-limiting step in the BR biosynthesis pathway (Nomura et al., 2001).

The Baeyer-Villiger oxidation creates a lactone at ring B of the steroid backbone, leading to the formation of BL from CS in *Arabidopsis* and tomato but not in rice (Kim et al., 2005b;

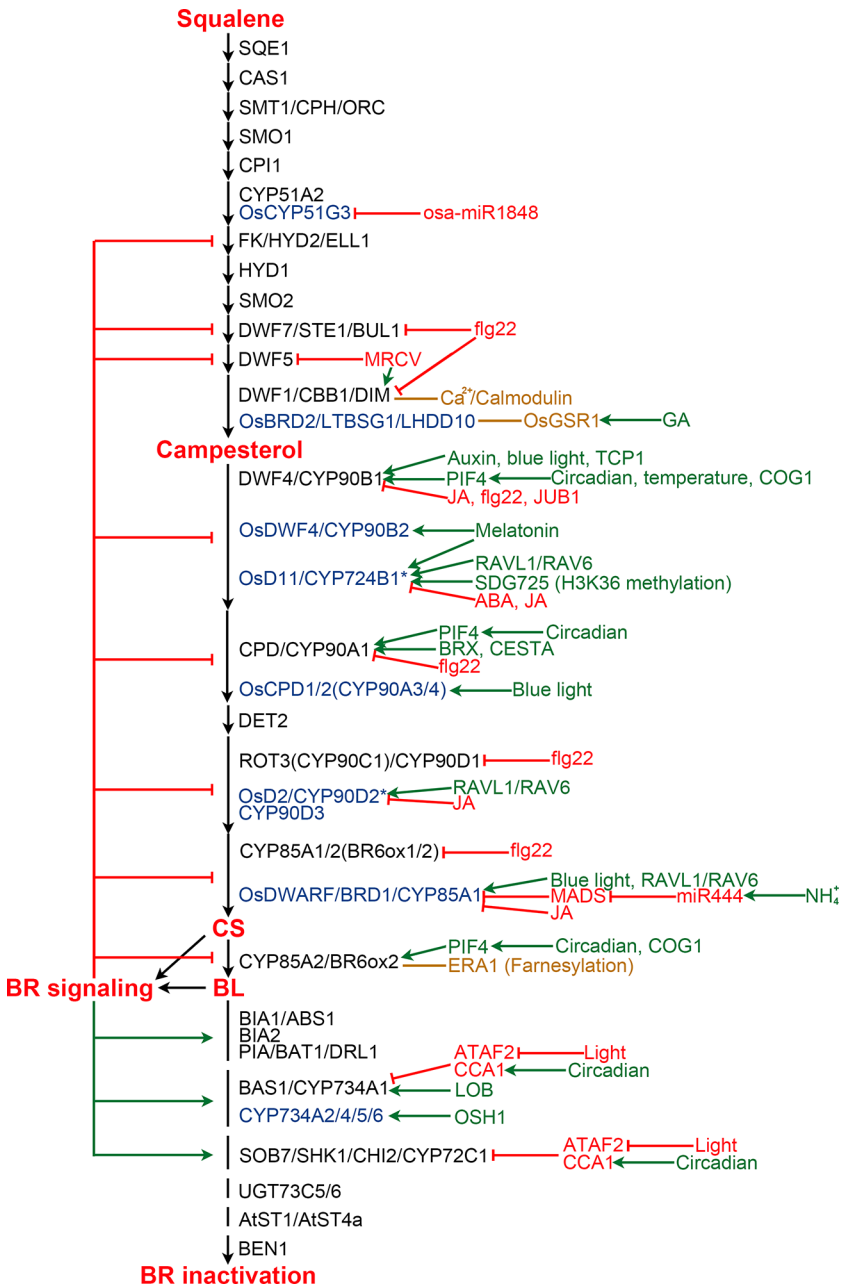

FIGURE 3 | Regulation of BR biosynthesis and catabolism in Arabidopsis and rice. BR biosynthesis pathway is shown from squalene to BL. Enzymes from Arabidopsis and rice are shown in black and blue colors, respectively. Enzymes marked by an asterisk are those with controversial functions. Enzymes from other plants are list in **Table 2** and **Table 3**. Green and red arrows indicate positive and negative regulation, respectively. Orange lines represent protein-protein interaction.

Nomura et al., 2005; Kim et al., 2008). Consistently, there is only one copy of CYP85A gene in rice, while there are two copies of CYP85As in *Arabidopsis* and tomato genomes. It has been elucidated that the extra CYP85A enzymes, CYP85A2/BR6ox2 in *Arabidopsis* and CYP85A3 in tomato, are responsible for the Baeyer-Villiger oxidation (Kim et al., 2005b; Nomura et al., 2005).

Regulation of BR Biosynthesis

BR biosynthesis is inhibited by the end product, CS or BL, *via* a feedback loop (**Figure 3**). Exogenous application of BL leads to down-regulation of multiple BR biosynthetic genes, while BR biosynthesis inhibitors induce the expression of these genes, suggesting feedback transcriptional regulation occurs at multiple steps of the BR biosynthesis pathway (Mathur et al., 1998; Hong et al., 2002; Hong et al., 2003; Tanabe et al., 2005; Tanaka et al., 2005). Now, it is clear that perception of BL by its receptor BRI1 and coreceptor BAK1 ultimately leads to the activation of a group of transcription factors, including BES1 and BZR1, in the nucleus (Li and Chory, 1997; Li et al., 2002; Wang et al., 2002; Yin et al., 2002). BES1 and BZR1 not only regulate the expression of thousands of genes involved in diverse processes during plant growth and development, but also are responsible for the feedback inhibition *via* directly binding to the promoter regions of multiple BR biosynthesis genes to repress their expression (He et al., 2005; Sun et al., 2010; Yu et al., 2011).

The BR biosynthesis pathway is regulated by various internal signaling molecules and by its end products (**Figure 3**). For example, auxin induces *DWF4* expression to increase endogenous BR level in *Arabidopsis* roots, partially by repressing the binding of BZR1 to the *DWF4* promoter (Chung et al., 2011). BREVIS RADIX (BRX) mediates auxin action on BR biosynthesis through activating the *CPD* expression in *Arabidopsis* (Mouchel et al., 2006). Gibberellins (GAs) have also been reported to play roles in regulating BR biosynthesis. OsGSR1, a GAST member (a GA-stimulated transcript) induced by GA and repressed by BR at a transcription level, interacts with DIM/DWF1 to modulate the BR level in rice (Wang et al., 2009). SPINDLY, an *O*-linked *N*-acetylglucosamine transferase negatively regulating GA signaling in rice, represses BR biosynthesis *via* an unknown mechanism (Shimada et al., 2006). JUNGBRUNNEN1 (JUB1), a NAC transcriptional regulator, acts at the nexus of BR-GA network by regulating a complex transcriptional module composed of key components of GA and BR pathways including *GA3ox1*, *DWF4* and the DELLA genes *GAI* and *RGL1* (Shahnejat-Bushehri et al., 2016). Moreover, the functional mechanism of JUB1 in regulating BR/GA biosynthesis and signaling is considerably conserved across species (Shahnejat-Bushehri et al., 2017). Jasmonic acid and abscisic acid were found to inhibit the expression of BR biosynthetic genes to antagonize BR functions (Ren et al., 2009; Gan et al., 2015; Li et al., 2019). Melatonin also plays a role in regulating BR biosynthesis. Block of its biosynthesis results in a decreased BR level in rice, while exogenously applied melatonin induces the expression of BR biosynthesis genes (Hwang and Back, 2018; Hwang and Back, 2019; Lee and Back, 2019).

Pieces of evidence support that light plays an important role in regulating BR biosynthesis. In rice aerial tissues, blue light promotes expression of *CYP85A1*/*BRD1*/*OsDWARF* and *OsCYP90A3*/*4*, thereby increasing CS level. While, far-red light, instead of blue light or red light, positively regulate BR biosynthesis in rice roots (Asahina et al., 2014). However, in *Arabidopsis*, blue light perception in aerial tissues enhances *DWF4* accumulation in the root tips (Sakaguchi and Watanabe, 2017; Sakaguchi et al., 2019). Moreover, the expression levels of *Arabidopsis CPD* and *CYP85A2*/*BR6ox2* display complex diurnal patterns (Bancos et al., 2006). A recent study revealed that BES1 inhibits the expression of BR biosynthesis genes during the day, while elevated PIF4 competes for BES1 resulting in de-repressed BR biosynthesis at dawn (Martinez et al., 2018). In addition, it was found that PIF5 acts redundantly with PIF4 to positively regulate BR biosynthesis. COG1, a Dof type transcription factor negatively regulating phytochrome signaling, can directly promote the expression of *PIF4* and *PIF5*. PIF4 and PIF5 then directly bind to the promoters of *DWF4* and *CYP85A2*/*BR6ox2* to enhance their expression, resulting in elevated levels of endogenous BRs (Park et al., 2003; Wei et al., 2017). It was demonstrated that PIF4 also activates the expression of BR biosynthesis genes in response to elevated temperatures to promote thermomorphogenic hypocotyl growth (Maharjan and Choe, 2011; Martinez et al., 2018).

BR biosynthesis is highly regulated by different environmental stimuli as well as light. For instance, ammonium (NH_4^+), one of the major nitrogen resources for plants, induces the accumulation of miR444, which then positively regulates rice BR biosynthesis *via* inhibiting its MADS-box targets and subsequently activating *OsBRD*1 expression (Jiao et al., 2020). Calmodulin, a Ca^{2+} sensor protein which plays an essential role in sensing and transducing environmental stimuli, can interact with DWF1 in a Ca^{2+}-dependent manner and control its function to regulate BR biosynthesis (Du and Poovaiah, 2005). Bacterial flagellin 22 triggers plant immunity responses, resulting in reduced expression of several BR biosynthetic genes, including *CPD*, *DWF4*, *BR6ox1/2*, *ROT3*, *DWF1*, and *DWF7* in *Arabidopsis* (Jimenez-Gongora et al., 2015). *Mal de Río Cuarto virus* (MRCV) causes severe diseases in several monocotyledonous crops. It was found that MRCV infection causes the up-regulation of *DIM*/*DWF1* but the down-regulation of *DWF5*, and significantly increased amount of BL in wheat (de Haro et al., 2019).

Several additional components regulating BR biosynthesis were identified from different plant species. However, their upstream signaling is yet to be elucidated in the future. In *Arabidopsis*, TCP1, a basic helix loop helix (bHLH) transcription factor, can directly bind to the promoter of *DWF4* to enhance its expression and promotes BR biosynthesis (Guo et al., 2010; An et al., 2011; Gao et al., 2015). CESTA, another bHLH transcription factor, positively regulates BR biosynthesis *via* promoting the expression of *CPD*

(Poppenberger et al., 2011). Farnesylation, a post-translational modification, of *Arabidopsis* CYP85A2/BR6ox2 was found to be essential for its subcellular localization and function. Loss of CYP85A2/BR6ox2 farnesylation results in reduced BL accumulation, similar to the mutation of CYP85A2/BR6ox2 (Northey et al., 2016; Jamshed et al., 2017). In rice, RAVL1 and RAV6, two homologous B3 transcription factors, mediate activation of both OsBRI1 and the BR biosynthetic genes that have antagonistic actions on BR levels to ensure the basal activity of the BR signaling and biosynthetic pathways (Il Je et al., 2010; Zhang et al., 2015). Rice microRNA osa-miR1848 mediates *OsCYP51G3* mRNA cleavage to regulate phytosterol and BR biosynthesis (Xia et al., 2015). SDG725, a H3K36 methyltransferase from rice, modulates the expression of *OsD11*, suggesting an important role of H3K36 methylation on BR biosynthesis (Sui et al., 2012). In addition, both SLG and XIAO, predicted to be a BAHD acyltransferase-like protein and a leucine-rich repeat protein like kinase (LRR-RLK), respectively, function as the positive regulators of BR biosynthesis *via* unknown mechanisms (Jiang et al., 2012; Feng et al., 2016). In wheat, TaSPL8 binds to the promoter of *CYP90D2/OsD2* to activate its expression and regulate leaf angle (Liu et al., 2019). In cotton, GhFP1, a bHLH transcription factor, directly binds to the promoters of *GhDWF4* and *GhCPD* to activate their expression (Liu et al., 2020b). In apple, MdWRKY9 directly represses *MdDWF4* transcription to inhibit BR biosynthesis (Zheng et al., 2018). MdNAC1 negatively modulates BR production probably by inhibiting the expression of *MdDWF4* and *MdCPD* (Jia et al., 2018).

CATABOLISM

BR Catabolism

Endogenous bioactive levels of BRs are also controlled by their catabolic processes. BR catabolism leads to decreased levels of bioactive BRs and attenuated signaling output. Elucidation of BR catabolism can help us, from a different aspect, to understand how plants regulate BR homeostasis for their optimal growth, development and environmental adaptations. Diverse modifications of BRs were revealed by various feeding experiments and analytic chemistry analyses, such as epimerization of C2 and C3 hydroxy groups, hydroxylation of C20, C25, and C26, side chain cleavage; sulfonation of C22; conjugation with fatty acids or glucose; acylation; demethylation; and so on (Fujioka and Yokota, 2003). At present, several BR inactivating reactions and some of their corresponding enzymes have been demonstrated in plants (**Table 3**). In *Arabidopsis*, at least 10 BR inactivating enzymes with different or similar biochemical mechanisms have been identified. Overexpression of these BR catabolic genes leads to BR deficiency, whereas loss of function of these enzymes results in elevated amounts of BL or CS in plants.

Hydroxylases

C26 hydroxylation is a relatively well characterized way of BR inactivation. *Arabidopsis* BAS1/CYP734A1 (formerly named CYP72B1) is the first reported BR C26 hydroxylase as revealed by the feeding experiment. It is able to convert both CS and BL to their C26 hydroxylated derivatives (Neff et al., 1999; Turk et al., 2003). Such modification possibly prevents the side chain of BRs from fitting into the binding pocket of the receptor, BRI1 (Hothorn et al., 2011; She et al., 2011). Tomato CYP734A7 can also convert CS and BL to their hydroxylated products, respectively (Ohnishi et al., 2006a). CYP734A orthologs from rice control endogenous bioactive levels of BRs by metabolizing both CS and its precursors (Sakamoto et al., 2011; Thornton et al., 2011). It is noteworthy that rice CYP734As can catalyze not only the hydroxylation but also the second and the third oxidations to produce aldehyde and carboxylate groups at C26 (Sakamoto et al., 2011). PAG1 from cotton and DcBAS1 from carrot may also inactivate bioactive BRs in a way similar to that of the *Arabidopsis* BAS1 (Yang et al., 2014; Que et al., 2019).

CYP72C1/SOB7/SHK1/CHI2, a homolog of BAS1/CYP734A1, was identified by three independent research groups in the same year. It acts redundantly with BAS1/CYP734A1 to modulate *Arabidopsis* photomorphogenesis and BR inactivation processes (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). However, CYP72C1 prefer to act

TABLE 3 | BR metabolism enzymes in different plant species.

Function	Species	Name	Reference
Acyltransferase	*Arabidopsis thaliana*	BIA1/ABS1	Roh et al., 2012; Wang et al., 2012
		BIA2	Zhang and Xu, 2018
		PIZ/BAT1/DRL1	Schneider et al., 2012; Choi et al., 2013; Zhu et al., 2013
C26 hydroxylase	*Arabidopsis thaliana*	BAS1/CYP734A1	Neff et al., 1999; Turk et al., 2003
	Lycopersicon esculentum	CYP734A7	Ohnishi et al., 2006a
	Oryza sativa	CYP734A2/4/5/6	Sakamoto et al., 2011
	Gossypium hirsuturm	PAG1	Yang et al., 2014
	Daucus carota	DcBAS1	Que et al., 2019
Sulfotransferase	*Brassica napus*	BNST3/4	Rouleau et al., 1999; Marsolais et al., 2004
	Arabidopsis thaliana	AtST1/AtST4a	Marsolais et al., 2007
Glycosyltransferase	*Arabidopsis thaliana*	UGT73C5	Poppenberger et al., 2005
		UGT73C6	Husar et al., 2011
Unknown	*Arabidopsis thaliana*	CYP72C1/SOB7/SHK1/CHI2	Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005
Unknown	*Arabidopsis thaliana*	BEN1	Yuan et al., 2007

on BR immediate precursors *via* an uncharacterized mechanism, which is different from CYP734A members that can inactivate both BL and CS through C26 hydroxylation (Thornton et al., 2010).

Glycosyltransferases

Glucosylation is one of the important regulatory mechanisms controlling hormone homeostasis *in planta*. CS and BL can be glucosylated at different positions. C2-, C3-, C22-, and C23-glucosylation of CS, and C2-, C3-, and C23-glucosylation of BL were confirmed, although the glucosylation profiles varied in different plant species (Soeno et al., 2006). 23-*O*-glucosylation of CS or BL was found to be predominant in *Arabidopsis*, which is catalyzed by two homologous UDP-glycosyltransferases named UGT73C5 and UGT73C6 (Poppenberger et al., 2005; Husar et al., 2011). Overexpression of *UGT73C5* or *UGT73C6* leads to a BR-deficient phenotype in *Arabidopsis*. Since these two functionally redundant genes are tightly linked, it is impossible to get high-order null mutant with traditional genetics to support the biochemical analysis results at the time when it was first published. Of course, using a CRISPR-Cas9 approach can solve such a problem at present time. In addition, enzymes mediating C2-, C3-, and C22- glucosylation of BRs in plants are still unknown.

BEN1, a Putative Reductase

BRI1-5 ENHANCED1 (BEN1) is also involved in BR inactivation in *Arabidopsis* (Yuan et al., 2007). Although the detailed biochemical mechanism has not been elucidated, strong genetic evidence supports that BEN1 functions as a BR inactivating enzyme. Gain of function of BEN1 severely enhances the *bri1-5* defective phenotype, while loss of function of BEN1 leads to an organ-elongation phenotype. Since *BEN1* encodes a dihydroflavonol 4-reductase (DFR)-like protein, it is hypothesized that BEN1 functions as a reductase to convert 6-oxo BR intermediates to their 6-deoxo counterparts (Yuan et al., 2007). It is noteworthy that the intronic T-DNA insertion in the *ben1-1* mutant is epigenetically regulated (Sandhu et al., 2013).

Acyltransferases

Three acyltransferases were found to decrease endogenous bioactive levels of BRs likely *via* different biochemical mechanisms in *Arabidopsis*. BRASSINOSTEROID INACTIVATOR 1 (BIA1)/ABNORMAL SHOOT1 (ABS1), a BAHD acyltransferase in *Arabidopsis*, was isolated by two independent research groups. Activation tagged mutants or transgenic plants overexpressing *BIA1*/*ABS1* display reduced levels of endogenous BRs and BR-deficient phenotypes that can be rescued by exogenous application of active BRs, indicating a possible role of BIA1/ABS1 in maintaining BR homeostasis (Roh et al., 2012; Wang et al., 2012). A more recent study demonstrated that BIA1 uses acetyl-CoA as a donor substrate to acylate CS, leading to the formation of monoacetylated and diacetylated CS (Gan et al., 2020). BIA2, a homolog of BIA1/ABS1 in *Arabidopsis*, also plays a role in BR inactivation possibly *via* the esterification of certain BRs (Zhang and Xu, 2018). PIZZA (PIZ)/BR-RELATED ACYLTRANSFERASE1 (BAT1)/DWARF AND ROUND LEAF1 (DRL1), an acyltransferase in *Arabidopsis*, was found to regulate BR homeostasis probably by converting BR intermediates into acylated inactive conjugates (Schneider et al., 2012; Choi et al., 2013; Zhu et al., 2013).

Sulfotransferases

BNST3 and BNST4, two homologous steroid sulfotransferases from *Brassica napus*, catalyze the *in vitro O*-sulfonation of BRs as well as mammalian estrogenic steroids and hydroxysteroids (Rouleau et al., 1999; Marsolais et al., 2004). They are stereospecific for 24-epiBRs, with a preference for 24-epicathasterone, an intermediate in the biosynthesis of 24-epiBL, which is different from other known metabolic enzymes that utilize CS and BL as substrates. However, BNST3 and BNST4 were also thought to be involved in BR inactivation since sulfonation of 24-epiBL leads to the absence of its biological activity in the bean second internode bioassay (Rouleau et al., 1999; Marsolais et al., 2004). AtST1, an *Arabidopsis* ortholog of BNST3 and BNST4, displays a similar specificity toward 24-epiBRs. Whereas, AtST4a, another steroid sulfotransferase from *Arabidopsis*, is specific for bioactive BR compounds (Marsolais et al., 2007). Genetic evidence to support the significance of these sulfotransferases in BR inactivation is still lacking (Sandhu and Neff, 2013).

Regulation of BR Catabolism

Plants evolved various mechanisms to control BR catabolism (**Figure 3**). Feedback regulation of key BR catabolic genes is one of these mechanisms. It was found that BL induces the expression of several BR catabolic genes, including *BIA1/ABS1*, *BIA2*, *PIZ/BAT1/DRL1*, *BAS1/CYP734A1*, and *SOB7/SHK1/CHI2* in *Arabidopsis*, *PAG1* in cotton, and *DcBAS1* in carrot (Tanaka et al., 2005; Roh et al., 2012; Zhu et al., 2013; Yang et al., 2014; Zhang and Xu, 2018; Que et al., 2019).

Besides the end products of the BR biosynthetic pathway, other phytohormones were also found to regulate the expression of the BR catabolic genes. For example, the expression of *BNST3/4* can be induced by salicylic acid (Rouleau et al., 1999). The expression of *PIZ/BAT1/DRL1* is induced by auxin but repressed by abscisic acid (Choi et al., 2013; Zhu et al., 2013). Moreover, ARF7, an auxin responsive transcription factor, can directly inhibit the expression of *BAS1/CYP734A1* to increase endogenous BR contents in *Arabidopsis*, providing sufficient evidence that auxin regulates BR catabolism (Youn et al., 2016).

Most of the abovementioned BR catabolic genes show different expression patterns under light and in darkness, indicating an important role of light in maintaining BR homeostasis by regulating the catabolic reactions. However, little is known about the detailed mechanisms. It has been found that PHYB, a red/far red-absorbing phytochrome, modulates *BAS1* expression in *Arabidopsis* shoot apex to inhibit phase transition (Sandhu et al., 2012). ATAF2, an *Arabidopsis* NAC transcription factor suppressed by light at a transcription level, modulates BR inactivation *via* directly binding to the promoter of *BAS1/CYP734A1* and *SOB7/SHK1/CHI2* to repress their expression (Peng et al., 2015). A more

recent study demonstrated that CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), a MYB transcription factor, interacts with ATAF2 and directly regulates the oscillation expression of *BAS1/CYP734A1* and *SOB7/SHK1/CHI2* (Peng and Neff, 2020).

Two more transcription factors were found to be involved in regulating BR catabolism. However, the upstream signaling is unknown. LATERAL ORGAN BOUNDARIES (LOB) activates *BAS1/CYP734A1* expression *via* directly binding to its promoter and consequently decrease BR accumulation to limit growth in *Arabidopsis* organ boundaries (Bell et al., 2012). OSH1, a KNOX transcription factor, promotes the expression of three homologous BR catabolic genes, *CYP734A2*, *CYP734A4*, and *CYP734A6*, to control local bioactive BR levels in rice shoot apical meristems (Tsuda et al., 2014).

GENERAL CONCLUSION

It has been about fifty years since BL was first discovered from *Brassica napus* pollen grains (Mitchell et al., 1970). Significant progress has been made in our understanding of BR biosynthesis and catabolism. Although the BR biosynthesis pathway displays a metabolic grid, the most dominant and efficient shortcut was established, with only eight and seven steps in *Arabidopsis* and rice, respectively (**Figure 2**). Moreover, enzymes catalyzing each reaction in the BR biosynthetic pathway, except for the C2 hydroxylation and the C3 redox reaction, have been identified by using analytical chemistry and molecular genetic approaches. Structural and physiological studies revealed that C2 and C3 positions are important for BR activity and perception by its receptor and coreceptor (Hothorn et al., 2011; She et al., 2011; Sun et al., 2013). Therefore, identification of C2 hydroxylase and C3 oxidase/reductase is essential for clarifying the whole picture of BR biosynthesis. CYP92A6/DDWF1 from pea was identified as the C2 hydroxylase, providing reference for study of C2 hydroxylase in *Arabidopsis*, rice, and other higher plants (Kang et al., 2001). As revealed by feeding experiments or anticipated from naturally occurring metabolites, various BR metabolic reactions were found (Fujioka and Yokota, 2003). However, little is known about the corresponding enzymes and the underlying mechanisms. Moreover, knowledge about how BR biosynthesis and catabolism are regulated, especially in a specific organ or tissue, by diverse internal and external cues is still very limited. Elucidating the mechanisms regulating BR homeostasis can help us to generate high-yield transgenic crops *via* manipulating bioactive BR contents. For example, modulating the expression of C22 hydroxylase, catalyzing the rate-limiting step in BR biosynthesis pathway, in different plant species indeed resulted in increased vegetative growth, yield, and tolerance (Choe et al., 2001; Sakamoto et al., 2006; Guo et al., 2010; Sakaguchi and Watanabe, 2017; Li et al., 2018; Zhou et al., 2018). It might not be possible to obtain optimal BR effects for all of the agronomic traits, since BRs control many aspects of plant growth and development, and responses to biotic and abiotic stresses. However, even if a subset of these traits can be improved by BRs, the accomplishment will be significant.

AUTHOR CONTRIBUTIONS

ZW prepared the manuscript. JL revised the manuscript.

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REVIEW
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Modes of Brassinosteroid Activity in Cold Stress Tolerance

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Cold stress is a significant environmental factor that negatively affects plant growth and development in particular when it occurs during the growth phase. Plants have evolved means to protect themselves from damage caused by chilling or freezing temperatures and some plant species, in particular those from temperate geographical zones, can increase their basal level of freezing tolerance in a process termed cold acclimation. Cold acclimation improves plant survival, but also represses growth, since it inhibits activity of the growth-promoting hormones gibberellins (GAs). In addition to GAs, the steroid hormones brassinosteroids (BRs) also take part in growth promotion and cold stress signaling; however, in contrast to Gas, BRs can improve cold stress tolerance with fewer trade-offs in terms of growth and yields. Here we summarize our current understanding of the roles of BRs in cold stress responses with a focus on freezing tolerance and cold acclimation pathways.

Keywords: steroid, resistance, frost, freezing, hormone, brassinosteroids, acclimation, abiotic stress

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INTRODUCTION

Cold stress represents a substantial risk for plant growth and development and impacts on plant distribution and crop production. Both chilling (>0°C) and freezing (<0°C) temperatures can cause damage, with the degree depending on the species and the developmental stage during exposure. Plants are most susceptible to frost during periods of active vegetative and reproductive growth, since growing, hydrated tissues are especially vulnerable to injury caused by freezing of cellular fluids (Nishiyama, 1995). Also at high risk is plant reproductive development, where both structural and functional abnormalities can lead to failed fruit and seed production (reviewed in Thakur et al., 2010; Albertos et al., 2019). Consequently, frost in spring during the bloom of fruit trees or stem elongation of winter cereal crops can result in a complete loss of harvest (Chmielewski et al., 2004; Augspurger, 2013).

It is therefore perhaps not surprising that cold stress has significant economic impact. For example, the United States in the mid 20th century experienced more economic losses due to frost damage than to any other weather-related phenomenon (White and Haas, 1975). As a land spanning a range of climates, many of the southern subtropical and warm temperate latitudes are areas of horticultural and agricultural significance. Various fruit crops in these areas are vulnerable to frost, as physical damage to produce hinders ideal growth and reduces yield quality

and quantity. Recent trends in winter-related damages also show an increasing number of incidents in the past century (**Figure 1**). In 2018, Harvey reported that up to 60% of locations across North America, Europe, East Asia, and parts of South America would see extreme weather events triple, much of which can be attributed to recent shifts in Arctic temperatures impacting the seasonal polar vortex.

Global warming is expected to further increase the risk of damage by frost in particular in mid-latitude zones where many major cropping areas lie. One such observation is the arctic amplification reduction of the pole–to–mid-latitude temperature gradient, predicted to cause more extreme weather events and extended conditions, such as early thaws, and sudden cold spells (Francis and Vavrus, 2012). The rising temperatures result in prolonged growing seasons, which delays cold hardening in fall and accelerates de-hardening in spring, increasing the potential for damage when early or late frosts occur (Rigby and Porporato, 2008; Augspurger, 2013; Hatfield and Prueger, 2015). Even in winter de-hardening could occur when temperatures rise periodically (Rapacz et al., 2017). Breeding for optimized cold stress tolerance may contribute to a possible solution, but has been mostly unsuccessful, as cold stress tolerance is usually correlated with impaired growth. This is supported widely by evidence that abiotic stress, including cold stress, represses growth, especially in scenarios of compounded stress or deprivation factors (Mittler, 2006). Growth repression is thought to free resources for other energy-demanding, stress-protective cellular reactions and systemic signaling (Mittler, 2002), although this hypothesis remains to be validated. Therefore, a thorough understanding of cold stress responses and how they are integrated with growth regulatory pathways is required for the design of targeted breeding approaches that aim to improve cold tolerance without trade-offs on growth or yield.

The repression of growth in response to cold involves effects on growth-promoting hormones and in this context, gibberellins (GAs) play an important role, although also other hormones are involved (reviewed in Eremina et al., 2016a). A reduction of GA levels and signaling activity contributes to restraint growth and enhances plant tolerance to several abiotic stress types including cold, drought, and osmotic stress (Achard et al., 2006, 2008; Magome et al., 2008). In addition to GAs, also the brassinosteroids (BRs) exhibit dual functions in growth control and abiotic stress protection. However, as opposed to GAs, there is evidence that BRs can promote both growth and resistance against certain abiotic stress types, which is intriguing since it may provide a means to increase abiotic stress tolerance with fewer trade-offs. A number of reviews have summarized progress in this area, providing an excellent overview of signaling events implicated in different abiotic stress types (reviewed in Divi and Krishna, 2009; Vriet et al., 2012; Planas-Riverola et al., 2019; Nolan et al., 2020). Here we have specifically focused on the role of BRs in cold stress responses and lay an emphasis on cold acclimation and freezing tolerance. While the resumption of growth after cold stress exposure is certainly also a relevant and exciting research area (reviewed in Vyse et al., 2019), evidence on the function of BRs in this process is still very

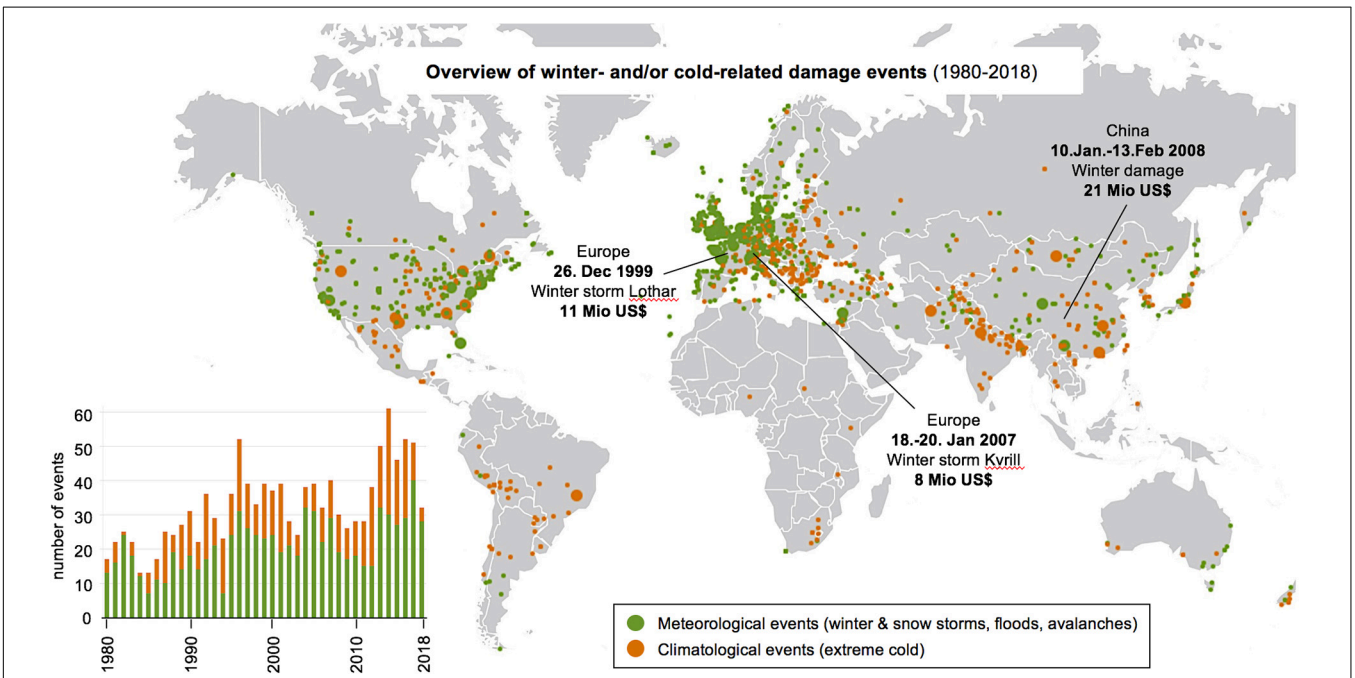

FIGURE 1 | Summary of winter storm and winter damage events from 1980 to 2018. Data were retrieved from the public database provided by Munich RE NatCatSERVICE in July 2020. The world map shows the location where damage occurred with meteorological events in green and climatological events in orange. Highlighted are the three most costly single events in this time span, with the costs of damage in Mio US$. The bar chart on the left shows the number of worldwide events per year.

limited (Pagter et al., 2017) and thus we do not expand on this topic here.

BRASSINOSTEROIDS AND THEIR ROLE IN PLANT GROWTH AND FROST TOLERANCE

Brassinosteroids are steroid hormones that are synthesized from the bulk sterol campesterol by multiple hydroxylation and oxidation events, which are catalyzed by different cytochrome P450 enzymes, including DWARF4 (DWF4), CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD), ROTUNDIFOLIA 3 (ROT3), and the CYP85A2 BR6ox2 (Clouse, 2011). The end products of BR biosynthesis castasterone (CS) and brassinolide (BL) are bioactive; they act in minute concentrations, which are closely monitored and adjusted. This is executed by regulation of genes involved in BR biosynthesis and catabolism, depending on the needs of intrinsic growth programs, but also according to requirements for growth adaptation and stress protection (reviewed in Clouse, 2011; Lv and Li, 2020). With respect to cold stress, there is evidence that the BR biosynthetic genes *DWF4*, *CPD*, and *BR6ox2* are rapidly downregulated by cold treatment (Eremina et al., 2016b). In particular, *BR6ox2* is repressed by one order of magnitude in plants exposed to 4°C, which is significant given that BR responses are usually only in the range of two to fourfold (Goda et al., 2002). Whether this repression of BR-biosynthetic genes also impacts BR levels and is of importance for BR-enabled effects in cold stress protection remains to be shown.

Castasterone and BL confer their bioactivity by binding to BR receptors of the BRASSINOSTEROID INSENSITIVE 1 (BRI1)-type, which initiates a phosphorylation-dependent signal transduction cascade that requires co-receptors, including BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and BRI1-KINASE INHIBITOR 1 (BKI1), multiple phosphatases, including BSU1, and kinases, the most-studied being the GSK3/shaggy-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2). BIN2 is a central repressor of BR signaling and, among other targets, also directly regulates BR-regulated transcription factors (TFs) that control BR-responsive gene expression (Kim and Wang, 2010). These TFs include different classical bHLH proteins such as BRASSINOSTEROID ENHANCED EXPRESSION 1-3 (BEE1-BEE3) and CESTA (CES), the BES1-INTERACTIVE MYC-LIKE (BIMs), and PHYTOCHROME INTERACTING FACTOR 4 (PIF4), but also atypical bHLH-type proteins, most importantly the BRI1-EMS-SUPPRESSOR 1/BRASSINAZOLE RESISTANT 1 (BES1/BZR1) subfamily (Wang et al., 2002; Yin et al., 2005; Bernardo-García et al., 2014; Khan et al., 2014).

Clear evidence for BRs being essential for plant development is the severe phenotypes of BR-deficient mutants. The most prominent features are dwarf growth with dark-green, cabbage-like leaves in the light, de-etiolated development in the dark, late flowering, and impaired fertility (reviewed in Clouse, 2011). Some of these defects are caused by malfunctioning cross-talk with GAs, since in certain plant species, including the model plant *Arabidopsis thaliana* and rice, BRs can promote GA biosynthesis (Tong et al., 2014; Unterholzner et al., 2015) and also interplay with GAs at the signaling level (reviewed in Tong and Chu, 2016; Unterholzner et al., 2016).

Severe growth defects represent a challenge when stress phenotypes are to be studied, since strong morphological alterations can impact stress perception. Therefore, multiple mutant settings and BR application studies were applied when the impact of BRs on freezing tolerance was assessed. This yielded solid evidence that BRs can improve frost tolerance. On the one hand, BR application increased survival rates of plants exposed to subzero temperatures (Kagale et al., 2007; Kim et al., 2010). On the other hand, and more importantly, *A. thaliana* mutants, deficient in various steps of BR signaling including the strong *bri1-1* and the weak *bri1-301* allele, and over-expression lines of *BIN2* and its homolog *ASKtheta*, showed decreased frost tolerance. In line, the *BRI1* over-expression line *35S:BRI1-GFP* and the higher-order *bin2-3 bil1 bil2* mutant, plants with constitutively active BR signaling, were more resistant to frost damage (Kim et al., 2010; Eremina et al., 2016b; Li et al., 2017). Interestingly, in addition to improving basal tolerance, BRs also contribute to acquired freezing tolerance in *A. thaliana*, which involves complex molecular and biochemical changes that are induced by low, but non-freezing temperatures, in a process termed cold acclimation.

BIOCHEMICAL AND CELLULAR EVENTS DURING COLD STRESS

Cold acclimation enables plants from temperate geographical zones to increase their basal levels of freezing tolerance through initiation of a multitude of biochemical and cellular changes. These changes are induced by cold, but non-freezing temperatures and include compositional changes to the cell wall and membrane, an activation of anti-oxidative mechanisms, and the synthesis and accumulation of cryoprotective solutes, amino acids, and proteins (Lissarre et al., 2014).

Frost damage can be caused by the freezing of soil waters leading to drought exposure, and by the freezing of cellular fluids, equally as problematic. The development of ice depends on the presence of ice nucleation sites, which may be intrinsically found in cells or cell walls, or formed by epiphytic bacteria found on leaves (Lindow et al., 1982). Although the formation of ice crystals is usually initiated in the cell walls and the intercellular space, it is the cellular water-deficit caused by both the lower chemical potential and vapor pressure of ice that actively dehydrates the cell. Water in the cytoplasm or vacuole moves down the potential water gradient toward extracellular ice, and across the plasma membrane (Buchanan et al., 2000). Freezing damage therefore induces cellular desiccation and rigidification of the cell membrane as it contracts and pulls away from the cell wall (Taiz et al., 2015; illustrated in **Figure 2**). Similar to the effects of drought stress, the symplast can lose about 90% of osmotically active water to the apoplast, putting already semi-dehydrated cells at risk for additional injury, in particular to further suffer membrane damage, which results in the loss of physical integrity of the cells (Taiz et al., 2015). On affected

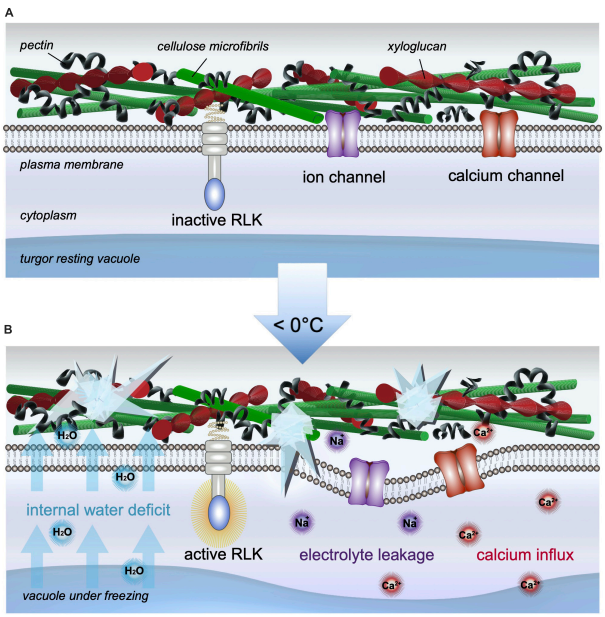

FIGURE 2 | Cellular changes induced by freezing stress. **(A)** At ambient temperatures, the cell wall and membrane, as well as other cellular compartments, are intact and the cell is in a normal resting state, which ensures water and cellular volume homeostasis. **(B)** In the event of freezing stress, ice formation in the apoplast induces a movement of water out of the cell, which results in an internal water deficit, mimicking dehydration. If exposure is prolonged, intracellular ice may form, rupturing the plasma membrane, causing activation of RLKs, electrolyte leakage, calcium influx, and cell death, when the membrane eventually pulls away from the cell wall.

plants, wilting and water-soaked areas appear; when dried these form necrotic lesions on leaves, fruits, or stems (Morel and Dangl, 1997). While one obvious consequence may include the depreciation of aesthetic worth, the nutritional value, longevity, and overall yield may ultimately be compromised.

Frost itself is not a solitary threat; temperatures above zero 0°C can be problematic, especially for species that stem from tropical and subtropical geographical zones. Chilling can induce disruption of photosynthetic pathways, which activates photochemical production of reactive oxygen species (ROS) that cause injury to DNA, proteins, and lipids (Gururani et al., 2015). To prevent harm, plants utilize ROS scavenging enzymes and antioxidants such as ascorbate and glutathione (Mittler et al., 2004). In addition, denaturation of proteins, loss of guard cell function, and increased tissue levels of CO_2 are observed (Allen and Ort, 2001). While chilling damage is evidently not attributed to ice crystal formation, it impacts on the plants ability to accommodate low temperatures with loss of membrane fluidity, resulting in membrane leakage (Verslues et al., 2006) and it appears that BRs can protect from the damaging effects of these events. BR application improved plant performance in the cold, which was shown for the chilling-sensitive species *Cucumis sativus* (cucumber), *Solanum lycopersicum* (tomato), *Oryza sativa* (rice), *Zea mays* (maize), and also for the cold-hardy plant *A. thaliana* (He et al., 1991; Katsumi, 1991; Kagale et al., 2007; Koh et al., 2007; Xia et al., 2009; Aghdam et al., 2012). Moreover, over-expression of *DWF4* conferred protective effects during germination and early seedling development of *A. thaliana* at 4°C (Divi and Krishna, 2009) and reduced chilling-induced oxidative damages in tomato (Xia et al., 2018). As a majority of high-value horticultural crops fall under the chilling-sensitive category, the function and efficacy of BR application demands a thorough understanding as to how each phase of injury may be transmitted, and this will be detailed here.

Physical and Structural Changes During Cold Stress: The Cell Wall

The primary cell wall consists of a hemicellulose, pectin, and a structural protein matrix, amid an integrated network of cellulose microfibrils. In a normalized metabolic state, the proteins act as reinforcing structural components, operating together similar to an exoskeleton layer, resisting forces of turgor pressure, and controlling cell expansion (Taiz et al., 2015). The cell wall not only works as a diffusion barrier for ions and macromolecules, but it limits the range of molecules that can reach the plasma membrane by selective permeability and hydrophobic interactions, maintained by an intrinsic negative charge. In the event of perceived chilling stress, the membrane becomes rigid as protein conformations change and complexes destabilize. The level of susceptibility to cold-dependent conformational changes also depends on the cell type; typically, thicker cells walls are found in sclerenchyma cells (epidermal cells, xylem, phloem, and tracheids). The type and function of a plant cell also influences whether it is composed of a primary wall or secondary wall, which vary in development and protein and polysaccharide profiles.

Cell expansion and compression resistance rely on the abundance of pectin, which is found at relatively high levels in primary cell walls. Alternately, secondary cell walls contain dense structures mostly of cellulose-hemicellulose and lignin. These polysaccharide components determine stability, flexibility, and permeability; in the context of BRs in cold stress, evidence suggests BR signaling pathways to be involved with cell wall remodeling mechanisms responsible for altering these features (Rao and Dixon, 2017). In maize, wheat, and rice, for instance, the expression of many xyloglucan transferase/hydrolase enzymes (XTHs) and expansin genes was reported to be regulated by BRs (Uozu et al., 2000). XTHs catalyze cleavage of xylogulcan polymers then transferring ends to other xyloglucan chains, while expansins loosen linkages between cellulose microfibrils initiating cell wall loosening, a protective reaction against abiotic stresses (Yennawar et al., 2006). Such structural flexibility may allow a plant to survive harsh conditions with extreme humidity or temperature fluctuations.

Brassinosteroids have also been found to play an essential role in secondary cell wall formation. In *A. thaliana*, it was shown that BR signaling regulates secondary wall development via BR-induced BES1 activation of VND6 and VND7 (Yang and Wang, 2016), which determine xylem cell transition to form tracheary elements and alter the expression of MYB TFs that regulate lignin biosynthesis (Ohashi-Ito et al., 2010). Moreover, in *A. thaliana*, a

loss of function of DIMINUTO1 (DIM1/DWF1), an enzyme that catalyzes an early step in BR biosynthesis, caused a phenotype with a significant reduction in lignin content and a lower lignin syringyl to guaiacyl ratio (Hossain et al., 2012). Lignin is the second most abundant carbon sink in plants; it is deposited in the secondary cell wall, augmenting cell wall rigidity and providing structural support, yet remaining clear of the primary cell wall (Karkonen and Koutaniemi, 2010); evidence that BRs can increase the accumulation of lignin has been implicated in the direct binding of BES1/BZR1 to promoter regions NAC and MYB TFs integral to lignin synthesis pathways (Benatti et al., 2012).

Alternately, pectin allows cell walls to remain firm, inhibiting collapse of the cellulose matrix, but also conferring flexibility, by forming hydrated gels, responsive to changes in polymer residues or pH (Voxeur and Hofte, 2016). In *A. thaliana*, BAK1 can interact with a plasma membrane receptor-like protein, RLP44, to repress pectin methylesterase inhibitor activity, reducing the rigidity of the pectic matrix and stimulating cell wall loosening in both basal and stressed conditions (Wolf et al., 2014). Although these implications of BR signaling in cell wall formation point to a significant contribution in cold stress tolerance, this area still requires further investigation.

In the event of cold exposure, both fluidity and strength of the cell wall influence internal cellular water conditions. Freezing especially alters the movement of water as intercellular ice forms. Ice formation occurs first in the apoplast where the negative water potential is far lower, eliciting further water movement down the gradient. Subsequently, an internal water deficit develops, mimicking the effects of cell dehydration (reviewed in Yamada et al., 2002; Le Gall et al., 2015). Moreover, freezing may induce intracellular ice crystal formation, and subsequently, wall destruction and cell death (reviewed in Tenhaken, 2015). As ice crystals grow, they puncture into the cytoplasm, rupturing membranes, and the membranes of nearby organelles. Both cellular desiccation and rigidification of the cell membrane continue as the membrane contracts and pulls away from the cell wall (Taiz et al., 2015). When the membrane then starts to break, an influx of calcium and electrolyte leakage occurs (illustrated in **Figure 2**), which is measurable and often serves as a quantifiable read-out for frost-induced damage.

Membrane Fluidity

Exposure to cold or frost affects not only the permeability, flexibility, and resilience of the primary cell wall but if severe, may damage the plasma membrane. Low temperatures induce a hardening of the cell membrane, and a number of studies on cold perception suggest a dependency on membrane fluidity changes (Markovskaya and Shibaeva, 2017). As a major site of freezing-induced injury, the plasma membrane undergoes structural changes, a consequence of cellular dehydration (reviewed in Ingram and Bartels, 1996). When stages of cellular desiccation progress, the plasma membrane draws inward, away from the cell wall and closer to organelle membranes, both altering and destabilizing the integrity of membrane components, predominantly lipids and proteins (reviewed in Thomashow, 1999).

Under ambient growth conditions, each membrane in the plant cell has characteristic heterogeneous lipid profiles, and each class of lipids an equally distinct fatty acid composition. One such class of lipids are sterols, and include campesterol; as BR precursors, and central membrane components, they regulate membrane fluidity and permeability of membranes by directly affecting the activity of membrane-bound or membrane-associated proteins. As such they play a range of roles, from mature membrane protein signaling to inducing hyperpolarization of the membrane in cell division (reviewed by Clouse, 2011). Altered sterol profiles in BR mutants may affect membrane structure, influencing how specific signaling proteins interact, and impacting the fluidity of the membrane in response to environmental cues (Clouse, 2002). Although the current knowledge of membrane fluidity and BR signaling focuses on membrane-bound receptor activity and downstream signaling targets, other cell interactions like the ER-localized Unfolded Protein Response have been recently described to play a role both in protecting reproductive development stages from extreme temperatures and BR-mediated responses by recruiting the membrane-associated TFs bZIP17 and bZIP2 (Che et al., 2010; Bao and Howell, 2017). Rigidification of the membrane and alteration of lipid profiles may be resolute structural responses to cold exposure; however, further investigation of membrane-compartment interactions in vulnerable developmental stages may provide insight into otherwise cryptic BR-mediated signaling.

Ca^{2+} Influx

A key molecular messenger prone to accumulation following cold perception is the divalent calcium cation Ca^{2+}. In the cell, cytosolic calcium is normally maintained at low resting concentrations to facilitate external and internal Ca^{2+} membrane transport. Organelles including the rough ER and the vacuole contain intracellular stores of Ca^{2+} ready for signal-induced mobilization. The primary calcium receptor, calmodulin, is a highly conserved Ca^{2+} calcium binding protein attached to the plasma membrane, also found in both nuclear and cytoplasmic compartments. In the event of cold perception, a signal initiates channel-mediated, inward calcium transport. These channels are either activated mechanically by cell wall rigidification, or through direct ligand binding (Lissarre et al., 2014). It has been suggested that Ca^{2+} spiking is regulated by downstream receptor-like kinases with leucine-rich-repeat domains similar to those of the BR receptors (Oldroyd and Downie, 2004) and there is evidence that BRs can impact on the activity of Ca^{2+} channels (Straltsova et al., 2015); albeit, the modes are still unknown.

In *A. thaliana,* CaM (calmodulin) binds in a Ca^{2+}-dependent manner to the cytoplasmic domain of BRI1 (Oh M. H. et al., 2012). Since BR signal transduction is initiated by hormone perception in the extracellular domain of BRI1, which then binds to BAK1, activating phosphorylation of cytoplasmic residues in the kinase domain by Ca^{2+}/CaM binding may attenuate kinase activity of BRI1 and influence subsequent signaling and downstream target regulation (Du and Poovaiah, 2005; Oh et al., 2009).

Ca^{2+} binding to CaM has also been shown to be critical for BR biosynthesis and plant growth since it was found that DIM/DWF1 is a Ca^{2+}/CaM-binding protein and that calmodulin-binding compromises DWF1 function *in planta* (Du and Poovaiah, 2005). DWF1 orthologs in other plant species have a similar Ca^{2+}/CaM binding motif, indicating that Ca^{2+}/CaM regulation of DWF1 and DWF1 homologs is conserved among plants (Du and Poovaiah, 2005). The possibility for a role of CaM in BR biosynthesis is also indicated by the fact that CaM over-expression lines show phenotypic features of plants over-expressing the BR biosynthetic gene *DWF4* (Du and Poovaiah, 2005). Consequently, the Ca^{2+}/CaM complex may regulate a wide range of factors on the biosynthesis path of BRs in addition to LRR RLK co-receptor activity.

ROS Species: Antioxidant Mobilization

Most reactions involving enzymatic kinetics interact with photosynthetic processes and metabolite accumulation to maintain a state of survival. ROS accumulate as a result of fewer scavenging enzymes and the disturbance of metabolic activity in response to abiotic stresses such as lowered temperatures or drought (Choudhury et al., 2017). Furthermore, over-reduction of the chloroplast electron chain may further increase ROS formation, leading to photoinhibition of PSI and PSII (Tjus et al., 2001). High concentrations of ROS lead to deterioration of membranes, causing membrane leakage of solutes, initiating a signal cascade responsive to the source of injury (Ruelland et al., 2009).

There is evidence that points to a BR function in the activation of cell-wall centered defense by ROS. In response to physical damage or pathogen inoculation, oxylipins, or oxygenated fatty acid products, might function as ROS signals to activate the BR pathway thereby reinforcing the cell wall defensively (Marcos et al., 2015). Furthermore, BRs may have an effect on bond integrity of monolignol polymers and phenolic acids in the cell wall by regulating antioxidant enzymes at both the transcriptional and post-transcriptional level (Li et al., 2016).

The exogenous application of BR increases the activity of antioxidant enzymes appreciably, including superoxide dismutase, catalase, ascorbate peroxidase, and peroxidase in grains exposed to high metal stress (reviewed in Kumar et al., 2015). This is thought to strengthen the mechanical properties of the wall by enhancing the covalent cross-linked components through combined peroxidase activity increase and ROS formation (Tenhaken, 2015). By utilizing the antioxidant defense system and facilitating cross-linking of phenolic compounds in the cell wall, BRs may orchestrate the alleviation of ROS-burst induced oxidative damage (Kumar et al., 2015).

Additionally, it has been reported that BRs play a role in the induced transcription of an NADPH oxidase-encoding gene, leading to increased levels of apoplastic H_2O_2. This rapid accumulation affects developmental and stress response activity by inducing biosynthesis of the plant hormone abscisic acid (ABA) and stomatal closure. By prolonging H_2O_2 accretion, it is suggested that BRs control ROS homeostasis to induce a level of plant stress tolerance (Xia et al., 2015). Moreover, BR-induced stimulation of antioxidant enzymes in response to high ROS levels appears to be relevant for ROS detoxification and thus plant survival following cold stress (Bajguz and Hayat, 2009; Liu et al., 2009).

Biochemical and Physiological Changes Toward Acclimation

While many economically important crops are considered cold-sensitive, chilling-resistant plants such as *A. thaliana* are able to grow and develop even in low temperatures of 0–12°C, albeit at reduced rates. This adaptive capacity can be explained by diverse biochemical and physiological changes both in cell structure, and production of sugars, fatty acids, and secondary metabolites (Ruelland et al., 2009). In *A. thaliana*, various amino-acids including asparagine, aspartate, glutamate, glutamine, and alanine accumulate in response to cold (Klotke et al., 2004). Although a range of solutes accumulate, localization and therefore function vary within the cell. The trisaccharide raffinose, for instance, translocates from the cytosol to the chloroplasts, thereby protecting photosystems against damage in freeze-thaw phases (Knaupp et al., 2011). Conversely, in the plasma membrane, it behaves dispensably by replacing water molecules in the hydration shell of the lipid headgroups, preventing injurious lipid phase shifts.

Compositional changes in cell membrane lipid profiles are one of a series of physiological adjustments to cold conditions. In many cases, de-polymerization of the cytoskeleton in combination with phospholipid desaturation in the membrane can create an adaptive circumstance where the membrane rigidification is partially counterbalanced (Tasseva et al., 2004). Other responses to chilling include the plant increasing phospholipids or cerebrosides to prevent further membrane apposition and collapse.

While modifications improving structural integrity of the cell alleviate physical destruction, the accumulation of secondary metabolites may involve more complex pathways relying on gene regulation, expression, and modes of signal transduction (Shinozaki et al., 2003). Phenylpropanoids are a large group of secondary metabolites that comprise flavonoids, ubiquitous compounds involved in both abiotic and biotic stress defense mechanisms (Janská et al., 2010). Although well known for its role in fruit and leaf tissue pigmentation, UV protection, and photosynthetic interactions, very little is known regarding flavonoid pathway regulation in plants exposed to low temperatures, or its activity related to hormone signaling pathways, such as BRs. Petridis et al. (2016) described the change of a phenylpropanoid accumulation profile in BEE1 and GFR (*G2-LIKE FLAVONOID REGULATOR*) mutants following low temperature exposure, defined by quercetins and scopolin accumulating less, and anthocyanins accumulating more than in wild-type. These phenotypes formed the basis of further work, which showed that BEE1 and GFR act as negative regulators of anthocyanin accumulation by inhibiting anthocyanin biosynthesis genes, via suppression of the bHLH protein encoding genes *TRANSPARENT TESTA8* (*TT8*) and *GLABROUS3* (*GL3*) (Petridis et al., 2016). While BRs that act

upstream of BEE1 have not been implicated into the complex rerouting of metabolic responses to low temperature, these data clearly imply BEE1 and potential redundantly acting factors as regulator(s), warranting further research in this field.

COLD STRESS SIGNALING EVENTS

COR Gene Regulation

In addition to the many changes that occur at the biochemical and morphological level, cold has a profound impact on the transcriptome; in *A. thaliana*, more than 2.500 genes are regulated by cold stress (Park et al., 2015). It can be assumed that the activation of cold responsive (*COR*) genes enables chilling stress protection and cold acclimation for increased freezing tolerance in capable plant species, although many of the described physiological events that take place when cold stress occurs have not been linked to up-stream signaling cascades as yet, and the sequence of events following cold stress exposure is often unclear.

COR gene regulation occurs in waves, is transient, and is realized by TFs. First wave TFs are activated at early time-points after cold perception and include ZAT10, ZAT12, HSFC1, CZF1, and ZF (Park et al., 2015). The by far best-characterized TFs that take part, however, are the C-REPEAT BINDING FACTORS (CBFs), also known as DROUGHT-RESPONSIVE ELEMENTS BINDING (DREB) factors, CBF1 (DREB1b), CBF2 (DREB1c), and CBF3 (DREB1a).

BR Impact on the CBF Regulon

The CBFs are AP2/ERF family TFs that bind to the C-repeat (CRT)/dehydration-responsive element (DRE), a motif found in promoters of genes activated by cold stress, drought, or high salt exposure (Park et al., 2015). In *A. thaliana*, the CBF regulon of *COR* genes comprises 133 induced genes, including *COR15A*, *COR15B*, *COR47*, *COR78/RD29a*, *KIN1*, and *KIN2* which are often used as read-outs, and 39 repressed genes (Park et al., 2015). In *cbf1,2,3* triple mutants, which were generated using the CRISPR/Cas9 technology, basal freezing tolerance and cold acclimation are strongly compromised, providing conclusive evidence for the important role CBFs play in cold stress response (Zhao et al., 2016). Over-expression of *CBFs* increases freezing tolerance, as well as tolerance to drought and salinity in various plant species (Jaglo-Ottosen et al., 1998; Achard et al., 2008); however, it additionally causes dwarfism (Achard et al., 2008; Hu et al., 2015). This is attributed to the fact that CBFs promote expression of *GA2ox3 and GA2ox6*, which encode enzymes that inactivate GAs, and of *RGL3*, a DELLA protein that represses GA responses (Achard et al., 2008; Park et al., 2015). Since the expression of *CBFs* and a large set of CBF-induced genes is constitutively decreased in the BR-deficient mutants *bri1-1* and *bri1-301* (Eremina et al., 2016b), whereas it is constitutively increased in *BRI1* over-expressing plants (Kim et al., 2010; Eremina et al., 2016b) and the *bin2-3 bil1 bil2* triple mutant (Li et al., 2017), there is clear evidence that BRs are required for *CBF* transcription in basal, non-acclimated conditions.

CBF expression is controlled by multiple upstream TFs (illustrated in **Figure 3**), the best-studied being the bHLH protein INDUCER OF CBF EXPRESSION 1/SCREAM 1 (ICE1/SCRM1)

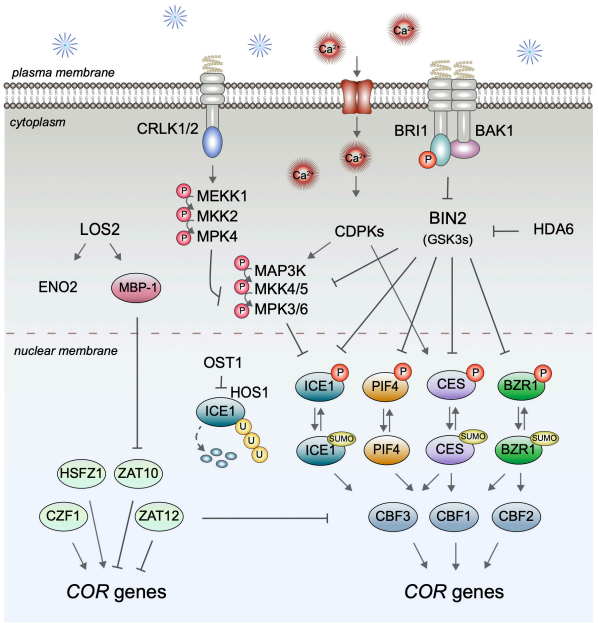

FIGURE 3 | BR signaling and its impact on *CBF* expression. In the presence of BRs, BRI1 binds to BAK1, to activate inhibition of BIN2-type kinases and thereby impair the phosphorylation and inactivation of the transcription factors ICE1, PIF4, CES, and BZR1 that control expression of specific *CBFs*. Cold may impact this pathway by multiple means, including effects of calcium-activated CDPKs, CRLK1/2-controlled MAP kinases, and cold-induced SUMOylation events. In addition to *CBF*-control, BR signaling also impacts CBF-independent modes of *COR* gene regulation, our understanding of which is summarized on the left. However, how this activity is realized is yet unknown.

and its homolog ICE2/SCRM2, which control *CBF3* abundance (Ye et al., 2019). Importantly, recently it has been shown that ICE1 is a BIN2 target (Ye et al., 2019) linking it to the BR signaling pathway. BIN2 can directly phosphorylate ICE1, which reduces ICE1 protein stability and transcriptional activity, and is thought to allow for a repression of ICE1 in later stages of cold stress responses, when, following cold-induction, *CBF* expression requires a return to basal levels. In addition to BR signaling, BIN2 kinase activity is also controlled by acetylation catalyzed by the histone deacetylase HDA6, which is required for freezing tolerance (To et al., 2011; Hao et al., 2016), and thus HDA6 may contribute to a cold stress control of BIN2 activity with potential relevance for ICE1 and other BIN2-substrates.

In addition to ICE1, also the BR-regulated bHLH proteins CES and the BEEs take part in *CBF* control. CES can directly bind to the promoters of all three CBFs *in vitro* and *in vivo* and induce their expression (Eremina et al., 2016b). Since in *ces bee1 bee2 bee3* quadruple mutants the expression of *CBF1* and *CBF3*, but not of *CBF2*, was significantly repressed, it appears that the CES/BEE subfamily of bHLH proteins preferentially regulates *CBF1* and *CBF3* mRNAs (Eremina et al., 2016b), although redundancy in function with other bHLH proteins, or tissue-specific control, may have masked *CBF2* effects in the quadruple mutant. Higher order mutant combinations with additional bHLH proteins that target *CBFs* will be required

to conclusively assess the relative contribution of the different bHLH proteins to *CBF* regulation. In this context, in addition to ICE1/2, also PIF4 and PIF7 should be considered, since both can control *CBF* expression (Lee and Thomashow, 2012), and PIF4 is a BIN2 target (Bernardo-García et al., 2014) that cooperates with BZR1 in transcriptional regulation (Oh E. et al., 2012).

ICE1 activity in CBF regulation is controlled by multiple upstream events with posttranslational modification being essential. The current postulation is that in response to cold stress ICE1 is phosphorylated by the ABA-regulated kinase OPEN STOMATA 1 (OST1). This inhibits ubiquitination by the UBQ E3 ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) and degradation, resulting in ICE1 accumulation (Ding et al., 2015). Later, ICE1 is phosphorylated by the MAP kinases MPK3/6 (Zhao et al., 2017) and also by BIN2, which promotes HOS1 interaction (Ye et al., 2019), and, following ubiquitination, is degraded by the proteasome. MPK3/6 activities in cold stress responses are controlled by MKK5 and an additional unknown MAPK kinase, and adjusted by the MAPK kinase YDA and the MEKK1-MKK1/2-MPK4 cascade (Li et al., 2017; Zhao et al., 2017). YDA represses MPK3/6 activities in a cold-induced manner (Zhao et al., 2017), which may enable adjustment of *CBF* expression and repression of the pathway once *COR* genes have been activated and CBF removal is required (Ramirez and Poppenberger, 2017). In this context interestingly, BRs are known to impact on the YDA-MKK4/5-MPK3/6 MAP kinase module. BIN2 can phosphorylate both YDA (Kim et al., 2012) and MKK4/5 (Khan et al., 2013) and thereby repress MPK3/6 activities. As yet this cross-talk has only been shown to be relevant for stomatal patterning events (Wang and Estelle, 2014), however, clearly it will be important to assess, if it also contributes to BR function in *CBF* transcription and freezing tolerance.

In addition to phosphorylation and ubiquitination, SUMOylation also takes part in cold stress responses. Following cold exposure, the overall SUMOylation of proteins in plants drastically increases, and it has been shown that ICE1 SUMOylation enhances its activity in *CBF3* activation (Miura et al., 2007). Similarly, BZR1 and CES are SUMOylated (Khan et al., 2014; Srivastava et al., 2020) and there is evidence that SUMOylation promotes CES activity in freezing tolerance (Eremina et al., 2016b). While the upstream SUMO E3 ligase that targets CES has remained unknown, SIZ1 is a suitable candidate since SIZ1 SUMOylates ICE1 and, in addition to many other roles, is also important in freezing tolerance (Miura et al., 2007). How SUMOylation of ICE1 is induced is unclear, although a phosphorylation-deficient ICE1 mutant is more readily SUMOylated in response to cold (Miura et al., 2011). Phosphorylation also counteracts SUMOylation of CES, and there is some evidence that calcium-dependent protein kinases (CDPKs) are involved (Khan et al., 2014). CDPKs have both Ca^{2+} sensing and responding activities and can thereby directly translate Ca^{2+} signals into phosphorylation events. They are activated transiently to regulate TF activities either independently or in cross-talk with MAPK signaling cascades (Boudsocq and Sheen, 2013). CDPKs are thought to act as positive regulators of cold stress responses since cold induces expression of certain OsCDPKs and over-expression of the OsCDPK7 in rice, thereby increasing resistance to cold, drought, and salinity (Saijo et al., 2000). Whether CDPK-mediated phosphorylation of CES or other BR-regulated TFs contribute to *CBF* control remains to be addressed.

Another BR-regulated TF which functions in *CBF* regulation is BZR1. BZR1 can bind to the promoters of both *CBF1* and *CBF2* and in the dominant *bzr1-1D* mutant *CBF1* and *CBF2* mRNA levels and freezing tolerance are increased. Moreover, in response to cold stress accumulation of the de-phosphorylated, active form of BZR1 was promoted (Li et al., 2017). In addition to *CBF* regulation in basal growth conditions, there is evidence that BZR1 and the CES/BEE proteins contribute to CBF independent routes of *COR* gene regulation, which are much less studied today, but certainly worth a consideration.

BRs in CBF-Independent Routes of *COR* Gene Regulation

The low temperature regulatory network beyond the CBF pathways is complex and highly interconnected and although it appears to account for the majority of cold stress responses in *A. thaliana* (Park et al., 2015), it has remained largely unstudied. One of the only CBF-independent signaling routes elucidated in some detail today is the control of *ZAT10* expression by LOS2, a bifunctional, cold-responsive locus that encodes both the TF MBP-1 and the enolase ENO2 (Lee et al., 2002; Kang et al., 2013; Eremina et al., 2015). However, neither up-stream events in LOS2 regulation by cold nor modes of ZAT10 activity in freezing tolerance are understood.

Brassinosteroids, in addition to promoting *CBF* expression in basal conditions, also take part in CBF-independent routes of *COR* gene activation and this likely accounts for the major part of their function in acquired freezing tolerance. A whole-transcriptome analysis of *bri1-301* plants following cold treatment identified non-CBF-regulon types of *COR* genes that depended on BRI1 activity. Importantly, there was a highly significant overlap of genes miss-regulated in both *bri1-301* and *ces bee1 bee2 bee3* quadruple mutant plants following cold exposure, providing evidence that the role of BRs in cold acclimation is conferred to a significant degree by the CES/BEE proteins (Eremina et al., 2016b). Among the *COR* genes that failed to be repressed in *bri1-301* and the *ces bee1 bee2 bee3* quadruple mutant, annotations involving cell cycle regulation, cell skeleton, and microtubule activity were overrepresented. This indicates that without a functional BR-CES/BEE module, plants are unable to decrease cell division and metabolism in the cold. Additionally, annotations for fatty acid and lipid synthesis, metabolism, and transport were over-represented (Eremina et al., 2016b) and it will therefore be interesting to see, if a role of CES/BEEs in these processes may contribute to their role in freezing tolerance.

Non-CBF-regulon targets of BZR1 include PYR1-LIKE 6 (PYL6), WRKY TF 6 (WRKY6), which play positive roles in ABA signaling, SENESCENCE-ASSOCIATED GENE 21 (SAG21), JASMONIC ACID CARBOXYL METHYLTRANSFERASE (JMT), and EPITHIOSPECIFIER MODIFIER1 (ESM1), which

are involved in defense responses, and SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), which is involved in flowering time control (Li et al., 2017). The relative contribution of CES/BEE and BZR1 down-stream targets to BR-conferred effects in freezing tolerance remains to be tested.

BRs and Cold Stress Perception

While significant progress was made in elucidating signal transduction cascades that plants utilize to respond to cold stress, very little is still known about low temperature perception. A number of candidates for temperature sensors have been put forward and changes in membrane fluidity have been favored for some time. Membranes are the primary site of temperature perception. Their rigidification in response to cold will therefore likely impact on the activity of membrane-bound receptors, although transcriptome analysis showed no differences in *COR* gene expression between mutants that differed in membrane lipid saturation in *A. thaliana* (Knight and Knight, 2012).

Candidates of membrane-bound proteins that could serve as cold sensors are Histidine kinase Hik33, Bacillus subtilis histidine kinase DesK, and the mechanosensory Ca^{2+}- and K^{+}-channels (Markovskaya and Shibaeva, 2017). Since low temperature induces a transient influx of Ca^{2+} ions into the cytoplasm (Kiegle et al., 2000; Knight and Knight, 2001; Scrase-Field and Knight, 2003), it is thought that calcium channels serve as multifunctional sensors (Medvedev, 2005) that sense stress-induced changes in plasma membranes, including changing fluidity. In rice, COLD1 was proposed as a cold sensor, since it was found to be essential for chilling tolerance and play a role in the activation of Ca^{2+}-channels in response to cold stress (Ma et al., 2015). Since there is evidence that BRs contribute to Ca^{2+}influx by impacting on the activity of Ca^{2+}-channels (Straltsova et al., 2015), it is conceivable that they could be taking part in cold perception modes, although this is another hypothesis that remains to be tested.

Another class of membrane-bound proteins, which likely contributes to the initiation of cold signaling, are calcium/calmodulin-regulated receptor-like kinases (CRLKs). In *A. thaliana*, CRLK1 and CRLK2 are required for the cold-induced induction of *CBFs* and act upstream of the MEKK1-MKK2-MPK4 module in the cold response pathway (Yang et al., 2010). Moreover, the COLD-RESPONSIVE PROTEIN KINASE 1 (CRPK1), which is activated by cold, was shown to phosphorylate 14-3-3 proteins that then shuttle from the cytoplasm to the nucleus to de-stabilize CBFs (Liu and Zhou, 2017). Since 14-3-3 proteins, through effects on BZR1 (Gampala et al., 2007) and BKI1 (Wang et al., 2011), are also involved in BR signaling in ambient conditions, it will be interesting to see if 14-3-3 proteins may also impact on BR activity in cold stress responses.

ENVIRONMENTAL IMPACT ON BR FUNCTION IN COLD STRESS RESPONSES

Temperature perception and signaling is strongly impacted by other environmental cues and also by intrinsic developmental programs. In particular, light is known to play an essential role in cold stress responses. Light is required for full cold acclimation (Wanner and Junttila, 1999) and induction of *CBF* expression (Kim et al., 2002; Soitama et al., 2008). A low red to far-red light ratio can increase *CBF* expression in a circadian-clock controlled manner even in the absence of cold, which is sufficient to improve freezing tolerance in *A. thaliana* (Franklin and Whitelam, 2007). In particular, the phytochrome light receptors PHYB and PHYD appear to be important in *CBF* regulation since in *phyB* and *pyhD* mutants the CBF-regulon is constitutively induced in *A. thaliana* (Franklin and Whitelam, 2007), tomato (Wang et al., 2016), and rice (He et al., 2017). Because PHYB regulates activity of PIF4 and PIF7, both of which are able to repress *CBF* expression in long-days (Lee and Thomashow, 2012), and PIF4 activity is also induced by BRs (Bernardo-García et al., 2014), there are indications for an interplay of light, cold, and BR signaling in *CBF* transcription. It remains to be tested, if this potential interplay may be mediated by a cooperation of BZR1 with PIF4, which as yet has only been shown to be relevant for growth control at ambient and high temperatures (Oh E. et al., 2012; Ibañez et al., 2018). Since light can also impact CBF-independent modes of cold stress responses (Lee et al., 2002), it is evident that much remains to be discovered about the complex cross-talk of light and cold stress responses in plants.

CONCLUDING REMARKS

In the last few years, notable progress has been made in our understanding of the exceptional capacities of BRs to promote both growth and cold stress responses. It appears that in contrast to other growth-promoting hormones such as the GAs, BRs have the capability to uncouple tolerance to cold stress, and related abiotic stress types, from trade-offs in terms of growth and yield. This may be executed, at least in part, by the ability of BRs to promote *CBF* expression (Eremina et al., 2016b; Li et al., 2017) and to promote GA biosynthesis and signaling (Tong and Chu, 2016; Unterholzner et al., 2016) at the same time, which could release the repressive activity of CBFs on GA activity in growth induction. However, in addition to CBF-dependent effects, it is clear that BRs also act by CBF-independent modes. Moreover, in addition to controlling TF activities, BRs may also impact upstream events including cold perception and down-stream events in physiological responses and morphological adaptations. It will be exciting to discover how these effects are realized, and explore if they can be utilized for improvements in crop production when cold stress occurs.

AUTHOR CONTRIBUTIONS

VR and BP contributed equally to all aspects of this work. Both authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Updates on BES1/BZR1 Regulatory Networks Coordinating Plant Growth and Stress Responses

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Brassinosteroids (BRs) play pivotal roles in the regulation of many dimensions of a plant's life. Hence, through extensive efforts from many research groups, BR signaling has emerged as one of the best-characterized plant signaling pathways. The key molecular players of BR signaling from the cell surface to the nucleus important for the regulation of plant growth and development are well-established. Recent data show that BRs also modulate plant responses to environmental stresses such as drought and pathogen infection. In this mini review, we present the recent progress in BR signaling specifically in the post-translational SUMO modification of BR's master regulators, BES1/BZR1. We also discuss recent findings on the crosstalk between BR, UV light, and jasmonic acid signaling pathways to balance growth during light stress and pathogen infections. Finally, we describe the current update on the molecular link between BR signaling and intracellular auxin transport that essential for plant development.

Keywords: brassinosteroid, BES1, BZR1, SUMOylation, jasmonic acid, auxin

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INTRODUCTION

Brassinosteroids (BRs) are plant steroid hormones that have a significant impact on plant growth and development, cell elongation, resistance to pathogens, and plant responses to environmental stresses. In the past decades, substantial progress in dissecting molecular mechanisms of the BR signaling pathway has been achieved using the model plant *Arabidopsis thaliana* (Nolan et al., 2020).

In *Arabidopsis*, BRs are perceived by a plasma membrane receptor, BRASSINOSTEROID INSENSITIVE1 (BRI1) leucine-rich repeat receptor-like kinase (LRR-RLK; Li and Chory, 1997; Wang et al., 2001; Kinoshita et al., 2005). BR binding to an extracellular LRR domain of BRI1 changes its conformation and allows the attachment of a required coreceptor, SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 (SERK3)/BRI1-ASSOCIATED KINASE1 (BAK1; Li et al., 2002; Nam and Li, 2002; Hothorn et al., 2011; She et al., 2011; Santiago et al., 2013). The formation of BRI1-BAK1 complex triggers a cascade of intracellular phosphorylation events involving several regulatory proteins, which eventually inhibit the activity of GSK3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2), a primary negative regulator of BR signaling pathway (Li et al., 2002). Inactivation of BIN2 initiates the accumulation of BRI1-EMS-SUPPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BZR1), two key transcription factors in BR signaling (Wang et al., 2002; Yin et al., 2002), and promotes their nuclear localization. When BRs are

low, BIN2 phosphorylates BES1 and BZR1, thus, prevents their nuclear localization, suppresses their DNA binding activity, and/or promotes their degradation (Nam and Li, 2002; Kim et al., 2009).

BES1 and BZR1 contain a basic helix-loop-helix (bHLH) like motif of DNA binding domain and are known as master regulators that modulate the expression of many target genes (He et al., 2005; Yin et al., 2005; Nolan et al., 2017a; Nosaki et al., 2018). These two transcription factors are subjected to various post-translational modifications that modulate their activity in response to environmental signals. They also have been identified as a node that connects BR signaling with other signaling pathways such as, light, auxin, and gibberellin (reviewed by Nolan et al., 2020). Here, we present updates on BR signaling with a focus on BES1 and BZR1 post-translational regulation and crosstalk between BR, jasmonic acid (JA), UV light, and auxin signaling pathways.

SUMOYLATION OF BES1 AND BZR1

Regulation of BES1 and BZR1 occurs in multiple ways. Phosphorylation of BES1/BZR proteins is well studied, and additional mechanisms that involve oxidation, ubiquitination, alternative splicing, and degradation are emerging (reviewed by Nolan et al., 2020). Recently, post-translational modification of BZR1 and BES1 by small ubiquitin-like modifier (SUMO) reportedly alters their functionality. SUMOylation is a reversible modification in which SUMO protein is covalently conjugated to its substrate through a series of biochemical reactions similar to ubiquitination. In plants, SUMOylation influences many fundamental cellular processes, including environmental stress responses by governing target proteins stability, protein-protein interaction, and subcellular localization (Morrell and Sadanandom, 2019).

Zhang et al. (2019) reported that the SUMOylation of BES1 by E3 ligase SIZ1 destabilizes and inhibits BES1 activity (**Figure 1A**). SIZ1 and BES1 physically interact *in vivo* and *in vitro*. Amino acids 219–288 in BES1, which includes the PEST domain implicated in BES1 degradation, are required for its interaction with SIZ1. Furthermore, the BES1 SUMOylation site is identified at Lysine(K)302 although this SUMOylation status is unaffected by brassinolide (BL) application. Compared to wild-type seedlings, the accumulation of BES1 and unphosphorylated BES1 protein is higher in a SIZ1 T-DNA insertion line (*siz1-2*). In the presence of cycloheximide, a protein synthesis inhibitor, BES1 protein in wild type is less stable than in *siz1-2* seedlings with or without BR, suggesting a negative role of SIZ1 in BES1 stability. In agreement, a cell-free degradation assay shows that without a 26S proteasome inhibitor (MG132), MBP-BES1 protein degradation is slower when incubated with total protein extracts from *siz1-2* seedlings than that of the wild type. Meanwhile, MG132 presence prevents MBP-BES1 degradation in both WT and *siz1-2* extracts. Together, these data show that the SIZ1-mediated SUMOylation promotes BES1 instability and stimulates its proteasome-dependent degradation.

BES1 transcriptional activity is modulated by SIZ1-mediated SUMOylation (Zhang et al., 2019). Using a luciferase (LUC) reporter assay in *Arabidopsis* protoplast, they investigated the promoter activity of two BR repressed genes, *DWAF4* and AT2G210, and one BR induced gene, *SAUR-AC1*. As expected, wild-type BES1 represses the activity of *DWF4*- and AT2G4210-LUC. Meanwhile, $BES1^{K302R}$ variant in which SIZ1 SUMOylation site is mutated, further represses the *DWF4*- and AT2G4210-LUC activity. Similarly, the activation level of *SAUR-AC1*-LUC activity is significantly higher in the presence of $BES1^{K302R}$ variant rather than wild-type BES1. To further confirm this finding, the binding ability of BES1 to the promoter of *DWAF4*, AT2G210, and *SAUR-AC1* was investigated. Chromatin immunoprecipitation (ChIP) assay results showed that the BES1 binding ability increases in the *siz1-2* compared to the wild type regardless of MG132. Based on these data, Zhang et al. (2019) suggested that the SUMOylation of BES1 by SIZ1 inhibits its transcriptional activity.

Contrary to BES1, a new study by Srivastava et al. (2020) revealed that SUMOylation of BZR1 at different sites stabilizes its activity and promotes growth, specifically in non-stress conditions. Meanwhile, salinity (salt stress) provokes BZR1 deSUMOylation through a SUMO protease, ULP1a, which leads to BZR1 degradation and plant growth suppression (**Figures 1B,C**). Salt stress fails to suppress root growth in a *ulp1a* mutant compared to wild-type since *ulp1a* mutant seedlings have longer root than wild-type seedlings in the presence of sodium chloride. Interestingly, *ulp1a* mutant is more tolerant to BR biosynthesis inhibitor brassinazole (BRZ), suggesting that the BR signaling in the *ulp1a* mutant is upregulated. To confirm the *ulp1a* BR response, the expression of BR biosynthesis genes and BR activated genes were investigated. BR availability activates BR induced genes under normal BR signaling, while BR biosynthesis genes are suppressed to maintain BR homeostasis. Indeed, in the *ulp1a* mutant, the expression of BR biosynthesis genes and BR activated genes decreases and increases, respectively, confirming that the ULP1a is negatively impacting BR signaling (Srivastava et al., 2020).

In silico analysis of BR signaling components reveals that the BZR1 and its homologs in other plant species, including BES1 in *Arabidopsis*, contain two conserved lysine residues, K280 and K320, which likely serve as putative SUMO conjugation sites. Co-immunoprecipitation assays in *Nicotiana benthamiana* capture the physical interaction between BZR1 and ULP1a. Since ULP1a is a SUMO protease, this interaction suggests that the ULP1a may promote deconjugation of SUMO in BZR1. Indeed, subsequent *in vitro* and *in vivo* experiments in planta demonstrated that the ULP1a specifically mediates deSUMOylation of BZR1 (Srivastava et al., 2020). It would be interesting to see if ULP1a could also act on BES1 since both BES1 and BZR1 proteins are highly identical.

Upon BL treatment, ULP1a protein is unstable, and SUMOylated BZR1 protein increases. On the other hand, in the presence of BRZ, ULP1a protein accumulates, and promotes deSUMOylation of BZR1. Furthermore, dephosphorylation level of wild-type BZR1 increases after BL treatment. However, the level of phosphorylation and dephosphorylation of the BZR1 SUMO-deficient form remain constant, indicating the significant role of SUMOylation to the accumulation of BZR1 (Srivastava et al., 2020).

BZR1 and ULP1a co-localize in the cytoplasm, and SUMOylation of BZR1 modulates its nuclear distribution (Liao et al., 2020;

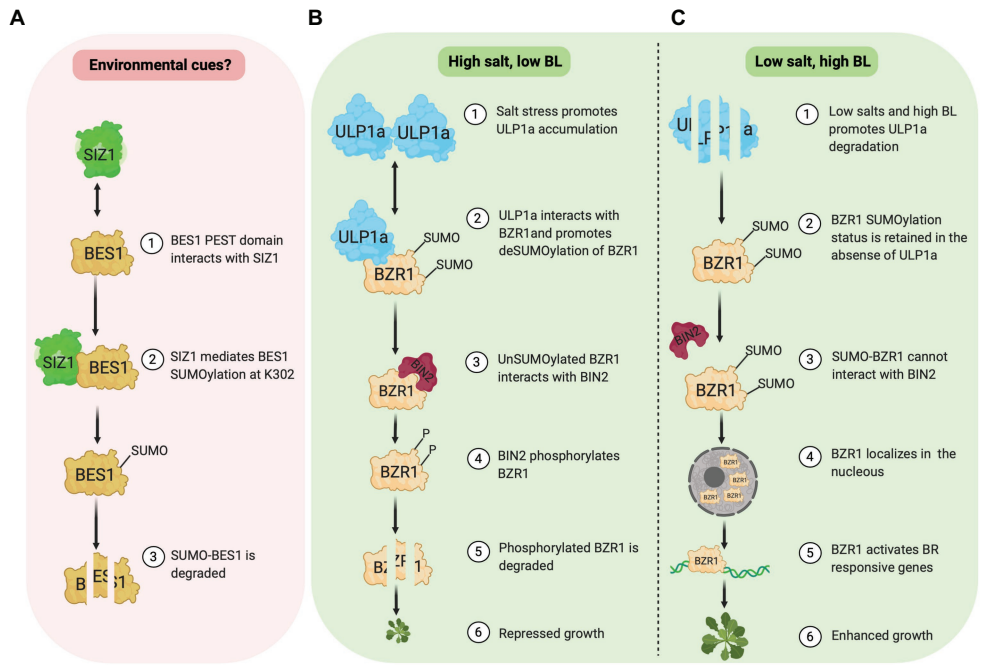

FIGURE 1 | SUMOylation of BES1 (pink box) and BZR1 (green box). **(A)** E3 ligase, SIZ1, mediates SUMOylation of BES1 at K302, which promotes BES1 degradation. **(B)** Under salt stress, ULP1a SUMO protease catalyzes deSUMOylation of BZR1 and BIN2 phosphorylates non-SUMOylated BZR1, which leads to BZR1 degradation. **(C)** Under non-salt stress condition, SUMO-BZR1 is stable and localizes in the nucleus to activate BR responsive genes.

Srivastava et al., 2020). While BL treatment increases nuclear-to-cytoplasmic (NP) ratio of wild-type BZR1, the NP ratio of BZR1 SUMO-deficient form is unchanged. In contrast, after BRZ treatment, the NP ratio of BZR1 SUMO-deficient form is rapidly decreased than that of the wild type. Interestingly, SUMOylation of BZR1 prevents its interaction with BIN2. The inability of BIN2 to interact with SUMO-BZR1 may be due to the overlap of SUMO site, K280, and K320, with BIN2 Interaction Motif, amino acids 309–320 (Peng et al., 2010), in BZR1. Since BIN2 is critical for phosphorylation which leads to BZR1 degradation, SUMOylation of BZR1 may play a significant role in maintaining BZR1 stability. DeSUMOylation of BZR1 by ULP1a is reportedly stimulated by salt stress (Srivastava et al., 2020). In this condition, ULP1a protein abundance is elevated, which results in decreasing SUMOylated BZR1 proteins. Thus, it is likely that the deSUMOylated form of BZR1 would be phosphorylated by BIN2 and degraded. This mechanism may be essential to control plant growth during salt stress (**Figure 1B**). A recent study showed that the BIN2 cooperates with calcium sensor SOS3/SCaBP8 to regulate SOS2 and BES1 to coordinate plant growth and growth recovery (Li et al., 2020b), suggesting that the BR and salt stress crosstalk at multiple targets.

CROSSTALK BETWEEN BR AND STRESS SIGNALING PATHWAYS

BRs are involved in plant responses to biotic and abiotic stresses (Kohli et al., 2019; Gupta et al., 2020) and crosstalk between BRs and other plant hormones is well-documented (Bostock, 2005; Nolan et al., 2020). To synchronize plant growth and defense to pathogen infection, BRs and jasmonic acid (JA) signaling pathways functionally interact. In rice, the presence of BL promotes susceptibility to the brown planthopper (BPH) pathogen and induces root susceptibility to the root-knot nematode *Meloidogyne graminicola* (Nahar et al., 2013; Pan et al., 2018). The increased sensitivity to *M. graminicola* is associated with an antagonistic interaction between BR and JA pathways. Upon BL treatment, JA-related genes transcripts are downregulated in the root. Furthermore, BR deficient mutant shows elevated endogenous JAs and decreased susceptibility to *M. graminicola* (Nahar et al., 2013). In contrast, upon BPH infestation, BR stimulates JA signaling while represses salicylic signaling pathway (Pan et al., 2018), suggesting a different mechanism of plant immunity responses.

Recent data from *Arabidopsis* revealed the molecular mechanisms underlying BR-JA pathway crosstalk that mediate the growth-defense trade-off (**Figures 2A,C**). Liao et al. (2020) discovered that BRs antagonize JA-activated defense mechanism *via* BES1, a master regulator in BR signaling pathway. Two routes in which BES1 represses JA-activated defense pathway were established. First, BES1 represses the expression of genes involved in the production of small cysteine-rich peptides with antimicrobial activity known as defensins (Thomma et al., 2002). Second, BES1 represses glucosinolate (GS) biosynthesis genes that produces GS compounds crucial for plant resistance to insect (Hopkins et al., 2009). In *bes1-D* mutant, defensin genes such as *PDF1.2a* and *PDF1.2b*, also a JA marker gene,

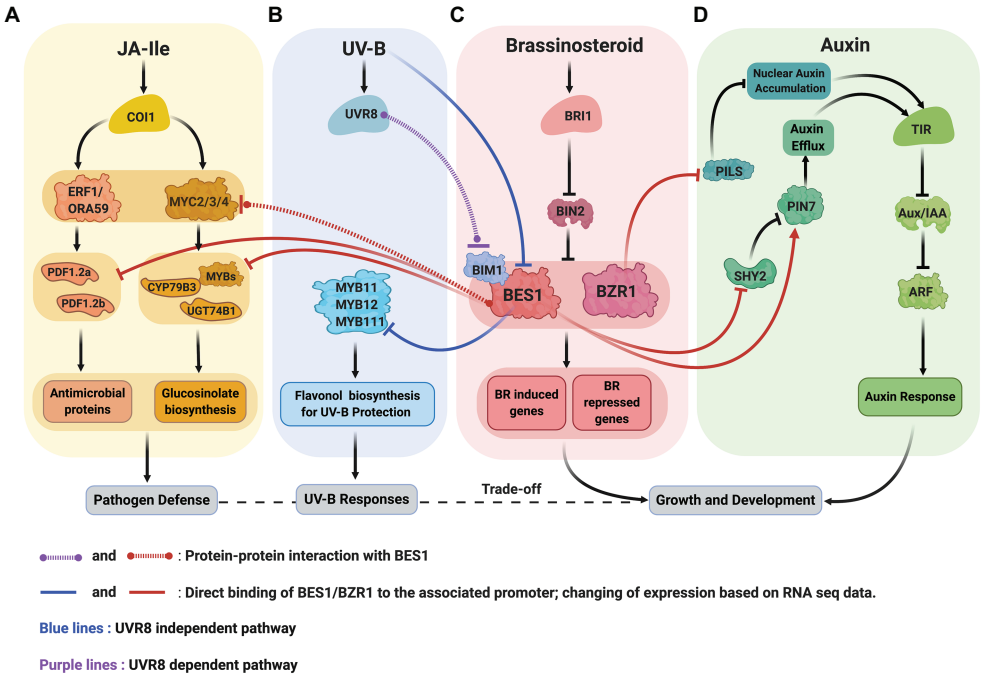

FIGURE 2 | Crosstalk between signaling pathway of BR (**C**, red), JA (**A**, yellow), UV-B (**B**, blue), and auxin (**D**, green). **(A,C)** BR and JA: BES1 restricts the production of defensin (antimicrobial proteins) by repressing the expression defensin genes, PDF1.2a and PDF1.2b. BES1 also negatively regulates the production of glucosinolates (GS) by inhibiting the transcriptional activity of MYC2, repressing GS biosynthesis transcription factors (MYBs) and genes (CYP79B3 and UGT74B1). **(B,C)** BR and UV-B: UV-B activated UVR8 and represses the function of BES1-BIM1 complexes to reduce the expression of BR induced genes to balance growth and stress (UVR8 dependent pathway). UV-B also reduces BES1 expression allowing the expression of MYB11, MYB12, and MYB111 transcription factors critical for flavonol biosynthesis (UVR8 independent pathway). **(C,D)** BR and auxin: BZR1 reduces the abundance of PILS protein (ER localized) resulting in auxin's nuclear accumulation. BES1 increases the abundance of PIN7 that involved in internal auxin flow in the embryo.

VPS1, are downregulated. Meanwhile, in *bzr1-1D* mutant, *PDF1.2b*, and *VPS1* transcripts are upregulated, suggesting that the BES1 has more dominant role in the suppression of JA-activated defense genes. A chromatin immunoprecipitation (ChIP)-qPCR, an electrophoretic mobility shift assay (EMSA), and a dual-luciferase (LUC) reporter assay confirmed that the BES1 directly binds the 3' downstream region of *PDF1.2a* and *PDF1.2b* and suppresses their expression.

Mutant of *bes1-D* is more susceptible to *Botrytis cinerea* infection than wild-type, while stable overexpression lines of *PDF1.2a* show improved resistance to *B. cinerea*. In response to *B. cinerea* invasion, the double mutant, *bes1-D* and *PDF1.2a* overexpression lines, is more similar to *PDF1.2a* overexpression lines than *bes1-D* (Liao et al., 2020). These genetic data support the antagonistic role of BES1 in JA mediated defense response.

The *bes1-D* mutant shows increased sensitivity to *Spodoptera exigua* (Liao et al., 2020). Furthermore, the level of glycosynolates (GS), which functions downstream of JA, significantly decreases in *bes1-D* mutant as well as the expression of indolic GS synthesis genes. BES1 modulates GS biosynthesis in two ways. First, the ChIP-qPCR and LUC reporter assay results demonstrated that the BES1 directly binds and represses the expression of indolic GS-biosynthetic genes, *CYTOCHROME P450 FAMILY79 SUBFAMILY B POLYPEPTIDE3 (CYP79B3)* and *UDP-GLUCOSYL TRANSFERASE 74B1 (UGT74B1)*, as well as the expression of *MYB34*, *MYB51*, and *MYB122* transcription factors that specifically regulate the expression indole-GS genes. Second, BES1 suppresses the transcriptional activity of MYC2 that involved in the activation of GS-biosynthetic genes. In the LUC reporter assay, the co-expression of both BES1 and MYC2 further represses the activity of *CYP79B3-LUC* and *UGT74B1-LUC* activity compared to MYC2 expression alone (Liao et al., 2020).

Consistent with the general theme that BES1/BZR1 functions as a hub to coordinate growth and stress responses, recent studies showed that the BES1 mediates UV-B stress responses at multiple levels (**Figures 2B,C**). First, UV-B receptor UVB8 represses the functions of BES1 and BIM1 and thus inhibit BR-induced plant growth (Liang et al., 2018). Secondly, the expression of *MYB11/12/111* genes required for flavonol biosynthesis is negatively regulated by BES1 under normal light condition and broad band UV-B reduces BES1 expression, allowing the production of flavonol for the UV-B stress protection (Liang et al., 2020).

CROSSTALK BETWEEN BR AND AUXIN SIGNALING PATHWAYS

Crosstalk between BRs and auxin are widely known, and the molecular link underlying this relationship has been reported.

For example, the interplay between auxin transcriptional regulators, the auxin response factor (ARF) family, and BR signaling components, such as BIN2 alters the expression of auxin-responsive genes. Phosphorylation of ARF2 eliminates its DNA binding and repressor activity. Furthermore, around 40% of differentially expressed genes in *arf2* loss-of-function mutant also are responsive to BRZ (Vert et al., 2008). Recently, BR signaling reportedly modulates intracellular auxin transport *via* auxin transporter proteins, PIN-FORMED (PIN), and PIN-LIKES (PILS) family proteins (Li et al., 2020a; Sun et al., 2020; **Figures 2C,D**).

Li et al. (2020a) showed that the interaction between BR signaling and auxin promotes root meristem development. BR-deficient mutants expressing auxin DR5::GFP marker exhibit lower fluorescence intensity in the seedling stage and reduced root meristem size. Upon exogenous auxin application, root meristem size, length, and cell number partially recovered. Furthermore, they showed that the BR elevates the expression level of PIN7. The PIN proteins are involved in cell-to-cell transport and intracellular auxin accumulation, which is essential for the root gravitropic response and aboveground organogenesis (Adamowski and Friml, 2015). PIN7, along with PIN1, PIN3, PIN4, and PIN5 are expressed in the embryo and determined the polarity of the embryonic axis (Friml et al., 2003). The upregulation of PIN7 in the BR treatment is mediated by BES1 that binds directly to the promoter of PIN7. BES1 also appears to regulate the expression of SHY2, a transcriptional repressor that inhibits the expression of PIN1, PIN3, and PIN7 (Ioio et al., 2008; Li et al., 2020a). EMSA and ChIP studies demonstrated that the SHY2 promoter is a direct target of BES1. BES1 participation in transcriptional regulation of SHY2 was shown by monitoring GUS expression driven by SHY2 promoter in BES1-RNAi and wild-type seedlings. The results indicated that the intensity of GUS signals is higher in BES1-RNAi mutants compared to wild-type. In line with this data, SHY2 transcript levels are significantly higher in BES1-RNAi lines than wild type. These results are consistent with the notion that BES1 negatively regulates SHY2. The connection between SHY2 and PINs was investigated by examining the expression of PIN1-GFP, PIN3-GFP, and PIN7-GFP driven by their native promoter in a loss-of-function mutant, *shy2-31*, and a wild-type background. The fluorescence signals of PIN1, PIN3, and PIN7 are higher in the *shy2-31* than wild type, which argue that the SHY2 inhibits the expression of those PIN proteins. Moreover, in the *shy2-31* background, BL further elevates fluorescence signals of PIN1, PIN3, and PIN7 while BRZ reduces the signals. Thus, Li et al. (2020a) conclude that in addition to direct regulation of *PIN7* by BES1, the expression of some of *PIN* genes including *PIN7* is partially regulated by BRs *via* BES1 and SHY2.

BR also regulates PILS-mediated intracellular auxin transport. PILS proteins evolve independently from PIN proteins, although they are structurally similar (Feraru et al., 2012). The role of PILS proteins in the developmental processes is mostly unknown. However, PILS proteins reportedly restrict nuclear auxin accumulation presumably by auxin retention in the ER resulted in spatially defined auxin minima that control organ growth responses (Feraru et al., 2019). PILS also integrates external signals such as light and temperature to the auxin signaling pathway (Sauer and Kleine-Vehn, 2019).

The connection between BR and PILS was revealed through forward-genetic screening in a PILS5 overexpressing strain (*PILS5*^{OE}) to identify secondary mutations with enhanced hypocotyl and root growth deficiency (Sun et al., 2020). The identified mutant, *imperial pils (imp)*, carries a secondary mutation that surprisingly mapped to the BRI1 gene. The identified mutation creates a single amino acid change, glycine to serine, and the impact of this mutation resembles that of the previously identified weak loss-of-function *bri1* mutant, *bri1-6*, or *bri1-119* (Sun et al., 2020).

BR signaling appears to suppress PILS transcript and protein levels. *In silico* analysis showed that the promoters PILS2, PILS3, and PILS5 are direct targets of BZR1 and BES1 (BZR2) and the binding of BZR1 to PILS2 and PILS5 promoter was confirmed by chromatin immunoprecipitation (ChIP)-sequencing (Sun et al., 2020). Upon BL application, the expression of GFP and GUS fused to the promoter of PILS2, PILS3, and PILS5 is repressed as well as the transcript level of endogenous PILS3 and PILS5. Similarly, pPILS5::GFP-GUS signals in roots increase in *bri1* loss-of-function mutants but decrease in BRI1 overexpressing lines and in the constitutive mutant, *bzr1-1D*. The later supports the notion that the BZR1 transcription factor may negatively regulate PILS5 expression. PILS5-GFP signals driven by 35S promoter declines upon BL treatment. In contrast, the same construct signals are increased in a 3-day-old seedling of *bri1*^{imp} (a *bri1* allele isolated from forward-genetic screening in *PILS5*^{OE}) than the wild type, suggesting a non-transcriptional regulation of PILS5 expression by BR signaling (Sun et al., 2020).

BR directly modulates PILS activity in intracellular auxin relocation, which subsequently contributes to the regulation of root organs. After BL treatment, nuclear auxin abundance is increased; meanwhile, PIL3 and PIL5 protein abundance are decreased, and PIL6 protein abundance, on the other hand, is only slightly reduced. In contrast, BL induction in a PIL6 constitutive line partially limits nuclear auxin accumulation. Furthermore, the triple mutant of PILS shows accelerated auxin signaling and hypersensitivity to BL application in the root. These data suggest that BRs increase nuclear auxin accumulation in the root by decreasing the presence of PILS proteins (Sun et al., 2020).

CONCLUSIONS

In harsh environmental conditions, plants cannot move around; thus, fine-tuning their growth and development using sophisticated internal signaling networks enable plants to survive and to conclude their life cycle. BR signaling is essential in regulating plant growth in response to environmental stresses. For example, *Arabidopsis* coordinates its growth and survival under drought *via* RD26 transcription factor that binds and antagonizes BES1 transcriptional activities to inhibit BR-mediated growth (Ye et al., 2017). BES1 also interacts with WRKY54 and AP2/ERF TINY transcription factors to balance growth

and drought response (Chen et al., 2017; Xie et al., 2019). Furthermore, activation of ubiquitin receptor protein DSK2 in drought prompts BES1 degradation through autophagy (Nolan et al., 2017b). New studies by Srivastava et al. (2020) and Sun et al. (2020) indicate that SUMO modifications of BZR proteins are essential not only in modulating the activity of these transcription factors but also crucial in adjusting plant growth under salt stress, as seen in the case of BZR1. It is quite intriguing that the SUMOylation of BES1 and BZR1 produces opposite outcomes, where BES is degraded while BZR1 is more stable. The different results could be due to the distinct SUMO target sites in each protein. Furthermore, it needs to be confirmed if SIZ1 and ULP1a could act on both BZR1 and BES1. While ULP1a mediated deSUMOylation strongly correlated with salt stress, it is still unclear if specific environmental cues dictate the SUMOylation of BES1 and BZR1. Recent studies also indicated that the light and nitrogen starvation can affect BZR1 phosphorylation status. In *Arabidopsis*, cryptochrome 1 (CRY1), a blue light receptor involved in the inhibition of hypocotyl elongation, interacts with BZR1 and suppresses BZR1 DNA binding ability. CRY1 also promotes the BZR1 phosphorylation to prevent its nuclear localization (He et al., 2019). Meanwhile, in tomato, to alleviate nitrogen deficiency stress, BR induces autophagy through BZR1 that directly binds to the promoter of ATG2 and ATG6 to increase their expression and promotes autophagosome formation. During nitrogen starvation, BZR1 was dephosphorylated especially in the first 5 days of nitrogen starvation (Wang et al., 2019). Investigating how different modifications collectively modify BES1/BZR1 in response to different environmental cues will be an important future area of research.

Molecular mechanism behind the antagonistic interaction between BR and JA signaling in response to pathogen attacks gives some clues on how plants balance their growth in this condition. It appears that the BES1 is emerging as a node that connects BR and JA signaling and it negatively modulates different aspect of JA induced plant defense depending on the type of pathogens. In response to *B. cinereal* infection, BES1 directly suppresses the expression of defensins genes, PDF1.2a and PDF1.2b. On the other hand, upon exposure to *S. exigua* herbivory infection, BES1 negatively modulates MYC2 transcriptional activities. BES1 also represses the expression of indol-GS synthetic genes, *CYP79B3* and *UGT74B1* and GS-associated transcription factors. This negative regulation mediated by BES1 may be crucial to maintain homeostasis of defensins and GS compounds that are increasing upon pathogen attacks (Liao et al., 2020).

Additional layers of hormone crosstalk have been depicted by how BR signaling modulates the intracellular auxin distribution. BR signaling is directly repressing the expression of PILS proteins that restrict nuclear auxin availability (Sun et al., 2020). BR signaling also modulates the expression of PIN7, which participates in polar auxin transport in the root meristem (Li et al., 2020a). However, the mechanistic basis of how specific BR signaling components influence PILS protein stability remains to be established. Nonetheless, these data reveal new insights on how plants synchronize BR and auxin signaling to support growth and development.

AUTHOR CONTRIBUTIONS

AK and YY conceived the idea and wrote the review. All authors contributed to the article and approved the submitted version.

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Regulation of Brassinosteroid Signaling and Salt Resistance by SERK2 and Potential Utilization for Crop Improvement in Rice

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The complex roles of the steroid hormone brassinosteroids (BRs) in many different yield- and stress-related traits make it difficult to utilize the hormones for crop improvement. Here, we show that SERK2 as a BR signaling component is a potentially useful candidate for BR manipulation in rice. We generated multiple mutant alleles of *SERK2* by CRISPR/Cas9 editing and show that knockout of *SERK2* results in a compact structure accompanied with increased grain size. SERK2 is localized on plasma membrane and can interact with OsBRI1, the BR receptor, suggesting its conserved role as co-receptor in BR signaling. Consistently, the mutant has impaired BR sensitivity compared to wild type. Notably, the mutant is highly sensitive to salt stress as evaluated by plant survival rate as well as transcriptome analysis, whereas has slightly increased sensitivity to ABA, the stress hormone. By contrast, overexpression of *SERK2* significantly enhances grain size and salt stress resistance, importantly, without affecting plant architecture. Furthermore, while salt suppresses *SERK2* transcription, the protein is greatly induced by salt stress. Taken together, we propose that the adverse condition induces SERK2 accumulation to enhance early BR signaling on plasma membrane in favor of the anti-stress response. Our results illustrate the great potentials of specific BR components such as SERK2 for crop improvement by utilizing flexible strategies.

Keywords: brassinosteroid, salt stress, rice, gain size, SERK2

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INTRODUCTION

Brassinosteroids (BRs) are a class of steroid hormones having chemical structures similar to those in animals or insects. Brassinolide (BL), one of the most active BRs, was identified forty years ago, quite later than the discovery of five other classical phytohormones including auxin, cytokinin, gibberellin, abscisic acid (ABA), and ethylene (Grove et al., 1979). BRs were thus considered as the sixth class of phytohormones. Although with a short history, our understanding of BR synthesis and signaling is rapidly progressed. So far, the BR signaling pathway could be one of the best characterized hormone signaling pathways in the model plant Arabidopsis (*Arabidopsis thaliana*). In the prevalent BR signaling model, BRs are perceived on plasma membrane by BRI1-BAK1 receptor complex and the signal is then transduced sequentially by BSK1 kinase, BSU1 phosphatase

and BIN2 kinase, eventually leading to activation of BZR1/BES1 transcriptional factors (Kim and Wang, 2010). Each of these components represents a family of protein members presuming to have at least partially redundant functions.

In recent years, many additional players, including a number of interacting proteins with the above-mentioned components, have been identified to be involved in BR signaling (Nolan et al., 2020). Interestingly, it has been shown that PP2a, a phosphatase, simultaneously targets BRI1 and BES1 to regulate BR signaling (Tang et al., 2011; Wu et al., 2011). In addition, some of the BR signaling components, such as BSU1-family members and BZR1-family proteins, could have other functions not associated with BR signaling (Maselli et al., 2014; Chen et al., 2019). Intriguingly, the downstream BIN2 can conversely phosphorylate and regulate BSK-proteins (Sreeramulu et al., 2013; Ren et al., 2019). Since BSK-proteins can interact with a number of BR-signaling components, the scaffold function of the proteins has been proposed (Ren et al., 2019). These studies largely advanced our understanding of the BR signaling, and suggested a complicated signal tuning system underlying the important hormones in plants.

In rice, BRs play predominant roles in regulating many important agronomic traits, including plant height, grain size, and leaf erectness. Thus, BRs are considered to have great potentials in crop improvement (Tong and Chu, 2018). Interestingly, a few studies have shown that rice could have a different BR signaling pathway, at least at some steps, from that in Arabidopsis. For example, PPKL1 has been shown to negatively regulate BR signaling, in contrast to its ortholog BSU1 in Arabidopsis (Gao et al., 2019). And also, BRs could have different functions in different species. For example, BRs significantly regulate photo-morphogenesis in Arabidopsis but appear to play a minor role in pea (Bishop, 2003). Therefore, to effectively utilize BRs in crops, the hormone functions should be carefully evaluated in each species.

Given that rice is grown in a largely different environment (i.e., temperature, water, light) compared with Arabidopsis, whether BRs differentially function in regulating stress responses in various species should be particularly concerned. However, despite the rapid progress made in BR-regulated plant growth and development, our understanding of BR-regulated stress response remains very limited, especially in rice. For example, several studies in Arabidopsis have respectively shown that BRs favor the plant tolerances to salt and cold (Sharma et al., 2013; Ye et al., 2019) but suppress drought tolerance (Chen et al., 2017). In addition, a number of studies in Arabidopsis also revealed that there exists multilayer crosstalk between BRs and ABA, the well-known stress hormone. For example, the ABA signaling phosphatase can dephosphorylate BIN2 to regulate plant growth and development (Wang et al., 2018), whereas BIN2 can phosphorylate both SnRK2 and ABI5, the ABA signaling components, to modulate stress responses and seed germination (Cai et al., 2014; Hu and Yu, 2014). However, little is known about how BRs regulates stress responses in rice.

BAK1, as the BR co-receptor, belongs to SERK-family proteins. Beside BR signaling, SERK-members play diverse roles in various biological processes such as embryo development, senescence, and plant immunity (Fan et al., 2016). In rice, OsBAK1 has also been suggested to play a conserve role as its ortholog in Arabidopsis, because knockdown or knockout of *OsBAK1* resulted in a compact plant structure and small grains (Li et al., 2009; Yuan et al., 2017). A couple of other proteins, such as a G protein and a remorin protein, have been shown to interfere with OsBRI1-OsBAK1 interaction to regulate BR signaling (Gui et al., 2016; Zhang et al., 2016). In addition, the role of SERKs in plant immunity has also been studied in rice (Chen et al., 2014; Zuo et al., 2014). However, solid genetic evidence supporting the roles of each SERK member in BR signaling or plant growth and development remains scarce. Despite of the intense crosstalk between BIN2 and ABA signaling components, whether and how SERKs, as potentially early BR signaling components, regulate stress responses remains largely unclear. Addressing this question has particular significance for utilizing BRs to simultaneously improve crop yield and stress resistance.

We previously proposed that molecular design utilizing functional specificities of BR-related components is one of potential strategies for BR application (Tong and Chu, 2018). In the scenery, manipulation of a specific BR component could obtain desired traits without negative effects or with even beneficial effects on other traits. In an attempt to screen this kind of genes, we have generated a panel of mutant defective of putative BR signaling genes by CRISPR/Cas9 editing. In this study, we evaluated the roles of *SERK2* and showed that *SERK2* could be one of the candidates meeting our requirement. The *serk2* mutants had reduced plant height and compact structure, whereas accompanied with increased grain size, thus the gene could be useful for engineering plant architecture without negative effect on grain size. On the other hand, we showed that overexpression of *SERK2* can simultaneously enhance salt resistance and grain size, without negative effect on plant architecture. Thus, the gene could be valuable for crop improvement in rice.

MATERIALS AND METHODS

Plant Materials and Growth Condition

A *japonica* rice (*Oryza sativa*) cultivar, Zhonghua11 (ZH11), was used as background for all the analyses. Plants were grown either in field under natural conditions or in a growth chamber (GXZ-800D) with settings: 30°C day and 28°C night, 10-h/14-h light/dark cycle. When grown in the chamber, half-strength Murashige and Skoog basal salt mixture (1/2MS) was used as nutrient source. Grain size is measured using an integrated photographing and analysis system (WSeen SC-G).

Gene Expression Analysis

Total RNA was isolated from the samples collected using Trizol (Invitrogen), and cDNA was prepared using a reverse transcription kit (Toyobo) following the product instructions. Quantitative real-time PCR (qRT-PCR) was performed on a LightCycler 96 system (Roche) using SYBR Green PCR mix (Roche). The gene expression levels were normalized to the

transcript level of the reference gene *Ubiquitin*. Primers used for qRT-PCR are listed in **Supplementary Table 1**

Vector Construction and Transgene

The *serk2* mutants were produced using CRISPR/Cas9 system (Lu et al., 2017) targeting 5′-TGGGACAATACCTAATGAA CTGG-3′ corresponding to 333-355 of the *SERK2* coding sequence. For overexpression analysis, the full-length cDNA of *SERK2* was amplified and then introduced into an empty vector named *p1300-35S-FLAG* by in-fusion technology (Clontech). See **Supplementary Table 1** for primers used for vector construction. The resulted vector *p1300-35S-SERK2-FLAG* was introduced into ZH11 callus to produce the overexpression plants by *Agrobacterium*-mediated method.

Subcellular Localization Analysis in Protoplast

Rice protoplasts were prepared according to the previous description (Bart et al., 2006). A vector *p2300-35S-SERK2-GFP* expressing SERK2-GFP fusion protein was used to transfecting rice protoplast. The empty vector expressing sole GFP was used as control and a vector expression plasma-membrane localized protein was used as reference. See **Supplementary Table 1** for primers used for vector construction. After one-night incubation, the protoplast cells were observed under a confocal fluorescence microscope (Zeiss) and those emitting fluorescence were photographed.

Split-luciferase Complementation Analysis

Empty vectors for this analysis have been introduced previously (Chen et al., 2008). SERK2 and OsBRI1 were introduced into the empty vectors, resulted in *p1300-split-nLUC* (nLUC-BRI1) and *p1300-split-cLUC* (cLUC-SERK2) respectively, which were used for luciferase complementation analysis to test the potential interactions in tobacco (*N. benthamiana*) leaves. See **Supplementary Table 1** for primers used for vector construction. The vectors were transformed into agrobacteria and the desired couples were mixed and then infiltrated into tobacco leaves as detailed previously. Chemiluminiscence was photographed using an imaging system equipped with a cold CCD (NightSHADE LB985).

Stress and Hormone Treatment

One-week-old seedlings were treated with 200 mM NaCl for 6-8 days and recovered for 3 days to count survival frequency, or treated for different times for transcription and protein analyses. Sterilized seeds were sown on 1/2MS agar medium with or without ABA (Sigma-Aldrich) supplementation and grown for 10 days prior to root and shoot measurements. Different concentrations of BL were used for BR sensitivity test. Briefly, BL was dissolved in ethanol for leaf inclination assay (0, 10, 100, and 1000 ng) and dissolved in DMSO for cultivation on agar medium (0, 0.1, and 1 μM) to test the shoot and root growth as described previously (Tong and Chu, 2017). For molecular analysis, one-week-old seedlings were treated with 1 μM BL for different times.

RNA Sequencing

Four samples, including ZH11, *serk2*, salt-treated ZH11, and salt-treated mutant (200 mM NaCl, 8 h), were prepared for transcriptome analyses. The aerial parts of one-week-old seedlings were harvested for total RNA extraction. The purified RNAs were used for library construction using a NEBNext Ultra RNA Library Prep Kit for Illumina (NEB). Three biological replicate libraries were prepared for each sample. Clustering of index-coded samples was performed on a cBot Cluster Generation System using a Nova-seq Cluster Kit (Illumina). The libraries were sequenced on the Illumina NovaSeq S6000 platform and 150 bp paired-end reads were generated. FDR (false discovery rate) value < 0.01 and log2FC (fold change) ≥ 1 were used to identify differentially expressed genes (DEGs). Analysis of overlapping DEGs was performed online using Venny[1]. Complete DEG lists used for overlapping analysis were provided in **Supplementary Tables 2, 3**.

Immunoblotting

The *SERK2*-overexpression plants were treated with salt or BL for different times, and the seedling shoots were harvested for immunoblotting analysis. Total proteins were extracted as described previously. Commercialized anti-Flag (1:2,000, Sigma-Aldrich) and anti-HSP (1:5,000, BPI) antibodies were used for immunoblotting analyses on a Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer's instructions.

RESULTS

The *serk2* Mutants Show Compact Structure but Increased Grain Size

The *serk2* mutants were generated by targeting a specific sequence within *SERK2* (*LOC_Os04g38480*) coding region (**Figure 1A**). A number of allelic mutants containing various mutations at the targeting site were obtained. Three representative lines containing frame-shift mutations, designated *serk2-1* to *serk2-3*, were selected for further analysis (**Figure 1A**). At the vegetative growth stage, a marked phenotype could be observed after 14-d growth. Both the seedling height and leaf angles were decreased compared with the wild type (**Figure 1B**). At the reproductive stage, the mutants exhibited a much more compact structure, with reduced plant height and erect leaves (**Figures 1C–F**). The decreases of mutant leaf angles were more obvious because at this stage the lamina joints of the wild-type plants greatly bended downward whereas the mutants had basically no bending (**Figures 1C–F**). Surprisingly, all the mutants had increased grain width, with slight change of grain length (**Figures 1G–I**), which are reminiscent of BR signaling mutant such as *dlt*, suggesting the involvement of SERK2 in BR responses. OsBAK1 is the closest homolog of SERK2 and has

[1]http://bioinfogp.cnb.csic.es/tools/venny/

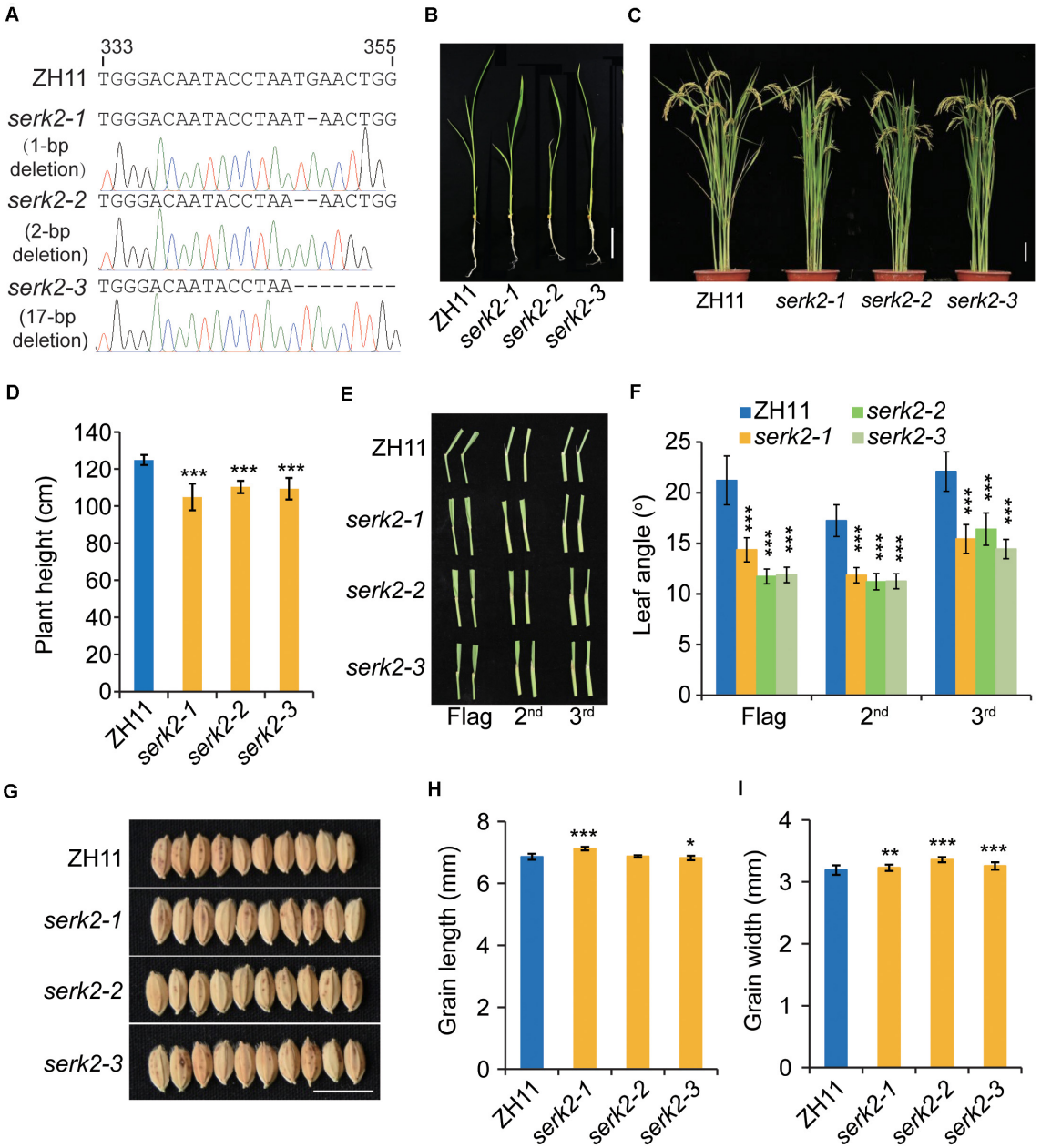

FIGURE 1 | The *serk2* mutants show compact structure accompanied with increased grain size. **(A)** Information of editing site and the mutations. The sgRNA sequence is shown with the position at the CDS indicated. The sequencing chromatograms are also shown. **(B)** Seedling phenotype after 14-d growth. Scale bar = 5 cm. **(C)** Gross morphology of ZH11 and *serk2* mutants. Scale bar = 10 cm. **(D)** Statistical data of the plant height. Bars indicate standard deviation (SD), $n = 15$, $^{***}p < 0.001$ by *t*-test. **(E)** Comparison of leaf angles in ZH11 and the mutants. The top three leaves were shown. **(F)** Statistical data of the leaf angles. Bars = SD, $n = 20$, $^{***}p < 0.001$ by *t*-test. **(G)** Grain morphology of ZH11 and *serk2* mutants. Scale bar = 1 cm. **(H,I)** Statistical data of the grain length **(H)** and grain width **(I)**. Bars = SD, $n = 50$, $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ by *t*-test.

been functionally characterized previously (Li et al., 2009; Yuan et al., 2017). We also edited *OsBAK1* gene using a same strategy, and found *osbak1* had a consistent BR-defective phenotypes as reported, including dwarfism, erect leaves, and decreased grain size (Li et al., 2009; Zuo et al., 2014; Yuan et al., 2017). Thus, it appears that SERK2 plays a relatively specific role in BR responses compared to OsBAK1. Given the beneficial effects in the mutants, *SERK2* could be valuable for engineering plant architecture.

The *serk2* Mutants Have Decreased BR Sensitivity

In rice, a high level of active BRs is able to induce leaf bending but inhibit shoot and root growth (Tong et al., 2014). To test

the BR sensitivities of *serk2* mutants, we treated plant seedlings with BL and then compared the growth of the three tissues with those of wild type. In lamina bending assay, while the wild type showed gradually enlarged leaf angles along with the increase of BL amount, the *serk2* mutants had basically no response to BL (**Figures 2A,B**). Similar results were obtained in growth inhibition analysis. The growths of both the shoot and root in the mutants were much less inhibited by BL compared to the cases in the wild type (**Figures 2C,D**). All these results strongly suggested the critical role of SERK2 in BR responses.

SERK2 Localizes on Plasma Membrane and Interacts With OsBRI1

Expression analysis by qRT-PCR showed that SERK2 was constitutively expressed in various tissues, with a preference in leaf blade and culm, but less in other tissues (**Figure 3A**). This expression pattern was somewhat consistent with the mutant phenotypes with main defections in leaf and culm. In addition, *SERK2* had greatly decreased expressions in the mutant (**Figure 3B**), implying that the dysfunctional *SERK2* transcripts were somehow suppressed in the mutants. However, we failed to detect increased expressions of the two BR synthetic genes, *D2* and *D11*, suggesting that the feedback regulation between BR signaling and synthesis somehow was not significant or not strong enough for detection in *serk2*. We also analyzed the subcellular localization of SERK2 by evaluating the fluorescence of SERK2-GFP fusion proteins in rice protoplast. The results showed that SERK2 was specifically localized on plasma membrane, where can be co-localized with OsBRI1 which serves as a plasma membrane marker (**Figure 3C**). Furthermore, we tested the potential interaction between SERK2 and OsBRI1 using split-luciferase complementation analysis, and detected the marked signal in tobacco leaves (**Figure 3D**). Together with the

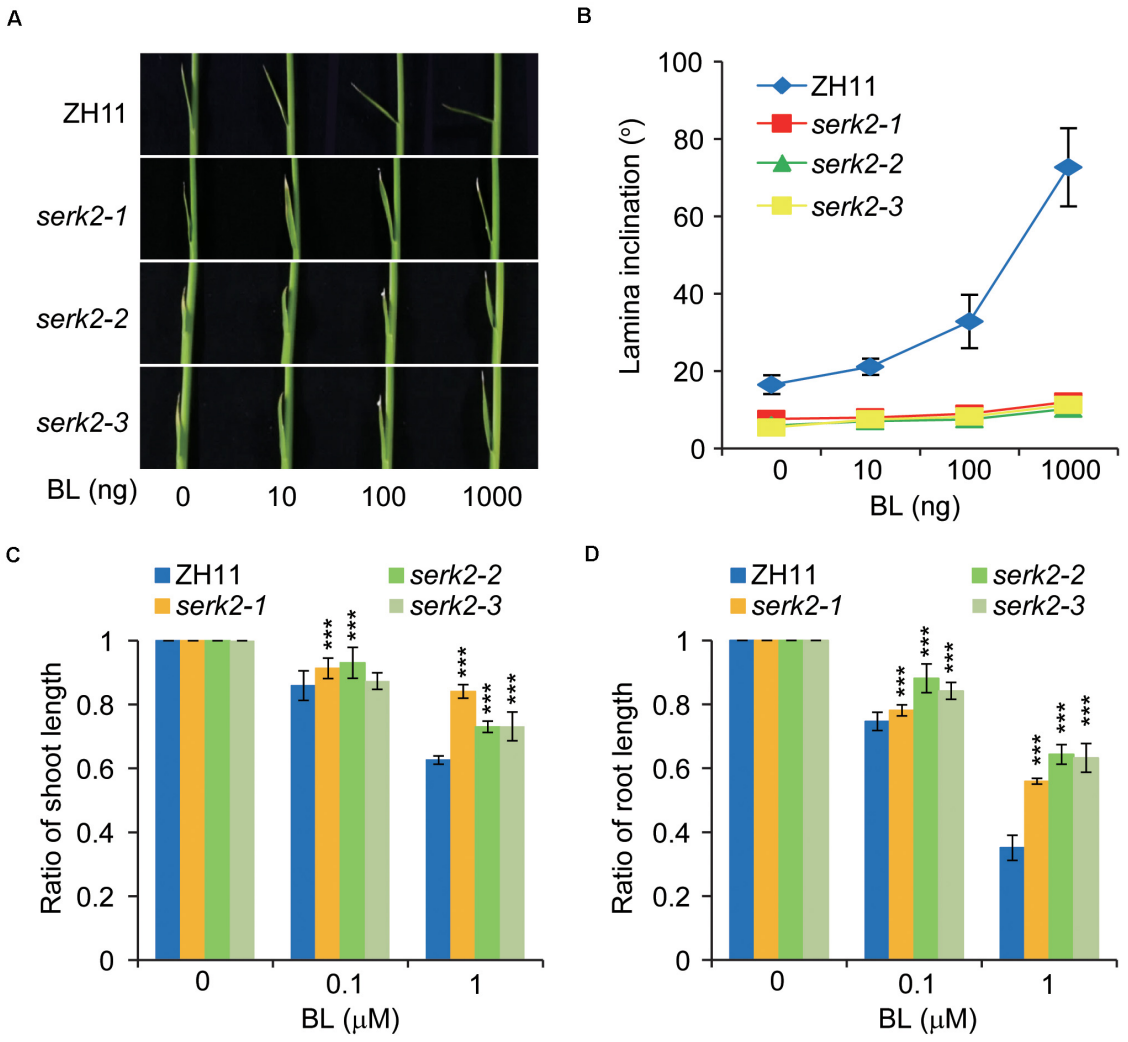

FIGURE 2 | The *serk2* mutants have decreased BR sensitivity. **(A,B)** Lamina bending analysis of the BR sensitivity with statistical data shown in **(B)**. Bars = SD, $n = 7$. **(C,D)** Growth inhibition of the shoot **(C)** and root **(D)** in response to BL in ZH11 and the mutants. Ratios between BL-treated plants and those without BL treatment are shown. Bars = SD, $n = 15$, $^{***}p < 0.001$ by *t*-test.

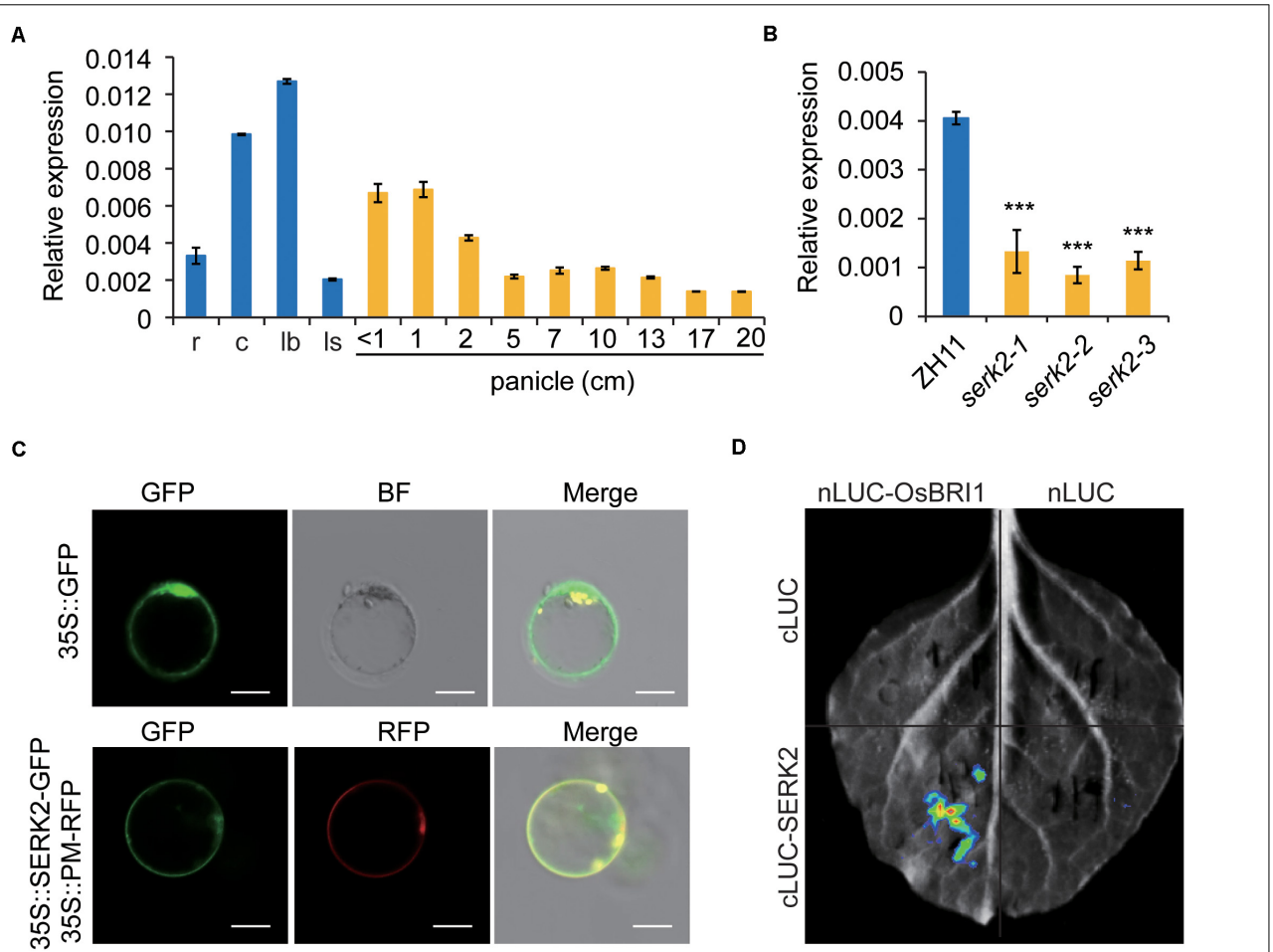

FIGURE 3 | Molecular characterization of SERK2. **(A)** Expression pattern of *SERK2* in various tissues. r, root; c, culm; lb, leaf blade; ls, leaf sheath. Bars = SD, $n = 3$. **(B)** Expression levels of *SERK2* in ZH11 and the mutants. Bars = SD, $n = 3$, $^{***}p < 0.001$ by *t*-test. **(C)** Subcellular localization of SERK2, showing a co-localization with plasma membrane marker protein (PM). Empty GFP vector was used as control. Scale bar = 10 mm. **(D)** Split-luciferase complementation analysis showing the interaction between SERK2 and OsBRI1.

mutant phenotypes, these analyses demonstrated that SERK2 plays a critical role in BR signaling in rice, possibly as a co-receptor of OsBRI1.

The *serk2* Mutants Show Decreased Survivability Under Salinity Stress

Next, we tested whether the mutant had altered resistance to salt stress. One-week-old seedlings were used for the analysis as at the time the mutant had little change of plant height. Strikingly, after high salt treatment (200 mM NaCl, 6 days) followed with recovery, all the three mutants showed greatly decreased survival frequencies compared to the wild type (**Figure 4A**). On average, the survival frequency of ZH11 was ∼65%, whereas of the mutant were ∼20–40%, strongly suggesting that SERK2 is required for plant resistance to salt stress (**Figure 4B**). To test whether *SERK2* is responsive to salt at molecular level, we treated ZH11 with salt, and then analyzed *SERK2* expression at different time points. Strikingly, *SERK2* was greatly suppressed by salt treatment after 1-h treatment, and the inhibitory effect could be kept to 24 h (**Figure 4C**). As control, expression of *SalT*, a salt induced gene (Zhang et al., 2000), was promoted by salt treatment (**Figure 4D**). Thus, *SERK2* is involved in salt stress response and positively regulates salt resistance.

The *serk2* Mutants Show Increased Sensitivity to ABA

The hypersensitivity of *serk2* mutants to salt prompted us to test whether the plants also have altered sensitivity to ABA, the well-known stress hormone. Without ABA, no significant difference was detected between the mutant and the wild type regarding the seed germination process (**Figure 5A**). However, when ABA (3 μM or 8μM) was supplemented, all the three allelic mutants showed decreased germination with detectable significance at the certain time points (84 h or 96 h) (**Figure 5A**), suggesting that the mutant has enhanced ABA sensitivities. Similar results were obtained when evaluating the ABA inhibitory effects on shoot and root growth. The inhibitory effects of ABA (3 μM or 6 μM) on either shoot and root in the mutant were greater than those in

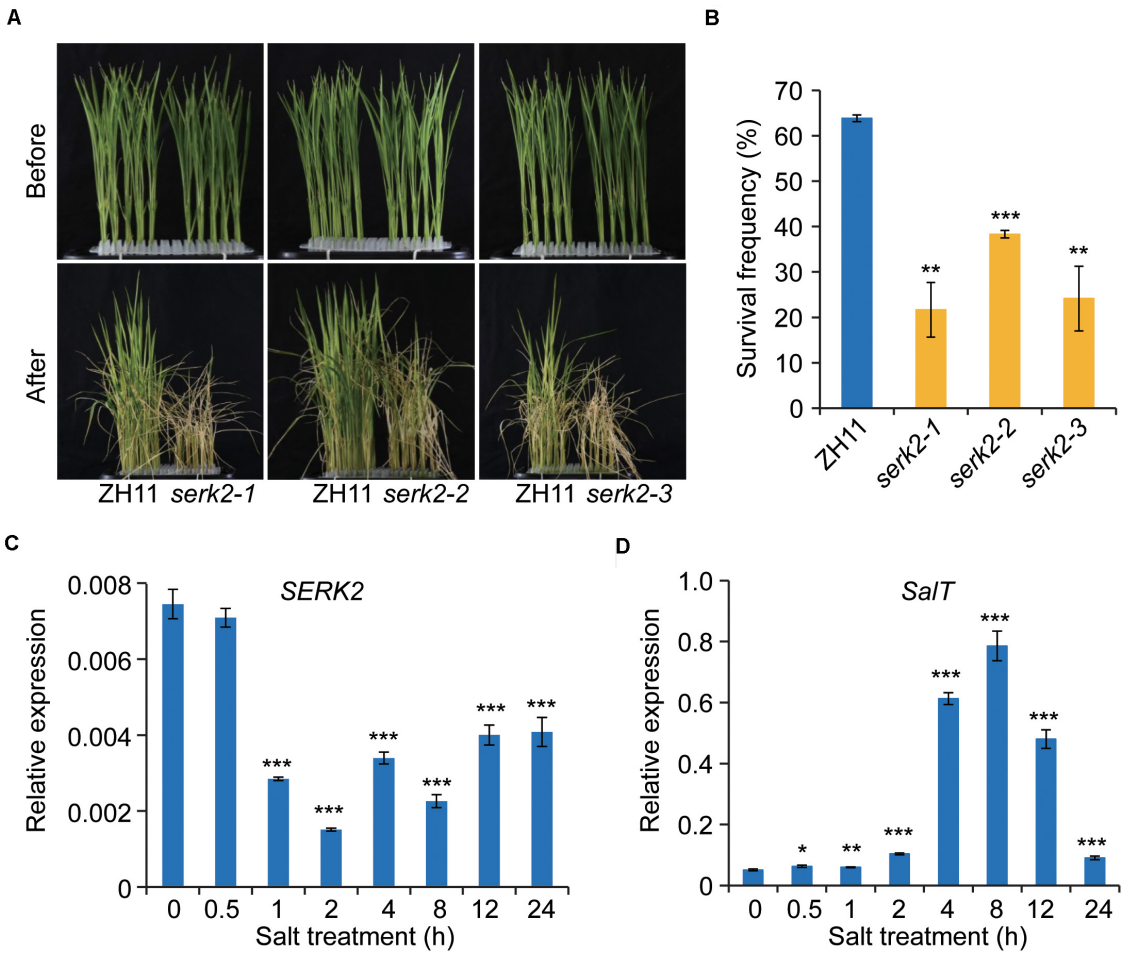

FIGURE 4 | *SERK2* regulates salt stress tolerance and is regulated by salt. **(A)** The *serk2* mutants have decreased surviving frequency after salt treatment. **(B)** Statistical data of the surviving frequency. Bars indicate standard error of the mean values, $n = 3$, $^{***}p < 0.001$ and $^{**}p < 0.01$ by *t*-test. **(C,D)** *SERK2* expression is suppressed by salt treatment. *SalT*, a known salt-induced gene, was used as control. Bars = SD, $n = 3$, $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ by *t*-test.

the wild type (**Figures 5B,C**). Given that enhanced ABA response should facilitate plant in cope with stress, these results indicated that the increased salt sensitivity of *serk2* mutants should be independent of ABA, and SERK2 as a BR signaling component could somehow affect ABA responses.

The *serk2* Mutants Are Hypersensitive to Salt Stress at Molecular Level

To verify the roles of SERK2 in stress responses, we performed transcriptome analysis to identify the salt-regulated genes in the wild type and the mutant respectively. Four samples, including ZH11, *serk2-1*, ZH11 treated with salt (200 mM NaCl, 8 h), and *serk2-1* treated with salt, were prepared and the seedling shoots were collected as materials for the analysis. The DEGs in salt-treated samples, namely salt-regulated genes, were used for the comparison (**Supplementary Tables 2, 3**). As results, we identified 586 salt-regulated genes in ZH11 background, but identified 975 in *serk2* background (**Figure 6A**). Intriguingly, most of these DEGs were up-regulated ones, and consistently, salt treatment apparently had upregulated more genes in *serk2* than in ZH11 (**Figure 6A**). We identified 329 overlapping DEGs between salt regulated genes in ZH11 and *serk2*. Notably, all of them were consistently regulated (**Figure 6B**). Among the salt upregulated DEGs in ZH11, 77.7% (279/359) were also upregulated by salt in *serk2* (**Figure 6B**). Importantly, the change folds of most overlapping DEGs in *serk2* were higher than those in ZH11 (**Figure 6C**). We selected three genes for verification, and found these genes indeed were much more obviously induced in the mutant than in the wild type (**Figure 6D**). Taken together, these analyses further confirmed that loss-of-function of *SERK2* enhances susceptibility of the mutant to salt treatment at molecular level.

Altered Expression of Salt-regulated Genes in the *serk2* Mutants

Compared to ZH11, we identified 129 DEGs from *serk2*, including 72 upregulated and 57 downregulated. As revealed by gene ontology (GO) analysis, these DEGs were markedly

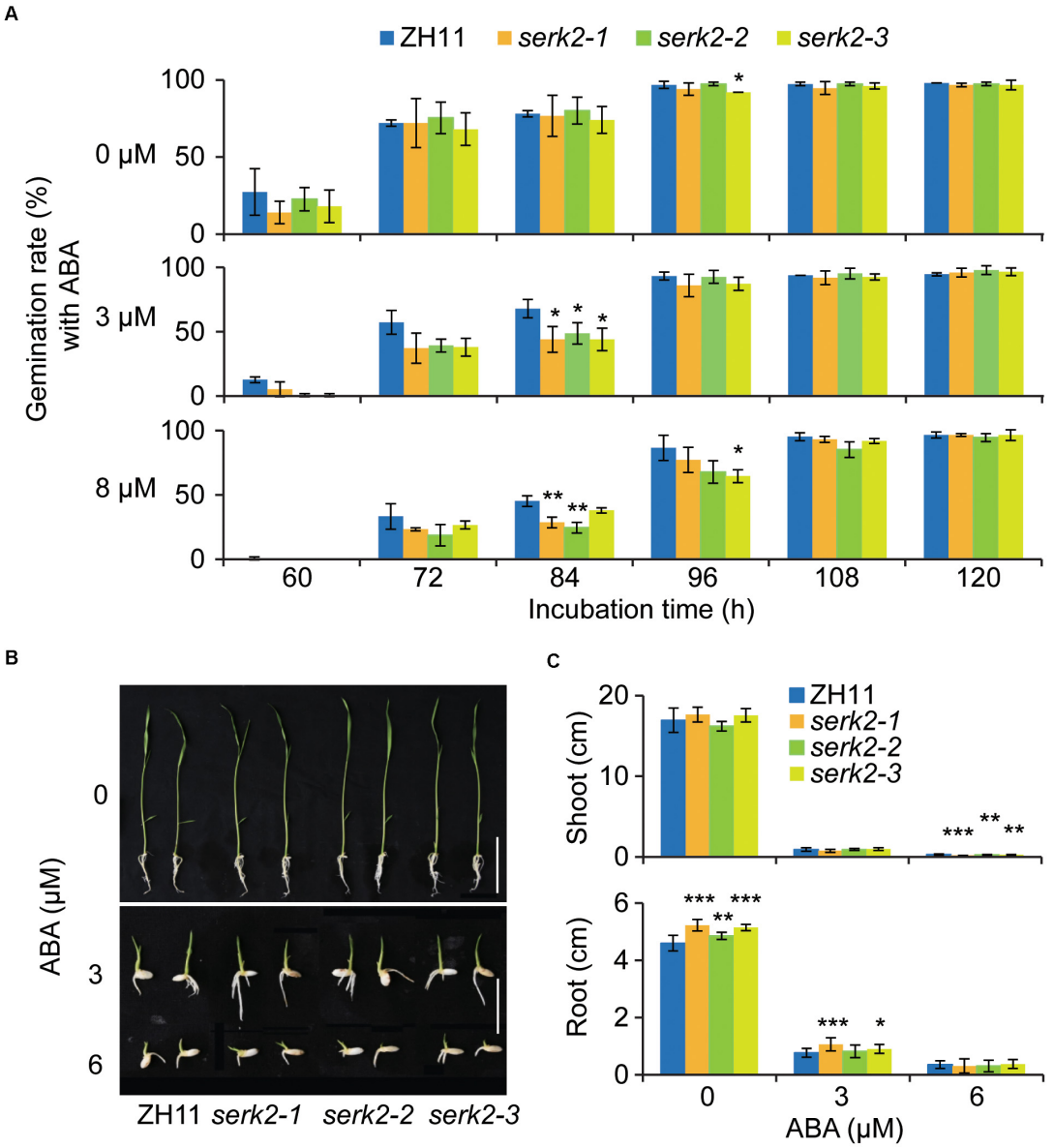

FIGURE 5 | The *serk2* mutants have slightly increased ABA sensitivity. **(A)** Germination test of ZH11 and the *serk2* mutants in response to ABA. Bars = SD, $n = 3$, $^{**}p < 0.01$, and $^{*}p < 0.05$ by *t*-test. **(B,C)** Shoot and root growth of ZH11 and the *serk2* mutants in response to ABA. Scale bar = 2 cm in **(B)**. In **(C)**, bars = SD, $n = 12$, $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ by *t*-test.

enriched in biological processes such as "growth" and "signaling" (**Figure 6E**), consistent with the roles of SERK2 in regulating plant growth and development and hormone signaling. In addition, the enrichment in GO terms such as "immune system process" and "response to stimulus" indicated that SERK2 is also involved in plant immunity, as has been reported previously (Chen et al., 2014), and stress responses, as revealed in this study (**Figure 6E**). Moreover, 20 of the DEGs were also differentially regulated in the salt treated ZH11 samples (salt_ZH11, **Figure 6F**). Notably, all these 20 DEGs, had consistent expression tendency, either upregulated or downregulated, in *serk2* and salt_ZH11 (**Figure 6F**). Thus, it appeared that loss-of-function of *SERK2* has more or less activated salt responses even without salt treatment. One possibility is that these genes represent a subset of salt responsive genes that are unfavorable for plant adaptability to salt stress.

Overexpression of *SERK2* Enhanced Grain Size and Salt Resistance With Little Effect on Plant Architecture

To gain more insight into *SERK2* function, we introduced a construct expressing SERK2-Flag fusion protein into ZH11 and obtained a number of transgenic lines. However, we failed to

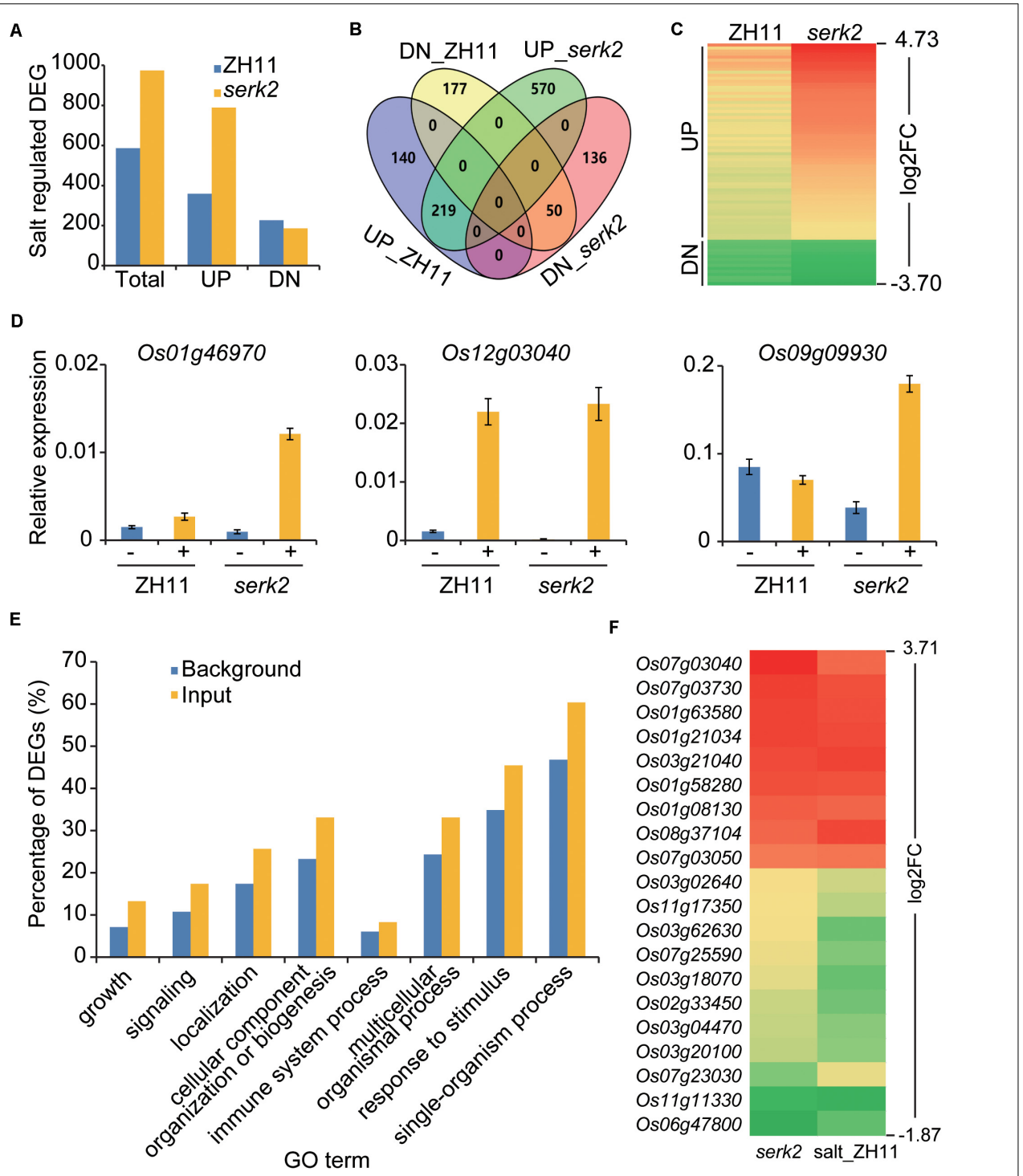

FIGURE 6 | The *serk2* mutants are more sensitive to salt at molecular level. **(A)** Salt-regulated DEGs in ZH11 and *serk2* identified by transcriptome analysis respectively. UP, upreuglated DEGs; DN, downregulated DEGs. **(B)** Overlapping analysis of the salt-regulated DEGs in ZH11 and *serk2*. DEG numbers are indicated in each section. This Venn diagram was generated online (https://bioinfogp.cnb.csic.es/tools/venny/). **(C)** Heatmap of the overlapping DEGs showing the logarithmic values of the fold changes (log2FC) in ZH11 and *serk2* upon salt treatment, illustrating the FC in *serk2* is generally higher than in ZH11. **(D)** Quantitative RT-PCR analysis of the three selected DEGs ± indicate with/without salt. Bars = SD, $n = 3$. **(E)** GO enrichment analysis of DEGs identified from *serk2* (input). Top eight GO terms identified in biological processes were shown. The percentages of the gene numbers in each GO term accounting for all gene numbers were shown as background. **(F)** Heatmap of the overlapping DEGs identified in both *serk2* and salt-treated ZH11 (salt_ZH11). Log2FCs were used for generating the map, illustrating the consistent expression tendencies of all the DEGs in the two samples.

observe a clear morphological difference compared to the wild type (**Figure 7A**). In two lines with enhanced *SERK2* expression, no difference was detected in term of plant height (**Figures 7B,C**). Regarding the leaf angle, we can only detect a slight increase of the second leaf (the flag leaf was counted as the first leaf), but not in the first and the third ones (**Figures 7D,E**). Immunoblotting analysis further confirmed the accumulation of SERK2-FLAG fusion proteins in both the two lines as well as two additional lines

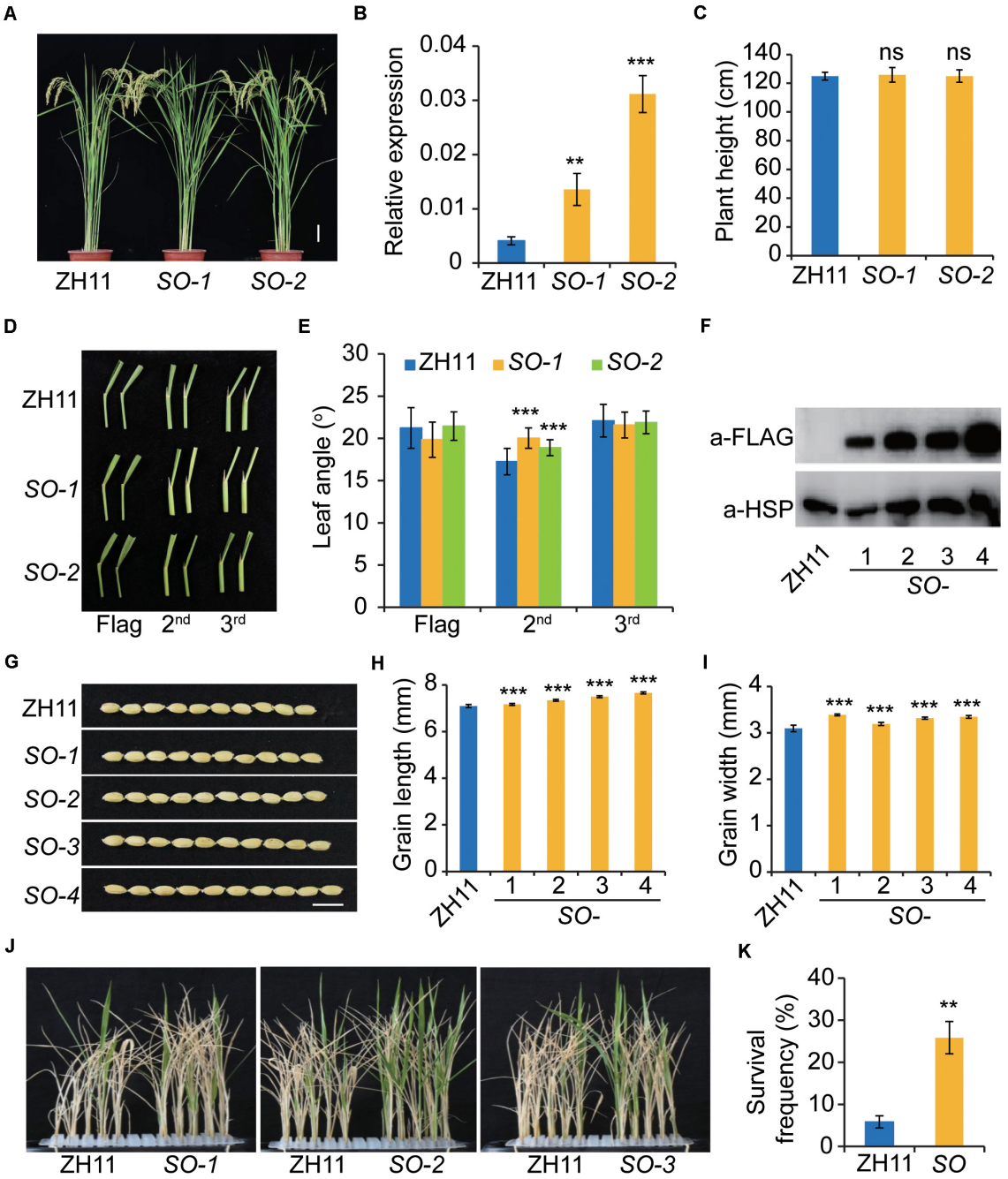

FIGURE 7 | Overexpression of *SERK2* simultaneously enhances grain size and salt resistance without affecting plant architecture. **(A)** Plant architecture of the *SERK2*-overexpressing plants. Scale bar = 10 cm. **(B)** Expression levels of *SERK2* in ZH11 and the transgenic plants. Bars = SD, $n = 3$, $^{***}p < 0.001$ and $^{**}p < 0.01$ by *t*-test. **(C)** Statistical data of the plant height. Bars = SD, $n = 15$. ns, no significant difference. **(D)** Leaf angles in ZH11 and the transgenic plants. Top three leaves are shown. *SO, SERK2*-overexpression lines. **(E)** Statistical data of the leaf angles. Bars = SD, $n = 20$, $^{***}p < 0.001$ by t-test. **(F)** SERK2-FLAG fusion proteins are accumulated in the transgenic plants. Detection of HSP is used as reference. **(G)** Grain morphology of ZH11 and the transgenic plants. Scale bar = 1 cm. **(H,I)** Statistical data of grain length **(H)** and grain width **(I)**. Bars = SD, $n = 50$, $^{***}p < 0.001$ by *t*-test. **(J,K)** Salt treatment showing the three independent transgenic lines have increased survival frequencies after salt treatment. Bars = SD, $n = 3$, $^{**}p < 0.01$ by *t*-test.

(**Figure 7F**). Notably, all these transgenic lines evaluated showed obviously increased grain length and width (**Figures 7G–I**). We also tested the stress adaptability of three lines and found that all of them showed consistently increased survival frequencies after high salt treatment (200 mM NaCl, 8 d) (**Figures 7J,K**). Thus, our results suggested that overexpression of *SERK2* is able to simultaneously promote grain size and stress resistance. The little effect on the plant architecture further enhances the feasibility of utilization of the gene for crop improvement.

SERK2 Protein Is Greatly Induced by Salt Stress

Since SERK2 positively regulates salt tolerance but is suppressed by salt at transcription level, we are curious how SERK2 is regulated by salt at protein level. When the overexpression lines were treated with high salt for different times, the SERK2-FLAG fusion proteins showed a dynamic expression pattern. Upon treatment for 1 h, the protein abundance was slightly suppressed by salt (**Figures 8A,B**). After that, the protein was gradually increased and was accumulated to a high level after 4- or 6-h-treatment (**Figures 8A,B**). However, when treated with BL, the fusion proteins showed unaltered or only slightly altered levels (**Figures 8C,D**). For both treatments, we obtained consistent results using two independent lines for the analysis. Thus, it appears that SERK2 is slightly inhibited and then greatly induced upon stress treatment. This result further demonstrates the involvement of SERK2 in salt stress responses. The eventually highly accumulation of the protein could favor the plant in cope with the adverse condition.

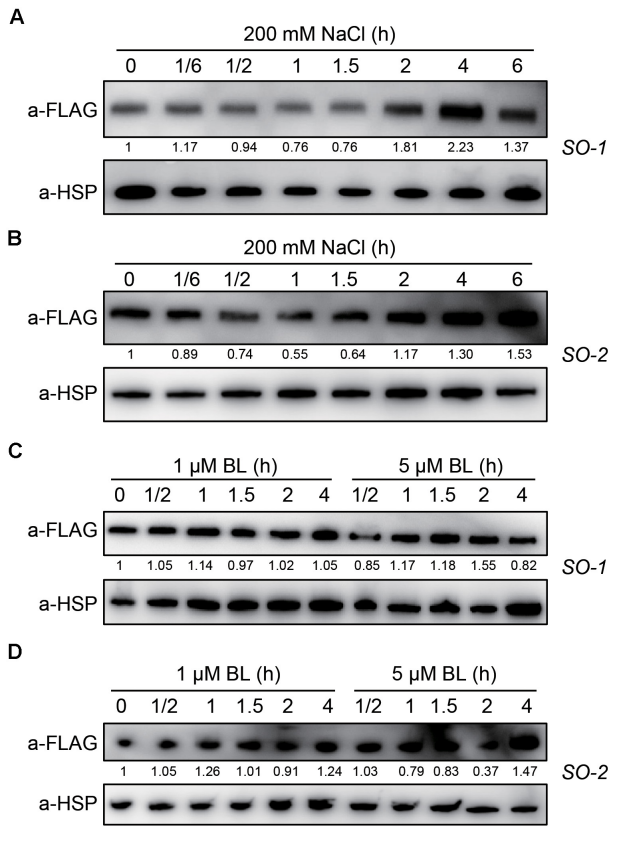

FIGURE 8 | SERK2 protein is greatly induced by salt treatment. **(A,B)** Evaluation of salt stress on SERK2-FLAG protein levels using two independent transgenic lines. Detection of HSP is used as reference. **(C,D)** Evaluation of BL on SERK2-FLAG protein levels using two independent transgenic lines. Detection of HSP is used as reference. Numbers indicate the relative intensities of each band, representing the relative abundances of SERK2-FLAG proteins.

DISCUSSION

BRs regulate many important agronomic traits such as plant height, leaf angle and grain size. However, the regulation of these traits is not simultaneously beneficial for crop improvement in the view of breeders. For example, deficiency of BRs could decrease plant height and leaf angle, which are associated with increased lodging resistance and photosynthetic efficiency, allowing high density planting, a major cultivation strategy for obtaining high yield (Sakamoto et al., 2006). However, BR deficiency could also impair grain size or weight, leading to reduced grain yield. Similarly, BR overdose could increase grain weight but this is usually accompanied with a loose plant architecture that is not preferred by farmers (Wu et al., 2008). To deal with this problem, one feasible approach is to identify specific BR components. Manipulation of this kind of BR components could obtain the desired traits without or with little effect on other traits (Tong and Chu, 2018). Considering each BR signaling step is executed by a family of genes, the genes within the family could have differential function or expression pattern. In this case, it's worthy to screen these family genes to identify the specific ones that regulate certain traits. Here, we showed that compared to OsBAK1, its homolog SERK2 functions more specifically in regulating leaf bending. Knockout of *OsBAK1* led to small grains (Yuan et al., 2017), but knockout of *SERK2* resulted in even increased grain size. Although both mutants show erect leaves, apparently *serk2* is a more applicable candidate for crop improvement. This result is also consistent with the gene expression pattern. According to the public database[2], *OsBAK1* has the highest expression in panicles, whereas *SERK2* has the highest expression in leaves.

Since BRs also play a critical role in stress responses, stress is another important aspect that should be considered when manipulation of BR genes. Our evaluation suggests that defective of BR signaling could decrease plant resistance to salt stress. Unlike most of the previous studies focusing on the downstream of BR signaling pathway (Cai et al., 2014; Hu and Yu, 2014; Wang et al., 2018; Ye et al., 2019), our study revealed that early BR signaling on plasma membrane is involved in stress responses. High salt is able to greatly induce SERK2 protein accumulation, suggesting that the adverse condition induces SERK2 accumulation to enhance early BR signaling on plasma membrane in favor of the anti-stress response. In addition, it appears that the slightly increased ABA sensitivity could not

[2]https://ricexpro.dna.affrc.go.jp/

explain the hypersensitivity of *serk2* to salt stress, implying the existence of an ABA-independent role of BR signaling in the stress responses. On the other hand, we show that overexpression of *SERK2* increases grain size as well as salt stress resistant ability. Importantly, the transgenic lines have little alteration of plant architecture including plant height and leaf angle, providing an alternative approach for *SERK2* utilization.

High yield and stress resistance are usually conflicting with each other due to the internal unknown compensation mechanism. However, high yield and high resistant crops are long-term breeding goals and also prime breeding targets. Phytohormones usually play important roles in both plant growth and development and stress responses. The dual roles of SERK2 in grain size and salt stress indicate that BRs have great potential for simultaneously improvement of grain yield and stress resistance, at least to salinity stress. Intriguingly, transcriptome analysis revealed that a subset of salt-regulated genes have consistently altered expression in *serk2* mutant, indicating that SERK2 regulates these genes to enhance plant resilience. Since SERK2 promotes BR signaling at the same time, one possibility is that these genes are also BR responsive genes by which BRs enhance salt resistance.

Considering the increased grain size in *SERK2*-overexpressing plants, the increased grain width in *serk2* mutants could be due to a compensation effect among the family members or feedback regulation on BR hemostasis in plants. Similarly, loss-of-function of *DLT*, a positive BR signaling regulator, also led to increased width, whereas overexpression of *DLT* resulted in increased grain size (Tong et al., 2009, 2012). These analyses, together with the previous studies (Tong et al., 2014; Xiao et al., 2017), suggested the existence of a highly elaborated BR balance system in plant, which may provide a potential explanation for the unaltered plant architecture in *SERK2*-overexpressing plants. Elucidation of this balancing system is critical for uncoupling different BR functions, thus facilitates the utilization of BR for crop improvement.

CONCLUSION

Salt stress induces SERK2 accumulation to enhance early BR signaling on plasma membrane in favor of the anti-stress response. Knockout of *SERK2* led to compact structure accompanied with decreased salt tolerance, whereas overexpression of *SERK2* is able to simultaneously enhance grain size and salt resistance without affecting plant architecture.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Genomics Data Center (https://bigd.big.ac.cn/) (accession: CRA003500).

AUTHOR CONTRIBUTIONS

ND performed most experiments with the assistance of the others. WY and DL assisted in the protein analysis. XZ and ZY assisted in phenotype analysis. WH and JL provided assistance in expression and transgenic analyses. YY participated in the transcriptome analysis. WM and MN assisted in stress treatment. ND and HT analyzed the data and wrote the manuscript. HT conceived and supervised the study. All authors contributed to the article and approved the submitted version.

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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.2020.621859/full#supplementary-material

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frontiers in Plant Science

REVIEW
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Brassinosteroid Signaling, Crosstalk and, Physiological Functions in Plants Under Heavy Metal Stress

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Brassinosteroids (BRs) are group of plant steroidal hormones that modulate developmental processes and also have pivotal role in stress management. Biosynthesis of BRs takes place through established early C-6 and late C-6 oxidation pathways and the C-22 hydroxylation pathway triggered by activation of the DWF4 gene that acts on multiple intermediates. BRs are recognized at the cell surface by the receptor kinases, BRI1 and BAK1, which relay signals to the nucleus through a phosphorylation cascade involving phosphorylation of BSU1 protein and proteasomal degradation of BIN2 proteins. Inactivation of BIN2 allows BES1/BZR1 to enter the nucleus and regulate the expression of target genes. In the whole cascade of signal recognition, transduction and regulation of target genes, BRs crosstalk with other phytohormones that play significant roles. In the current era, plants are continuously exposed to abiotic stresses and heavy metal stress is one of the major stresses. The present study reveals the mechanism of these events from biosynthesis, transport and crosstalk through receptor kinases and transcriptional networks under heavy metal stress.

Keywords: BR biosynthetic pathway, BR signaling, transcription, heavy metal, stress, hormone crosstalk

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Abbreviations: ABI1/2, abscisic acid-insensitive 1 and 2; ABRE, abscisic acid response elements; ACO, 1-aminocyclopropane-1-carboxylic acid (ACC)-oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase enzyme; APOX, ascorbate peroxidase; BIN2, brassinosteroid-insensitive 2; BZR1, brassinazole-resistant 1; BZR1/BES1, brassinazole-resistant 1/BRI1-EMS suppressor1; CAT, catalase; Cd, cadmium; CKX, CK oxidases/dehydrogenases; Cr, chromium; Cu, copper; DHAR, dehydroascorbate reductase; EBL, 24-epibrassinolide; GPOX, guaiacol peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; HBL, 28-homobrassinolide; Hg, mercury; IPT, isopentenyltransferases; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; Mn, manganese; Ni, nickel; NPR1, non-expressor of pathogenesis-related genes 1; Pb, lead; POD, peroxidase; PP2C, protein phosphatase 2C; PPO, polyphenol oxidase; ROS, reactive oxygen species; Sb, antimony; SOD, superoxide dismutase; Zn, zinc.

INTRODUCTION

Brassinosteroids (BRs) were discovered 40 years ago, and since then an enormous amount of work has been done to illuminate their role in plant physiology (Peres et al., 2019). BRs are poly-hydroxylated steroids that regulate developmental processes such as cell division, cell cycle, elongation, morphogenesis, reproduction, senescence and stress-protective responses (Clouse and Sasse, 1998). They function especially as master switches in triggering the metabolic response to noxious environmental conditions (Bajguz, 2010). BRs are considered as derivatives of 5α-cholesterol, but they vary in structure due to the carbon side-chains. They are present in all parts of plants but are mostly found in seeds and pollen (Bartwal and Arora, 2020). Caño-Delgado et al. (2004) suggested that as animal steroid hormones bind to nuclear receptors that modulate gene expression, BRs also bind to receptors on the cell surface initiating a signal cascade leading to alterations in gene expression. Recent progress in understanding the BR signaling pathways supports the idea that they do not follow a linear path but rather undergo crosstalk with other hormones to combat stress conditions (Nolan et al., 2017). BRs also provide tolerance to plants against abiotic stressors by modulating the activity of enzymatic and non-enzymatic antioxidants (Sharma A. et al., 2016; Sharma et al., 2017; Bartwal and Arora, 2020; Chi et al., 2020; Rattan et al., 2020; Chen et al., 2021). BRs are also reported to stimulate the formulation of phytochelatins as reported by Bajguz, 2002. Reports suggested that brassinolides along with the lead are responsible for the increase in the synthesis of phytochelatins in *Chlorella vulgaris*. To overcome the harmful effects of heavy metals, BRs interact with other hormones like auxin, cytokinin, abscisic acid (ABA), ethylene, and jasmonic acid (JA). In this review we have discussed in detail the molecular mechanism of BR biosynthesis, signaling, and the role of transcriptional networks in the response to heavy metal stress in plants, and the ways that BRs crosstalk with other phytohormones to prevent heavy metal damage.

STRUCTURE AND DISTRIBUTION OF BRs

Brassinosteroids share structural resemblance with animal steroids like cholestane, ergo- and stigmastane. The majority of BRs are 5α-cholestane derivatives and structural variation among them emerges because of C-substitutions within the side chains (Ali, 2019). They can be found in all plant parts, yet most of the synthesis occurs in the meristematic zones of seeds and pollen grains. These BR categories represent more than eighty plant species including angiosperms, pteridophytes, algae, and bryophytes (Yokota, 1999). Based on the number of carbon atoms and alkyl groups within side chains, BRs are divided into C-27, -28, and -29 steroids present freely or in conjugation with fatty acids and sugars (Fujioka and Yokota, 2003). Forty to fifty percent of BRs are C-28. The great diversity in the patterns of cyclic groups as well as side chains is mainly responsible for the conversion of BRs into active analogs such as 24-epibrassinolides and 28-homobrassinolides (Kang and Guo, 2011). BRs along with analogs like typhasterol, brassinolide, and castasterone are widely distributed within specialized plant structures like pollens, seeds, flowers, roots, shoots, leaves and stems. The greatest concentration of BRs (100 ng per g fresh weight) have been recorded within pollen grains and seeds compared to 0.01 and 0.1 ng per g fresh weight within shoots and leaves, respectively.

More than 69 different BRs and derivatives have been identified to date from various plants (Bajguz and Tretyn, 2003) including *Castanea crenata* and *Catharanthus roseus*. Along with various BRs there are five conjugates of BRs which are found together with 8 metabolites and 137 analogs of BRs (Liu et al., 2017). The distribution of BRs in various plant families from maximum to minimum runs from angiosperms to gymnosperms, pteridophytes, bryophytes, and algae (Bajguz and Tretyn, 2003). It is well documented that sterols within plants are transformed into brassinolide through teasterone, typhasterol as well as castasterone via the isoprenoid pathway in association with acetyl coA, isopentenyl pyrophosphate (IPP), geranyl pyrophosphate (GPP), mevalonate and farnesyl pyrophosphate (FPP) (Symons et al., 2008). Brassinolides are highly effective forms of BRs that are produced as the end product of BRs synthesis (Zhao and Li, 2012). According to a more limited description, among the metabolites produced during the biosynthesis of BR-lactones, only those that have been formed from 22α and 23α-dihydroxylation would be considered as true BRs, the rest being placed in the category of BR precursors (Zullo and Kohout, 2004). BRs containing 23-epoxy groups, 23-glycosidic groups, 23-ester groups, 3-oxygenated-5α-cholestane-23α diols along with alkyl derivatives were also considered natural BRs (Zullo, 2018). As reported, the bioactive potential of BRs lies with the side chains of rings (Zullo and Adam, 2002). Employment of the rice laminal inclination assay for ring structures showed that 22α and 23α dihydroxy BRs were active as 28-epibrassinolides (Watanabe et al., 2001). However, 23-dehydrogenation or conjugation of side chains may disrupt the biological activity (Watanabe et al., 2001). Further studies demonstrated that the biological activity of BRs was regulated by other active sites (Liu et al., 2017). A wide spectrum of structural variability was seen in ring A with fifteen types of structures starting from Δ2, 3-unsaturated to conjugated BRs. Even if analysis of the structural variation does not reveal the probable structures of this ring, it is assumed to belong to the category of BRs (Zullo, 2018). The biological functionality of BRs possessing rings increases in the order from 3β-hydroxy to 3α-dihydroxy rings (Liu et al., 2017). This variation in rings correlates with the biosynthetic pathway of BRs, suggesting that higher oxidation states are enhanced from the 6-deoxo and 6α-hydroxy forms toward the 6-oxo and 7-oxa lactone states (Vriet et al., 2013). It has been observed that there is decline in the functionality from 2α, 3α toward 2β, 3β and it is suggested that this decline is due to reason that α-oriented hydroxyl group that is present at C-2 is important for its activity (Zou et al., 2020). BRs have also been classified according to B-rings: 6-oxo-7-oxalactonic BRs (28-homobrassinolides, 2α,3β-dihydroxylated: 3-epibrassinolide, 2α,3α-dihydroxylated: brassinolide, 3β-hydroxylated: 3-epi-2-deoxybrassinolide, dolicholide), 6-oxo or 6-keto BRs (2α,3α-dihydroxylated: castasterone, 28-homocastasterone,

25-methylcastasterone, 2β,3α-dihydroxylated: 2-epicastasterone, 2α,3β-dihydroxylated: 3-epicastasterone, 2,3-diepi-25-methyl dolichosterone, 3β-monohydroxylated: teasterone, 28-homotyph asterol, 23-dehydro-2-epicastasterone, 1α,2α,3β-dihydroxylated: 1α-hydroxy-3-epicastasterone, 1α,2α,3β-dihydroxylated: 1α-hydroxy-3-epicastasterone, 23α-conjugates: 23-*O*-β-*D*-glucopy ranosyl-25-methyldolichosterone, 3-dehydro: 3-dehydrotea sterone), 6α-hydroxy BRs (6α-hydroxycastasterone), 6-deoxo BRs (2α,3α-dihydroxylated: 6-deoxocastasterone, 6-deoxo-28-homodolichosterone, 6-deoxo-24-epicastasterone, 3α-monohy droxylated: 6-deoxotyphasterol, 6-deoxo-28-homotyphasterol, 6-deoxo-28-norteasterone, 3-dehydro-6-deoxo-28-norteasterone), respectively (Zullo and Bajguz, 2019).

BIOSYNTHETIC PATHWAYS OF BRs

Genetic and biochemical studies have identified most of the enzymes associated with BR synthesis up to now, but the molecular mechanism of BR biosynthesis and release needs further work to provide a deeper understanding of the regulation of its synthesis, catalysis and conjugation. These activities were explained in a coordinated manner by Japanese scientists who systematically elucidated the biosynthetic pathways concerning BRs within plant cells (Fujioka and Sakurai, 1997). Their data illustrated that biosynthesis of brassinolide (28-epibrassinolide) was linked to two main pathways, the early and late C6 oxidation pathways (Fujioka et al., 1998). The plant steroid, campesterol, was thought to be the progenitor of brassinolides on account of the side chain as well as the bioactive potential. The molecular structure and concurrence of teasterone, typhasterol, and castasterone also suggested that brassinolides were synthesized from campesterol (Yokota et al., 1991). The BR-specific campesterol precursor is initially transformed into campestanol, after which the early as well as late C6-pathways of oxidation, also termed as campestanol-dependent pathways, are activated. The enzymes required for this biosynthetic process include DET2, DWF4, cytochrome monooxygenases (Cyp P450), ROT3, BAS1, BR oxidase 2(BRox2), constitutive photomorphogenesis and dwarfism (CPD), and BR oxidase 6(BR6ox1) (Bhanu, 2019). The synthesis of castasterone and brassinolide requires the enzyme Cyp450 (CYP85A2), that also contributes to the rate limiting step for the conversion of 6-deoxycatasterone into castasterone and finally into brassinolide (Kim et al., 2005). Thus, by acting on several intermediates upstream, DWF4 can bifurcate the pathway at campesterol to generate an initial C-22 hydroxylation pathway (Fujioka and Yokota, 2003).

The BR biosynthetic pathway is generally triterpenoid in nature; therefore, the triterpene squalane can also be a precursor for BR synthesis, where it gets cyclized to cycloartenol using specialized enzymatic machinery (Hartmann, 1998). It is mainly formed via condensation of two molecules of FPP using NADPH as reducing agent (Heldt and Piechulla, 2011). In addition, plant sterols containing different alkyl groups at C24 like cholesterol and sitisterol, as well as campesterol, can also act as precursors for different BRs (Fujioka and Yokota, 2003). The biosynthetic pathway of BRs is limited at various steps through regulatory feedback processes to maintain endogenous BR homeostasis by the expression of genes for BR degradation. For example, expression of the *CPD*, *DET2*, and *DWARF4* genes is known to get modulated under such conditions. Also, those genes functioning in the C-22 hydroxylase pathway become active during the DWF4- or CPD-induced cascade depending on the availability of substrates. DWF4 can also bind large substrates like campesterol, 4-en-3-one, campestanol, and 6-oxo campesterol to enter the C-22 oxidation biosynthetic pathway. Likewise, CPD can metabolize campesterol and 22, 23-dihydroxy campesterol via parallel pathways. After campesterol hydroxylation by DWF4, the intermediates formed are altered and enter the 6-oxidation pathway. It was also assumed that *CYP90A1/CPD* encoding C23 hydroxylases converted teasterone into castasterone (Szekeres et al., 1996; Chung and Choe, 2013). *CYP90A1/CPD* was reported to be active in the C3 oxidation pathway as revealed through GC-MS analysis (Ohnishi et al., 2012). The transformation of 6-deoxo teasterone to 6-deoxo dehydroteasterone can also occur through CYP90A1/CPD activation. However, 22-hydroxycampesterol accumulation was observed along with reduced 6-deoxo 3-deoxyeasterone and 6-deoxo castasterone in CPD-knockout mutants. By this, it was inferred that the campestanol in-mediated pathway was principally involved in BR biosynthesis. CYP90C1/ROT3 and CYP90D1 were also important in C23 hydroxylase synthesis. This adds a new dimension to the BR synthesis pathway through conversion of 22-hydroxy-ergost-4-en-3-one and 22-hydroxy-5α-ergostan 3-one into C23 hydoxylated forms (Bishop, 2007). During the early stage of the C6 oxidation pathway, oxidation at C-6 occurs before the onset of DWF4-induced C22 hydroxylation. Studies were done with *Arabidopsis* CYP85A1 and CYP85A2 on the C-6 oxidation pathway that showed conversion of 6-deoxoteasterone and 6-deoxocastasterone into typhasterol and castasterone (Kim et al., 2005). CYP85A2 was also observed to induce the conversion of 6-deoxotyphasterol into brassinolides through castasterone by a downstream pathway. An investigation in CYP85A1 and CYP85A2 double mutants of endogenous BR levels suggested that increased levels of 6-oxocampesterol were present at the initial steps of BR-biosynthesis, revealing the activity of an unknown enzyme (Kwon et al., 2005). It is pertinent to mention here that CYP85A2-induced brassinolide synthesis only occurs in dicots. In rice, which is a monocot, brassinolides were not detected even in BRI1 mutants (Yamamuro et al., 2000). Also, monocots have only single copy *CYP85A* gene, while, dicots have duplicate genes (Kim et al., 2008). The evidence shows that castasterone is the product formed using these mechanistic pathways (Kim et al., 2008), but the additional genetic and biochemical strategies that enhance BR biosynthesis also need to be determined.

The accumulation of BZR1 (brassinazole-resistant 1) and BRI1 EMS suppressor BES1/BZR2 reduced the expression of BR biosynthetic genes (Bartwal and Arora, 2020).*BAS1*, which encodes PhyB-activated tagged suppressor 1, is the foremost BR-inactivating gene in plants for maintaining optimal hormone levels. Mutations in the pathway for BR synthesis have also been implicated in male sterility, leaf curl, and dwarfing and BR-induced signal transduction is essential in plant metabolism

(Bartwal and Arora, 2020). Earlier studies on mutants showed that exogenous application of BRs in wild type plants restored mutant BR-biosynthetic genes, while this did not occur in strains where BR signaling was hindered. Later studies were conducted on mutants of *Arabidopsis,* to elucidate the BR-signaling and transcriptional regulatory process. It was reported that leucine-rich repeated receptors and protein kinases (LRR-RLK) and BRI1 were the primary receptors for BRs. The resulting signal cascade led to trans-phosphorylation of BRI1 and BAK1, and further downstream signal transduction events (Cheng et al., 2017). **Figure 1** represents the detailed biosynthetic pathway of BRs showing the early C-6 oxidation pathway, late C-6 oxidation pathway and early C-22 oxidation pathway.

REGULATION OF BR BIOSYNTHESIS

Transcriptional Regulation

With evolution, plants have developed strategies for optimal growth with minimal energy input. When BRs levels are adequate, the regulatory mechanism of positive and negative feedback loops are activated to down-regulate the endogenous BR levels. The endogenous BR level is either regulated by the down-regulation of BR biosynthetic genes or by the inactivation of already available bioactive BRs. Exogenous BL application down-regulates the BR biosynthetic genes, DWF-4, CPD, BR6ox1, and ROT-3 (rotundifolia 3), but up-regulates Brz, an inhibitor of BR synthesis. Exogenous BL also up-regulates BAS-1, another inhibitor of BR synthesis and down-regulates BES/BZR, a key transcription factor in the BR biosynthesis and signaling network (Yu et al., 2011).

DWF-4 genes expressed in bacterial cells were found to not be allosterically regulated. Exogenous application of epi-BR or Brz inhibitors of BR biosynthesis altered the functioning of DWF-4 genes and the transcription of DWF-4 exceeded the threshold levels, even in BR signaling mutants. The accumulation of DWF-4 mRNA was observed after Brz application, but BL reduced DWF-4 mRNA. Some other genes like *CPD, BR6ox1*, and *ROT-3* were transcriptionally regulated by BL and Brz, but *DET-2* was sensitive to Brz only (Tanaka et al., 2005). The sterol methyltransferase 2 (*SMT2*) and Dwarf1(*DWF1*) genes related to sterol biosynthesis are sensitive to both BL and Brz, but the sterol C-14 reductase, FACKEL (FK), is responsive to Brz only. In Bri1 mutants exposure to BL and Brz can trigger the expression of DWF-4 but the other genes required the normal BRI1 for transcriptional regulation (Tanaka et al., 2005).

Wang et al. (2002) reported that the two downstream factors BES and BZR controlled the whole BR regulation pathway. BZR1 is involved in transcriptional regulation of multiple BR-biosynthesis genes necessary for triggering specific growth processes and in down-regulating these genes by negative feed-back mechanism (He et al., 2005). At low BL levels, BIN2 phosphorylates BZR1, which inactivates it and blocks initiation of the BR signaling cascade. Normal BL levels initiate the BR signaling cascade, which either activates or inhibits the expression of intermediate genes. Thus, starting from campesterol as the primary precursor for BL formation, and proceeding through the activation or inactivation of BR-related genes, there exists a regulatory feed-back mechanism for controlling BR biosynthesis (**Figure 2**).

Key BR Biosynthetic Enzymes and Their Transcriptional Regulation

Regulation of *CPD* (Constitutive Photo-Morphogenesis) and Dwarfism

In the brx^{s} mutant of *Arabidopsis*, expression of the *CPD* gene is arrested, which results in defective cell division and altered root growth (Mouchel et al., 2006). Normal BRX is required for *CPD* expression, as evidenced by exogenous BL application or by over-expression of CPD genes that convert the mutant root trait into the wild type one (Poppenberger et al., 2011). CESTA (CES), a basic helix-loop helix (bHLH) transcription factor, is a well-known trigger of CPD gene expression in *Arabidopsis*. CES binds to the CPD promoter region at the G-Box sequence elements and modulates its transcription.

Regulation of DWF4

Data from ChIP (chromatin immunoprecipitation) assays has shown that the expression of DWF4 can be regulated by TCP1 via direct or indirect binding to the DWF-4 promotor. TCP1 expression is up-regulated by BL treatment but down-regulated by the application of Brz (Guo et al., 2010).

D2, D11, and BRD1 Regulation

RAVL-1 and BRI-1 exhibit similar activity in loss of function and gain of function BR synthesis mutants. In-vivo and in-vitro experiments revealed that RAVL-1 bound to the B-box motif of the BR biosynthetic genes, *D2*, *D11*, *BRD-1*, and *OsBRI1*, and controlled their expression (Je et al., 2010).

Auxin-Mediated BR Biosynthesis

In Arabidopsis, auxin can induce the expression of DWF4. Treatment with the auxin, indole acetic acid (IAA), or its synthetic analog, 2,4-D, up-regulates the expression of DWF4 and other intermediates such as 22-OHCR and 22-OH-3, and typically enhances endogenous BR levels in roots (Chung et al., 2011; Yoshimitsu et al., 2011). Thus, auxin signaling is required for independent DWF4 regulation with respect to BR biosynthesis. BZR1 binds strongly to the BRRE motif on the DWF-4 promotor with or without BL treatment. The DWF4 promotor was found to have multiple regulatory sequences including one Aux/IAA response element (Aux/IAA-RE, TGTCTC) (Ulmasov et al., 1997), three BRRE motifs (Chung et al., 2011) and one Aux/IAA-RE-like element (TGTGCTC) (Hagen and Guilfoyle, 2002). The auxin response factor, ARF-7, also directly bound to the DWF4 promotor. At high auxin concentrations, the inhibitor of Aux/IAA was degraded with the release of ARF-7, which then bound to the DWF-4 promotor and initiated the expression of BR response and BR biosynthetic genes. It was also found that BZR1 left its binding site to accommodate the binding of ARF-7. CHX treatment masked the expression of DWF-4, which suggests that some other transcription factor must be required to mediate auxin-dependent DWF-4 regulation. One study conducted at a higher

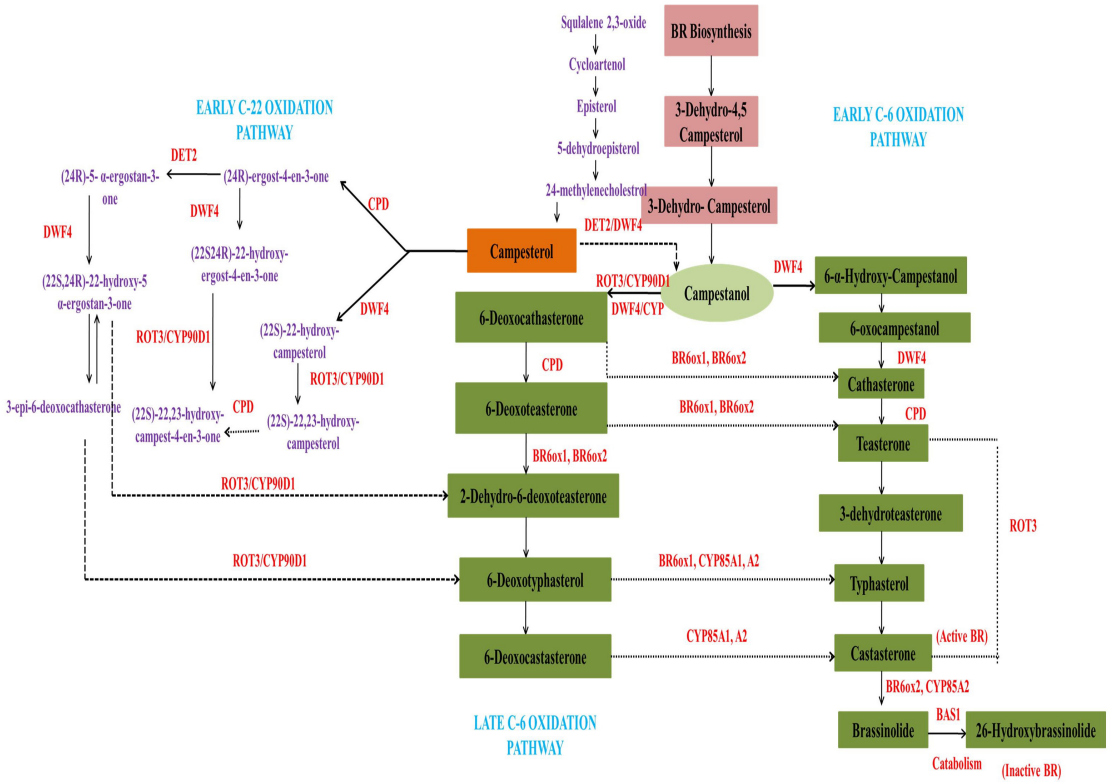

FIGURE 1 | Schematic representation of the BR biosynthetic pathway, showing the early C-6 oxidation pathway, the late C-6 oxidation pathway and the early C-22 oxidation pathway.

temperature (29°C) supported the hypothesis that auxin is involved in the expression of DWF-4 (Maharjan and Choe, 2011). Seedlings of a DWF-4 proGUS line showed increased DWF-4 gene expression at the higher ambient temperature. Hypocotyl elongation is stimulated by auxin, and higher temperature increases the synthesis and transport of auxins (Gray et al., 1998). Hypocotyl elongation was arrested in BR-biosynthetic mutants, and it was concluded that high BR levels were required for hypocotyl elongation under temperature stress (Nemhauser et al., 2004).

Role of BZR1 and Other Transcriptional Regulators

Brassinosteroids are recognized by the receptor kinase, BRASSINOSTEROID-INSENSITIVE 1 (BRI1), and the subsequent downstream signal cascade activates the brassinazole-resistant 1 (BZR1) family of transcription factors, which plays a vital role in brassinosteroid-regulated gene expression (Guo R. et al., 2013). When the levels of BRs are low, BZR1 is phosphorylated by the BRASSINOSTEROID-INSENSITIVE 2 (BIN2) GSK3-like kinase. As a result, its DNA-binding activity is lost and it remains in the cytoplasm. When BR levels are high, BZR1 is dephosphorylated by protein phosphatase 2A (PP2A). Activation of BRI1 results in a sequence of phosphorylation reactions which activates brassinosteroid-signaling kinases (BSKs), cytoplasmic kinase (CDG1) and members of the BRI1 Suppressor 1 (BSU1) family. This phosphatase activity leads to the inactivation of glycogen synthase kinase 3 (GSK3) through dephosphorylation of tyrosine. Dephosphorylated BZR1 is translocated into the nucleus where it regulates various target genes (Bai et al., 2012). It was documented in *Arabidopsis* that BRZ1 accumulated in the nucleus at elevated temperatures where it induced the expression of growth-promoting genes. BZR1 induced the expression of PIF4 by binding to its promoter and acting as a temperature-dependent positive growth regulator (Ibañez et al., 2018). A recent study revealed that organ size in *Arabidopsis* is regulated by BZR1. Enhanced expression of *Zea mays* BZR1 resulted in phenotypes of enlarged cotyledons, and increased size of floral organs, rosette leaves and seeds in transgenic *Arabidopsis* (Zhang et al., 2020).

BZR1 and BES1 have been documented as positive regulators of freezing tolerance. Gain-of-function mutants (bzr1-1D and bes1-D) of the transcription factors, BZR1 and BES1, showed increased tolerance to freezing, and the accumulation of dephosphorylated BZR1 upon cold treatment (Li et al., 2017). Chen et al., 2019 revealed that BZR1 was essential for locule development in *Arabidopsis*. Disruption of all *BZR*s resulted in generation of a hextuple mutant (*bzr-h*) which showed vegetative growth phenotypes that were similar to those of the BR receptor null mutant. Yin et al. (2018) demonstrated the role of BRZ1 in heat stress tolerance in tomato by regulating the feronia (FER)

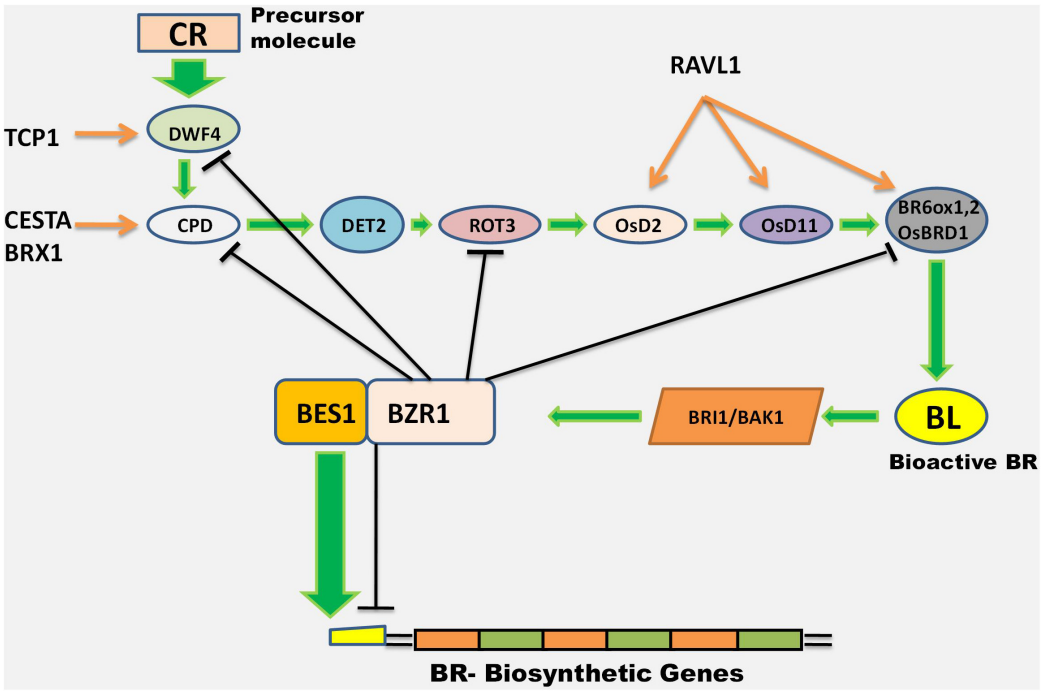

FIGURE 2 | Outline of the transcriptional regulation of BR biosynthetic genes. BZR1 controls the expression of these genes through a negative feed-back loop (shown by black arrows). BES1, another downstream transcription factor, positively regulates the expression of BR-biosynthetic genes (shown as green and brown arrows) (It is a conclusion diagram based on reports in corresponding section "Transcriptional Regulation").

homologs. Mutations in BZR1 impeded the respiratory burst oxidase homolog 1 (*RBOH1*) generation of hydrogen peroxide (H_2O_2) in the apoplast and heat tolerance. Addition of exogenous H_2O_2 restored the heat tolerance of the tomato *bzr1* mutant, and enhanced expression of BZR1 increased the generation of apoplastic H_2O_2 and improved heat stress tolerance.

Role of Biosynthetic Inhibitors

Brassinazole (Brz) was reported as the first brassinosteroid biosynthesis inhibitor. *Arabidopsis thaliana* treated with Brz showed the phenotype of a brassinosteroid mutant but application of brassinolide restored wild type activity (Nagata et al., 2000). The triazole compound, propiconazol (Pcz), is a potent BR metabolism inhibitor in plants. *Arabidopsis* seedlings treated with Pcz showed various BR-deficient phenotypes like reduced primary root growth, reduced size of cotyledons, and epinastic growth of cotyledons. However, seedlings co-treated with Pcz and 24-epibrassinolide showed improved root length compared to controls (Hartwig et al., 2012). YCZ-18 is another BR inhibitor that targets C23-hydroxylation in the BR biosynthetic pathway. Wild-type *Arabidopsis* plants grown in medium supplemented with YCZ-18 had short opened cotyledons. A decline in endogenous BR levels was also observed in *Arabidopsis* treated with YCZ-18 (Oh et al., 2015a). A pyrimidine-type fungicide, fenarimol, was also reported to inhibit BR biosynthesis in *Arabidopsis* and cause loss of etiolation and dwarfism in the dark (Oh et al., 2015b). An imidazole fungicide, Imazalil used as a post-harvest antifungal agent binds to CYP51 and inhibits fungal biosynthesis of ergosterol. In *A. thaliana*, hypocotyl shortening was observed with Imazalil treatment, which was reversed by application of 24-epibrassinolide (Rozhon et al., 2019). It has been reported by Bajguz, 2019 that brassinozole (BRz) decreases the level of BRs in the leaves of Barley and this effect is reversed by the application of BRs exogenously. Voriconazole, fenpropimorph, and fluconazole are also some of the BR inhibitors that inhibit the synthesis of cycloeucalenol-obtusifoliol isomers that is responsible for decreasing the synthesis of BRs (Rozhon et al., 2019).

ADVANCES IN BR SIGNAL TRANSDUCTION

The metabolic pathways associated with BRs are related to successive signaling networks. Therefore, an inclusive understanding of BRs homeostasis in plants is imperative for establishing a comprehensive view of BR signaling cascades (Chung and Choe, 2013). The BR-linked pathways have been elucidated over the past decades and the studies revealed a complex BR signaling network with a crucial role in proper growth and development of plants (Anwar et al., 2018). The signaling cascade initiated by BR was categorized into three steps: (i) BR recognition and early activation of BRI1 receptor kinases, (ii) inactivation of BIN2 inhibitors, phosphatases, and kinases, and (iii) regulation of transcriptional factors such as BES1 and BZR1 (Clouse, 2011).

Brassinosteroid Recognition and Early Activation of BRI1 Receptor Kinases

The BRs are initially recognized by brassinosteroid-insensitive 1 (BRI1) receptor kinase on the surface of the cells (Chakraborty et al., 2015). BRI1 is comprized of a leucine-enriched repeat receptor kinase with an extra-cellular domain that binds BRs and trans-phosphorylates signals. BRI1 is associated with a co-receptor, somatic embryogenesis receptor kinase (SERK), and belonging to a smaller LLRK family. BRI1 and SERK in combination form an active intricate complex that stimulates a downstream signal network involving activation of a wide array of kinases and phosphatases. Consequently, many transcription factors are activated which in turn alter the expression of specific genes (Belkhadir et al., 2012). Exogenously supplemented BRs bind to BRI1 and induce an association with BRI1-associated receptor kinase 1 (BAK1) and a disassociation of BRI1 kinase inhibitor 1 (BKI1). BKI1 is a membrane-bound negative regulator of BR signals, which combines with BRI1 and is responsible for prevention of co-receptor interference. It has been widely reported in *Oryza sativa* and *A. thaliana* plants (Jiang et al., 2015). Another protein, OsREM4.1, has similar functions to BRI1. It was identified in *O. sativa* and has corroborated that disruption of the BRI1/SERK complex suppresses downstream BR signaling. The expression of OsREM4.1 was positively modulated by ABA and elevated in response to increased ABA levels, and showed significant participation of the bZIP transcription factor (Gui et al., 2016). Various studies support the hypothesis that BRI1 is a multi-function kinase with the ability to phosphorylate Ser, Thr, and Tyr residues and it was affirmed that phosphorylation of Tyr residue is vital for specific aspects of BR signaling in plants (Oh et al., 2009). BRI1 was localized to the plasma membrane as a homodimer. It is ligand-independent and responsible for stabilization and initiation of BR binding and signaling (Albrecht et al., 2008). BAK1 (also termed SERK3) has been associated with phosphorylation of BRI1 and BAK1 both in-vivo and in-vitro (Wang et al., 2008). SERK4, also termed as BAK1-like (BKK1), has been shown to interact with BRI1 in-vivo in a BR-dependent manner (He et al., 2007). Another LRR RLK, FLS2, interacts with BAK1 and augments its function in plant defense (Bar et al., 2010). Karlova et al. (2006) observed a significant role for SERK1 in embryogenesis where it heterodimerizes with BRI1 and augments BR signaling.

In order to expound the entire mechanism of phosphorylation and oligomerization of BRI1 and BAK1 in response to exogenously supplemented BR, Wang et al. (2008) expressed various combinations of kinase-inactive and wild-type *Arabidopsis* BRI1 and BAK1 in the same transgenic plant. It was concluded that an active BRI1 was responsible for BR-dependent association of the pair, and not BAK1 kinase. Additionally, it was observed that when BAK1-green fluorescent protein (GFP) was highly expressed in the bri1-1 null mutant background, the phosphorylation was drastically lowered in BAK1-GFP. In-vitro studies indicated that BAK1 positively triggered BRI1 activity and that they can transphosphorylate each other at specific residues (Wang et al., 2008). The above studies with LC-MS/MS assays, biochemical analysis and functional characterization have aided in development of a new sequential transphosphorylation model of BRI1/BAK1 interaction. These studies further suggested that plant receptor kinases have certain properties similar to those of TGF-b and RTKs receptor kinases present in mammals, although they have plant-specific characteristics (Wang et al., 2008). Similar to BRI1, Tyr residue are also autophosphorylated and mutation studies revealed that Tyr phosphorylation regulated the expression of a BAKI subset in-vivo (Oh et al., 2010).

Inactivation of BIN2 – Role of Inhibitors, Phosphatases, and Kinases

Increased BR signaling activity results in de-activation of brassinosteroidinsensitive-2(BIN2) kinase, which is considered the principal effect of BR signaling (Chung and Choe, 2013). Sequence analysis of BIN2 revealed significant similarity with mammalian glycogensynthasekinase3 (GSK3). There was also similarity to a member of a small family of ten related genes identified in *Arabidopsis* that have an essential role in BR signal transduction (Rozhon et al., 2010). The inactivation of BR results in activation of two related transcription factors, BRI-EMS suppressor-1(BES1) (Yin et al., 2005) and brassinazole-resistant 1 (BZR1) (He et al., 2005). BES1 is also known as BZR2 (Wang et al., 2002). More recent studies of downstream BR signaling in *Arabidopsis* have elucidated the association between BRI1 and BAK1 activation (Kim and Wang, 2010). Another observation by Wang and Chory (2006) using a yeast two-hybrid screen for BRI1 integrators suggested that BRI1 kinase inhibitor-1 (BKI1) acted as a negative modulator of BR signaling. In the absence of BAK1 it bound to BRI1 and inactivated its function and prevented further binding to BAK1.

Exogenous BR treatment results in dissociation of BKI1 from the outer cell surface and further inhibits the BR signaling network. Association of BRI1 and BKI1 was demonstrated in-vivo and in-vitro, which resulted in BKI1 phosphorylation and interaction with recombinant BRI1-CD in vitro. More recent observations showed that BKI1 interacted with BRI1 through a C-terminal 20-residue conserved segment (Jaillais et al., 2011). They additionally indicated that a peptide surrounding this binding site suppressed the association of BRI1 with BAK1. A Lys-Arg-enriched domain present within the BKI1 directed the protein toward the plasma membrane. This localization consequently phosphorylated Tyr-211 within the motif and released BKI1 from the plasma membrane in a BRI1- and BR-dependent manner. Over-expression of a BKI1-Y211F mutant construct in transgenic *Arabidopsis* consequently resulted in extremely dwarfed plants. This protein was reportedly membrane bound and found at the same location even after BR supplementation. This observation suggests that upon recognition, BR phosphorylates BRI1 on Tyr-211, resulting in its disassociation from the membrane. This permits BRI1 to freely associate with BAK1 and initiate BR signaling (Lemmon and Schlessinger, 2010; Lim and Pawson, 2010).

A proteomic screening of BR-modulated proteins identified a plethora of members of receptor-like cytoplasmic kinase families such as RLCK-XII, specifically BR-signaling kinases (BSKs). BSKs

are a direct substrate of BRI1 and up-regulate BR signaling (Tang et al., 2008). BSKs have a presumed trans-membrane sequence with N-myristylation sites that are probably responsible for directing their membrane localization. Additionally, BSK1 and BSK3 have been observed to interact with BRI1 in-situ in the absence of other ligands. BRI1 phosphorylated BSK1, most likely on Ser-230, resulting in activation and its release from the receptor complex (Tang et al., 2008). This phosphorylated BSK1 interacted with BRI suppressor-1 (BSU1) phosphatase which then negatively modulated BIN2 (Kim et al., 2009). Ample biochemical and genetic evidence affirmed that BSK1 binding to BSU1 activated BSU1-mediated dephosphorylation of phosphoTyr-200 in BIN2, resulting in suppression of the BR signaling cascade (Kim et al., 2009).

Regulation of Transcriptional Factors, BES1 and BZR

Brassinazole-resistant 1(BZR1) and BRI 1-EMS suppressor-1 (BES1) are the chief transcriptional factors activated when BR binds to BRI1. BZR1 and BES1 (also known as BZR2) regulate a number of genes involved in various physiological processes (Peres et al., 2019). BIN2 (brassinosteroid insensitive-2), a GSK3/serine-threonine protein kinase, plays a key role in regulation of BZR1 AND BES1. In the BR-mediated signaling cascade, BIN2 acts as negative regulator by repressing the activity of BZR1 and BES1. In the absence of BR signaling, cytoplasmic CDG1 and BSKs interact with inactive BRI1 to prevent binding of BAK1 to the receptor. BES1 and BZR1 are phosphorylated by BIN2, and the phosphorylated transcription factors are expelled from the nucleus and retained in the cytoplasm by 14-3-3 proteins (Chung and Choe, 2013). The dephosphorylated BIN2 may be involved in degradation of BZR1 and BES1. Concomitantly, in the presence of BR, BRI1 recognized the signal followed by association with BAK1 and disassociation from BKI1. Auto- or trans-phosphorylation of BRI1 and BAK1 activates the main receptor, which is followed by phosphorylation of BSKs and CDG1. Activated BSKs and CDG1 stimulate BSU1 phosphatase, which dephosphorylates the major repressor protein, BIN2. Inactivation of BIN2 positively affects BZR1 and BES1 by terminating their degradation. In the absence of BIN2, phosphatase 2A dephosphorylates BZR1 and BES2 in the cytoplasm. The dephosphorylated transcription factors are than translocated to the nucleus where they affect expression of various BR-mediated genes. The mechanism of BRs signaling is illustrated in the **Figure 3**.

INTERACTION OF BES1 AND BZR1 WITH OTHER TRANSCRIPTIONAL FACTORS–INTEGRATION OF SIGNALING NETWORKS

In the BR-mediated signaling cascade, BES1 and BZR1 interact with the target genome product and play important roles in regulating gene expression. BES1/ BZR1 can act as an inducer as well as a repressor in the BR signaling pathway. BES1 binds to the CANNTG sequences (E-box) to stimulate gene expression, whereas BZR1 interacts with the CGTGT/CG sequences (BRRE) to suppress gene expression (He et al., 2005). MYB30, a BES1 target interacts with BES1 to strengthen BR induced signals. MYBL2 also functions as an important regulator in BR signaling. It is phosphorylated and stabilized by BIN2 kinase, thus playing an effective role in the transcriptional process of BR (Ye et al., 2012). Studies revealed that in the BR-repressed condition, there were more BRRE expressed, whereas E-boxes showed enhanced expression in BR-induced genes (Yu et al., 2011). The E-box interacts with BES1 and different transcription factors and cofactors. Interactions between BES1, PIL6, GLK1, and GLK2 have been associated with the transcriptional network pathway that triggers BR-induced gene expression. BZR1 interacted with PIF4 (a target gene product) to form a heterodimer that recognized a promoter element, CACGTG (G-box) (Oh et al., 2012). Interaction of BZR1 and PIFs are very important for normal growth of hypocotyls. Evidence has been obtained by studying mutants of PIF4 and the homologs (pif1 pif3 pif4 pif5), which showed reduced plant growth in darkness and inhibition of BR-induced growth. Plants have up to 2,000 target genes common for both BZR1 and PIF4, which also have various PREs involved in cell elongation (Guo H. et al., 2013). In the GA signaling pathway, in the absence of GA, the DELLA protein is stably localized to the nucleus and binds to PIFs and BZR, impeding the genome binding activity. An active GA signal leads to degradation of the DELLA protein thereby activating PIF and BZR, which target specific genes (Liu et al., 2018). Pioneer studies have revealed that in inhibition of chloroplast BES1 plays a role through GLK1 and GLK2. Inhibition of chloroplasts causes enlarged plastoglobules and other alterations in the structure of the organelle (Yu et al., 2011).

ROLE OF BRs IN PLANTS UNDER HEAVY METAL STRESS

Plants survival is threatened in several ways but heavy metal stress is one of the most important concerns in agricultural research. The effects of metal contamination on plants is primarily ameliorated by hormones such as the BRs, that scavenge reactive oxygen species (ROS) and activate the antioxidant defense enzymes, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APOX), glutathione reductase (GR), guaiacol peroxidase (GPOX), and glutathione-S-transferase (GST) (Bücker-Neto et al., 2017). BRs help the plant to become metal-tolerant, thereby increasing crop yield and quality (Vriet et al., 2012). The bioactive BRs, 28-homobrassinolide (HBL) and 24-epibrassinolide (EBL), are part of a system called assisted phytoremediation, which helps the plant to eliminate toxic metals (Barbafieri and Tassi, 2011). BRs reduce the uptake of heavy metals by altering cell membrane permeability and also induce a group of defensive enzymes. They stimulate the production of stress-proteins through actions on anti-stress genes due to increased expression of ATPase (Madhan et al., 2014). External application of BRs induces transient H_2O_2 formation, which activates MAPK, leading to

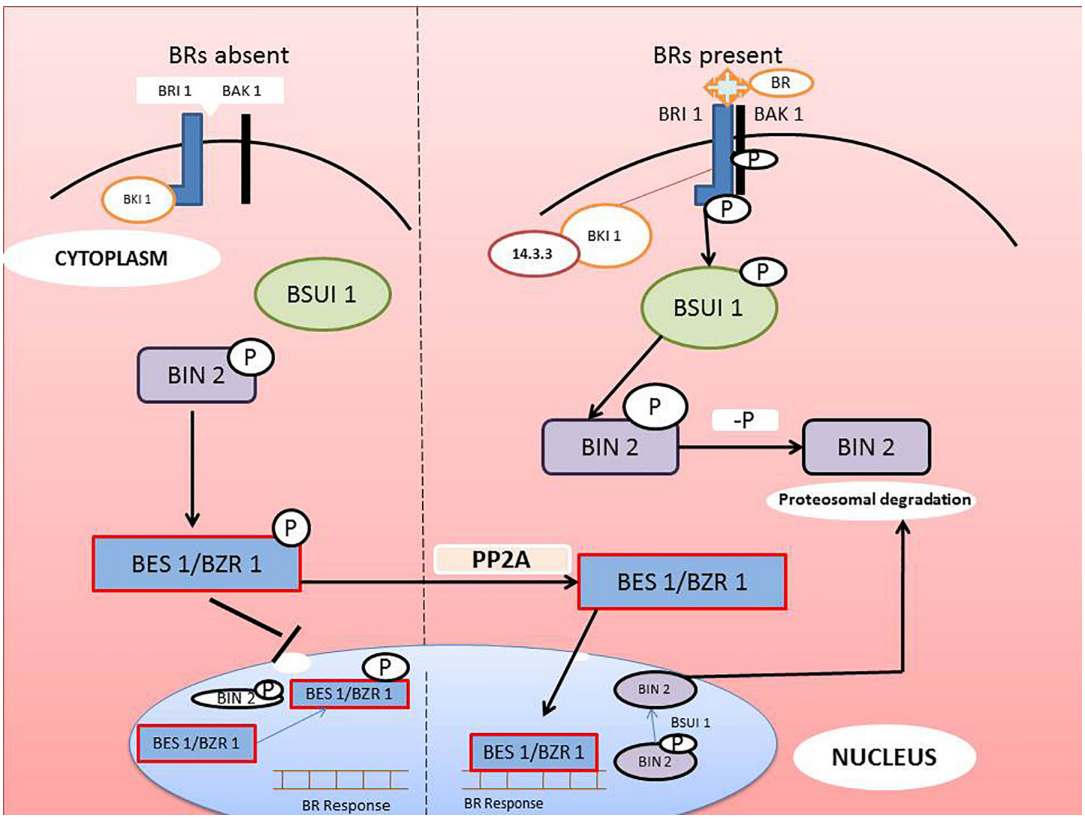

FIGURE 3 | Molecular mechanism of BRs signaling from recognition at the cell surface to transcriptional activation of specific genes in the nucleus (modified from Chung and Choe, 2013).

the production of NADPH oxidase (Jiang et al., 2012) and upregulation of stress-proteins and defensive enzymes that curtail the metal stress (Yin et al., 2016). Jiang et al. (2012) suggested that low doses of EBR (0.1–0.15 μM) suppressed photosynthetic efficiency. According to Xia et al. (2014), 0.1 μM EBR stimulated stomatal opening while 1.0 μM EBR facilitated the closing of stomata (**Figure 4**).

The mechanism of regulation of heavy metal toxicity via BRs involves: (a) stimulating H_2O_2 production, (b) scavenging ROS through boosting the defensive antioxidant system, (c) up-regulating MAPK expression, and (d) alleviating metal toxicity by increasing the concentration of potassium and sodium ions, proline, antioxidants, and osmolytes (Rajewska et al., 2016). Some reports indicated that NADPH oxidase was an important apoplastic source of H_2O_2 from conversion of O_2^- by superoxide dismutase in the plasma membrane of plant cells, which further increased H^{++} ATPase activity via upregulation of the CsHA gene (Jakubowska et al., 2015). Thus, while uptake of toxic metals can negatively affect plant cell membranes by ROS peroxidation of lipids and oxidation of proteins, phytohormones like BRs boost the level of antioxidants and defensive enzymes to ameliorate this toxicity and restore normal osmoregulation (Shahzad et al., 2018). The BR-specific inhibitor, brassinazole (Brz), and the bioactive brassinosteroid, 24-brassinolide (EBL), were applied for alleviating the toxicity of metals, and restoring the photosynthetic machinery and defense system of *A. thaliana* (Wu et al., 2019). BRs are vital for plant cellular homeostasis. They restore CO_2 absorption and enhance antioxidant capacity, thus overcoming the toxic effects of heavy metals (Ahammed et al., 2020). BRI1 is a BR receptor on the plasma membrane (Nolan et al., 2020) that activates the signaling cascade up-regulating the expression of transcription factors, which enhance the transcription of brassinosteroid genes (Tong and Chu, 2018; Planas-Riverola et al., 2019). Increased expression of these genes enhances the endogenous level of BRs that help in mitigating metal stress (Xia et al., 2018), but the mechanism remains unclear. **Table 1** summarizes the role of brassinosteroids in regulation of physiological and biochemical responses of plants growing under metal toxicity.

BRs CROSSTALK WITH OTHER PHYTOHORMONES UNDER HEAVY METAL STRESS

Brassinosteroids play a diverse and vital role in regulating plant metabolism because of their synergy with other plant hormones such as auxin, cytokinins (CK), ethylene, polyamines (PA), gibberellins (GA), salicylic acid (SA), JA, and ABA (Ohri et al., 2019). The bioactive BRs (HBL, EBL) can protect the plant from

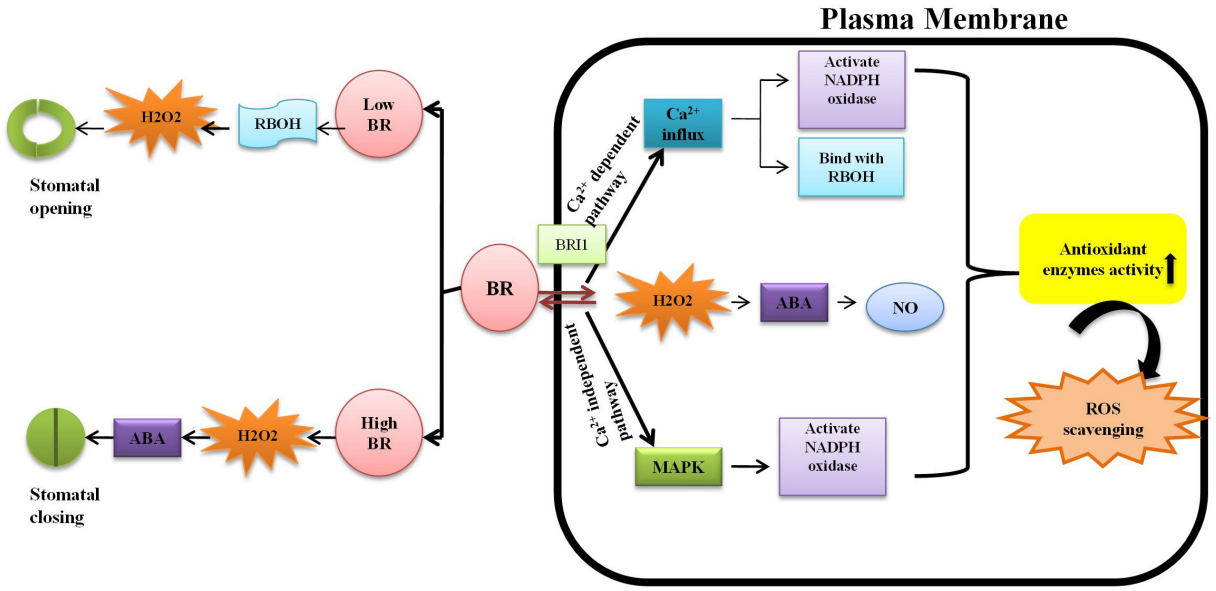

FIGURE 4 | Different doses of Brassinosteroid regulates the opening and closing of stomata: at low concentration of BR activate respiratory burst oxidase homolog (RBOH) which further increases the level of H_2O_2 resulting in opening of stomata whereas at high concentration, it causes the accumulation of H_2O_2 and also reducing the uptake of K^+ ions in guard cells lead to the activation of abscisic acid (ABA) cause closure of stomata (Daszkowska-Golec and Szarejko, 2013). Brassinosteroid hormone received by a specific receptor, brassinosteroid insensitive1 (BR1), on plasma membrane. Furthermore, there is increase in Ca^{2+} infux that activates the NADPH oxidase and then bind with EF-motif of RBOH enhancing the production of ROS (Xia et al., 2015); another pathway is upregulation of mitogen-activated protein kinase (MAPK) which further scavenge the reactive oxygen species (ROS); BR also stimulates the production of nitric oxide (NO) resulting the enhancement in antioxidative defense system (Zhang et al., 2011).

toxic metals by assisted phytoremediation (Barbafieri and Tassi, 2011). BRs reduce the uptake of toxic metals by altering cell permeability and reduce damage by activating defensive enzymes. The mechanism of BRs signaling and its interplay with other hormones at the molecular level (Peres et al., 2019) is illustrated in **Figure 5**.

Brassinosteroids and Ethylene

Brassinosteroids stimulate the synthesis of ethylene by activating the expression of ACO (1-aminocyclopropane-1-carboxylic acid oxidase) and ACS (1-aminocyclopropane-1-carboxylic acid (ACC)-synthase enzyme) genes (**Figure 5**). BR regulates ethylene synthesis at the transcriptional and post-transcriptional level by increasing the ACS5 protein half-life (Hansen et al., 2009). BR regulates ethylene biosynthesis negatively or positively via a dose-dependent pathway (Lv et al., 2018). Some reports showed that exogenous application of BR enhanced the ripening of banana (*Musa acuminata* L.) by elevating the expression of MaACS1, MaACO13, and MaACO14 genes and regulating ethylene production (Guo et al., 2019). BR acts at the post-transcriptional level by increasing the expression of ACS2 and ACS4 in *Solanum lycopersicum* (Zhu et al., 2015). BR also stabilizes other ACS proteins like ACS6, ACS9 and ACS5 by degrading the 26S proteasome (Hansen et al., 2009). BR indirectly regulates the hyponastic growth by regulating expression of the ROT3/CYP90C1 gene to increase ethylene synthesis (Polko et al., 2013). The overproduction of BR genes (BRI1 and DWARF) enhances the biosynthesis of ethylene in tomato (Li and He, 2016; Nie et al., 2017).

Brassinosteroids and Gibberellins

The interplay between BRs (BZR1/BES1) and gibberellin (DELLA) genes is complex (Sun et al., 2010; Sun, 2011; Tong et al., 2014; Hu et al., 2017), reflecting interaction with both proteins and DNA (Li Q.F. et al., 2018; Li W. et al., 2018). The exogenous application of BR activates BZR1, which upregulates the expression of GA20ox genes and enhances GA production (Stewart Lilley et al., 2013). The DELLA gene has an inhibitory effect on the transcriptional activity of BZR1, but GA induces the degradation of DELLA (Bai et al., 2012). Therefore, both GA and BR affect expression of the target gene, BRZ1 (**Figure 5**). Thus, BES1/BRZ1 promotes production of GA, resulting in greater degradation of DELLA (Ross and Quittenden, 2016). There is direct interaction between DELLA and BZR1 that inhibits the binding of BZR1 to DNA. This suppresses the signal that is required for maintaining the etiolation of seedlings and elongation of cells (Li and He, 2013). The level of ROS-scavenging enzymes increases because of the interaction between DELLA protein and BRZ1 under heavy metal stress (Achard et al., 2008).

Brassinosteroids and Auxins

Both BRs and auxins are considered "master regulators" with synergistic effects on plant growth and development (Chaiwanon and Wang, 2015). BIN2-mediated phosphorylation reduces the

TABLE 1 | Some of the reports studied on role of brassinosteroids under heavy metal stress.

S.no.	Plant species	Metal concentration	Brassinosteroid concentration	Effect	References
1	*Brassica juncea*	150 mg/kg Mn	10^{-8} M EBL	Enhances ROS production, increases photosynthesis rate, restores stomatal opening and reduces electrolyte leakage	Hussain et al., 2019
2	*Glycine max* L.	20 μM Zn	100 nM EBL	Improves photosystem II; mitigates zinc stress by boosting antioxidant system and nutritional content; restores chloroplast membranes	dos Santos et al., 2020
3	*Vitis vinifera* L.	2 gL^{-1} Zn ($ZnSO_4 \cdot 7H_2O$)	0.4 mg L^{-1} EBL	Increases photosynthetic rate and promotes grape productivity	Tadayon and Moafpourian, 2019
4	*Pisum sativum*	150 mg L^{-1} Cd	10^{-7} M EBL	Decreases methylglyoxal and hydrogen peroxide; alleviates electrolyte leakage; enhances glyoxylase I content and nutrient uptake by roots and shoots	Jan et al., 2018
5	*Brassica juncea* L.	2 mM Pb	10^{-8} M EBL	Eliminates Pb toxicity and increases protein content by reducing H_2O_2 and MDA	Dalyan et al., 2018
6	*Vitis vinifera* L.	120 μM Cu	0.10 mg L^{-1} EBL	Stimulates antioxidant system and alleviates oxidative damage by up-regulating activity of AsA-GSH cycle	Zhou et al., 2018
7	*Solanum lycopersicum*	3 and 9 mg kg^{-1} Cd	10^{-8} M HBL	Mitigates toxic effects of Cd on solanum seedlings by enhancing enzymes of photosystem II, carbohydrate and nitrogen assimilation	Singh and Prasad, 2017
8	*Arabidopsis thaliana*	50 μM Sb	10^{-3} M EBL	Mitigates toxic effects of Sb by activating antioxidant system	Wu et al., 2019
9	*Cucumis sativus*	10 μM Cd ($CdCl_2$)	10 nM EBL	Enhances NADPH oxidase activity which causes accumulation of hydrogen peroxide and activation of the antioxidant system against Cd stress	Jakubowska and Janicka, 2017
10	*Solanum nigrum*	100 μM Ni($NiSO_4 \cdot 6H_2O$)	1 μM EBL	Raises Ni stress tolerance in *Solanum* by enhancing SOD activity; but, can cause down-regulation of APOX and CAT	Soares et al., 2016
11	*Oryza sativa* L.	0.5 mM Cr	0.1 nM EBL	Upregulates expression of CAT, APOX, and GR, thus increasing metal tolerance level in rice seedlings	Sharma P. et al., 2016
12	*Cajanus cajan* (L.) *Millsp.*	7.5 mM Al^{3+}	0.5–2 μM EBL	Restores cellular homeostasis by reducing ROS and alleviating aluminum toxicity	Madhan et al., 2014
13	*Vigna radiata*	200 mg kg^{-1} Zn	10^{-8} M EBL	Improves plant growth by enhancing antioxidant activity	Mir et al., 2015
14	*Brassica juncea*	3 mM Mn	10^{-8} M EBL	Upregulates antioxidant defense system and photosynthetic efficiency of *Brassica* seedlings	Fariduddin et al., 2015
15	*Helianthus annuus*	80 μM Cu ($CuSO_4 \cdot 5H_2O$)	100 μM EBL	Increases growth and metabolism of sunflowers	Filová et al., 2013
16	*Phaseolus vulgaris* L.	1 mM Cd^{2+} ($CdCl_2$)	5 μM EBL	Increases activity of POD, GR, SOD, and CAT; down regulates MDA activity	Rady, 2011
17	*Raphanus sativus* L.	5 mM Zn($ZnSO_4.7H_2O$)	2 μM HBL/EBL	Upregulates expression of SOD, APOX, POD, GR, and CAT; increases proline and chlorophyll content; restore nitrate reductase level in radish plants	Ramakrishna and Rao, 2015

(Continued)

TABLE 1 | Continued

S.no.	Plant species	Metal concentration	Brassinosteroid concentration	Effect	References
18	*Brassica juncea*	0.75, 0.25, and 0.5 mM Cu	10^{-7} M and 10^{-9} M 24-EBL	Enhances antioxidant enzyme activity and reduces copper toxicity	Poonam et al., 2014
19	*Brassica juncea*	0.2 mM Cd	Endogenous	Upregulates 28-homobrassinolide in Brassica seedlings; enhances antioxidant enzyme production	Kapoor et al., 2014
20	*Vigna radiata*	100 or 150 mg kg^{-1} $Ni(NiCl_2)$	10^{-6} M EBL	Activates peroxidase, catalase and superoxide dismutase; boosts proline content for nodulation and growth	Yusuf et al., 2012
21	*Raphanus sativus* L.	1.2 mM Cr(VI) (K_2CrO_4)	10^{-9} M EBL/1 mM spermidine	Stimulates production of plant hormones, IAA and ABA, and antioxidant enzymes, GR, SOD, CAT, and GPOX; increases content of proline, sugars, phytochelatins and pigments by decreasing MDA and H_2O_2	Choudhary et al., 2012a
22	*Raphanus sativus*	0.25 mM Cu ($CuSO_4{\cdot}5H_2O$)	10^{-9} M EBR/1 mM spermidine	Increases seedling growth; boosts antioxidant system by up-regulating phytohormones, IAA and ABA	Choudhary et al., 2012b
23	*Solanum lycopersicum*	3–10 mg/kg Cd	10^{-8} M EBL/HBL	Increases antioxidant enzymes and photosynthetic pigments	Hayat et al., 2012
24	*Lycopersicon esculentum*	0.5-1.5 nM	10^{-7}, 10^{-9},	Enhances Cd tolerance by up-regulating antioxidant system and protein content; decreases PPO and GST activity.	Sharma et al., 2014
		Cd+Hg	10^{-11} M HBL		
25	*Brassica juncea* L.	2 mM Ni	10^{-9} M EBL 10^{-11} M EBL	Increases DHAR and GR activities of MDHAR and SOD enzymes	Kanwar et al., 2012
26	*Lycopersicon esculentum*	3–12 mg/kg Cd	10^{-8} M EBL/HBL	Restores photosynthetic efficiency; boosts antioxidant defense response against Cd stress	Hasan et al., 2011
27	*Raphanus sativus* L.	1.0 mM Ni	10^{-9} M EBL	Mitigates metal-induced oxidative damage and activates antioxidant enzymes	Sharma et al., 2011
		1.0 mM Ni	10^{-7} M EBL		
		1.5 mM Ni	10^{-9} M EBL		
28	*Triticum aestivum*	50 and 100 μM Ni	0.01 μM HBL	Improves antioxidant system and upregulates CAT, POD, and SOD	Yusuf et al., 2011
29	*Lycopersicon esculentum*	100 or 200 μM	2 μM EBL	Increases expression of GSH, AsA and defense enzymes like SOD, GR, CAT, and APOX	Rady and Osman, 2012
		Pb^{2+} /Cd^{2+}			
30	*Eucalyptus urophylla*	2.5 μM Fe (deficiency)	100 nM EBR	Increases consumption, transport and accumulation of iron (Fe) and other micronutrients in roots, leaves and stems	Lima et al., 2018
		250 μM Fe (control)			

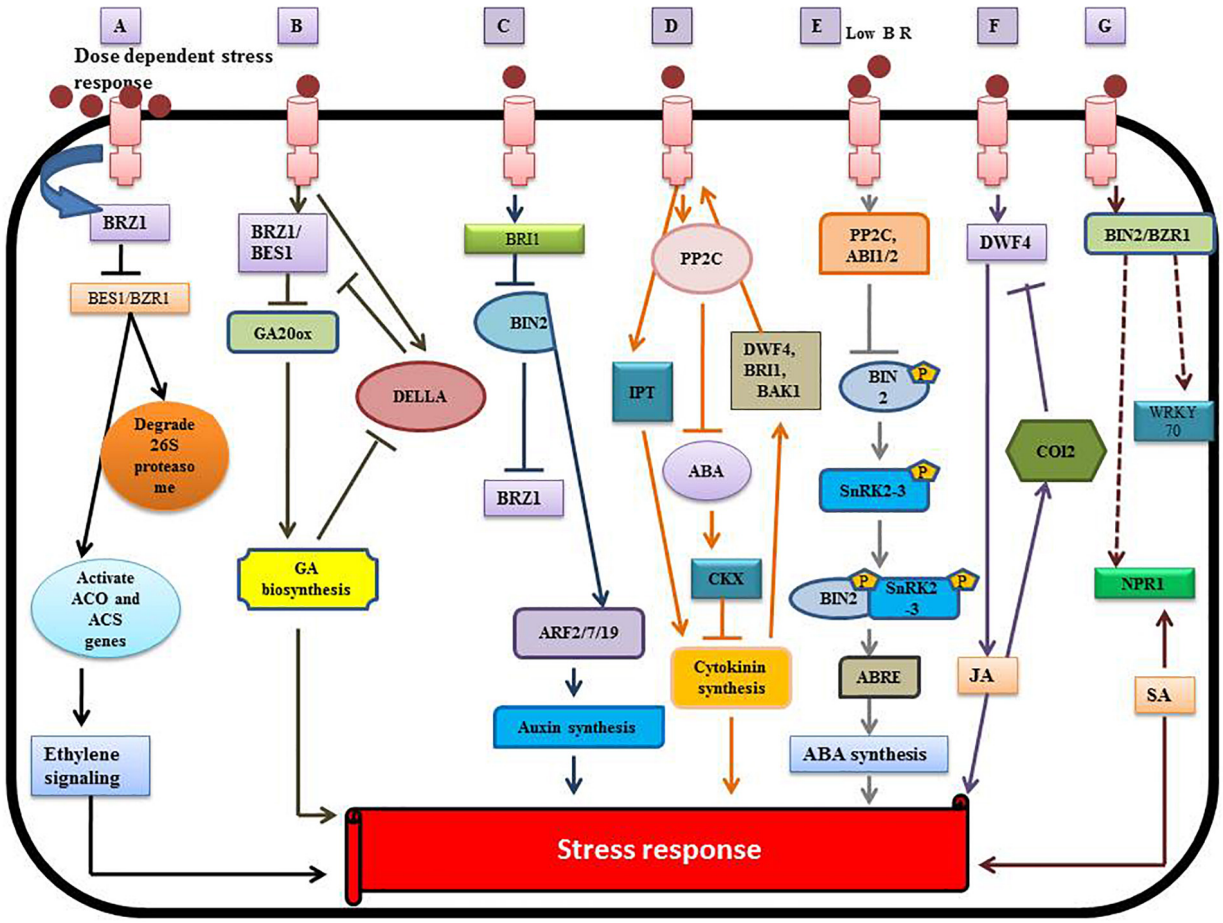

FIGURE 5 | Crosstalk between BRs and other phytohormones. **(A)** Interplay between BR and ethylene. **(B)** Interaction between BR and gibberellin (GA). **(C)** Brassinosteroid – auxin crosstalk. **(D)** Brassinoster oid-cytokinin interaction. **(E)** Brassinosteroid-Abscisic acid (ABA) interplay. **(F)** Brassinosteroid-jasmonic acid (JA) crosstalk. **(G)** Brassinosteroid-salicylic acid (SA) [modified from Saini et al. (2015), Peres et al. (2019), and Ohri et al. (2019)], involving the regulation of various transcription factors.

inhibitory effect of auxin response factors (ARF2) (Vert et al., 2008) that leads to the enhancement of ARF promoter activities and stimulates the expression of BR-regulated genes promoting auxin synthesis. The indole-3-acetic acid/auxin (IAA/AUX) genes are also involved in BR-regulated auxin production (Li Q.F. et al., 2018; Li W. et al., 2018). The expression of genes associated with auxin transport such as PIN3, PIN4, PIN7, and LAX are suppressed by the BR signaling pathway (Nemhauser et al., 2004). This crosstalk suggests an interplay between auxin and BR in heavy metal tolerance through the auxin transport genes. Wang et al., 2015 reported that the movement and local concentration of auxin was regulated by the expression of CYP79B2, the ABCB family, Yucca (YUC), the PIN family, the Gretchen Hagen (GH3) genes, and phosphoribosyl anthranilate transferase 1 (PAT1), in response to heavy metals (Hacham et al., 2012). The auxin secretion transporters, PIN7 and PIN4, that regulate the movement and distribution of auxin, are managed by BES1 (Paponov et al., 2005). In the root elongation zone, BIN2 stimulates the post-transcriptional modification of ARF, and BZR1 transcriptionally activates ARF expression and auxin-responsive genes (Tian et al., 2018). Both ARF and auxin-related genes were suppressed by BZR1 in the quiescent region of the root (Chaiwanon and Wang, 2015). It has been reported in *A. thaliana* that auxin treatment increases DWF4 expression, which stimulates production of BR through the auxin-induced Bravis radix (BRX) gene (Chung et al., 2011); there is feedback inhibition of DWF4 by BR. Several reports discussed the adversary aspect of auxin and BR in controlling BR synthesis genes and the DWF4 gene (Maharjan et al., 2011).

Brassinosteroids and Cytokinins

Cytokinins (CKs) are adenine-derived compounds, which regulate the plant growth processes under abiotic stress (Perilli et al., 2010). CKs stimulate the defense mechanism that mitigates heavy metal toxicity and restores the photosynthetic apparatus. Piotrowska-Niczyporuk et al., 2012 reported the alleviation of Cd toxicity by enhancing photosynthesis efficiency and the level of primary metabolites. Two key enzymes regulate the biosynthesis of CKs: isopentenyltransferases (IPTs) which

promote synthesis of CKs and CK oxidase/dehydrogenases (CKXs) which suppress genes involved in CK synthesis. Both enzymes target the responses mediated by BR (Werner and Schmülling, 2009). Exogenous application of BR leads to overexpression of CKX3 and ectopic expression of BRI1, which increases the leaf and root length. This suggests crosstalk between BR and CKs, which may be involved in enhancing crop yield (Yuldashev et al., 2012). Some reports showed that interaction between BR and CKs resulted in the accumulation of anthocyanins (Yuan et al., 2014). ABA is also involved in the interaction between BR and CKs, as it suppresses BR synthesis during metal toxicity (Zhang et al., 2009). Therefore, there are three hormones interacting in a complex way that remains unclear.

Brassinosteroids and Abscisic Acid

Abscisic acid is known to be a "sensing element" for abiotic stress that protects the plant from various kinds of stress (Fujii and Zhu, 2009). The BR and ABA interplay depend upon the regulation of gene expression and modulation of protein activity. The complex formed by the merging of histone deacetylase19 (HDAC19), topless (TPL/TPR) and BRI1-EMS suppressor1 (BES1) affects the E-box promoter causing suppression of ABA insensitive 3 (ABI3) gene expression in the presence of BR. There is also repression of ABI3 and ABI5 gene expression through the interaction of the BZR1 transcription factor with the ABI5 G-Box promoter sequences. This reduces the stress response by down regulating the ABA-regulated gene expression. BIN2 promotes activation of SnRK2.3 that stimulates the stress response at low levels of BR. ABA-related gene expression is upregulated through phosphorylation of the ABI5 transcription factor by BIN2 (Wang et al., 2018). Some reports also showed auto-stimulation of downstream expression of ABA-related SnRK2s genes and kinase activity (Belin et al., 2006; Yunta et al., 2011).

Brassinosteroids and Jasmonic Acid

CORONATINE INSENSITIVE 1 (COI1) is an F-box protein responsible for JA signaling and responses in *A. thaliana* (Yan et al., 2009; Peng et al., 2011) reported that blocking JA signaling caused an accumulation of anthocyanins in *Arabidopsis* under the influence of brassinazole. Upregulation and downregulation of JA transcript factors as well as signaling genes depended upon the relative concentration of BR in plants (Peng et al., 2011). The exogenous application of JA decreased expression of the BR signaling gene, OSBRI1 and the BR biosynthesis gene, OsDWF4, revealing the interplay between JA and BR in *O. sativa* (Nahar et al., 2013).

Brassinosteroids and Salicylic Acid

Activation of systemic acquired resistance (SAR) is a putative role of SA during abiotic and biotic stress (Roychoudhury et al., 2016). Divi et al., 2010, studied the induction of BR-mediated stress tolerance by regulating BIN2 and BZR1. During the stress response, the OsWRKY45 transcription factor was expressed in the presence of SA (Huangfu et al., 2016). Some reports revealed that the metabolite composition was affected by the cumulative action of both SA and BR (24-epibrassinolide) leading to a decline in lead toxicity (Kohli et al., 2018). The interplay between BR and SA may be due to the NPR1 gene (non-expressor of pathogenesis-related genes 1) which stimulates expression of the SA-related genes involved in plant defense (Ohri et al., 2015). NPR1 also regulates the BR signaling genes, BIN2 and BZRI, which induces stress tolerance in plants (Divi et al., 2010).

Brassinosteroids and Polyamines

Polyamines are associated with disease and the aging process. Previous studies reported that PAs did not affect the biosynthesis and signaling of BR (Anwar et al., 2015), butco-treatment with BR and PAs improved tolerance against copper stress. Choudhary et al. (2012b), reported enhanced Cu tolerance in radish through exogenous application of EBR and spermidine (Spd), which decreased the uptake of Cu and upregulated the expression of RsCOPT2 and RsCOPT1 genes. BR controls the spermidine level in plants, which further raises the concentration of putrescine as required for stress tolerance as well as diminishing cadaverine levels to counterbalance the oxidative burst (Takahashi and Kakehi, 2010). Pál et al., 2017, showed that in cadmium toxicity, Pas caused phytochelatin production in *O. sativa*. Recent reports suggest that the crosstalk between BR and PAs requires modulation of the expression of various enzymes associated with PA synthesis and its interplay with other plant hormones. As summarized in recent findings, this crosstalk between BRs and other phytohormones as well as their interaction showed both positive and antagonistic effects in response to stress.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we have discussed the molecular mechanisms involved in the biosynthesis of the BRs, the roles of various transcriptional factors on gene expression, the interactions with auxin and other molecules, and the modulating effects of BES1/BZR1 in the biosynthesis of BRs. The molecular mechanism of BR signaling from its recognition on the plasma membrane to the transcriptional activation of specific genes in the nucleus has also been reviewed in detail. As a leading example of the importance of the BRs, we examined their role in heavy metal stress and their crosstalk with other phytohormones under stress conditions. But more work needs to be done for a detailed understanding of how BRs are transported out of cells and how they protect the plant during oxidative stress. There is a knowledge gap that needs attention to unravel the interactions between these phytohormones and various metabolites and transcription factors, which will add a new direction to the study of stress responses in plants.

AUTHOR CONTRIBUTIONS

AS, ML, and RB designed the outline and revised the initial draft. All authors were equally involved in writing of current version.

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