Genome-Wide Expression and Network Analyses of Mutants in Key Brassinosteroid Signaling Genes

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Research article

Keywords: Brassinosteroid signaling, expression analysis, systems biology, network analysis, Arabidopsis

Posted Date: September 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-75547/v1

Abstract

Background

Brassinosteroid (BR) signaling regulates plant growth and development in concert with other signaling pathways. Although many genes have been identified that play a role in Brassinosteroid (BR) signaling, the biological and functional consequences of disrupting those key BR genes still requires detailed investigation.

Results

Here we performed phenotypic and transcriptomic comparisons of *A. thaliana* lines carrying a loss-of-function mutation in *BRI1* gene, *bri1-5*, that exhibits a dwarf phenotype along with its three activation-tag suppressor lines that were able to partially revert the *bri1-5* mutant phenotype to a WT phenotype, namely *bri1-5/bri1-1D*, *bri1-5/brs1-1D, bri1-5/bak1-1D.* From the three investigated *bri1-5* suppressors, *bri1-5/bak1-1D* was the most effective suppressor at the transcriptional level. All three *bri1-5* suppressors showed altered expression of the genes in the abscisic acid (ABA signaling) pathway, indicating that ABA likely contributes to the partial recovery of the wild type phenotype in these *bri1-5* suppressors. Network analysis revealed crosstalk between BR and other phytohormone signaling pathways, suggesting that interference with one hormone signaling pathway affects other hormone signaling pathways. In addition, differential expression analysis suggested the existence of a strong negative feedback from BR signaling on BR biosynthesis and also predicted that *BRS1*, rather than being directly involved in signaling, is likely responsible for providing an optimal environment for the interaction between BRI1 and its ligand.

Conclusions

Our study provides insights into the molecular mechanisms and functions of key brassinosteroid (BR) signaling genes, especially *BRS1*.

Background

Brassinosteroids (BRs) are essential plant hormones, regulating multiple processes amongst which plant growth, flowering, senescence, and seed germination [1]. BR biosynthetic and signaling mutants display aberrant morphological phenotypes such as dwarfism, reduced fertility, impaired photomorphogenesis and altered vascular development [2, 3]. Where the phenotypes of mutants in BR biosynthetic genes can be rescued by the application of exogenous BRs, this is not the case for strains carrying mutations in genes responsible for BR signal perception and transduction. Hence these latter strains are referred to as BR insensitive (*bri*) mutants [1, 3]. In the BR signaling pathway, BRs are perceived by membrane-localized leucine-rich-repeat-receptor kinase BRI1 or by the BRI1-like homologs, BRL1 and BRL3 [4, 5]. After binding to BRs, BRI1 and its co-receptor BRI1-Associated Receptor Kinase 1 (***BAK1***) phosphorylate each other. This results in triggering a cytoplasmic phosphorylation/dephosphorylation signaling cascade which deactivates the GSK3-like kinase BRASSINOSTEROID INSENSITIVE 2 (**BIN2**) through dephosphorylating

[6, 7]. Upon BIN2 deactivation, the downstream transcription factors, BRASSINAZOLE-RESISTANT1 (***BRZ1***) and BR-INSENSITIVE-EMS-SUPPRESSOR1 (***BES1***), are dephosphorylated by PP2A (PHOSPHATASE 2A). This results in their disassociation from 14-3-3 proteins, causing them to get activated and regulating a range of downstream genes involved in various aspects of plant growth and development [8-10]. In the absence of BRs, BIN2 is active (phosphorylated) and it prevents the activation of ***BRZ1*** and ***BES1.*** Because BRI1 is the core receptor of BRs, mutants of *BRI1* have been used as genetic background to identify suppressors, i.e. other genes that when mutated, suppress the *bri1* phenotype and thus may play a role in brassinosteroid signaling. For example, the function of *BZR1* has been unveiled by using the null allele of *BRI,* ***bri1-116*** [11]. The weak mutant of *bri1*, ***bri1-5,*** can be rescued by overexpression of ***BAK1*** and *BRI* Suppressor 1 (***BRS1***) [3, 12]. BRS1 is a secreted member of the serine carboxypeptidase (SCP) family [3]. The fact that overexpression of *BRS1*can suppresses two weak BRI1 extracellular domain mutants, *bri1-5* and *bri1-9*, but not the strong cytoplasmic domain mutant *bri1-1*, implies that *BRS1*, unlike the downstream genes, *BZR1* or *BES1*, may function upstream of the BR signaling pathway or in a close regulatory relationship with *BRI1* [3]. Moreover, three of the five overexpressed *BRS1*'s homologs amongst which *ECS1 (*Extra Carpels and Seeds 1) can also partially suppress the phenotype of the *bri1-5* mutant observed in leaves and increases carpal and seed numbers, confirming the role of *BRS1* and its homologs in the BR signaling [13]. Yet, the detailed mechanism of how *BRS1* potentially interacts with other BR genes in order to maintain balance in BR signaling is still unknown.

Some genes involved in BR signaling are also involved in other processes, such as stress response, and act independently of the presence of BRs. Several studies found that *bes1-1D* and *bzr1-1D* backgrounds are not responsive to exogenous BRs, suggesting that *BES1* and *BZRI* have also other functions than BR signaling [14, 15]. In another study, BAK1 works together with Flagellin-Sensitive 2 (FLS2) during pathogen defense programmed cell death independently of BR signaling [14, 16-18]. In addition, SERK1 and SERK2, the homologs of BAK1 play a role in male microsporogenesis, also independently of BR signaling [19]. Some *bri1* mutants show in addition to reduced growth, an increased stress-tolerance, further confirming the complexity and dosage sensitivity of brassinosteroid signaling and regulation [20, 21]. Transcriptomic study and gene network analysis has shown to be effective in uncovering the expression and biological consequences of gene mutants, and have successfully been applied to study several BR genes such as *BRI1* and *BES1* [22]. Therefore, in the present study we applied a similar strategy to elucidate the role of *BRI1*, *BAK1* and *BRS1* in regulation/restoring response to BRs and/or other independent functions

Results

bri1-5/bak1-1D, *bri1-5/brs1-1D* and *bri1-5/bri1-1D* partially reconstitute *bri1-5* gene expression

To better understand the molecular mechanisms of key BR signaling genes, we performed a phenotypic screening and expression analysis of *bri1-5* and its three activation-tag suppressors along with their corresponding wild type. Two suppressor strains *bri1-5/bak1-1D, bri1-5/brs-1D* were obtained from [12]

and an additional *bri1-5/bri1-1D* mutant was selected in the framework of the current study (see Materials and methods). Sequencing the *BRI1* flanking region from the suppressor *bri1-5/bri1-1D* showed that the activation tag was inserted 534 bp downstream of *BRI1* gene (Figure 1, A). All suppressor mutants were shown to indeed overexpress the activation tagged gene as confirmed by Real-Time qPCR (RT-qPCR) (Table S1). Phenotypically, all light-grown *bri1-5* suppressors (*bri1-5/ bak1-1D, bri1-5/ brs1-1D and bri1-5/ bri1-1D)* lines displayed larger seedlings and enhanced epidermal cell length as compared to the *bri1-5* mutant (Figure 1-B, Figure 2). We observed that the *bri1-5/bak1-1D* line best approximated wild type epidermal cell length (Figure 2). To gain insight into which pathways in each of the studied lines were responsible for the restoration of the *bri1-5* phenotype towards the wild type phenotype, we performed gene expression analysis. All suppressor lines, together with their corresponding WT and with the *bri1-5* background were sampled atTo assess the reproducibility of the expression analysis, we measured the extent to which the expression profiles of replicate samples were similar using Principal Component Analysis (PCA): PCA indeed showed that most of the variation in gene expression between the samples could be assigned to differences in genetic background and not to differences between replicates, confirming the reproducibility. (Figure S1). In addition, microarray results were confirmed using RT-qPCR for a selected set of genes (Figure S2).

We determined for each mutant line its differential expression versus the same common reference i.e. the expression state in WS2 (Figure 3) and used these values to assess to what extent the different lines share the same/different differentially expressed genes (aberrantly expressed versus the WT control) (see vendiagram, Figure 3). In this vendiagram genes that for instance are differentially expressed in the *bri1-5* mutant, but not in any of the suppressor lines represent the genes that are affected by the *bri1-5* mutation and that could be restored by overexpression of any of the genes being overexpressed in the suppressor lines. Genes that are in the intersection of a suppressor line and *bri1-5* represent genes of which the aberrant expression in the *bri1-5* background could not be restored in the respective suppressor line with which the overlap was observed. The venndiagram represented in Figure 3 and the scatter-plots in Figure S3 (A-C) show that of all suppressor lines, *bri1-5/bak1-1D* could restore the largest number of genes that were affected in expression in the *bri1-5* (about two-thirds of the genes that were differentially expressed in *bri1-5* were no longer differentially expressed in *bri1-5/bak1-1D*). This is in line with its observed phenotypic behavior as indeed *bri1-5/bak1-1D* seems to also phenotypically best compensate for the *bri15* mutation. Both the *bri1-5/brs1-1D* and *bri1-5/bri1-1D* lines could recover the same one-third of *bri1-5* affected genes (see vendiagram Figure 3). The latter is also illustrated in Figure S3 panel A-C which show that *bri1-5/brs1-1D* and *bri1-5/bri1-1D* (R^2 = 0.20) are the most similar from an expression impact point of view (i.e. affecting the same genes), followed by *bri1-5/brs1-1D* and *bri1-5/bak1-1D* (R^2 = 0.12), whereas *bri1-5/bri1-1D* and *bri1-5/bak1-1D* display the lowest similarity (R^2 =0.029) (Figure S3 D-F). This high similarity between *brs1-1D* and *bri1-1D* indicates that overexpression of *BRI1* and *BRS1* in a *bri1-5* background restores the expression of the same genes and hence that *BRI1* and *BRS1* must have close roles in the BR signaling pathway.

Identification of restoring and compensatory pathways

We assumed that if the suppressor strains compensate the phenotype of the *bri1-5* mutant, they could do so because they either restore the pathways disrupted in the *bri1-5* mutant to wild type levels or they alter the pathways that compensate for the *bri1-5* affected pathways. Both mechanisms could be potentially reflected in the expression data. To identify restored and compensatory pathways shared by all suppressor mutants, we first focused on genes with altered expression in the *bri1-5* mutant, but not in any of the suppressor mutants (group A**,** 118 presumably restored genes) and vice versa genes that were not differentially expressed in the *bri1-5* mutant, but in all suppressor strains (group B**:** 23 presumable compensatory genes) (Figure 3). Next to identifying genes involved in compensatory/restoring pathways shared by all mutants, we also extracted the genes that would be involved in *brs1-1D* specific compensatory mechanisms (groups C, 333 genes) because as compared to *BAK1* and *BRI1*, the role of the *BRS1* gene in BR signaling is yet less characterized. Another gene set we analyzed in-depth consists of the genes that are affected in the *bri1-5* mutant, but that were not recovered by any of the suppressors (149 genes, group D). These genes could explain the residual phenotypic differences between the *bri1-5* mutant, the suppressor strains and the wild type. To identify how the genes of the compensatory and restoring groups act together in pathways, we performed GO and network analysis (see Materials and Methods).

Restoring and compensatory pathways activated in all suppressor lines

Genes restored to wild type state by all suppressors (Group A) were enriched for 'defense response' and 'hormone response' (Additional file 1). Compensatory genes, (group B, i.e. genes that are significantly differentially expressed in all three *bri1-5* suppressors, but not in *bri1-5* mutant) are enriched in 'response to abscisic acid (ABA) signaling' including six responsive genes to ABA (*DTX50, HVA22D, GRP23, COR15B, SAG113, HAB1*), indicating that ABA signaling has been affected to compensate the *bri1-5* deficiency in all suppressor strains (hypergeometric test, p-value =5.5e-06, Additional file 1). To identify the link between the restoring and compensatory pathways, we mapped the genes of both group A and B on the interaction network and used Phenetic to extract sub-networks. Phenetic identifies 6 sub-networks connecting the genes of group A and B. Note that these subnetworks contain, next to the genes of group A and B also connector genes. These are genes that are not differentially expressed themselves but that are still recovered by the network analysis because of their high connectivity with genes of group A/B. As they are needed to connect genes of group A/B, they are most likely involved in the same process as represented by genes of group A/B. Five of these subnetworks showed enrichment in known GO functions (being enriched in respectively ABA, ethylene, auxin, cytokinin and ROS signaling (Figure 4). This indicates that these pathways contribute to recovering *bri1-5* signaling deficiency.

In-depth analysis shows that **the subnetwork enriched in ABA signaling (Figure 4, network 1)** contains two main negative regulators of ABA signaling**,***HAI1* and *HAB1*, which are up-regulated in all *bri1-5* suppressor lines compared to wild type, but not differentially expressed in the *bri1-5* mutant (compensatory genes). Interestingly, the list of compensatory genes (group B) contains 6 targets of the ABA signaling pathway (enrichment p-value: 5.5e-6; *DTX50, HVA22D, GRP23, COR15B, SAG113, HAB1*), indicating that ABA signaling has indeed been negatively affected in the suppressor strains to compensate for the *bri1-5*

signaling deficiency. These 6 additional genes could not be connected by Phenetic on the interaction network, implying they are quite distantly located from each other in the network and hence likely belong to different biological pathways.

The aforementioned negative regulators of ABA signaling, *HAI1* and *HAB1*, belong to the protein phosphatase 2C (PP2C) gene family which has nine members in total (*HAI2*, *HAB2*, *HAB1*, *HAI3*, *PP2CA*, *ABA1*, *AHG1*, *ABI2*, *AHI1*). In our analysis, most of the PP2C genes appeared to be differentially expressed in at least two suppressors, but they failed to pass the strict FDR threshold for the third suppressor to be included in group B (Table S3). PP2C is known to repress ABI5, the main activator of ABA signaling [23]. PP2C is also known to represses BIN2 activity [6, 7]. As BIN2 activates ABI5 by phosphorylating SnRKs [6, 7], repressing ABA signaling by PP2C via blocking SnRKs phosphorylation seems to compensate for the deficiency in BRI1 mediated signaling. **The subnetwork enriched in ABA signaling (network 1)** also contains members of the *PYR/PYL/RCAR* family as connector genes (*RCAR10*, *SRK2B*, *SRK2G*, *RCAR4*, *RCAR13*, *PYL8*, *RCAR14*, *RCAR12*, *RCAR11*, *PYL9*, *RCAR6*, *SRK2D*). The PYR/PYL/RCAR family constitutes the receptor of ABA signaling and promotes the activation of SnRKs by repressing PP2C [15, 24]. The fact that the SnRKs and PYR/PYL/RCAR genes were identified as connector genes implies that they are likely involved in the pathways that connect the genes of group A/B. They are most likely not primarily regulated at expression level given their role in phosphorylation-mediated signaling [7, 25]. This explains why they were detected as connector genes and not retrieved by differential expression analysis.

We could not find any link in the literature to explain how PP2C can be up-regulated by brassinosteroid signaling in order to repress ABA signaling. Our network identified a TF (DREB1B) of which the aberrant expression in the *bri1-5* mutant is restored in all suppressor lines. According to the KEGG pathway, DREB1B directly regulates PYR/PYL/RCAR [26]. Hence, this TF might the missing link between PPC2 and BR signaling. This aforementioned TF could also be a good candidate to explain the crosstalk between ABA and ethylene signaling (**subnetwork 2)** in response to BR signaling as this TF links the subnetwork 1 to subnetwork 2. Indeed the ethylene signaling pathway seems to be resorted by all suppressors (Figure 4, subnetwork 2).

Next to the ABA and ethylene subnetwork, also subnetworks related to other hormone signaling processes like auxin (subnetwork3), cytokinin (subnetwork5) and ROS signaling (subnetwork6) were detected. It is well known that crosstalk between these phytohormone signaling pathways exists [27, 28]. Hence interfering with one pathway e.g. ABA signaling through BR signaling might affect other phytohormone pathways as well. Overall the subnetwork enriched in ABA signaling contains mostly genes of group B (compensatory genes) whereas the subnetworks related to the other phytohormone signaling pathways belong to group A (restoring genes). Based on their differential expression behavior we can conclude that ABA signaling is mostly a compensatory pathway, whereas the other hormone signaling pathways contribute to restoring pathways that were affected in the *bri1-5* strain.

Negative feedback of BR signaling on BR biosynthesis:

Group D (genes differentially expressed in the *bri1-5* lines, but not fully restored by any suppressors) consisted of four cytochrome P450 genes (*CYP90C1*, *DWF4*/ *CYP90B1*, *CYP85A2* and *CYP90A1*). These were aberrantly up-regulated in *bri1-5* and in all three suppressor mutants, although to different degrees (gradually stronger upregulated in *bri1-5* than in the suppressors). This indicates that the aberrant expression of these genes in the *bri1-5* mutant could only be partially compensated for in the suppressor mutants. Cytochrome P450 genes play a role in BR biosynthesis by converting the sterol 'campesterol' to BRs [29]. So the fact that mutations in BR signaling genes affect the expression of BR biosynthetic genes indicates that a negative feedback exists of BR signaling on BR biosynthesis.

If indeed a negative feedback exists between BR signaling and biosynthesis, this feedback should be reflected in quantitative differences in overexpression of the BR signaling and biosynthesis genes in the *bri1-5* and suppressor mutants. The better the signaling can be restored in the suppressors (as reflected by the phenotype), the less we expect the expression of the BR biosynthesis to be aberrant. We indeed found that the expression of the BR-biosynthesis genes (*CYP90C1*, *CYP90A1*, *CYP85A2*, *CYP90B1*) are less affected in the strains that better mimic the wild type phenotype (see Figure 5, the best suppressor of *bri1-5*, *bri1-5*/ *bak1-D,* shows the lowest upregulation of the biosynthesis genes). This further supports the existence of negative feedback from BR regulation on BR biosynthesis: a more sustained BR signaling results in decreased BR biosynthesis, whereas suboptimal BR signaling is compensated for by higher transcriptional activity of BR biosynthetic genes.

BRs possibly involved in providing an optimal environment for BRI1 and ligand binding

Unlike for BRI1 and BAK1, much less is known about the role of BRS1 in BR signaling. Here we had a closer look at genes of group D which are exclusively differentially expressed in *bri1-5/brs1-1D* mutant and hence comprise compensatory pathways specific for *bri1-5/brs1-1D*. GO enrichment showed that the genes uniquely involved in compensating for the *bri1-5* mutant by the *brs1-1D* suppressor (in group C, 333 genes form Venn diagram), are overrepresented in iron ion homeostasis/ferroxidase activity (down-regulated) (p-value 3.0e-09), and glutathione transferase (up-regulated) (Figure 6). As in the ferroxidase reaction, four H^+ are used to catalyzes the oxidization of Fe^{2+} to Fe^{3+}, repressing this reaction results in the accumulation of H^+ which can be transported to the apoplast via plasma-membrane pumping mediated by ATPase (H+-ATPase transporters) [30]. Accordingly, we found that the main inhibitor of H^+-ATPase transporters, *CBC1*, was significantly down-regulated in *bri1-5/brs1-1D (*log-fold -1.7, adj p-value 8.36e-06) but not in the other suppressors. This implies that H^+-ATPase transporters are more active in *bri1-5/brs1-1D* to export H^+ from cytosol into apoplast and making the apoplast more acidic. In line with this hypothesis, the up-regulated glutathione transferase activity in the *brs1-1D* mutant might be essential to compensate for the more acidic environment and would be required for redox homeostasis. The observed acidification could generate a cellular environment that improves BRI1-BRs binding or BRI1-BAK1 dimerization and hence restoration of the *bri1-5* mutant phenotype.

Link between stress response and BR signaling

According to literature, there is crosstalk between BR signaling and other hormone signaling in response to stress, especially via ABA and auxin signaling [27]. In the absence of BRs (or low amounts of BRs), BIN2, activates ABA and auxin signaling, resulting in the induction of stress response genes [6, 7, 15]. On the other hand, some stress-response genes are known to be targets of BZR1 and BES1 [7, 15] (Figure 7), indicating that also when BR levels are high, stress response genes can be activated. These observations show that balanced BR levels are needed for normal growth and that deviation from the optimal levels (either too high or too low) would activate stress response mechanisms. We observed that by partially recovering *bri1-5* signaling deficiency by suppressors mutants, the transcript level of some stress-response genes is restored to normal but other stress-response genes become induced (Additional file 1: GO enrichment for genes exclusively differentially expressed in each suppressor from the venndiagram, "GO_only_bri1-1D", "GO_only_bak1-1D", "GO_only_brs1-1D"). This observation is in line with this complex effect of BR and BR signaling on stress response pathways.

Discussion

In this study, we explored the alteration of gene expression in BR signaling mutants to better understand brassinosteroid signaling and functions of those key BR genes.

Crosstalk between BR and other hormone signaling and stress response

Our analysis identified ABA signaling as a compensatory pathway, and hormone signaling pathways related to ethylene, auxin, cytokinin, and ROS as pathways partially restoring the *bri1-5* expression phenotype to WT levels. The crosstalk between BR signaling and signaling by ABA observed in our study is in line with the literature [31]. The serine-threonine kinases SnRK2.2 is the main positive regulator of ABA signaling by regulating key TFs such as *SLAC1*, *KAT1* [32, 33]. SnRK2.2 gets inactivated after dephosphorylation by PHOSPHATASE 2C (PP2C) (Figure 7). In the presence of ABA, the complex of PYR/PYL/RCAR inactivates the PP2C by blocking its substrate's entry [34]. The activated SnRK2 phosphorylate ABI5 leading to the activation of downstream ABA-dependent mechanisms. In the absence of BRs (or BR signaling deficiency like *bri1-5*), SnRK2.3 can mimic the presence of ABA in triggering ABA signaling, once it is phosphorylated by BIN2 [23]. This means that repressing ABA signaling (repressing SnRK2) can compensate for the BR signaling deficiency. In line with our result, a recent study showed that overexpression of *ABI1* or *ABI2,* which encodes the negative regulator of ABA signaling could promote BR signaling [31]. Network analysis suggested that all *bri1-5* suppressor strains suppressed the ABA signaling (SnRK2) through upregulating its negative regulator, PP2C (Figure 7). We could not find any evidence on a molecular mechanism that might explain the observed up-regulation of PP2C, but forwarded based on our analysis, the DREB1B as a TF can explain the PP2C up-regulation and that could mediate the crosstalk between the observed ABA-ethylene response and BR signaling.

Links between BR signaling and auxin we observed are also supported by literature and is in line with the observed phenotype of the *bri1-5* mutant. ARFs (auxin response factors) are transcription factors that affect root and shoot elongation [28]. In the presence of BRs, BZR1 and BES1 enhance the DNA-binding

activity of the auxin response factors ARF6 and ARF7 to promote auxin response [35, 36]. This explains the restored auxin-related pathways in the suppressor strains. However, it remains unclear whether the observed restored auxin signaling was the result of the activation of BZR1/BES1 or whether the suppressors employed other pathways to restore the auxin signaling. In the other hand, at low concentrations of BRs, BIN2 enhances the DNA-binding activity of the auxin response factors ARF2, ARF7, and ARF19 through phosphorylation. This results in growth and root elongation in the absence of BRs [33, 37]. Hence, optimal plant growth and development regulated by auxin signaling requires a balanced level of BR signaling. Like with ABA and auxin, also crosstalk between BR and respectively ethylene and cytokinins have been reported [27].

Negative feedback between BR signaling and BR biosynthesis.

In addition, our results show that a negative feedback exists between BR signaling and BR biosynthesis. This hypothesis was already made by [2] who explained the observed accumulation of BR precursors in *bri1* mutants by the presence of a negative effect of the BR signaling proteins BZR1/BES1 on the BR-biosynthesis pathway. Although it cannot be excluded that the previously reported accumulation of BR precursors in *bri1-5* mutants results from the inability of the mutant lines to use available BRs, our observation in BR signaling mutants suggests that the observed accumulation is indeed rather due to aberrant regulation of BR biosynthesis: it seems that non-aberrant BR signaling is required for homeostasis of appropriate *levels* of *endogenous* BRs.

Our results also showed that the level of negative feedback depended on the degree to which the suppressor could compensate for the phenotypic difference between the *bri1-5* and WT. The better the BR signaling was restored (as reflected by the phenotype), the less pronounced the observed effects on the BR biosynthetic genes. This observation also supports the hypothesis made by [6] that BIN2 would regulate BR-biosynthesis through phosphorylating CESTA, a transcription factor that regulates BR-biosynthesis (Figure 7).

BRS possibly involved in providing an optimal environment for BRI1 and ligand binding

Our analysis of the genes/pathways that are uniquely involved in the *bri1-5/brs1-D* suppressor line to compensate for the *bri1-5* mutant showed that BRS1 is involved in the acidification of the apoplast environment. We hypothesize that this acidification could contribute to an improved BRI1-BRs binding or BRI1-BAK1 dimerization and hence restoration of the *bri1-5* mutant phenotype. In vitro studies have indeed shown that BRs preferentially bind to BRI1 in an acidic cell wall environment (PH < 5) [24, 38]. It has also been suggested that changing PH environment by endocytosis of BRI1 from the plasma membrane into the cytosol reduces the affinity of BRI1 to BRs and would terminate BR signaling [24]. The same ligand-receptor mechanism has been reported in animal cells [39]. In addition, acidification of the apoplast is the major requirement for increasing cell wall extensibility, which controls extension growth and can also be a compensatory pathway in *brs1-1D* [40]. These observations support that the *brs1-D* mutant can restore BR signaling by creating an acidic environment and providing the optimal conditions for either BRI1–ligand binding or BRI1-BAK1 dimerization along with improving cell wall extensibility. This

might also explain the very similar genome-wide expression impact of *brs1-1D* and *bri1-1D* and explains why overexpression of *BRS1* can suppress two weak BRI1 extracellular domain mutants, *bri1-5* and *bri1-9*, but not the strong cytoplasmic domain mutant *bri1-1.*

The *bri1-5/brs1-D* suppressor line also induces glutathione transferase activity which is necessary for redox homeostasis. This link between BR and redox signaling is in line with the literature [41]. An oxidative environment induces BZR1 activity and promotes the interaction of BZR1 with ARF6 and PIF4 [41]. Since a loss-of-function mutant of BRS1 shows no obvious phenotype [3] but its gain-of-function mutant does and partially restores *bri1-5* signal deficiency, it can be suggested that BRS1 does not have a regulatory role and only provides a better condition for triggering the BR signaling by making the apoplast environment more acidic.

BR signaling and stress response

Because BR signaling regulates response to a wide spectrum of stresses [15], it is not unexpected we observed that stress response genes were affected in the mutants that interfered with BR signaling. At the low level of BRs (or BR signaling deficiency e.g. *bri1-5*) the activated SnRK2 by BIN2 mimics the presence of ABA, activates ABI5 and finally regulates stress response genes (Figure 7). In the presence of BRs, BZR1/BES1 inhibits ABI5 and can terminate the ABA signaling. On the other hand, BZR1/BES1 can regulate the expression of stress response independent of ABA (Fig7). Therefore, some stress response genes are regulated by BIN2 at low levels of BRs through ABA signaling, while other stresses are controlled by BZR1/BES1 at high levels of BRs independent of ABA signaling. We observed that by partially recovering *bri1-5* signaling deficiency in *bri1-5* suppressors strains, the transcript level of some stress-response genes is restored to normal, but of other stress-response genes it becomes induced. This is in line with literature which suggests that balanced BR levels are needed for normal growth and that deviation from the optimal levels (either too high or too low) would activate stress response mechanisms. The need for optimal BR homeostasis might also explain why some gain-of-function mutants (e.g. *bes1-D*) described in the literature or why treatment with exogenous BR give rises to a phenotypic response that is worse than the one observed in the *bri1-5* mutant (shorter root) [42]. This further confirms that an appropriate balance in BR signaling is essential to guarantee coherent cross-talk between hormones signaling networks and any aberration of this optimal level leads to activation of stress response genes.

In addition, there is evidence that BAK1 plays a role in regulating stress-response pathways independently from BR signaling [16]. Since BAK1 usually works as a coreceptor and serves to promote cross-phosphorylation leading to downstream signaling, the existence of other stress-sensor receptors interacting with BAK1 cannot be excluded.

Conclusions

In this study, we performed expression and network analysis to provide more insight into the BR signaling by taking advantage of the availability of mutants for keys genes in BR signaling. Our results suggest that ABA signaling plays a significant role in restoring the *bri1-5* dwarf phenotype. Other phytohormones

resorted toward wild type expression level by all *bri1-5* suppressors that confirm the crosstalk between BR and other phytohormones signaling. The negative feedback from BR signaling on BR biosynthesis was also confirmed by quantitative evidence. In addition, a new function for *BRS1* was suggested and it seems to have an indirect function in BR signaling. However, our study is limited to transcriptome analysis and BR signaling is likely regulated to a large extent at post transcriptional level (i.e. phosphorylation). By using network analysis we can partially deal with this missing information but still restricted by the incompleteness of current interaction networks. More extensive validation studies are required to confirm our hypotheses. Furthermore, we used samples from the seedling stage and it might be of interest to repeat the experiment in later stages to confirm the results.

Material And Methods

Expression profiling experiment and differential expression analysis

The two activation tagging suppressors of *bri1-5,bri1-5/bak1-1D* and *bri1-5/brs1-1D* were obtained from our previous study [12]. An additional activation tagging suppressor line *bri1-5/ bri1-1D* was here generated in *bri1-5* as we previously described [3]. Wild type (WS2), the loss-of-function BR mutant (*bri1-5)* and its three suppressor mutants (*bri1-5/ brs1-1D*, *bri1-5/ bak1-1D*, *bri1-5/ bri1-1D)* were grown in light under normal condition for seven days. All mutants are generated from the WS2 ecotype background. Microarray analysis of all genotypes was performed with three biological replicates. Total RNA of 7-day whole seedlings was isolated, labeled and hybridized with an Arabidopsis ATH1 genome array according to the Affymetrix instructions (www.affymetrix.com). Scanning of the array was performed using the Agilent GeneArray Scanner. The *data are available in GEO (*GSE70843). The cel files were preprocessed using the AFFY package (background correction, quantile normalization and probe value summarization (RMA normalization)) [43]. The arrayQualityMetrics package was used to check the quality of the normalized expression values [44]. All samples passed the quality check. Non-unique probes were removed and the expression value of genes was calculated as the average of the expression measured by the probes that covered the gene. The consistency between replicate samples was assessed using PCA. Differential expression was calculated by comparing the samples of the mutated lines with those obtained from the wild type (WS2) using the Limma package [45]. To define differentially expressed genes, the absolute fold change and false discovery rate (FDR) threshold were set at 1.5 and 0.05, respectively. Further, ten differentially expressed genes were randomly selected and confirmed by RT-qPCR following standard protocol [46]. The primers used for qPCR are presented in Table S2. Go enrichment was performed using topGO package [47].

Network analysis

A high confidence Arabidopsis interaction network was compiled from the following sources: 64185 FunTFBS regulatory interactions were obtained from PlantRegMap [45], 96827 protein-protein interactions from AtPIN [48] and 34003 metabolic interactions from KEGG [26]. This resulted in a final number of 182748 interactions between 21263 unique genes. In this integrated network, nodes

represented genes, and edges the interactions between the genes. To construct a weighted network, we used a local connectivity measure that considers first-order neighbors. To calculate the weight between a source (S) and target (T) node, we counted the number of interactions between S and T with a maximum path length of 2 edges. This count was divided by the total number of first neighbors of both S and T. To avoid assigning a high score to genes with a small number of neighbors, a max operator was added to the denominator. This ensures that sparsely connected genes in the network (genes that are connected only by one edge to the rest of the network) obtain a relatively lower score.

$$score_{S\text{-}T} = \frac{\#\text{ Edges with max length 2 between S and T}}{max\left(\sqrt{N_S * N_T}, 10\right)}$$

with N_s and N_t being the number of first-order neighbors of S and T, respectively. Subsequently, a smoothing formula was applied that down weights the relative impact of the more connected components in the network. $weight_{s\_t} = \frac{e^{score(s\_t)}}{e}$. This to avoid that the molecular layers with an intrinsically more connected character would dominate the results (for instance cliques obtained from protein-protein interactions). This weighted interaction network was used together with the query gene list (list of differentially expressed genes) in Phenetic [49, 50]. Phenetic aims at connecting as many genes as possible from the query list on the interaction network in the most parsimonious way (using the least number of edges). By enforcing such a parsimonious solution, Phenetic detects subnetworks in which genes from the query list are closely connected. Such connected components can be viewed as proxies of pathways. Phenetic was run in the downstream mode with the following parameters: min cost: 0.1; max cost: 5; step size: log scale between max and min cost with 28 steps; Path-length=4; k-best paths: 20. For each edge cost, the highest-scoring subnetwork was selected and a stability score was computed for all the subnetworks with this edge cost. For each cost, the subnetwork is rejected if either it has a low stability score (minimal stability score is 0.5) or is too large (max size is 80). The final subnetwork was extracted as a combination of all these "best networks" that passed the tests.

Abbreviations

BR: Brassinosteroid

BRI: BR insensitive

BRL1: BRI1-like homologs

WT: Wild type

BAK1: BRI1-Associated receptor kinase

ABA: Abscisic acid

BIN: Brassinosteroid insensitive

BRZ: Brassinazole-Resistant

BES: Br-Insensitive-Ems-Suppressor

PP: Phosphatase

BRS: BRI suppressor

SCP: Serine carboxypeptidase

ECS: Extra Carpels and seeds

FLS: Flagellin-Sensitive

RT-qPCR: Real time quantitative polymerase chain reaction

PCA: Principal component analysis

ROS: Reactive oxygen species

FDR: False discovery rate

TF: Transcription Factor

FunTFBS: Functional transcription binding site

AtPIN: *Arabidopsisthaliana* protein interaction network

Declarations

Availability of Data and Materials

The microarray *datasets have been deposited* in *GEO* under accession number GSE70843. Requests for resources, codes and material should be directed to and will be fulfilled by the Lead Contact, Jia Li (lijia@lzu.edu.cn).

Funding and Acknowledgments

This work was supported by the National Basic Research Program of China [grant 31720103902 and 31530005 to J.L., grant 31700197 to T.S.], Youth Innovation Promotion Association of the Chinese Academy of Sciences [grant 2019335 to T.S.] and the Ministry of Science, Research and Technology, Iran [grant to R.SR]. The work was also supported by grants of the Fonds Wetenschappelijk Onderzoek-Vlaanderen (FWO) [G046318, G.0371.06, and 3G045620] and UGent Bijzonder onderzoeksfonds.

Authors' Contributions:

Conceptualization: J.L, T.S., R.S.R., K.M.; Methodology: T.S., R.S.R.; Investigation: D.Z., X.G., J.Y.; Writing – Original Draft: T.S., R.S.R; Writing – Review & Editing: J.L., K.M.; Funding Acquisition: J.L., K.M, R.S.R., T.S.; Resources: J.L.; Supervision: K.M., J.L. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

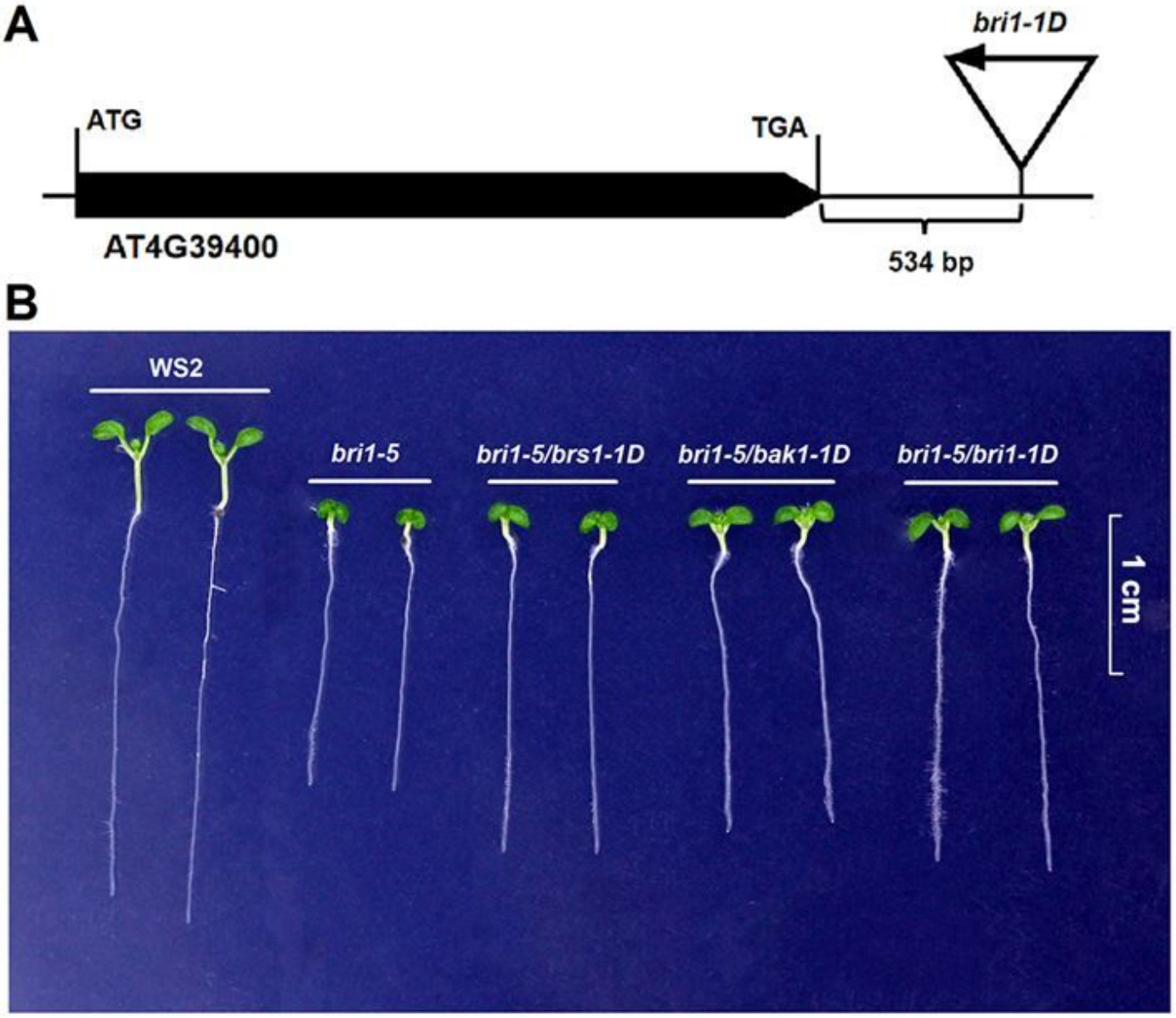

Figure 1

T-DNA insertion site for bri1-1D and phenotype of plants used for expression profiling The T-DNA insertion site for bri1-1D (A) and phenotypes for light-grown 7-day seedlings including WS2, bri1-5, bri1-5/brs1-1D, bri1-5/bak1-1D and bri1-5/bri1-1D (B).

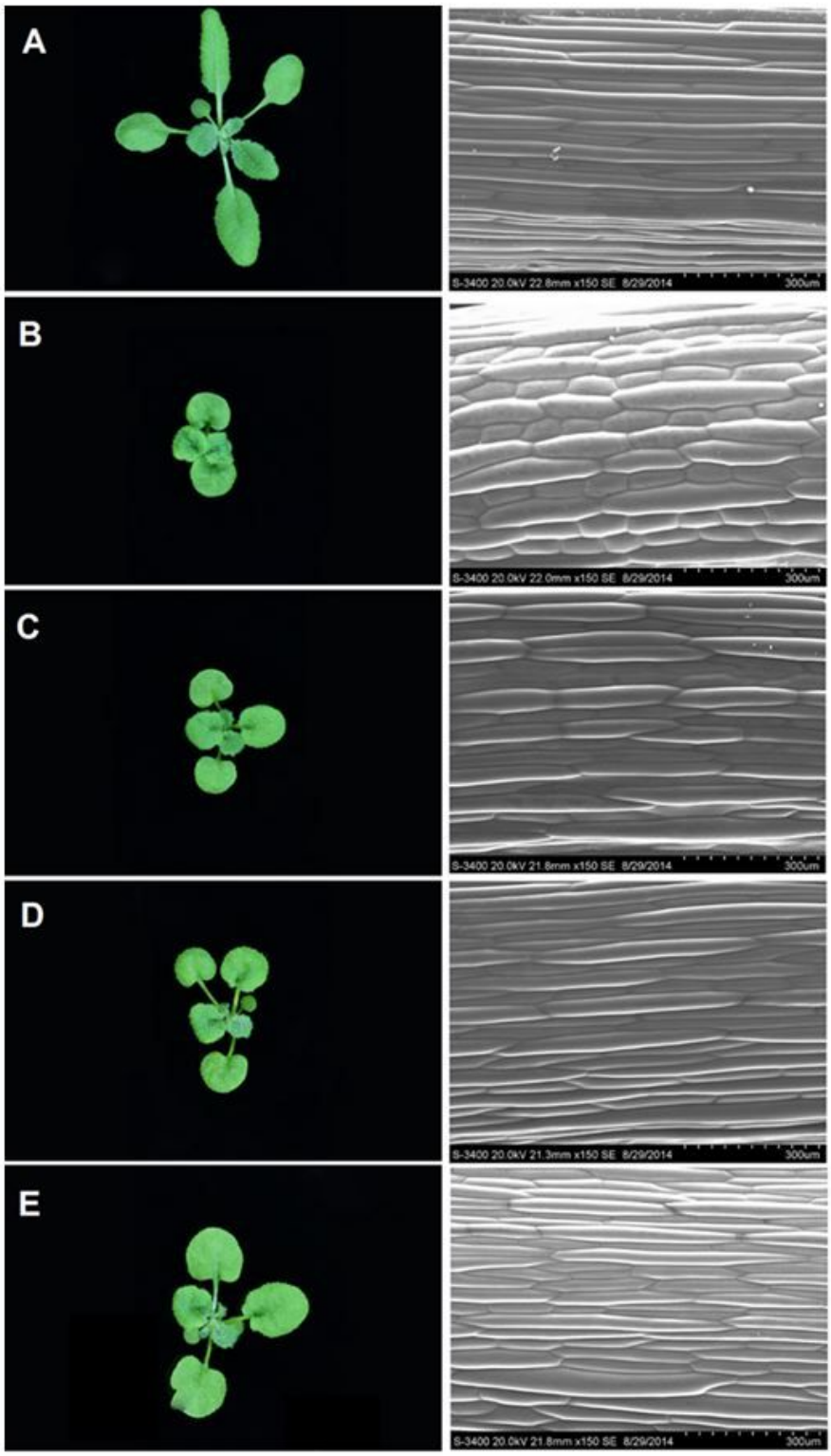

Figure 2

Phenotypes and epidermal cell length of plants Phenotypes of the whole plant and epidermal cell length of light-grown seedlings from WS2 (A), bri1-5 (B), bri1-5/brs1-1D (C), bri1-5/bak1-1D (D) and bri1-5/bri1-1D (E) are selected after 15 days of germination for better visualization of epidermal cell distinctions.

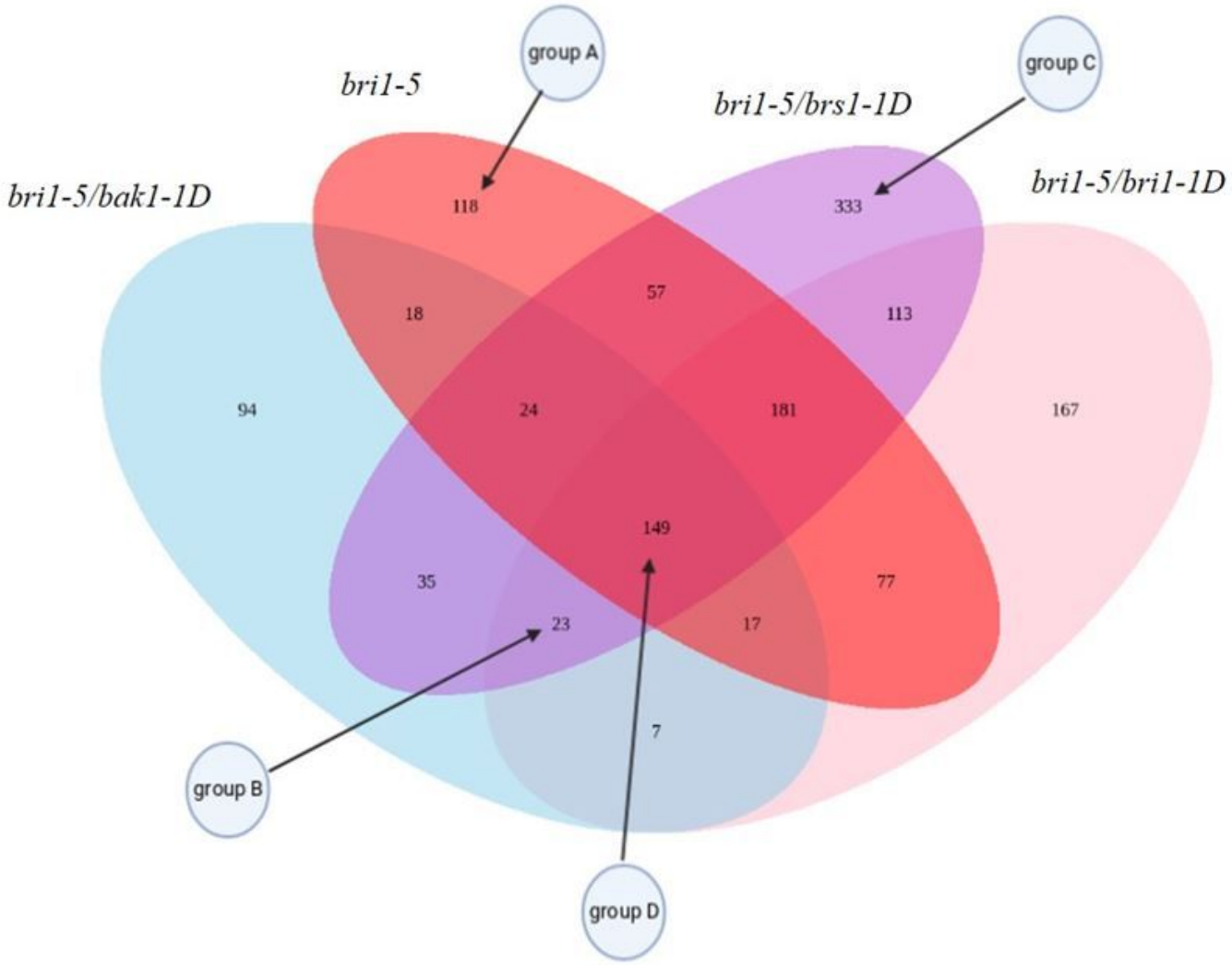

Figure 3

Venndiagram of differentially expressed genes (DEGs) compared between bri1-5 and its three suppressors Group A: restored genes by all suppressors; Group B: genes that are differentially expressed in all suppressors but not in bri1-5 (compensatory genes); Group C: genes that are elusively differentially expressed in brs1-1D mutant. Group D: genes that are not restored by any suppressors.

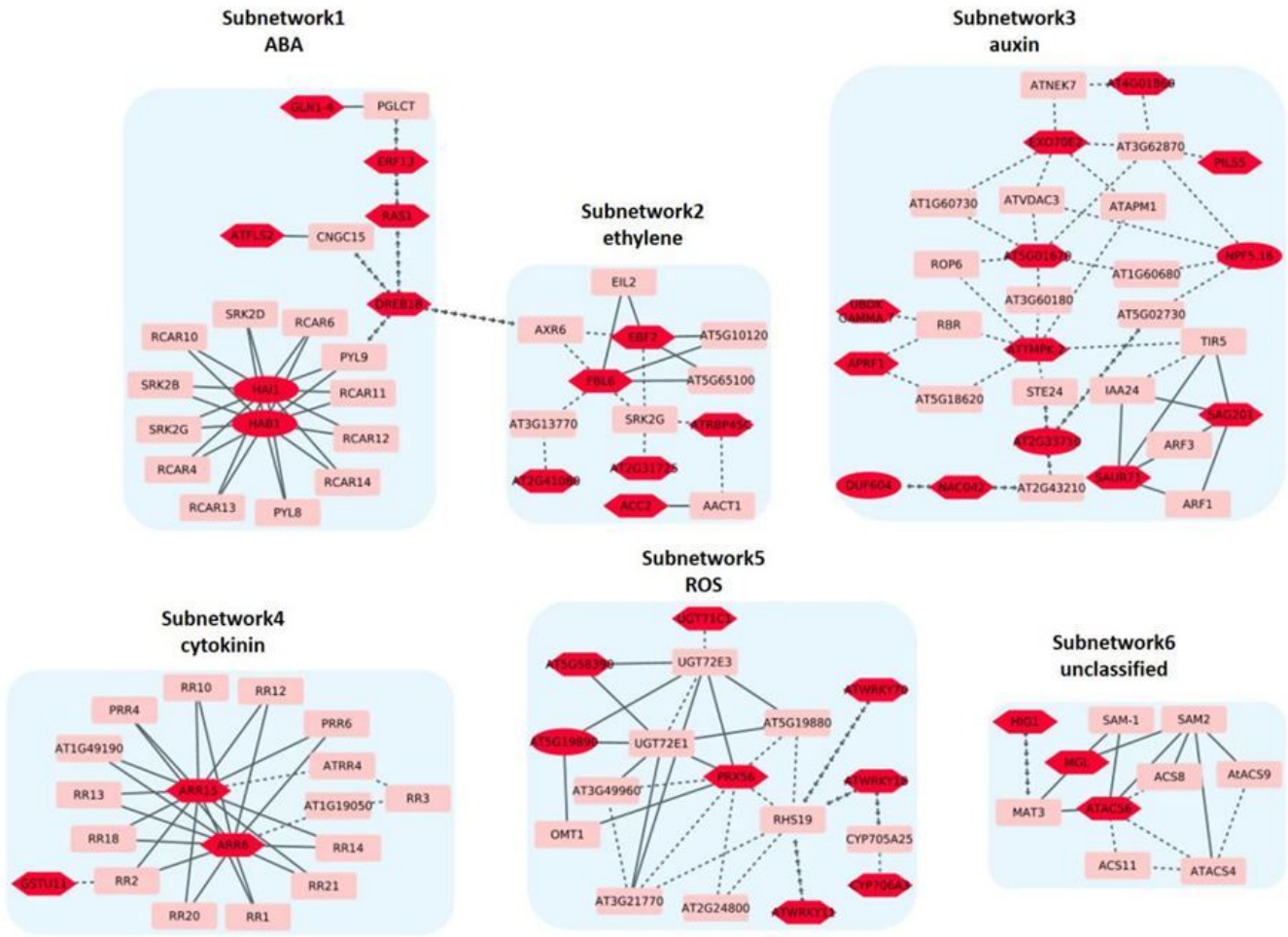

Figure 4

Sub-networks identified by Phenetic Sub-networks identified by Phenetic representing different phytohormone pathways that were identified by mapping and connecting the genes of group A (compensatory genes) and B (restored genes) on the interaction network. Query genes (group A: diamond and B: hexagons) are indicated in red color. Connector genes that were not identified as differentially expressed but identified by Phenetic on the paths that connect the differentially expressed genes are shown in light pink.

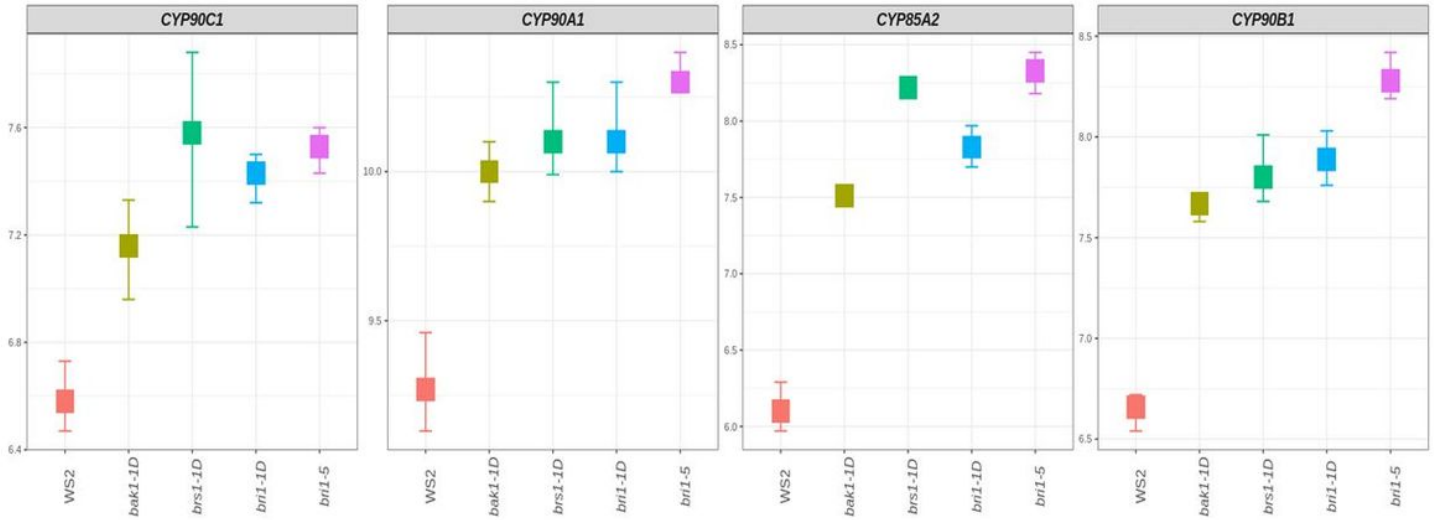

Figure 5

Comparing the mean expression values of BR-biosynthesis genes in the bri1-5 mutant and suppressor lines For each line (WS2, bri1-5, and suppressors) the average log2 expression values of gene expression are given for the indicated brassinosteroid biosynthesis genes. The squares indicate the mean and bars shows the 95 percent confidence interval for the mean. The main BR-biosynthesis genes are up-regulated in bri1-5 mutant and all suppressors. The plots show that BR-biosynthesis genes are less affected in the line (bri1-5/bak1-1D) that best suppress the bri1-5 phenotype.

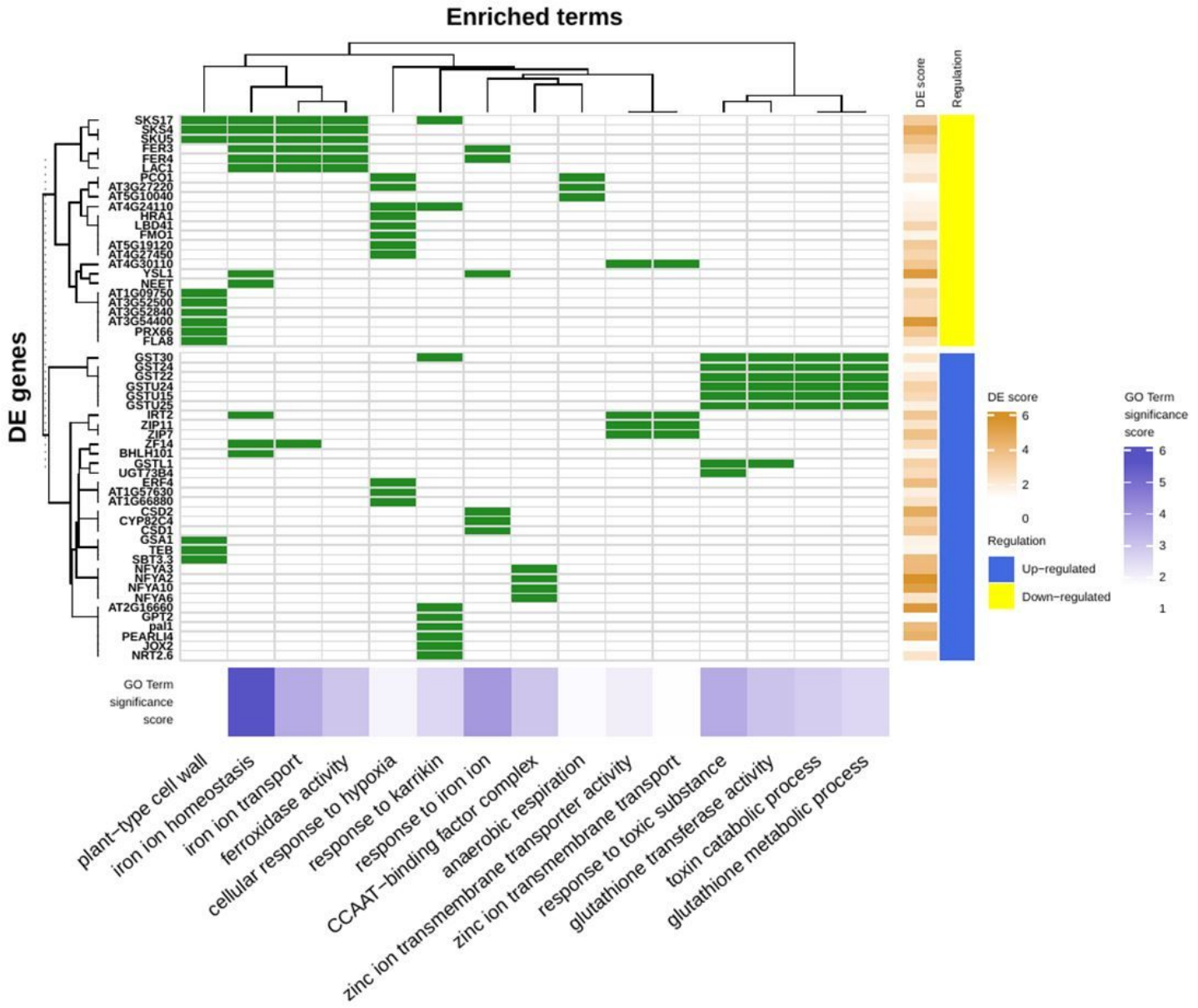

Figure 6

GO enrichment for differentially expressed genes (DEGs) exclusively in bri1-5/brs1-D compare to WS2 The over-represented GO terms and DEG are shown on the x-axis and left-side y-axis, respectively. The green color shows the corresponding gene is present in the indicated GO term and white means not; “DE score” reflects the degree of log fold changes (differential expression compare to WS2); “Regulation” represents down (yellow) and up (blue) regulation for the corresponding gene. The small bottom heat map shows the significant over-representation value for GO terms based on p-value in the hypergeometric test.

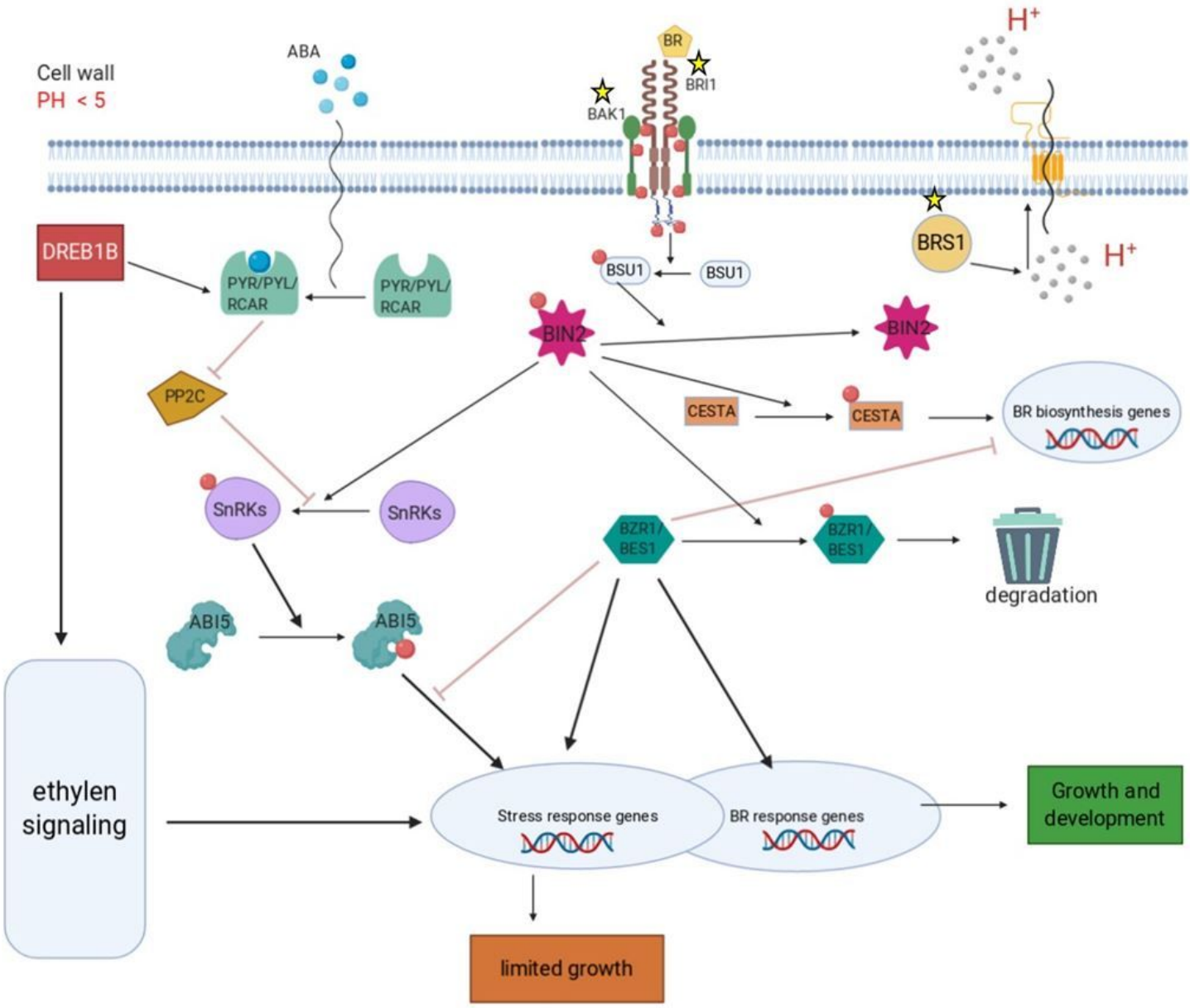

Figure 7

Schematic overview of the results of this study in the context of the BR signaling cascade The figure provides a simplified scheme of BR signaling based on (Planas-Riverola et al, 2019; Gruszka D, 2013; Belkhadir and Jaillais, 2014). The studied genes in this work are indicated by a yellow star. Binding of BRs to the BRI1/BAK1 receptor triggers the phosphorylation/dephosphorylation signaling cascade that leads to the deactivation (dephosphorylation) of BIN2. Phosphorylated BIN2 deactivates the repressing activity of BZR1/BES1 which results in repressing the BR target genes directly or indirectly. The effects of BIN2 and BZR1/BES1 on BR-biosynthesis genes are depicted. The overlap between stress-response and BR response genes and the dual effect of BZR1/BES1 on stress response genes also is shown. The hypothetical inferred role for BRS1 based on DE and GO enrichment results in generating a more acidic environment that provides a better condition for BRI/BAK1/BR binding is shown on the top right-hand

side. The compensatory pathway resulting in the over-expressing expression of PP2C mediated by ABA is shown on the left-hand side.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xlsx