Brassinosteroid's multi-modular interaction with the general stress network customizes stimulus-specific responses in Arabidopsis

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Abstract

Adaptation to fluctuating environmental conditions is a universal feature of plant life, governed by fundamental mechanisms optimizing resource allocation. This balance is achieved in part through tightly regulated communication networks between growth and stress response signaling pathways. Understanding the communication modules between brassinosteroids (BRs), the ubiquitous hormones known to control growth and stress adaptation, and the general stress response (GSR), a rapid and transient transcriptional output in response to perturbations, provides an optimal platform to unravel new facet(s) of plant stress adaptation. Here, we explore communication facets of BR with GSR via *in planta* quantification of the GSR in Arabidopsis expressing luciferase driven by a functional GSR *cis*-element, the Rapid Stress Response Element (4xRSRE:LUC). We establish that application of exogenous BR suppresses microbe-associated molecular pattern-activated GSR, but enhances the wound-triggered GSR. The enhanced wound-activated GSR in BR-treated plants results in a greater wound-induced resistance to *Botrytis cinerea*. A combination of molecular genetics using BR signaling mutants and application of an activator of BR signaling, bikinin, confirms these results and places the chief point of BR-GSR interaction downstream of potential membrane receptor circuitry. These results support a multi-modular interaction between BRs and stress signaling, instrumental in customizing stimulus-specific responses in Arabidopsis.

Highlight

Brassinosteroids (BR) modulate the general stress response (GSR)

Exogenously applied BR reduces flg22-, but enhances wound-induced GSR

Wound-activated GSR in BR-treated plants heightens resistance to *Botrytis cinerea*

Point of BR-GSR interaction is downstream of membrane receptor circuitry

Multiple interaction points between BRs and stress signaling tailor plant responses

Keywords

Brassinosteroid (BR), cross-hormonal communication, general stress response (GSR), hypoxia, microbe-associated molecular pattern (MAMP), wound-induced resistance.

Abbreviations

ABA: abscisic acid, BAK1: BRI1 associated kinase 1, BES: BRI1 EMS suppressor1, BIK: botrytis induced kinase 1, BIN2: brassinosteroid insensitive2, BKI1: BRI1 kinase inhibitor1, BL: brassinolide, BR: brassinosteroid, BRI1: brassinosteroid insensitive 1, BSK: brassinosteroid signaling kinase, BSU: BRI1 suppressor 1, BZR1: brassinazole resistant 1, CCD: charge-coupled device, CDG: constitutive differential growth 1, CrRLK1L: *Catharanthus roseus* receptor like kinase 1-like, COI1: Coronatine-Insensitive 1, DAMP: damage associated molecular pattern, DMSO: dimethyl sulfoxide, ERF1: Ethylene Response Factor 1, flg22: flagellin22, GA: giberellic acid, GSK3: glycogen synthase kinase, GSR: general stress response, IAA: indole acetic acid, JA: jasmonic acid, LUC: luciferase, MAMP: microbe-associated molecular pattern, MAPK: mitogen-activated protein kinase, MeJA: methyl jasmonate, OPDA: 12-oxo phytodienoic acid, ORA59: Octadecanoid-Responsive Arabidopsis AP2/ERF 59, PEPR: peptide receptor, PDF1.2: Plant Defensin 1.2, PR1: pathogen responsive 1, PRR: pattern recognition receptor, RAP2.12: related to *apetala 2.12*, ROS: reactive oxygen species, RSRE: rapid stress response element, SA: salicylic acid, SAR: systemic acquired resistance, TF: transcription factor, UBI: ubiquitin, VSP2: vegetative storage protein 2, WAK1: wall-associated kinase 1, WIR: wound-induced resistance.

1. Introduction

Plants are sessile organisms exposed to constant environmental challenges, and as such evolved numerous adaptive strategies to cope with inevitable abiotic and biotic stresses. One such adaptation strategy relies on metabolic rechanneling; alteration in growth and developmental patterns to provide resources required to successfully adapt to a myriad of environmental challenges. This shift in

resource allocation is dependent on a repertoire of intricate networks, among them hormone-mediated signaling that in turn is contingent upon the suite of hormones present and the nature of environmental challenges perceived.

The general stress response (GSR) is the rapid (within 5 minutes) and transient reprogramming of the transcriptome, inducing genes responsive to a wide range of environmental challenges [1, 2]. Study of the GSR revealed a *cis*-regulatory element overrepresented in the promoters of GSR-associated genes, dubbed the Rapid Stress Response Element (RSRE). Additionally, bioinformatics analyses confirmed overrepresentation of the RSRE in rapidly and transiently upregulated genes in response to biotic and abiotic stresses [3]. The establishment of stress-induced rapid and transient LUC expression driven by two RSRE-containing promoters (*ERF18* and *CAF1like*) led to the generation and functional validation of a synthetic promoter consisting of four copies of the RSRE driving LUC expression. Plants expressing this construct respond to biotic and abiotic stresses similarly to those containing a full-length promoter harboring the RSRE, whereas the synthetic promoter with four copies of a mutated version of the element, mRSRE, does not [1, 3]. These findings established the 4XRSRE:LUCIFERASE (4XRSRE:LUC) reporter line as an optimal experimental tool for examining GSR, which has since proven to be instrumental for detailed temporal and spatial visualization and quantification of the GSR [1, 3, 4]. These efforts specifically demonstrated dependency of the GSR on secondary messengers including Ca^{2+} and reactive oxygen species (ROS) implicated in initial biotic and abiotic signaling cascades [3, 4].

Hormone signaling has also been implicated in GSR regulation. Specifically, it is demonstrated that the reporter line exogenously treated with various stress-associated plant hormones, including SA, methyl-JA, and ABA displayed significant induction of the RSRE only in response to ABA [3]. However, molecular genetic approaches established an accelerated RSRE response peak and enhanced basal activation levels in the absence of external stress in a mutant line, *mekk1-5*, which

accumulates constitutively high levels of the stress hormone salicylic acid [3, 4]. These reports support the notion of crosstalk between selected hormones and induction of the GSR, thereby affording the rationale to explore potential facets of hormone-GSR signaling, with specific emphasis on brassinosteroids.

Brassinosteroids (BRs) are plant steroid hormones known to alter growth and development independently or interdependently with other growth promoting hormones such as gibberellic acid (GA) and auxin [5-10]. The transduction of brassinosteroid (BR) signaling is initiated by BR binding to the extracellular domain of a cell-surface receptor kinase, brassinosteroid insensitive1 (BRI1), leading to activation of the intracellular kinase domain, recruitment of BRI1 associated kinase 1 (BAK1), and sequential transphosphorylation events between the kinase domains of these two enzymes (Supplementary Fig. S1) [11-13]. BRI1 phosphorylation of brassinosteroid signaling kinase1 (BSK1), and constitutive differential growth1 (CDG1), results in activation of BRI1 suppressor1 (BSU1), a phosphatase that inactivates brassinosteroid insensitive2 (BIN2), leading to proteasome-mediated degradation of BIN2 [14-16]. Once relieved from BIN2-mediated suppression, two homologous transcription factors, brassinazole resistant1 (BZR1) and BRI1 EMS suppressor 1 (BES1) are dephosphorylated, move into the nucleus, and bind to the promoters of their target genes, leading to transcription of BR responsive genes [17, 18].

In addition to growth promotion, BR-mediated signaling is also associated with altered resistance to a range of biotic and abiotic stresses. For example, BRs are known to modulate the efficiency of innate immunity responses to microbe associated molecular patterns (MAMPs) [19-22]. In plants, MAMPs are perceived by Pattern Recognition Receptors (PRRs), often in association with the co-receptor BAK1 [23]. This initiates responses including phosphorylation of botrytis-induced kinase 1 (BIK1), which phosphorylates the NADPH oxidase RBOHD, leading to a rapid and transient production of apoplastic reactive oxygen species [24]. MAMP perception also leads to a burst of intracellular Ca^{2+} ,

activation of mitogen-activated protein kinase (MAPK) cascades and a large-scale transcriptional reprogramming, through mechanisms currently not fully understood [25]. It is known that BR signaling exerts an inhibitory effect on the MAMP-induced ROS burst, gene transcription, and late defense responses [19, 20, 26]. This inhibitory effect on defense responses however is not mediated through the shared co-receptor BAK1, illustrating interaction points other than BAK1 in BR and immune response pathways [19, 26]. This interaction is in part mediated through BR-induced altered abundance of stress-responsive transcription factors [27], as well as through an interaction between BR-responsive and stress-responsive transcription factors [26, 28]. However, some evidence suggests that BAK1 may act as mediator of a separate interaction leading to synergistic activities of BRs on MAMP responses [20]. The intricacies of BR signaling in plant-pathogen interaction led to the proposal that a pathogen's virulence is in part determined by its ability to alter biosynthesis or signaling pathway of this and other central hormones [20, 29].

In contrast to BR-MAMP communication, less is known about the timing and mechanistic basis of BR interaction with abiotic stress signaling network. It has been demonstrated that BR application improves plant health and survival under various stresses such as heavy metal, extreme temperatures, drought, and flooding [27, 30-33]. Furthermore, it has been suggested that the protective effect of BL under these diverse unfavorable conditions may be a result of enhanced antioxidant enzyme and metabolite production in plants treated with or over-accumulating BR [33]. Nonetheless, not only are the mechanisms of recognition and the signal transduction pathways for many of these stresses not well understood, the nature of interaction between BR signaling and stress perception and ultimately GSR induction has not been explored.

Here we have utilized 4XRSRE:LUC reporter plants to examine the role of BR in modulating the GSR. Specifically, we show that while BR signaling suppresses the MAMP-triggered GSR as expected, it enhances the wound-activated GSR. This effect is more pronounced in systemic (distal)

than in treated tissues, and is upheld in long-term stress adaptation through enhanced wound-induced resistance to *Botrytis cinerea*. Response to application of bikinin, an inhibitor of BIN2 kinase and by extension an inducer of BR signaling, as well as mutants with altered BR signaling both support this conclusion and extend it to show that BR signaling suppress hypoxia-induced GSR, while providing additional evidence for a role of BAK1 in BR-stress crosstalk in response to specific type of assault. Finally, the BR-mediated enhancement of the wound-activated GSR is not accompanied by changes in the levels of stress-inducible hormones or transcript levels of hormone-marker genes, suggesting specific BR-stress response interactions.

Altogether, these results demonstrate the dependency of BR-mediated modulation of the GSR on the nature of stresses perceived and further support a central role for multi-modular functionality of BRs with general stress network, customizing stimulus-specific responses in Arabidopsis.

2. Materials and methods

2.1 Plant growth conditions

Arabidopsis thaliana seeds were surface-sterilized and plated on ¼ strength Murashige and Skoog media, 0.8% phytoagar. Plates were stratified 3-5 days at 4°C, and grown for two weeks in a growth chamber set at 23°C, 16 hours light/8 hours dark.

2.2 Luciferase assays

Plates were sprayed with 1mM luciferin, 0.01% Tween 20 at 20-16 hours prior to imaging. Three to four hours prior to imaging plates were moved from growth chamber to lab bench, and lightly re-sprayed with luciferin. For chemical treatments, a 6µL drop of chemical was applied to the treated leaf, each chemical with respective solvent control: brassinolide, 1µM with 0.1% DMSO control; bikinin, 30µM with 0.2% DMSO control; MeJA, 1µM with 0.00015% silwet control; SA, 10µM with 0.00015% silwet control; IAA, 1µM with 0.001% ethanol control; ABA, 10µM with water control; GA, 1µM with water control. Subsequently, treated leaves were wounded using a hemostat through

the drop of chemical. In the case of flg22 treatments leaves were not wounded, but the chemical drop was prepared in 0.5 μ M flg22, 0.01% Silwet L-77 solution. In the case of hypoxia sterile MQ water was poured slowly onto plates sufficient to cover hypocotyls and lower leaves of plants without complete submergence. Plants were then imaged using a cooled Charge-Coupled Device (CCD) camera in a light-tight box, controlled by the ANDOR SOLIS v. 15 image analysis software. Quantification of luciferase activity was performed as described previously [3, 4]. It is of note that special attention was paid on selection of brassinosteroid signaling mutant plants that are compromised in growth. Those plants large enough to permit local and systemic regions of interest were specifically selected for luciferase quantification. At the two-week time point considered for imaging, this included most seedlings.

2.3 *Botrytis cinerea* infection assays

Inoculations of detached leaves were performed as previously described [34, 35], with some modification. Briefly, the two largest leaves from three-week old plants were either not wounded, wounded using a hemostat [1], or wounded through a 6 μ L drop of 1 μ M BL or 0.1%DMSO (control). Plants were incubated for 24 hours under a clear cover with dim light to maintain high humidity. Subsequently, treated leaves were detached from the plant and placed on 1% Phytoagar (PlantMedia). A 5 μ L drop of 50% organic grape juice containing 5x10⁴ *Botrytis cinerea* B05.10 spores/mL was placed on the wound site on each leaf. The detached leaves were covered and assayed for lesion area at 48 hours post-inoculation using imageJ.

2.4 Hormone extraction

Hormone extraction was performed as described previously [35, 36] . Briefly, internal standards of dihydro-JA, deuterated SA, deuterated IAA, and deuterated ABA were added to extractions of approximately 200mg fresh weight of plant tissue. Hormones in plant extract were methyl-ester derivatized to facilitate analysis by gas chromatography-mass spectrometry operated in electronic

ionization mode. Peak area at hormone elution time was normalized to relevant standard peak area (dtSA for SA, dhJA for JA, dtIAA for IAA, dtABA for ABA and OPDA) for quantification.

2.5 qRT-PCR

Ninety minutes post wounding and application of a drop of either BL or DMSO on a single leaf, the aerial portions of two week-old seedlings were collected and flash-frozen in liquid nitrogen. RNA was extracted using the Quick-RNA mini-prep kit from Zymo Research, and cDNA was prepared using the Superscript III Reverse Transcriptase from Life Technologies. Next, qRT-PCR was performed using 6uL of SYBR green mix (Qiagen). Each reaction was performed on 8 independent biological replicates per genotype, with 2 technical replicates for each biological replicate, and expression levels were normalized to RHIP1, At4g26410 [37]. Primer sequences are presented in Table S1, reaction conditions and analyses were as previously described [1, 38].

2.6 Statistics

All statistics were performed in R. Comparisons were first done using one-way ANOVAs, with independent variables of experimental replicate and treatment (or genotype). In the case of two-way comparisons, the p-value for treatment in the ANOVA is reported [39]. For comparisons among three or more treatments Tukey's honest significant difference $p < 0.05$ was used to separate sample means.

3. Results

3.1 Brassinosteroid application exerts a stress-specific effect on the GSR

The ability of BR signaling to modify innate immunity responses led us to compare and contrast BR potency on induction of GSR by multiple stimuli. Application of the most potent BR, brassinolide (BL), led to the discovery of a role for BRs in immune responses [40]. Therefore, we examined activation of the GSR in 4xRSRE:LUC reporter plants treated exogenously with BL as compared with dimethyl sulfoxide (DMSO) solvent control treatment. We induced biotic stress by application

of flg22, a commonly used MAMP consisting of a 22 amino acid fragment of bacterial flagellin [41], and abiotic stress initially via a mechanical stress, wounding.

As expected based on the previously reported ability of exogenously applied BL to suppress the flg22-induced ROS burst, transcription of flg22 responsive genes, and callose deposition [19, 20], flg22-induced GSR was also modestly but significantly suppressed by BL treatment (Fig. 1A,B), without any detectable alteration in timing of the peak response (Fig. 1B). Interestingly, comparison of RSRE activity in wounded mock and BL treated plants showed modest but significantly higher local and systemic LUC activity in BL treated plants, again with no change in the pattern or peak activation time (Fig. 1A and C). This is unlikely to be due to additive effects of BL and wounding on the RSRE, as supported by the observation that application of BL on an intact unwounded leaf caused a slight but marginally significant increase in RSRE:LUC expression, not at a comparable scale to the BL modulation of the response in wounded leaf (Supplementary Fig. S2). Interestingly, BL-mediated wound enhancement, as well as flg22 suppression of RSRE was most evident in systemic tissues, defined here as the two brightest untreated leaves (Fig. 1A-C).

3.2 BR enhancement of wound-induced GSR enhances long-term stress adaptation

BR-mediated inhibition of MAMP responses inhibits pattern-triggered immunity (PTI), resistance of MAMP-treated plants to subsequent infection [26]. By analogy, we sought to test whether the BR-mediated enhancement of wound responses could augment wound-induced resistance (WIR). WIR refers to the phenomenon that wounding temporarily enhances plant resistance to necrotrophic fungal pathogens [34, 42-44]. We therefore tested for wound-induced resistance to *Botrytis cinerea* in BL-treated vs control plants. Specifically, we wounded plants treated with BL or DMSO control, followed by 24 hours recovery in high humidity, and subsequent inoculation with *Botrytis cinerea* in a detached leaf assay (Fig. 2). These data show that BL-treated leaves consistently display significantly smaller lesion size at 48 hours post-inoculation than the DMSO controls, revealing that

enhanced short-term GSR in BL-treated plants corresponds with augmented long-term fungal defense.

3.3 Bikinin supports a membrane-receptor independent BR-GSR crosstalk

Having observed a BR enhancement of wounding response both in the GSR and WIR, we sought to clarify the nature of the BR-wound interaction. It has been reported that the transcriptional regulator BZR1 is a key interaction point for BR-mediated suppression of immune signaling [26]. However, given the observed BL induction of wounding-activated GSR as opposed to suppression of MAMP-mediated GSR, we questioned whether this disparity may be due to interaction of the wound-induced GSR with an earlier step in BR signaling, specifically membrane receptors and the co-receptor BAK1. Wounding is likely perceived by many membrane receptors that recognize Damage-Associated Molecular Patterns (DAMPS), including wall-associated kinase (WAK1), peptide receptor1 and 2 (PEPR1 &2), and the *Catharanthus roseus* receptor-like kinase 1 like (CrRLK1L) family [45, 46]. This notion is supported by the established interaction of PEPR with BAK1, and connections between BR and cell wall integrity signaling [47-49]. Thus, to clarify the basis of the aforementioned disparity of BR effect, we utilized bikinin, an inhibitor of a subset of glycogen synthase kinases 3 type kinases (GSK3s), including BIN2. This chemical induces BR signaling downstream of the BR receptor [50]. Similar to BL application, bikinin treatment together with wounding led to enhanced local and systemic wound-responsive GSR (Fig 3 A, C). To confirm this chemical is acting similarly in the GSR as in other flg22-induced responses, we also co-applied bikinin with flg22 and observed attenuated systemic flg22-elicited GSR (Fig. 3A, B), similarly to BL co-application. Also similarly to BL, bikinin treatment alone or together with silwet caused no or only marginal effects on local or systemic GSR response (Supplementary Fig. S2). Thus, the result of bikinin application indicates that, like the BR/MAMP interaction, the BR/wound interaction point is

downstream of BR receptors, thus arguing against GSK3-independent and receptor-dependent BR-mediated modulation of wound-induced GSR.

3.4 Brassinosteroid signaling mutants suggest a potential second point of interaction

To eliminate the possibility of artifacts from exogenous chemical application, we next utilized BR-signaling mutants, crossing the 4XRSRE:LUC parent plants to two mutants with enhanced BR-signaling: *BRI1^{sud1}*, a hyperactive allele of the BR receptor [20], and *BAK1^{elg}*, a BAK1 mutant with enhanced BR signaling [51-53]. Additionally and as a counter balance, we also performed crosses between the 4XRSRE:LUC parent plant and two mutants with reduced BR signaling: *P_{UBQ}:BKI1*, overexpressing an inhibitor of BRI1 [54], and *BRI1^{K911E}*, a kinase-dead mutant in the BR receptor [55] (Fig. 4A). Upon generation of homozygous lines containing RSRE, for simplicity here still referred to as the corresponding BR signaling mutants line, we quantified basal levels as well as peak local and systemic 4xRSRE:LUC expression in each of these backgrounds in response to wounding, and upon application of flg22 (Figs. 4B and S2).

In general, the data exhibit similar trends to that observed with exogenous hormone application, with some noted exceptions, particularly in lines with enhanced BR signaling. Specifically, despite the similar hypersensitivity of *BAK1^{elg}* and *BRI1^{sud1}* to BL, these lines display opposing GSR phenotypes. *BRI1^{sud1}* displays the expected enhancement of systemic wound-induced GSR, but also exhibits enhanced flg22-activated GSR, significantly only in systemic tissue (Fig. 2B). In contrast, *BAK1^{elg}* displays a consistently reduced GSR compared to the parent line. This differential response is also observed in silwet treated plants, resulting in slight reduction in silwet-induced local GSR in *BAK1^{elg}* and an increase in silwet-induced systemic GSR in *BRI1^{sud1}* (Supplementary Fig. S2). BAK1 activity is altered differentially in these lines, possibly explaining the two distinct RSRE response phenotypes observed in *BAK1^{elg}* and *BRI1^{sud1}* lines.

It is noteworthy that both reduced BR signaling mutants, $BRI1^{K911E}$ and $P_{UBQ}:BKI1$, display reduced systemic wound response, while only $P_{UBQ}:BKI1$ exhibits statistically significantly enhanced systemic flg22-induced GSR (Fig. 4B). As with exogenous BL application (Fig. 1), effects on GSR responses in these two signaling mutants were generally more pronounced in systemic/distal tissues than in local. As an exception, $BRI1^{K911E}$ exhibits no altered response to flg22, but displays significantly reduced local and systemic wound responses as compared to 4xRSRE:LUC parent line.

3.4 Reduced BR signaling mutants have an enhanced hypoxia-induced GSR

The difference between wounding, an abiotic stress, and MAMP-response, a biotic stress, suggested a possible general abiotic vs. biotic difference in BR-GSR crosstalk. In order to investigate this possibility, we developed a protocol for induction of a secondary abiotic stress, hypoxia via a mild waterlogging treatment of Arabidopsis seedlings grown on agar plates (Fig. 5A). Unlike flg22 and wounding, hypoxia is sensed intracellularly by N-end rule mediated turnover of hypoxia-responsive TFs such as RAP2.12 under aerobic conditions, as well as by organelle-derived and low-energy signals including ROS [56, 57]. Thus, this stress additionally provides a mechanism to test the possible roles of BAK1-mediated crosstalk in enhanced BR signaling lines.

Induced hypoxia triggered a mild transcriptional activation of RSRE with different peak time and values than those resulting from wounding or application of flg22. The hypoxia-mediated induction of RSRE reaches approximately 25% of the levels observed in wounded or flg22 treated seedlings, at a delayed peak time of 2 to 2.5 hours, as opposed to the 1.5 to 2 hour peak time induced by wounding and flg22 (Fig. 5B and C). This lag in peak time in waterlogged seedlings could correspond to the period necessary for the establishment of hypoxia. Unexpectedly, the trend of hypoxia-mediated RSRE activation in BR signaling mutants more closely resembles the response observed upon flg22 application rather than wounding. Specifically, RSRE response in BR-hypersensitive $BRI1^{sud1}$ and $BAK1^{elg}$ are not significantly different than parent plants, whereas BR-hyposensitive $BRI1^{K911E}$ and

P_{UBQ}:BKI1 display an enhanced RSRE response (Fig. 5D). This suggests that while BR signaling suppresses the hypoxia-induced GSR, further enhancement of BR signaling does not suppress it further. Indeed, the observed increase in hypoxia-triggered GSR in BR-deficient lines may not be due to BR suppression of stress response, but rather to a loss of the protective effect of BR signaling, since it has been shown that BR application enhances long-term plant health under hypoxia [58, 59]. Furthermore, the similar response of BRI1^{sud1} and BAK1^{elg}, both to each other and to parent line, supports the notion that differences in BAK1 status may underlie the differences of these genotypes in response to wounding and flg22.

3.5 Hormone crosstalk networks and the GSR

The stress-specific interaction between BR signaling and the GSR suggested a possible role for hormone crosstalk in observed responses. Indeed, extensive crosstalk is known to exist between BR and other known hormone signaling cascades associated with the tested stresses. In order to investigate whether the observed effects of BR were due to interaction with these or other hormones, we examined and compared the effect of a panel of hormones in modulating the wound-induced GSR. Specifically, wounded 4XRSRE:LUC plants were treated with the stress inducible hormones salicylic acid (SA), methyl jasmonate (MeJA), and abscisic acid (ABA), as well as the growth-promoting hormones indole acetic acid (IAA) and gibberellic acid (GA), in parallel with their corresponding mock treated controls (Fig. 6). Although ethylene signaling would help to understand the submergence phenotypes, ethylene as a gaseous hormone cannot be applied to wounded leaves. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is often used to stimulate ethylene signaling, however unfortunately ACC is not significantly converted to ethylene on the timescale of wound-triggered RSRE activation [60]. These data identified MeJA as the only hormone that consistently and significantly increased the wound-induced GSR (Fig. 6). However, unlike that of BL, the effect of MeJA was significant solely in the local treated leaf and not in systemic tissues.

Next, we tested whether BL enhancement of the wound-induced GSR is exerted via altering JA levels. Specifically, we quantified levels of JA as well as the other hormones tested for GSR modulation, with the exception of GA, in plants wounded and treated with BL vs. corresponding controls (Supplementary Fig. S3). We additionally quantified 12-OPDA, the signaling-active JA precursor, but found no significant changes in the levels of any of these hormones. Furthermore, we extended these analyses to BR-signaling mutants, specifically examining the levels of SA, JA, 12-OPDA, IAA and ABA in wounded and unwounded *BRI1^{sud1}*, *BAK1^{elg}*, *BRI1^{K911E}*, and *P_{UBQ}:BKI1* lines. Similarly, with the exception of *BAK1^{elg}* no consistent differences in measured hormone levels among mutants with enhanced or reduced BR signaling were observed (Supplementary Fig. S4). Interestingly however, the signaling mutant *BAK1^{elg}* accumulates significantly higher basal JA and conversely lower levels of 12-OPDA, as compared to the control. In addition, *BAK1^{elg}* accumulates higher levels of SA than the parent line.

Hormone communication networks are not always restricted to alterations in hormone level, but include changes in responsiveness to endogenous and exogenous cues, through transcription factor interaction or other interaction points within the signaling cascade [51, 61]. Accordingly, we tested transcript levels of the SA-reporter gene, Pathogen Responsive 1 (PR1, Figure 7A). JA response is divided into two mutually antagonistic branches [62], associated with pathogen and wounding/insect response (Figure 7B). We thus tested marker genes for both the pathogen-response (Plant Defensin 1.2, PDF1.2) and the wound-response branch of JA signaling (Vegetative Storage Protein 2, VSP2) [21] (Fig. 7C). Although increased BR synthesis has been reported to result in altered expression of marker genes for these pathways [63], we found no change in expression levels of these marker genes in BL-treated vs DMSO controls.

Collectively, these data suggest that although application of MeJA, similar to BL, enhances the wound-induced GSR, the effect of BL does not appear to be mediated simply through interaction with the stress-associated SA or JA signaling cascades, but is rather an independent stress interaction.

4. Discussion

4.1 Multi-modular effects of BL on the GSR

In order to survive in the ever-challenging environment, plants have evolved a myriad of mechanisms to sense and react to stress, including tradeoffs in growth to divert resources to stress response. This is accomplished by communication among the various involved pathways. Recently, an example of this process has been described: the inhibition of MAMP responses under co-application of the growth-associated hormone BL [19]. By utilizing reporter plants which express luciferase during the transient transcriptional reprogramming of the general stress response, we here expand this observation and provide several novel insights into BR-stress communication in early response to stresses beyond MAMP perception. Chief among these is the observation that exogenous BL enhances the wound response, suggesting rather than a simple growth vs. stress response pivot, a more complicated stress-specific BR signaling mechanism. The more nuanced view of stress response-growth communication suggested by this specificity is in line with ecological models of “costs of resistance,” recognizing that growth and immunity are not always in competition but interact in multiple ways to optimize fitness [64]. The enhanced wound response associated with brassinosteroid signaling is confirmed via bikinin application, BR signaling mutants, and wound-induced resistance. Several other observations are made possible in this reporter system, including differences in the degree of BL effect, as well as consistently more significant effects in systemic than in treated tissues.

BL coapplication leads to a strong reduction in flg22-induced ROS production [19], but a comparatively mild effect on flg22-triggered GSR. Several scenarios could explain this difference; in one scenario, this could be the result of differences in the experimental assays; the GSR in 4xRSRE:LUC reporter plants is examined in whole seedlings treated with a drop of elicitor, whereas the ROS burst is typically assayed by floating leaf discs on elicitor and hormone solution. The ROS burst itself is less extreme when measured in intact seedlings, without the wounding inherent to preparing leaf discs [65, 66], suggesting that signaling pathways are differentially altered by these conditions. Alternatively and/or in addition, other GSR upstream signals may not be affected by BR, diluting the effect of the ROS burst and leading to a smaller net effect on GSR activation. This scenario is supported by the ineffectiveness of BL application to alter MAPK signaling cascades upstream of RSRE activation [4, 26].

The more significant effect of BL in systemic/distal tissues than in the local treated leaf, both in suppressing MAMP-triggered GSR and in enhancing wound-activated GSR, is a clear indication of the involvement of one or a consortium of signals mobilized from the site of signal perception to the distal untreated tissue. Although BRs do not seem to be transported long-distance in a plant [67], the production of ROS, Ca^{2+} burst, and an electric signal all propagate rapidly in response to stress [68], as does a signal induced by PEPs, recognized as DAMPS by PEPR1 and 2 [69]. Lipid-based stress signaling, especially oxylipins are also considered as mobile signals, associated with SAR [70]. This may therefore suggest that local BR signaling modulates one or more of these mobile signals.

4.2 BR-oxylipin crosstalk in the GSR

Although our results show that the effect of BR on the GSR are not mediated through jasmonates or other tested hormones, several observations suggest that more remains to be discovered concerning BR-oxylipin crosstalk in stress signaling. These include the more significant effect in systemic tissues, discussed in section 4.1, and the increased resistance to *Botrytis cinerea*. Although wound-

induced resistance does not require JA, SA, or ethylene [34], resistance to necrotrophic pathogens is associated with oxylipin, and particularly jasmonate signaling [71]. Indeed BR signaling has been shown to enhance oxylipin synthesis [72] and signaling at multiple levels and vice versa [73]. Loss of BRI1, while not affecting JA levels, leads to a reduction in the signaling-active JA-Ile conjugate [74]. Finally, BR signaling has been shown to alter levels of JA-response transcription factors as well as JA response reporter genes, physiological sensitivity to exogenous JA, and plant response to stresses involving JA signaling, such as insect herbivory [63, 74].

Although we did not observe changes in JA levels or tested reporter genes upon BL co-treatment, rather than a lack of connection this may be a reflection of the intricacies of BR/JA communication including differing points of interaction in long- versus short-term responses

4.3 BAK1 in BR-GSR interactions

Although competition for BAK1 was initially a tempting mechanism for BR inhibition of MAMP signaling, it has been shown that this is not the primary mechanism [19]. Furthermore, the results presented herein using bikinin confirm that enhancement of wound response, like inhibition of flg22 response, must occur downstream of membrane receptors. It is of note that effects of bikinin might be due to inhibition of other GSK3-type kinases, and also potential roles of BIN2 outside BR signaling cannot be ruled out. Regardless, in contrast to bikinin the use of brassinosteroid signaling mutants suggests that BAK1-mediated crosstalk does have a role to play in BR-stress response crosstalk.

One obvious example is the BAK1^{elg} mutant. This mutant in BAK1's extracellular domain is hyper-responsive to BL due to due to BAK1-mediated stabilization of BRI1-BL-BAK1 complexes [52, 75]. The reduced response to both wounding and flg22 in this line may thus be mediated by reducing the potential of BAK1^{elg} for dimerization with MAMP and wound-responsive PRRs. Indeed, BAK1^{elg} mutants over accumulate both JA and SA, while conversely containing lower levels of 12-OPDA than parent plants. These differences may be due to disruption of one of the documented functions of

BAK1 in hormone and immune responses and control of cell death signaling [76] in the BAK1^{elg} mutant, or may suggest alternative direct or indirect roles in maintaining hormone homeostasis under stressed and unstressed conditions. By contrast, in response to hypoxia, which is sensed independently of membrane receptors, BAK1^{elg} does not have an altered GSR in comparison with parent line. Thus, in the artificial situation of the BAK1^{elg} mutant, disrupted crosstalk between BR and multiple other signaling pathways may be BAK1-mediated.

Another possible line of evidence for BAK1-mediated crosstalk is the enhanced GSR of BRI1^{sud1} to both wounding and flg22. Unlike BRI1 overexpression or exogenous hormone application, each of which inhibits flg22 responses [19, 20], BRI1^{sud1} also enhances the flg22-triggered ROS burst [20]. The hypersensitivity of BRI1^{sud1} to BL is the result of stabilization of the BRI1^{sud1} hormone binding pocket [12], which has been suggested to prime the shared co-receptor BAK1 [20], resulting in enhanced responsiveness to membrane receptor sensed stresses. Alternatively, BRI1^{sud1} may have reduced or altered levels of “resting state” receptor, hypothesized to involve cytoplasmic domain-mediated dimerization, leading to increased availability/activity of BAK1 for FLS2, and consequently increased flg22 responses [77]. As with BAK1^{elg}, the hypoxia response of BRI1^{sud1} is not significantly different than parent plants, suggesting a membrane-receptor mediated interaction in the case of this mutant.

Thus, although exogenous chemical application clearly shows that membrane receptor-mediated crosstalk is not the only means of BR-GSR interaction, the use of membrane receptor mutants and an intracellularly-sensed stress suggests that BAK1-mediated crosstalk may play a role under certain conditions.

5. Conclusion

Collectively, the results presented herein highlight the importance of multimodular functions of BR in tailoring GSR to the nature of stress encountered. Specifically we have shown that just as BL

application diminishes MAMP-induced ROS and transcriptional response, the MAMP-induced GSR is attenuated in BL-treated and bikinin-treated plants. In line with this, mutants with reduced BL signaling show an enhanced flg22- and hypoxia-induced GSR. This is in contrast to the wound-activated GSR, which is enhanced by BL and bikinin application, and in a BL-hypersensitive mutant, but suppressed in BR-hyposensitive mutants. Enhanced wound responses with BL application are maintained in enhanced wound-induced resistance to *Botrytis cinerea*. Although the primary interaction point is downstream of membrane receptors, BAK1-mediated crosstalk may play a role under certain circumstances. No change in JA level or signaling was observed after BL treatment, but the prominent role of this hormone in wound signaling, and previously published BR/JA interaction, suggests that JA signaling may yet play some role in the effect of BL. In all instances, the GSR is more significantly altered in systemic than local treated tissues, suggesting involvement of one or more mobile signals. The schematic (Fig. 8) highlights the complexity of the BR-stress communication network and proposes possible avenues of connection between concerted array of BR signaling and the GSR, central to understanding the complex communication circuitry mediating tailoring of plant stress responses.

Conflict of interest

The authors declare no conflict of interest

Acknowledgements

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Authors and Contributors

MB performed the work. MB and KD drafted the manuscript. MB, KD, AD, and JC designed the experiments and analyzed the results.

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Figure legends

Figure 1. Brassinolide (BL) suppresses the systemic flg22-induced general stress response (GSR) while enhancing the wound-induced GSR.

(A) Representative false-color images show peak LUC expression of Rapid Stress Response cis-Element reporter plants (4xRSRE:LUC) in response to wounding (W) or flg22 (used at 0.5 μ M) with brassinolide (BL, an active brassinosteroid, used at 1 μ M) or solvent control of DMSO, with the bar above showing conversion from intensity to color. Circles show local (red) and systemic (white) tissues, as quantified in (B) and (C). (B) BL suppresses the peak flg22-activated GSR while not affecting the pattern of activation. (C) Conversely, BL enhances the wound-induced GSR without affecting the pattern. Both effects are more pronounced in systemic activation (lower chart) than the treated leaf (upper chart). Data are the mean $\pm$ SEM, $n \geq 90$ across three experimental repeats, and p values are derived from a one-way ANOVA blocking by experiment on peak (90min) values.

Figure 2. BL application enhances wound-induced resistance to *Botrytis cinerea*.

(A) Representative images of detached leaves 48 h post inoculation with a drop of *Botrytis cinerea* spores. Twenty-four h prior to inoculation leaves were either left intact (not wounded, NW), wounded with a hemostat (W), or wounded through a drop of BL or DMSO. (B) Measurements of lesion size 48 h post inoculation exhibit reduced susceptibility to *Botrytis cinerea* following wounding, enhanced in wounding with DMSO and further enhanced in wounding with BL. The notched boxplot represents population distribution (box and whiskers) with median and confidence intervals (notches) of lesions on at least 90 leaves over three independent experiments. Non-overlapping notches are strong evidence (95% confidence) that samples are statistically different [78]. Additionally, letters represent significantly different values (Tukeys HSD test, $p < 0.05$).

Figure 3. Bikinin exerts opposing effects on flg22- vs. wound-induced GSR.

(A) Representative false-color images show peak RSRE:LUC expression in response to wounding (W) or flg22 (used at 0.5 μ M) with, bikinin (a brassinosteroid signaling activator, 30 μ M) or solvent control of DMSO, with the bar above showing conversion from intensity to color. Circles show local (red) and systemic (white) tissues, as quantified in (B) and (C). (B) Bikinin suppresses the peak flg22-activated GSR while not affecting the pattern of activation. (C) Conversely, bikinin enhances the wound-induced GSR without affecting the pattern. Both effects are more pronounced in systemic activation (lower chart) than the treated leaf (upper chart). Data are the mean $\pm$ SEM, $n \geq 90$ across three experimental repeats, and p values are derived from a one-way ANOVA blocking by experiment on peak (90min) values.

Figure 4. The GSR is altered in brassinosteroid signaling mutant backgrounds.

(A) Representative images of 4xRSRE:LUC (P), expressing BRI1^{sud1} (enhanced BR signaling), BAK1^{elg} (enhanced BR but reduced MAMP), BRI1^{K911E}, and P_{UBQ}:BKI1 (reduced BR signaling) seedlings (B) Peak (90 min) LUC activity in the various genotypes in response to wounding treatment or flg22 application. Data are the mean $\pm$ SEM, $n \geq 90$ across three experimental repeats. Letters represent statistically differences (Tukeys HSD test, $p < 0.05$).

Figure 5. Hypoxia induces the GSR, differentially in BR signaling mutants.

(A) Hypoxia is induced via waterlogging, covering cotyledons and lower leaves of *Arabidopsis thaliana* grown on plates. (B) Representative false-color images show RSRE:LUC expression at time point 0, peak expression, and in the course of experiment. Circles show local (red) and systemic (white) tissues, as quantified in (C) and (D). (C) Hypoxia (right axis) induces the RSRE less than wounding (left axis) with a later peak of activation, highlighted by box on graph. (D) BR signaling mutants display differential GSR to hypoxia stress. Data are the mean $\pm$ SEM, $n \geq 90$ across three experimental repeats. For (D) letters represent statistically differences (Tukeys HSD test, $p < 0.05$) derived from a one-way ANOVA blocking by experiment at time point 2hrs 10min.

Figure 6. Methyl Jasmonate enhances local 4xRSRE:LUC activity in response to wounding.

Local and systemic wound response in 4xRSRE:LUC expressing plants 90 min post wounding and treatment with MeJA, SA, IAA, ABA, and GA with corresponding mock controls (H₂O or EtOH). Data are the average of at least 90 plants over 3 to 5 experimental repeats, $\pm$ SEM, and p values are derived from a one-way ANOVA blocking by experiment on peak (90min) values.

Figure 7. BL treatment does not significantly alter expression of stress hormone-marker genes.

(A) Expression levels of SA-response gene *PR1* in plants treated with BL and the respective control DMSO. (B) Simple schematic of JA response branches and respective marker genes, and (C) expression levels of *PDF1.2* and *VSP2* in plants treated with BL and the respective control DMSO. Each bar is the average of 8 biological replicates, $\pm$ SEM, and p values are derived from a two-tailed Students t-test.

Figure 8. Brassinosteroid signaling exerts opposing effects on the microbe-associated molecular pattern (MAMP) vs. wound-induced general stress response (GSR).

Wounding, brassinosteroids, and MAMPS are all perceived by receptors in the plasma membrane, leading to downstream signaling cascades. This signaling network includes among other signals a burst of reactive oxygen species (ROS) and intracellular Calcium ions as well as mitogen-activated protein kinase (MAPK) activation for wounding and MAMP, and a well-defined phosphorylation cascade in the case of brassinosteroids. In contrast, hypoxia is sensed intracellularly via N-end rule degradation of transcription factors such as RAP12.2. Downstream of BIN2, brassinosteroid signaling enhances the wound-induced GSR while suppressing MAMP-triggered GSR locally and systemically. In each case brassinosteroid interaction with early stress signaling affects respective subsequent immune responses. BR signaling suppresses hypoxia-induced GSR.

Fig 1

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Fig. 6

fig7

Fig. 8

Graphical Abstract