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The IBI1 Receptor of β-Aminobutyric Acid Interacts with VOZ Transcription Factors to Regulate Abscisic Acid Signaling and Callose-Associated Defense

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ABSTRACT

External and internal signals can prime the plant immune system for a faster and/or stronger response to pathogen attack. β-aminobutyric acid (BABA) is an endogenous stress metabolite that induces broad-spectrum disease resistance in plants. BABA perception in Arabidopsis is mediated by the aspartyl tRNA synthetase IBI1, which activates priming of multiple immune responses, including callose-associated cell wall defenses that are under control by abscisic acid (ABA). However, the immediate signaling components after BABA perception by IBI1, as well as the regulatory role of ABA therein, remain unknown. Here, we have studied the early signaling events controlling IBI1-dependent BABA-induced resistance (BABA-IR), using untargeted transcriptome and protein interaction analyses. Transcriptome analysis revealed that IBI1-dependent expression of BABA-IR against the biotrophic oomycete Hyaloperonospora arabidopsidis is associated with suppression of ABA-inducible abiotic stress genes. Protein interaction studies identified the VOZ1 and VOZ2 transcription factors (TFs) as IBI1-interacting partners, which are transcriptionally induced by ABA but suppress pathogen-induced expression of ABA-dependent genes. Furthermore, we show that VOZ TFs require nuclear localization for their contribution to BABA-IR by mediating augmented expression of callose-associated defense. Collectively, our study indicates that the IBI1-VOZ signaling module channels pathogen-induced ABA signaling toward cell wall defense while simultaneously suppressing abiotic stress-responsive genes.

Key words: IBI1, priming, abscisic acid, β-aminobutyric acid, callose, E-MTAB-8720

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INTRODUCTION

Plants can acquire broad-spectrum disease resistance after perception of stress-indicating signals. To avoid the costs of constitutive defense expression, plants have evolved defense priming (Conrath et al., 2006; Ahmad et al., 2010), which allows for a faster and/or stronger immune response after subsequent pathogen attack (Zimmerli et al., 2000; Pastor et al., 2013). Priming can be elicited by external and internal stress stimuli, including microbe-associated molecular patterns and endogenous immune signals (Conrath et al., 2006). One metabolite that has garnered much interest over recent years is β-aminobutyric acid (BABA). Application of this non-proteinogenic amino acid primes systemic plant defenses that are controlled by both salicylic acid (SA)-dependent and SA-independent pathways, resulting in broad-spectrum resistance against biotrophic and necrotrophic pathogens (Zimmerli et al., 2000; Ton et al., 2005; Cohen et al., 2016).

While the resistance-inducing properties of BABA have been known for decades (Papavizas, 1964; Cohen et al., 2016), it was only recently discovered that this compound accumulates in stress-exposed plants (Thevenet et al., 2017), indicating that...
it acts as an endogenous stress metabolite (Baccelli and Mauch-Mani, 2017). This discovery is consistent with our previous finding that the aspartyl tRNA synthetase (AspRS) IBI1 in Arabidopsis thaliana (Arabidopsis) acts as an enantiomer-specific receptor of BABA (Luna et al., 2014). While the primary function of AspRS enzymes is the charging of tRNA$_{\text{Aasp}}$ with L-aspartic acid (L-Asp) for protein biosynthesis, Luna et al. (2014) showed that the biologically active R-enantiomer of BABA, which is structurally similar to L-Asp, binds in planta to IBI1 to activate defense priming and disease resistance. It was proposed that binding of R-BABA to the L-Asp-binding domain induces a secondary defense activity by IBI1 (Luna et al., 2014). This mode of action was recently confirmed by our finding that site-directed mutagenesis of the L-Asp binding domain of IBI1 blocks BABA-induced resistance (BABA-IR; Buswell et al., 2018).

Apart from broad-spectrum resistance, BABA also induces plant stress, which at high concentrations can result in growth reduction (Wu et al., 2010; Luna et al., 2014). This undesirable side effect has hampered commercial exploitation of BABA in crop protection schemes. It has been demonstrated in Arabidopsis that BABA-induced stress is caused by the inhibitory activity of R-BABA on cellular AspRS activity, leading to enhanced accumulation of uncharged tRNA$_{\text{Aasp}}$. This induces phosphorylation of the eukaryotic initiation factor eIF2z by the protein kinase GCN2, which alters cellular metabolism through translational regulation (Lageix et al., 2008), ultimately resulting in a plant stress response that is associated with growth inhibition. Since the gcn2-1 mutant was found to be more tolerant to BABA-induced stress but unaffected in BABA-IR, it was concluded that the GCN2-dependent pathway does not contribute to BABA-IR (Luna et al., 2014). Conversely, mutants in IBI1 were not only impaired in BABA-IR but were also hypersensitive to BABA-induced stress. This contrasting response to BABA was explained by the fact that ibi1 mutants have strongly reduced AspRS activity, which makes them more prone to BABA-induced accumulation of uncharged tRNA$_{\text{Aasp}}$ and GCN2-dependent stress (Luna et al., 2014). Hence, IBI1 controls BABA-IR and BABA-induced stress via separate pathways.

Compared with the GCN2-dependent pathway controlling BABA-induced stress, less is known about the early signaling steps controlling IBI1-dependent BABA-IR. While it is known that BABA primes multiple immune responses that become active at different stages of pathogen infection, there is no direct mechanistic link between IBI1 perception of BABA and defense priming. In addition to relatively late-acting SA-dependent defense mechanisms (Zimmerli et al., 2000; Ton et al., 2005; Van der Ent et al., 2009), BABA also primes early-acting cell wall defenses, which are under control by abscisic acid (ABA) (Ton and Mauch-Mani, 2004; Flors et al., 2008; Pastor et al., 2013). However, how BABA-activated IBI1 regulates ABA and SA signaling to mediate augmented defense induction after pathogen attack remains unclear. While there is ample evidence that ABA suppresses SA-dependent immunity (Audenaert et al., 2002; Mohr and Cahill, 2003; Anderson et al., 2004; de Torres-Zabala et al., 2007; Yasuda et al., 2008; Fan et al., 2009; Ding et al., 2016; Berens et al., 2019), other studies have demonstrated a positive role for ABA in plant immunity, particularly with regard to callose-associated cell wall defense (Ton and Mauch-Mani, 2004; Ton et al., 2005; Adie et al., 2007; García-Andrade et al., 2011; Oide et al., 2013; Hok et al., 2014).

**RESULTS**

**IBI1 Regulates Global Gene Responses to BABA**

To obtain a global impression of IBI1-dependent gene expression during the expression of BABA-IR, we analyzed the transcriptome of water (control)- and BABA-treated Columbia-0 (Col-0; wild-type) and ibi1-1 mutant plants after challenge inoculation with water (mock) or Hpa conidiospores. Three biological replicates per treatment-genotype combination were collected at 2 days post inoculation (dpi), which represents an early time point in the interaction, when Hpa conidiospores are starting to penetrate the epidermal cell layer. To verify a primed immune response at this stage, we analyzed Hpa-inoculated leaves by epifluorescence microscopy to determine the percentage of callose-rich papillae arresting Hpa germ tubes. As expected, BABA-treated Col-0 plants displayed a statistically significant increase in the percentage of callose-arrested Hpa germ tubes compared with water-treated Col-0, whereas the ibi1-1 mutant failed to express this augmented...
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Three-week-old plants were soil-drenched with water or BABA (10 mg/l) and challenged with conidiospores of 
_Hya_loperonospora arabidopsis (Hpa) 2 days later. Replicate shoot samples for RNA extraction and analysis of callose deposition were collected at 2 dpi. Samples for the analysis of Hpa colonization were collected at 5 dpi. (A) Quantification of the effectiveness of callose depositions in arresting Hpa colonization at the epidermal cell layer. Hpa-induced callose was analyzed in aniline blue/calcofluor-stained leaves by epifluorescence microscopy. Shown are percentages of callose-arrested and non-arrested conidiospores (see Supplemental Figure 7 for details). Different letters indicate statistically significant differences in class distribution between samples (Fisher’s exact tests + FDR; \( p \lt 0.05 \); \( n = 110–130 
\) conidiospores). (B) BABA-induced resistance (BABA-IR) against Hpa. Pathogen colonization was determined by microscopy analysis of trypan blue-stained leaves. Leaves were assigned to different Hpa colonization classes (I: no growth; II: hyphal growth; III: hyphal growth + conidiophores; IV: extensive hyphal growth + conidiophores + conidiospores; see Supplemental Figure 6 for details). Different letters indicate statistically significant differences in class distribution between samples (Fisher’s exact tests + FDR; \( p \lt 0.05 \); \( n = 25–30 
\) leaves). (C) Principal component analysis (PCA) of relative gene expression values (Arabidopsis Gene ST 1.1 array). Biologically replicated samples (\( n = 3 
\)) from Col-0 (blue) and ibi1-1 (red) are highlighted within the same PCA plot for each of the four experimental conditions (water + mock, BABA + mock, water + Hpa, and BABA + Hpa). (D) Numbers of differentially expressed genes (DEGs) between Col-0 and ibi1-1 under the four different (pre-)treatment conditions (linear model + FDR; \( q < 0.01 \)). Red bars indicate downregulated DEGs in ibi1-1; green bars indicate upregulated DEGs in ibi1-1.

Transcriptome analysis was based on the Arabidopsis Gene 1.1 ST array (Affymetrix), which contains 600 941 gene probes of 28 501 genes. To verify that the experimental design of our transcriptome experiment was suitable to detect a global response to Hpa, we first analyzed the differences in gene expression between mock- and Hpa-inoculated wild-type plants that had not been treated with BABA. This analysis identified 477 differentially expressed genes (DEGs), of which 446 showed >2-fold induction by Hpa (Supplemental Table 1). Such response is comparable with previous transcriptome studies of Hpa-infected Arabidopsis (Huibers et al., 2009; López Sánchez et al., 2016), thus confirming that the timing of sample collection and general conditions of our experiment were suitable for detection of a transcriptional host response to Hpa.

We next investigated the global impact of IBI1 on BABA- and Hpa-inducible defense responses. Unsupervised principal component analysis (PCA) revealed clustering of samples according to treatments and genotypes (Figure 1C). To visualize the contribution of IBI1 under the different experimental conditions, we highlighted samples from similarly treated Col-0 and ibi1-1 plants within the same PCA plot across all four conditions (Figure 1C). Differences between Col-0 and ibi1-1 were most pronounced after pre-treatment with BABA, irrespective of the secondary challenge treatment, indicating that IBI1 plays a major regulatory role in the transcriptomic response to BABA.

Comparative Transcriptome Analysis Separates Stress-Related Gene Expression from Defense-Related Gene Expression during BABA-IR

To identify the genes that are under transcriptional control by IBI1, we selected differentially expressed genes (DEGs) between Col-0 and ibi1-1 under each of the four experimental conditions (i.e., water + mock, BABA + mock, water + Hpa, and BABA +
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Hpa). Under stress-free conditions (water + mock), there were only five DEGs between Col-0 and ibi1-1 (Figure 1D and Supplemental Table 2). This low number of differential gene expression is consistent with the lack of developmental growth phenotypes of ibi1-1. Similarly, we only detected 15 DEGs in unprimed plants after Hpa inoculation, supporting our observations that ibi1-1 is not majorly affected in basal resistance against Hpa (Luna et al., 2014; Figure 1D). By contrast, we detected 166 DEGs between Col-0 and ibi1-1 after BABA + mock treatment, and 172 DEGs after BABA + Hpa treatment (Figure 1D and Supplemental Table 2), confirming that IBI1 controls a relatively large set of BABA-responsive genes.

Since the ibi1-1 mutant is not only impaired in BABA-IR but is also hypersensitive to BABA-induced stress (Luna et al., 2014; Buswell et al., 2016), the DEGs after BABA treatment can be related to either BABA-induced stress or BABA-augmented defense against Hpa. To separate the stress-related genes from the defense-related genes, we selected the 136 DEGs that are unique to BABA + mock treatment (Figure 2A), during which Col-0 and ibi1-1 develop different levels of BABA-induced stress—but do not express different levels of Hpa-induced defense. Hierarchical clustering by the expression profiles of these 136 genes revealed that samples from BABA-treated ibi1-1 plants clustered apart from all other samples, irrespective of secondary inoculation treatment (mock or Hpa; Figure 2B). By contrast, samples from water- and BABA-treated Col-0 after Hpa inoculation (BABA + Hpa and water + Hpa) clustered closest to those from water-treated ibi1-1 plants after Hpa inoculation (water + Hpa; Figure 2B). Thus, the 136 DEGs responding to BABA + mock treatment only show similar expression patterns in both resistant and susceptible plants, indicating that these genes are unrelated to plant resistance, but rather mark the genotypic differences in BABA-induced stress tolerance. Therefore, to select for genes that are related to BABA-augmented defense against Hpa, we removed all stress-related genes from the group of 172 DEGs after BABA + Hpa treatment. The resulting 143 DEGs, which are unique for the BABA + Hpa condition (Figure 2A), represent IBI1-controlled genes that are associated with BABA-augmented defense expression against Hpa.

**BABA-IR against Hpa Is Associated with IBI1-Dependent Suppression of ABA-Dependent Signaling**

Hierarchical clustering of the 143 defense-related DEGs revealed two distinct gene clusters with opposite expression patterns between Col-0 and ibi1-1 after BABA + Hpa treatment (Figure 2C and Supplemental Table 2B). Statistical gene ontology (GO)-term enrichment analysis revealed that the cluster displaying enhanced expression in ibi1-1 compared with Col-0 is strongly enriched with genes related to ABA signaling and abiotic stress tolerance (Figure 2C). By contrast, there was no enrichment for any GO terms among the 136 genes that were differentially expressed in response to BABA only. Hence, IBI1-dependent repression of ABA-inducible genes is associated with BABA-IR against Hpa and not BABA-induced stress. To examine the significance of ABA signaling in BABA-IR against Hpa, we tested the effects of co-application of BABA with ABA on the level of BABA-IR. To this end, 2-week-old Col-0 and ibi1-1 plants were soil-drenched with BABA or water, sprayed with 10 μM ABA or mock solution at 1 day after BABA treatment, and challenged with Hpa at 2 days after BABA treatment. Analysis of Hpa colonization at 5 dpi revealed that ABA treatment completely repressed BABA-IR in Col-0 against Hpa (Figure 2D). Similar results were obtained with a 10-fold higher concentration of ABA (100 μM; Supplemental Figure 1A). Furthermore, reversing the order of ABA and BABA application did not change the outcome of this experiment (Supplemental Figure 1B). Hence, ABA represses BABA-IR against Hpa. Together with the results of our transcriptome analysis, this strongly indicates that BABA-IR against Hpa requires IBI1-dependent repression of ABA signaling.

**IBI1 Interacts with the Transcription Factors VOZ1 and VOZ2**

Based on our previous finding that IBI1 moves from the ER to the cytoplasm during BABA-IR against Hpa (Luna et al., 2014), we hypothesized that the increased pool of cytoplasmic IBI1 mediates the BABA-augmented defense response by interacting with defense-regulatory proteins. To identify such IBI1-interacting proteins, we screened two different cDNA libraries of Arabidopsis by Y2H analysis, using the full-length IBI1 protein as a bait. The first screen was based on a cDNA library from 1-week-old Arabidopsis seedlings while the second screen used a cDNA library from a mixture of Arabidopsis tissues at different developmental stages. Together, both screens yielded 25 putative interactors (Supplemental Figure 2A and Supplemental Table 3). Since BABA-IR against Hpa manifests itself in the leaves, we first selected candidates that are expressed in leaves, using publicly available gene expression data from the eFP Browser (Schmid et al., 2005; Winter et al., 2007; Kleipkova et al., 2016). From the remaining 15 candidates, we selected three high-confidence interactors that had previously been shown to localize to the cytoplasm (Hooper et al., 2017), which is the subcellular location of IBI1 during expression of BABA-augmented defense against Hpa. Apart from IBI1 itself, we identified the VOZ TFs VOZ1 and VOZ2 (Supplemental Figure 2B), which are functionally redundant to each other (Yasui et al., 2012; Nakai et al., 2013). In planta interactions of IBI1–IBI1 and IBI1–VOZ2 were confirmed using bimolecular fluorescence complementation (BiFC) assays. Co-infiltration of Nicotiana benthamiana leaves with Agrobacterium tumefaciens strains expressing complementary IBI1–YFP (yellow fluorescent protein) constructs (IBI1 autointeraction), as well as complementary IBI1–YFP and VOZ2–YFP constructs (IBI1–VOZ2 interaction), elicited a strong YFP fluorescence signal for each reciprocal combination (Figure 3A). By contrast, the four negative controls (IBI1–YFP constructs co-infiltrated with the empty vector strains and IBI1–YFP constructs co-infiltrated with complementary YFP fusion constructs of the protein kinase OAK) failed to elicit YFP fluorescence.

**Mutations in VOZ1 and VOZ2 Attenuate BABA-IR against Hpa**

The VOZ1 and VOZ2 TFs have previously been reported to repress ABA-dependent gene expression and abiotic stress tolerance (Nakai et al., 2013). Since our transcriptome analysis had revealed that BABA-IR against Hpa is associated with IBI1-dependent suppression of ABA-dependent abiotic stress genes (Figure 2), we investigated the role of VOZ1 and VOZ2 in BABA-IR against Hpa. Because both VOZ genes are functionally redundant to each other (Yasui et al., 2012; Nakai et al., 2013), we tested BABA-IR in the voz1-2/voz2-2

VOZ1/2 Control ABA-Dependent Callose Defense

with Hpa at 2 days after BABA treatment. Analysis of Hpa colonization at 5 dpi revealed that ABA treatment completely repressed BABA-IR in Col-0 against Hpa (Figure 2D). Similar results were obtained with a 10-fold higher concentration of ABA (100 μM; Supplemental Figure 1A). Furthermore, reversing the order of ABA and BABA application did not change the outcome of this experiment (Supplemental Figure 1B). Hence, ABA represses BABA-IR against Hpa. Together with the results of our transcriptome analysis, this strongly indicates that BABA-IR against Hpa requires IBI1-dependent repression of ABA signaling.
double mutant (voz1/2-2) and compared its resistance phenotype with Col-0 and ibi1-1. As expected, BABA-treated Col-0 plants showed almost complete protection against Hpa, whereas ibi1-1 failed to express BABA-IR (Figure 3B). Notably, while BABA-treated voz1/2-2 still expressed a statistically significant resistance response to Hpa, the level of BABA-IR was attenuated compared with the wild type, as evidenced by a statistically higher level of Hpa colonization in BABA-treated voz1/2-2 compared with BABA-treated Col-0. Hence, the IBI1-interacting VOZ proteins contribute to BABA-IR against Hpa.

The voz1/2-2 Mutant Is Affected in Priming of SA-Induced Gene Expression

BABA-IR against Hpa is based on priming of both SA-dependent and SA-independent defenses (Ton et al., 2005). Since the voz1/2-2 mutant was only partly impaired in...
BABA-IR (Figure 3B), we hypothesized that one of these mechanisms is affected. Since the voz1/2-2 mutant had previously been reported to be compromised in systemic acquired resistance (SAR) against Pseudomonas syringae (Nakai et al., 2013), which is predominantly based on priming of SA-dependent defenses (Kohler et al., 2002), we examined BABA-induced priming of the SA-inducible PR1 gene in Col-0 and voz1/2-2 plants. To this end, replicate leaf samples were collected from water- and BABA-treated plants at 8 h after challenge treatment with SA. In water-treated (unprimed) plants, the level of PR1 induction by SA was similar in both genotypes (Figure 4A), indicating that voz1/2-2 is unaffected in basal SA sensitivity. Furthermore, BABA-treated Col-0 displayed statistically increased levels of SA-induced PR1 expression compared with water-treated Col-0 plants, confirming that BABA primes SA-induced gene expression (Zimmerli et al., 2000; Ton et al., 2005). While SA-induced PR1 induction in BABA-treated voz1/2-2 plants was marginally enhanced compared with water-treated voz1/2-2 plants, this effect was not statistically significant (Figure 4A). Hence, the voz1/2-2 mutant is partially affected in the priming of SA-induced PR1 expression by BABA.

To determine the impact of the attenuated priming of SA-induced gene expression in voz1/2-2 on BABA-IR against Hpa, we introgressed the NahG gene into the voz1/2-2 background, which prevents endogenous SA accumulation (Gaffney et al., 1993; Delaney et al., 1994). If the reduced BABA-IR response of voz1/2-2 was caused by its defect in priming of SA-defense gene expression (Figure 4A), introgression of NahG in voz1/2-2 would not further compromise BABA-IR. Accordingly, we compared levels of BABA-IR against Hpa in Col-0, NahG, voz1/2-2, and NahG voz1/2-2. As reported previously (Zimmerli et al., 2000; Ton et al., 2005), BABA-IR against Hpa in NahG plants is largely unaffected, due to earlier-acting cell wall defenses that operate independently of SA (Figure 4B). While the voz1/2 mutant showed attenuated BABA-IR against Hpa compared with Col-0 and NahG plants, NahG voz1/2-2 plants displayed an even stronger reduction in BABA-IR, which was statistically significant (Figure 4B). These results indicate that the attenuated BABA-IR response of voz1/2 is not due to its impaired priming of SA-inducible gene expression but rather due to a defect in other, SA-independent, resistance mechanisms.

VOZ1 and VOZ2 Contribute to BABA-IR against Hpa by Mediating Augmented Expression of Callose-Associated Cell Wall Defense

Since priming of SA-dependent defenses did not have a major contribution to VOZ1/2-dependent BABA-IR against Hpa (Figure 4B), we investigated the involvement of SA-independent cell wall defenses. To this end, the effectiveness of callose depositions in water- and BABA-treated Col-0 and voz1/2-2 was determined at 3 days after Hpa inoculation. Epifluorescence microscopy analysis of the percentage of callose-arrested germ tubes confirmed that BABA-treated Col-0 plants show a
VOZ1/2 Control ABA-Dependent Callose Defense

Figure 4. The Signaling Role of VOZ1 and VOZ2 in BABA-IR.

(A) The voz1/2-2 mutant is partially compromised in BABA-induced priming of salicylic acid (SA)-induced PR1 expression. Leaves of water- and BABA-treated Col-0 and voz1/2-2 were sprayed with 1 mM SA at 2 days after soil-drench treatment of 2-week-old plants with water or BABA (5 mg/l). Shoots were harvested at 8 h after SA treatment for qRT–PCR analysis. Data shown are mean expression values (±SEM) of PR1 expression relative to water-treated mock-sprayed Col-0 plants. Different letters indicate statistically significant differences in expression (two-way ANOVA + Tukey’s multiple comparisons test; p < 0.05; n = 4).

(B) Introgresion of the NahG gene in the voz1/2-2 mutant background further reduces its attenuated BABA-IR response, indicating that SA-dependent defense does not play a major role in VOZ1/2-dependent BABA-IR against Hpa. Two-week-old Col-0, NahG, voz1/2-2, and NahG voz1/2-2 plants were soil-drenched with water or 5 mg/l BABA and inoculated with Hpa 2 days later. Disease progression was assessed at 5 dpi by assigning trypan blue-stained leaves to different Hpa colonization classes (Supplemental Figure 6). Different letters indicate statistically significant differences in class distribution between samples (Fisher’s exact tests + FDR; p < 0.05; n = 55–71 leaves).

(C) The voz1/2-2 mutant is impaired in BABA-induced priming of callose-associated cell wall defense. Two-week-old Col-0, ibi1-1, and voz1/2-2 plants were soil-drenched with water or 5 mg/l BABA and inoculated with Hpa 2 days later. Callose effectiveness was analyzed at 3 dpi in aniline blue/calcofluor-stained leaves by epifluorescence microscopy. Shown are percentages of callose-arrested and non-arrested germ tubes at the epidermal cell layer (Supplemental Figure 7). Letters indicate statistically significant differences between samples (Fisher’s exact tests + FDR; p < 0.05; n = 31–105 conidia/sprouts).

statistically significant augmentation in callose-associated defense against Hpa (Figure 4C). By contrast, BABA-treated voz1/2-2 plants, like ibi1-1 plants, failed to express this augmented cell wall defense (Figure 4C). Hence, the voz1/2-2 mutant is impaired in BABA-induced priming of callose-associated cell wall defense against Hpa.

VOZ2 Must Locate to the Nucleus for Its Contribution to BABA-IR against Hpa

Pathogen-induced callose deposition requires activity by the callose synthase PMR4 in Arabidopsis (Nishimura et al., 2003), which is regulated at the post-translational level (Flors et al., 2008; Ellinger et al., 2013; Ellinger and Voigt, 2014). Accordingly, it has been proposed that this defense layer is regulated independently of gene transcription (Van der Ent et al., 2009), which is difficult to reconcile with our finding that VOZ TFs regulate this defense (Figure 4C). Therefore, we investigated whether the role of VOZ2 in BABA-IR depends on its nuclear localization as a TF or whether it involves an alternative cytoplasmic function. To this end, we first characterized the subcellular localization of VOZ2 in the voz1/2-1 double mutant expressing GFP-VOZ2 (p35S:GFP-VOZ2; Yasui et al., 2012) under all four experimental conditions (water + mock, BABA + mock, water + Hpa, and BABA + Hpa). Even though p35S:GFP-VOZ2 plants expressed wild-type levels of BABA-IR (Supplemental Figure 3A), epifluorescence microscopy of 4’,6-diamidino-2-phenylindole (DAPI)-stained leaves only revealed cytoplasmic localization of GFP-VOZ2 and no detectable colocalization with the nucleus (Figure 5A). In an independent experiment, confocal laser scanning microscopy did not detect noticeable changes in cytoplasmic localization of GFP-VOZ2 between the four different experimental conditions (Supplemental Figure 3B). However, as has been reported previously (Yasui et al., 2012), the amount of active GFP-VOZ2 in the nucleus may be too low for nuclear detection by microscopy. To examine the role of potentially low amounts of VOZ2 in the nucleus, we quantified BABA-IR in voz1/2-1 plants overexpressing VOZ2 fused to either a nuclear localization sequence (VOZ2-NLS) or a nuclear export sequence (VOZ2-NES, Yasui et al., 2012). VOZ2-NLS-expressing plants showed wild-type levels of BABA-IR against Hpa and also displayed increased basal resistance compared with water-treated Col-0 plants (Figure 5B).
contrast, VOZ2-NES plants showed attenuation in BABA-IR similar to that of voz1/2-2 plants and showed wild-type levels of basal resistance (Figure 5B). Thus, despite the fact that we only detected GFP-VOZ2 fluorescence in the cytoplasm, nuclear localization of VOZ2 is critical for its contribution to BABA-IR against Hpa.

**VOZ1/2 Are Transcriptionally Induced by ABA but Repress Hpa-Induced ABA Abiotic Stress Signaling during BABA-IR**

To investigate the signals controlling VOZ gene expression, we first consulted publicly available transcriptome data (eFP Browser; Winter et al., 2007). While none of the VOZ genes showed transcriptional induction by SA, VOZ1 was transcriptionally induced by ABA, and both TF genes showed transient induction by salt, drought, and osmotic stress. To verify this effect of ABA on VOZ gene expression under our experimental growth conditions, we sprayed shoots of 2-week-old plants with 100 μM ABA (1% Silwet) and collected replicate samples 8 h later for qRT–PCR analysis. Both VOZ1 and VOZ2 showed statistically significant levels of induction by ABA (Figure 6A). Since VOZ1 and VOZ2 have been reported to suppress ABA-inducible genes during abiotic stress exposure (Nakai et al., 2013), this indicates that both TFs act in a negative signaling loop to control excessive induction of genes controlling abiotic stress tolerance.

Our transcriptome analysis had shown that BABA-IR against Hpa involves IBI1-dependent repression of ABA-inducible genes (Figures 2C and 2D). Accordingly, we hypothesized that VOZ1 and VOZ2 mediate augmented cell wall defense during BABA-IR by repressing ABA-dependent defense against abiotic stress, which is known to antagonize plant immunity (Yasuda et al., 2008; Bostock et al., 2014; Berens et al., 2019). To investigate this hypothesis, we profiled the transcription of six ABA-responsive genes in Col-0, voz1/2-2, and 35S:VOZ2 plants at 24 h after shoot treatment with control solution or 100 μM ABA. This gene set included genes showing IBI1-dependent repression during BABA-IR against Hpa (HAI1, HAI2, ABI1, NAC019; Figure 2C and Supplemental Table 2), the ABA-inducible marker gene ABI2, and the ABA-dependent TF gene CBF4, which had previously been reported to be repressed by VOZ1/2 (Nakai et al., 2013). As expected, qRT–PCR profiling of replicate samples showed increased induction of these ABA marker genes in voz1/2-2 and reduced induction in 35S:VOZ2 compared with Col-0 plants (Figure 6B). Although we did not detect a statistically significant induction of the VOZ1 and VOZ2 genes by Hpa at the time points of our qRT–PCR experiments (data not shown), mining of publicly available transcriptome data from a time-course experiment by Wang et al. (2011) revealed that infection by virulent Hpa transiently induces both VOZ1 and VOZ2 (Supplemental Figure 4). To further test our hypothesis that VOZ1 and VOZ2 repress ABA-dependent abiotic stress signaling during BABA-IR against Hpa, we profiled the expression of the six ABA marker genes in water- and BABA-pre-treated Col-0 and voz1/2-2 after mock or Hpa inoculation. At 4 dpi, the ABA-responsive genes showed transcriptional induction by Hpa, which was stronger in voz1/2-2 plants (Figure 6C). Moreover, BABA pre-treatment completely prevented the induction of ABA marker genes by Hpa in Col-0 but not voz1/2-2 (Figure 6C). Hence, VOZ1 and VOZ2 repress Hpa-induced expression of ABA response genes, and this effect is augmented during BABA-IR against Hpa.

**DISCUSSION**

The resistance-inducing activities of BABA have been known for decades (Jakab et al., 2001; Cohen et al., 2016). However, the biological relevance of this resistance response has long...
remained unclear. Recently, Thevenet et al. (2017) reported that plant stress exposure induces endogenous BABA accumulation. Together with the discovery that the AspRS protein IBI1 acts as an in planta receptor of BABA (Luna et al., 2014; Buswell et al., 2018), these recent results strongly suggest that BABA acts as an endogenous immune signal in plants. Other studies have shown that BABA primes various immune responses, including SA- and NPR1-dependent defenses (Zimmerli et al., 2000; Ton and Mauch-Mani, 2004; Ton et al., 2005; Van der Ent et al., 2009; Pastor et al., 2013), as well as early-acting cell wall-localized defenses, such as apoplastic reactive oxygen species accumulation and callose deposition that are controlled by ABA signaling (Ton and Mauch-Mani, 2004; Ton et al., 2005; Flors et al., 2008). However, the immediate signaling steps by which the BABA-bound IBI1 mediates augmented defense expression, as well as the signaling role of ABA therein, have remained unresolved. Our study has identified two IBI1-interacting TFs, VOZ1 and VOZ2, which contribute to BABA-IR by mediating augmented expression of early-acting callose-associated defense at the cell wall (Figures 3 and 4). Both TFs are transcriptionally inducible by ABA but repress pathogen-induced ABA signaling during BABA-IR (Figure 6). Thus, our study has identified the VOZ TFs as novel signaling components in IBI1- and ABA-dependent expression of cell wall defense.

The role of ABA in plant immunity has remained controversial (Ton et al., 2009; Berens et al., 2019). Our study points to a model that reconciles the controversy about ABA in plant immunity and explains the complex signaling role of ABA in BABA-IR (Figure 7). During BABA perception, the molecule binds to the L-Asp-binding domain of the IBI1 receptor (Luna et al., 2014; Buswell et al., 2018), which primes this protein for pathogen-induced translocation from the ER membrane to the cytoplasm (Luna et al., 2014). Upon Hpa infection, the pathogen stimulates ABA signaling as a virulence strategy to suppress pattern-triggered immunity (PTI; Asai et al., 2014), which elicits enhanced expression of VOZ1 and VOZ2 genes (Figure 6A and Supplemental Figure 4). Since induction of PTI genes causes ER stress (Wang et al., 2005; Moreno et al., 2012; Korner et al., 2015), we propose that the Hpa-suppressed PTI during the early stages of infection is sufficient in BABA-primed cells to trigger moderate levels of ER stress and so allow for cytoplasmic translocation of IBI1, where it interacts with the ABA-induced...
pool of defense-regulatory VOZ1/2 TFs. Through yet unknown mechanisms, this interaction activates nuclear defense activity by the TFs (Figure 5B), which antagonizes ABADependent immune suppression by Hpa (Figure 6C) and facilitates augmented expression of callose-associated PTI (Figure 4C). In unprimed cells this IB1/VOZ1/2-dependent signaling cascade is delayed, as IB1 without BABA is less responsive to pathogen-induced cytoplasmic translocation (Luna et al., 2014), resulting in basal resistance that is too weak to prevent infection. It is worth noting that pathogen-induced production of endogenous BABA does not appear to have a significant contribution to basal resistance against Hpa, since control-treated wild-type plants displayed levels of Hpa colonization similar to those in ib1-1 plants (Figures 1B, 2D, 3B, and 5B). Thevenet et al. (2017) showed that BABA concentrations in Arabidopsis leaves increased significantly within 1 and 2 dpi with P. syringae and Plectosphaerella cucumerina, respectively, whereas endogenous BABA concentrations did not increase until 5 dpi with Hpa. Considering that BABA-augmented cell wall defense against Hpa is effective from 2–3 dpi, we conclude that Hpa employs specific effectors that suppress endogenous accumulation of BABA. This hypothesis also explains the relatively weak impact of the ib1-1 mutation on Hpa-induced gene expression in water-treated plants (Figure 1D).

The voz1/2 mutant was not only affected in callose-associated cell wall defense (Figure 4C), but also showed a defect in BABA-induced priming of SA-induced expression of the PR1 gene (Figure 4A). This latter result supports earlier findings that voz1/2-2 plants are affected in SA-dependent basal acquired resistance and SAR against P. syringae pv. tomato DC3000 (Nakai et al., 2013). While SA-dependent defenses are important for basal resistance and SAR against Hpa (Delaney et al., 1994; Ton et al., 2002), the function of VOZ1/2 in priming of SA-induced gene expression did not have a major contribution to BABA-IR against Hpa, since SA non-accumulating voz1/2-2 NahG plants showed a stronger reduction in BABA-IR than voz1/2 plants (Figure 4B). Unlike ib1-1 plants, SA non-accumulating NahG voz1/2-2 plants still expressed a residual level of BABA-IR against Hpa (Figure 4B), indicating activity of additional VOZ1/2- and SA-independent defense layers, which act at different stages of infection than relatively early callose-associated defense and relatively late SA-dependent defense, respectively. This conclusion also supports the wider notion that BABA-IR is based on priming of PTI (Po-Wen et al., 2013), which constitutes a multitude of different defense layers that become active at different stages of infection (Zipfel and Robatzek, 2010; Bigeard et al., 2015).

Our Y2H screens for IB1-interacting proteins identified 25 different protein candidates, including three high-confidence interactors which in other studies have been confirmed to be localized in the cytoplasm (Supplemental Figure 2 and Supplemental Table 3). It is, however, possible that IB1 also interacts with proteins in other cellular compartments during BABA-IR. For instance, our confocal microscopy analyses of IB1 and VOZ2 cannot exclude the possibility that the IB1–VOZ complex transiently moves to the nucleus, where it interacts with other
defense-regulatory proteins. In this regard, the D111/G-patch domain-containing protein is interesting, which showed a high-confidence interaction with IB1 and is predicted to be targeted to the nucleus (Supplemental Table 3). The corresponding At5G26610 gene was recently found to be translationally regulated during effector-triggered immunity and to regulate the hypersensitive cell death response (HR) (Yoo et al., 2020). In this context, it is possible that a transient interaction between the IB1–VOZ complex and the D111/G-patch protein in the nucleus increases HR-related cell death and Hpa resistance. Furthermore, considering that the default localization of IB1 is the ER (Luna et al., 2014), it is equally possible that IB1 undergoes relevant interactions with ER-localized proteins. Of particular interest is the interaction with the ER-localized FAH2 protein (Supplemental Table 3). This fatty acid hydroxylase is required for the activity of Bax Inhibitor-1 (BI-1), which represses ER stress-related cell death (Watanabe and Lam, 2008; Nagano et al., 2012). Based on our model that PTI-related ER stress triggers IB1 translocation to the cytosol (Figure 7), it is tempting to speculate that binding of BABA to IB1 affects FAH2-dependent BI-1 activity, resulting in increased sensitivity to PTI-related ER stress and augmented IB1 translocation to the cytoplasm during pathogen infection.

Although our study has identified a new regulatory module in plant acquired immunity, there are still unresolved questions about the role of ABA in BABA-IR against stresses other than Hpa. For example, the suppressive activity of IB1 and VOZ1/2 on abiotic stress genes (Figures 2C, 6B, and 6C) seems difficult to reconcile with previous observations that BABA induces tolerance against drought and salt stress (Jakab et al., 2005). This could in part be explained by the fact that the signaling pathways controlling BABA-IR highly depend on the challenging stress (Zimmerli et al., 2000). For instance, while BABA primes for enhanced induction of the ABA-dependent RAB18 gene after salt stress exposure (Jakab et al., 2005), this priming effect is not apparent after inoculation with Alternaria brassicicola or P. cactorum (Ton and Mauch-Mani, 2004). Hence, the role of ABA in BABA-IR differs depending on the challenging stress, and it is possible that other IB1-interacting proteins control these stress-specific augmented defenses in BABA-primed plants. Future research is needed to investigate how BABA manipulates the crosstalk between environmental signaling pathways. In the face of global climate change, a better understanding of the crosstalk between biotic and abiotic stress signaling is crucial for the breeding of crops that are able to cope simultaneously with drought, heat, pests, and diseases.

**METHODS**

**Plant Material and Growth Conditions**

*A. thaliana* (ecotype Col-0) for bioassays and gene expression assays were grown on a mixture of Levington M3 soil and sand (2:1 [v/v]), at 8.5-h light (115–140 μmol/m²/s, 21°C) and 15.5-h darkness (19°C), and at 65%–80% relative humidity. *N. benthamiana* plants were grown at 15.5-h light (115–140 μmol/m²/s, 21°C) and 8.5-h darkness (19°C) in controlled growth chambers. Leaf infiltration assays were performed on 4- to 6-week-old plants (i.e., before plants developed flowers). Seeds of the voz1-2 voz2-2 (voz1/2-2) mutant were kindly provided by M. Sato (Kyoto Prefectural University), voz1-1 voz2-1 (voz1/2-1), voz1/2-1 SSS:GFP-VOZ2, voz1/2-1 3SS:VOZ2-NLS, and voz1/2-1 3SS:VOZ2-NES seeds were kindly provided by T. Kohchi (Kyoto University). To generate homozygous NahG voz1/2-2 lines, we crossed Col-0 NahG (line B15) reciprocally with the voz1/2-2 double mutant. F2 progeny were genotyped by end-point PCR to select for individuals homozygous for the voz1-2 and voz2-2 7-T-DNA mutations and carrying the NahG gene. Two homozygous voz1/2-2 F2 plants from each reciprocal cross with the strongest PCR band for NahG were selected for qPCR quantification of NahG DNA. Both plants displayed ~2-fold higher levels of NahG than their hemizygous F1 parents (Supplemental Figure 5A), suggesting that they were homozygous for NahG. Fourteen individual plants in the F3 progeny from one line (F3-1) were tested for the presence of the NahG gene (Supplemental Figure 5B), confirming that this line is no longer segregating for the NahG gene and thus homozygous for all three mutations/insertions. Seeds from this homozygous NahG voz1/2-2 line were tested for BABA-IR against Hpa (Figure 4B).

**Induced Resistance Assays**

Resistance against the biotrophic oomycete *H. arabidopsidis* (strain WAC09) was assessed microscopically in leaves of 3- to 4-week-old plants after soil-drenching plants with water or a racemic mixture of R/S-BABA (Sigma-Aldrich, #A44207), as described previously (Buswell et al., 2010). The BABA concentrations applied (5–10 mg/l) were sufficient to induce near complete levels of Hpa resistance, but low enough to prevent stress symptoms and growth reduction in wild-type plants (Col-0). To ensure that enough leaf material for the transcriptomic analysis could be harvested, plants were 1 week older than the plants used in subsequent bioassays. At this older age, Col-0 plants require a higher BABA concentration to reach near complete levels of Hpa resistance. Accordingly, the final BABA concentrations in the soil were 10 mg/l for transcriptome analysis and 5 mg/l in all subsequent bioassays. Plants were inoculated with a suspension of *Hpa* conidiospores (10⁶ spores/ml) at 2 days after soil-drench treatment. Leaf samples were harvested at 5–7 dpi for trypan blue staining and scored for *Hpa* colonization by assigning individual leaves to four distinct colonization classes (Supplemental Figure 6): I = healthy leaf, no sporulation; II = hyphal growth, less than eight conidiophores; III = hyphal growth, and more than eight conidiophores; IV = extensive hyphal growth, conidiophores and oospores present. To assess the effectiveness of *Hpa*-elicited callose depositions, we collected samples at 2–3 dpi for aniline blue/ calcofluor staining, as described previously (Ton et al., 2005). Stained leaves (>10 different leaves from independent plants) were analyzed by UV epifluorescence microscopy (Olympus BX51; light source: CoolLED eP2-3; 330 nm wide band excitation filter, 400 nm LP emission filter, 400 nm dichromatic filter) and scored for numbers of callose-arrested versus unarrested germ tubes at the epidermal cell layer (Supplemental Figure 7). Statistical differences in class distributions of *Hpa* colonization and callose defense efficiency were determined by pairwise Fisher’s exact tests with Benjamini–Hochberg false discovery rate (FDR) correction (Benjamini and Hochberg, 1995), using the R package “fifer” (https://cran.r-project.org/package=fifer).

**qRT–PCR Assays**

For qRT–PCR quantification of gene expression, shoot samples from three to four biological replicates per treatment/genotype combination were snap-frozen in N2(l) and homogenized. Each biological replicate consisted of 4–10 seedlings/pot. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, cat. no. 74904), according to the manufacturer’s protocol. RNA was treated with DNase (RNase free DNase, Promega, #M6101), and cDNA synthesis was performed from 800 ng of total RNA, using oligo(dT) primers with SuperScript III reverse transcriptase (Invitrogen, 18080085), according to the manufacturer’s protocol. The cDNA was diluted 4–10× in nuclease-free water before qPCR in a RotorGene Q real-time PCR cycler (Qiagen; Q-Rex Software v1.0), using the RotorGene SYBR Green Kit (Qiagen, cat no. 204074) and gene-specific primer pairs at a final concentration of 250 nM (see Supplemental Table 4 for primer sequences). Specificity of primers was verified by dissociation melting curve analysis. For each primer pair, PCR efficiencies (1 + E)
Molecular Plant were determined by averaging machine-estimated PCR efficiencies of all replicate reactions per experiment. For each replicate sample, fold-change values in comparison with an arbitrary calibrator sample were calculated as \( 1 + \frac{E_{q1} - E_{q2}}{E_{c1} - E_{c2}} \), where \( C_t \) (take-off cycle) was defined as the cycle at which the increase in fluorescence is 20% of the peak increase in fluorescence (Qiagen, 2012). Fold-change values were normalized to the averaged fold-change values of three housekeeping genes: GAPDH (At1g13440), UBC21 (At5g25780), and SAND family protein (At2g83930) (Czechowski et al., 2005). For each replicate sample, these corrected fold-change values were normalized to the averaged values of the control samples (e.g., water-treated and mock-inoculated Col-0 plants), as indicated in figure legends. Statistically significant differences in gene expression were determined by Student’s t-tests or ANOVA (\( n = 3 \)), as indicated in the figure legends.

Yeast Two-Hybrid Analysis

The *IBI1* coding sequence (amino acids 1–558) (At4g31180; NCBI reference NM_119268.4) without STOP codon was PCR-amplified from pENTR-IBI1 and cloned either into pB29 as an N-terminal fusion to LexA DNA-binding domain (IBI1-LexA; first screen), or into pB43 as an N-terminal fusion to the Gal4 DNA-binding domain (IBI1-Gal4; second screen). The LexA construct was used as a bait to screen a random-primed cDNA library from etiolating Arabidopsis seedlings; the Gal4 construct was cloned into pGADT7-RecAB, resulting in a collection of Y2H prey colonies. Yeast Two-Hybrid Analysis

Three-week-old Col-0 and *ibi1-1* plants were cultivated, treated, and inoculated as described above. Three biological replicates (each consisting of pooled leaves from ~20 plants from one pot) were collected at 2 dpi and snap-frozen in N\(_2\)(l) for total RNA extraction. RNA hybridization to Arabidopsis Gene ST 1.1 arrays (median 22 probes/genome, Affymetrix, #901913) was performed at the Nottingham Arabidopsis Stock Center. Microarray data were extracted, quality-checked, and normalized by robust multiarray average (RMA), using R Bioconductor software (version 3.2; Huber et al., 2015). Prior to normalization, an image of each microarray was produced using the package “affy” (version 1.48.0; Gautier et al., 2004) to verify absence of bubbles and smears. Normalization was performed by the RMA algorithm in package “oligo” (version 1.34.2; Carvalho and Irizarry, 2010), which implements background correction, log transformation, and quantile normalization of the raw hybridization values, thereby assigning identical statistical properties to the distribution of hybridization signals from each microarray. A linear model was fit to the normalized data to obtain an expression value for each probe set (irizarry et al., 2003). Annotations of probe sets and transcripts were retrieved from the NetAffx database (Liu et al., 2003), using the “oligo” package. PCA at the transcript level across all 24 microarrays was performed, using the package “arrayQualityMetrics” (Kaufmann et al., 2009). DEGs between Col-0 and *ibi1-1* at each experimental condition were selected by pairwise comparisons of normalized hybridization values, using the “limma” package (linear model + Benjamini–Hochberg FDR; \( q < 0.01 \); no cut-off value for fold change applied). Hierarchical clustering of DEGs was performed using the software MeV version 4.9.0 (Saeed et al., 2006), after row-normalization of gene expression values to the average across all samples. GO-term analysis of DEGs was performed using the web tool Gorilla (Eden et al., 2009; http://cbl-gorilla.cs.technion.ac.il/; accessed 11/02/2015).

Microscopy Analysis of GFP-VOZ2

Microscopy analysis of GFP-VOZ2 subcellular localization was examined by epifluorescence microscopy and confocal microscopy of *voz1-2/1* plants overexpressing GFP-VOZ2 (p3SS:GFP-VOZ2; Yasui et al., 2012). Leaves for epifluorescence microscopy analysis were harvested at 4 dpi for DAPI staining, as described by Borg et al. (2019). In brief, samples were incubated in DAPI solution (1.5 \( \mu \)g/ml) for 15 min and washed with water, and individual leaves were mounted on microscope slides. Images were taken with a Leica DM6 B upright microscope (light source: CoolLED pE-2; GFP: 470/40 nm excitation filter, 525/50 nm emission filter, 495 nm dichroic filter; DAPI: 350/50 nm excitation filter, 460/50 nm BP emission filter, 400 nm LP dichroic filter), using Leica LAS X software. For confocal laser scanning microscopy, plants were harvested at 2 dpi. Images were taken in unprocessed intact leaves using an Olympus FV1000 confocal laser scanning microscope (excitation, 488 nm argon laser; emission filter, 510–550 nm).

ACCESSION NUMBERS

Microarray data have been deposited at EMBL (E-MTAB-8720). Sequence data from this article can be found in The Arabidopsis Information Resource data library (www.arabidopsis.org) under the following accession numbers: TAIR: At3g41180 (IBI1); At1g28520 (VOZ1); At2g42400 (VOZ2); At4g26080 (AB11); At5g57050 (AB12); At5g59220 (HAI1); At1g07430 (HAI2); At1g52890 (NAC019); At5g51990 (CBF4); At2g14610 (PR1).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at Molecular Plant Online.
VOZ1/2 Control ABA-Dependent Callose Defense

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AUTHOR CONTRIBUTIONS
J.T. conceived the project; R.E.S. and J.T. designed and supervised experiments; R.E.S., G.W., E.G., P.J., E.L., and J.T. performed bioassays; R.E.S., J.S., and E.L. analyzed the microarray data; R.E.S. and J.T. wrote the manuscript. All authors reviewed and approved the final manuscript.

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