The Ankyrin-Repeat Transmembrane Protein BDA1 Functions Downstream of the Receptor-Like Protein SNC2 to Regulate Plant Immunity[1][C][OA]

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Plants utilize a large number of immune receptors to recognize pathogens and activate defense responses. A small number of these receptors belong to the receptor-like protein family. Previously, we showed that a gain-of-function mutation in the receptor-like protein SNC2 (for Suppressor of NPR1, Constitutive2) leads to constitutive activation of defense responses in *snc2-1D* mutant plants. To identify defense signaling components downstream of SNC2, we carried out a suppressor screen in the *snc2-1D* mutant background of Arabidopsis (*Arabidopsis thaliana*). Map-based cloning of one of the suppressor genes, *BDA1* (for *bian da*; "becoming big" in Chinese), showed that it encodes a protein with amino-terminal ankyrin repeats and carboxyl-terminal transmembrane domains. Loss-of-function mutations in *BDA1* suppress the dwarf morphology and constitutive defense responses in *snc2-1D npr1-1* (for *nonexpressor of pathogenesis-related genes1,1*) and also result in enhanced susceptibility to bacterial pathogens. In contrast, a gain-of-function allele of *bda1* isolated from a separate genetic screen to search for mutants with enhanced pathogen resistance was found to constitutively activate cell death and defense responses. These data suggest that BDA1 is a critical signaling component that functions downstream of SNC2 to regulate plant immunity.

Plants have evolved two types of immune receptors to recognize different spectra of pathogen molecules and initiate downstream defense responses. Pathogen-associated molecular pattern (PAMP) receptors recognize conserved microbial molecules collectively known as PAMPs (Boller and Felix, 2009). Most of the known PAMP receptors, such as FLS2, EFR, and CERK1, belong to the receptor-like kinase family (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Miya et al., 2007; Wan et al., 2008). The receptor-like kinase BAK1 functions as a coreceptor of FLS2 and EFR in the perception of bacterial flagellin and translation elongation factor EF-Tu (Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010; Roux et al., 2011). In rice (*Oryza sativa*), CERK1 functions together with the lysin motif (LysM) domain protein CEBiP to recognize the fungal cell wall component chitin (Shimizu et al., 2010). Together with two other LysM domain proteins, LYM1 and LYM3, Arabidopsis (*Arabidopsis thaliana*) CERK1 is also involved in the perception of bacterial peptidoglycan (Willmann et al., 2011). The other class of immune receptors is composed of plant Resistance (R) proteins, which have evolved to detect pathogen-specific effector molecules secreted to suppress host defense and promote virulence. The majority of plant *R* genes encode nucleotide-binding site Leu-rich-repeats proteins (Rafiqi et al., 2009).

Receptor-like proteins (RLPs) containing extracellular Leu-rich repeats, a single transmembrane motif, and a short cytoplasmic tail form one of the largest protein families in plants. A small number of RLPs, such as the tomato (*Solanum lycopersicum*) Cf and Ve proteins and the apple (*Malus domestica*) HcrVf2 protein, were shown to function as R proteins (Jones et al., 1994; Kawchuk et al., 2001; Belfanti et al., 2004; Fradin et al., 2009). Interestingly, Ve1-mediated resistance to *Verticillium* requires BAK1 (Fradin et al., 2009, 2011). Two other RLPs, tomato Eix1 and Eix2, function as receptors for the fungal ethylene-inducing xylanase (Ron and Avni, 2004). The Arabidopsis genome encodes 57 RLPs (Wang et al., 2008). The best studied ones include CLAVATA2 and TOO MANY MOUTHS, both functioning as critical regulators of plant development (Jeong et al., 1999; Nadeau and Sack, 2002).

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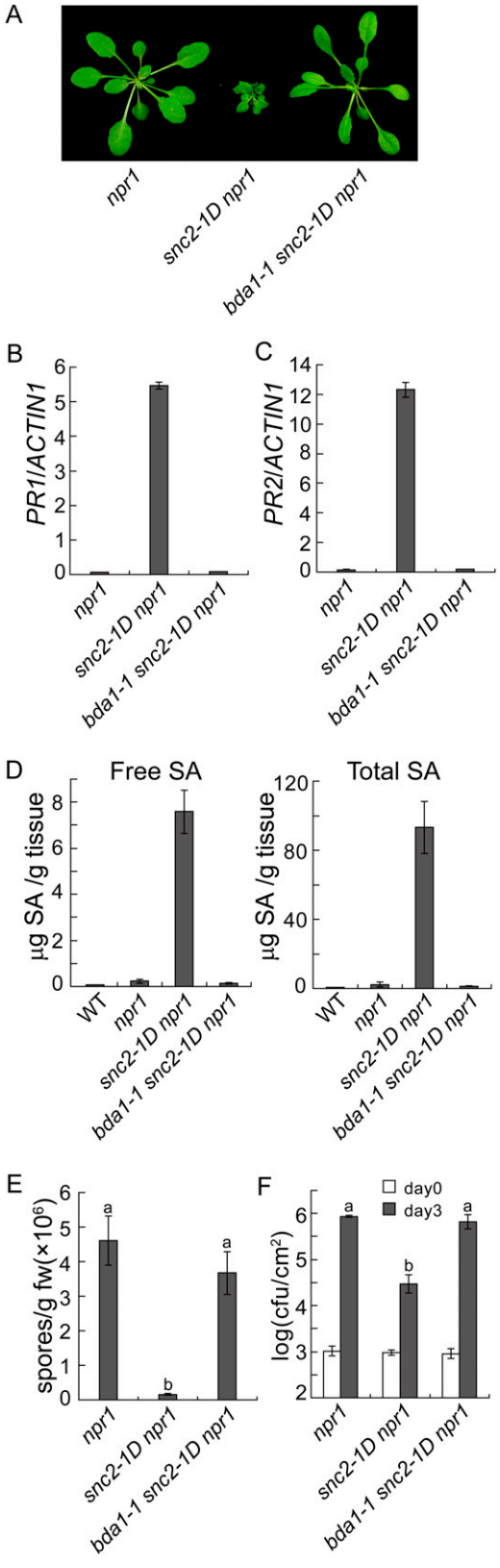

Figure 1. Characterization of the *bda1-1 snc2-1D npr1-1* triple mutant. A, Morphology of *npr1-1*, *snc2-1D npr1-1*, and *bda1-1 snc2-1D npr1-1*. Plants were grown on soil and photographed approximately 4 weeks after planting. B and C, Expression of *PR1* (B) and *PR2* (C) in *npr1-1*, *snc2-1D npr1-1*, and *bda1-1 snc2-1D npr1-1* as determined by qPCR. RNA was extracted from 12-d-old seedlings grown on one-half-strength Murashige and Skoog medium. Values were normalized to the expression of *ACTIN1*. Error bars represent SD from three measurements. D, Free and total SA levels in *npr1-1*, *snc2-1D npr1-1*, and *bda1-1 snc2-1D npr1-1* plants. Error bars represent SD from four measurements. WT, Wild type. E, Growth of *H.a.* Noco2 on *npr1-1*, *snc2-1D npr1-1*, and *bda1-1 snc2-1D npr1-1*. Sixteen-day-old seedlings were sprayed with *H.a.* Noco2 spores (50,000 spores mL^{-1}). Infection was scored 7 d after inoculation. The values presented are averages of three measurements ± SD. Statistical differences among the samples are labeled with different letters ($P < 0.001$). fw, Fresh weight. F, Growth of *P.s.t.* DC3000 on *npr1-1*, *snc2-1D npr1-1*, and *bda1-1 snc2-1D npr1-1*. Five-week-old plants were infiltrated with a bacterial suspension at an optical density at 600 nm of 0.0002. The values presented are averages of four replicates ± SD. Statistical differences among the samples are labeled with different letters ($P < 0.001$). cfu, Colony-forming units. [See online article for color version of this figure.]

Recently, the RLP SNC2 was identified as a critical defense regulator in Arabidopsis (Zhang et al., 2010). Loss-of-function mutations in SNC2 result in enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*P.s.t.*) DC3000.

snc2-1D (for *suppressor of npr1, constitutive2-1D*) is a semidominant gain-of-function mutation conferring constitutively activated defense responses in Arabidopsis (Zhang et al., 2010). The *snc2-1D* mutant plants exhibit dwarf morphology, accumulate high levels of salicylic acid (SA), express high levels of *PR* (*Pathogenesis-Related*) genes, and display enhanced resistance to pathogens. This mutant was identified from a screen searching for mutants with constitutively activated defense responses that are independent of NPR1 (for Nonexpressor of Pathogenesis-Related Genes1), an essential component of SA-induced gene expression and pathogen resistance (Dong, 2004). Because very little is known about how RLP-activated defense signals are transduced intracellularly, the identification of *snc2-1D* provides a very useful tool for dissecting defense pathways downstream of the RLP.

To study signal transduction pathways downstream of SNC2, a suppressor screen was performed in the *snc2-1D* mutant background and a large number of suppressor mutants named *bda* (for *bian da*; "becoming big" in Chinese) were identified (Zhang et al., 2010). *BDA2* encodes the WRKY transcription factor WRKY70. Mutations in *WRKY70* suppress the mutant morphology and defense gene expression but not the increased accumulation of SA in *snc2-1D npr1-1*, suggesting that WRKY70 is a critical regulatory component of the SA-independent defense pathway in *snc2-1D npr1-1*.

In this study, we report the characterization and positional cloning of *BDA1*. *BDA1* encodes a novel protein with an N-terminal ankyrin-repeat domain and a C-terminal transmembrane domain. It is structurally related to ACD6, a known positive regulator of plant

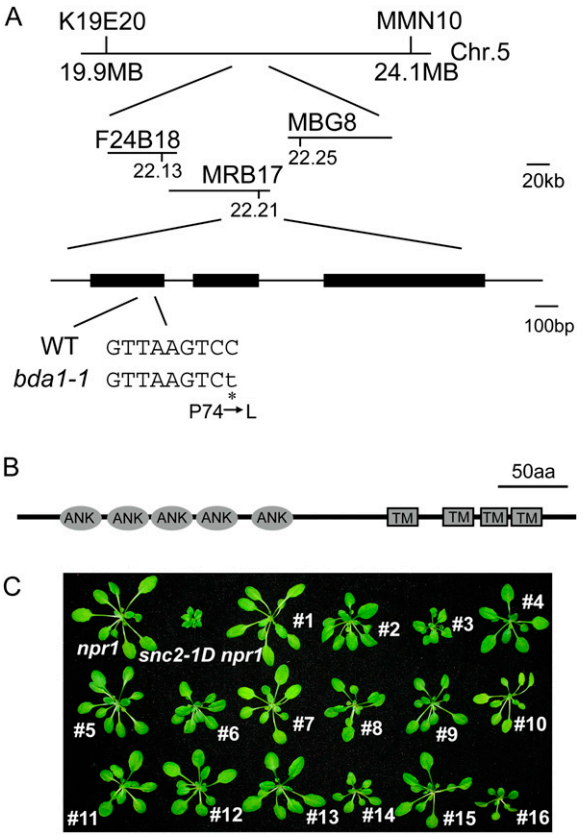

Figure 2. Positional cloning of *BDA1*. A, Map position and mutation location in *bda1-1*. WT, Wild type. B, Predicted protein structure of BDA1. aa, Amino acids; ANK, ankyrin repeat; TM, transmembrane domain. C, Morphological phenotypes of additional *bda1 snc2-1D npr1-1* mutant alleles. Plants were grown on soil and photographed approximately 4 weeks after planting. Plants 1 to 16, *bda1-1* to *bda1-16* in the *snc2-1D npr1-1* background. [See online article for color version of this figure.]

defense (Lu et al., 2003). Loss-of-function mutations in *BDA1* suppress the dwarf morphology and constitutive defense responses in *snc2-1D npr1-1* and result in enhanced susceptibility to pathogens. By contrast, a gain-of-function allele of *BDA1* constitutively activates cell death and defense responses, suggesting that BDA1 is a critical regulator of plant immunity.

RESULTS

Identification and Characterization of *bda1-1 snc2-1D npr1-1*

The *bda1-1 snc2-1D npr1-1* triple mutant was identified from a suppressor screen of *snc2-1D npr1-1* as described previously (Zhang et al., 2010). The dwarf morphology of *snc2-1D npr1-1* was completely suppressed in the triple mutant (Fig. 1A). Real-time PCR (qPCR) analysis of the expression of defense marker genes *PR1* (Fig. 1B) and *PR2* (Fig. 1C) showed that the constitutive expression of both genes in *snc2-1D npr1-1* is completely suppressed in the triple mutant. In addition, increased accumulation of SA and enhanced resistance to the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 and the bacterial pathogen *P.s.t.* DC3000 in *snc2-1D npr1-1* are also suppressed in *bda1-1 snc2-1D npr1-1* (Fig. 1, D–F). Taken together, *bda1-1* completely suppresses the mutant morphology as well as constitutive defense responses in *snc2-1D npr1-1*.

Positional Cloning of *BDA1*

To map the *bda1-1* mutation, *bda1-1 snc2-1D npr1-1* (in the ecotype Columbia [Col-0] background) was crossed with Landsberg *erecta* (L*er*) to generate a segregating mapping population. Crude mapping using the F2 progeny showed that the *bda1-1* mutation is located between markers K19E20 and MMN10 on chromosome 5. Further fine-mapping narrowed the *bda1-1* mutation to a region of approximately 80 kb between markers F24B18 and MRB17 (Fig. 2A). Sequence analysis of genes in this region in *bda1-1* identified a single C-to-T mutation in *At5g54610*, which results in an amino acid change from Pro-74 to Leu. Sequence analysis using SMART (http://smart.embl-heidelberg.de/) showed that At5g54610 encodes a new protein with five ankyrin repeats at the N terminus and four transmembrane domains at its C terminus (Fig. 2B).

To determine whether there are additional *bda* mutants that also contain mutations in the gene, *At5g54610* was amplified from other *bda* mutants by PCR and sequenced. Fifteen additional mutant alleles of *At5g54610* were identified (Table I; Fig. 2C), each containing a unique missense mutation. These results provide further evidence that *BDA1* is *At5g54610*.

Table I. *A list of bda1 mutant alleles*

Allele	Mutation[a]	Amino Acid Change
bda1-1	C221 to T	Pro-74 to Leu
bda1-2	G650 to A	Gly-217 to Glu
bda1-3	G157 to A	Ala-53 to Thr
bda1-4	C220 to T	Pro-74 to Ser
bda1-5	C386 to T	Pro-129 to Leu
bda1-6	G415 to A	Gly-139 to Arg
bda1-7	G418 to A	Glu-140 to Lys
bda1-8	C437 to T	Thr-146 to Met
bda1-9	G568 to A	Ala-190 to Thr
bda1-10	C628 to T	Arg-210 to Trp
bda1-11	G629 to A	Arg-210 to Gln
bda1-12	G664 to A	Asp-222 to Asn
bda1-13	G862 to A	Ala-288 to Thr
bda1-14	C908 to T	Thr-303 to Ile
bda1-15	G973 to A	Glu-325 to Lys
bda1-16	G1162 to A	Gly-388 to Arg

[a]Location of the nucleotide change in the coding sequence.

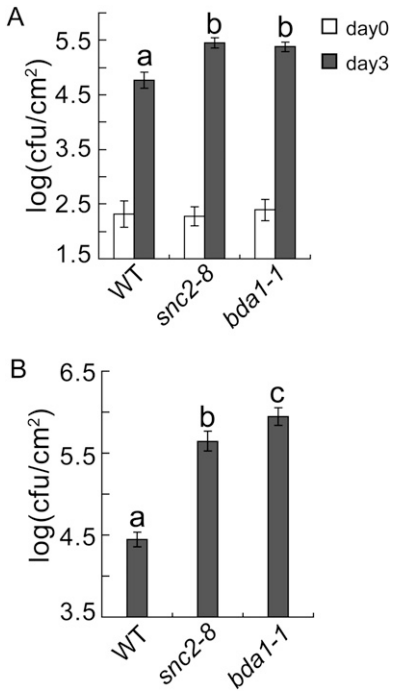

Figure 3. *bda1-1* exhibits enhanced susceptibility to *P.s.t.* DC3000. A, Growth of *P.s.t.* DC3000 on wild-type (WT), s*nc2-8*, and *bda1-1* plants. Leaves of 5-week-old plants were infiltrated with a bacterial suspension at an optical density at 600 nm of 0.0002. The values presented are averages of four replicates ± SD. cfu, Colony-forming units. Statistical differences among the samples are labeled with different letters ($P < 0.001$). B, Growth of *P.s.t.* DC3000 on wild-type, s*nc2-8*, and *bda1-1* plants. Leaves were collected 2 d after spray inoculation with a bacterial suspension at an optical density at 600 nm of 0.2. The values presented are averages of six replicates ± SD. Statistical differences among the samples are labeled with different letters ($P < 0.001$).

BDA1 Is Required for Basal Resistance against *P.s.t.* DC3000

To determine whether *BDA1* is required for pathogen resistance, we crossed *bda1-1 snc2-1D npr1-1* with wild-type Col-0 and isolated the *bda1-1* single mutant in the F2 progeny. We also obtained a transferred DNA insertion mutant, GABI_883C03, from the Nottingham Arabidopsis Stock Centre. Unfortunately, the transferred DNA insertion located in the second intron of *BDA1* does not affect its expression. When *bda1-1* and *snc2-8*, a loss-of-function *snc2* mutant (Zhang et al., 2010), were inoculated with *P.s.t.* DC3000 by infiltration, both mutants supported modestly higher growth of the pathogen compared with the wild type (Fig. 3A). Spray inoculation of these mutants with *P.s.t.* DC3000 results in considerably higher bacterial growth than that observed in wild-type plants (Fig. 3B). These data suggest that, like *SNC2*, *BDA1* is required for basal resistance against *P.s.t.* DC3000.

BDA1 Is Required for Resistance against Nonpathogenic Bacteria

To determine whether *SNC2* and *BDA1* are required for PAMP-mediated resistance against nonpathogenic bacteria, we challenged *snc2-8* and *bda1-1* with *P.s.t.* DC3000 *hrcC*, a *P.s.t.* DC3000 mutant defective in type III protein secretion (Wei et al., 2000). In the absence of effector-triggered immunity, PAMP-triggered immunity plays a predominant role in resistance against the bacteria. As shown in Figure 4A, growth of *P.s.t.* DC3000 *hrcC* after bacterial infiltration was modestly higher in *snc2-8* and *bda1-1* compared with that in wild-type plants. Interestingly, both of the mutants supported much higher growth of the pathogen than the wild type when the plants were spray inoculated with *P.s.t.* DC3000 *hrcC* (Fig. 4B). Growth of *P.s.t.* DC3000 *hrcC* in *snc2-8* was always higher than in *bda1-1* regardless of the inoculation method. These data suggest that both SNC2 and BDA1 are required for resistance against nonpathogenic bacteria.

A Gain-of-Function Allele of *BDA1* Constitutively Activates Cell Death and Defense Responses

From a separate genetic screen to search for mutants with enhanced disease resistance against *H.a.* Noco2 (Bi et al., 2010), we obtained a semidominant mutant with a lesion-mimic phenotype (Fig. 5A). The mutant was mapped to the same region where BDA1 is located. Sequencing analysis revealed that it contains a mutation in *BDA1* that changes Gly-330 to Glu in one of the transmembrane domains. As a result, we named this mutant *bda1-17D*. In *bda1-17D*, both *PR1* and *PR2*

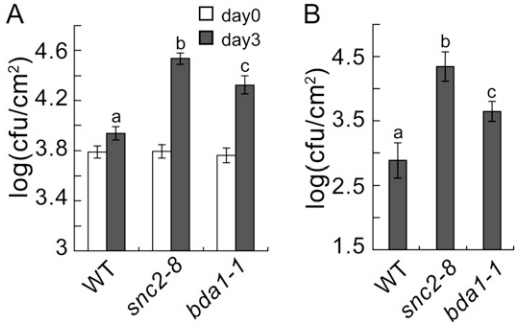

Figure 4. PAMP-triggered immunity in s*nc2-8* and *bda1-1*. A, Growth of *P.s.t.* DC3000 *hrcC* on wild-type (WT), s*nc2-8*, and *bda1-1* plants. Leaves of 5-week-old plants were infiltrated with a bacterial suspension at an optical density at 600 nm of 0.002. The values presented are averages of four replicates ± SD. cfu, Colony-forming units. Statistical differences among the samples are labeled with different letters ($P < 0.001$). B, Growth of *P.s.t.* DC3000 *hrcC* on wild-type, s*nc2-8*, and *bda1-1* plants. Leaves were collected 2 d after spray inoculation with a bacterial suspension at an optical density at 600 nm of 0.2. The values presented are averages of six replicates ± SD. Statistical differences among the samples are labeled with different letters ($P < 0.001$).

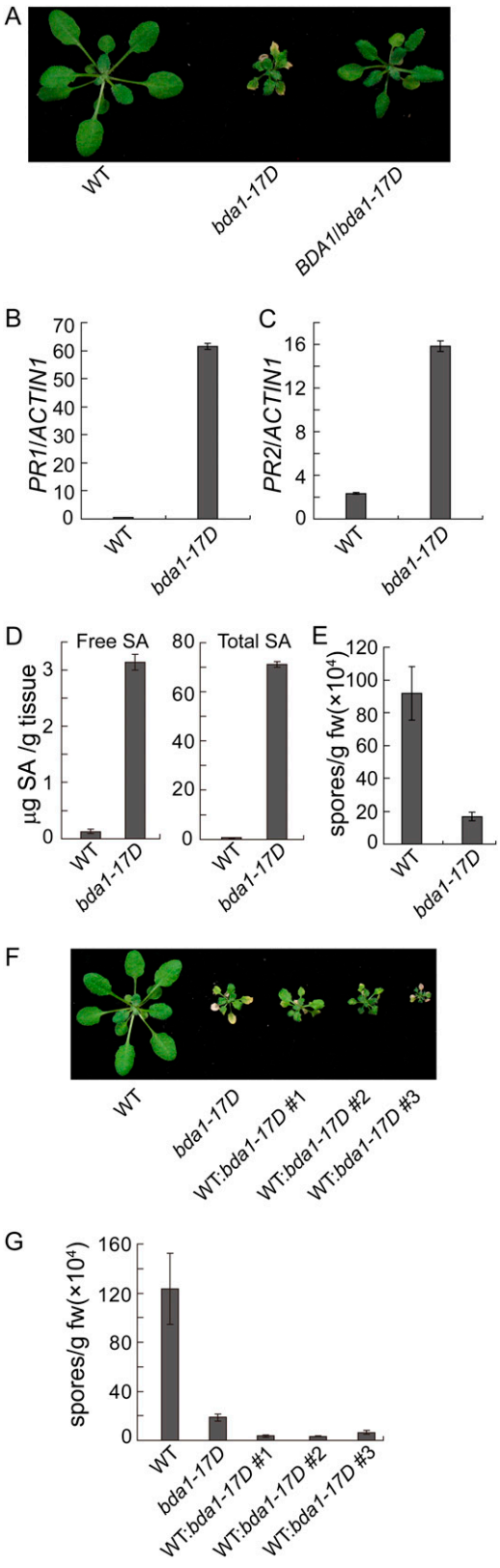

Figure 5. *bda1-17D* constitutively activates cell death and defense responses. A, Phenotypes of wild-type (WT), *bda1-17D*, and *BDA1*/*bda1-17D* heterozygous plants. Plants were grown on soil and photographed approximately 4 weeks after planting. B and C, Expression of *PR1* (B) and *PR2* (C) in the wild type and *bda1-17D* determined by qPCR. RNA was extracted from 25-d-old soil-grown plants. Values were normalized to the expression of *ACTIN1*. Error bars represent SD from three measurements. D, Free and total SA in the wild type and *bda1-17D*. Error bars represent SD from four measurements. E, Growth of *H.a.* Noco2 on the wild type and *bda1-17D*. Sixteen-day-old seedlings were sprayed with *H.a.* Noco2 spores (50,000 spores mL^{-1}). Infection was scored 7 d after inoculation. The values presented are averages of three measurements ± SD. fw, Fresh weight. F, Morphology of 4-week-old soil-grown wild-type, *bda1-17D*, and transgenic lines expressing the *bda1-17D* mutant gene in the wild-type background. G, Growth of *H.a.* Noco2 on wild-type and transgenic lines expressing the *bda1-17D* mutant gene. Sixteen-day-old seedlings were sprayed with *H.a.* Noco2 spores (50,000 spores mL^{-1}). Infection was scored 7 d after inoculation. The values presented are averages of three measurements ± SD. [See online article for color version of this figure.]

are constitutively expressed at high levels (Fig. 5, B and C). Analysis of SA levels in *bda1-17D* showed that it accumulates high levels of SA (Fig. 5D). When challenged with *H.a.* Noco2, *bda1-17D* displayed enhanced resistance to the pathogen (Fig. 5E). These data suggest that defense responses are constitutively activated in *bda1-17D*.

To test whether the *bda1-17D* mutation causes the cell death and constitutive defense responses, a genomic clone containing the *bda1-17* mutation was constructed and transformed into wild-type plants. About one-half of the transgenic lines with the mutant transgene exhibit *bda1-17D*-like morphology. We also made transgenic plants expressing wild-type *BDA1* under the control of its native promoter. Among about 30 transgenic lines obtained, none of them display *bda1-17D*-like morphology. Three representative *bda1-17D* transgenic lines are shown in Figure 5F. When these transgenic lines were challenged with *H.a.* Noco2, they all displayed enhanced resistance to *H.a.* Noco2 (Fig. 5G), suggesting that the *bda1-17D* mutation is responsible for the constitutive defense responses in *bda1-17D* mutant plants.

BDA1 Functions Upstream of NPR1 and WRKY70

In *snc2-1D*, two parallel defense pathways were activated, one dependent on SA and NPR1 and the other dependent on WRKY70 (Zhang et al., 2010). To determine whether the constitutive defense responses in *bda1-17D* are dependent on NPR1 and WRKY70, we generated the *bda1-17D npr1-1* and *bda1-17D wrky70-1* double mutants and the *bda1-17D npr1-1 wrky70-1* triple mutant. As shown in Figure 6A, the dwarf morphology of *bda1-17D* is partially suppressed in the double mutants and completely suppressed in the triple mutant. qPCR analysis showed that the constitutive *PR1* expression in *bda1-17D* is mainly dependent on NPR1, with very little contribution from WRKY70

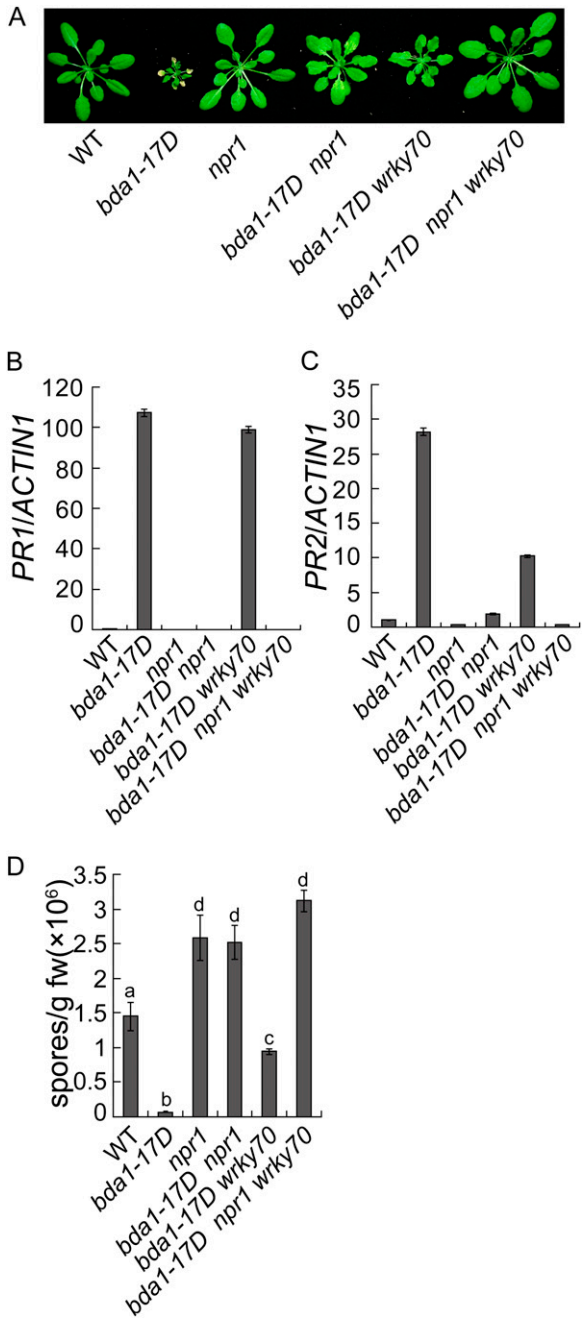

Figure 6. *bda1-17D* activates both NPR1-dependent and WRKY70-dependent defense responses. A, Morphology of wild-type (WT), *bda1-7D*, *npr1-1*, *bda1-17D npr1-1*, *bda1-17D wrky70-1*, and *bda1-17D npr1-1 wrky70-1* plants. Plants were grown on soil and photographed when they were approximately 4 weeks old. B and C, Expression of *PR1* (B) and *PR2* (C) in the indicated genotypes as determined by qPCR. Values were normalized to the expression of *ACTIN1*. RNA was extracted from 25-d-old soil-grown plants. Error bars represent SD from three measurements. D, Growth of *H.a.* Noco2 on the indicated genotypes. Sixteen-day-old seedlings were sprayed with *H.a.* Noco2 spores (10,000 spores mL^{-1}). Infection was scored 7 d after inoculation. The values presented are averages of three measurements ± SD. Statistical differences among the samples are labeled with different letters ($P < 0.05$). fw, Fresh weight. [See online article for color version of this figure.]

(Fig. 6B). In contrast, the expression of *PR2* is reduced in both *bda1-17D npr1-1* and *bda1-17D wrky70-1* and completely blocked in *bda1-17D npr1-1 wrky70-1* (Fig. 6C), suggesting that both NPR1 and WRKY70 contribute to the up-regulation of *PR2* in *bda1-17D*. Analysis of resistance to *H.a.* Noco2 showed that the enhanced resistance in *bda1-17D* is dependent on both NPR1 and WRKY70, with greater contribution from NPR1 (Fig. 6D). Taken together, these data suggest that BDA1 functions upstream of NPR1 and WRKY70.

DISCUSSION

From a suppressor screen of *snc2-1D npr1-1*, we identified BDA1 as an essential component of SNC2-mediated defense responses. Mutations in BDA1 suppress the dwarf morphology as well as defense responses in *snc2-1D npr1-1*. Loss of BDA1 function also results in enhanced susceptibility to *P.s.t.* DC3000. In addition, a gain-of-function allele of *BDA1* was found to constitutively activate cell death and defense responses. These data suggest that BDA1 is a critical regulator of plant immunity.

When *bda1-1* and *snc2-8* loss-of-function mutants were challenged with the nonpathogenic bacterium *P.s.t.* DC3000 *hrcC*, bacterial growth in the mutants was significantly higher than in wild-type plants, suggesting that SNC2 and BDA1 play important roles in PAMP-triggered immunity. It is possible that SNC2

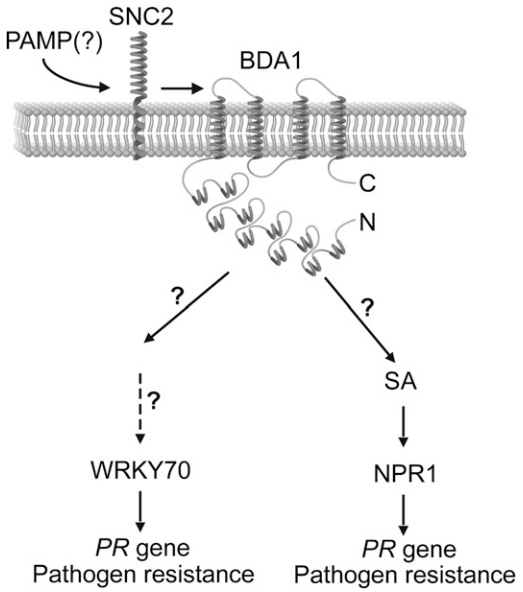

Figure 7. A working model for the role of BDA1 in SNC2-mediated immunity. BDA1 functions as a signaling component to transduce an unknown PAMP signal perceived by SNC2. Most likely through an association with other proteins, it activates two downstream defense pathways, one dependent on WRKY70 and the other dependent on SA and NPR1.

serves as a receptor or coreceptor of an unidentified bacterial PAMP signal and that BDA1 functions downstream of SNC2 to activate PAMP-triggered defense responses. Compared with inoculation by infiltration, greater differences in bacterial growth were found between wild-type and mutant plants upon spray inoculation with *P.s.t.* DC3000 or *P.s.t.* DC3000 *hrcC*. This indicates that SNC2 and BDA1 may also play critical roles in stomatal defense against bacterial pathogens.

BDA1 encodes a protein with ankyrin repeats and transmembrane domains. In Arabidopsis, there are 37 predicted ankyrin-repeat transmembrane proteins (Becerra et al., 2004). Among these proteins, only ACD6 and ITN1 have previously been characterized. ACD6 was shown to function as a positive regulator of SA signaling in local defense responses (Lu et al., 2003), whereas ITN1 is involved in salt stress tolerance (Sakamoto et al., 2008). How ACD6 and ITN1 are regulated is unknown. Our study suggests that BDA1 transduces signals perceived by the RLP SNC2 to activate downstream defense responses. It will be interesting to test whether ACD6 and ITN1 function as downstream components of other RLPs in Arabidopsis.

Ankyrin repeats are generally involved in protein-protein interactions (Sedgwick and Smerdon, 1999). One of the best studied plant ankyrin-repeat proteins is NPR1 (Cao et al., 1997; Ryals et al., 1997), which regulates SA-induced *PR* gene expression and pathogen resistance through its associations with TGA transcription factors (Zhang et al., 1999, 2003; Després et al., 2000). What the ankyrin-repeat domains of ACD6 and ITN1 interact with is unknown. We were not able to detect interactions between SNC2 and BDA1 by coimmunoprecipitation and bimolecular fluorescence complementation analysis. It is likely that other proteins are also involved in transducing the signal from SNC2 to BDA1. BDA1 and ACD6 share very low sequence identity in their ankyrin-repeat domains. Most likely, they regulate plant defense responses through interactions with distinct proteins. Identification of proteins interacting with BDA1 and ACD6 will lead to a better understanding of how they regulate plant defense responses.

In *bda1-17D*, a single amino acid change in the second transmembrane domain results in the activation of cell death and defense responses. *bda1-17D* has very similar mutant phenotypes to *acd6-1*, which also exhibits spontaneous cell death, expresses high levels of *PR-1*, and displays enhanced pathogen resistance (Rate et al., 1999). Interestingly, the gain-of-function mutation in *acd6-1* also occurs in one of the transmembrane domains (Lu et al., 2003). These data suggest that the transmembrane domains of BDA1 and ACD6 play very important roles in the negative regulation of these proteins. One possibility is that these transmembrane domains interact with their negative regulators and that the mutations in *bda1-17D* and *acd6-1* disrupt these interactions.

Our study identified BDA1 as a critical signaling component downstream of SNC2. A working model is proposed in Figure 7. When an unknown PAMP signal is perceived by SNC2, it activates the transmembrane ankyrin protein BDA1. Once it is activated, BDA1 probably recruits additional signaling components through its ankyrin-repeat domain to activate SA synthesis and WRKY70-dependent defense gene expression. It is unclear whether BDA1 also functions in regulating plant defense responses that are independent of SNC2. Future research on other BDA genes will provide us with more information on how SNC2 and BDA1 regulate plant defense responses.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) *npr1-1*, *snc2-1D npr1-1*, *snc2-8*, and *wrky70-1* were described previously (Cao et al., 1994; Ulker et al., 2007; Zhang et al., 2010). Loss-of-function alleles of *bda1* were identified from an ethyl methanesulfonate-mutagenized *snc2-1D npr1-1* population by screening for mutants of increased size. *bda1-17D* was identified from an ethyl methanesulfonate-mutagenized population in the Col-0 background by searching for mutants with increased resistance to *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 (Bi et al., 2010).

To obtain transgenic plants expressing the *bda1-17* mutant gene, a genomic DNA fragment containing the *bda1-17* mutant gene was amplified from *bda1-17* mutant plant DNA by PCR using primers 5′-CGGGGTACCGTCGACCTCCA-CAAAATGCATGTCAAG-3′ and 5′-CGGAATTCGAGCTCTGTGGGGATTC-AATACTATAGC-3′ and then cloned into pCAMBIA1305. The resulting plasmid was transformed into *Agrobacterium tumefaciens* and subsequently into wild-type Col-0 plants by floral dipping (Clough and Bent, 1998).

Mutant Characterization

To determine the expression levels of *PR1* and *PR2*, RNA was extracted from 0.1 g of leaf tissue using the RNAiso reagent (Takara). Reverse transcription was carried out using the Moloney murine leukemia virus reverse transcriptase (Takara). qPCR was performed on the complementary DNA samples using the SYBR Premix Ex Taq II kit (Takara). Primers for amplification of *PR1*, *PR2*, and *ACTIN1* were described previously (Zhang et al., 2003). SA was extracted from leaf tissues and measured by HPLC as described previously (Li et al., 1999).

H.a. Noco2 infection experiments were performed by spraying plant seedlings with spores of *H.a.* Noco2 at different concentrations. Plants were subsequently kept in a growth chamber with 95% humidity at 18°C under 12-h-light/12-h-dark cycles. Infections were scored 7 d later by counting the spores with a hemocytometer as described previously (Bi et al., 2010). For *Pseudomonas syringae* pv *tomato* DC3000 infections, plants were grown at 23°C under 12-h-light/12-h-dark cycles. Leaves of 5-week-old plants were inoculated by infiltrating or spraying with a bacterial suspension. Bacterial growth was determined by plating the bacteria on Kings B plates.

Positional Cloning of BDA1

To map *bda1-1*, the *bda1-1 snc2-1D npr1-1* triple mutant in the Col-0 background was crossed with wild-type L*er*. Plants homozygous for *snc2-1D* in the F2 population were identified by PCR and used for linkage analysis. For fine-mapping of the *bda1-1* mutation, recombinants between markers K19E20 and MMN10 were identified by PCR and the phenotypes of the recombinants were determined by analyzing the segregation of the *snc2-1D* morphological phenotype in their progeny. For mapping *bda1-17D*, *bda1-17D* in the Col-0 background was crossed with wild-type L*er*. Plants exhibiting *bda1-17D*-like morphology in the F2 population were used for linkage analysis. All markers used were designed according to the Monsanto Arabidopsis polymorphism

and L*er* sequence collection (Jander et al., 2002). Marker primers used include K19E20, 5′-GACAAGAACCACATGAGAGC-3′ and 5′-GTTATGTGTACACTT-CAGGTC-3′; MMN10, 5′-AGCTGCAATAATGCCAAAGG-3′ and 5′-GAAC-CATCACCACTGGTGAG-3′; and MBG8, 5′-GCTAAGAAAGTAGAAAGCCG-3′ and 5′-ATGGTATCTCACCAATGGGG-3′. These markers were designed using insertion/deletion polymorphisms. Markers F24B18 and MRB17 are based on a single nucleotide polymorphism. The common, Col-specific, and L*er*-specific primers for F24B18 are 5′-GGAAGGCAGAGATTATAGAC-3′, 5′-GAG-CAAAGCTTTGATGTACCA-3′, and 5′-GAGCAAAGCTTTGATGTACCG-3′, respectively. The common, Col-specific, and L*er*-specific primers for MRB17 are 5′-GAGTTCACAAGAGAAGACGT-3′, 5′-CTCTCACAAATCTGGGCATG-3′, and 5′-CTCTCACAAATCTGGGCATA-3′, respectively.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_124842 (BDA1).

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