Arabidopsis CALMODULIN-BINDING PROTEIN 60b plays dual roles in plant immunity

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ABSTRACT

Arabidopsis SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g) are two master transcription factors that regulate many defense-related genes in plant immunity. They are required for immunity downstream of the receptor-like protein SUPPRESSOR OF NPR1-1, CONSTITUTIVE 2 (SNC2). Constitutive defense responses in the gain-of-function autoimmune snc2-1D mutant are modestly affected in either sard1 or cbp60g single mutants but completely suppressed in the sard1 cbp60g double mutant. Here we report that CBP60b, another member of the CBP60 family, also functions as a positive regulator of SNC2-mediated immunity. Loss-of-function mutations of CBP60b suppress the constitutive expression of SARD1 and enhanced disease resistance in cbp60g-1 snc2-1D, whereas over-expression of CBP60b leads to elevated SARD1 expression and constitutive defense responses. In addition, transient expression of CBP60b in Nicotiana benthamiana activates the expression of the pSARD1::luciferase reporter gene. Chromatin immunoprecipitation assays further showed that CBP60b is recruited to the promoter region of SARD1, suggesting that it directly regulates SARD1 expression. Interestingly, knocking out CBP60b in the wild-type background leads to ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)-dependent autoimmunity, suggesting that CBP60b is required for the expression of a guardee/decoy or a negative regulator of immunity mediated by receptors carrying an N-terminal Toll-interleukin-1 receptor-like domain.

Key words: Arabidopsis, plant immunity, CBP60b, CBP60g, SARD1, salicylic acid

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INTRODUCTION

Plants have evolved sophisticated innate immune systems to fight against various pathogens (Jones and Dangl, 2006) (Zhou and Zhang, 2020). Upon infection, plasma membrane–localized pattern recognition receptors recognize pathogen/microbe-associated molecular patterns, which are conserved molecules associated with groups of pathogens, to initiate pattern-triggered immunity (PTI) (Monaghan and Zipfel, 2012). To facilitate colonization, pathogens have evolved various effector proteins to interfere with PTI (Dou and Zhou, 2012). Meanwhile, plants evolved resistance proteins, mostly in the nucleotide-binding leucine-rich-repeat (NLR) protein family, to perceive the pathogen effectors and activate effector-triggered immunity (van Wersch et al., 2020). Activation of PTI and effector-triggered immunity at local infection sites leads to the development of systemic acquired resistance (SAR) in distal parts of the plant (Sun and Zhang, 2021).

One class of plant pattern recognition receptors belongs to the receptor-like protein (RLP) family. RLPS usually have a short cytoplasmic tail, a single transmembrane motif, and an extracellular leucine-rich-repeat domain involved in ligand binding. One of the Arabidopsis RLPS, SNC2 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE 2), contributes to defense against bacteria (Zhang et al., 2010b). Loss of SNC2 results in enhanced susceptibility to the bacterial pathogen Pseudomonas syringae pv. tomato DC3000, whereas a gain-of-function mutation in snc2-1D results in constitutive Pathogenesis-Related (PR) gene expression, elevated levels of the plant defense hormone salicylic acid (SA), and enhanced resistance to the oomycete pathogen Hyaloperonospora arabidopsidis (Hpa) Noco2 (Zhang et al., 2010b). Owing to constitutively activated immunity, snc2-1D plants exhibit a dwarf morphology.

SAR DEFICIENT 1 (SARD1) and CALMODULIN BINDING PROTEIN 60g (CBP60g) are two master transcription factors in plant...
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immunity (Sun et al., 2015). Induction of SA biosynthetic genes such as ISOCHORISIMATE SYNTHASE 1 (ICS1), ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5), and AVRPPHB SUSCEPTIBLE 3 (PBS3) during pathogen infection is coordinately regulated by SARD1 and CBP60g (Zhang et al., 2010a; Wang et al., 2011; Sun et al., 2015). Pathogen-induced SA accumulation is almost completely blocked in sard1 cbp60g double-mutant plants (Zhang et al., 2010a; Wang et al., 2011). In addition, SARD1 and CBP60g are also required for activation of the biosynthesis of N-hydroxy-pipolic acid, a signaling molecule important for both local resistance and SAR (Sun et al., 2020). They coordinately regulate pathogen-induced expression of N-hydroxy-pipolic acid biosynthesis genes such as AGD2-like defense response protein 1 (ALD1), SAR deficient 4 (SARD4), and Flavin-dependent monoxygenase 1 (FMOT) (Sun et al., 2018, 2020). Both SARD1 and CBP60g belong to a small protein family with eight members in Arabidopsis. Unlike CBP60g and other members of the family that have calmodulin (CaM) binding activity (Wang et al., 2009; Reddy et al., 2002), SARD1 does not bind CaM and is primarily regulated at the transcriptional level, as its overexpression is sufficient to activate downstream defense gene expression (Zhang et al., 2010a). Another member of the family, CBP60a, was shown to function as a negative regulator of plant immunity (Truman et al., 2013). How CBP60a negatively regulates plant immunity is unknown.

Both SARD1 and CBP60g contribute to autoimmunity of snc2-1D (Sun et al., 2015). Although the upregulation of ICS1 and accumulation of SA in snc2-1D are only slightly reduced in cbp60g-1 snc2-1D and sard1-1 snc2-1D double mutants, they are largely blocked in the cbp60g-1 sard1-1 snc2-1D triple mutant. snc2-1D has a dwarf morphology and is much smaller than the wild type. The cbp60g-1 snc2-1D and sard1-1 snc2-1D double mutants are slightly larger than snc2-1D, whereas the cbp60g-1 sard1-1 snc2-1D triple mutant exhibits wild-type morphology. These findings suggest that there are two parallel pathways downstream of SN2C, one dependent on SARD1 and the other requiring CBP60g (Sun et al., 2015). How the plasma membrane–localized SNC2 transduces signals to SARD1 or CBP60g is largely unknown.

To identify components involved in SARD1-dependent defense signaling downstream of SN2C, we performed a suppressor screen in the cbp60g-1 snc2-1D background. Here we report the identification and characterization of one of the suppressors, bda7-1 (bian da 7; bian da means “becoming big” in Chinese). Positional cloning revealed that BDA7 encodes another member of the CBP60 family, CBP60b, which positively regulates the expression of SARD1.

RESULTS

Identification and characterization of bda7-1 cbp60g-1 snc2-1D

To identify regulators of SARD1-dependent defense signaling in SNC2-mediated immunity, we mutagenized cbp60g-1 snc2-1D and searched for mutants that suppressed its dwarf morphology. bda7-1 was one of the mutants identified. As shown in Figure 1A, bda7-1 cbp60g-1 snc2-1D is intermediate in size compared with wild-type Col-0 and cbp60g-1 snc2-1D. Quantitative RT-PCR analysis showed that the elevated expression of PR1 and ICS1 in cbp60g-1 snc2-1D is fully blocked by bda7-1 (Figure 1B and 1C). Consistent with the ICS1 expression, both free and total SA (Figure 1E) levels are considerably lower in bda7-1 cbp60g-1 snc2-1D than in cbp60g-1 snc2-1D. In addition, the enhanced resistance to Hpa Noco2 observed in cbp60g-1 snc2-1D is lost in bda7-1 cbp60g-1 snc2-1D (Figure 1F). Together, these data indicate that bda7-1 suppresses the autoimmunity of cbp60g-1 snc2-1D.

Map-based cloning of BDA7

When bda7-1 cbp60g-1 snc2-1D was backcrossed with cbp60g-1 snc2-1D, F1 plants showed morphology similar to that of cbp60g-1 snc2-1D (Supplemental Figure 1), indicating that bda7-1 is recessive. To map bda7-1, bda7-1 cbp60g-1 snc2-1D (in the Col-0 background) was crossed with Landsberg erecta. In the F2 population, plants homozygous for both cbp60g-1 and snc2-1D with dwarf morphology were selected for linkage analysis. bda7-1 was initially mapped to a region between the simple sequence length polymorphism markers MBG8 and MUB3 on chromosome 5, then subsequently fine-mapped to a ø-0.8-Mb region between MUA2 and MMN10 (Supplemental Figure 2).

To identify BDA7, DNA from 50 F2 plants of a size similar to that of bda7-1 cbp60g-1 snc2-1D obtained from a cross between bda7-1 cbp60g-1 snc2-1D and cbp60g-1 snc2-1D was pooled and sequenced by Illumina sequencing. A single candidate gene within the ø-0.8-Mb mapped region, AT5G57580, was found, which carries a homozygous C-to-T mutation in its coding sequence (Figure 2A and Supplemental Figure 3), resulting in the substitution of Leu148 with Phe (Figure 2A).

Loss of CBP60b suppresses the autoimmunity of cbp60g-1 snc2-1D

To test whether AT5G57580 is indeed required for the autoimmunity of cbp60g-1 snc2-1D, AT5G57580 was knocked out by CRISPR-Cas9 in the cbp60g-1 snc2-1D background. As shown in Figure 2B, the newly generated AT5G57580 CRISPR alleles suppressed cbp60g-1 snc2-1D, similar to bda7-1. Sanger sequencing confirmed that these lines carry homozygous deletion mutations in BDA7 (Supplemental Figure 4). Similar to bda7-1, these deletion mutants also suppress the constitutive expression of PR1 and ICS1 (Figure 2C and 2D), elevated SA levels (Supplemental Figure 5), and enhanced resistance to Hpa Noco2 in cbp60g-1 snc2-1D (Figure 2E), suggesting that AT5G57580 is required for the constitutive immune responses of cbp60g-1 snc2-1D.

To further confirm that suppression of the cbp60g-1 snc2-1D mutant phenotype in bda7-1 cbp60g-1 snc2-1D is caused by the mutation in AT5G57580, we transformed a construct expressing wild-type AT5G57580 with a C-terminal 3x hemagglutinin (HA) tag under the control of its native promoter into bda7-1 cbp60g-1 snc2-1D. As shown in Supplemental Figure 6, the AT5G57580-3HA transgenic lines have a morphology similar to that of cbp60g-1 snc2-1D, indicating that AT5G57580-3HA can complement the bda7-1 mutation. Because AT5G57580 encodes CBP60b, we renamed bda7-1 as cbp60b-4.
The Leu148 residue in CBP60b is highly conserved in all CBP60 family members except CBP60a (Supplemental Figure 7A), which functions as a negative regulator of plant immunity (Truman et al., 2013). Notably, from the same cbp60g-1 snc2-1D suppressor screen, three sard1 alleles that carry mutations on Gly143 were found to suppress the autoimmunity of cbp60g-1 snc2-1D (Supplemental Figure 7B). This Gly residue is also highly conserved and is only two amino acids from the conserved Leu site. These data suggest that the region around Leu148 is critical for the functions of CBP60 family proteins.

**CBP60b regulates SARD1 expression**

As the elevated ICS1 expression and SA levels in cbp60g-1 snc2-1D are suppressed by bda7-1 (Figure 1C–1E), we examined whether the expression of SARD1 is affected in the cbp60b cbp60g-1 snc2-1D mutants. As shown in Figure 3A, the expression level of SARD1 is much higher in cbp60g-1 snc2-1D than in the wild type, and this elevated SARD1 expression is fully suppressed in cbp60b cbp60g-1 snc2-1D, suggesting that CBP60b functions upstream of SARD1. We also examined the expression levels of FM01, another target gene of SARD1. Consistent with the expression levels of SARD1, upregulation of FM01 in cbp60g-1 snc2-1D is also blocked in cbp60b cbp60g-1 snc2-1D (Figure 3B). These data suggest that CBP60b functions upstream of SARD1.

As CBP60b is predicted to be a transcription factor, we tested whether CBP60b binds to the promoter of SARD1. A chromatin
immunoprecipitation (ChIP)-qPCR assay was performed on transgenic plants expressing CBP60b-3HA under its own promoter in the cbp60b-4 cbp60g-1 snc2-1D background. Compared with the non-transgenic negative control cbp60g-1 snc2-1D/C24, 4.6-fold enrichment of the DNA around 1.0 kb upstream of the start codon and 2.8-fold enrichment of the DNA around 0.3 kb upstream of the start codon were observed in samples from the CBP60b-3HA transgenic line (Figure 3C), indicating that CBP60b is recruited to the SARD1 promoter region.

To further test whether CBP60b can activate the expression of SARD1, a plasmid expressing a luciferase reporter gene under the control of the SARD1 promoter (pSARD1::Luc) was transformed into Nicotiana benthamiana, together with a 35S::CBP60b-3HA construct. Co-transformation of 35S::CBP60b-3HA with pSARD1::Luc resulted in increased expression of the luciferase reporter (Figure 3D and 3E), suggesting that overexpression of CBP60b in N. benthamiana leads to activation of SARD1 expression. Together, these data suggest that CBP60b serves as a transcriptional activator of SARD1.

Overexpression of CBP60b leads to upregulation of SARD1 and enhanced disease resistance in Arabidopsis

To further test the hypothesis that CBP60b regulates SARD1 expression, we overexpressed CBP60b under the 35S promoter in the wild-type Col-0 background. Among a number of CBP60b lines with high levels of CBP60b-3HA protein (Supplemental Figure 8), two lines, CBP60b-3HA-OX #21 and #27, were selected for further analysis. Both lines exhibit dwarfism and dark green leaves (Figure 4A). The expression levels of SARD1 are significantly higher in the CBP60b-OX transgenic lines than in the wild-type control (Figure 4B), further supporting the role of CBP60b in regulating SARD1 expression. Consistent with the elevated SARD1 expression levels, these transgenic lines showed enhanced resistance to Hpa Noco2 (Figure 4C).

cbp60b single mutants exhibit constitutively activated immune responses

The requirement for CBP60b in the constitutive defense responses of cbp60g-1 snc2-1D indicates that CBP60b serves as a positive regulator of plant immunity. To our surprise, when we isolated the cbp60b-4 single mutant from the F2 population of a cross between cbp60b-4 cbp60g-1 snc2-1D and Col-0, the cbp60b-4 plants displayed a dwarf morphology with dark green and abnormal leaves (Figure 5A). cbp60b-4 cbp60g-1 double-mutant plants isolated from the same F2 population showed an even more dramatic phenotype, as they were seedling-lethal and grew only two cotyledons at room temperature (Figure 5A). To rule out the possibility that the unexpected phenotypes originated from random background mutations in cbp60b-4...
we generated cbp60g-1 snc2-1D, we generated cbp60b deletion mutants in wild-type Col-0 and cbp60g-1 backgrounds by CRISPR-Cas9. The cbp60b deletion mutants cbp60b-5 and cbp60b-6 showed morphology identical to that of cbp60b-4 (Figure 5A). In addition, the cbp60b deletion mutants in the cbp60g-1 background exhibited morphology similar to that of cbp60b-4 cbp60g-1 (Figure 5A). These data confirm that the dwarfism observed in cbp60b-4 and cbp60b-4 cbp60g-1 is caused by the mutation in CBP60b.

Next, we tested whether defense responses are activated in the cbp60b single and cbp60b cbp60g double mutants. As shown in Figure 5B, the expression levels of PR1 were dramatically increased in cbp60b-5 and cbp60b-6 single mutants and were even higher in the cbp60b cbp60g-1 double mutants. cbp60b and cbp60b cbp60g-1 mutants also exhibited elevated ICS1 expression (Figure 5C). Consistently, SA levels were much higher in cbp60b mutants than in the wild type (Figure 5D and 5E). In addition, cbp60b mutants showed strong resistance to...
Hpa Noco2 (Figure 5F). These observations suggest that knocking out CBP60b leads to constitutive activation of defense responses.

We further examined whether mutations in SARD1 affect the autoimmunity of cbp60b cbp60g-1. cbp60b cbp60g-1 sard1-1 triple mutants were generated by knocking out CBP60b in the cbp60g-1 sard1-1 double mutant using CRISPR-Cas9. Similar to the cbp60b cbp60g-1 double mutant, cbp60b cbp60g-1 sard1-1 triple mutants were seedling-lethal and grew only two cotyledons (Supplemental Figure 9A). Unlike cbp60b cbp60g-1, cotyledons of cbp60b cbp60g-1 sard1-1 plants also showed visible lesions (Supplemental Figure 9A).

Positive regulators of plant immunity are often monitored or guarded by NLR receptors (Cui et al., 2015). To test whether the constitutive defense responses in cbp60b are caused by activation of NLR-mediated immunity, we crossed cbp60b-5 with eds1-24, a CRISPR-deletion knockout mutant of EDS1, a gene known to be required for defense signaling mediated by Toll/interleukin-1 receptor domain-containing NLRs (TNLs) (Aarts et al., 1998). The cbp60b-5 eds1-24 double mutant showed wild-type-like morphology (Figure 5G). In addition, constitutive PR1 expression and enhanced resistance to Hpa Noco2 were completely suppressed in cbp60b-5 eds1-24 (Figure 5H and 5I), indicating that the autoimmunity of cbp60b is dependent on EDS1 and that TNL-mediated immunity is probably activated upon loss of CBP60b function.

As NLR-mediated autoimmunity can often be suppressed by high temperature (van Wersch et al., 2016), we tested whether the dwarf phenotypes of cbp60b, cbp60b cbp60g-1, and cbp60b cbp60g-1 sard1-1 can be suppressed by growth at 28 °C. As shown in Supplemental Figure 9B, cbp60b exhibited wild-type morphology at 28 °C, whereas cbp60b cbp60g-1 and cbp60b cbp60g-1 sard1-1 plants were of intermediate size and were able to grow true leaves at 28 °C.

**DISCUSSION**

SARD1 and CBP60g play critical roles in the transcriptional regulation of plant immunity (Sun et al., 2015). Unlike CBP60g, which requires binding of CaM for its activation (Wang et al., 2009), SARD1 is primarily regulated at the transcriptional level. The autoimmune mutant snc2-1D provides a unique system in which to uncover components that regulate SARD1 expression, as the constitutive defense response in cbp60g-1 snc2-1D is mainly dependent on SARD1 (Sun et al., 2015). From a suppressor screen of cbp60g-1 snc2-1D, we identified CBP60b as a positive regulator of SARD1 transcription. CBP60b is targeted to the promoter region of SARD1 and is required for its upregulation in cbp60g-1 snc2-1D. Overexpression of CBP60b leads to elevated SARD1 expression and constitutive defense responses. In addition, transient expression of CBP60b in N. benthamiana activates the expression of the pSARD1-Luc reporter gene, confirming that CBP60b positively regulates the expression of SARD1.
Figure 5. Knocking out CBP60b activates EDS1-dependent defense responses.  
(A and G) Morphology of 3-week-old soil-grown plants of the indicated genotypes under long-day conditions. Scale bar, 1 cm.  
(B and C) Expression levels of PR1 (B) and ICS1 (C) in the indicated genotypes normalized to those of ACTIN1. Error bars represent standard deviations. Letters indicate statistical differences (P < 0.05, Student’s t-test; n = 3).  
(legend continued on next page)
CBP60b belongs to the same protein family as SAR1D and CBP60g (Reddy et al., 2002), which share a highly conserved central domain with DNA-binding activity but have divergent sequences at the N and C termini (Wang et al., 2009; Zhang et al., 2010a). Unlike SAR1D and CBP60g, which are strongly induced during pathogen infection (Wang et al., 2009; Zhang et al., 2010a), CBP60b is constitutively expressed in different tissues (Reddy et al., 2002). CBP60b was identified as a CaM-binding protein, suggesting that its activity may be influenced by Ca2+ levels (Reddy et al., 2002). SAR1D was previously shown to activate ICS1 expression through a GAATTT motif in its promoter (Sun et al., 2015). CBP60b shares ~80% sequence similarity with SAR1D in the middle domain. Whether it binds to a similar DNA sequence in activating SAR1D expression remains to be determined.

Surprisingly, defense responses are constitutively activated in the cbp60b single mutants. The constitutive defense response in cbp60b is blocked by knocking out EDS1, suggesting that loss of CBP60b leads to activation of TNL-mediated immune signaling. It is possible that CBP60b is required for the expression of a negative regulator of TNL-mediated immune signaling. More likely, CBP60b may be directly or indirectly monitored by a TNL, and loss of its function triggers activation of this unidentified TNL and downstream defense responses, as critical immune regulators are often targeted by pathogen effector proteins and guarded by plant NLR receptors (Cui et al., 2015; Kourelis and Van Der Hoorn, 2018). This is supported by the partial requirement for the TNL SNC1 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1) in the autoimmunity of cbp60b (Li et al., 2021). Although no pathogen effector has been reported to target CBP60b, SAR1D and CBP60g were shown to be targets of the Verticillium effector VdSCP4 (Qin et al., 2018).

Compared with cbp60b, the cbp60b cbp60g-1 double mutants show an even more dramatic autoimmune phenotype, suggesting that CBP60b and CBP60g play partially redundant roles in the transcriptional regulation of the putative guardee/decoy recognized by the unknown TNL. In cbp60b, the expression of the putative guardee/decoy is partially blocked, leading to activation of TNL-mediated immunity. Loss of both CBP60b and CBP60g leads to further reduction in its expression and stronger defense responses. Interestingly, the severe dwarf phenotype of cbp60b cbp60g-1 was not observed in the snc2-1D background. It is possible that snc2-1D can also activate CBP60b- and CBP60g-independent expression of the putative guardee/decoy, thereby compensating for the loss of CBP60b and CBP60g.

A working model is proposed based on our findings (Figure 6). CBP60b promotes the expression of SAR1D and contributes to SNC2-mediated immunity. It is required for the expression of a yet-to-be-identified guardee/decoy of a TNL. Disruption of the expression of the putative guardee/decoy results in activation of TNL-mediated immunity. It is of future interest to identify this guardee/decoy that is guarded by SNC1 and another TNL(s).

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

All Arabidopsis thaliana mutants were in the Col-0 ecotype background unless otherwise specified. The cbp60g-1 snc2-1D, cbp60g-1, and cbp60g-1 snc1-1 plants were described previously (Zhang et al., 2010a; Sun et al., 2015). cbp60b-4 and cbp60b-4 cbp60g-1 plants were isolated from a cross between cbp60b-4 cbp60g-1 snc2-1D and Col-0. cbp60b-5 and cbp60b-6 are two independent deletion lines generated by CRISPR-Cas9. CBP60b-3HA-OX #21 and #27 are two independent overexpression lines generated by transforming Col-0 with Agrobacterium carrying pCambia1300-3SS-CBP60b-3HA. eds1-24 is a CRISPR mutant line with both Edwards1 and ED51B genes deleted, as described previously (Tian et al., 2020). cbp60b-5 eds1-24 was obtained from the F2 population of a cross between cbp60b-5 and eds1-24. Primers used for genotyping are listed in Supplemental Table 1.

Plants were grown on soil under long-day conditions (16 h light/8 h dark) with a light intensity of ~100 μmol/m2/s at 22°C unless otherwise specified. Plants for quantitative RT-PCR were grown on plates with 1/2 Murashige and Skoog (MS) medium and 1% sucrose for 2 weeks. Plants used for SA quantification were grown on soil for 4 weeks under short-day conditions (8 h light/16 h dark).

**Constructs for generation of deletion mutants and transgenic plants**

The CRISPR-Cas9 system used to generate cbp60b mutants was described previously (King et al., 2014). Two guide RNAs were designed to target CBP60b genomic DNA and generate a deletion ~1 kb in size. A PCR fragment containing the guide RNA sequences was amplified from the pCBC-DT12 vector using primers At5g57580 BspF10 and At5g57580-BspR0 and subsequently inserted into the pHEE401 vector using the Bsal site. The derived plasmid was transformed into E. coli DH10B and later into Agrobacterium GV3101 by electroporation. Arabidopsis plants were transformed with Agrobacterium carrying the plasmid by the floral dip method (Clough and Bent, 1998). T1 plants were analyzed for deletion in CBP60b by PCR with the primers listed in Supplemental Table 1. Homozygous deletion mutants were obtained in the T2 generation.

To overexpress CBP60b, CBP60b was amplified from genomic DNA using primers AT5G57580-attkpnfl and AT5G57580-nstopBamH1-R. The PCR fragment was digested with KpnI and BamHI and then ligated into the pCambia1300-3SS-3HA vector. The derived plasmid was transformed into E. coli DH10B and later into Agrobacterium GV3101. Wild-type Col-0 plants were transformed with Agrobacterium carrying the plasmid by the floral dip method. Homozygous transgenic lines were obtained in the T2 generation.

For transgene complementation, a genomic DNA fragment containing CBP60b was amplified with primers AT5G57580-Kpnfl and AT5G57580-nstopBamH1-R. The PCR fragment was inserted into a pBasta-3HA vector
derived from pCambia1305 to obtain the pAt5g57580::At5g57580-3HA construct. The pAt5g57580::At5g57580-3HA/cbp60b-4 cbp60g-1 snc2-1D transgenic lines were obtained by transforming cbp60b-4 cbp60g-1 snc2-1D plants with Agrobacterium carrying the pAt5g57580::At5g57580-3HA plasmid.

Mutagenesis and suppressor screen
Approximately 5000 cbp60g-1 snc2-1D mutant seeds were mutagenized with 20 mM ethyl methanesulfonate (EMS) as described previously (Li and Zhang, 2016). In brief, seeds were soaked in the EMS solution for 16 h. Afterward, the EMS solution was removed and the seeds were washed with 100 mM Na2S2O3 three times and then with ddH2O three times. The mutagenized seeds were plated on 1/8 MS plates and later transplanted to the soil when the seedlings were about 10 days old. M2 plants were grown on soil and screened for plants that were larger than cbp60g-1 snc2-1D. Any plants with a larger size were collected for further analysis.

Genetic mapping, mapping by sequencing, and identification of bda7-1
Traditional mapping of bda7-1 was performed using the F2 population from a cross between the bda7-1 cbp60g-1 snc2-1D mutant (in the Col-0 background) and Landsberg erecta. In the F2 generation, any plants homozygous for both snc2-1D and cbp60g-1 were selected for linkage analysis using simple sequence length polymorphism markers as previously described (Zhang et al., 2007). These plants were first analyzed with markers throughout the five chromosomes, thereby mapping bda7-1 to the lower arm of chromosome 5. Afterward, fine-mapping was performed using both F2 and F3 plants with additional markers to narrow down the mapped region to ~0.8 Mb.

Figure 6. A working model of CBP60b in plant immunity.
CBP60b contributes to SNC2-mediated immunity by promoting SARD1 expression. It is also required for the expression of an unknown gene (X), which encodes a guardee/decoy of a TNL(s) or a negative regulator of Toll/interleukin-1 receptor signaling. Activation of SNC2-mediated defense responses also leads to CBP60b-independent expression of gene X.

For mapping by sequencing, bda7-1 cbp60g-1 snc2-1D was backcrossed with cbp60g-1 snc2-1D. In the F2 population, about 50 plants that showed morphology similar to that of bda7-1 cbp60g-1 snc2-1D were selected and pooled for genomic DNA extraction. The genomic DNA was subsequently sequenced using the Illumina HiSeq platform to generate 150-bp paired-end reads. Single-nucleotide polymorphisms were called from the next-generation sequencing data using the Genome Analysis Toolkit (GATK v.3.5-0) (McKenna et al., 2010) following the variant discovery pipeline from GATK Best Practices suggested by the Broad Institute (DePristo et al., 2011). In brief, the raw sequences were trimmed and filtered with PRINSEQ lite 0.20.4 to remove any unwanted sequences (Schmieder and Edwards, 2011). The sequences were then mapped to the Tair10 Arabidopsis reference genome with BWA MEM (Li, 2013). The alignments were then processed by GATK HaplotypeCaller to call variants. All identified variants were then merged using PICARD tools (http://broadinstitute.github.io/picard). After variant calling, we identified the bda7-1 mutation by looking for homozygous single-nucleotide polymorphisms in the exons of genes in the ~0.8-Mb region of chromosome 5 to which bda7-1 was mapped.

Chromatin immunoprecipitation analysis
ChIP-qPCR assays were performed as previously described (Sun et al., 2015). The chromatin complexes containing CBP60b-3HA were immunoprecipitated using anti-HA antibody (Roche, Basel, Switzerland) and Protein A/G Agarose beads (GE Healthcare, Chicago, IL, USA). The immunoprecipitated DNA was analyzed by qPCR using gene-specific primers, which are listed in Supplemental Table 1.

Dual-reporter assay
The dual-reporter assay was performed by transforming N. benthamiana with the reporter constructs together with different effector constructs, pSARD1::Luc was described previously (Ding et al., 2018). A pUBQ1-driven Renilla luciferase reporter was included as an internal control. Agrobacterium carrying the reporter construct pSARD1::Luc, the internal control pUBQ1::Renilla, and the effector construct 35S::CBP60b-3HA or 35S::GFP-3HA was first cultured in liquid LB and then resuspended in 10 mM MgCl2. Leaves of N. benthamiana were co-infiltrated with Agrobacterium carrying the indicated construct combinations with final concentrations of OD600 = 0.2 (pSARD1::Luc), OD600 = 0.05 (pUBQ1::Renilla), and OD600 = 0.5 (35S::CBP60b-3HA or 35S::GFP-3HA). To image the luminescence intensity, N. benthamiana leaves were infiltrated with 1 mM luciferin 40 h after inoculation with the Agrobacterium and then imaged under the Gel Doc XR+ system (Bio-Rad) with the Blot mode. To quantify the promoter activity, areas of N. benthamiana leaves infiltrated with Agrobacterium were collected 40 h after inoculation. The Dual-Luciferase reporter assay system (Promega) was used to measure the activity of firefly luciferase and Renilla luciferase sequentially using a BioTek Synergy 2 Multi-Mode
RNA extraction, reverse transcription, and qPCR

Plants for gene expression assays were grown on 1/2 MS plates for 14 days under long-day conditions. Approximately 50 mg of plant tissue from three or four individual seedlings of the indicated genotypes was collected as a single sample. Three biological replicates were analyzed for each genotype. RNA extraction was performed using the EZ-10 Spin Column Plant RNA Miniprep Kit (Bio Basic, Toronto, ON, Canada). RNA was reverse transcribed into cDNA with OneScript reverse transcriptase (Applied Biological Materials, Richmond, BC, Canada). qPCR was performed on the total cDNA using SYBR Premix Ex Taq II (Takara, Shiga, Japan). Primers for qPCR are listed in Supplemental Table 1.

SA extraction and quantification

The procedure for SA extraction and measurement was reported previously (Sun et al., 2015). Approximately 100 mg of plant tissue was collected from two or three individual plants of the indicated genotypes as a single sample. Plant tissue was ground into a fine powder with liquid nitrogen, resuspended with 600 µl of 90% methanol, and sonicated for 20 min to release SA. After centrifugation at 12 000 g for 10 min, the supernatant was collected, and the pellets underwent a second round of extraction by addition of 500 µl of 100% methanol and sonication for another 20 min. The supernatants from both extractions were combined and dried by vacuum. For free SA quantification, 500 µl of 5% (w/v) trichloroacetic acid was added to the dry samples, vortexed, and sonicated for 5 min. For total SA measurement, 100 µl of β-glucosidase solution (80 units/ml in 0.1 M NaAc [pH 5.2]) was added to the dry samples, vortexed, and sonicated for 5 min and then incubated at 37°C for 90 min to cleave SAG into free SA before adding 500 µl of 5% (w/v) trichloroacetic acid. After centrifugation at 12 000 g for 15 min, the supernatant was collected and extracted with 500 µl of extraction buffer (ethylacetate acid:cyclopentane:isopropanol, 100:99:1, v/v/v) three times. Each time, after centrifugation at 12 000 g for 10 min, the organic phase was collected and combined into a new tube. The combined organic phase was then dried by vacuum. The dry sample was resuspended with 200 µl of mobile phase (0.2 M KAc, 0.5 mM EDTA [pH 5]) by vortexing and sonication for 5 min. After the final centrifugation at 12 000 g for 5 min, the supernatant was collected and used to determine the amount of SA by high-performance liquid chromatography.

Pathogen infection assay

Hpa Noco2 infection was performed by spray-inoculating 2-week-old seedlings with spores in water (50 000 spores/ml). Inoculated seedlings were covered with a transparent lid and grown in a plant chamber at 18°C with a relative humidity of ~80%. Infection was scored at 7 days post-inoculation by counting conidia spores with a hemocytometer. Four or five individual plants were pooled as a single sample. Four biological replicates were included for each genotype.

Statistical analysis

Error bars in all of the figures represent standard deviations. The number of biological replicates is indicated in the figure legends. Statistical comparisons among different samples were performed either by one-way ANOVA with Tukey’s honestly significant difference post hoc test or by Student’s t-test, as reported in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental information is available at Plant Communications Online.

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CBP60b in plant immunity

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