The CALMODULIN-BINDING PROTEIN60 Family Includes Both Negative and Positive Regulators of Plant Immunity

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Two members of the eight-member CALMODULIN-BINDING PROTEIN60 (CBP60) gene family, CBP60g and SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1 (SARD1), encode positive regulators of plant immunity that promote the production of salicylic acid (SA) and affect the expression of SA-dependent and SA-independent defense genes. Here, we investigated the other six family members in Arabidopsis (Arabidopsis thaliana). Only cbp60a mutations affected growth of the bacterial pathogen Pseudomonas syringae pv maculicola ES4326. In contrast to cbp60g and sard1 mutations, cbp60a mutations reduced pathogen growth, indicating that CBP60a is a negative regulator of immunity. Bacterial growth was increased by cbp60a only in the presence of CBP60a, while the increase in growth due to sard1 was independent of CBP60a, suggesting that the primary function of CBP60a may be to counter the repressive effect of CBP60a. In the absence of pathogen, levels of SA as well as of several SA-dependent and SA-independent pathogen-inducible genes were higher in cbp60a plants than in the wild type, suggesting that the enhanced resistance of cbp60a plants may result from the activation of immune responses prior to pathogen attack. CBP60a bound calmodulin, and the calmodulin-binding domain was defined at the C-terminal end of the protein. Transgenes encoding mutant versions of CBP60a lacking the ability to bind calmodulin failed to complement null cbp60a mutations, indicating that calmodulin-binding ability is required for the immunity-repressing function of CBP60a. Regulation at the CBP60 node involves negative regulation by CBP60a as well as positive regulation by CBP60g and SARD1, providing multiple levels of control over the activation of immune responses.

Plant innate immunity depends on the recognition of pathogen attack, activation of a complex signaling network, and expression of defense genes. One type of recognition is mediated by pathogen recognition receptors in the plasma membrane that bind molecules characteristic of microbes, called microbe-associated molecular patterns (MAMPs), resulting in pattern-triggered immunity (PTI). Adapted pathogens produce effector proteins that promote virulence by targeting the receptors themselves or critical components of the defense signaling machinery. A second type of pathogen recognition involves the detection of pathogen effectors by plant resistance proteins, resulting in effector-triggered immunity (ETI; Jones and Dangl, 2006). In PTI, pathogen recognition is followed rapidly by early responses, including the influx of Ca2+ into the cytoplasm and nucleus (Gust et al., 2007; Ranf et al., 2011, 2012), an oxidative burst (Gómez-Gómez et al., 1999), deposition of callose at the cell wall (Gómez-Gómez et al., 1999), and activation of a mitogen-activated protein kinase cascade (Zhang and Klessig, 1998; Nühse et al., 2000; Zhang et al., 2000). After a few hours, the signaling hormones ethylene, jasmonic acid-Ile conjugate, and salicylic acid (SA) are produced and mediate the expression changes of a large number of genes (Dodds and Rathjen, 2010). The efficacy of PTI can be observed by pretreating plants with a MAMP, such as the peptide flg22 derived from bacterial flagellin, and then challenging with a virulent pathogen (Zipfel et al., 2004; Tsuda et al., 2009). Pathogen growth is reduced in plants pretreated with the MAMP, presumably due to the activation of defenses prior to pathogen challenge. ETI responses often include a form of programmed cell death called the hypersensitive response as well as production of ethylene, jasmonic acid-Ile conjugate, and SA and associated changes in gene expression (Dodds and Rathjen, 2010).

The pathogen studied in this work, Pseudomonas syringae pv maculicola strain ES4326 (Pma ES4326), is a moderately virulent pathogen not known to trigger an ETI response in Arabidopsis (Arabidopsis thaliana; Dong et al., 1991). It does elicit a strong SA response, which is known to be important for limiting pathogen growth, as plant mutants with defects in SA production or SA signaling allow much more bacterial growth than wild-type
plants (Glazebrook et al., 1996). Wild-type levels of SA production require the related lipase-like genes ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and PHYTOALEXIN DEFICIENT4 (PAD4), which are also required for many SA-independent gene expression changes in response to infection (Falk et al., 1999; Jirage et al., 1999; Wiemer et al., 2005; Wang et al., 2008). EDS1 and PAD4 indirectly increase the expression of SALICYLIC ACID INDUCTION DEFICIENT2/ISOCORISMATE SYNTHASE1 (SID2/ICS1), which encodes isochorismate synthase, an enzyme required for SA synthesis (Wildermuth et al., 2001). The SA signal is detected by the NON-EXPRESSOR OF PATHOGENESIS RELATED GENES3 (NPR3) and NPR4 proteins, resulting in the translocation of NPR1 to the nucleus, where it controls gene expression through physical interaction with transcription factors (Zhang et al., 1999; Mou et al., 2003; Dong, 2004; Fu et al., 2012).

SA signaling is also affected by two members of the CALMODULIN-BINDING PROTEIN60 family, CBP60g and SARD1 (Wang et al., 2009, 2011; Zhang et al., 2010). Both genes are induced by flg22 treatment or Pma ES4326 infection, with the response of CBP60g being faster than that of SARD1 (Wang et al., 2009, 2011). Single mutations in either gene cause modest increases in the growth of Pma ES4326 (Wang et al., 2009, 2011). Double cbp60g sard1 mutants allow greatly increased growth of Pma ES4326 and reduced levels of SA (Wang et al., 2011). The effect on bacterial growth is larger than that of sild2, while the effect on SA is less than that of sild2, indicating that the mutations also affect an SA-independent defense response (Wang et al., 2011).

Expression profiling of plants 24 h after Pma ES4326 infection revealed this SA-independent defense. The genes whose expression was affected in cbp60g sard1 plants included the SA-dependent genes as well as a subset of the SA-independent genes whose expression is affected in pad4 and eds1 plants (Wang et al., 2011). This analysis placed the CBP60g/SARD1 signaling node between the PAD4/EDS1 and SA nodes in the immune signaling network (Wang et al., 2011). CBP60g bound calmodulin (CaM) in vitro, while SARD1 did not (Wang et al., 2009, 2011).

The CaM-binding domain of CBP60g lies near the N-terminal end (Wang et al., 2009). Transgenes encoding site-directed mutants that could not bind CaM failed to complement the Pma ES4326 growth phenotype, indicating that CaM binding is required for the function of CBP60g in immunity (Wang et al., 2009). CBM is a small protein consisting almost entirely of four so-called “EF-hand” domains (Chin and Means, 2000). Each EF-hand binds one Ca2+ ion with micromolar affinity, causing a conformational change in the protein (Chin and Means, 2000). CaM mediates Ca2+ signals by controlling the activities of other proteins by binding to them in a Ca2+-dependent manner (Chin and Means, 2000). In the case of CBP60g, CaM binding occurred only in the presence of Ca2+ (Wang et al., 2009). These results suggest that CBP60g constitutes a link between the Ca2+ influx that occurs early after pathogen recognition and the subsequent production of SA and expression of SA-independent immunity genes (Wang et al., 2009). It was proposed that SARD1 may provide a similar function in the activation of defenses, but in a Ca2+-independent manner, allowing the persistence of defense after Ca2+ returns to the resting level (Wang et al., 2011).

Both CBP60g and SARD1 were shown to be located in the nucleus and to bind to the SID2 promoter in chromatin immunoprecipitation assays (Zhang et al., 2010). Using electrophoretic mobility shift assays, the central portions of both proteins were found to bind to an oligonucleotide of sequence GAAATTTTTG (Zhang et al., 2010). A portion of this sequence, GAAATTT, was overrepresented in the promoters of 25 genes whose pathogen-induced expression was found to be reduced in cbp60g sard1 plants (Wang et al., 2011). The results suggest that CBP60 proteins may directly bind to the promoters of the genes they control, thereby affecting their transcription (Zhang et al., 2010).

The extent of the CBP60g/SARD1 regulon was explored by mining expression profiling data obtained using the ATH1 microarray. Experiments in which CBP60g and SARD1 were coexpressed with SID2 were selected. Within these experiments, genes strongly coexpressed with any of the 44 genes whose expression was affected in cbp60g sard1 plants were selected. This gene set was then clustered based on the strength of coexpression. The effects of cbp60g sard1 on the expression of representative genes from clusters enriched with GAAATTT promoter motifs were determined by quantitative real-time (qRT)-PCR. While the expression of some genes whose promoters were enriched in GAAATTT motifs was reduced in cbp60g sard1 plants, the expression of other genes was unaffected, and the expression of some genes was increased. These results indicate that the enrichment of GAAATTT motifs is not a sufficient condition for CBP60g/SARD1-dependent induction of gene expression (Truman and Glazebrook, 2012).

CBP60g and SARD1 have also been implicated in responses to abiotic stresses. Both genes are induced during the response to cold, with the kinetics following those of SID2, which is also induced (Kim et al., 2013). It is not yet known whether CBP60g and SARD1 are required for the cold induction of SID2. Overexpression of CBP60g resulted in increased tolerance to drought stress and abscisic acid, while cbp60g plants showed increased sensitivity (Wan et al., 2012).

The Arabidopsis CBP60 gene family contains eight members. In view of the known roles of CBP60g and SARD1 in responses to biotic and abiotic stress, we decided to test the other six family members for roles in biotic stress responses by testing the effects of mutations in these genes on the growth of Pma ES4326. Only cbp60a mutants had a substantial phenotype, in that bacterial growth was reduced relative to wild-type plants. Levels of SA and the expression of selected pathogen-inducible genes were elevated in cbp60a plants in the absence of pathogen. The CaM-binding domain of CBP60a was located at the C terminus of the protein, in contrast to CBP60g, whose CaM-binding domain lies near the
N terminus. The CaM-binding ability of CBP60a was required for complementation of the reduced bacterial growth phenotype of cbp60a null mutants. In contrast to CBP60g and SARD1, CBP60a is a repressor of immunity.

RESULTS
CBP60a Negatively Affects Plant Immunity

As cbp60g and sard1 mutations compromise plant immunity, allowing enhanced growth of Pma ES4326, we decided to test mutations in the other six CBP60 family members for effects on Pma ES4326 growth. As shown in Figure 1A, two independent cbp60a transfer DNA (T-DNA) insertion alleles resulted in reduced growth of Pma ES4326, indicating that the immunity to this pathogen is enhanced in cbp60a plants. Among the other cbp60 T-DNA insertion mutations tested, mutations in cbp60c and cbp60d caused very small but statistically significant increases in Pma ES4326 growth. These small differences were not pursued further and could be due to secondary mutations in the T-DNA lines, as additional alleles were not tested. Wild-type CBP60a constructs, containing the native introns and promoter, with C-terminal hemagglutinin (HA) tags, complemented the Pma ES4326 growth phenotype of the cbp60a-1 mutant, further confirming that mutations in cbp60a cause enhanced resistance (Fig. 1B).

The growth of two other P. syringae strains, virulent P. syringae pv. tomato strain DC3000 (Pto DC3000) and an isogenic avirulent strain expressing an effector recognized by the R protein RPS2, Pto DC3000 avrRpt2, was also reduced in cbp60a plants (Fig. 1, C and D). Thus, CBP60a acts as a repressor of immunity, in contrast to CBP60g and SARD1, which are activators of immunity. CBP60a, CBP60g, and SARD1 define a clade distinct from the other five CBP60 genes (Wang et al., 2011), and each member of this clade plays a role in immunity.

The level of CBP60a mRNA was induced by Pma ES4326 inoculation, but to a much lesser extent than CBP60g or SARD1. The expression of CBP60a was not affected by the cbp60g, sard1, and sid2 mutations, indicating that it is controlled independently of CBP60g, SARD1, and SA. The expression of CBP60g and SARD1 was not affected in cbp60a plants (Supplemental Fig. S1).

The Effect of cbp60a on Pma ES4326 Growth Is Epistatic to cbp60g But Additive with sard1

To explore the genetic relationships among the three CBP60 family members with effects on plant immunity, we constructed all three double mutants and the triple mutant and compared the growth of Pma ES4326 in plants of all cbp60 genotypes and the Columbia (Col-0) wild type. Figure 2 shows that the cbp60a single mutant showed reduced growth, while cbp60g and sard1 mutations increased growth, consistent with Figure 1 and previous results (Wang et al., 2009, 2011). Interestingly, growth in the cbp60a cbp60g double mutant was lower than in Col-0 and indistinguishable from cbp60a cbp60d and cbp60a sard1 mutants (Fig. 2, A, B, and C).

Figure 1. Growth of P. syringae is reduced in cbp60a plants. A, Pma ES4326. B, Complementation of cbp60a-1 by CBP60a. C, Pto DC3000. D, Pto DC3000 avrRpt2. Bacterial titers were determined immediately (Day 0) or 3 d (Day 3) after inoculation of wild-type Col-0, cbp60a-1, cbp60a-2, cbp60b, cbp60c, cbp60d, cbp60e, cbp60f, Comp #1 (complementation line cbp60a-1:CBP60a-HA #1), Comp #2 (an independent complementation line cbp60a-1:CBP60a-HA #2), and pad4 plants. Data were obtained in two (A and B) or three (C and D) independent experiments, each with four (Day 0) or 12 (Day 3) biological replicates, combined with mixed linear models. Error bars indicate SE, and asterisks indicate q < 0.05 for the comparison with Col-0. cfu, Colony-forming units.
growth in cbp60a. The effect of cbp60g in a wild-type background was 0.4 log₁₀ units, while the effect of cbp60g in a cbp60a background was indistinguishable from 0 (Fig. 2B), indicating that the effect of cbp60a is epistatic to that of cbp60g. In contrast, growth in the double cbp60a sard1 mutant was intermediate between that in cbp60a and sard1. The effects of sard1 in the wild-type background and in a cbp60a background were not significantly different, indicating that cbp60a and sard1 have independent effects on Pma ES4326 growth (Fig. 2C).

Growth in cbp60a cbp60g sard1 was slightly lower than in cbp60g sard1. These data are consistent with the idea that CBP60g is needed to counter the suppressive effect of CBP60a on immunity, while the effects of CBP60a and SARD1 are independent.

To test for an effect on PTI, plants of each genotype were treated with flg22 or mock treated and inoculated with Pma ES4326 1 d later. Pathogen titers were determined after a further 2 d. The extent of PTI was defined as the difference in pathogen titer in mock-pretreated plants versus flg22-pretreated plants. As shown in Supplemental Figure S2, cbp60a and cbp60a cbp60g plants had reduced PTI. This was due entirely to the increased resistance of mock-treated plants, as titers in flg22-pretreated plants were indistinguishable from Col-0. Increased PTI was observed in cbp60a cbp60g sard1 plants, as the increased growth in mock-treated plants was slightly larger than the increased growth in flg22-pretreated plants. We conclude that cbp60g and sard1 mutations do not compromise PTI. Formally, cbp60a does compromise PTI, but this is due to decreased bacterial growth in mock-treated plants rather than increased growth in flg22-pretreated plants, which is the phenotype usually associated with reduced PTI.

SA Levels Are Elevated in cbp60a Plants

Immunity-related increases in SA levels are reduced in cbp60a and sard1 plants. As cbp60a plants are more resistant to P. syringae, while cbp60g and sard1 plants are more susceptible, we hypothesized that SA levels might be high in cbp60a plants. To test this, we measured SA levels in wild-type plants and all the combinatorial cbp60 mutants 24 h after mock inoculation or inoculation with Pma ES4326. Each sample was divided into two parts. Total SA was measured in one part by including β-glucosidase to hydrolyze salicylic acid glucoside (SAG) before the determination of SA, yielding measurements of total SA. In the other part, β-glucosidase was omitted, yielding measurements of free SA. SAG was determined as the difference between the total and free SA values. As shown in Figure 3, total SA levels were higher in cbp60a plants than in cbp60g and sard1 single mutants (Wang et al., 2011) was not reproduced here, likely due to an unknown variation in experimental conditions. No significant differences in free SA levels were observed between cbp60a and corresponding CBP60a genotypes, while the effects of cbp60a on SAG levels were analogous to the effects on total SA, except that the difference between Col-0 and cbp60a was not significant (Supplemental Fig. S5). Evidently, the difference in total SA levels caused by cbp60a is due to its effect on SAG levels, rather than free SA. Taken together, these data show that
CBP60a Represses Immunity

Expression Levels of Selected Immunity-Related Genes Are Elevated in cbp60a Plants

CBP60g and SARD1 are involved in the transcriptional regulation of SID2 and SA-dependent genes. To determine whether CBP60a affects the expression of SA-dependent genes, we determined expression levels of the SA marker gene PATHOGENESIS RELATED1 (PR-1) as well as SID2. Loss of CBP60a singly or in a cbp60g background caused the up-regulation of PR-1 in plants 24 h after mock inoculation but did not affect expression following inoculation with Pma ES4326 (Fig. 4A). Thus, the baseline or starting point for SA-associated defenses, which play a critical role against biotrophic pathogens, is elevated. Expression of the key SA biosynthesis gene SID2 was also elevated in mock-inoculated cbp60a plants (Fig. 4B). These expression patterns mirror the accumulation of SA in response to mock inoculation. However, neither SID2 nor PR-1 expression was significantly altered in Pma ES4326-infected cbp60a cbp60g sard1 plants compared with cbp60g sard1, in contrast to measurements of total SA.

In a previous study, we clustered genes that were coexpressed with known CBP60g/SARD1-dependent genes. For representative genes whose promoters are enriched with the GAAATT motif thought to be bound by CBP60g and SARD1, we found that CBP60g and SARD1 acted as inducers, repressors, or had no effect on gene expression. Here, we determined the expression levels of these genes in Col-0, cbp60a, cbp60g sard1, and cbp60a cbp60g sard1 plants (Fig. 4B). Strikingly, for eight of these nine genes, as well as PR-1, the cbp60a mutation resulted in elevated expression levels compared with wild-type Col-0 following mock inoculation. This indicates that CBP60a is involved in the blanket suppression of these immune response genes, even though CBP60g/SARD1 affected these genes in a variety of ways. Genes such as At1g51890 and PINOID BINDING PROTEIN1 (PBPI) are up-regulated in mock-inoculated cbp60g sard1 plants, although there does not appear to be any additive effect of cbp60a in the level of gene expression in mock-inoculated plants. With SID2, FLAVIN MONOOXYGENASE1 (FMO1), and PR-1, the repression of expression in mock-inoculated plants by CBP60a is CBP60g/SARD1 dependent, and the transcript level in the triple mutant is similar to that in the wild type. At1g64610, At2g32030, SYNTAXIN OF PLANTS122 (SYP122), and At5g47150 have intermediate expression levels in cbp60g sard1 that are indistinguishable from either the wild type on the lower side or cbp60a/cbp60a cbp60g sard1 on the higher side. For Pma ES4326-inoculated plants, the comparison of Col-0 and the cbp60a single mutant is not significantly different for any of the selected genes. Curiously, the cbp60a cbp60g sard1 triple mutant exhibits down-regulation in comparison with cbp60g sard1 for FMO1, PBPI, and SYP122, genes that are, respectively, suppressed, induced, or unchanged in cbp60g sard1 compared with the wild type. In summary, these data show several patterns of CBP60a effects on gene expression. For nearly all GAAATT-enriched genes tested, expression in cbp60a plants is higher than in Col-0 plants in the absence of pathogen. While the primary effect of cbp60a on gene expression in mock-inoculated plants is positive, in Pma ES4326-inoculated plants the only significant effects were negative, suggesting that CBP60a can act as a repressor or activator of gene expression depending on whether plants are responding to a pathogen attack.

CBP60a Binds CaM through a C-Terminal Domain

CBP60a family members a to e are known to bind CaM (Reddy et al., 2002). The C-terminal domain was predicted to contain the CaM-binding site based on sequence similarity (Reddy et al., 2002). To test this, we fused full-length CBP60a to glutathione S-transferase (GST) and expressed it using an isopropylthio-β-galactoside-inducible system in Escherichia coli. CaM binding was tested using an immunoblot with bovine CaM conjugated to biotin. CBP60a bound CaM (Supplemental Fig. S4), and no binding was observed in the presence of 50 mM EGTA, indicating that binding is Ca2+ dependent. To define the CaM-binding domain, we fused various fragments of CBP60a to a His tag, expressed them in E. coli, and tested for CaM binding. A 120-amino acid (aa) fragment from the C-terminal end was sufficient for binding (Supplemental Fig. S4). To further refine the
position of the CaM-binding domain, fragments of the C-terminal 120 aa were fused to GST and expressed in E. coli. As shown in Figure 5A, only fragments containing the C-terminal 31 aa bound CaM, and this fragment was sufficient for CaM binding (Fig. 5B). Immunoblotting with anti-GST antibody showed that all the protein fusions were expressed (Fig. 5B). The 31-aa sequence was tested for likely CaM-binding regions using the Calmodulin Target database (Yap et al., 2000). This suggested that the region from Trp-455 to Val-467 might constitute a CaM-binding domain (Fig. 5C). Generally, CaM-binding domains are disordered regions that form amphiphilic helices upon CaM binding, with CaM binding to the hydrophobic sides (Rhoads and Friedberg, 1997). Using the Heliquest algorithm (Gautier et al., 2008), we found that the region from Gly-448 to Leu-465 could form an amphiphilic helix. To locate the CaM-binding site, we created six mutant versions of the C-terminal 31 aa of CBP60a fused to GST, each with one hydrophobic amino acid changed to Lys, and fused them to GST. CaM-binding assays on fusion proteins expressed in E. coli showed that L458K, V461K, and L465K abolish CaM binding, indicating that these Leu and Val residues form part of the CaM-binding site, thus precisely defining its location at the C-terminal end of the protein (Fig. 5E).

CaM Binding Is Required for the Function of CBP60a in Immunity

To test for a requirement of CaM binding for the function of CBP60a in repressing immunity, we constructed site-directed mutants of CBP60a encoding proteins that do or do not retain CaM-binding activity and tested them for complementation of the Pma ES4326 growth phenotype of cbp60a plants. A genomic clone of CBP60a including the promoter and ending with a C-terminal Myc tag was used to create the mutants. We

Figure 4. Immunity gene expression levels in cbp60a mutants. qRT-PCR measurements are given for gene expression 24 h after mock or Pma ES4326 inoculation (OD600 = 0.01). A, PR-1 expression in all plants with all combinations of cbp60a genotypes. B, Expression of the indicated genes in Col-0, cbp60a, cbp60g sard1, and cbp60a cbp60g sard1. Note the different scales on the y axes for different genes. Data from four (or seven for SID2) biological replicates were merged using a mixed linear model, and the mean log2 ratio to ACTIN2 expression was plotted along with the se. Letters denote significantly different groups, with lowercase used for mock-treated plants and uppercase used for infected plants, with q ≤ 0.05 for A or P ≤ 0.05 for B.
constructed mutants V461K and L465K that do not bind CaM and mutants F459K and V467K that do bind CaM. These constructs were then introduced into cbp60a plants, and transgenic progeny carrying one or two copies of the insertions were selected by quantitative PCR. Growth of Pma ES4326 was determined in Col-0, cbp60a, and two or three independent transgenic lines for each construct. As shown in Figure 6A, cbp60a plants carrying the wild-type CBP60a construct unexpectedly allowed significantly more bacterial growth than wild-type plants. All five cbp60a lines expressing versions of CBP60a that cannot bind CaM allowed growth significantly lower than the cbp60a lines expressing wild-type CBP60a, and two lines were significantly lower than Col-0. Growth in the two cbp60a lines expressing the F459K mutant was indistinguishable from growth in cbp60a plants expressing wild-type CBP60a, while growth in the cbp60a lines carrying V467K was similar to that in the V461K and L465K lines. We were unable to detect the proteins produced from the CBP60a transgenes in the cbp60a plants by immunoblotting. However, the expression of transcripts was measured by qRT-PCR (Fig. 6B). The expression of wild-type CBP60a was higher than in Col-0, and the expression of V467K in line 2 was very low, but all other transgenes were expressed at levels similar to Col-0. All the CBP60a proteins were similarly stable when transiently expressed from the strong 35S promoter in Nicotiana benthamiana. Thus, it is unlikely that differences in effects on bacterial growth were due to differences in transgene expression or protein stability. As cbp60a lines expressing either mutation that eliminated CaM-binding activity allowed reduced bacterial growth relative to cbp60a lines expressing wild-type CBP60a, but growth was not reduced to the level in cbp60a plants, we conclude that the loss of CaM binding greatly reduces the function of CBP60a as a repressor of immunity but may not completely abolish it. It seems likely that the V467K mutation interferes with CBP60a function in a manner independent of CaM binding.

DISCUSSION AND CONCLUSION

Plants with cbp60a mutations are significantly more resistant to P. syringae than wild-type plants. Pathogen growth is a highly summarized phenotype, presumably resulting from the combined effects of many biological processes influencing immunity. In the absence of pathogen, cbp60a plants have higher levels of SA, in the form of SAG, as well as higher expression of nine of ten Pma ES4326-inducible genes assayed. Except for PR-1 and ARABINOGALACTAN PROTEINS, these genes do not require SA for induced expression (Wang et al., 2008), indicating that, like CBP60g and SARD1, CBP60a affects SA levels and the expression of a suite of polar residues, purple indicates uncharged polar residues, gray indicates Ala and Gly, and light blue indicates His. Residues determined to be required for CaM binding are indicated by asterisks. E, CaM binding of full-length CBP60a proteins with the indicated mutations fused to GST.
SA-independent defense genes. In the absence of pathogen, immune responses in cbp60a plants are at a higher level than in wild-type plants, likely explaining the increased resistance observed.

We explored the relationships among CBP60a, CBP60g, and SARD1 by comparing phenotypes of single, double, and triple mutants. For Pma ES4326 growth, the phenotype of cbp60a cbp60g plants was indistinguishable from that of cbp60a, indicating that CBP60g impacts growth only when CBP60a is present. In contrast, the effects of cbp60a and sard1 were additive, indicating that CBP60a and SARD1 act independently. These observations suggest that CBP60g counteracts the immune-suppressing effect of CBP60a. However, similar relationships were not observed in SA levels or in the expression pattern of PR-1. The effects of single cbp60 mutations on these phenotypes are small or undetectable, so the effects on bacterial growth may result from as yet unknown responses and/or from the combined effects of small changes in many defense responses.

Our targeted survey of the influence of CBP60a on gene expression revealed a consistent repression of the basal expression of genes with GAAATT-rich promoters. This repression did not hold sway over expression levels in infected leaves, a condition where the expression of CBP60g is very strongly induced, possibly titrating out any antagonistic interaction with the more modestly up-regulated CBP60a. Intriguingly, CBP60g and SARD1 also repress the basal expression of some of these genes, with CBP60a and CBP60g/SARD1 acting redundantly as a brake on their expression. The genes selected for this study are all induced by Pma ES4326 infection, and no combination of CBP60 mutations abolished this up-regulation, implicating other, as yet undefined, transcriptional regulators as critical for their response. The interaction of CBP60a with other regulators may explain the capacity for CBP60a to act both as a repressor and an activator of FMO1, PBP1, and SYPI22 expression under different conditions. That this activator role is only observed in the absence of CBP60g and SARD1 implies some competition for access to these other regulators.

In the complementation experiments shown in Figure 6A, a transgene encoding wild-type CBP60a, tagged at the C terminus with Myc and expressed from...
the native promoter in cbp60a plants, increased Pma ES4326 growth above the level in wild-type plants. A similar transgene carrying the F459K mutation, which does not affect CaM binding, had a similar effect. However, the CBP60a transgene used in the experiment shown in Figure 1B, which was also expressed from the native promoter but had an HA C-terminal tag, did not have this effect. It is possible that the enhancement of bacterial growth by the Myc-tagged CBP60a protein was caused by some alteration of protein function due to the Myc tag.

Our data show that CBP60a is a CaM-dependent negative regulator of immunity, affecting SA levels and SA-independent gene expression. There are other CaM-dependent negative regulators of immunity. The transcription factor known as CALMODULIN BINDING TRANSCRIPTION ACTIVATOR3 (CAMTA3) and ARABIDOPSIS THALIANA SIGNAL RESPONSIVE 1 represses SA accumulation and other defense responses by binding to the promoter of EDS1, reducing its transcription (Du et al., 2009). Combining camta3 mutations with mutations in the other two family members, camta1 and/or camta2 mutations result in even higher SA levels, increased expression of CBP60g and SARD1, and severe dwarfism (Kim et al., 2013). The CaM-binding NAC (NO APICAL MERISTEM, ARABIDOPSIS THALIANA TRANSCRIPTION ACTIVATION FACTOR, CUP-SHAPED COTYLEDON) transcription factor CBNAC acts as a transcriptional repressor, and binding to DNA is enhanced by CaM (Kim et al., 2007). CBNAC binds to the PR-1 promoter, and the cbnac1 mutant is more resistant to Pto DC3000 and has increased expression of PR-1 (Kim et al., 2012). Thus, at least three distinct proteins mediate CaM-dependent repression of immunity in the absence of pathogen attack. It is not clear how this occurs. There is Ca\(^{2+}\) influx into the cytoplasm and nucleus, which occurs rapidly after pathogen recognition, and this likely promotes CaM binding to its targets. However, repression is occurring in the absence of pathogen, so there must be enough Ca\(^{2+}\) and CaM present in the absence of pathogens to allow function of the CBP60a, CAMTA, and CBNAC repressors.

For plants to effectively respond to pathogen attack, repression of defense by CBP60a and other repressors must be removed. We propose the following model as one explanation of how this might occur (summarized in Fig. 7). In the absence of pathogen, CBP60a is repressing immunity and CBP60g and SARD1 have low activity. If a microbe activates a PTI response, the associated Ca\(^{2+}\) spike further enhances the repression of EDS1-dependent immunity, including SA synthesis. This prevents the activation of a full immune response, which is detrimental to growth, every time a plant detects a microbe. If the microbe is, in fact, a pathogen, the MAMP signal may be stronger, persist longer, and/or include an additional signal that leads to the expression of CBP60g, which is induced by 9 h in response to a strong MAMP signal or Pma ES4326 infection. CBP60g removes repression by CBP60a, consistent with the data in Figure 2 showing that cbp60g is epistatic to cbp60a. The action of CBP60g is enhanced by elevated Ca\(^{2+}\) due to CaM binding. The continued presence of the pathogen requires an even stronger response, mediated by the induction of SARD1 expression by 24 h after pathogen attack, resulting in continued activation of defenses in a CaM-independent manner.

![Figure 7](image-url)
manner. In this model, there must also be mechanisms for the relief of suppression by CAMTAs and CBNAC. The finding that three CBP60 family members play roles in immune signaling, with one having a suppressive effect and two having inducing effects, begs the question of what evolutionary forces drove this arrangement. There are at least two attractive hypotheses. One possibility is that it provides more stringent control of defense activation. In the absence of pathogen attack, immune responses are low due to combined effects of suppression by CBP60a, insufficient Ca2+ to activate CBP60g by CaM binding, and low expression levels of CBP60g and SARD1. Once a pathogen has been detected, these factors reverse, with CBP60g/CaM removing the effect of CBP60a, maintenance of the response by CaM-independent SARD1, and increased expression of CBP60g and SARD1, encoding the activators. This system likely confers a much greater dynamic range than could be achieved through controlling the activity of a single family member. Another possible explanation is that this CBP60 system provides resilience against pathogen attack. The plant immune system is subject to evolutionary pressure from pathogens, which evolve to overcome immune responses, thus providing a measure of resilience in the face of effector attack.

**MATERIALS AND METHODS**

**Plant Genotypes, Growth Conditions, and Pathogen Inoculation**

Wild-type Col-0, pale-1 (At1g52430; Jirage et al., 1999), cbp60g-1 (At5g26920, SALK_023199), sard1-1 (At1g73805, SALK_138476), cbp60a-l (At1g62570, SALK_124410), cbp60e-2 (SALK_036308), cbp60f (At5g39780, SAIL_40090), cbp60c (At2g18750, SALK_005511), cbp60d (At4g31000, SAIL_576D02), and sid2-2 (At1g74710; Wildermuth et al., 2001) Arabidopsis (Arabidopsis thaliana) plants were grown in autoclaved BM2 germinating mix (Berger) in a growth chamber at 22°C and 75% relative illumination. Where not otherwise specified, "cbp60e" refers to cbp60e-1. Plants used in experiments were 4 to 5 weeks old. *Pseudomonas syringae* was cultured in King’s B medium at room temperature as described (Tsuda et al., 2008). For bacterial growth assays, *Pma* ES4326, *Pto DC3000*, and *Pto DC3000 avrRpt2* were suspended in 5 mM MgSO4 at optical density at 600 nm (OD600) = 0.001 and infiltrated into the abaxial sides of leaves using a needleless 1-mL syringe. Flg22 peptide was used at 1 μM and introduced by infiltration. Determination of bacterial titers was as described previously (Tsuda et al., 2008).

**Quantification of SA and mRNA**

For determination of SA, plants were inoculated with *Pma* ES4326 at OD600 = 0.01 or mock inoculated. Total and free SA were determined by gas chromatography-mass spectrometry as described previously (Tsuda et al., 2008). mRNA levels were determined by qRT-PCR, as described previously (Truman and Glazebrook, 2012), using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) in a LightCycler 480 (Roche).

Primers used for ACTIN2, CBP60a, CBP60g, SARD1, PR-1, SID2, FM01, ARABINOGALACTIN PROTEINS, At1g51890, At1g64610, PRP1, At1g93200, SYPI22, and At1g41750 are provided in Supplemental Table S1.

**Cloning and Site-Specific Mutagenesis of CBP60a**

To create an epitope-tagged genomic clone of *CBP60a* for the complementation experiment shown in Figure 1B, the genomic sequence of At5g62570, including introns and 2.986 bp upstream from the translation start codon, was amplified by PCR using KOD Hot Start DNA polymerase (Novagen) and TA cloned into vector pCR3 according to the manufacturer’s instructions (Invitrogen). This clone was then integrated into the Gateway-compatible binary vector pBG301 (Earley et al., 2006) using the LR reaction, thereby adding a C-terminal HA tag. To test for CaM binding, the coding sequence of *CBP60a*, without introns, was amplified from complementary DNA, cloned into pCR8, and recombined into the Gateway destination vector pDEST15 (Invitrogen), creating a N-terminal fusion to GST. To create the D1 to D6 constructs used in Supplemental Figure S2, portions of *CBP60a* were PCR amplified, cloned into pCR8, and recombined into Gateway destination vector pDEST17, creating N-terminal fusions to a His epitope tag. To create subconstructs D6_1 and D6_2 shown in Figure 5, fragments were amplified by PCR and cloned into pDEST15 as described above. To define the CaM-binding domain within fragment D6_6, the desired sequence changes were created in primers used to amplify linear versions of D6_6 in pDEST15, which were then ligated with T4 DNA ligase, generating plasmids with the desired sequence changes. To create point mutants in the context of the full-length protein, for use in the complementation experiments shown in Figure 6, the genomic *CBP60a* construct in pCR8 was amplified using primers containing the desired mutations, as described above. The point mutants were recombined into the Gateway binary vector pEG303 (Earley et al., 2006), thereby adding a Myc tag at the C terminus. DNA sequences of all cloned PCR products were verified by sequencing. For transient expression in *Nicotiana benthamiana*, wild-type and mutant constructs in pCR8 were recombined into pFC203 (Earley et al., 2006), thereby adding the 35S promoter and an N-terminal HA tag. These plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101(pMP90), which was infiltrated into *N. benthamiana*. After 2 d, tissue was collected and used for immunoblot analysis.

**Arabidopsis Transformation and Determination of Transgene Copy Number**

*A. thaliana* strain GV3101(pMP90) was used to transform Arabidopsis by dip inoculation as described (Clough and Bent, 1998). For estimation of transgene copy number, three to four leaves from each 4-week-old primary transformant were collected and homogenized in liquid nitrogen using a mortar and pestle in 0.5 mL of extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, and 1.3% SDS). Samples were incubated at 65°C for 10 min, and DNA was precipitated by adding 130 μL of 5 μL potassium acetate. Samples were then treated with 10 μg mL−1 RNase, ethanol precipitated, and washed, and DNA concentrations were determined. The copy number of the BAR transgene relative to that of the single-copy gene, PEN1, was determined by quantitative PCR experiments as described (Bubner and Baldwin, 2004) using SYBR Green PCR Master Mix (Invitrogen) following the manufacturer’s protocol. Experimental readouts were obtained using a LightCycler 480 (Roche). Primers used in these experiments are listed in Supplemental Table S1. Estimated transgene copy numbers for all transgenic lines described here are listed in Supplemental Table S2.

**Statistical Analysis**

For all experiments yielding quantitative data, experiments were repeated multiple times. Generally, one plant of each genotype studied was included in each pot assay, with eight pots to each plate. Data from multiple experiments were combined using mixed-effect linear models including genotype and treatment as fixed effects and experiment, flat, and pot as random effects. Factors that did not significantly contribute to a particular model, as determined by Akaike’s Information Criterion, were excluded. When appropriate, a multiple testing correction was used, utilizing the procedure of Benjamini and Hochberg (1995) to control the false discovery rate. In these cases, the corrected significance, rather than uncorrected p values, were used. All statistical analyses were carried out in the R programming environment using the lme4 package (Bates and Maechler, 2010; R Development Core Team, 2013).

Web addresses for bioinformatics tools are as follows: Calmodulin Target database, http://calcium.ubnres.utoronto.ca/cdbf/cdbf/home.html;
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of CBP60a genes in various genetic backgrounds.

Supplemental Figure S2. Flg22-induced PTI in cbp60 mutants.

Supplemental Figure S3. Levels of free SA and SAG in various cbp60 genotypes 24 h following mock inoculation or inoculation with Pma1 ES426.

Supplemental Figure S4. Delineation of the CaM-binding domain of CBP60a to the C-terminal 120 aa.

Supplemental Table S1. Primers used for PCR.

Supplemental Table S2. Copy numbers of transgenes in transgenic lines.

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LITERATURE CITED


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