Expression and Functional Roles of the Pepper Pathogen–Induced bZIP Transcription Factor CabZIP2 in Enhanced Disease Resistance to Bacterial Pathogen Infection

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Submitted 9 October 2015. Accepted 10 February 2015.

A pepper bZIP transcription factor gene, *CabZIP2*, was isolated from pepper leaves infected with a virulent strain of *Xanthomonas campestris* pv. *vesicatoria*. Transient expression analysis of the CabZIP2-GFP fusion protein in *Nicotiana benthamiana* revealed that the CabZIP2 protein is localized in the cytoplasm as well as the nucleus. The acidic domain in the N-terminal region of *CabZIP2* that is fused to the GAL4 DNA-binding domain is required to activate the transcription of reporter genes in yeast. Transcription of *CabZIP2* is induced in pepper plants inoculated with virulent or avirulent strains of *X. campestris* pv. *vesicatoria*. The *CabZIP2* gene is also induced by defense-related hormones such as salicylic acid, methyl jasmonate, and ethylene. To elucidate the in vivo function of the *CabZIP2* gene in plant defense, virus-induced gene silencing in pepper and overexpression in *Arabidopsis* were used. *CabZIP2*-silenced pepper plants were susceptible to infection by the virulent strain of *X. campestris* pv. *vesicatoria*, which was accompanied by reduced expression of defense-related genes such as *CaBPR1* and *CaAMP1*. *CabZIP2* overexpression in transgenic *Arabidopsis* plants conferred enhanced resistance to *Pseudomonas syringae* pv. *tomato* DC3000. Together, these results suggest that *CabZIP2* is involved in bacterial disease resistance.

As plants are sessile organisms, they are constantly contiguous to unfavorable environmental conditions, such as drought, high salinity, and pathogens. Plants defend themselves against various pathogens, including bacteria, fungi, viruses, and nematodes. The activation of defense mechanisms is initiated by the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (Jones and Dangl 2006). Pathogen recognition by plants activates signal transduction pathways that

may involve efflux of ions, generation of reactive oxygen species, and induction of defense-related genes, ultimately leading to resistance to pathogens. Although defense mechanisms in response to plant-microbe interactions have been widely studied, the mechanisms underlying the functional modifications caused by pathogen infection are unclear. Plants have evolved diverse defense mechanisms to overcome attacks from pathogens. One of these defense mechanisms is to generate defense-related hormones and antimicrobial proteins by increased expression of defense-related genes (Blée 2002; Chen et al. 2009; Lee et al. 2008). The plant defense response to pathogens is regulated by multiple signal transduction pathways, with significant overlap between gene expression patterns induced against different pathogens (Jones and Dangl 2006; Lee et al. 2006b, 2008).

In the defense-activated signal transduction pathways, genes are regulated by the interaction of *cis*-acting elements in their promoters with multiple transcriptionally or posttranslationally activated transcription factors (Llorca et al. 2014; Thatcher et al. 2012). Transcription factors have specific characteristics, such as nuclear localization, DNA-binding to promoter regions, transcriptional activation or repression, and interaction with other transcription factors or cofactors, which are necessary to regulate target gene expression (Eulgem et al. 2000; Jakoby et al. 2002; Llorca et al. 2014). These transcription factors are then involved in adaptation to unfavorable environmental conditions, such as pathogen infection or osmotic stress (Froidure et al. 2010; Fu and Dong 2013; Lee et al. 2006a). Several transcription factor families, each containing a distinct type of *cis*-acting element, such as WRKY, ERF, RAV, bZIP, or MYB, have been implicated in plant defense responses, mainly because knock-out or overexpressing mutants exhibited resistant or susceptible phenotypes to different stresses (Kesarwani et al. 2007; Lee et al. 2006a; Li et al. 2014; Sohn et al. 2006; Zhu et al. 2014).

bZIP proteins, one of the largest families of transcription factors in plants, are characterized by a basic region, which is necessary for DNA binding, and a leucine zipper motif, which is responsible for dimerization (Corrêa et al. 2008; Pogenberg et al. 2014). bZIP genes have been reported in all eukaryotes, including *Saccharomyces cerevisiae*, *Homo sapiens*, and *Arabidopsis thaliana* (17, 56, and 75 bZIP proteins, respectively). bZIP is required for homo- or heterodimerization of the protein prior to binding to the promoter region of genes (Baloglu et al. 2014; Corrêa et al. 2008; Llorca et al. 2014; Wang et al. 2011). The potential interactions of each bZIP factor in relation to homo- or heterodimerization are determined by the charge distribution and amino acid position of the zipper region (Llorca

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Nucleotide and amino acid sequences data reported here have been deposited in the GenBank database under accession numbers KC795276 and AHI85726, respectively.

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*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures and one supplementary table are published online.

et al. 2014; Pogenberg et al. 2014). Genetic and molecular studies of bZIP factors in plants have shown that they regulate diverse biological processes, such as seed maturation, senescence, and defense responses to biotic and abiotic stresses (Alves et al. 2013; Jakoby et al. 2002; Zhang et al. 2003). The involvement of bZIP factors in the plant defense response to pathogens is well established (Blanco and Judelson 2005; Lee et al. 2006a; Pitzschke et al. 2009). A common bZIP protein that is linked to defense responses is comprised of the TGA protein, which binds to the *activation sequence-1 (as-1)* element found in pathogenesis-related (PR) genes, including the systemically acquired resistance (SAR) marker gene *PR1* (Després et al. 2003; Fu and Dong 2013; Kim and Delaney 2002; Pitzschke et al. 2009; Zhang et al. 2003).

In a previous study, we isolated and characterized bZIP transcription factor *CabZIP1* (Lee et al. 2006a). We reported that transcripts of *CabZIP1* were accumulated in pepper leaf tissues infected by bacterial pathogens and that *CabZIP1* was involved in plant development. Furthermore, overexpression of *CabZIP1* in *Arabidopsis* enhanced resistance to pathogens and the plants exhibited increased tolerance to abiotic stresses, including drought and high salinity. In this study, we identified and functionally characterized a pepper bZIP transcription factor, *CabZIP2*, which was isolated from a pepper leaf cDNA library using pathogen-inducible cDNA as a probe. To investigate the functional role of the *CabZIP2* protein as a *trans*-acting factor, GAL4 fusion proteins were analyzed in yeast. Using *CabZIP2*-silenced pepper plants and *CabZIP2* overexpressing transgenic *Arabidopsis* plants, we evaluated the *in vivo* functions of *CabZIP2* based on the expression profile of the pepper *CabZIP2* gene in response to bacterial pathogens and abiotic elicitors. Our data suggest that *CabZIP2* is involved in the expression of defense-related genes during pathogen infection. These findings suggest that the *CabZIP2* transcription factor acts as a positive regulator of defense signaling.

RESULTS AND DISCUSSION

Isolation and sequence analysis of the *CabZIP2* gene.

To isolate novel pathogen-induced pepper genes, we performed differential hybridization screening of a cDNA library constructed using leaves infected with an avirulent strain, Bv5-4a, of *X. campestris* pv. *vesicatoria*, and the putative pepper pathogen-induced candidate gene was isolated (Jung and Hwang 2000). We designated this gene *CabZIP2* (*Capsicum annuum* bZIP transcription factor 2), based on sequencing and amino acid alignment (Fig. 1). The *CabZIP2* clone consists of 1,243 bp, and the deduced amino acid sequence encodes a protein of 344 amino acids containing a nuclear localization signal (Fig. 1). Sequence analysis revealed that *CabZIP2* contains an N-terminal region that includes an acidic domain, a bZIP region, and a C-terminal region. The bZIP domain, composed of 59 amino acids and present in the central region of *CabZIP2*, was extensively characterized in various plant proteins. Amino acid sequence alignments and comparison of *CabZIP2* with six bZIP transcription factor members in different plant species revealed that these bZIP proteins have similar leucine zipper structural signatures (Fig. 1). The predicted *CabZIP2* (accession number AH185726) shares 86.3% identity with the potato VIP1-like protein (accession number XP_006362824), 85.2% identity with the tobacco RSG protein (accession number BAA97100) and tomato VIP1-like protein (accession number XP_004237777), 60.2% identity with the pepper bZIP transcriptional activator (accession number ACB30357), 53.53% identity with the *Arabidopsis* VIP1 protein (accession number NP_001237194), and 52.3% identity with the soybean bZIP131 protein (accession number NP_001237194). These sequence differences were reflected

in a phylogenetic tree, and *CabZIP2* was grouped in the same clade with six VIP1-like proteins containing *CabZIP* (accession number ACB30357; 73% identity and 85% similarity) (Supplementary Fig. S1). In particular, *CabZIP1* (accession number AAX20030; Lee et al. 2006a), isolated in our previous study, shares 19.4% identity with *CabZIP2* and was located in a different clade, indicating *CabZIP2* is a novel gene isolated from pepper.

Subcellular localization of *CabZIP2*.

To determine the subcellular localization of the *CabZIP2* protein, we fused the green fluorescent protein (GFP) sequence to *CabZIP2* cDNA under the control of the 35S promoter (Fig. 2). As shown in Figure 2, expression of the 35S:*CabZIP2*-GFP fusion protein in *N. benthamiana* epidermal cells generated GFP signals in the nucleus and cytoplasm, coincident with 4',6-diamidino-2-phenylindole staining, indicating that *CabZIP2* is a cytosolic- as well as a nuclear-localized protein. Figure 1 shows that *CabZIP2* has high homology to VIP1-like proteins in other plant species. Djamei et al. (2007) suggested that VIR1 is relocated from the cytoplasm to the nucleus by flg22, a bacterial PAMP. To determine whether the localization of *CabZIP2* was affected by bacterial PAMP, *N. benthamiana* leaves expressing 35S:*CabZIP2*-GFP were treated with flg22, while localization of *CabZIP2* was not changed (Supplementary Fig. S2). A fluorescent signal was detected in the nucleus and cytoplasm, indicating that PAMP did not change *CabZIP2* localization. Although the localization of *CabZIP2* protein was not affected by this PAMP, it is possible that the levels of *CabZIP2* proteins present are sufficient for function in the nucleus.

Transactivation assay of *CabZIP2* in yeast.

Transcription factors have an activation domain that is enriched in either proline, glutamine, or acidic amino acids. However, not all activation domains fit into these categories (Cantin et al. 2003; Jakoby et al. 2002; Schwechheimer et al. 1998a and b). To determine whether the *CabZIP2* protein could function as a *trans*-activator in yeast, a series of deletion constructs of the *CabZIP2* gene were fused to the vector for the yeast GAL4 DNA binding domain (GAL4DB) to be expressed under the control of the *Saccharomyces cerevisiae* *ADHI* promoter (Fig. 3A). The fusion proteins were expressed in *Saccharomyces cerevisiae* AH109, which carries integrated copies of nutritional reporter genes (*ADE1* and *HIS3*) under the control of GAL4-responsive promoters (bait:full length [BF]: 1 to 344, bait:N-terminal domain [BN]: 1 to 130, bait:bZIP domain [BZ]: 131 to 260, and bait:C-terminal domain [BC]: 261 to 334). Transcription activation of these constructs was confirmed by growing yeast cells in selection medium (SD [−Ade, −His, −Leu, −Trp]). *CabZIP2* contains an acidic domain in its N-terminal region, similar to that in other bZIP transcription factors. Acidic transactivation domains have universal functions in eukaryotes (Hahn 1993; Schwechheimer et al. 1998b). Yeast cells carrying the constructs containing BF or BN grew well on the selection medium (Fig. 3A), indicating that expression of the *ADE1* and *HIS3* reporter genes was activated by the N-terminal domain of *CabZIP2*. The N-terminal region including the acidic domain of *CabZIP2* functions as a transactivation domain in yeast. In contrast, the constructs expressing the bZIP region (BZ: 131 to 260) and the C-terminal region (amino acids 261 to 334) did not cause yeast growth in the selection medium. These results suggest that the N-terminal region including the acidic domain of *CabZIP2* is responsible for its ability as a potential transcriptional activator in yeast.

Several studies have reported that bZIP transcription factors have dimerization preferences from different species, including *Homo sapiens*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* (Deppmann et al. 2006). Unlike most other bZIP transcription factors, subfamily I members are predicted to form homodimers rather than heterodimers. Since *CabZIP2* belongs

to subfamily I of bZIP proteins, its homodimerization was verified by a bimolecular fluorescence complementation (BiFC) assay. As shown in Figure 3B, An *Agrobacterium*-mediated transient expression assay in *N. benthamiana* leaves was performed to confirm the homodimerization of CabZIP2. Coexpression of *CabZIP2*:SPYNE with *CabZIP2*:SPYCE resulted in yellow fluorescence only in the cytoplasm and nucleus. This result indicates that CabZIP2 presumably functions as a homodimer. Protein dimerization of bZIP pairs in this family of transcription factors is mediated by the leucine zipper motif, which is required for the recognition of *cis*-acting elements (Deppmann et al. 2006; Llorca et al. 2014). In contrast, some bZIP transcription factors lose their DNA-binding activity when they form heterodimers with certain partners (Kang et al. 2010).

Induction of *CabZIP2* gene expression in pepper leaves by bacterial pathogen infection and abiotic elicitors.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to examine whether *CabZIP2* is

constitutively expressed in pepper tissues, such as leaf, stem, root, and flower (Supplementary Fig. S3). *CabZIP2* was found to be constitutively expressed in all these tissues and the expression levels of *CabZIP2* were relatively high especially in the stem and root tissues. To determine whether *CabZIP2* is induced by compatible and incompatible interactions of pepper with *X. campestris* pv. *vesicatoria*, the expression levels of *CabZIP2* were analyzed in pepper leaves inoculated with virulent and avirulent strains of *X. campestris* pv. *vesicatoria* (Fig. 4A). Pepper leaves inoculated with virulent strain Ds1 did not show any visible symptoms 24 h after inoculation, whereas chlorotic and necrotic symptoms were visible 6 days after inoculation. In contrast, pepper leaves infected with avirulent strain Bv5-4a exhibited a hypersensitive response 18 h after inoculation (Lee and Hwang 1996). This different disease response results in the differential induction of *CabZIP2*. In the compatible interaction, *CabZIP2* transcripts were first detected 2 h after inoculation, with maximal levels at 6 h. In the incompatible interaction, the *CabZIP2* transcripts were more rapidly and strongly accumulated

Fig. 1. Alignment of amino acid sequences of pepper *CabZIP2* cDNA. Comparison of the deduced amino acid sequences of the pepper CabZIP2 protein with the bZIP transcription factor from *Solanum tuberosum* (accession number XP_006362824), *Nicotiana tabacum* (BAA97100), *Solanum lycopersicum* (XP_004237777), *Capsicum annuum* (ACB30357), *Arabidopsis thaliana* (NP_001237194), and *Glycine max* (NP_001237194) proteins. White letters on black indicate identical amino acid residues. The acidic domain (AD), nuclear localization signal (NLS), and bZIP domain are represented by black boxes. Gaps introduced to maximize the alignment of homologous regions are marked by dashes.

compared with that observed during the compatible interaction. The *CabZIP2* gene was detectable 0.5 h after inoculation, followed by a slight and gradual reduction of expression. The defense signals generated during the earlier stages of the plant-

Fig. 2. Subcellular localization of the CabZIP2 protein using transient expression of the 35S:*CabZIP2-GFP* construct in *Nicotiana benthamiana*. Transient expression of 35S:*CabZIP2-GFP* in *N. benthamiana* was examined 48 h after transformation, under a Zeiss LSM 700 confocal laser-scanning microscope. 4',6-Diamidino-2-phenylindole staining indicates the positions of nuclei in cells. The scale bar represents 10 μ m.

microbe interactions are required for an effective defense response against the pathogen and may be integrated into one of the terminal pathways that leads to the transcriptional activation of PR genes (Lippok et al. 2007; Boyle and Després 2010). In the compatible and incompatible interactions, strong expression of *CabZIP2* occurred at 0.5 and 2 h after inoculation, respectively. In our earlier studies, transcription of pepper PR genes, including *CaBPR1* and *CaAMP1*, was induced by bacterial pathogens (Hong et al. 2005; Lee et al. 2008); therefore, we compared expression patterns of these genes with *CabZIP2* (Supplementary Fig. S4). Expressions of *CaBPR1* and *CaAMP1* occurred after the induction of *CabZIP2*. Furthermore, *CabZIP2* transcripts were induced more rapidly and strongly during incompatible interactions as compared with that in compatible interactions, which suggests that the *CabZIP2* gene may be involved in the defense response.

The plant hormones salicylic acid (SA), ethylene (ET), and methyl jasmonate (MeJA) are already known as defense-related hormones that may accumulate during pathogen infection and are involved in the signal transduction pathway (Alazem and Lin 2014). To assess the effect of these hormones on *CabZIP2* expression, we applied SA, ET, or MeJA to pepper leaves. SA has been reported to be a key component in the signal transduction pathway leading to plant resistance to biotrophic pathogens (Lazebnik et al. 2014). Treatment with SA strongly upregulated *CabZIP2* within 0.5 to 12 h after treatment (Fig. 5B). Following ET treatment, *CabZIP2* transcripts were first detected after 0.5 h and increased rapidly between 6 and 12 h after treatment (Fig. 5C). In MeJA-treated pepper leaves, *CabZIP2* transcripts started to accumulate 0.5 h after treatment

Fig. 3. Functional analysis of the CabZIP2 protein. **A**, Transactivation of GAL4-responsive transcription by the full-length and truncated forms of the *CabZIP2* gene, using a GAL4 yeast system. Derivatives of yeast strain AH 109, harboring plasmids that encode GAL4-*CabZIP2*, were grown on SD medium (–Leu, –Trp; right panel) lacking adenine and histidine (–Ade, –His, –Leu, –Trp; left panel). Plates were incubated at 28°C for 5 days. BD = binding domain; AD = activation domain. **B**, Analysis of homodimerization of CabZIP2 proteins by the bimolecular fluorescence complementation assay. *CabZIP2*-35S-SPYNE(R)173 coexpressed with *CaBZIP2*-35S-SPYCE(M) in the leaves of *Nicotiana benthamiana* by agroinfiltration. The fluorescence indicates interaction between CabZIP2 proteins. Images were obtained from the yellow fluorescent protein channel, bright field, or a merged picture of the two. White bar = 10 μ m.

and reached peak levels at 2 to 12 h (Fig. 5D). These defense-related hormones are known to trigger disease resistance in plants (Adie et al. 2007; Clarke et al. 2000; Ferrari et al. 2003). The expression of *CabZIP2* was induced more rapidly and extensively in the incompatible interactions and following treatment with defense hormones, which suggests that the *CabZIP2* gene may be involved in the defense response.

Enhanced susceptibility of *CabZIP2*-silenced pepper plants to *X. campestris* pv. *vesicatoria* infection.

Since the experiments described above show that *CabZIP2* gene expression in pepper leaves is activated by *X. campestris* pv. *vesicatoria* infection, and abiotic elicitor treatment to trigger defense signaling (Fig. 4), we examined the role of *CabZIP2* in the defense response of pepper plants to bacterial pathogens, using the virus-induced gene silencing (VIGS) technique. The generation of stable transgenic pepper plants by overexpressing or knock-out genes of interest is very difficult, and VIGS analysis provided a fast and effective means to knock-down phenotypes and allowed us to elucidate the function of interesting genes in pepper plants (Lee et al. 2010; Lim and Lee 2014). Pepper seedlings were inoculated with recombinant *Tobacco rattle virus* (TRV) silencing constructs. Phenotypic differences were not observed in plants containing an empty-vector control (TRV:00) or in *CabZIP2*-silenced (TRV:*CabZIP2*) pepper plants during growth and development (data not shown). The efficiency of VIGS was analyzed by quantitative (q)RT-PCR using *CabZIP2* empty-vector control and *CabZIP2*-silenced pepper leaves 12 h after inoculation with virulent (Ds1) and avirulent (Bv5-4a) strains of *X. campestris* pv. *vesicatoria* (Fig. 5A). *CabZIP2* expression was compromised in *CabZIP2*-silenced pepper leaves with or without inoculation of *X. campestris* pv.

vesicatoria. Since *CabZIP2* functions as a transcriptional activator (Fig. 3) and the VIP1 transcription factor regulates the expression of *PR1* (Djamei et al. 2007), we examined by RT-PCR whether *CabZIP2* silencing affects the induction of defense-related genes (Fig. 5A). The expression levels of *CaBPR1* (Hong et al. 2005) and *CaAMP1* (Lee et al. 2008) were remarkably reduced in *CabZIP2*-silenced pepper leaves as compared with empty-vector control plants.

To confirm whether the expression of defense-related genes, including *CabZIP2*, reflected bacterial proliferation within the plants, bacterial growth was measured in empty-vector control and *CabZIP2*-silenced pepper at 0, 3, or 5 days postinoculation (dpi) (Fig. 5B and C). In the compatible interaction, silencing of *CabZIP2* not only compromised the expression of some defense-related genes, it also increased susceptibility of plants to infection by the virulent strain of *X. campestris* pv. *vesicatoria* (Fig. 5B). *CabZIP2*-silenced plants exhibited significantly higher bacterial proliferation than control plants at 3 or 5 dpi. Consistent with the induction of *CabZIP2* expression by infection of *X. campestris* pv. *vesicatoria* and treatment of abiotic elicitors, these findings strongly support the notion that *CabZIP2* may be required for the resistance of pepper plants to virulent bacterial pathogens. In the incompatible interaction, bacterial proliferation was not distinguished in *CabZIP2*-silenced pepper leaves compared with empty-vector control pepper leaves (Fig. 5C). Although *CabZIP2* expression was rapidly and strongly induced in pepper leaves by avirulent *X. campestris* pv. *vesicatoria* infection, *CabZIP2* silencing did not significantly alter the susceptibility of pepper plants to avirulent *X. campestris* pv. *vesicatoria* infection, indicating that *CabZIP2* plays a key role in basal resistance rather than in resistance gene-mediated resistance. Together, these results indicate that *CabZIP2* affects the expression of other

Fig. 4. Relative expression of the *CabZIP2* gene in pepper plants. **A**, Expression of *CabZIP2* in pepper leaves at various time points after inoculation with the virulent strain Ds1 and the avirulent strain Bv5-4a of *Xanthomonas campestris* pv. *vesicatoria* at the four-leaf stage. **B** to **D**, Expression of *CabZIP2* in pepper leaves at various time points after treatment with salicylic acid (**B**), ethylene (**C**), and methyl jasmonate (**D**). Relative expression levels ($\Delta\Delta CT$) of the *CabZIP2* gene were normalized to the geometric mean of *18s rRNA*, *Actin1*, and *EF1a* expression as internal control genes and were compared with each value for mock-treated leaves. Data are expressed as the mean $\pm$ standard error from three independent experiments.

defense-related genes and plays a crucial role in the defense response of pepper plants to bacterial pathogens.

Enhanced resistance of *CabZIP2*-OX (overexpressing) transgenic plants to bacterial pathogen.

To further investigate the effect of *CabZIP2* in the defense response in vivo, we generated *CabZIP2* *Arabidopsis* transgenic plants that overexpress the entire precursor under the control of a strong constitutive 35S promoter (Fig. 6). To verify *CabZIP2*-OX transgene expression in independent homozygous T₃ lines, we performed RT-PCR analysis using genomic mRNA from the wild-type plants and from the *CabZIP2*-OX plants. PCR products were not detected for the wild-type plants, while the *CabZIP2* gene was amplified from three *CabZIP2*-OX plants, which were used for further phenotypic analysis (Fig. 6A). The phenotype of *CabZIP2*-OX plants was indistinguishable from wild-type plants under normal growth conditions (data not shown). To examine whether overexpression of *CabZIP2* was able to enhance disease resistance, we inoculated both the wild-type and *CabZIP2*-OX plants with *P. syringae* pv. *tomato* DC3000, which causes symptoms similar to bacterial speck disease of tomato and *Arabidopsis* (Whalen et al. 1991). Seven days after inoculation, wild-type plants exhibited typical chlorotic symptoms on inoculated leaves, whereas symptoms were not visible on any leaves of the *CabZIP2*-OX lines (Fig. 6B). To confirm that these macroscopic disease symptoms reflect bacterial proliferation in the plants, bacterial growth in the leaf tissues was examined at 0, 1, 3, and 5 dpi (Fig. 6C). The bacterial growth was not any

different in wild-type and *CabZIP2*-OX plants at 0 or 1 dpi. The *CabZIP2*-OX plants showed a 15- to 30-fold reduction in bacterial growth at 3 dpi. At 5 dpi, the bacterial population in wild-type plants was only slightly reduced and the *CabZIP2*-OX plants showed 18- to 20-fold less bacterial growth of *P. syringae* pv. *tomato* DC3000, as compared with that in the wild-type plants. These results indicate that overexpression of *CabZIP2* confers enhanced resistance to bacterial pathogens. Enhanced resistance of the *CabZIP2* transgenic lines to the virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000 presumably arises from the overexpression of the pepper *CabZIP2* gene. In our previous study, *CabZIP1*-OX plants also exhibited a phenotype that was resistant to *P. syringae* pv. *tomato* DC3000. However, *CabZIP1* is a different bZIP transcription factor from *CabZIP2* (Lee et al. 2006a).

To investigate whether this enhanced resistance to the bacterial pathogen in *CabZIP2*-OX plants is related to SA-dependent PR gene expression, we compared the expression levels of *PR1* and *NPR1* in wild-type and *CabZIP2*-OX plants (Fig. 7). The transcripts of these genes were more-accumulated in leaves of *CabZIP2*-OX than in leaves of wild-type plants. However, the expression levels of *PDF1.2* and *LOX* genes, which are related to JA, were not significantly different in wild-type and *CabZIP2*-OX plants. These results indicate that a SA-dependent defense mechanism may contribute to *CabZIP2*-OX enhanced disease resistance to bacterial pathogen infection.

In conclusion, we have shown that the *CabZIP2* protein localizes to both the cytoplasm and the nucleus, in which it acts as a transcriptional activator. Silencing of *CabZIP2* led to an

Fig. 5. Enhanced susceptibility of *CabZIP2* gene-silenced pepper plants to *Xanthomonas campestris* pv. *vesicatoria* infection. **A**, Expression of *CabZIP2* and pepper defense-related genes in empty-vector control (TRV:00) and *CabZIP2* gene-silenced (TRV: *CabZIP2*) pepper plants 12 h after inoculation with the *X. campestris* pv. *vesicatoria* virulent strain Ds1 and the avirulent strain Bv5-4a. *18S rRNA* served as a control. H = untreated healthy leaves, V = virulent strain Ds1, A = avirulent strain Bv5-4a, *CaAMP1* = pepper antimicrobial protein 1, and *CaBPR1* = pepper basic PR1. Relative expression levels ($\Delta\Delta CT$) of the *CabZIP2* gene were normalized to the geometric mean of *18S rRNA*, *Actin1*, and *EF1 α* expression as internal control genes and were compared with the values for nontreated leaves. Data are expressed as the mean $\pm$ standard error from three independent experiments. **B** and **C**, Bacterial growth in empty-vector control and *CabZIP2* gene-silenced pepper leaves at 0, 3, and 5 days after inoculation with the *X. campestris* pv. *vesicatoria* virulent strain Ds1 (**B**) and avirulent strain Bv5-4a (10^4 CFU ml⁻¹) (**C**). Experiments were repeated three times with similar results. Data are expressed as the mean $\pm$ standard deviation from three independent experiments. Asterisks indicate significant difference as determined by Student's *t* test ($P < 0.05$).

enhanced susceptibility of pepper plants to the bacterial pathogen, whereas ectopic expression of the *CabZIP2* transgenic *Arabidopsis* plants induces enhanced resistance to *P. syringae* pv. *tomato* DC3000. Together, these results suggest that *CabZIP2* may function as a transcriptional activator of the defense response against bacterial pathogens in pepper plants. However, the mechanisms involved in the activation of the defense response by *CabZIP2* remain unclear. Furthermore, stable transgenic pepper plants, in which *CabZIP2* is knocked-out, are required to better understand the function of this gene.

MATERIALS AND METHODS

Plant material and growth conditions.

Pepper (*Capsicum annuum* L. cv. Nockwang), *Arabidopsis thaliana* (ecotype Col-0), and *Nicotiana benthamiana* plants were grown at a 9:1:1 ratio of peat moss, perlite, and vermiculite under fluorescent light ($130 \text{ mol photons m}^{-2} \text{ s}^{-1}$) at 24°C with 60% humidity and a 16-h light and 8-h dark cycle. *Arabidopsis thaliana* seeds were surface-sterilized with 70% ethanol for 1 min prior to in vitro culture. After the seeds were treated with 2% sodium hydroxide for 10 min, they were

washed 10 times in sterile distilled water and were sown on Murashige and Skoog (MS) (Murashige and Skoog 1962) agar supplemented with 1% sucrose for 2 weeks at 24°C in a growth chamber exposed to a 16-h light and 8-h dark cycle.

Pathogen inoculation.

Strains of *Xanthomonas campestris* pv. *vesicatoria* that are virulent (Ds1) and avirulent (Bv5-4a) to pepper were used in this study. To prepare bacterial suspensions for inoculation of pepper leaves, bacteria were cultured for 16 h in yeast-nutrient broth (5 g of yeast extract, 8 g of nutrient broth, and 1 liter of H_2O) at 28°C . Bacterial suspensions were adjusted to a concentration of 10^8 CFU ml^{-1} with sterile tap water prior to inoculation. Pepper plants at the six-leaf stage were inoculated by infiltrating bacterial suspensions into the abaxial side of fully expanded leaves. The control healthy plants also were mock-inoculated by infiltrating sterile tap water into the leaves. The mock-inoculated and bacteria-inoculated pepper plants were incubated in a growth room as described previously (Lee and Hwang 2009).

The virulent pathogen *P. syringae* pv. *tomato* DC3000 was used in this study. To prepare bacterial suspensions for inoculation on *Arabidopsis* leaves, bacterial strains were cultured overnight in King's B medium broth at 28°C . Leaves of 5-week-old plants were syringe-inoculated with the bacterial suspension of *P. syringae* pv. *tomato* DC3000 ($1 \times 10^5 \text{ CFU ml}^{-1}$) in 10 mM MgCl_2 . After inoculation, *Arabidopsis* plants were maintained under 100% humidity for 24 h and were then raised in a growth chamber under the same conditions. The density of the bacterial population was determined by plating serial dilutions on King's B medium supplemented with kanamycin (50 g ml^{-1}) and rifampicin (50 g ml^{-1}) at 28°C . Data are means $\pm$ standard deviations of the log (CFU cm^{-2}) of three replicates. Bacterial growth in the infected leaves of each plant was determined according to the method of Katagiri et al. (2002).

Fig. 6. Enhanced resistance of *CabZIP2*-overexpressing (OX) transgenic *Arabidopsis* plants to *Pseudomonas syringae* pv. *tomato* DC3000 infection. **A**, Reverse transcription-polymerase chain reaction analysis of *CabZIP2* expression in wild type and the *CabZIP2*-OX transgenic lines. *Actin8* was used as internal control gene. **B**, Disease symptoms developed on the leaves of wild-type or transgenic plants 7 days after infiltration with *P. syringae* pv. *tomato* DC3000 (10^5 CFU ml^{-1}). **C**, Bacterial growth in the leaves of wild-type and transgenic plants at different time points after infiltration with *P. syringae* pv. *tomato* DC3000 (10^5 CFU ml^{-1}). Values are the mean $\pm$ standard deviation from three independent experiments. Statistical analysis was performed with analysis of variance test ($P < 0.05$) and different letters indicate significant differences.

Fig. 7. Relative expression of (pathogenesis related) PR genes in leaves of wild-type and transgenic plants after infiltration with *Pseudomonas syringae* pv. *tomato* DC3000 (10^5 cell/ml). Relative expression levels ($\Delta\Delta\text{CT}$) of each gene were normalized to the geometric mean of *Actin2*, *Actin8*, and *EF1 α* as internal control genes. Values are the mean $\pm$ standard deviation from three independent experiments. Asterisks indicate significant difference as determined by Student's *t* test ($P < 0.05$).

Treatments with plant hormones.

Pepper plants at the six-leaf stage were enclosed in stoppered 500-ml glass bottles, and ethylene was injected to yield a final concentration of 5 l per liter. MeJA (100 lM) and SA (5mM) were sprayed onto pepper plants at the six-leaf stage. Pepper plants treated with MeJA were incubated in a vinyl bag. Control plants were sprayed with water. The treated pepper plants were incubated in a growth room at 27°C ± 1°C under approximately 80 lmol photons m⁻²s⁻¹ (white fluorescent lamps) for 16 h a day.

RNA isolation, RT-PCR, and real-time qRT-PCR analysis.

Total RNA was isolated from pepper leaf tissues infected with bacterial pathogen or abiotic plant hormones, using an RNeasy mini kit (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer's instructions. To remove genomic DNA, all RNA samples were treated with RNA-free DNase. After quantification using a spectrophotometer, 1 l of total RNA was used to synthesize cDNA using a Transcript First Strand cDNA synthesis kit (Roche, Indianapolis, IN, U.S.A.), according to the manufacturer's instructions. In parallel, cDNAs were synthesized without reverse transcription and were subjected to qRT-PCR to rule out the possibility of contamination by genomic DNA in the cDNA samples. The synthesized cDNA was amplified in a CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.) with iQ SYBR Green Supermix and specific primers. Every reaction was performed in triplicate. The PCR was performed as follows: 95°C for 5 min, 45 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. The relative expression value of each gene was calculated by the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). The relative levels of the target transcripts were normalized to those of *18S rRNA*, *Actin1*, and *EF1 α* , which were included as internal controls.

Subcellular localization of CabZIP2:GFP fusion protein.

The coding region of the *CabZIP2* gene without the stop codon was inserted into the GFP-fused binary vector p326GFP. *Agrobacterium tumefaciens* GV3101 carrying the construct was combined with the p19 strain (1:1 ratio; optical density at 600 nm [OD₆₀₀] = 0.5) and was coinfiltrated into fully expanded leaves of 5-week-old *N. benthamiana*. For microscopic analyses, leaf discs were cut 2 days after infiltration. Cells of the lower epidermis were analyzed by the confocal laser-scanning microscope LSM 700 (Carl Zeiss, Oberkochen, Germany) operated with LSM Image Browser software. For studying flg22-induced relocation of CabZIP2 protein, 10 lM flg22 peptide was infiltrated into leaves expressing CabZIP2-GFP at least 1 h before analysis.

Transactivation analysis of CabZIP2 in yeast.

The coding sequences for the full-length and truncated *CabZIP2* mutants were cloned into the pGBKT7 vector (Clontech, Palo Alto, CA, U.S.A.), which contains a GAL4 DNA-binding domain. Transactivation by CabZIP2 was examined in *Saccharomyces cerevisiae* AH109, in which *Ade1* and *His3* reporter genes were driven by *GAL4* promoters. Yeast cells were transformed with expression vectors that carried *GAL4-CabZIP2* fusion genes, as described in the manufacturer's instructions (Clontech, Palo Alto, CA, U.S.A.). After selection on SD (-Trp, -Leu) medium, the transformed yeast cells were transferred to SD (-Ade, -His, -Leu, -Trp) medium to confirm transcriptional activation.

VIGS.

The TRV-based VIGS system was used to knock down expression of the *CabZIP2* gene in pepper plants (Kim and Hwang 2012). To silence *CabZIP2*, a 254-bp fragment of *CabZIP2* cDNA was inserted into the pTRV2 vector. *Agrobacterium tumefaciens* GV3101 harboring pTRV1 was combined with pTRV2:00 or

pTRV2:*CabZIP2* (1:1 ratio) and was coinfiltrated into the fully expanded cotyledons of pepper plants (OD₆₀₀ = 0.2 for each construct). Plants were placed in a growth room under the same conditions as described above.

BiFC analysis.

To generate the BiFC constructs, full-length cDNAs with no stop codon of *CabZIP2* were subcloned into 35S-SPYNE(R)173 and 35S-SPYCE(M) vectors. For transient expression, *Agrobacterium tumefaciens* GV3101 strains harboring each construct were mixed with the p19 strain to avoid gene silencing and were infiltrated into the abaxial side of 5-week-old *N. benthamiana* leaves using a 1-ml needleless syringe. For microscopic analyses, leaf discs were cut 4 days after infiltration. The lower epidermis cells were analyzed by confocal microscopy (model Zeiss 510 UV/Vis Meta) operated with LSM Image Browser software.

Generation of the CabZIP2-OX transgenic plants.

The full-length cDNA sequence of *CabZIP2* was integrated into the pK2GW7 binary vector to induce constitutive expression of the *CabZIP2* gene under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter in *Arabidopsis*. The *CabZIP2* binary vectors were introduced into *Agrobacterium tumefaciens* GV3101 via electroporation. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* with the *CabZIP2* gene was carried out using the floral-dip method (Clough and Bent 1998). For selection of transgenic lines, seeds harvested from the putative transformed plants were sowed on MS agar plates containing 50 l of kanamycin per milliliter.

ACKNOWLEDGMENTS

This work was supported by a grant from the Next-Generation BioGreen 21 Program (number 01100501).

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