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Promoter analysis of the pepper antimicrobial protein gene, *CaAMP1*, during bacterial infection and abiotic stress

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Abstract In a previous study, we isolated and functionally characterized a novel antimicrobial protein gene with a high level of antimicrobial activity, *CaAMP1,* from pepper leaves infected with *Xanthomonas campestris* pv. v*esicatoria.* In this study, a series of 5′-deletions in the region −1190 bp upstream of the transcriptional start site of the *CaAMP1* gene were analyzed in tobacco to determine the activity of the *CaAMP1* promoter fused to the β-glucuronidase (GUS) reporter gene, using an *Agrobacterium*-mediated transient expression assay. The *CaAMP1* gene and promoter were locally and systemically induced by microbial infection. Several biotic and abiotic stress-related *cis*-acting elements, including GT1 box, W box, MYB, RAV1, ERE, and LTRE, were localized in the upstream promoter region of the *CaAMP1* gene. The −1190, −967 and −626 bp regions of the *CaAMP1* promoter activated by *Pseudomonas syringae* pv. *tabaci* infection, were simultaneously activated by treatments with salicylic acid and methyl jasmonate. The −1190 bp *CaAMP1* promoter was also activated by abscisic acid, NaCl, and low temperature. Expression of the pepper transcription factor, CARAV1, but not CAZFP1, contributed to the activation of the *CaAMP1* promoter. Deletion analysis of the *CaAMP1* promoter suggested that some novel *cis*-acting elements are necessary for the induction of *CaAMP1* expression during pathogen exposure and that environmental stresses may reside in the genomic sequence upstream of the *CaAMP1* gene between −626 bp and −425 bp.

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Introduction

Plants respond to unfavourable changes in their environments caused by biotic or abiotic stresses, such as pathogen infection, wounding, drought, and cold, with rapid activation of intracellular and extracellular signalling. Transcriptional control of biotic or abiotic stress-inducible genes is crucial to plant response to pathogen infection or environmental stresses.

A number of signalling molecules, including salicylic acid (SA), ethylene, jasmonic acid (JA), and abscisic acid (ABA), amplify and regulate the expression of pathogenesis-related (PR) genes (Reymond and Farmer 1998; Schenk et al. 2003; Lin et al. 2007; Asselbergh et al. 2008). SA, in particular, is crucial for the signal transduction pathway leading to systemic acquired resistance (SAR), which is accompanied by the expression of SAR genes (Ward et al. 1991). SAR is characterized by a rapid increase in oxidative burst, an increase in the level of endogenous SA, and transcriptional activation of PR genes, as well as enhanced resistance to pathogens (Ryals et al. 1996; Lee and Hwang 2005).

A large number of PR genes have been identified in plants, and their promoters have been thoroughly studied (Kenton et al. 2000; Brown et al. 2003). The *cis*-acting elements, which are localized in the gene promoter and are responsible for the activation or repression of gene expression, have been identified in many PR gene promoters (Shinshi et al. 1995; Lee and Hwang 2006; Lee et al. 2007). The promoters of pathogen-inducible genes in plants have multiple *cis*-acting elements, including W, GCC, and as-1 boxes (Hao et al. 1998; Rushton and Somssich 1998; Eulgem et al. 2000; Ohme-Takagi et al. 2000). The *cis*-acting elements are recognized by transcription factors (Rushton et al. 2002). Interactions between transcription factors and *cis*-acting elements constitute a key step in the regulation of plant gene expression in response to pathogen attack or environmental stresses. Defence-related transcription factors and *cis*-acting elements appear to be highly conserved among divergent plant species (Rushton and Somssich 1998; Rushton et al. 2002). Several transcription factors play significant roles in defence, including WRKY, ERF, bZIP, RAV, and zinc-finger protein (Rushton and Somssich 1998; Singh et al. 2002; Lee et al. 2006). WRKY plays an important role in regulating genes associated with plant defence responses (Kim and Zhang 2004; Turck et al. 2004). Many PR genes contain W-box *cis*-acting elements in their promoters. W-box sequences are specifically recognized by WRKY proteins and are necessary for the inducible expressions of these genes (Rushton et al. 2002).

Some PR genes encode anti-microbial proteins, such as glucanase, chitinase, and defensin (Broglie et al. 1991; Datta et al. 1999; Gao et al. 2000), whereas others encode proteins with regulatory functions in the defence signalling pathways. The *CaAMP1* gene encoding an antimicrobial protein has recently been demonstrated to be differentially expressed in pepper (*Capsicum annuum* L.) leaf tissues infected by *Xanthomonas campestris* pv. *vesicatoria* (Lee et al. 2008). *CaAMP1* transcripts accumulate in the pepper leaf and stem tissues during salt and drought stresses. Purified CaAMP1 protein exhibits a high level of antimicrobial activity. Transgenic approaches have been used to verify the effects of ectopic expression of genes encoding elicitors of defence responses (Keller et al. 1999) and antimicrobial proteins (DeGray et al. 2001; Li et al. 2001). Overexpression of *CaAMP1* in *Arabidopsis* confers enhanced disease resistance and environmental stress tolerance (Lee et al. 2008; Lee and Hwang 2009). These findings raise questions regarding which *CaAMP1* promoter region or regions is essential for the activation of gene expression due to pathogen infection and environmental stress treatments.

Analysis of the regulation of *CaAMP1* may provide insight into the regulation of *CaAMP1* expression under biotic and abiotic stresses. In this study, we analyzed how the pepper *CaAMP1* promoter regulates the expression and possible function of *CaAMP1*. To identify the stress-responsive regions of the *CaAMP1* promoter, several 5′-deletions of the *CaAMP1* gene promoter region were each fused to a β-glucuronidase (GUS) reporter gene for use in an *Agrobacterium*-transient assay in tobacco plants. The transient assay is used in a wide variety of plant science experiments, including protein expression (Andrews and Curtis 2005), sub-cellular localization (Koroleva et al. 2005; Earley et al. 2006), and the analysis of promoters (Baudry et al. 2004; Lee and Hwang 2006; Lee et al. 2007). The possible functions of several putative *cis*-acting elements found within the identified regulatory region of the *CaAMP1* gene are discussed.

Materials and methods

Plant materials and growth conditions

Pepper (*Capsicum annum* L.) cv. Hanbyul was used in this study. The seeds were sown in a plastic tray (55×35×15 cm) containing a steam-sterilized soil mix (peat moss, perlite, and vermiculite, 5:3:2, v/v/v), sand, and loam soil (1:1:1, v/v/v). Six pepper seedlings were transplanted at the two-leaf stage to a plastic pot (5×15×10 cm) containing the soil mix and were used for bacterial inoculation.

Tobacco plants (*Nicotiana tabacum* L., cultivar Xanthi nc) were grown in a 9:1:1 mixture of peat moss, perlite, and vermiculite. Tobacco plants were used for agroinfiltration at the six-leaf stage. Pepper and tobacco plants were raised in a growth room at 25±2°C for 16 h per day.

Pathogen inoculation

X. campestris. pv. *vesicatoria* strains Ds1 and Bv5-4a, virulent and avirulent to the pepper cultivar Hanbyul, respectively, as well as the non-pathogenic *P. syringae*

pv. *fluorescens* (ATCC 13525), were used in this study. To prepare bacterial suspensions for inoculation into pepper leaves, we cultured *X. campestris* pv. *vesicatoria* strains overnight in yeast-nutrient (YN) broth (5 g yeast extract, 8 g nutrient broth, and 1 L H_2O) at 28°C. Bacterial suspensions were adjusted to 10^8 colony-forming units (cfu) ml^{-1} with sterile tap water prior to inoculation. Pepper plants were inoculated at the two-leaf stage by infiltrating bacterial suspensions into the abaxial side of fully expanded leaves. Healthy control plants were also mock-inoculated via the infiltration of sterile tap water into the leaves. The mock-inoculated and bacteria-inoculated pepper plants were then incubated in a growth room, as described previously (Lee and Hwang 1996).

P. syringae pv. *tabaci* KACC13525 was grown overnight at 28°C in King B medium. Bacterial suspensions were adjusted to 10^8 cfu ml^{-1} with 10 mM $MgCl_2$ before inoculation. Six-week-old tobacco plants were inoculated via the infiltration of bacterial suspensions into the abaxial side of fully-expanded leaves using a needleless syringe. Mock-inoculated and bacteria-inoculated tobacco plants were maintained in a moist chamber at 28°C for 24 h and then placed in a growth room until sampled.

Treatment with abiotic elicitors and environmental stresses

To characterize the induction of promoter activity of the *CaAMP1* gene by the defence signal molecules, we treated tobacco plants with ethylene, salicylic acid (SA), methyl jasmonate (MeJA), ethylene or abscisic acid (ABA). For the SA, MeJA, and ABA treatments, the tobacco plants were sprayed with 1 mM sodium salicylate, 50 μM MeJA, and 100 μM ABA in distilled water. Tobacco plants treated with MeJA were then incubated in vinyl bags. The control plants were sprayed with water.

To determine the degree to which promoter activation occurred under different environmental stresses, tobacco plants were exposed to environmental stresses, including salinity and low temperature. For the high salinity treatment, the tobacco plants were removed from the soil, and their roots were soaked in 200 mM NaCl. To induce low-temperature stress, the plants were transferred to an incubator at 4°C. The treated pepper plants were then incubated in a growth room at 25±1°C for 16 h per day.

RNA gel blot analysis

Total RNA was prepared from pepper leaves, according to the guanidine isothiocyanate method (Chomczynski and Sacchi 1987). Isolated RNA was stored in diethyl pyrocarbonate (DEPC)-treated sterile water at −70°C until used. Twenty micrograms of RNA were denatured, separated onto 1.2% agarose-formaldehyde gels, and transferred onto nylon membranes (Hybond N^+, Amersham: Little Chalfont, UK), according to the manufacturer's recommendations. The blotted RNA was then cross-linked to the membranes via UV illumination. ^{32}P-labeled gene probes were constructed with a random prime kit (Boehringer Mannheim: Mannheim, Germany). Pre-hybridization and hybridization were performed at 65°C in 5% (w/v) dextran sulfate, 0.25 M disodium phosphate, 7% (w/v) sodium dodecyl sulfate (SDS), and 1 mM EDTA, with a pH of 7.2. The membranes were washed twice with 2X SSC and 0.1% SDS for 10 min each at room temperature, followed by three washes with 0.1X SSC and 0.1% SDS for 5 min each at 65°C. The hybridized blots were then exposed to X-ray film.

Isolation of the *CaAMP1* gene promoter

Fragments of the promoter region of the *CaAMP1* gene were obtained using a Universal Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). The pepper genomic DNA was isolated from the pepper leaves according to the methods described by Lee et al. (2000). The genomic DNA was digested with *Avi*II, *Dra*I, *Eco*RV, *Pvu*II, *Stu*I, *Nru*I, *Sca*I, and *Ssp*I, all of which make blunt cuts at 6-bp sequences. Genome walker adaptors (AP1 and AP2) were then ligated to the restricted products for 18 h at 16°C. Using the genome walker library, we constructed primary and nested PCR with the *CaAMP1* gene-specific primer, and the Advantage Genomic Polymerase mixture (Clontech). The gene-specific primers were designed from the *CaAMP1* cDNA sequence. The nested PCR product was purified on a 1.5% agarose gel, and subcloned into the pCR^{R}2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The cloned vector was sequenced and analyzed using the PLACE Web Signal Scan database at the Advanced Biosciences Computing Center.

Promoter deletion-GUS constructs

The reporter constructs used for the transient expression assays in this study were prepared according to the following procedure. To construct the −1190, −967, −626, −424, and −267 to −1 *CaAMP1*::GUS plasmid, we amplified the *CaAMP1* promoter regions using the following forward primers: 5′-cccgggatttcgtgcattgtgcaggtatca-3′ (-1190), 5′-cccgggggctagtcaactgatttggaccac-3′ (-967), 5′-ggattcgg actaattgagcgcc acgtgttg-3′ (-626), 5′-ggatcccgaaggatc caata ataccatgcc-3′ (−424), and 5′-ggatccttattcttattccg attattatct-3′ (−267). The same reverse primer, 5′-aagctttttgattaac aaattaaactattt-3′, was used for all constructs. The resultant constructs were digested with *Hin*dIII-*Bam*HI, and cloned into the pBI101 vector (Clontech). All constructs were verified by sequencing. The constructs were then transferred into the *A. tumefaciens* strain, EHA105, via electroporation.

Agrobacterium-mediated transient expression assay

Agrobacterium-mediated, transient expression assays were conducted according to a modified version of the method described by Yang et al. (2000). *A. tumefaciens* strain, EHA105, containing serially-deleted promoter and GUS constructs were grown on YEP medium (10 g Bacto peptone, 10 g yeast extract, 5 g NaCl, 15 g agar/ H_20 l) supplemented with rifampicin (60 μg/ml) and kanamycin (50 μg/ml). A single *Agrobacterium* colony was inoculated into 3 ml of YEP broth, and cultured at 28°C for 2 days. Post-culturing was conducted in 50 ml of fresh YEP at 28°C overnight. *Agrobacterium* cells were harvested after centrifugation for 15 min at 3000x *g*, suspended in infiltration media [0.1x MS salts, 0.1x B5 vitamins, 20 mM MOPS, pH 5.4, 1% (w/v) glucose, 2% (w/v) sucrose, 200 μM acetosyringone], and adjusted to an OD_{600} of 0.8 for infiltration into the tobacco leaves. After infiltration of the *Agrobacterium* suspension into the tobacco leaves using a needleless syringe, the tobacco plants were maintained in a moist chamber at 26°C for 48 h. All treatments were applied 48 h after agroinfiltration into the tobacco leaves.

Transactivation assay

The coding regions for the two pepper transcription factor genes, *CAZFP1* (accession no. AF539746) and *CARAV1* (accession no. AF478458), were inserted into the plant expression vector pBIN35S to construct the effector plasmid. The plant expression vector pBIN35S has a CaMV 35 S promoter, multiple cloning sites, and a nopaline synthase terminator. Four 5′-serially deleted promoter-GUS fusion cassettes were used as reporter plasmids. The two different *Agrobacterium* EHA105 strains (each OD_{600}=0.8) containing effector and reporter plasmids, respectively, were mixed at a 1:1 ratio, and co-infiltrated into tobacco leaves. The infiltrated tobacco plants were maintained in a moist chamber at 26°C for 48 h and harvested for GUS activity analysis after incubation in a growth chamber for 24 h.

GUS activity assay

GUS activity was assessed by either fluorometric assays or histochemical staining (Jefferson 1987). For the fluorometric GUS assay, we homogenized leaf discs in 1 ml of extraction buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% (w/v) sarcosyl (w/v), 10 mM β-mercaptoethanol). The homogenate was centrifuged for 10 min at 12,000x *g* at 4°C, and the GUS activity of the supernatant was assessed according to the method described by Jefferson (1987). GUS activity was normalized to the protein concentration in each of the crude extracts and was calculated as the *p*mol of 4-methylumbelliferone (4-MU) produced per minute, per milligram of soluble protein. Protein content was assessed by the Bradford (1976) method, using bovine serum albumin (BSA) as a standard.

For histochemical staining, tobacco leaf tissues were vacuum-infiltrated with a staining solution containing 1 mg ml^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 100 mM phosphate buffer, 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA, at a pH of 7.0, and was incubated for 16 h at 37°C. After staining, the tissue sections were fixed via incubation in a solution consisting of 5% formaldehyde, 5% acetic acid, and 50% ethanol for 4 h at room temperature and cleared by sequential incubation in 60, 80, and 100% ethanol for 1 h each at room temperature.

Results

Genomic structure of the *CaAMP1* gene

We generated deletions of the −1190 bp-long region upstream of *CaAMP1* to characterize the promoter

sequences involved in *CaAMP1* transcription. The putative *cis*-acting elements in the promoter region were searched using PLACE, a database for PLAnt Cis-acting Elements (http://www.dna.affrc.go.jp). Several putative regulatory motifs that are homologous to the *cis*-acting elements required for the activation of defence-related genes in plants were identified in the promoter region of the *CaAMP1* gene (Fig. 1). They included one ERE, two GT-1, two LTRE, eight MYB binding sites, three RAV boxes, and four W-box elements. Among the identified *cis*-acting elements, there were several pathogen-responsive *cis*-acting elements, including a W-box and a RAV box (Kagaya et al. 1999; Chen et al. 2002), along with ethylene and environmental stress-responsive elements, such as ERE, LTRE, and MYB binding sites (Abe et al. 1997; Dunn et al. 1998; Finkelstein and Lynch 2000). To analyze the promoter of the *CaAMP1* gene, we fused the promoter region of the *CaAMP1* gene to the GUS reporter and analyzed GUS activity by the *Agrobacterium*-mediated transient assay in tobacco plants (Fig. 1). In an attempt to characterize the promoter sequences that regulate the transcription of *CaAMP1*, we generated deletions in the promoter region of *CaAMP1,* which was fused to the GUS coding gene (Fig. 1).

Local and systemic activation of the *CaAMP1* gene and promoter by pathogen infection

In previous studies, we revealed that the transcription of *CaAMP1* was strongly induced at the initial infection sites of pepper leaf tissues in response to *X. campestris* pv. *vesicatoria* (Lee et al. 2008). To further establish whether *CaAMP1* gene expression is systemically induced in pepper plants, we characterized the transcription patterns of the *CaAMP1* gene in both local (primary) and systemic (secondary) leaves. The characterization was performed at different time points after inoculation with either *X. campestris* pv. *vesicatoria* or non-pathogenic *P. fluorescens* into the primary pepper leaves (Fig. 2a). *CaAMP1* mRNA levels were significantly elevated in both local and systemic pepper leaf tissues, after infection of the primary leaves by virulent, avirulent or non-pathogenic bacteria strains. Using an *Agrobacterium*-mediated, transient expression assay, we analyzed the activity of GUS driven by the *CaAMP1* promoter constructs in tobacco leaves, 6 h after inoculation with *P. syringae* pv. *tabaci* KACC 10388 (Fig. 2b). The *CaAMP1* promoter (−1190, −967, and −626 bp) and GUS constructs yielded strong activity (4.81–15.52 fold induction) of GUS expression in response to *P. syringae* pv. *tabaci* infection. Deletion derivatives, −424 and −267 bp, showed low levels of promoter activity (0.91–1.34 fold induction).

To determine whether the *CaAMP1* promoter-GUS gene expression was local or systemic, GUS activity under the control of the −626 bp promoter was noted in the uninoculated, systemic (secondary) leaves, as well as in the inoculated, lower (primary) leaves at different time points after inoculation (Fig. 2c). Bacterial infection was shown to induce GUS activity

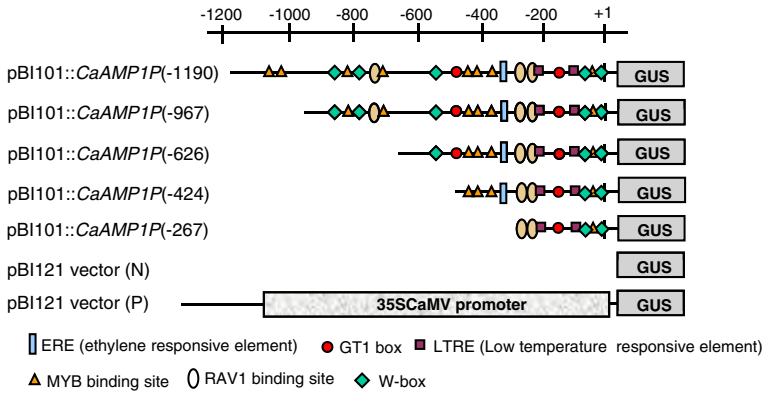

Fig. 1 Schematic diagram of different *CaAMP1* promoter::GUS fusions. Promoter fragments of different lengths were inserted into pBI101 to construct the GUS expression vectors. The DNA construct sequences shown describe the fusion of the *GUS* gene to the *CaAMP1* promoter. The putative *cis*-acting elements are represented by symbols. pBI101.1, a negative vector control containing the GUS (β-glucuronidase) reporter gene; pBI121, a positive vector control containing the CaMV 35S promoter fused to the *GUS* reporter gene

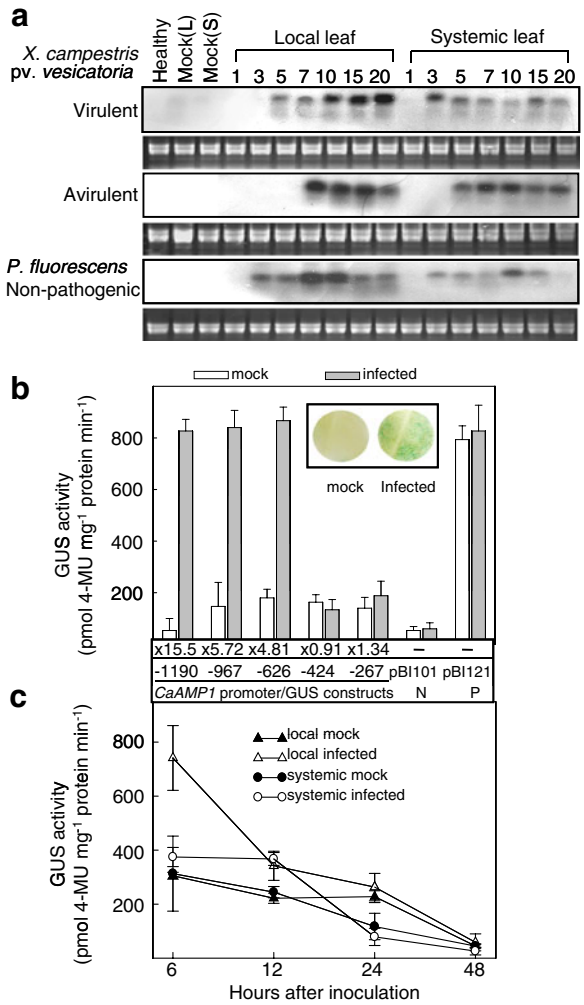

Fig. 2 **a** Time courses of mRNA expression of the pepper *CaAMP1* gene in local (primary) and systemic (secondary) leaves during compatible and incompatible interactions of pepper with *Xanthomonas campestris* pv. *vesicatoria* or *Pseudomonas fluorescens*. H: healthy leaves, h: hours after inoculation. **b** Effects of the 5′ deletions on transient expression of the *CaAMP1* promoter-GUS gene in tobacco leaves 6 h after mock-inoculation with 10 mM $MgCl_2$, or inoculation with *P. syringae* pv. *tabaci*. The photos are of mock and pathogen-infected leaves stained with 5-bromo-4-chloro-3-indoly-β-D-glucuronide (X-Gluc). Numbers under the bars indicate fold increases in induction of GUS activity after pathogen infection versus mock inoculation. **c** Time course showing the induction of GUS activity in local (primary) and infected, or systemic (secondary) and non-infected leaves by the *CAbZIP1* promoter (−626) in tobacco leaves after infection by *P. syringae* pv. *tabaci*. GUS activity was analyzed fluorometrically and is displayed quantitatively in pmol 4-MU mg^{-1} protein min^{-1}. Data are expressed as the means ± standard deviations of three independent experiments. N, negative control plasmid (pBI101.1) harboring the *GUS* reporter gene; P, positive control plasmid (pBI121) containing the CaMV 35S promoter fused to the *GUS* reporter gene

driven by the *CaAMP1* promoter in both the local and systemic leaves. However, induction levels in the uninoculated, systemic leaves were relatively low compared to the levels observed in locally inoculated leaves (Fig. 2c).

Activation of the *CaAMP1* promoter by plant hormones

To assess the promoter sequences of the *CaAMP1* gene involved in the induction of GUS activity by abiotic elicitors, we tested five chimeric *CaAMP1* promoter-GUS constructs based on the induction of GUS expression in tobacco leaves treated with SA (5 mM), MeJA (100 μM), ethylene (5 μl/l) or ABA (100 μM) (Fig. 3). The patterns of GUS induction by SA and MeJA were very similar to those observed in the tobacco leaves inoculated with *P. syringae* pv. *Tabaci* (Fig. 2b). GUS activity driven by the *CaAMP1* promoter (−1190, −967 and −626 bp) and GUS constructs increased over five-fold compared to the mock-treated controls, due to the SA, MeJA and ethylene treatments. No significant induction of GUS activity by SA or MeJA was observed in tobacco leaf tissues harboring the *CaAMP1* promoter (−424 and −267) and GUS constructs. These results suggest that the *CaAMP1* promoter region between −626 and −424 bp is necessary for, but may not be sufficient for, transcriptional expression of GUS by pathogen infection, as well as SA and MeJA treatments. The *CaAMP1* promoter (−1190) and GUS construct was effective in activating the induction of GUS activity by treatment with ABA (Fig. 3c). This indicates that the *CaAMP1* promoter regions required for pathogen, SA, and MeJA responses are different from those required for ABA response.

Activation of the *CaAMP1* promoter by salt and cold stresses

CaAMP1 transcripts were recently shown to accumulate in response to environmental stresses (Lee and Hwang 2009). In this study, we attempted to determine whether the promoter sequences of the *CaAMP1* gene are involved in the induction of GUS activity by environmental stresses. Treatment with NaCl and low temperature for 24 h induced GUS activity (Fig. 4). The tobacco leaves that harbored the *CaAMP1* promoter (−1190 bp) and GUS construct

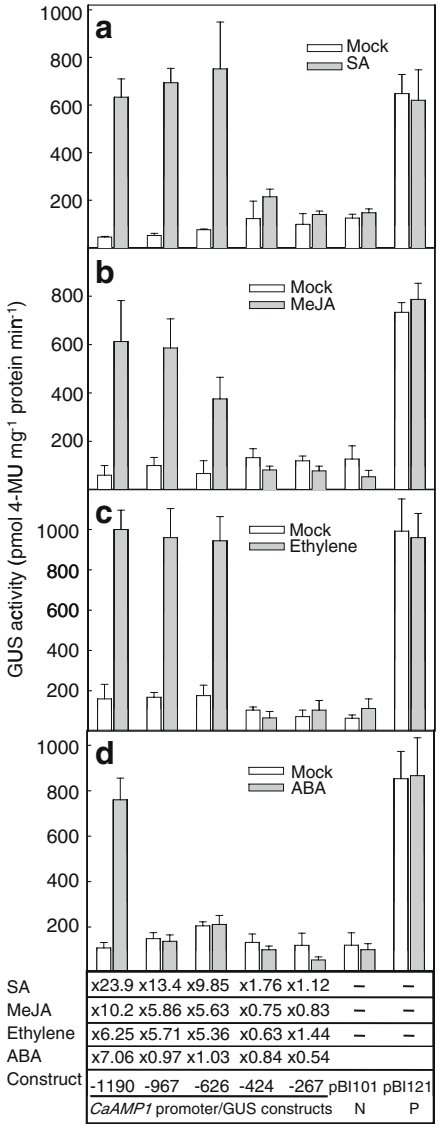

Fig. 3 Effects of salicylic acid, methyl jasmonate, and abscisic acid on transient expression of the *CaAMP1* promoter-GUS gene in tobacco leaves. Activity of the 5′ deletions of the *CaAMP1* promoter was examined in tobacco plants treated with 2 mM salicylic acid (SA) (**a**), 50 μM methyl jasmonate (MeJA) (**b**), 5 μl/l ethylene (**c**) and 100 μM abscisic acid (ABA) (**d**). GUS activity, analyzed fluorometrically, is displayed quantitatively in pmol 4-MU mg $^{-1}$ protein min $^{-1}$. Data are expressed as means ± standard deviations of three independent experiments. N, negative control plasmid (pBI101.1) harbouring the *GUS* reporter gene; P, positive control plasmid (pBI121) containing the CaMV 35S promoter fused to the *GUS* reporter gene. Numbers under the bars indicate fold increases in the induction of GUS activity versus mock treatment

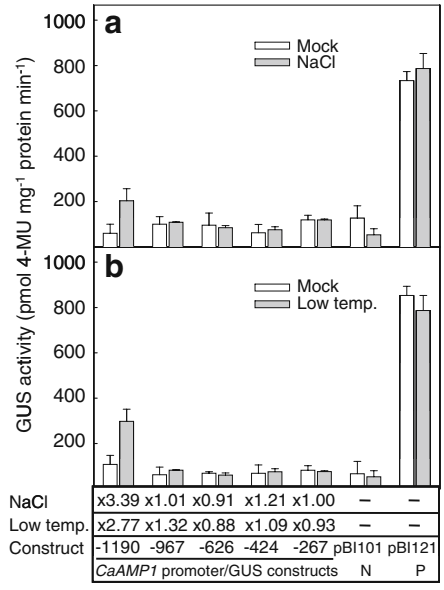

Fig. 4 Effects of plant hormone and environmental stresses on transient expression of the *CaAMP1* promoter-GUS gene in tobacco leaves. Activity of the 5′ deletions of the *CaAMP1* promoter was examined in tobacco plants treated with 300 mM NaCl (**a**) and low temperature (**b**). GUS activity, analyzed fluorometrically, is displayed quantitatively in pmol 4-MU mg $^{-1}$ protein min $^{-1}$. Data are expressed as means ± standard deviations of three independent experiments. N, negative control plasmid (pBI101.1) harbouring the *GUS* reporter gene; P, positive control plasmid (pBI121) containing the CaMV 35S promoter fused to the *GUS* reporter gene. Numbers under the bars indicate fold increases in the induction of GUS activity after each osmotic stress versus mock treatment

exhibited robust GUS activity. By contrast, other *CaAMP1* promoters (−967, −626, −424, and −267 bp) and GUS constructs did not activate the expression of the GUS reporter gene in response to NaCl and low temperature.

Transactivation of the *CaAMP1* promoter by transcription factors CAZFP1 and CARAV1

In previous studies, we were surprised to find that *CAZFP1* and *CARAV1* transcription factors were expressed in pepper leaf tissues infected by *X. campestris* pv. *vesicatoria* and *Phytophthora capsici*, suggesting that these transcription factors might regulate the expression of defence-related genes in the pepper plant (Kim et al. 2004; Sohn et al. 2006). Here, the ability of these two transcription factors to activate the *CaAMP1* promoter, as measured by their expression of a *GUS* reporter gene, was assayed based

on the *Agrobacterium*-mediated transient expression of the co-transformed plasmids in tobacco leaves (Fig. 5). The *CaAMP1* promoter (−626 bp):*GUS* reporter gene construct was only markedly expressed in the tobacco leaves when *CARAV1* was transiently overexpressed under the control of the 35 S CaMV promoter (Fig. 5). However, no significant induction of GUS activity by *CAZFP1* was detected in tobacco leaf tissues that harbored the *CaAMP1*. This suggests that *CARAV1*, but not *CAZFP1*, is sufficient for the activation of the *CaAMP1* gene.

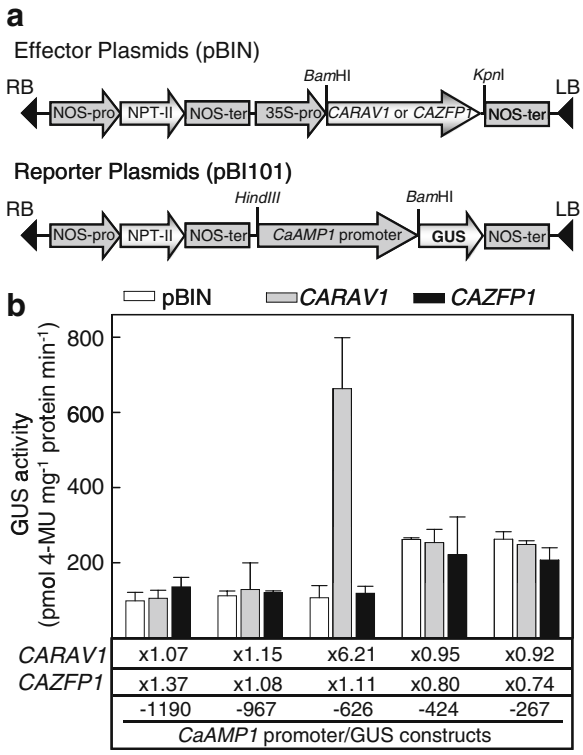

Fig. 5 **a** Schematic representations of the effector constructs (35S:*CARAV1* or 35S:*CAZFP1*) and reporter construct (*CaAMP1* promoter-GUS) for the *Agrobacterium*-mediated transient expression assay. RB, right border; LB, left border; NOS-pro, nopaline synthase promoter; NOS-ter, nopaline synthase terminator; NPTII, neomycin phosphotransferase; 35S-pro, CaMV 35S promoter. **b** Effects of pepper transcription factors CARAV1 and CAZFP1 on transient expression of the *CaAMP1* promoter-GUS gene in tobacco leaves. GUS activity was measured in tobacco leaves co-infiltrated with Agrobacteria harboring the reporter construct of the *CaAMP1* promoter-GUS gene, and the effector constructs of the pepper RAV gene (*CARAV1*) or zinc-finger protein (*CAZFP1*). The GUS activity analyzed fluorometrically is displayed quantitatively in pmol 4-MU mg $^{-1}$ protein min $^{-1}$. Data are represented as means ± SD of three independent experiments. Numbers under the bars indicate fold increases in the induction of GUS activity versus mock treatment

Discussion

In recent studies, we isolated and functionally analyzed the pepper *CaAMP1* gene from pepper leaves infected by the avirulent strain of *X. campestris* pv. *vesicatoria* (Lee et al. 2008). The *CaAMP1* gene, encoding a novel antimicrobial protein active against various bacterial and fungal plant pathogens, was strongly induced in the leaf tissues of pepper plants during pathogen attacks (Lee et al. 2008) and environmental stresses (Lee and Hwang 2009). To characterize the promoter region of *CaAMP1*, we tested for functional regions of the promoter using a set of chimeric genes constructed through the fusion of several portions of the *CaAMP1* promoter to the GUS reporter gene.

Promoter activation of the *CaAMP1* gene by biotic and abiotic stresses was proposed to modulate *CaAMP1* expression in pepper leaf tissues. The sequences located between −626 and −425 bp upstream of the *CaAMP1* gene were shown to play a crucial role in activating the *CaAMP1* promoter by bacterial infection, and treatment with MeJA, ethyoene and SA. This suggested that the minimal *cis*-regulatory sequences responsive to infection with *P. syringae* pv. *tabaci*, and SA, MeJA and ethylene treatments may be located in the 202-bp region between −626 and −425 bp of the *CaAMP1* promoter. One W-box, which is known as a *cis*-acting element capable of binding to WRKY transcription factors, was located within −626 to −425 bp of the *CaAMP1* promoter region. WRKY transcription factors unique to plants have been shown to be induced by both pathogen infection and SA treatment (Eulgem et al. 2000; Yu et al. 2001). Most of the WRKY proteins in *Arabidopsis*, including AtWRKY6 and AtWRKY29, are transcription activators, whereas some WRKYs function as transcription repressors (Asai et al. 2002; Robatzek and Somssich 2002). However, we did not find the well-known *cis*-acting element that is response to MeJA. These results suggest that a new *cis*-acting element for responding to MeJA is located within −626 to −425 bp of the *CaAMP1* promoter region.

CaAMP1 expression was differentially induced at the systemic sites of pepper leaf tissues during compatible

and incompatible interactions with *X. campestris* pv. *vesicatoria*. In systemic tissues, transcriptional up-regulation of genes involved in signal transduction leads to systemic acquired resistance (SAR) to subsequent pathogen attacks (Schenk et al. 2003; Lee and Hwang 2005). A strong and systemic accumulation of *CaAMP1* transcripts occurred in response to infection by an avirulent strain of *X. campestris* pv. *vesicatoria*, indicating the involvement of *CaAMP1* in the establishment of the SAR against pathogens in pepper plants (Lee and Hwang 2005). The systemic induction of the *CaAMP1* promoter suggests that signals mediating the activation of the promoter are translocated to distant tissues after primary infection of lower tobacco leaves with avirulent bacterial pathogens. SAR has been extensively studied in tobacco and *Arabidopsis*, where the expression was accompanied by an increase in SA levels (Ward et al. 1991; Uknes et al. 1993). The systemic induction of *PR* genes by pathogen infection occurs via a SA- or JA-dependent signal transduction pathway (Ward et al. 1991; Manners et al. 1998). Here, we showed that the exogenous application of SA, MeJA and ethylene were effective in activating the same promoter region of *CaAMP1* required during bacterial infection. These results suggest that the *cis*-acting elements important for defence signalling may be located within −626 to −425 bp of the *CaAMP1* promoter region.

The *cis*-acting elements of the *CaAMP1* promoter that are activated by treatment with ABA, NaCl, and low temperature, may reside between −1190 and −968 bp of the promoter. The *cis*- and *trans*-acting factors involved in ABA and osmotic stress-induced gene expression have been previously analyzed (Ingram and Bartels 1996; Bray 1997; Shinozaki and Yamaguchi-Shinozaki 1997). A conserved sequence, PyACGTGGC, was identified as an ABA-responsive element (ABRE) in the promoters of the ABA-responsive genes (Guilitinan et al. 1990). However, the *CaAMP1* promoter does not include the *cis*-acting element ABRE. Thus, other *cis*-acting elements, including MYB (CACATG) and MYC (TGGTTAG) recognition sites, may contribute to the activation of drought- and ABA-regulated genes (Abe et al. 1997). Two MYB boxes were located in the 223 bp region between −1190 and −967 bp of the *CaAMP1* promoter, suggesting that the MYB elements may play a critical role in the induction of the *CaAMP1* gene by ABA and osmotic stresses.

Transcription factors display tissue, developmental, and stress-inducible expression patterns (Riechmann 2002). Positive or negative regulation associated with any *cis*-acting element is dependent upon the specific transcription factors present in the cell types. Expression of the pepper transcription factors *CAZFP1* (Cys2/His2-type zinc-finger) and *CARAV1* (RAV) were induced by a number of biotic and abiotic stress stimuli (Kim et al. 2004; Sohn et al. 2006), which resulted in the activation of some defence-related genes, such as *CASAR82A* or *CAPIP2* (Lee and Hwang 2006; Lee et al. 2007). The *cis*-acting elements capable of binding to the Cys2/His2-type zinc-finger proteins are two AGT core motifs separated by approximately 13 base pairs (Takatsuji and Matsumoto 1996). We did not detect any significant induction of GUS activity by *CAZFP1* in tobacco leaf tissues harbouring *CaAMP1*. This indicates that the *CaAMP1* promoter region does not possess functional *cis*-acting elements for the transcription factor *CAZFP1*. The *CARAV1* gene contains highly conserved AP2 and B3 DNA-binding domains. RAV1 protein, a DNA-binding protein, binds to both bipartite recognition sequences RAV1-A (CAACA) and RAV1-B (CACCTG) (Kagaya et al. 1999). Three RAV1-A sites were found in the *CaAMP1* promoter region, whereas a RAV1-B site was not detected. However, *GUS* activity was shown to be strongly driven by the *CaAMP1* promoter (−626):GUS construct compared to the mock-treated control. These results show that novel binding sites other than the RAV domains may be present in the *CaAMP1* promoter to activate transcription. In addition, CARAV1 did not activate the −1190 and the −967 promoter regions, which are activated by pathogen infection and defence-related hormones. These results suggest that different mechanisms are used by pathogen infections and CARAV1 to activate the *CaAMP1* promoter.

Overall, analysis of the *CaAMP1* promoter revealed that its regulation is complex, with positive and negative regulatory regions. We identified six *cis*-acting elements in the promoter region that varied in their regulatory properties, being either positive or negative, and that caused changes in GUS activity of different magnitudes when deleted, depending on the biotic and abiotic stresses observed in the plants. Taken together, we found that the *CaAMP1* gene and promoter are activated locally and systemically by

bacterial infection, as well as by plant hormones and environmental stresses. Differential expressions of functional regions of the *CaAMP1* promoter were observed during biotic or abiotic stresses. Several biotic and abiotic stress-related *cis*-acting elements, including GT1 box, W box, MYB, RAV1, ERE, and LTRE, were localized within the upstream promoter region of the *CaAMP1* gene. In addition, analysis of the *CaAMP1* promoter provided a clue as to the possible existence of novel *cis*-acting elements for the CARAV1 transcription factor involved in mediating *CaAMP1* expression during the defence response to pathogens or environmental stresses. Further identification of the putative *cis*-acting elements of transcription factors specifically binding to the *CaAMP1* promoter will be necessary for gaining insight into the positive or negative regulation of *CaAMP1* gene expression during biotic and abiotic stresses.

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