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Functional Analysis of Pepper Cys_2/His_2-Type Zinc-Finger Protein Promoter Region in Response to Bacterial Infection and Abiotic Stresses in Tobacco Using *Agrobacterium*-Mediated Transient Assay

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The promoter region flanking the 5' *CAZFP1* coding region was isolated from the genomic DNA of *Capsicum annuum*. To identify the upstream region of the *CAZFP1* gene required for promoter activity, a series of CAZFP1 promoter deletion derivatives was created. Each deletion construct was analyzed by *Agrobacterium*-mediated transient transformation in tobacco leaves after infection by *Pseudomonas syringae* pv. *tabaci*, or treatment with methyl jasmonate (MeJA), ethylene, abscisic acid (ABA), salicylic acid (SA), cold and wounding. Promoter fragments of 685 bp or longer showed 7-fold or greater induction after *P. s.* pv. *tabaci* infection and MeJA treatment. The *CAZFP1* full-length promoter (-999 bp) also showed 6-fold induction in response to ethylene. The transiently transformed tobacco leaves with the *CAZFP1* full length promoter fused-GUS gene showed more than 5-fold induction in response to SA, ABA and cold. These results suggest that the *CAZFP1* promoter contains responsive elements for pathogen, MeJA, ethylene, SA, ABA and cold.

Keywords : *Agrobacterium*-mediated transient transformation, Cys_2/His_2-type zinc finger protein, GUS gene, *Nicotiana tabacum*, promoter region

Plants have constitutive and induced defense mechanisms to protect themselves from pathogen attack. In response to pathogen infection, plants operate specific signal transduction pathways, resulting in hypersensitive response (HR), generation of reactive oxygen species (ROS), *de novo* synthesis of pathogenesis-related (PR) and systemic acquired resistance (SAR) (van der Biezen and Jones, 1998; Yang et al., 1997). Plant signaling molecules, such as ethylene, salicylic acid (SA) and jasmonic acid (JA), which accumulate in plants during pathogen attack, are involved in regulatory pathways mediating defense responses (Grazebrook, 2001). These regulatory pathways require the coordination of highly specific DNA-protein interaction and protein-protein interactions, most of which are not fully understood (Onate-Sanchez and Singh, 2002).

The Cys_2/His_2-type zinc-finger protein was first discovered in the *Xenopus* transcription factor IIIA (TFIIIA) and stand for an important class of eukaryotic transcription factors (Miller et al., 1985). The Cys_2/His_2-type zinc-finger is a sequence of $CX_{2\text{-}4}CX_3FX_3QALGGHX_{3\text{-}5}H$, in which two cysteines and two histidines tetrahedrally coordinate a zinc atom to form a compact finger structure containing a α-helix and an antiparallel β-sheet (Pavletich and Pabo, 1991). Another structural feature of Cys_2/His_2-type zinc-finger protein is that the zinc-finger motifs contain a sequence, QALGGH, in the putative DNA-containing surfaces. This conserved sequence motif that is quite highly conserved in many TFIIIA-type zinc finger proteins and is highly critical for DNA-binding activity has so far been reported only in plants (Kubo et al., 1998; Takasuji, 1998).

A number of Cys_2/His_2-type zinc-finger proteins have been known to play various regulatory roles, such as developmental regulation, tissue specific expression and stress-related regulation. SUPERMAN is best characterized in terms of its genetic function in flower development in *Arabidopsis* (Sakai et al., 1995). *Arabidopsis* ATZFPs are similar to SUPERMAN, in which have only one zinc-finger motif and are expressed predominantly in vegetative tissue (Tague et al., 1995, 1997). Petunia EPF1 is considered a potential candidate for the organ- and stage-specific activator of EPSPS gene (Takatsuji et al., 1992). Some of the Cys_2/His_2-type zinc-finger proteins have been implicated in the regulation of tolerance for various stress responses. Overexpression of ZPT 2-3 in transgenic petunia plants increases tolerance to dehydration (Sugano et al., 2003). SCOF-1 functions as a positive regulator of the cold-regulated (*COR*) gene and enhances cold tolerance in soybean (Kim et al., 2001). AZFs and STZ are involved in a water-stress response in an abscisic acid (ABA)-dependent or -independent pathway in *Arabidopsis* (Sakamoto et al., 2000). *Arabidopsis* RHL41 has a key role in the acclimatization response to changes in light intensity (Iida et al., 2000).

We have previously identified that Cys_2/His_2-type zinc-

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finger transcription factor, *CAZFP1*, functions as a pathogen-induced early-defense gene in *Capsicum annuum* (Kim et al., 2004). The CAZFP1 protein was a nuclear targeting protein, which functions as a transcriptional regulator. The full-length *CAZFP1* had no transcriptional activation activity, whereas the C-terminal region of *CAZFP1* had transactivation activity. The *CAZFP1* transcripts were constitutively expressed in the pepper stem, root, flower and red fruit, but were not detectable in the leaf and green fruit. The *CAZFP1* transcripts accumulated earlier than the *CABPR1* (PR-1) gene in the incompatible interaction of the pepper leaves with *X. campestris* pv. *vesicatoria*. The *CAZFP1* transcripts were significantly induced in the systemic, uninoculated leaf tissues early after inoculation with bacterial pathogens, but gradually declined thereafter. The *CAZFP1* transcripts were localized, and confined to the phloem cells of the vascular bundle in the pepper leaf midrib in response to *C. coccodes* infection, ethylene and abscisic acid. The *CAZFP1* gene was also induced much earlier by abiotic elicitors and environmental stresses, compared to the *CABPR1* gene. Overexpression of the *CAZFP1* gene in the Arabidopsis transgenic plants was also demonstrated to confer enhanced resistance to *Pseudomonas syringae* pv. *tomato* DC3000 and tolerance to drought stress.

In this study, we describe the isolation and functional analysis of the *CAZFP1* promoter regions. Deletion analysis with GUS-reporter gene was performed using an *Agrobacterium*-mediated transient assay to identify regions of the *CAZFP1* promoter regulating transcription in tobacco leaves in response to *P. s.* pv. *tabaci*, abiotic elicitors and environmental stresses. GUS histochemical staining also was conducted in tobacco leaves inoculated with *P. s.* pv. *tabaci*. To our knowledge, this is the first report analyzing the promoter regions of Cys_2/His_2-type zinc-finger proteins.

Materials and Methods

Isolation of the *CAZFP1* promoter region. Fragments of promotor regions of the *CAZFP1* gene were obtained using an Universal Genome Walker Kit (Clontech, Palo Alto, USA). Total genomic DNA was isolated from pepper leaves using hexadecyltrimethylammonium and ultracentrifugation. Genomic DNA was digested with *Dra*I, *Eco*RV, *Pvu*II, *Stu*I, *Nru*I, *Sca*I and *Ssp*I, which recognize a 6-bp and make a blunt end. Genome Walker adaptors were ligated to the restricted product for 18 h at 16°C. Using this genomic library, primary and nested PCR were achieved with the *CAZFP1* specific primer and Advantage genomic polymerase mixture (Clontech, Palo Alto, USA). Nested PCR product was purified from 1.5% agarose gel and subcloned into pCR^{R}2.1-TOPO vector (Invitrogen, Carlsbad, USA). The cloned vector was sequenced and analysed with PLACE (http://www.dna.affrc.go.jp) database at the Advanced Biosciences Computing Center (Higo et al., 1999).

Promoter deletion-GUS constructs. The 999-bp *CAZFP1* promoter region was subcloned into pCR^{R}2.1-TOPO vector (Invitrogen, Carlsbad, USA) and the 5' deletion constructs were generated from the 999-bp *CAZFP1* promoter using the PCR (Perkin-Elmer, Norwalk, CT). Forward primers were designed to correspond to the -999, -685, -485 and -249 sequences of *CAZFP1* promoter and the reverse primer was located in the 3' end of *CAZFP1* promoter containing the translational start site. Deletion constructs were subcloned into pCR^{R}2.1-TOPO vector and all constructs were sequenced to confirm their identity. Each of deletion constructs was excised by *Bam*HI-*Hin*dIII and linked to the GUS reporter gene by ligation into *Bam*HI-*Hin*dIII digested pBI101, a binary vector containing the neomycin phosphotransferase II gene that confers kanamycin resistance.

Agroinfiltration of tobacco leaves. *Agrobacterium*-mediated transient assays were performed according to the method of Yang et al. (2000). *Agrobacterium tumefaciens* strain EHA 105 containing individual promoter constructs was streaked on YEP solid medium (1 g yeast extract, 5 g beef extract, 5 g peptone, 5 g sucrose and 2 mM $MgSO_4$ in 1 L H_2O) supplemented with 1.8% agar, rifampicin (60 μg/ml) and kanamycin (50 μg/ml), and grown at 28°C for 2 days. *Agrobacteria* were grown in 3 ml YEP broth with antibiotics to a stationary phase for 2 days at 28°C. Cultures were diluted 1:100 into 100 ml of fresh YEP broth plus antibiotics and grown overnight at 28°C. Cells were pelleted and washed once in infiltration media [0.1X MS salts, 0.1X B5 vitamins, 20 mM MOPS (pH 5.4), 1% glucose, 2% sucrose, 200 μM acetosyringone] (Rathjen et al., 1999), then pelleted again and adjusted to a final OD_{600} of 0.8 in infiltration media for agroinfiltration.

Agrobacterium-mediated transient transformation was conducted on near-fully expanded tobacco (*Nicotiana tabacum* cv. Xanthi nc) leaves that still attached to the intact plants. Bacterial suspension was inoculated by pressure infiltration using a plastic syringe. After agroinfiltration, tobacco plants were covered with transparent plastic bags and maintained in a growth chamber at 22°C under 16 h light for 48 h.

Gus activity assays. All treatments were carried out 48 h after agroinfiltration into tobacco leaves. Agroinfiltrated tobacco leaves were inoculated with *P. s.* pv. *tabaci* KACC 10388 (1×10^8 cfu/ml) by pressure infiltration using a plastic syringe. Mock treatment was performed with infiltration of sterile tap water. Tobacco leaves also were treated with 100 μM methyl jasmonate (MeJA), 5 μl/L ethylene, 5 mM salicylic acid (SA), 100 μM ABA, 4°C

cold and wounding at 48 h after agroinfiltration, as previously described (Kim and Hwang, 2000). Tobacco leaf tissues inoculated with *P. s.* pv. *tabaci* KACC 10388 or treated with MeJA and ethylene were harvested for measurement of GUS activity at 3 h and 18 h after inoculation or treatment. Tobacco leaves treated with SA, ABA, cold and wounding were harvested for measurement of GUS activity at 3 h.

Fluorometric GUS assays were performed according to the method of Jefferson et al. (1987). Each leaf sample was ground in 1 ml GUS extraction buffer [50 mM NaH_2PO_4 (pH 7.0), 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100 and 10 mM 2-mercaptoethanol. After centrifugation for 10 min (12,000 *g*, 4°C), 100 μl of supernatant was mixed with 400 μl of GUS assay solution (2 mM 4-methylumbelliferyl-D-glucuronide in GUS extraction buffer). A 100 μl of mixture was immediatedly removed and added into 2 ml of stop buffer (0.2 M Na_2CO_3). The rest of mixture was incubated at 37°C for 60 min. To obtain a concentration of 4-methylumbelliferone (4-MU) for each sample, background fluorescence (reaction at zero time) was substracted. Protein concentrations were determined according to Bradford method (Bradford, 1976). GUS activity is given in nmol 4-MU per minute per milligram of protein.

Histochemical GUS staining was performed using agroinfiltrated tobacco leaves. Tobacco leaves were inoculated with *P. s.* pv. *tabaci* (1×10^8 cfu/ml) and sterile tap water at 48 h after agroinfiltration. The leaf tissues were harvested for the GUS histochemical staining. Histochemical assays were performed on leaf tissues with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) as a substrate (Jefferson et al., 1987). After staining, the leaf samples were fixed in 37% formaldehyde-5% acetic acid-20% ethanol solution for 10 min, and cleared with 50% ethanol for 30 min and 100% ethanol overnight.

Results

Isolation of the promoter region of a zinc-finger protein (*CAZFP1*) gene from pepper. The pepper *CAZFP1* promoter was isolated by PCR using the adapter-ligated fragment libraries of pepper genomic DNA as a template, along with an adapter primer and a gene specific primer. When it was analyzed on an agarose gel, a fragment of 1.0 kb was detected and cloned for sequencing. The *CAZFP1* promoter was 999 bp in length (Fig. 1). To investigate potential regulatory *cis*-acting elements, the *CAZFP1* promoter was analyzed with PLACE (http://www.dna.affrc.go.jp) database at the Advanced Biosciences Computing Center (Higo et al., 1999). A typical TATA (TTATATA) box is located at -207 bp upstream of the translational initiation codon. Several putative regulatory motifs, such as MYBST1, RAV1AAT, ERELEE4, E-box, W-box and GT-1, which are homologous to the *cis*-acting elements identified in plants, were found in the *CAZFP1* promoter. MYBST1 (GGATA) element is a binding consensus site for a potato MYB homologue (Baranowskij et al., 1994). RAV1AAT element (CAACA) is a binding consensus sequence of *Arabidopsis* transcription factor, RAV1 (Kagaya et al., 1999). ERELEE4 (AXTTCAAA) is an ethylene responsive element (ERE) of tomato E4 gene (Montgomery et al., 1993) and carnation GST1 gene (Itzhaki et al., 1994). E-box (CAXXTG) is a promoter element of napA storage protein gene of *Brassica napus* (Stalberg et al., 1996). W-box (TTGAC), promoter of Arabidopsis NPR1 gene, is specifically recognized by salicylic acid-induced WRKY DNA binding protein (Yu et al., 2001). GT-1 element (GGTTAA) is a GT-1 binding site in many light-regulated genes (Buchel et al., 1999). However, the functions of these putative regulatory elements for expression of the *CAZFP1* gene in pepper remain to be determined.

Transient expression of the *CAZFP1* promoter-GUS gene in tobacco leaves. To identify the upstream region of the *CAZFP1* gene required for promoter activity, we constructed four deletion derivatives (-999, -685, -485 and -249) from the 5' end of the *CAZFP1* gene promoter region, followed by their fusion to the GUS reporter gene (Fig. 2). *Agrobacterium*-mediated transient transformation was conducted in tobacco leaves with each of the 5' deletion constructs of the *CAZFP1* gene promoter region, a negative control (pBI101) and a positive control (pBI121).

The GUS activity driven by the *CAZFP1* gene promoter was analyzed in tobacco leaves at 3 h and 18 h after either inoculation with *P. s.* pv. *tabaci* or treatment with MeJA and ethylene (Fig. 3). The *CAZFP1* promoter (-999 bp) yielded strong inducibility (5.9-8.2 fold induction) of GUS gene at 3 h and 18 h after *P. s.* pv. *tabaci* infection. The -685 deletion derivative exhibited slight reduction in the *CAZFP1* promoter activity (5.3-7.2 fold induction), compared to the -999 deletion one. No significant promoter activity was found in the deletion derivatives -485 and -249. Methyl jasmonate induced a strong activity of the *CAZFP1* promoter at the -999 (7.0-8.0 fold induction) and -685 (4.5-5.5 fold induction) deletion fragments. However, further deletions to -485 bp and -249 bp abolished almost all activity and inducibility (1.6-2.2 fold induction) of the *CAZFP1* promoter. These data suggest that *P. s.* pv. *tabaci*- and MeJA-responsive sequences of *CAZFP1* promoter are mainly localized in the region between the positions -999 and -485. The transient assay of the *CAZFP1* full-length promoter (-999) also showed strong activity and significant induction (5.7-7.3 fold induction) in response to ethylene treatment. However, significant decline of promoter activity

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                          attttttttggctttctcaaacatcatacaaaaataaaaa      -999
                                        DOF                  MART-Box
agagtttaaaaatcaaaacacctaaaagtaagttaattcaaacacccacttattcttaca            -960
                                DOF        ERELEE4
atgtattttgtaaaaggaggaaaataactttatgaataatgtgttttagaaaaacttgc             -900
            DOF             DOF
tgaaatggagaactatatacaatcgatattgttagaaatatgaatgtggggttgtgggtg            -840
 E-Box
tgagtggagaaggcacaatcaatataaaataaaatttgtgtagcttaattttcttatttt            -780

cactagataaaccaagttgtttcttataatttaaaaaattaacccataattatgaaaca             -720
                                                GT1
ttattttcctttgtaccaaatacacctttaagcgagaaatctttttctcccctattttg             -660
                            DOF            DOF
tcaactaagcaataaaagagatttttccctttccccattacaaaataaatgaaaacacc             -600
W-Box         DOF            DOF
gcgtggggcttgttgtgtttctttccgagtacgtctttgagatttgaattttgacagagt            -540
           RAV1AAT  DOF            DOF     ERELEE4 W-Box
ccatggtcttactagtctactgtcttacctcactcaaaataactcccccctttttggttg            -480
                                                     DOF
cttcctttgcaaagcccacaacaaaatttagtcatttacacttcacttccaagtggcact            -420
                  RAV1AAT                                E-Box
ccaacgactatttctttttccaacgtgctccactctccctttccctctttattttaatta            -360
            DOF                          DOF     DOF
ttaataatttataattttcaattcaataacttgagtaacctatactatcctacttcttac            -300
                                                MYBST1
tctttcccacgcaacttccgatttttcccccctttatatatcccacaaactcccttcttc            -240
 DOF                              TATA MYBST1
actcattcactactcaaaaacttctctatactcatcaatccaataaaccttatacagcct            -180

gctttcatcttctggacactcattaaactaacaacttcacacaactcaaaatcttcgcta            -120
 DOF
cttacttacatcttctagaatagtcactagaaccagtaactttatacaacggatatcgat             -60
                                        DOF        MYBST1
ATGGCACTTGAAGCTTTGAATTCTCCAACTGGTACACCAACTCCGCCACCGTTTCAATTT             60
M  A  L  E  A  L  N  S  P  T  G  T  P  T  P  P  P  F  Q  F               20
GAGAGCGACGGCCAACAGCTTCGATATATCGAAAACTGGAGGAAGGGAAAGAGATCTAAA            120
E  S  D  G  Q  Q  L  R  Y  I  E  N  W  R  K  G  K  R  S  K               40
AGGTCACGCAGCATGGAGCACCAGCCTACTGAGGAAGAATACTTAGCGCTTTGTTTGATC            180
R  S  R  S  M  E  H  Q  P  T  E  E  E  Y  L  A  L  C  L  I               60
ATGCTTGCACGTAGCGGTGGCTCCGTTAATCATCAACGATCTCTACCACCGCCGGCTCCG            240
M  L  A  R  S  G  G  S  V  N  H  Q  R  S  L  P  P  P  A  P               80
GTGATGAAACTGCACGCGCCGTCGTCATCATCGGCGGCGGAGGAGGAGAAGGAGAAGATG            300
V  M  K  L  H  A  P  S  S  S  S  A  A  E  E  E  K  E  K  M              100
GTGTATAAGTGTTCGGTTTGTGGTAAGGGATTTGGGTCTTATCAAGCTTTAGGTGGACAC            360
V  Y  K  C  S  V  C  G  K  G  F  G  S  Y  Q  A  L  G  G  H              120
AAAGCTAGTCACCGGAAACTCGTACCCGGCGGAGATGATCAGTCAACTACCTCCACAACC            420
K  A  S  H  R  K  L  V  P  G  G  D  D  Q  S  T  T  S  T  T              140
ACTAACGCAACCGGAACAACAACCTCCGTTAACGGCAACGGCAACAGAAGTGGAAGGACT            480
T  N  A  T  G  T  T  T  S  V  N  G  N  G  N  R  S  G  R  T              160
CACGAGTGTTCGATTTGTCACAAGTGTTTTCCCACTGGACAAGCTTTAGGTGGACACAAA            540
H  E  C  S  I  C  H  K  C  F  P  T  G  Q  A  L  G  G  H  K              180
AGGTGTCACTACGACGGCGGTATCGGTAACGGAAACGCTAACAGTGGCGTTAGTGCTAGC            600
R  C  H  Y  D  G  G  I  G  N  G  N  A  N  S  G  V  S  A  S              200
GTTGGAGTGACGTCATCGGAGGGTGTGGGGTCCACAGTCAGTCACCGGGATTTCGACTTG            660
V  G  V  T  S  S  E  G  V  G  S  T  V  S  H  R  D  F  D  L              220
AACATTCCGGCGTTGCCGGAATTCTGGCTGGGATTTGGTTCCGGCGAAGATGAGGTGGAG            720
N  I  P  A  L  P  E  F  W  L  G  F  G  S  G  E  D  E  V  E              240
AGTCCACATCCGGCGAAGAAATCGCGGTTATGTTTGCCTCCAAAATATGAATTATTTCAA            780
S  P  H  P  A  K  K  S  R  L  C  L  P  P  K  Y  E  L  F  Q              260
CATTAAtgggaatttgattgttaggatttactattttggtagacaaaattatactatgta            840
H  *                                                                    261
agttttaattttcattgtgggtgggagcaaaatttttaattttttgtctatagacctagc            900
tagttactaatagcaaaaattcaattgattgattt                                     935
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Fig. 1. Nucleotide sequences of the pepper *CAZFP1* genomic region and putative *cis*-acting elements. The translational start sites are shown in bold types and the termination codon is marked by an asterisk (*). Motifs with significant similarity to the previously identified *cis*-acting elements are underlined and the names are given under the elements.

and inducibility (0.4-3.0 fold induction) was observed in the transformants carrying -685, -485 and -249 promoters. This result suggests that ethylene-responsive elements of *CAZFP1* promoter are mainly localized in the region between the positions -999 and -685. GUS histochemical staining also was performed in tobacco leaves at 3 h after either inoculation with *P. s.* pv. *tabaci* or mock (Fig. 3C). Histochemical GUS detection result in the staining of the area infiltrated with the *P. s.* pv. *tabaci* in the tobacco leaves transiently expressing the both -999 and -685 bp promoter

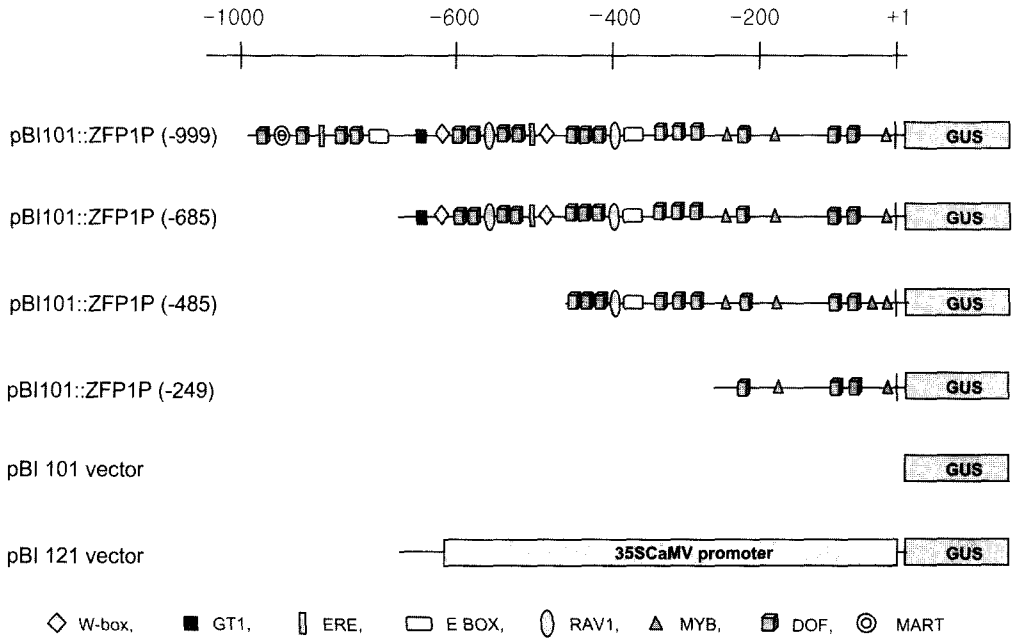

Fig. 2. Schematic diagram of the *CAZFP1* promoter-GUS constructs for assaying reporter gene expression in tobacco. Four *CAZFP1* promoter regions (-249 bp, -485 bp -685 bp and -999 bp) were amplified by PCR and fused to the GUS gene in plasmid pBI101. The numbers of each construct indicate the distance from the *CAZFP1* translation start site. Plasmids pBI101 and pBI121 were used as negative and positive controls, respectively. The putative *cis*-acting elements are represented by symbols : ◇ W-box : TTGAC ■ GT-1 : GGTTAA ▯ ERE : AXTTCAAA ▭ E-box : CAXXTG ⬯ RAV1 : CAACA ▲ MYB : GGATA ▣ DOF : AAAG ◎ MART : TTXTXTTXTT

region-GUS genes.

The *CAZFP1* gene was induced in pepper leaves by salicylic acid, abscisic acid, wounding and cold treatments (Kim et al., 2004). The transient expression assay of deletion derivatives of the *CAZFP1* gene promoter was performed in tobacco leaves after treatment with these abiotic stresses (Fig. 4). The transiently transformed tobacco leaves carrying the *CAZFP1* -999 promoter GUS gene showed strong GUS expression in response to SA (8.8 fold induction), ABA (4.8 fold induction) and cold (8.0 fold induction). By contrast, the -685 deletion derivative of the *CAZFP1* promoter was not effective in inducing the GUS expression by treatment with SA (2.6 fold induction), ABA (1.4 fold induction) and cold (1.0 fold induction). However, wounding did not significantly induce the activity of both -999 full-length promoter and -685 deletion derivative (2.1 and 1.4 fold induction, respectively). The deletion analyses of the *CAZFP1* promoter indicate that SA-, ABA-, cold-responsive elements may be located in the region between positions -999 and -685.

Discussion

In the previous study, we have isolated and functionally characterized Cys_2/His_2-type zinc-finger protein (*CAZFP1*) gene from pepper (*C. annuum* L., cv. Hanbyul) (Kim et al., 2004). The *CAZFP1* transcripts were rapidly induced in pepper plants by various pathogen infection, plant hormones, abiotic elicitors and environmental stresses. Overexpression of the *CAZFP1* gene in the Arabidopsis transgenic plants was also demonstrated to confer enhanced resistance to *P. s.* pv. *tomato* DC3000 and tolerance to drought stress. To further understand the mechanism of the *CAZFP1* gene expression in the present study, the -999 bp flanking promoter region of the *CAZFP1* gene was isolated and characterized from the genomic DNA of *C. annuum*.

Agrobacterium-mediated transient transformation has been used to analyze foreign gene expression (Kapila et al., 1997), gene silencing (Baulcombe, 1999) and gene-for-gene interaction (Frederick et al., 1998; Scofield et al., 1996). More recently, the *Agrobacterium*-mediated transient expression assay has been demonstrated to be a simple and efficient method for the quantitative analysis of plant promoter and *cis*-element/*trans*-factor interaction *in vivo* (Yang et al., 2000). We used fully expanded 6-week-old tobacco leaves for agroinfiltration, because it gave excellent transformation efficiency and minimize assay variations (Yang et al., 2000).

The -999 and -685 *CAZFP1* promoter produced high levels of induced GUS gene expression at 3 h and 18 h after *P. s.* pv. *tabaci* infection or methyl jasmonate treatment (Fig. 3). The *CAZFP1* promoter region of 685 bp-upstream

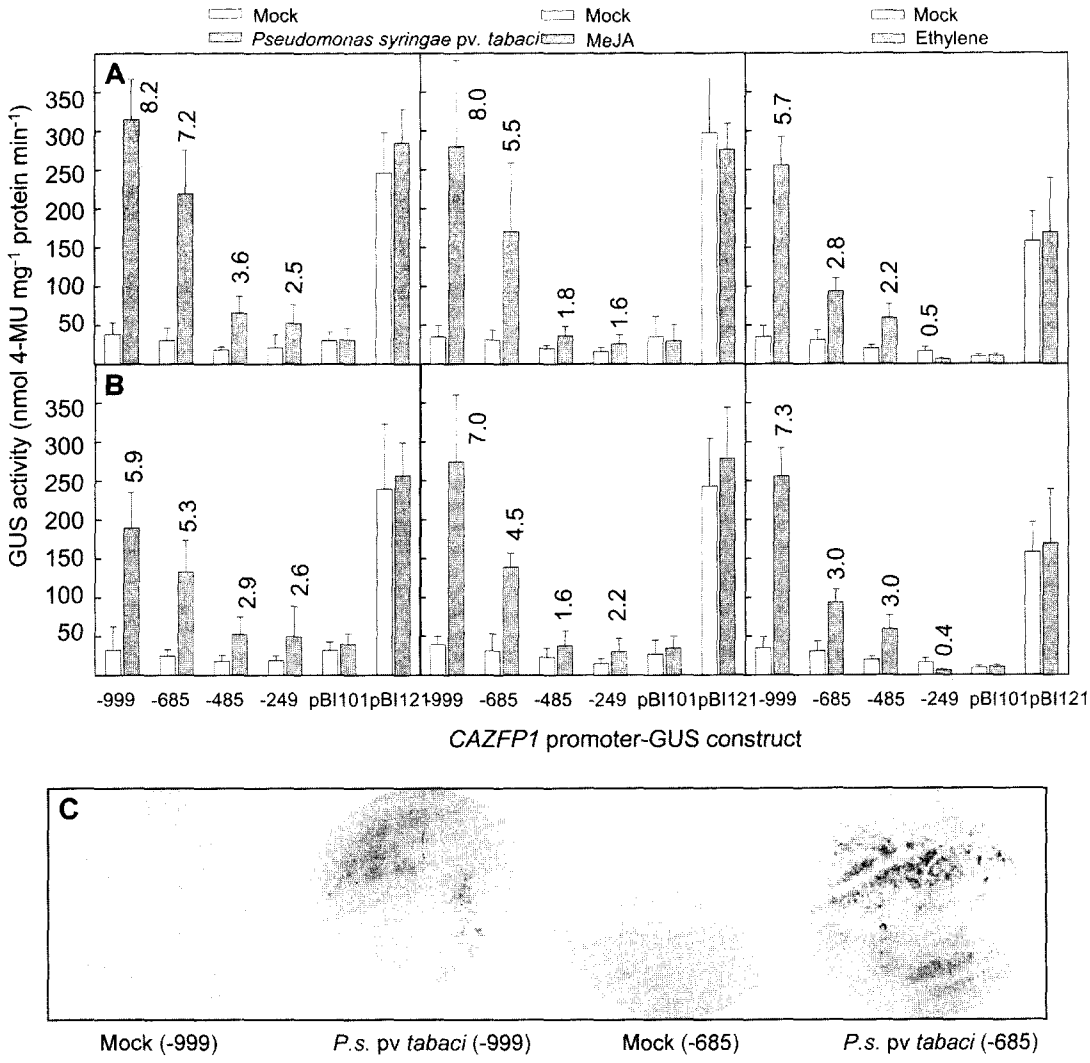

Fig. 3. Transient expression of the *CAZFP1* promoter-GUS gene in tobacco leaves at 3 h (**A**) and 18 h (**B**) after *Pseudomonas syringae* pv. *tabaci* infection, methyl jasmonate (MeJA) and ethylene treatments. Transient transformation was conducted by agroinfiltration of negative vector control (pBI101, N), positive vector control (pBI121, P), *CAZFP1* promoter deletion derivatives (-999, -685, -485 and -249 bp) in tobacco leaves. Values are means ± standard errors of three independent experiments. (**C**) Identification of GUS activity by histochemical staining in tobacco leaves transiently expressing the *CAZFP1* promoter-GUS gene. Promoter deletion derivatives (-999 and -685 bp) were tested for responsiveness to ether mock or *P. s.* pv. *tabaci* infection.

sequence was sufficient for regulating expression of the GUS reporter gene induced by *P. s.* pv. *tabaci* infection. Two W-boxes, WRKY transcription factor binding sites, were identified between the positions -486 and -490, and between the positions -597 and -601 within 685 bp. Pathogen-induced WRKY DNA-binding proteins has been known to recognize various W-box elements with a TGAC core sequence that are present in promoters of many defense-related genes in plants (Rushton and Somssich, 1998; Rushton et al., 1996; Yu, et al., 2001). These results suggest that bacterial pathogen (*P. s.* pv. *tabaci*)-responsive sequence of the *CAZFP1* promoter may be W-box elements mediated by WRKY transcription factor.

The -999 and -685 *CAZFP1* promoters were strongly activated upon application of methyl jasmonate. However, no JERE (jasmonate- and elicitor-responsive element, AGACCGCC) was found in the *CAZFP1* promoter. Methyl jasmonate-mediated GUS expression may be mediated via an alternative *cis*-element in the *CAZFP1* promoter region between -685 and -485. In response to ethylene treatment, the *CAZFP1* full length promoter (-999) was strongly activated to induce the GUS gene expression, but its deletion derivates (-685, -485 and -249) were not effective for the promoter activity. ERELEE4 (AXTTCAAA) was found to be an ethylene responsive element (ERE) of tomato E4 gene (Montgomery et al., 1993) and carnation

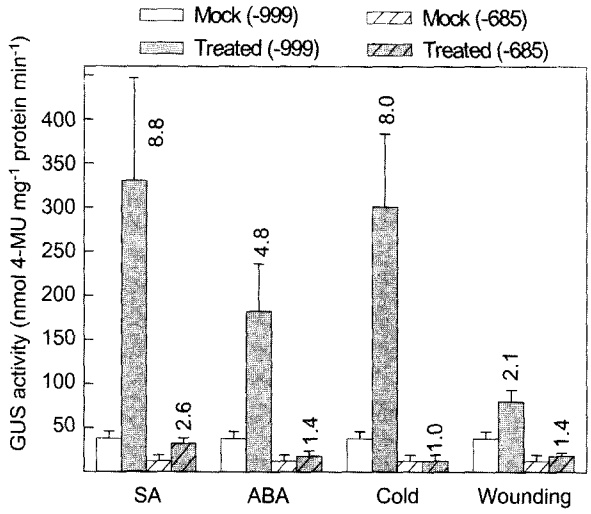

Fig. 4. Transient expression of the *CAZFP1* promoter-GUS gene in tobacco leaves at 3 h after treatment with salicylic acid (SA), abscisic acid (ABA), cold and wounding. Transient transformation was conducted by agroinfiltration of *CAZFP1* promoter derivatives (-999 and -685 bp) in tobacco leaves. Values are means ± standard errors of three independent experiments.

GST1 gene (Itzhaki et al., 1994). As shown in Figure 1, ERELEE4, located in the promoter region between -926 and -919, seems likely to function as an ethylene responsive element of the *CAZFP1* promoter.

Salicylic acid-, abscisic acid- and cold-responsive elements may also be located in the region between -999 and -685 (Fig. 4). A DOF binding sequence (AAAG) was shown to be SA-responsive (Kang and Singh, 2000). The W-box element with a TGAC core sequence is involved in the SA-inducible responses (Yu et al., 2001). The *CAZFP1* -999 promoter GUS gene showed strong GUS expression in response to SA. However, the -685 deletion derivative of the *CAZFP1* promoter was not effective in activating the GUS by treatment with SA. These results imply that 4 DOF binding sequences in the *CAZFP1* promoter region between -999 and -685 may play an important role in regulation of the *CAZFP1* expression during SA induction. E-box element (CANNTG) was known to be involved in the activation of gene expression by osmotic stresses (Stalberg et al., 1996). There are two E-box elements in the *CAZFP1* promoter region between -839 and -834 and between -371 and -366. The -999 promoter, which carried a -839 and -834 E-box, showed significant GUS-activity and inducibility upon ABA and cold stresses, whereas the -685 promoter, which lacked a -839 and -834 E-box, exhibited a weak induction of GUS activity (Fig. 4). Thus, the positive *cis*-acting elements that may drive the expression of the *CAZFP1* gene inducible by osmotic stresses, such as ABA and cold, appear to reside between -999 and -685. Any ABA- and cold-responsive elements, such as ABRE (abscisic acid-responsive element, ACGTGGC) and CRT/DRE (C-repeat/drought-responsive element, TGGCCGAC), were not found in the *CAZFP1* promoter region between -999 and -685. Therefore, ABA and cold-mediated *CAZFP1* expression in the *CAZFP1* promoter region between -999 and -685 may be controlled via E-box element. The *CAZFP1* full length promoter (-999) showed lower GUS activity and inducibility in response to the wounding (Fig. 4), which suggest that wounding-responsive elements may be located in the upstream region of the *CAZFP1* -999 promoter.

Taken together, the *CAZFP1* promoter regions fused with GUS-reporter gene is activated by pathogen infection, abiotic elicitors and environmental stresses in tobacco. The *CAZFP1* promoter sequence possesses at least two distinct promoter regions not only for pathogen- and MeJA-responsive elements, but also for ethylene, SA, ABA and cold responsive elements. Further deletion analyses will be required to elucidate the *cis*-acting elements of the *CAZFP1* promoter region.

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