

# The promoter of the pepper pathogen-induced membrane protein gene *CaPIMPI* mediates environmental stress responses in plants

Jeum Kyu Hong · Byung Kook Hwang

Received: 6 July 2008 / Accepted: 10 September 2008 / Published online: 21 October 2008  
© Springer-Verlag 2008

**Abstract** The promoter of the pepper pathogen-induced membrane protein gene *CaPIMPI* was analyzed by an *Agrobacterium*-mediated transient expression assay in tobacco leaves. Several stress-related *cis*-acting elements (GT-1, W-box and ABRE) are located within the *CaPIMPI* promoter. In tobacco leaf tissues transiently transformed with a *CaPIMPI* promoter- $\beta$ -glucuronidase (*GUS*) gene fusion, serially 5'-deleted *CaPIMPI* promoters were differentially activated by *Pseudomonas syringae* pv. *tabaci*, ethylene, methyl jasmonate, abscisic acid, and nitric oxide. The -1,193 bp region of the *CaPIMPI* gene promoter sequence exhibited full promoter activity. The -417- and -593 bp promoter regions were sufficient for *GUS* gene activation by ethylene and methyl jasmonate treatments, respectively. However, *CaPIMPI* promoter sequences longer than -793 bp were required for promoter activation by abscisic acid and sodium nitroprusside treatments. *CaPIMPI* expression was activated in pepper leaves by treatment with ethylene, methyl jasmonate, abscisic acid,  $\beta$ -amino-*n*-butyric acid, NaCl, mechanical wounding, and low temperature, but not with salicylic acid. Overexpression of *CaPIMPI* in *Arabidopsis* conferred hypersensitivity

to mannitol, NaCl, and ABA during seed germination but not during seedling development. In contrast, transgenic plants overexpressing *CaPIMPI* exhibited enhanced tolerance to oxidative stress induced by methyl viologen during germination and early seedling stages. These results suggest that *CaPIMPI* expression may alter responsiveness to environmental stress, as well as to pathogen infection.

**Keywords** *Capsicum annuum* · Osmotic stress · Oxidative damage · Plasma membrane protein · Promoter activation · Transgenic *Arabidopsis*

## Abbreviations

ABA	Abscisic acid
BABA	$\beta$ -Amino- <i>n</i> -butyric acid
ERE	Ethylene-responsive element
GUS	$\beta$ -Glucuronidase
MeJA	Methyl jasmonate
MV	Methyl viologen
OX	Overexpression
PR	Pathogenesis-related
SA	Salicylic acid
SNP	Sodium nitroprusside

The nucleotide sequence data reported here has been deposited in the GenBank database under the accession number DQ356279.

J. K. Hong  
Department of Horticulture,  
College of Life Sciences and Natural Resources,  
Jinju National University, 150 Chilamdong, Jinju,  
Kyungnam 660-758, Republic of Korea

B. K. Hwang (✉)  
Laboratory of Molecular Plant Pathology,  
School of Life Sciences and Biotechnology, Korea University,  
Anam-dong, Sungbuk-ku, Seoul 136-713, Republic of Korea  
e-mail: bkhwang@korea.ac.kr

## Introduction

Adverse environmental conditions such as pathogen and herbivore attack, or high salinity and drought stresses, limit plant growth and drastically reduce plant productivity. Biotic and abiotic stresses trigger depolarization of the plasma membrane and changes in membrane potentials, and plants respond by transmitting defense signals (Gelli et al. 1997; Pike et al. 1998; Krol et al. 2003). Plasma membrane-associated proteins, which are

involved in plant homeostasis and stress responses, comprise a subfamily of membrane proteins localized predominantly in organelles such as vacuoles, the Golgi apparatus, the endoplasmic reticulum, and the nucleus. Structurally distinct transmembrane domains have been found in plasma membrane proteins, and their number and location are highly variable among plant species. For example, two transmembrane domains have been found in *Arabidopsis* NDR1 (Century et al. 1997), and the barley Mlo protein and the cold-regulated wheat COR-413 protein were found to have seven and nine transmembrane domains, respectively (Breton et al. 2003; Bhat et al. 2005). Only transmembrane helix structures are sufficient to address the biological roles of plasma membrane proteins, including COR-413 and wpi6 (Breton et al. 2003; Imai et al. 2005).

Large proteins localized in plasma membranes are involved in plant stress tolerance in adverse environmental conditions (Shi and Zhu 2002; Véry and Sentenac 2002; Hussain et al. 2004; Takano et al. 2005). The rice Xa21 and tomato Cf-9 resistance proteins are involved in the recognition of bacterial avrXa21 and fungal Avr9 proteins, respectively, in plasma membrane to mediate rapid defense signaling. The plasma membrane-localized  $K^+$  and  $Ca^{2+}$  channels play a pivotal role in plant nutrition and cell signaling during abiotic stress (Véry and Sentenac 2002).  $Na^+$ / $H^+$  antiporters localized in plasma membrane and tonoplast are involved in maintaining cellular homeostasis during high salt stress (Shi and Zhu 2002; Shi et al. 2003; Yokoi et al. 2005), and overexpression of these antiporters confers salt tolerance in *Arabidopsis* (Aspe et al. 1999; Shi et al. 2003). The *Arabidopsis* P-type ATPase HMA2 and boron transporter BOR1, which regulate transport of these metals in the plasma membrane, are required for heavy metal homeostasis (Hussain et al. 2004; Takano et al. 2005). HMA2 was also suggested to influence cadmium detoxification (Hussain et al. 2004). More recently, *Arabidopsis* and rice proteins that belong to the integral and peripheral plasma membrane protein family have been identified by proteomics analyses (Alexandersson et al. 2004; Marmagne et al. 2004; Tanaka et al. 2004; Chen et al. 2007). However, the roles of plasma membrane proteins in cellular adaptation and developmental cues in plants are still poorly understood.

Several *cis*-acting promoter elements are indispensable for the regulation of defense-related gene expression during biotic and abiotic stress. These elements, such as the W-box, the GCC-box, and CRT/DRE, have been identified in stress- and hormone response-related promoters in several plant species and have been investigated by deletion analysis (Eyal et al. 1993; Eulgem et al. 1999, 2000; Shinozaki et al. 2003). The W-box (TTGACC), the ethylene-responsive GCC-box (AGCCGCC), and the salicylic acid-respon-

sive (SA) *as-1* element (TGACG) are also involved in plant disease defense (Eyal et al. 1993; Jupin and Chua 1996; Eulgem et al. 2000). The W-box is a binding site for transcription factors in the WRKY family. Ethylene-inducible pathogenesis-related (PR) gene expression is modulated by ERF transcription factors containing the AP2 domain, which specifically interact with a GCC-box characterized in *Arabidopsis* and tobacco (Ohta et al. 2000; Oñate-Sánchez and Singh 2002). Recently, several pepper (*Capsicum annuum*) PR promoters were isolated and functionally characterized by *Agrobacterium*-mediated transient expression in tobacco plants (Hong et al. 2005; Jung et al. 2005; Hong and Hwang 2006). However, there is little information about how defense-related promoters of plasma membrane protein genes are regulated in response to pathogen infection and abiotic elicitors.

We previously demonstrated that the pepper *CaPIMPI* gene, which encodes a plasma membrane protein, is differentially expressed in leaf tissues during compatible and incompatible interactions with *Xanthomonas campestris* pv. *vesicatoria* (Hong et al. 2008). Overexpression of *CaPIMPI* also alters resistance to bacterial and oomycete pathogens. In this study, we analyzed *CaPIMPI* expression and promoter activation by biotic and abiotic stimuli. We found that *CaPIMPI* transcripts accumulated in pepper leaf tissues upon treatment with abiotic defense elicitors. The *CaPIMPI* promoter region was essential for gene expression activated by pathogen infection and defense elicitor treatment. *CaPIMPI* overexpression (OX) in transgenic *Arabidopsis* plants altered osmotic and oxidative stress tolerance.

## Materials and methods

### Plants and growth conditions

Pepper (*Capsicum annuum*, cv. Nockkwang) seeds were sown in a soil mix (peat moss/perlite/vermiculite, 5/3/2, v/v/v). Plants were grown in a growth room at  $25 \pm 1^\circ\text{C}$  and  $70 \mu\text{mol photons/m}^2 \text{s}^{-1}$  illumination under a 16-h-light/8-h-dark regime. Plants at the 6-leaf stage were used for treatment with various agents. Tobacco (*Nicotiana tabacum*, cv. Xanthi-nc) seeds were sown in the same soil mix and grown under the same conditions. Tobacco leaves at the 6-leaf stage were used for *Agrobacterium*-mediated transient gene expression. Wild-type (Col-0) and *CaPIMPI*-overexpression (OX) transgenic *Arabidopsis thaliana* seeds (Hong et al. 2008) vernalized for 4 days at  $4^\circ\text{C}$  were sown on a potting soil mix (compost soil/perlite/vermiculite, 3/1/1, v/v/v). *Arabidopsis* plants were raised in a growth chamber at  $24^\circ\text{C}/19^\circ\text{C}$  (day/night) with a 12-h photoperiod ( $100 \mu\text{mol photons/m}^2 \text{s}^{-1}$ ).

## Pathogen inoculation

Tobacco leaves infiltrated with *Agrobacterium* harboring the binary vector pCAMBIA1381 with *CaPIMP1* promoter deletion constructs were inoculated with a suspension ( $2 \times 10^8$  cfu/ml) of *Pseudomonas syringae* pv. *tabaci* using a syringe without a needle. Control plants were mock-inoculated by infiltration with 10 mM MgCl<sub>2</sub>. Mock- and bacteria-infected tobacco plants were maintained in a moist chamber at 26°C for 12 h prior to the GUS activity assay.

## Isolation of genomic sequence and promoter of the *CaPIMP1* gene

Pepper genomic DNA was extracted from leaf tissues following the method of Hong et al. (2000). A genomic fragment containing the *CaPIMP1* gene was amplified by PCR using degenerate primers, primer, 5'-CTATTTTAGTTG AATAGACAAAGTGAA-3' (forward), and 5'-AAACA TAATTTCTCGAAACTG-3' (reverse), based on the 5'- and 3'-untranslated regions of the *CaPIMP1* cDNA. PCR amplification was performed with initial denaturation at 95°C for 2 min followed by 35 cycles of incubation at 95°C for 1 min, 54°C for 30 s, and 72°C for 2 min, with final extension at 72°C for 10 min. PCR products were cloned into the vector pCR2.1-TOPO (Invitrogen). Genomic DNA sequences were aligned and compared with the *CaPIMP1* cDNA nucleotide sequence. The Genome Walker Universal Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) was used to isolate the *CaPIMP1* promoter region with antisense *CaPIMP1*-specific primers, 5'-AGC ATAAAAGTCCTTAAACTTGTATTTGA-3' and 5'-AA ATGTTTCTGACAAAATTTTCATAGTTT-3' for primary and secondary nested PCR, respectively, according to the manufacturer's instructions. The generated PCR product was cloned into pCR2.1-TOPO and sequenced. *CaPIMP1* promoter sequences were analyzed by the PLACE Web Signal Scan program (Higo et al. 1999).

## Promoter deletion-GUS constructs

A *CaPIMP1-GUS* construct was generated by fusing a *CaPIMP1* promoter fragment (from -1193 to -1 bp, where the first nucleotide of the initiating ATG is designated +1) to the coding region of the *GUS* reporter gene in pCAMBIA 1381. Serially 5'-deleted *CaPIMP1-GUS* constructs were created by PCR, using the full-length promoter fragment as a template with the reverse oligonucleotide primer VI (5'-CCATGGTTCACCTTGTCTATTCAACT AAA-3', with a *NcoI* restriction site at the 5'-end) with five forward oligonucleotides: primer I (5'-GAATTCACCTGT GAGAAATAGTTTGTAGT-3'), primer II (5'-GAATTC CTTATTTCTTTCAAAGCTTA-3'), primer III (5'-GAA

TTCTATATTCGATCAATATTCAAGAA-3'), primer IV (5'-GAATTCCTTAATAGGATGAAAATACATA-3') or primer V (5'-GAATTCATTATGTTGTTTGAACAAC G-3'), each containing an *EcoRI* restriction site at the 5'-ends. Each fragment was digested with *EcoRI/NcoI* and subcloned into *EcoRI/NcoI*-digested pCAMBIA 1381 to generate five promoter deletion derivatives. All constructs were verified by nucleotide sequencing. Each promoter-GUS fusion construct was introduced into *Agrobacterium tumefaciens* strain EHA105 via electroporation.

## *Agrobacterium*-mediated transient expression assay

Assays of the *CaPIMP1* promoter-GUS constructs were performed in tobacco leaves using the method of Hong et al. (2005). *A. tumefaciens* EHA105 harboring each of the serially deleted promoter-GUS constructs was grown on yeast extract peptone medium (10 g yeast extract, 10 g Bacto peptone, 5 g NaCl, 15 g agar/l) supplemented with rifampicin (60 µg/ml) and kanamycin (50 µg/ml). *Agrobacterium* was cultured at 28°C and harvested by centrifugation for 15 min at 6,000×g, resuspended in infiltration media [0.1× MS salts, 0.1× B5 vitamins, 20 mM MOPS, pH 5.4, 1% (w/v) glucose, 2% (w/v) sucrose, 200 µM acetosyringone (Sigma-Aldrich, St Louis, MO)], and adjusted to an OD<sub>600</sub> of 0.7. After infiltration of *Agrobacterium* suspension into abaxial surfaces of tobacco leaves using a syringe without a needle (Kim et al. 2007), the tobacco plants were maintained in a moist chamber at 26°C for 48 h, followed by *P. syringae* pv. *tabaci* inoculation and abiotic elicitor treatments for GUS activity analysis.

## GUS activity measurement

GUS activity in *Agrobacterium*-mediated, transiently expressed tobacco leaves was measured as described by Jefferson et al. (1987). Tobacco leaf tissues were homogenized in 1 ml extraction buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% (w/v) sodium laurylsarcosine, 10 mM β-mercaptoethanol]. After centrifuging for 10 min at 12,000×g at 4°C, the supernatant was transferred to a fresh microtube. The fluorogenic reaction was carried out in a 1-ml volume with 1 mM 4-methylumbelliferyl-β-D-glucuronide (Duchefa Biochemie, Haarlem, The Netherlands) in the extraction buffer supplemented with a 0.1-ml aliquot of protein extract supernatants. GUS activity was normalized to protein concentration in each of the crude extracts and was expressed as nmol 4-methylumbelliferone min/mg protein. Total protein in sample extracts was quantified using bovine serum albumin as a standard, according to the method of Bradford (1976). The GUS measurement was repeated at least three times with similar results.

## Abiotic elicitor treatments

Ethylene treatment was performed by placing the pepper plants in a tight glass chamber, where 5  $\mu\text{l/l}$  ethylene was applied by injecting the gas, via a syringe, through a rubber septum in the chamber. Pepper plants sprayed with 100  $\mu\text{M}$  methyl jasmonate (MeJA) were packed in a vinyl bag. For abscisic acid (ABA) treatment, the pepper plants were removed from soil. The roots were carefully washed with tap water and then soaked in 100  $\mu\text{M}$  ABA. For salicylic acid (SA) treatment, 5 mM SA was foliar-sprayed onto pepper plants. For  $\beta$ -amino-*n*-butyric acid (BABA) treatment, 20 mM BABA in water was foliar-sprayed onto pepper plants. For wounding stress, the leaves were pricked with a needle. For low-temperature treatment, pepper plants were placed at 4°C. For NaCl treatment, pepper plants grown in plastic pots containing compost soil mix were gently removed from the soil and their roots were immersed in 400 mM NaCl. At various time points, pepper leaves treated with abiotic elicitors were harvested, frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$  until used for RNA blot analyses.

To investigate the activation of the *CaPIMPI* promoter by treatment with abiotic elicitors, tobacco leaves infiltrated with *Agrobacterium* harboring *CaPIMPI* promoter-*GUS* constructs were sprayed with 100  $\mu\text{M}$  ABA or 100  $\mu\text{M}$  sodium nitroprusside (SNP). Tobacco plants were sprayed with water as a mock-treatment. To monitor ethylene responsiveness of the *CaPIMPI* promoter, 10  $\mu\text{l/l}$  of ethylene gas was injected into a glass chamber containing tobacco plants. Tobacco plants sprayed with 100  $\mu\text{M}$  MeJA were sealed with a transparent plastic bag. Treated tobacco plants were placed in a growth room for 12 h and then immediately frozen in liquid nitrogen for GUS activity assays.

## RNA gel blot analysis

Total RNA was isolated from pepper and *Arabidopsis* using the guanidium-acid phenol method (Chomczynski and Sacchi 1987; Chung et al. 2007) and Trizol reagent (Invitrogen, Carlsbad, CA, USA), respectively. Ten micrograms of total RNA was separated on 1.2% agarose/formaldehyde gels, blotted onto Tropilon-Plus nylon membranes positively charged (Applied Biosystems, Bedford, MA, USA), and hybridized overnight with 14-dCTP-biotin-labeled *CaPIMPI* cDNA (accession no. DQ356278) in the hybridization buffer (1 mM EDTA, 7% SDS, 250 mM  $\text{Na}_2\text{HPO}_4$ , and 5% dextran sulfate) at 65°C. After hybridization, the nylon membranes were washed as previously described (Hong et al. 2005). Biotin was detected via chemiluminescence with CDP-*Star* substrate according to manufacturer's protocol (Applied Biosystems, Bedford, MA, USA). The

membranes were exposed to X-ray film. All RNA blot analyses were repeated at least three times.

## Evaluation of *Arabidopsis* responses to abiotic elicitors

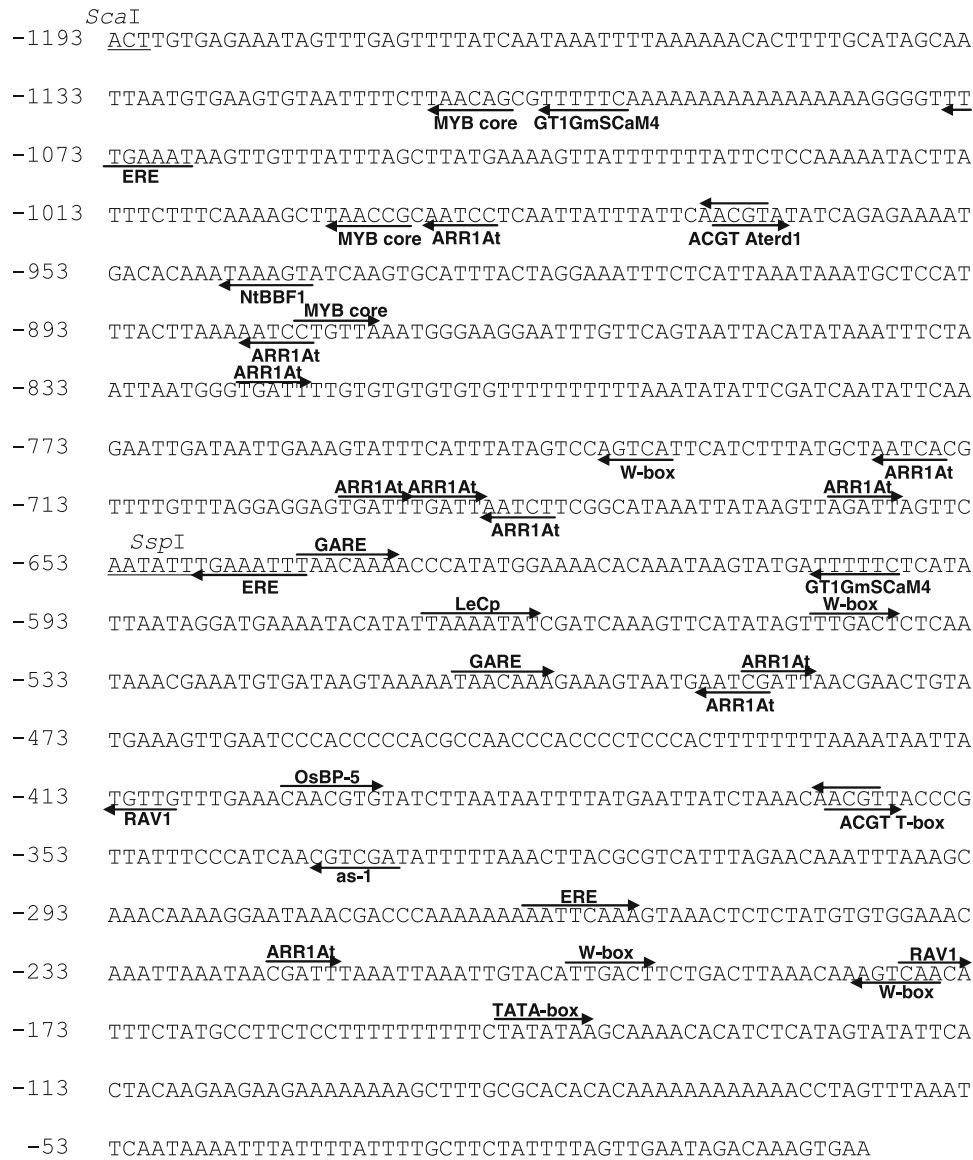
*Arabidopsis* seeds sown on basal MS medium containing 400 mM mannitol, 200 mM NaCl, and 2.5  $\mu\text{M}$  ABA were maintained at 4°C for 4 days, and germination (emergence of radicles) was scored daily. *Arabidopsis* seedlings were grown in 1 $\times$  MS agar medium supplemented with 1% sucrose in a growth chamber for 4 days after sowing and transferred to 1 $\times$  MS agar medium supplemented with mannitol, NaCl, or ABA. *Arabidopsis* seeds sown on MS medium containing 100  $\mu\text{M}$  methyl viologen (MV) were maintained at 4°C for 2–4 days, and germination (emergence of radicles) was scored daily. To monitor seedling development, *Arabidopsis* seedlings were grown in 1 $\times$  MS agar medium supplemented with 1% sucrose in a growth chamber for 7 days after sowing and transferred to 1 $\times$  MS liquid medium supplemented with MV at different concentrations. Germination and seedling growth assays were repeated at least three times with similar results.

## Results

### Sequence analysis of the *CaPIMPI* gene

The *CaPIMPI* genomic sequence was isolated and compared with the *CaPIMPI* cDNA sequence, which revealed that it contains three exons and two introns of 698 and 859 bp in length (data not shown). All deduced intron/exon junctions possess the consensus GT/AG splice sites. The nucleotide sequence data in this study appear under the accession number DQ356279 in the DDBJ/EMBL/GenBank nucleotide database.

An upstream region including the putative promoter sequence of the *CaPIMPI* gene was isolated from pepper genomic DNA, and sequence analysis with the PLACE program revealed several motifs that are found in most eukaryotic promoters for gene expression and regulation (Fig. 1). Potential regulatory elements associated with hormone- and stress-related responses found in other plant promoters were located within the *CaPIMPI* promoter: two GT-1 elements, three MYB transcription factor-binding sites, four W-boxes, three ethylene responsive elements (EREs), two ACGT elements, eleven cytokinin-regulated transcription factor ARR1-binding sites, and two gibberellin-responsive elements. The presence of these motifs indicates that *CaPIMPI* may be regulated by various *cis*-acting elements within the promoter as well as corresponding *trans*-acting factors.



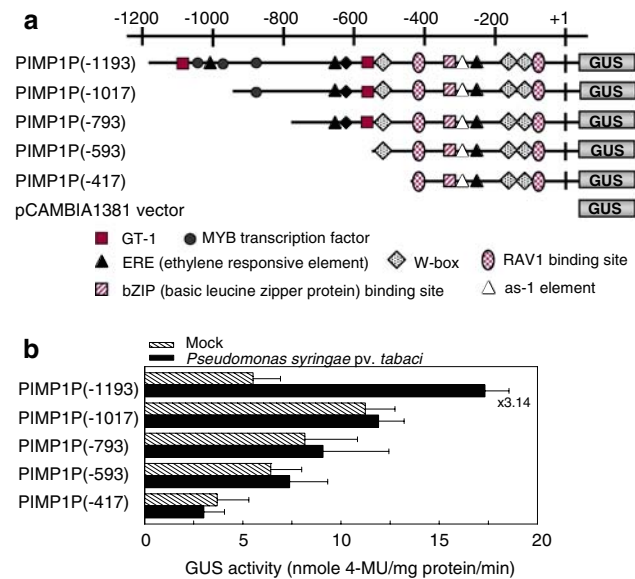
**Fig. 1** Nucleotide sequence of 5'-flanking promoter regions and putative *cis*-acting elements of the *CaPIMP1* gene. ACGTATERd1, ACGT sequence required for the etiolation-induced expression of *erd1* (early responsive to dehydration) in *Arabidopsis* (Simpson et al. 2003); ARR1At, ARR1-binding element (Sakai et al. 2000; Ross et al. 2004); BP5, OsBP-5 (a MYC protein) binding site in the rice *Wx* promoter (Zhu et al. 2003); ERE, ethylene-responsive element of the tomato *E4* and carnation *GST1* genes (Montgomery et al. 1993; Itzhaki et al. 1994); GARE, GA-responsive element (Ogawa et al. 2003); GT1GmSCaM4, GT-1 motif found in the promoter of soybean CaM

isoform, SCaM-4 (Park et al. 2004); LeCp, TAAATAT element in the *LeAcS2* gene (Matarasso et al. 2005); MYB core, binding site for all animal MYBs and the *Arabidopsis* MYB proteins AtMYB1 and AtMYB2 (Urao et al. 1993); NtBBF1, tobacco Dof protein binding site in the *Agrobacterium rhizogenes rolB* gene (Baumann et al. 1999); RAV1At, binding consensus sequence for the *Arabidopsis* transcription factor RAV1 (Kagaya et al. 1999); W-box, binding site for the WRKY transcription factor (Eulgem et al. 2000)

Activation of the *CaPIMP1* promoter by bacterial infection and defense signaling molecules

To determine the minimal promoter sequence of the *CaPIMP1* gene required for promoter activity, five promoter fragments beginning -1,193, -1,017, -793, -593, and -417 bp upstream of the translational initiation site were fused to the *GUS* reporter gene (Fig. 2a). Tobacco

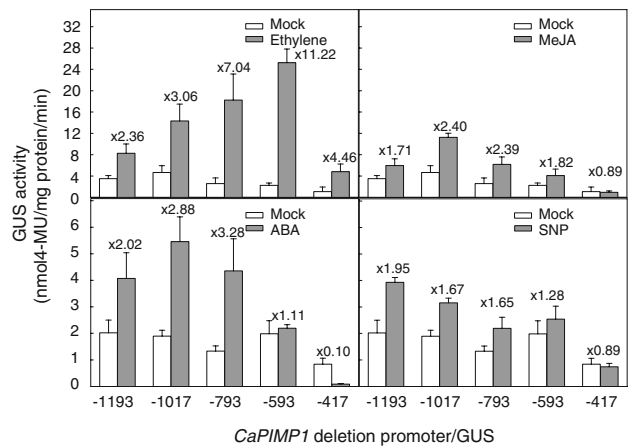
leaves were infiltrated with *Agrobacterium* harboring these constructs, and GUS activity expressed in response to bacterial infection and various signal molecules was analyzed by quantitative fluorometry. Twelve hours after inoculation with *P. syringae* pv. *tabaci* (Fig. 2b), tobacco leaf tissues harboring the -1,193 bp promoter construct exhibited a threefold higher GUS activity than did mock-inoculated leaves. However, further deletion of the promoter permitted



**Fig. 2** **a** Schematic representation of *CaPIMP1* promoter constructs for assaying GUS ( $\beta$ -glucuronidase) expression in tobacco leaves. The serially 5'-deleted promoter constructs of the *CaPIMP1* gene were fused to the *GUS* reporter gene in the vector pCambia1381. **b** *CaPIMP1* promoter activation in response to *Pseudomonas syringae* pv. *tabaci* infection in tobacco leaf tissues transiently transformed with 5'-*CaPIMP1*-*GUS* chimeric constructs. Tobacco leaves were infiltrated with a bacterial suspension of *P. syringae* pv. *tabaci* ( $2 \times 10^8$  cfu/ml 10 mM  $MgCl_2$ ) or with 10 mM  $MgCl_2$  as a mock-inoculation. GUS activity was analyzed fluorometrically and expressed as nmoles 4-methylumbelliferone (MU)/mg protein  $min^{-1}$ . Data are means  $\pm$  standard deviations from three independent assays of tobacco leaf extracts

induction of GUS activity in response to *P. syringae* pv. *tabaci*.

Treatments with ethylene, MeJA, ABA, and SNP for 20 h proved sufficient to trigger GUS expression driven by *CaPIMP1* promoter constructs (Fig. 3). Ethylene treatment distinctively induced expression driven by all promoter regions between  $-1,193$  and  $-417$  bp. Promoter deletion to  $-1,017$  bp led to a twofold induction of GUS activity. Further deletions to  $-793$  and  $-593$  bp were more effective for ethylene-mediated GUS activation, resulting in 7- and 11-fold increases, respectively. The  $-417$  bp promoter construct showing a 4.5-fold increase also was sufficient for the induction of GUS activity by ethylene. The ethylene-induced GUS activity levels driven by the *CaPIMP1* promoter were relatively higher than those by other signal molecules, such as ABA, SNP, and MeJA. All *CaPIMP1* promoter fusions except for the  $-417$  bp construct were responsive to MeJA treatment. ABA induced a twofold increase in GUS activity in tobacco leaves harboring the  $-1,193$  bp promoter construct. Deletion to  $-1,017$  and  $-793$  bp regions resulted in roughly threefold increases in GUS activity by ABA treatment. However, significant GUS activity was not observed in ABA-treated tobacco leaves



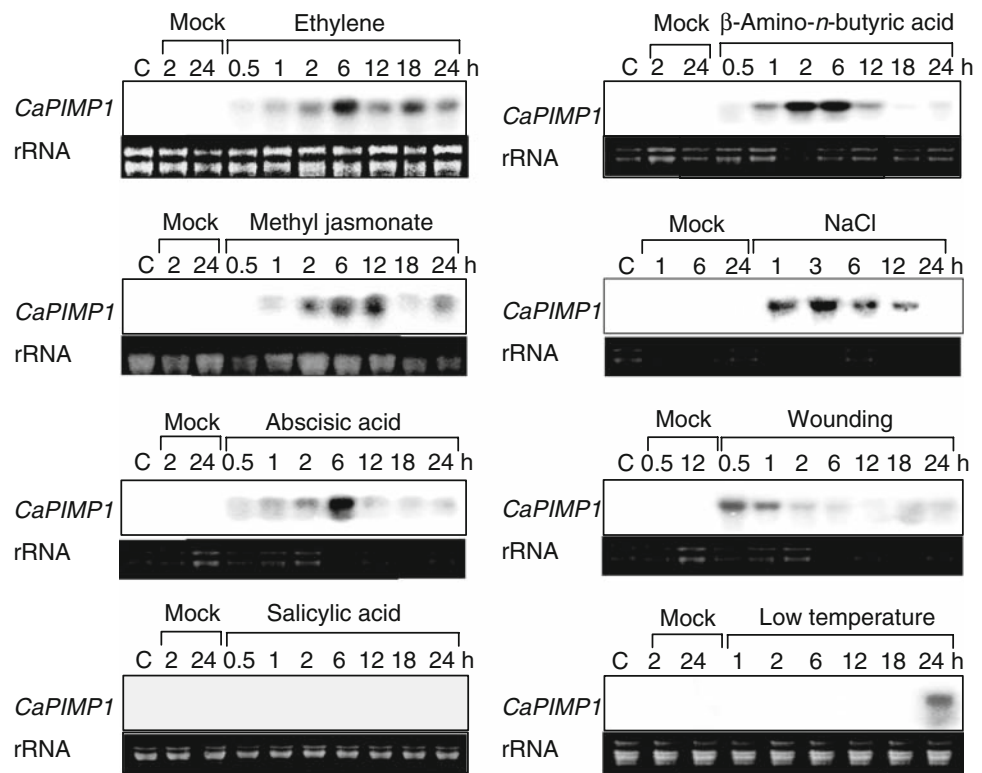
**Fig. 3** *CaPIMP1* promoter activation in response to ethylene, methyl jasmonate (MeJA), abscisic acid (ABA), and sodium nitroprusside (SNP) applied to tobacco leaf tissues transiently transformed with *CaPIMP1*-*GUS* constructs. The numbers over the bars indicate the fold increase in induction of GUS activity after chemical treatment versus mock-treatment. Data are means  $\pm$  standard deviations from three independent assays of tobacco leaf extracts

harboring the  $-593$ - and  $-417$  bp *CaPIMP1* promoter fusions. Treatment with SNP, a nitric oxide donor, also induced GUS expression in tobacco leaves harboring the  $-1,193$ ,  $-1,017$ , and  $-793$  bp regions of the *CaPIMP1* promoter. SNP-induced GUS activity gradually decreased following further promoter deletion to  $-417$  bp. GUS activity was abolished by deletion of the *CaPIMP1* promoter to  $-593$  and  $-417$  bp.

*CaPIMP1* gene expression in pepper leaves treated with abiotic elicitors

To evaluate the effect of signal molecules on *CaPIMP1* expression, ethylene, MeJA, ABA, SA, and BABA were exogenously applied to pepper plants at the 6-leaf stage (Fig. 4). Treatment with ethylene, MeJA, ABA, and BABA activated the *CaPIMP1* gene. Transcription began 1 h after ethylene treatment, increased up to 6 h and slightly decreased over 24 h. *CaPIMP1* expression was also transiently induced 2–12 h after treatment with MeJA and ABA. *CaPIMP1* transcripts were detected 1 h after BABA treatment, with a peak of induction at 2–6 h. To determine whether environmental stresses affect *CaPIMP1* expression, pepper plants were exposed to NaCl, wounding, and low temperature. The *CaPIMP1* gene was rapidly activated within 1 h after NaCl treatment, and expression drastically declined by 12 h and disappeared by 24 h. The *CaPIMP1* gene was markedly expressed within 30 min following mechanical wounding, and thereafter gradually diminished in pepper leaf tissues. In response to cold stress, *CaPIMP1* transcripts accumulated in pepper leaves 24 h after low-temperature treatment.

**Fig. 4** RNA gel blot analysis of *CaPIMP1* expression in leaf tissues of pepper plants treated with *ethylene*, *MeJA*, *ABA*, *SA*, *BABA*, *mechanical wounding*, and *low temperature*. The rRNA in agarose gels was stained with ethidium bromide to show equal loading of RNA. Similar results were obtained in three independent experiments. *C* healthy controls



**Enhanced sensitivity to osmotic stress and ABA of *CaPIMP1*-OX *Arabidopsis***

We monitored seed germination and seedling growth of *Arabidopsis* to examine the responses of transgenic plants to osmotic stress and ABA (Fig. 5). Most (80–90%) wild-type seeds germinated in 400 mM mannitol, 200 mM NaCl, and 2.5 μM ABA within 7 days after sowing, whereas germination of *CaPIMP1*-OX transgenic seeds was severely reduced under the same conditions (Fig. 5a). In contrast, there was no difference in development for wild-type and transgenic seedlings when they were treated with mannitol, NaCl, or ABA (Fig. 5b).

**Enhanced tolerance to oxidative stress of *CaPIMP1*-OX *Arabidopsis***

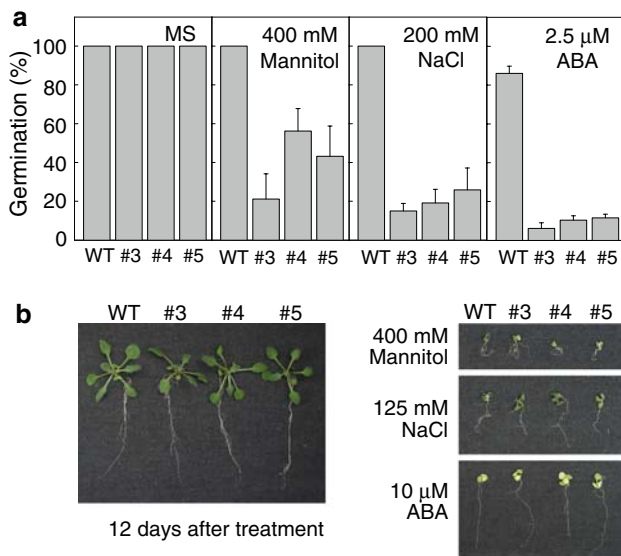
Methyl viologen (MV), a redox-cycling herbicide that propagates cellular reactive oxygen species, was used to evaluate the tolerance of *CaPIMP1*-OX transgenic plants to oxidative stress. Transgenic plants were more resistant than wild-type plants to MV-mediated oxidative stress during the germination and early seedling stages. Following 100 μM MV treatment, the transgenic lines germinated to a higher extent than did the wild-type seeds (Fig. 6a): after 4 days, 75% of the transgenic seeds but only 10% of the wild-type seeds germinated. Lower dosages (0.4–0.8 μM) of MV severely retarded post-germination growth of both

wild-type and transgenic plants, and this effect was more pronounced in wild-type plants (Fig. 6b, c). Some wild-type seedlings became bleached and died. Oxidative stress responses of 4-day-old wild-type and transgenic seedlings were not distinctively different after treatment with 0.5 μM MV (Fig. 6d). However, transgenic lines #4 and #5 exhibited slightly higher tolerance to 1.0 μM MV compared to the wild-type plants.

**Discussion**

To elucidate the molecular basis of *CaPIMP1* gene expression, we analyzed its genomic organization and promoter activity in this study. We further investigated the biological functions of *CaPIMP1* during osmotic and oxidative stresses in *CaPIMP1*-OX transgenic *Arabidopsis* plants.

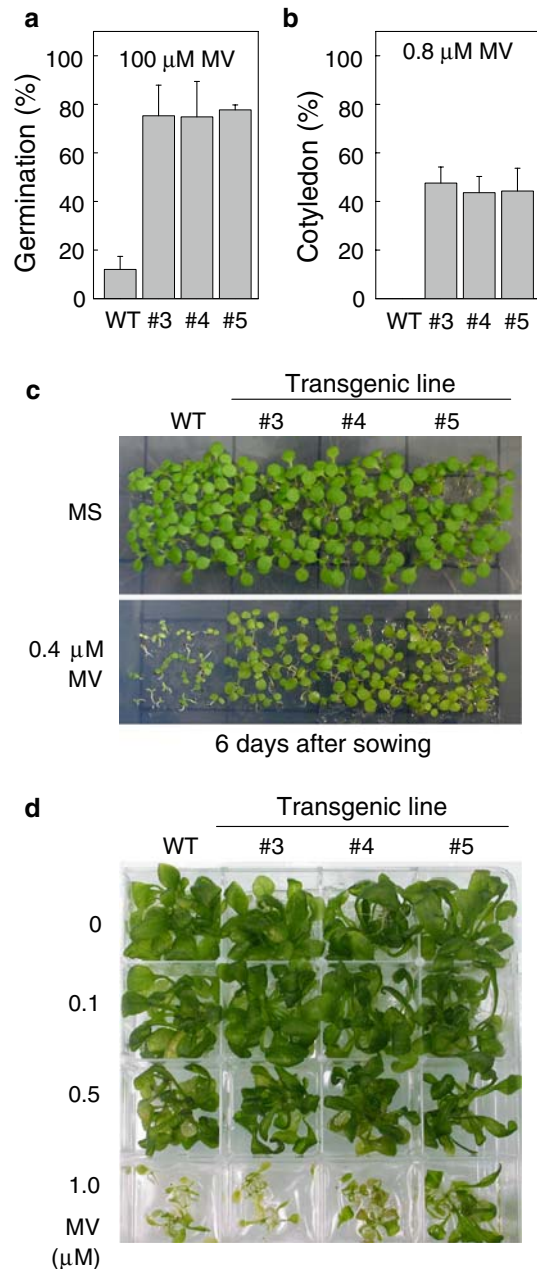
*CaPIMP1* expression was induced by pathogen and abiotic elicitors. Several putative *cis*-acting elements, such as the ACGT-box and W-box, were found by computational analysis to reside in the *CaPIMP1* promoter, and these elements may be responsible for *CaPIMP1* expression by pathogen infection and abiotic elicitors. The –1,193 bp *CaPIMP1* promoter was sufficient to drive GUS activity in tobacco leaf tissues infected with *P. syringae* pv. *tabaci*. *Cis*-acting elements essential for activation in response to *P. syringae* pv. *tabaci* infection may reside between –1,193 and –1,017 bp. Only a GT-1 element identified in



**Fig. 5** Enhanced sensitivity to osmotic stresses and abscisic acid (ABA) of *CaPIMP1*-OX transgenic *Arabidopsis* plants during germination and early seedling development. **a** Germination rates of wild-type (WT) and transgenic lines #3, #4 and #5 on  $1\times$  MS medium containing 400 mM mannitol, 200 mM NaCl and 2.5  $\mu$ M ABA 6 days after sowing. Germination was scored when the radicle tips had fully emerged from the seed coats. The data are the mean  $\pm$  standard deviations of three independent experiments in the evaluation of 100 seeds. **b** Seedling development of wild-type and transgenic lines on  $1\times$  MS agar medium containing 400 mM mannitol, 125 mM NaCl and 10  $\mu$ M ABA 12 days after sowing

the soybean calmodulin gene promoter activated by pathogen infection and NaCl stress was also found within the *CaPIMP1* promoter region from  $-1,103$  to  $-1,098$  bp (Park et al. 2004). The presence of this GT-1 element suggests that it may function in *CaPIMP1* promoter activation in response to bacterial infection. Another GT-1 element was also identified at  $-605$  to  $-599$  bp, indicating that this GT-1 element may not be sufficient for *CaPIMP1* promoter activation by *P. syringae* pv. *tabaci* infection. Three W-boxes and one as-1 element were found within the  $-1017$  *CaPIMP1* promoter region. These elements have been suggested to be binding sites for the SA-dependent and pathogen-induced transcription factors WRKY and TGA, respectively (Jupin and Chua 1996; Eulgem et al. 2000). However, promoter constructs containing these *cis*-acting elements were not activated by *P. syringae* pv. *tabaci* infection.

In this study, the minimal promoter region was demonstrated to be differently located for *CaPIMP1* activation by abiotic elicitors, ABA, SNP, ethylene, and MeJA. The ABA-responsive, bZIP transcription factor-binding ACGT-box, and EREs were found in the *CaPIMP1* promoter region. The  $-593$  bp deletion construct did not respond to ABA treatment, although there are ABA-responsive bZIP and MYB binding sites in this region



**Fig. 6** Enhanced tolerance to oxidative stress of *CaPIMP1*-OX transgenic *Arabidopsis* plants during germination and early seedling development. **a** Germination rates of wild-type (WT) and transgenic lines on  $1\times$  MS medium containing 100  $\mu$ M methyl viologen (MV) 6 days after sowing. The data are the means  $\pm$  standard deviations of three independent experiments in the evaluation of 100 seeds. **b** Cotyledon formation of wild-type (WT) and transgenic lines on  $1\times$  MS medium containing 0.8  $\mu$ M MV 6 days after sowing. The data are the means  $\pm$  standard deviations of three independent experiments in the evaluation of 40 seedlings. **c** Seedling development of wild-type (WT) and transgenic lines on  $1\times$  MS medium containing 0.4  $\mu$ M MV 6 days after sowing. **d** MV tolerance of seedling plants of transgenic lines. Wild-type and transgenic lines were germinated and grown in  $1\times$  MS agar medium in the absence of MV for 4 days. Seedlings were transplanted to liquid medium containing different MV concentrations. Photographs were taken after exposure to MV for 12 days



(Urao et al. 1993, 2000). This observation suggests that the bZIP element identified within the *CaPIMPI* promoter may not function in the activation of *CaPIMPI* deletion promoters. Three EREs were found in the *CaPIMPI* promoter. A 5'-deletion of the *CaPIMPI* promoter to -593 bp resulted in a gradual induction of ethylene-responsive promoter activity, indicating that putative *cis*-acting elements bound by transcriptional repressors may exist between -1,193 and -593 bp. These transcriptional repressors may tightly control *CaPIMPI* gene expression by ethylene-mediated signaling. Deletion of the promoter to -417 bp drastically reduced ethylene-responsive promoter activity. An ERE in the -257 bp fragment was sufficient to activate the promoter; however, a lack of putative ERE(s) between -593 and -417 bp responsible for *CaPIMPI* promoter activation may reduce promoter activity. Interestingly, the -793 deletion retained the ERE at the -640 bp site, leading to a gradual increase in promoter activity. The GCC-box-like jasmonic acid-responsive element or other jasmonic acid-responsive elements (Menke et al. 1999; Xu and Timko 2004) were not found in the *CaPIMPI* promoter region. However, the *CaPIMPI* promoter was sufficient for MeJA-induced activation, suggesting that there are unidentified novel jasmonic acid-responsive *cis*-acting elements in the *CaPIMPI* gene promoter region. Synergistic and antagonistic interactions of various *cis*-acting elements for *CaPIMPI* promoter activation remain to be elucidated.

Plasma membrane proteins are involved in the recognition and transduction of endogenous hormonal signals (Blakeslee et al. 2005). *CaPIMPI* expression may be dependent on ethylene, MeJA, and ABA. However, SA had no effect on *CaPIMPI* gene expression in pepper leaves. Induction of disease resistance-related plasma membrane proteins by plant hormones has not been reported. Cold-regulated plasma membrane protein genes are induced in wheat and rice by ABA treatment (Breton et al. 2003; Imai et al. 2005; Morsy et al. 2005). Inducible *CaPIMPI* may be efficient at mediating and enhancing plant defense responses against abiotic stresses.

Environmental stresses, including wounding or exposure to low temperature or high NaCl induced *CaPIMPI* expression in pepper plants. Multispanning transmembrane proteins in several plant species have been shown to be regulated by cold stress (Breton et al. 2003). Pathogenesis-related genes isolated from pepper plants were shown to be induced by exogenous hormone treatment and environmental stresses (Jung et al. 2003; Lee and Hwang 2005; Hong and Hwang 2005, 2006). Analysis of transgenic *Arabidopsis* overexpressing basic PR-1, chitinase, lipid transfer protein, and the Cys2/His2 zinc-finger transcription factor indicates that these pepper pathogenesis-related proteins are involved in environmental stress tolerance

(Kim et al. 2004; Hong and Hwang 2005, 2006; Jung et al. 2005; Lee and Hwang 2006). Recently, we found that *CaPIMPI* is also rapidly induced by infection with *X. campestris* pv. *vesicatoria*, and that *CaPIMPI* overexpression in transgenic *Arabidopsis* alters disease resistance (Hong et al. 2008). These studies support the possibility that the CaPIMPI protein is also involved in abiotic stress signaling in pepper plants, as well as in disease resistance.

Overexpression of *CaPIMPI* in transgenic *Arabidopsis* results in increased bacterial resistance to *P. syringae* pv. *tomato*, but enhanced disease susceptibility to the oomycete biotroph *Hyaloperonospora parasitica* (Hong et al. 2008), suggesting distinct roles for *CaPIMPI* in diverse interactions of pathogens with host plants. Interestingly, ectopic expression of the *CaPIMPI* gene in *Arabidopsis* also caused altered responses to high osmotic stress and oxidative damage during germination and seedling development in this study. *CaPIMPI* overexpression in transgenic plants may negatively regulate ABA-related signaling, but positively enhance oxidative stress signaling. Interestingly, negative effect of *CaPIMPI*-overexpression on ABA-mediated signaling was only shown at the seed germination stage, which may be due to the difference in physiology between germination and seedling growth. It is not evident why overexpression of *CaPIMPI* results in increased tolerance to oxidative stress. The *CaPIMPI* gene may participate in oxidative burst-mediated disease resistance, which is supported by previous studies of environmental stress perception and of plant antioxidant systems (Foyer and Noctor 2005). Oxidative damage in plants caused by MV may be due to the excess generation of superoxide radicals, which are normally detoxified into oxygen and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by superoxide dismutase (Apel and Hirt 2004). Nevertheless, exogenous application of H<sub>2</sub>O<sub>2</sub>, causing oxidative stress, did not distinctively affect the germination of *CaPIMPI*-OX *Arabidopsis* seeds and early seedling development (data not shown), suggesting that CaPIMPI may function differently against different sources of reactive oxygen species in plant cells.

In conclusion, we suggest that *CaPIMPI* promoter activation by pathogen infection and abiotic elicitor treatment is sufficient to regulate both disease resistance and abiotic stress tolerance in plants. The *CaPIMPI* gene may be involved in plant tolerance to a broad spectrum of plant stresses. Further dissection of the *CaPIMPI* promoter will reveal the presence of unidentified *cis*-acting elements for promoter activation by pathogen- and abiotic stimuli. Together with our previous studies of altered disease resistance of *CaPIMPI*-OX *Arabidopsis*, these findings emphasize the need to continue elucidating the distinct roles of *CaPIMPI* in disease resistance and abiotic stress tolerance.

**Acknowledgments** This research was supported by a grant (CG1133) from the Crop Functional Genomics Center of the 21st Century, Frontier Research Program, funded by the Ministry of Science and Technology, Korea.

## References

- Alexandersson E, Saalbach G, Larsson C, Kjellbom P (2004) *Arabidopsis* plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Physiol* 45:1543–1556
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399
- Aspe MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter in *Arabidopsis*. *Science* 285:1256–1258
- Baumann K, De Paolis A, Costantino P, Gualberti G (1999) The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the rolB oncogene in plants. *Plant Cell* 11:323–334
- Bhat RA, Miklis M, Schmelzer E, Schulze-Lefert P, Panstruga R (2005) Recruitment and interaction dynamic of plant penetration resistance components in a plasma membrane microdomain. *Proc Natl Acad Sci USA* 102:3135–3140
- Blakeslee JJ, Peer WA, Murphy AS (2005) Auxin transport. *Curr Opin Plant Biol* 8:494–500
- Bradford MM (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Breton G, Danyluk J, Charron JBF, Sarhan F (2003) Expression profiling and bioinformatics analyses of a novel stress-regulated multispansing transmembrane protein family from cereals and *Arabidopsis*. *Plant Physiol* 132:64–74
- Century KS, Shapiro AD, Repetti PP, Dahlbeck D, Holub E, Staskawicz BJ (1997) *NDR1*, a pathogen-induced component required for *Arabidopsis* disease resistance. *Science* 278:1963–1965
- Chen F, Yuan Y, Li Q, He Z (2007) Proteomic analysis of rice plasma membrane reveals proteins involved in early defense response to bacterial blight. *Proteomics* 7:1529–1539
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
- Chung E, Oh SK, Park JM, Choi D (2007) Expression and promoter analyses of pepper *CaCDPK4* (*Capsicum annuum* calcium dependent protein kinase 4) during plant defense response to incompatible pathogen. *Plant Pathol J* 23:76–89
- Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. *Trends Plant Sci* 5:199–206
- Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE (1999) Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. *EMBO J* 18:4689–4699
- Eyal Y, Meller Y, Lev-Yadan S, Fluhr R (1993) A basic-type PR-1 promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. *Plant J* 4:225–234
- Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17:1866–1875
- Gelli A, Higgins VJ, Blumwald E (1997) Activation of plant plasma membrane Ca<sup>2+</sup>-permeable channels by race-specific fungal elicitors. *Plant Physiol* 113:269–279
- Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database. *Nucleic Acids Res* 27:297–300
- Hong JK, Choi DS, Kim SH, Yi SY, Kim YJ, Hwang BK (2008) Distinct roles of the pepper pathogen-induced membrane protein gene *CaPIMPI* in bacterial disease resistance and oomycete disease susceptibility. *Planta* 228:485–497
- Hong JK, Hwang BK (2005) Functional characterization of PR-1 protein,  $\beta$ -1, 3-glucanase and chitinase genes during defense response to biotic and abiotic stresses in *Capsicum annuum*. *Plant Pathol J* 21:195–206
- Hong JK, Hwang BK (2006) Promoter activation of pepper class II basic chitinase gene, *CACHi2*, and enhanced bacterial disease resistance and osmotic stress tolerance in the *CACHi2*-overexpressing *Arabidopsis*. *Planta* 223:433–448
- Hong JK, Jung HW, Kim YJ, Hwang BK (2000) Pepper gene encoding a basic class II chitinase is inducible by pathogen and ethephon. *Plant Sci* 159:39–49
- Hong JK, Lee SC, Hwang BK (2005) Activation of pepper basic PR-1 gene promoter during defense signaling to pathogen, abiotic and environmental stresses. *Gene* 356:169–180
- Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. *Plant Cell* 16:1327–1339
- Imai R, Koike M, Sutoh K, Kawakami A, Torada A, Oono K (2005) Molecular characterization of a cold-induced plasma membrane protein gene from wheat. *Mol Genet Genomics* 274:445–453
- Itzhaki H, Maxson JM, Woodson WR (1994) An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-S-transferase (*GSTI*) gene. *Proc Natl Acad Sci USA* 91:8925–8929
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 13:3901–3907
- Jung HW, Kim KD, Hwang BK (2005) Identification of pathogen-responsive regions in the promoter of a pepper lipid transfer protein gene (*CALTP1*) and the enhanced resistance of the *CALTP1* transgenic *Arabidopsis* against pathogen and environmental stresses. *Planta* 221:361–373
- Jung HW, Kim WB, Hwang BK (2003) Three pathogen-inducible genes encoding lipid transfer protein from pepper are differentially activated by pathogens, abiotic, and environmental stresses. *Plant Cell Environ* 26:915–928
- Jupin I, Chua NH (1996) Activation of the CaMV *as-1 cis*-element by salicylic acid: Differential DNA-binding of a factor related to TGA1a. *EMBO J* 15:5679–5689
- Kagaya Y, Ohmiya K, Hattori T (1999) RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. *Nucleic Acids Res* 27:470–478
- Kim BS, Kim YC, Shin KS, Kim JH (2007) Near-isogenic lines for genes conferring hypersensitive resistance to bacterial spot in chili pepper. *Plant Pathol J* 23:155–160
- Kim SH, Hong JK, Lee SC, Sohn KH, Jung HW, Hwang BK (2004) *CAZFP1*, Cys<sub>2</sub>/His<sub>2</sub>-type zinc-finger transcription factor gene functions as a pathogen-induced early-defense gene in *Capsicum annuum*. *Plant Mol Biol* 55:883–904
- Krol E, Dziubinska H, Trebacz K (2003) Low-temperature induced transmembrane potential changes in liverwort *Conocephalum conicum*. *Plant Cell Physiol* 44:527–533
- Lee SC, Hwang BK (2005) Induction of some defense-related genes and oxidative burst is required for the establishment of systemic acquired resistance in *Capsicum annuum*. *Planta* 221:790–800
- Lee SC, Hwang BK (2006) CASAR82A, a pathogen-induced pepper SAR8.2, exhibits an antifungal activity and its overexpression enhances disease resistance and stress tolerance. *Plant Mol Biol* 61:95–109

- Marmagne A, Roue MA, Ferro M, Rolland N, Alcon C, Joyard J, Garin J, Barbier-Brygoo H, Ephritikhine G (2004) Identification of new intrinsic proteins in *Arabidopsis* plasma membrane proteome. *Mol Cell Proteomics* 3:675–691
- Matarasso N, Schuster S, Avni A (2005) A novel plant cysteine protease has a dual function as a regulator of 1-aminocyclopropane-1-carboxylic Acid synthase gene expression. *Plant Cell* 17:1205–1216
- Menke FLH, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *EMBO J* 18:4455–4463
- Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL (1993) Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. *Proc Natl Acad Sci USA* 90:5939–5943
- Morsy MR, Almutairi AM, Gibbons J, Yun SJ, de los Reyes BG (2005) The *OsLti6* genes encoding low-molecular-weight membrane proteins are differentially expressed in rice cultivars with contrasting sensitivity to low temperature. *Gene* 344:171–180
- Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S (2003) Gibberellin biosynthesis and response during *Arabidopsis* seed germination. *Plant Cell* 15:1591–1604
- Ohta M, Ohme-Takagi M, Shinshi H (2000) Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. *Plant J* 22:29–38
- Oñate-Sánchez L, Singh KB (2002) Identification of *Arabidopsis* ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. *Plant Physiol* 128:1313–1322
- Park HC, Kim ML, Kang YH, Jeon JM, Yoo JH, Kim MC, Park CY, Jeong JC, Moon CB, Lee JH, Yoon HW, Lee SH, Chung WS, Lim CO, Lee SY, Hong JC, Cho MJ (2004) Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol* 135:2150–2161
- Pike SM, Ádám AL, Pu XA, Hoyos ME, Laby R, Beer SV, Novacky A (1998) Effects of *Erwinia amylovora* harpin on tobacco leaf cell membranes are related to leaf necrosis and electrolyte leakage and distinct from perturbations caused by inoculated *E. amylovora*. *Physiol Mol Plant Pathol* 53:39–60
- Ross EJ, Stone JM, Elowsky CG, Arredondo-Peter R, Klucas RV, Sarath G (2004) Activation of the *Oryza sativa* non-symbiotic haemoglobin-2 promoter by the cytokinin-regulated transcription factor, ARR1. *J Exp Bot* 55:1721–1731
- Sakai H, Aoyama T, Oka A (2000) Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. *Plant J* 24:703–711
- Shi H, Zhu JK (2002) Regulation of expression of the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene AtNHX1 by salt stress and abscisic acid. *Plant Mol Biol* 50:543–550
- Shi H, Lee B, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nat Biotechnol* 21:81–85
- Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* 6:410–417
- Simpson SD, Nakashima K, Narusaka Y, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Two different novel cis-acting elements of *erd1*, a *clpA* homologous *Arabidopsis* gene function in induction by dehydration stress and dark-induced senescence. *Plant J* 33:259–270
- Takano J, Miwa K, Yuan L, von Wirén N, Fujiwara T (2005) Endocytosis and degradation of BOR1, a boron transporter of *Arabidopsis thaliana*, regulated by boron availability. *Proc Natl Acad Sci USA* 102:12276–12281
- Tanaka N, Fujita M, Handa H, Murayama S, Uemura M, Kawamura Y, Mitsui T, Mikami S, Tozawa Y, Yoshinaga T, Komatsu S (2004) Proteomics of the rice cell: systematic identification of the protein populations in subcellular compartments. *Mol Gen Genomics* 271:566–576
- Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell* 5:1529–1539
- Urao T, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc Natl Acad Sci USA* 97:11632–11637
- Véry AA, Sentenac H (2002) Cation channels in the *Arabidopsis* plasma membrane. *Trends Plant Sci* 7:168–175
- Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM (2005) Differential expression and function of *Arabidopsis thaliana* NHX Na<sup>+</sup>/H<sup>+</sup> antiporters in the salt stress response. *Plant J* 30:529–539
- Zhu Y, Cai XL, Wang ZY, Hong MM (2003) An interaction between a MYC protein and an EREBP protein is involved in transcriptional regulation of the rice *Wx* gene. *J Biol Chem* 278:47803–47811
- Xu B, Timko M (2004) Methyl jasmonate induced expression of the tobacco putrescine *N*-methyltransferase genes requires both GCC-box and GCC-motif elements. *Plant Mol Biol* 55:743–761