

Pepper pectin methylesterase inhibitor protein CaPMEI1 is required for antifungal activity, basal disease resistance and abiotic stress tolerance

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Abstract Pectin is one of the main components of the plant cell wall that functions as the primary barrier against pathogens. Among the extracellular pectinolytic enzymes, pectin methylesterase (PME) demethylesterifies pectin, which is secreted into the cell wall in a highly methylesterified form. Here, we isolated and functionally characterized the pepper (*Capsicum annuum* L.) gene *CaPMEI1*, which encodes a pectin methylesterase inhibitor protein (PMEI), in pepper leaves infected by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). *CaPMEI1* transcripts are localized in the xylem of vascular bundles in leaf tissues, and pathogens and abiotic stresses can induce differential expression of this gene. Purified recombinant CaPMEI1 protein not only inhibits PME, but also exhibits antifungal activity against

some plant pathogenic fungi. Virus-induced gene silencing of *CaPMEI1* in pepper confers enhanced susceptibility to *Xcv*, accompanied by suppressed expression of some defense-related genes. Transgenic *Arabidopsis CaPMEI1*-overexpression lines exhibit enhanced resistance to *Pseudomonas syringae* pv. *tomato*, mannitol and methyl viologen, but not to the biotrophic pathogen *Hyaloperonospora parasitica*. Together, these results suggest that *CaPMEI1*, an antifungal protein, may be involved in basal disease resistance, as well as in drought and oxidative stress tolerance in plants.

Keywords Pectin methylesterase inhibitor protein · *Capsicum annuum* · Antifungal activity · Disease resistance · Drought tolerance · Oxidative stress tolerance

Soo Hyun An and Kee Hoon Sohn contributed equally to this study.

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Introduction

Plant cell wall, the first barrier of defense against invading pathogens, is composed of cellulose microfibrils cross-linked by hemicellulose, pectin and extensin. Pectin comprises a highly heterogeneous group of polymers that includes homogalacturonans and rhamnogalacturonans I and II (Willats et al. 2001a, b). In pectin polymers, the galacturonic acid carboxyl groups can be methylesterified by a group of pectinases. These galacturonic acid methyl esters are hydrolyzed by pectin methylesterase (PME) (Hagerman and Austin 1986; Pelloux et al. 2007). Many physiological processes, such as fruit maturation, microsporangogenesis, seed germination and pollen growth, are affected by the degree to which PME changes the methylesterification of galacturonic acid (Tieman and Handa 1994; Kagan-Zur et al. 1995; Ren and Kermodé 2000; Bosch et al. 2005). The number and distribution of free and

unesterified galacturonate carboxyl groups along the homogalacturonan chain has a great influence on the pectin properties and cell wall firmness (Willats et al. 2001a, b).

Plant pathogens hydrolyze the cell wall components of plants using extracellular pectinolytic enzymes (Collmer and Keen 1986), and PME is found in many plant pathogenic bacteria and fungi (Asoufi et al. 2007). The black filamentous fungus *Aspergillus niger* secretes a set of pectin-degrading enzymes that include PME, polygalacturonase and pectin lyase, and these decompose the plant cell wall to establish infection and absorb nutrients from the host (de Vries and Visser 2001). In soft rot disease caused by *Erwinia chrysanthemi*, bacterial PME activity is induced during infection of *Saintpaulia* plants; however, PME-deficient mutants are noninvasive to the host cells (Boccaro and Chatain 1989). PMEs have also been found in higher plants; they play significant roles in physiological processes and interactions with pathogens (Micheli 2001). In tobacco plants, host cell pectin methylesterases are required for the tobacco mosaic virus movement protein to transfer the viruses between host cells (Chen et al. 2000). Furthermore, PME-degraded polygalacturonans are associated with recognition of fungal pathogens (Wietholter et al. 2003). PME is also involved in symbiosis-specific functions (Lievens et al. 2002). For example, plant PME isoenzymes may undergo organism-specific post-translational processing for structural and functional integrity during interactions with various microorganisms (Micheli 2001).

The PME enzyme activity is modulated specifically by inhibitor proteins such as the pectin methylesterase inhibitor (PMEI; Micheli 2001). Moreover, the PMEIs that inhibit demethylesterification of highly heterogeneous polymers (pectins) are the plant invertase inhibitor-related proteins, which are inhibitors of important metabolic enzymes (Koch 1996). Plant invertase inhibitor-related proteins play key roles in wounding, the plant defense reaction and developmental transitions (Rausch and Greiner 2004), as well as during osmotic stress, senescence and seed development (Greiner et al. 1998, 1999). Investigation of gain- and loss-of-function mutants of tobacco cell wall invertase inhibitor (*NtCIF*) protein demonstrated that these inhibitor proteins play a role in seed development (Rausch et al. 1998). Overexpression of the tobacco vacuolar invertase inhibitor protein (*NtVIF*) gene in transgenic potatoes is of potential use in the field of food technology (Greiner et al. 1998). However, there is little known about the in vivo functions of PMEI protein.

Proteinaceous inhibitors have been purified from kiwi (*Actinidia deliciosa*) (Giovane et al. 2004), *Arabidopsis* (Wolf et al. 2003; Raiola et al. 2004), rice (Han et al. 2005) and the jelly fig (*Ficus awkeotsang* cv. Makino) (Jiang et al. 2001, 2002). The kiwi PMEI is specific for PME (Balestrieri et al. 1990) and is active against PMEs from several

plants, including kiwi, orange, apple, tomato, apricot, carrot, potato and banana (Ly-Nguyen et al. 2004). Four Cys residues conserved in several isoforms of PMEI are involved in the formation of disulfide bridges (Camardella et al. 2000). PME and PMEI form a stoichiometric 1:1 complex, in which the interaction between the PME and the inhibitor occurs in close proximity to the putative active site (Di Matteo et al. 2005). Since PME activity can be modulated by pH, the stability of the PME–PMEI complex is also affected by pH (Denès et al. 2000). Crystallographic work has revealed that an α -helical hairpin motif plays a structurally important role in PMEI activation (Hothorn et al. 2004). Many cDNAs encoding PMEIs have been isolated and functionally characterized from plants (Rausch and Greiner 2004); however, their role in plant defense remains relatively unknown.

To date, the functional analyses of genes associated with defense responses in plants have utilized reverse-genetics approaches based on loss-of-function via double-stranded RNA interference (Robertson 2004) or gain-of-function via transgenic gene expression (Clough and Bent 1998). Virus-induced gene silencing (VIGS) has been proven to be a useful method for assessing the function of target genes in *Solanum* species (Brigneti et al. 2004). In particular, VIGS studies have been used to investigate disease resistance signaling and defense-related genes such as *SGTI* (Liu et al. 2002c; Peart et al. 2002b), *EDS1* (Liu et al. 2002b; Peart et al. 2002a) and *NPRI/NIMI* (Liu et al. 2002b) in *Nicotiana benthamiana*. *AtPGIP1* is among the genes encoding pectic enzyme-related proteins; it encodes polygalacturonase (PG)-inhibiting protein (PGIP), and an antisense *AtPGIP1* gene was used to silence its expression in transgenic *Arabidopsis* plants (Ferrari et al. 2006). Previous studies have revealed that overexpression of two closely related genes, *AtPGIP1* and *AtPGIP2*, conferred resistance against *Botrytis cinerea* infection (Ferrari et al. 2003). Silencing of *AtPGIP1* resulted in enhanced susceptibility to infection, as well as reduced activity of PGIP (Ferrari 2006). However, gene-silencing techniques such as VIGS and antisense RNA have not yet been used to investigate the role played in plant defense by other PMEI-encoding genes.

Here, we used a macroarray technique to isolate and functionally characterize a pectin methylesterase inhibitor gene, *CaPMEI1*, from a cDNA library of pepper (*Capsicum annuum* L.) leaves infected with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*; Jung and Hwang 2000). Local and systemic induction of *CaPMEI1* was investigated in pepper plants following inoculation with pathogenic and non-pathogenic bacteria. We also examined the involvement of *CaPMEI1* in defense-related signal transduction cascades via exogenous application of abiotic elicitors to pepper plants. Recombinant *CaPMEI1* proteins were expressed in *E. coli* and exhibited antifungal activity against plant pathogenic fungi. Since it is

difficult to transform pepper plants, we performed gene silencing and *CaPMEII* overexpression in pepper and *Arabidopsis*, respectively, to identify the cellular functions of the *CaPMEII* gene. The functional data obtained by VIGS and transgenic ectopic expression of *CaPMEII* suggest that this pepper pectin methylesterase inhibitor is involved in plant defense and abiotic stress responses.

Materials and methods

Plant materials and growth conditions

Pepper (*Capsicum annuum* L. cv. Nockwang) plants were grown at 28°C under a 16 h day at 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Plants were seeded into a plastic tray (55 \times 35 \times 15 cm^3) containing steam-sterilized soil mix (peat moss, perlite and vermiculite; 5:3:2, v/v/v) and loam soil (1:1, v/v). At the two-leaf stage, seedlings were transplanted into plastic pots (5 \times 15 \times 10 cm^3) containing the soil mix previously described.

Arabidopsis thaliana (ecotype Columbia) plants were grown in pots containing vermiculite, peat moss and perlite (1:1:0.5, v/v/v) in a growth chamber under a 12 h light/12 h dark photoperiod (130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 24°C and 60% relative humidity. Prior to sowing, the seeds were surface-sterilized using 1% sodium hypochlorite and vernalized at 4°C for 3 days to break dormancy.

Pathogens, inoculation procedures, disease rating and tissue staining

Xanthomonas campestris pv. *vesicatoria* (*Xcv*) strains Ds1 and Bv5-4a were used in this study. Bacteria were cultured overnight in yeast-nutrient (YN) broth (5 g L^{-1} yeast extract, 8 g L^{-1} nutrient broth) at 28°C. Prior to inoculation, bacteria were harvested by centrifugation and resuspended in sterile tap water (10⁸ cfu mL^{-1}). Pepper plants were inoculated at the six-leaf stage by infiltrating the bacterial suspension into the abaxial side of fully expanded leaves using an atomizer. Infected plants were then incubated for 16 h at 28°C in a moist chamber with 100% relative humidity. Bacteria-infected leaves were sampled at various time intervals after inoculation. To evaluate systemic induction in the upper leaves, bacterial suspensions (10⁸ cfu mL^{-1}) were infiltrated into the lower leaves of pepper plants at the two-leaf stage using a needleless syringe. The bacterial strains used for this study included: virulent and avirulent strains of *X. campestris* pv. *vesicatoria* (Ds1 and Bv5-4a); a non-pathogenic strain (*Pseudomonas fluorescens* ATCC13525); and *Escherichia coli* JM109.

Leaves of 6-week-old *Arabidopsis* plants were infiltrated with a suspension (OD₆₀₀ = 0.001) of virulent *Pseudomonas*

syringae pv. *tomato* strain DC3000 (*Pst* DC3000). The bacteria were cultured overnight at 28°C and suspended in 10 mM MgCl_2 . To determine bacterial growth, leaf discs were cut from infected leaves at different time intervals after inoculation. Bacterial growth was monitored by performing serial dilutions onto KB agar containing 100 $\mu\text{g mL}^{-1}$ rifampicin. Each experiment was replicated three times.

Hyaloperonospora parasitica isolate Noco2 was propagated by weekly subculturing on 7- to 10-day-old *Arabidopsis* seedlings. The 7-day-old seedlings were inoculated with an *H. parasitica* asexual inoculum (5 \times 10⁴ conidiosporangia mL^{-1}). The seedlings inoculated with *H. parasitica* were covered with a plastic dome to maintain a high relative humidity (80–100%) and grown in a growth chamber at 17°C. Seven days after inoculation, disease rating was scored for more than 50 plants per treatment. A visual disease rating was expressed as the number of sporangiophores on each cotyledon and was divided into five classes: 0–5, 6–10, 11–15, 16–20 and >20 sporangiophores per cotyledon. The cotyledons from inoculated plants were stained with lactophenol-trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol and 10 mg trypan blue, dissolved in 10 mL of distilled water) to assess *H. parasitica* growth. At 2–5 days after inoculation, the infected cotyledons were boiled for 5 min in the staining solution and destained overnight in chloral hydrate (2.5 g chloral hydrate dissolved in 1 mL distilled water). The destained cotyledons were subsequently mounted in 70% glycerol for microscopic observation.

Isolation and sequence analysis of pathogen-induced cDNAs

To construct a pathogen-induced cDNA library, pepper leaves were inoculated with the avirulent strain *X. campestris* pv. *vesicatoria* Bv5-4a. The pathogen-induced cDNA library was constructed using 5 $\mu\text{g poly(A)}^+$ mRNA extracted from inoculated pepper leaves (Kim and Hwang 2000). To isolate pathogen-inducible cDNAs from the pepper cDNA library, we performed differential hybridization, as described previously by Jung and Hwang (2000). Digoxigenin (DIG)-labeled, single-stranded cDNA probes were generated from total RNA of healthy and Bv5-4a-infected leaves using RT-PCR. Nylon membranes were pre-hybridized at 65°C for 3 h in 5 \times SSC, 0.1% sodium lauroylsarcosine, 0.02% SDS and 1% blocking reagent (Boehringer Mannheim, Mannheim, Germany). Hybridization was then performed overnight at 65°C in the same buffer with single-stranded cDNA probes. Hybridized membranes were rinsed twice for 5 min with 2 \times SSC and 0.1% SDS at room temperature, and twice for 10 min with 0.1 \times SSC and 0.1% SDS at 65°C. The hybridization signals were detected according to the manufacturer's protocol (Boehringer Mannheim).

We selected cDNA clones that were expressed strongly in pathogen-infected leaves, compared with those of healthy leaves. Clones were sequenced with an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, USA). Sequencing results were analyzed using BLAST (National Center for Biotechnology Information; Altschul et al. 1997).

Treatment with abiotic elicitors and environmental stresses

The leaves of pepper plants at the six-leaf stage were sprayed with 5 mM salicylic acid (SA), 100 μ M methyl jasmonate (MeJA) or 100 μ M abscisic acid (ABA). Pepper plants treated with methyl jasmonate were sealed tightly in plastic bags. For ethylene treatment, whole plants were removed from soil, and then placed in a water-containing glass chamber, followed by injection of ethylene gas (5 μ L L⁻¹). For cold stress treatment, plants were placed at 4°C in a cold room. For wounding stress, the leaves were pricked with needles. To impose drought stress, the plants were removed from the soil and then incubated at room temperature without water. H₂O₂ treatment was performed by spraying leaves with 100 mM H₂O₂ solution. Leaves treated with various elicitors and abiotic stresses were removed from the plants, frozen in liquid nitrogen and stored at -70°C until used for RNA isolation.

RNA isolation and RNA gel blot analysis

Total RNA was extracted from pepper leaves, stems, roots, flowers and fruits using the guanidine isothiocyanate method (Chomczynski and Sacchi 1987). Frozen tissues (1 g) were ground to a powder and homogenized in 10 mL extraction buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate [pH 7.0], 0.55% [w/v] *N*-laurylsarcosine and 0.1 M 2-mercaptoethanol). A mixture of 2 M sodium acetate (pH 4.0), water-saturated phenol and chloroform-isoamylalcohol (24:1) was added to the homogenate, followed by precipitation. Total RNA (20 μ g) was separated by 1.2% formaldehyde-agarose gel electrophoresis and then blotted onto Hybond-N⁺ membranes (Amersham, Buckinghamshire, UK). Transferred RNA was fixed to the membrane using UV cross-linking.

The 3' UTR region of *CaPMEII* was amplified for use in the generation of a gene-specific probe. The primers used for amplification of the *CaPMEII* gene-specific region were 5'-CATGGGTAAGTGCTGCCTTGACGGAC-3' and 5'-GTTAACAAATGCATA TGGAACATTT-3'. The *CaBPR1* coding region was amplified with the primers 5'-ATGGGACACTCTAATATTGCC-3' and 5'-GACATCAGTTGGAAGTTCCAA-3'. The *CaSAR82A* coding region was amplified using the primers 5'-ATGGTTTCCAAA

GT AGTATTTTTATTT-3' and 5'-TATGCTTAACAAT TACTACTGAATA TAATC-3'. PCR-amplified products were ³²P-labeled using a random priming kit (Boehringer, Mannheim). Hybridization was performed overnight at 65°C in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) sodium dodecyl sulfate (SDS) and 1 mM EDTA. Following hybridization, the membranes were washed twice for 10 min with 2× SSC and 0.1% SDS at room temperature, and then twice for 5 min with 0.1× SSC and 0.1% SDS at 65°C. Equal loading of RNA was confirmed by ethidium bromide-staining of ribosomal RNA.

In situ RNA localization

In situ RNA localization was performed as described previously (Lee et al. 2000). Leaf tissue was fixed for 2 h in a solution of 1× phosphate-buffered saline (PBS), 4% paraformaldehyde and 1 μ L mL⁻¹ Triton X-100 by vacuum infiltration for 10 min and shaking for 2 h at room temperature. The fixed samples were washed with 1× PBS, dehydrated through a graded ethanol and xylol series, and then embedded in liquid paraplast at 57°C (Sherwood Medical, St. Louis, MO, USA). Paraplast-embedded sections (10 μ m in thickness) were placed on glass slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA) and incubated at 42°C. Sections were deparafinated using xylene and 1-propanol, followed by rehydration with serial dilutions of ethanol. Tissue samples were treated with 0.01 M Tris-HCl (pH 8.0) and 1% bovine serum albumin (BSA) for 10 min, followed by incubation in 100 mM Tris-HCl solution (pH 8.0) containing proteinase K (5 mg mL⁻¹) and 50 mM EDTA, for 30 min at 37°C. Sections were treated with 0.25% acetic anhydride in 100 mM triethanolamine (pH 8.0) for 10 min at room temperature to inhibit non-specific signals. Digoxigenin (DIG)-labeled probes were prepared using the Dig High Prime DNA Labeling and Detection kit, according to the manufacturer's instructions (Boehringer Mannheim). Sections were prehybridized and hybridized at 42°C in 50% formamide, 4× SSC, 150 μ g mL⁻¹ tRNA and 0.5% blocking reagent (Boehringer Mannheim). After hybridization, the sections were washed twice with 50% formamide and 4× SSC at 42°C, twice with 4× SSC and then once with diethyl pyrocarbonate (DEPC)-treated water. The DIG signal was detected, according to the manufacturer's instructions (Boehringer Mannheim). Color reactions were developed overnight at 37°C with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and reactions were stopped with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Sections were photographed with Kodak ISO 100 film under a bright field Olympus BH-2 microscope (Olympus, Tokyo, Japan). To demonstrate the specificity of in situ hybridization, control hybridizations were performed without DIG-labeled probes.

Purification of recombinant CaPMEI1 protein

The *CaPMEI1* coding region, including the stop codon, was amplified by PCR using the forward and reverse primers 5'-GAATTCATGGAAGGTGGCAATTTTCT-3' and 5'-CTCGAGTAGCCGTGAAGGGCAGCCAGACG-3', respectively. Amplification products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA), which was digested with *EcoRI* and *XhoI* and ligated into the similarly digested pET32a (Novagen, Madison, WI, USA).

Escherichia coli BL21 (DE3) pLysS (Novagen), which is defective for thioredoxin reductase, was used as a host for recombinant protein expression. Cultures were started from single colonies, grown in LB broth at 37°C, and then diluted 1:100 at OD₆₀₀ = 0.6. After dilution, bacteria were grown to a density of OD₆₀₀ = 0.6, induced with 10 mM IPTG and grown for a further 5 h at 37°C. Cells were harvested by centrifugation for 15 min at 5,000g, extracted with denaturation buffer (8 M urea, 20 mM sodium phosphate buffer [pH 7.8], 500 mM NaCl) and disrupted by sonication. Following centrifugation at 5,000g for 15 min at 4°C, the supernatant was loaded onto a 1.5 mL column of Ni-NTA resin (Qiagen, Hilden, Germany), which was washed with an initial denaturing wash buffer (8 M urea, 20 mM sodium phosphate buffer [pH 6.0], 500 mM NaCl), followed by a second denaturing wash buffer (8 M urea, 20 mM sodium phosphate buffer [pH 5.3], 500 mM NaCl). Bound fusion protein was eluted with a final denaturing elution buffer (8 M urea, 20 mM sodium phosphate buffer [pH 4.0], 500 mM NaCl). Recombinant proteins were dialyzed against a buffer containing 10 mM Tris (pH 8.0) and 0.1% Triton X-100, according to the manufacturer's protocol. As a control, thioredoxin was purified using a native buffer that did not contain urea. The purified CaPMEI1 recombinant protein was digested for 16 h at room temperature with recombinant enterokinase (1 U/5 µg recombinant protein; Novagen).

SDS polyacrylamide gel electrophoresis

CaPMEI1 was dissolved in 1× SDS sample buffer (0.9 g glycerol, 5% SDS, 1% bromophenol blue, 0.1 mL mercaptoethanol and 1 L H₂O), separated by 12% SDS-PAGE as described previously (Laemmli 1970), and stained with Coomassie Brilliant Blue R-250. Molecular weights were estimated using 6.5 to 200.5 kDa marker proteins (Bio-Rad, Hercules, CA, USA).

Pectin methylesterase enzyme inhibitor assay

The inhibitory effect of CaPMEI1 on the enzymatic activity of pectin methylesterase (PME) was assayed under standard conditions (Grsic-Rausch and Rausch 2004). The reac-

tion mixture comprised 894 µL 0.4 mM NAD in 50 mM phosphate buffer (pH 7.5), 80 µL 5% (w/v) pectin (Sigma) in H₂O, 8 µL formaldehyde dehydrogenase (0.35 U; Sigma) and 8 µL alcohol oxidase (1.0 U; Sigma). After mixing, the reaction was started with the addition of 10 µL (7.8 mU) PME from orange peel (Sigma). To analyze CaPMEI1 inhibition of PME, 1 µL inhibitor solution (0.5 mg CaPMEI1 fusion protein, 10 mM Tris buffer [pH 7.5], 0.1 M NaCl) was mixed with 10 µL PME (7.8 mU) and 0.5 µL 3 M K-acetate buffer (pH 5.3), followed by incubation for 15 min at room temperature. All reaction temperatures were maintained at 25°C. Reaction rates were recorded continuously at 340 nm using a DU650 UV-visible spectrophotometer (Beckman, Fullerton, CA, USA). PME–PMEI interactions were determined by measuring the rate of NADH formation per minute at pH 7.5 and 25°C.

In vitro antifungal activity

To determine the antifungal activity of CaPMEI1, we examined its effect on plant pathogenic fungi. *Fusarium oxysporum* f.sp. *matthioli* and *Alternaria brassicicola* were incubated on potato dextrose agar (PDA) at 28°C for 1–2 weeks; *B. cinerea* was incubated at 20°C for 1–2 weeks. Fungi were grown in 48-well plates (Cell Wells™, Corning Glass Works, Corning, NY, USA) containing sterile 4× potato dextrose broth (PDB, 100 µL) and a range of concentrations of purified CaPMEI1 protein (0–500 µg mL⁻¹). Spore suspensions (10⁴ spores mL⁻¹) were prepared and 100 µL of inoculum was added to each microwell. Plates containing *B. cinerea* were incubated at 20°C; the other fungi were incubated at 28°C for 4–7 days.

To determine the inhibitory effect of CaPMEI1 on spore germination and hyphal growth, CaPMEI1 (0.1–500 µg mL⁻¹) or the thioredoxin control were added to *F. oxysporum* f.sp. *matthioli* spore suspensions (10⁵ spores mL⁻¹), placed on glass slides and incubated for 12 h at 28°C. After 6 h incubation, 100 germinated spores were examined using a haemocytometer and the lengths of 50 individual hyphae were determined. The experiment was repeated three times.

Plasmid construction and plant transformation

The *CaPMEI1* coding region was PCR-amplified without stop codon using the following primers to generate *XbaI* and *BamHI* sites: 5'-TCTAGAATGGAAGGTGGCA ATT TTCTACA-3' and 5'-GGATCCGCGTGAAGGGCAG CCAGACGGT -3'. The fragment was inserted into pCR2.1-TOPO (Invitrogen) and digested with *XbaI* and *BamHI*. The construct was confirmed by sequencing. To generate the reporter construct p35S-*CaPMEI1*-GFP, the fragment was inserted into the *XbaI*–*BamHI* sites of the

binary vector p35S-smGFP. p35S-smGFP was generated by fusing the gene encoding smGFP from the vector 326-GFP into pBIN35S digested with *Hind*III and *Eco*RI. The CaMV35S promoter-*CaPMEII*-smGFP construct was introduced into the *Agrobacterium tumefaciens* strain EHA105 using electroporation.

Transgenic *Arabidopsis* plants were generated by floral-dipping wild-type (Col-0) plants into an *A. tumefaciens* culture containing the appropriate construct (Clough and Bent 1998). *Agrobacterium* was grown at 28°C and 250 rpm in YEP medium (10 g Bacto peptone, 10 g yeast extract, 5 g NaCl) supplemented with kanamycin (25 µg ml⁻¹). Cells were harvested by centrifugation for 20 min at 5,500g and resuspended in inoculation media containing 5.0% sucrose and 0.05% Silwet L-77 (OSi Specialties, Inc., Danbury, CT, USA) to OD₆₀₀ = 0.8. *Arabidopsis* plants with 2–10 cm long bolts were inoculated by dipping into an *Agrobacterium* suspension (OD₆₀₀ = 0.8) and then left in the dark overnight, prior to return to a growth chamber. Seeds from the transformed *Arabidopsis* plants were collected and screened for kanamycin resistance.

Virus-induced gene silencing (VIGS)

The TRV-based VIGS system was used for silencing of *CaPMEII* in pepper plants, as described previously (Liu et al. 2002a; Chung et al. 2004). The pepper *CaPMEII* coding region was cloned into pTRV2 to generate the construct pTRV2:*CaPMEII*. The fully extended cotyledons of 2-week-old pepper seedlings were co-infiltrated with *A. tumefaciens* GV3101 carrying pTRV1 or pTRV2:*CaPMEII* (OD₆₀₀ = 0.2 for each construct). Plants were placed in a growth room at 25°C under a 16 h light/8 h dark photoperiod, to allow for growth and viral spread. Experiments were performed 5–6 weeks after the induction of silencing.

RT-PCR analysis

The RT reactions (20 µL) were performed at 42°C with total RNA (2 µg), oligo p(dT)₁₅ primer (Roche, Mannheim, Germany) and AMV reverse transcriptase (Roche). Aliquots (1 µL) of the RT reaction products were used for RT-PCR analysis with the following gene-specific primers: 5'-CAG GATGCAACACTCTGGTGG-3' and 5'-ATCAAAGGC CGGTTGGTC-3' for *CaBPRI* (accession no. AF053343); 5'-TGTCGAAGGTGGTCC AATAAA-3' and 5'-TAGA CAGAAGGATTGGCGAGG-3' for *CaPRI0* (accession no. AF244121); 5'-ATCTGTACCAGCTTGCACGTGT-3' and 5'-CCCTCACTGTGGCCT TGG-3' for *CaPOAI* (accession no. AF442387); and 5'-CAGGGAGATGAAT TCTGA GGC-3' and 5'-CATATGAACCTCTATGGA TTTCTG-3' for *CaSAR82A* (accession no. AF313766). RT-PCR conditions were 95°C for 10 min and 30 cycles of

95°C for 30 s, 52°C for 30 s, and 72°C for 1 min and 30 s. Single bands for PCR products were confirmed on an agarose gel.

Drought stress treatment and evaluation

For germination tests, seeds from *Arabidopsis* wild-type and *CaPMEII*-OX transgenic lines were surface-sterilized and placed on MS media (Murashige and Skoog 1962) containing 200 or 600 mM mannitol. The seeds were maintained at 4°C for 48 h under dark conditions to synchronize germination, and then transferred to a growth chamber. Experiments were repeated at least three times, using approximately 100 seeds.

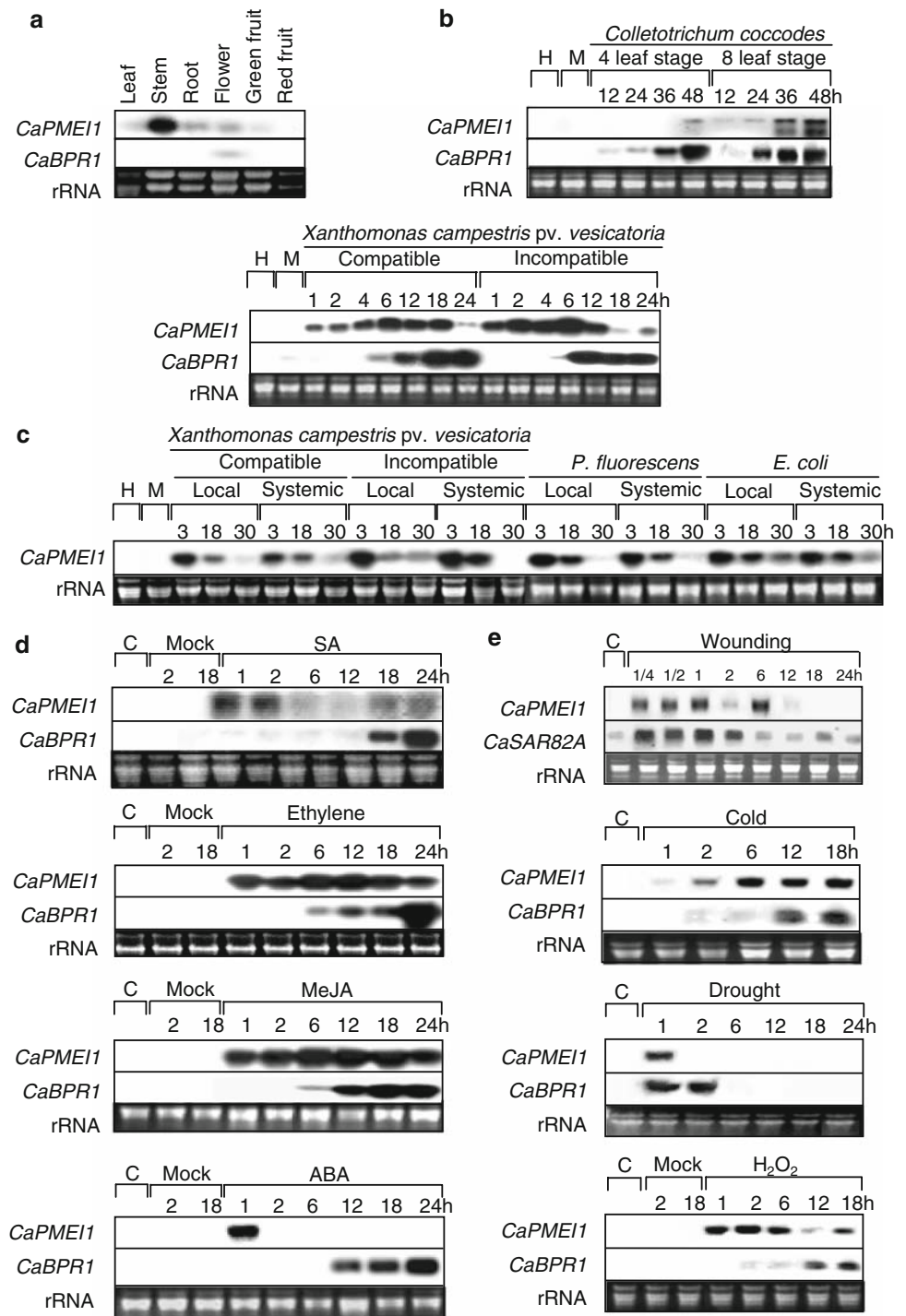
Surface-sterilized seeds were also used for examination of relative root length. Seedlings were grown on plates placed vertically in growth chambers and root length was estimated for 14 days. To assess mannitol tolerance, seedlings were grown for 7 days in 1× MS agar media supplemented with 1% sucrose, and then transferred to wells containing 1× MS liquid media supplemented with 100, 200, 300 or 400 mM mannitol. For drought treatment, 4-week-old soil-grown plants were deprived of water for 15 days and then re-watered on day 16. To minimize experimental variation, both the transgenic and control plants were grown in the same tray. Experiments were repeated at least three times.

For transpiration rate measurements, leaves of 4-week-old plants were detached and maintained at room temperature. Leaf weight was determined every 20 min for 2 h, and then every 1 h thereafter. Each measurement was performed using eight leaves. Experiments were repeated at least three times with similar results.

Oxidative stress treatment and evaluation

For analysis of oxidative stress tolerance, surface-sterilized seeds from *Arabidopsis* wild-type, vector control and *CaPMEII*-OX transgenic lines were placed on MS medium containing different concentrations of methyl viologen (MV; Sigma). After synchronized germination, seeds were maintained in a growth chamber. To investigate whether or not MV treatment caused retardation of seedling development, 7-day-old seedlings were randomly transferred into well-plates containing 1× MS liquid medium supplemented with 0.05, 0.1 or 0.5 µM MV, and then grown for a further 2 weeks. To determine leaf senescence during MV treatment, fully expanded leaves were detached from 4-week-old plants and rinsed briefly with 70% ethanol. The detached leaves were floated on MS medium containing MV for 24 h following extraction with liquid nitrogen. Their chlorophyll content was measured spectrophotometrically according to the formula ($C = 5.24 \times A_{664} + 22.24 \times A_{648}$), where C is the

Fig. 2 RNA gel blot analysis of expression of *CaPMEI1*, *CaSAR82A* and *CaBPR1* in pepper plants. Membranes were hybridized with probes from the 3' UTR region of pepper *CaPMEI1* cDNA or full-length *CaBPR1* cDNA. Equal loading (20 μ g) was verified by visualizing RNA on a gel stained with ethidium bromide. *H* healthy, *M* mock-inoculated or mock-treated. **a** *CaPMEI1* expression in various organs of pepper plants. **b** Expression of *CaPMEI1* and *CaBPR1* in pepper leaves at various time intervals after inoculation with *C. coccodes* or virulent strain Ds1 (compatible interactions with pepper: susceptible response) and avirulent strain Bv5-4a (incompatible interactions with pepper: resistant response) of *Xcv*. **c** *CaPMEI1* expression in lower (local) infected and upper (systemic) uninfected leaves at various time intervals after inoculation with the virulent and avirulent strains Ds1 and Bv5-4a of *Xcv*, *P. fluorescence* ATCC13525 and *E. coli* JM109. The lower leaves of pepper plants were inoculated at the 6-leaf stage. For the mock-inoculation, the lower leaves were infiltrated with 10 mM $MgSO_4$. **d** Expression of *CaPMEI1* and *CABPR1* in pepper leaves at various time intervals after treatment with salicylic acid (SA, 5 mM), methyl jasmonate (MeJA, 100 μ M), ethylene (5 μ l L^{-1}) and abscisic acid (100 μ M). **e** Expression of *CaPMEI1* and *CaSAR82A* in pepper leaves at various time intervals after treatment with drought, wounding, cold and H_2O_2



interactions with pathogens, leaves were inoculated with *C. coccodes* and *Xcv* Ds1 (virulent, compatible), as well as *Xcv* Bv5-4a (avirulent, incompatible). *CaPMEI1* transcripts were not detected in leaves mock-inoculated with sterilized water (Fig. 2b, c). During fungal infection at the four- and eight-leaf stages, *CaPMEI1* expression was induced at 48 and 12–48 h after inoculation, respectively, suggesting that the adult plant stage is more resistant to *C. coccodes* (Fig. 2b, upper panel). *CaPMEI1* transcripts increased rap-

idly within 30 min after infection by both the virulent (Ds1) and avirulent (Bv5-4a) strains of *Xcv* (Fig. 2b, lower panel). High transcript levels accumulated by 18 h after infection with the virulent strain (Ds1) that causes a susceptible response; however, they started to decline rapidly by this time following infection with the avirulent strain (Bv5-4a) that causes a resistant response. *CaBPR1* (*Capsicum annuum basic PR1*) expression was observed to confirm the success of individual inoculations. *CaBPR1* transcripts

were detected at 6 h after inoculation with the virulent and avirulent strains of *Xcv* and increased gradually during the 24 h following inoculation (Fig. 2b).

Local and systemic expression of *CaPMEII* in infected pepper plants

The local and systemic *CaPMEII* expression was analyzed in pepper leaves inoculated with *Xcv* and the non-pathogenic bacteria *Pseudomonas fluorescens* ATCC13525 and *E. coli* JM109 (Fig. 2c). In leaves infected with the virulent (Ds1) and avirulent (Bv5-4a) strains of *Xcv*, *CaPMEII* transcripts were detected 3 h after inoculation and levels decreased thereafter. Infection of lower (local) leaves by either *Xcv* strain induced systemic accumulation of *CaPMEII* transcripts in the upper leaves. The systemic induction by the avirulent strain (Bv5-4a) infection was stronger than that by the virulent strain (Ds1) infection. Local or systemic induction of *CaPMEII* transcripts was not observed 30 h after infection. In response to the non-pathogenic bacteria *P. fluorescens* and *E. coli*, *CaPMEII* transcripts were detected 3 h after inoculation and decreased subsequently in both local and systemic leaves.

CaPMEII induction following treatment with plant hormones

Plant hormones are involved in defense-related signal transduction pathways (Hammond-Kosack and Jones 1996; Chung et al. 2007). We examined whether or not *CaPMEII* expression is induced by plant hormones using pepper leaves treated with salicylic acid (SA), ethylene, methyl jasmonate (MeJA) or abscisic acid (ABA) (Fig. 2d). Salicylic acid strongly induced expression of *CaPMEII* at 1 and 2 h, as well as between 18 and 24 h after treatment. Following treatment with ethylene and MeJA, *CaPMEII* expression was strongly induced for 24 h. In addition, strong *CaPMEII* induction was observed 1 h after treatment with ABA.

In comparison, SA only induced *CaBPR1* transcription 18–24 h after treatment (Fig. 2d), although transcripts were detected 6 h after treatment with ethylene and MeJA. In response to ethylene treatment, *CaBPR1* transcripts increased rapidly between 18 and 24 h after treatment, but increased gradually between 6 and 24 h after MeJA treatment. *CaBPR1* expression was induced between 2 and 12 h after ABA treatment.

CaPMEII induction by abiotic stresses

Abiotic stresses resulted in differential induction of *CaPMEII* in pepper leaves (Fig. 2e). The *CaPMEII* gene was responsive to mechanical injury, and expression was observed between 15 min and 6 h after wounding, although

no transcription was detected thereafter. *CaPMEII* transcription also increased between 2 and 18 h after cold treatment. *CaPMEII* transcription was induced at 1 h of drought stress, but was undetectable thereafter. In response to H₂O₂ treatment, *CaPMEII* transcript levels increased for the first 6 h and declined thereafter. The marker genes *CaBPR1* and *CaSAR82A* were used for comparison, as they also exhibit differential induction by these abiotic stresses.

In situ localization of *CaPMEII* transcripts

We performed an in situ hybridization using a *CaPMEII* probe to examine spatial expression of *CaPMEII* in pepper leaf tissues (Fig. 3). No hybridization signals were observed in non-treated leaves (Fig. 3a, b). Intense localization of *CaPMEII* transcripts was observed in the xylem area of the vascular bundle in non-treated stems (Fig. 3c). *CaPMEII* transcripts were detected in the vascular bundle of leaves inoculated with *C. coccodes* (Fig. 3e) and in the vascular bundles and upper epidermis of leaves treated with ethylene (Fig. 3g). No transcript signals were observed in any pepper tissues hybridized with the *CaPMEII* sense DIG-labeled RNA probe (Fig. 3b, d–f). These in situ hybridization results are supported by the temporal expression patterns observed in the RNA gel blot analysis of *CaPMEII* expression (Fig. 2).

Inhibition of pectin methylesterase (PME) activity by *CaPMEII* protein

To prepare recombinant *CaPMEII* protein, the *CaPMEII* coding region was PCR-amplified and cloned into pET32a. *E. coli* strain BL21 (DE3) pLysS was used as a host for the recombinant expression of the *CaPMEII* construct and an empty vector control, yielding recombinant *CaPMEII* and thioredoxin, respectively. The purified *CaPMEII* (Fig. 4a, lane 5) and thioredoxin (Fig. 4a, lane 3) were examined by SDS-PAGE analysis. The purified thioredoxin–*CaPMEII* fusion protein formed a single band with an apparent molecular mass of 42 kDa (Fig. 4a, lane 5). Following cleavage of thioredoxin by enterokinase digestion, the purified *CaPMEII* protein (Fig. 4a, lane 6) was used for biological function determination. The cleaved recombinant *CaPMEII* protein was found exclusively in the insoluble fraction as inclusion bodies (data not shown). After attempting unsuccessfully to renature the cleaved protein using Triton X-100 as a detergent (Kim and Hwang 1994), we performed the PME inhibition assay using crude and purified *CaPMEII* fusion proteins.

CaPMEII contains a pectin methylesterase inhibitor (PMEI) domain (Fig. 1) and shares similarity with a plant pectin methylesterase inhibitor related to regulation of pectin degradation (Raiola et al. 2004). In addition, PMEIs

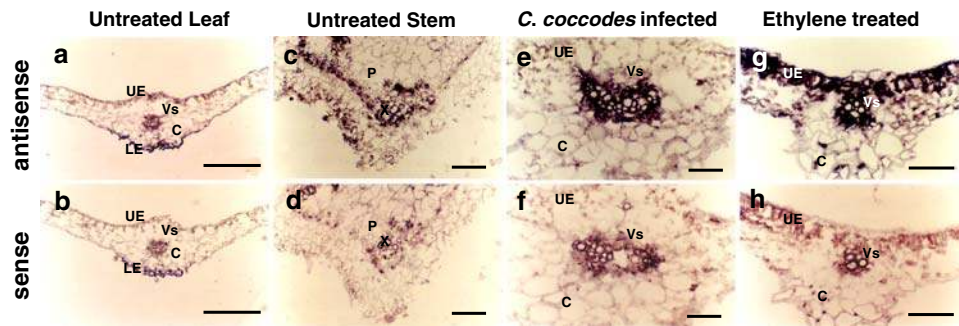


Fig. 3 In situ localization of *CaPMEII* transcripts in pepper leaf and stem tissues. Cross sections of leaf tissues were hybridized with *CaPMEII* antisense (**a, c, e, g**) and sense (**b, d, f, h**) DIG-labeled RNA probes, and then photographed under bright-field conditions. The tran-

script signal is purple. **a, b** Untreated leaf tissues. **c, d** Untreated stems. **e, f** Leaf tissues at 24 h after inoculation with *C. coccodes*. **g, h** Leaf tissues treated with $5 \mu\text{L}^{-1}$ ethylene. *P* phloem, *X* xylem, *UE* upper epidermis, *LE* lower epidermis, *Vs* vascular bundle, *C* cortical cell

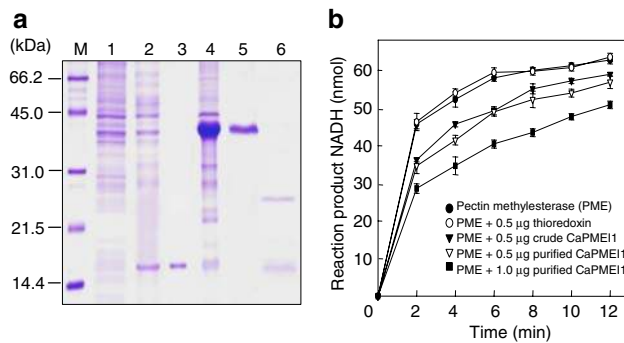


Fig. 4 Inhibition of pectin methylesterase (PME) activity by the pepper pectin methylesterase inhibitor (CaPMEI). **a** Recombinant CaPMEI expression in *E. coli* BL21 (DE3) pLysS. Cells were grown in LB media, and recombinant protein expression was induced with 1 mM IPTG. *M*, molecular marker (kDa); Lane 1, Uninduced *E. coli* BL21 cell extracts; Lane 2, Soluble fraction of *E. coli* BL21 cell extracts encoding thioredoxin protein after IPTG induction; Lane 3, Purified thioredoxin; Lane 4, Crude protein extracts of *E. coli* cells producing the thioredoxin–CaPMEI1 fusion protein after IPTG induction; Lane 5, Purified thioredoxin–CaPMEI1 fusion protein; Lane 6, Cleaved CaPMEI1 and thioredoxin proteins following enterokinase digestion. Protein staining was performed using Coomassie brilliant blue. **b** Inhibition of PME activity following treatment with CaPMEI1. For the inhibition assay, crude CaPMEI1 (0.5 μg) was mixed with 7.8 mU of orange peel PME in a total volume of 11 μL , and then preincubated at 25°C for 15 min, followed by addition to the reaction solution

have been found to play crucial roles in plant and microbial enzyme regulation of pectin degradation by de-esterification (Raiola et al. 2004). To determine whether or not orange peel PME activity is inhibited in the presence of crude CaPMEI1 fusion protein, we used the PME enzyme assay to measure the effect of PME inhibitor on the formation of NADH (Fig. 4b). Both crude and purified CaPMEI1 (ca. 0.5 μg) strongly inhibited PME enzyme activity, and a higher concentration of purified CaPMEI1 (ca. 1.0 μg) exhibited the strongest inhibition. However, thioredoxin alone did not exhibit any inhibitory effect. These results suggest that CaPMEI1 functions as an inhibitor of PME activity.

Antimicrobial activity of CaPMEI1

CaPMEI1 exhibited antifungal activity against the three plant pathogenic fungi examined: *F. oxysporum* f.sp. *matthiole*, *A. brassicicola* and *B. cinerea* (Fig. 5a). The thioredoxin–CaPMEI1 fusion protein ($50 \mu\text{g mL}^{-1}$) suppressed mycelial growth of the three plant pathogenic fungi, whereas thioredoxin alone did not. Furthermore, the purified thioredoxin–CaPMEI1 ($500 \mu\text{g mL}^{-1}$) fusion protein inhibited spore germination and hyphal growth of *F. oxysporum* f.sp. *matthiole*, whereas thioredoxin alone did not (Fig. 5b). Increasing concentrations of recombinant thioredoxin–CaPMEI1 fusion protein increasingly inhibited both spore germination and hyphal growth (Fig. 5c).

Enhanced susceptibility of *CaPMEII*-silenced pepper plants to *Xcv* infection

Since inoculation with *Xcv* strongly induced *CaPMEII* expression in pepper plants, we performed the virus-induced gene silencing (VIGS) technique (Liu et al. 2002a; Chung et al. 2004) to examine its cellular function during pathogen infection. The full-length ORF of *CaPMEII* was used to construct pTRV2:*CaPMEII*. Five to six weeks after induction of silencing, empty vector control (TRV:00) and *CaPMEII*-silenced (TRV:*CaPMEII*) pepper plants were inoculated with virulent and avirulent strains Ds1 and Bv5-4a of *Xcv*, respectively. To assess the efficiency of VIGS, *CaPMEII* transcript levels were examined by RT-PCR (Fig. 6). *CaPMEII* transcripts were nearly undetectable in both non-inoculated empty vector control and *CaPMEII*-silenced plants. However, at 12 h after inoculation with virulent and avirulent *Xcv*, we observed strong induction of *CaPMEII* in the empty vector control plants, whereas only weak or undetectable transcript levels were found in *CaPMEII*-silenced plants. These results confirm the effective silencing of the target gene in pepper plants.

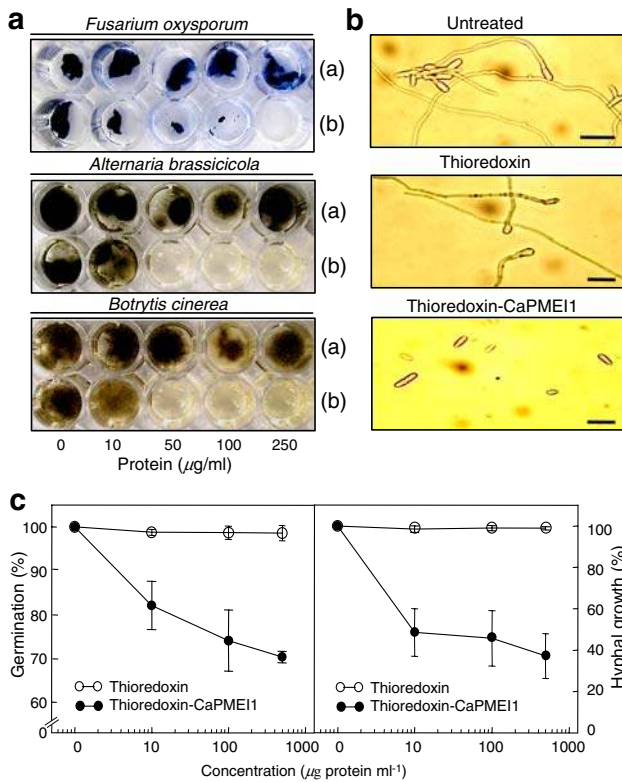


Fig. 5 Assay of thioredoxin–CaPMEII fusion protein antimicrobial activity. **a** Inhibitory effects of the CaPMEII–thioredoxin fusion protein on mycelial growth of the plant pathogenic fungi *F. oxysporum* f.sp. *matthioli*, *A. brassicicola* and *B. cinerea*. In each plate, the upper wells (a) were treated with purified thioredoxin and the lower wells (b) were treated with the purified thioredoxin–CaPMEII fusion protein. **b** Inhibition of germination and hyphal growth of *F. oxysporum* f.sp. *matthioli*. Fungal spores were allowed to germinate and grow in 100 μL potato dextrose broth medium alone (top), or with 500 $\mu\text{g mL}^{-1}$ thioredoxin (middle) or 500 $\mu\text{g mL}^{-1}$ thioredoxin–CaPMEII fusion protein (bottom). Photographs were taken after incubation for 10 h at 28°C. Bars 20 μm . **c** Inhibition of spore germination and hyphal growth of *F. oxysporum* f.sp. *matthioli* by CaPMEII. The percentage of germinated spores and the length of fungal hyphae were determined by light microscopy. Data represent means \pm SD from three independent experiments

To determine whether or not the expression of defense-related genes is affected by bacterial infection in the silenced plants, we used RT-PCR to analyze transcript levels of some defense-related genes (Fig. 6). In the empty vector control plants, both *CaBPR1* (basic pathogenesis-related protein 1) and *CaPR10* (putative ribonuclease-like protein) were strongly induced by virulent and avirulent *Xcv* infection. However, induction of these genes was slightly reduced in *CaPMEII*-silenced plants infected with virulent *Xcv*, but was unaffected in plants infected with avirulent *Xcv*. Thus, *CaPMEII* gene silencing did not alter the induction of *CaPOA1* (ascorbate peroxidase) or *CaSAR82A* (SAR 8.2) in response to *Xcv* infection.

To examine the function of the *CaPMEII* gene in basal defense or gene-for-gene resistance, empty vector control

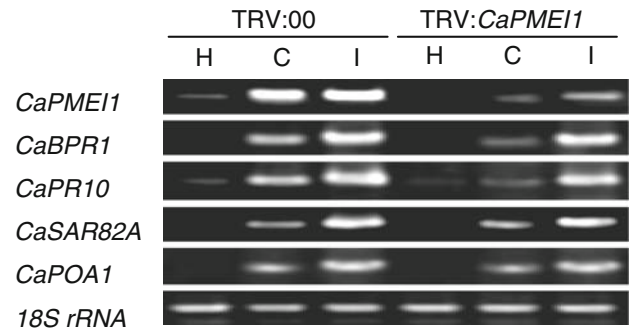


Fig. 6 RT-PCR analysis of expression of *CaPMEII* and several defense-related genes in empty vector control (TRV:00) and *CaPMEII* gene-silenced (TRV:CaPMEII) pepper plants 12 h after inoculation with the virulent (Ds1; C, compatible) and avirulent (Bv5-4a; I, incompatible) strains of *Xcv* (5×10^6 cfu mL^{-1}). 18S rRNA levels were visualized as a loading control. This experiment was repeated three times with similar results. H healthy leaves, *CaBPR1* basic pathogenesis-related protein 1, *CaPR10* putative ribonuclease-like protein, *CaPOA1* ascorbate peroxidase 1, and *CaSAR82A* SAR8.2

and *CaPMEII*-silenced pepper plants were infected with virulent or avirulent *Xcv* (Fig. 7). *CaPMEII* gene silencing significantly increased susceptibility to virulent *Xcv* infection, but not to avirulent *Xcv* infection. The *CaPMEII*-silenced pepper leaves exhibited more severe disease symptoms 5 days after virulent *Xcv* inoculation than did empty vector control plants, and these symptoms were accompanied by severe chlorosis and enlarged water-soaked lesions (Fig. 7a). However, we did not observe any phenotypical changes in the cell death response of *CaPMEII*-silenced plants following avirulent *Xcv* inoculation (Fig. 7a). At 3 days after inoculation with virulent *Xcv*, bacterial growth was tenfold greater in *CaPMEII*-silenced plants than in the empty vector controls (Fig. 7b). However, silencing of *CaPMEII* conferred a slightly enhanced susceptibility to avirulent *Xcv*. These findings suggest that CaPMEII may function in basal resistance of pepper plants against *Xcv* infection, rather than gene-for-gene resistance.

Enhanced resistance of *CaPMEII*-OX plants to *Pst* DC3000

The *p35S-CaPMEII-GFP* and *p35S-GFP* overexpression (OX) plants were generated by transformation of *A. thaliana* using the floral dipping method (Clough and Bent 1998). Plants transformed with the empty vector *p35S-GFP* were used as a control. Seeds (T_1) were collected from each transformed plant and screened for resistance to kanamycin. Northern blot analysis was performed on T_1 plants exhibiting kanamycin resistance to determine the integrity of the inserted transgene. Three T_2 plants showing strong *CaPMEII* expression were selected for analyses in planta (Fig. 8a).

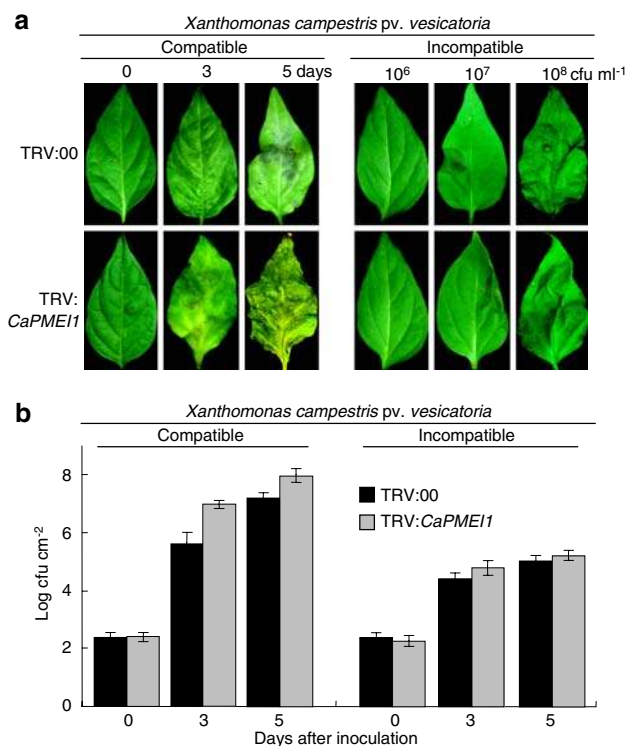


Fig. 7 Enhanced disease susceptibility of *CaPMEI1*-silenced pepper plants to infection by the virulent *Xcv* strain Ds1, but not the avirulent *Xcv* strain Bv5-4a. **a** Disease symptoms developed on the leaves at different time points after inoculation with the virulent *Xcv* strain Ds1 (5×10^6 cfu mL⁻¹) and the avirulent *Xcv* strain Bv5-4a (various bacterial concentrations). **b** Bacterial growth in leaves of empty vector control (TRV:00) or *CaPMEI1*-silenced (TRV:*CaPMEI1*) pepper plants at different time points after inoculation with the virulent *Xcv* strain Ds1 or the avirulent *Xcv* strain Bv5-4a (10^4 cfu mL⁻¹). Data represent the mean \pm SD from three independent experiments

To determine the contribution of *CaPMEI1* to *Arabidopsis* resistance, wild-type and *CaPMEI1*-OX plants were inoculated with virulent *P. syringae* pv. *tomato* DC3000 (*Pst*; 10^5 cfu mL⁻¹). Bacterial titers were determined 4 days after inoculation. *CaPMEI1*-OX plants exhibited much less growth of *Pst* bacteria than wild-type or empty vector control plants (Fig. 8b). In the transgenic plants, this reduced bacterial multiplication was most pronounced at 4 days after inoculation. At 6 days after inoculation, wild-type (Col-0) and empty vector control plants developed typical chlorotic symptoms in the infected leaves, whereas the transgenic plants displayed few disease symptoms (Fig. 8c).

Expression of defense-related genes in *CaPMEI1*-OX plants

To gain insight into the role played by *CaPMEI1* in *PR* gene induction, we examined the expression patterns of the well-established marker genes *AtPR1a*, *AtPR2* and

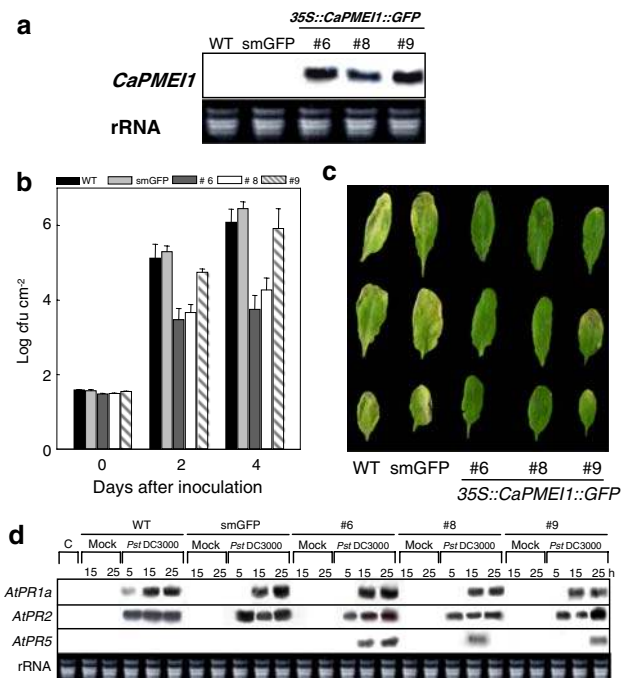


Fig. 8 Responses of wild-type (Col-0) *Arabidopsis* and *CaPMEI1*-OX transgenic plants to infection with *P. syringae* pv. *tomato* DC3000. **a** RNA gel blot analysis confirming *CaPMEI1* overexpression (OX) in the transgenic *Arabidopsis* lines. Total RNA (10 μ g) was loaded into each lane. The 3' UTR region of pepper *CaPMEI1* cDNA was used as a probe. **b** Growth of *Pst* DC3000 in the leaves of wild-type and transgenic plants. The mature leaves of the 6-week-old plants were infiltrated with a *Pst* DC3000 suspension (10^5 cfu mL⁻¹), and the degree of bacterial growth was rated at 0, 2 and 4 days after inoculation. **c** Disease symptoms on leaves of 6-week-old plants infiltrated with virulent *Pst* DC3000 (10^5 cfu mL⁻¹). **d** Expression of pathogen-related (*PR*) genes in transgenic plants. Northern blot analyses were performed with 10 μ g total RNA prepared from 5-week-old leaves of the wild-type (WT), vector control (smGFP) and transgenic (*CaPMEI1*::smGFP) plants. The samples were collected at 5, 15 and 25 h following pathogen infiltration with a suspension of the virulent strain *Pst* DC3000 (10^5 cfu mL⁻¹)

AtPR5 in the defense responses of wild-type, vector control and the *CaPMEI1*-OX *Arabidopsis* plants infected with virulent *Pst* DC3000 (Fig. 8d). The expression of *AtPR1a* and *AtPR2* was very similar in the *CaPMEI1*-OX, wild-type and vector control plants. *AtPR5* expression was not induced in either un-inoculated or inoculated, wild-type or vector control plants. However, there was significant expression of *AtPR5* in the transgenic *CaPMEI1*-OX plants at 15 and 25 h after *Pst* DC3000 infection. *AtPR1a*, *AtPR2* and *AtPR5* expression is known to be regulated via the SA-dependent pathway in *Arabidopsis* (Uknes et al. 1992), and in *nahG* plants, lack of SA reduces expression of these three *PR* genes during pathogenesis (Delany et al. 1994; Nawrath and Metraux 1999). In our experiments, *AtPDF1.2* (defensin) transcripts were not detected in wild-type, vector control or *CaPMEI1*-OX plants (data not shown).

Responses of wild-type and *CaPMEII*-OX plants to *Hyaloperonospora parasitica*

We examined whether or not ectopic *CaPMEII* expression in *Arabidopsis* plants affected resistance to the virulent biotrophic oomycete pathogen *H. parasitica* isolate Noco2 (Fig. 9). Over 100 seedlings of both wild-type (Col-0) and *CaPMEII*-OX transgenic lines were inoculated with spores of *H. parasitica* isolate Noco2 (5×10^4 conidiosporangia mL⁻¹). At 7 days after inoculation, over 50 plants in each line were sampled to estimate the disease and assess the degree of asexual sporulation, which was quantified and expressed as the mean number of sporangiophores per cotyledon.

As shown in Fig. 9a, the cotyledons of wild-type and transgenic seedlings responded to *H. parasitica* infection by stimulating similar levels of asexual parasite sporulation. Their trypan blue-stained cotyledons also exhibited abundant hyphae, conidiospores and oospores (Fig. 9a). Quantitative disease ratings are shown in Fig. 9b. Both the transgenic and the wild-type plants showed a high level of susceptibility to *H. parasitica* isolate Noco2, and similar

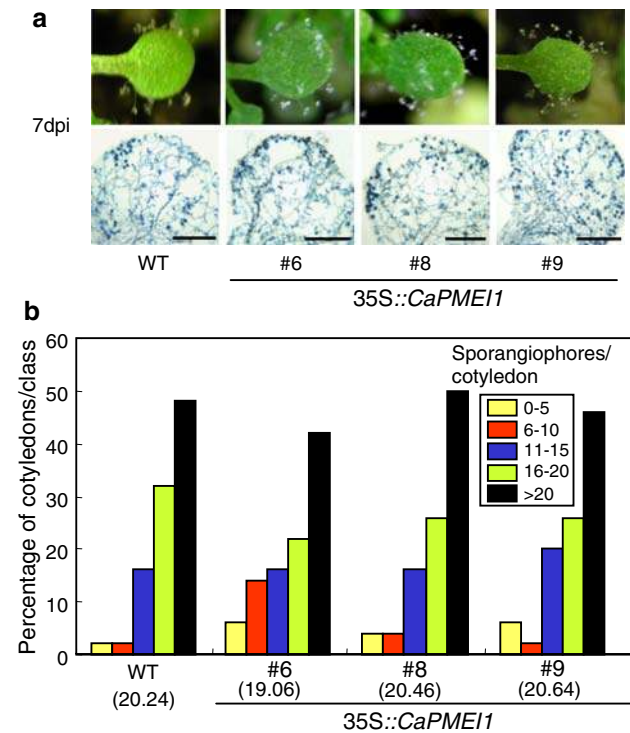


Fig. 9 Responses of wild-type (Col-0) *Arabidopsis* and *CaPMEII*-OX transgenic plants to infection with *H. parasitica* isolate Noco2. **a** Disease symptoms and trypan blue-stained pathogen structures on 7-day-old cotyledons of wild-type and transgenic plants 7 days after inoculation; dpi days post-inoculation. Bars 0.5 mm. **b** Quantification of asexual sporangiophores per cotyledon for at least 50 cotyledons of wild-type and transgenic plants 7 days after inoculation. The average number of sporangiophores produced on the cotyledons of wild-type and transgenic lines are shown below each of the lines tested

levels of heavy asexual sporulation (>20 sporangiophores per cotyledon) were observed on both lines.

Drought tolerance of *CaPMEII*-OX plants

To investigate a possible role for *CaPMEII* in the dehydration response, we tested seed germination and seedling growth under osmotic stress. The seeds of wild-type, vector control and *CaPMEII*-OX plants were placed on MS media supplemented with various concentrations of mannitol. We observed no significant differences in seed germination between these lines (Fig. 10a). However, treatment with 200 and 600 mM mannitol strongly inhibited germination in the wild-type and vector control plants compared with the *CaPMEII*-OX plants.

We also tested the sensitivity of root growth to osmotic stress (Fig. 10b). Drought tolerance was observed during post-germination growth. The root growth of wild-type and vector control seedlings was inhibited in the presence of 150 and 200 mM mannitol. However, root elongation of *CaPMEII*-OX plants was less sensitive to mannitol-induced osmotic stress and in comparison with wild-type and vector control plants, mutant seedlings grew well in liquid medium supplemented with 100 mM mannitol (Fig. 10c).

In addition, we noticed that adult *CaPMEII*-OX plants exhibited enhanced drought tolerance. Following 14 days without water, wild-type and vector plants had withered severely, whereas the *CaPMEII*-OX plants remained healthy. To determine the effect of *CaPMEII* on survival, these plants were rewatered on day 16; wild-type and vector control plants died, whereas the transgenic lines survived (Fig. 10d). Next, we examined transpiration rates by measuring fresh weight loss in detached leaves. The leaves of *CaPMEII*-OX lines exhibited slightly slower water loss than those of wild-type or vector control plants (Fig. 10e). Together, these results indicate that *CaPMEII* overexpression enhanced water stress resistance.

Oxidative tolerance of *CaPMEII*-OX plants

To investigate the response of *CaPMEII*-OX plants to oxidative stresses, seeds of wild-type, vector control and *CaPMEII*-OX plants were exposed to MS medium containing methyl viologen (MV) (Fig. 11). Treatment with 5 or 10 μM MV significantly inhibited germination of wild-type and vector control seeds compared with *CaPMEII*-OX seeds (Fig. 11a). Seven-day-old seedlings were transferred to a medium containing different MV concentrations (0–0.5 μM) and grown for 2 weeks. Wild-type and vector control seedlings turned white and started to die after 15 days of MV treatment, but *CaPMEII*-OX lines were much less affected by the treatment (Fig. 11b). Furthermore, these

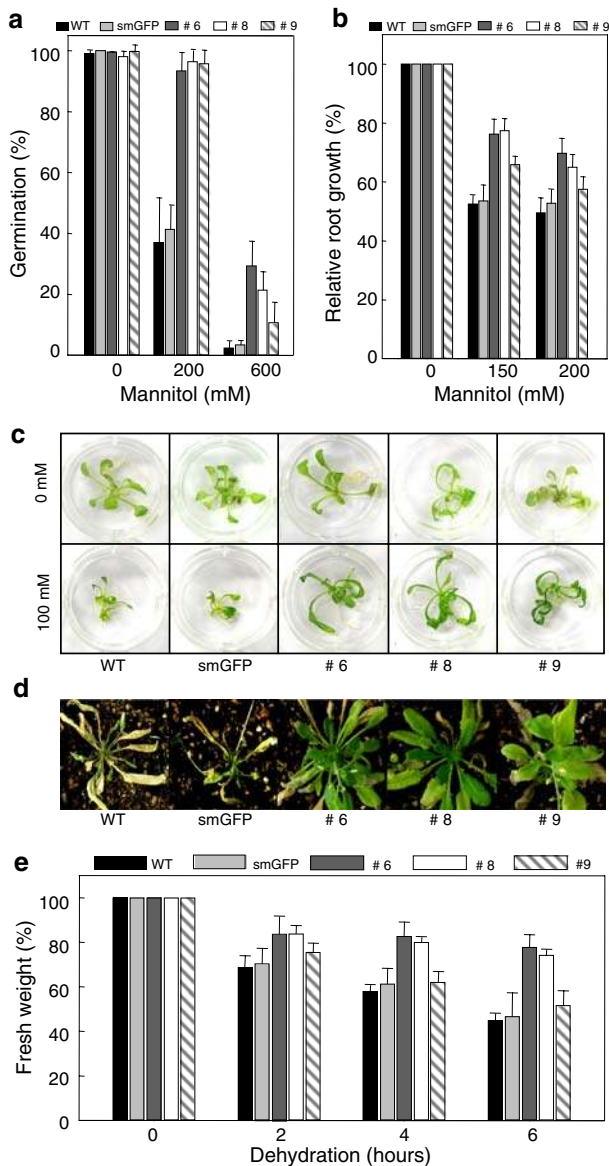


Fig. 10 Transgenic *Arabidopsis CaPMEII-OX* lines exhibit enhanced tolerance to drought stress. **a** Seed germination in wild-type, smGFP and transgenic plants on the MS media containing 0, 200 and 600 mM mannitol. The data represent the mean \pm SD of 100 seeds for each line tested. **b** Relative root length of wild-type, smGFP and transgenic lines in MS agar medium containing different concentrations of mannitol. Three independent experiments were performed with 40 seedlings of both wild-type and transgenic lines. **c** Drought tolerance test of transgenic seedlings. Wild-type, smGFP and transgenic lines were germinated and grown in $1 \times$ MS agar medium. Each seedling was transferred to liquid medium containing 100 mM mannitol. **d** Wild-type, smGFP and the *CaPMEII* transgenic *Arabidopsis* plants after 15 days without water. **e** Water loss from the excised leaves of wild-type, smGFP and transgenic plants. Data represent the mean \pm SD from three independent experiments

differences in plant phenotype were also observed with respect to the higher fresh weight of transgenic seedlings compared to those of wild-type and vector control plants (Fig. 11c). Detached leaves of 4-week-old plants were

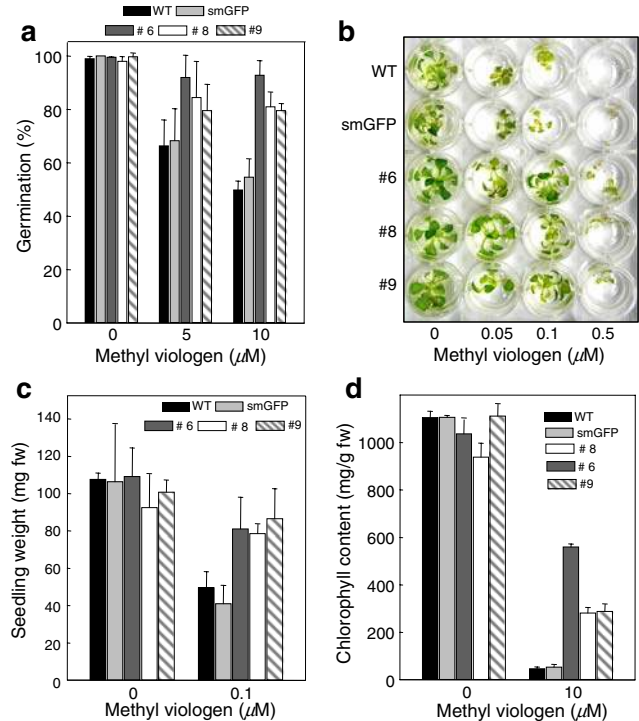


Fig. 11 Transgenic *Arabidopsis CaPMEII-OX* lines exhibit tolerance to oxidative stress. **a** Effects of methyl viologen on the seed germination of transgenic lines. Seeds from wild-type, vector control and transgenic lines were plated on media with or without methyl viologen (MV, 5 and 10 μ M) and incubated for 3 days. The data represent mean \pm SD of 100 seeds for each line tested. **b** Phenotypes of wild-type, vector control and transgenic line seedlings treated with different concentrations of MV. **c** Fresh weights of seedlings grown in the indicated concentrations of MV for 2 weeks. The results are presented as the average fresh weight per seedling. Data represent mean \pm SD from three independent experiments. **d** Chlorophyll content of MV-treated leaves of wild-type, vector control and transgenic plants, which were floated on 0, 0.05, 0.1 and 0.5 μ M MV in MS medium and then incubated for 24 h in a growth chamber

treated with 10 μ M MV for 24 h, after which chlorophyll content was measured (Fig. 11d). The leaves of transgenic lines treated with MV retained more chlorophyll than those of wild-type or vector control plants.

Discussion

Plants possess a diverse range of cell wall-modified enzymes, which are post-transcriptionally regulated by numerous inhibitor-related proteins (Tymowska-Lalanne and Kreis 1998; Rausch and Greiner 2004). Several invertase inhibitor-related proteins have been isolated from higher plants such as kiwi, tobacco and *Arabidopsis* (Greiner et al. 1998; Wolf et al. 2003; Giovane et al. 2004; Raiola et al. 2004). Attempts to characterize the activity of invertase inhibitor proteins from plant species other than kiwi or *Arabidopsis* have been either unsuccessful or

resulted in the isolation of invertase inhibitors that share structural similarities with pectin methylesterase inhibitor proteins (PMEI), but which represent completely different target enzymes (Greiner et al. 1998; Scognamiglio et al. 2003). In this study, we identified and functionally characterized a novel pepper *CaPMEII* gene encoding a PMEI. This CaPMEII protein contains the four cysteine residues that are conserved among other PMEI proteins (Camardella et al. 2000; Sato et al. 2000). These residues are expected to be engaged in two disulfide bridges, which constitute a common structural motif within the PMEI domain (Camardella et al. 2000).

At the molecular level, *CaPMEII* expression was induced in pepper leaves by infection with bacterial pathogens and treatment with plant hormones such as SA, ethylene, MeJA and ABA. In particular, these hormone treatments strongly induced *CaPMEII* transcription, suggesting that this gene may be involved in the early stages of the active defense responses to bacterial pathogen infection and exogenous treatment with plant hormones.

In enzymatic assays, purified CaPMEII proteins significantly inhibited activity of plant pectin methylesterase (PME). In addition, CaPMEII exhibited antifungal activity against a broad range of plant pathogenic fungi, including *F. oxysporum* f.sp. *matthiolae*, *A. brassicicola* and *B. cinerea*. To penetrate the cuticular layer, plant fungal pathogens produce plant cell wall-degrading enzymes such as polygalacturonase, pectin lyase and cellulase (Collmer and Keen 1986). An aggressive *Phaeosphaeria nodorum* isolate was shown to produce high amounts of xylanase, cellulase, polygalacturonase and butyrate esterase in vitro (Lalaoui et al. 2000). Recently, it was found that plant PMEIs do not inhibit the PMEs produced by plant pathogens (Giovane et al. 2004; Di Matteo et al. 2005). However, *Arabidopsis* plants expressing either *AtPMEI-1* or *AtPMEI-2* showed reduced infection by *B. cinerea* (Lionetti et al. 2007). This finding suggests that the increased level of pectin methylesterification caused by overexpression of *AtPMEI-1* results in the inhibition of fungal endopolygalacturonase activity. Thus, the increase in PMEI activity resulted in reduced accessibility for fungal pectin degrading-enzymes and hence provided increased resistance to pathogens (Boudart et al. 1998; Lionetti et al. 2007). Consistent with these findings, our results suggest that CaPMEII may function as part of a new group of plant pectin methylesterase inhibitors, which restrict fungal pathogen infection in plants.

We used virus-induced gene silencing (VIGS) to investigate the effect of *CaPMEII* loss-of-function in pepper plants during *Xcv* infection. The *CaPMEII*-silenced plants were susceptible to *Xcv* infection, and in particular to infection with the virulent strain, which resulted in enhanced bacterial growth and reduced *PR1* and *PR10* gene expression. Basal resistance is activated during the compatible

bacterial interaction which restricts the spread of pathogens in the host plants to a certain extent (Glazebrook 2001). In addition, basal resistance is also effective in retarding proliferation of a wide range of microbial pathogens (Chisholm et al. 2006), but it is dependent upon SA accumulation (Cao et al. 1994, 1997; Kinkema et al. 2000). Therefore, we conclude that *CaPMEII* expression may be involved in basal resistance by triggering downstream *PR* gene induction in pepper plants.

To determine the effect of CaPMEII gain-of-function in *planta*, we generated the *CaPMEII-OX Arabidopsis* transgenic lines and investigated their response to *P. syringae* and *H. parasitica* infection, because these well-known model pathogens have been used extensively for the study of disease resistance mechanisms in *Arabidopsis* (Quirino and Bent 2003; Slusarenko and Schlaich 2003). The *CaPMEII-OX* lines were resistant to *Pst* DC3000 infection, but not to infection by the biotrophic oomycete *H. parasitica*, which uses living cells as a nutrient source during the infection cycle (Alfano and Collmer 1996; Heath 2002). Moreover, as *CaPMEII* transcripts localize intensively in the xylem of vascular bundles in leaf tissues, they may not affect the accessibility of host plant cells to *H. parasitica*. In contrast, we hypothesize that intercellular growth of *P. syringae* may be restricted by the extracellular secretion of CaPMEII into host cells, resulting in triggering of the basal resistance response.

We found that fungal growth was inhibited by treatment with the recombinant CaPMEII protein in vitro, suggesting that CaPMEII may interfere directly with pathogen infection of host plants. Plant pathogenic microorganisms have been shown to produce a variety of pectinolytic enzymes that macerate and kill plant tissues (Collmer and Keen 1986). Cell wall fragments released by these pectinolytic enzymes may elicit the plant defense response (D'Ovidio et al. 2004). The polygalacturonase-inhibiting protein (PGIP) plays an important role in the recognition and inhibition of fungal polygalacturonase (PG). Overexpression of PGIPs in *Arabidopsis* not only significantly reduces disease symptoms, but also enhances defense gene activation during pathogen infection (Ferrari et al. 2003). Thus, we suggest that CaPMEII can disrupt invading pathogenic microorganisms by inhibiting pectin methylesterases produced by these pathogens.

The reduced bacterial growth observed in *CaPMEII-OX Arabidopsis* lines may result from the expression of SA-inducible genes and *CaPMEII* overexpression. The SA-inducible genes such as *PR1*, *PR2* and *PR5* are activated in the SA defense pathway (Uknes et al. 1992). *PR5* proteins are similar to thaumatin, which is a sweet-tasting protein from *Thaumatococcus daniellii* (Hu and Reddy 1997), and several pathogens can induce these proteins in a wide range of plant species (Ward et al. 1991; Hu and Reddy 1997;

Reuber et al. 1998). Members of the PR5 group have been shown to exhibit antifungal activity against a broad spectrum of fungal pathogens (Coca et al. 2000) and to participate in the coordinated induction of systemic acquired resistance (SAR) against TMV (Ward et al. 1991). Therefore, the concomitant induction of PR genes may contribute to the enhanced resistance of *CaPMEI1-OX Arabidopsis* plants to bacterial pathogens.

The *CaPMEI1-OX Arabidopsis* lines showed a strong tolerance to drought stress, and *CaPMEI1* overexpression resulted in reduced transpiration and enhanced root elongation. In contrast, transgene overexpression did not cause any obvious phenotypic differences under optimal growing conditions. Dehydration factors such as a mannitol and polyethyleneglycol (PEG) have been used to evaluate the effects of decreased water availability and simulate drought conditions in wild-type and *CaPMEI1-OX Arabidopsis* plants (Gupta and Kaur 2005; Verslues et al. 2006). Other plant invertase inhibitor-related protein genes such as *NtCIF* and *NtVIF* are also strongly induced by treatment with PEG or ABA (Rausch and Greiner 2004). However, the molecular and genetic roles played by PMEIs in drought and osmotic stress tolerance remain poorly understood.

Since plant responses to different abiotic stresses may be related to the accumulation of ROS and the mechanisms for their detoxification, the role played by ROS in stress signaling has been studied extensively (Apel and Hirt 2004). Methyl viologen binds to the thylakoid membranes of chloroplasts and in the presence of light, transfers electrons to O₂ in a chain reaction causing continuous formation of superoxide radicals and oxidative stress (Asada 1996). The *Arabidopsis CaPMEI1-OX* lines exhibited tolerance to oxidative stress, both during seed germination and seedling growth. This tolerance to oxidative stress may reduce the damage caused by other stresses via the antioxidizing system, which suggests that *CaPMEI1* overexpression results in detoxification of endogenous superoxide.

Here, we have determined that *CaPMEI1* from pepper plays a role as an antifungal protein and has an inhibitory effect on PME. Furthermore, we have shown that *Arabidopsis CaPMEI1-OX* lines are resistant to bacterial pathogens. In addition, they exhibit tolerance to drought and oxidative stress. In conclusion, these multivariate functions of *CaPMEI1* provide valuable insights into understanding the physiological significance of PMEIs in plant disease resistance and abiotic stress tolerance.

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