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) demethylsterifies 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

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 methylesters 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 Kermode 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 (Boccara 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 methylsterases 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 (Wietzholder 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 methylsterase inhibitor (PMEI; Micheli 2001). Moreover, the PMEIs that inhibit demethylsterification 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 (Balestri-eri 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). Crystalllographic 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 NPR1/NIM1 (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 At-PGIP1 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 methylsterase inhibitor gene, CaPMEII, from a cDNA library of pepper (Capsicum annum L.) leaves infected with Xanthomonas campestris pv. vesicatoria (Xcv). Local and systemic induction of CaPMEII was investigated in pepper plants following inoculation with pathogenic and non-pathogenic bacteria. We also examined the involvement of CaPMEII in defense-related signal transduction cascades via exogenous application of abiotic elicitors to pepper plants. Recombinant CaPMEII 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 CaPMEI1 overexpression in pepper and Arabidopsis, respectively, to identify the cellular functions of the CaPMEI1 gene. The functional data obtained by VIGS and transgenic ectopic expression of CaPMEI1 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 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\). Plants were seeded into a plastic tray (55 x 35 x 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 x 15 x 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 \( \mu \)mol photons m\(^{-2}\) 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 (10\(^6\) 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 times intervals after inoculation. To evaluate systemic induction in the upper leaves, bacterial suspensions (10\(^8\) cfu mL\(^{-1}\)) were infiltrated into the lower leaves of pepper plants at the two-leaf stage using a needless 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 (OD\(_{600}\) = 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 \( \mu \)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 x 10\(^4\) 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-trypsin blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol and 10 mg trypsin 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 \( \mu \)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× SSC, 0.1% sodium laurylsarco sine, 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× SSC and 0.1% SDS at room temperature, and twice for 10 min with 0.1× SSC and 0.1% SDS at 65°C. The hybridization signals were detected according to the manufacturer’s protocol (Boehringer Mannheim).

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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 μM methyl jasmonate (MeJA) or 100 μM 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 μL 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, by incubation in 100 mM Tris–HCl solution (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 deparaffinized 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 0.1% SDS 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 μL 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 μm in thickness) were placed on glass slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA) and incubated at 42°C. Sections were deparaffinized 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 μg 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′-GAATTCATGGAGGTGCCATTTTCT-3′ and 5′-CTCGAGTGCGTGAAAGGCGGAGCGAAGCG-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 5,000 g, 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 5,000 g 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 reaction 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. matthioliæ and Alternaria brassicicola were incubated in 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. matthioliæ 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′-TCTAGAATGGAGGTGCCATT TTCTACA-3′ and 5′-GGATCCCGCCGTGAAGGCGCAG 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 HindIII and EcoRI. The CaMV35S promoter-CaPMEI1-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 5,500 g 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 CaPMEI1 in pepper plants, as described previously (Liu et al. 2002a; Chung et al. 2004). The pepper CaPMEI1 coding region was cloned into pTRV2 to generate the construct pTRV2:CaPMEI1. The fully extended cotyledons of 2-week-old pepper seedlings were co-infiltrated with A. tumefaciens GV3101 carrying pTRV1 or pTRV2:CaPMEI1 (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 (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 GATGCAACACTCTTGTTG-3’ and 5’-ATCAAGGG CGTTTGGTC-3’ for CaBPR1 (accession no. AF053343); 5’-TGTCCAAGGGGTGGTC-3’ and 5’-ATAG CAGAAGATTGGCGAGG-3’ for CaPR10 (accession no. AF244121); 5’-ATCTGTACCAGCTTGACGT-3’ and 5’-CCCTCACTGTGGCCT TGG-3’ for CaPOA1 (accession no. AF442387); and 5’-CAGGGAAGTAGTAAT TCTGA GCC-3’ and 5’-CATATGAACTCTATGGAT TTCTG-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 CaPMEI1-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 CaPMEI1-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 (Ca + b = 5.24 × A₆₆₄ + 22.24 × A₅₄₈), where C is the
chlorophyll concentration (Ca + b) in micrograms per mL and A is absorption (Lichtenthaler 1987).

Results

Isolation and sequence analysis of CaPMEI1 cDNA in pepper

To identify the molecular mechanisms involved in plant defense against the invasion of microbial pathogens, we performed a differential hybridization screening of a cDNA library constructed from pepper leaves undergoing an incompatible interaction with Xcv (Jung and Hwang 2000). PR-1, PR-10, SAR8.2 and Myb transcription factor were among the pathogen-induced genes isolated by this screen. In addition, we isolated a full-length cDNA (834 bp) designated CaPMEI1 (Capsicum annuum pectin methylesterase inhibitor protein, accession no. AF477956), which contains a 54 bp 5′ untranslated sequence and a 174 bp 3′ untranslated region. The predicted open reading frame encodes a full-length protein of 200 amino acid residues, with an estimated molecular mass of 21.39 kDa and an isoelectric point (pI) of 6.51.

BLAST searches were performed between the predicted amino acid sequence of CaPMEI1 (accession no. AF477956) from C. annuum and the EMBL/GenBank database. These sequence analyses revealed that CaPMEI1 contains a conserved pectin methylesterase inhibitor (PMEI) domain of 156 amino acids (Fig. 1). Four cysteine residues were conserved in all the aligned proteins (Fig. 1), and these are predicted to be involved in the formation of disulfide bridges (Camardella et al. 2000). The predicted amino acid sequence of CaPMEI1 is 80% identical to that of Nicotiana tabacum DC1.2 protein (accession no. BAA95794) and has 50% identity with Arabidopsis thaliana ripening-related protein (accession no. BAA97200), 36% with Pinus radiata pectinesterase homolog (accession no. T08112) and 24% with A. thaliana invertase homolog (accession no. NP_201267).

Organ-specific expression of CaPMEI1 in pepper tissues

RNA gel blot analysis was performed to examine the organ-specific expression of CaPMEI1 in pepper plants. Although high levels of CaPMEI1 transcription were observed in stem tissues, only relatively low levels of expression were found in leaf, root, flower and green fruit (Fig. 2a). CaPMEI1 transcripts were not detected in red fruit.

CaPMEI1 induction by pathogen infection

To assess the accumulation of CaPMEI1 transcripts in pepper leaves during the compatible and incompatible
interactions with pathogens, leaves were inoculated with *C. coccodes* and *Xcv* Ds1 (compatible interactions with pepper) and Xcv Ds1 and Bv5-4a (incompatible interactions with pepper). *CaPMEI1* expression 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. *CaPMEI1* expression in lower (local) and upper (systemic) uninfected leaves at various time intervals after inoculation with the virulent and avirulent strains Ds1 and Bv5-4a of Xcv, *P. fluorescens* 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 MgSO₄. *CaBPR1* expression of *CaPMEI1* and *CaBPR1* in pepper leaves at various time intervals after treatment with salicylic acid (SA, 5 mM), methyl jasmonate (MeJA, 100 μM), ethylene (5 μL L⁻¹) and abscisic acid (100 μM). *CaPMEI1* and *CaSAR82A* in pepper leaves at various time intervals after treatment with drought, wounding, cold and H₂O₂ 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 annum 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 CaPMEI1 in infected pepper plants**

The local and systemic CaPMEI1 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*, CaPMEI1 transcripts were detected 3 h after inoculation and levels decreased thereafter. Infection of lower (local) leaves by either *Xcv* strain induced systemic accumulation of CaPMEI1 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 CaPMEI1 transcripts was not observed 30 h after infection. In response to the non-pathogenic bacteria *P. fluorescens* and *E. coli*, CaPMEI1 transcripts were detected 3 h after inoculation and decreased subsequently in both local and systemic leaves.

**CaPMEI1 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 CaPMEI1 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 CaPMEI1 at 1 and 2 h, as well as between 18 and 24 h after treatment. Following treatment with ethylene and MeJA, CaPMEI1 expression was strongly induced for 24 h. In addition, strong CaPMEI1 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.

**CaPMEI1 induction by abiotic stresses**

Abiotic stresses resulted in differential induction of CaPMEI1 in pepper leaves (Fig. 2e). The CaPMEI1 gene was responsive to mechanical injury, and expression was observed between 15 min and 6 h after wounding, although no transcription was detected thereafter. CaPMEI1 transcription also increased between 2 and 18 h after cold treatment. CaPMEI1 transcription was induced at 1 h of drought stress, but was undetectable thereafter. In response to H2O2 treatment, CaPMEI1 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 CaPMEI1 transcripts**

We performed an in situ hybridization using a CaPMEI1 probe to examine spatial expression of CaPMEI1 in pepper leaf tissues (Fig. 3). No hybridization signals were observed in non-treated leaves (Fig. 3a, b). Intense localization of CaPMEI1 transcripts was observed in the xylem area of the vascular bundle in non-treated stems (Fig. 3c). CaPMEI1 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 CaPMEI1 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 CaPMEI1 expression (Fig. 2).

**Inhibition of pectin methylesterase (PME) activity by CaPMEI1 protein**

To prepare recombinant CaPMEI1 protein, the CaPMEI1 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 CaPMEI1 construct and an empty vector control, yielding recombinant CaPMEI1 and thioredoxin, respectively. The purified CaPMEI1 (Fig. 4a, lane 5) and thioredoxin (Fig. 4a, lane 3) were examined by SDS-PAGE analysis. The purified thioredoxin–CaPMEI1 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 CaPMEI1 protein (Fig. 4a, lane 6) was used for biological function determination. The cleaved recombinant CaPMEI1 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 CaPMEI1 fusion proteins.

CaPMEI1 contains a pectin methylsterase inhibitor (PMEI) domain (Fig. 1) and shares similarity with a plant pectin methylsterase inhibitor related to regulation of pectin degradation (Raiola et al. 2004). In addition, PMEIs...
Fig. 3  In situ localization of CaPMEI1 transcripts in pepper leaf and stem tissues. Cross sections of leaf tissues were hybridized with CaPMEI1 antisense (a, c, e, g) and sense (b, d, f, h) DIG-labeled RNA probes, and then photographed under bright-field conditions. The transcript 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 µL 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 (CaPMEI1). a Recombinant CaPMEI1 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. mathiole, A. brassicicola and B. cinerea (Fig. 5a). The thioredoxin–CaPMEI1 fusion protein (50 µg mL⁻¹) suppressed mycelial growth of the three plant pathogenic fungi, whereas thioredoxin alone did not. Furthermore, the purified thioredoxin–CaPMEI1 (500 µg mL⁻¹) fusion protein inhibited spore germination and hyphal growth of F. oxysporum f.sp. mathiole, 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 CaPMEI1-silenced pepper plants to Xcv infection

Since inoculation with Xcv strongly induced CaPMEI1 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 CaPMEI1 was used to construct pTRV2:CaPMEI1. Five to six weeks after induction of silencing, empty vector control (TRV:00) and CaPMEI1-silenced (TRV:CaPMEI1) pepper plants were inoculated with virulent and avirulent strains Ds1 and Bv5-4a of Xcv, respectively. To assess the efficiency of VIGS, CaPMEI1 transcript levels were examined by RT-PCR (Fig. 6). CaPMEI1 transcripts were nearly undetectable in both non-inoculated empty vector control and CaPMEI1-silenced plants. However, at 12 h after inoculation with virulent and avirulent Xcv, we observed strong induction of CaPMEI1 in the empty vector control plants, whereas only weak or undetectable transcript levels were found in CaPMEI1-silenced plants. These results confirm the effective silencing of the target gene in pepper plants.
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 CaPMEI1-silenced plants infected with virulent Xcv, but was unaffected in plants infected with avirulent Xcv. Thus, CaPMEI1 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 CaPMEI1 gene in basal defense or gene-for-gene resistance, empty vector control and CaPMEI1-silenced pepper plants were infected with virulent or avirulent Xcv (Fig. 7). CaPMEI1 gene silencing significantly increased susceptibility to virulent Xcv infection, but not to avirulent Xcv infection. The CaPMEI1-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 phenotypic changes in the cell death response of CaPMEI1-silenced plants following avirulent Xcv inoculation (Fig. 7a). At 3 days after inoculation with virulent Xcv, bacterial growth was tenfold greater in CaPMEI1-silenced plants than in the empty vector controls (Fig. 7b). However, silencing of CaPMEI1 conferred a slightly enhanced susceptibility to avirulent Xcv. These findings suggest that CaPMEI1 may function in basal resistance of pepper plants against Xcv infection, rather than gene-for-gene resistance.

Fig. 6 RT-PCR analysis of expression of CaPMEI1 and several defense-related genes in empty vector control (TRV:00) and CaPMEI1 gene-silenced (TRV:CaPMEI1) pepper plants 12 h after inoculation with the virulent (Ds1; C, compatible) and avirulent (Bv5-4a; I, incompatible) strains of Xcv (5 × 10⁶ 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.
To determine the contribution of CaPMEI1 to Arabidopsis resistance, wild-type and CaPMEI1-OX plants were inoculated with virulent *P. syringae* pv. *tomaoto* 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 *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 CaPMEI1-OX plants to Hyaloperonospora parasitica

We examined whether or not ectopic CaPMEI1 expression in Arabidopsis plants affected resistance to the virulent biotrophic oomycte pathogen H. parasitica isolate Noco2 (Fig. 9). Over 100 seedlings of both wild-type (Col-0) and CaPMEI1-OX transgenic lines were inoculated with spores of H. parasitica isolate Noco2 (5 × 10^5 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 levels of heavy asexual sporulation (>20 sporangiophores per cotyledon) were observed on both lines.

Drought tolerance of CaPMEI1-OX plants

To investigate a possible role for CaPMEI1 in the dehydration response, we tested seed germination and seedling growth under osmotic stress. The seeds of wild-type, vector control and CaPMEI1-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 CaPMEI1-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 CaPMEI1-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 CaPMEI1-OX plants exhibited enhanced drought tolerance. Following 14 days without water, wild-type and vector plants had withered severely, whereas the CaPMEI1-OX plants remained healthy. To determine the effect of CaPMEI1 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 CaPMEI1-OX lines exhibited slightly slower water loss than those of wild-type or vector control plants (Fig. 10e). Together, these results indicate that CaPMEI1 overexpression enhanced water stress resistance.

Oxidative tolerance of CaPMEI1-OX plants

To investigate the response of CaPMEI1-OX plants to oxidative stresses, seeds of wild-type, vector control and CaPMEI1-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 CaPMEI1-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 CaPMEI1-OX lines were much less affected by the treatment (Fig. 11b). Furthermore, these
Fig. 10 Transgenic Arabidopsis CaPMEI1-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 ± 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 1x MS agar medium. Each seedling was transferred to liquid medium containing 100 mM mannitol. d Wild-type, smGFP and the CaPMEI1 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 ± SD from three independent experiments.

Fig. 11 Transgenic Arabidopsis CaPMEI1-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 μM) and incubated for 3 days. The data represent mean ± 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 ± 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 μM MV in MS medium and then incubated for 24 h in a growth chamber treated with 10 μM 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; Raiola et al. 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 CaPMEI1 gene encoding a PMEI. This CaPMEI1 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, CaPMEI1 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 CaPMEI1 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 CaPMEI1 proteins significantly inhibited activity of plant pectin methylesterase (PME). In addition, CaPMEI1 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 CaPMEI1 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 CaPMEI1 loss-of-function in pepper plants during *Xcv* infection. The CaPMEI1-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 CaPMEI1 expression may be involved in basal resistance by triggering downsteam PR gene induction in pepper plants.

To determine the effect of CaPMEI1 gain-of-function in planta, we generated the CaPMEI1-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 CaPMEI1-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 CaPMEI1 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 CaPMEI1 into host cells, resulting in triggering of the basal resistance response.

We found that fungal growth was inhibited by treatment with the recombinant CaPMEI1 protein in vitro, suggesting that CaPMEI1 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 CaPMEI1 can disrupt invading pathogenic microorganisms by inhibiting pectin methylesterases produced by these pathogens.

The reduced bacterial growth observed in CaPMEI1-OX *Arabidopsis* lines may result from the expression of SA-inducible genes and CaPMEI1 overexpression. The SA-inducible genes such as *PR1*, *PR2* and *PR5* are activated in the SA defense pathway (Üknes 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;
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 polyethylene glycol (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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