An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens

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Abstract

Phenylalanine ammonia-lyase (PAL) has a crucial role in secondary phenylpropanoid metabolism and is one of the most extensively studied enzymes with respect to plant responses to biotic and abiotic stress. Here, we identified the pepper (Capsicum annuum) PAL (CaPAL1) gene, which was induced in pepper leaves by avirulent Xanthomonas campestris pv. vesicatoria (Xcv) infection. CaPAL1-silenced pepper plants exhibited increased susceptibility to virulent and avirulent Xcv infection. Reactive oxygen species (ROS), hypersensitive cell death, expression of the salicylic acid (SA)-dependent marker gene CaPR1, SA accumulation, and induction of PAL activity were significantly compromised in the CaPAL1-silenced pepper plants during Xcv infection. Overexpression (OX) of CaPAL1 in Arabidopsis conferred increased resistance to Pseudomonas syringae pv. tomato (Pst) and Hyaloperonospora arabidopsidis infection. CaPAL1-OX leaves exhibited restricted Pst growth, increased ROS burst and cell death, and induction of PR1 expression and SA accumulation. The increase in PAL activity in healthy and Pst-infected leaves was higher in CaPAL1-OX plants than in wild-type Arabidopsis. Taken together, these results suggest that CaPAL1 acts as a positive regulator of SA-dependent defence signalling to combat microbial pathogens via its enzymatic activity in the phenylpropanoid pathway.

Key words: Arabidopsis, defence, pepper, phenylalanine ammonia-lyase, Xanthomonas campestris pv. Vesicatoria.

Introduction

Plants have evolved multiple defence signalling pathways to cope with adverse environmental conditions and pathogen attack (Jones and Dangl, 2006). The levels of secondary metabolites such as phenylpropanoids are controlled in response to environmental cues (Dixon and Paiva, 1995; Payyavula et al., 2012). The evolutionary emergence of the phenylpropanoid pathway in plants is an important adaptation that enables plant defence against abiotic and biotic stresses (Ferrer et al., 2008). Phenylpropanoid compounds are precursors to a wide range of phenolic compounds, such as flavonoids, isoflavonoids, anthocyanins, plant hormones, phytoalexins, and lignins (Dixon and Paiva, 1995; La Camera, et al., 2004). Phenylpropanoids have important functions in several different pathways: in plant defence against pathogens and predators, in protection from UV irradiation, in signal transduction and communication with other organisms, and as regulatory molecules (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995; Ferrer et al., 2008; Vogt, 2010). Phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine (Vogt, 2010). Phenylalanine ammonia-lyase (PAL) catalyses the non-oxidative deamination of phenylalanine to trans-cinnamate. This is the first
step in the phenylpropanoid pathway, and is an important regulation point between primary and secondary metabolism (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995; Huang et al., 2010; Vogt, 2010). PAL is an inducible enzyme that responds to biotic and abiotic stresses such as pathogens, UV irradiation, and low temperature (Dixon and Paiva, 1995; MacDonald and D’Cunha, 2007). PAL plays an important role in plant defence; it is involved in the biosynthesis of salicylic acid (SA), an essential signal involved in plant systemic resistance (Mauch-Mani and Slusarenko, 1996; Nugroho et al., 2002; Chaman et al., 2003). PAL gene expression responds to a variety of environmental stresses, including pathogen infection, wounding, nutrient depletion, UV irradiation, and extreme temperatures (Edwards et al., 1985; Liang et al., 1989a, b; Huang et al., 2010; Payyavula et al., 2012; Jin et al., 2013).

Molecular genetics methods have been used to silence or disrupt PAL genes for the functional analysis of their roles in plant development and responses to external stimuli (Pallas et al., 1996; Rohde et al., 2004; Huang et al., 2010). In Arabidopsis thaliana, PAL is encoded by a small gene family with four members, denoted PAL1–PAL4 (Raes et al., 2003; Huang et al., 2010). PAL1, PAL2, and PAL4 are strongly expressed in inflorescent stems, a tissue rich in lignifying cells, whereas the PAL3 transcript is expressed at a very low level (Raes et al., 2003). Phenotypic analysis of thePAL1 and PAL2 genes was performed for both single and double mutants. The growth and development phenotypes of the pal1 and pal2 single mutants were not significantly different from the growth and development phenotypes of wild-type (WT) plants. The pal1/pal2 double mutant did show phenotypic differences from that of WT plants, such as infertility, a significant reduction in lignin accumulation, and alteration in secondary cell-wall ultrastructure (Rohde et al., 2004). Recently, morphology-based genetic analysis has been conducted on pal1/pal2 double mutants and pal1/pal2/pal3/pal4 quadruple mutants. The pal1/pal2 double mutant produced slightly yellow seeds due to the lack of condensed tannin pigments in the seed coat, which was deficient in anthocyanin pigments in various plant tissues, and it was highly sensitive to UV-B light. These results suggest that PAL1 and PAL2 have important and redundant roles in flavonoid biosynthesis (Huang et al., 2010). The pal1/pal2/pal3/pal4 quadruple mutant had a stunted phenotype, substantially reduced levels of SA accumulation, and increased susceptibility to the virulent bacterial pathogen Pseudomonas syringae (Huang et al., 2010).

PAL-suppressed transgenic tobacco has undeveloped systemic acquired resistance, which correlates with the reduction of SA levels in both inoculated and uninoculated leaves (Pallas et al., 1996). The PAL inhibitor 2-aminohydroxynaphthoic acid decreases the pathogen- or elicitor-induced SA accumulation in potato (Solanum tuberosum), cucumber (Cucumis sativus), and Arabidopsis (Meuwly et al., 1995; Mauch-Mani and Slusarenko, 1996; Coquoz et al., 1998). Mauch-Mani and Slusarenko (1996) proposed that the production of SA precursors is a major function of PAL in the resistance of Arabidopsis to Hyaloperonospora arabidopsidis (Hpa).

In this study, we identified the pepper (Capsicum annuum) PAL gene, CaPALI, using the macroarray method (Jung and Hwang, 2000). CaPALI was upregulated in pepper leaves by infection with avirulent Xanthomonas campestris pv. vesicatoria (Xcv) Bv5-4a. CaPALI loss of function and gain of function were characterized using virus-induced gene silencing (VIGS) in pepper plants (Choi et al., 2007) and ectopic overexpression in Arabidopsis plants (Kim and Hwang, 2011). CaPALI-silenced pepper plants exhibited increased susceptibility to Xcv infection. CaPALI silencing promoted Xcv growth but suppressed pathogen-induced PAL activity in pepper leaves. Suppression of CaPALI induction in silenced leaves reduced the expression of the SA-dependent gene CaPR1, but did not reduce the expression of the jasmonic acid-dependent gene CaDEF1 (defensin). CaPALI silencing also compromised the SA accumulation, oxidative burst, and cell death during Xcv infection. Constitutive overexpression of CaPALI in Arabidopsis conferred increased PAL activity, increased reactive oxygen species (ROS) burst and cell death, induction of PR1 expression and SA accumulation, reduced susceptibility to Pseudomonas syringae pv. tomato (Pst), and increased basal resistance to infection by the obligate biotrophic oomycete Hpa. Together, these results provide convincing evidence for the involvement of pepper CaPALI in plant defence against pathogen attack. This suggests the potential significance of PAL genes in plant immune responses.

Materials and methods

Plant materials and growth conditions

Pepper (C. annuum L., cv. Nockwang) plants were grown in plastic pots containing a soil mix (peat moss:perlite:vermiculite, 2:1:1, v/v/v) in a growth room at 28 °C with a day length of 16 h and a light intensity of 70 μmol photons m⁻² s⁻¹. All A. thaliana lines used in this study were the ecotype Columbia (Col-0). Seeds were surface sterilized and cold treated for 3 d at 4 °C before they were sown in the soil mix. Plants were grown in the soil mix in an environmental growth chamber at 24 °C under 14 h light/10 h dark cycles with a photosynthetic flux of 130 μmol photons m⁻² s⁻¹ and 60% relative humidity.

Pathogen inoculation

Xcv strains Ds1 and Bv5-4a, which are virulent and avirulent to pepper plants (cv. Nockwang), respectively, were used in this study. The bacteria were cultured in yeast nutrient (YN) broth (5 g l⁻¹ of yeast extract and 8 g l⁻¹ of nutrient broth), harvested by centrifugation at 10000 g for 2 min, and resuspended in sterilized tap water. To prepare the inoculum for inoculation, the bacterial suspension was diluted to an appropriate density. Leaves of pepper plants at the six-leaf stage were inoculated by infiltrating the bacterial suspension using a syringe without a needle (Choi and Hwang, 2011).

Pst DC3000 and DC3000 (avrRpm1), which are virulent and avirulent to Arabidopsis Col-0, respectively, were grown in King’s B broth (10 g l⁻¹ of peptone, 1.5 g l⁻¹ of K₂HPO₄, 15 g l⁻¹ of glycerol, and 5 g l⁻¹ of MgSO₄). Leaves of Arabidopsis plants were infiltrated with Pst in 10 mM MgCl₂ using a syringe without a needle.

Hpa isolate Noco2, which is virulent to Arabidopsis ecotype Col-0, was used in this study. Spore suspension (5 × 10⁶ conidiospores ml⁻¹) was sprayed onto 7-d-old seedlings. Inoculated plants were domed with a plastic wrap to maintain moisture. The numbers of
sporangioles and spores on cotyledons were counted to assess disease severity at 7 d after inoculation.

Isolation and sequence analysis of CaPAL1 cDNA

A cDNA library was constructed from poly(A) mRNA of pepper leaves inoculated with the incompatible Xcv Bv5-4a strain. Pathogen-inducible cDNAs were screened using the macroarray method of Jung and Hwang (2000). Among the screened defence response genes, the PAL homologue, CaPAL1 (GenBank accession no. KF279696), was selected based on the BlastX algorithm of the NCBI website (Altschul et al., 1997). Amino acid sequence alignment of CaPAL1 and other PAL orthologues was performed using ClustalX (Thompson et al., 1997).

Genomic DNA gel blot analysis

For Southern blot analysis, genomic DNA was extracted from pepper leaves using Plant DNAzo® Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Twenty micrograms of genomic DNA was digested with restriction endonucleases EcoRI and HindIII, subjected to electrophoresis through 0.7% agarose gels, and blotted onto Hybond-N+ membranes (Amersham, Little Chalfont, UK), followed by cross-linking under UV illumination. CaPAL1 cDNA probes were [32P]dCTP labelled using Klenow enzyme (Roche, Mannheim, Germany). The membranes were hybridized to [32P]-labelled probe at 65 °C overnight (Jung et al., 2003).

RNA gel blot and real-time reverse transcription (RT)-PCR analyses

Total RNA was extracted from pepper and Arabidopsis plants with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. To generate the CaPAL1 gene-specific probe for the northern blot, the full-length CaPAL1 cDNA was [32P]dCTP labelled using Klenow enzyme. RNA gel blot and real-time RT-PCR analyses were conducted following standard procedures, as described previously (Choi and Hwang, 2011; Choi et al., 2012).

For real-time RT-PCR analysis, cDNA was synthesized from 1 μg of total RNA using avian myeloblastosis virus reverse transcriptase (Roche), synthesized oligo(dT)15 primer, and dNTPs (Takara, Shiga, Japan). The real-time PCR was performed for 45 cycles using 1 μl of cDNA as template. The PCR conditions were 94 °C for 5 s, 72 °C for 90 s; and 72 °C for 10 min for the final extension. The primers used for RT-PCR were 5′-GGGTTTGTGGCAACATCATACAGG-G3′ (forward) and 5′-ATTTGCCAATCTCTTCTGACTCTGGC-3′ (reverse) for CaPAL1, 5′-CATCGAGAAGGACTTGTACGG-3′ (forward) and 5′-GATGGACCTGAGCTGTCATAC-3′ (reverse) for CaPRI, 5′-CAGGATGCAAACACTCTGTGG-3′ (forward) and 5′-ATCAGGGGCTTGTGGTC-3′ (reverse) for CaPR1, 5′-ACAGGAGTTGATGCTGATGGAC-3′ (forward) and 5′-TCAGGACGACTATCAACAC-3′ (reverse) for CaDEF1, 5′-AACGGCGCTACCTCAGCA-3′ (forward) and 5′-ACCCATCCAAGGTTCA-3′ (reverse) for the 18S rRNA gene in pepper.

VIGS

pTRV (tobacco rattle virus)-based VIGS was conducted to generate CaPAL1 knockdown (TRV:CaPAL1) pepper plants (Liu et al., 2002; Choi et al., 2007). The N-terminal fragment of CaPAL1 cDNA (5′-546bp) was inserted into pTRV2. pTRV1 and pTRV2:00 or pTRV2:CaPAL1 in Agrobacterium tumefaciens GV3101 were co-infiltrated into the fully expanded cotyledons of pepper plants. Gene knockdown efficiency in CaPAL1-silenced pepper plants was confirmed by RT-PCR from the leaves infected with Xcv.

Plant transformation

The cDNA fragment of CaPAL1 was cloned into the pBIN35S plant binary vector. The primer pairs 5′-GAGATTCTGAATGG CATCAACATTGC-3′ (forward) and 5′-GAGAGATCCCGCT TCCAAGATCTCAA-3′ (reverse) were used in the PCR to create XbaI- and BamHI-sited CaPAL1 cDNA. The resulting binary plasmid was transformed into A. tumefaciens strain GV3101 by electroporation. Agrobacterium-mediated transformation of Arabidopsis ecotype Col-0 was performed using the floral dipping method of Clough and Bent (1998). Transformants were selected on 0.5× Murashige and Skoog (MS) agar plates containing 50 μg ml−1 of kanamycin. Successful transformation was confirmed by RT-PCR using the CaPAL1 gene-specific primer described above. Among the selected transgenic plants, lines #2, #3, and #7 were used in this study.

PAL activity assay

Total proteins were extracted from pepper and Arabidopsis leaves using 100 mM phosphate buffer (pH 6.0) containing 2 mM EDTA, 4 mM dithiothreitol, and 2%/w/v polyvinylpyrrolidone. PAL activity in the leaf extract was determined by the method of Song and Wang (2011), with slight modifications. Briefly, the protein extract (0.2 ml) was incubated at 30 °C for 60 min with 2 ml of 0.01 M borate buffer (pH 8.7) and 1 ml of 0.02 M l-phenylalanine (pre-dissolved in 0.01 M borate buffer pH 8.7). Absorbance was measured at 290 nm before and after incubation. One unit of activity (katal) was defined as the amount of PAL that produces 1 mole of cinnamic acid in 1 s and was expressed as katal mg−1 of protein. A reaction without the substrate was used as a blank control. Triplicate assays were performed for each extract.

Measurement of H₂O₂ and ion conductivity

H₂O₂ levels in pepper leaves were quantified using a xylenol orange assay (Gay et al., 1999; Choi et al., 2007). The xylenol orange assay reagent was freshly prepared. Two hundred microliters of solution [25 mM FeSO₄ and 25 mM (NH₄)₂SO₄ or 2.5 mM H₂SO₄] was added to 20 ml of 125 μM xylenol orange in 100 mM sorbitol. Eight leaf discs (0.4 cm in diameter) were excised from pepper leaves and floated on 1 ml of distilled water for 1 h. One hundred microliters of the supernatant was immediately added to 1 ml of xylenol orange assay reagent. The reaction mixture was incubated for 30 min at room temperature, and H₂O₂ production was monitored by measuring the absorbance at 560 nm using a DU 650 spectrophotometer (Beckman, Urbana, IL, USA).

Ion conductivity was measured using a SensION7 conductivity meter (Hach, Loveland, CO, USA) to quantify cell death in pepper leaves. Leaf discs (1.2 cm in diameter) were excised from pepper leaves inoculated with Xcv and washed for 30 min in 20 ml of distilled water. Ion conductivity was monitored after incubation of the leaf discs for 3 h in freshly prepared distilled water (20 ml).

SA measurement

SA and SA glycoside were extracted and measured from pepper and Arabidopsis leaves, as described previously (Aboul-Soud et al., 2004; Kim and Hwang, 2011). Leaf tissues (0.5 g) were extracted in 1 ml of 90% methanol following homogenization in liquid nitrogen. 3-Hydroxybenzoic acid (Sigma) was used as an internal standard. SA extracts were analysed automatically through a fluorescence detector (excitation at 305 nm and emission at 405 nm) in reversed-phase high-performance liquid chromatography in a Waters 515 system (Waters, Milford, MA, USA) with a C18 column.

Results

Isolation and sequence analyses of CaPAL1

To isolate pathogen-inducible genes from the pepper cDNA library, differential hybridization was performed based on a
macroarray method (Jung and Hwang, 2000). Among the isolated clones, a full-length cDNA encoding a PAL homologue in pepper plants was selected and designated CaPAL1 based on the BlastX algorithm of the NCBI website (Altschul et al., 1997). The full-length CaPAL1 cDNA is 2318 bp, and contains a 70 bp 5’ untranslated region, a 2154 bp coding region that encodes a protein of 717 aa, and a 94 bp 3’ untranslated region (Supplementary Fig. S1A at JXB online). The deduced amino acid sequence of the CaPAL1 cDNA sequence was compared with plant PAL proteins of tomato, potato, tobacco, Ipomoea, and Arabidopsis. The sequence identities ranged from 82 to 93%. The PAL domain was placed at aa 199−215 in the CaPAL1 sequence (Supplementary Fig. S1B). The strict conservation of a PAL domain suggests that CaPAL1 may act as a PAL enzyme in nitrogen metabolism, phenylpropanoid biosynthesis, and alkaloid biosynthesis in pepper plants (Fritz et al., 2006).

To determine how many PAL genes are in pepper, pepper genomic DNA was digested with restriction enzymes EcoRI and HindIII, which do not have the recognition sites within the CaPAL1 cDNA. Hybridization of the EcoRI and HindIII genome digests with the full-length CaPAL1 cDNA showed three and four bands in the range of 2.7–7.1 kb, respectively (Supplementary Fig. S2 at JXB online). The full-length CaPAL1 cDNA (2154 bp) was used as a positive hybridization control. These results indicated that the CaPAL gene family is composed of three to four genes in pepper plants.

CaPAL1 is induced by avirulent Xcv infection

Expression of the CaPAL1 gene in different pepper organs was examined by RNA gel blot analysis. CaPAL1 transcript levels were barely detectable in stem and flower tissues, but expression was constitutively much higher in roots. Healthy leaf and fruit tissues did not constitutively express CaPAL1 (Supplementary Fig. S3A at JXB online). We investigated whether CaPAL1 is induced in pepper leaves by virulent or avirulent Xcv infection (Supplementary Fig. S3B). Infection with the avirulent Xcv Bv5-4a (incompatible interaction), but not with the virulent Xcv Ds1, strongly induced CaPAL1 in pepper leaves. CaPAL1 induction remained extremely high 5–25 h after avirulent Xcv Bv5-4a infection. CaPAL1 transcripts were not detected in mock-inoculated leaves. The virulent Xcv Ds1 infection did not effectively induce CaPAL1 in pepper leaves compared with the induction in response to avirulent Xcv Bv5-4a infection.

CaPAL1 silencing in pepper confers increased susceptibility to Xcv infection

To investigate loss of function of CaPAL1 in pepper plants, we silenced CaPAL1 in pepper plants using the tobacco rattle virus (TRV) VIGS technique (Liu et al., 2002; Chung et al., 2004; Kim and Hwang, 2011). To increase the specificity of silencing, the 5’ partial sequence (546 bp) of the CaPAL1 coding region was used to construct pTRV2:CaPAL1.

Silencing of CaPAL1 in pepper plants resulted in a susceptible response to Xcv infection (Fig. 1). The disease phenotypes of CaPAL1-silenced plants were compared with those of empty-vector control plants after inoculation with virulent and avirulent Xcv at 10⁷ and 10⁸ colony-forming units (cfu) ml⁻¹, respectively. CaPAL1-silenced pepper leaves exhibited more severe chlorotic lesions 7 d after virulent Xcv inoculation compared with that of the empty-vector control. The susceptible regions were intensely fluorescent under UV illumination, indicating the increased accumulation of fluorescent phenolic compounds in silenced leaves (Fig. 1A). Localized hypersensitive response (HR)-like cell death was initiated in the empty-vector control leaves 2 d after inoculation with 10⁷ cfu ml⁻¹ of avirulent strain Bv5-4a; however, the HR-like cell death response significantly decreased in CaPAL1-silenced leaves during infection. Under UV illumination, dark yellow fluorescent lesions (characteristic of HR) were more numerous in the empty-vector control leaves than those in CaPAL1-silenced leaves (Fig. 1A). These disease phenotypes correlated with the proliferation of Xcv in silenced leaves. Xcv growth in CaPAL1-silenced leaves was significantly higher than that in empty-vector control leaves 3 d after inoculation with 5 × 10⁴ cfu ml⁻¹ virulent and avirulent Xcv strains (Fig. 1B).

We next investigated whether CaPAL1 silencing affected PAL activity in pepper leaves during Xcv infection (Fig. 1C). Avirulent (incompatible, Bv5-4a) Xcv infection induced significantly higher PAL activity than did virulent (compatible, Ds1) Xcv infection in both the empty-vector control and CaPAL1-silenced leaves. The induction of PAL activity by Xcv infection was distinctly compromised in CaPAL1-silenced leaves compared with that in empty-vector control leaves (Fig. 1C). These results indicated that CaPAL1 silencing promotes Xcv growth and suppresses pathogen-induced PAL activity in pepper leaves.

CaPAL1 silencing compromises the oxidative burst and cell death during Xcv infection

We analysed ROS and the HR cell death in empty-vector control and CaPAL1-silenced leaves during Xcv infection (Fig. 2). Silencing of CaPAL1 significantly attenuated the production of H₂O₂ by virulent or avirulent Xcv infection (Fig. 2A). The visual scores of the cell death phenotype were substantiated by an electrolyte leakage assay. The level of ion (electrolyte) leakage was significantly lower in CaPAL1-silenced leaves than that in empty-vector control leaves during virulent Xcv infection. Notably, the induction of ion leakage drastically declined in CaPAL1-silenced pepper leaves 48 h after the avirulent Xcv infection (Fig. 2B). Taken together, virulent Xcv infection did not significantly induce different phenotypes with respect to H₂O₂ production and cell death; however, significantly reduced H₂O₂ production and cell death were observed in the CaPAL1-silenced leaves during avirulent Xcv infection.

VIGS of CaPAL1 alters pepper defence-related gene expression and SA accumulation

To assess the efficiency of VIGS, CaPAL1 transcript levels were monitored by real-time RT-PCR in empty-vector
control (TRV:00) and CaPAL1-silenced (TRV:CaPAL1) pepper leaves infected with Xcv (Fig. 3). The CaPAL1 transcript levels decreased significantly in the CaPAL1-silenced pepper leaves during virulent or avirulent Xcv infection, indicating that CaPAL1 silencing was effective in suppressing CaPAL1 expression (Fig. 3).

Real-time RT-PCR analyses were performed to investigate whether silencing of CaPAL1 altered the expression of defence-related genes in pepper during Xcv infection (Fig. 3). Silencing of CaPAL1 significantly compromised the induction of CaPAL1 and SA-dependent CaPR1 (basic PR1) by virulent or avirulent Xcv infection. Consequently, the levels of CaPR1 induced in silenced plants were distinctly lower than those induced in the empty-vector control plants during Xcv infection. Silencing of CaPAL1 significantly inhibited CaDEF1 (defensin) expression in the healthy pepper leaves. However, the CaDEF1 levels during infection were similar in both empty-vector control and CaPAL1-silenced pepper leaves, although CaDEF1 was less expressed in silenced leaves 12 h after inoculation with the compatible Xcv (Fig. 3). Suppression of the induction of the SA-dependent marker gene CaPR1 by CaPAL1 silencing was clearly observed among the tested defence genes.

To investigate whether the effect of CaPAL1 silencing on SA accumulation was related to defence response, we analysed levels of free SA and total SA (free SA plus Glc-conjugated SA) in leaves of the empty-vector and CaPAL1-silenced pepper plants infected with compatible Ds1 (virulent) and incompatible Bv5-4a (avirulent) strains of Xcv (Fig. 4). CaPAL1 silencing significantly compromised the induction of SA accumulation during avirulent and virulent Xcv infection, although there was no significant difference in the SA induction between the empty-vector and silenced plants 24 h after inoculation with the virulent Xcv. Taken together, these results indicated that CaPAL1 and CaPR1 are involved in SA-dependent defence signalling during Xcv infection.

**Increased resistance of Arabidopsis CaPAL1-OX transformants to Pst**

The Arabidopsis CaPAL1-overexpression (OX) lines #2, #3, and #7, which constitutively expressed CaPAL1, were used to investigate CaPAL1 gain of function in planta (Fig. 5A). We tested whether overexpression of the CaPAL1 gene in Arabidopsis increased the resistance to the hemi-biotrophic bacterium Pst DC3000, and DC3000 strains harbouring
avrRpm1. Severe disease symptoms were observed on the leaves of WT plants but not on CaPAL1-OX leaves 2 d after inoculation with virulent Pst DC3000 (10^7 cfu ml^{-1}) (Fig. 5B). Severe HR phenotypes against avirulent Pst DC3000 (avrRpm1) were observed in CaPAL1-OX leaves compared with those in WT plants (Fig. 5B). All CaPAL1-OX lines exhibited significantly lower bacterial growth compared with that in WT plants 3 d after inoculation with virulent and avirulent Pst DC3000 (10^7 cfu ml^{-1}) (Fig. 5C).

We next analysed PAL activity in WT and CaPAL1-OX Arabidopsis plants during infection with Pst DC3000 and Pst DC3000 (avrRpm1) (Fig. 5D). The levels of PAL activity were slightly higher in healthy leaves of CaPAL1-OX plants compared with those in WT plants. As expected, infection with virulent Pst DC3000 or avirulent Pst DC3000 (avrRpm1) induced significantly higher PAL activities in CaPAL1-OX transgenic leaves compared with the induction of PAL activities in WT plants.

CaPAL1 overexpression in Arabidopsis stimulates oxidative burst and cell death during Pst infection

We investigated whether CaPAL1 overexpression altered the oxidative burst and cell death response during Pst infection (Fig. 6). H_2O_2 accumulation and cell death were quantified using the xylenol orange method and ion conductivity, respectively. H_2O_2 accumulation in CaPAL1-OX leaves was higher than that in WT leaves 12−48 h after inoculation with Pst DC3000 and DC3000 (avrRpm1) (Fig. 6A). As a cell death indicator, electrolyte leakage from leaf tissues in CaPAL1-OX plants was distinctly stimulated by Pst infection, compared with that in leaf tissues of WT plants (Fig. 6B). The avirulent DC3000 (avrRpm1) infection rapidly and strongly induced ion leakage from CaPAL1-OX leaves 12 h after bacterial infiltration.

CaPAL1 overexpression in Arabidopsis stimulates defence-related gene expression and SA accumulation during Pst infection

RT-PCR analyses showed that constitutive CaPAL1 overexpression was confirmed in leaves of transgenic Arabidopsis plants (Fig. 7). Both virulent Pst DC3000 and avirulent DC3000 (avrRpm1) infections induced significantly higher expression of SA-dependent AtPR1 and the ROS-producing NADPH oxidase AtRbohD in leaves of CaPAL1-OX plants than in leaves of WT plants. By contrast, the levels of the oxidative stress-induced marker gene AtGST1 and the jasmonic acid-dependent marker gene AtPDF1.2 were similar in WT and CaPAL1-OX transgenic leaves (Supplementary Fig. S4 at JXB online).
PALs are one of the most extensively studied enzymes in plant biology. They catalyze the first step in the phenylpropanoid pathway, which leads to the synthesis of diverse natural products such as hydroxycinnamic acid derivatives, stilbenes, and flavonoids. PAL activities are abundant in root tissues, barely detectable in stem and flower tissues, and not constitutively expressed in leaves, green fruit, and red fruit of healthy pepper plants. Suppression of CaPAL1 expression in pepper plants results in abnormal phenotypes such as delayed root formation and an underdeveloped root system. These data suggested that CaPAL1 expression significantly increases PAL activity, which triggers the immune response in plants.

**Discussion**

PALs are encoded by a multi-gene family in plant species such as *Arabidopsis* (Wanner *et al.*, 1995; Cochrane *et al.*, 2004), *Solanum lycopersicum* (Guo and Wang, 2009), *Bambusa oldhamii* (Hiuch et al., 2010a, b), and *Phyllostachys edulis* (Gao *et al.*, 2012). PALs are one of the most extensively studied plant enzymes, particularly with respect to their responses to a variety of biotic and abiotic stress (Dixon and Paiva, 1995; MacDonald and D’Cunha, 2007). The biological functions of the PAL genes have been investigated through gene silencing in tobacco and disruption of the *PAL1, PAL2, PAL3, and PAL4* genes in *Arabidopsis* (Elkind *et al.*, 1990; Pallas *et al.*, 1996; Rohde *et al.*, 2004; Huang *et al.*, 2010).

In this study, we isolated and functionally characterized the PAL gene (*CaPAL1*) in pepper (*Capsicum annuum*) plants. Based on the observed results, *CaPAL1* was proposed to function as a positive regulator of plant innate immunity. To determine the effect of the loss-of-function or gain-of-function PAL gene in pepper plants, we generated *CaPAL1* knockdown pepper plants using the VIGS technique (Liu *et al.*, 2002) and *CaPAL1-OX* transgenic *Arabidopsis* using *Agrobacterium*-mediated transformation (Clough and Bent, 1998). First, the strict conservation of a PAL domain in the deduced amino acid sequence of CaPAL1 suggested that CaPAL1 may function as a PAL enzyme in pepper plants. The suppression or induction of PAL activity in plants during pathogen infection was confirmed by biochemical assays (Song and Wang, 2011) on the *CaPAL1*-silenced pepper plants and the *CaPAL1-OX* transgenic *Arabidopsis* plants. These data suggested that *CaPAL1* expression significantly increases PAL activity, which triggers the immune response in plants.

PAL catalyses the first step of the phenylpropanoid pathway and the synthesis of diverse natural products based on the phenylpropane skeleton, such as hydroxycinnamic acid, stilbenes, and flavonoids, which fulfill many essential roles in higher plants (MacDonald and D’Cunha, 2007). *CaPAL1* transcripts are abundant in root tissues, rarely detectable in stem and flower tissues, and not constitutively expressed in leaves, green fruit, and red fruit of healthy pepper plants. Suppression of PAL in *Salvia miltiorrhiza* plants results in abnormal phenotypes such as delayed root formation and an underdeveloped root system (Song and Wang, 2011), indicating that PAL is required for root development in plants. Consistent with these results, the constitutive expression of *CaPAL1* in pepper roots supports the hypothesis that PAL activity may be required for...
Fig. 5. Increased resistance of CaPAL1-OX Arabidopsis plants to Pst DC3000 and DC3000 (avrRpm1) infection. (A) RT-PCR analysis of CaPAL1 expression in leaves of WT and CaPAL1-OX transgenic plants. AtACT1 expression was visualized as a control. (B) Disease symptoms developed on the leaves 2 d after inoculation (10^7 cfu ml^-1). (C) Bacterial growth in the leaves of WT and CaPAL1-OX transgenic plants 0 and 3 d after inoculation (10^5 cfu ml^-1). (D) PAL activity in leaves of WT and CaPAL1-OX transgenic plants infected with Pst (10^7 cfu ml^-1). Data are the means±SD from three independent experiments. Statistical significances according to Fisher’s protected LSD test (P<0.05) are indicated by different letters above the data points. (This figure is available in colour at JXB online.)

Fig. 6. Increased ROS burst and cell death in leaves of CaPAL1-OX transgenic Arabidopsis plants infected with Pst DC3000 and DC3000 (avrRpm1). (A) Quantification of H_2O_2 from leaf tissues. (B) Quantification of electrolyte leakage from leaf tissues. Statistical significances according to Fisher’s protected LSD test (P<0.05) are indicated by different letters above the data points.
proper root formation and development. The upregulation of CaPAL1 in pepper leaves by avirulent Xcv infection suggests that CaPAL1 plays a crucial role in the induction of plant defence in response to microbial pathogens.

Phenylpropanoid compounds have been proposed to play crucial roles in plant defence to microbial pathogens based on the correlation between rates of phenylpropanoid accumulation and expression of resistance in vivo (Dixon and Paiva, 1995; La Camera et al., 2004). CaPAL1 silencing in pepper plants confers increased susceptibility to Xcv infection. Some phenylpropanoid compounds accumulate to high levels in plants that are resistant to an invading pathogen (Dixon, 2001; La Camera et al., 2004). These pathogen-induced phenylpropanoids, such as pterocarpans, isoflavans, stilbenes, and coumarins, act as phytoalexins, which have antimicrobial activity against plant-pathogenic fungi and bacteria (Dixon and Paiva, 1995; Barber et al., 2000; La Camera et al., 2004). The induction of PAL activity by Xcv infection was significantly compromised in CaPAL1-silenced pepper plants compared with that in empty-vector control plants. The level of PAL activity in CaPAL1-silenced plants was lower than that in the empty-vector control plants. These results suggested that PAL activity in pepper plants contributes to the basal and R gene-mediated resistance to Xcv infection.

SA-dependent signalling controls activation of sophisticated plant defence mechanisms to ward off attacks from microbial pathogens (Delaney et al., 1994; Lu, 2009; Vlot et al., 2009; Robert-Seilaniantz et al., 2011). CaPAL1 silencing in pepper leaves significantly compromised the induction of SA accumulation and SA-dependent CaPRI expression during avirulent and virulent Xcv infection. CaPAL1 overexpression in Arabidopsis strongly stimulated SA induction as well as AtPRI expression in leaves of the transgenic plants infected with both virulent Pst DC3000 and avirulent Pst DC3000 (avrRpm1). These results suggest that CaPAL1 acts as a positive regulator of SA-dependent defence signalling and downstream defence gene expression. It has been proposed that PAL activity is important for pathogen-induced SA formation in plants (Pallas et al., 1996; La Camera et al., 2004). The levels of free SA produced in pathogen-inoculated leaves of PAL-silenced Nicotiana tabacum plants were lower than those in the WT control plants (Pallas et al., 1996). The PAL inhibitor 2-aminoindan-2-phosphonic acid abolishes pathogen- or pathogen elicitor-induced SA accumulation in potato, cucumber, and Arabidopsis (Meuwly et al., 1995; Lu, 2009; Slusarenko, 1996; Coquez et al., 1998). Two pathways of SA biosynthesis have been proposed in plants (Chen et al., 2009).
One is the phenylpropanoid pathway controlled by PAL as the first enzyme (Huang et al., 2010), and the other is the chorismate pathway controlled by isochorismate synthase (ICS) (Wildermuth et al., 2001; Catinot et al., 2008; Chen et al., 2009). Previous evidence from Arabidopsis has indicated that the chorismate pathway is the most important for SA biosynthesis after stress stimuli (Garcion et al., 2008). In fact, knockout in the two ICS genes (ics1 ics2 double mutant) in Arabidopsis completely abolishes SA accumulation after stress (Garcion et al., 2008). On the other hand, plants knocked out in the four PAL genes from Arabidopsis (pal1 pal2 pal3 pal4 quadruple mutant), which retain 10% of PAL activity, also showed a reduction in the basal levels of SA (up to 25% of WT) and in the pathogen-induced levels (50% of WT) (Huang et al., 2010). However, whether SA is produced via the chorismate pathway in pepper remains to be determined.

In this study, VIGS of CaPAL1 altered pepper defence-related gene expression. Suppression of the induction of the SA-dependent marker gene CaPRI by CaPAL1 silencing was apparent among the tested defence-related genes, suggesting that CaPAL1 is required for SA-dependent defence signalling during Xcv infection. The oxidative burst and H$_2$O$_2$ accumulation seem to be essential for the establishment of plant immunity (Alvarez et al., 1998; Apel and Hirt, 2004; Choi et al., 2007). Consistent with these results, VIGS of CaPAL1 compromised H$_2$O$_2$ accumulation and cell death during Xcv infection, suggesting that CaPAL1 expression triggers oxidative burst and cell death to promote the plant immune response.

Constitutive overexpression of CaPAL1 in transgenic Arabidopsis lines was used to investigate whether CaPAL1-OX affects the defence response and PAL activity when expressed epigenetically. CaPAL1-OX plants showed increased resistance to both virulent and avirulent strains of Pseudomonas syringae pv. tomato (Pst) and to virulent Hpa Noco2. PAL activity in CaPAL1-OX Arabidopsis was significantly higher than that in non-inoculated WT plants. PAL activity was increased in
both CaPALI-OX and WT plants after Pst infection. These results suggested that constitutive expression of CaPALI may lead to the induction of PAL activity in response to Pst infection. Although PAL activity in CaPALI-OX transgenic plants was significantly higher than that in WT plants, the levels of induction of PAL activity were similar in CaPALI-OX and WT Arabidopsis infected with virulent Hpa Noco2. PAL activity in rosette leaves was generally higher than that in cotyledons, suggesting that the phenylpropanoid pathway may be more active in rosette leaves than in cotyledons. During Pst infection, the induction of ROS accumulation and ion leakage were higher in CaPALI-OX plants. Expression of the SA-dependent AtPR1 and the ROS-producing NADPH oxidase AtRbohD in leaves of CaPALI-OX plants was higher than that in WT plants. AtRbohD was proposed to be required for ROS accumulation in the plant defence response (Torres et al., 2002). Collectively, these results suggest that the CaPAL gene is required for the induction of SA-dependent defence signalling events in plants.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. (A) Nucleotide and deduced amino acid sequences of pepper CaPALI cDNA encoding a class family of phenylalanine ammonia-lyase. (B) Comparison of the deduced amino acid sequence of CaPALI with phenylalanine ammonia-lyase from other plant species.

Supplementary Fig. S2. Genomic DNA gel blot analysis of CaPALI. Genomic DNA from pepper plants was digested with restriction enzymes EcoRI and HindIII. CaPALI open reading frame cDNA was used as a positive control. Red arrowheads indicate the genomic DNA fragments hybridized with [32P]dCTP-labelled CaPALI probe.

Supplementary Fig. S3. RNA gel blot analyses of CaPALI expression in pepper plants at the six-leaf stage. (A) Constitutive expression of CaPALI in various organs of pepper plants. (B) Time course of induction of CaPALI in leaf tissues infected with virulent strain Ds1 and avirulent strain Bv5-4a of Xanthomonas campestris pv. vesicatoria (10^7 cfu ml^{-1}). The RNA gel blot was hybridized with [32P]-labelled CaPALI probe.

Supplementary Fig. S4. Real-time RT-PCR analyses of the expression of AtGST1 and AtPDF1.2 in WT and CaPALI-OX Arabidopsis plants infected with Pseudomonas syringae pv. tomato (Pst) DC3000 and Pst DC3000 (avrRpm1). AtGST1, glutathione S-transferase; AtPDF1.2, plant defensin 1.2. Arabidopsis ACT1 was used as an internal control. The control sample was normalized to 1. Data are the means±SD from three independent experiments. Statistical significances according to Fisher’s protected LSD test (P<0.05) are indicated by different letters above the data points.

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