RESEARCH PAPER

The *Capsicum annuum* class IV chitinase ChitIV interacts with receptor-like cytoplasmic protein kinase PIK1 to accelerate PIK1-triggered cell death and defence responses

Dae Sung Kim*, Nak Hyun Kim and Byung Kook Hwang†

Laboratory of Molecular Plant Pathology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea

*Present address: The Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK.

† To whom correspondence should be addressed. E-mail: bkhwang@korea.ac.kr

Received 28 April 2014; Revised 15 December 2014; Accepted 17 December 2014

Abstract

The pepper receptor-like cytoplasmic protein kinase, CaPIK1, which mediates signalling of plant cell death and defence responses was previously identified. Here, the identification of a class IV chitinase, CaChitIV, from pepper plants (*Capsicum annuum*), which interacts with CaPIK1 and promotes CaPIK1-triggered cell death and defence responses, is reported. CaChitIV contains a signal peptide, chitin-binding domain, and glycol hydrolase domain. CaChitIV expression was up-regulated by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) infection. Notably, avirulent *Xcv* infection rapidly induced CaChitIV expression in pepper leaves. Bimolecular fluorescence complementation and co-immunoprecipitation revealed that CaPIK1 interacts with CaChitIV in planta, and that the CaPIK1–CaChitIV complex is localized mainly in the cytoplasm and plasma membrane. CaChitIV is also localized in the endoplasmic reticulum. Transient co-expression of CaChitIV with CaPIK1 enhanced CaPIK1-triggered cell death response and reactive oxygen species (ROS) and nitric oxide (NO) bursts. Co-silencing of both CaChitIV and CaPIK1 in pepper plants conferred enhanced susceptibility to *Xcv* infection, which was accompanied by a reduced induction of cell death response, ROS and NO bursts, and defence response genes. Ectopic expression of CaPIK1 in *Arabidopsis* enhanced basal resistance to *Hyaloperonospora arabidopsidis* infection. Together, the results suggest that CaChitIV positively regulates CaPIK1-triggered cell death and defence responses through its interaction with CaPIK1.

Key words: Cell death, class IV chitinase, defence, pepper, *Xanthomonas campestris* pv. *vesicatoria*.

Introduction

Plants are exposed to a constant and diverse array of potential microbial pathogens and have developed the ability to protect themselves from pathogen attack by the early detection of disease-causing agents (Kenrick and Crane, 1997; Jones and Dangl, 2006). Recognition of microbial pathogens activates defence responses, including activation of mitogen-activated protein kinase (MAPK) cascades and accumulation of reactive oxygen species (ROS) and nitric oxide (NO), and activation of transcriptional factors, leading to the timely expression of pathogenesis-related (PR) genes (Chisholm et al., 2006; van Loon et al., 2006; Asai et al., 2008; Kim and Hwang, 2011; Choi et al., 2012; Meng and...
Zhang, 2013). The hypersensitive response (HR) is the most effective and best known plant response to pathogen attacks. It is a form of programmed cell death (PCD) in which cells around the infection site undergo rapid necrosis. The HR is associated with a co-ordinated and integrated set of metabolic modifications that are integral to hindering the further progress of pathogens, as well as to enhancing the ability of the host to limit subsequent infection by various pathogens (Greenberg, 1997; van Loon and Strien, 1999; Greenberg and Yao, 2004). The HR is activated by intracellular resistance (R) proteins which recognize effector proteins derived from avirulent pathogens, so-called effector-triggered immunity (ETI). ETI is generally characterized by the induction of HR at the site of infection and of systemic acquired resistance (SAR) at distal sites (Dangl and Jones, 2001; Chisholm et al., 2006; Yao and Greenberg, 2006).

Protein kinases are well-characterized, essential proteins that act through phosphorylation as diverse key enzymes in signal transduction (Stone and Walker, 1995). A growing body of evidence highlights the importance of protein kinases in various aspects of plant immunity (Dardick et al., 2007; Meng and Zhang, 2013). Plant receptor-like cytoplasmic protein kinases (RLCKs) belong to the superfamily of receptor-like kinases (RLKs). Well-known RLCKs include PBS1, PBL1, and BIK1 from Arabidopsis thaliana (L.) Heynh, and Pto, Pti, and Tpk1b from tomato (Solanum lycopersicum L.), which regulate plant immunity against biotrophic and necrotrophic pathogens (Martin et al., 1993; Zhou et al., 1995; Swiderski and Innes, 2001; AbuQamar et al., 2008; Zhang et al., 2010). In a previous study (Kim and Hwang, 2011), the pepper receptor-like cytoplasmic protein kinase, CaPIK1, which mediates signalling of cell death and defence responses to microbial pathogens was identified. CaPIK1 expression in pepper plants (Capsicum annuum L.) triggers immune responses including ROS and NO bursts, as well as callose deposition, ultimately leading to HR-like cell death.

Plants produce many types of chitinases, which catalyse the degradation of chitin, a linear polymer of N-acetyl-D-glucosamine (GlcNAc). Chitinases are grouped into seven classes based upon their primary structure (Collinge et al., 1993; Neuhau et al., 1996; Gomez et al., 2002; Wiweger et al., 2003). Different chitinase classes are defined depending on sequence similarities and the presence of an N-terminal cysteine-rich domain, usually referred to as hevein-like domain or chitin-binding domain (CBD), which is separated from the catalytic domain by a hinge region (Gomez et al., 2002). Only chitinases of classes I and IV possess a CBD. Chitinases in class IV are phylogenetically related to classes I and II chitinases (Gomez et al., 2002; Wiweger et al., 2003). Collinge et al. (1993) proposed that class IV chitinases evolved from class I through a series of four deletions, one of which removed a vacuole-targeting sequence; as a result, class IV chitinases are secreted to the apoplast rather than targeted to vacuoles.

It is known that plant chitinases play important roles in defence against pathogenic attacks (Gomez et al., 2002; Hong and Hwang, 2002; Hieta et al., 2004) and stress response (Hong and Hwang, 2006; Takenaka et al., 2009), and in growth and development (Wiweger et al., 2003). Many chitinases have been classified as pathogenesis-related proteins of the PR-1, PR-8, PR-4, and PR-11 families (Neuha et al., 1996). Antifungal activity has been reported for chitinases that contain an N-terminal CBD (classes I and IV) and also for enzymes that lack such a domain (Schlumbaum et al., 1986; Gomez et al., 2002). There is increasing evidence that transgenic plants constitutively overexpressing chitinases exhibit elevated resistance to pathogens (Broglie et al., 1991; Grison et al., 1996; Shin et al., 2008). Plant class IV chitinases are mainly involved in regulating resistance to fungal pathogens (Hieta et al., 2004). There is some evidence that class IV chitinases are implicated in other processes, such as the response to abiotic stress (Gerhardt et al., 2004) and defence against bacterial pathogens (Gerhardt et al., 1997). However, our knowledge of the cellular mechanisms by which class IV chitinases activate plant cell death and innate immunity is still limited, and functional studies of class IV chitinases are needed to provide evidence for their distinct functions.

Previously, the pathogen-induced CaPIK1 (pepper receptor-like cytoplasmic protein kinase) was identified as a positive regulator of plant cell death and defence responses (Kim and Hwang, 2011). In the current study, the pepper class IV chitinase, CaChitIV, which interacts with CaPIK1 in yeast and in planta, was isolated and functionally characterized Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) experiments revealed that CaPIK1 interacts with CaChitIV in planta, with the CaChitIV–CaPIK1 complex being localized mainly to the cytoplasm and plasma membrane. CaChitIV is secreted to the apoplastic region via the endoplasmic reticulum (ER). Transient co-expression of CaChitIV with CaPIK1 enhanced the CaPIK1-triggered cell death response and ROS burst, as well as NO burst. Virus-induced gene silencing (VIGS) of CaChitIV or/and CaPIK1 in pepper plants conferred enhanced susceptibility to Xanthomonas campestris pv. vesicatoria (Xcv) infection. In contrast, heterologous CaChitIV overexpression in Arabidopsis enhanced basal resistance to Hyaloperonospora arabidopsis (Hpa) infection. The results suggest that CaChitIV positively regulates ROS and NO burst, leading to plant cell death and defence responses through its interaction with CaPIK1.

Materials and methods

Plant growth and pathology assays

Pepper (Capsicum annuum L., cv Nockwang) and tobacco (Nicotiana benthamiana) plants were grown in soil mix (peat moss/perlite/vermiculite, 2:1:1, v/v/v) at 26 °C with a photoperiod of 16 h at a light intensity of 130 μmol m−2 s−1 and 60% relative humidity in an environmental growth chamber. Arabidopsis thaliana wild-type (ecotype Columbia, Col-0) and transgenic seeds were surface-sterilized with ethanol and washed, before undergoing imbibition at 4 °C for 3 d to overcome dormancy. Plants were grown in soil mix at 24 °C under long-day conditions (16 h light/8 h dark cycle) or under short-day conditions (12 h light/12 h dark) at a light intensity of 130 μmol m−2 s−1 and 60% relative humidity in an environmental growth chamber.
Virulent Ds1 and avirulent Bs5-4a strains of Xcv (Kim et al., 2010) were cultured overnight in yeast nutrient broth (5 g L⁻¹ yeast extract, 8 g L⁻¹ nutrient broth), harvested, re-suspended in sterile tap water to a concentration of 5 × 10⁸ cfu ml⁻¹, and used to infiltrate fully expanded pepper leaves. To inoculate Arabidopsis leaves, Pseudomonas syringae pv. tomato (Pst) DC3000 and DC3000 (avrRpm1) were grown in King’s B broth (10 g L⁻¹ peptone, 1.5 g L⁻¹ K₂HPO₄, 15 g L⁻¹ glycerol, and 5 g L⁻¹ MgSO₄). Bacterial cultures were diluted to the appropriate density and infiltrated into plant leaves. The infected leaves were sampled at various time points for bacterial growth assay, RNA isolation, and histochemical assay.

Spores of Hpa isolate Noco2, known to be virulent to Arabidopsis ecotype Col-0, were collected in sterile tap water containing 0.05% Tween-20 from infected cotyledons and leaves. Spore suspensions (5 × 10⁴ conidiaospores ml⁻¹) were sprayed onto 7-day-old Arabidopsis seedlings, infected plants were covered with plastic wrap to maintain moisture, and the number of sporangiophores on cotyledons was counted to assess disease severity 7 d after infection. Infected cotyledons were sampled for histochemical assay after 3 d.

Yeast two-hybrid screening

Yeast two-hybrid screening was conducted using the GAL4 system, according to the manufacturer’s instructions (Matchmaker™ GAL4 Two-Hybrid System 3, Clontech, CA, USA). The full-length CaPIK1 coding regions were amplified using PCR and cloned into the EcoRI/BamHI restriction sites of the bait vector pGBK7T, which includes the GAL4 DNA-binding domain (BD). A yeast two-hybrid cDNA library was constructed in the prey vector pGADT7, which contains a GAL4 activation domain (AD), using cDNA constructed from pepper leaves infected with the Xcv avirulent strain Bs5-4a.

Constructs were introduced into yeast strain AH109 using the lithium acetate-mediated transformation method, and transformants were arrayed on interaction selection media [SD-Adenine (Ade)-Histidine (His)-Leucine (Leu)-Tryptophan (Trp)], supplemented with 40 μg ml⁻¹ 5-bromo-4-chloro-3-indoyl-α-D-galactoside (X-Gal), to score growth and colony colour as indicators of protein–protein interactions.

Bimolecular fluorescence complementation (BiFC) analysis

BiFC analyses were conducted as described previously (Walter et al., 2004). For the BiFC constructs, cDNAs encoding CaPIK1 and CaChitIV without termination codons were amplified using PCR and recombined into the binary vectors pSPYNE and pSPYCE; harboring YFPN and YFP (yellow fluorescent protein N- and C-termini), respectively, under the control of the Cauliflower mosaic virus (CaMV) 35S promoter, resulting in CaPIK1-YFPN and CaChitIV-YFP. Agrobacterium tumefaciens strain GV3101 was transformed with the BiFC constructs, and cultures were co-infiltrated into N. benthamiana leaves. Three days after infiltration with Agrobacterium, leaves were visualized using an LSM5 Exciter confocal laser-scanning microscope (Carl Zeiss, Germany) with excitation at 488 nm and emission at 505–530 nm.

Green fluorescent protein (GFP) fluorescence microscopy

For GFP constructs, the CaChitIV coding region and the signal peptide-deleted CaChitIV (CaChitIVASP) were PCR amplified and introduced into Xhol/BamHI sites of the binary vector pBIN35S:326-GFP to generate a C-terminal soluble-modified GFP (smGFP)-tagged fusion protein. For particle bombardment, onion (Allium cepa L.) epidermis was bombarded with gold particles coated with plasmids using a Bio-Rad (Hercules) PDS-1000/He particle delivery system. Bombarded specimens were incubated for 24 h on 0.5× Murashige and Skoog (MS) agar medium and observed using a LSM 5 Exciter confocal laser-scanning microscope (Carl Zeiss, Germany) with excitation at 488 nm and emission at 505–530 nm.

Agrobacterium-mediated transient expression of smGFP-tagged constructs in N. benthamiana leaves was used. CaChitIV-GFP or CaChitIVASP-GFP constructs under control of the CaMV 35S promoter were introduced into A. tumefaciens strain GV3101 by electroporation. Three days after infiltration with Agrobacterium, epidermal cells of N. benthamiana leaves were observed using a confocal laser-scanning microscope, as described above. The presence of GFP-tagged proteins was confirmed by immunoblotting using anti-GFP antibody.

Immunoblotting

For Co-IP, total proteins were extracted from leaves using immunoprecipitation buffer [50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, and protease inhibitor cocktail (Roche, Mannheim, Germany)]. Insoluble debris was pelleted by centrifuging leaf extracts at 15 000 g for 30 min at 4°C. The soluble protein extracts were incubated with monoclonal anti-cMyc or anti-HA agarose conjugates (Sigma-Aldrich, St Louis, MO, USA) overnight. Beads were collected and washed three times with wash buffer [50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, and protease inhibitor cocktail (Roche)]. Eluted proteins were analysed using immunoblotting with anti-cMyc or anti-HA peroxidase conjugates. Immunodetection was performed using the WEST-ZOL plus protein gel blot detection system, according to the manufacturer’s instructions (INTRON, Seoul, Korea).

RNA gel blot and quantitative reverse transcription–PCR (RT–PCR) analyses

Total RNA was extracted from pepper plants using IsoI-RNA lysis reagent (5 Prime, Gaithersburg, MD, USA), according to the manufacturer’s instructions. Total RNA (20 μg) was denatured by heating at 65°C for 10 min in a formaldehyde gel loading buffer and then separated by electrophoresis on 7.4% formaldehyde/L.2% agarose gels. Gels were immersed in denitoxin water for 30 min and RNA transferred to Hybond™-N+ membranes (Amersham, Little Chalfont, UK), followed by cross-linking under UV illumination.

To generate the CaChitIV gene-specific probe, full-length CaChitIV cDNA was labelled with [32P]dCTP using the Klenow fragment of DNA polymerase 1 (Roche). Membranes were prehybridized and then hybridized overnight with the probe at 65°C. After hybridization, the membranes were washed twice with 2× SSC, 0.1% SDS for 10 min at room temperature and once with 0.1× SSC, 0.1% SDS for 15 min. The membranes were exposed to X-ray film (Agfa, Mortsel, Belgium).

For real-time RT–PCR analysis was prepared using 1 μg of total RNA, 500 ng of oligo d(T)15 (primer), and Moloney murine leukaemia virus reverse transcriptase at 42°C for 1 h. Real-time PCR was performed with 1 μl of cDNA as a template and 45 reaction cycles, using iQTM SYBR Green Supermix and an iCyclerIQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to the manufacturer’s instructions. To normalize the transcript levels, C. annuum 18S rRNA and CaACTIN expression was monitored as reference genes in each reaction. The gene-specific primers used for the quantitative real-time RT–PCR analysis are listed in Supplementary Table S1 available at JXB online.

Agrobacterium-mediated transient expression in pepper plants

The cMyc-tagged CaPIK1 and HA-tagged CaChitIV were cloned into the pBIN35S plant binary vector, and A. tumefaciens strain GV3101 was transformed with the resulting plasmids using electroporation. The overnight cultures were centrifuged, harvested cells were diluted to OD₆₀₀=1.0 in infiltration buffer (10 mM MES, 10 mM MgCl₂, pH 5.7), and acetoxymer-ino was added to a final concentration of 200 μM. The bacterial suspensions were infiltrated into the leaves of pepper plants at the six-leaf stage using a needleless syringe.
**Virus-induced gene silencing (ViGS)**

*Toxin tomato leaf virus* (ToxTV)-based ViGS of *CaChitIV* and *CaPIK1* was performed in pepper plants (Liu et al., 2002; Chung et al., 2004). To elevate silencing specificity, 188 bp and 195 bp of non-conserved 5′ untranslated regions (UTRs) of the genes were PCR amplified and cloned into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA, USA), followed by their digestion with EcoRI. The eluates were inserted into the EcoRI site of pTRV2 to generate TRV2:CaChitIV and TRV2:CaPIK1 constructs, respectively. Two weeks after sowing, expanded cotyledons of pepper plants were co-infiltrated with *A. tumefaciens* strain GV3101 transformed with ViGS vectors pTRV1 and pTRV2 containing *CaChitIV-UTR* and *CaPIK1-UTR*.

**Arabidopsis transformation**

To induce constitutive *CaChitIV* overexpression (OX) in *Arabidopsis*, transgenic plants were generated using the floral dipping method (Clough and Bent, 1998). The *CaChitIV* coding region was amplified and inserted into XbaI/BamHI sites of the binary vector pBIN35S under the control of the CaMV 35S promoter (Choi et al., 2012). A pBIN35S:CaChitIV construct was introduced into *A. tumefaciens* strain GV3101 through electroporation. Transformants were selected on 0.5× MS agar plates containing 50 μg ml⁻¹ kanamycin. Three transgenic *Arabidopsis* lines (#1, #2, and #3) were confirmed using RT-PCR analysis.

**Measurement of ion conductivity, H₂O₂, and NO bursts**

Cell death was quantified by ion conductivity measurement. At various time points following bacterial infiltration, eight leaf discs (1.4 cm in diameter) were excised and washed for 30 min in 20 ml of distilled water. After incubation for 3 h in 20 ml of distilled water, ion conductivity was measured using a Sension7 conductivity meter (Hach, Loveland, CO, USA).

H₂O₂ production in pepper leaves was quantified using the xylenoI orange assay (Choi et al., 2007). Briefly, the xylenoI orange assay reagent was freshly prepared: 200 μl of a solution [25 mM FeSO₄, 25 mM (NH₄)₂SO₄ in 2.5 M H₂SO₄] was added to 20 ml of 125 μM xylenoI orange in 100 mM sorbitol. Eight leaf discs (0.5 cm²) were floated on 1 ml of solution [25 mM FeSO₄, 25 mM (NH₄)₂SO₄ in 2.5 M H₂SO₄] for 30 min at room temperature. H₂O₂ production was monitored by measuring the absorbance at 560 nm using a DU 650 spectrophotometer (Beckman, Urbana, IL, USA). A standard curve was generated from measurements obtained from serial dilutions of H₂O₂ from 100 nmol to 100 μmol.

NO production was monitored using the NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma). Leaves were infiltrated with 200 mM sodium phosphate buffer (pH 7.4) including 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma). Leaves were sliced into discs 0.5 cm in diameter and floated on 1 ml of solution [25 mM FeSO₄, 25 mM (NH₄)₂SO₄ in 2.5 M H₂SO₄] was added to 20 ml of 125 μM xylenoI orange in 100 mM sorbitol. Eight leaf discs (0.5 cm²) were floated on 1 ml of distilled water in a microtube for 1 h. After centrifugation for 1 min at 12 000 g, 100 μl of supernatant was added to 1 ml of xylenoI orange assay reagent. The mixture was incubated for 30 min at room temperature. H₂O₂ production was monitored by measuring the absorbance at 560 nm using a DU 650 spectrophotometer (Beckman, Urbana, IL, USA). A standard curve was generated from measurements obtained from serial dilutions of H₂O₂ from 100 nmol to 100 μmol.

**Results**

*CaPIK1 interacts with CaChitIV in yeast and in planta*

The pepper receptor-like cytoplasmic protein kinase gene (*CaPIK1*) was previously isolated from pepper leaves infected with *Xcv* (Kim and Hwang, 2011). To identify proteins interacting with *CaPIK1*, *CaPIK1* was used as bait to screen a pepper cDNA library generated from avirulent *Xcv*-infected leaves using a GAL4-based yeast two-hybrid screen. Among the clones identified by screening, a pepper class IV chitinase, *CaChitIV* (accession no. KJ649334), was selected for further characterization as an interacting partner of *CaPIK1* (Supplementary Fig. S1 at *JXB* online).

The specific interaction between *CaPIK1* and *CaChitIV* was verified by a vector change and re-transformation protocol. After recovering initial fusion constructs (BD/CaPIK1 and AD/CaChitIV) from positive colonies, pepper *CaPIK1* and *CaChitIV* cDNAs were re-introduced into pGADT7 and pGBKKT7, respectively, to produce AD/CaPIK1 and BD/CaChitIV. The murine p53 (BD/p53), human lamin C (BD/Lam), and SV40 large T antigen (AD/T) were used as interaction controls. All the yeast constructs containing the indicated combinations of plasmids grew on synthetic dropout (SD) medium lacking leucine (L) and tryptophan (T). The yeast combination BD/CaPIK1 and AD/CaChitIV grew well on plates lacking adenine, histidine, leucine, and tryptophan (SD-AHLT), as did the combination BD/CaChitIV and AD/CaPIK1; both combinations showed blue colour on the X-α-Gal plate, as did a positive control harbouring BD/p53 and AD/SV40-T (Fig. 1A). This indicates that *CaPIK1* specifically interacts with *CaChitIV* in the GAL4-based yeast two-hybrid system.

The interaction of *CaPIK1* and *CaChitIV* in planta was examined using BiFC analysis (Walter et al., 2004). BiFC vectors (pSPYNE and pSPYCE) containing YFP N and YFP C, respectively, were used to construct CaPIK1–YFP N and CaChitIV–YFP C, or vice versa. Interactions between the fusion proteins were visualized in *N. benthamiana* leaves using *Agrobacterium*-mediated transient co-expression. Combinations of CaChitIV–YFP N and CaPIK1–YFP C, or vice versa, were observed in the cytoplasm and the plasma membrane, indicating that *CaPIK1* binds to *CaChitIV* in plant cells (Fig. 1B). A combination of bZIP63–YFP N and bZIP63–YFP C was used as a nuclear-localized BiFC control (Walter et al., 2004; Fig. 1B).

The *CaPIK1* and *CaChitIV* interaction in planta was further confirmed using Co-IP (Fig. 1C). CMyc-tagged *CaChitIV* and/or HA-tagged *CaPIK1* were transiently expressed in *N. benthamiana* leaves. Three days after infiltration, proteins were extracted from leaves and incubated with monoclonal anti-CMyC agarose conjugates to immunoprecipitate *CaChitIV*. After immunoprecipitation, potential *CaChitIV* and *CaPIK1* complexes were separated using SDS–PAGE. *CaPIK1*-HA was detected only when co-expressed with *CaChitIV*-CMyC. These results indicate that *CaPIK1* physically interacts with *CaChitIV* in plant cells.

**Sequence and expression analysis of *CaChitIV* in pepper**

*CaChitIV* (accession no. KJ649334) is a 990 bp cDNA encoding a chitinase of 277 amino acids (Supplementary Fig. S1 at *JXB* online). A BLAST search found that the *CaChitIV* protein sequence closely resembled other plant
CaChitIV in CaPIK1-triggered cell death

chitinases (Supplementary Fig. S2A), being 82% identical to Nicotiana tabacum chitinase (accession no. BAF44533), 61% identical to Oryza sativa chitinase (accession no. NP_001053186), 57% identical to Zea mays chitinase (accession no. NP_001158904; Chaudet et al., 2004), and 53% identical to Picea abies chitinase (accession no. AY270017; Ubhayasekera et al., 2009). As shown in Supplementary Fig. S2B, CaChitIV contains a signal peptide with the initiation methionine (amino acids 1–28), a CBD (amino acids 30–61), and a glycol hydrolase domain (glyco-hydro-19; amino acids 77–277), indicating that CaChitIV encodes an extracellular chitinase.

It was previously shown that CaPIK1 is constitutively expressed in flowers but either not at all or weakly in leaves, fruits, stems, and roots of healthy pepper plants (Kim and Hwang, 2011). However, CaPIK1 expression is strongly induced in pepper leaves by infection with virulent (Ds1) and avirulent (Bv5-4a) strains of Xcv (Kim and Hwang, 2011). In the present study, RNA gel blot analysis was used to investigate transcriptional regulation of CaChitIV, a CaPIK1-interacting partner, in pepper plants.

CaChitIV was constitutively expressed in flowers but detected only at relatively low levels in leaves, stems, green fruits, and red fruits (Fig. 2A). It was next investigated whether CaChitIV transcription is altered by Xcv infection (Fig. 2B). Infection with avirulent (incompatible) Bv5-4a Xcv rapidly and strongly induced expression of CaChitIV in pepper leaves. In contrast, weak induction of CaChitIV expression was seen in mock-inoculated leaves and in leaves infected with virulent (compatible) Xcv Ds1.

CaChitIV localizes to the endoplasmic reticulum

The first 18 amino acids of CaChitIV form an N-terminal signal peptide (Supplementary Fig. S2B at JXB online). Such signal peptides cause proteins to be targeted to the secretory pathway through organelles including the ER, Golgi body, or endosomes (Blobel and Dobberstein, 1975; Crowley et al., 1994; Johnson et al., 2013). To determine the subcellular localization of CaChitIV, C-terminal smGFP-tagged CaChitIV and signal peptide-deleted CaChitIV ΔSP were constructed. Using Agrobacterium-mediated transient expression, CaChitIV and CaChitIV ΔSP fusion proteins with GFP were expressed in N. benthamiana.
At 48 h after infiltration with *Agrobacterium*, CaChitIV:GFP expression was observed exclusively in polygonal net-like structures (Fig. 3A); however, by 72 h after infiltration, GFP signals were strongly detected in the cell periphery and apoplastic regions. Transient expression of CaChitIVASP:GFP in epidermal cells of *N. benthamiana* leaves was similar to that of the non-fused GFP (00:GFP) control, which was dispersed throughout the cytosol and nucleus (Fig. 3A). In onion epidermal cells, CaChitIV:GFP was localized as a membrane-bound spot at the cell periphery 24 h after bombardment (Supplementary Fig. S3A, B at *JXB* online); whereas, after 48 h, GFP signals were detected exclusively at the cell periphery and apoplastic regions.

The subcellular distribution of CaChitIV:GFP in net-like structures (Fig. 3A) resembles that of plant ER marker proteins, such as *Arabidopsis* Ca2+-ATPase, isoform 2 protein (ACA2p), and *A. thaliana* wall-associated kinase 2 (AtWAK2) (He *et al.*, 1999; Bracha *et al.*, 2002). To investigate whether CaChitIV:GFP also localizes to the ER, the ER marker, ER-rk CD3-959, which was created by adding the AtWAK2 signal peptide to the N-terminus of the mCherry fluorescent protein and the ER retention signal, His-Asp-Glu-Ler, to its C-terminus (Nelson *et al.*, 2007), was used. 00:GFP,CaChitIV:GFP or CaChitIVΔSP:GFP were transiently co-expressed with ER-rk CD3-959 in leaf epidermal cells of *N. benthamiana* (Fig. 3B). Co-localization of the fusion proteins, CaChitIV:GFP and ER-rk CD3-959, was observed indicating that CaChitIV:GFP localizes to the ER. In contrast, free-GFP and CaChitIVASP:GFP were detected only in the cytoplasm and nucleus of the same leaves (Fig. 3B). Transient expression of 00:GFP, CaChitIV:GFP, and CaChitIVΔSP:GFP in *N. benthamiana* leaves was confirmed by immunoblotting with GFP antibodies (α-GFP; Fig. 3C). Collectively, these results indicate that the signal peptide CaChitIV targets the protein to the ER.

**Co-expression of CaChitIV with CaPIK1 promotes CaPIK1-triggered cell death and defence responses**

Transient expression of CaPIK1 in pepper leaves triggers early defence responses, including ROS and NO bursts, and ultimately leads to HR-like cell death (Kim and Hwang, 2011). In the present study, it was investigated whether co-expression of CaChitIV with CaPIK1 in pepper leaves influenced the CaPIK1-triggered cell death response (Fig. 4).

Transient expression of empty vector or CaChitIV did not trigger the cell death response (Fig. 4A). Electrolyte leakage from pepper leaves co-expressing CaPIK1 and CaChitIV was significantly greater than that from leaves expressing CaPIK1 alone (Fig. 4B), indicating an enhanced level of necrosis. Moreover, co-expression of CaChitIV with CaPIK1 effectively enhanced CaPIK1-triggered HR-like cell death responses (Fig. 4A, C), indicating that CaChitIV positively regulates cell death induction by CaPIK1. Cell death responses were classified based on a 0–3 scale: 0, no cell death (<10%); 1, weak cell death (10–30%); 2, partial cell death (30–80%); and 3, full cell death (80–100%) (Choi and Hwang, 2011). The effect of co-expression of CaChitIV with CaPIK1 on the level of cell death was higher than that of CaPIK1 expression alone (Fig. 4C). The synergistic effects of CaChitIV on CaPIK1-mediated cell death in pepper were further investigated in leaves by co-expressing CaPIK1 at a...
low inoculum density (OD\textsubscript{600}=0.2) with different inoculum concentrations (OD\textsubscript{600}=0.05, 0.1, and 0.5) of \textit{CaChitIV}. Increasing the inoculum density of \textit{CaChitIV} enhanced cell death levels, trypan blue-stained cell death response, and electrolyte leakage (Fig. 4D, E). Expression of \textit{CaPIK1} and proteins was confirmed by immunoblot analysis (Fig. 4F). Increased inoculum concentration of \textit{CaChitIV} gradually increased the level of \textit{CaChitIV} expression in pepper leaves.

In addition, the effect of co-expressing \textit{CaChitIV} with \textit{CaPIK1} on ROS (Fig. 5A) and NO bursts (Fig. 5B) in pepper leaves was investigated. ROS are known to act as regulators of PCD in animal and plant cells (Jabs, 1999; Jones, 2001; Doyle et al., 2010) and NO is a reactive nitrogen species acting as an intermediate in multiple signalling pathways in plants (Besson-Bard et al., 2008). Levels of H\textsubscript{2}O\textsubscript{2} and NO gradually increased in pepper leaves transiently co-expressing \textit{CaPIK1} and \textit{CaChitIV}, in proportion to the original inoculum density of \textit{Agrobacterium}. Cell death levels were rated based on a 0–3 scale: 0, no cell death (<10%); 1, weak cell death (10–30%); 2, partial cell death (30–80%); and 3, full cell death (80–100%). (E) Electrolyte leakage from leaf discs at different time points following infiltration with different inoculum concentrations. (F) Immunodetection of \textit{CaPIK1-cMyc} and \textit{CaChitIV-HA} expression following infiltration with different inoculum concentrations; immunoblotting was performed with cMyc antibody (\textit{α-cMyc}) and HA antibody (\textit{α-HA}), respectively. Equal protein loading is shown by Ponceau S staining. For B, C, and E, data are the means ±standard deviations from three independent experiments. Statistically significant differences in B, C, and E, according to Fisher’s least significant difference (LSD) test (P<0.05), are indicated by the letters above the data points, hai, hours after infiltration. (This figure is available in colour at JXB online.)
plants in which expression of CaChitIV, CaPIK1, or both CaChitIV and CaPIK1 had been silenced. Expression of CaChitIV and/or CaPIK1 was significantly down-regulated during Xcv infection in pepper leaves in which CaChitIV and/or CaPIK1 was silenced, indicating that CaChitIV and/or CaPIK1 were efficiently silenced (Fig. 9). It was observed that growth of virulent and avirulent Xcv reached significantly higher levels in leaves from gene-silenced plants than in leaves from empty vector control plants (Fig. 7A). Notably, silencing both CaChitIV and CaPIK1 allowed the proliferation of virulent Ds1 and avirulent Bv5-4a strains of Xcv over and above the effect of silencing each gene separately. This indicates that CaChitIV expression contributes to the CaPIK1-mediated basal defence and HR-like cell death response.

The cell death and defence phenotypes were substantiated by an electrolyte leakage assay (Fig. 7B). Avirulent Xcv infection resulted in a higher level of electrolyte leakage from leaf discs than the virulent Xcv infection; however, electrolyte leakage from leaves in which CaChitIV, CaPIK1, or CaChitIV and CaPIK1 were silenced was significantly less than from leaves containing the empty vector control, following both virulent and avirulent Xcv infection. Notably, silencing of both CaChitIV and CaPIK1 significantly reduced electrolyte leakage from leaf discs infected with Xcv. To determine whether the silencing of CaChitIV and/or CaPIK1 inhibited ROS accumulation, H2O2 production in pepper leaves was quantified using the xylene orange assay (Choi et al., 2007). At the early stage of Xcv infection, significantly lower levels of H2O2 accumulated in leaves in which expression of both

---

Silencing CaChitIV and/or CaPIK1 in pepper plants increases susceptibility to Xanthomonas campestris pv. vesicatoria infection

To investigate the effect of loss of CaChitIV and/or CaPIK1 function, VIGS (Liu et al., 2006) was used to generate pepper leaves co-expressing empty vector control or CaChitIVASP in combination with CaPIK1 was significantly lower than that from leaves co-expressing CaChitIV and CaPIK1 (Fig. 6C). Thus expression of CaChitIVASP in the cytoplasm and nucleus does not increase levels of CaPIK1-triggered cell death.

---

**Fig. 6.** Transient co-expression of CaChitIVASP with CaPIK1 does not enhance the CaPIK1-triggered cell death response in pepper leaves. (A) Cell death phenotypes in pepper leaves transiently expressing empty vector control, CaChitIV, or CaChitIVASP in combination with CaPIK1 2 d after infiltration with Agrobacterium. Cell death levels were rated based on a 0–3 scale: 0, no cell death (<10%); 1, weak cell death (10–30%); 2, partial cell death (30–80%); and 3, full cell death (80–100%). (B) Quantification of cell death in pepper leaves treated as in A. (C) Electrolyte leakages from leaf discs at the stated time points following infiltration. Data in B and C are the means ±standard deviations from three, independent experiments. Statistically significant differences between treatments, according to the LSD test (P<0.05), are indicated by the letters above the bars. i, Empty vector; ii, CaPIK1/Empty; iii, CaPIK1/CaChitIV; iv, CaPIK1/CaChitIVASP. hai, hours after infiltration. (This figure is available in colour at JXB online.)
CaChitIV and CaPIK1 was silenced than in leaves containing the empty vector control or leaves in which CaChitIV or CaPIK1 had been silenced (Fig. 7C). To determine whether the silencing of CaChitIV and/or CaPIK1 inhibited NO accumulation, NO production in pepper leaves was visualized using the NO-sensitive dye DAF-2DA (Fig. 8A). During both virulent and avirulent Xcv infection, significantly lower levels of NO accumulated in both CaChitIV- and CaPIK1-silenced leaves than in the empty vector control and CaChitIV- or CaPIK1-silenced leaves (Fig. 8B). Together, these results indicate that CaChitIV and CaPIK1 co-expression triggers pathogen-induced hypersensitive cell death, and ROS and NO bursts in pepper leaves.

To investigate whether silencing of CaChitIV and/or CaPIK1 affects the expression of defence-related genes in pepper, quantitative real-time RT-PCR analysis was performed (Fig. 9; Supplementary Fig. S4 at JXB online). Expression values of these genes were normalized by the expression levels of C. annuum CaACTIN and 18S rRNA as reference genes. CaPIK1 silencing significantly compromised the induction of CaPRI (PR1) and CaDEFI (defensin), but not CaChitIV (chitinase IV), during virulent and avirulent Xcv infection. In contrast, induction of these three defence response genes during Xcv infection was not reduced.
in CaChitIV-silenced plants (Fig. 9). However, silencing of both CaChitIV and CaPIK1 significantly inhibited induction of these defence response genes following infection. These results indicate that although CaPIK1 expression positively regulates expression of defence-related genes in pepper, CaChitIV may not play a significant role in the regulation of downstream defence gene expression.

Overexpression of CaChitIV in Arabidopsis reduces susceptibility to Hyaloperonospora arabidopsidis infection

To investigate whether increased expression of CaChitIV affected resistance to Hpa infection, wild-type (Col-0) and transgenic Arabidopsis plants overexpressing CaChitIV (CaChitIV-OX) were inoculated with Hpa isolate Noco2 (Fig. 10). RT–PCR analysis showed that CaChitIV was constitutively overexpressed in leaves of transgenic lines #1, #2, and #3 (Supplementary Fig. S5 at JXB online).

More vigorous growth of Hpa isolate Noco2 was observed on cotyledons of wild-type seedlings than on seedlings from CaChitIV-OX lines (Fig. 10A). Seven days after inoculation, Hpa had produced significantly fewer conidiospores when grown on the cotyledons of CaChitIV-OX seedlings than on wild-type cotyledons (Fig. 10B). Hpa-infected cotyledons were grouped into five classes based on the number of sporangiophores per cotyledon: 0, 1–10, 11–20, 21–30, 31–40, and >41. The incidence of classes containing lower numbers of sporangiophores was significantly higher for CaChitIV-OX lines than for wild-type plants (Fig. 10C). Collectively, these results indicate that CaChitIV overexpression confers increased basal resistance to Hpa infection in Arabidopsis.

Discussion

In a previous work, it was reported that the receptor-like cytoplasmic protein kinase, CaPIK1, acts as a positive regulator to trigger an HR-like cell death response in pepper plants, as well as accumulation of ROS and NO (Kim and Hwang, 2011). Here, evidence is provided that the pepper class IV chitinase, CaChitIV, interacts with CaPIK1 in yeast and in planta. Using Agrobacterium-mediated transient co-expression of CaChitIV and CaPIK1, a critical role for CaChitIV in CaPIK1-triggered cell death and defence responses was revealed.

It is well known that plant chitinases function in plant defence responses to fungal and oomycete pathogen infection (Collinge et al., 1993; Kim and Hwang, 1994; Lee et al., 2000). Chitinase is a catalytic enzyme responsible for the hydrolysis of chitin, a linear polymer of GlcNAc and an important structural component of the fungal cell wall (Wubben et al., 1992; Nielsen et al., 1993). However, chitinase gene expression in plants is also induced by infection with viruses, bacteria, and oomycetes that do not contain chitin or related structures (Metraux et al., 1988; Hong et al., 2000; Hong and Hwang, 2002; Ott et al., 2006). Here, strong induction of CaChitIV expression was shown at an early stage of infection with avirulent Xcv Bv5-4a carrying AvrBsT that induces cell death and defence responses in pepper (Kim et al., 2010). However, infection with a virulent strain of Xcv does not induce expression of CaChitIV. Induced chitinases may be utilized as a positive regulator of early basal resistance. Non-pathogenic, saprophytic, and avirulent bacteria triggered early basal resistant responses, such as induction and accumulation of chitinases in tobacco plants, whereas virulent bacterial infection suppressed chitinase activity (Ott et al., 2006), indicating that virulent bacteria have molecular mechanisms to circumvent early basal resistance and so ensure their survival in host tissues. However, little is known about how chitinases regulate basal resistance and HR-like cell death in response to infection with virulent bacterial pathogens.

Co-expression of CaChitIV with CaPIK1 accelerated the CaPIK1-triggered cell death response. The presence of
CaChitIV in CaPIK1-triggered cell death

and assist in defence response to pathogens (Besson-Bard et al., 2008; Perchepied et al., 2010). The enhanced ROS and NO bursts seen in pepper leaves co-expressing CaPIK1 and CaChitIV support the suggestion that ROS and NO act as signalling radicals in plant cell death and defence responses, including MAPK activation, expression of defence-related genes, and cell wall thickening via callose accumulation (Torres and Dangl, 2005; Asai et al., 2008).

The TRV-based VIGS system (Liu et al., 2002; Chung et al., 2004) was used to investigate the effect of losing CaChitIV and/or CaPIK1 function on cell death-mediated defence signalling in pepper plants. Expression of CaChitIV and/or CaPIK1 was significantly down-regulated during Xcv infection in pepper leaves in which CaChitIV and/or CaPIK1 was silenced. Silencing either CaChitIV or CaPIK1, as well as both CaChitIV and CaPIK1, significantly enhanced Xcv growth but compromised the cell death response, ROS and NO accumulation, and defence response gene induction in pepper leaves during compatible and incompatible Xcv infections. Notably, co-silencing of both genes was much more effective at suppressing CaPIK1-triggered cell death, ROS and NO accumulation, and defence response gene induction than silencing either separately. It is concluded that CaChitIV expression enhances CaPIK1-triggered basal defence and HR-like cell death response in pepper leaves and is required for bacterial disease resistance. It has been proposed that recognition of pathogen-associated molecular patterns (PAMPs) by plant receptor-like kinase produces PAMP-triggered immune (PTI) responses, including oxidative bursts, callose deposition, and defence gene induction (Zipfel et al., 2004). It is suggested that, in pepper plants, activation of ROS bursts by the receptor-like cytoplasmic protein kinase, CaPIK1, acting together with the class IV chitinase, CaChitIV, triggers cell death and defence responses, resulting in reduced growth of Xcv. Silencing of CaChitIV alone did not compromise induction of the defence response genes CaPR1 (PR1) (Kim and Hwang, 2000) and CaDEF1 (defensin) (Do et al., 2004), supporting the idea that CaChitIV expression assists CaPIK1-triggered cell death and defence responses. Moreover, CaChitIV expression may not positively regulate downstream defence genes in pepper.

The results provide the first evidence that CaChitIV specifically interacts with CaPIK1 in yeast and in planta. Moreover, it is shown that CaChitIV functions as an enhancer of CaPIK1-triggered cell death and defence responses. However, it remains to be clarified how secreted CaChitIV acts in the processes resulting in plant cell death and how signal transduction pathways triggered by CaPIK1 regulate the activation of CaChitIV for plant defence.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Nucleotide and predicted amino acid sequences of pepper CaChitIV cDNA.

Figure S2. (A) Comparison of the deduced amino acid sequence of CaChitIV with sequences of class IV chitinases
from tobacco, grapevine, Arabidopsis, rice, maize, and Norway spruce (B) A schematic diagram of domains in CaChitIV.

Figure S3. Subcellular localization of CaChitIV in onion epidermal cells following biolistic transformation

Figure S4. Quantitative real-time–RT–PCR analysis of relative gene expression of CaChitIV, CaPIK1, CaPR1, and CaDEF1 in pepper plants infected with virulent Ds1 (compatible) or avirulent Bv4-4a (incompatible) strains of Xanthomonas campestris pv. vescatoria.

Figure S5. RT–PCR analysis of expression levels of CaChitIV in leaves from transgenic Arabidopsis empty vector control lines (00) and CaChitIV-OX lines #1, #2, and #3.

Table S1. Gene-specific primers for plasmid constructs used in this study.

Acknowledgements

This work was carried out with the support of the ‘Cooperative Research Program for Agriculture Science & Technology (Project No.PJ00802701)’, Rural Development Administration, Republic of Korea.

References


Hong JK, Jung HW, Kim YJ, Hwang BK. 2000. Pepper gene encoding a basic class II chitinase is inducible by pathogen and ethephon. Plant Science 159, 39–49.


Zhou JM, Loh YT, Bressan RA, Martin GB. 1995. The tomato gene Pt1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. Cell 83, 925–935.