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

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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, CaPK1, which mediates signalling of cell death and defence responses to microbial pathogens was identified. CaPK1 expression in pepper plants (Capsicum annum 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; Neuhaus et al., 1996; Gomez et al., 2002; Wiweger et al., 2002). 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 class 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 targeted 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; Hietala 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-3, PR-4, PR-8, and PR-11 families (Neuhaus 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 (Schlumberg 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 (Hietala 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 CaPK1 (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 CaPK1 in yeast and in planta, was isolated and functionally characterized. Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) experiments revealed that CaPK1 interacts with CaChitIV in planta, with the CaChitIV–CaPK1 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 CaPK1 enhanced the CaPK1-triggered cell death response and ROS burst, as well as NO burst. Virus-induced gene silencing (VIGS) of CaChitIV or/and CaPK1 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 CaPK1.

Materials and methods

Plant growth and pathology assays

Pepper (Capsicum annum 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 environmentally controlled growth room.

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 environmentally controlled growth chamber.
Virulent Ds1 and avirulent Bv5-4a strains of Xcr (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 × 10⁸ conidiospores ml⁻¹) were sprayed onto 7-day-old Arabidopsis seedlings, infected plants were covered with plastic wrap to maintain moisture, and the number of sporangioles 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 pGBK7T7, 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 Bv5-4a.

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

**Bioluminescence complementation assay (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 YFP⁺ and YFP⁻ (yellow fluorescent protein N- and C-termini), respectively, under the control of the Cauliflower mosaic virus (CaMV) 35S promoter, resulting in CaPIK1-YFP⁺ 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 Exci confocal laser-scanning microscope (Carl Zeiss, Germany) with excitation at 514 nm and emission at 525–600 nm.

**Green fluorescent protein (GFP) fluorescence microscopy**

For GFP constructs, the CaChitIV coding region and the signal peptide-deleted CaChitIV (CaChitIVΔSP) were PCR amplified and introduced into XbaI/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 Exci 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 CaChitIVVASP: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.

**Immuno blotting**

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)]. Total RNA was extracted with Tri-reagent (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, 100 mM EDTA, 0.1% Triton X-100, and protease inhibitor cocktail (Roche)]. Eluted proteins were analysed using immunoblotting with anti-c-Myc 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 Isol-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/1.2% agarose gels. Gels were immersed in deionized 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 pre-hybridized 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).

cDNA for real-time RT–PCR analysis was prepared using 1 μg of total RNA, 500 ng of oligo dT(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.
**Tobacco rattle virus (TRV)-based VIGS of CaChitIV and CaPIK1**

To induce constitutive CaChitIV overexpression (OX) in *Arabidopsis*, transgenic plants were generated using the floral dipping protocol (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. Transformsants 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 (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 12.5 μM DAF-2DA, using a needleless syringe, and were incubated for 1 h at the dark room temperature. Fluorescence from diaminofluorescein (DAF-2T), the reaction product of DAF-2DA for 1 h in the dark at room temperature. Fluorescence from diaminofluorescein was 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 supernatant was added to 1 ml of xylol orange assay reagent. The mixture was incubated for 30 min at room temperature. H₂O₂ production in pepper leaves was quantified using the xylol orange assay (Choi et al., 2007). Briefly, the xylol orange assay reagent was freshly prepared: 200 μl of solution [25 mM FeSO₄, 25 mM (NH₄)SO₄ in 2.5 M H₂SO₄] was added to 20 ml of 125 μM xylol orange in 100 mM sorbitol. Eight leaf discs (0.5 cm²) were floated on 1 ml of xylol 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.

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 12.5 μM DAF-2DA, using a needleless syringe, and were incubated for 1 h in the dark room temperature. Fluorescence from diaminofluorescein was 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 xylol orange assay reagent. The mixture was incubated for 30 min at room temperature. H₂O₂ production in pepper leaves was quantified using the xylol orange assay (Choi et al., 2007). Briefly, the xylol orange assay reagent was freshly prepared: 200 μl of solution [25 mM FeSO₄, 25 mM (NH₄)SO₄ in 2.5 M H₂SO₄] was added to 20 ml of 125 μM xylol 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 xylol 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 pGBKTT7, 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 YFPN and YFPc, respectively, were used to construct CaPIK1-YFPN and CaChitIV-YFPc, or vice versa. Interactions between the fusion proteins were visualized in *N. benthamiana* leaves using *Agrobacterium*-mediated transient co-expression. Combinations of CaChitIV–YFPN and CaPIK1–YFPc, 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–YFPN and bZIP63–YFPc 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

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 sfGFP-tagged CaChitIV and signal peptide-deleted CaChitIV (CaChitIVΔSP) were constructed. Using Agrobacterium-mediated transient expression, CaChitIV and CaChitIVΔSP fusion proteins with GFP were expressed in N. benthamiana.

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
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* Ca^{2+}-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
low inoculum density (OD$_{600}$=0.2) with different inoculum concentrations (OD$_{600}$=0.05, 0.1, and 0.5) of CaChitIV. Increasing the inoculum density of CaChitIV enhanced cell death levels, trypan blue-stained cell death response, and electrolyte leakage (Fig. 4D, E). Expression of CaPIK1 and proteins was confirmed by immunoblot analysis (Fig. 4F). Increased inoculum concentration of CaChitIV gradually increased the level of CaChitIV expression in pepper leaves.

In addition, the effect of co-expressing CaChitIV with 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$_2$O$_2$ and NO gradually increased in pepper leaves transiently co-expressing CaPIK1 and CaChitIV, in proportion to the original inoculum density of Agrobacterium.

CaChitIVΔSP:GFP expression was restricted to the cytoplasm and nuclei of leaf epidermal cells of N. benthamiana (Fig. 3B). It was therefore investigated whether co-expression in pepper leaves of CaChitIVΔSP with CaPIK1 affected the CaPIK1-triggered cell death response (Fig. 6). In contrast to co-expression with CaChitIV, co-expression of CaPIK1 with CaChitIVΔSP did not enhance the CaPIK1-triggered HR-like cell death response (Fig. 6A, B). Electrolyte leakage from pepper
leaves co-expressing empty vector control or CaChitIVΔSP in combination with CaPIK1 was significantly lower than that from leaves co-expressing CaChitIV and CaPIK1 (Fig. 6C). Thus expression of CaChitIVΔSP in the cytoplasm and nucleus does not increase levels of CaPIK1-triggered cell death.

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 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 CaPIK1 and CaChitIV 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 xylenol 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
**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 **CaDEF1** (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.

**Fig. 7.** Silencing of **CaChitIV** and/or **CaPIK1** in pepper plants enhances susceptibility to infection with virulent Ds1 (compatible) and avirulent Bv5-4a (incompatible) strains of *Xanthomonas campestris* pv. *vesicatoria* (Xcv). (A) Bacterial growth in leaves of pepper plants infiltrated with empty vector control (TRV:00) or with gene silencing constructs TRV:**CaChitIV**, TRV:**CaPIK1**, or TRV:**CaChitIV*/TRV:**CaPIK1**, and then infected with Xcv (5 × 10⁷ cfu ml⁻¹). (B) Quantification of electrolyte leakage from leaves infected with Xcv (5 × 10⁷ cfu ml⁻¹). (C) Quantification of H₂O₂ accumulation in leaves infected with Xcv (5 × 10⁷ cfu ml⁻¹). Data are means ± standard deviations from three, independent experiments. Letters above the bars indicate statistically significant differences between treatments, according to the LSD test (*P*<0.05).

**Fig. 8.** Microscopic images and quantification of NO production in empty vector control and **CaChitIV** and/or **CaPIK1**-silenced pepper leaves infected with virulent (compatible) Ds1 and avirulent (incompatible) Bv5-4a strains of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) (5 × 10⁷ cfu ml⁻¹). (A) Visualization of the leaf areas 18 h after infiltration with Xcv using a confocal microscope. Mock: infiltrated with 10 mM MgCl₂. Scale bars=100 μm. (B) Quantification of fluorescence intensities in leaves by colour histogram analysis. Data are means ± standard deviations from 30 randomly taken pictures. Letters above the bars indicate statistically significant differences between treatments, according to the LSD test (*P*<0.05).
CaChitIV-OX Hpa were inoculated with isolate Noco2 to investigate whether increased expression of CaChitIV reduces susceptibility to Hpa infection. 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 (Métrax 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 avirulent bacterial pathogens.

Co-expression of CaChitIV with CaPIK1 accelerated the CaPIK1-triggered cell death response. The presence of
between groups, according to the LSD test (P < 0.05). Experiments. Different letters indicate statistically significantly differences in B and C are means ±standard deviations from three independent ±standard deviation is shown below each of the lines tested. Values plants 7 d after inoculation. The mean number of sporangiophores sporangiophores on at least 50 cotyledons of wild-type and CaChitIV-OX cotyledons of wild-type (WT) and CaChitIV did not enhance the cytoplasm and nucleus. Co-expression with ΔCaChitIV protein was indeed localized mainly in the ER; in contrast, ER. A subcellular localization assay revealed that CaChitIV be secreted to the extracellular, apoplastic region via the signal peptide led to the prediction that CaChitIV would be secreted to the extracellular, apoplastic region via the ER. A subcellular localization assay revealed that CaChitIV protein was indeed localized mainly in the ER; in contrast, CaChitIVΔASP-GFP, lacking the signal peptide, localized to the cytoplasm and nucleus. Co-expression with CaChitIVVASP did not enhance the CaPIK1-triggered HR-like cell death response. These results suggest that it is localization of CaChitIV to the ER that is responsible for its enhancement of CaPIK1-triggered cell death.

Expression of CaPIK1 protein in planta maintained a steady-state level following inoculation with a given dose of Agrobacterium (OD_{600}=0.2); however, CaChitIV levels increased with the cell density of the inoculum, indicating that CaPIK1 and CaChitIV do not affect each other at the protein level. When CaPIK1 and CaChitIV were co-expressed in pepper leaves, induction of ROS and NO accumulation gradually increased, in line with increasing density of CaChitIV-containing inoculum. Such an enhancement of ROS and NO bursts following increased CaChitIV expression may lead to the promotion of CaPIK1-triggered cell death, as ROS are known to work synergistically with NO to stimulate PCD 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 immunity (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 CaDEFI in pepper plants infected with virulent Ds1 (compatible) or avirulent Bv5-4a (incompatible) strains of Xanthomonas campestris pv. vesicatoria.

**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.PJ00820701)’, Rural Development Administration, Republic of Korea.

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