A pepper (*Capsicum annuum* L.) metacaspase 9 (*Camc9*) plays a role in pathogen-induced cell death in plants

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SUMMARY

Metacaspases, which belong to the cysteine-type C14 protease family, are most structurally similar to mammalian caspases than any other caspase-like protease in plants. *Atmc9* (*Arabidopsis thaliana* metacaspase 9) has a unique domain structure, and distinct biochemical characteristics, such as Ca^{2+} binding, pH, redox status, *S*-nitrosylation and specific protease inhibitors. However, the biological roles of *Atmc9* in plant–pathogen interactions remain largely unknown. In this study, a metacaspase gene present as a single copy in the pepper genome, and sharing 54% amino acid sequence identity with *Atmc9*, was isolated and named *Capsicum annuum* metacaspase 9 (*Camc9*). *Camc9* encodes a 318-amino-acid polypeptide with an estimated molecular weight of 34.6 kDa, and shares approximately 40% amino acid sequence identity with known type II metacaspases in plants. Quantitative reverse transcription-polymerase chain reaction analyses revealed that the expression of *Camc9* was induced by infections of *Xanthomonas campestris* pv. *vesicatoria* race 1 and race 3 and treatment with methyl jasmonate. Suppression of *Camc9* expression using virus-induced gene silencing enhanced disease resistance and suppressed cell death symptom development following infection with virulent bacterial pathogens. By contrast, overexpression of *Camc9* by transient or stable transformation enhanced disease susceptibility and pathogen-induced cell death by regulation of reactive oxygen species production and defence-related gene expression. These results suggest that *Camc9* is a possible member of the metacaspase gene family and plays a role as a positive regulator of pathogen-induced cell death in the plant kingdom.

INTRODUCTION

Plant metacaspases are candidates originally proposed to be the functional homologues of mammalian caspases (van der Hoorn, 2008; Mottram *et al.*, 2003; Rawlings *et al.*, 2012; Woltering *et al.*, 2002). Subsequently, characterization of plant metacaspases was

extensively performed in *Arabidopsis*, tobacco, tomato and wheat (Bonneau *et al.*, 2008; Hao *et al.*, 2007; Hoeberichts *et al.*, 2003; Wang *et al.*, 2012). Plant metacaspases are divided into types I and II. Type I metacaspases have an extension in their N-terminal domain including a zinc finger domain, whereas type II metacaspases have no such extension, but have a linker region between the putative large (p20) and small (p10) subunits (Uren *et al.*, 2000; Vercammen *et al.*, 2004).

In the last decade, a number of reports have investigated the biological function of plant metacaspases. The *Arabidopsis* type I metacaspases, metacaspase 1 (*Atmc1*) and 2 (*Atmc2*), have opposing roles associated with *LSD1* during cell death as positive and negative regulators, respectively (Coll *et al.*, 2010, 2011). In *Arabidopsis*, a type II metacaspase, *Atmc4*, is involved in programmed cell death (PCD) induced by the fungal toxin fumonisin B1, and the lysine-225 residue of *Atmc4* is critical for the Ca^{2+} dependence of its catalytic activation (Tsiatsiani *et al.*, 2011; Watanabe and Lam, 2011b). However, the *Picea abies* type II metacaspase (*mcll-Pa*) is required for developmental PCD, oxidative stress-induced cell death and cleaves endogenous Tudor staphylococcal nuclease (Bozhkov *et al.*, 2005; Sundström *et al.*, 2009).

Among type II metacaspases, *Arabidopsis* metacaspase 9 (*Atmc9*) is distinct from the other five type II metacaspases. *Atmc9* has a unique domain structure, autoprocessing of proenzymes in bacterial or yeast cells, regulation by *S*-nitrosylation and specific protease inhibitors compared with the other type II metacaspases (Vercammen *et al.*, 2004, 2006). Because of the unique structural and biochemical characteristics, *Atmc9* has been classified into a distinct subgroup from other type II metacaspases (Watanabe and Lam, 2011b). However, the biological role of *Atmc9* remains to be identified.

In this study, a *Capsicum annuum* metacaspase 9 (*Camc9*), which has strongest sequence similarity to *Atmc9*, was isolated and its function was characterized. Gene expression, and gain- and loss-of-function studies revealed that *Camc9* plays a role in pathogen-induced cell death via the regulation of reactive oxygen species (ROS) production and defence-related gene expression in plants. These results provide an insight into the role of plant metacaspase 9 as a positive regulator of pathogen-induced cell death.

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RESULTS

Camc9 is a type II metacaspase that is a homologue of Atmc9

Putative proteases were isolated using bioinformatics tools from the pepper expressed sequence tag (EST) database to investigate their function in plant–pathogen interactions (Kim *et al.*, 2008). One hundred and fifty-three pepper cDNAs were selected as protease representatives by protease domain prediction based on the MEROPS classification (Rawlings *et al.*, 2012). The 153 representative protease genes from pepper cDNA were selected and cloned into a *Tobacco rattle virus* (TRV) ligation-independent cloning vector for a loss-of-function study (Dong *et al.*, 2007). To identify the roles of proteases in cell death and the immune response against bacterial pathogens, protease-silenced plants were infiltrated with *Pseudomonas syringae* pv. *tomato* T1 (*Pst* T1), which causes hypersensitive response (HR) cell death, and *P. syringae* pv. *tabaci* (*P.s. tabaci*), which causes wild fire disease on *Nicotiana*

benthamiana (Melotto *et al.*, 2008; Oh *et al.*, 2008). There were various phenotypes related to development and defence (Bae *et al.*, unpublished data). Among these proteases, a gene with a caspase domain was selected and designated as *C. annuum* metacaspase 9 (*Camc9*) for further characterization. The predicted open reading frame of *Camc9* encodes a full-length protein of 318 amino acid residues, with an estimated molecular weight of 34.6 kDa.

Camc9 has the peptidase subfamily C14B domain (metacaspase) characteristic of the caspase superfamily based on the MEROPS database (Rawlings *et al.*, 2012). The alignment of the corresponding protein sequences is shown in Fig. 1A, and the corresponding phylogenetic tree is shown in Fig. 1B. Although Solanaceae metacaspases are reported, *Camc9* is most similar to *Atmc9* (Hoeberichts *et al.*, 2003). When compared with the full-length protein sequences, *Camc9* shares 54% amino acid sequence identity with *Atmc9*. Furthermore, *Camc9* contains a shorter linker region between the putative p20 and p10 subunits, like *Atmc9*. In addition, on alignment of multiple plant metacaspases, *Camc9* has a conserved histidine/cysteine catalytic dyad in

Fig. 1 Sequence analysis of *Capsicum annuum* metacaspase 9 (*Camc9*) and corresponding phylogenetic tree. (A) Comparison of amino acid sequences of *Camc9* with homologues from *Arabidopsis thaliana*, *Solanum lycopersicum* and *Nicotiana benthamiana* using the CLUSTALW program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The putative catalytic histidine (His) and cysteine (Cys) dyad is marked by asterisks (*). The putative autoprocessing site of *Camc9* is marked by a line. (B) Phylogenetic tree with *Camc9* and metacaspase genes from various plants. Bootstrap consensus phylogenetic tree representing similarities of metacaspases in amino acid sequences from *C. annuum*, *A. thaliana*, *N. benthamiana*, *Picea abies* and *S. lycopersicum*. The scale bar indicates the genetic distance based on branch lengths. The GenBank accession numbers are indicated in parentheses.

the putative p20 subunit domain, which is predicted for caspases (Vercammen *et al.*, 2004). The residues YKSK (177) may be the putative Camc9 autoprocessing site based on Atmc9, whose autoprocessing site is ITSR (183) (Tsiatsiani *et al.*, 2011; Vercammen *et al.*, 2004) (Fig. 1A). Based on these results, Camc9 is considered as a member of the metacaspase gene family in the plant kingdom that has most similarity to Atmc9.

Tissue-specific and pathogen-responsive expression of Camc9

Although Camc9 was similar to Atmc9 in protein sequence levels, the gene expression patterns of Camc9 were different from those of Atmc9, which showed relatively high expression levels in all the tested *Arabidopsis* tissues and organs (Watanabe and Lam, 2011a). The Camc9 expression level was highest in pepper flowers when compared with leaf. Expression levels in other tissues, including stem, root, fruit and seedling, were moderate (Fig. 2A). To analyse the transcript levels of Camc9, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) were performed with Camc9-specific primers under the conditions described in the experimental procedures.

To study the role of Camc9 in plant-pathogen interactions, transcript levels of Camc9 were detected by qRT-PCR of ECW30R plants following inoculation with *Xanthomonas campestris* pv. *vesicatoria* race 1 (Xcv1, incompatible) or race 3 (Xcv3, compatible) bacterial pathogens (Kousik and Ritchie, 1996). The expression of Camc9 in response to incompatible and compatible interactions was pleiotropic, but not significantly different (Fig. 2B). We also investigated the expression of Camc9 following treatment with the defence signal molecules, salicylic acid (SA), methyl jasmonate (MeJA) and ethephon (ET), in pepper leaves. By RT-PCR analysis, the transcript levels of Camc9 were not elevated by SA or ET treatment in pepper leaves (Fig. S1, see Supporting Information). However, elevated levels of Camc9 transcript were detected during the time course of MeJA treatment in *C. annuum* (Fig. 2C). The expression of Camc9 was increased approximately five-fold at 6 h after MeJA treatment. The successful infiltrations of MeJA, SA and ET were verified by monitoring the transcript levels of *CaPinII* (protease inhibitor II, MeJA and ET) and *CaBPR1* (basic PR1, SA) as positive markers of MeJA, SA and ET treatments, respectively (Yeom *et al.*, 2011a) (Fig. S1). These results indicated that the expression of Camc9 is regulated by both developmental stage and environmental cues, such as MeJA.

Silencing of Camc9 affects pathogen-induced cell death

We examined the effect of silencing Camc9 using virus-induced gene silencing (VIGS) to identify the roles of Camc9 in plant cell death. VIGS was performed with the N-terminus (TRV2-Camc9-N)

Fig. 2 Expression of *Capsicum annuum* metacaspase 9 (Camc9) in various tissues and following pathogen inoculation. (A) Organ-specific expression of Camc9 was analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The values were normalized to *CaActin* and are estimated relative to this control in leaves. R, root; S, stem; L, leaf; F, flower; IMG, immature green fruit; MR, mature green fruit; SD, seedling. (B) Expression of Camc9 in pepper leaves inoculated with *Xanthomonas campestris* pv. *vesicatoria* race 3 (Xcv3) (compatible, black bar) and Xcv1 (incompatible, grey bar) at approximately 2×10^8 colony forming units (CFUs)/mL. *CaActin* expression was used to normalize expression. (C) Expression of Camc9 after treatment with methyl jasmonate (MeJA, 100 μ M). *CaActin* expression was used to normalize expression. hpi, hours post-inoculation.

or C-terminus (TRV2-Camc9-C) of Camc9 in *C. annuum* plants (Fig. 3A). No visible phenotypic difference was observed between Camc9-silenced and control plants during vegetative growth (Fig. S2, see Supporting Information). To investigate the effects of silencing Camc9 on pathogen-induced cell death, plants were inoculated with Xcv3 at an optical density at 600 nm ($OD_{600\text{ nm}}$) of 0.2. Cell death symptoms induced by Xcv3 (compatible) were delayed in Camc9-silenced plants compared with controls. The control plants showed disease symptoms at 5 days post-inoculation (dpi), whereas Camc9-N- and Camc9-C-silenced plants presented disease symptoms at 7 dpi (top panel of Fig. 3B). Trypan blue staining was carried out to assess cell death, and revealed that the cell death of control plants was 30% higher than that of Camc9-N- or Camc9-C-silenced plants (bottom panel of Fig. 3B,C). To investigate the silencing degree of Camc9, the N-terminus, C-terminus and intermediate part of Camc9 were determined by RT-PCR (Fig. 3D).

Fig. 3 Virus-induced gene silencing (VIGS) of the *Capsicum annuum* metacaspase 9 (*Camc9*) gene in pepper plants. (A) Diagrams of *Camc9*-silencing constructs targeting the N- and C-termini or the intermediate part (I) of *Camc9*. (B) The disease symptoms induced by the pathogen *Xanthomonas campestris* pv. *vesicatoria* race 3 [*Xcv3*, optical density at 600 nm ($OD_{600\text{ nm}}$) = 0.01] were observed at 0, 2, 5 and 6 days post-inoculation (dpi) (top panel). Trypan blue staining at 0, 2, 5 and 6 dpi of the same area as the photographs (bottom panel). (C) Relative intensity of trypan blue-stained peppers. Values are means $\pm$ standard deviation (SD) ($n = 3$). Relative intensity was calculated relative to the control. (D) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression levels of *Camc9* in the *Camc9*-N- and *Camc9*-C-silenced peppers. Total RNA was extracted from the silenced plants and subjected to RT-PCR analysis with *Camc9*-N, *Camc9*-I and *Camc9*-C gene-specific primers to detect *Camc9* transcripts.

The same silencing constructs were used to silence *Camc9* orthologues in *N. benthamiana*, which belongs to the Solanaceae family, like pepper, and is widely used for functional analysis of plant–pathogen interactions. To evaluate the effects of silencing on cell death, *N. benthamiana* was inoculated with *Pst* T1 ($OD_{600\text{ nm}} = 0.1$) or *P.s. tabaci* ($OD_{600\text{ nm}} = 0.005$), which induce hypersensitive cell death and disease symptoms, respectively. We could not find any time difference of the HR caused by the incompatible pathogen (*Pst* T1). The control and *Camc9*-silenced plants induced hypersensitive cell death within 24 h (data not shown). As shown in Fig. 4A, the disease symptoms induced by *P.s. tabaci* (compatible) were delayed significantly, as in *Camc9*-N- and *Camc9*-C-silenced pepper plants (Fig. 3B). The ion leakage of *Camc9*-C-silenced *N. benthamiana* plants was lower than that of control plants (Fig. 4B). The confirmation of the silencing of *Camc9* was determined by qRT-PCR (Fig. 4C). We cloned *Camc9* in a homologous manner in *N. benthamiana* and carried out VIGS in *N. benthamiana* plants. *Pseudomonas syringae* pv. *tabaci* was infiltrated into *Nbmc9*-C-silenced plants, the infiltration area was measured for ion leakage and the degree of silencing of *Nbmc9* was confirmed by qRT-PCR (Fig. S3, see Supporting Information). The same results were obtained when silencing *Camc9* in *N. benthamiana* plants (Fig. 4). These results may indicate that *Camc9* plays a role as a positive regulator of plant cell death induced by pathogen infection.

Overexpression of *Camc9* enhances disease susceptibility and pathogen-induced cell death

Plant cell death induced by a compatible pathogen was delayed in *Camc9*-silenced *N. benthamiana*. We tested whether the overexpression of *Camc9* could enhance disease susceptibility. *Agrobacterium* carrying *Camc9* constructs was used for transient overexpression in *N. benthamiana* (Fig. 5A). Overexpression of *Camc9* showed enhancement of cell death induced by *P.s. tabaci*, as indicated by the increased ion leakage of *Camc9*-overexpressing parts relative to pMBP1-overexpressing parts as

Fig. 4 Virus-induced gene silencing (VIGS) of the *Capsicum annuum* metacaspase 9 (*Camc9*) gene in *Nicotiana benthamiana* plants. (A) *Pseudomonas syringae* pv. *tabaci* (*P.s. tabaci*) was diluted to an optical density at 600 nm ($OD_{600\text{ nm}}$) of 0.005, and infiltrated after silencing into a whole leaf of *Tobacco etch virus* (TRV)-dGFP (as a control) and *Camc9* -C in *N. benthamiana* plants. The white circle indicates the pathogen infiltration area. Photographs were taken at 3 days post-inoculation (dpi). (B) Conductivity from leaf discs corresponding to the white circles was measured at 3 dpi. Values are means $\pm$ standard deviation (SD) ($n = 3$). Asterisks indicate significant differences relative to the TRV-dGFP control as determined by Student's *t* -test (** $P < 0.01$). (C) Expression of *Camc9* was analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The values were normalized to *CaActin* and are calculated relative to this control in leaves.

control (Fig. 5B). Confirmation of the transient overexpression of *Camc9* was determined by qRT-PCR (Fig. 5C).

To confirm the function of the *Camc9* gene, *Camc9* -transgenic *N. benthamiana* plants, constitutively expressing *Camc9* under the control of the cauliflower mosaic virus 35S promoter, were also generated using *Agrobacterium tumefaciens* -mediated transformation. Three stable T2 transgenic plant lines were selected from a total of nine lines and characterized to identify the roles of *Camc9* overexpression in plant defence following *P.s. tabaci* inoculation (Fig. 5D,E). Similar to the transient expression

(Fig. 5A), the disease symptoms induced by *P.s. tabaci* were also enhanced in *Camc9* -transgenic plants with increased ion leakage, as shown in Fig. 5F.

The cell death pathway includes complicated processes, such as ROS bursts, regulation of hormones and protease activation (Rao *et al.* , 2000; Solomon *et al.* , 1999). To investigate ROS production in *Camc9* -transgenic plants, 3,3'-diaminobenzidine (DAB) staining was carried out following infiltration of *P.s. tabaci* . Heavily DAB-stained leaves relative to control plants were observed in *Camc9* -transgenic plants at 6 h post-infiltration (Fig. 6A,B). To examine the expression of defence-related genes in the inoculated plants, qRT-PCR was performed with gene-specific primers (Table S1, see Supporting Information). A decreased level of *PR1a* was observed in *Camc9* -transgenic plants relative to controls. Overexpression of *Camc9* also down-regulated the transcript levels of trypsin proteinase inhibitor (*TPI*) and ascorbate peroxidase genes, such as *APX1* and *APX2* (Yeom *et al.* , 2011b; Yoon *et al.* , 2009) (Fig. 6C). These results suggest that overexpression of *Camc9* may cause the down-regulation of ROS-modulating and defence-related gene expression, and, as a result, lead to an enhancement of pathogen-induced cell death via increased levels of ROS. Together with gene silencing data, these results also further confirm the positive role of *Camc9* in pathogen-induced cell death in plants.

DISCUSSION

In this study, we isolated *Camc9* from pepper ESTs for representative proteases by protease domain prediction. Bioinformatic analysis provided strong evidence that *Camc9* belongs to the type II metacaspase gene family. In particular, the protein domain structure of *Camc9* is similar to that of *Atmc9* , with the conserved catalytic dyad of histidine and cysteine of caspases and an arginine/lysine-specific proteolytic activity (Vercammen *et al.* , 2006). *Capsicum annuum* has 15 putative members of the metacaspase gene family, which is very high compared with other metazoan genomes (Coll *et al.* , 2011; Tsiatsiani *et al.* , 2011) (Fig. S4, see Supporting Information).

Vacuolar processing enzyme (VPE) has been suggested to have caspase 1 activity and its role is essential for virus-induced cell death (Hatsugai *et al.* , 2004). Although VPE has biological functions of caspase-1, it is structurally unrelated to the caspase family (Hara-Nishimura and Hatsugai, 2011). Recently, the type II metacaspases have been reported as negative or positive regulators of PCD triggered by abiotic or biotic stress (Tsiatsiani *et al.* , 2011). However, there is little evidence that metacaspases are involved in disease symptoms caused by pathogens.

Our findings suggest that *Camc9* may play a key role in pathogen-induced cell death, like caspase 1 in mammals, based on the expression pattern of *Camc9* induced by compatible and incompatible pathogens (Fig. 2B). In addition, silencing of *Camc9*

Fig. 5 Overexpression of *Capsicum annuum* metacaspase 9 (*Camc9*) enhances disease symptoms. (A) *Pseudomonas syringae* pv. *tabaci* (*P.s. tabaci*) was serially diluted from an optical density at 600 nm (OD_{600 nm}) of 0.5 to OD_{600 nm} of 0.05, and infiltrated into one half of a whole leaf after transient overexpression of pMBP1 control (left part of the leaf) and pMBP1-*Camc9* (right part of the leaf) in *Nicotiana benthamiana*. White and black circles indicate the infiltration areas of *P.s. tabaci*. Red circles represent the infection area of INF1 (cell death inducer) as the positive control. Photographs were taken at 3 days post-inoculation (dpi). (B) Disease symptoms were calculated using ion leakage in leaves transiently overexpressing *Camc9*. Asterisks indicate significant differences relative to the Tobacco rattle virus (TRV)-dGFP control as determined by Student's *t*-test (***P* < 0.01, ****P* < 0.001). (C) Leaf samples were harvested at the indicated times after agroinfiltration containing pMBP1 or pMBP1-*Camc9* constructs, and total RNA was isolated for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The values were normalized to *NbActin* and calculated relative to the pMBP1 control. (D) Photographs of enhanced disease symptoms of *Camc9*-transgenic plants (lines 2, 3 and 4) infected with *P.s. tabaci*. Photographs were taken at 2 dpi. (E) Transcript levels of *Camc9* in *Camc9*-transgenic plants. (F) Ion leakage measurement of *P.s. tabaci* infiltration area in *Camc9*-transgenic plants at 2 dpi. Each of the tests was assayed three times. Different letters indicate significant differences at the 95% level by Duncan's multiple range tests. Values are means $\pm$ standard deviation (*n* = 3). Similar results were obtained from at least three experiments. One representative experiment is shown.

delayed cell death symptoms induced by compatible pathogens (Figs 3 and 4). In contrast with the VIGS result, overexpression of *Camc9* caused increased susceptibility to pathogen-induced cell death (Figs 5 and 6). These results imply that *Camc9* positively influences bacterial pathogen-induced cell death in plants. However, we could not find any phenotypic differences by silencing or overexpression of the *Camc9* gene in *N. benthamiana* plants (Fig. S2). These results indicate that *Camc9* is primarily involved in defence mechanisms, rather than developmental processes, of plants.

ROS are important signalling molecules that are rapidly generated after the recognition of pathogens in plants (Besson-Bard *et al.*, 2008; Bolwell and Daudi, 2009; Hong *et al.*, 2008). The recognition of pathogen- or microbe-associated molecular patterns by the host leads to rapid activation of defence mechanisms, such as cell wall reinforcement by callose deposition, the production of ROS and the induction of numerous defence-related genes (Kim *et al.*, 2005; Nomura *et al.*, 2005). In a previous study, type 1 metacaspases (*Atmc1*, *Atmc2*) have been reported to regulate developmental plant cell death related to *LSD1*, caused by oxidative stress (Coll *et al.*, 2010). Furthermore, *Atmc9* has been shown to be S-nitrosylated at the active site, which is related to the production of ROS (Belenghi *et al.*, 2007; Mannick *et al.*, 1999; Spoel and Loake, 2011).

Our results suggest that *Camc9* increases the level of ROS during plant-pathogen interaction by decreasing the transcript levels of APX genes, such as *APXT1* and *APX1* (Fig. 6C). In transgenic tobacco, the ROS-scavenging APX enzymes increase protec-

tion against methyl viologen-induced oxidative stress (Allen *et al.*, 1997). In addition, *PR1a* expression is suppressed in *Camc9*-transgenic plants (Fig. 6C). It is known that *PR1a* overexpressed in tobacco has antifungal activity and increases tolerance to *Perono-*

Fig. 6 Overexpression of *Capsicum annuum* metacaspase 9 (*Camc9*) enhances reactive oxygen species (ROS) production and the expression of defence-related genes. (A) 3,3'-Diaminobenzidine (DAB) staining in *Camc9*-transgenic *Nicotiana benthamiana* following *Pseudomonas syringae* pv. *tabaci* (*P.s. tabaci*) inoculation [optical density at 600 nm ($OD_{600\text{ nm}}$) = 0.005] at 6 h post-inoculation (hpi). Cell death on the leaves was verified visually by DAB staining. (B) Quantification of H_2O_2 intensity by DAB staining is described in the experimental procedures section. Values represent means and standard deviation from at least two independent experiments with four replications. Different letters indicate significant differences at the 95% level by Duncan's multiple range tests. (C) Overexpression of *Camc9* in *N. benthamiana* induces defence-related genes. Leaf samples were harvested at the indicated times after inoculation of *P.s. tabaci*, and total RNA was isolated for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The values were normalized to *NbActin* and calculated relative to the pMBP1 control. Values are means $\pm$ standard deviation ($n = 3$). Asterisks indicate significant differences relative to the empty vector control (pMBP1) as determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). DAI, days after inoculation.

spora tabacina and *Phytophthora parasitica* var. *nicotianae* (Alexander *et al.*, 1993). Taken together, the results of this study suggest that *Camc9* may influence ROS production and basal defence mechanisms by triggering the down-regulation of defence-related gene expression in plants.

In conclusion, we provide evidence that *Camc9*, probably a unique member of the metacaspase gene family, positively influences pathogen-induced cell death by controlling the production of ROS and the expression of defence-related genes. Furthermore, *Camc9* is not involved in developmental PCD, but plays key roles in plant defence mechanisms against pathogens. These findings may place *Camc9* as a candidate for the functional orthologue of mammalian caspase 1, which plays a role in pyroptosis-like cell death in the plant kingdom.

EXPERIMENTAL PROCEDURES

Plant materials

Capsicum annuum cv. Bukang, cv. 'ECW30R' and *Nicotiana benthamiana* seeds were germinated in a growth chamber at 28 °C under dark conditions. After germination, seeds were transferred into a plastic tray (55 $\times$ 35 $\times$ 15 cm³) and grown in a growth chamber at 25 °C under a cycle of 16 h of light and 8 h of dark.

Bacterial infection

The bacterial pathogens *Xcv1* (AvrBs3) and *Xcv3* (AvrBs3) for *C. annuum* and *Pst T1* and *P.s. tabaci* for *N. benthamiana* (Oh *et al.*, 2008) were grown in YEP medium (10 g/L yeast extract, 10 g/L bacto-peptone, 5 g/L NaCl) and resuspended in 10 mM MgCl₂ for inoculation. The bacterial suspensions were pressure infiltrated into *C. annuum* and *N. benthamiana* leaves using a 1-mL needleless syringe.

Cloning and sequence analysis of *Camc9*

Camc9 was obtained from the Bukang flower cDNA in the *C. annuum* EST database (Kim *et al.*, 2008). For the cloning of full-length cDNA, PCR was performed to amplify *Camc9* using gene-specific primers (Table S1). The PCR product was cloned using the pCR® 8/GW/TOPO® TA cloning kit (Invitrogen, Carlsbad, CA, USA). The *Camc9* sequence was confirmed by DNA sequencing using an Applied Biosystems 3730xl DNA Sequencer (Applied Biosystems, CA, USA) at the National Instrumentation Center for Environmental Management (Seoul, South Korea). The alignment of *Camc9* was performed by CLUSTALW2 using multiple sequence alignment (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), National Center for Biotechnology Information BLAST search (Altschul *et al.*, 1997) and the T-Coffee multi-alignment tool (<http://www.ebi.ac.uk/Tools/msa/tcofee/>).

VIGS of *Camc9* and *Nbmc9*

For VIGS, *Camc9*-N (targeting the N-terminus of *Camc9*) and *Camc9*-C (targeting the specific C-terminus of *Camc9*) were prepared by PCR with specific primers (Table S1) and cloned into pTRV2 using ligation-independent TRV vector cloning (Chung *et al.*, 2004; Dong *et al.*, 2007). Transformed *Agrobacterium* carrying TRV1, TRV2-*CaPDS*, TRV2-*dGFP*, TRV2-*Camc9*-N, TRV2-*Camc9*-C or *Nbmc9*-C was grown in 5 mL YEP medium containing rifampicin (50 mg/mL) and kanamycin (50 mg/mL) overnight. The transformed *Agrobacterium* was harvested by centrifugation at $13 \times 10^3 \times g$ for 15 min at 20 °C, resuspended to $OD_{600\text{ nm}} = 0.7$ (*C. annuum*) or $OD_{600\text{ nm}} = 0.3$ (*N. benthamiana*) in 10 mM $MgCl_2$ with 150 μM acetosyringone, and incubated at 22 °C with shaking for 4 h. *Agrobacterium* with TRV1, TRV2-*GFP*, TRV2-*CaPDS*, TRV2-*Camc9*-N, TRV2-*Camc9*-C or *Nbmc9*-C was mixed at a 1:1 ratio and infiltrated into cotyledons of *C. annuum* or *N. benthamiana* plants.

RNA extraction, RT-PCR and qRT-PCR

Total RNA was extracted from *C. annuum* and *N. benthamiana* using TRIzol (Invitrogen) as described in the manufacturer's instructions. First-strand cDNA was synthesized using 5 μg of total RNA with Oligo (dT) primers and SuperScript II reverse transcriptase (Invitrogen) for RT-PCR and qRT-PCR. Oligonucleotides were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3>) to amplify the target sequences. qRT-PCR was performed with a Rotor-Gene 6000 apparatus (Qiagen, Valencia, CA, USA) using SYBR Green (Invitrogen) and a LightCycler® 1536 Real-Time PCR System (Roche, Mannheim, Germany). All calculations and statistical analyses were performed as described in the manufacturer's instructions. To normalize the data, the expression level of *Actin* was used as an endogenous control for *C. annuum* and *N. benthamiana*.

Cell death measurements

Cell death was quantified by ion leakage by measuring the conductivity of leaf discs (Yeom *et al.*, 2011a). For conductivity tests, six discs per leaf (1 cm in diameter) were floated on 6 mL of distilled water for 4 h at room temperature. Electrical conductivity was measured using a conductivity meter (Model 455C; Istek, Seoul, South Korea). The experiments were repeated three times with independently treated samples.

Environmental and abiotic stress treatments

For chemical treatments, *C. annuum* cv. Bukang plants were sprayed with 5 mM SA in 10% acetone, 100 μM MeJA or 5 mM ET in water. *Capsicum annuum* plants treated with chemicals were covered with plastic bags for 3 h. Leaves were collected at the indicated time points, frozen in liquid nitrogen and stored at -70 °C until use for RNA extraction.

Agrobacterium-mediated transformation of *N. benthamiana*

Seeds were germinated on Murashige and Skoog agar medium and kept in a plant growth chamber under a 16-h photoperiod at 25 °C for 2 weeks before transformation. Full-length *Camc9* cDNA was cloned into the pMBP plant gene expression vector (Oh *et al.*, 2010). The recombinant vector was transferred to the *Agrobacterium* strain GV2260 by the freeze-thaw method (Chen *et al.*, 1994). Cotyledons from 2-week-old *N. benthamiana* seedlings germinated on Murashige and Skoog medium were used for co-cultivation with *Agrobacterium* to generate transgenic plants (Yi *et al.*, 2010). Kanamycin-selected transgenic plants were grown in a plant growth chamber, and further selected by measuring the expression levels of *Camc9* with specific primer by RT-PCR.

Staining of hydrogen peroxide (H₂O₂) with DAB and trypan blue

The detection of H₂O₂ was measured as described by Thordal-Christensen *et al.* (1997). Leaves were infiltrated in 1 mL DAB in 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) (pH 5.5) under vacuum for 20 min and incubated for 12 h in the dark. The samples were incubated in 100% ethanol in a 65 °C oven for 12 h for chlorophyll destaining. The DAB-stained H₂O₂ was visualized as a brown coloration. To detect plant cell death, leaves were stained with lactophenol/trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol, 10 mL deionized distilled water), as described by Koch & Slusarenko (1990). For trypan blue staining, the leaves were vacuum treated twice for 5 min in the staining solution, incubated overnight and then destained in chloral hydrate (2.5 g/mL).

Accession numbers

Sequence data for *Camc9* and *Nbmc9* referred to in this article can be found in the GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank/>) under the following accession numbers: *C. annuum* metacaspase 9 (KC597255); *N. benthamiana* metacaspase 9 (KC597256).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Expression levels of *Capsicum annuum* metacaspase 9 (*Camc9*) were determined by reverse transcription-polymerase chain reaction (RT-PCR) using *Camc9*-specific primers after various treatments in *C. annuum* cv. Bukang. To analyse the expression of *Camc9*, pepper plants were treated with salicylic acid (5 mM), ethephon (3 mM) or methyl jasmonate (100 μ M). The housekeeping gene *CaActin* was used to normalize expression levels.

Fig. S2 Virus-induced gene silencing (VIGS) phenotype of *Capsicum annuum* metacaspase 9 (*Camc9*)-N- and *Camc9*-C-silenced *C. annuum*.

Fig. S3 (A) Diagram of *Nicotiana benthamiana* caspase 9 (*Nbmc9*) silencing construct targeting the C-terminus of *Nbmc9*. (B) *Pseudomonas syringae* pv. *tabaci* was diluted to an optical density at 600 nm ($OD_{600\text{ nm}}$) of 0.005, and infiltrated into a whole

leaf of *Tobacco rattle virus* (TRV)-*dGFP* (as a control) and *Nbmc9*-C-silenced *N. benthamiana* plants. Ion leakage from the infiltration area was measured at 3 days post-inoculation (dpi). Values are means $\pm$ standard deviation ($n = 3$). (C) Expression of *Nbmc9* was analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The values were normalized to *NbActin* and are calculated relative to that in leaves. Asterisks indicate significant differences relative to the TRV-*dGFP* control, as determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig. S4 Phylogenetic tree with *Capsicum annuum* metacaspase 9 (*Camc9*) and metacaspase genes from *Arabidopsis thaliana*, *Nicotiana tabacum*, *Nicotiana benthamiana*, *Solanum lycopersicum* and *Capsicum annuum* (from GenBank database <http://www.ncbi.nlm.nih.gov/GenBank/>). The scale bar indicates the genetic distance based on branch lengths. Putative *C. annuum* metacaspases are labelled as p*Camc1* to p*Camc15* (Kim *et al.*, unpublished data). The GenBank accession numbers are indicated in parentheses.

Table S1 Primer sequences used in the study.