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BIOTIC AND ABIOTIC STRESS

Expression of a *metacaspase* gene of *Nicotiana benthamiana* after inoculation with *Colletotrichum destructivum* or *Pseudomonas syringae* pv. *tomato*, and the effect of silencing the gene on the host response

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Abstract Metacaspases are cysteine proteinases that have homology to caspases, which play a central role in signaling and executing programmed cell death in animals. A type II metacaspase cDNA, *NbMCA1*, was amplified from *Nicotiana benthamiana* infected with *Colletotrichum destructivum*. It showed a peak in expression at 72 h post-inoculation corresponding with the switch to necrotrophy by *C. destructivum*. Inoculation of *N. benthamiana* with an incompatible bacterium, *Pseudomonas syringae* pv. *tomato*, which should induce a non-host hypersensitive response (HR), did not result in an increase in *NbMCA1* expression at the time of necrosis development at 20–24 h postinoculation. Virus-induced silencing of *NbMCA1* resulted in three to four times more lesions due to *C. destructivum* compared with leaves inoculated with the PVX vector without the cloned metacaspase gene or inoculated with water only. However, virus-induced silencing of *NbMCA1* did not affect the HR necrosis or population levels of *P. syringae* pv. *tomato*. Although this metacaspase gene does not appear to be involved in the programmed cell death of non-host HR resistance to *P. syringae*, it does affect the susceptibility of *N. benthamiana* to *C. destructivum* indicating a function in a basal defense response. Possible roles of *NbMCA1*could be in degrading virulence factors of the pathogen, processing pro-proteins involved in stress responses, eliminating damaged proteins created during stress, and/or degrading proteins to remobilize amino acids to fuel de novo synthesis of proteins involved in stress adaptations.

Keywords Hemibiotrophy · Hypersensitive response · Metacaspase · Necrosis · Virus-induced gene silencing

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Introduction

Caspases are members of the C14 family of cysteine proteinases that specifically target sites containing aspartate residues (Cohen 1997). Caspases have been extensively studied because of their central role in signaling and executing programmed cell death (PCD) in animals (Cryns and Yuan 1998). PCD also occurs during plant development and the response of plants to different stresses (Beers et al. 2000). Although caspase-like proteinase activities have been identified in plants (del Pozo and Lam 1998; Solomon et al. 1999), no true plant caspase has been identified from them. However, plants and fungi have metacaspases, which are a family of caspase-related proteases that have a p20-like subunit containing a histidine and cysteine catalytic diad (Uren et al. 2000). Plant metacaspases are arginine/lysine-specific cysteine proteinases and thus do not have aspartate-specific proteolytic activity like animal caspases (Vercammen et al. 2004; Watanabe and Lam 2005). Thus, they are not able to cleave animal caspase-specific substrates, suggesting that they are not responsible for plant caspase-like enzyme activity. Despite this, plant metacaspases can trigger PCD, such as during embryogenesis in arabidopsis, where PCD was found to be dependent on the enzyme's proteinase activity (Bozhkov et al. 2005). Also, two arabidopsis metacaspase genes complemented a metacaspase yeast mutant that mediates PCD, indicating that plant metacaspases can activate downstream protein-

ases with caspase-like activity required for PCD (Watanabe and Lam 2005). Thus, plant metacaspases appear to play a parallel role as animal caspases in PCD.

Relatively little is known about metacaspases in plant–pathogen interactions. Armando et al. (2003) found that a metacaspase gene, *AtMCA1*, was expressed in wounded and *Pseudomonas syringae*-infected arabidopsis leaves, and suggested that they participate as caspase-like enzymes during PCD. Watanabe and Lam (2004) examined three type I and six type II metacaspase genes from arabidopsis and showed that two type I metacaspase genes were up-regulated following infiltration by *P. syringae* pv. *maculicola*, while expression of two type II metacaspases was not significantly affected. These metacaspase genes were hypothesized to be involved in the activation of PCD. In tomato, one type II metacaspase gene, *LeMCA1*, rapidly increased during cell death induced by *Botrytis cinerea* (Hoeberichts et al. 2003). More direct evidence for a role in plant–pathogen interactions comes from an examination of arabidopsis mutants, which showed that there was increased susceptibility in type I metacaspase mutants and decreased susceptibility in type II metacaspase mutants following inoculation by several *Botrytis* species (van Baarlen et al. 2007).

Nicotiana benthamiana is becoming widely used by molecular biologists because it exhibits more pronounced and longer lasting virus-induced gene silencing (VIGS) than other plants, which permits a high-throughput forward genetics approach for obtaining functional genomics information (Lu et al. 2003). VIGS is a post-transcriptional gene silencing mechanism for sequence-specific targeting and degradation of plant RNA that spreads systemically in the plant, and it has been successfully used in analyzing the function of a number of genes of *N. benthamiana* (Robertson 2004), including genes involved in plant–pathogen interactions (Baulcombe et al. 2002).

A hemibiotrophic pathogen of *N. benthamiana* is *Colletotrichum destructivum*, which has a biotrophic phase of infection followed by a necrotrophic phase, resulting in the formation of water-soaked spots on leaves and petioles that develop into small necrotic lesions by 72 h post-inoculation (HPI) (Shen et al. 2001). In contrast, inoculation of *N. benthamiana* with *P. syringae* pv. *tomato* DC3000 results in an incompatible interaction, which triggers a non-host hypersensitive response (HR) within 24 HPI (Espinosa et al. 2003). *Pseudomonas syringae* pv. *tomato* DC3000 is a pathogen of tomato and arabidopsis causing bacterial speck disease (Preston 2000), and several studies have been done on its incompatible interaction with *N. benthamiana* (Rommens et al. 1995; Scofield et al. 1996; Sessa and Martin 2000; Espinosa et al. 2003).

This work describes a metacaspase cDNA, *NbMCA1*, amplified from *N. benthamiana* leaves infected with *C. destructivum*. The expression of this gene was examined following a compatible interaction of *N. benthamiana* with *C. destructivum* and an incompatible interaction with *P. syringae* pv. *tomato*. A portion of *NbMCA1* was then cloned into a PVX-based VIGS vector (Ruiz et al. 1998) to determine if silencing the gene would affect the response of *N. benthamiana* to *C. destructivum* or *P. syringae* pv. *tomato*. Silencing may reduce resistance to *P. syringae* pv. *tomato* as the HR is associated with resistance and PCD (del Pozo and Lam 1998; Heath 2000). In contrast, it has been suggested that some pathogens induce host PCD to create dead plant tissue as a nutritional source during disease development (Dickman et al. 2001; Richael et al. 2001), and thus silencing a metacaspase may increase resistance by reducing the amount of nutrients to the pathogen.

Materials and methods

Biological materials and pathogen inoculations

Nicotiana benthamiana plants were grown with 16 h light (150 μmol m^{-2} s^{-1}) at 25°C and 8 h dark at 17°C. Seedlings of *N. benthamiana* at the sixth true-leaf stage were used for fungal or bacterial inoculation. *C. destructivum* N150 P3 (Chen et al. 2002) was cultured for 10 days on sodium chloride-yeast extract-sucrose agar medium (SYAS) (Mandanhar et al. 1986) at 22°C under continuous fluorescent light. Conidia were harvested to make a 1×10^5 conidia/ml suspension in sterile distilled water, which was sprayed onto whole plants. These plants were then incubated at room temperature in containers to maintain high humidity. *P. syringae* pv. *tomato* DC3000 was grown overnight in King's medium B (KB) (King et al. 1954) at 22°C and suspended in 10 mM $MgCl_2$ to 10^8 CFU per ml. The bacterial suspension was infiltrated into each of the two youngest fully developed *N. benthamiana* leaves per plant using a needle-less syringe (Klement 1963).

The area of the inoculated leaves was measured with a leaf area meter (Model 3100, LI-COR, Lincoln, NE, USA). For quantifying *C. destructivum* infection, the number of lesions was counted at 60 HPI, and the lesions per cm^2 was calculated. For quantifying *P. syringae* pv. *tomato*, 1-cm-diameter leaf discs at 24 HPI were ground in 1 ml 10 mM $MgCl_2$ and then dilution-plated onto KB to determine the number of colony forming units. For both pathogens, two inoculated leaves from each of the three replicate plants per treatment were used.

RNA extraction

Leaf samples were immediately frozen in liquid nitrogen and stored at –80°C. Total RNA was prepared following

the method of Chen et al. (2000), except that phenol and chloroform were added just prior to the homogenization buffer, which was composed of 200 mM Tris base, 400 mM KCl, 200 mM sucrose, 35 mM $MgCl_2 \cdot 6H_2O$ and 25 mM EDTA, pH 9.0. The RNA was resuspended in 25–50 μL DEPC-treated dH_2O and stored at –80°C.

Sequence alignments

Putative metacaspase amino acid sequences were obtained from the GenBank NR database (http://www.ncbi.nlm.nih.gov), The Institute for Genomic Research (TIGR) (http://www.tigr.org), Phytome (http://www.phytome.org), The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org), and the Beijing Genomics Institute (BGI) (ftp://ftp.genomics.org.cn/pub/ricedb/SynVs9311/Syngenta/Sequence/FgeneSH). The sequence names and origins are listed in Table 1. The sequences were aligned with CLUSTALX (Thompson et al. 1997) using default parameters to assess sequence identity and locate conserved regions for designing the primers described below. A dendrogram was created that also included the predicted protein sequence of *NbMCA1*, which was obtained in this study. This unrooted dendrogram was generated with the Neighbor-Joining-bootstrap procedure of CLUSTALX using 1,000 bootstrap replications.

Reverse transcriptase PCR (RT-PCR)

Except for the 3′-RACE described below, single-stranded cDNA was synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) and oligo (dT) primer with total RNA following the manufacturer's instructions. All PCR reactions were performed using a Touchgene Thermo Cycler (Techne, Princeton, NJ, USA). Except where noted, all RT-PCR reactions were done in 15 μL volumes with 2 μL cDNA, 0.25 units *Taq* polymerase (Promega, Madison, WI, USA), 10× *Taq* polymerase buffer, 2 mM dNTPs, 2.5 mM Mg^{2+}, and 1.0 mM of each primer. The PCR conditions were 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 62°C for 1 min, and 72°C for 1 min, and a final extension period of 10 min at 72°C.

A degenerate forward primer, CYP11F1, 5′-GGTGAA GAHGATGAYACTGG and a degenerate reverse primer, CYP11R1, 5′-CCTGAACCRGCATAAACCTC, (H = A/C/T, R = A/G, Y = T/C) were designed based on conserved regions of the plant metacaspase nucleotide sequences: *LeMCA1* (AAM51555) from *Lycopersicon esculentum*, *HbLAR* (AAD13216) from *Hevea brasiliensis* and *At1g79340* (AAL85992) from *Arabidopsis thaliana*. The primers were used in RT-PCR with cDNA from leaves of *N. benthamiana* at 96 HPI with *C. destructivum*. A second forward primer, CYP11F3, 5′-TTCAACAAACTACAG CACGG, was designed based on the sequence of the RT-PCR product that had been amplified with CYP11F1 and CYP11R1, and was paired with the anchor primer supplied in a 5′/3′ RACE kit (Roche, Indianapolis, IN, USA). The RT-PCR fragment obtained with the CYP11F3 primer and 5′/3′ RACE anchor primer was purified following electrophoresis in 1% TAE agarose gels using the GENECLEAN II Kit (Q-BIOgene, Montreal, QUE), and then cloned into pGEMT-easy (MBI Fermentas, Burlington, ON, Canada).

Relative RT-PCR

Relative RT-PCR was done in 15 μL reaction volumes as described previously except that 0.5 mM of each translation elongation factor 1α (EF-1α) primer and 1.2 mM of each metacaspase-specific primer was used. The EF-1α forward primer, TobefIS 5′-CTCCAAGGCTAGGTATGA TG, and reverse primer, TobefA 5′-CTTGGTGGTGCA TCTCAAC, were designed to amplify a RT-PCR product of 375 bp (Dean et al. 2002). The metacaspase forward primer, CYP11F2, 5′-CACAAGCAAGGCGACGATGA, and reverse primer, CYP11R3, 5′-CTGAATCGCGTTGC TTAGAG, were designed based on the combined sequence of the portion of the metacaspase gene obtained by RT-PCR with primers CYP11F1 and CYP11R1 as well as the portion obtained by RT-PCR with primers CYP11F3 and the 5′/3′ RACE anchor primer. Primers CYP11F2 and CYP11R3 yielded a 630 bp RT-PCR product.

The PCR conditions were 94°C for 3 min followed by 25 cycles of 94°C for 30 s, 62°C for 1 min, and 72°C for 1 min, and a final extension period of 10 min at 72°C. The RT-PCR products were separated in 1% TAE agarose gels, and the images were saved as TIF electronic image files for quantification using NIH Image (Scion Corporation, Frederick, MD, USA). The band intensities were determined for both the metacaspase and EF-1α RT-PCR products, and compared for each gel lane. Relative expression was determined by taking a ratio of the band intensity of metacaspase over the band intensity of the EF-1α. Four plants were assessed at each time point following inoculation with *C. destructivum*. To confirm the identity of the RT-PCR products, the *NbMCA1* RT-PCR product at 96 HPI with *C. destructivum* were excised after electrophoresis in 1% TAE agarose gels, purified with GENECLEAN II (Q-BIOgene) and then sequenced (Laboratory Services Division, University of Guelph, Guelph, ON, Canada).

Gene silencing

The RT-PCR fragment cloned into pGEMT-easy (MBI Fermentas) was digested with *NotI* and then subcloned into pGR106 (Jones et al. 1999), which was also digested with

Table 1 Cysteine proteinase amino acid sequences of metacaspase families used in sequence analyses

GenBank accession	Type[a]	Species	Gene name (locus tag)
AAP84706	Type I	*Arabidopsis thaliana*	*AMC1* (AT1G02710)
AAP84707	Type I	*Arabidopsis thaliana*	*AMC2* (AT4G25110)
AAP84708	Type I	*Arabidopsis thaliana*	*AMC3* (AT5G64240)
TC7963	Type I	*Nicotiana benthamiana*	*NbMCA2*
TC8186	Type I	*Nicotiana benthamiana*	*NbMCA3*
XP_470787[b]	Type I	*Oryza sativa*	OsFCC010005
XP_470792	Type I	*Oryza sativa*	OsFCC010006
XP_470794	Type I	*Oryza sativa*	OsFCC010007
XP_470796[b]	Type I	*Oryza sativa*	OsFCC010009
–	Type II	*Oryza sativa*	OsFCC034713
–	Type II	*Oryza sativa*	OsFCC026625
–	Type II	*Pinus taeda*	PTAE9659 (from Phytome)
–	Type II	*Solanum tuberosum*	STUB1528 (from Phytome)
AAD13216	Type II	*Hevea brasiliensis*	*HbLAR*
AAM51555	Type II	*Lycopersicon esculentum*	*LeMCA1*
AAP84709	Type II	*Arabidopsis thaliana*	*AMC4* (AT1G79310)
AAP84710	Type II	*Arabidopsis thaliana*	*AMC7* (AT1G79340)
AAP84711	Type II	*Arabidopsis thaliana*	*AMC8* (AT1G16420)
AAP84712	Type II	*Arabidopsis thaliana*	*AMC9* (AT5G04200)
AAP84713	Type II	*Arabidopsis thaliana*	*AMC5* (AT1G79320)
AAP84714	Type II	*Arabidopsis thaliana*	*AMC6* (AT1G79330)
BE642699	Type II	*Ceratopteris richardii*	CRIC1930 (from Phytome)
CAD59226	Type II	*Picea abies*	mcII
DQ084024	Type II	*Nicotiana benthamiana*	*NbMCA1*
NP_915811	Type II	*Oryza sativa*	OsFCC003462
XP_475477[b]	Type II	*Oryza sativa*	OsFCC014526
XP_475478	Type II	*Oryza sativa*	OsFCC014527

No GenBank accession number (Gene name used in Fig. 2 instead)

[a] Type I metacaspases are characterized by a prodomain with a proline-rich repeat motif and a Zn finger motif typical of plant proteins, such as LSD-1, which functions in the hypersensitive response pathway. Type II metacaspases have a conserved insertion of approximately 180 amino acid directly C terminal to the metacaspase p20-like subunit (Uren et al. 2000)

[b] This GenBank sequence was most similar to the sequence found in the predicted protein set of the *O. savtiva* Nipponbare genome version 9311 dated 20 January, 2005, but was not identical

NotI. The pGR106 construct was electroporated into *A. tumefaciens* GV3101, and *N. benthamiana* leaves were inoculated following Takken et al. (2000). The *A. tumefaciens* cultures contained either PVX with a fragment of *NbMCA1* (PVX-*NbMCA1*), PVX without any insert as an empty vector control (PVX vector) or water only as a control for *A. tumefaciens*. A toothpick was used to inoculate *A. tumefaciens* at six sites per leaf along the main vein of the two youngest fully developed leaves of *N. benthamiana* at the fourth true-leaf stage. At 20 days after inoculation with *A. tumefaciens*, the plants had another four fully developed leaves, and one set of plants were spray inoculated with *C. destructivum* as previously described. The number of lesions per cm^2 of leaf for the two youngest fully developed leaves was determined 60 h after spraying. To test the effect of silencing on *P. syringae* pv. *tomato*, the two youngest fully developed leaves of another set of plants were infiltrated with a bacterial suspension using a needle-less syringe as previously described, and the number of CFU per cm^2 leaf was determined 24 h later. Four plants per treatment were assessed for disease and gene expression.

To test for silencing of *NbMCA1* at 20 days after inoculation with *A. tumefaciens*, the two youngest fully developed leaves per plant were harvested from another set of plants, and relative RT-PCR analysis was done as previously described. This set of plants was only used for relative RT-PCR analysis. To assess the specificity of silencing, the expression of another metacaspase gene of *N. benthamiana*, *NbMCA2*, as well as two other cysteine

proteinases, *NbCYP1* (DQ084022) and *NbCYP2* (DQ084023) (Hao et al. 2006), were examined by relative RT-PCR prior to pathogen inoculation as previously described except with using different primers. A 830 bp portion of *NbMCA2* was amplified with primers MCA2F1 5′-CCTGCTCTCCATAATCCTCA and MCA2R1 5′-ATA CCAGAACGAGGACGATG designed from the sequence of TC7963 (http://www.tigr.org). Based on the primers described by Hao et al. (2006), a 391 bp portion of *NbCYP1* was amplified with primers CYP4F3, 5′-GCGGA TCTTGCTGGACATTC and CYP4R1 5′-GGGAGTATT GCCACATTCGG, and a 310 bp portion of *NbCYP2* was amplified with primers CYP5F1, 5′-CGAAACCGAAA CCGACGA and CYP5R1 5′-GTCACGCCAGTCATAAT CAG. Quantification and confirmation of the identity of the RT-PCR products was done as previously described.

Results

Amplification and sequencing of NbMCA1

A pair of degenerate primers was designed based on conserved regions of the nucleotide sequence of *LeMCA1* (AAM51555) and its two mostly closely related metacaspase genes, *HbLAR* (AAD13216) and *At1g79340* (AAL85992). These conserved regions correspond to the sequences identified by Uren et al. (2000) as being highly conserved among caspases and metacaspases. The primers were used to amplify a single RT-PCR product of 700 bp from cDNA produced from leaves of *N. benthamiana* at 96 HPI with *C. destructivum*. Cloning of the RT-PCR product and subsequent sequence analysis of two of the clones revealed the same sequence, which had 87% identity with *LeMCA1* from *L. esculentum* (Table 1). The *N. benthamiana* sequence was used in 3′ RACE to amplify a 610 bp RT-PCR product, using cDNA from leaves of *N. benthamiana* at 96 HPI with *C. destructivum*. This yielded a consensus sequence with a total length of 1,150 bp which was designated *NbMCA1* (GenBank accession number DQ084024). Based on its alignment with *HbLAR*, the 5′-end of the *NbMCA1* sequence matched the 3′-end of the highly conserved p20-like subunit of plant metacaspases (Uren et al. 2000). The p20 subunit contains the catalytically active site for caspases (Hoeberichts et al. 2003; Uren et al. 2000).

A search of the *N. benthamiana* Gene Index at TIGR (http://www.tigr.org/tdb/potato/) using TBLASTX did not reveal any sequences identical to *NbMCA1*, but did show similarity (expect value < 3E-4) to TC7963, which is composed of nine ESTs and has a tentative annotation as a caspase family protein. This putative metacaspase from the TIGR database was designated *NbMCA2*. *NbMCA1* also had similarity (expect value < 7E-3) to TC8186, which is composed of seven ESTs and also has a tentative annotation as a caspase family protein, and this was designated *NbMCA3*. Although *NbMCA2* and *NbMCA3* had low similarity to *NbMCA1*, a 54 amino acid portion that was most similar with over 35% identity contained the highly conserved p20-like subunit of metacaspases, including the conserved cysteine and histidine catalytic sites of cysteine proteinases (Uren et al. 2000), indicating that *NbMCA2* and *NbMCA3* are also metacaspases.

The predicted protein sets of the genomes of *O. sativa* cv. Nipponbare and *A. thaliana* ecotype "Columbia" were mined for metacaspases using predicted plant metacaspase protein sequences from GenBank as the query sequences in Standalone BLAST version 2.2.6 (Altschul et al. 1997). These sequences plus the predicted *N. benthamiana* metacaspase proteins and several other putative metacaspases were aligned and a dendrogram was produced (Fig. 1). In this dendrogram, metacaspase sequences from rice and arabidopsis were scattered among various groups, providing evidence that metacaspases arose before the divergence of dicots from monocots. When genomes of other plant species are completed, they are likely to show genes interspersed among the various groups of rice and arabidopsis metacaspases, demonstrating the ancient origin and diversity of this multigene family. The major division in this dendrogram was between types I and II metacaspases which

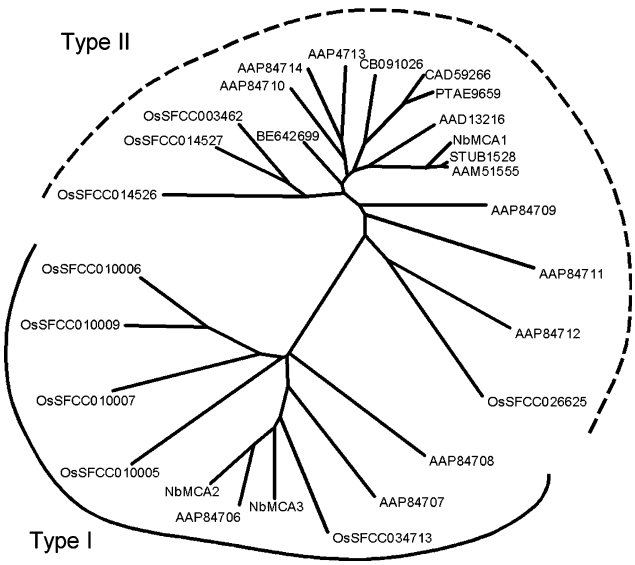

Fig. 1 Comparison of metacaspase sequences from *Arabidopsis thaliana*, *Ceratopteris richardii*, *Hevea brasiliensis*, *Lycopersicon esculentum*, *Nicotiana benthamiana*, *Picea abies*, *Pinus taeda*, *Oryza sativa* and *Solanum tuberosum* using Neighbor-Joining analysis to produce an unrooted tree. A description of each sequence is provided in Table 1. Amino acid sequences were aligned using CLUSTALX and a tree generated with the bootstrap NJ-tree procedure of CLUSTALX

clustered separately from each other with 100% bootstrap support (Fig. 1). *NbMCA1* clustered with type II metacaspases, and *NbMCA2* and *NbMCA3* clustered with type I metacaspases (Fig. 1).

The division of plant metacaspases into types I and II is based on sequence similarities within their caspase-like domain and their overall domain structure (Uren et al. 2000). Type I metacaspases have a prodomain with a proline-rich repeat motif and a Zn finger motif typical of plant proteins, such as LSD-1, that functions in the HR pathway, whereas type II metacaspases lack those prodomain features, but have a conserved insertion of approximately 180 amino acids directly C-terminal to their p20-like subunit (Uren et al. 2000). An examination of these characteristics confirmed that *NbMCA1* is a type II metacaspase, whereas *NbMCA2* and *NbMCA3* are type I metacaspases.

Within type II metacaspases, *NbMCA1* grouped with metacaspases of *L. esculentum* (AAM51555), *H. brasiliensis* (AAD13216) and *Solanum tuberosum* (STUB1528) with 97% bootstrap support, and these were separate from other groups of type II metacaspases of *A. thaliana* and *O. sativa*. Phylogenetically, the genera of *Lycopersicon, Solanum* and *Nicotiana* are close as they all belong to the Solanaceae, and so it was not unexpected that these type II metacaspases were most similar with high bootstrap support. However, a sister group to the metacaspases from solanaceaeous plants and *Hevea* contained two conifer sequences (*Picea abies*, CAD59226 and *Pinus taeda*, PTAE9659) and a cycad sequence (*Cycas rumphii*, CRUM1400) with 98% bootstrap support, which indicates that this particular group of type II metascaspases arose before the separation of gymnosperms and angiosperms, possibly over 500 million years ago (Hedges 2002). There was also a more distantly related sequence from a true fern (*Ceratopteris richardii*, BE642699) among type II metacaspases, demonstrating that the type II metacaspases arose at least prior to the appearance of seed plants.

NbMCA2 and *NbMCA3* grouped with *A. thaliana* (AAP84706 and AAP84707) and *O. sativa* (Os-SFCC034713) type I metacaspases with 100% bootstrap support. The remaining type I metascaspases were represented by predicted proteins of *A. thaliana* and *O. sativa*, again reflecting a diverse multigene family.

Expression of NbMCA1 in *N. benthamiana* following inoculation with *C. destructivum* or *P. syringae* pv. *tomato* DC3000, and the effect of silencing NbMCA1 on disease development

Transcripts of *NbMCA1* were detected in healthy *N. benthamiana* leaves by relative RT-PCR. Following infection with *C. destructivum*, expression of *NbMCA1* showed a slow progressive increase peaking at 72 HPI (Fig. 2). At 72 HPI, expression of *NbMCA1* was approximately 40% greater than that at 0 HPI. There was a slight decline at 96 HPI, but that difference was not significant. For the interaction with *P. syringae* pv. *tomato* DC3000, relative expression of *NbMCA1* showed a transient but significant 48% increase from 0 to 3 HPI (Fig. 3). Infiltration with buffer alone resulted in a 31% increase in *NbMCA1* expression at 3 HPI only indicating that changes in expression at 3 HPI were at least partially due to a wound response. At 3 HPI, the leaves appeared slightly wilted, but the leaf regained turgidity by 6 HPI. No necrosis was observed until 20–24 HPI. By 6 HPI, the expression level declined but still remained significantly higher than that at 0 HPI for the remainder of the experiment.

PVX-mediated VIGS of *NbMCA1* in *N. benthamiana* resulted in a significant decrease in *NbMCA1* expression compared to the water and PVX vector controls (Table 2; Fig. 4). Silencing appeared to be relatively specific as there was no significant difference in the expression of another metacaspase gene, *NbMCA2*, or two other cysteine proteinase genes, *NbCYP1* and *NbCYP2*, in *NbMCA1*-silenced plants compared to the water and PVX vector controls (Table 2). *NbCYP1* and *NbCYP2* are papain-like cysteine proteinases (family CA1) with less than 3% identity to *NbMCA1*. *NbMCA2* is the most similar metacaspase gene to *NbMCA1* currently available at the TIGR *N. benthamiana* Gene Index (http://www.tigr.org/tdb/potato) with 9.2% protein identity over their entire lengths.

The *NbMCA1*-silenced plants had three to four times more water-soaked lesions induced by *C. destructivum*

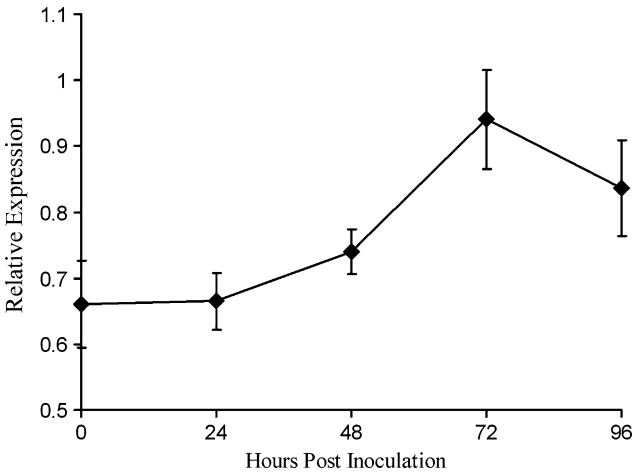

Fig. 2 Relative RT-PCR of *NbMCA1* expression in *N. benthamiana* leaves inoculated with *C. destructivum*. The amount of *NbMCA1* mRNA was determined relative to the amount of the translation elongation factor 1α, *NbEF-1α* . Mean relative expression values are shown with standard error bars calculated from three replications

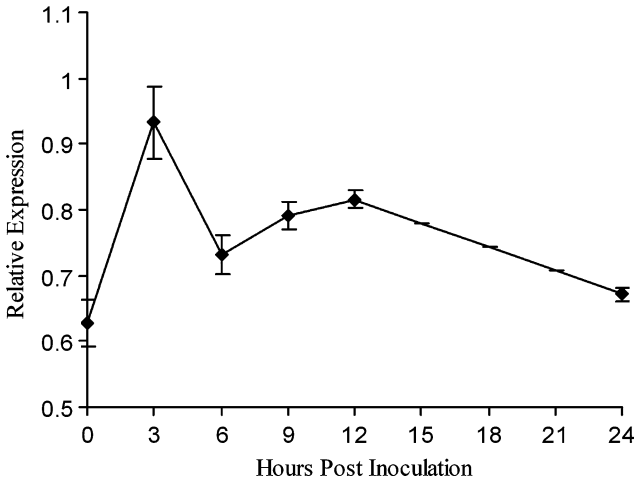

Fig. 3 Relative RT-PCR of *NbMCA1* gene expression in *N. benthamiana* inoculated with *P. syringae* pv. *tomato* DC3000. The amount of *NbMCA1* mRNA was determined relative to the amount of the translation elongation factor 1α, *NbEF-1α*. Mean relative expression values are shown with standard error bars calculated from three replications

Table 2 Relative RT-PCR analysis of silencing of *NbMCA1* and related genes in *N. benthamiana*

Treatment[1]	Relative gene expression[2]			
	NbMCA1	*NbMCA2*	*NbCYP1*	*NbCYP2*
Water control	0.55 a	0.85 a	1.04 a	1.00 a
PVX vector control	0.51 a	0.42 a	1.02 a	0.91 a
NbMCA1-silenced	0.12 b	0.51 a	0.95 a	0.84 a

[1] *NbMCA1*-silenced and PVX vector control *N. benthamiana* plants were toothpick inoculated with *A. tumefaciens* inoculum containing potato virus X (PVX) in its Ti plasmid with or without a fragment of *NbMCA1* construction in PVX vector. Water control plants were wounded by toothpick without *A. tumefaciens* inoculum

[2] Expression of *NbMCA1, Nb MCA1, NbCYP1 and NbCYP2* in water control, PVX vector control or *NbMCA1*-silenced *N. benthamiana* as determined by relative RT-PCR 20 days after *A. tumefaciens* inoculation and prior to inoculation with either *C. destructivum* or *P. syringae* pv. *tomato*. Each measurement represents the mean relative expression from at least three experiments. Means followed by a letter in common indicates that they are not statistically different according to the protected least significant difference test at $P < 0.05$

compared to the water and PVX vector controls (Table 3). There were no differences in the appearance of the water-soaked lesions among the different treatments. Although the populations of *P. syringae* pv. *tomato* DC3000 were higher in *NbMCA1*-silenced plants at 24 HPI inoculation compared to either the water or PVX controls, the difference was not significant (Table 4). There were also no significant differences among the treatments in the timing or appearance of the hypersensitive response due to infiltration of *P. syringae* pv. *tomato* DC3000.

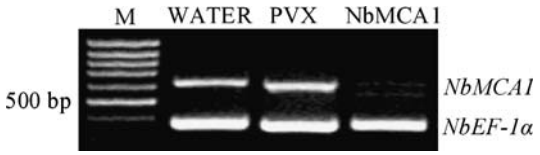

Fig. 4 Relative RT-PCR of virus-induced gene silencing of *NbMCA1*. RT-PCR gel showing the co-amplification of *NbMCA1* and a constitutive control, *NbEF-1α* (translation elongation factor 1α). *Lane* M is the 100 bp ladder. The other *lanes* are WATER, which is the water control where leaves were wound inoculated with water instead of *A. tumefaciens*; PVX, which is the vector control where leaves were wound inoculated with *A. tumefaciens* containing potato virus X cloned in a Ti plasmid; and NbMCA1, where leaves were wound inoculated with *A. tumefaciens* containing potato virus X along with a portion of *NbMCA1* cloned in a Ti plasmid

Discussion

A new plant metacaspase cDNA, *NbMCA1*, was amplified from leaves of *N. benthamiana* during the necrotrophic phase of infection by *C. destructivum* using a pair of degenerate primers. *NbMCA1* is part of a small gene family of metacaspases in *N. benthamiana*, as there appears to be at least two additional metacaspase genes, which were identified among a collection of 26,918 input ESTs that form the basis of Release 2.0 of the *N. benthamiana* Gene Index (http://www.tigr.org/tdb/potato/). All of the ESTs comprising the tentative consensus sequences of *NbMCA2* and *NbMCA3* were obtained from RNA of *N. benthamiana* from callus, roots in liquid culture or leaves subjected to heat stress, cold stress or challenge with incompatible bacterial pathogens (*P. syringae* pv. *tomato*, *Xanthomonas campestris* pv. *campestris*, *P. syringae* pv. *phaseolicola* and *X. campestris* pv. *vesicatoria*).

Plant metacaspases have been divided into types I and II based on the sequence similarity within their caspase-like domain and their overall domain structure (Uren et al. 2000). A dendrogram of various type I and type II metacaspases from plants showed that the sequences of the two types are clearly distinguishable from each other. Nine metacaspase genes have been described from the genome of *A. thaliana* ecotype "Columbia" (*AMC1–9*) with three type I genes and six type II genes. There appears to clustering of some of these genes in the genome as the locus tags for the type II metacaspases *AMC4* to *AMC7* were AT1G79310 to AT1G79340 on chromosome 1. With metacaspase sequences from GenBank as query sequences, nine metacaspases (five type I and four type II) were found in the genome of *O. sativa* cv. Nipponbare using Standalone BLAST. Like arabidopsis and rice, *N. benthamiana* probably has a small gene family of both type I and type II metacaspases.

For the interaction with *P. syringae* pv. *tomato* DC3000, there was a transient increase in the expression of *NbMCA1*

Table 3 Lesion number in water control, PVX vector control or *NbMCA1*-silenced *N. benthamiana* plants inoculated with *C. destructivum* at 60 HPI

Treatment[1]	Lesions per square centimeter[2]
Water control	0.45 a
PVX vector control	1.33 a
NbMCA1-silenced	4.05 b

[1] *N. benthamiana* were wounded-inoculated using a toothpick without any *A. tumefaciens* (water control), or with *A. tumefaciens* containing PVX in a Ti plasmid without an insert (PVX vector) or with a fragment of *NbMCA1* (*NbMCA1*-silenced). Plants were grown for 20 days after *A. tumefaciens* treatment and then inoculated with *C. destructivum*

[2] Lesion numbers per square centimeter were means pooled from two separate experiments with a total of 20 replications. All samples were collected at 60 HPI on 5-week-old plants. Means followed by a letter in common indicates that they are not statistically different according to the protected least significant difference test at $P < 0.05$

Table 4 Bacterial populations in water control, PVX vector control or *NbMCA1*-silenced *N. benthamiana* plants inoculated with *P. syringae* pv. *tomato* DC3000 at 24 HPI

Treatment[1]	CFU per square centimeter[2]
Water control	5.92×10^8 a
PVX vector control	6.97×10^8 a
NbMCA1-silenced	9.38×10^8 a

[1] *N. benthamiana* plants were inoculated with *A. tumefaciens* containing PVX in a Ti plasmid without an insert (PVX vector), inoculated with a fragment of *NbMCA1* (*NbMCA1*-silenced) or wounded with a toothpick without any *A. tumefaciens* (water control)

[2] Colony forming units (CFU) per square centimeter were means pooled from three separate experiments with a total of 27 replications. All sample were collected at 24 HPI on 5-week-old plants. Means followed by a letter in common indicates that they are not statistically different according to the protected least significant difference test at $P < 0.05$

at 3 HPI due to the effect of infiltration, but the expression level declined by 6 HPI to a level slightly above that at 0 HPI and then remained relatively constant as the resistance response developed. No peak in *NbMCA1* expression was observed immediately preceding or during the PCD-associated HR necrosis at 20–24 HPI. In contrast, transcripts of two arabidopsis type I metacaspases, *AtMCP1a* and *AtMCP1b*, were up-regulated at 24 HPI with an incompatible *P. syringae* strain suggesting that some metacaspase genes are induced during the activation of the HR PCD (Watanabe and Lam 2004).

Following infection with *C. destructivum*, expression of *NbMCA1*did not significantly change from 0 to 48 HPI, which is during the biotrophic phase when *C. destructivum* penetrates and grows as multilobed vesicles in the initially infected leaf epidermal cell (Chen et al. 2002). During this phase, the host cells remain alive with no visible sign of host cell degeneration. At 72 HPI, expression of *NbMCA1* significantly increased, and between 60 and 72 HPI, secondary hyphae was observed from the tips of the multilobed vesicles, penetrating adjacent cells and producing host necrosis that is clearly visible in infected cells (Shen et al. 2001). Although the necrotic lesions expanded after 72 HPI, expression of *NbMCA1* relative to the constitutive control did not increase from 72 to 96 HPI. By comparison, *LeMCA1* mRNA levels were undetectable in healthy tomato leaves but increased at 16 and 32 HPI as primary necrotic lesions formed in a susceptible interaction with the necrotrophic fungus, *B. cinerea* (Hoeberichts et al. 2003). As disease progression stalled at 48 HPI, expression of *LeMCA1* decreased, but this was followed by increases at 72 and 96 HPI as lesions expanded to cover the whole leaf (Hoeberichts et al. 2003). Therefore, expression of *LeMCA1* was consistently linked to the appearance of host cell death, unlike *NbMCA1* which showed increased expression with the beginning but not the latter stages of the necrotrophic phase of infection by *C. destructivum*.

Cloning a portion of *NbMCA1* into a PVX-based VIGS vector resulted in significantly reduced expression of *NbMCA1* in the two youngest fully developed leaves of *N. benthamiana* compared with that of the controls by 20 days after inoculation with the recombinant virus. However, this silencing had no apparent effect on the HR of such leaves to *P. syringae* pv. *tomato* DC3000. The HR is a resistance response to pathogen attack that is a relatively well characterized form of PCD in plants (del Pozo and Lam 1998; Heath 2000). Therefore, if *NbMCA1* played an important role in PCD during non-host HR resistance of *N. benthamiana* to *P. syringae* pv. *tomato* DC3000, then reducing *NbMCA1* expression by VIGS should reduce the amount of HR necrosis. Although the induction of defense mechanisms can be uncoupled from HR cell death (Lam and del Pozo 2000), the population levels of the incompatible bacteria might also be higher in the silenced plants as the inoculated tissue could be less resistant because of less effective PCD. The lack of a significant difference among *NbMCA1*-silenced plants compared with the controls in either the timing and degree of HR necrosis or the size of the bacterial populations in inoculated leaves at 24 HPI indicates that *NbMCA1* is not an important regulator of PCD during the HR of *N. benthamiana* to *P. syringae* pv. *tomato* DC3000. The discovery that PVX-mediated VIGS of two vacuolar processing proteases of *N. benthamiana*, that have limited sequence identity to caspases but have caspase activity, resulted in suppression of the HR caused by tobacco mosaic virus demonstrates that there are proteinases other than metacaspases that are important in the PCD of the HR (Hatsugai et al. 2004).

In contrast, virus-induced silencing of *NbMCA1* resulted in an increase in the susceptibility of *N. benthamiana* leaves to *C. destructivum*. In susceptible interactions, necrotrophic pathogens may induce plant cells to initiate PCD to kill themselves thus creating dead plant tissue that the pathogen can use as a source of nutrition (Dickman et al. 2001; Richael et al. 2001). If *NbMCA1* is involved in inducing PCD to create dead host tissue for use during the necrotrophic phase by *C. destructivum*, then reducing its expression by VIGS should have resulted in the plants becoming less susceptible because there would be less metacaspase available for pathogen-induced host PCD. However, this was not the case as *NbMCA1*-silenced *N. benthamiana* exhibited several times more water-soaked lesions of *C. destructivum* compared to the water and PVX controls.

In other plant–pathogen interactions, metacaspases appear to be involved in host PCD (van Baarlen et al. 2007). Mutants of five arabidopsis type II metacaspases, MCA2 to MCA6, were less susceptible to *B. cinerea* and *B. tulipae*, which are pathogenic to arabidopsis, and it was believed that those enzymes are involved in inducing PCD. Thus, the type II metacaspases of arabidopsis appear to have different functions in at least some plant–pathogen interactions compared with *NbMCA1*. *NbMCA1* may act more similarly to the type I metacaspases of arabidopsis, MCA7 and MCA8, since mutants of those metacaspases were more susceptible to infection by *Botrytis* species. One difference, however, is that the arabidopsis type I metacaspase mutants also showed accelerated senescence, which was not observed in the *NbMCA1*-silenced plants. It appears that different metacaspases may play different roles depending upon the plant–pathogen interaction.

The increased susceptibility of *NbMCA1*-silenced *N. benthamiana* to *C. destructivum* indicated that reducing the amount of this metacaspase lowered some aspect(s) of host resistance, and thus *NbMCA1* may play a role in basal resistance to *C. destructivum* rather than in pathogen-induced PCD. During infection by *C. destructivum*, *NbMCA1* may have a direct role in host defenses by affecting a virulence factor of the pathogen. Alternatively, *NbMCA1* could encode a proteinase that has an indirect role in the host response by functioning to activate and process proproteins involved in stress responses (Shimada et al. 1994), eliminate damaged proteins created by oxidative stress (Palma et al. 2002) or degrade proteins to remobilize amino acids to fuel de novo synthesis of enzymes associated with stress adaptation (Forsthoefel et al. 1998). Those roles would be more similar to that of a subgroup of human caspases that are involved in processing cytokines rather than inducing PCD, and cytokine-processing caspases have different cleavage site specificities than the caspases that induce PCD (Reed 2000). Whether it functions directly or indirectly in plant defense or pathogen-induced stress responses, *NbMCA1* has a significant role in fungal disease resistance.

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