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Role of two cysteine proteinases in the susceptible response of *Nicotiana benthamiana* to *Colletotrichum destructivum* and the hypersensitive response to *Pseudomonas syringae* pv. *tomato*

L. Hao, T. Hsiang, P.H. Goodwin *

Department of Environmental Biology, University of Guelph, Guelph, Ont. N1G 2W1, Canada

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Abstract

Two cysteine proteinase genes of the papain family, *NbCYP1* and *NbCYP2*, were amplified from cDNA of *Nicotiana benthamiana* leaves infected with the hemibiotrophic fungus *Colletotrichum destructivum*. Both genes showed peak expression corresponding with the switch from biotrophic to necrotrophic growth by *C. destructivum* at 72 h post-inoculation (HPI). For *N. benthamiana* inoculated with the incompatible bacterium, *Pseudomonas syringae* pv. *tomato*, expression of *NbCYP1* significantly decreased at 12 HPI, whereas *NbCYP2* expression increased at 3 HPI. Expression of both genes then returned to near pre-inoculation levels and remained constant as necrosis later appeared due to a non-host hypersensitive response (HR). Virus-induced gene silencing of *NbCYP1* and *NbCYP2* resulted in increased susceptibility of *N. benthamiana* to *C. destructivum* but did not affect the HR necrosis or population levels of *P. syringae* pv. *tomato*. These two cysteine proteinase genes do not appear to be involved in the programmed cell death of the HR resistance to *P. syringae* pv. *tomato*, but they are involved in limiting the host's susceptibility to *C. destructivum*.

Keywords: Anthracnose; Papain; Plant disease; Tobacco

1. Introduction

Cysteine proteinases have been reported to be involved in many processes in plants, including proprotein processing [1], protein turnover [2,3], programmed cell death (PCD) [4,5] and degradation of stress-damaged proteins to fuel de novo synthesis of enzymes associated with stress adaptations [6]. Plant stresses associated with changes in expression of cysteine proteinase genes include low or high temperatures [7], drought [8], salt [6], wounding [9,10] and diseases [10–13].

Cysteine proteinases can be grouped into 63 subfamilies belonging to six clans: C, CA, CD, CE, CF and CH [merops.sanger.ac.uk, 14]. Papain family cysteine proteinases (family C1 in the CA clan) occur ubiquitously in living organisms, and the MEROPS database lists more than 50 papain homologs in 26 plant species, making it the largest and most widely represented plant proteinase family in that database [merops.sanger.ac.uk, 15]. Some papain family cysteine proteinases from plants appear to be involved in plant–pathogen interactions. For example, a papain cysteine proteinase gene from potato, *StCathB*, was up-regulated during the interaction with either a compatible or incompatible race of *Phytophthora infestans*, and expression of a tomato papain cysteine proteinase gene, *Rcr3*, was elevated following infection with *Cladosporium fulvum* and was shown by mutant analysis to be required for *Cf2*-dependent resistance to *C. fulvum* [12,16]. These results suggest that cysteine proteinases in the papain family may be involved in both compatible and incompatible plant–pathogen interactions.

Thus far, no papain family cysteine proteinases have been described from *Nicotiana benthamiana*, which has been promoted as a model plant for fast forward genetics through the use of virus-induced silencing (VIGS) [17]. VIGS is a post-transcriptional gene silencing mechanism for sequence-specific targeting and degradation of mRNAs in plant cells, and although it appears that all plants can undergo VIGS, it is more extensive, pronounced and longer lasting in *N. benthamiana* than in other plants [18]. *N. benthamiana* is susceptible to the hemibiotrophic

* Corresponding author. Tel.: +1 519 824 4120x52754; fax: +1 519 837 0442.
E-mail address: pgoodwin@uoguelph.ca (P.H. Goodwin).

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fungal pathogen, *Colletotrichum destructivum*, resulting in anthracnose disease that is characterized by the appearance of water-soaked lesions three days after inoculation [19]. The infection process can be divided into an initial biotrophic phase followed by a necrotrophic phase. In contrast, resistance occurs when *N. benthamiana* is inoculated with *P. syringae* pv. *tomato* due to a non-host hypersensitive response (HR) [20].

To determine if cysteine proteinases play a role in plant disease, two cysteine proteinase genes of *N. benthamiana*, *NbCYP1* and *NbCYP2*, were cloned, and their expression was examined after inoculation with *C. destructivum* and *P. syringae* pv. *tomato* by relative RT-PCR [21]. The role of these two genes in disease was studied by silencing each gene with VIGS using a PVX vector [22] and then determining the response of the silenced *N. benthamiana* plants to inoculation with *C. destructivum* and *P. syringae* pv. *tomato*.

2. Materials and methods

2.1. Biological materials and pathogen inoculations

N. benthamiana plants were grown with 16 h fluorescent light (150 μmol m^{-2} s^{-1}) at 25 °C and 8 h dark at 17 °C. *N. benthamiana* seedlings at the 6th true-leaf stage were used for fungal or bacterial inoculations. *C. destructivum* N150 P3 [23] was cultured for 10 days on sodium chloride-yeast extract-sucrose agar medium (SYAS) [24] at 22 °C under continuous light. Entire plants were sprayed with a suspension of 1×10^5 conidia per mL of sterile distilled water, and then incubated at room temperature in containers to maintain high humidity. *Pseudomonas syringae* pv. *tomato* DC3000 was grown overnight in King's medium B (KB) [25] at 22 °C and suspended in 10 mM $MgCl_2$ to 10^8 cfu/mL. The bacterial suspension was infiltrated into each of the two youngest fully developed *N. benthamiana* leaves using a needle-less syringe [26].

The area of the inoculated leaves was measured with a leaf area meter (Model 3100, LI-COR, Lincoln, NE). For extraction of RNA for relative RT-PCR, leaf samples were then immediately frozen in liquid nitrogen, and stored at −80 °C. For quantifying *C. destructivum* infection, the number of lesions was counted at 60 HPI, and expressed on a per cm^2 basis. For quantifying *P. syringae* pv. *tomato* infection, leaf discs of 1 cm diameter collected at 24 HPI were ground in 1 mL of 10 mM $MgCl_2$ and then dilution-plated onto KB to determine the number of colony forming units. For both pathogens, two inoculated leaves on each of the three replicate plants per treatment were used.

2.2. RNA extraction

Total RNA was prepared following the method of Chen et al. [27], except that phenol:chloroform was added before 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.

2.3. Sequence alignments

Cysteine proteinase amino acid sequences from members of the Solanaceae were obtained from the GenBank NR database, and their names and accession numbers are listed in Table 1.

Table 1
Cysteine proteinase amino acid sequences from the papain family (family CA1) used in the sequence analyses in this study

GenBank accession		Group name	Source	Gene name
Protein	Nucleotide			
AAC49361	U31094	Aleurain	*Petunia* × hybrida	*PeTh3*
AAD29084	AF082181	Glycinain	*Solanum melongena*	*SmCP*
AAD48496	AF172856	Brassicain	*Lycopersicon esculentum*	*TDI-65*
AAD56028	AF181567	Brassicain	*Solanum chacoense*	*CYP1*
AAF63517	AF242733	Aleurain	*Capsicum annuum*	*S1-35*
AAM00234	AF359422	Cathepsin B	*Nicotiana tabacum*	Cathepsin B like
AAM19207	AF493232	Rcr3 peptidase	*Lycopersicon pimpinellifolium*	*Rcr3*
AAM19208	AF493233	Rcr3 peptidase	*L. esculentum*	*Rcr3pen*
AAM19209	AF493234	Rcr3 peptidase	*L. esculentum*	*Rcr3esc*
BAA96501	AB032168	Aleurain	*N. tabacum*	*NTCP-23*
CAA57522	X81995	Cathepsin B	*Nicotiana rustica*	Cathepsin B like
CAA78361	Z13959	Glycinain	*N. tabacum*	*NTCP-7*
CAA78365	Z13964	Glycinain	*N. tabacum*	*NTCP-8*
CAA78403	Z14028	Glycinain	*L. esculentum*	*LCYP-2*
CAA88629	Z48736	Aleurain	*L. esculentum*	*Cyp-3*
CAB16317	Z99173	Brassicain	*N. tabacum*	*TCPR1*
CAB44983	AJ242994	Glycinain	*N. tabacum*	*cpr2*
CAB53515	AJ245924	Brassicain	*Solanum tuberosum*	*CYP*
P20721	M21444	Brassicain	*L. esculentum*	*CYSL LYCES*
S60479	X81995	Cathepsin B	*N. rustica*	Cathepsin B like
DQ084022	DQ084022	Aleurain	*Nicotiana benthamiana*	*NbCYP1*
DQ084023	DQ084023	Glycinain	*N. benthamiana*	*NbCYP2*
NP071967	NM022522	Caspase	*Rattus norvegicus*	Caspase 2

The sequences were aligned with CLUSTALX [28] using default parameters to identify related genes and locate conserved regions for designing the primers described below. A dendrogram was created that also included the predicted protein sequences of *NbCYP1* and *NbCYP2* derived from the sequences of their unigenes determined in this study, as well as a rat cysteine proteinase from the GenBank NR database that served as an outgroup (Table 1). For the dendrogram, the CLUSTALX alignment was subjected to distance analysis using the PHYLIP programs SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE [29] with the neighbor-joining algorithm and 1000 bootstrap replications.

2.4. 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) 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). RT-PCRs were done in 15 μL reaction volumes with 2 μL cDNA, 0.75 units *Tsg* polymerase (Biobasic, Toronto, ON), 10× *Tsg* polymerase buffer, 2 mM dNTPs, and 2.5 mM Mg^{2+}, with 1.0 mM of each primer. Primers were designed using the program GeneRunner (Hastings Software, Hastings, NY).

For the amplification of *NbCYP1*, an alignment of three closely related cysteine proteinase nucleotide sequences, GenBank accessions Z48736, U31094 and AB032168 (Table 1), was made with CLUSTALX [28] using default parameters to identify conserved regions for the design of the forward primer, CYP4F2 5′-GCTTGTGGACTGTGCTGGAG. Primer CYP4F2 was paired with the 3′-RACE PCR anchor primer, 5′-GACCACGCGTATCGATGTCGAC, supplied in a 5′/3′-RACE kit (Roche, Indianapolis, IN). RACE cDNA synthesis was done using an oligo (dT)-anchor primer and Superscript II reverse transcription enzyme (Invitrogen, Burlington, ON). DNA amplification was done at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min and a final extension period of 10 min at 72 °C. The RT-PCR fragment of *NbCYP1* obtained with the CYP4F2 primer and the PCR anchor primer was purified using GENECLEAN II (Q-BIOgene, Montreal, QUE) and cloned into pGEMT-easy (MBI Fermentas, Burlington, ON) for subsequent sequencing at the Laboratory Services Division, University of Guelph, Guelph, ON.

For the amplification of *NbCYP2*, a forward primer, CYP5F2, 5′- CGGACTTACCTTGGACTTCA, was designed based on the sequence of EST #160 that was previously obtained from a suppression subtraction cDNA library of *N. benthamiana* inoculated with C. *orbiculare* at 72 HPI (Goodwin, unpublished). This was paired with a reverse primer, CYP5R2, 5′-TCACAGTAGAGACCATGGCA, which was designed in a conserved region identified following an alignment of the sequence of EST#160 with five closely related cysteine proteinase genes, GenBank accessions AJ242994, Z13964, Z13959, Z14028 and AF082181 (Table 1). The sequences were aligned with CLUSTALX [28] using default parameters. PCR conditions were 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min and a final extension period of 10 min at 72 °C. The RT-PCR fragment of *NbCYP2* obtained with CYP5F2 and CYP5R2 primers was purified using GENECLEAN II (Q-BIOgene), cloned into pGEMT-easy (MBI Fermentas) and sequenced (Laboratory Services Division).

2.5. Relative RT-PCR

Relative RT-PCR was done following Dean et al. [21] in 15 μL reaction volumes with 2 μL cDNA, 0.75 units *Tsg* polymerase (Biobasic), 10× *Tsg* polymerase buffer, 2 mM dNTPs, and 2.5 mM Mg^{2+}, with 0.5 mM translation elongation factor 1α (EF-1α) primer pair and 1.0 mM of each cysteine proteinase primer described below. The translation elongation factor 1α, which catalyzes the first step of the elongation stage of protein synthesis, has been shown to be expressed constitutively in leaves of *N. benthamiana* after inoculation with *C. destructivum* [21].

Forward primer, TobefS 5′-CAAGTATGCCTGGGTGCT and reverse primer, TobefA 5′-CTTGGTGGTTGCATCTCAAC were designed by Dean et al. [21] to amplify an RT-PCR product of 700 bp to serve as an internal constitutive control for comparison of gel band intensity.

For relative RT-PCR of *NbCYP1*, forward primer CYP4F3, 5′- GCGGATCTTGCTGGACATTC, which was 90 bp upstream from CYP4F2, was designed from a conserved region among three cysteine proteinase genes, GenBank accessions Z48736, U31094 and AB032168. Primer CYP4F3 was paired with the reverse primer, CYP4R1 5′-GGGAGTATTGCCACATTCGG, which was designed based on the sequence of the cysteine proteinase gene amplified by RT-PCR with primer CYP4F2 and the PCR anchor primer from *N. benthamiana* at 96 HPI with *C. destructivum*. Primer pair, CYP4F3 and CYP4R1, gave a RT-PCR product of 391 bp.

For relative RT-PCR of *NbCYP2*, forward primer, CYP5F1, 5′-CGAAACCGAAACCGACGA, which is 225 bp upstream of CYP5F2, and reverse primer, CYP5R1, 5′- GTCACGCCAGTCATAATCAG, which is 640 bp upstream of CYP5R2, were designed based on the sequence of the EST #160. The primer pair gave a RT-PCR product of 310 bp.

Relative RT-PCR was done at 94 °C for 3 min followed by 25 cycles of 94 °C for 30 s, 60 °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% Tris–acetate/EDTA buffer (TAE) agarose gels, and the images were saved as TIF electronic image files for quantification using NIH image (Scion Corporation, Frederick, MD). The band intensities were determined for both the cysteine proteinase and elongation factor 1α RT-PCR products, and compared for each gel lane. The relative expression of the cysteine proteinase genes was determined by taking a ratio of the band intensity of cysteine proteinase over the band intensity of the elongation factor 1α. To check that the end point number of amplification cycles was

not too large for quantification, the cDNA samples were also amplified with five fewer cycles, and the quantification results were compared to demonstrate that the quantification was not significantly different. Four plants were assessed at each time point following inoculation with *C. destructivum*. To confirm the identity of the RT-PCR products, the bands of the *NbCYP1* and *NbCYP2* RT-PCR products 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).

2.6. Gene silencing

The cloned RT-PCR fragment of *NbCYP1* obtained with primers, CYP4F2 and 3′-RACE anchor primer, and the cloned RT-PCR fragment of *NbCYP2* obtained with primers, CYP5F2 and CYP5R2, were digested from pGEMT-easy using *Not*I, and subcloned into pGR106 [22], which was also digested with *Not*I. The pGR106 construct was transformed into *E. coli* DH5α and selected on LB agar containing 50 mg L^{-1} kanamycin. The plasmid was extracted from *E. coli* and then transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and selected on LB agar containing 50 mg L^{-1} kanamycin and 5 mg L^{-1} tetracycline. The antibiotic resistant GV3101 colonies were checked for inserts by PCR. Transformed *A. tumefaciens* was grown at 28 °C on LB agar containing kanamycin and tetracycline for 48 h before being used to inoculate the plants.

A. tumefaciens inoculations were done following Thomas et al. [30]. A toothpick was used to inoculate *A. tumefaciens* at six sites per leaf along the main veins of the two largest leaves of *N. benthamiana* at the 4th true-leaf stage. The *A. tumefaciens* cultures contained either PVX with a fragment of *NbCYP1* (PVX-*NbCYP1*) or *NbCYP2* (PVX-*NbCYP2*) or without any insert as an empty PVX vector control. An additional control consisted of inoculation with water only, as a control for the *A. tumefaciens* inoculation. At 20 days after *A. tumefaciens* inoculation, the plants were sprayed either with a spore suspension (1×10^5 conidia/mL) of *C. destructivum* or infiltrated with a 10^8 cfu/mL suspension of *P. syringae* pv. *tomato*. Relative RT-PCR analysis of gene silencing was done prior to these inoculations and at 48 HPI for *C. destructivum* or 24 HPI for *P. syringae* pv. *tomato*. Four plants were assessed per treatment for disease and gene expression.

3. Results

3.1. NbCYP1 and NbCYP2 amplification and sequence analyses

An alignment of the nucleotide sequences of three closely related cysteine proteinases, *NTCP-23*, *Cyp-3* and *PeTh3* (Table 1), from plants in the Solanaceae revealed conserved regions for designing a primer that was paired with a 3′-RACE PCR anchor primer to amplify a single RT-PCR product of 750 bp from cDNA obtained from leaves of *N. benthamiana* at 96 HPI with *C. destructivum*. Cloning and subsequent sequence analysis of five of the clones revealed only one unique sequence, which was most similar at 98% identity to *NTCP-23* (Table 1). A search of the TIGR *N. benthamiana* Gene Index (http://www.tigr.org), which is based on 26,994 input sequences, showed that TC7311, composed of 20 overlapping ESTs, had an identical overlapping region with the 750 bp RT-PCR product. A unigene was generated from the sequences and designated *NbCYP1* (GenBank accession no. DQ084022).

EST #160 was from a suppression subtraction cDNA library of *N. benthamiana* at 72 HPI with C. *orbiculare* that had previously been constructed in the laboratory. EST#160 was 502 bp and included the putative start codon. It had a predicted amino acid sequence that was most similar at 98% identity to *cpr2* (Table 1) from *N. tabacum*. A primer designed from EST#160 was paired with a reverse primer, which was designed in a conserved region shared between *cpr2* and closely related sequences, *NTCP-8*, *LCYP-2* and *SmCP* (Table 1), to yield a RT-PCR product of 716 bp from cDNA obtained from leaves of *N. benthamiana* at 96 HPI with *C. destructivum*. Cloning and subsequent sequence analysis of two of the clones revealed only one sequence with the overlapping regions having 100% identity with EST#160. A comparison of those sequences with the TIGR *N. benthamiana* Gene Index revealed 100% identity in the overlapping regions with TC7406, which is compiled from 10 ESTs. A unigene of 1372 bp was created from these sequences and was designated *NbCYP2* (GenBank accession no. DQ084023).

A comparison was made of 20 amino acid sequences from plants in the Solanaceae comprised of cysteine proteinases from the papain family (clan CA) that were available in the GenBank NR database, as well as a rat cysteine proteinase that was included as an outgroup (Table 1). Five clusters were observed

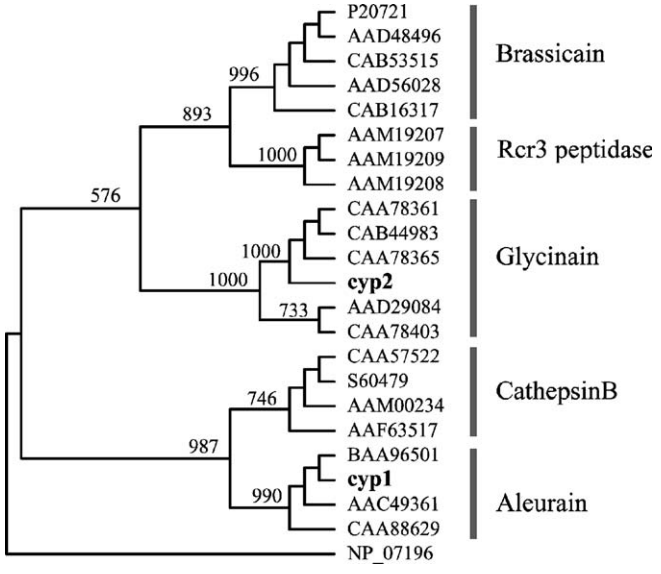

Fig. 1. Comparison of papain family cysteine proteinase sequences from Solanaceae. *Rattus norvegicus* caspase 2 (NP_071967) was included as an outgroup. Amino acid sequences were aligned using CLUSTALX, and then subjected to bootstrap in the distance analysis using the neighbor-joining algorithm as implemented in PHYLIP. The bootstrap values represent that number of times out of 1000 that the branch was supported by bootstrap analysis, and only bootstraps values for major branches are shown.

in the dendrogram, all having between 88% and 100% bootstrap support (Fig. 1). These five clusters correspond to aleurains, brassicains, cathepsins, glycinains, and Rcr3 peptidases based on their comparison to the papain family cysteine proteinases in the MEROPS peptidase (http://merops.sanger.ac.uk/). Phylogenetic analysis of *NbCYP1* with these other cysteine proteinases showed that it clustered with members of the aleurain group with 99% bootstrap support, and *NbCYP2* clustered with members of the glycinain group with 100% bootstrap support (Fig. 1).

3.2. Expression of NbCYP1 and NbCYP2 in N. benthamiana following inoculation with C. destructivum and P. syringae pv. tomato

The expression patterns of both *NbCYP1* and *NbCYP2* were very similar during the course of infection by *C. destructivum* (Fig. 2). Expression declined slightly by 24 HPI, but there was no significant change from pre-inoculation levels (0 HPI) until 72 HPI, when there was a significant peak in expression. Both genes then showed a decline in expression at 96 HPI (Fig. 2). Necrotic lesions were first observed between 60 and 72 HPI which then proceeded to expand in diameter.

Expression of *NbCYP1* and *NbCYP2* differed following inoculation with *P. syringae* pv. *tomato* (Fig. 3). The relative expression level of *NbCYP1* fluctuated slightly during the first 12 h following inoculation, and the relative expression was not significantly different from its pre-inoculation level by 24 HPI. For *NbCYP2*, there was a small, but significant, increase in relative expression at 3 HPI, but then remained statistically unchanged up to 24 HPI. The inoculated leaves appeared wilted around 3 HPI but then recovered, and HR-related necrosis was visible around 20–24 HPI.

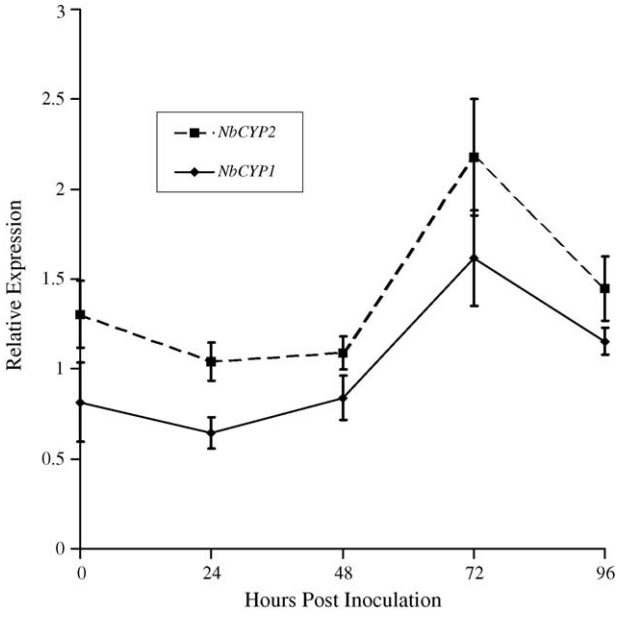

Fig. 2. Relative RT-PCR of *NbCYP1* and *NbCYP2* expression in *N. benthamiana* leaves inoculated with *C. destructivum*. The quantity of *NbCYP1* and *NbCYP2* mRNA was determined relative to the amount expression of *NbEF-1α*. Means are shown with standard error bars calculated from three or four replications.

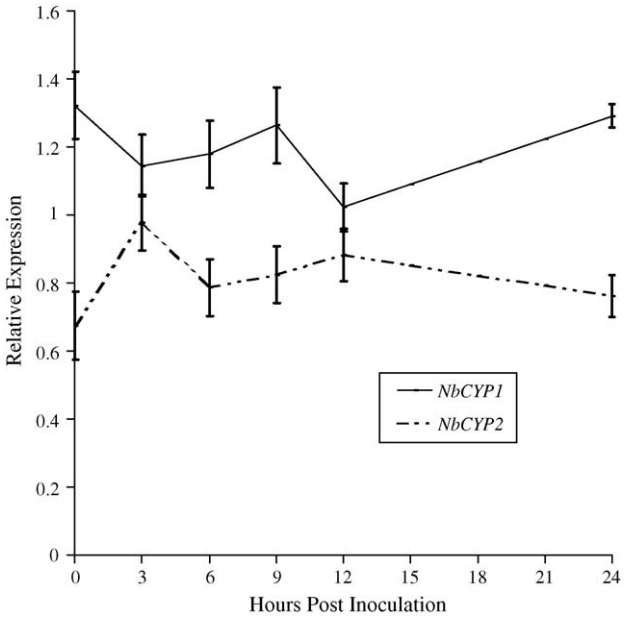

Fig. 3. Relative RT-PCR of *NbCYP1* and *NbCYP2* expression in *N. benthamiana* inoculated with *P. syringae* pv. *tomato*. The quantity of *NbCYP1* and *NbCYP2* mRNA was determined relative to the amount expression of *NbEF-1α*. Means are shown with standard error bars calculated from three replications.

3.3. Silencing NbCYP1 in N. benthamiana and the effect on the interactions with C. destructivum and P. syringae pv. tomato

Both prior and following inoculation with *C. destructivum*, the *NbCYP1* RT-PCR product relative to that of the control EF-1α product was significantly less in *NbCYP1*-silenced plants compared to that of the water and PVX vector controls (Fig. 4).

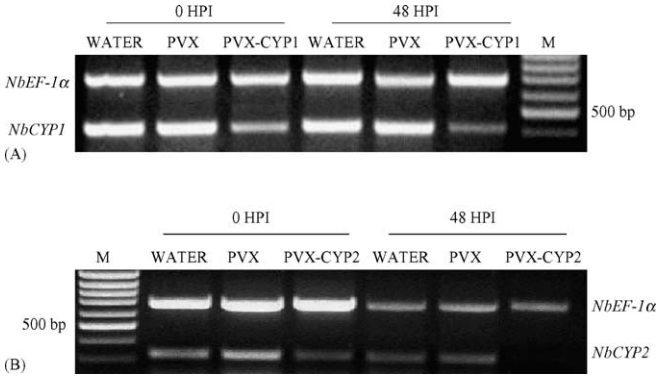

Fig. 4. RT-PCR of *NbCYP1* and *NbCYP2* expression in *N. benthamiana* relative to that of *NbEF-1α*. Relative RT-PCR gel of *NbCYP1* (A) and *NbCYP2* (B) co-amplified with *NbEF-1α* at pre-inoculation (0 HPI) and approximately the time of conversion from biotrophy to necrotrophy (48 HPI) by *C. destructivum*. Lanes are: WATER, which is the water control wounded by toothpick without *A. tumefaciens* inoculum, PVX, PVX-CYP1 and PVX-CYP2, which are the virus vector control wounded by toothpick with *A. tumefaciens* inoculum containing potato virus X (PVX) in a Ti plasmid without an insert or else with a fragment of *NbCYP1* or a fragment of *NbCYP2*, respectively. Lane M is the 100 bp ladder.

Table 2
Relative RT-PCR analysis of *NbCYP1*, *NbCYP2* and *NbMCA1* expression in water control, PVX vector control, *NbCYP1*-silenced (A) and *NbCYP2*-silenced (B) *N. benthamiana* prior and post-inoculation with *C. destructivum* or *P. syringae* pv. *tomato*

Treatment[a]	Relative expression[b]								
	Pre-inoculation			*C. destructivum*			*P. syringae* pv. *tomato*		
	NbCYP1	*NbCYP2*	*NbMCA1*	*NbCYP1*	*NbCYP2*	*NbMCA1*	*NbCYP1*	*NbCYP2*	*NbMCA1*
(A)									
Water control	1.16 a	1.09 a	0.54 a	1.02 a	1.06 a	0.67 a	0.99 a	1.15 a	0.57 a
PVX vector control	1.14 a	1.03 a	0.49 a	1.45 a	1.14 a	0.58 a	1.10 a	1.17 a	0.48 a
NbCYP1-silenced	0.69 b	1.06 a	0.54 a	0.37 b	1.11 a	0.53 a	0.29 b	1.20 a	0.46 a
(B)									
Water control	0.93 a	0.76 a	0.60 a	0.71 a	0.83 a	0.52 a	1.15 a	0.67 a	0.46 a
PVX vector control	0.87 a	0.71 a	0.57 a	0.78 a	0.90 a	0.47 a	1.08 a	0.68 a	0.64 a
NbCYP2-silenced	0.93 a	0.21 b	0.57 a	0.82 a	0.21 b	0.53 a	1.13 a	0.12 b	0.66 a

Means in the same column followed by the same letter are not significantly different according to the protected L.S.D. test at $p < 0.05$.

[a] Silenced and PVX vector control plants were toothpick inoculated with *A. tumefaciens* inoculum containing potato virus X (PVX) in a Ti plasmid with or without a fragment of *NbCYP1* or *NbCYP2*. Water controls were wounded by toothpick without *A. tumefaciens* inoculum.

[b] Gene expression in water control, PVX vector control or silenced *N. benthamiana* was determined by relative RT-PCR prior to inoculation and following infection at the time of the conversion from biotrophy to necrotrophy with *C. destructivum* N150 and around the time of necrosis with *P. syringae* pv. *tomato* DC3000. Each measurement represents the mean relative expression from at least three experiments.

Quantification of the bands showed that *NbCYP1* expression was significantly lower before and after inoculation with *C. destructivum* or *P. syringae* pv. *tomato* DC3000 in *NbCYP1*-silenced plants than in the water and PVX vector controls (Table 2). Silencing appeared to be relatively specific as there was no significant difference in the expression of *NbCYP2* or a more distantly related cysteine proteinase of *N. benthamiana*, *NbMCA1* (GenBank accession no. DQ084024), in *NbCYP1*-silenced plants compared to the controls (Table 2). A sequence comparison of the region of *NbCYP1* in the silencing vector with the sequences of *NbCYP2* and *NbMCA1* showed no contiguous regions of 21 nt or longer with 100% identity, and the overall nucleotide identity in silenced region was 51% between *NbCYP1* and *NbCYP2* and 41% between *NbCYP1* and *NbMCA1*.

A significantly greater number of water-soaked lesions was observed on leaves of the *NbCYP1*-silenced plants after inoculation with *C. destructivum* compared to the water and PVX vector controls (Table 3). Although there were more lesions on the PVX vector control than the water control, the difference was not significant (Table 3). There were no differences in the appearance of the lesions among the plants with the different treatments.

After inoculation with *P. syringae* pv. *tomato*, there was no significant difference among the plants in the timing or the appearance of the HR necrosis. Bacterial populations in inoculated leaves at 24 HPI were not significantly different between the water control, PVX vector control and *NbCYP1*-silenced treatments (Table 4).

3.4. Silencing NbCYP2 in N. benthamiana and the effect on the interactions with C. destructivum and P. syringae pv. tomato

Compared to water and PVX vector controls before and after inoculation with *C. destructivum*, the RT-PCR product of *NbCYP2* was greatly reduced in *NbCYP2*-silenced plants (Fig. 4). Quantification of the bands showed that relative

Table 3
Lesion number in water control, PVX vector control, *NbCYP1*-silenced or *NbCYP2*-silenced *N. benthamiana* at 60 HPI with *C. destructivum*

Treatment[a]	Lesions per square centimeter[b]
Water control	0.31 a
PVX vector control	1.28 a
NbCYP1-silenced	4.31 b
NbCYP2-silenced	3.30 b

Means in the same column followed by the same letter are not significantly different according to the protected L.S.D. test at $p < 0.05$.

[a] *N. benthamiana* plants were inoculated with *A. tumefaciens* containing PVX in a Ti plasmid without an insert (PVX vector), inoculated with a fragment of *NbCYP1* or *NbCYP2* (*NbCYP1*- or *NbCYP2*-silenced) or wounded by toothpick without any *A. tumefaciens* (water control).

[b] 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.

Table 4
Bacterial populations in water control, PVX vector control, *NbCYP1*-silenced or *NbCYP2*-silenced *N. benthamiana* at 24 HPI with *P. syringae* pv. *tomato*

Treatment[a]	CFU per square centimeter[b]
Water control	6.98×10^8 a
PVX vector control	5.92×10^8 a
NbCYP1-silenced	7.68×10^8 a
NbCYP2-silenced	9.23×10^8 a

Means in the same column followed by the same letter are not significantly different according to the protected L.S.D. test at $p < 0.05$.

[a] *N. benthamiana* plants were inoculated with *A. tumefaciens* containing PVX in a Ti plasmid without an insert (PVX vector), inoculated with a fragment of *NbCYP1* or *NbCYP2* (*NbCYP1*-or *NbCYP2*-silenced) or wounded with a toothpick without any *A. tumefaciens* (water control).

[b] Colony forming units (CFU) per square centimeter were means pooled from two separate experiments with a total of 27 replications. All samples were collected at 24 HPI on 5-week-old plants.

NbCYP2 expression was significantly lower in *NbCYP2*-silenced plants than in the water and PVX vector controls both pre- and post-inoculation with *C. destructivum* or *P. syringae* pv. *tomato* (Table 2). Silencing appeared to be specific as there was no significant difference in the expression of *NbCYP1* and *NbMCA1* in *NbCYP2*-silenced plants compared to the controls (Table 2). A comparison of the nucleotide identity between the silenced regions of *NbCYP2* and *NbCYP1* was described above, and the total nucleotide identity between the silenced regions of *NbCYP2* and *NbMCA1* was 41% without any contiguous regions of 21 nt or longer having 100% identity.

After inoculation with *C. destructivum*, there were significantly greater numbers of water-soaked lesions on leaves of the *NbCYP2*-silenced plants compared to the water and PVX vector control plants. Although there were more lesions on the PVX vector control than the water control, this difference was not significant (Table 3). There were no differences in the appearance of the lesions among the different treatments.

The populations of *P. syringae* pv. *tomato* at 24 HPI were not significantly different between the water control, PVX vector control and *NbCYP2*-silenced plants (Table 4). There was also no significant difference among the plants in the timing of the occurrence of necrosis or in the appearance of the hypersensitive response.

4. Discussion

Two novel cysteine proteinase genes, *NbCYP1* and *NbCYP2*, were cloned from cDNA of *N. benthamiana* leaves, and both showed altered expression during a susceptible interaction with *C. destructivum* and a non-host resistant interaction with *P. syringae* pv. *tomato*. Leaf cell death occurs both during invasion by *C. destructivum* and the HR to *P. syringae* pv. *tomato*, but *NbCYP1* and *NbCYP2* expression did not show the same patterns over the course of these two interactions, demonstrating that there are differences during the progression of plant cell death between susceptible and resistant reactions.

Following inoculation with *C. destructivum*, expression of both *NbCYP1* and *NbCYP2* remained at or slightly below that of non-inoculated leaves up to 48 HPI. By 24 HPI, conidial germination and appressorial formation has occurred, and then up to approximately 48 HPI, multi-lobed infection vesicles grow in the initially infected cell, constituting the biotrophic phase of infection [19,23]. The necrotrophic phase of infection began between 48 and 72 HPI when secondary hyphae developed from the tips of the multi-lobed vesicles that then penetrated the host cell wall [19]. The start of the necrotrophic phase corresponded with an increase in *NbCYP1* and *NbCYP2* expression from 48 to 72 HPI. This expression pattern implies that increased *NbCYP1* and *NbCYP2* expression was associated with the initial development of host necrosis. However, as expression of the two genes declined at 96 HPI, it appears that they are not associated with the continuation of necrosis during lesion expansion. The decline in expression of *NbCYP1* and *NbCYP2* at 96 HPI is not due to loss of mRNA in the leaves caused by necrosis as expression in relative RT-PCR is taken relative to that measured for a constitutively expressed translation elongation factor 1α gene [21].

Changes in *NbCYP1* and *NbCYP2* expression following *P. syringae* pv. *tomato* inoculation were much less than that observed with *C. destructivum*. *P. syringae* pv. *tomato* DC3000 is a pathogen of Arabidopsis and tomato [31] and causes a non-host HR in *N. benthamiana* [20]. Following infiltration of *N. benthamiana* leaves with *P. syringae* pv. *tomato*, *NbCYP1* and *NbCYP2* expression fluctuated, but they were generally similar to pre-inoculation levels and never showed the level of change in expression observed in the *C. destructivum* interaction.

NbCYP1 is in the aleurain group of cysteine proteinases and is most similar to *NTCP-23* from *N. tabacum*, which was also down-regulated during the HR due to TMV, but mRNA of *NTCP-23* continued to decrease to reach its lowest level during the formation of HR necrosis [10]. *NTCP-23* was also down-regulated by wounding but was up-regulated by natural leaf senescence [10]. *NTCP-23* has been suggested to be localized to the vacuole and to function in nitrogen (amino acid) remobilization [10]. Another related gene in the aleurain group is *S1-35* from *Capsicum annuum*, which showed increased expression during TMV-induced HR [13].

NbCYP2 is in the glycinain group of cysteine proteinases and is most similar to a drought inducible cysteine proteinase gene from *N. tabacum*, *cpr2* [8]. However, *cpr2* has thus far not been studied in relation to any plant diseases. Two other related genes in the glycinain group, *NTCP-7* and *NTCP-8*, from *N. tabacum* were up-regulated by wounding but not by the HR to TMV [9]. *NTCP-7* and *NTCP-8* were hypothesized to function in the degradation of intracellular proteins, which are no longer capable of or necessary for the correct functioning of the cellular processes [9].

The roles of *NbCYP1* and *NbCYP2* in the susceptible response to *C. destructivum* and resistance response to *P. syringae* pv. *tomato* were examined by silencing these gene using PVX-mediated VIGS. VIGS provided significant advantages in studying the function of these genes, since cysteine proteinases are important during plant development [6,32], and thus VIGS allowed for the plants to be grown in an unaltered state prior to silencing in order to avoid unintended effects on development. PVX-mediated VIGS was found to be an effective means of silencing *NbCYP1* and *NbCYP2*. Because VIGS can affect genes with 80% or higher identity [17], closely related genes may have been affected. However, silencing of *NbCYP1* or *NbCYP2* did not have a significant effect on the expression of the other gene as determined by relative RT-PCR, nor did their silencing affect expression of a more distantly related cysteine proteinase of *N. benthamiana*, *NbMCA1*.

In susceptible plant–pathogen interactions, necrotrophic pathogens have been hypothesized to induce PCD in order to control plant cell death so that the pathogen can obtain adequate nutrients to multiply and spread [33]. Cysteine proteinases have been demonstrated to be involved in PCD, where they can selectively degrade and eliminate damaged or denatured proteins [5]. If PCD has a role in susceptible plant–pathogen interactions, then suppression of cysteine proteinases involved in PCD by VIGS should result in decreased or delayed plant cell

necrosis (i.e., reduced susceptibility). However, lowering the expression of *NbCYP1* and *NbCYP2* by VIGS resulted in *N. benthamiana* being more, rather than less, susceptible to *C. destructivum*, which is not consistent with *NbCYP1* and *NbCYP2* functioning in PCD during necrotrophy. Also, the timing of lesion development and the appearance of the lesions were not significantly altered by silencing *NbCYP1* and *NbCYP2*, which is consistent with them having a role in limiting infection rather than in the progression of necrotrophy. It thus appears that *NbCYP1* and *NbCYP2* contributes in some way to executing defenses against *C. destructivum*.

In resistant plant–pathogen interactions, the HR has clearly been established as a form of PCD [34]. Although expression of *NTCP-23* in tobacco was suppressed during the HR to TMV [10], many other papain family cysteine proteinases are induced during the HR. This includes *cyp* in potato [11], *S1-35* in pepper [13] and *StCathB* in potato [16]. In addition, expression of cystatin, an inhibitor of papain family cysteine proteinases, suppressed cell death from a non-host HR in tobacco due to avirulent *P. syringae* pv. *maculicola* [35] and blocked HR cell death from avirulent *P. syringae* pv. *glycinea* in soybean [5]. However, there was no major induction of either *NbCYP1* or *NbCYP2* during the non-host HR to *P. syringae* pv. *tomato*, and silencing their expression did not reduce or abolish the HR cell death. These results do not support a role for *NbCYP1* and *NbCYP2* during the HR to *P. syringae* pv. *tomato*.

Members of the papain family of cysteine proteinases in plants have been postulated to have dual functions, either specialized in PCD or acting more generally in protein degradation [15]. Some papain-type cysteine proteinases function in routine intracellular protein turnover [36], processing cell wall proteins in developing seeds [4], accelerating remobilization of nitrogen, carbon, and minerals to actively growing areas [37] and degrading and mobilizing storage proteins during germination [38]. During stress, papain-type cysteine proteinases may also act to selectively degrade and eliminate damaged or denatured proteins that have been modified by free radicals or other oxidants [3], and degrade proteins to provide materials for de novo synthesis of enzymes associated with short or long term stress adaptations [2]. Therefore, *NbCYP1* and *NbCYP2* may function in resistance responses by a variety of means, such as processing a pro-protein required for resistance, remobilizing nitrogen, carbon and minerals for enzymes and other compounds needed by resistance mechanisms, or eliminating pathogen-damaged plant proteins.

For plant diseases, van der Hoorn and Jones [39] proposed that plant proteinases may function in pathogen perception, signal transduction or execution of plant defenses. The authors noted that since papain family proteinases accumulate at high levels around sites of infection, they are good candidates for executing defense responses. Certain cysteine proteinases are believed to have a role in defense execution by helping to degrade pathogen virulence molecules and activate defensive enzymes from pro-proteins [39]. Other cysteine proteinases, like Rcr3 of tomato, may have direct antimicrobial activities for basal pathogen defense, but Rcr3 is also a target for the Avr2 protein of *C. fulvum*, which inhibits it causing a conformational change in Rcr3 that can trigger an HR if the resistance gene protein, Cf-2, is present [40]. Perhaps the enzymes encoded by *NbCYP1* and *NbCYP2* have one of these functions.

In conclusion, two papain family cysteine proteinase genes of *N. benthamiana*, *NbCYP1* and *NbCYP2*, have been found to respond significantly in expression and play functional roles in the susceptible response to *C. destructivum* but not in the resistant response to *P. syringae* pv. *tomato*. Both genes can limit the amount of disease in a susceptible interaction, indicating that the gene products of *NbCYP1* and *NbCYP2* have direct or indirect roles in host defense responses to *C. destructivum*.

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