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The involvement of two epoxide hydrolase genes, *NbEH1.1* and *NbEH1.2*, of *Nicotiana benthamiana* in the interaction with *Colletotrichum destructivum*, *Colletotrichum orbiculare* or *Pseudomonas syringae* pv. *tabaci*

C. P. Wijekoon[A], *P. H. Goodwin*[A,B] *and T. Hsiang*[A]

[A]Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada.
[B]Corresponding author. Email: pgoodwin@uoguelph.ca

Abstract. Epoxide hydrolase hydrates epoxides to vicinal diols in the phyto-oxylipin peroxygenase pathway resulting in the production of epoxy alcohols, dihydrodiols, triols and epoxides, including many lipid epoxides associated with resistance. Two epoxide hydrolase genes from *Nicotiana benthamiana* L., *NbEH1.1* and *NbEH1.2*, were amplified from coding DNA of leaves during a susceptible response to the hemibiotrophic pathogens, *Colletotrichum destructivum* O'Gara, *Colletotrichum orbiculare* Berk. and Mont. von Arx. or *Pseudomonas syringae* pv. *tabaci* Wolf and Foster, or the hypersensitive resistance response to *P. syringae* pv. *tabaci* expressing *avrPto*. Increases in expression of *NbEH1.1* generally occurred during the late biotrophic and necrotrophic stages in the susceptible responses and before the hypersensitive response. *NbEH1.2* expression was not significantly induced by *C. orbiculare* but was induced by *C. destructivum*, *P. syringae* pv. *tabaci* and *P. syringae* pv. *tabaci* expressing *avrPto*, although to a lesser degree than *NbEH1.1*. Virus-induced gene silencing of *NbEH1.1* delayed the appearance of lesions for *C. destructivum*, reduced populations of *P. syringae* pv. *tabaci* and increased populations of *P. syringae* pv. *tabaci* expressing *avrPto*. The importance of epoxide hydrolase during pathogen attack may be related to its roles in detoxification, signalling, or metabolism of antimicrobial compounds.

Additional keywords: hemibiotrophy, hypersensitive response, oxylipin, virus-induced gene silencing.

Introduction

In plants, phyto-oxylipins are synthesised by the lipoxygenase pathway, where oxygen is incorporated into the fatty acids, linoleic or linolenic acid, to create hydroperoxides (Blee 1998, 2002). These hydroperoxides can enter the hydroxyperoxide lyase pathway to produce traumatin (a wound hormone), allene oxide synthase pathway to produce jasmonic acid (a signalling molecule) or peroxygenase pathway to produce epoxides, epoxy alcohols, dihydrodiols and triols. Epoxide hydrolase (EH) is the second enzyme in the peroxygenase pathway hydrating epoxides to vicinal diols. EHs are present in all organisms and belong to a sub-category of hydrolytic enzymes that include esterases, proteases, dehalogenases and lipases (Beetham *et al*. 1995).

In animals, the products of EH have many different activities, such as regulation of inflammation, xenobiotic detoxification and drug metabolism (Newman *et al.* 2005). Plant EHs appear to play a role in the biosynthesis of monomers of cutin, a polymer that accumulates in cell walls of damaged tissues (Blee 1998). Cutin is important in plant development, protection from environmental stresses and disease resistance. Plant EHs may also contribute to disease resistance by producing oxylipin phytoalexins (Ohta *et al*. 1991; Hamberg and Hamberg 1996). Phytoalexins are pathogen-induced low-molecular-weight antimicrobial compounds often associated with the hypersensitive response (HR) and other defences (Hammerschmidt 1999). Plant oxylipins have antimicrobial activity against a wide range of plant pathogenic bacteria and fungi (Prost *et al*. 2005). In addition, epoxides are usually unstable and reactive causing toxicity to cells, and thus another role for EHs in plants would be to break down epoxides accumulating during stress into more stable and less reactive compounds (Murray *et al*. 1993).

NtEH-1, an EH gene from *Nicotiana tabacum* L., has been the subject of several studies. Its induction was associated with HR to tobacco mosaic virus (TMV), and it was believed to be involved in affecting signalling of resistance or detoxifying compounds produced during the oxidative burst of the HR (Guo *et al*. 1998). For tobacco inoculated with different bacteria, increased *NtEH-1* expression was associated with the HR as well as basal resistance, which is not associated with the HR (Szatmari *et al*. 2006). Increased *NtEH-1* expression, along with that of several other genes, was also associated with stress resistance produced by sublethal doses of the redox-active compound, methyl viologen, and EH activity was presumed to be involved in detoxification (Vranová *et al*. 2002). *NtEH-1* was also among a group of genes induced in BY-2 cell cultures going into the stationary growth phase, and the enzyme was hypothesised to be involved in the response to extracellular signals (Matsuoka *et al*. 2004). Although these studies indicate that *NtEH-1* may be involved in detoxification, signalling, HR

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and basal disease resistance, these conclusions have thus far been based only on examinations of *NtEH-1* expression.

A close relative of *N. tabacum* is *Nicotiana benthamiana* L., which has been proposed to be a model plant for studying gene function by virus-induced silencing (VIGS) (Baulcombe 1999, 2004). VIGS can silence genes in mature plants in 10–20 days after inoculation with a recombinant virus, does not require stable plant transformation, can silence one or more genes at a time, and does not need a full length clone of the targeted gene to be silenced (Robertson 2004). Examples of the use of VIGS in the study of genes associated with diseases of *N. benthamiana* include examinations of N-mediated signalling resistance to TMV (Liu *et al*. 2002), glutathione S-transferases in the susceptibility to *Colletotrichum orbiculare* and *Colletotrichum destructivum* (Dean *et al.* 2002) and cysteine proteinases in the susceptibility to *C. destructivum* and HR resistance to *Pseudomonas syringae* pv. *tomato* (Hao *et al*. 2006).

Because of the potential importance of *NtEH-1* in disease resistance, the goal of this research was to find a homologue of *NtEH-1* in *N. benthamiana*, examine its response to fungal and bacterial infection, and then silence the gene through VIGS using a tobacco rattle virus (TRV)-based vector to assess its role in resistance and susceptibility. The susceptible responses of *N. benthamiana* that were investigated were anthracnose caused by the hemibiotrophic fungi, *C. destructivum* and *C. orbiculare* (Shen *et al*. 2001*a*, 2001*b*), and wildfire caused by the hemibiotrophic bacterium, *Pseudomonas syringae* pv. *tabaci* (Lucas 1965). In addition, a resistance response was examined in *N. benthamiana* expressing the resistance gene, *Pto*, which induces a HR when it interacts with *P. syringae* pv. *tabaci* expressing a type III secretion system effector encoded by *avrPto* (Rommens *et al*. 1995).

Materials and methods

Biological materials

Wild type and *Pto*-transformed (Rommens *et al.*1995) *Nicotiana benthamiana* L. were grown to the eighth true-leaf stage with 16 h light (150 μmol m^{-2} s^{-1}) at 25°C and 8 h dark at 17°C. Strain N150P3 of *Colletotrichum destructivum* O' Gara and ATCC20767P1 of *Colletotrichum orbiculare* Berk. and Mont. von Arx (Chen *et al*. 2003) were cultured on sodium-chloride-yeast-extract- sucrose-agar medium (SYAS) (Manandhar *et al*. 1986) at 22°C under continuous fluorescent light. Conidia were washed from 7–12 day-old plates, and plants were sprayed with a suspension of 10^5 conidia mL^{-1} for *C. destructivum* and 2×10^6 conidia mL^{-1} for *C. orbiculare*, and then incubated at room temperature in containers to maintain high humidity. The lesion number was counted, and the area of each leaf was measured using a leaf area meter (Model 3100; LI-COR, Lincoln, NB, USA) to calculate lesions per cm^2 leaf.

Pseudomonas syringae pv. *tabaci* Wolf and Foster was grown overnight in King's medium B (KB; King *et al*. 1954) at 22°C. A suspension of 10^6 colony forming units (CFU) mL^{-1} for *P. syringae* pv. *tabaci* 11528R or 10^8 CFU mL^{-1} for *P. syringae* pv. *tabaci* containing *avrPto* in 10 mM $MgCl_2$ was infiltrated with a needle-less syringe into each of the two youngest fully developed leaves of wild type or *Pto*-transformed *N. benthamiana*, respectively. After incubation at 16 h light (150 μmol m^{-2} s^{-1}) at 25°C and 8 h dark at 17°C, 0.785 cm^2 leaf discs were excised, ground in 1 mL 10 mM $MgCl_2$, serially diluted in 10 mM $MgCl_2$ and then plated onto KB agar. Bacterial populations were assessed as CFU per cm^2 leaf.

Sequence alignments

EH protein sequences from members of the Solanaceae were obtained from the Dana Farber Cancer Institute (DFCI) Gene Indices (http://compbio.dfci.harvard.edu/, accessed 10 July 2007) by a keyword search for epoxide hydrolase, and are listed in Table 1. The sequences were aligned with CLUSTALX (Chenna

Table 1. Source, accession number and gene name of epoxide hydrolase predicted protein sequences from members of the Solanaceae with at least 100 amino acid length

Source	Accession number[A]	Gene name
Capsicum annuum L.	TC3801	–[C]
C. annuum	TC3802	–
C. annuum	TC4755	–
C. annuum	TC5349	–
C. annuum	TC5838	–
Lycopersicon esculentum L.	TC178498	–
L. esculentum	TC174348	–
L. esculentum	TC186942	–
L. esculentum	TC170114	–
L. esculentum	TC171270	–
L. esculentum	TC171911	–
L. esculentum	TC186355	–
L. esculentum	TC190171	–
L. esculentum	TC185647	–
L. esculentum	TC181374	–
Nicotiana benthamiana L.	EU700053	*NbEH1.1*
N. benthamiana	TC9495	*NbEH1.2*
N. benthamiana	EU779658[B]	*NbEH2.1*
N. benthamiana	EU779659[B]	*NbEH2.2*
Nicotiana tabacum L.	TC7691	*NtEH-1*
N. tabacum	TC7022	–
N. tabacum	TC4399	–
Solanum tuberosum L.	AAA81893	*StEH9.2*
S. tuberosum	AAA81891	*StEH5.3*
S. tuberosum	TC149040	–
S. tuberosum	TC150343	–
S. tuberosum	TC141127	–
S. tuberosum	TC144147	–
S. tuberosum	TC139523	–
S. tuberosum	TC132944	–
S. tuberosum	TC152206	–
S. tuberosum	TC146241	–
S. tuberosum	TC134015	–
S. tuberosum	TC156302	–
S. tuberosum	TC153402	–
S. tuberosum	TC155598	–
S. tuberosum	TC145540	–
S. tuberosum	TC152273	–
S. tuberosum	TC137677	–
S. tuberosum	TC150329	–

[A]Tentative consensus (TC) sequences were obtained from the DFCI Gene Indices (http://compbio.dfci.harvard.edu/). Other sequences were obtained from GenBank (www.ncbi.nlm.nih.gov/).
[B]TC1982 was separated into two genes: EU779658 and EU779659.
[C]None provided.

et al. 2003) using default parameters, and a dendrogram was generated using the neighbour-joining algorithm with 1000 bootstrap replications in CLUSTALX.

3′ rapid amplification of cDNA ends (RACE) PCR

Based on the grouping observed in a dendrogram of EH sequences, the following nucleotide sequences were chosen: *N. benthamiana* (TC9495), *Solanum tuberosum* L. (TC137677, TC144147 and TC139523), *Lycopersicon esculentum* L. (TC170114), *Capsicum annuum* (TC3801, TC3802 and TC4755) and *Nicotiana tabacum* L. (TC7691). These sequences were aligned in CLUSTALX, and conserved regions were used to design a forward 3′ RACE primer, NbEH1RACE (5′-TCCGAACWGCATTGCAGGT-3′). Primer specifications and suitability were tested using the program GeneRunner (Hastings Software, Hastings, NY, USA).

For all RNA extractions, leaf samples were immediately frozen in liquid nitrogen at 0–96 h post inoculation (HPI) for *C. destructivum* and *Pseudomonas syringae* pv. *tabaci*, 0–120 HPI for *C. orbiculare*, and 0–24 HPI for *P. syringae* pv. *tabaci* expressing *avrPto*. Samples were stored at −80°C. RNA extraction was done as per Hao *et al*. (2006). The RNA was resuspended in 25–50 μL DEPC-treated dH_2O and stored at −80°C. Single-stranded coding DNA (cDNA) was synthesised from RNA using 3′ RACE oligo dT-anchor primer (5′-GACCAC GCGTATCGATGTCGACTTTTTTTTTTTTTTTV-3′) (V = A, C or G) following the manufacturer's instructions (Roche, Indianapolis, IN, USA). All PCRs were performed using a Touchgene Thermo Cycler (Techne, Princeton, NJ, USA). PCR was done in 15 μL with 2 μL cDNA, 0.75 units *Tsg* polymerase (Biobasic, Markham, ON, CA), 10× *Tsg* polymerase buffer, 2 mM dNTPs, 2.5 mM $MgSO_4$, 1.0 mM NbEH1RACE primer and 1.0 mM 3′ RACE anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′) supplied in the 5′/3′ RACE kit (Roche). 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 10 min at 72°C. A PCR product was excised after electrophoresis in 1.2% TAE agarose gels, purified with the GENECLEAN II Kit (Q BIOgene, Montreal, Quebec, Canada) and sequenced at Laboratory Services Division, (University of Guelph, Guelph, Ontario, Canada).

Relative reverse transcription (RT)–PCR

Relative RT–PCR was done following Dean *et al*. (2002). Single-stranded cDNA was synthesised using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Burlington, Ontario, Canada) and oligo (dT) primer (Promega, Madison, WI, USA) using total RNA following the manufacturer's instructions.

A forward primer NbEH1F1 (5′-AGTAGCGGATGCAGGA TTTC-3′) was designed in a conserved region among the EH genes that had been used to design primer NbEH1RACE, and it was predicted to be 504 bp upstream of NbEH1RACE. This was paired with the gene-specific reverse primer NbEH1.1R1 (5′-TCTTTCACCATTCCACTT-3′) designed from the 3′ UTR of the 3′ RACE product (designated *NbEH1.1*), or the gene-specific reverse primer NbEH1.2R1 (5′-CTTATTTGAATA CTATGGTAGG-3′) designed from the 3′ UTR of TC9495 (designated *NbEH1.2*). As an internal constitutive control, the translation elongation factor 1α (*NbEF1α*) forward primer, TobefS (5′-CTCCAAGGCTAGGTATGATG-3′) and reverse primer, TobefA (5′-CTTCGTGGTTGCATCTCAAC-3′) were used to obtain a 370 bp RT–PCR product (Dean *et al*. 2002). The choice of *NbEF1α* was made as Dean *et al*. (2002) had shown that the expression of *NbEF1α* was constitutively expressed in infected leaves, and Nicot *et al*. (2005) had demonstrated that the translation elongation factor 1-α gene in another solanaceous plant, potato, was the most stable among seven different housekeeping genes examined during biotic and abiotic stresses.

Relative RT–PCR was done in 15 μL with 2 μL cDNA, 0.75 units *Tsg* polymerase (Biobasic), 10× *Tsg* polymerase buffer, 2 mM dNTPs, 2.5 mM Mg^{2+}, 0.5 mM TobefS, 0.5 mM TobefA, and 1.0 mM of each EH primer described above. Amplification was done at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min and a final 10 min at 72°C. RT–PCR products were separated in 1.2% TAE agarose gels, and the images were scanned and saved as TIFF files. The band intensities were determined for both the EH and EF1α RT–PCR products using NIH Image (Scion Corporation, Frederick, MD, USA), and the ratio of band intensities was calculated for each gel lane. For all RT–PCR quantifications, at least four replications were done using two different RNA samples from two independent inoculations.

To confirm that the number of cycles was not excessive for quantification, relative RT–PCR was also done as above with 30 cycles, and the expression patterns were compared with those obtained with 35 cycles. No significant differences were found. To confirm the identity of the RT–PCR products, the bands for *NbEH1.1* and *NbEH1.2* at 48 HPI with *P. syringae* pv. *tabaci* were excised, purified and sequenced as described previously.

Virus induced gene silencing (VIGS)

The 3′ RACE product was cloned into pGEMT-easy (MBI Fermentas, Burlington, Ontario, Canada), and then digested with *EcoR1* for subcloning into the *EcoR1* site of pTRV2 (Dinesh-Kumar *et al*. 2003). The construct was transformed into *Escherichia coli* DH5α, and selected on Luria Broth (LB) agar containing 50 mg L^{-1} kanamycin. The plasmid was extracted from *E. coli* and electroporated into *Agrobacterium tumefaciens* strain GV3101. Colonies growing on LB agar amended with 50 mg L^{-1} kanamycin and 5 mg L^{-1} tetracycline were screened for inserts with NbEH1RACE primer and 3′ RACE anchor primer (Roche) using the 3′ RACE PCR conditions described above.

Cultures of *A. tumefaciens* containing either pTRV2 with the 3′ RACE product of *NbEH1.1* or pTRV2 with the green fluorescent protein gene (GFP) from pEZS-CL (Yoshimoto *et al*. 2004) as a vector control were grown at 28°C for 48 h on LB agar containing kanamycin and tetracycline. Infiltration of the two first fully matured leaves of three (for relative gene expression) or eight (for disease assessment) plants at the fourth true-leaf stage was done with equal numbers of *A. tumefaciens* cells containing pTRV-RNA1 with either pTRV2-*NbEH1.1* or pTRV2-GFP as described by Liu *et al*. (2002). As an additional control, an equal number of plants were also infiltrated with 10 mM $MgCl_2$. A repeated

experiment was done for disease assessment, and two more repeated experiments were done for relative RT–PCR.

At 14–15 days after *A. tumefaciens* inoculation, the plants were inoculated with *C. destructivum*, *C. orbiculare* or *P. syringae* pv. *tabaci* as described above. Disease severity and relative RT–PCR was assessed after fungal or bacterial inoculation on the first fully mature leaves as described above. Disease severity was assessed at 64–96 HPI for *C. destructivum*, *C. orbiculare* or *P. syringae* pv. *tabaci*, and assessed at 12 HPI for *P. syringae* pv. *tabaci* containing *avrPto*.

Statistical analysis

Data analysis of the relative RT–PCR results from silencing of *NbEH1.1* and analysis of the disease assessment in $MgCl_2$ control, TRV vector control and the *NbEH1.1*-silenced plants was done using PROC GLM (analysis of variance) as implemented in SAS (SAS Institute, Cary, NC, USA). Analysis of variance was conducted to look for a significant ($P < 0.05$) interaction between the main treatment effect (e.g. gene expression or disease severity) and experimental repetition. If the interaction term was not significant, then the data for all three repeated experiments were combined for statistical analyses. Where significant treatment effects were found ($P < 0.05$), the means were separated by the test of LSD at $P = 0.05$.

Results

NbEH1.1 *amplification and sequence analysis*

An alignment was made of EH nucleotide (nt) sequences from plants in the Solanaceae in the DFCI Gene Indices (http://compbio.dfci.harvard.edu/, accessed 10 July 2007) showing the highest identity to *NTEH-1*. The sequences were from *N. tabacum* (*NtEH-1* = TC7691), *N. benthamiana* (TC9495), *C. annuum* (TC3801, TC3802 and TC4755), *S. tuberosum* (TC144147 and TC139523) and *L. esculentum* (TC170114) (Table 1). A highly conserved region in the sequences was used to design the primer NBEH1RACE, which was paired with a 3′ RACE anchor primer to amplify a single RT–PCR product of 490 bp from cDNA of *N. benthamiana* leaves at 72 HPI with *P. syringae* pv. *tabaci*. This sampling time was chosen as preliminary results indicated that it was the time after infection when the strongest band could be obtained using those primers. The sequence of the 3′ RACE PCR product had 96% nt identity with *NTEH-1* in contrast to only 51% nt identity with TC9495 from *N. benthamiana*. This novel EH gene was designated *NbEH1.1* and TC9495 was designated *NbEH1.2*.

A forward primer (NbEH1F1) was designed in a conserved region among the *NtEH-1* homologues, and paired with a reverse primer (NbEH1R1) designed in a region of the 3′ UTR of *NbEH1.1* that differed from *NbEH1.2*. RT–PCR with primers NbEH1F1 and NbEH1R1 resulted in a 657 bp band using cDNA from the 72 HPI interaction with *P. syringae* pv. *tabaci*. The sequence had a 52 bp overlap with 100% nt identity to the 3′ RACE sequence of *NbEH1.1*. Joining the two sequences resulted in a contig of 1012 bp for *NbEH1.1*.

Epoxide hydrolase sequence comparison

A comparison was made of the predicted protein sequences of *NbEH1.1* and 39 other EHs from plants in the Solanaceae (Table 1). The EH sequences formed two major clades, which were named EH1 and EH2 (Fig. 1). Within the EH1 clade, there were two subclusters, A and B, with high and low bootstrap support (85% and 51%, respectively), while the EH2 clade had moderate bootstrap support (61%). Both *NbEH1.1* and *NbEH1.2* were in subcluster A in the EH1 clade. A BLASTN search of the DFCI *N. benthamiana* Gene Index (Release 2.0, http://compbio.dfci.harvard.edu/) with *NbEH1.1* as the query sequence revealed a more distantly related EH sequence, TC1982; however, a closer examination of the 10 EST sequences comprising TC1982 showed that it was composed of at least two related genes rather than a single gene. These two EH genes were designated *NbEH2.1* and *NbEH2.2*, and both can be found in the EH2 clade.

It appears that many plants usually have both EH1 and EH2 type sequences. In addition to *N. benthamiana*, there are both EH1 and EH2 type sequences in *N. tabacum*, *C. annuum*, *L. esculentum* and *S. tuberosum* (Fig. 1). However, this is not limited to plants belonging to the Solanaceae. A partial search of EH sequences from the Phytome database (http://www.phytome.org/, accessed 10 July 2007) and the DFCI Gene Indices (http://compbio.dfci.harvard.edu/) showed a wide range of plants that had both EH1 and EH2 type sequences. This included a gymnosperm, *Pinus taeda* L., and the dicotyledonous angiosperms, *Aquilegia formosa* Fisch. ex DC., *Aquilegia pubescens* Coville, *Brassica napus* L., *Citrus clementina* Hort. ex Tan., *Gossypium hirsutum* L., *Liriodendron tulipifera* L., *Mesembryanthemum crystallinum* L., *Phaseolus vulgaris* L., and *Vitis vinifera* L., which each belong to a different plant family. Also, both EH1 and EH2 type sequences were found in the monocotyledonous angiosperms, *Allium cepa*, *Hordeum vulgare*, *Oryza sativa* and *Triticum aestivum*. However, an exception was the *Arabidopsis thaliana* (L.) Heynh. genome, which contains 33 EH genes that are all of the EH2 type (http://www.arabidopsis.org/, accessed 10 July 2007).

Expression of NbEH1.1 *and* NbEH1.2 *in* N. benthamiana *following infection by* C. destructivum, C. orbiculare *and* P. syringae *pv.* tabaci

Expression of *NbEH1.1* did not change significantly until there was a significant increase at 72 HPI with *C. destructivum* (Fig. 2*A*). This correlated with the appearance of small water-soaked spots at approximately 60 HPI. Expression of *NbEH1.2* was significantly greater than the water control only at 24 HPI with *C. destructivum*, which is when the fungus is at the penetration and early biotrophic phase (Fig. 2*B*). No expression of *NbEH1.1* or *NbEH1.2* was detectable in the water control (Fig. 2).

A significant increase in *NbEH1.1* expression began at 24 HPI with *C. orbiculare* continually increasing to a peak at 72 HPI (Fig. 3*A*). The peak in expression correlated with the appearance of small water-soaked spots at 64–72 HPI. In contrast, expression of *NbEH1.2* was never significantly different from the water control after infection with *C. orbiculare* (Fig. 3*B*). No expression of *NbEH1.1* or *NbEH1.2* was detectable in the water control (Fig. 3).

Expression of *NbEH1.1* first significantly increased at 48 HPI with *P. syringae* pv. *tabaci* and then continued to increase at progressively faster rates (Fig. 4*A*). In contrast, expression of

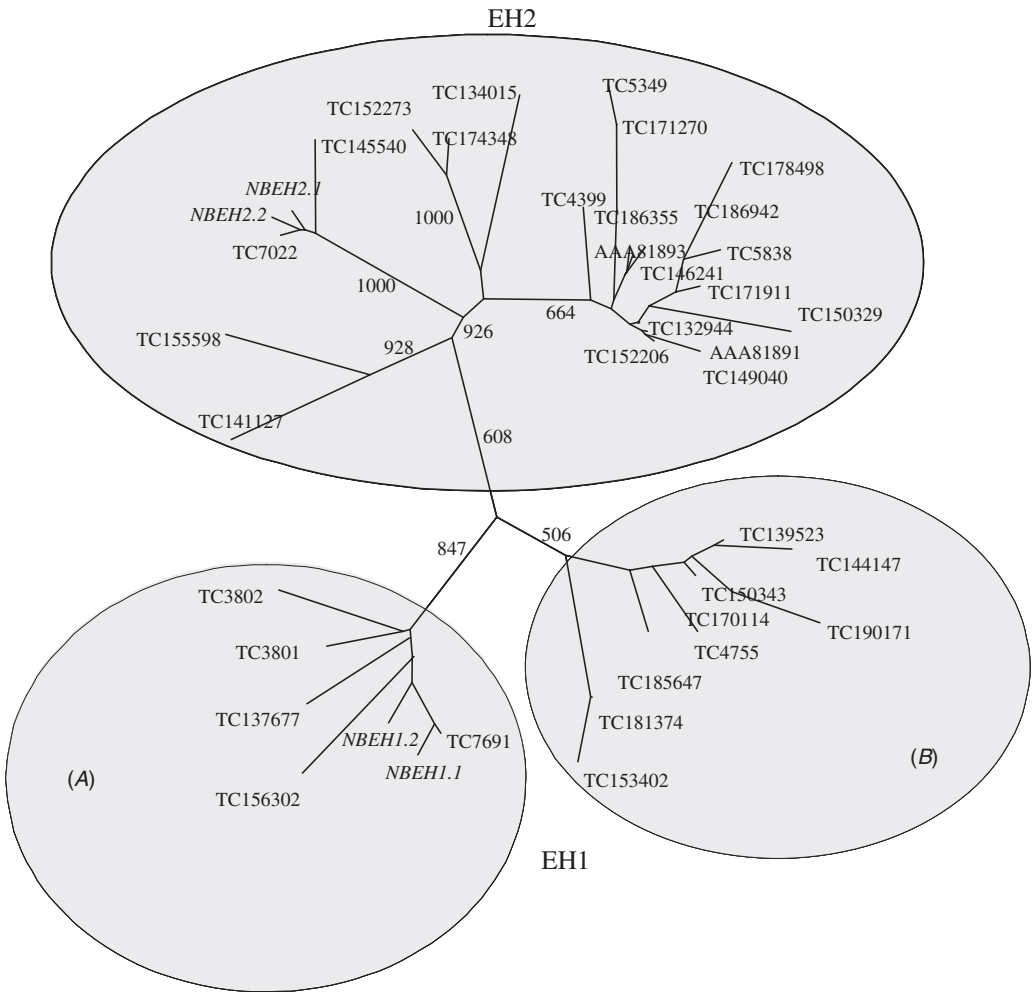

Fig. 1. Comparison of α/β hydrolase fold family epoxide hydrolase (EH) sequences from plants in the Solanaceae. Amino acid sequences were aligned using CLUSTALX, and bootstrap analysis conducted using the NJ-bootstrap procedure of CLUSTALX. The values near major branches represent that number of times out of 1000 that the branch was supported by bootstrap analysis, and only branches with over 50% support are marked. The two EH1 subclusters and the EH2 cluster are marked in grey.

NbEH1.2 first significantly increased at 24 HPI peaking at 48 HPI (Fig. 4*B*). For *P. syringae* pv. *tabaci*, the significant increases in both *NbEH1.1* and *NbEH1.2* expression began before the appearance of water-soaking that developed at approximately 72 HPI. No expression of *NbEH1.1* or *NbEH1.2* was detectable in the buffer infiltration control (Fig. 4).

Expression of *NbEH1.1* in *N. benthamiana* (*Pto*) inoculated with *P. syringae* pv. *tabaci* (*avrPto*) increased after infiltration with a peak at 15 HPI (Fig. 5*A*). The decline from the peak expression corresponded with visible HR necrosis at 16–18 HPI. Expression of *NbEH1.2* did not show such a peak in expression but only fluctuated somewhat after an initial significant increase at 3 HPI (Fig. 5*B*). No expression of *NbEH1.1* or *NbEH1.2* was detectable in the buffer infiltration control (Fig. 5).

To confirm the identity of the EH RT–PCR product, bands amplified from cDNA at 72 HPI with *P. syringae* pv. *tabaci* using the primers for relative RT–PCR of *NbEH1.1* or *NbEH1.2* were directly sequenced. The sequences had 100% nt identity with the corresponding regions of *NbEH1.1* and *NbEH1.2*.

Silencing NbEH1.1 *in* N. benthamiana *and the response to inoculation with* C. destructivum *or* C. orbiculare

TRV-mediated VIGS of *NbEH1.1* in *N. benthamiana* decreased *NbEH1.1* expression to non-detectable levels, whereas considerable expression was detected in the buffer and TRV-GFP controls at 72 HPI with *C. destructivum* (Fig. 6; Table 2). Expression of *NbEH1.2* was also tested to see if it was affected by silencing *NbEH1.1*, but there was no detectable expression in the buffer and TRV vector controls or in *NbEH1.1*-silenced plants. However, *NbEH2.1* expression was detected and was not affected by silencing *NbEH1.1* indicating the relative specificity of silencing (Table 2).

Silencing of *NbEH1.1* expression was also observed at 72 HPI with *C. orbiculare* compared with the buffer and TRV-GFP controls (Fig. 6; Table 2). No detectable *NbEH1.2* expression

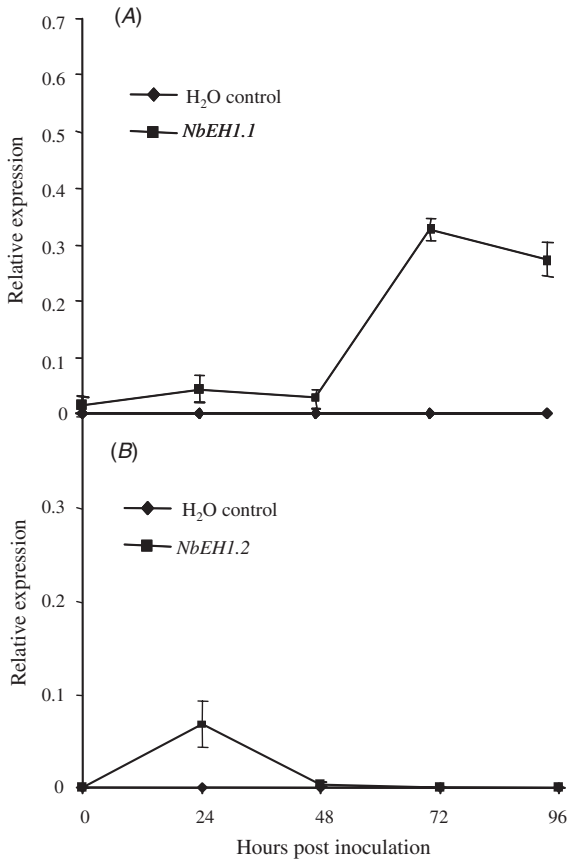

Fig. 2. Relative reverse transcription (RT)–PCR of (*A*) *NbEH1.1* and (*B*) *NbEH1.2* expression in *Nicotiana benthamiana* leaves inoculated with *Colletotrichum destructivum*. The levels of *NbEH1.1* mRNA were determined relative to the level of expression of *NbEF-1*α. Means are shown with standard error bars calculated from three replications.

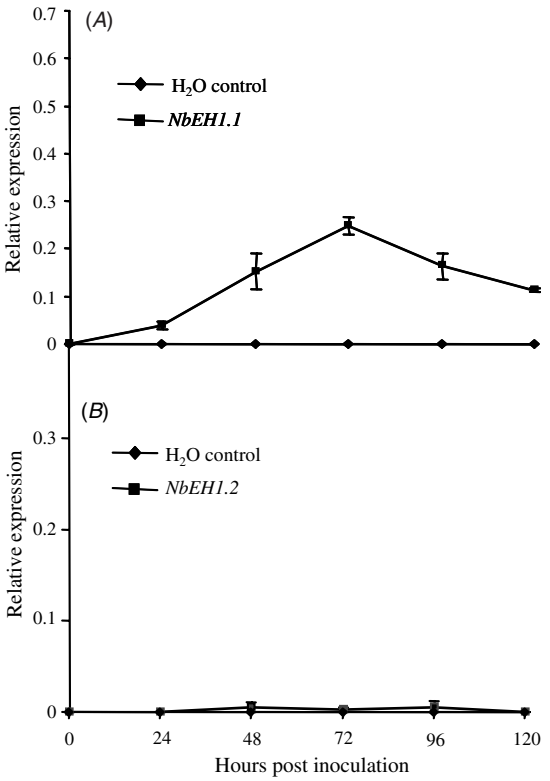

Fig. 3. Relative reverse transcription (RT)–PCR of (*A*) *NbEH1.1* and (*B*) *NbEH1.2* expression in *Nicotiana benthamiana* leaves inoculated with *Colletotrichum orbiculare*. The levels of *NbEH1.1* and *NbEH1.2* messenger RNA were determined relative to the level of expression of *NbEF-1*α. Means are shown with standard error bars calculated from three replications.

was found in the buffer control, TRV vector control or *NbEH1.1*-silenced plants inoculated with *C. orbiculare*, but *NbEH2.1* expression was detected and was not affected by silencing of *NbEH1.1*.

NbEH1.1-silenced plants had significantly fewer water-soaked lesions on leaves at 64 HPI with *C. destructivum* compared with the controls, but by 72 HPI, lesion number was not significantly different between *NbEH1.1*-silenced and non-silenced plants (Table 3). This indicates that the development of necrosis was delayed for a significant number of infection sites. Although large numbers of water-soaked lesions became visible several hours later in *NbEH1.1*-silenced plants compared with the controls, there was no difference in the appearance of the lesions with the different treatments. In contrast, there was no significant difference in the lesion number on leaves of *NbEH1.1*-silenced plants compared with the controls at either 64 or 72 HPI for *C. orbiculare* (Table 3).

Silencing NbEH1.1 *in* N. benthamiana *and the response to inoculation with* P. syringae *pv.* tabaci *or* P. syringae *pv.* tabaci (avrPto)

TRV-mediated VIGS of *NbEH1.1* resulted in a significant decrease in *NbEH1.1* expression compared with the buffer and TRV-GFP controls at 72 HPI during the susceptible response of wild type *N. benthamiana* to *P. syringae* pv. *tabaci* (Fig. 6; Table 4). TRV-mediated VIGS of *NbEH1.1* also resulted in significantly less *NbEH1.1* expression in *NbEH1.1*-silenced *N. benthamiana* (*Pto*) plants compared with the controls at 15 HPI during the HR resistance to *P. syringae* pv. *tabaci* (*avrPto*) (Fig. 6; Table 4). Silencing appeared to be relatively specific for these susceptible and resistant responses as there was never a significant difference in the expression of *NbEH1.2* in the *NbEH1.1*-silenced plants compared with the controls (Fig. 6; Table 4).

After inoculation with *P. syringae* pv. *tabaci*, bacterial populations at 72 HPI were significantly lower in *NbEH1.1*-silenced wild type *N. benthamiana* compared with the $MgCl_2$ control or the TRV-GFP control (Table 5). Despite the lower bacterial population, the timing and appearance of necrosis in the susceptible response did not differ between silenced and non-silenced plants. In the resistant response, silencing *NbEH1.1* resulted in significantly higher population levels of *P. syringae* pv. *tabaci* (*avrPto*) at 12 HPI in the *NbEH1.1*-silenced *N. benthamiana* (*Pto*) compared with the $MgCl_2$ control or the TRV-GFP control (Table 5). There were no differences between silenced and non-silenced plants in the timing and appearance of HR necrosis.

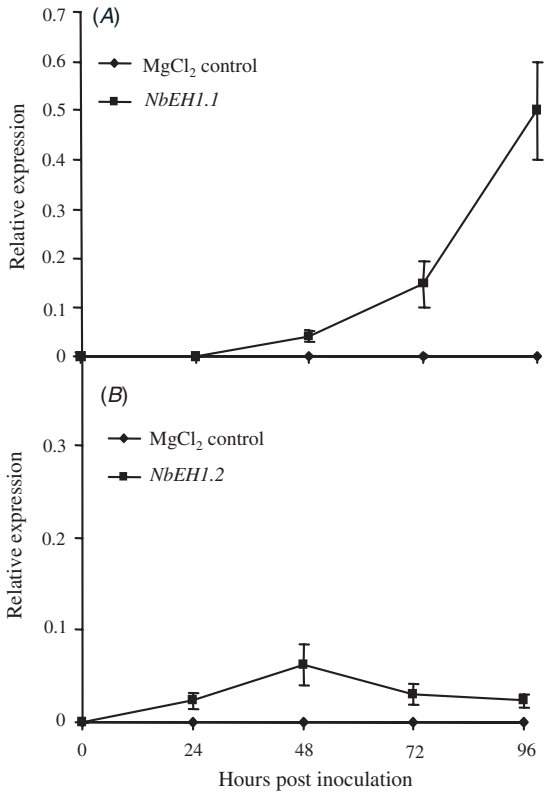

Fig. 4. Relative reverse transcription (RT)–PCR of (*A*) *NbEH1.1* and (*B*) *NbEH1.2* expression in *Nicotiana benthamiana* leaves inoculated with *Psuedomonas syringae* pv. *tabaci*. The messenger RNA levels of *NbEH1.1* and *NbEH1.2* were determined relative to the level of expression of *NbEF-1*α. Means are shown with standard error bars calculated from three replications.

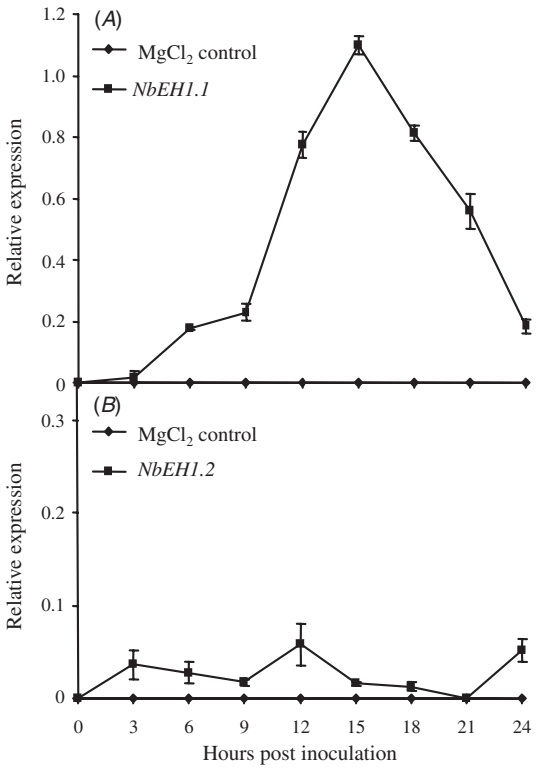

Fig. 5. Relative reverse transcription (RT)–PCR of (*A*) *NbEH1.1* and (*B*) *NbEH1.2* expression in *Nicotiana benthamiana* (*Pto*) leaves inoculated with *Pseudomonas. syringae* pv *tabaci* (*avrPto*). The levels of *NbEH1.1* and *NbEH1.2* messenger RNA levels were determined relative to the level of expression of *NbEF-1*α. Means are shown with standard error bars calculated from three replications.

Discussion

An EH gene, *NbEH1.1*, was identified in *N. benthamiana* that has relatively high sequence identity to *NtEH-1* from *N. tabacum*. In the DFCI *N. benthamiana* Gene Index, three other *N. benthamiana* EH genes were identified with much less similarity to *NtEH-1*. However, it is likely that there are additional EH genes in *N. benthamiana*, since other solanaceous plants, such as *L. esculentum* and *S. tuberosum*, have 10 and 18 tentative consensus sequences for EH, respectively, in the DFCI Gene Indices.

At 24 HPI with *C. destructivum*, only expression of *NbEH1.2* significantly increased, followed by a decline. At that time, the fungus was penetrating the cuticle with an appressorium, although some infections have begun producing a vesicle in epidermal cells beginning the biotrophic phase (Shen *et al.* 2001*a*). This suggests that, unlike *NbEH1.1*, *NbEH1.2* expression may be related to penetration of the cuticle and/or early biotrophy. Components of the cuticle include cutin monomer epoxides, such as 9,10-epoxy-18-hydroxystearic acid, that are produced by EH (Kolattukudy 1981). In leaves of *Citrus jambhiri* Lush., expression of an EH gene, *RlemEH*, was induced during appressorial penetration of the cuticle by the virulent fungus, *Alternaria alternata*, and *RlemEH* might help in reinforcing and repairing cuticle damage caused by penetration

Table 2. Relative reverse transcription (RT)–PCR analysis of silencing of *NbEH1.1*, *NbEH1.2* and *NbEH2.2* using $MgCl_2$ control, tobacco rattle virus (TRV) vector control (TRV-green fluorescent protein gene, GFP) and *NbEH1.1*-silenced *Nicotiana benthamiana* at 72 HPI with *Colletotrichum destructivum* or *Colletotrichum orbiculare*

Pathogen	Treatment[A]	Relative gene expression[B]		
		NbEH1.1	*NbEH1.2*	*NbEH2.2*
C. destructivum	$MgCl_2$ control	0.37 a	0.00 a	0.28 a
C. destructivum	TRV-GFP control	0.16 b	0.00 a	0.29 a
C. destructivum	TRV-*NbEH1.1*	0.00 c	0.00 a	0.30 a
C. orbiculare	$MgCl_2$ control	1.64 a	0.00 a	0.39 a
C. orbiculare	TRV-GFP control	0.96 b	0.00 a	0.34 a
C. orbiculare	TRV-*NbEH1.1*	0.04 c	0.00 a	0.38 a

[A]*NbEH1.1*-silenced and TRV vector control *N. benthamiana* plants were inoculated with *Agrobacterium tumefaciens* containing TRV in a tumor inducing (Ti) plasmid with a GFP insert or a fragment of *NbEH1.1*. $MgCl_2$ control plants were inoculated with buffer only.

[B]Expression of *NbEH1.1*, *NbEH1.2* and *NbEH2.1* in $MgCl_2$ control in $MgCl_2$ control, TRV vector control or *NbEH1.1*-silenced *N. benthamiana* was determined by relative RT–PCR following infection with *C. destructivum* or *C. orbiculare*. Each value represents the mean relative expression from at least three experiments. Means for each pathogen in the same column followed by the same letter are not significantly different according to the protected LSD test at $P = 0.05$.

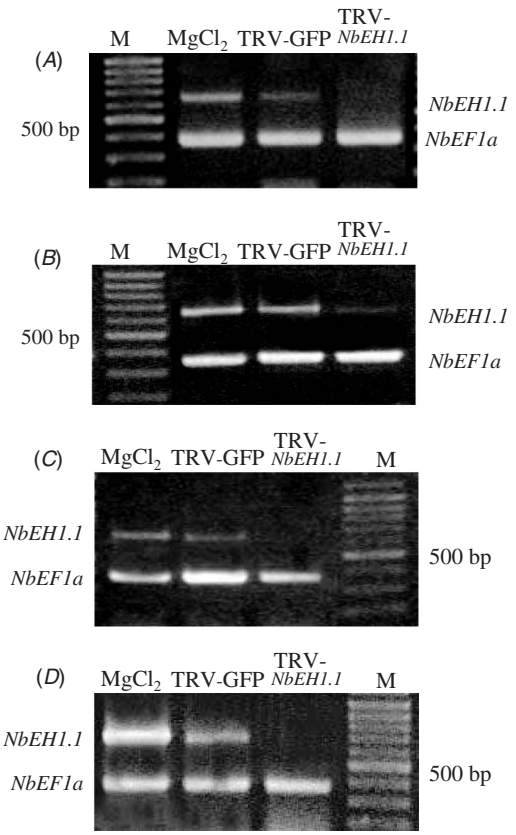

Fig. 6. Gel pictures of relative reverse transcription (RT)–PCR of *NbEH1.1* in *NbEH1.1*-silenced *Nicotiana benthamiana* and $MgCl_2$ and tobacco rattle virus (TRV)-green fluorescent protein gene (GFP) controls. *NbEH1.1* was co-amplified with *NbEF-1*α using coding DNA from the interaction at 72 h post inoculation (HPI) with (*A*) *Colletotrichum destructivum*, (*B*) *Colletotrichum orbiculare* or (*C*) *Pseudomonas syringae* pv. *tabaci* or (*D*) 15 HPI with *P. syringae* pv. *tabaci* (*avrPto*). Lanes are: $MgCl_2$, which is the buffer only infiltration control, TRV-GFP, which is the virus vector control with *Agrobacterium tumefaciens* containing pTRV2 with a green fluorescent protein gene and TRV-*NbEH1.1* which is the *A. tumefaciens* containing pTRV2 with a fragment of *NbEH1.1*, and M, which is the 100 bp ladder.

(Gomi *et al*. 2003). *Colletotrichum destructivum* penetration also involves appressoria, and thus *NbEH1.2* may also be involved in reinforcing the cuticle.

Expression of *NbEH1.1* remained low during penetration and the biotrophic phase of *C. destructivum* when multi-lobed vesicles were growing and invaginating the initially infected leaf epidermal cell (Shen *et al*. 2001*a*). However, at approximately 60 HPI, secondary hyphae developed from the ends of the vesicles to penetrate adjacent cells and produce water soaked spots, correlating with the timing of increased *NbEH1.1* expression. It appears that *NbEH1.1* expression is induced by changes in host cells due to fungal necrotrophy, but not penetration or even extensive biotrophy, making it more likely that it is associated with responses like catabolism of epoxides to non-toxic forms or production of antimicrobial compounds, rather than cuticle reinforcement (Ohta *et al*. 1991; Murray *et al*. 1993; Gomi *et al*. 2003).

In contrast to the *C. destructivum* infection, *NbEH1.1* expression increased at a relatively constant level when

Table 3. Lesion number in $MgCl_2$ control, tobacco rattle virus (TRV) vector control, *NbEH1.1*-silenced *Nicotiana benthamiana* at 64 and 72 h post inoculation (HPI) with *Colletotrichum destructivum* or 72 and 96 HPI with *Colletotrichum orbiculare*

Treatment[A]	*C. destructivum*		*C. orbiculare*	
	Lesions/cm^2 at 64 HPI[B]	Lesions/cm^2 at 72 HPI[B]	Lesions/cm^2 at 72 HPI[B]	Lesions/cm^2 at 96 HPI[B]
$MgCl_2$ control	0.96 a	1.25 a	0.23 a	0.31 a
TRV-GFP control	0.80 a	1.01 a	0.18 a	0.38 a
TRV-*NbEH1.1*	0.32 b	0.97 a	0.19 a	0.35 a

[A]*NbEH1.1*-silenced and TRV vector control *N. benthamiana* plants were inoculated with *Agrobacterium tumefaciens* containing TRV in a tumor inducing (Ti) plasmid with a green fluorescent protein gene (GFP) insert or a fragment of *NbEH1.1*. $MgCl_2$ control plants were inoculated with buffer only.

[B]Lesions/cm^2 were means from two experiments with a total of 16 replications collected from 5-week-old plants. Means in the same column followed by the same letter are not significantly different according to the protected LSD test at $P = 0.05$.

Table 4. Relative reverse transcription (RT)–PCR analysis of silencing of *NbEH1.1* and *NbEH1.2* in $MgCl_2$ control, tobacco rattle virus (TRV) vector control and *NbEH1.1*-silenced *Nicotiana benthamiana* at 72 h post inoculation (HPI) with *Pseudomonas syringae* pv. *tabaci* or at 15 HPI with *P. syringae* pv. *tabaci* (*avrPto*)

Treatment[A]	Relative gene expression[B]			
	P. syringae pv. *tabaci*		*P. syringae* pv. *tabaci* (*avrPto*)	
	NbEH1.1	*NbEH1.2*	*NbEH1.1*	*NbEH1.2*
$MgCl_2$ control	0.49 a	0.57 a	0.74 a	0.68 a
TRV-GFP control	0.21 b	0.56 a	0.38 b	0.65 a
TRV-*NbEH1.1*	0.00 c	0.58 a	0.00 c	0.63 a

[A]*NbEH1.1*-silenced and TRV vector control *N. benthamiana* plants were inoculated with *A. tumefaciens* containing TRV in a tumor inducing (Ti) plasmid with a green fluorescent protein gene (GFP) insert or a fragment of *NbEH1.1*. $MgCl_2$ control plants were inoculated with buffer only.

[B]Expression of *NbEH1.1*, *NbEH1.2* and *NbEH2.1* in $MgCl_2$ control, TRV vector control or *NbEH1.1*-silenced *N. benthamiana* or *N. benthamiana* (*Pto*) was determined by relative RT–PCR following infection with *P. syringae* pv. *tabaci* or pv. *tabaci* (*avrPto*), respectively. Each value represents the mean relative expression from at least three experiments. Means within the same column followed by the same letter are not significantly different according to the protected LSD test at $P = 0.05$.

C. orbiculare grew biotrophically, producing large primary hyphae that penetrate and invaginate the cytoplasm of multiple host cells (Shen *et al*. 2001*b*). After approximately 72 HPI, necrotrophy began with thin secondary hyphae growing through host cells producing water-soaked spots and visible lesions. Penetration of multiple host cells occurs during the biotrophic phase of *C. orbiculare* but not for *C. destructivum* (Shen *et al*. 2001*a*, 2001*b*). Therefore, one explanation for the difference in *NbEH1.1* expression is that as *C. orbiculare* spreads from cell to cell by large primary hyphae, it degrades some of the cell wall, which could generate plant cell wall-based signal molecules that can affect host gene expression (Vidhyasekaran 2008).

Table 5. Bacterial populations in $MgCl_2$ control, tobacco rattle virus (TRV) vector control, *NbEH1.1*-silenced *Nicotiana benthamiana* at 72 h post inoculation (HPI) with *Psuedomonas syringae* pv. *tabaci* or *N. benthamiana* (*Pto*) at 12 HPI with *P. syringae* pv. *tabaci* (*avrPto*)

Treatment[A]	*P. syringae* pv. *tabaci* CFU/cm^{2}[B]	*P. syringae* pv. *tabaci* (*avrPto*) CFU/cm^{2}[B]
$MgCl_2$ control	1.71×10^7 a	5.50×10^6 a
TRV-GFP control	1.44×10^7 a	2.53×10^6 a
TRV-*NbEH1.1*	9.55×10^6 b	3.33×10^7 b

[A]*NbEH1.1*-silenced and TRV vector control *N. benthamiana* or *N. benthamiana* (*Pto*) plants were inoculated with *Agrobacterium tumefaciens* containing TRV in a tumor inducing (Ti) plasmid with a GFP insert or a fragment of *NbEH1.1*. $MgCl_2$ control plants were inoculated with buffer only.

[B]Colony forming units (CFU)/cm^2 were means from two experiments with a total of 16 replications collected from 5-week-old plants. Means in the same column followed by the same letter are not significantly different according to the protected LSD test at $P = 0.05$.

Unlike the interaction with *C. destructivum*, expression of *NbEH1.2* always remained low with *C. orbiculare*. The reason for the difference in *NbEH1.2* expression during penetration between the interactions with *C. destructivum* and *C. orbiculare* is unclear since penetration by both fungi occurs with appressoria and penetration pegs.

Pseudomonas syringae has a Type III Secretion System (TTSS) to secrete effectors directly into plant cells that are essential for biotrophic bacterial multiplication in the apoplast of plant tissue (Mudgett 2005). A major role for TTSS effectors is to perturb host cell metabolism to create more favourable conditions for the pathogen, typically by suppressing defence processes (Da Cunha *et al*. 2007). However, increased *NbEH1.1* expression was first observed during biotrophic growth of *P. syringae* pv. *tabaci* in the apoplast, indicating that the plant had detected the pathogen. At approximately 72 HPI, the bacteria became necrotrophic degrading host cell walls and spreading into the host cytoplasm from the apoplast producing symptoms (Lucas 1965). This marked the start of an accelerated increase in *NbEH1.1* expression. In contrast, expression of *NbEH1.2* was induced earlier than *NbEH1.1* and peaked during biotrophic phase. It is therefore possible that *NbEH1.1* expression could be associated with stress or defence responses later in the interaction as bacterial populations become larger, while *NbEH1.2* expression could be associated with cutin synthesis earlier in the interaction. Cutin would not be involved in the same way for infections by *P. syringae* compared with *C. destructivum* because the bacterium does not directly penetrate the cuticle. However, cells in the substomatal cavity, which is invaded once bacteria enter thorough stomata, are covered with a cuticle membrane, and cutin-related fatty acids can repress TTSS gene expression (Xiao *et al*. 2004).

The interaction of *N. benthamiana* (*Pto*) with *P. syringae* pv. *tabaci* (*avrPto*) (Rommens *et al*. 1995) results in an HR when the TTSS effector encoded by *avrPto* enters the cell and interacts with its target that is guarded by Pto (Ronald 1992). During the HR, expression of *NbEH1.1* was induced with a peak before cell death that was much higher than in any of the compatible interactions examined in this study. In contrast, *NbEH1.2* expression does not appear to have a close relationship to the HR as *NbEH1.1* expression showed only a slight increase during the HR.

NtEH-1 expression, like *NbEH1.1* expression, was also upregulated before the appearance of necrosis in the HR to *P. syringae* pv. *syringae* and TMV (Malamy *et al*. 1992; Guo *et al*. 1998; Szatmari *et al*. 2006). One difference, however, is that *NtEH-1* expression was not induced in the susceptible response to TMV (Guo *et al*. 1998), but *NbEH1.1* expression was induced during all the susceptible responses in the current study. This may be related to the biotrophic nature of TMV verses the hemibiotrophic pathogens examined in current study. Szatmari *et al*. (2006) also found that expression of *NtEH-1* was induced in the susceptible response of *N. tabacum* to the hemibiotroph, *P. syringae* pv. *tabaci*, but to a lower level than in resistance responses.

Silencing of *NbEH1.1* was done by VIGS. An advantage of VIGS is that it allows plants to be grown to a mature state before silencing, thus avoiding unintended effects of silencing a gene on plant development. This is important for EHs as cutin biosynthesis is involved in plant development during germination, seed development and differentiation of meristematic tissues (Stapleton *et al*. 1994; Arahira *et al*. 2000; Edqvist and Farbos 2000).

TRV-mediated VIGS was very effective in lowering levels of *NbEH1.1* expression. Since VIGS can affect genes with 80% or higher identity (Baulcombe 1999), genes closely related to *NbEH1.1* may have been affected. The gene known to be most closely related to *NbEH1.1* is *NbEH1.2*, with 62% nt identity. In interactions where *NbEH1.2* expression could be detected, silencing *NbEH1.1* did not significantly affect its expression or that of a more distantly related gene, *NbEH2.1*, showing that *NbEH1.1* silencing was relatively specific.

For *NbEH1.1*-silenced plants infected with *C. destructivum*, fewer lesions were observed at 64 HPI, but by 8 h later, lesion numbers were similar to the controls. Thus, silencing *NbEH1.1* caused a delay in the appearance of the necrotrophic phase. Reduction of *NbEH1.1* expression should result in less EH activity and thus higher levels of epoxides and epoxy fatty acids, such as epoxyhydroxy acids. Epoxyhydroxy acids, the main decomposition product of alkoxy radicals, have antifungal activity (Kato *et al*. 1983), and so silencing *NbEH1.1* may have resulted in increased levels of inhibitory compounds that slowed fungal development thus delaying symptom appearance.

Although infection by *C. destructivum* was affected by silencing, there was no effect when *NbEH1.1*-silenced plants were inoculated with *C. orbiculare*. The earlier and more gradual increase in *NbEH1.1* expression in plants inoculated with *C. orbiculare* compared with *C. destructivum* indicates that host epoxide production responds differently during infections by those two fungi, which could be related to why *NbEH1.1* silencing did not affect the interactions in the same way.

For the infection by *P. syringae* pv. *tabaci*, silencing *NbEH1.1* resulted in a significant inhibition of pathogen growth suggesting that, like with *C. destructivum*, reduced EH levels resulted in increased amounts of epoxides with antimicrobial activity. Epoxides with antibacterial activity could contribute to bacterial basal resistance. For example, a caryophyllene-4,5-epoxide from *Pilgerodendron uviferum* (D. Don) Florin

showed antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* (Solis *et al*. 2004). It was hypothesised that *NtEH-1* was involved in basal resistance to *P. syringae* pv. *tabaci* and *A. tumefaciens*, as well as the response to the symbiont, *Sinorhizobium meliloti* (Szatmari *et al*. 2006). These compatible bacteria were believed to limit basal resistance, since induction of *NtEH-1* was less than that caused by HR or non-HR inducing incompatible bacteria.

In contrast, silencing *NbEH1.1* resulted in higher bacterial populations during the HR in the gene-for-gene interaction of *N. benthamiana* (*Pto*) with *P. syringae* pv. *tabaci* (*avrPto*). As the HR is related to enzymatic lipid peroxidation and rapid formation of toxic epoxides (Gobel *et al*. 2003), Guo *et al*. (1998) speculated that *NtEH-1* was highly induced to detoxify harmful metabolites produced during the oxidative burst, thus, helping to limit host cell death during the HR to TMV. However, silencing of *NbEH1.1*, which is very similar to *NtEH-1*, did not result any significant change in HR associated cell death. Guo *et al*. (1998) also proposed that *NtEH-1* may affect jasmonic acid synthesis and thus, defence signalling. This explanation would be compatible with our results, where silencing *NbEH1.1* resulted in reduced HR resistance as indicated by greater bacterial growth.

Another explanation for silencing resulting in greater growth of *P. syringae* pv. *tabaci* (*avrPto*) during the HR is that silencing *NbEH1.1* may have reduced the production of certain EH-produced oxylipin phytoalexins associated with the HR. An example of an oxylipin phytoalexin produced by EH is 13-L-hydroperoxy-9,11(Z,E)-octadecadienoic acid, which is formed in rice leaves during the HR to an incompatible race of *Magnaporthe grisea* (Herbert) Barr, and is able to inhibit conidial germination and germ tube growth (Ohta *et al*. 1991).

This is the first evidence that an EH gene can play a direct role in a plant-pathogen interaction. Each of the pathogens tested in this study induced a unique pattern of *NbEH1.1* expression, and silencing *NbEH1.1* resulted in no effect, inhibition or greater growth of the pathogen depending upon the interaction. Because of its potential roles in detoxification, signalling, as well as breakdown and synthesis of antimicrobial compounds, the role of EH in different plant-microbe interactions may be varied and complex.

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