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RESEARCH PAPER

Induction of glutathione *S*-transferase genes of *Nicotiana benthamiana* following infection by *Colletotrichum destructivum* and *C. orbiculare* and involvement of one in resistance

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Abstract

Four glutathione *S*-transferase (GST) genes, *NbGSTU1*, *NbGSTU2*, *NbGSTU3*, and *NbGSTF1*, were amplified from cDNA of *Nicotiana benthamiana* leaves infected with *Colletotrichum destructivum* using primers based on conserved regions of *N. tabacum* GST sequences. Expression of *NbGSTU1 and NbGSTU3* increased progressively during infection by either *C. destructivum* or *Colletotrichum orbiculare*, except for a slight decrease by *NbGSTU1* late in the infection, whereas *NbGSTU2 and NbGSTF1* expression remained relatively constant. Each of the four genes was cloned into a PVX vector for virus-induced gene silencing, and reduced expression of the four genes was detected by RT-PCR. A statistically significant increase in susceptibility of *N. benthamiana* to infection following gene silencing was found only for *NbGSTU1*-silenced plants, which had 130% more lesions and 67% more colonization by *C. orbiculare* compared with control plants. These results demonstrate that the different GST genes respond in different ways to fungal infection, and at least one plant GST gene has an important role in disease development.

Key words: Fungal infection, glutathione *S*-transferase, hemibiotrophy, virus-induced gene silencing.

Introduction

During infection by fungal pathogens, plant cells respond by expressing a battery of disease response genes, which can result in the production of various toxic plant products, including active oxygen species and phytoalexins (Marrs, 1996; Lamb and Dixon, 1997). In addition, an invading fungus may produce stress-inducing chemicals, such as phytotoxins, resulting in significant stress and damage to the host cells. One response of plants is the increased expression of glutathione *S*-transferase (GST) genes following infection by pathogens (Mauch and Dudler, 1993; Hahn and Strittmatter, 1994; Wagner *et al*., 2002).

GSTs are dimeric enzymes that catalyse the conjugation of electrophilic molecules to glutathione (GSH). In plants, these conjugates are sequestered in the vacuole where they are further processed and detoxified (Gullner and Komives, 2001; Dixon *et al*., 2002). In addition to catalysing GSH conjugation reactions, GSTs can function as carriers of auxin and phenylpropanoids, transporters of anthocyanin into the vacuole, and enzymes in tyrosine catabolism (Droog *et al*., 1995; Mueller *et al*., 2000; Yu and Facchini, 2000; Smith *et al*., 2003; Kitamura *et al*., 2004). GSTs can serve as signalling molecules, activating phenylpropanoid metabolism following exposure to UV light (Loyall *et al*., 2000). Stress-inducible GSTs also have glutathione peroxidase activity, thereby protecting cells from oxidative injury by detoxifying organic hydroperoxides of fatty and nucleic acids (Dixon *et al*., 2002). Organic peroxides are created in plants during processes such as photosynthesis, pathogen attack (Mauch and Dudler, 1993), detoxification of microbial toxins (Edwards *et al*., 2000), and detoxification of phytoalexins produced during the hypersensitive response (Li *et al*., 1997). If not reduced, these peroxides will be converted to cytotoxic derivatives that can damage plant cells.

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Abbreviations: GST, glutathione *S*-transferase; HPI, hours post-inoculation; PVX, potato virus X; PR, pathogenesis-related; RT-PCR, reverse transcriptase PCR; EF-1α, translation elongation factor 1α; VIGS, virus-induced gene silencing.

Based on sequence similarity, plant GSTs have been divided into classes *phi* and *tau*, which are found exclusively in plants, and classes *theta* and *zeta*, which are found in all five kingdoms (Edwards *et al*., 2000). *Nicotiana* species are good subjects for examining GSTs because nine GSTs have already been studied in *Nicotiana tabacum* (van der Zaal *et al*., 1987, 1991; Takahashi *et al*., 1989; Takahashi and Nagata, 1992*a*, *b*; Ezaki *et al*., 1995), and a GST gene has also been studied in *N. plumbaginifolia* (Dominov *et al*., 1992). Although *N. tabacum* GSTs are known to be involved in responses to cold, salt stress, and aluminum toxicity (Roxas *et al*., 1997; Ezaki *et al*., 2001), little is known about their role during pathogen infection.

Using heterologous primers based on *N. tabacum* GST sequences, four GSTs were amplified and cloned from *N. benthamiana*. The expression of these four GST genes was determined in response to infection by the causal agent of tobacco anthracnose, *Colletotrichum destructivum* (Shen *et al*., 2001*a*), as well as *C. orbiculare*, which can also infect *Nicotiana* spp. (Shen *et al*., 2001*b*). Both of these fungi produce intracellular hemibiotrophic infections in *N. benthamiana* (Shen *et al*., 2001*a*, *b*). To determine if the four GST genes are involved in the host response to fungal infection, they were silenced by virus-induced gene silencing (VIGS) using a potato virus X (PVX) gene-silencing vector (Ruiz *et al*., 1998). The susceptibility of the plants to infection by *C. destructivum* and *C. orbiculare* following silencing was then determined.

Table 1. *Glutathione* S*-transferase protein sequences used in this study*

Name	GenBank accession	GST class[a]	Species
ApiC	P46440	*Phi*	*Nicotiana tabacum*
At103-1a	P46421	*Tau*	*Arabidopsis thaliana*
C-7	CAA45741	*Tau*	*Nicotiana tabacum*
CpGSTU	T09781	*Tau*	*Carica papaya*
EeGSTZ	P57108	*Zeta*	*Euphorbia esula*
ERD11	S39541	*Phi*	*Arabidopsis thaliana*
ERD13	S39542	*Phi*	*Arabidopsis thaliana*
GmGSTU	T06239	*Tau*	*Glycine max*
GST1	CAC19475	*Zeta*	*Arabidopsis thaliana*
Gst1rat	NP_445745	*Theta*	*Rattus norvegensis*
Gst2	S35268	*Phi*	*Arabidopsis thaliana*
GST27	S52037	*Phi*	*Zea mays*
GSTA1	P30111	*Phi*	*Triticum aestivum*
HmGSTF	P46423	Phi	*Hyoscyamus muticus*
Hsp26-A	P32110	*Tau*	*Glycine max*
NbGSTF1	AY206005 (this study)	*Phi*	*Nicotiana benthamiana*
NbGSTU1	AY206006 (this study)	*Tau*	*Nicotiana benthamiana*
NbGSTU2	AY206007 (this study)	*Tau*	*Nicotiana benthamiana*
NbGSTU3	AY206008 (this study)	*Tau*	*Nicotiana benthamiana*
Nt103	Q03664	*Tau*	*Nicotiana tabacum*
Nt103-1	Q03662	*Tau*	*Nicotiana tabacum*
Nt103-35	Q03663	*Tau*	*Nicotiana tabacum*
Nt107	Q03666	*Tau*	*Nicotiana tabacum*
ParA	P25317	*Tau*	*Nicotiana tabacum*
ParB	P30109	*Phi*	*Nicotiana tabacum*
ParC	P49332	*Tau*	*Nicotiana tabacum*
PGST2	AF118925	*Tau*	*Papaver somniferum*
Prp1-1	T07595	*Tau*	*Solanum tuberosum*
SR8	P28342	*Zeta*	*Dianthus caryophyllus*
TA-GSTZ1	T06333	*Zeta*	*Triticum aestivum*

[a] Glutathione *S*-transferase classification based on sequence similarity and intron location following Edwards *et al*. (2000).

Materials and methods

Biological materials and inoculations

Nicotiana benthamiana plants were grown at 22 °C in Pro-mix (Premier Horticulture Inc., Red Hill, PA) until the 8-leaf stage, with a photoperiod of 8/16 h dark/light at 120 μmol m^{-2} s^{-1}. *Colletotrichum orbiculare* isolate A20767P1 and *C. destructivum* isolate N150P3 (Chen *et al*., 2003*a*) were cultured on potato dextrose agar (Difco Laboratories, Detroit, MI) or SYAS (Mandanhar *et al*., 1986) at 20 °C under continuous fluorescent lighting, and conidia were harvested after 7–12 d. After the plants had been placed in a plastic-lined container for 3 h, they were sprayed with a 2×10^6 conidia ml^{-1} suspension and incubated in the plastic-lined container. After 72–96 h, leaf samples were collected and immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Primer design and cloning of GST and other genes from N. benthamiana

To design conserved primers, GST nucleotide and protein sequences from the classes *phi*, *tau*, and *zeta* were obtained from GenBank (Table 1). The protein sequences were aligned using Clustal X (Thompson *et al*., 1997). The mammalian *theta* GST from *Rattus norvegensis* (Gst1-rat) was used as an outgroup. Dendrograms were generated using both distance and parsimony methods. The aligned sequences were subjected to bootstrapping using the program SEQBOOT in the PHYLIP package (Felsenstein, 1989). The 100 bootstrap replicates were then examined using the PHYLIP programs PROTPARS and PROTDIST. The distance matrices from PROTDIST were then analysed with the PHYLIP program NEIGHBOR using the Neighbor–Joining algorithm. The data sets were analysed with PHYLIP CONSENSE to obtain bootstrap values that represent the consistency of tree branching patterns, and dendrograms were created using the DRAWGRAM program.

Based on the groups of sequences observed in the protein alignment, nucleotide sequences were selected for further analysis and primer design. Primers Gst1S (5′-GATGGCAGAAGTGAAGTTG-3′) and Gst1A (5′-CTCCTAGCCAAAATSCCA-3′) were designed based on *N. tabacum* GSTs in cluster *tau* 1 (Fig. 1). Primers Gst2S (5′-YTRSARATGAAYCCWRTY-3′) and Gst2A (5′-SAGSWARRG-ACTTWGMRAC-3′) were designed based on *N. tabacum* GSTs in cluster *tau* 2 (Fig. 1). Primers Gst3S (5′-CTGGKGAWCACA-AGAAGS-3′) and Gst3A (5′-GCCAARATATCAGCACACC-3′) were designed based on *N. tabacum* GSTs in cluster *phi* (Fig. 1). Degenerate bases are coded as follows: Y=C or T/U, M=A or C, K=G or T/U, W=A or T, S=C or G, R=A or G. As there were no known *N. tabacum* GSTs in the cluster *zeta*, no primers were designed for this group.

PCR amplifications were performed in 15 μl reactions with 1 μl cDNA from *N. benthamiana*, 0.04 U μl^{-1} *Tsg* polymerase (Biobasic, Toronto, ON), 1× *Tsg* polymerase buffer, 2 mM dNTPs, 2.5 mM Mg^{2+}, and 0.5 μM primers. RNA was extracted according to Chen *et al*. (2000) and reverse transcribed into cDNA using Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Burlington,

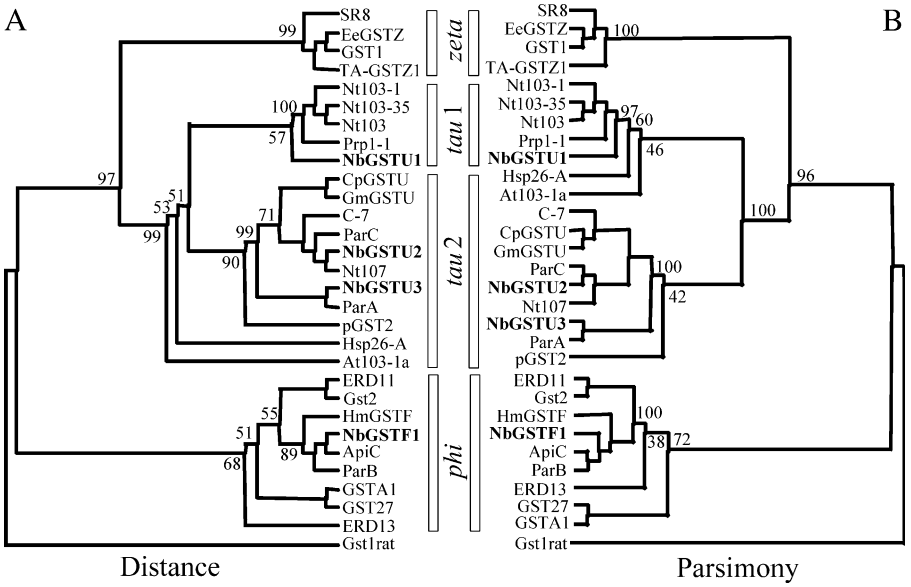

Fig. 1. Comparison of GST sequences using distance (A) and parsimony (B) methods in PHYLIP. Full descriptions of the sequences can be found in Table 2, and group classification is based on Edwards *et al*. (2000). A *theta* GST from *Rattus norvegensis* (Gst1rat) was included as the outgroup. Protein sequences were aligned with the program Clustal X, and the data were then bootstrapped with the PHYLIP program SEQBOOT. Bootstrap datasets were analysed with PHYLIP PROTDIST followed by NEIGHBOR for distance analysis (A) and with PROTPARS for parsimony analysis (B). PHYLIP CONSENSE was then used to produce final consensus trees with bootstrap values out of 100 shown near major branches.

ON) according to the manufacturer's instructions. Amplification conditions were 3 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, and one cycle for 10 min at 72 °C. All RNA samples used for reverse transcription were tested for the presence of genomic DNA by using them directly as the PCR template, prior to cDNA synthesis, under the same PCR conditions. PCR of the cDNA was performed in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT).

The amplification products were purified as described in Sambrook *et al*. (1989) and cloned into the TA vector, pGEMT-easy (Promega, Madison, WI) to obtain the plasmids, pGST1, pGST2-16, pGST2-17, and pGST3. Based on the sequence of the inserts in the plasmids, the genes were designated *NbGSTU1*, *NbGSTU2*, *NbGSTU3*, *and NbGSTF1*, respectively. The GenBank accession numbers of the sequences are listed in Table 1. The predicted protein sequences of all four genes were aligned with the 26 GST sequences mentioned earlier using Clustal X, and dendrograms were constructed using the PHYLIP programs.

Primers TobefiS (5′-CTCCAAGGCTAGGTATGATG-3′) and TobefA (5′-CTTCGTGGTTGCATCTCAAC-3′) were designed from conserved regions of the translation elongation factor 1α (EF-1α) genes of *N. tabacum* and *N. paniculata*. Primers Pr1aS (5′-TGGSATTTRTTCTCTTTTCAC-3′) and Pr1aA (5′-CCTGGAGGATCATAGTTGC-3′) were designed from conserved regions of the *N. tabacum* and *N. glutinosa* acidic PR (pathogenesis-related) 1a genes. Primers Pr2S (5′-CATCACAGGGTTCGTTTAGGA-3′) and Pr2A (5′-GGGTTCTTGTTGTTCTCATCA-3′) were designed from conserved regions of the *N. tabacum*, *Lycopersicon esculentum*, and *Solanum tuberosum* basic PR2 genes. These genes were amplified and cloned as previously described. The identity of these genes was confirmed by sequencing, and they were designated *NbEF-1α*, *NbPR1a*, and *NbPR2*, respectively.

Relative RT-PCR analysis

For relative RT-PCR (reverse transcriptase PCR), the gene of interest was co-amplified with primers TobefiS and TobefA as described by Dean *et al*. (2002) in order to include a constitutively expressed *NbEF-1α* gene as an internal control in each reaction. Primers Gst1S (5′-GATGGCAGAAGTGAAGTTG-3′) and Gst1A (5′-CTCCTAGCCAAAATSCCA-3′) were used to amplify *NbGSTU1*, and primer Gst216iA2 (5′-TAGGCATAAAACCAGCTGTAGT-3′) or primer Gst217iA2 (5′-CATAAGAATAAAACCAACTAGTAA-3′) was paired with primer GST216/7iS (5′-TGAGTACATTGAWGAAGTTTGG-3′) to amplify *NbGSTU2 and NbGSTU3*, respectively. Primer Gst3iS (5′-GGCTTCAAGATTAACCTGGGA-3′) was paired with primer Gst3A (5′-GCCAARATATCAGCACACC-3′) for relative RT-PCR of *NbGSTF1*. The identity of the RT-PCR products was confirmed by direct sequencing.

Quantification of the relative RT-PCR products was performed as described by Dean *et al*. (2002). PCR reactions were performed in 15 μl reactions with 1 μl cDNA from *N. benthamiana*, 0.04 U μl^{-1} *Tth* polymerase (Interscience, Markham, ON), 1× *Tth* polymerase buffer, 2 mM dNTPs, 5.0 mM $MgCl_2$, 0.5 μM EF-1α primers and 1.0 μM GST gene-specific primers. Amplification conditions were as previously described. RNA samples were tested for the presence of genomic DNA as described above. PCR of the cDNA was performed in a GeneAmp PCR System 2400. The relative RT-PCR was repeated three times for each GST gene using different RNA samples from different fungal inoculations for each replication. The data were subjected to analysis of variance, and Fisher's Protected LSD at P=0.05 was used to separate the means.

Silencing of GST genes and effect on susceptibility

To amplify internal fragments from pGST1, pGST2-16, pGST2-17, and pGST3, primer Gst1iA (5′-CTCTTGCTCCTCTCCTTT-3′) was paired with Gst1S, primer Gst216iA (5′-CCAATCAGAGCAATATCCAC-3′) was paired with Gst216/7iS, primer Gst217iA (5′-CCCTTTACTAGTTGGTTTTATT-3′) was paired with Gst216/7iS, and primer Gst3iA (5′-AAGCACTCACACGACGGC-3′) was paired with Gst3S, respectively. These fragments were purified and cloned into pGEMT-easy vectors. They were then digested with *Not*I (MBI Fermentas, Burlington, ON), and cloned into the PVX-based VIGS vector, pGR106, which is a derivative of pGR107 (Jones *et al*., 1999). The pGR106-GST clones were transformed into *E. coli* strain

DH5α and then into *Agrobacterium tumefaciens* strain GV3101 via electroporation. Transformed *A. tumefaciens* was grown at 28 °C on LB agar containing 50 mg l^{-1} kanamycin and 5 mg l^{-1} tetracycline.

At the 6-leaf stage (5–6 cm tall), *N. benthamiana* plants were inoculated using a toothpick at four sites per leaf along the main veins of the two largest leaves. The toothpick inoculum contained *A. tumefaciens* with either a pGR106-GST construct, a pGR106 vector without an insert (PVX-vector control) or water instead of *A. tumefaciens* (water control). After 2 weeks, plants were tested for gene silencing by relative RT-PCR and inoculated with conidial suspensions of *C. orbiculare* (5×10^5 conidia ml^{-1} or *C. destructivum* (1×10^5 conidia ml^{-1}) as described previously. At 96 h post-inoculation (HPI) for *C. orbiculare*-inoculated and 72 HPI for *C. destructivum*-inoculated plants, three or four leaves were collected for lesion counts and to determine fungal biomass, or the tissue was immediately frozen in liquid nitrogen, stored at −80 °C and later used for RT-PCR analysis of silencing. The leaf area was measured using the Leaf Area Meter, Model 3100 (Li-Cor, Lincoln, NE). The biomass of GFP-marked strains of *C. orbiculare* and *C. destructivum* was quantified in the leaves according to Chen *et al.* (2003*a*). These procedures were done two or more times for *C. destructivum* and *C. orbiculare*, and two sets of at least five plants were assessed in each procedure. The data were subjected to analysis of variance, and Fisher's Protected LSD at P=0.05 was used to separate the means.

Results

Cloning of four GST genes from N. benthamiana

Based on alignments of *N. tabacum* GST genes obtained from GenBank, three pairs of degenerate primers were designed in conserved regions to amplify GST fragments from *N. benthamiana* cDNA taken at 72 HPI with *C. destructivum*. Single PCR products of the predicted sizes were cloned and sequenced, and both distance and parsimony analyses of the predicted protein sequences showed that they all belonged to the predicted groups of GST genes (Fig. 1). One cloned gene, designated *NbGSTU1*, was most similar to other members of the *tau* 1 group of GST sequences, and two other clones, designated *NbGSTU2 and NbGSTU3*, were most similar to the *tau* 2 group of GST sequences. A fourth clone, designated *NbGSTF1*, was most similar to the *phi* group of GST sequences. In both distance and parsimony analysis, bootstrap support for groups *zeta* and *tau* was over 99% (Fig. 1). Group *phi* showed greater variation among its members with bootstrap support at 68% and 72% in distance and parsimony analyses, respectively (Fig. 1).

Role of GST in the response to infection by C. destructivum

Relative RT-PCR was done for each of the four GST genes, and sequencing of the RT-PCR products confirmed the specificity of the amplifications for each GST gene using leaf samples infected with *C. destructivum* as well as *C. orbiculare* that is described later. Following infection by *C. destructivum*, the relative expression of *NbGSTU1* increased 5-fold to a maximum at 96 HPI followed by a significant decrease of 30% (Fig. 2). *NbGSTU3* expression increased at a similar rate to *NbGSTU1* up to 96 HPI. However, unlike *NbGSTU1*, *NbGSTU3* expression did not show a significant decrease at 120 HPI. By contrast, expression levels of *NbGSTU2 and NbGSTF1* changed relatively little following infection (Fig. 2). *NbPR1a* expression increased from 24 to 96 HPI and then declined from 96 to 120 HPI (Fig. 3). *NbPR2* expression increased progressively from 0 to 120 HPI when the expression level was five times higher than that prior to inoculation by *C. destructivum* (Fig. 3). The *NbPR2* gene expression was induced faster and showed a greater increase by 120 HPI than any of the GST genes tested.

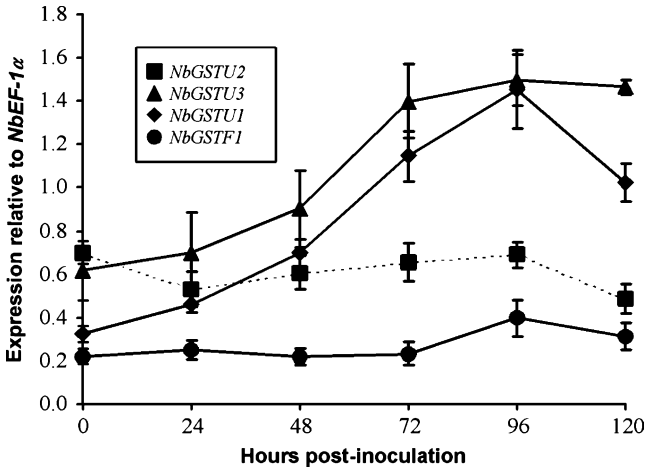

Fig. 2. Relative expression of *NbGSTU1*, *NbGSTU2*, *NbGSTU3*, and *NbGSTF1* following infection of *Nicotiana benthamiana* by *Colletotrichum destructivum*. The relative expression levels after inoculation were calculated by dividing GST band intensity by that of the co-amplified *NbEF-1*α. Standard error bars represent the variation of three relative RT-PCR experiments.

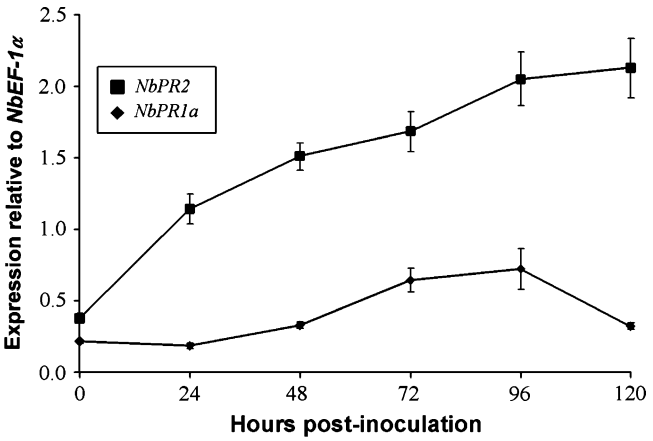

Fig. 3. Relative expression of *NbPR1a* and *NbPR2* in *Nicotiana benthamiana* following infection by *Colletotrichum destructivum*. The relative expression levels after inoculation were calculated by dividing the PR band intensity by that of the co-amplified *NbEF-1*α. Standard error bars represent the variation of three relative RT-PCR experiments.

Table 2. *Expression of the* tau *GSTs of* Nicotiana benthamiana, NbGSTU1, NbGSTU2, *and* NbGSTU3, *in control and GST-silenced plants as determined by relative RT-PCR for leaves of non-inoculated plants (NI) and plants inoculated with* Colletotrichum orbiculare *(CO) or* C. destructivum *(CD)*

Silencing treatment[a]	Relative *NbGSTU1* expression[b]			Relative *NbGSTU2* expression[b]			Relative *NbGSTU3* expression[b]		
	NI	CO	CD	NI	CO	CD	NI	CO	CD
Water	0.56 a	1.14 a	0.94 a	0.92 a	0.99 a	0.65 a	0.66 a	1.14 a	1.33 a
PVX-vector	0.52 ab	0.90 a	0.96 a	0.76 a	0.78 a	0.61 a	0.69 a	1.05 a	1.11 a
GST-silenced	0.27 b	0.43 b	1.05 a	0.29 b	0.31 b	0.32 b	0.41 b	0.63 b	0.64 b

[a] *Nicotiana benthamiana* plants were pin-pricked with *Agrobacterium tumefaciens* containing potato virus X (PVX) in the Ti plasmid without an insert (PVX-vector control) or with a fragment of *NbGSTU1*, *NbGSTU2*, *NbGSTU3*, or *NbGSTF1*, respectively (GST-silenced). Water controls were wounded by toothpick with water instead of *A. tumefaciens*. Plants were grown for 2 weeks after PVX treatment to allow silencing to spread in the plant.

[b] RNA was extracted at the time of conversion from biotrophy to necrotrophy for plants inoculated with *C. orbiculare* (96 h) or *C. destructivum* (72 h). Relative RT-PCR of *NbGSTU1*, *NbGSTU2*, or *NbGSTU3* co-amplified with *NbEF-1*α was done for non-inoculated (0 h) or fungal-inoculated plants, and band intensities were quantified. Each measurement represents the mean relative expression from at least three experiments. Means in the same column followed by a letter in common are not significantly different using Fisher's Protected LSD (P <0.05).

At 0 HPI, prior to fungal inoculation, all the GST genes except *NbGSTU1 in* GST-silence plants showed significant reductions in expression compared with either the water or PVX-vector controls, with average reductions of 57% and 58%, respectively (Tables 2, 3). Although *NbGSTU1*expression was significantly less than the water control, it was not significantly less than the PVX vector control.

For plants inoculated with *C. destructivum*, all the genes showed silencing at 72 HPI except *NbGSTU1*, which appeared not to have any silencing (Table 2). Among the three which showed silencing, *NbGSTF1* had the highest level of silencing with an average 21% expression compared with water and PVX vector controls (Table 2). *NbGSTU2 and NbGSTU3* had expression levels around 50% compared with the controls (Table 2). Cross silencing among the three *tau* GSTs was observed only for expression of *NbGSTU3*, which was at 0.38±0.04 in *NbGSTU2*-silenced plants following inoculation with *C. destructivum*. No cross silencing was observed among the *tau* GSTs when *NbGSTF1*was silenced.

When inoculated with *C. destructivum*, none of the GST-silenced plants had significantly different lesion numbers compared with the PVX-vector control, but the *NbGSTU2*, *NbGSTU3*, and *NbGSTF1*-silenced plants did have significantly more lesions than the water control (Table 4). GFP measurements revealed that all of the infected GST-silenced plants contained a significantly greater biomass of *C. destructivum* than the water control, but none had a significantly greater biomass of *C. destructivum* than the PVX-vector control.

Role of GST in the response to infection by C. orbiculare

Following infection by *C. orbiculare*, the relative expression of *NbGSTU1* progressively increased 10-fold from 0 to 96 HPI before declining slightly at 120 HPI (Fig. 4). The relative expression of *NbGSTU3* also increased at a similar rate to that of *NbGSTU1* throughout the infection, except that it continued to increase at 120 HPI (Fig. 4). Expression of *NbGSTU2* remained relatively unchanged during the infection, except for a small increase at 96 HPI followed by a decrease at 120 HPI. Expression of *NbGSTF1* increased slightly, but continually, throughout the infection cycle, becoming approximately 1.5-fold higher at 120 HPI than at 0 HPI (Fig. 4). By comparison, the expression levels of *NbPR1a* did not increase until between 24–48 HPI, after which the expression remained relatively unchanged (Fig. 5). The increase in *NbPR1a* expression due to *C. orbiculare* infection was less than that of *NbGSTU1* and *NbGSTU3* (Figs 4, 5). The relative expression of *NbPR2* doubled from 0 to 24 HPI but then remained relatively unchanged until it increased again at 96 to 120 HPI, (Fig. 5).

Table 3. *Expression of the* phi *GST of* Nicotiana benthamiana, NbGSTF1, *in control and GST-silenced plants as determined by relative RT-PCR for leaves of non-inoculated plants (NI) and plants inoculated with* Colletotrichum orbiculare *(CO) or* C. destructivum *(CD)*

Silencing treatment[a]	Relative *NbGSTF1* expression[b]		
	NI	CO	CD
Water	0.36 ab	0.41 a	0.48 a
PVX-vector	0.59 a	0.34 a	0.48 a
NbGSTF1-silenced	0.11 b	0.09 b	0.10 b

[a] *Nicotiana benthamiana* plants were pin-pricked with *Agrobacterium tumefaciens* containing potato virus X (PVX) in the Ti plasmid without an insert (PVX-vector control) or with a fragment of *NbGSTF1*. Water controls were wounded by toothpick with water instead of *A. tumefaciens*. Plants were grown for 2 weeks after PVX treatment to allow silencing to spread in the plant.

[b] RNA was extracted at the time of conversion from biotrophy to necrotrophy for plants inoculated with *C. orbiculare* (96 h) or *C. destructivum* (72 h). Relative RT-PCR of *NbGSTF1* co-amplified with *NbEF*-1α was done for non-inoculated (0 h) or fungal-inoculated plants, and band intensities were quantified. Each measurement represents the mean relative expression from at least three experiments. Means in the same column followed by a letter in common are not significantly different using Fisher's Protected LSD (P <0.05).

Table 4. *Fungal biomass and number of lesions cm^{-2} in control and GST-silenced* Nicotiana benthamiana *after inoculation with* Colletotrichum orbiculare *or* C. destructivum

Silencing treatment[a]	*C. orbiculare*[b]		*C. destructivum*[b]	
	Lesions (cm^{-2})	Fungal biomass (cm^{-2}) (RFU)	Lesions (cm^{-2})	Fungal biomass (cm^{-2}) (RFU)
Water	0.32 a	1882 a	0.94 a	962 a
PVX-vector	0.90 a	2372 a	1.68 ab	1720 b
NbGSTU1-silenced	2.08 b	3963 b	1.63 ab	1466 b
NbGSTU2-silenced	1.00 a	2548 a	2.37 b	1868 b
NbGSTU3-silenced	0.71 a	2906 ab	2.34 b	1704 b
NbGSTF1-silenced	0.90 a	2323 a	2.79 b	1736 b

[a] *Nicotiana benthamiana* plants were pin-pricked with *Agrobacterium tumefaciens* containing potato virus X (PVX) in the Ti plasmid without an insert (PVX-vector control) or with a fragment of *NbGSTU1*, *NbGSTU2*, *NbGSTU3*, or *NbGSTF1*. Water controls were wounded by toothpick without any *A. tumefaciens*. Plants were grown for 2 weeks after PVX treatment to allow for silencing to spread.

[b] Plants were inoculated with a GFP-marked strain of *C. orbiculare* or *C. destructivum*, and leaf samples were taken at the time of conversion from biotrophy to necrotrophy (72 h for *C. destructivum*, 96 h for *C. orbiculare*). Number of lesions per leaf was divided by the leaf area to determine the number of lesions cm^{-2} leaf. Fungal biomass was measured as the fluorescence of GFP per leaf divided by the leaf area and is expressed as relative fluorescence units (RFU) cm^{-2} leaf. Each measurement represents the mean from at least three experiments, each involving 10 leaves per treatment. Means in the same column followed by different letters are significantly different using Fisher's Protected LSD (P <0.05).

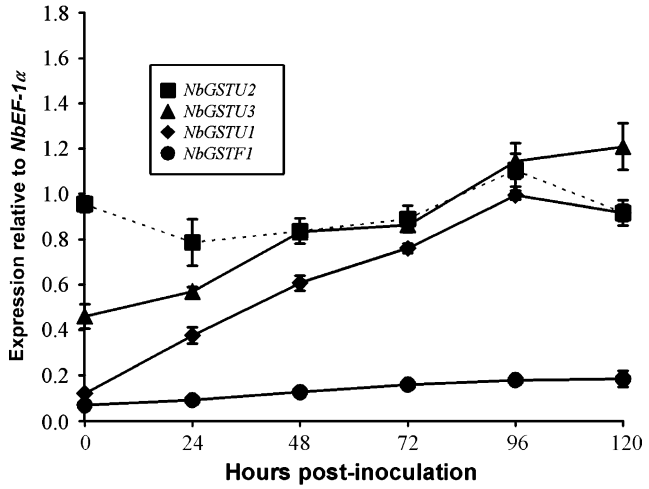

Fig. 4. Relative expression of *NbGSTU1*, *NbGSTU2*, *NbGSTU3*, and *NbGSTF1* following infection of *Nicotiana benthamiana* by *Colletotrichum orbiculare*. The relative expression levels after inoculation were calculated by dividing GST band intensity by that of the co-amplified *NbEF-1α*. Standard error bars represent the variation of three relative RT-PCR experiments.

The level of expression of all of the four GST genes in the silenced plants was significantly reduced compared with the water and PVX vector controls at 96 HPI following infection by *C. orbiculare* (Tables 2, 3). The levels of silencing in the *C. orbiculare*-inoculated plants were quite

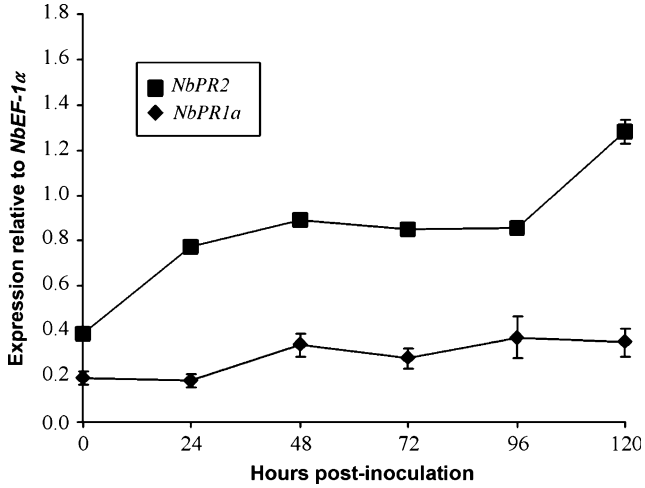

Fig. 5. Relative expression of *NbPR1a* and *NbPR2 in Nicotiana benthamiana* following infection by *Colletotrichum orbiculare*. The relative expression levels after inoculation were calculated by dividing the PR band intensity by that of the co-amplified *NbEF-1α*. Standard error bars represent the variation of three relative RT-PCR experiments.

similar to those prior to inoculation with the greatest level of silencing at approximately 24% of the control levels for *NbGSTF1*, 36% for *NbGSTU2*, 43% for *NbGSTU1*, and 58% for *NbGSTU3* (Tables 2, 3). Some cross silencing among *NbGSTU1*, *NbGSTU2*, and *NbGSTU3* was observed. Expression of *NbGSTU3* was at 0.49±0.08 in *NbGSTU1*-silenced plants, expression of *NbGSTU3* was at 0.59±0.04 in *NbGSTU2*-silenced plants, and expression of *NbGSTU2* was at 0.87±0.16 in *NbGSTU3*-silenced plants following inoculation with *C. orbiculare*. However, no cross silencing was observed for any of the three *tau* GSTs when *NbGSTF1*was silenced.

When inoculated with *C. orbiculare*, *NbGSTU1*-silenced plants had a significantly greater number of lesions compared with both the water and PVX-vector controls (Table 4). However, plants silenced for *NbGSTU2*, *NbGSTU3*, and *NbGSTF1* did not show any altered susceptibility to *C. orbiculare*. As an indicator of the amount of fungal biomass in the *C. orbiculare*-inoculated plants, the amount of GFP expressed by *C. orbiculare* was measured and was also found to be significantly higher in *NbGSTU1*-silenced plants compared with the controls and the other GST-silenced plants (Table 4). None of the other inoculated GST-silenced plants had a greater biomass of *C. orbiculare* than the controls.

Discussion

Based on the response of plant GSTs to stress and chemical treatments, it is believed that GST expression is primarily regulated at the transcriptional level (Dixon *et al.*, 2002). Expression of both *NbGSTU1* and *NbGSTU3* showed a major increase following infection by *C. destructivum*

and *C. orbiculare*. The changes in *NbGSTU1* and *NbGSTU3* expression due to infection were similar to each other, except that *NbGSTU1* expression declined late in both interactions whereas *NbGSTU3* expression did not. The inducibility of *NbGSTU1* and *NbGSTU3* after infection contrasted with the lack of induction of *NbGSTU2* and *NbGSTF1* implying that the first two genes are more likely than the latter two to be involved in the plant response to infection by *C. orbiculare* and *C. destructivum*.

By comparison, expression of both acidic *NbPR1a* and basic *NbPR2* from *N. benthamiana* increased following infection, with *NbPR2* showing a greater increase. The expression of acidic PR1a genes is dependent on the accumulation of salicylic acid, which probably acts as a signalling molecule (Malamy *et al*., 1990), whereas basic PR2 genes are regulated by ethylene, a gaseous signalling molecule that is produced during interactions of various types of pathogens and their plant hosts (Kombrink and Somssich, 1997). Ethylene production in *N. tabacum* was induced by *C. destructivum* infection beginning at 24 HPI, peaking at 48 HPI, and then followed by a second peak at 120 HPI (Chen *et al*., 2003*b*). The rapid production of ethylene may explain the rapid induction of *NbPR2*, *NbGSTU1*, and *NbGSTU3* expression. Ethylene treatment induced the expression of a GST gene, *AtGSTF2*, in *Arabidopsis thaliana* (Smith *et al*., 2003). Although none of the four GSTs followed precisely the pattern of expression of acidic *NbPR1a* or basic *NbPR2*, the patterns of *NbGSTU1* and *NbGSTU3* expression were more similar to that of *NbPR2*, indicating a possible role for ethylene in the induction of those two GST genes.

Following silencing treatment of the four *N. benthamiana* GST genes by VIGS, an examination of gene expression in the silenced plants showed that the four GST genes were always silenced compared with the water and PVX vector controls in the *C. orbiculare*-infected plants, but *NbGSTU1* was not silenced in the *C. destructivum*-infected plants. This may reflect the higher level of infection by *C. destructivum*, which may have disrupted the plant cells more than *C. orbiculare*, thus affecting the metabolic machinery needed for silencing. A higher level of infection could also explain why higher levels of *NbPR1*, *NbPR2*, *NbGSTU1*, and *NbGSTU3* expression were generally observed in the *C. destructivum* versus the *C. orbiculare* interaction.

NbGSTU1-silenced plants inoculated with *C. orbiculare* exhibited an increase in both fungal biomass and the number of lesions compared with the water and PVX vector control plants. Therefore, *NbGSTU1* appeared to play a significant role in the plant response to the pathogen. However, genes with approximately 80% or higher similarity can also be silenced by the same VIGS construct (Baulcombe, 1999). Expression of *NbGSTU3* was also reduced in the *NbGSTU1*-silenced plants following inoculation with *C. orbiculare*, and it is possible that other genes closely related to *NbGSTU1* may also have been silenced, contributing to the altered disease reaction due to the effects of the *NbGSTU1–VIGS* construct. However, silencing of the other three GST genes, which mostly achieved similar or greater degrees of reduced target gene expression without causing cross silencing of *NbGSTU1*, did not show a significant effect on disease susceptibility compared with both the water and PVX vector controls. This demonstrates that not all of the *N. benthamiana* GST genes play a role in disease susceptibility. The most surprising was the lack of an effect of silencing of *NbGSTU3* by VIGS on the susceptibility of the plants to *C. destructivum* or *C. orbiculare* since this gene showed a similar amount of induction as *NbGSTU1* following infection by either fungus. This demonstrates that although induced gene expression may indicate involvement, it still is necessary that gene expression be altered, such as by VIGS during an infection process, to demonstrate the significance of a gene in the plant response to fungal infection.

The lack of a change in disease severity compared with the PVX vector control when *NbGSTU1*-silenced plants were inoculated with *C. destructivum* was surprising considering that *C. destructivum* and *C. orbiculare* both follow an intracellular hemibiotrophic mode of infection. One possible reason for this difference is that there was no significant reduction in *NbGSTU1* transcript levels in *NbGSTU1*-silenced plants inoculated with *C. destructivum*, whereas silencing was observed in the *C. orbiculare*-inoculated plants. These two fungi appear to differ mainly in their manner of biotrophic growth. *C. orbiculare* infects and forms large primary hyphae growing through multiple epidermal cells, while *C. destructivum* forms a multi-lobed infection vesicle, which remains in one epidermal cell (Shen *et al*., 2001*a*, *b*). The infection strategy of *C. orbiculare* may result in a greater amount of host cell stress. Another difference is the greater virulence of *C. destructivum* to *N. benthamiana*, which may overwhelm the plant's defence and stress responses, and therefore *NbGSTU1* may not be able to play an important role in the host response.

Other fungal–plant interactions have shown altered GST expression due to pathogen attack, and a variety of roles have been proposed for the GST genes in the host response. In potato, *Prp1-1*, a *tau* GST, was induced 2 HPI with *Phytophthora infestans* with maximum expression between 48 and 56 HPI (Hahn and Strittmatter, 1994). They speculated that *Prp1-1* was induced during the disease as a result of auxin produced by *P. infestans*, which competitively binds PRP1-1, thereby inhibiting GST function and causing an increased need for GST. In wheat, a *phi* GST, *Gsta1*, was induced dramatically by 2 HPI with *Erysiphe graminis* f. sp. *tritici*, and the expression level remained high for at least 2 d in both compatible and incompatible interactions (Mauch and Dudler, 1993). The proposed function of GSTA1 involved the detoxification of organic peroxides to prevent continuing cell death caused by free radicals produced during the hypersensitive response in the

incompatible interaction. In the compatible interaction, GSTA1 was proposed to detoxify active oxygen species produced as the plant was damaged by the pathogen. After inoculation of *A. thaliana* with a compatible or incompatible strain of *Peronospora parasitica*, higher expression of *phi*, *tau*, and *zeta* GST genes was observed, and these may have roles in restricting cellular damage by functioning in antioxidative reactions (Wagner *et al*., 2002). Treatment of poppy cell suspension cultures with a fungal elicitor extracted from *Botrytis* spp resulted in the induction of a class *phi* GST 1 h after exposure to the elicitor, and the GST was believed to be involved in the translocation or metabolism of phenylpropanoids both as part of the normal developmental physiology and the defence response (Yu and Facchini, 2000).

This report is the first to show that a plant GST gene plays a role in susceptibility to fungal infection. *NbGSTU1*, possibly in conjunction with closely related GST genes, could affect disease development in *N. benthamiana* following infection by *C. orbiculare* in several possible ways. For example, toxins have been reported from several *Colletotrichum* species and have been isolated from mulberry leaves infected by *C. dematium* (Bailey *et al*., 1992; Yoshida *et al*., 2000). GSTs are active in the process of binding of xenobiotics to produce less toxic metabolites, and chickpea GSTs may be involved in detoxifying two toxins produced by the chickpea blight fungus, *Ascochyta rabei* (Hamid and Strange, 2000). Therefore, *NbGSTU1* might act by conjugating and detoxifying toxins produced by *C. orbiculare*. *NbGSTU1* could also possibly reduce infection by *C. orbiculare* by maintaining auxin homeostasis. Auxin production by plant pathogenic fungi may be involved in pathogenesis, and it has been shown that higher auxin production by *C. gloeosporioides* f. sp. *aeschynomene* increases virulence (Cohen *et al*., 2002). A potato GST was competitively bound by auxin produced by *P. infestans* (Hahn and Strittmatter, 1994), and maintenance of auxin homeostasis has been proposed to be the role for the GST genes, *ParB* and *Nt107*, in *N. tabacum* (Droog *et al*., 1995; Takahashi and Nagata, 1992*a*). Many plant GSTs also have glutathione peroxidase activity that detoxifies cytotoxic alkenals and lipid hydroperoxides (Mauch and Dudler, 1993). Gullner and Komives (2001) concluded that although GSTs appear to have a variety of functions in plant metabolism, the most likely role for GSTs in pathogen-infected plants was to suppress necrosis by detoxifying lipid hydroperoxides produced by peroxidation of membranes. This may also apply to the role of GST in limiting *C. orbiculare* infection in *N. benthamiana*.

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References

Bailey JA, O'Connell RJ, Pring RJ, Nash C. 1992. Infection strategies of *Colletotrichum* species. In: Bailey JA, Jeger MJ, eds. Colletotrichum: *biology*, *pathology and control*. Wallingford, UK: CAB International, 88–120.

Baulcombe D. 1999. Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology* **2,** 109–113.

Chen GYJ, Jin S, Goodwin PH. 2000. An improved method for the isolation of total RNA from *Malva pusilla* tissues infected with *Colletotrichum gloeosporioides* f.sp. *malvae*. *Journal of Phytopathology* **148,** 57–60.

Chen N, Hsiang T, Goodwin PH. 2003*a*. Use of green fluorescent protein to quantify the growth of *Colletotrichum* during infection of tobacco. *Journal of Microbiological Methods* **53,** 113–122.

Chen N, Goodwin PH, Hsiang T. 2003*b*. The role of ethylene during the infection of *Nicotiana tabacum* by *Colletotrichum destructivum*. *Journal of Experimental Botany* **54,** 2449–2456.

Cohen BA, Amsellem Z, Maor R, Sharon A, Gressel J. 2002. Transgenically enhanced expression of indole-3-acetic acid confers hypervirulence to plant pathogens. *Phytopathology* **92,** 590–596.

Dean JD, Goodwin PH, Hsiang T. 2002. Comparison of relative RT-PCR and Northern blot analyses to measure expression of β-1,3-glucanase in *Nicotiana benthamiana* infected with *Colletotrichum destructivum*. *Plant Molecular Biology Reporter* **20,** 347–356.

Dixon DP, Lapthorn A, Edwards R. 2002. Plant glutathione transferases. *Genome Biology* **3,** 3004.1–3004.10.

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH. 1992. Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. *The Plant Cell* **4,** 451–461.

Droog F, Hooykaas P, van der Zaal B. 1995. 2,4-Dichlorophenoxyacetic acid and related chlorinated compounds inhibit two auxin-regulated type-III tobacco glutathione *S*-transferases. *Plant Physiology* **107,** 1139–1146.

Edwards R, Dixon D, Walbot B. 2000. Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. *Trends in Plant Science* **5,** 193–198.

Ezaki B, Katsuhara M, Kawamura M, Matsumoto H. 2001. Different mechanisms of four aluminum (Al)-resistant trangenes for Al toxicity in *Arabidopsis*. *Plant Physiology* **127,** 918–927.

Ezaki B, Yamamoto Y, Matsumoto H. 1995. Cloning and sequencing of the cDNAs induced by aluminium treatment and Pi starvation in cultured tobacco cells. *Physiologia Plantarum* **93,** 11–18.

Felsenstein J. 1989. PHYLIP—Phylogeny Inference Package (Version 3.2). *Cladistics* **5,** 164–166.

Gullner G, Komives T. 2001. The role of glutathione and glutathione-related enzymes in plant–pathogen interactions. In: Grill D, Tausz M, Kok L, eds. *Significance of glutathione in plant adaptation to the environment*. Dordrecht, NL: Kluwer Academic Publishers, 207–239.

Hahn C, Strittmatter G. 1994. Pathogen-defense gene *prp1-1* from potato encodes an auxin responsive glutathione *S*-transferase. *European Journal of Biochemistry* **226,** 619–626.

Hamid K, Strange RN. 2000. Phytotoxicity of solanapyrones A and B produced by the chickpea pathogen *Ascochyta rabiei* (Pass.) Labr. and the apparent metabolism of solanapyrone A by chickpea tissues. *Physiologial and Molecular Plant Pathology* **56,** 235–244.

Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe D. 1999. RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *The Plant Cell* **11,** 2291–2301.

Kitamura S, Shikazono N, Tanaka A. 2004. *TRANSPARENT TESTA 19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *The Plant Journal* **37,** 104–114.

Kombrink E, Somssich IE. 1997. Pathogenesis-related proteins and plant defense. In: Carroll GC, Tudzynski P, eds. *The Mycota V*. A. *Plant relationships*. Berlin, DE: Springer-Verlag, 107–128.

Lamb C, Dixon RA. 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 251–275.

Li Z, Alfenito M, Rea P, Walbot V, Dixon R. 1997. Vacuolar uptake of the phytoalexin medicarpin by the glutathione conjugate pump. *Phytochemistry* **45,** 689–693.

Loyall L, Uchida K, Braun S, Furuya M, Frohnmeyer H. 2000. Glutathione and a UV light-induced glutathione *S*-transferase are involved in signalling to chalcone synthase in cell cultures. *The Plant Journal* **25,** 237–245.

Malamy J, Carr JP, Klessig DF, Raskin I. 1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250,** 1002–1004.

Mandanhar J, Hartman G, Sinclair J. 1986. *Colletotrichum destructivum*, the anamorph of *Glomerella glycines*. *Phytopathology* **76,** 282–285.

Marrs K. 1996. The functions and regulation of glutathione *S*-transferases in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 127–158.

Mauch F, Dudler R. 1993. Differential induction of distinct glutathione *S*-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiology* **102,** 1193–1201.

Mueller LA, Goodman CD, Silady RA, Walbot V. 2000. AN9, a petunia glutathione *S*-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. *Plant Physiology* **123,** 1561–1570.

Roxas V, Smith R, Allen E, Allen R. 1997. Overexpression of glutathione *S*-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nature Biotechnology* **15,** 988–991.

Ruiz M, Voinnet O, Baulcombe D. 1998. Initiation and maintenance of virus-induced gene silencing. *The Plant Cell* **10,** 937–946.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. Plainview, NY: Cold Spring Harbor Laboratory Press.

Shen S, Goodwin P, Hsiang T. 2001*a*. Infection of *Nicotiana* species by the anthracnose fungus, *Colletotrichum orbiculare*. *European Journal of Plant Pathology* **107,** 767–773.

Shen S, Goodwin P, Hsiang T. 2001*b*. Hemibiotrophic infection and identity of the fungus, *Colletotrichum destructivum*, causing anthracnose of tobacco. *Mycological Research* **105,** 1340–1347.

Smith AP, Nourizadeh SD, Peer WA, Xu J, Bandyopadhyay A, Murphy AS, Goldsbrough PB. 2003. *Arabidopsis* AtGSTF2 is regulated by ethylene and auxin, and encodes a glutathione *S*-transferase that interacts with flavonoids. *The Plant Journal* **36,** 433–442.

Takahashi Y, Kuroda H, Tanaka T, Machida Y, Takebe I, Nagata T. 1989. Isolation of an auxin-regulated gene cDNA expressed during the transition from G_0 to S phase in tobacco mesophyll protoplasts. *Proceedings of the National Academy of Sciences, USA* **86,** 9279–9283.

Takahashi Y, Nagata T. 1992*a*. *ParB*: an auxin regulated gene encoding glutathione *S*-transferase. *Proceedings of the National Academy of Sciences, USA* **89,** 56–59.

Takahashi Y, Nagata T. 1992*b*. Differential expression of an auxin-regulated gene, *parC*, and a novel related gene, *C-7*, from tobacco mesophyll protoplasts in response to external stimuli and in plant tissues. *Plant and Cell Physiology* **33,** 779–787.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24,** 4876–4882.

van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR. 1991. Promoters of auxin-induced genes from tobacco can lead to auxin-inducible and root tip-specific expression. *Plant Molecular Biology* **16,** 983–998.

van der Zaal E, Memelink J, Mennes A, Quint A, Libbenga K. 1987. Auxin-induced mRNA species in tobacco cell cultures. *Plant Molecular Biology* **10,** 145–157.

Wagner U, Edwards R, Dixon DP, Mauch F. 2002. Probing the diversity of the *Arabidopsis* glutathione *S*-transferase family. *Plant Molecular Biology* **49,** 515–532.

Yoshida A, Hiradate S, Fujii Y, Shiriata A. 2000. *Colletotrichum dematium* produces phytotoxins in anthracnose lesions of mulberry leaves. *Biochemistry and Cell Biology* **90,** 285–291.

Yu M, Facchini PJ. 2000. Molecular cloning and characterization of a type III glutathione *S*-transferase from cell suspension cultures of opium poppy treated with a fungal elicitor. *Physiologia Plantarum* **108,** 101–109.