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

J. D. Dean, P. H. Goodwin* and T. Hsiang

Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 10 October 2004; Accepted 28 February 2005

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.
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, 1992a, 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., 2001a), as well as C. orbiculare, which can also infect Nicotiana spp. (Shen et al., 2001b). Both of these fungi produce intracellular hemibiotrophic infections in N. benthamiana (Shen et al., 2001a, 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.

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., 2003a) 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 2×106 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 PRONTDIST. 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>GenBank accession</th>
<th>GST class</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApIC</td>
<td>P46440</td>
<td>Phi</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>At103-1a</td>
<td>P46421</td>
<td>Tau</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>C-7</td>
<td>CAA45741</td>
<td>Tau</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>CpGSTU</td>
<td>T09781</td>
<td>Tau</td>
<td>Carica papaya</td>
</tr>
<tr>
<td>EeGSTZ</td>
<td>P57108</td>
<td>Zeta</td>
<td>Euphorbia esula</td>
</tr>
<tr>
<td>ERD11</td>
<td>S39541</td>
<td>Phi</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>ERD13</td>
<td>S39542</td>
<td>Phi</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>GnGSTU</td>
<td>T06239</td>
<td>Tau</td>
<td>Glycine max</td>
</tr>
<tr>
<td>GST1</td>
<td>CAC19475</td>
<td>Zeta</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>Gst1rat</td>
<td>NP_445745</td>
<td>Theta</td>
<td>Rattus norvegensis</td>
</tr>
<tr>
<td>Gst2</td>
<td>S35268</td>
<td>Phi</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>GST27</td>
<td>S52037</td>
<td>Phi</td>
<td>Zea mays</td>
</tr>
<tr>
<td>GSTA1</td>
<td>P30111</td>
<td>Phi</td>
<td>Triticum aestivum</td>
</tr>
<tr>
<td>HmGSTF</td>
<td>P46423</td>
<td>Phi</td>
<td>Hyoscyamus muticus</td>
</tr>
<tr>
<td>Hsp26A</td>
<td>P32110</td>
<td>Tau</td>
<td>Glycine max</td>
</tr>
<tr>
<td>NbGSTF1</td>
<td>AY206005</td>
<td>Phi</td>
<td>Nicotiana benthamiana</td>
</tr>
<tr>
<td>NbGSTU1</td>
<td>AY206006</td>
<td>Tau</td>
<td>Nicotiana benthamiana</td>
</tr>
<tr>
<td>NbGSTU2</td>
<td>AY206007</td>
<td>Tau</td>
<td>Nicotiana benthamiana</td>
</tr>
<tr>
<td>NbGSTU3</td>
<td>AY206008</td>
<td>Tau</td>
<td>Nicotiana benthamiana</td>
</tr>
<tr>
<td>Nt103</td>
<td>Q03664</td>
<td>Tau</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>Nt103-1</td>
<td>Q03662</td>
<td>Tau</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>Nt103-35</td>
<td>Q03663</td>
<td>Tau</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>Nt107</td>
<td>Q03666</td>
<td>Tau</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>ParA</td>
<td>P25317</td>
<td>Tau</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>ParB</td>
<td>P30109</td>
<td>Phi</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>ParC</td>
<td>P49332</td>
<td>Tau</td>
<td>Nicotiana tabacum</td>
</tr>
<tr>
<td>PGST2</td>
<td>AF118925</td>
<td>Tau</td>
<td>Papaver somniferum</td>
</tr>
<tr>
<td>Prp1-1</td>
<td>T07595</td>
<td>Tau</td>
<td>Solanum tuberosum</td>
</tr>
<tr>
<td>SR8</td>
<td>P28342</td>
<td>Zeta</td>
<td>Dianthus Caryophyllus</td>
</tr>
<tr>
<td>TA-GSTZ1</td>
<td>T06333</td>
<td>Zeta</td>
<td>Triticum aestivum</td>
</tr>
</tbody>
</table>

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

Based on the groups of sequences observed in the protein alignment, nucleotide sequences were selected for further analysis and primer design. Primers Gst1S (5'-GATTGCGACAAGGATGATTG-3') and Gst1A (5'-CTCTAGGCCAAAATSCCA-3') were designed based on N. tabacum GSTs in cluster tau 1 (Fig. 1). Primers Gst2S (5'-YTRSRATGAYCCWRTY-3') and Gst2A (5'-SAGSWRRG-ACCTTGMRAC-3') were designed based on N. tabacum GSTs in cluster tau 2 (Fig. 1). Primers Gst3S (5'-CTGKKGAWCACAAGAGS-3') and Gst3A (5'-GCCAARATATCAGCACAC-3') were designed based on N. tabacum GSTs in cluster phi (Fig. 1). Degenerate bases are coded as follows: Y=C 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 Mg2+, and 0.5 μM primers. RNA was extracted according to Chen et al. (2000) and reverse transcribed to cDNA using Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Burlington,
Glutathione S-transferases and fungal resistance

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.

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 GST genes of *N. benthamiana* 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.

**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 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.

**Silencing of GST genes and effect on susceptibility**

To amplify internal fragments from pHST1, pHST2-16, pHST2-17, and pHST3, primer Gst1S (5’-CTCTGTTGGTTGCAATCTCAAC-3’) were designed from conserved regions of the translation elongation factor 1α (EF-1α) genes of *N. tabacum* and *N. paniculata*. Primers Pr1S (5’-TGG-SATTTRTTCTCTTTTTCAC-3’) and Pr1A (5’-CCTGGAAGATTCAATCTCAAC-3’) were designed from conserved regions of the *N. tabacum* and *N. glutinosa* acidic PR (pathogenesis-related) 1a genes. Primers Pr2S (5’-CATCACAGGCTTTTGAAGCTT-3’) and Pr2A (5’-GGGGTCAGTGGTTTGCATCTCAAC-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, NbPR1α, 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’-GATGGCGAGAAGTAGTTG-3’) and Gst1A (5’-CTCCATGCGGAAAATSCCA-3’) were used to amplifyNbGSTU1, and primer Gst216iA2 (5’-TGGGCTAATAACCCAGCTTGATG-3’) or primer Gst217iA2 (5’-CATCAGATAATACCCAGCAGCTGAA-3’) was paired with primer GST216/7iS (5’-TGATACATTGGAAGTGTGG-3’) to amplifyNbGST2 and NbGSTU3, respectively. Primer Gst3S (5’-GGCTCAAGATACCCGGA-3’) was paired with primer Gst3A (5’-GCCAARATATACGACACG-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 Tth polymerase (Interscience, Markham, ON), 1× Tth polymerase buffer, 2 mM dNTPs, 5.0 mM MgCl2, 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 pHST1, pHST2-16, pHST2-17, and pHST3, primer Gst1iA (5’-CTCTGTTGGTTGCAATCTCAAC-3’) were designed from conserved regions of the translation elongation factor 1α (EF-1α) genes of *N. tabacum* and *N. paniculata*. Primers Pr1S (5’-TGG-SATTTRTTCTCTTTTTCAC-3’) and Pr1A (5’-CCTGGAAGATTCAATCTCAAC-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, NbPR1α, 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’-GATGGCGAGAAGTAGTTG-3’) and Gst1A (5’-CTCCATGCGGAAAATSCCA-3’) were used to amplifyNbGSTU1, and primer Gst216iA2 (5’-TGGGCTAATAACCCAGCTTGATG-3’) or primer Gst217iA2 (5’-CATCAGATAATACCCAGCAGCTGAA-3’) was paired with primer GST216/7iS (5’-TGATACATTGGAAGTGTGG-3’) to amplifyNbGST2 and NbGSTU3, respectively. Primer Gst3S (5’-GGCTCAAGATACCCGGA-3’) was paired with primer Gst3A (5’-GCCAARATATACGACACG-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 Tth polymerase (Interscience, Markham, ON), 1× Tth polymerase buffer, 2 mM dNTPs, 5.0 mM MgCl2, 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.
DH5a 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 (5×10⁷ conidia ml⁻¹) or C. destructi-
vum (1×10⁵ conidia ml⁻¹) as described previously. At 96 h post-
imoculation (HPI) for C. orbiculare-inoculated and 72 HPI for C. destructivuminoculated 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. (2003a). 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.
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. *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).
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 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 (Kombrik 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.*, 2003b). 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 some 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*-silent 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.*, 2001a, 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 *Phtophthora 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 development of membranes. This may also apply to the role of GST in limiting C. orbiculare infection in N. benthamiana.

Acknowledgement

This work was supported by the Natural Sciences and Engineering Research Council of Canada.

References


