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Colletotrichum gloeosporioides infection induces differential expression of glutathione S-transferase genes in *Malva pusilla*

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Abstract. Among a collection of 840 expressed sequence tags of *Malva pusilla* leaves infected with *Colletotrichum gloeosporioides* f. sp. *malvae* (Cgm), a total of four different glutathione S-transferase (GST) (EC 2.5.1.18) genes were identified, each showing a different pattern of expression following infection. *MpGSTU1* and *MpGSTU2* were members of the class tau GSTs, *MpGSTF1* was a member of the class phi GSTs, and *MpGSTZ1* belonged to the class zeta GSTs. Infection by Cgm occurs by a hemibiotrophic process with an initial biotrophic phase preceding the necrotrophic phase and the appearance of symptoms. Expression of *MpGSTZ1* progressively increased during infection, corresponding directly with the growth of the pathogen. Expression of *MpGSTU2* was similar to that of *MpGSTZ1*, except for a greater increase during the late necrotrophic phase. *MpGSTU1* expression remained relatively constant throughout the infection, whereas *MpGSTF1* expression was induced primarily during the conversion from the biotrophic to necrotrophic phases of infection. Incubation of healthy mallow leaves in the dark resulted in decreased expression of *MpGSTF1* and *MpGSTU1*, but not *MpGSTZ1* and *MpGSTU2*. The differential expression patterns indicate that these mallow GST genes play a variety of roles in healthy and fungal-infected leaf tissue.

The nucleotide sequences reported in this paper have been submitted to GenBank under the accession numbers AY206003, AY206001, AY206002, AY206000 and AY205999.

Keywords: glutathione S-transferase; hemibiotrophy.

Introduction

Glutathione S-transferases (GSTs) are enzymes best known for their ability to conjugate glutathione (GSH) to electrophilic molecules, and in plants, this is followed by sequestration of the conjugates into vacuoles where they are further metabolized (Cole and Edwards 2000). Several functions of GSTs in plant metabolism have been proposed, including catalysis of GSH conjugation reactions with certain plant products, binding and transport of phytochemicals between cellular compartments, and catalysis of GSH-dependent biotransformation reactions (Edwards *et al.* 2000; Dixon *et al.* 2002).

Plant GSTs have been divided into four groups, phi, tau, zeta and theta, based on sequence homology. The two largest GST groups, phi and tau, are found exclusively in plants and catalyse GSH-dependent detoxification and peroxidation reactions or function as ligandins (Edwards *et al.* 2000). The zeta and theta GST groups also have conserved GSH-dependent activities but do not involve the conjugation of GSH (Droog 1997). Zeta GSTs catalyse isomerase reactions, whereas theta GSTs likely function as peroxidase enzymes (Edwards *et al.* 2000).

Although phi and tau GSTs are well known for their role in the detoxification of xenobiotics, such as herbicides (Edwards *et al.* 2000), little is known about any GSTs in plant–pathogen interactions. GSTs may detoxify organic peroxides, which are highly reactive molecules that can be produced during pathogen attack (Mauch and Dudler 1993). Hahn and Strittmatter (1994) proposed that GSTs might be induced in plants as a result of increased levels of auxins produced by fungal pathogens. Thus far, GSTs have been found to be induced following infection of potato by *Phytophthora infestans*, wheat by *Erysiphe graminis* f. sp. *hordei* and *Arabidopsis* by *Peronospora parasitica* (Mauch and Dudler 1993; Hahn and Strittmatter 1994; Wagner *et al.* 2002). These are all pathogens that produce haustoria as feeding structures, and primarily or exclusively feed biotrophically (i.e. on living host cells).

Colletotrichum gloeosporioides f. sp. *malvae* (Cgm) was developed as a fungal biocontrol agent (Makowski and

Abbreviations used: Cgm, *Colletotrichum gloeosporioides* f. sp. *malvae*; ESTs, expressed sequence tags; GSH, glutathione; GST, glutathione S-transferase; HPI, hours post-inoculation.

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Mortensen 1992) for controlling the weed, *Malva pusilla* (round-leaved mallow, referred to here as mallow). Cgm has an intracellular hemibiotrophic infection strategy that involves the penetration of host tissue by an appressorium with an infection peg, followed by the development of large primary hyphae, which grow biotrophically within the epidermal cells (Wei *et al.* 1997). At approximately 72 h post-inoculation (HPI), Cgm develops thinner secondary hyphae, which feed necrotrophically (i.e. on host cells previously killed by the pathogen), and disease symptoms appear (Wei *et al.* 1997).

The molecular basis of this interaction is being examined (Goodwin 2001). A cDNA library was constructed from Cgm-infected mallow at 72 HPI (Jin *et al.* 1999). At this point in the interaction, the infection is in the process of switching from the biotrophic to the necrotrophic phase (Wei *et al.* 1997). Expressed sequence tags (ESTs) were obtained from 840 clones, and seven of these showed homology with plant GSTs. To determine if the expression of one or more of these mallow GST genes was affected during infection by Cgm, they were characterized and their expression levels were observed by relative reverse transcriptase polymerase chain reaction (RT–PCR), which provides accuracy comparable to northern blots, but because it is based on PCR, RT–PCR is much more sensitive and requires much less RNA (Dean *et al.* 2002).

Materials and methods

Biological materials and inoculations

The Cgm isolate, Biomal™, and mallow seeds were provided by Dr K. Mortensen (Agriculture and Agri-Food Canada, Regina, SK). The fungus was cultured on potato dextrose agar (Difco Laboratories, Detroit, MI, USA) at 20°C under continuous fluorescent lighting. *Malva pusilla* plants were grown as previously described (Wei *et al.* 1997), and inoculated with conidia as described by Shih *et al.* (2000) after a 24-h pre-incubation in darkness.

Sequence analyses of round-leaved mallow GST genes

The mallow GSTs were aligned with 18 other GST protein sequences with ClustalX (Thompson *et al.* 1997). Seventeen of these sequences were from various plant species and were chosen on the basis of their high similarity to the mallow GSTs or because they were known to be pathogen-induced (Table 1). The outgroup was from *Rattus norvegensis*. Maximum parsimony analysis was conducted using PHYLIP programs (Felsenstein 1989). A dendrogram was generated using PHYLIP PROTPARS, and bootstrap analysis (PHYLIP SEQBOOT and CONSENSE) was used to provide confidence limits on tree topology. The dendrogram was produced using PHYLIP DRAWGRAM with modifications in Corel Presentations.

RNA extraction and RT–PCR

Mallow leaf tissue was excised and frozen at –80°C. RNA was extracted from the tissues using the RNeasy kit (Qiagen, Mississauga, ON, Canada), according to the manufacturer's instructions. In a 20-µL reaction, 1 µg total RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Burlington, ON, Canada) into cDNA as recommended by the manufacturer, and stored at –20°C until needed.

Table 1. Source of glutathione S-transferase proteins used in dendrogram (Fig. 1)
Glutathione S-transferase classification based on sequence similarity and intron location following Edwards *et al.* (2000). Selection of sequences for this study was based on S = similarity to sequences from *Malva pusilla* in this study, P = pathogen-induced, or E = elicitor-induced

Name	GenBank accession	GST class	Species	Reference (selection)
AtGSTF2	CAA53051	phi	*Arabidopsis thaliana*	Wagner *et al.* 2002 (P)
AtGSTF6	BAA04553	phi	*A. thaliana*	Wagner *et al.* 2002 (P)
AtGSTU19	CAA10060	tau	*A. thaliana*	Wagner *et al.* 2002 (P)
AtGSTZ1	AAG30131	zeta	*A. thaliana*	Wagner *et al.* 2002 (P)
CpGSTU1	T09781	tau	*Carica papaya*	Direct submission (S)
DcSR8	P28342	zeta	*Dianthus caryophyllus*	Meyer *et al.* 1991 (S)
EeGSTZ1	P57108	zeta	*Euphorbia esula*	Direct submission (S)
Gm24D	T06239	tau	*Glycine max*	Direct submission (S)
GmHSP26A	P32110	tau	*G. max*	Czarnecka *et al.* 1988 (S)
GST-RAT	NP445745	theta	*Rattus norvegensis*	Pemble and Taylor 1992
HmGSTU1	P46423	phi	*Hyoscyamus muticus*	Bilang *et al.* 1993 (S)
MpGSTF1	AY206005	phi	*Malva pusilla*	This study
MpGSTU1	AY206001	tau	*M. pusilla*	This study
MpGSTU2	AY206002	tau	*M. pusilla*	This study
MpGSTZ1	AY206003	zeta	*M. pusilla*	This study
NtParA	P25317	tau	*Nicotiana tabacum*	Takahashi *et al.* 1989 (S)
NtparB	BAA01394	phi	*N. tabacum*	Takahashi and Nagata 1992*a* (S)
NtparC	CAA45740	tau	*N. tabacum*	Takahashi and Nagata 1992*b* (S)
PsGST2	AF118925	tau	*Papaver somniferum*	Yu and Facchini 2000 (E)
StGST1	T07595	tau	*Solanum tuberosum*	Hahn and Strittmatter 1994 (P)
TaGSTA1	P30111	phi	*Triticum aestivum*	Dudler *et al.* 1991 (P)
TaGSTZ1	T06333	zeta	*T. aestivum*	Subramaniam *et al.* 1999 (S)

PCR of the cDNA was performed in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). The 15-μL reactions contained 1 μL cDNA, 0.6 U *Tsg* polymerase (Biobasic, Toronto, ON, Canada), 1 × *Tsg* polymerase buffer, 2 mM dNTPs, and 2.5 mM Mg^{2+}, with 0.5 μM elongation factor-1 primers and one pair of 1.0 μM GST primers (Table 2). Except for EST 578, amplification was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension period at 72°C for 10 min. The PCR conditions for EST 578 were the same except for an annealing temperature of 58°C. All RNA samples to be used for reverse transcription were tested for the presence of genomic DNA by using them as the PCR template, prior to cDNA synthesis, under the same PCR conditions.

Products of RT–PCR were separated in 1.4% agarose gels, scanned and saved as TIF files (Tagged Image File Format) for quantification using NIH image (Scion Corporation, Frederick, MD, USA). The optical density was determined for both the GST and elongation factor-1 bands for each gel lane, and the GST expression value was determined from a ratio of the GST band intensity to the elongation factor-1 band intensity (Dean *et al.* 2002). Linear regression was applied to the data using the MINITAB program (State College, PA, USA) and then these values were pooled to generate a graph. All RT–PCR results are an average from three separate experiments with different RNA preparations.

Results

Identification of mallow GST genes

BlastX analysis with the GenBank non-redundant database of 840 ESTs from Cgm-infected mallow leaves at 72 HPI was used to identify ESTs 199, 287, 442, 443, 577, 578 and 695 as putative GST genes and EST 354 as a putative translation elongation factor-1α gene. The sequences of ESTs 442, 443 and 695 were identical, as were those of ESTs 577 and 578. Using the nomenclature proposed by Edwards *et al.* (2000), ESTs 199, 287, 442 and 578 were designated *MpGSTZ1*, *MpGSTU1*, *MpGSTU2* and *MpGSTF1*, respectively (GenBank accession nos. AY206003, AY206001, AY206002, AY206000, respectively). EST 354 was designated as *MpEF-1α* (GenBank accession no. AY205999). *MpGSTZ1*, *MpGSTU1*, *MpGSTU2* encode complete proteins of 218, 219 and 219 amino acids in length, respectively, with predicted masses of 24.8, 25.2 and 25.3 kDa, respectively. The *MpGSTF1* sequence encodes for only 166 amino acids, and based on a comparison with GST27 from *Zea mays* (GenBank accession no. S52037), the predicted MpGSTF1 protein sequence appeared to be missing approximately the first 55 amino acids.

Table 2. Sequences of the primers used for RT–PCR analysis and their PCR fragment sizes

Primer name	Sequence	Gene	Fragment size (base pairs)
S2-199	ctcttgctcctaccgtatca	*MpGSTZ1*	393
A-199	ctcaagcgcatcaaagcc		
S-287	ggattgctttggctgaga	*MpGSTU1*	283
A-287	ccatactctgctcccttgtt		
S-442	cctctgcttccttccgat	*MpGSTU2*	413
A-442	gttccctccaagtttctc		
S-578	tgtggagggaagtggaagc	*MpGSTF1*	256
A-578	cttcttggacggtgtggc		
S-353	atggatgcttgctacccc	*MpEF-1α*	714
A-353	catcttcaccattcctgc		

GST sequence analysis

Parsimony analysis of the predicted proteins (Fig. 1) placed MpGSTZ1 in a clade with four other GST genes with 100% bootstrap support; these genes included DsSR8 and TaGSTZ1, which have both been previously identified as zeta GST genes (Droog 1997; Subramaniam *et al.* 1999). MpGSTF1 was found in a clade with five other GSTs with 100% bootstrap support (Fig. 1), including AtGSTF6 (= ERD11) and NtParB, which have been previously classified as phi GST genes (Droog 1997). Both MpGSTU2 and MpGSTU3 were placed in a clade with a group of eight other GST genes with 100% bootstrap support, including NtParA, NtParC and StGST1, which have all previously been classified as phi GST genes (Droog 1997).

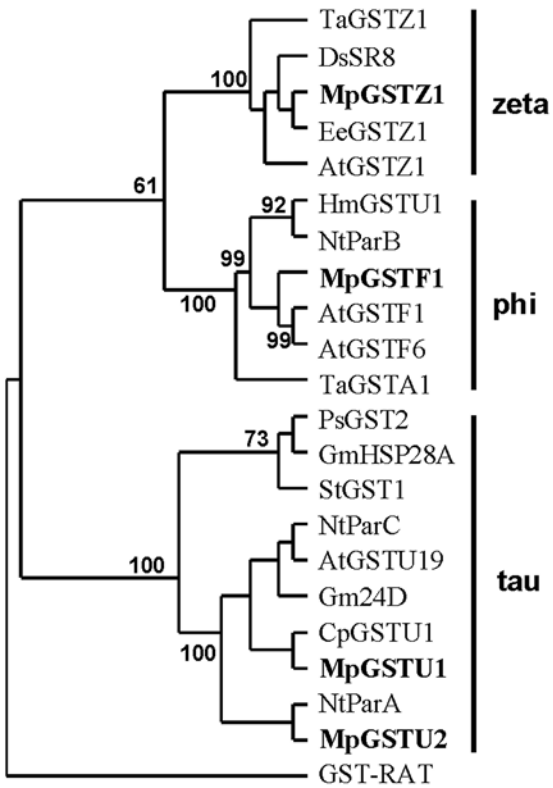

Fig. 1. Relationships between 21 plant GST proteins from different GST classes, inferred by maximum parsimony analysis. *Rattus norvegensis* gst1 (GST–RAT) was included as an outgroup. All GST proteins analysed are listed in Table 1. In this majority-rule consensus tree, bootstrap values over 70% (out of 1000 bootstrap replications) are shown on the major tree branches. Sequences produced in this study are shown in bold.

RT–PCR analysis of gene expression

Amplification of *MpGSTZ1*, *MpGSTU1*, *MpGSTU2*, *MpGSTF1* and *MpEF-1α* from cDNA from healthy and infected leaf tissue resulted in PCR fragments of the predicted sizes (Figs 2–5). Relative quantification of GST gene expression was determined by including a constitutively-expressed internal control, *MpEF-1α*, in each relative RT–PCR to normalize the GST expression data (Dean *et al.* 2002).

Relative RT–PCR analysis of *MpGSTF1* showed that its expression was reasonably constant until 48 HPI and then increased 5-fold, to a peak at 72 HPI (Fig. 2). This was followed by a 50% decrease in relative expression at 96 HPI. An R^2 value of 0.59 (P<0.05) between expression and time was calculated from 0–72 HPI, reflecting a significant increase in expression as a result of Cgm infection. *MpGSTU1* relative expression remained constant throughout the infection process (Fig. 3), unlike those of *MpGSTF1*, *MpGSTU2* or *MpGSTZ1* (Figs 2, 4, 5), which all started at relatively low levels, but then showed higher relative levels between 72–120 HPI. A linear regression analysis of *MpGSTU1* expression and HPI revealed an R^2 value near zero, suggesting that there was no change in *MpGSTU1* transcript levels as a result of Cgm infection. *MpGSTU2* expression remained relatively low until 72 HPI, after which expression increased rapidly, particularly from 96–120 HPI (Fig. 4). An R^2 value of 0.64 (P<0.05) suggested that the increase in *MpGSTU2* expression was associated with the Cgm infection. *MpGSTZ1* relative expression increased slightly but steadily up to 72 HPI, followed by a rapid increase at 96 HPI, then remained at the same level up to 120 HPI (Fig. 5). Linear regression analysis of *MpGSTZ1* expression against HPI produced a significant R^2 value of 0.803 (P<0.05). A comparison of the relative levels of *MpGSTZ1* expression with Cgm *actA* expression, which has been used to measure the biomass of the fungus during infection (Jin *et al.* 1999), showed that the increase in *MpGSTZ1* expression correlated closely with the *actA* expression (Fig. 6), with an R^2 value of 0.80 (P<0.001).

Among the four genes, only *MpGSTU2* showed a large and significant increase in expression at 120 HPI, when most of the mallow tissue was dead or damaged. The rapidly senescing state of the mallow leaves by that time made RNA extraction difficult and was probably the reason why the largest standard errors in relative expression were found at 120 HPI for all four genes.

Because expression of some GSTs has also been reported to be induced by UV light (Loyall *et al.* 2000), relative RT–PCR of the GST expression levels was performed after plants were placed in darkness for 0, 2 or 24 h (Fig. 7). The expression of both *MpGSTZ1* and *MpGSTU2* remained unchanged following transfer to the dark, whereas expression of *MpGSTU1* and *MpGSTF1* decreased by approximately 50% and 75%, respectively, when plants were placed in the dark for 2 h, but there was no further change in their expression from 2 to 24 h.

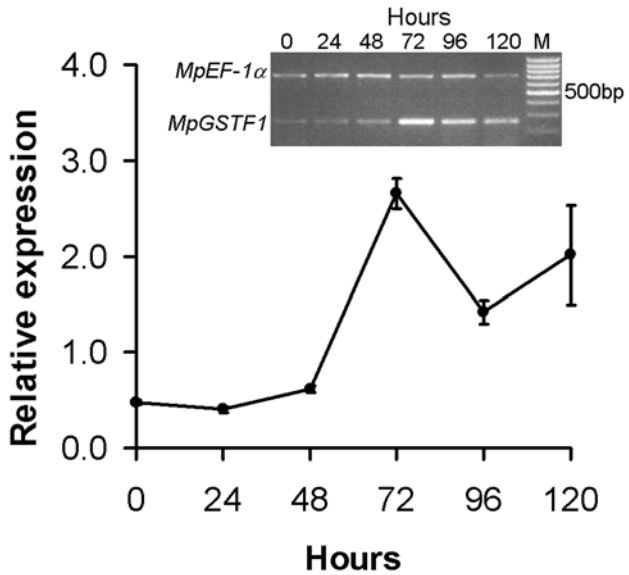

Fig. 2. Relative expression of *MpGSTF1* following infection of *Malva pusilla* by *Colletotrichum gloeosporioides* f. sp. *malvae*. The relative expression levels at 0, 24, 48, 72, 96 and 120 h post-inoculation were calculated by dividing the band intensity for GST by that of the co-amplified *MpEF-1α.* Standard error bars represent the variation of three relative RT–PCR experiments. Inset shows representative picture of gel bands for *MpGSTF1* (256 bp) and *MpEF-1α* (370 bp). M = 100 base pair ladder.

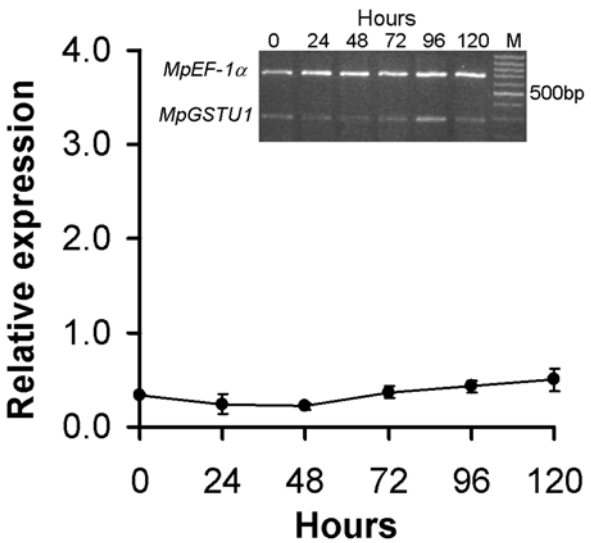

Fig. 3. Relative expression of *MpGSTU1* following infection of *Malva pusilla* by *Colletotrichum gloeosporioides* f. sp. *malvae*. The relative expression levels at 0, 24, 48, 72, 96 and 120 h post-inoculation were calculated by dividing the band intensity for GST by that of the co-amplified *MpEF-1α.* Standard error bars represent the variation of three relative RT–PCR experiments. Inset shows representative picture of gel bands for *MpGSTU1* (283 bp) and *MpEF-1α* (370 bp). M = 100 base pair ladder.

Discussion

EST libraries are useful not only to discover new genes, but they can also provide information on the level of gene expression because they represent a sample of the genes being expressed at a particular time (Ohlrogge and Benning 2000). Multiple GST sequences have been identified in soybean infected with *Sclerotinia sclerotiorum*, *Brassica napus* infected with *Leptosphaeria maculans*, and wheat spikes infected by *Fusarium graminearum* (Fristensky *et al.* 1999; McGonigle *et al.* 2000; Kruger *et al.* 2002). In the mallow–Cgm interaction described here, seven GST sequences were found in a collection of 840 ESTs. The repeated appearance of multiple GST sequences in these different EST collections suggests that GSTs are commonly induced in plants following fungal infection.

Although GSTs in plants are well known for their ability to detoxify xenobiotics, such as herbicides (Droog 1997), little is known about other functions of plant GSTs. In addition to being induced by plant pathogens, GST genes are also induced by auxin, cytokinin, salicylic acid, ethylene, hydrogen peroxide, temperature, dehydration and salt, suggesting that they have a wide diversity of functions (Dixon *et al.* 2002).

Although GSTs are divided into several classes, putative GST function is only weakly correlated with GST classification (McGonigle *et al.* 2000). There appears to be no major functional differences between the tau and phi GST groups, but some zeta GSTs appear to have a unique function in tyrosine catabolism (Dixon *et al.* 2000). Because plant GSTs are induced by a variety of stress stimuli, it has been speculated that they may act as signaling molecules (Loyall *et al.* 2000) or as glutathione peroxidases, which reduce organic hydroperoxides of fatty and nucleic acids (Dixon *et al.* 2002). Organic hydroperoxides are created in plants during processes such as photosynthesis (Creissen *et al.* 1999), 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 hydroperoxides will be converted to cytotoxic derivatives. It has also been suggested that GSTs can act as carrier proteins for auxin and phenylpropanoids, such as flavonoids (Droog *et al.* 1995; Mueller *et al.* 2000), as well as enzymes in the catabolism of tyrosine (Dixon *et al.* 2000).

Several plant GST genes have been reported to be induced by fungal pathogens. Expression of *prp1-1*, a tau GST from potato, was induced by *P. infestans* within 2 HPI, reaching a maximum expression between 48 and 56 HPI, and then remained high during the remainder of the infection (Hahn and Strittmatter 1994). Hahn and Strittmatter (1994) speculated that *prp1-1* was induced during disease as a result of auxin produced by *P. infestans*; auxin competitively binds PRP1-1, thereby inhibiting GST function. For wheat infected with *E. graminis* f. sp. *tritici*, a phi GST, *Gsta1*, was also induced dramatically by 2 HPI, and the expression level remained high for at least 2 d in both compatible and incompatible interactions (Mauch and Dudler 1993). GSTa1

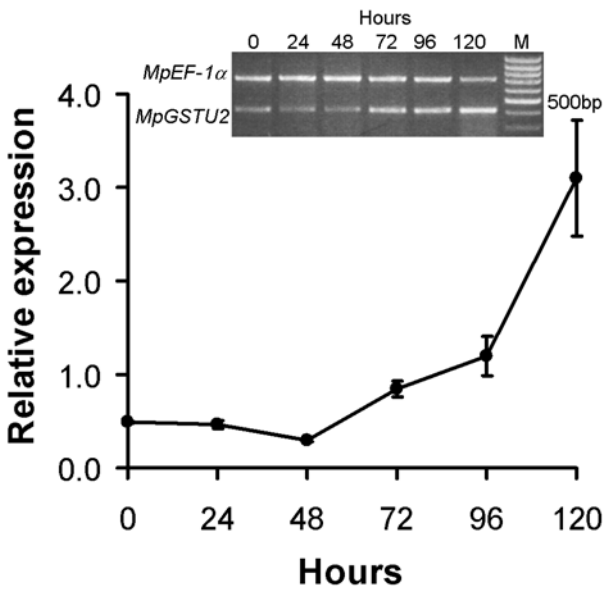

Fig. 4. Relative expression of *MpGSTU2* following infection of *Malva pusilla* by *Colletotrichum gloeosporioides* f. sp. *malvae*. The relative expression levels at 0, 24, 48, 72, 96 and 120 h post-inoculation were calculated by dividing the band intensity for GST by that of the co-amplified *MpEF-1α*. Standard error bars represent the variation of three relative RT–PCR experiments. Inset shows representative picture of gel bands for *MpGSTU2* (413 bp) and *MpEF-1α* (370 bp). M = 100 base pair ladder.

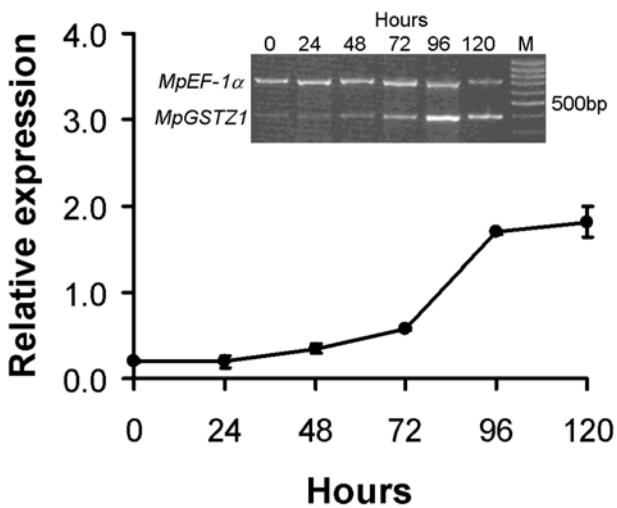

Fig. 5. Relative expression of *MpGSTZ1* following infection of *Malva pusilla* by *Colletotrichum gloeosporioides* f. sp. *malvae*. The relative expression levels at 0, 24, 48, 72, 96 and 120 h post-inoculation were calculated by dividing the band intensity for GST by that of the co-amplified *MpEF-1α*. Standard error bars represent the variation of three relative RT–PCR experiments. Inset shows representative picture of gel bands for *MpGSTZ1* (393 bp) and *MpEF-1α* (370 bp). M = 100 base pair ladder.

was proposed to function in the detoxification of organic peroxides produced during infection. Among 10 GST genes of *A. thaliana* tested, one zeta, one tau and two phi GST genes showed increased expression by 3 d after inoculation in a susceptible interaction with *P. parasitica*, whereas only the tau and zeta GST genes were transiently induced in a resistant interaction 2 d after inoculation (Wagner *et al.* 2002). These genes were believed to be involved in the detoxification of oxidative stress products.

The infection of mallow by Cgm occurs very differently from those of *P. infestans*, *E. graminis* f. sp. *tritici* or *P. parasitica* The latter are all biotrophic pathogens, which produce specialized structures, known as haustoria, for feeding upon living plant cells (Agrios 1997). If necrosis occurs, then it appears after the pathogen has already fed biotrophically for a period of time (Agrios 1997). However, Cgm is an intracellular hemibiotrophic pathogen with only the early stages of infection involving biotrophy, but without the production of haustoria (Bailey *et al.* 1992). In the later stages of infection, necrotrophy occurs with host tissue being killed prior to invasion and feeding by the pathogen. Therefore, host responses could be very different for tissues infected with Cgm compared with haustoria-producing fungi.

MpGSTZ1 expression increased only slightly until the end of the biotrophic phase but then greatly increased at the time of conversion to necrotrophy at 72–96 HPI and remained relatively high in the necrotrophic phase. These changes were very similar to the expression of the Cgm *actA* gene, which has been used to measure fungal growth during infection (Jin *et al.* 1999). This suggests that *MpGSTZ1* expression was directly responding to the amount of stress occurring at all times during infection, when the fungus grew both biotrophically and necrotrophically. Therefore, it appears that as each host cell became affected by Cgm, *MpGSTZ1* expression was induced, regardless of whether the fungus fed upon living host cells or the fungus killed and then fed upon the dead host cells. One explanation for this could be that the growth of the fungus always resulted in the creation of active oxygen species and thus organic hydroperoxides were continually being produced (Mauch and Dudler 1993).

Similar to *MpGSTZ1*, expression of *MpGSTU2* also increased as the fungus grew, but this increase occurred later than that of *MpGSTZ1*, and was still increasing at 120 HPI when most of the tissue was dead or damaged. The similarity in the patterns of expression of *MpGSTU2* and *MpGSTZ1* following inoculation indicates that these genes may be responding to the same effects of the fungus on the host cells. The similarity in the response of *MpGSTU2* and *MpGSTZ1* following dark treatment also suggests that the two genes may be associated with similar processes. However, expression of *MpGSTU2* did differ from that of *MPGSTZ1* by continuing to increase late in the interaction (i.e. 120 HPI) when leaf tissue was still undergoing necrosis but fungal growth, as indicated by *actA* expression, had slowed.

MpGSTF1 expression increased faster in the late biotrophic phase than *MpGSTZ1* or *MpGSTU2*, but this was transitory with a decline in expression as necrotrophy developed. Therefore, the expression of this gene appears to be related to changes to host cells as the fungus converts from biotrophy to necrotrophy. Among the four mallow GSTs, the expression of *MpGSTF1* relative to the *MpEF-1α* showed the highest peak. In contrast, the relative expression of *MpGSTU1* was much lower than any of the other mallow

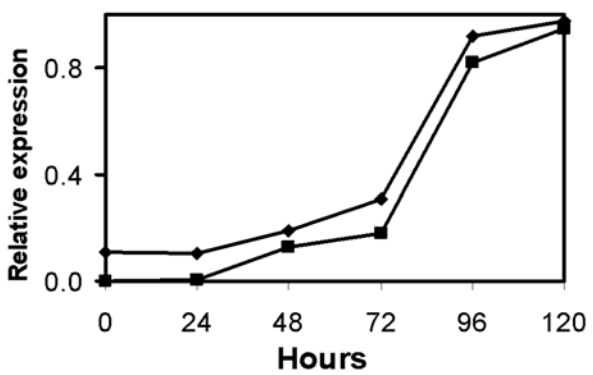

Fig. 6. A comparison of the expression pattern of *MpGSTZ1* with *Colletotrichum gloeosporioides* f. sp. *malvae* biomass during infection as determined by the level of the fungal *actA* gene (Jin *et al.* 1999). *MpGSTZ1* expression (◆) was measured relative to *MpEF-1α*, and *actA* expression (■) was measured relative to total RNA. A linear regression analysis of *MpGSTZ1* on *actA* was performed, and an R^2 value of 0.80 was calculated, suggesting a strong relationship between fungal biomass and *MpGSTZ1* expression ($y = -11.5 + 0.844x$).

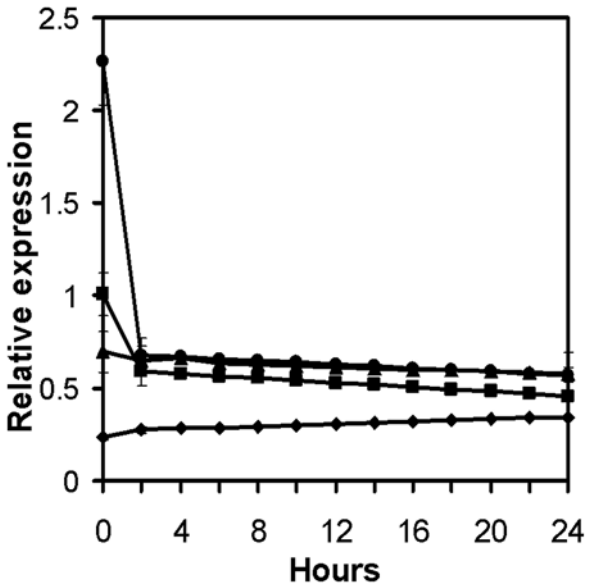

Fig. 7. Relative expression of GST genes in *Malva pusilla* following incubation in the dark. The relative expression of *MpGSTZ1* (◆), *MpGSTU1* (■), *MpGSTU2* (▲) and *MpGSTF1* (●) after 0, 2 and 24 h in the dark was calculated by dividing GST expression by that of the co-amplified *MpEF-1α*. Standard error bars represent the variation of three relative RT–PCR experiments.

GST genes and showed no induction during the disease, indicating that *MpGSTU1* may play a minor role, if any, during pathogenesis.

Expression of the four mallow GSTs was also detected in healthy plants, suggesting that they have functions unrelated to disease. Photosynthesis produces oxidative stress in plant cells, which leads to the production of organic peroxides (Creissen *et al.* 1999). *MpGSTF1* and *MpGSTU1* expression decreased when plants were incubated in the dark, which may be due to a decrease in the production of organic peroxides. This supports the possibility of roles for *MpGSTU1* and *MpGSTF1* in the detoxification of organic peroxides, but not for *MpGSTZ1* and *MpGSTU2*, whose expression was not affected by incubation in the dark.

This study has revealed that at least three mallow GSTs were differentially induced following attack by Cgm, with patterns of expression that were unlike those previously reported for other plant GSTs during disease. Because Cgm is a hemibiotrophic pathogen with a distinct mode of invasion (Wei *et al.* 1997; Jin *et al.* 1999), these patterns revealed that host GST gene expression did not begin to increase greatly until fungal biotrophy was ending and necrotrophy was developing. By comparing host GST expression with fungal growth, using a constitutively expressed fungal actin gene as a marker, it could be demonstrated that the level of expression of at least one plant GST gene, *MpGSTZ1*, was directly related to amount of fungal growth. As GST genes occur in large gene families, plants are able to produce differential GST responses to a variety of stresses, such as fungal infection (Wagner *et al.* 2002). Since a number of plant GST genes have been observed to be induced in such a wide variety of plants following various fungal infections, they likely have universal roles in the responses of plants to disease.

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