Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the rice brown planthopper, *Nilaparvata lugens*

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A novel glutathione S-transferase (GST)-based pyrethroid resistance mechanism was recently identified in *Nilaparvata lugens* [Vontas, Small and Hemingway (2001) Biochem. J. **357**, 65–72]. To determine the nature of GSTs involved in conferring this resistance, the GSTs from resistant and susceptible strains of *N. lugens* were partially purified by anion exchange and affinity chromatography. The majority of peroxidase activity, previously correlated with resistance, was confined to the fraction that bound to the affinity column, which was considerably elevated in the resistant insects. A cDNA clone encoding a GST (*nlgst1-1*) – the first reported GST sequence from Hemiptera with up to 54% deduced amino-acid identity with other insect class I GSTs – was isolated from a pyrethroid-resistant strain. Northern analysis showed that *nlgst1-1* was overexpressed in resistant insects. *nlgst1-1* was expressed in *Escherichia coli*, purified and characterized. The ability of the recombinant protein to bind to the *S*-hexylglutathione affinity matrix, its substrate specificities and its immunological properties confirmed that this GST was one from the elevated subset of *N. lugens* GSTs. Peroxidase activity of the recombinant *nlgst1-1* indicated that it had a role in resistance, through detoxification of lipid peroxidation products induced by pyrethroids. Southern analysis of genomic DNA from the resistant and susceptible strains indicated that GST-based insecticide resistance may be associated with gene amplification in *N. lugens*.

Key words: gene amplification, GST-peroxidase activity, pyrethroid resistance.

INTRODUCTION

Glutathione S-transferases (GSTs) are a major family of detoxification enzymes. They catalyse the conjugation of the tripeptide glutathione to the electrophilic centre of lipophilic compounds, thereby increasing their solubility and aiding excretion from the cell. They possess a wide range of substrate specificities, including endogenous substrates, such as reactive unsaturated carbonyls, electrophilic aldehydes, reactive DNA bases, epoxides and organic hydroperoxides produced *in vivo* as the breakdown products of macromolecules during periods of oxidative stress [1,2]. Mammalian GSTs have been classified into eight cytosolic classes and one microsomal class [3–5], whereas only three classes of insect GSTs (classes I–III) have been described to date [6,7].

The GSTs in insects are primarily of interest because of their role in insecticide resistance. They are involved in the O-dealkylation or O-dearylation of organophosphorus insecticides [8,9], as a secondary mechanism in the detoxification of organophosphate metabolites [10] and in the dehydrochlorination of organochlorines [11]. Although the involvement of GSTs in pyrethroid-resistant insects had been indicated in many insects [12–14], the mechanism by which GSTs confer resistance to this insecticide class has only recently been elucidated [15,16]. In contrast with other insecticides, GSTs do not metabolize pyrethroid molecules. They confer resistance by detoxifying lipid peroxidation products induced by pyrethroids, thereby protecting tissues from oxidative damage [16] and/or by binding pyrethroid molecules in a sequestration mechanism, thus offering a passive protection [15].

The molecular mechanism by which elevated GST activity occurs has not been fully deciphered in any insect species [17]. *Trans*-acting regulators were observed in *Aedes aegypti*, although the nature of this regulatory factor is not known [18], while elevation of GST activity due to enhanced mRNA stability has been reported in *Drosophila* [19]. Gene amplification has been observed in *Musca domestica* [20] but the significance of this is unclear, as the genomic organization of *M. domestica* GSTs is extremely complex [21].

In a pyrethroid-resistant *Nilaparvata lugens* strain (SRC), elevated GSTs with a predominant peroxidase function are responsible for a large part of its resistance to the pyrethroid insecticide lambda-cyhalothrin [16]. The aim of the present study was to characterize the elevated GSTs from *N. lugens*. We report the cloning of the first GST gene from the insect order Hemiptera and show that this gene is involved in conferring resistance to pyrethroids.

EXPERIMENTAL

Reagents

Q-Sepharose Fast Flow, phenyl-Sepharose Fast Flow and PD-10 columns were purchased from Pharmacia. Amicon Centriprep 10 and Centricon 10 units were purchased from Amicon (Stonehouse, Glos., U.K.). Lambda-cyhalothrin [*RS*-α-cyano-3-phenoxybenzyl(*Z*)-(1*R*,3*R*)-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethyl-cyclopropanecarboxylate] and 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) were purchased from British

Abbreviations used: GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; CB, Cibacron Blue; *p*-NPA, *p*-nitrophenol acetate; *p*-NPB, *p*-nitrophenyl bromide; CHP, cumene hydroperoxide; t-bHP, t-butyl hydroperoxide; DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane; RACE, rapid amplification of cDNA ends; poly(A)$^+$, polyadenylated; RFLP, restriction-fragment-length polymorphism.

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Greyhound (Birkenhead, Merseyside, U.K.). Standard protein markers, biochemicals and all other reagents including the TRI reagent were purchased from Sigma.

Planthopper strains

The pyrethroid-resistant SRC *N. lugens* strain, obtained after laboratory selection with lambda-cyhalothrin, and the parental insecticide-susceptible strain (SR) were used in the present study [16].

Purification of GSTs

The *N. lugens* GSTs from the resistant and the susceptible strains were partially purified from crude adult homogenates by sequential Q-Sepharose and *S*-hexylglutathione agarose affinity chromatography, as described by Prapanthadara et al. [22]. Briefly, 10 g of *N. lugens* adults were homogenized in 25 mM Bis-Tris propane buffer, pH 6.5, containing 15 mM DTT and 25 μM PMSF (buffer A). The homogenate was centrifuged at 10000 ***g*** for 20 min, filtered and applied on to a 30 ml Q-Sepharose column equilibrated in buffer A. The protein was eluted with a 0–0.3 M NaCl gradient in equilibration buffer. The majority of GST activity in the eluate was found to be concentrated in a single peak. Subsequently, it was applied directly on to a 20 ml *S*-hexylglutathione agarose column equilibrated with buffer A. The affinity column was washed with buffer A containing 0.2 M NaCl and the bound protein was eluted with 5 mM *S*-hexylglutathione. Fractions with GST activity were pooled and concentrated in Amicon Centriprep 10 units and buffers were exchanged using PD-10 columns, according to the manufacturer's instructions. Enzymes were stored in 50 mM sodium phosphate buffer containing 25 mM dithiothreitol and 40% (w/v) glycerol. Stored enzyme at -20 °C was stable for several weeks.

Biochemical assays

The methods of Habig, Pabst and Jacoby [23] were used to measure GST activity with 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), *p*-nitrophenol acetate (*p*-NPA), *p*-nitrophenyl bromide (*p*-NPB) and ethacrynic acid (EA). Se-independent glutathione peroxidase activity was determined with cumene hydroperoxide (CHP) and t-butyl hydroperoxide (t-bHP) by the method of Simmons et al. [24]. Protein was assayed by using the Bio-Rad protein assay kit, with BSA as the standard protein [25]. Kinetic parameters (V_{max} and K_m) were estimated for CDNB-conjugating activity by nonlinear regression using the Micromath Scientist program (MicroMath Scientific, Salt Lake City, UT, U.S.A.). Simple inhibition studies with lambda-cyhalothrin and IC_{50} determinations with different GST inhibitors were performed as detailed previously [16,22]. Inhibition kinetics of CDNB-conjugating activity of GSTs with three different lambda-cyhalothrin concentrations (5, 10 and 25 μM) were determined at 0.05–1 mM CDNB concentrations (with GSH held constant at saturating concentration), or variable GSH concentrations (0.25–2 mM) with CDNB held constant. The initial rate of reaction was used to construct a double-reciprocal plot of $1/V$ versus $1/S$, and the mode of inhibition was determined as described by Cornish-Bowden [26]. DDT dehydrochlorinase activity and HPLC analysis of DDT metabolites were conducted as described previously [22].

Each experiment including the purification procedure was performed at least three times for both resistant and susceptible strains. All biochemical data were subjected to analysis of variance (ANOVA). Either Scheffé's method or Dunnet's method was used as the *post hoc* test. The differences described in the Results section and shown in the Figures were statistically significant at $P < 0.01$, unless otherwise stated.

Electrophoresis, native size determination, isoelectric focusing and Western blot analysis

Enzyme purity and subunit size were determined by SDS/PAGE, using the PhastGel 10–15 gradient (Pharmacia) with low-range standard molecular-mass markers (Bio-Rad). For isoelectric focusing (IEF), PhastGel IEF gels 4–6.5 (Pharmacia) with IEF standard protein markers were used. Native size was determined by gel filtration chromatography on a Sephadex G75-50 column, and the molecular mass of the native GST was determined with reference to standard gel filtration molecular-mass markers according to the manufacturer's instructions. For the Western blots, enzyme preparations resolved by SDS/PAGE were blotted on to Hybond ECL membranes (Amersham Corp.). Semi-dry electrophoretic transfer of proteins was achieved with a Bio-Rad Trans-blot SD Semi-Dry Transfer Cell, as described by Small and Hemingway [27]. The filter was probed with a GST polyclonal antibody raised against the recombinant class I GST, *aggst1-6* from *Anopheles gambiae* at a dilution 1:2000 in TBS [28] and detected with the enhanced chemiluminescence detection system (Amersham Corp.).

Extraction of DNA, total RNA and mRNA, and cDNA synthesis

Total RNA was isolated from individual or batches of *N. lugens* adult females using the TRI reagent as described by Vontas et al. [29]. The RNA was treated with DNase RQ1 (Promega) to remove any contaminating genomic DNA. After re-extraction with TRI reagent, first-strand cDNA synthesis was carried out using the SuperScript II protocol (Gibco BRL) and an oligo-(dT) adaptor primer [5′-GACTCGAGTCGACATCGA-$(dT)_{17}$-3′]. Polyadenylated [poly$(A)^+$] RNA was isolated from total RNA with the PolyATract mRNA Isolation System IV (Promega), following the manufacturer's instructions. Genomic DNA was isolated from *N. lugens* adults as described by Vaughan et al. [30].

Isolation of *nlgst1-1* cDNA

The degenerate PCR strategy for insect GSTs detailed by Ranson et al. [31] was used to amplify an orthologous region from *N. lugens* cDNA. A 121 bp PCR product was isolated, subcloned into T-easy vector (Promega) and sequenced in both directions. DNA sequences were analysed using DNA Star software (Lasergene). Alignment of the isolated fragment to the sequences of other insect GSTs indicated that this encoded a partial GST cDNA. The 3′ end of the cDNA was amplified by PCR with a specific forward primer [5′-CGACTCGCTGTACCCAAAGG-AC] and the adaptor primer [5′-GACTCGAGTCGACATCGA]. The 5′ end of the cDNA sequence for *nlgst1-1* was obtained using a modification of a 5′-RACE (rapid amplification of cDNA ends) system (Gibco BRL Life Technologies). First-strand cDNA synthesis was performed as above. After RNA degradation with RNAse H and purification of the single-stranded cDNA, the cDNA was tailed at the 3′ end (5′ end of the mRNA) with dCTP using terminal deoxytransferase. The adapter primer (AAP), provided with the kit, anneals to this polyC tail. PCR was performed with the AAP primer and gene-specific reverse primer [5′-GAAAGACAAGAGCATCGCCAAG]. PCR reactions contained 3 mM $MgCl_2$, 0.5 mM dNTPs, 0.4 μM of each primer and 2.5 units of HotStar Taq polymerase (Qiagen) in the manufacturer's reaction buffer; reactions were carried out at 95 °C for 15 min followed by 35 cycles of 50 °C for 30 s, 72 °C for

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Table 1 Purification summary of GSTs from the SRC and SR strains of *N. lugens*

Activity was determined with CDNB as substrate. Units are given in μmol/min per mg of protein.

	Susceptible (SR)				Resistant (SRC)			
Preparation	Total protein (mg)	Total activity	Specific activity	Purification (×)	Total protein (mg)	Total activity	Specific activity	Purification (×)
10000 supernatant	94	25.09	0.267	1	96	39.36	0.41	1
Q-Sepharose								
Peak I	17	18.05	1.06	3.9	19	28.2	1.48	3.6
S-Hexylglutathione								
Peak II	10.08	7.49	0.743	2.8	9.68	8.2	0.847	2.06
Peak III	0.228	4.11	18.02	67.5	0.326	7.33	22.4	55

1 min and 94 °C for 30 s, and finally 72 °C for 5 min. A second round of PCR was performed with a nested primer [5′-GTCC-TTTGGGTACAGCGAGTCG] and the UAP primer provided with the kit, under the same conditions as those for the first round of PCR. PCR products were cloned into T-easy and sequenced in both directions.

The forward primer [5′-ATGCCAATTGATCTGTACTACG] and the reverse primer [5′-TTACTTTCCGGTCATAGCCTTG] encompassing the putative start codon and termination codon respectively of the *nlgst1-1* cDNA were used to amplify the full coding region from cDNA and genomic DNA isolated from both the SR and the SRC strains. PCR reactions contained 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 μM of each primer and 2.5 units of PFU polymerase (Stratagene) in the manufacturer's reaction buffer; reactions were carried out at 95 °C for 2 min followed by 35 cycles of 55 °C for 30 s, 72 °C for 1 min and 94 °C for 30 s, and finally 72 °C for 5 min. PCR products were purified using Microcon YM-100 centrifugal filter devices (Millipore) and sequenced in both directions.

Phylogenetic analysis of insect GSTs was performed as described by Ranson et al. [7].

Southern and Northern hybridization

Separate 10 μg aliquots of genomic DNA were digested to completion with *Eco*RV or *Pvu*II, size-fractionated by electrophoresis on a 0.8 % agarose gel, transferred to a charged nylon membrane (Amersham Corp.) and hybridized with a ^{32}P-labelled full-length *nlgst1-1* probe (specific radioactivity $> 2 \times 10^6$ c.p.m./μg) at 60 °C for 16 h in hybridization buffer [5 × Denhardt's solution, 6 × SSC, 0.1 % (w/v) SDS, 0.1 % (w/v) sodium pyrophosphate, 5 % (w/v) polyethylene glycol 8000, and 100 μg/ml boiled, sheared herring sperm DNA]. The final washes were at 65 °C in 0.1 × SSC and 1 % (w/v) SDS for 30 min. For Northern blot analysis, poly(A)$^+$ RNA extracted from resistant and susceptible adults was separated by formaldehyde denaturing gel electrophoresis and transferred to nylon membranes by capillary action. The hybridization and washing followed the conditions described for Southern analysis. A probe generated from the *N. lugens* tubulin gene (*nltub1*) was used as the loading control [29]. All probes were generated using a random primer oligolabelling kit (Pharmacia), following the manufacturer's instructions.

In vitro expression of *nlgst1-1*

The *nlgst1-1* was expressed *in vitro* by inserting a cDNA encoding the putative full-length coding sequence into the plasmid expression vector pET3a (Novagen) as described by Ranson et al. [28]. Briefly, the coding region of *nlgst1-1* was amplified in a PCR reaction using SRC cDNA as template, PFU polymerase (Stratagene) and the primers, which contained the initiation and termination codons of the gene, preceded by *Bam*HI sites. The full-length cDNA obtained was sequenced to ensure that no errors had been introduced during PCR amplification, ligated into the *Bam*HI site of the pET3a vector (Novagen) and the resulting expression construct was used to transform *Escherichia coli* BL21(DE3)pLysS. PCR screening using the T7 promoter primer and a reverse-gene-specific primer identified colonies containing the appropriate insert in the correct orientation. Expression was induced by the addition of 0.4 mM isopropyl β-D-thiogalactoside. The cells were harvested by centrifugation, freeze-thawed, re-suspended in 50 mM Tris/HCl (pH 7.5), 1 mM EDTA and 10 mM mercaptoethanol and disrupted by sonication. The recombinant *nlgst1-1* was purified by affinity chromatography on *S*-hexylglutathione–agarose as described above. Only trace amounts of native *E. coli* GSTs without detectable GST activity are co-eluted from the affinity column [28], under the purification conditions described above.

RESULTS

GST purification

Anion exchange Q-Sepharose chromatography gave a 3.5-fold purification with 75 % recovery of GSTs activity (Table 1) eluted in a single peak (Figure 1, peak I). Stepwise elution (rather than gradient) did not improve GST activity resolution. Affinity purification of peak I GST on an *S*-hexylglutathione column produced a single sharp peak with high GST activity for CDNB (Figure 1, peak III). A significant proportion of GST activity passed through the affinity column (peak II) and, as confirmed by passage of this fraction through an additional affinity column, the lack of binding was not due to overloading of the original affinity matrix. Protein from the affinity-purified activity (peak III) gave a single band on gradient SDS/PAGE with a molecular size of $M_r = 23\,500$, while the unbound activity gave a number of faint bands including bands of similar size to that expected for GSTs (Figure 2). A summary of purification data for the resistant and the susceptible *N. lugens* strains is presented in Table 1. CDNB-conjugating GST activity of the affinity-purified isoenzyme (peak III) was approx. 2-fold higher in the resistant SRC strain, in comparison with the susceptible one, while a marginal increase was observed in the unbound fraction (peak II). Furthermore, the bound fraction accounted for the majority of GST peroxidase activity (indicated by the substrates CHP and t-bHP; Table 2) previously correlated with resistance [16]. Therefore, this subset of GST activity (peak III) is implicated in pyrethroid resistance.

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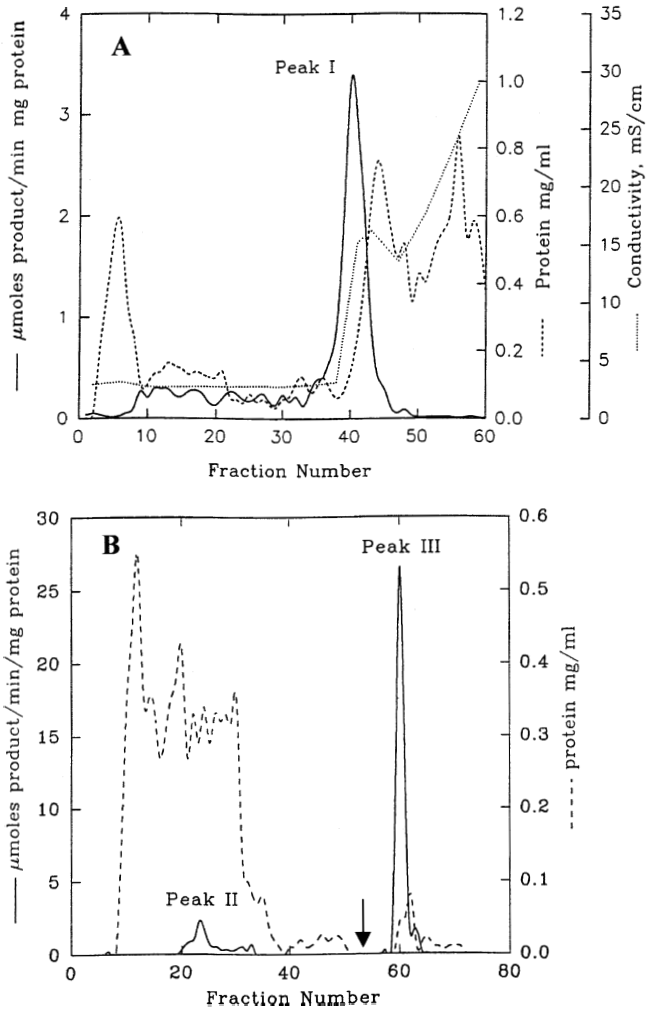

Figure 1 Partial purification of *N. lugens* GSTs by sequential anion-exhange Q-Sepharose and *S*-hexylglutathione affinity chromatography

A typical chromatography elution profile from the pyrethroid-resistant SRC strain. (**A**) The elution profile for GST activity and protein for Q-Sepharose chromatography (fraction size, 2 ml). (**B**) The elution profile for the *S*-hexylglutathione chromatography. The eluting buffer was changed to 5 mM *S*-hexylglutathione at the position of the vertical arrow (fraction size, 1 ml). GST activity was determined with CDNB and units are given in μM CDNB-GSH conjugate · min^{-1} · (mg protein)$^{-1}$. A similar elution profile occurred for enzymes prepared from the SR strain.

The affinity-purified GST isoenzyme from the resistant strain had 22% higher specific activity toward CDNB in comparison with the susceptible one. The difference in the amount of protein recovered from the resistant and susceptible insects accounts for the majority of elevated activity observed (Table 1), indicating that elevated GST activity is mainly associated with a quantitative change in the enzyme. The affinity-purified fraction (peak III), from the resistant strain, had slightly higher peroxidase activity in comparison with the susceptible strain (results not shown). IC_{50} inhibition plots of the affinity-purified fractions (peak III) from the resistant and the susceptible strains showed similar inhibitor sensitivities with EA, Cibacron Blue (CB) and *S*-hexylglutathione, and a heterogeneous nature, indicating the presence of multiple GSTs or allelic variants within this subset of GST activity (results not shown). Hydroxylapatite and phenyl-Sepharose column chromatography did not resolve GST peak III further. Isoelectric focusing indicated an acidic pI in the range 5.1–5.4.

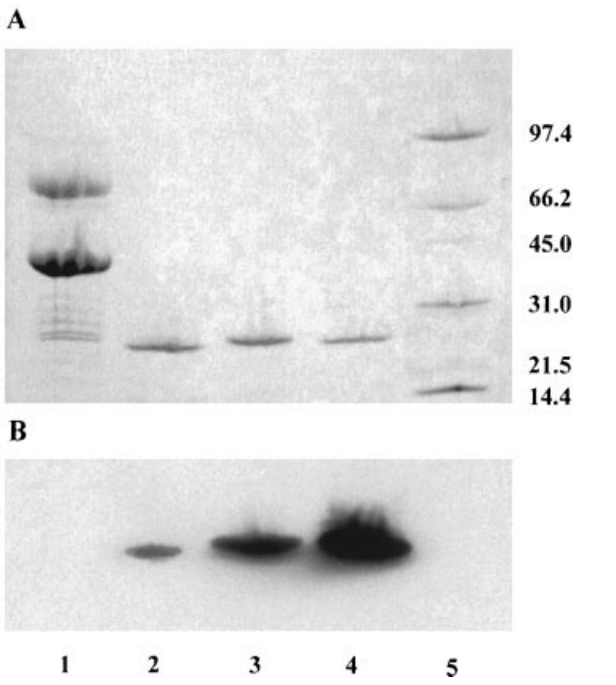

Figure 2 Cross-immunoreactivity of purified and recombinant *nlgst1-1* from *N. lugens*

The partially purified peak II (lane 1) and peak III (lane 2) and the recombinant *nlgst1-1 N. lugens* GSTs (lane 3) were resolved by gradient SDS/PAGE on 10–15% acrylamide gels. Lane 4 contains recombinant *aggst1-6* and lane 5 contains the molecular mass marker. Approximately 5 μg of protein was loaded into lane 1 and 500 ng of protein into lanes 2–4. Lane 5 contains molecular-mass markers. One gel (**A**) was stained with Coomassie Blue R-250, while an identical gel (**B**) was semi-dry-blotted on to a nitrocellulose membrane and probed with antisera raised against *aggst1-6*.

Table 2 Substrate specificities of partially purified natural and purified recombinant GSTs from *N. lugens*

	Enzyme activity			
Substrate	Peak I	Peak II	Peak III	*nlgst1-1*
CDNB	1480 ± 210	847 ± 140	22400 ± 3200	141000 ± 16400
DCNB	1.8 ± 0.3	1.3 ± 0.3	13.1 ± 2.0	90.0 ± 12.1
CHP	8.1 ± 1.4	2.5 ± 0.7	129.7 ± 20.9	510.2 ± 41.7
t-bHP	1.6 ± 0.3	0.6 ± 0.1	27.5 ± 6.1	79.8 ± 10.9
p-NPA	0.5 ± 0.2	1.6 ± 0.3	11.6 ± 1.8	50.7 ± 9.1
p-NPB	n.d.*	n.d.	n.d.	n.d.
EA	n.d.	n.d.	n.d.	n.d.

* n.d., activity was not detected. The data are the means ± S.E.M. for five separate experiments.

Cloning of a class I GST from *N. lugens*

By applying a PCR strategy using degenerate primers for insect class I GSTs and utilizing RACE techniques, we obtained the full-length sequence of *nlgst1-1* with an open reading frame of 651 bp. *nlgst1-1*, the first GST from Hemiptera, was up to 54% identical with class I GSTs from other insects (Table 3), and is therefore designated as a class I GST [1]. An alignment of *nlgst1-1* with representative insect GSTs is shown in Figure 3. Figure 4 shows a phylogenetic tree, illustrating the relationship between these amino-acid sequences based on a CLUSTAL W amino-

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Table 3 Percentage identity of the deduced amino-acid sequence of *nlgst1-1* (*N. lugens*, Hepiptera) with other insect and human GSTs

Species	Sequence	Insect (I–III) or mammalian group	Identity (%)
Anopheles gambiae (Diptera)	*Aggst1-2*	I	43.5
Anopheles gambiae (Diptera)	*Aggst2-1*	II	11.6
Anopheles gambiae (Diptera)	*Aggst3-1*	III	33.3
Drosophila melanogaster (Diptera)	*DmgstD1*	I	54.5
Drosophila melanogaster (Diptera)	*Dmgst2*	II	12.5
Drosophila melanogaster (Diptera)	*Dmgst3*	III	33.9
Musca domestica (Diptera)	*Mdgst1*	I	54.3
Musca domestica (Diptera)	*Mdgst2*	II	13.9
Plutella xylostella (Lepidoptera)	*Pxgst3*	I	33.8
Manduca sexta (Lepidoptera)	*Msgst1*	I	31.0
Lucilia cuprina (Lepidoptera)	*Lugst1*	I	54.3
Choristoneura fumiferana (Lepidoptera)	*Cfgst*	II	14.8
Blattella germanica (Blattodea)	*Bggst*	II	14.0
Human Theta	*GstT2*	Theta	27.3

acid alignment. The deduced amino acids of *nlgst1-1* cDNA were 100 % identical in resistant and susceptible strains. Sequencing of genomic DNA indicated that the gene is intronless.

Expression of recombinant *nlgst1-1*

The *nlgst1-1* cDNA was expressed in *E. coli* (Figure 5). Comparison of CDNB-conjugating activity of crude cell extracts indicated that the recombinant enzyme encoded a catalytically active GST. Induction of the expression constructs resulted in high yields of the recombinant protein (4–6 mg from 50 ml culture), which was purified by affinity chromatography for further characterization. The ability of the recombinant protein to bind to the *S*-hexylglutathione affinity matrix indicates that it belongs to the elevated subset of GST activity (peak III). The purified recombinant *nlgst1-1* was homogeneous after SDS/PAGE (Figure 2) with a subunit size of approx. 25 kDa. This is slightly larger than that predicted (23800 Da on the basis of its amino-acid composition), due to a 1.4 kDa vector-derived tag that is present on the N-terminal of the expressed protein. The electrophoretic mobility of the recombinant enzyme was equivalent to (or slightly greater than) that of the affinity-purified isoenzyme (peak III). The native molecular mass of *nlgst1-1* as estimated by filtration chromatography was 45 kDa in the range expected for a homodimer.

Substrate specificities and kinetic properties of *nlgst1-1*

The specific activity of *nlgst1-1* with the general GST substrate CDNB was high [141 μM · min^{-1} · (mg protein)$^{-1}$; Table 2] and comparable to the activities described for other class I insect GSTs from *M. domestica* (*mdgst1*), *An. gambiae* (*aggst1-6*), *Lucillia cuprina* (*lcgst1*) and *D. melanogaster* (*gstD1*) [20,28,38,43]. Peroxidase activities of insect class I GSTs vary significantly, with activity being undetectable in *aggst1-5*, very low in *lcgst1* and at comparable levels in *nlgst1-1*, *aggst1-6* and *gstD1*. A previous study suggested that peroxidase activity of

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LucilGST1   --MDFYYLPGSTPYHSVLMTAKALGI-ELNKKLLNLQAGEHLKPEFLKINPQHTIPTLVD
MdGST1       -MDFYYLPGSAPCRSVLMTAKALGI-ELNKKLLNLQAGEHLKPEFLKINPQHTIPTLVD
DmGST1      -MVDFYYLPGSSPCRSVIMTAKAVGV-ELNKKLLNLQAGEHLKPEFLKINPQHTIPTLVD
NlGST1-1    MPIDLYYVPGSAPCRNVLLAAKAVGV-DLNLKLTDLKSGQHLTPEFIKLNPQHNVPTLDD
AgGST1-2    -MLDFYYLPGSAPCRAVQMVAEAVHV-KLNLKYLDLMAGAHRSPQFTKLNPQRTIPTLVD
MsexGST1    MVMTLYKLDASPPARAVMMVIEALKIPDVEYIDVNLLEGSHLSEEFTKMNPQHTVPLLKD
PlxGST3     --MKLYKLDMSPPARATMMVAEALGV-KVDTVDVNLMKGDHTTPEYLKKNPIHTVPLLED
                                                              *

LucilGST1   GDFALWESRAIMVYLVEKYGKNDS-LFPKCPKKRAVINQRLYFDMGTLYKSFADYYYPQI
MdGST1      GDFALWESRAIMVYLVEKYGKTDS-LFPKCPKKRAVINQRLYFDMGTLYKSFADYYYPQI
DmGST1      NGFALWESRAIQVYLVEKYGKTDS-LYPKCPKKRAVINQRLYFDMGTLYQSFANYYYPQV
NlGST1-1    NGFVLNESRAIMTYLADQYGKDDS-LYPKDPKKRAKVNQRLYFDMGTLYQSFGDAYYPHM
AgGST1-2    GSLVLSESRAALIYLCDQYGDEDNDWYPRDTIQRAIVNQRLFFDACVLYPRFADFYHPQV
MsexGST1    DDFLVWDSHAIAGYLVSKYGADDS-LYPTDPKKRAIVDQRLHFDSGILFPALRGSLEPVI
PlxGST3     GDLILHDSHAIVTYLVDKYGKSDA-LYPKDVKKRAQVDQKLYLDATILFPRLRAVTFLIF

LucilGST1   FAKAPA-DPELYKKMEAAFDFLNTFLEGHQYVAGDSLTVADLALLASVSTF--EVAGFDF
MdGST1      FAKAPA-DPELFKKIETAFDFLNTFLKGHEYAAGDSLTVADLALLASVSTF--EVASFDF
DmGST1      FAKAPA-DPEAFKKIEAAFEFLNTFLEGQDYAAGDSLTVADIALVATVSTF--EVAKFEI
NlGST1-1    FGGAPL-DEDKKKKLGDALVFLDGFLEKSAFVAGEDLTLADLAIVASISTI--EAVEYDL
AgGST1-2    FGNAAP-DGRKRLAFEKAVELLNIFLSEHEFVAGSKMTIADISLFATLATA--CTLGFIL
MsexGST1    FWGETAFRPECLEKVRKGYDFAEKFL-TSTWMAGEEFTVADICCVASISTMNDIIVPIDE
PlxGST3     TEGLKKPSDKMLKDIEEAYSILNSFLSTSKYLAGDQLSLADISAVATVTSL-VYVLPLDE
                                      *       *       *

LucilGST1   SKYANVAKWYANAKTVAPGFDEN-WEGCLEFKKFFN------
MdGST1      SKYPNVAKWYANLKTVAPGWEEN-WAGCLEFKKYFG------
DmGST1      SKYANVNRWYENAKKVTPGWEEN-WAGCLEFKKYFE------
NlGST1-1    SPYKNINSWYSKVKAAAPGYKEANEEGAKGFGQMFKAMTGK-
AgGST1-2    RPYVHVDRWYVTMVASCPGAQAN-VSGAKEFLTYK-------
MsexGST1    NTYPKLSAWLERCSQLDVYKKKN-APGNDLCKDLVASKLS--
PlxGST3     AKYPKVTAWLKTMKDLPFVKSKN-EPGVTQSGQWITSSLTSK
```

Figure 3 Alignment of the deduced amino-acid sequence of *nlgst1-1* cDNA with other insect class I GSTs

Gaps introduced to maximize sequence identity are shown by a horizontal dash. Amino-acid residues marked with an asterisk (below) are present in all known insect GSTs and are thought to be critical in determining the GST fold. All insect sequences were retrieved from Genbank (Lucil, *Lucilia cuprina*; Md, *Musca domestica*; Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Ae, *Aedes aegypti*; Msex, *Manduca sexta*; Cv, *Culicodies variipennis*; Plx, *Plutella xylostalla*). The *nlgst1-1* sequence has been deposited in the GenBank®/EMBL Data Bank (accession number AF448500).

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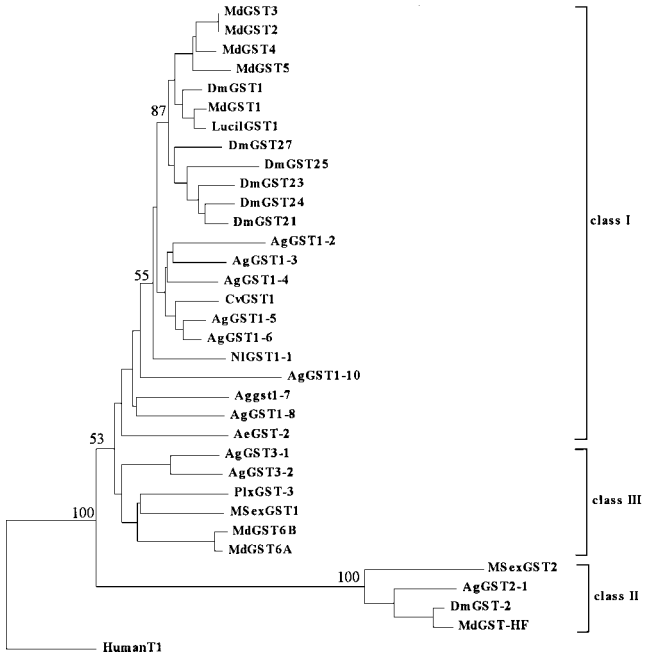

Figure 4 Dendrogram illustrating the genetic relationships among insect GSTs representatives from different orders

Amino-acid sequences were aligned by using CLUSTAL W and the tree was constructed by the neighbour-joining method program from a similar matrix of pairwise comparisons made by using the Jukes–Canton algorithm. The tree was rooted with the human class Theta *gstT1* [42]. Selected bootstrap values from 500 replicate trees are shown (as percentage values) at dendrogram nodes. All insect sequences were retrieved from GenBank® (see Figure 3 legend).

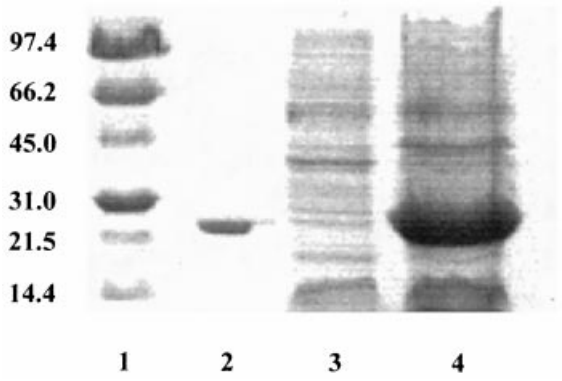

Figure 5 SDS/PAGE gel of cell lysates and purified *nlgst1-1*

Lane 1 contains molecular-mass markers, lane 2 contains approx. 2 μg of the purified *nlgst1-1*, and lanes 3 and 4 contain *nlgst1-1* expression constructs prepared with the insert in the correct or reverse orientation respectively within the pET3a vector and induced with isopropyl-β-D-galactopyranoside.

N. lugens GSTs is positively correlated with resistance [16]. Therefore the levels of peroxidase activity of *nlgst1-1* with the model organic peroxide substrates CHP and t-bHP indicates that this enzyme may have a role in resistance.

nlgst1-1 had low levels of DDT dehydrochlorinase activity [14.1 nmol of 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDE) formed per mg of protein], characteristic of class I GSTs, showing that GSTs from this insect may also metabolize DDT, as found earlier in *D. melanogaster* [11] and *An. gambiae* [31].

Table 4 Kinetic parameters for the recombinant *nlgst1-1*

The units used are V_{max} [μmol · min · (mg protein)$^{-1}$], K_m (mM), k_{cat} (s^{-1}). Values are the means ± S.E.M. for three independent experiments. Kinetic parameters of class I recombinant GSTs from *An. gambiae aggst1-6* [28] and *D. melanogaster dmgst1* [44] are given for comparison.

Kinetic parameter	*nlgst1-1*	*aggst1-6*	*dmgst1*
V_{max}	162 ± 9.2	247	58
K_m GSH	0.656 ± 0.07	0.807	0.28
K_m CDNB	0.264 ± 0.02	0.123	0.80
k_{cat}	118	97.39	28.3–38.3

Recombinant *nlgst1-1* had a higher specific activity with all substrates than the affinity-purified fraction (Table 2). This difference may result from the partial inactivation of the *N. lugens* isoenzymes during purification and/or the presence of many GSTs or allelic variants with lower substrate specificity in the affinity-purified fraction. The steady-state kinetics of the recombinant enzyme was studied with various concentrations of GSH and CDNB. The reaction followed Michaelis–Menten kinetics and the kinetic parameters were determined and compared with other insect GSTs (Table 4). Inhibition studies with three GST inhibitors (EA, CB, and *S*-hexylglutathione) indicated the homogeneity of *nlgst1-1* with inhibition characteristics of homodimeric enzyme (symmetrical IC_{50} plots, with a maximum slope of approx. −0.58 at the point of inflection) and IC_{50} values of 0.04 μM for EA, 0.20 μM for CB and 0.77 μM for *S*-hexylglutathione. The percentage inhibition of *nlgst1-1* by lambda-cyhalothrin on CDNB-conjugating activity was low (up to 20 % at the solubility limits of the insecticide), in the range determined for the affinity-purified fractions from both SRC and SR strains (results not shown). Lineweaver–Burk double-reciprocal plots of lambda-cyhalothrin inhibition with varying CDNB or GSH concentrations showed an uncompetitive type of inhibition, since the apparent V is decreased, with the apparent V/K_m unchanged (or both apparent V and K_m decreased by the same factor). This type of inhibition suggests that the insecticide does not bind to the active site of *nlgst1-1*.

Immunological cross-reactivity relationships

As shown in Figure 5 the antiserum raised against class I GSTs from *An. gambiae* (*aggst1-6*) cross-reacted with the recombinant *nlgst1-1* and the affinity-purified isoenzyme from peak III, verifying that they are immunologically related, as indicated by their biochemical characteristics (ability to bind the affinity matrix and activity against CDNB, CHP and t-bHP). There was no evidence for cross-reactivity with GSTs in the unbound fraction (peak II). The different intensity of the signal obtained from the affinity-purified isoenzyme in comparison with the recombinant *nlgst1-1*, indicates the presence of multiple GSTs or allelic variants within the affinity-purified subset, as suggested by the heterogeneous biochemical properties (IC_{50} plots) of this GST activity subset (peak III).

Northern and Southern blots

A single transcript of approx. 0.9 kb was detected using poly(A)$^+$ RNA prepared from adults of both susceptible and resistant *N. lugens* strains. Considerably more *nlgst1-1* mRNA was present in

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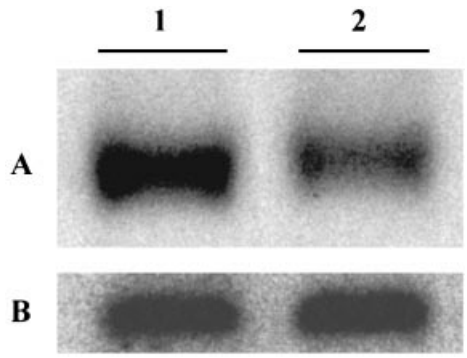

Figure 6 A Northern blot showing *nlgst1-1* gene expression in the SRC (lane 1) and SR (lane 2) strains of *N. lugens*

Poly(A)$^+$ RNA (2 μg/lane) isolated from *N. lugens* adults was fractionated on a formaldehyde-agarose gel and transferred to a nylon membrane. (**A**) Blot probed with ^{32}P-labelled *nlgst1-1* cDNA. (**B**) Blot reprobed using a labelled *nltub1* probe.

the resistant SRC than in the susceptible SR strain (Figure 6). This is not due to unequal loading as confirmed by re-probing the blot using a labelled *nltub1* probe (Figure 6).

Southern blots of genomic DNA digested with *Eco*RV or *Pvu*II (restriction sites absent from the coding region of *nlgst1-1*) were probed with *nlgst1-1* cDNA and each revealed multiple hybridizing fragments. More stringent hybridization conditions

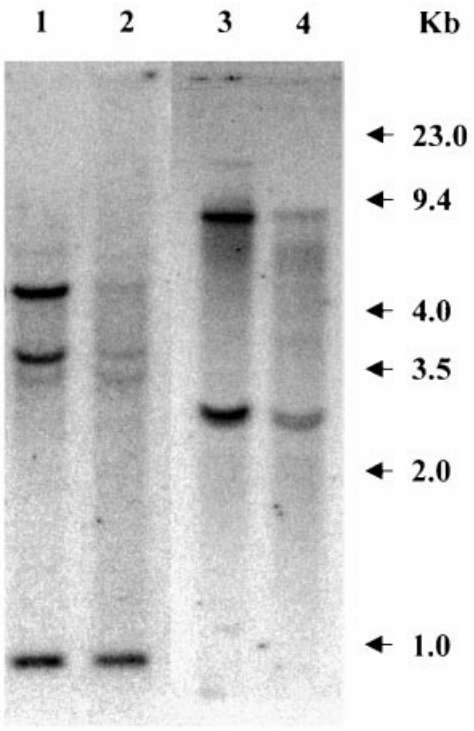

Figure 7 Southern blot of equal amounts of SRC and SR genomic DNA hybridized with *nlgst1-1* cDNA probe

Lanes 1 and 2 are SRC and SR genomic DNA respectively, digested with *Eco*RV, and lanes 3 and 4 are SRC and SR genomic DNA digested with *Pvu*II. After transfer to nylon membranes, the DNA was hybridized with ^{32}P-labelled *nlgst1-1* cDNA.

did not alter the number of bands detected (results not shown), suggesting that multiple copies of *nlgst1-1* or very similar GSTs are present in the *N. lugens* genome. An identical restriction-fragment-length polymorphism (RFLP) pattern was found in SR and SRC strains, suggesting that the genomic DNA environment of GST is similar in the two strains. Four bands were detected in *Eco*RV digests, two of which (3.6 and 5 kb) showed a markedly higher intensity of signal in the insecticide-resistant SRC strain compared with the susceptible SR (Figure 7). The identical intensity of the 1 and 3.4 kb bands shows that this difference is not due to unequal loading (Figure 7). Similarly, two (2.5 and 8.5 kb) out of the three bands gave a stronger hybridization signal in the *Pvu*II digests of the resistant strain SRC (Figure 7).

DISCUSSION

Elevated GSTs with a predominant peroxidase activity confer pyrethroid resistance in *N. lugens* [16]. To determine the nature of GSTs involved, GSTs from resistant and susceptible strains of *N. lugens* were partially purified by sequential anion exchange and affinity chromatography. The profile of GST isoenzymes prepared from the two strains was similar and two main peaks of GST activity were resolved after the *S*-hexylglutathione affinity matrix. The bound fraction of activity (peak III) appeared as a single 23.5 kDa band on an SDS/PAGE and the unbound fraction (peak II) consisted of a number of faint bands of similar size to that expected for GSTs. CDNB-conjugating GST activity of the affinity-purified peak III, which accounted for the majority of GST peroxidase activity of *N. lugens* GST previously correlated with pyrethroid resistance [16], was elevated by approx. 2-fold in the resistant strain, while only a marginal increase occurred in peak II activity. Hence, the bound fraction of activity (peak III) was indicated as being associated with pyrethroid resistance. Further biochemical purification of this subset of activity was intractable; therefore a molecular cloning approach was applied to the study of *N. lugens* GSTs.

A novel cDNA species *nlgst1-1* encoding a GST was isolated from a pyrethroid-resistant strain of *N. lugens*, by exploiting similarities with other insect GSTs. Phylogenetic analysis assigned *nlgst1-1*, the first GST from the order Hemiptera, to class I insect GSTs. The open reading frame of *nlgst1-1* had an identical amino-acid composition in the resistant SRC and the susceptible SR strains and was uninterrupted by introns, which has been observed in most, but not all, class I insect GSTs [44,45].

Northern analysis of *nlgst1-1* showed considerably higher expression levels in resistant than in susceptible insects. To establish whether *nlgst1-1* was catalytically active and likely to be involved in conferring resistance, it was expressed in *E. coli*. The recombinant *nlgst1-1* protein was retained by the *S*-hexylglutathione affinity matrix and had high CDNB and peroxidase activity with the substrates CHP and t-bHP, which indicates that it is a member of the elevated subset of GST activity (peak III). Cross-immunoreactivity studies verified this recombinant GST as belonging to the affinity-bound, elevated subset of *N. lugens* GSTs.

The main association between elevated GSTs and pyrethroid resistance in *N. lugens* was through detoxification of lipid peroxidation products induced by pyrethroids [16]. The putative function of GSTs, in preventing or repairing oxidative damage, is by conjugating organic hydroperoxides, oxidized lipids, oxidized DNA bases and activated compounds, such as epoxides, introduced after pyrethroid exposure. Although the effect of the elevation of one GST on the total GST activity may be low, especially when measured with model substrates, this elevation could result in high levels of insecticide resistance [7]. Peroxidase

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activity of the elevated affinity-purified GST subset (peak III) and *nlgst1-1* with two model organic hydroperoxide substrates indicates that these GSTs have a potential role in resistance. Recently, Kostaropoulos et al. [15] suggested that GSTs might facilitate the binding of pyrethroid molecules with other enzymes involved in the degradation of pyrethroids, or act as binding proteins in a similar way to higher vertebrates providing passive protection. The former is yet to be proven by demonstration of such enhanced metabolism in pyrethroid-resistant insects with elevated GSTs. In *N. lugens*, both resistant and susceptible strains had a similar rate of lambda-cyhalothrin metabolism, providing no evidence that the elevated GSTs have a metabolic role in resistance [16]. Although *in vitro* analysis showed that *N. lugens* GSTs do not sequester pyrethroid molecules [16], we cannot totally exclude a possible reversible binding *in vivo* that might decrease the levels of free insecticide in a passive way, adding to the insecticide resistance. However, similar and marginal rates of sensitivity to inhibition of the affinity-purified GSTs from the susceptible and resistant strains with lambda-cyhalothrin (results not shown) and the type of inhibition of the overexpressed *nlgst1-1* by the insecticide make this hypothesis unlikely.

RFLP patterns suggested identical genomic DNA environments for *nlgst1-1* from the SRC and SR strains. The selectively higher intensity signal of certain bands in the resistant strain compared with the susceptible strain suggests that an underlying mechanism of GST elevation in the SRC strain is gene amplification. Furthermore, signal elevation in the Southern and Northern blots is approximately similar in the SRC strains, suggesting that gene amplification may account for the difference in GST expression observed. The elevation in total GST activity cannot be directly compared with the degree of *nlgst1-1* overexpression and genomic amplification, as the effect of a many-fold up-regulation may be only a few-fold increase in the overall activity. GST elevation due to gene amplification is in contrast with up-regulation mechanisms of other insect GSTs, such as the *trans*-acting transcriptional regulators mechanism implicated in *A. aegypti* [18] or the mRNA stabilization in *Drosophila*, but in line with GST gene amplification reported previously in *M. domestica* [20]. High levels of gene amplification have been demonstrated for esterase-based organophosphate resistance in an *N. lugens* strain of the same origin [29].

In conclusion, the localization of *nlgst1-1* in the elevated subset of GSTs from a pyrethroid-resistant *N. lugens* strain, its peroxidase activity and its overexpression in resistant insects suggests that *nlgst1-1* is involved in insecticide resistance. Its most likely role is through detoxification of lipid peroxidation products induced by pyrethroids [16]. Our data suggest that the underlying mechanism of GST elevation in the pyrethroid-resistant strain is gene amplification. The possibility that the amplified gene is not *nlgst1-1* itself but a closely related gene, although unlikely given the good correlation between the biochemical properties of recombinant *nlgst1-1* and the elevated GSTs in the resistant strain, cannot be discounted at this stage. Further studies on the class I GST family of *N. lugens* will help resolve this issue.

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