Insect Molecular Biology (2000) **9**(6), 655–660

Comparison of esterase gene amplification, gene expression and esterase activity in insecticide susceptible and resistant strains of the brown planthopper, *Nilaparvata lugens* (Stål)

J. G. Vontas, G. J. Small and J. Hemingway

Cardiff School of Biosciences, Cardiff University, PO Box 915, Cardiff CF10 3TL, Wales, UK

Abstract

Organophosphorus and carbamate insecticide resistance in *Nilaparvata lugens* is based on amplification of a carboxylesterase gene, Nl-EST1. An identical gene occurs in susceptible insects. Quantitative real-time PCR was used to demonstrate that Nl-EST1 is amplified 3–7-fold in the genome of resistant compared to susceptible planthoppers. Expression levels were similar to amplification levels, with 1–15-fold more Nl-EST1 mRNA in individual insects and 5–11-fold more Nl-EST1 mRNA in mass whole body homogenates of resistant females compared to susceptibles. These values corresponded to an 8–10-fold increase in esterase activity in the head and thorax of individual resistant insects. Although amplification, expression and activity levels of Nl-EST1 in resistant *N. lugens* were similar, the correlation between esterase activity and Nl-EST1 mRNA levels in resistant individuals was not linear.

Keywords: carboxylesterase, gene amplification, quantitative PCR, *Nilaparvata lugens*.

Introduction

The brown planthopper, *Nilaparvata lugens*, is a major pest of rice in Asia. As a consequence of intensive chemical control measures, resistance to organophosphorus and carbamate insecticides has been selected in numerous localities (Hemingway *et al*., 1999). The underlying mechanism of resistance to these insecticides is elevation of a carboxylesterase N1-EST1 (Small & Hemingway, 2000b; Karunaratne *et al*., 1999). Unlike *Culex quinquefasciatus*, where the amplified esterase genes differ from their non-amplified counterparts (Karunaratne *et al*., 1995), the non-amplified homologue of Nl-EST1 in susceptible *N. lugens* is identical (Small & Hemingway, 2000b) suggesting that the differential expression of this esterase is the primary mechanism affecting the level of resistance to organophosphates and carbamates.

Received 7 February 2000; accepted following revision 23 August 2000. Correspondence: Prof. Janet Hemingway, Cardiff School of Biosciences, Cardiff University, PO Box 915, Cardiff CF10 3TL, Wales, UK. Tel.: 44 2920 874621; fax: 44 2920 874305; e-mail: Hemingway@cardiff.ac.uk

Results

Prior to quantitative real-time PCR assays, reactions amplifying the *N. lugens* tubulin and Nl-EST1 esterase were optimized for Mg^{2+} and primer concentrations. This was especially important for the reactions amplifying Nl-EST1, which gave nonspecific products at lower Mg^{2+} concentrations and at higher primer concentrations (Fig. 1).

Figure 2 gives the raw fluorescence data profiles (Fig. 2A,B) and standard curves (Fig. 2C,D) for the control plasmids containing the esterase gene (*pEst*) and tubulin gene fragment (*pTub*). Replicate PCR reactions were reproducible and the standard curves were linear for all but the lowest (10 fg) concentrations of plasmid for both inserts. A representative analysis of esterase quantitative PCR is given in Fig. 3. PCR products of the correct size and identity were found in all reactions, and all PCR reactions with both genomic and cDNA fell well within the plasmid standard curves, with the exception of that from some individual female susceptible Sri Lanka-S genomic DNA samples. The identical melting curves for the esterase plasmid insert and unknown samples indicate that a single specific product, identical to the sequence of the plasmid insert, was obtained from the *N. lugens* cDNA and genomic DNA for both the Sri Lanka-R and Sri Lanka-S strains (see Fig. 3B,D for representative melting curves).

Quantification of genomic gene copy number

At least ten tubulin genes occur in insects. In both individual insects and genomic DNA from pooled homogenates there were no significant differences between the gene copy

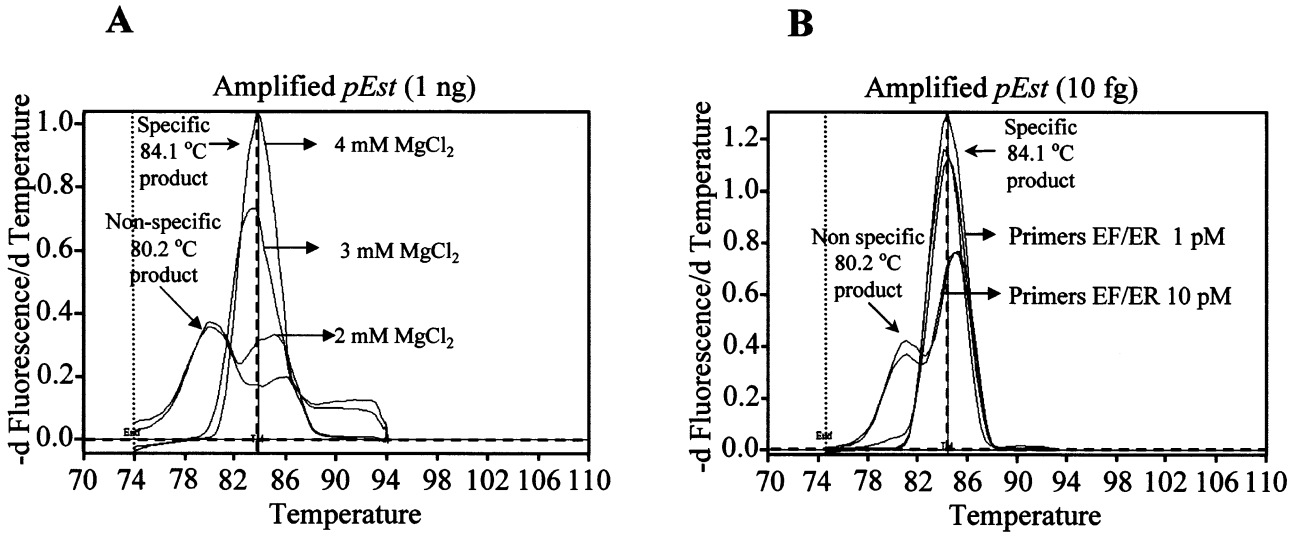

Figure 1. Optimization of Lightcycler conditions of plasmid *pEst* for Mg^{2+} concentration (A) and primer concentration (B) prior to the quantification of NI-EST1. Melting peaks of nonspecific and specific products are indicated with varying conditions.

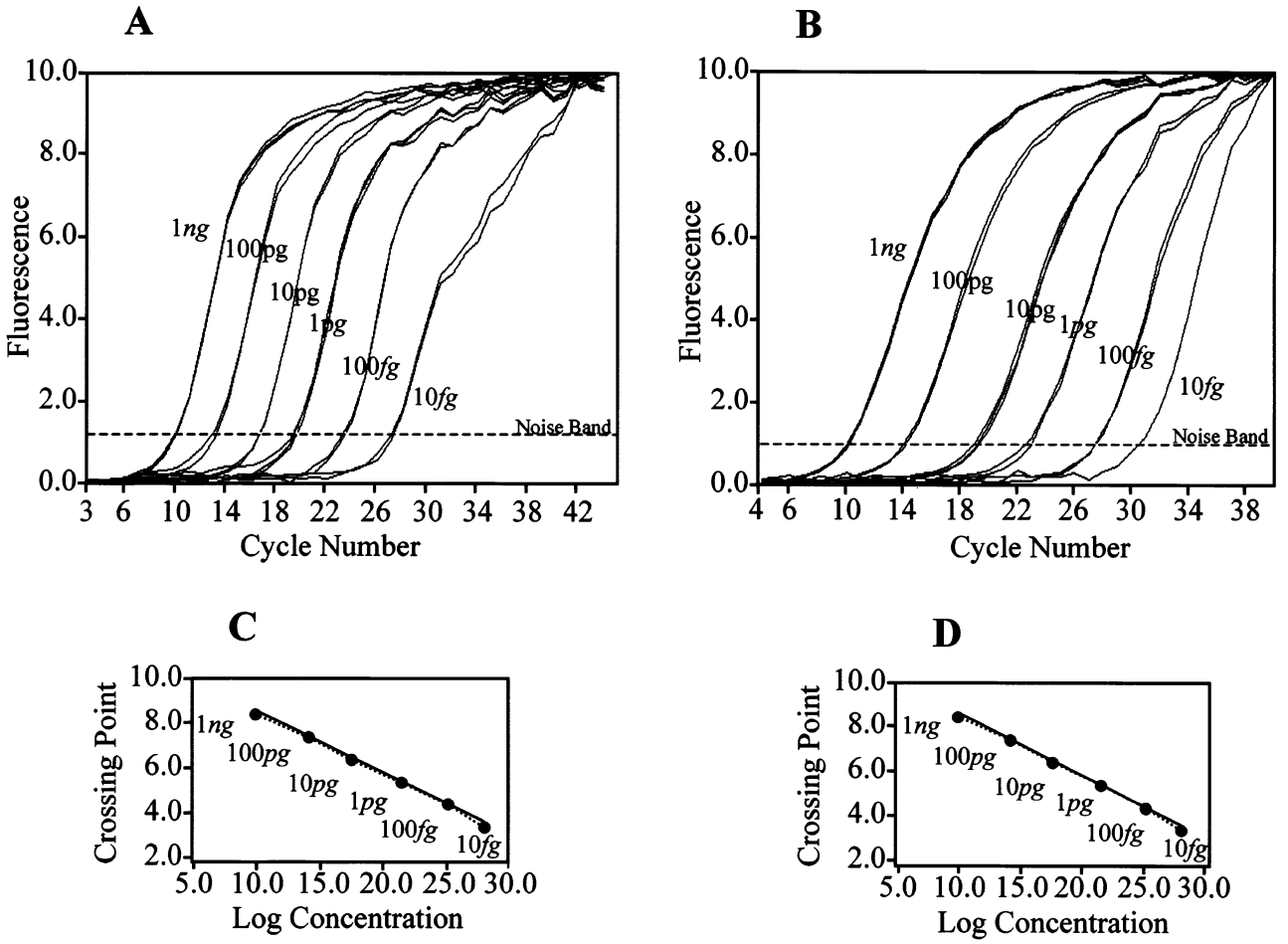

Figure 2. Raw fluorescence data profiles for quantitative PCR using the control plasmids *pEst* (containing the *N. lugens* NI-EST1 esterase gene) (A) and *pTub* (containing the *N. lugens* tubulin gene fragment) (B) and the standard curves for *pEst* (C) and *pTub* (D).

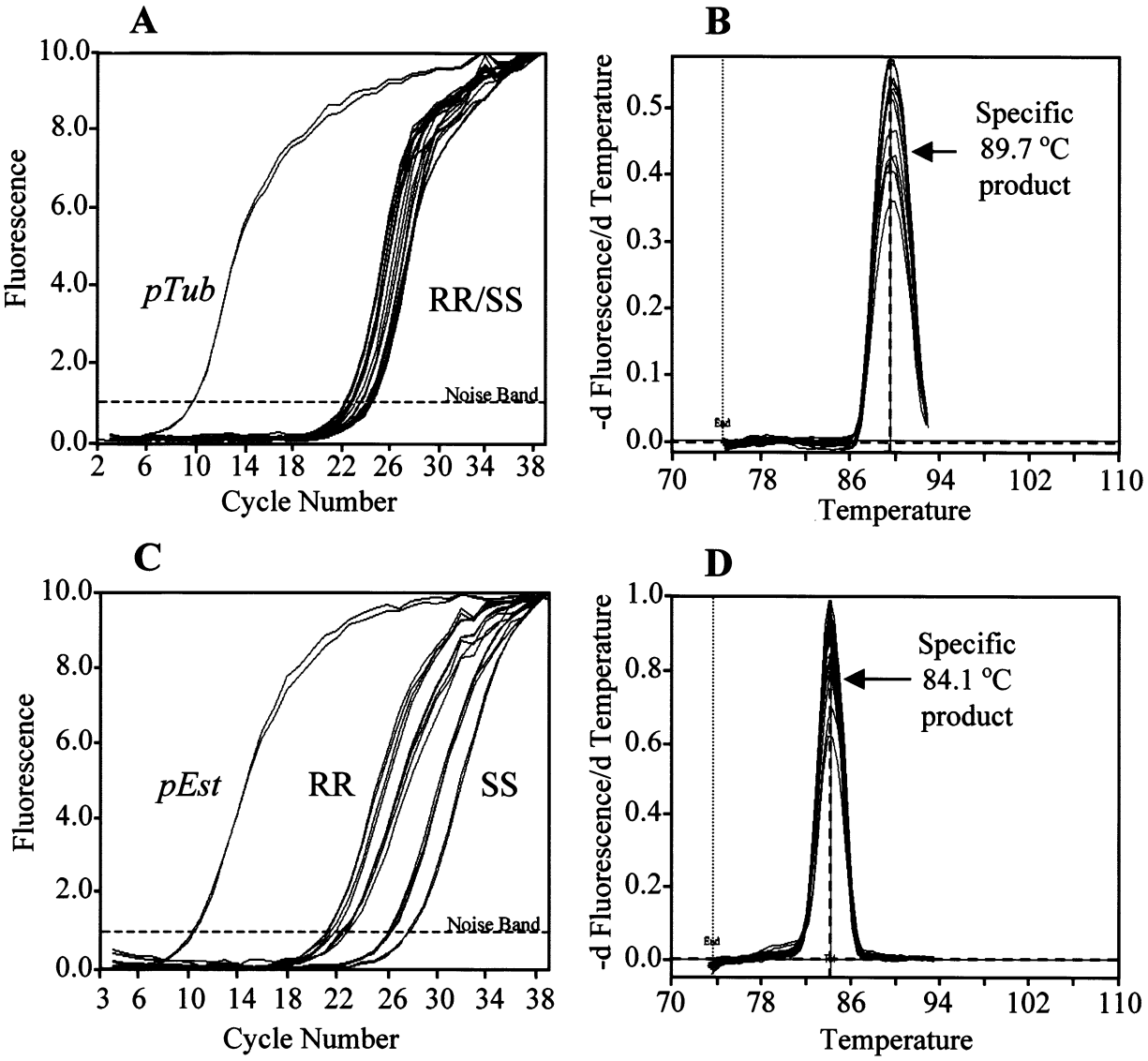

Figure 3. Representative data for the quantification of esterase NI-EST1 mRNA expression levels from nine Sri Lanka-R (RR) and six Sri Lanka-S (SS) adult females. (A) Graph of PCR fluorescence profiles against cycle number with the Lightcycler for individuals for tubulin (TF/TR primers). (B) Melting curves of the tubulin PCR products by using TF/TR primers. (C) Graph of PCR fluorescence profiles against cycle number with the Lightcycler for individuals for NI-EST1 (EF/ER primers). (D) Melting curves of the NI-EST1 PCR products by using EF/ER primers. Each set of PCR reactions included control reactions containing 1 ng tubulin plasmid (*pTub*) and NI-EST1 plasmid (*pEst*).

number of tubulin genes from insecticide resistant and susceptible insects. In DNA from the pooled samples of susceptible insects the esterase/tubulin copy number ratio was 1 : 10, suggesting that the esterase is present as a single copy in the Sri Lanka-S strain. The ratio in individuals could not be measured due to the weak signal from insecticide susceptible Sri Lanka-S insects, hence genomic DNA from six independent mass homogenates of ten to fifteen insects was used. Figure 4 gives the esterase gene copy numbers relative to those of the tubulin genes for the six homogenates used as templates from each strain. Esterase gene copy number was one-half to one-third that of the tubulin genes in the resistant Sri Lanka-R strain, suggesting that even in its amplified format it is present at a lower copy number than the tubulin genes. Genomic esterase amplification level in the Sri Lanka-R DNA was 3–7-fold greater than that in the susceptible Sri Lanka-S *N. lugens* strain.

Quantification of cDNA copy number

It was possible to estimate the copy number of both the esterase and tubulin message in individual females from both insecticide resistant and susceptible *N. lugens*. In the majority of Sri Lanka-R individuals the level of esterase message was significantly higher than that for tubulin (Fig. 5),

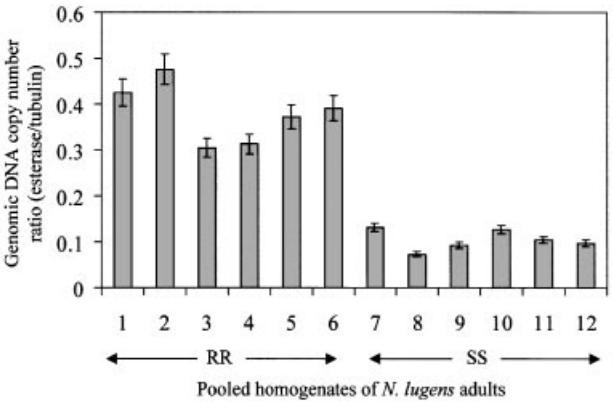

Figure 4. Estimates of esterase gene copy number in the Sri Lanka-S (SS) and Sri Lanka-R (RR) strains of *Nilaparvata lugens* relative to the tubulin genes from the same pooled homogenate. Values are means ± standard errors.

whilst in Sri Lanka-S the reverse was true, with the majority of individuals having levels of tubulin message greater than that for esterase.

Between individual resistant females (Fig. 5) there was a marked variability in estimates of esterase message, whilst the level of tubulin message remained relatively constant. The pooled cDNA from Sri Lanka-R had 5–11-fold more esterase message than that from Sri Lanka-S, while resistant Sri Lanka-R individuals had 1–15-fold more esterase message than Sri Lanka-S females.

Comparison of esterase activity with gene copy number and cDNA copy number

Esterase activity estimated from the pooled homogenates from heads of ten planthoppers and the number of copies of Nl-EST1 in the genome (Fig. 6A) were higher for all Sri Lanka-R homogenates than in any of the Sri Lanka-S homogenates. There was a nonlinear relationship between activity and Nl-EST1 copy number within the Sri Lanka-R strain. A similar nonlinear relationship was also seen between esterase activity in the head and thorax of individual Sri Lanka-R females and esterase mRNA levels in the abdomen of the same individual (Fig. 6B). Several individuals had higher levels of activity than that expected from a linear model. However, overall esterase activity in mass homogenates of Sri Lanka-R was 8–10-fold greater than that of the susceptible strain, which is similar to the level of message elevation seen in Sri Lanka-R compared to Sri Lanka-S homogenates.

Discussion

Attempts to quantify esterase gene copy number in aphids with amplified esterase-based resistance mechanisms have proved difficult. Semi-quantification of dot-blots of genomic DNA suggested an eightfold amplification between susceptible and highly resistant aphids (Field *et al.*, 1988), while a quantitative assay of probe binding showed 5–11-fold more esterase gene sequences in the resistant compared to the susceptible aphid (Field *et al.*, 1996). However, later data showed that these probes hybridized to several genes in susceptible aphids, leading to a revision of amplified esterase E4 copy number estimates of 20–90-fold in resistant aphids, linked to a 64-fold increase in E4 enzyme estimated immunologically (Field *et al.*, 1999). Quantitative real-time PCR has been used to show that in *C. quinquefasciatus*, two esterases, which are coamplified in a 1 : 1 ratio, are differentially expressed (Paton *et al.*, 2000). Here we use the same methodology to estimate Nl-EST1 amplification levels in resistant *N. lugens*, and relate Nl-EST1 copy number to expression and activity levels of this esterase and to resultant insecticide resistance in adult planthoppers.

Previous estimates of esterase gene amplification linked with insecticide resistance are ≈ 80 copies of the E4 esterase in R3 strains of the aphid *Myzus persicae* (Field *et al.*, 1999), ≈ 20 copies for Est$\beta1^1$ in the TEM-R strain (Guillemaud *et al.*, 1997) and ≈ 80 copies of Est$\alpha2^1$ and Est$\beta2^1$ in the Pel RR strain of *C. quinquefasciatus* (Paton *et al.*, 2000). A 3–7-fold level of esterase gene amplification in resistant *N. lugens*

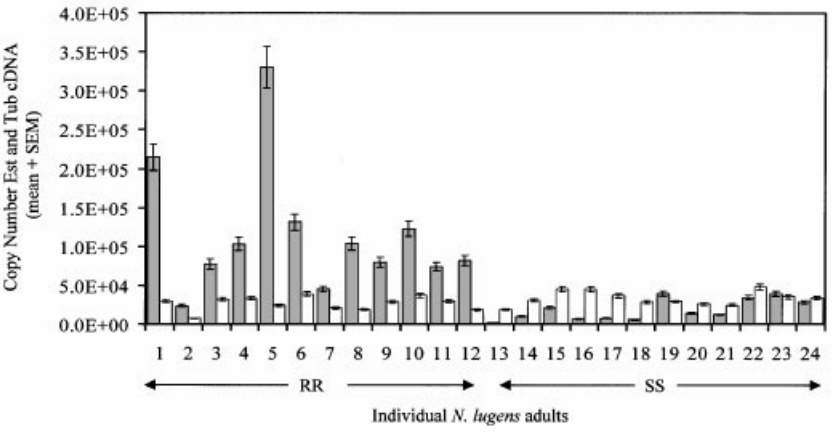

Figure 5. mRNA quantification in individual resistant Sri Lanka-R (RR) and susceptible Sri Lanka-S (SS) *Nilaparvata lugens* females for the esterase (shaded) and tubulin (unshaded) genes from the same individual insects.

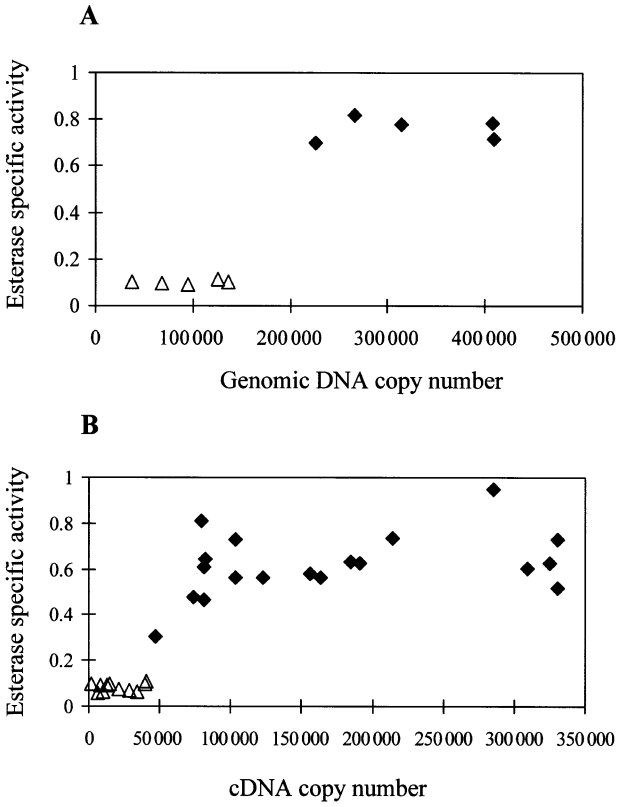

Figure 6. Mean esterase specific activity in the heads of Sri Lanka-S (open triangle) and Sri Lanka-R (solid diamond) strains of *Nilaparvata lugens* relative to gene (A) and cDNA (B) copy number. Homogenates of ten to fifteen insect thoraxes and abdomens were used in the determination of gene copy number and mean esterase specific activity was calculated from assays of separate homogenates of the heads of the same insects. Homogenates of individual thoraxes and abdomens were used, respectively, in the determination of cDNA copy number and mean esterase specific activity calculated from assay of individual heads of the same insects. Units of specific activity are μmoles ρ-nitrophenol acetate/min/mg protein.

is much lower than the estimates in the other two species. This is reflected in the lower levels of insecticide resistance in *N. lugens*, and it is likely that further selection of these laboratory colonies, which had been maintained for several years without insecticide pressure prior to this study, would increase the amplification levels.

The real-time quantitative PCR system utilized in this study is more specific than some earlier methods used for quantification. In addition to the quantification of template copy number, the technique separates products based on differences in the position and shape of the PCR products melting curve, which is a function of product length and GC content. Hence, the binding of probes to multiple templates, which complicate accurate estimations of amplification for the aphid E4 esterase, is not an issue.

The lack of a direct correlation between esterase activity and esterase gene amplification levels in individual females and in pooled homogenates of females may be due to variations in the localization and stability of the amplified esterase in the planthoppers with physiological state or with age. Although only adult females were used, the age of these insects was not known. In resistant aphids there is a proportionate relationship between the level of gene amplification and the amount of esterase enzyme protein in all but revertant clones of aphids (where a lack of DNA methylation results in loss of enzyme expression) (Field *et al.*, 1999). If activity is taken as a crude measure of the amount of Nl-EST1 present, then the relationship between the level of gene amplification and the amount of Nl-EST1 in *N. lugens* would appear to be more complex. The stability of Nl-EST1 may be influenced by the extensive differential glycosylation of this protein (Small & Hemingway, 2000a).

Experimental procedures

Strains

The resistant and susceptible strains of *N. lugens* were derived from a heterogeneous field population collected from Batalogoda, Sri Lanka in 1980, as described by Small & Hemingway (2000a).

Esterase activity assay

To compare esterase activity and expression levels in the same insect, the head was separated from the thorax and abdomen of individual female *N. lugens*. The head was homogenized in 100 μl of sodium phosphate buffer (0.05 M pH 7.2) and microfuged. The supernatant was used for replicates of esterase and protein assays as described previously (Hemingway *et al.*, 1997). Only those individuals from the Sri Lanka-R strain showing high esterase activities, and those individuals from the susceptible strain showing low esterase activities were used in the quantitative real-time PCR assays.

Extraction of planthopper RNA and cDNA synthesis

RNA was extracted individually from the separated thorax and abdomen or from multiple whole *N. lugens* females using TRI reagent (Sigma) following the manufacturer's protocols. The RNA pellet was re-suspended in nuclease-free water and treated with DNase RQ1 (Promega) [20 μl RNA, 10 μl 10 × DNAse buffer, 4 μl DNAse RQ1, 2 μl RNasin ribonuclease inhibitor (Promega) and 64 μl water incubated at 37 °C for 15 min], re-extracted with TRI reagent and the resultant pellet taken up in nuclease-free water.

First strand cDNA synthesis was carried out using the Super-Script II protocol (Gibco/BRL). Genomic DNA, from the same individuals that the cDNA had been prepared from, was extracted by the same TRI reagent protocol, washed twice in 0.3 M sodium citrate/10% ethanol and once in 75% ethanol, air-dried and re-suspended in 20 μL of 8 mM sodium hydroxide. The isolated DNA was treated with RNaseA, extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol and 3 M NaAc.

Extraction of planthopper genomic DNA

The abdomen and thorax of ten to fifteen individuals from the Sri Lanka-R strain showing high esterase, and the same number of individuals from the susceptible strain showing low esterase activities were pooled separately and genomic DNA extracted as

described previously (Vaughan *et al*., 1995). DNA concentration was determined spectrophotometrically and adjusted to 100 mg/ml.

Preparation of tubulin and esterase standard plasmids

Control plasmids containing a fragment of a tubulin gene (*pTub*) and the Nl-EST1 gene (*pEst*) were used as standards in quantitative real-time PCR. A primer pair was designed to conserve regions within a single exon of three insect tubulin genes. A single 196 bp product was amplified with these primers using either *N. lugens* cDNA or pooled 15 Sri Lanka-S genomic DNA, and the tubulin primers were used to amplify a section of a *N. lugens* tubulin gene [1 μl DNA (100 mg/ml), 1.5 μl $MgCl_2$, 1 μl dNTPs, 4 μl of each primer, 5 μl Platinum taq DNA polymerase buffer, 33 μl water and 0.5 μl Platinum taq DNA polymerase (Gibco/BRL)]. The PCR product was purified using a Microcon YM-100 centrifugal filter device (Millipore) and ligated into the pGEM-T Easy vector plasmid following the manufacturer's instructions (Promega). The resultant plasmid was transformed into *E. coli* XL1-Blue cells. Overnight plasmid cultures were purified using the QIAprep Spin Miniprep Kit Protocol. The insert in three different plasmids was sequenced in both directions to confirm its identity as tubulin. The sequence of the inserts in all three plasmid preparations was identical and had 75–84% identity to other insect tubulin genes. One of the plasmids containing the tubulin gene insert was used to establish a standard curve on the LightCycler. The plasmid containing a 1644 bp esterase gene insert, *pEst*, was described previously (Small & Hemingway, 2000b).

Quantitative real-time PCR

The oligonucleotide primers used for amplification of the tubulin gene were 5′-CATGATGGCCGGGTGCGA (forward, TF) and 5′-GTTGCTCTCGGCCTCGGTGA (reverse, TR) and for the Nl-EST-1 esterase gene were 5′-TGAATTGCTCGGCTGACACCTC (forward, EF) and 5′-ATCTCCTTCGACCGGCATTATTTC (reverse, ER). Prior to the quantitative assays, both the tubulin and esterase reaction were optimized for Mg^{2+} concentration and primer concentration. For the tubulin reaction, 3 mM Mg^{2+} and a primer concentration of 10 pM was optimal and for the esterase reaction 4 mM Mg^{2+} and 1 pM of primers. For all reactions, 1 μl of cDNA synthesis reaction was used as the template. For reactions containing genomic DNA a 1 μl sample (100 mg/ml) was used as the template. Reaction mixtures contained 50 mM Tris, 0.2 mM dNTPs, 0.2 U *Taq* polymerase and a 1 : 20 000 dilution of SYBR Green1 dye. PCR reactions were carried out in glass capillaries in the LightCycler, with forty cycles of 95 °C for 1 s, 62 °C (esterase) or 55 °C (tubulin) for 3 s and 72 °C for 14 s, followed by one cycle of continuous monitoring of fluorescence from 72 to 95 °C to generate the melting curve of the PCR product. The specificity of each amplified PCR product was assessed by melting point analysis cycle and subsequent agarose gel electrophoresis of the whole PCR reaction mixture.

Standard curves representing the fluorescence profiles for serial dilutions of plasmid (1 ng–10 fg) were converted to gene copy number; 1 fg of the esterase plasmid contained 131.6 copies of the target insert, while 1 fg of the tubulin plasmid contained 265.3 copies. As the primers for both the tubulin and esterase genes were internal to a single exon, the same standard curves could be used for both genomic and cDNA esterase and tubulin quantification. Each sample of cDNA or genomic DNA template was analysed at least twice and a standard 1 ng plasmid control was run on each occasion to control for variance in PCR conditions.

Acknowledgements

This work was funded by a Greek NATO Fellowship to J.V. and a BBSRC Award to J.H. We thank Keith Sexton for technical assistance with real-time PCR assays.

References

Field, L.M., Blackman, R.L., Tyler-Smith, C. and Devonshire, A.L. (1999) Relationship between amount of esterase and gene copy number in insecticide resistant *Myzus persicae* (Sulzer). *Biochem J* **339**: 737–742.

Field, L.M., Devonshire, A.L. and Forde, B.G. (1988) Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem J* **251**: 309–312.

Field, L.M., Devonshire, A.L. and Tyler-Smith, C. (1996) Analysis of amplicons containing the esterase genes responsible for insecticide resistance in the peach-potato aphid *Myzus persicae* (Sulzer). *Biochem J* **313**: 543–547.

Guillemaud, T., Makate, N., Raymond, M., Hirst, B. and Callaghan, A. (1997) Esterase gene amplification in *Culex pipiens*. *Insect Mol Biol* **6**: 319–327.

Hemingway, J., Karunaratne, S.H.P.P. and Claridge, M.F. (1999) Insecticide resistance spectrum and underlying resistance mechanisms in tropical populations of the brown planthopper (*Nilaparvata lugens*) collected from rice and the wild grass *Leersia hexandra*. *Int J Pest Mngmt* **45**: 215–223.

Hemingway, J., Penilla, R.P., Rodriguez, A.D., James, B.M., Edge, W., Rogers, H. and Rodriguez, M.H. (1997) Resistance management strategies in malaria vector mosquito control. A large scale trial in Southern Mexico. *Pest Sci* **51**: 375–382.

Karunaratne, S.H.P.P., Hemingway, J., Jayawardena, K.G.I., Dassanayaka, V. and Vaughan, A. (1995) Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide-resistant and susceptible *Culex quinquefasciatus* mosquitoes. *J Biol Chem* **270**: 31 124–31 128.

Karunaratne, S.H.P.P., Small, G.J. and Hemingway, J. (1999) Characterization of the elevated esterase-associated insecticide resistance mechanism in *Nilaparvata lugens* (Stål) and other planthopper species. *Int J Pest Mngmt* **45**: 225–230.

Paton, M.G., Karunaratne, S.H.P.P., Giakoumaki, E., Roberts, N. and Hemingway, J. (2000) Quantitative analysis of gene amplification in insecticide resistant *Culex* mosquitoes. *Biochem J* **346**: 17–24.

Small, G.J. and Hemingway, J. (2000a) Differential glycosylation produces heterogeneity in elevated esterases associated with insecticide resistance in the brown planthopper *Nilaparvata lugens* Stål. *Insect Biochem Mol Biol* **30**: 443–453.

Small, G.J. and Hemingway, J. (2000b) Molecular characterisation of the amplified carboxylesterase gene associated with organophosphorous insecticide resistance in the brown planthopper, *Nilaparvata lugens*. *Insect Mol Biol*, in press.

Vaughan, A., Rodriguez, M. and Hemingway, J. (1995) The independent gene amplification of indistinguishable esterase B electromorphs from the insecticide resistant mosquito *Culex quinquefasciatus*. *Biochem J* **305**: 651–658.