Insect Molecular Biology (2000) **9**(6), 647–653

Molecular characterization of the amplified carboxylesterase gene associated with organophosphorus insecticide resistance in the brown planthopper, *Nilaparvata lugens*

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Abstract

Widespread resistance to organophosphorus insecticides (OPs) in *Nilaparvata lugens* is associated with elevation of carboxylesterase activity. A cDNA encoding a carboxylesterase, Nl-EST1, has been isolated from an OP-resistant Sri Lankan strain of *N. lugens*. The full-length cDNA codes for a 547-amino acid protein with high homology to other esterases/lipases. Nl-EST1 has an N-terminal hydrophobic signal peptide sequence of 24 amino acids which suggests that the mature protein is secreted from cells expressing it. The nucleotide sequence of the homologue of Nl-EST1 in an OP-susceptible, low esterase Sri Lankan strain of *N. lugens* is identical to Nl-EST1. Southern analysis of genomic DNA from the Sri Lankan OP-resistant and susceptible strains suggests that Nl-EST1 is amplified in the resistant strain. Therefore, resistance to OPs in the Sri Lankan strain is through amplification of a gene identical to that found in the susceptible strain.

Keywords: *Nilaparvata lugens*, carboxylesterase, insecticide resistance.

Introduction

The brown planthopper *Nilaparvata lugens* (Stål) is a sporadic but serious pest of rice in the temperate regions of Asia (Paik, 1977). It occurs throughout Asia and Australia, although there is debate over whether different species occur in the two continents. *N. lugens* became a major tropical rice pest in the 1970s following the introduction of modern high yielding rice cultivars, coupled with increased insecticide use (Dyck & Thomas, 1979). Organophosphorus, carbamate and pyrethroid insecticides are used on rice pests throughout Asia, and resistance to them has been reported from numerous localities (Hemingway *et al*., 1999). An elevated esterase-based mechanism is the major form of resistance in many rice pests, including the small brown planthopper *Laodelphax striatellus* (Sakata & Miyata, 1994), the green rice leafhopper *Nephotettix cincticeps* (Hasui & Ozaki, 1984) and *N. lugens* (Chen & Sun, 1994).

In *N. lugens*, purified elevated esterase appears as two β-naphthyl acetate specific bands on native gradient polyacrylamide gel electrophoresis (PAGE); one band is intensely staining and diffuse and the other, of slightly lower mobility, is more focused but less intense (Small & Hemingway, 2000; Karunaratne *et al*., 1999). On IEF gels these esterases appeared as a ladder of bands ranging in pI from 4.7 to 5.0. Glycobiological analysis suggests that these isozymes arise by differential post-translational glycosylation of a nascent protein (Small & Hemingway, 2000).

In the aphid *Myzus persicae* and the mosquito *Culex quinquefasciatus*, gene amplification is the mechanism underlying esterase elevation in insecticide-resistant insects (Field & Devonshire, 1997; Field *et al*., 1999; Hemingway & Karunaratne, 1998; Hemingway *et al*., 1998). The current study was undertaken to determine the molecular basis of elevated esterase-based resistance in *N. lugens*, to provide a model agricultural sexually reproducing pest system in which to study the evolution and spread of this resistance, to contrast with the clonally reproducing aphid model.

Results

Screening of an amplified Sri Lanka-R cDNA library with a depleted polyclonal Sri Lanka-R esterase antiserum yielded

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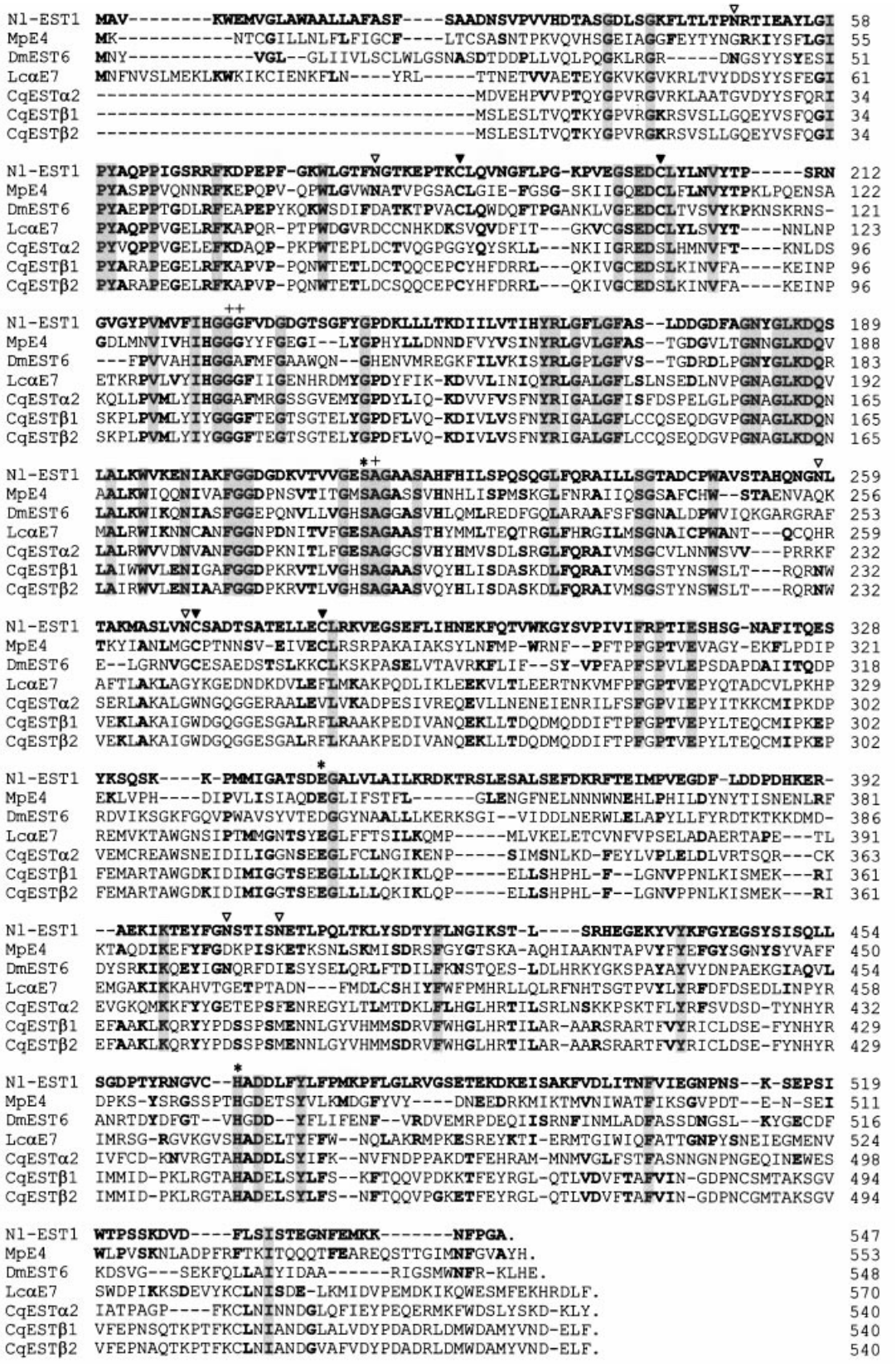

Figure 1. An alignment of the predicted amino acid sequence of Nl-EST1 with five esterases involved organophosphorus insecticide resistance, the amplified E4 esterase of *Myzus persicae* (MpE4; Field *et al.*, 1993), the amplified Estα2[1] (CqEstα2; Vaughan & Hemingway, 1995), Estβ1[2] (CqEstβ1; Vaughan *et al.*, 1995) and Estβ2[1] (CqEstβ2; Vaughan *et al.*, 1995) of *Culex quinquefasciatus* and the unamplified LcαE7 of *Lucilia cuprina* (Newcomb *et al.*, 1997). Est 6, a member of the β-cluster from *Drosophila melanogaster* (DmEST6; Oakeshott *et al.*, 1987) is also included. The alignment was determined using the MegAlign program of DNASTAR and altered by eye. Bold type indicates identity with Nl-EST1, while shading indicates identity across all seven sequences. The amino acid residues predicted to be members of the catalytic triad are marked with an asterisk, those predicted to contribute to the oxyanion hole are marked with a plus sign, the cysteine residues in Nl-EST1 predicted to form disulphide bridges are indicated with a filled triangle and the possible N-glycosylation sites are marked with an open triangle.

more than fifty positive clones. cDNAs from five positive plaques, chosen randomly, revealed four inserts of ≈ 1.7 kb and a fifth of ≈ 1.9 kb. Both inserts were sequenced completely in both directions. The cDNA sequences of the two clones were truncated at the 5′-end and were identical apart from an additional 71 bp at the 5′-end and a longer poly(A) tail in the 1.9 kb clone. They encoded a carboxylesterase gene with an open reading frame at its 5′-end, which terminated at a stop codon. PCR, performed on the 5′-end of the primary screen cDNAs, isolated the 5′-end of the gene. These PCR products overlapped exactly with that of the two partial length cDNAs and added an additional 139 bp to the 5′-end of the sequence including the AUG methionine start codon. The full-length cDNA had an open reading frame of 1937 base pairs and coded for a protein of 547 amino acids (Fig. 1). A putative polyadenylation signal (AAUAAA) occurred 220 bp after the predicted stop codon (GENBANK accession no. AF302777). The inferred Sri Lanka-R Nl-EST1 protein has a calculated molecular weight of 60 kDa and a pI of 5.56. Analysis of the Nl-EST1 amino acid sequence using the computer program SIGNALP indicated a signal peptide of 24 amino acids, with a cleavage site between amino acids 24 and 25. The program PSORT II found no motifs to target the protein for transport into cell organelles or for retention in the endoplasmic reticulum. Therefore, the enzyme is probably secreted. Analysis of the inferred amino acid sequence by the program PSITE identified six possible N-glycosylation sites at positions 49, 84, 258, 268, 402 and 407 (Fig. 1). The site at position 268, occurs in a hydrophobic domain (predicted by the method of Kyte & Doolittle (1982) within the PROTEAN program) and is next to a cysteine residue which is predicted by alignment with other esterases to form a disulphide bridge, hence it would not be accessible for glycosylation. The remaining sites occur in hydrophilic domains at or near turn regions [as predicted by the method of Chou & Fassman (1978)] making them readily accessible for glycosylation. PSITE identified several possible sites for the phosphorylation of serine, threonine and tyrosine residues. It is possible that phosphorylation could account for the discrepancy between the deduced size of the protein (60 kDa) and its size estimated by SDS-PAGE (67 kDa) (Small & Hemingway, 2000). The size of the Sri Lanka-R esterase in the control incubation without CIAP was estimated to be 67.4 kDa (Fig. 2). Incubation with CIAP for 4, 24 and 48 h produced two additional bands of 60.7 and 57.0 kDa. This suggests that at least two phosphorylation sites are utilized in the mature protein.

The predicted Sri Lanka-R Nl-EST1 contains all invariant residues required in the assembly of an active esterase/lipase (Cygler *et al.*, 1993). Essential active site residues, by alignment of Nl-EST1 with other members of the esterase/lipase family, are predicted to be Ser215, Glu345 and His466 (Fig. 1). Ser215 forms part of the sequence motif

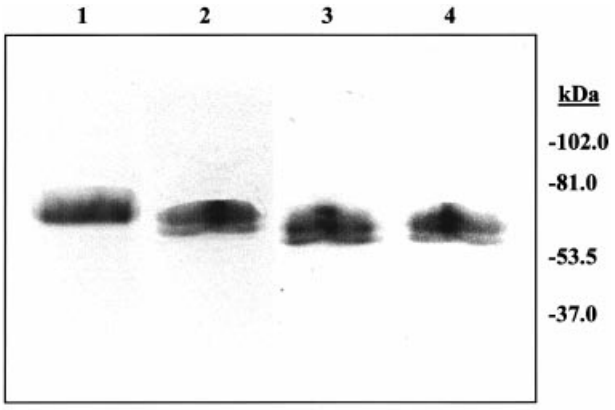

Figure 2. Purified Nl-EST1 dephosphorylated with calf intestinal alkaline phosphatase (CIAP) 20 mg of Nl-EST1 was incubated with CIAP for 4 h (lane 2), 24 h (lane 3) and 48 h (lane 4), separated on a 10–15% gradient SDS-polyacrylamide gel and stained with Coomassie Blue. Lane 1 is Nl-EST1 incubated under identical conditions without CIAP. Molecular weight standards were run on adjacent lanes.

Gly-Xaa-Ser-Xaa-Gly (Nl-EST1 = Gly213-Asp214-Ser215-Ala216-Gly217) which is conserved in all esterases/lipases (Cygler *et al.*, 1993). The multi-alignment of esterases/lipases predicts that Ala216, together with Gly133 and Gly134, form the oxyanion hole of the active site (Fig. 1). The two intramolecular disulphide bridges conserved in nearly all esterases/lipases would join Cys89 with Cys110 and Cys269 with Cys281 in N1-EST1 (Fig. 1). The Sri Lanka-R Nl-EST1 contains all 24 residues that are invariant across 29 active esterases/lipases (Cygler *et al.*, 1993).

The inferred amino acid sequence of Nl-EST1 is most similar to that of the E4 esterase of *Myzus persicae* with which it shares 38% identity. Its identity with other insect carboxylesterases is in the range 32–35%. The program PHD aligned the Sri Lanka-R esterase with forty-seven other proteins, all of which are from the esterase/lipase family and all of which have a pair-wise sequence identity of at least 30%.

Using primers designed from the coding and noncoding regions of the Nl-EST1 cDNA, overlapping fragments of a homologous cDNA were amplified from individual Sri Lanka-S adults by RT-PCR. The cDNA obtained was 100% identical to that of the Nl-EST1 cDNA. No products were obtained when Sri Lanka-S total RNA was used as the template for PCR reactions, showing that fragments amplified during PCR were not due to contamination of the reactions with Sri Lanka-R cDNA or with genomic DNA.

The 1.9 kb Sri Lanka-R esterase cDNA was used as a probe for Southern blot analysis of *Eco*RI and *Pvu*II restriction digests of equal amounts of DNA extracted from the insecticide-resistant Sri Lanka-R and the insecticide-susceptible Sri Lanka-S strains. After hybridization and high stringency washing of the blot, the probe bound to several

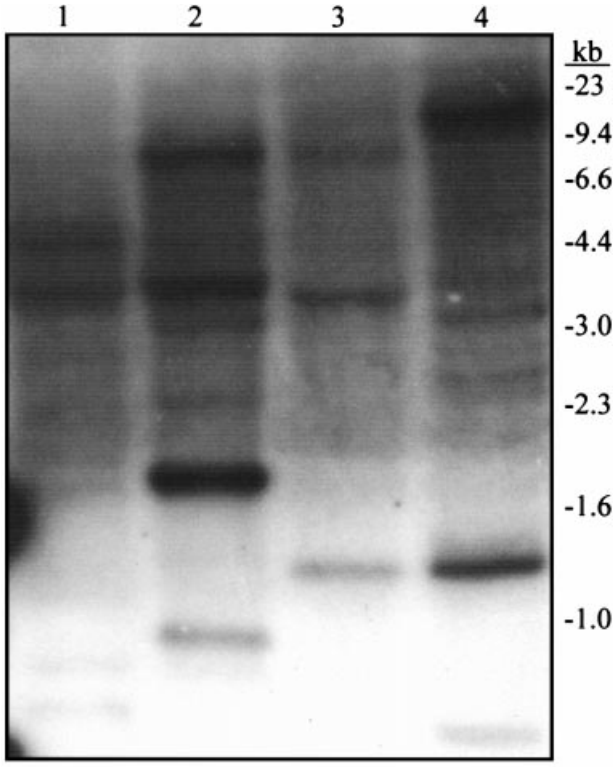

Figure 3. Southern blot of equal amounts of Sri Lanka-S and Sri Lanka-R genomic DNA digested with *Eco*RI and *Pvu*II and hybridized with a Sri Lanka-R Nl-EST1 cDNA probe. Lanes 1 and 2 are Sri Lanka-S and Sri Lanka-R genomic DNA digested with *Eco*RI and lanes 3 and 4 are Sri Lanka-S and Sri Lanka-R genomic DNA digested with *Pvu*II.

bands in both the Sri-Lanka R and Sri-Lanka S digested DNA (Fig. 3). In the *Eco*RI digested Sri Lanka-R DNA there are three bands of high intensity at 8 kb, 3.7 kb and 1.8 kb, and several other bands of lower intensity. The 8 kb and 1.8 kb bands only occur in the *Eco*RI digested Sri Lanka-R DNA. The 3.7 kb is present in the *Eco*RI digested Sri Lanka-S DNA but at a lower intensity than in the Sri Lanka-R DNA. In the *Pvu*II digested Sri Lanka-R DNA there are two high intensity bands at 12 kb and 1.3 kb whilst there are three lower intensity bands in the *Pvu*II digested Sri Lanka-S DNA of 8 kb, 3.5 kb and 1.3 kb. The higher intensity signal in the insecticide-resistant strain compared with the susceptible suggests that the underlying mechanism of esterase elevation in the Sri Lanka-R strain is gene amplification. Analysis of the Nl-EST1 cDNA sequence for restriction sites reveals that there is one *Pvu*II site that cuts between nucleotides 730 and 731, and no *Eco*RI sites. The number of hybridizing bands in the *Pvu*II and *Eco*RI digested Sri Lanka-S DNA therefore suggests the presence of introns within the genomic sequence. The difference in the RFLP pattern of Nl-EST1 in Sri Lanka-S and Sri Lanka-R may be associated with changes in sequence brought about by the amplification event.

Discussion

All esterase cDNA clones chosen randomly following screening of the Sri Lanka-R cDNA library with the depleted polyclonal Sri Lanka-R antiserum had identical overlapping sequences. This suggests that a single esterase gene has been amplified in resistant insects. Much of the heterogeneity of the elevated esterases in *N. lugens* is explained in terms of differential glycosylation of sites with sialic acid-containing glycans. However, two isoforms of elevated esterase were still detected on IEF gels following complete deglycosylation (Small & Hemingway, 2000). The difference in pI between the deduced protein both with and without the signal peptide at the N-terminus is only 0.007, which is not sufficient to account for the difference in pI of the esterase isoforms on IEF. This indicates a further post-translational modification of the protein giving rise to these two isoforms. The N-glycosylation sites at positions 49 and 84 align with a site in the Dm Est6 and MpE4 esterases, respectively. However, although most of the possible N-glycosylation sites of the insect esterases are similarly placed in hydrophilic domains, there is otherwise no correlation between site placements in these enzymes.

The calculated molecular weight of the inferred Nl-EST1 protein lies at the lower end of the 60–70 kDa range of most esterases (Cygler *et al.*, 1993) and is significantly smaller than the size of the elevated *N. lugens* esterase, estimated by SDS-PAGE, for either the glycosylated or deglycosylated enzyme. Alkaline phosphatase treatment of Nl-EST1 enhanced the electrophoretic migration on SDS-PAGE to give two bands with molecular weights of 60.7 and 57.0 kDa. These bands correspond to the predicted protein sizes of 60 and 57.6 kDa with and without the cleavable signal peptide. Alkaline phosphatase treatment also enhanced the electrophoretic migration of acetylcholinesterase isolated from various mammalian sources under denaturing conditions and raised the isoelectric point of some of the treated acetylcholinesterase molecules (Grifman *et al.*, 1997). The latter effect might explain the fact that, whilst the pI of 5.56 for Nl-EST1 is well within the range pH 4.7–6.5 which is typical of carboxylesterases (Heymann, 1980), the pI for the deglycosylated enzyme, estimated by isoelectric focusing, was slightly lower than that predicted. Of the twenty-two possible phosphorylation sites of Nl-EST1 identified by the programme PSITE, only two, both casein kinase II phosphorylation sites, aligned with similar sites in other insect esterases. The first site (positions 173–176) aligns with a site in the *M. persicae* E4 and *D. melanogaster* EST6 and the other (positions 342–345) aligns with a site in *L. cuprina* Lcα$E7^{1}$ and *C. quinquefasciatus* Estβ1^{2} and Estβ2^{1}. It has been hypothesized that phosphorylation in acetylcholinesterase may offer a rapid feedback mechanism that can compensate for impairments in cholinergic neurotransmission

due to exposure to both natural and xenobiotic compounds, including organophosphate and carbamate insecticides, modulating the hydrolytic activity of the enzyme and enabling acetylcholine hydrolysis to proceed under such challenges (Grifman *et al*., 1997).

Nl-EST1 from five individual insecticide-susceptible, low esterase activity strain Sri Lanka-S adults was identical in sequence to Sri Lanka-R esterase. In the aphid *Myzus persicae*, resistance is also due to the amplification of an identical gene to that in insecticide-susceptible insects (Devonshire, 1977). However, the aphids primarily reproduce asexually, unlike *N. lugens*. In contrast, in the sexually reproducing mosquito *Culex quinquefasciatus* the amplified esterases differ from, and code for, proteins which have a higher affinity for organophosphates than their susceptible equivalents (Small *et al*., 1998).

Experimental procedures

Planthopper strains

Two strains of *N. lugens* were used, both of which were derived from a heterogeneous population sampled from rice plants in Batalagoda, Sri Lanka in 1980. Adult malathion selection at the LT_{80} level for five generations resulted in Sri Lanka-R, a strain homogeneous for an elevated esterase-based organophosphorus insecticide resistance mechanism. A strain homozygous for low esterase activity, Sri Lanka-S, was derived by single family selection of the parental strain for five generations. Sri Lanka-R was 8.5-fold more resistant to malathion than Sri Lanka-S at the LT_{50} level (Hemingway *et al*., 1999).

Production of a polyclonal antiserum

A polyclonal antiserum was raised in a New Zealand White rabbit by the method of Vaughan & Hemingway (1995) using nondenatured elevated *N. lugens* esterases purified from the Sri Lanka-R strain by the method of Small & Hemingway (2000). The resultant antiserum was depleted of antibodies cross-reactive with XL1-Blue MRF′ bacterial proteins by the method of Sambrook *et al*. (1989). This antiserum had a high level of specificity to the elevated Sri Lanka-R esterases in Western blots of crude homogenates.

Isolation of cDNA clones for the N. lugens *esterase*

Construction and screening of a cDNA library from Sri Lanka-R. Total RNA was isolated from 0.5 g (wet weight) of adults. Insects were ground under liquid nitrogen and the resultant powder vortexed with 0.5 ml of TRI reagent (Sigma). Thereafter, RNA isolation followed the manufacturer's methodology. mRNA was isolated with the PolyATract mRNA Isolation System IV (Promega). cDNA was synthesized with the Riboclone cDNA Synthesis System (Promega) using an oligo-(dT) adaptor primer [5′ GACTCGAGTCGACATCGA-$(dT)_{17}$ 3′]. First and second strands were synthesized, purified by ethanol precipitation and dissolved in a small volume of water. The cDNA was blunt-ended and then size-selected (> 400 base pairs) on a Sephacryl S-400 spin column (Pharmacia Biotech Inc.). After *Eco*RI linker addition (Promega) and subsequent phosphorylation, 150 ng of cDNA was ligated into 1 μg Lambda ZAP II prepared arms (Stratagene) and packaged with Gigapack III Gold extracts (Stratagene). The library was screened with the depleted polyclonal Sri Lanka-R esterase antiserum at a dilution of 1 : 500 following the *pico*Blue immunoscreening kit methodology (Stratagene).

Positive clones were purified by four rounds of screening. *In vivo* excision of the pBluescript SK(–) phagemid from the Lambda ZAP II vector was carried out following the manufacturer's methodology (Stratagene). Two clones that differed in size by 208 bp were sequenced with universal primers complemented with subcloning of endonuclease-digested cDNA fragments and with primers specific to the Sri Lanka-R esterase clones. This allowed the sequencing of both strands of the insert cDNAs from the plasmid.

PCR of cDNA 5′-ends. Using a universal forward primer to the pBluescript SK(–) phagemid and a reverse primer (5′-GCAAAC-ACTTGGTGGGCTC-3′) positioned close to the 5′-end of the longer of the cDNAs originally isolated from the cDNA library, PCR was performed directly on cDNA/Lambda ZAP II clones isolated during the primary library screen. PCR reactions (50 μl) contained 20 ng cDNA/vector DNA, 100 ng of each primer, 0.5 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer) and 1× PCR Buffer II (Perkin Elmer). Reactions were heated to 95 °C for 10 min followed by thirty-five cycles of amplification (95 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min). Several of these clones gave PCR products that were larger in size than those obtained from the two clones already sequenced. Two independent PCR products were purified using Microcon YM-100 centrifugal filter devices (Millipore) and sequenced in both directions.

Reverse transriptase-PCR (RT-PCR) amplification of the homologous planthopper esterase from Sri Lanka-S. Five Sri Lanka-S adults were homogenized individually in 150 μl of TRI reagent (Sigma) and total RNA isolated following the manufacturer's methodology. The resultant RNA pellet was resuspended 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]. After treatment, the RNA was re-extracted with TRI reagent and resuspended in nuclease free water. First strand cDNA was synthesized using an oligo $(dT)_{15}$ primer (Promega) and SuperScript II reverse transcriptase (Gibco BRL). PCR primers designed from the Nl-EST1 cDNA sequence (both coding and noncoding) were used to amplify a homologous gene from the Sri Lanka-S cDNA. The position of these primers on the Nl-EST1 sequence and their direction of amplification can be seen in Fig. 4. PCR conditions were as described above for the PCR of cDNA 5′-ends. Identical PCR reactions were run with aliquots of total RNA to check for genomic DNA contamination. PCR products were purified using Microcon YM-100 centrifugal filter devices (Millipore) and sequenced in both directions.

Genomic DNA studies. A Sri Lanka-R Nl-EST1 cDNA fragment was used as a probe to determine the haplotype of the esterase genes from the Sri Lanka-R and Sri Lanka-S strains. Genomic DNA was isolated from adults by the method of Vaughan *et al*. (1995). Ten micro-grams of genomic DNA was digested to completion with *Eco*RI or *Pvu*II and separated by gel electrophoresis through 0.8% (w/v) agarose. The DNA was transferred to charged nylon membranes (Amersham Corp.) and hybridized with a ^{32}P-labelled Sri Lanka-R esterase cDNA probe (specific activity

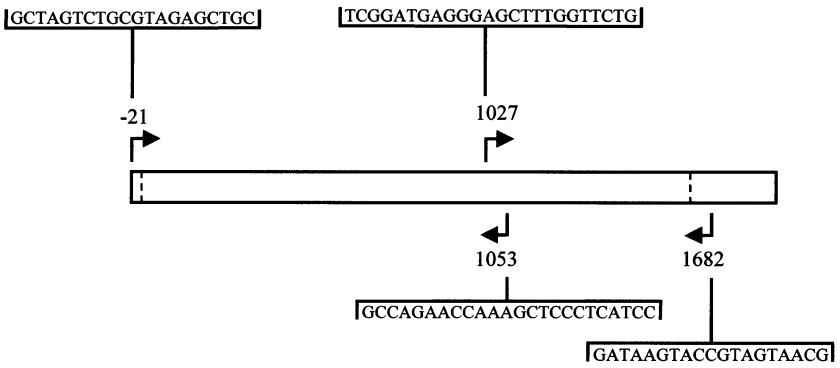

Figure 4. The nucleotide sequence, position and direction of amplification of primers designed from the Sri Lanka-R Nl-EST1 cDNA and used in RT-PCR reactions with Sri Lanka-S mRNA and subsequent sequencing of PCR products. The dashed lines indicate the positions of the start and stop codons on the Sri Lanka-R Nl-EST1 cDNA. GENBANK™ accession no. AF302777.

> 2×10^6 c.p.m./μg) at 65 °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) PEG 8000, and 100 μg/ml boiled, sheared herring sperm (DNA). The final washes were at 65 °C in 0.1 × SSC and 0.1% (w/v) SDS for 20 min.

Phosphorylation Sri Lanka-R Nl-EST1. To investigate possible phosphorylation of Nl-EST1 protein, purified by the method of Small & Hemingway (2000), was treated with calf intestinal alkaline phosphatase (CIAP) (Promega). Protein (2 mg) was incubated with 2 U CIAP in 1× CIAP buffer in a 20-μl reaction at 37 °C. A 5 μl aliquot was withdrawn after 4 h and after 24 h. A further 2 U CIAP was added to the reaction and incubation continued up to 48 h. A control reaction containing 2 mg protein and 20 μl 1 × CIAP buffer was incubated under the same conditions without CIAP. All aliquots were made up to 2% SDS and 5% β-mercaptoethanol, boiled for 5 min and put on ice. 4 μl of each aliquot was applied to a 10–15% gradient Phastgel (Pharmacia) with molecular weight markers (Sigma) and subjected to SDS-PAGE using the Phastsystem (Pharmacia) (250 V, 10 mA, 3 W, 15 °C, 60 Vh). Proteins were visualized by staining with Coomassie Blue. The size of the esterase after treatment was estimated using its relative mobility and the linear regression equation for the molecular weight markers.

Acknowledgements

This work was supported by a BBSRC Award to J.H. We thank Gareth Lewis for technical assistance with sequencing.

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