Analysis of amplicons containing the esterase genes responsible for insecticide resistance in the peach-potato aphid *Myzus persicae* (Sulzer)

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The amplification of genes encoding an insecticide-detoxifying esterase (E4) in the peach-potato aphid *Myzus persicae* is one of the few examples where this genetic phenomenon has been shown to be involved in the response of an intact higher organism to artificial selection. Here we report quantitative and qualitative studies of the repeat units (amplicons) containing the E4 genes in a highly resistant aphid clone. Initial studies to quantify esterase sequences showed a 5–11-fold increase in resistant aphids compared with susceptible aphids, suggesting the presence of 10–22 gene copies per diploid genome. A more incisive analysis by pulsed-field gel electrophoresis confirmed the presence of about 12 copies of the E4 gene and showed them to be on about 24 kb amplicons, arranged as a tandem array of direct repeats. This, together with previous results from crossing experiments and with recent *in situ* hybridization studies, confirms that the E4 gene amplification in this aphid clone is heterozygous at a single locus. However, these data show that the gene amplification alone cannot account for the approx. 60 times higher levels of E4 protein and its mRNA present in this aphid clone, and therefore resistance must involve changes in both esterase gene copy number and gene expression.

INTRODUCTION

Gene amplification in eukaryotes can occur in a developmentally regulated way, to synthesize large amounts of a particular gene product as and when required, or as a sporadic spontaneous event [1]. The latter is a rare genetic occurrence, but when the amplified sequences contain advantageous genes, the amplification may be selected by appropriate environmental conditions. The most widely studied examples are in cultured cells, where treatment with cytotoxic drugs rapidly selects for cells carrying amplified genes that encode either the target proteins [e.g. dihydrofolate reductase (DHFR) or the multifunctional protein **c**arbamoyl phosphate synthetase/**a**spartate trans-carbamylase/**d**ihydro-orotase (CAD)] or proteins involved in detoxification (e.g. P-glycoprotein, an efflux pump) [2]. Amplifications can also occur *in vivo*, as with oncogenes in tumour tissues and genes encoding insecticide-detoxifying enzymes in mosquitoes and aphids [3]. Amplification in tumours is a somatic event and may be similar to amplification in cultured cells, less is known about the germline amplification in whole insects.

A major contribution to understanding the mechanism of amplification has come from studies of the structure and chromosomal location of the amplified DNA. Amplified genes in cultured cells can be on extrachromosomal elements or integrated into chromosomes as homogeneously staining regions, and the unit of DNA that increases in copy number during amplification has been termed an amplicon [4]. Such amplicons are generally much larger that the gene under selection and are probably formed by multiple recombination events [5]. Initially amplicons can be very large but then as copy number increases there is progressive and extensive loss of co-amplified DNA [2,6,7]. Amplified insecticide-resistance genes in insects are integrated into the chromosomes [8] but the amplicon structure is not known.

Early methods of assessing amplicon size involved estimating the DNA content of homogeneously staining region and dividing by the repetition factor [e.g. 9] or staining restriction digests and summing the sizes of visible discrete bands [e.g. 10]. Renaturation within the gel in order to visualize amplified fragments [11] has also been used widely [e.g. 12], and, more recently, the distance between *in situ* hybridization signals has been measured [13,14].

In 1987 Borst et al. [15] made use of rare-cutting restriction enzymes together with pulsed-field gel electrophoresis (PFGE) to show that DHFR amplicons in mouse cell lines were initially 550 kb and homogeneous, but with further amplification became heterogeneous units of 250, 350 and 550 kb. Subsequently a minimum size (140 kb) of DHFR amplicons was measured in cultured mosquito cells [16].

Here we report the use of PFGE for qualitative and quantitative studies of the amplicons containing genes encoding the insecticide-detoxifying esterase E4 in a naturally occurring insecticide-resistant clone of the peach-potato aphid, *Myzus persicae*.

MATERIALS AND METHODS

Aphid clones

Two parthenogenetic clones of *M. persicae* were studied. The susceptible clone, US1L, has no elevated esterase or amplified genes, whereas the very resistant (R_3) clone, 794J, has about 60 times more E4 protein and mRNA [17] and has amplified E4 genes linked to a heterozygous chromosomal translocation typical of very resistant aphids [8]. The amplified E4 gene spans approx. 4.3 kb, including seven introns, and restriction mapping

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Abbreviations used: PFGE, pulse-field gel electrophoresis; DHFR, dihydrofolate reductase.
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of flanking DNA shows that the repeat unit must be larger than 15 kb [18]. The cDNA encoding E4 has been cloned and sequenced [19].

For the quantification studies, where even slight contamination would have had a major impact on the results, the purity of the aphid cultures was given special attention. Individual aphids were placed on single Chinese cabbage leaves, which had been confirmed as aphid-free, in Blackman boxes [20]. After they had produced clonal offspring, the adults were removed and their esterase levels confirmed by immunoassay. Their F_1 offspring were then reared to adulthood before DNA extraction. For PFGE analysis, where larger numbers were required, aphids reared as above were transferred to Chinese cabbage plants previously fumigated with nicotine [20].

DNA preparation and restriction digests

DNA for analysis by conventional agarose-gel electrophoresis was extracted from aphids as described previously [17], quantified using a fluorimeter and Hoechst 33258 dye and digested with restriction enzymes. For PFGE, aphids (1 g) were ground carefully in a Dounce homogenizer (with a B pestle) in 10 ml of ice-cold PBS (0.8 % NaCl, 0.02 % KCl, 0.14 % Na_2HPO_4, 0.02 % KH_2PO_4) containing 500 mM EDTA and filtered through two layers of muslin. The filtrate was centrifuged at 1500 g for 5 min at 4 °C and the pellet resuspended in the same buffer (0.5 ml) and mixed at 37 °C with 0.5 ml of 1 % low-gelling-temperature agarose (Sea Plaque). After setting in moulds (10 mm × 7 mm × 1.5 mm) the agarose plugs were incubated in 1 mg/ml Pronase in NDS buffer (1 % N-lauryl sulphate, 500 mM EDTA, pH 9.5) [21] at 50 °C for 48 h. The plugs were then washed twice for 2 h in ice-cold NDS and stored in this buffer at 4 °C. Before DNA digestion each plug was washed 3 times for 30 min in ice-cold TE (10 mM Tris/HCl, pH 8, 1 mM EDTA), cut into three pieces and incubated for 30 min in the appropriate restriction enzyme buffer. The buffer was then replaced with fresh buffer containing restriction enzyme (50 units for complete digestions or less for partial digestions, see below) and the plugs were incubated at 37 °C or 50 °C for 2–4 h.

PFGE

Plugs containing digested aphid DNA were inserted into the wells of 1.5 % agarose gels and electrophoresed using a 'waltzer' apparatus [21]. The conditions used for each gel are described in the Figure legends. Size markers were *Saccharomyces cerevisiae* YP148 chromosomes and λcI857 multimers.

Detection of esterase sequences

DNA was transferred from agarose gels by Southern blotting to Hybond-N nylon membrane (Amersham) and probed with radiolabelled (^{32}P) cloned E4 genomic sequences as described previously [22]. Details of the probe are given in the Results section.

Quantification of radiolabel bound to nylon membranes

The amount of ^{32}P-labelled esterase probe that had hybridized to aphid DNA sequences on nylon membranes was measured using a Molecular Dynamics Phosphor Imager. The membrane was exposed for 24 h and the intensities measured using Image Quant software. Measurements are expressed in arbitrary units.

RESULTS

Preliminary studies showed that the amplified DNA in 794J aphids appeared to have a simple structure compared with some other resistant aphid clones. This clone was therefore chosen for our initial analysis.

Quantification of E4 copy number in resistant aphid cone 794J

Although the susceptible (S) allele, corresponding to the amplified E4 gene, has not been cloned, a comparison of restriction fragments has shown that when enzymes that cut within the E4 gene are used in conjunction with an E4 probe, there are fragments common to the DNA of both susceptible and 794J aphids (results not shown). One such fragment, spanning 1.7 kb of the E4 gene from the *Eco*RI site in exon 3 to the *Kpn*I site in intron 5 [22], was cloned from 794J aphids and used to probe US1L and 794J DNA digested with the same two restriction enzymes (*Eco*RI and *Kpn*I). Figure 1 shows that the amount of the probe bound is approximately equivalent for US1L and a corresponding 8-fold dilution of 794J DNA.

In order to quantify the probe bound to the 1.7 kb esterase fragment, the amount of ^{32}P on the membrane was measured using a Phosphor Imager. The amount of ^{32}P in two other bands, A and B (see Figure 1), was also measured and the results are given in Table 1. These bands appear to result from the probe binding to non-amplified related sequences (possibly other esterase genes), and were therefore used as internal standards to normalize the DNA loading between tracks. Using fragment A as a standard gives an increase in binding to 794J DNA of
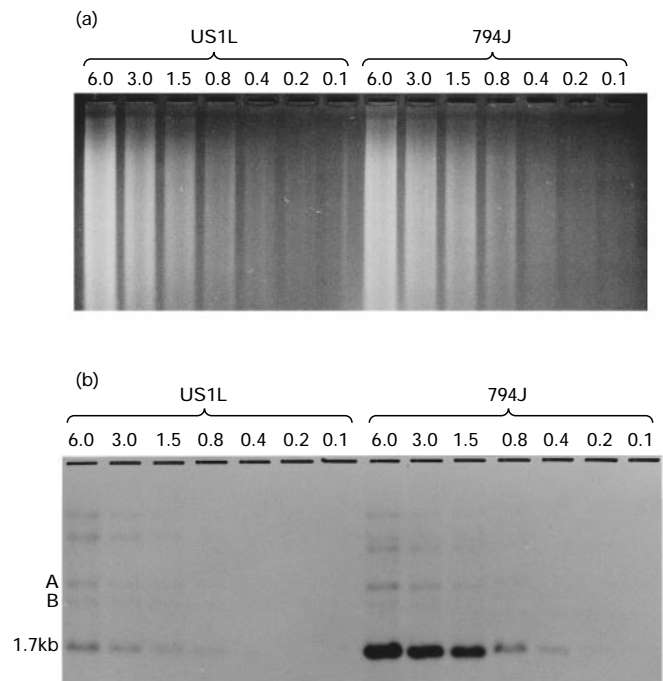

Figure 1   DNA (0.1–6.0 μg) extracted from susceptible (US1L) and resistant (794J) aphids, digested with *Eco*RI and *Kpn*I and electrophoresed for 16 h in 0.8 % agarose gels at 1 V/cm

(**a**) Ethidium bromide-stained gel; (**b**) Southern-blot-probed with the cloned 1.7 kb *Eco*RI–*Kpn*I E4 fragment.

Table 1   Amount of ^{32}P bound to nylon membranes in the region of the 1.7 kb *Eco*RI–*Kpn*I esterase fragment and two other bands, A and B (see Figure 1)

	^{32}P bound (arbitrary units)								
	US1L			794J					
DNA (μg) ...	6.0	3.0	1.5	6.0	3.0	1.5	0.8	0.4	0.2
1.7 kb	6947	3265	1983	58 395	37 230	24 593	11 120	4044	1741
A	2362	—	—	3526	2155	—	—	—	—
B	1154	—	—	930	736	—	—	—	—

approx. 5- and 6-fold for the 6 μg and 3 μg tracks respectively; similarly using fragment B gives about 11- and 8-fold increases. From these data we can conclude that the DNA from the resistant 794J aphid clone has between 5 and 11 times more esterase sequences than US1L. In a duplicate experiment, using different DNA preparations, a similar range of values was obtained. Thus, assuming that susceptible aphids have two copies of the S allele (*M. persicae* is diploid), 794J must have between 10 and 22 E4 genes, and, to be consistent with inheritance studies [23] and *in situ* hybridizations [8], these must all be on one homologous chromosome.

Analysis of E4 amplicons by PFGE

In situ hybridization [8] has indicated that the amplified E4 genes in resistant aphid clone 794J occur at a single heterozygous locus on the short translocated element of chromosome 3 (794J is clone 3980 in ref. [8]). If the genes are arranged as a tandem array of direct repeats, then digesting the DNA with a restriction enzyme that cuts once per amplicon should result in the amplified E4 sequences being on a single fragment, the size of the amplicons. Partial digests with the same enzyme should give a ladder of bands with a size increment corresponding to the amplicon size. Furthermore, if the probe binds on one side of the restriction site, the number of bands will represent the number of amplicons present.

Previous mapping studies of the amplified E4 gene and its flanking DNA had shown a *Sal*I site 1.6 kb upstream of the ATG start codon and no other *Sal*I site within about a 15 kb region spanning the E4 gene [22]. Probing *Sal*I digests of 794J DNA (using an excess of enzyme) with the 1.7 kb *Eco*RI–*Kpn*I E4 probe gave an approx. 23 kb band (results not shown). Partially digested DNA separated by PFGE gave the same band and an additional 11 larger bands (48–290 kb) with an average incremental size of 24.3 kb (Figure 2). As the probe binds on one side of the *Sal*I site (i.e. downstream), this suggests that there are 12 copies of the amplicon, in agreement with the quantification studies. In Figure 2 the probe also binds to esterase sequences on undigested, or very infrequently digested, DNA which either remains in the plug or migrates to the limit of resolution of the gel. The double band at this limit is always present for undigested or partially digested DNA and coincides with a region of high DNA intensity as judged by staining with ethidium bromide. Full digestion with *Sal*I (or any other restriction enzyme) removes this DNA and eliminates esterase probe binding in this region. The double band thus corresponds to random-sized large DNA fragments migrating together, as seen on all agarose gels, whether PFGE or conventional.

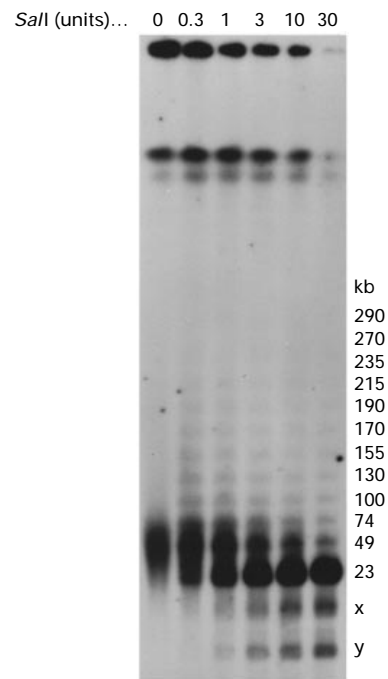When the 794J DNA was digested with 1 or more units of *Sal*I

*Sal*I (units)...   0 0.3 1 3 10 30

kb
290
270
235
215
190
170
155
130
100
74
49
23
x
y

Figure 2   Southern-blot analysis of DNA from resistant (794J) aphids, digested with *Sal*I, subjected to PFGE (for 25 h at 150 V with an effective pulse time of 16 s) and probed with the cloned 1.7 kb *Eco*RI/*Kpn*I E4 fragment

Sizes of bands were assessed relative to size markers (λ ladder).

there were also two bands smaller and less intense than the 23 kb amplified band (i.e. X and Y in Figure 2). These could represent the end of the amplified array, or the unamplified homologue, or possibly other single-copy esterase genes with homology to the probe.

If 794J aphids have 12 copies of the E4 gene on about 24 kb amplicons in a tandem array at a single genetic locus, restriction enzymes that do not cut within the amplicon should produce a single fragment contaning all of the amplified genes, with a minimum size of approx. 300 kb. This is indeed the case for a number of enzymes: *Bss*HII and *Sst*II give fragments of 350 and 320 kb respectively (Figure 3) and *Bgl*I, *Mlu*I and *Nae*I give bands of about 300 kb (results not shown). Thus Figure 4 shows the most likely arrangement of E4 amplicons in 794J aphids.
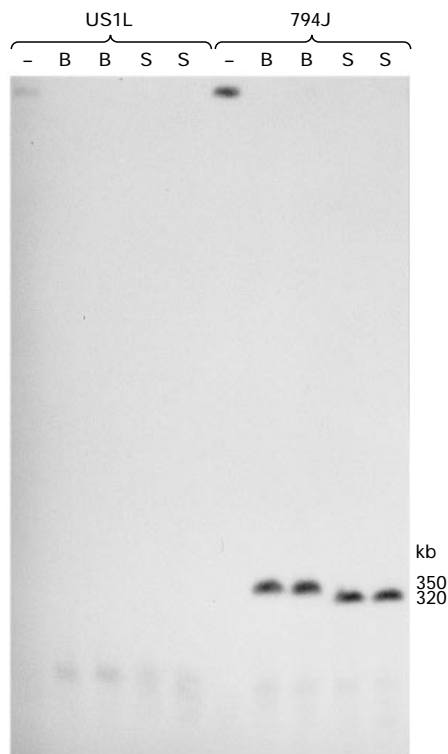
Figure 3 Southern-blot analysis of DNA from susceptible (US1L) and resistant (794J) aphids either undigested (−) or digested in duplicate with *Bss*HII (B) or *Sst*II(S), PFGE (for 27 h at 150 V with an effective pulse time of 60 s) and probed with the cloned 1.7 kb *Eco*RI-*Kpn*I E4 fragment
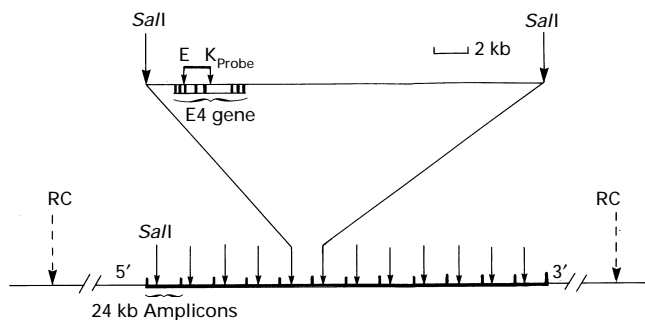

Figure 4 Proposed arrangement of E4 amplicons in 794J aphids

Unbroken arrows indicate *Sal*I restriction sites and broken arrows the approximate position for rare-cutting (RC) enzymes (*Bss*HII, *Sst*II, *Bgl*I, *Mlu*I and *Nae*I).

DISCUSSION

These results show that the highly resistant *M. persicae* clone, 794J, has about 12 copies of the E4 gene, arranged as direct repeats in tandem array on approx. 24 kb amplicons. This is consistent with *in situ* hybridization studies which have detected a single heterozygous locus for the amplified E4 sequences of 794J [8]. How typical is this of other resistant *M. persicae* clones with amplified E4 genes? Restriction mapping of the E4 locus has shown that the E4 gene and about 15 kb of flanking DNA is indistinguishable for *M. persicae* clones of wide geographic origin [22], indicating that each has amplicons of similar structure,

although the copy number may vary in line with observed differences in resistance levels. *In situ* hybridization studies have shown that three other very resistant clones have amplified E4 sequences at the same single heterozygous locus as 794J, but a fifth clone has two additional sites of amplified E4 genes (on chromosomes 2 and 5) [8]. The same study also showed that amplified aphid genes encoding a very closely related esterase FE4 are at two loci in one clone (heterozygous on chromosome 1 and homozygous on chromosome 2) and three loci in another (one homozygous and one heterozygous on chromosome 1 and one homozygous on chromosome 3). These two clones have a common close-range (< 15 kb) restriction map at the FE4 locus (differing from that of E4 genes [22]) suggesting that amplicon structure can be conserved even when the amplified genes are spread around the genome.

The size of the esterase amplicons in *M. persicae* is small (about 24 kb) compared with that reported for amplicons in cell cultures. However, it is comparable with the size seen for repeated genes such as the ribosomal RNA genes: 12 and 17 kb in *Drosophilia melanogaster*, 44 kb in humans. There may be constraints on the size that can exist in an intact organism, which do not apply to cell lines. For example, it is likely that the amplification occurred at random in *M. persicae* and was selected because of its ability to confer insecticide resistance, and this would mean that it must be transmitted via both mitosis and meiosis.

The finding that there are only about 12 copies of the E4 gene in 794J aphids means that gene amplification alone cannot account for the approx. 60-fold increases in E4 enzyme and its mRNA found in this aphid clone [17]. Clearly, in this case at least, E4 gene amplification must be accompanied by either increased gene transcription and/or greater stability of E4 mRNA. It is known that amplified E4 genes are expressed in the presence of DNA methylation in and around the genes and are silent when methylation is lost [24,25]. It is therefore possible that methylation could also play a role in overexpression of the amplified genes. Further direct evidence of methylation affecting E4 gene transcription is clearly required, and analysis of other resistant aphid clones with various levels of E4 enzyme will clarify the relative contributions of gene amplification and increased gene expression to insecticide resistance in *M. persicae*.

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