Relationship between amount of esterase and gene copy number in insecticide-resistant *Myzus persicae* (Sulzer)

Linda M. FIELD*[1], Roger L. BLACKMAN†, Chris TYLER-SMITH‡ and Alan L. DEVONSHIRE*

*IACR-Rothamsted, Harpenden, Herts. AL5 2JQ, U.K., †Natural History Museum, Cromwell Road, London SW7 5BD, U.K., and ‡Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

Overproduction of the insecticide-degrading esterases, E4 and FE4, in peach-potato aphids, *Myzus persicae* (Sulzer), depends on both gene amplification and transcriptional control, the latter being associated with changes in DNA methylation. The structure and function of the aphid esterase genes have been studied but the determination of their copy number has proved difficult, a common problem with gene amplification. We have now used a combination of pulsed-field gel electrophoresis and quantitative competitive PCR to determine relative esterase gene copy numbers in aphid clones with different levels of insecticide resistance (R_1, R_2 and R_3). There are approx. 4-fold increases between susceptible, R_1, R_2 and R_3 aphids, reaching a maximum of approx. 80 times more genes in R_3; this gives proportionate increases in esterase protein relative to susceptible aphids. Thus there is no overexpression of the amplified genes, in contrast with what was thought previously. For *E4* genes, the loss of 5-methylcytosine is correlated with a loss of expression, greatly decreasing the amount of enzyme relative to the copy number.

Key words: DNA methylation, gene amplification.

INTRODUCTION

In the peach-potato aphid, *Myzus persicae* (Sulzer), insecticide resistance can occur by the amplification of genes encoding the detoxifying esterases E4 or FE4 [1,2]. There is good evidence that the *E4* and *FE4* genes (approx. 5 kb) are part of an esterase gene family (with *E4* approx. 19 kb upstream of *FE4*) [3], situated near the subtelomeric repetitive DNA of autosome 1 [4]. The amplification of *E4* genes occurs in aphids with an A1,3 chromosome translocation and, in all but one of the cases so far studied, fluorescence *in situ* hybridization (FISH) has shown that the amplified genes are situated at a single heterozygous site on autosome 3^T [5], which was probably moved there from autosome 1 during the reciprocal exchange [4]. In one aphid clone, 794J, with amplified *E4* at 3^T, the genes are on a series of direct, head-to-tail repeat units (amplicons) of approx. 24 kb [6]. The one aphid clone (4156) so far found with multiple sites of amplified *E4* has genes on autosomes 3^T, 5 and 2 [5]. In contrast, amplified *FE4* genes are found in aphids of apparently normal karyotype and are widely distributed around the genome (from three to eight sites [4,5]) from their putative original location on autosome 1 [4]. The amplified *E4* and *FE4* sequences contain 5-methylcytosine (5mC) within and downstream of the genes and, for *E4*, the loss of this 5mC is associated with a loss of both esterase gene expression and insecticide resistance [7–9].

The amount of esterase protein present in individual aphids can be measured accurately by an immunoassay with antiserum against E4 [10] and used to characterize aphids as either susceptible (S) or resistant at one of three broad levels, R_1, R_2 or R_3, with an approx. 4-fold increase in enzyme between each level. In contrast, it has proved very difficult to quantify the esterase gene copy number. Early studies on the binding of an E4 cDNA to dot-blots of dilutions of aphid genomic DNA showed an approx. 8-fold increase between S and R_3 [1]; a subsequent quantitative assay of probe binding to an *E4* genomic fragment indicated that an R_3 clone (794J) had between 5 and 11 times more esterase gene sequences than susceptible aphids [11]. It was assumed that the susceptible aphid diploid genome has two copies of the S allele and consequently the R_3 copy number should be between 10 and 22. This was supported by the finding that pulsed-field gel electrophoresis (PFGE) of DNA from R_3 aphids, digested with rare-cutting restriction enzymes, gave a single 300 kb fragment containing *E4* genes, which would be able to accommodate approx. 12 copies of the 24 kb repeat [11]. However, in the light of what is now known about the presence of an esterase gene family [3] in susceptible aphids, these results must be reassessed. The probe used in the quantitative binding assay [11] would detect not only *E4* but also the adjacent *FE4* gene, both of which occur together in susceptible aphids, and probably also the two known related S_1E4 and S_2E4 sequences in susceptible aphids [3]. Thus the wild-type esterase copy number detected by the probe would be a minimum of 4 (i.e. diploid for E4 and FE4) and could be 8 (if S_1E4 and S_2E4 were not co-allelic). This would mean that the copy number in the amplified R_3 clone would be between 20 and 90, i.e. $(5–11) \times 4$ or $(5–11) \times 8$, a result much more in line with the approx. 64-fold increase in E4 enzyme. Uncertainty in interpreting dot-blots to measure the copy number of the B1 esterase gene in *Culex* mosquitoes has also been pointed out by Callaghan et al. [12].

The use of quantitative competitive PCR (QC-PCR) for measuring levels of mRNA species has been evaluated and described by Gilliland et al. [13] and Becker-André [14]. The mRNA species are transcribed into cDNA, and PCRs are done in the presence of a dilution series of a competitive template that uses the same primers as the target cDNA but can be distinguished after the amplification. The competitor or 'mimic' can be the

Abbreviations used: 5mC, 5-methylcytosine; FISH, fluorescence *in situ* hybridization; gDNA, genomic DNA; PFGE, pulsed-field gel electrophoresis; QC-PCR, quantitative competitive PCR.

[1] To whom correspondence should be addressed (e-mail Lin.Field@bbsrc.ac.uk).

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same sequence containing a small mutation [15,16] or non-homologous DNA ligated to sequences complementing the same primers [17]. Alternatively, cloned genomic DNA (gDNA) can be used with primers that span a small intron so that the cDNA will give a smaller band than the competitor gDNA. The point of equivalence, i.e. the point at which there is a 1:1 ratio (and the amount of PCR product is equal), will be where the concentration of unknown cDNA exactly matches that of the added competitor [13].

Here we report the use of a QC-PCR assay in the opposite configuration to quantify gene copy number in gDNA from a range of aphid clones, by using primers spanning intron 2 of the *E4* and *FE4* genes and variable amounts of cloned E4 cDNA as competitor. The amount of cDNA at the equivalence point will be correlated with, and reflect, the esterase gene copy number. We have used this technique, in conjunction with further PFGE studies, to reassess the levels of *E4* and *FE4* amplification in resistant aphids and to relate this to the amount of enzyme, the type of amplified gene present (as determined by a new PCR-based diagnostic described here) and the methylation of the esterase sequences.

MATERIALS AND METHODS

Aphid clones

Parthenogenetic cultures (clones) of *M. persicae* were reared on Chinese cabbage leaves in small boxes [18] at 20 °C with a 16 h light/8 h dark regime. Each was established from a single aphid taken from a 'stock' clone, each of which had in turn originated from single aphids collected from the field: clones US1L, T1V, 948B, 951A, 1076A, 1171D, 1261A, 1301H, 1302M, 1303A, 1305A and 1306A from the U.K.; 1090Z and 1260Y from Greece; FrR from France; 800F from Italy; and 975A from Hungary. Clone 4156 and its descendants (242DT, 97N, 271D, 246T and 95T) were the result of a breeding programme as detailed in [19].

Measurement of esterase

The activity of E4 or FE4 was assayed with naphth-1-yl butyrate for seven individuals of each clone in microplates after being trapped with a specific IgG and after the removal of other esterases by washing the plates with PBS/Tween [10]. The absorbance in each well was measured at 620 nm. In line with previously published results, readings were not corrected for the background absorbance that would be obtained in a well with all reactants except the aphid homogenate. This background (0.05 A_{620} unit) is insignificant for resistant aphids but susceptible aphids seem to have approximately twice their true E4/FE4 esterase activity (e.g. 0.09 rather than 0.04; see Figure 4). Similarly, the dynamic range of the assay is compressed at the high values given by R_3 aphids owing to the inability of the plate reader to record absorbance values over 3.0. Within these constraints, the activities given in Figures 4 and 5 broadly reflect the molar amount of E4/FE4 esterase protein in an aphid (0.4–25 pmol/mg of aphid), as measured by titration with a radiolabelled site-directed ligand [20].

Isolation and quantification of DNA

Twenty aphids from each clone were homogenized in extraction buffer; the DNA was purified [9] and then treated with RNase and extracted twice with phenol/chloroform (1:1, v/v) and twice with chloroform before precipitation with ethanol. The amount of DNA was calculated from taking 1 A_{260} unit as 50 μg/ml DNA; its purity was judged from the A_{260}/A_{280} ratio (must be more than 1.7). The DNA was then diluted to 10 ng/μl and the concentration was confirmed by spotting on agarose gels alongside herring sperm DNA standards, and staining with ethidium bromide.

Detection of methylated esterase sequences

The presence of 5mC within and downstream of the amplified esterase sequences was detected by differences in *Msp*I and *Hpa*II restriction fragments as described previously [7–9].

Diagnosis of amplified esterase genes

Distinguishing between E4 and FE4 by staining for esterase activity after PAGE can give equivocal results, so a new PCR-based diagnostic test was developed to detect the presence and type of amplified esterase gene. It made use of the finding that amplified *E4* genes have an approx. 1.7 kb insert at the 5′ end that is absent from *FE4* [3]. A primer, common to both genes, just upstream of the insert and pointing downstream (5′-GGG-AATTTTGGATTCT-3′) was used in conjunction with two primers pointing upstream: one within the insert (5′-GGC-GGACCTGACGACTC-3′) that would give only a product for E4 (572 bp) and one within intron 2 of both *E4* and *FE4* (5′-TGGTTGGGATCTAGGG-3′) that would give an 865 bp product for *FE4* and potentially a 2.5 kb product for *E4* (spanning the insert), but amplification of the latter could be prevented by limiting the extension time of the PCR.

Each 25 μl PCR amplification contained 50 ng of template DNA, 200 μM dNTPs, 1.5 mM $MgCl_2$, 30 ng of common primer, 15 ng of each gene-specific primer, 0.2 unit of *Taq* polymerase (1 unit catalyses the incorporation of 10 nmol of deoxyribonucleotides in 30 min at 70 °C) and buffer supplied with the enzyme. The reaction conditions were 94 °C for 30 s, followed by addition of the enzyme and then 35 cycles of 94 °C for 30 s, 45 °C for 120 s, and 72 °C for 60 s. The products were separated on 1% (w/v) agarose gels and stained with ethidium bromide. The technique was developed and evaluated on DNA from aphids known to be susceptible (no amplified genes) or to contain amplified *E4* or *FE4*, as judged by the presence of either a 2.2 kb (*E4*) or 1.8 kb (*FE4*) *Msp*I restriction fragment from their DNA [7]. Aphids with amplified *E4* always gave a 572 bp PCR product, those with *FE4* yielded a 865 bp product, and susceptible aphids gave small, and similar, amounts of both products, which is consistent with the presence of both unamplified genes (results not shown but see the Results section for products from clones used in the present study). The other esterase genes, S_1E4 and S_2E4, seem not to interfere with this assay and it is quicker and more reliable than a previously published PCR-restriction enzyme technique [21].

QC-PCR for *E4* and *FE4* genes

Competitive PCRs were performed with primers spanning intron 2 of both the *E4* and *FE4* genes (5′-GAACCACAACCCGT-ACAG-3′ and 5′-CCAAAATAGTATCCGCCG-3′). Each reaction contained 50 ng of gDNA from the test aphid and 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 or 6.4 pg of *E4* competitor cDNA, a full-length cDNA cloned in pBluescript [2] purified by CsCl centrifugation [22], quantified by spectrophotometry and then linearized. For these very dilute solutions of cDNA it was necessary to store individual aliquots of the amount needed for each reaction (at −20 °C) because freeze–thawing stocks resulted in rapid degradation. In addition to the template DNA species,

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each 25 μl reaction contained 30 ng of each primer and the same components as described above. The conditions were 94 °C for 30 s, followed by addition of the *Taq* polymerase and then 35 cycles of 94 °C for 30 s, 50 °C for 120 s, and 72 °C for 180 s. For gDNA the PCR product was 338 bp, and for cDNA it was 215 bp. As the concentration of cDNA increased, the products changed from being predominantly 338 bp to being 215 bp; the point at which the amounts were judged to be the same was designated the 'equivalence' point (see the Results section).

PFGE

Agarose plugs containing digested aphid DNA were prepared and subjected to electrophoresis as described previously [11] to identify the large fragments generated by rare-cutting restriction enzymes.

RESULTS AND DISCUSSION

Identification of amplified *E4* or *FE4* esterase genes and DNA methylation

The PCR-based diagnostic test for distinguishing between *E4* and *FE4* was used to characterize the 23 aphid clones in the present study. A typical result is given for 14 of the clones in Figure 1. The single 572 bp product in clones 951A, 1301H, 1306A, 1303A, 1305A and 1261A identifies the presence of amplified *E4* genes; the single 865 bp fragment in clones 800F, 1171D, 948B, 1302M, FrR and 1260Y shows that these have amplified *FE4*. For clones US1L and 1076A the presence of both bands at low intensity is characteristic of susceptible clones with no amplified genes, in which both single-copy genes give a product. It should be noted that clones with amplified *E4* or *FE4* genes also have a single copy of the other gene but this does not give a detectable product, presumably because the PCR preferentially detects the multi-copy gene. For the other clones tested, 975A and 1090Z had amplified *FE4*, and T1V, 4156 and its descendants, 246T, 95T, 97N, 271D, 242DT, all had *E4* (results not shown).

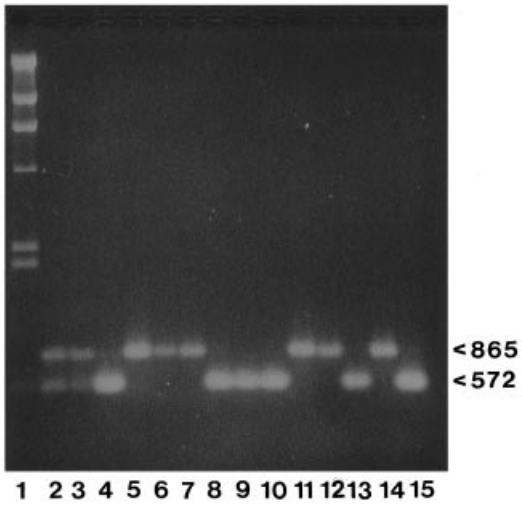

Figure 1 Products of PCR amplifications with *E4*/*FE4*-specific primers

DNA was extracted from aphid clones 1076A, US1L, 951A, 800F, 1171D, 948B, 1301H, 1306A, 1303A, 1302M, FrR, 1305A, 1260Y and 1261A (lanes 2–15 respectively) and PCRs were done with a common 5′ primer and two 3′ *E4*- or *FE4*-specific primers as described in the Materials and methods section. Lane 1 contained λ *Hin*dIII markers. The numbers at the right are sizes of fragments in bp.

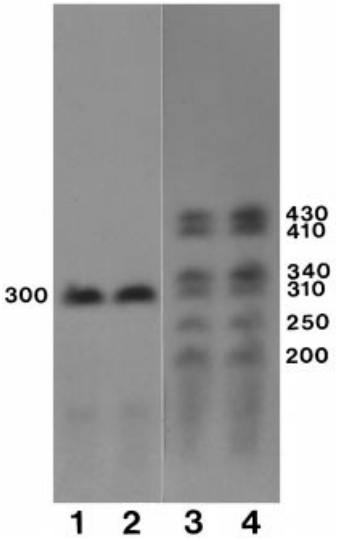

Figure 2 Southern blot analysis of DNA from R_3 aphids with either *E4* genes (lanes 1 and 2) or *FE4* genes (lanes 3 and 4)

DNA from aphid clones 794J (lanes 1 and 2) and 800F (lanes 3 and 4) was digested in duplicate with *Sst*II; the fragments were separated on a 1.5% (w/v) agarose gel by PFGE (for 27 h at 150 V with an effective pulse time of 60 s) and the blot was probed with a cloned 1.7 kb *Eco*RI–*Kpn*I *E4* fragment [10]. Unrelated samples between tracks 2 and 3 have been excised from the photograph. Sizes of fragments in kb (indicated at the left and the right) were judged by comparison with size markers.

The *Msp*I/*Hpa*II diagnostic for the presence of 5mC [7–9] in amplified esterase genes showed that the *E4* genes were methylated in clones T1V, 1305A, 1261A and 4156 but not in 1301H, 1306A and 1303A; in all clones with amplified *FE4* the genes were methylated (results not shown).

Assessment of gene amplification by PFGE

Clone 800F has R_3 levels of esterase and amplified *FE4* genes at three loci, two homozygous and one heterozygous [5]. Southern blot analysis with the use of PFGE on 800F DNA digested with the rare-cutting restriction enzyme *Sst*II is shown in Figure 2 alongside the previously published [11] result for an R_3 clone (794J), with amplified *E4* genes.

The DNA for 800F gives six bands, which could represent the five known sites plus an additional one undetected by FISH [5], or possibly the array of genes at one of the sites is cut into two fragments by the *Sst*II enzyme. Either way, the total number of copies of the repeat unit containing the *FE4* genes can be estimated. For *FE4*, restriction mapping has shown that the repeat unit (amplicon) is approx. 20 kb, in contrast with the 24 kb repeat for *E4* [6]. Allowing 50 kb for non-repeat DNA flanking the blocks of amplicons, the number of 20 kb repeat units would be 19, 18, 14, 13, 10 and 7 on the six fragments, giving a total of approx. 80 copies of the *FE4* gene in 800F.

The PFGE result for the *E4* R_3 clone, 794J, reported previously [11] and reproduced in Figure 2 shows only a single 300 kb fragment, previously considered to contain approx. 12 copies of the 24 kb amplicon containing the *E4* gene. Thus the PFGE

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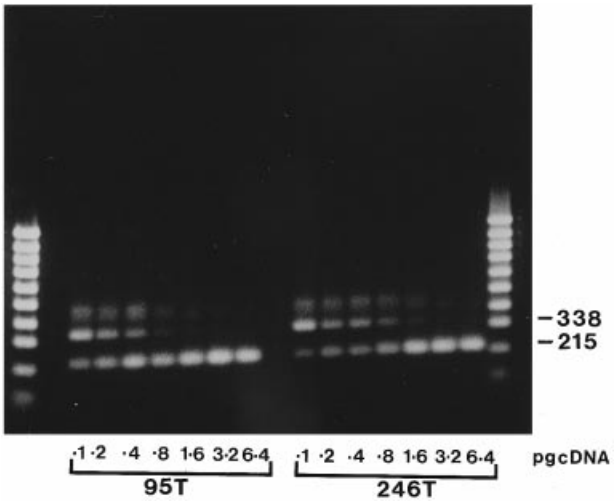

Figure 3 QC-PCR on two aphid clones

Shown are PCR products from amplifications containing 50 ng of gDNA from aphid clones 95T and 246T and a doubling series of amounts of *E4* cDNA as competitor. Lane 1 is a 100 bp ladder. The numbers at the right are sizes of fragments in bp.

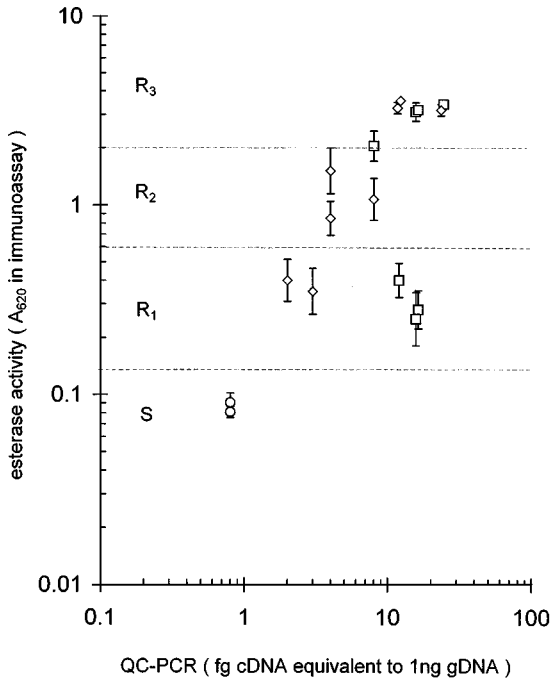

Figure 4 Relationship between QC-PCR and esterase activity in field-collected clones of *M. persicae*

Esterase activities (means ± S.D.) in 17 aphid clones are plotted against the amount of E4 cDNA equivalent to 1 ng of gDNA in a QC-PCR experiment (see the text). Symbols: ◇, clones with amplified *FE4* genes; □, clones with amplified *E4* genes; ○, susceptible clones (no amplified genes). Logarithmic scales are used because cDNA was diluted serially for the QC-PCR, and esterase activity measurements in clonal groups of aphids are known to be log-normally distributed [28]. Bands of esterase activity corresponding to S, R_1, R_2 and R_3 are denoted by dashed lines. Where QC-PCR gave the same value for two clones, the data are slightly offset either side of the value for clarity of presentation.

results might suggest that R_3 aphids with *E4* have considerably fewer gene copies than those with *FE4*. However, it is possible that the 300 kb band in the R_3 *E4* aphids is the result of several blocks of 12 copies at the same locus (and indistinguishable by FISH) being cut into the same-sized fragment owing to intervening *Sst*II sites. This interpretation is supported by the more intense binding of the probe to the 300 kb fragment in the *E4* clone relative to the individual *FE4* bands (Figure 2); indeed, the total binding to the *FE4* fragments approximates that of the single *E4* band.

To compare the amplification levels in aphids with *E4* rather than *FE4*, we have used QC-PCR to assess relative gene copy numbers.

Analysis of QC-PCR data

Typical results for the QC-PCR analysis of 50 ng of gDNA from two aphid clones (95T and 246T) are shown in Figure 3. For 95T there is a clear 'equivalence' point at 0.2 pg of cDNA (lane 4), where both products are of equal intensity. However, for 246T, the 'equivalence' point is between 0.2 and 0.4 pg of cDNA (Figure 3, lanes 12 and 13) and was taken as the mid-point, at 0.3 pg. In this way an amount of cDNA equivalent to 50 ng of gDNA was ascertained for each clone tested.

Relationship between esterase activity and gene copy number in field-collected aphid clones

QC-PCR established 'equivalence' points for the DNA from 17 field-collected aphid clones; these, expressed as fg of cDNA equivalent to 1 ng of gDNA, are plotted against esterase activities in Figure 4.

Fourteen of the clones show a proportionate relationship irrespective of the form of gene (*E4* or *FE4*) amplified. The other three *E4* clones have much less esterase enzyme than others with similar QC-PCR results. These three R_1 clones, 1301H, 1306A and 1303A, were shown to have little DNA methylation and are therefore characteristic of 'revertant' clones with unmethylated, under-expressed amplified E4 genes [7–9]. All three had been collected as R_1 aphids in 1996 in the U.K., showing that reversion had occurred in the field and that it had remained at this R_1 level in culture; indeed, in attempting to find clones with low levels of *E4* gene amplification, we found three additional U.K. revertant clones (results not shown), but none with low gene copy number.

For the 14 aphid clones showing a linear relationship between esterase activity and gene copy number, those with low or intermediate levels of esterase (R_1 and R_2) all have amplified *FE4* genes, whereas the high R_2 and R_3 levels are associated with both *E4* or *FE4* (Figure 4), i.e. the relative copy number is the same, regardless of whether *E4* or *FE4* genes are amplified. Because the PFGE results indicate that there are 80 copies in *FE4* R_3 aphids, those with comparable QC-PCR measurements should also have a similar copy number of *E4* genes. There is no way of relating the QC-PCR results to absolute copy number by comparison with susceptible aphids because we do not know whether the primers used will bind to the two other esterase genes (S_1E4 and S_2E4) known to be present in *M. persicae* [3]. However, an estimate of 80 copies in R_3 aphids is in line with our reassessment of probe binding in Southern blots of gDNA (see the Introduction section). It also means that there is no need to invoke over-expression as well as gene amplification to explain the levels of esterase associated with resistance.

Distribution of amplified *E4* genes

We have only ever found one clone with amplified *E4* genes at more than one locus [5]. This R_2 clone, 4156, has *E4* genes at the normal 3^T site and also at sites on autosomes 2 and 5, with all

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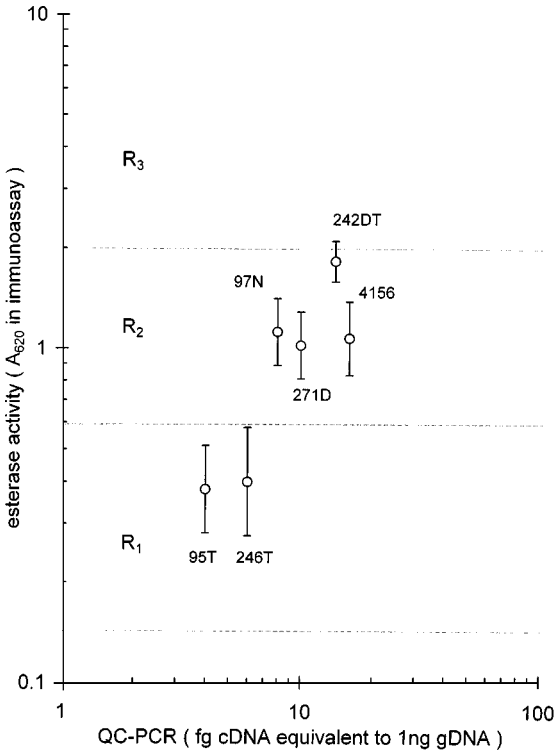

Figure 5 Relationship between QC-PCR and esterase activity in *M. persicae* clone 4156 and its descendants

Esterase activity plotted against the amount of E4 cDNA equivalent to 1 ng of gDNA in a QC-PCR experiment (see the text and the legend to Figure 4). All clones have amplified *E4* genes as follows: 4156, 2 3^T 5; 242DT, 3^T 5; 97N, 2 5; 95T, 3^T 2; 271D, 5; 246T, 3^T.

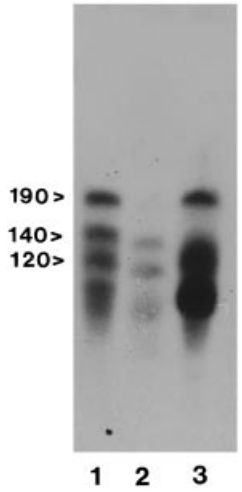

Figure 6 Southern blot analysis of DNA from aphid clones with amplified *E4* genes

DNA was digested with *Bss*HII; the fragments were separated by PFGE for 28 h at 150 V with an effective pulse time of 40 s, then probed with a cloned 1.7 kb *Eco*RI–*Kpn*I *E4* fragment [10]. Sizes of fragments in kb (indicated at the left) were judged by comparison with size markers. Lanes 1, 2 and 3 were from clones 4156, 95T and 271D respectively.

three loci being heterozygous. We have done QC-PCR on the DNA from clone 4156 and its descendants that have inherited different *E4* loci (Figure 5). Clone 242DT has sites on autosomes 3^T and 5, 97N on 5 and 2, 271D on 5, 246T on 3^T and 95T on 3^T and 2 [19]. From Figure 5 it can be estimated that the site on autosome 5 has about twice as many copies as that on 3^T, with very few copies at the autosome 2 site. This agrees with the intensity of signal observed by FISH on 4156 and its descendants, where the autosome 5 site was the brightest and the autosome 2 site was very weak [5].

Thus, like *FE4* genes, *E4* can be found in different numbers at different sites around the genome; however, this is much rarer for *E4*. This might be because clones with the A1,3 translocation rarely contribute to the sexual phase of the aphid [19] and therefore the *E4* site on 3^T tends to be protected from the effects of meiotic recombination.

These QC-PC data are also supported by PFGE results for clones 4156, 95T and 271D (Figure 6). Clone 4156 has three bands of approx. 190, 140 and 120 kb; the 190 kb band is present in clone 271D, which only has site 5, and the 140 and 120 kb bands are present in 95T, which has sites 3^T and 2. (The smaller, diffuse areas of probe binding, especially in track 3, are typical of those seen in some of our PFGE experiments, apparently arising from DNA degradation.) From the sizes of the bands there will be approx. six, four and three amplicons with *E4* genes at sites 5, 3^T and 2 respectively. Thus clone 4156 has a total of approx. 13 *E4* genes, which is broadly in line with an R_2 clone having approx. one-quarter as many copies as an R_3 aphid.

If R_3 aphids have 80 copies of the 24 kb *E4* amplicon, they will have approx. 2 Mb of amplified DNA at the heterozygous 3^T locus. The diploid genome of *M. persicae* has been reported as 0.64 pg, or approx. 600 Mb [23], so the amplified region constitutes approx. 0.3 % of the genome. Cytogenetic analysis of aphid chromosomes cannot resolve the chromosome structure associated with these large amplified regions. However, analysis of polytene chromosomes from *Culex* mosquitoes, with an estimated 32-fold [24] or 250-fold [25] amplification of the esterase B gene, has shown that the amplified array is clustered in a single extended chromosomal region (ECR) of chromosome 2 associated with a DNA 'puff'.

The results presented here have clarified the relationship between esterase gene copy number and enzyme level in resistant *M. persicae*. A wide range of copy numbers can exist for both *E4* and *FE4*, which gives, in the absence of reversion, a proportionate increase in esterase level and consequent resistance. The maximum copy number so far found is similar for both *E4* and *FE4* genes, reaching approx. 80 copies in R_3 aphids. There is apparently full expression of methylated genes; in revertants there is loss of methylation and much decreased expression. We have found this complete loss of methylation only for *E4* genes, possibly as a result of their chromosomal location (i.e. on 3^T). Highly repetitive sub-telomeric DNA translocated from autosome 1 on to 3^T [26] could be involved in the loss of *E4* gene expression; such position-effect variegation is a well-known phenomenon in *Drosophila* and other organisms [27].

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