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

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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, R, and R). There are approx. 4-fold increases between susceptible, R, and S aphids, reaching a maximum of approx. 80 times more genes in R.; 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 1 [5], which was probably moved there from autosome 1 during the reciprocal exchange [4]. In one aphid clone, 794J, with amplified E4 at 3, 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, 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, R, or R, 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-blot of dilutions of aphid genomic DNA showed an approx. 8-fold increase between S and R; [1]; a subsequent quantitative assay of probe binding to an E4 genomic fragment indicated that an R 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 copy number should be between 10 and 22. This was supported by the finding that pulsed-field gel electrophoresis (PFGE) of DNA from R, 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, E4 and S, E4 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, E4 and S, E4 were not allelic). This would mean that the copy number in the amplified R, clone would be between 20 and 90, i.e. (5–11) × 4 or (5–11) × 8, a result much more in line with the approx. 64-fold increase in E4 enzyme. Uncertainty in interpreting dot-blot 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
same sequence containing a small mutation [15,16] or non-

246T and 95T) were the result of a breeding programme as

1305A and 1306A from the U.K.; 1090Z and 1260Y from

from single aphids collected from the field: clones US1L, T1V,

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and extracted twice with phenol

...buffer; the DNA was purified [9] and then treated with RNase

Twenty aphids from each clone were homogenized in extraction

radiolabelled site-directed ligand [20].

Diagnosis of methylated esterase sequences

The presence of 5mC within and downstream of the amplified

Diagnosis of amplified esterase genes

Distinguishing between E4 and FE4 by staining for esterase

QC-PCR for E4 and FE4 genes

Competitive PCRs were performed with primers spanning intron

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designated the ‘equivalence’ point (see the Results section). The point at which the amounts were judged to be the same was changed from being predominantly 338 bp to being 215 bp; the 215 bp. As the concentration of cDNA increased, the products gDNA the PCR product was 338 bp, and for cDNA it was 215 bp. It should be noted that clones with amplified genes, in which both single-copy genes give a product, presumably because the PCR preferentially detects the multi-copy gene. For the other clones tested, no amplified genes, in which both single-copy genes give a product, bands at low intensity is characteristic of susceptible clones with one or possibly the array of genes at one of the sites is cut into two or more fragments by the SstII 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, giving a total of approx. 80 copies of the FE4 gene in 800F.

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 E4 and 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).

Assessment of gene amplification by PFGE

Clone 800F has R₉ 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 SsrII is shown in Figure 2 alongside the previously published [11] result for an R₉ 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 SsrII 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 R₉ 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...
results might suggest that $R_1$ aphids with $E4$ have considerably fewer gene copies than those with $FE4$. However, it is possible that the 300 kb band in the $R_1$ $E4$ 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 SstII 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 $R_3$ and $R_4$ 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 ($S,E4$ and $S,E4$) 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 $R_1$ clone, 4156, has $E4$ genes at the normal 3’ site and also at sites on autosomes 2 and 5, with all.
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\(^{\text{T}}\) and 5, 97N on 5 and 2, 271D on 5, 246T on 3\(^{\text{T}}\) and 95T on 3\(^{\text{T}}\) 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\(^{\text{T}}\), 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\(^{\text{T}}\) tends to be protected from the effects of meiotic recombination.

These QC-PCR 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\(^{\text{T}}\) 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\(^{\text{T}}\) and 2 respectively. Thus clone 4156 has a total of approx. 13 E4 genes, which is broadly in line with an R\(_{3}\) clone having approx. one-quarter as many copies as an R\(_{1}\) 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\(^{\text{T}}\) 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\(^{\text{T}}\)). Highly repetitive sub-telomeric DNA translocated from autosome 1 on to 3\(^{\text{T}}\) [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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