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Molecular evidence that insecticide resistance in peach–potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene

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cDNA clones for the esterase (E4) responsible for broad insecticide resistance in peach–potato aphids (*Myzus persicae* Sulz.) were isolated and used to study the molecular basis of resistance. Increased esterase synthesis by resistant aphids was found to be associated with amplification of the structural gene for the esterase (E4 or its closely related variant, FE4), the degree of amplification being correlated with the activity of the esterase and the level of resistance. Hybridization of the cDNA clones to genomic Southern blots showed that only some of the esterase-related restriction fragments are amplified. Qualitative differences between restriction patterns in different clones of resistant aphids correlated with the presence or absence of a specific chromosome translocation and with production of E4 or FE4.

INTRODUCTION

The biochemical basis of insecticide resistance has been identified in many species, but generally the molecular events responsible are poorly understood. For example, an increase in insecticide-degrading activity is a common resistance mechanism [1], but in most cases it is not known whether this arises from mutant enzymes or from the increased production of an enzyme already present in susceptible insects. However, in the peach–potato aphid (*Myzus persicae* Sulz.) it has been established that resistance results from the increased synthesis of an esterase (E4) that both hydrolyses and sequesters insecticidal esters [2]. The enzyme is a glycoprotein of M_r 65000, with a polypeptide component of 57000, but in some strains it occurs as a variant form (FE4) differing very slightly in both the M_r of its polypeptide (58000) and its catalytic-centre activity [3]. The esterase content in a series of seven *M. persicae* clones doubles between each successive variant, leading to the hypothesis that gene duplication (or amplification) is the underlying mechanism [4], and this is supported by further studies showing that resistant aphids have elevated levels of E4 mRNA [5].

We report here the isolation of two E4 cDNA clones and their use in studying the genetic basis of the increased esterase production responsible for resistance.

MATERIALS AND METHODS

Aphid clones

Of the four standard aphid variants, S, R_1, R_2 and R_3 (equivalent to V1, V4, V16 and V64 of [4]) used in the present work, R_1 aphids overproduce FE4 and have the normal karyotype found in S, whereas the strongly resistant variants, R_2 and R_3, overproduce E4 and are heterozygous for an A1,3(autosomes 1 and 3) translocation believed to be associated with the regulation of esterase production [6].

In addition, we also studied nine other independent clones established from populations on field and glasshouse crops; three had the normal karyotype and esterase pattern (FE4) of R_1 and six were translocated and gave the esterase pattern (E4) of R_2 and R_3.

Construction of a cDNA library from R_3 aphids and its screening for putative E4 cDNA clones

Poly(A)$^+$ RNA was prepared from 1 g of R_3 aphids by phenol extraction followed by two passages through oligo(dT)–cellulose [5]. The poly(A)$^+$ RNA was size-fractionated by centrifugation through 5 ml sucrose gradients, {5–20 % (w/v) dissolved in 95 % (v/v) dimethyl sulphoxide and 4 % (v/v) formamide [7]}, and 20 fractions (250 μl) were collected. The RNA in each fraction was precipitated, washed, dried and re-dissolved in 20 μl of sterile water; 2 μl of each fraction was translated in a rabbit reticulocyte-lysate cell-free system in the presence of 20 μCi of L-[^{35}S]methionine. The fraction most enriched for E4 mRNA was identified by immunoprecipitation of the translation products using an E4-specific polyclonal antiserum, followed by polyacrylamide-gel electrophoresis in the presence of SDS and fluorography [5].

Poly(A)$^+$ RNA (2 μg), from the fraction most enriched for E4 mRNA, was used as the template to synthesize double-stranded cDNA, which was cloned in the restriction-endonuclease-*Pst*1 site of pUC8 according to the protocol of Heidecker & Messing [8]. The resulting cDNA library of 1200 recombinants in *Escherichia coli* (JM83) was screened by differential hybridization to identify clones that hybridized strongly with ^{32}P end-labelled R_3 poly(A)$^+$ RNA, but weakly, or not at all, with S poly(A)$^+$ RNA, initially using colony blots [9] and then on a smaller number of selected clones, with isolated plasmid DNA.

Hybrid-arrested translation (HART) to identify E4 cDNA clones

Inserts from three putative E4 cDNA clones were excised from pUC8 (with *Eco*RI and *Hin*dIII), ligated

Abbreviations used: poly(A)$^+$, polyadenylated; HART, hybrid-arrested translation.

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into bacteriophage M13-mp18 and -mp19 (replicative form) DNA and propagated in *E. coli* JM101. Single-stranded DNA was purified from phage supernatants of M13-mp18 or -mp19 and each of the sub-clones [10]. Sub-clones that had the cDNA in the +strand of the phage were identified by the ability of the single-stranded DNA, when immobilized on nylon filters, to hybridize with ^{32}P-labelled RNA from R_3 aphids. Single-stranded DNA (2 μg) from M13 and the selected clones was then hybridized for 20 min at 60 °C with 2 μg of poly(A)$^+$ RNA from R_3 aphids [in 100 mM-KCl/20 mM-Hepes (pH 7)/1 mM-EDTA]. The incubations were treated with RNAase H to destroy RNA–DNA hybrids [11], and the remaining RNA was ethanol-precipitated, dried, and used to direct the synthesis of protein in a rabbit reticulocyte system. Control non-hybridized poly(A)$^+$ RNA was treated in the same way throughout. Translation products were immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis; the gel was dried and fluorographed at −80 °C [15].

Probing aphid DNA dot blots with E4 cDNA

DNA prepared from 1 g of aphids [9] of each variant S, R_1, R_2 and R_3 was sheared by three passages through a syringe needle (18 gauge) and boiled for 10 min. A series of dilutions (in 2 × SSC; 1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0) containing 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg of aphid DNA were adjusted to 8 μg of total DNA using sheared and boiled herring sperm DNA. These were loaded on to a nylon filter (Biodyne A) using a Hybridot apparatus (BRL, Gibco, Paisley, Renfrewshire, Scotland, U.K.) and washed through with 2 × SSC. The DNA was denatured on the filter with NaOH, neutralized, and then baked at 80 °C for 2 h according to the Biodyne protocol. After pre-hybridizing [50% formamide/5 × SSC/50 mM-sodium phosphate (pH 6.5)/0.1% SDS/herring sperm DNA (250 μg·ml^{-1})/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin] at 42 °C for 2 h, the filter was incubated with ^{32}P-labelled nick-translated plasmid DNA (200 ng of pMp31 in 5 ml of the same buffer) with shaking at 42 °C for 16 h. The filter was washed at 65 °C in 2 × SSC/0.1% SDS (3 × 30 min) and then in 0.1 × SSC/0.1% SDS (30 min) and autoradiographed at −80 °C using intensifying screens and Kodak X-Omat S film, preflashed to improve its linearity of response [12].

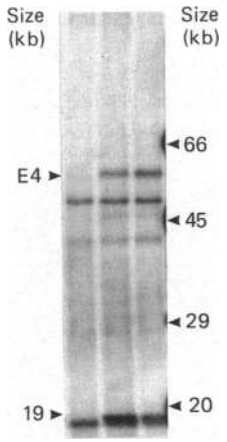

Fig. 1. Identification of an E4 cDNA clone by HART

A fluorograph from polyacrylamide-gel electrophoresis of immunoprecipitated proteins produced in translations of R_3 poly(A)$^+$ RNA (lane 3) and after hybridization with single-stranded DNA of M13:31 (lane 1) or M13-mp18 (lane 2) is shown.

Probing Southern blots of aphid DNA with E4 cDNA

DNA (10 μg) prepared from S, R_1, R_2 and R_3 aphids was digested to completion with *Eco*RI (50 units in 40 μl for 2 h) and electrophoresed on 1%-(w/v)-agarose gels in Tris/acetate buffer (0.4 M-Tris base/0.2 M-sodium acetate/20 mM-EDTA, pH 7.8) at 1 V·cm^{-1} for 16 h. The gel was stained with ethidium bromide to confirm digestion and uniformity of loading. The DNA was denatured (2 × 30 min in 1.5 M-NaCl/0.5 M-NaOH), neutralized (60 min in 3 M-sodium acetate, pH 5.5) and then blotted [9] on to a nylon filter (Biodyne A) overnight with the use of 20 × SSC. The filter was baked at 80 °C for 90 min and then pre-hybridized, hybridized, washed and autoradiographed as for dot blots, except that the stringency of hybridization was increased by raising the formamide concentration to 58% (v/v).

RESULTS

Identification of E4 cDNA clones

Initial screening of the cDNA library by differential colony hybridization identified 20 clones that hybridized more strongly to poly(A)$^+$ RNA from R_3 aphids than to poly(A)$^+$ RNA from S aphids. Since the RNA from R_3 aphids is > 50-fold enriched for the E4 mRNA [5], these 20 clones (pMp21–40) were selected as putative E4 clones. After repeating the differential hybridization on plasmid DNA purified from each of the 20 clones, three (pMp24, pMp26 and pMp31) were chosen for further study.

The identity of the three putative E4 cDNA clones was examined further using hybrid-arrested translation (HART). To facilitate the preparation of single-stranded DNA for the HART assay, the cDNA insert from each plasmid was cloned into phage M13 to give the sub-clones M13:24, M13:26 and M13:31. Single-stranded DNA from each sub-clone was tested for its ability to arrest the translation of E4 mRNA in the reticulocyte-lysate system. Fig. 1 shows that M13 DNA alone had no effect on the immunoprecipitated preteins, whereas M13:31 specifically arrested translation into E4 (M_r 57000). Of the other two clones screened by this technique, M13:24 also arrested translation, but M13:26 had no effect (results not shown).

These results confirmed that pMp24 and pMp31 were E4 cDNA clones. Other bands present were also seen in immunoprecipitated control translations with no RNA. Both cDNA clones arrested not only the translation of the E4 mRNA, but also that of a polypeptide of M_r 19000 (Fig. 1) previously observed in translations of poly(A)$^+$ RNA from R_3 aphids [5]. The significance of this polypeptide is not known, although it is clearly related to E4 both immunologically and in its mRNA sequence. A Northern blot of R_3 poly(A)$^+$ RNA, probed with pMp24, gave only one band of about 2 kb (not shown), a size appropriate for the E4 polypeptide (M_r 57000). Thus the 19000-M_r polypeptide appears to be encoded by an mRNA of the same size as the E4 mRNA. Further detailed analysis of the cDNA clones is required to establish whether the same mRNA species encodes

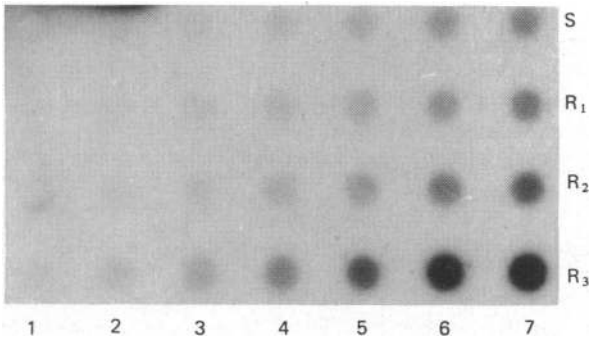

Fig. 2. Assay for E4-related sequences in total DNA from susceptible and resistant aphic clones

DNA prepared from S, R_1, R_2 and R_3 aphids was loaded on to a nylon filter at 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg (dots 1–7 respectively). The filter was hybridized with ^{32}P-labelled pMp31 DNA, washed and autoradiographed.

both polypeptides (through occasional premature termination of translation [13]), or whether two distinct, but closely related, mRNAs are involved.

Restriction analysis indicated that the cDNA inserts in pMp24 and pMp31 are about 0.9 and 0.7 kb respectively, equivalent to about 45 and 35% respectively of the estimated size of the E4 mRNA.

Use of E4 cDNA clones to study aphid esterase genes

Dot blots of genomic DNA, prepared from each of the four aphid clones (S, R_1, R_2 and R_3) were probed with ^{32}P-labelled pMp31 to assay for the abundance of E4-related sequences (Fig. 2). DNA from resistant aphids hybridized more strongly than DNA from susceptible aphids, and the signal intensity increased with increasing levels of resistance. As expected from the 4-fold difference in their E4 levels [4], the binding of the probe showed a 4-fold increase between R_2 and R_3. However, the differences were less apparent at lower resistance levels, probably because they were masked by hybridization to non-amplified sequences common to all strains (see below).

Southern blots of *Eco*RI digests of genomic DNA from the four aphid clones (S, R_1, R_2 and R_3) probed with pMp31 (Fig. 3) again showed amplification of esterase sequences. Furthermore, amplification in the different clones was associated with changes in the restriction pattern. Thus R_2 DNA (lane 2) lacked the 8-kb fragment detected as a faint band in S DNA (lane 1), but had a new, more strongly hybridizing, 4-kb fragment, whereas R_2 and R_3 aphid DNA did not have the 4-kb band, but had amplification of the 8-kb band. The relative intensities of the amplified fragments (4 kb or 8 kb) in the four aphid clones appeared from visual inspection to correspond to the relative amounts of their esterases (1:4:16:64 in S, R_1, R_2 and R_3 respectively).

Amplification of the 8-kb *Eco*RI fragment is not the only feature characteristic of the restriction pattern of R_2 and R_3 DNAs. A novel 15-kb fragment was present in both (Fig. 3, lanes 3 and 4), associated with a reduced intensity of the 10-kb band compared with S and R_1. In each case the combined intensities of the 15-kb and 10-kb bands were similar to the intensity of the 10-kb band in S or R_1.

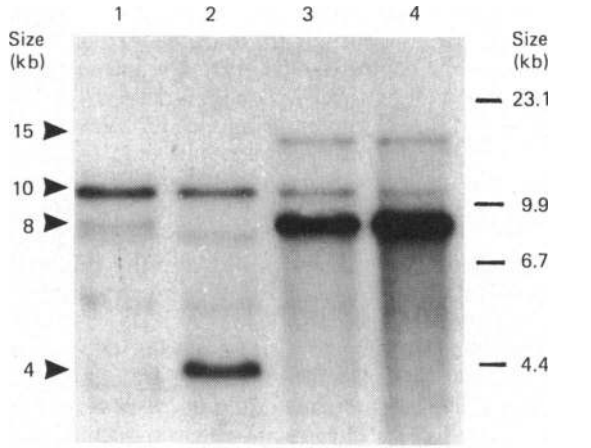

Fig. 3. Autoradiograph showing E4-related restriction fragments in aphid DNA

DNA from 5 (lane 1), R_1 (lane 2), R_2 (lane 3) and R_3 (lane 4) aphid clones (10 μg) was digested with *Eco*RI, electrophoreised on an agarose gel and blotted on to a nylon filter. The filter was hybridized with ^{32}P-labelled pMp31, washed and autoradiographed.

The qualitative *Eco*RI restriction pattern of R_1 DNA (Fig. 3, lane 2) was also found in the three other non-translocated resistant-aphid clones examined (of widely different origins), and that of R_2/R_3 aphids (Fig. 3, lane 3 and 4) was found in the six other translocated aphid clones examined (results not shown), suggesting that the restriction patterns are related to both karyotype and esterase form and are not simply the result of random polymorphism.

DISCUSSION

Gene amplification is a well-established mechanism by which mammalian cell cultures develop resistance to cytotoxic drugs [14]. Amplification can affect the amount of either a target protein, as with dihydrofolate reductase/methotrexate [15] and 'CAD/PALA' [carbamyl-phosphate synthetase–aspartate transcarbamylase–dihydroorotase/*N*-(phosphonacetyl)-L-aspartate] [16], or the P-glycoprotein that gives broad cross-resistance to drugs by enhancing their elimination from the cell [17]. The present work establishes that insecticide resistance in *M. persicae* is accompanied by amplification of the esterase structural gene and that this is associated with qualitative differences between strains in *Eco*RI restriction fragments. One set of fragments is produced in the four non-translocated clones and another set in the eight translocated clones studied. This correlation between karyotype and restriction pattern indicates close linkage between the A1,3 translocation [6] and the events leading to the overproduction of the esterase. The differences in banding pattern of E4-related DNA also correspond to the production of one form of esterase (FE4) in non-translocated clones and another (E4) in translocated clones.

The Southern blots of aphid DNA cannot be interpreted with the same certainty as similar data from cell cultures [18], because the aphid clones originated in the field and may have arisen independently, rather than

sequentially, as in the cell-culture-selection experiments. However, the 15-kb fragment in translocated aphids may be equivalent to the new bands appearing after drug selection of cell cultures and believed to result from the formation of novel joints during the amplification [14,18]. The 15-kb fragment in aphids could thus arise from the 10-kb fragment of non-translocated aphids. The apparent heterozygosity of the 10-kb and 15-kb fragments in very resistant aphids correlates with the heterozygosity of the A1,3 translocation in these aphids; on this interpretation the 15-kb fragment could be linked to the translocation and generated by it.

The data have much in common with studies of amplification in cell cultures. Thus, by analogy, the 4- and 8-kb *Eco*RI fragments could correspond to multiple esterase gene copies grouped together in tandem arrays, rather than scattered throughout the genome, with the 10- and 15-kb fragments arising from one of the non-amplified terminal regions together with its different flanking sequence on either A1 or A3 respectively. Although the 4- and 8-kb fragments could arise entirely from within the E4/FE4 genes themselves (including introns), they might alternatively be part of a much larger sequence that has been amplified as a conserved unit. The amplification unit (amplicon) in mammalian cell cultures is typically larger than 100 kb [14], and amplification is thought to occur in two stages. First, an amplifiable unit is generated by a process involving DNA rearrangement leading to a new restriction pattern, and then the unit increases in number, but remains unchanged in structure [19]. Further characterization of the present example in *M. persicae* requires more extensive restriction mapping and analysis of genomic clones.

Despite a good understanding of gene amplification in cell cultures, little is known of its occurrence and role in the resistance of intact higher organisms to environmental stress. The findings presented here constitute a further economically important exception to the view that gene amplification in response to toxic stress is essentially an aberrant process that occurs in cultured cells [20]. Our preliminary evidence [4] that gene amplification is responsible for overproduction of an insecticide-degrading enzyme by *M. persicae* was the first indication that it could mediate the evolution of resistance in intact organisms, and the present data confirm this hypothesis. Although it has been suggested that this may be a common mechanism by which insects develop resistance [1], the only other molecular evidence of gene amplification is in mosquitoes [21]. If gene amplification proves to be a common basis of resistance, it will have important implications for understanding the evolution of resistant populations, since amplification and its associated instability can occur at very high frequency, enabling resistance to arise quickly and to show flexibility in response to selection pressure. This should be considered when building evolutionary models of the development of resistance.

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