A molecular diagnostic for endosulfan insecticide resistance in the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae)

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Abstract

The coffee berry borer *Hypothenemus hampei* (Ferrari) has recently evolved high levels of resistance to endosulfan and other cyclodiene-type insecticides in New Caledonia. During population outbreaks this has contributed to levels of infestation of coffee berries reaching up to 90%. Using degenerate primers in the polymerase chain reaction (PCR) we have amplified a section of the cyclodiene resistance gene *Rdl* from *H. hampei*. This gene codes for a γ-aminobutyric acid (GABA) gated chloride ion channel. Here we report that resistant strains of *H. hampei* carry exactly the same single amino acid replacement (alanine to serine) as that found in resistant *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae). A molecular diagnostic based upon PCR-mediated amplification of specific alleles (PASA) is described. This technique is capable of detecting resistance or susceptibility in adults, larvae or eggs but not in susceptible females carrying resistant sperm. Its potential use in field monitoring is discussed.

Introduction

The coffee berry borer *Hypothenemus hampei* (Ferrari) is a scolytid pest of coffee. Mated adult females tunnel into the coffee berry and produce large numbers of larvae which damage the berry and diminish the value of the crop. In New Caledonia this pest has recently developed resistance to the cyclodiene-type insecticides endosulfan and lindane, contributing to large population outbreaks resulting in up to 90% infestation of coffee berries (Brun *et al.*, 1989).

Following our recent cloning of the cyclodiene resistance gene *Rdl* (*Resistance to dieldrin*) from the genetic model *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) (ffrench-Constant *et al.*, 1991) we wished to examine if resistance was conferred by the same mechanism in *H. hampei*. The cyclodiene resistance gene codes for a subunit of a γ-aminobutyric acid (GABA) gated chloride ion channel or GABA receptor (ffrench-Constant *et al.*, 1993a). GABA is the major inhibitory neurotransmitter in both invertebrates and vertebrates (Kuffler & Edwards, 1965; Usherwood & Grundfest, 1965; Otsuka *et al.*, 1966). In *D. melanogaster* resistance is conferred by a single amino acid replacement (alanine302 > serine) in the proposed second membrane-spanning domain of the GABA receptor protein, the region thought to line the chloride ion channel pore (ffrench-Constant *et al.*, 1993a).

Here we describe PCR amplification and cloning of DNA coding for the putative second membrane-spanning region of *Rdl* from susceptible and resistant *H. hampei*,

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identification of the same resistance-associated amino acid replacement as that found in *D. melanogaster* and its correlation with resistance in several strains. As this amino acid replacement is caused by a single nucleotide substitution we have developed a molecular diagnostic, based upon PCR-mediated amplification of specific alleles (PASA) (Sommer *et al.*, 1992), as a monitoring technique capable of genotyping individual insects.

Materials and methods

Insect strains

Strains were collected and reared in the laboratory on artificial diet. Resistant strains were selected to apparent homozygosity by 13 generations of selection with endosulfan. Susceptible strains were from Canala and La Foa, New Caledonia and Côte d'Ivoire, Africa. Resistant strains, all from New Caledonia, were collected in Neavin Valley, Ponérihouen; Kokengone and Paola Valleys, Touho.

Cloning of H. hampei *Rdl*

For cloning, genomic DNA was isolated from *H. hampei* strains as previously described for *Drosophila* (ffrench-Constant *et al.*, 1993b). Amplification of DNA within exon 7, which codes for the first and second putative membrane spanning domains of the protein, was carried out using 'nested' degenerate primers in two separate rounds of PCR. The first round was performed using the degenerate primers F5 (forward) and R6 (reverse) making an expected product of size 161 base pairs (bp). The second round amplified a smaller fragment (80 bp) from within the first PCR product by using the same reverse primer (R6) but with a new forward primer F6 designed from sequence between F5 and R6 (fig. 1a). PCR reaction conditions for use of the degenerate primers have been described elsewhere (Thompson *et al.*, 1993b). PCR products were cloned into the vector pCRII (Invitrogen) and sequenced as double stranded templates using SequenaseII (Bethesda Research Laboratories) according to the manufacturer's instructions. In contrast to other insects (Thompson *et al.*, 1993b), single PCR products were obtained from both pairs of PCR primers, thus both the long (161 bp) and short (80 bp) products (fig. 1) were cloned and sequenced.

Design of PASA diagnostic

PASA relies upon the specific amplification of one allele in preference to others at a given magnesium concentration within the PCR reaction (Sommer *et al.*, 1992). A forward, allele-independent, primer was made from just within the proposed exon/intron boundary of exon 7 of the *H. hampei Rdl* gene from sequence obtained within the long (161 bp) PCR product (sequence data not shown). This intron/exon boundary was assumed to be the same as that found in *Drosophila* (ffrench-Constant & Rocheleau, 1992). A primer specific for the resistance allele was made by placing the resistance-associated nucleotide substitution at the 3' end of a reverse PCR primer, whereas the primer corresponding to the susceptible allele carries the susceptible sequence at this point (fig. 2). For PASA, DNA was prepared from individuals of *H. hampei* by homogenizing in 100 µl STE buffer per insect (0.1 M NaCl, 10 mM Tris-HCl pH8, 1 mM EDTA pH8) in the wells of a microtitre plate (ffrench-Constant & Devonshire, 1987), denaturing at 95°C for 5 min, plunging on ice and adding 4 µl directly to the PCR reaction. PCR was performed as previously described (Steichen & ffrench-

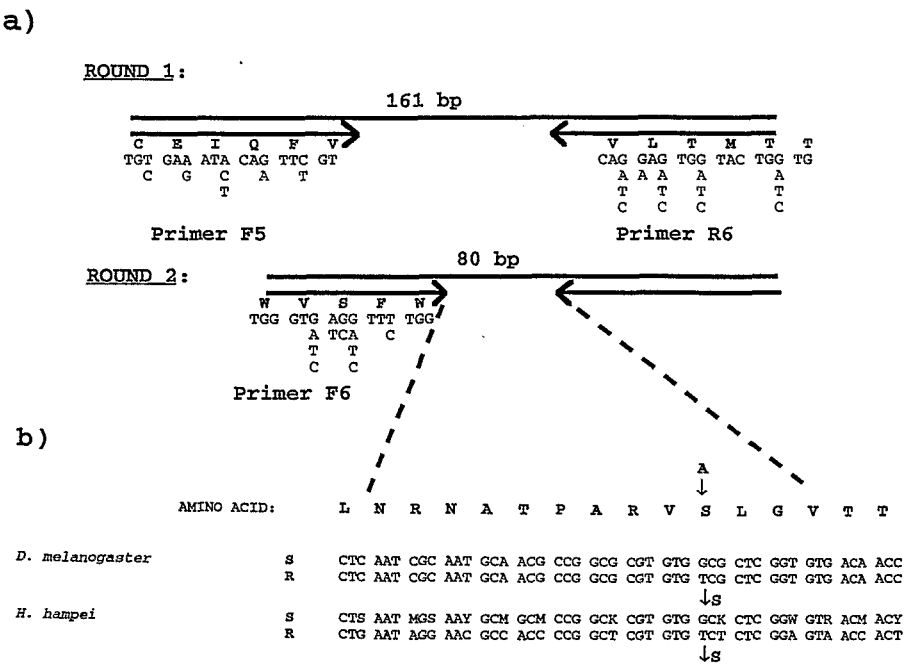

Fig. 1. a) Relative locations and sequence of the nested PCR primers used to amplify DNA from exon 7 of the *Hypothenemus hampei Rdl* gene; b) comparison of the nucleotide and predicted amino acid sequences of susceptible and resistant *Drosophila melanogaster* with those from *H. hampei*. Some nucleotide degeneracy was found at third base positions in susceptible alleles. This is coded as R, A or G; S, C or G; Y, C or T; M, A or C; K, G or T and W, A or T.

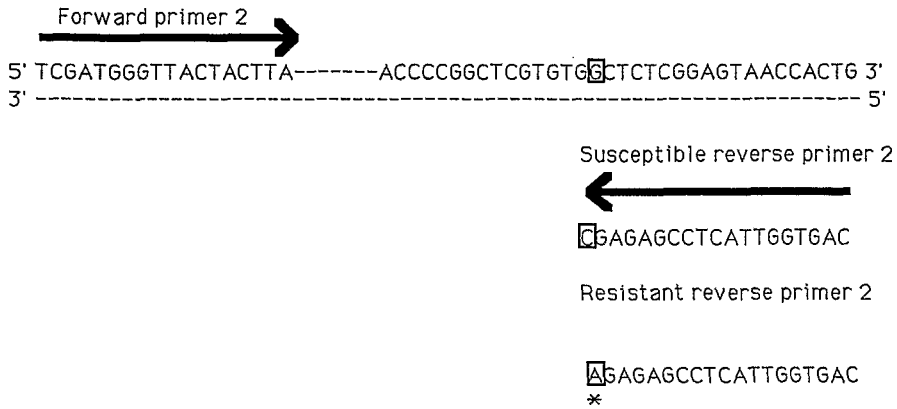

Fig. 2. Sequence and relative location of PASA primers used in specific amplification of cyclodiene resistant or susceptible alleles of *Rdl* from *Hypothemus hampei*. The nucleotide sequence from a resistant strain is shown above with the location and the sequence of the PASA primers below. The boxed nucleotide corresponds to the 3' end of the allele specific primers and an asterix indicates the substitution within the resistant primer specifically complementing the resistance associated mutation.

Constant, 1993) except that the polymerase used was from the thermophilic bacterium *Thermus flavus* (Epicentre Technologies). The concentration of magnesium in the PCR reaction was then varied until selective amplification of either the resistant or susceptible allele occurred.

Results and discussion

Resistance associated mutation

Sequencing of the proposed second membrane spanning region of *Rdl* from a resistant strain showed that the same alanine to serine mutation as that found in *D. melanogaster* was present in *H. hampei* (fig. 1b). Magnesium concentrations giving selective amplification of resistant and susceptible alleles were 1.5 and 1.0 mM respectively. Successful PASA amplification of the same resistance 'allele' from adults of two other homozygous resistant strains collected from varying locations suggests that the same mutation is present in all the resistant strains examined (fig. 3). The same susceptible 'allele' was also amplified from all susceptible strains examined, despite the observation of significant sequence heterogeneity between cloned susceptible alleles within the primer (fig. 1). However, due to the difficulty in ensuring that *H. hampei* strains are homozygous for the *same* susceptible allele, it is not possible to definitely ascertain if the susceptible PASA primer successfully amplified *all* the susceptible alleles present. Testing of this primer on a wider series of sequenced susceptible alleles may therefore be necessary in order to guarantee their diagnostic capabilities on a wider range of strains.

By performing two separate PCR reactions, with susceptible and resistant primers, on DNA from strains or individuals it will thus also be possible to identify heterozygous (*RS*) individuals, as in *Drosophila* (Steichen & ffrench-Constant, 1993). These findings have implications for the conservation of insecticide resistance associated mutations between insect orders, the number of times resistance may have arisen in *H. hampei* populations in New Caledonia, and the use of a molecular diagnostic for the detection of resistance in the coffee berry borer.

The finding that the cyclodiene resistance-associated mutation is the same in *H. hampei* as in *D. melanogaster* supports our previous observations of the conservation of the same alanine to serine replacement within species from three different orders of insect; the yellow fever mosquito *Aedes aegypti* Linnaeus (Diptera: Culicidae) (Thompson *et al.*, 1993a), the house fly *Musca domestica* Linnaeus (Diptera: Muscidae), the red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) and the American cockroach *Periplaneta americana* Linnaeus (Dictyoptera: Blattidae) (Thompson *et al.*, 1993b). The observation that a single 'allele' was present in all resistant strains examined is also similar to our findings in *D. melanogaster* where the same mutation appears to occur in all populations worldwide (ffrench-Constant *et al.*, 1993b). This degree of conservation

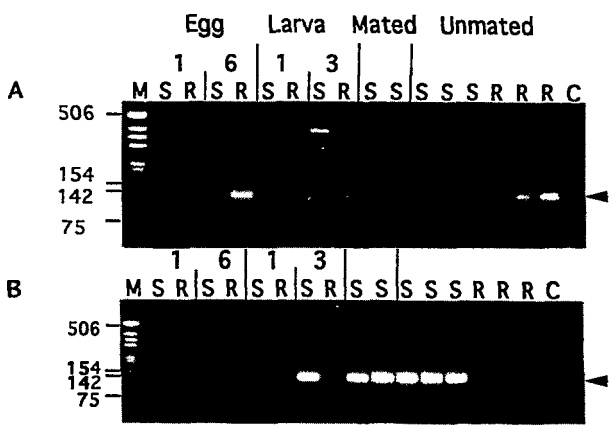

Fig. 3. PASA products from various lifestages of susceptible and resistant strains of *Hypothenemus hampei*. The same DNA was analysed independently with a) resistant and b) susceptible allele specific primers. Lanes show alternating PCR products from DNA extracted from susceptible (S) and resistant (R) eggs (lanes 1-2, one egg; 3-4, six eggs), larvae (lanes 5-6, one larva; 7-8, 3 larvae), susceptible adult females mated to resistant males (lane 9-10), unmated adults (lane 11, La Foa; 12, Côte d'Ivoire, 13, Canala; 14, Paola Valley; 15, Kokengone Valley; 16, Ponérihouen) and a no DNA negative control (lane 17). PCR products were run on a 4% agarose gel, stained with ethidium bromide and visualized under UV light.

within a species could arise from a single mutation event and subsequent spread of a single resistance allele, as revealed by the partial sequencing of a number of resistance alleles from *D. melanogaster* (R. Roush, C. Aquadro & R. ffrench-Constant, unpublished data). However, such conservation of amino acid replacements at this one position (alanine302 in the *D. melanogaster* sequence) in the gene *between* species infers that only replacements of this single residue are capable of causing sufficient resistance and still maintaining adequate receptor function. It is therefore difficult to readily separate the effects of a possible single origin of this mutation within *H. hampei* populations in New Caledonia, from functional constraints on the GABA receptor protein which only permit replacement of alanine302.

Detailed sequencing of a larger number of resistant and susceptible haplotypes will therefore be necessary in order to determine the number of susceptible and resistant alleles present and to estimate how many times resistance has evolved independently. However, as resistance was only detected in *H. hampei* populations in 1987 and because New Caledonia may still be the only location globally in which resistance is found, the coffee berry borer will form an excellent model system in which to examine the number of times cyclodiene resistance arises within a population and how it spreads. Further, conservation of the resistance associated mutation between strains of *H. hampei* will greatly facilitate the monitoring of the spread of resistance within populations using molecular diagnostics.

PASA diagnostic and its potential use

The resistant and susceptible alleles were both selectively amplified in DNA made from individual adults, resistance was also detected from a number of *H. hampei* eggs and larvae (fig. 2b). Increasing the concentration of DNA added following extraction from eggs or larvae will probably allow for resistance detection in individual immatures. Susceptible females mated with resistant males did not give significant PASA products from the presence of resistant sperm, thereby overcoming the concern that females mated to other genotypes would lead to misclassifications.

The technique, as currently developed, therefore has a number of potential advantages for use in resistance monitoring:

1. Resistance can be detected in all lifestages facilitating the early detection of resistance in field infestations. In contrast, insecticide bioassays have been standardized on adults (Brun *et al.*, 1990, 1991).
2. PASA does not rely upon the detection of a resistance associated restriction enzyme polymorphism unlike the PCR/restriction endonuclease (PCR/REN) diagnostic described for *Drosophila* (ffrench-Constant *et al.*, 1993b).
3. The potential exists for the detection of the presence or absence of resistance in DNA extracted from pooled samples of insects. In this case, although the frequency of resistance in individual populations would not be obtained, analysis of pooled samples would allow for the processing of a larger number of insects from more locations and thus allow for more efficient detection of resistance at low frequencies in a geographical survey. Although in the present study resistant male sperm could not be detected in susceptible females, suggesting a lower limit of detection, preliminary experiments with DNA from mixtures of adult *Drosophila* (J. Steichen and R. ffrench-Constant, unpublished data) suggest that a single resistant heterozygote can be detected within up to 10,000 susceptibles.
4. Extrinsic and intrinsic factors associated with the variability of bioassays (Brown & Brogdon, 1987; ffrench-Constant & Roush, 1990) are avoided. Following modification of the PASA technique to the 96 well microtitre plate or smaller format (Garner *et al.*, 1993) the performance of an extra set of reactions to genotype *RS* individuals will not be a significant rate limiting step in analysis. This technique will therefore compare favourably to the effort necessary in performing two discriminating doses to separate both *SS* from *RS* and *RS* from *RR* genotypes in an insecticide bioassay.

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