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Méthods

Methods for characterising resistance to carbamates, pyrethroids and neonicotinoids in *Myzus Persicae*

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The peach-potato aphid is a pest of several plant species. The use of plant protection products to limit the spread of this aphid in various crops can result in the selection of resistant individuals. Modification of the protein targeted by the insecticide is one of the most easily detected resistance mechanisms using tests relying on molecular biology methods. This article presents three methods of detecting various mutations that cause resistance to three insecticides very commonly used against the aphid: carbamates, pyrethroids and neonicotinoids.

Introduction

The peach-potato aphid, *Myzus persicae*, is highly polyphagous as it is capable of colonising over 400 different wild and cultivated plant species (herbaceous plants, fruit trees, shrubs, etc.). This phytophagous insect causes direct damage through its feeding punctures that weaken the plant and deform the leaves. It also causes indirect damage that can be highly destructive, since through its punctures, it is a virus vector that can transmit over 120 plant viruses affecting various plants and trees (CMV: Cucumber Mosaic Virus, CaMV: Cauliflower Mosaic Virus, Plum Pox virus affecting stone-fruit trees). In addition, the excretion of honeydew by the aphids promotes the growth of a fungus (*Fumago salicina*) known as sooty mould that makes the affected organs unfit for sale. *M. persicae* is also characterised by a complex biological cycle, stemming from its ability to reproduce both sexually and asexually and the fact that this cycle varies depending on the colonised plant and geographic location.

By virtue of its characteristics (asexual and sexual reproduction, polyphagia, virus vector), *M. persicae* is a highly destructive pest for numerous agricultural crops. The use of insecticides is one way of controlling this pest. And yet the repeated application of active substances can lead to the development of resistance to these products. For example, in the *M. persicae* species, resistance to several classes of insecticides has been reported. Several resistance mechanisms have been described, affecting four classes of insecticides approved for use against this pest in France (organophosphorous compounds, pyrethroids, carbamates, neonicotinoids). Resistance to insecticides in *M. persicae* is linked to two main types of mechanisms:

- **metabolic resistance** induced by the duplication of a gene that leads to increased production of the corresponding protein. The overexpressed protein is an enzyme capable of breaking down one or more active substances. For example, in *M. persicae*, carboxylesterases (E4 and FE4) are involved in moderate resistance to a broad spectrum of insecticides (carbamates, pyrethroids, organophosphorous compounds) (Devonshire *et al.,* 1982) while cytochrome P450 (Puinean *et al.,* 2010) is involved only in moderate resistance to neonicotinoids;
- **so-called target resistance**, caused by modification of the protein targeted by the insecticide, has also been described. This resistance mechanism is generally responsible for a very sharp decline in insecticide efficacy. Three main classes of insecticides are affected by this type of resistance: pyrethroids, carbamates and more recently neonicotinoids.

Regarding carbamates, a mutation responsible for a high level of resistance has been identified on the encoding gene for acetylcholinesterase 2 (Nabeshima *et al.,* 2003), a target of this insecticide class. At protein level, this mutation, referred to as MACE for "Modified acetylcholinesterase", occurs due to substitution of a phenylalanine for a serine at amino acid 431 of acetylcholinesterase 2 (S431F).

For pyrethroids, three mutations can cause target resistance to this class in *M. persicae*. They affect the insecticide's target: the voltage-dependent sodium channel. These types of resistance are called kdr (knock down-resistance) and s-kdr (super kdr). At protein level, the kdr mutation involves substitution of a phenylalanine for a leucine at position 1014 of the protein (L1014F). So-called s-kdr mutations affect codon 918 and involve a methionine mutation. Several substitutions have been described, for example, methionine can be replaced by a threonine (M918T) (Martines-Torres *et al.,* 1999). This mutation is always found in association with the kdr mutation (L1014F). The second substitution, which has been described more recently, involves a methionine-to-leucine replacement (Fontaine *et al.,* 2011). It has thus far always been found in the absence of kdr.

For neonicotinoids, the mutation involves substitution of a threonine for an arginine at position 81 (R81T) of the β1 subunit of the nicotinic acetylcholine receptor, the target protein for this insecticide class (Bass *et al.,* 2011).

As part of surveillance plans developed by the French DGAL (Directorate General for Food), the Resistance to plant protection products unit (RPP) monitors the development and spread of resistance to plant protection products in crop pests. In this context, for *M. persicae*, various analysis tools have been developed to detect four of the mutations causing resistance to insecticides in this insect. One of these tools can simultaneously detect resistance to carbamates caused by the MACE mutation of acetylcholinesterase and resistance to pyrethroids linked to the kdr mutation. A second tool seeks to detect the M918L mutation affecting the sodium channel involved in high resistance to pyrethroids. The last method presented here is used to detect the R81T mutation, in the β1 subunit of the nicotinic acetylcholine receptor (nAChR), which causes resistance to neonicotinoids.

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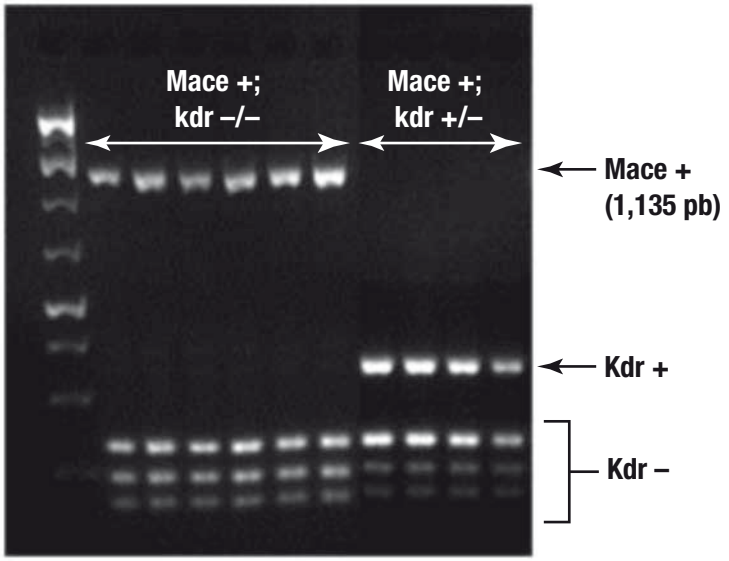
Simultaneous detection of the modified acetylcholinesterase (MACE) and sodium channel kdr mutations involved respectively in resistance to carbamates and pyrethroids

This technique relies on multiplex PCR for amplifying a fragment of the sodium channel gene (para) containing the 1014 codon and amplifying a fragment of the acetylcholinesterase 2 gene (ace2) only when the S431F mutation occurs. Successful amplification of the para gene is a positive DNA extraction control in the absence of the mutated allele of the ace2 gene. For the kdr mutation, the presence or absence of mutation is then tested for by enzyme digestion, which can distinguish between the wild-type allele (two restriction sites, and three 159 pb, 193 pb and 256 pb fragments) and the mutated allele (one restriction site, and two 256 pb and 352 pb fragments) (Cassanelli *et al.,* 2005). Detection of the kdr allele indicates whether the aphid is heterozygous, homozygous susceptible or homozygous resistant (Figure 1). The method for detecting the MACE allele only indicates whether the allele is present or not and does not provide information about the individual's heterozygous or homozygous status. Since these two MACE and kdr resistance alleles are dominant, if they are found in the heterozygous state this therefore produces a phenotype with respective resistance to carbamates and pyrethroids.

Detection of M918L sodium channel mutations involved in resistance to pyrethroids

QPCR (quantitative PCR) with TaqMan probes is used for detecting the M918L mutation. The primers and probes were designed based on nucleic sequences and advice was kindly provided by Mr. Williamson (Rothamsted Research). The probe for detecting the wild-type allele (918M) is bound to fluorochrome Cy3 at the 5' end and BHQ2 at the 3' end. The probe for detecting the resistant allele (918L) is bound to fluorochrome FAM at the 5' end and BHQ1 at the 3' end. To increase specificity, each probe was designed with three LNAs (Locked Nucleic Acids). This technique can detect two alleles of the voltage-dependant sodium channel. It can determine whether the aphid is homozygous [MM], heterozygous [ML] or homozygous [LL] for codon 918. Following qPCR, after verification of the amplification curves, the probes' end-point fluorescence ratios are compared with one another (Figure 2) in order to define the genotype of each aphid.
Since the 918L resistance allele is dominant, if it is found in the heterozygous state it therefore produces a phenotype with resistance to pyrethroids.
The method presented here cannot detect the other mutation that can affect codon 918 (M918T). This mutation can be detected by a qPCR method (Anstead *et al.,* 2004). Multiplex testing with the three probes to simultaneously detect the three possible codons at position 918 (responsible for amino acids methionine, leucine and threonine) has not produced satisfactory results. Furthermore, other mutations affecting codon 918 have recently been highlighted (unpublished data).



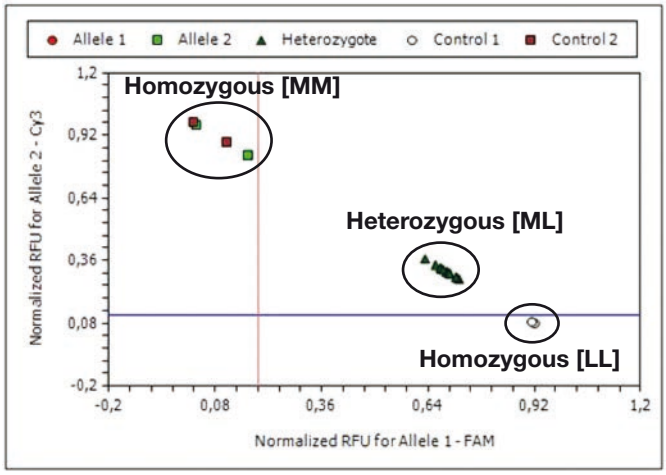
PCR was performed with the Multiplex PCR Master Mix kit (Qiagen), 200 nM of each primer for detection of the S431F mutation in the ACE2 gene (AchE-F2 from Cassanelli *et al.* 2005 and Mace-R-Rev: TCAGCTGCCAATAATAAAATATAA) and 200 nM of each KDR-F1 and KDR-R4 primer (Cassanelli *et al.*, 2005) for amplification of the fragment in the *para* gene.

An initial denaturation phase at 94°C for 15 minutes was followed by 30 cycles at 94°C for 30 seconds, 60°C for 90 seconds and 72°C for 90 seconds. The 30 cycles were followed by a final extension of 5 minutes at 72°C.

Figure 1. Migration profiles, on a 2.5% agarose gel, of digestion products for the simultaneous detection of the modified acetylcholinesterase (MACE) and sodium channel kdr mutations.

Mace +; kdr –/–: *individuals with the MACE allele and without the mutated 'kdr' allele.*
Mace –; kdr +/–: *individuals without the MACE allele and heterozygous for the mutated 'kdr' allele.*

Méthods



Primers and probes for TaqMan PCR, the nucleotides in brackets are Locked Nucleic Acids (LNA):

qMP SKDR-F:
GTGGCCCACACTGAATCTTTTAAT

qMP SKDR-R:
ACAAACGTTAGGTTACCCAAAGCA

Probe MPskdr-SBIS:
Cy3-ATGGTTCGACCC[+A][+T][+T]AT-[BHQ2]

Probe MPskdr-R- 918L:
FAM-ATGGTTCGACC[+A][+A][+T]AT-[BHQ1]

qPCR is performed in a final volume of 25 µL with 12.5 µL of Jumpstart Taqman (Sigma-Aldrich), 200 nM of each primer, 400 nM of each probe, 4.5 mM of MgCl2 and 1 µL of DNA. The PCR cycle consists of an initial denaturation phase of 2 minutes at 94°C and then 40 cycles with denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and then extension at 60°C for 45 seconds.

Figure 2. Result of aphid genotyping for codon 918 by TaqMan probe to detect the wild-type allele and the mutated 918L allele.
*The x-axis and y-axis show the fluorescence intensities of the two FAM and Cy3 fluorochromes used to distinguish between the three genotypes: **Homozygous [MM]:** aphid without a mutation at codon 918; **Heterozygous [ML]:** aphid having only one mutated allele at codon 918 (substitution of a leucine for methionine); **Homozygous [LL]:** aphid with the two mutated alleles at codon 918.*

Sense sequence for wild-type individual

5' - TAGTTCTAACTTATTGCCTGCAGCTATTAAAATATCCAATTAAATAATGT

GTCTTAATATTGTTTTATTGTTTAATGAAAAGAGTCAAATAATGAAATCAAAC

3' - AAAAGAGTCAAATAATGAAATCAAAC

***MPB1F-SmlI** sense primer* →

GTTTGGTTGAGACTTGTGAGTAACCTACTTAATATATATATTATATATATATA
GTTTGCTTGA – 5'

Base degenerated to create the restriction site

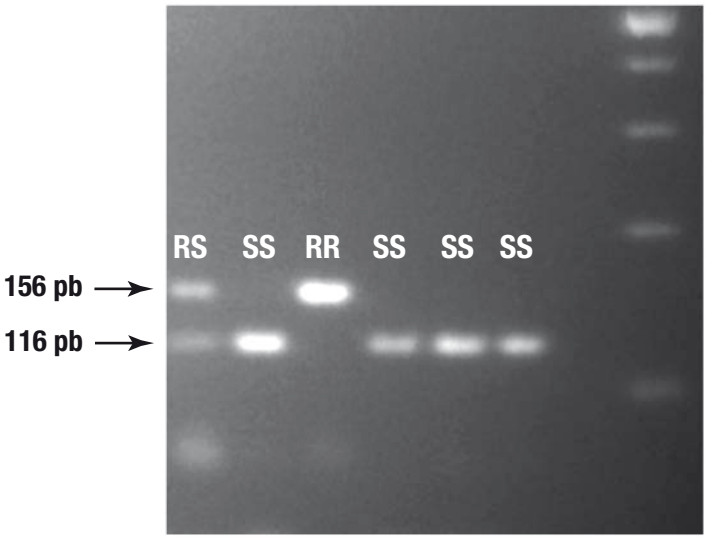
TTTATTTTCAGTTTGTAAACTATAAAATTAAAAAATAAACAGTTTCCTTTCTA

← ***MPB1TMR** anti-sense primer*

3' –ATAATCTGAAGGACTGGCG– 5'
ACGTATTAGACTTCCTGACCGC– 3'

Figure 3. dCAPS-PCR, position of the MPB1F-SmlI and MPB1TMR primers on the β1 subunit of the nicotinic acetylcholine receptor (nAChR) containing codon 81.
In red the mutated base for the dCAPS primer. In blue the base concerned by the R81T mutation.

Méthods



PCR is performed in a final volume of 25 µL with 200 µM of dNTPs, 300 nM of each primer, 2 mM of MgCl2, 0.1 µg/µl of BSA, 1.5 units of Taq and 1µL of DNA.

The PCR cycle consists of an initial denaturation phase of 5 minutes at 94°C and then 35 cycles including denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and then extension at 72°C for 30 seconds. PCR ends with a final extension of 5 minutes at 72°C. Digestion of 10 µL of PCR products is performed with 5 SmlI enzyme units at 55°C overnight.

Figure 4. Migration profiles, on a 3% agarose gel, of the dCAPS-PCR digestion products, to detect the R81T mutation affecting the β1 subunit of the nicotinic acetylcholine receptor (nAChR).

Homozygous SS: *aphid without a mutation at codon 81;* ***Heterozygous RS:*** *aphid having only one mutated allele at codon 81 (substitution of a threonine for arginine);* ***Homozygous RR:*** *aphid with the two mutated alleles at codon 81.*

As a result, when analyses are undertaken with the two specific probes for the 918M and 918L alleles, for individuals with no fluorescence measurements or with ambiguous results, sequencing of a portion of the sodium channel gene containing codon 918 is performed in order to accurately determine the genotype.

Detection of R81T mutations in the β1 subunit of the nicotinic acetylcholine receptor (nAChR) involved in resistance to neonicotinoids

The method used is a dCAPS (Derived Cleaved Amplified Polymorphic Sequence) PCR. It relies on the creation of a restriction site when the allele is non-mutated. This site is created with a primer placed next to codon 81, one of whose bases, which is not complementary to the sequence for amplifying, results in the creation of a restriction site when the allele is non-mutated (Figure 3). Enzyme digestion can distinguish between the wild-type allele (116pb and 37pb) and the mutated allele lacking a restriction site (156pb) (Figure 4). In heterozygous individuals, the R81T mutation indicates decreased susceptibility to neonicotinoids but to a lesser extent than in homozygous individuals. This allele therefore appears co-dominant (unpublished data).

Conclusion

Myzus persicae is an ideal species for examining resistance to insecticides. Found on numerous crops, it is subject to various forms of phytosanitary pressure. Its biological cycle, during which sexual reproduction (allowing for genetic recombination) can alternate with asexual reproduction cycles (leading to rapid multiplication of the most advantageous genotypes), is an evolutionary advantage. The tools presented here allow for the targeted detection of some of the known resistance mechanisms in this aphid. They have been designed to meet the need to examine resistance alleles in *M. persicae* for three main classes of insecticides. However, the absence of a tested resistance allele does not necessarily indicate that an individual is susceptible to an insecticide. An aphid can have other resistance mechanisms not detected by any of the methods described here. Only insecticide susceptibility testing undertaken in a laboratory, by spraying or ingestion of an insecticide in controlled conditions, can exhaustively determine whether an aphid has a susceptible or resistant phenotype. Molecular tools, which are less burdensome to implement, can be used to determine the presence or absence of an allele recognised as causing resistance to one or more

Méthods

active substances. Their advantage lies in the fact that they can detect several mechanisms against different active substances, in the same individual. It should be noted however that considering the evolving capacities of this pest and changes in the use of insecticide classes, these analytical methods are likely to change or be replaced with new ones. For example, for resistance to pyrethroids, the development of an HRM (high-resolution melting) analytical method aiming to identify the various mutations affecting codon 918 of the para gene is currently being studied with a view to more exhaustive detection of the various alleles involved in resistance to pyrethroids.

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