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Development of SCAR/CAPS markers linked to tolerance/sensitivity to *Fusicoccum* in almond

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SUMMARY – *Fusicoccum amygdali* is responsible for *Fusicoccum* canker, a damaging and economically important disease in almond (*Prunus dulcis* Mill.) orchards around the Mediterranean region. Fourteen almond cultivars classified as tolerant (7) or sensitive (7) to *Fusicoccum* were screened with 120 RAPD primers. Polymorphic bands were assessed for co-segregation with tolerant or sensitive phenotypes using 30 seedlings obtained from a 'Masbovera' (tolerant) x 'Lauranne' (sensitive) cross. Three RAPD markers linked to tolerance (OPD-19_{300} and OPA-08_{900}) or sensitivity (OPD-19_{500}) to *Fusicoccum* in almond were identified. SCAR primers were developed but polymorphism between the two groups was lost. The possibility of developing CAPS will be analysed.

Key words: Almond, *Fusicoccum* markers, RAPD, SCAR, CAPS.

RESUME – *"Développement de marqueurs SCAR/CAPS liés à la tolérance/sensibilité à* Fusicoccum *chez l'amadier".* Fusicoccum amygdali *est le responsable du chancre du* Fusicoccum*, une maladie dégénérative ayant des répercussions économiques importantes sur les vergers d'amandiers (*Prunus dulcis *Mill.) autour de la région méditerranéenne. Quatorze cultivars d'amandiers classifiés comme tolérantes (7) ou sensibles (7) au* Fusicoccum *ont été examinés avec 120 primers de RAPD. Des bandes polymorphes ont éte évaluées pour la co-ségrégation avec des phénotypes tolérants ou sensibles en utilisant 30 jeunes plantes obtenues à partir d'un croisement de 'Masbovera' (tolérante) et 'Lauranne' (sensible). Trois marqueurs de RAPD liés à la tolérance (OPD-19_{300} et OPA-08_{900}) ou à la sensibilité (OPD-19_{500}) de l'amandier au* Fusicoccum *ont été identifiés. Des primers de SCAR ont été développés mais le polymorphisme existant entre les deux groupes s'est perdu. La possibilité de développement de CAPS sera analysée.*

Mots-clés : *Amandier, marqueurs moléculaires pour* Fusicoccum*, RAPD, SCAR, CAPS.*

Introduction

Fusicoccum canker or constriction canker is a fungal disease caused by *Fusicoccum amygdali* Delacr., when in a perfect stage it is classified as *Phomopsis amygdali* (Del.) Tuset and Portilla. This is a widespread fungal disease in almond orchards in the Mediterranean region being responsible for important economic losses. This disease affects mainly twigs of the lower part of the trees, causing canker on shoots and necrosis on leaves (Barbé, 1993).

The development of *Fusicoccum* canker is favoured mainly by high nitrogen levels in the soil and by wet weather, since this disease is transmitted just by rain. The diffusion of this disease is very slow but it's eradication from an orchard is very difficult and expensive (Romero and Vargas, 1981).

The development of molecular markers linked to the tolerance/sensitivity phenotype could be of high value because, in the field, it takes several years for the plant to develop symptoms.

Several techniques like RAPD, ISSR, AFLP and RFLP have been used to identify markers linked to characteristics of agronomic interest in several plants (Scovel *et al*., 1998; Gill *et al*., 1998; Tacconi *et al*., 2001).

In this study, the RAPD (Randomly Amplified Polymorphic DNA) technique and bulked DNA analysis were used to select markers associated to either tolerance or sensitivity to *Fusicoccum*. The RAPD markers were sequenced and converted to SCAR (Sequence Characterised Amplified Region) markers, which are more reproducible. The segregation of SCARs was tested, however, the specificity of the association to tolerance or sensitivity to *Fusicoccum* was lost. The possibility of developing CAPS (Cleaved Amplified Polymorphic Sequence) through the analysis of SCAR sequences obtained for tolerant and sensitive almond cultivars will be studied.

A segregating population of 140 seedlings derived from a cross of a very sensitive cultivar ('Lauranne') with a very tolerant one ('Primorskyi') is being assessed in the field for phenotypic characterisation and will later be used for confirmation of the results obtained.

Materials and methods

Plant material

Fourteen cultivars (Table 1) classified as sensitive (7 cultivars) or tolerant (7 cultivars) phenotypes to *Fusicoccum*, have been used, to bulked DNA analysis and SCAR/CAPS development.

Table 1. List of tolerant and sensitive cultivars obtained from IRTA, Centre de Mas Bové, Spain

Tolerant cultivars	Sensitive cultivars
'Ardechoise'	'Achaak'
'Cristomorto'	'Desmayo Largueta'
'Glorieta'	'Ferragnès'
'Masbovera'	'Lauranne'
'Nonpareil'	'Marcona'
'Primorskyi'	'Steliette'
'Texas'	'Tuono'

Thirty seedlings obtained from the cross of 'Masbovera' (tolerant) x 'Lauranne' (sensitive), identified as tolerant or sensitive, were used for co-segregation studies.

All the plants were growing in the field under natural contamination conditions.

DNA extraction

Total DNA was extracted from leaves collected in the field. The DNA was isolated following the method described by Doyle and Doyle (1987) with a few modifications.

For bulked DNA analysis, 2 DNA bulks were constructed, each using the 7 cultivars with either tolerance (T) or susceptibility (S) for the disease.

RAPD analysis

PCR was performed as described by Williams *et al.* (1990) with a few modifications. One hundred and twenty RAPD primers (OPA, OPB, OPC, OPD, OPE and OPF primers, from Operon Technologies) were tested to screen for tolerance/sensitivity associated markers.

This primer evaluation was performed in three different stages: (i) the bulked DNA of the tolerant (T) and sensitive (S) cultivars was used; (ii) the primers that produced polymorphism between the two

DNA bulks (tolerant-sensitive) were tested again for each cultivar, individually, and the ones that kept the polymorphism were selected; and (iii) co-segregation studies were performed for the markers associated to tolerance or sensitivity.

Cloning and sequencing of PCR products

Three fragments associated to tolerance or sensitivity to *Fusicoccum* were identified.

The selected PCR products were isolated from a 2% agarose gel and purified using the QIAquick™Gel extraction Kit (Qiagen).

The fragments were cloned by ligation into pCR®2.1 vector (Invitrogen) and transformation of INVα F' *E. coli* competent cells.

Plasmid DNAs were isolated and purified using Wizard™Plus Minipreps DNA Purification System (Promega).

DNA sequencing was performed at MWGAG Biotech in Germany.

SCAR/CAPS development

For each of the 3 markers associated to tolerance or sensitivity to *Fusicoccum*, 2 sets of SCAR primers (18 to 22 nucleotides) were design based on the RAPD markers sequences, OPA-08_{Tol900}, OPD-19_{Sen500} and OPD-19_{Tol300}.

Each set of SCAR primers was tested, for the maintenance of polymorphism, on the 14 cultivars used for bulked DNA analysis (Table 1). PCR conditions were the same as the ones used for RAPD analysis, but using the SCAR primers in the reaction mixture and using optimized annealing temperatures for each set of SCAR primers.

Cloning and sequencing of SCAR fragments is underway to be used for CAPS development.

Results and discussion

Of the 120 RAPD primers screened using the bulked DNA of tolerant (T) and sensitive (S) cultivars, 50 originated polymorphic bands discriminating between the two groups of plants. These 50 primers were evaluated on a second screening using the individual DNA of each of the 14 cultivars (7 tolerant and 7 sensitive). Four possible molecular markers, were amplified using three RAPD primers, OPA-08 (Fig. 1A, B), OPA-20, OPD-19. Two of the fragments obtained with these primers were apparently associated to the *Fusicoccum* disease tolerance and 2 were associated to susceptibility.

The co-segregation studies with the 4 selected fragments, OPA-08_{Tol900}, OPA-20_{Sen370}, OPD-19_{Sen500} and OPD-19_{Tol300}, originated 76,7%, 33,3%, 56,7% and 70,0% of co-segregation with the expected phenotype, respectively. Based on these results the OPA-20_{Sen370} fragment was eliminated from the subsequent studies since it had a very low co-segregation percentage (33,3%).

The fragments OPA-08_{Tol900}, OPD-19_{Sen500} and OPD-19_{Tol300}, were cloned and sequenced. DNA sequences were analysed using BLAST at NCBI and low homologies were found with several proteins but none specifically related to resistance mechanisms.

The conversion of RAPD to SCAR markers resulted in a loss of polymorphism (data not shown), this loss of polymorphism as been reported in similar studies (Jacobs *et al.*, 1996; Tacconi *et al.*, 2001).

We are now developing CAPS from the SCAR fragments obtained. SCAR markers sequences are going to be analysed to search for differences between tolerant and sensitive sequences, with

subsequent selection of specific enzymes to be applied for CAPS markers development associated to tolerance or sensitivity to *Fusicoccum*.

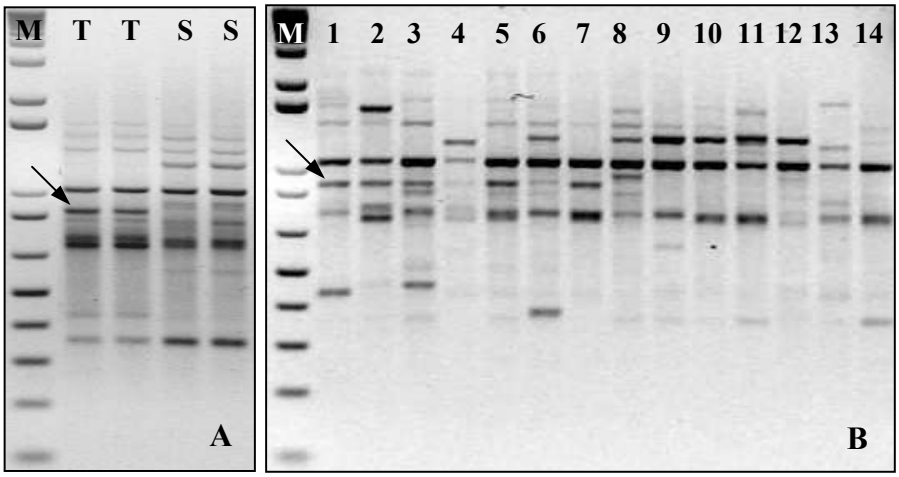

Fig. 1. RAPD banding pattern of tolerant and sensitive cultivars obtained with the RAPD primer OPA-08. (A) using bulked DNA of tolerant (T) and sensitive (S) cultivars. (B) using DNA of individual cultivars. Tolerant cultivars: 1-'Primorskyi'; 2-'Ardechoise'; 3-'Masbovera'; 4-'Glorieta'; 5-'Cristomorto'; 6-'Texas'; 7-'Nonpareil'. Sensitive cultivars: 8-'Ferragnès'; 9-'Marcona'; 10-'Desmayo Largueta'; 11-'Tuono'; 12-'Achaak'; 13- 'Lauranne ' ; 14- 'Steliette'. M-DNA ladder 1KbPlus (Gibco-BRL). The arrows indicate the discriminating bands between the two groups of cultivars.

Further investigations including the co-segregation study of a progeny (very tolerant) 'Primorskyi' x 'Lauranne' (very sensitive) with 140 seedlings is necessary to validate the results obtained so far.

The identification of molecular markers linked to tolerant/susceptible phenotypes will be useful for plant certification, as a selection tool in breeding programs and to assist in the establishment of collections with plants tolerant to *Fusicoccum*.

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