NOTE / NOTE

Isolation of a strawberry gene fragment encoding an actin depolymerizing factor-like protein from genotypes resistant to *Colletotrichum acutatum*

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Abstract: Actin depolymerizing factors (ADFs) have been recently implicated in plant defense against pathogenic fungi, associated with the cytoskeletal rearrangements that contribute to establish an effective barrier against fungal ingress. In this work, we identified a DNA fragment corresponding to a part of a gene predicted to encode an ADF-like protein in genotypes of *Fragaria ananassa* resistant to the fungus *Colletotrichum acutatum*. Bulked segregant analysis combined with AFLP was used to identify polymorphisms linked to resistance in hybrids derived from the cross between the resistant cultivar 'Sweet Charlie' and the susceptible cultivar 'Pájaro'. The sequence of one out of three polymorphic bands detected showed significant BLASTX hits to ADF proteins from other plants. Two possible exons were identified and bioinformatic analysis revealed the presence of the ADF homology domain with two actin-binding sites, an N-terminal phosphorylation site, and a nuclear localization signal. In addition to its possible application in strawberry breeding programs, these finding may contribute to investigate the role of ADFs in plant resistance against fungi.

Key words: anthracnose, *Fragaria ananassa*, molecular markers, plant disease resistance.

Résumé : Les facteurs de dépolymérisation de l'actine (ADFs) ont récemment été impliqués dans la défense des plantes vis-à-vis des champignons pathogènes en étant associés à des réarrangements cytosquelettiques qui contribuent à établir une barrière à l'entrée des champignons. Dans ce travail, les auteurs identifient un fragment d'ADN correspondant à une partie d'un gène qui coderait pour une protéine de type ADF chez des génotypes du *Fragaria ananassa* qui sont résistants au champignon *Colletotrichum acutatum*. Une analyse de ségrégants en masse (« bulked segregant analysis » ou BSA) à l'aide de marqueurs AFLP a été employée pour identifier des polymorphismes liés à la résistance chez des hybrides dérivés d'un croisement entre le cultivar résistant 'Sweet Charlie' et le cultivar sensible 'Pájaro'. La séquence d'un des trois amplicons ainsi détectés a montré une homologie significative avec des protéines ADF chez d'autres plantes suite à une analyse BLASTX. Deux exons possibles ont été identifiés et une analyse bioinformatique a révélé la présence d'un domaine ADF-H avec deux sites de liaison à l'actine, d'un site de phosphorylation N-terminal et d'un signal de localisation nucléaire. En plus de son utilisation en amélioration génétique du fraisier, cette découverte pourrait contribuer à l'investigation du rôle des ADF dans la résistance aux champignons.

Mots-clés : anthracnose, *Fragaria ananassa*, marqueurs moléculaires, résistance des plantes aux maladies.

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Actin depolymerizing factors (ADFs) are members of the ADF/cofilin family that includes a variety of low molecular mass actin-binding proteins in eukaryotic cells. The ADF/cofilin proteins have the ability to regulate actin polymerization and depolymerization: a crucial process in the reorganization of cytoskeleton. Plant ADFs are implicated in pollen tube elongation (Chen et al. 2003), root formation (Thomas and Schiefelbein 2002), cell expansion (Dong et al. 2001), and

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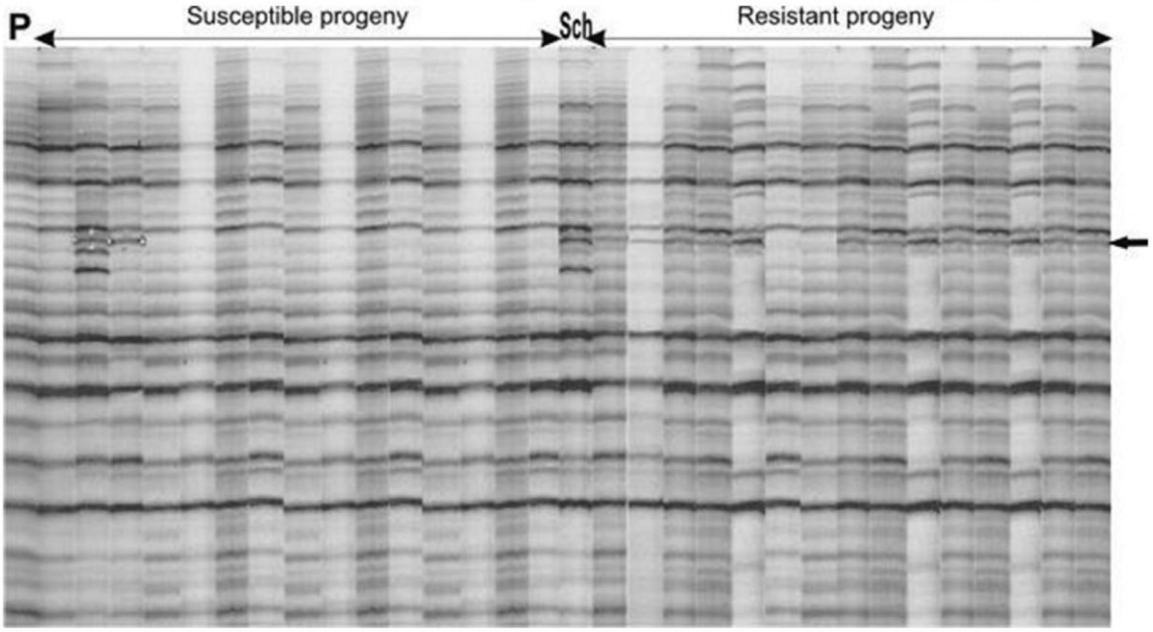
Table 1. AFLP primer combinations that allowed the identification of the 13 bands present in resistant parent and bulk.

	AFLP primer combinations	
	Primer *Eco*RI + 3 (5′-3′)	Primer *Mse*I + 3 (5′-3′)
1	GACTGCGTACCAATTC**AGG**	GATGAGTCCTGAGTAA**CTT**
*2	GACTGCGTACCAATTC**AAC**	GATGAGTCCTGAGTAA**CAG**
*3	GACTGCGTACCAATTC**ACC**	GATGAGTCCTGAGTAA**CAA**
*4	GACTGCGTACCAATTC**ACC**	GATGAGTCCTGAGTAA**CAT**
5	GACTGCGTACCAATTC**ACT**	GATGAGTCCTGAGTAA**CAG**
6	GACTGCGTACCAATTC**ACT**	GATGAGTCCTGAGTAA**CAC**
7	GACTGCGTACCAATTC**ACC**	GATGAGTCCTGAGTAA**CTT**
8	GACTGCGTACCAATTC**AGG**	GATGAGTCCTGAGTAA**CTA**
9	GACTGCGTACCAATTC**AAG**	GATGAGTCCTGAGTAA**CTA**
10	GACTGCGTACCAATTC**AAG**	GATGAGTCCTGAGTAA**CAG**
11	GACTGCGTACCAATTC**ACG**	GATGAGTCCTGAGTAA**CAG**
12	GACTGCGTACCAATTC**AGC**	GATGAGTCCTGAGTAA**CAG**
13	GATGAGTCCTGAGTAA**ACG**	GATGAGTCCTGAGTAA**CTT**

Note: Asterisks indicate the primer combinations that generated the bands PS130, PS250, and PS450. Nucleotides in bold represent the different base combinations at the 3′ end of each primer.

Fig. 1. AFLP gels of progenitors: susceptible and resistant hybrids obtained with *Eco*RI + ACC and MseI + CAT primer combinations. The arrow indicates the PS450 band. P corresponds to 'Pájaro' (susceptible) and Sch to 'Sweet Charlie' (resistant) progenitors.

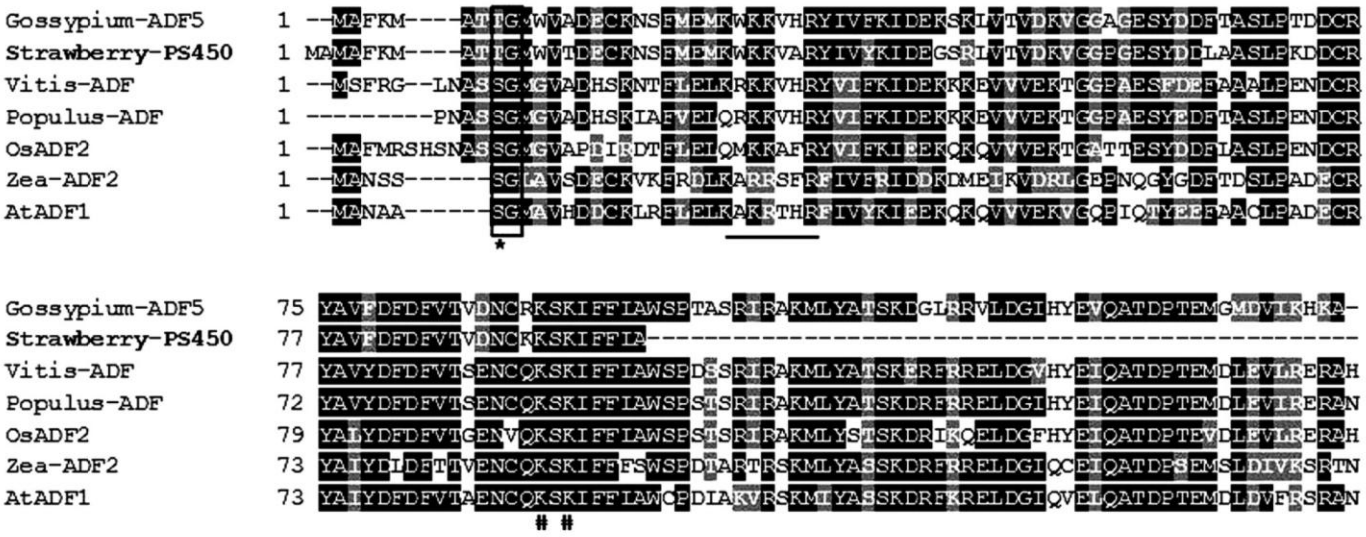


cold acclimation (Ouellet et al. 2001). There are also reports concerning the role of ADFs in nonhost and basal plant resistance (Miklis et al. 2007). ADFs are thought to act at the first line of defense against fungal pathogens by means of cell polarization, which involves rearrangements of the actin cytoskeleton, translocation of the cytoplasm and the nucleus, and local deposition of cell wall materials at the site of invasion (Schmelzer 2002; Brueggeman et al. 2009).

In higher plants, ADFs/cofilins have been referred to as ADFs (Staiger 2000), and cDNAs encoding ADF proteins have been cloned from several species, but in strawberry no ADF genes have been reported yet.

Strawberry (*Fragaria ananassa*) is an important horticultural crop in many countries, and anthracnose, caused by fungi of the genus *Colletotrichum*, is one of the most damaging diseases that affect this crop. With the aim to detect molecular markers linked to resistance against *Colletotrichum acutatum*, we used a progeny of 80 hybrids derived from the cross of *F. ananassa* cultivars 'Pájaro' × 'Sweet Charlie' that were highly susceptible and resistant, respectively (Ramallo et al. 2000). Inoculation assays, disease assessment, and statistical analyses were performed as in Salazar et al. (2007). The susceptibility (disease severity rating: DSR) was scored on a scale ranging from 1 to 5 using a disease index based on petiole symptoms according to Delp and Milholland (1980). The experimental design was randomized with eight plants (four inoculated and four control) per genotype and per experimental unit. Within the F_1 segregating population, the DSR ranged between 1 and 5. Out of the 80 hybrids, 15 exhibited a DSR between 1–1.25 and were considered as re-

Fig. 2. Multiple amino acid sequence alignment of the predicted amino acid strawberry sequence (PS450) with ADFs from other plants performed with Clustal X. Annotated sequences used were as follows: *Gossypium hirsutum* ADF5 (ABD66507), *Vitis vinifera* ADF isoform1 (XP_002278882), *Populus tremula* × *Populus alba* ADF (AAD23407), *Oriza sativa* ADF2 (NP_001051449), *Zea mays* ADF2 (NP_001105590), and *Arabidopsis thaliana* ADF1 (NP_190187). Residues that are essential for actin monomer and actin filament binding are boxed. Hash marks indicate the residues necessary for specific binding to filamentous actin. Asterisk indicates the phosphorylation site. Residues corresponding to the nuclear localization signal are underlined.



sistant genotypes, whereas 19 showed a DSR between 4.5–5; therefore, they were considered highly susceptible.

To identify polymorphisms linked to resistance, bulked segregant analysis (BSA) (Michelmore et al. 1991) and AFLP (Vos et al. 1995) were performed. Genomic DNA was extracted from 100 mg of young leaves of each genotype using the Nucleon Phytopure kit (Amersham Pharmacia Biotech, UK Ltd., England). Two bulks comprising equal amounts of DNA from the 15 resistant and 15 of the 19 highly susceptible plants were employed. AFLP experiments were conducted according to Vos et al. (1995) using the AFLP Analysis System I kit (Life Technologies, Gaithersburg, Maryland, USA). Primers containing one selective nucleotide (*Eco*RI + A and *Mse*I + C) for the first amplification and three selective nucleotides (*Eco*RI + 3 and *Mse*I + 3) for the second amplification were used (Vos et al. 1995). The *Eco*RI + 3 primers were labeled with γ [^{33}P]-ATP (3000 µCi/mmol) (NEN Life Sciences, England). Restriction, adapter ligation, preamplification, and selective amplification were performed according to the manufacturer's protocol. Amplification products were separated on 6% polyacrylamide denaturing sequencing gels that were later exposed to X-ray film (Kodak Biomax-MR). From a total of 64 primers combinations (eight *Eco*RI + 3 and eight *Mse*I + 3) tested on the susceptible and resistant bulks and parents, 13 combinations (Table 1) allowed the identification of 13 bands present only in the resistant parent and bulk, and these were further used for AFLP analysis of the 15 individuals constituting each bulk. Finally, 3 of the 13 bands were present in 'Sweet Charlie' and in the majority of the resistant individuals. These bands named PS130 (130 bp), PS250 (250 bp), and PS450 (450 bp) were obtained with the primer combinations 2, 3, and 4, respectively (see Table 1). Bands PS130 and PS250 were present in 12 resistant genotypes, whereas band PS450 was detected in 13 resistant hybrids (Fig. 1). All three bands were detected only in 2 of the 15 susceptible hybrids. Candidate polymorphic DNA fragments from each of the resistant genotypes were excised from the gel, eluted, and reamplified. Amplicons were cloned in the T/A-cloning vector pGEM-T Easy (Promega Corp., Madison, Wisconsin, USA). Three of the recombinant bacterial colonies from each band were selected for sequencing and bioinformatic analysis. Thus, sequences of 39 inserts of the PS130 band, 39 inserts of the PS250 band, and 42 of the PS450 band were analysed.

The ORF finder program (http://www.ncbi.nlm.nih.gov/) was used to search open reading frames. Comparisons of PS130 and PS250 DNA partial sequences against those annoted in the GenBank NR Database, using BLASTX (Altschul et al. 1990), revealed not significant homology with known sequences.

The PS450 band showed a sequence common to 39 of the 42 fragments analysed (GenBank accession numbers HQ877449 and HQ877450), that had significant Blast-X hits in the GenBank NR Database to ADF proteins from *Glicine max*, *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, and *Vitis vinifera*. The deduced amino acid sequence showed identity scores ranging between 93% and 60%, with E values comprised between 1×10^{-37} and 6×10^{-44}. The gene prediction programs SPL and FGENESH 2.6 (www.softberry.com) (Solovyev et al. 1994) were used to identify possible exons and splicing sites with the dicot-Arabidopsis training set. The resulting two exons were then used to predict the amino acid sequence which, when compared to GenBank with Blast-P, confirmed the homology with many others ADFs from the plant kingdom. The CDSearch analysis (at NCBI) of the deduced 93 amino acid strawberry sequence identified two actin-binding sites, an N-terminal phosphorylation site, and a nuclear localization signal corresponding to the ADF homology domain present in all ADFs/cofilins. In our sequence,

two of the six residues (T^{10} and G^{11}) that compose the N-terminal actin binding site were found. These residues correspond to the most highly conserved residues in this region (Lappalainen et al. 1998). Two of the four residues that compose the specific F-actin binding site correspond to K^{86} and K^{88} in our sequence. The presence of the phosphorylation site, reported to be the main regulatory ADF/cofilin function (Miklis et al. 2007), was confirmed by the NetPhos 2.0 Server program (Blom et al. 1999) and mapped at the residue T^{10} of our sequence. The nuclear localization signal has been located in a loop between K^{26} and R^{32}, close to the amino terminus as reported by Maciver and Hussey (2002). The iPSORT algorithm (Bannai et al. 2002) failed to predict any signal of mitochondrial targeting or chloroplast transit peptides.

The multiple amino acid sequence alignment of the strawberry sequence with other plant ADFs constructed by Clustal X (Thompson et al. 1997) revealed a high degree of conservation of the mentioned functional motifs (Fig. 2).

It should be noted that the PS450 fragment was found in the resistant individuals of our population and absent in almost all the susceptible genotypes. If we assume that this sequence corresponds to an ADF gene, it should be present in all individuals. The absence of the band in susceptible genotypes could be due to mutations within restriction sites or insertion/deletions that modify the size and position of the fragment in the AFLP pattern. The fact the ADF sequence was found in most of the resistant individuals suggests that this gene may be somehow associated to the resistance of strawberry against *C. acutatum*.

In summary, a DNA fragment of a putative ADF gene in strawberry was identified. It is significant that it was found in individuals resistant to the fungus *C. acutatum*. This finding may be useful for the development of a molecular marker for breeding programs and could contribute to understanding the role of some of the factors involved in plant resistance against fungi.

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