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Methylation of the S_f locus in almond is associated with *S*-RNase loss of function

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Abstract. Self-compatibility in almond (*Prunus amygdalus* Batsch) is attributed to the presence of the S_f haplotype, allelic to and dominant over the series of *S*-alleles controlling self-incompatibility. Some forms of the S_f haplotype, however, are phenotypically self-incompatible even though their nucleotide sequences are identical. DNA from leaves and styles from genetically diverse almond samples was cloned and sequenced and then analyzed for changes affecting S_f variants. Epigenetic changes in several cytosine residues were detected in a fragment of 4700 bp of the *5'* upstream region of all self-compatible samples of the S_f allele, differentiating them from all self-incompatible samples of S_f analyzed. This is the first report of DNA methylation in a Rosaceae species and appears to be strongly associated with inactivation of the S_f allele.

Keywords. Almond – Self-compatibility – DNA methylation – Epigenetics – *S*-RNase upstream region.

La méthylation du locus* S_f *chez l'amandier est associée avec la perte de fonction de la* S*-RNase

Résumé. *L'auto-compatibilité chez l'amandier (*Prunus amygdalus *Batsch) est attribuée à la présence du haplotype* S_f*, allélique avec et dominant sur la série d'allèles S qui contrôlent l'auto-incompatibilité. Néanmoins, quelques formes du haplotype* S_f *sont phénotypiquement auto-incompatibles malgré l'identité des séquences de nucléotides. Le DNA de feuilles et de styles de différents génotypes d'amandier a été cloné, séquencé et après analysé pour changes pouvant affecter les variantes de l'allèle* S_f*. Changes epigénétiques pour quelques résidus de cytosine ont été détectés dans un fragment de 4700 bp de la région 5' upstream de tous les génotypes auto-compatibles de l'allèle* S_f*, ce qui les différencie de tous les génotypes auto-incompatibles de l'allèle* S_f *analysés. Ce résultat est le premier rapport sur la méthylation du DNA dans une espèce des Rosacées et semble être fermement associé avec l'inactivation de l'allèle* S_f*.*

Mots-clés. *Amandier – Auto-compatibilité– Métilation du DNA – Épigénétique – Région upstream de la* S*-RNase.*

I – Introduction

Almond (*Prunus amygdalus* Batsch) shows gametophytic self-incompatibility, controlled by a single polymorphic locus. Most almond cultivars are self-incompatible and the few exceptions that have been identified as self-compatible have been shown to carry a mutation at the *S*-locus referred to as S_f (Socias i Company, 1990). Bošković *et al*. (1997) showed that in almond the *S* alleles code for stylar proteins with RNase activity whereas S_f does not. Quantitative real-time PCR analysis has revealed transcripts of S_f-RNase in the style tissue of a SI cultivar ('Vivot', $S_{23}S_f$) as well as the absence of the S_f-RNase transcripts in a SC cultivar ('Blanquerna', S_8S_f). Because of this differential transcription of the S_f-RNase, the S_f allele conferring SI has been designated S_{fa} (active), whereas the same S_f allele conferring SC has been designated S_{fi} (inactive) (Kodad *et al.,* 2009; Fernández i Martí *et al*., 2010). Sequencing confirmed that S_{fa} and S_{fi} were identical in the coding region and in their 5'-flanking regions (Fernández i Martí *et al*., 2010). Similarly, the TATA-Box and IB-like motifs did not reveal any difference in the *cis*-element in the active relative to inactive S_f types (Fernández i Martí *et al*., 2010).

The absence of S_f nucleotide sequence differences in SC and SI phenotypes, suggests that either modifier genes can strongly affect the expression or that epigenetic changes are involved. Consequently, our objective was to determine if DNA methylation of the S_f allele was associated with its differential phenotypic expression in almond.

II – Material and methods

1. Plant material

Six almond genotypes, containing both homozygotes and heterozygotes for the S_f haplotype, were included for analysis (Table 1). Genotypes were selected based on previous genetic and phenotypic characterization of the S_f allele established by pollen tube growth and field pollination studies. The S_f alleles analyzed could be traced back to two different origins: the Italian region of Puglia for the standard self-compatible form exemplified by the heirloom cultivar 'Tuono', and the island of Majorca where S_f types showing self-incompatible phenotypes have been identified (Fernández i Martí *et al*., 2010).

Table 1. Almond genotypes included in the methylation analysis

Genotype	S-genotype	Phenotype	Origin
'Blanquerna'	$S_8 S_f$	SC	Genco × AS-1
'Vivot'	$S_{23} S_f$	SI	Local of Majorca, Spain
'Soleta'	$S_{23} S_f$	SC	'Blanquerna' × 'Belle d'Aurons'
'Ponç'	$S_{23} S_f$	SI	Local of Majorca, Spain
A2-199	$S_f S_f$	SC	C-1322 ['Tuono' × 'Genco'] ⊗
M-2-16	$S_f S_f$	SC	M-2-2 ['Tuono' × 'Ferragnès'] ⊗

SC: self-compatible; SI: self-incompatible.

2. DNA methylation

Total DNA was extracted from leaves and styles at stage D (Felipe, 1977) using the procedure of Doyle and Doyle (1991). Once extracted, DNA was was submitted to the DNA bisulphite modification treatment 'MethylEasy' (Human Genetic Signatures, Brisbane, Australia). This kit has been designed to efficiently convert cytosine to uracil and to reduce DNA degradation and loss without decreasing the conversion of C to U residues. The converted DNA was then used for PCR amplification, cloning and sequencing.

For PCR amplification, specific primers were designed in this work. The thermal cycling program was as follows: an initial denaturation at 95ºC for 1 min followed by 35 cycles of 94ºC for 30 s, annealing for 30 s of 53ºC and extension at 72ºC for 1 min, ending with a 5 min extension at 72ºC. The PCR products were cloned using the pGEM-T-Easy Vector System (Promega, Madison, WI, USA). Cloning and sequencing of the whole region of 4700 bp in the 5' upstream were initially performed for 'Blanquerna' and 'Vivot' and methylation changes over the whole sequence in both cultivars were carefully analyzed. Subsequently, only those samples showing a methylation change were used for cloning and sequencing the remaining genotypes.

III – Results and discussion

Cloning and sequencing of the PCR products obtained confirmed that the bisulphite treatment worked properly in all DNA tested. In the partial sequence shown in Fig. 1, the presence of several cytosine residues can be observed in the upstream region of genomic DNA of 'Blanquerna' ob-

tained from pistil before the bisulphite treatment. However, after the treatment, sequencing of all clones from 'Blanquerna' showed that almost all cytosines were converted to thymines with a single exception (large arrow).

Fig. 1. Nucleotide sequence using the primer pair CIPABF4/CIPABR4 of 'Blanquerna' showing that the sequence from pistil DNA with bisulphite treatment is the same as that of genomic DNA from pistil without bisulphitetreatment, with the single exception of the conversion of cytosines to thymines (black arrows). The large (red) arrow shows where the methylation of the cytosine residue appeared.

After confirming the efficacy of the treatment, every nucleotide was carefully compared between the two sequences for a length of 4700 bp in the upstream region, as well as in the first and second intron of the S_f haplotype (Fig. 2). Among all sequences obtained, no changes were produced in the introns of the S_f gene; however four cytosine residues were not able to be converted to thymine in the upstream region of the SC cultivars indicating the presence of methylation. Fig. 2 shows the whole sequence of the unconverted S_f upstream region where the four methylation points have been identified, as well as the four primer pairs overlapping these methylated cytosines.

The first methylated nucleotide was located approximately 4275 bp upstream to the start codon in all samples from leaf as well as pistil tissue. This methylated region was stable in all five clones obtained from the SC cultivar 'Blanquerna'. The other three cytosine epi-mutations were located 3200, 2400 and 650 bp upstream of the start codon. Analysis of the DNA samples of the SI cultivars 'Vivot' and 'Ponç' showed that all cytosine residues were converted to thymine in all the clones obtained.

The differential expression of the two S_f haplotypes was initially attributed to a possible mutation (Kodad *et al*., 2009) and the present results suggest that this mutation could be an epigenetic change. Thus, when the S_f-RNase sequence is methylated, its expression is inhibited resulting in an SC phenotype, as occurs in 'Blanquerna', 'Soleta' and the two homozygous SC selections. When S_f-RNase sequences do not show methylated cytosines, the RNase remains active, resulting in a SI genotype and phenotype, as in 'Vivot' and 'Ponç'. The original S_f haplotype would have been in an active form, thus conferring SI, as with other *S* haplotypes. Epigenetic change through DNA methylation resulted in loss of RNase function and so a novel SC phenotype in a in an otherwise SI species such as almond.

While the S_f alleles examined were derived from two distinct Mediterranean regions, they may be identical by descent from the original pool of Mediterranean germplasm. The epigenetic change appears to have taken place in the Italian region of Puglia, where most SC cultivars have been described (Socias i Company, 1990). Additionally, all SC almond genotypes identified so far have shown an identical S_f allele (Fernández i Martí *et al*., 2010) and are of Mediterranean origin and so probably related to the Puglia almond population.

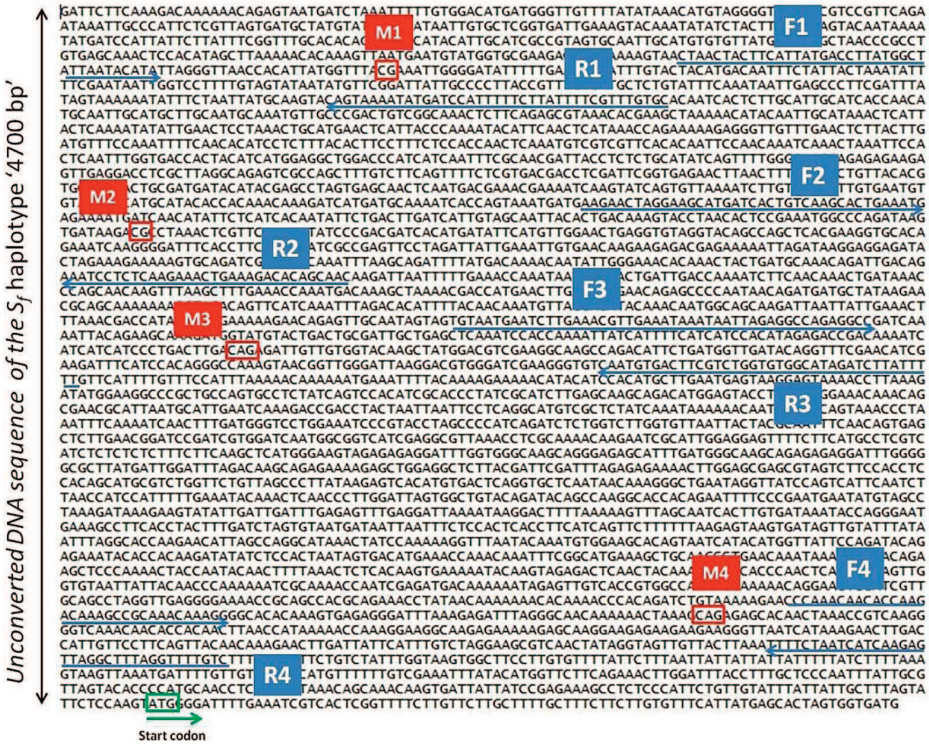

Fig. 2. Detection of methylated sites in the 5' untranslational region of S_{fi}-RNase. Primer positions overlapping the cytosine residues are indicated by blue arrows. These primers identify the best primer pair for limiting the region where the cytosine base is non-methylated.

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References

Bošković R., Tobutt K.R., Batlle I. and Duval H., 1997. Correlation of ribonuclease zymograms and incompatibility genotypes in almond. In: *Euphytica,* 97, p. 167-176.

Doyle J.J. and Doyle J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. In: *Phytochemistry Bulletin,* 19, p. 11-15.

Felipe A.J., 1977. Almendro: estados fenológicos. In: *Información Técnica Económica Agraria,* 27, p. 8-9.

Fernández i Martí A., Hanada T., Alonso J.M., Yamane H., Tao R. and Socias i Company R., 2010. The almond S_f haplotype shows a double expression despite its comprehensive genetic identity. In: *Scientia Horticulturae,* 125, p. 685-691.

Kodad O., Socias i Company R., Sánchez A. and Oliveira M.M., 2009. The expression of self-compatibility in almond may not only be due to the presence of the S_f allele. In: *Journal of the American Society for Horticultural Science,* 134, p. 221-227.

Kodad O., Alonso J.M., Fernández I Martí A., Oliveira M.M. and Socias i Company R., 2010. Molecular and physiological identification of new *S*-alleles associated with self-(in)compatibility in local Spanish almond cultivars. In: *Scientia Horticulturae,* 123, p. 308-311.

Socias i Company R., 1990. Breeding self-compatible almonds. In: *Plant Breeding Reviews*, 8, p. 313-318.