A possible activation of the Sf allele in almond

Fernández i Martí A., Alonso J.M., Kodad O., Socias i Company R.

in

Zakynthinos G. (ed.).
XIV GREMPA Meeting on Pistachios and Almonds

Zaragoza : CIHEAM / FAO / AUA / TEI Kalamatas / NAGREF
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 94

2010
pages 95-100

A possible activation of the S_f allele in almond

A. Fernández i Martí, J.M. Alonso, O. Kodad and R. Socias i Company

Unidad de Fruticultura, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA)
Av. Montañana 930, 50059 Zaragoza (Spain)

Abstract. Self-compatibility has become the primary objective of most almond (*Prunus amygdalus* Batsch) breeding programmes in order to avoid the problems related to the gametophytic self-incompatibility (GSI) system present in almond, as in other species of the genus *Prunus*, belonging to the Rosaceae family. Self-incompatibility is a mechanism in flowering plants which prevents self-fertilization and promotes out-crossing. The *S* locus encodes for ribonucleases expressed in the style (*S*-RNase) and for F-box proteins expressed in the pollen (SFB). Interactions between both genes are suspected of being involved in determining the specific self pollen and with same haplotype rejection. As this mechanism is still not fully understood, a progeny of 90 seedlings from the cross 'Vivot' ($S_{23}S_{f?}$) × 'Blanquerna' ($S_fS_{f?}$) was studied because the microscopic observation of pollen tube growth after self-pollination during several years showed an unexpected self-incompatible behaviour in most seedlings. Although the zymograms from stylar ribonucleases and the PCR products using specific and consensus primers allowed distinguishing the individuals with SI or SC genotype, the expression of the S_f allele was not the same in all cases, as shown by the SI phenotype of many seedlings with a SC genotype, suggesting a possible differential expression of S_f. Cloning and sequencing of the SFBs in the two parents may allow to determine whether they have a different protein expression and also to closely study the pollen-expressed F-box genes in almond.

Keywords. Almond – *Prunus* – self-(in)compatibility – *S*-RNase.

Une activation possible de l'allèle S_f chez l'amandier

Résumé. *L'auto-compatibilité est devenue l'objectif prioritaire de la plupart des programmes d'amélioration génétique de l'amandier (*Prunus amygdalus *Batsch) pour éviter les problèmes liés à la présence du système d'auto-incompatibilité gamétophytique de l'amandier, comme chez d'autres espèces du genre* Prunus*, appartenant à la famille Rosaceae. L'auto-incompatibilité est un mécanisme des plantes qui empêche leur auto-fertilisation en favorisant le croisement entre les différents génotypes. Le locus* S *code pour une ribonucléase exprimée dans le style (S-RNase) et aussi pour une protéine exprimée dans le pollen (SFB). Les interactions entre les deux gènes sont suspectées d'être déterminantes du pollen spécifique ainsi que du pollen avec le même haplotype. Comme ce mécanisme n'est pas encore bien compris, une famille de 90 individus provenant du croisement 'Vivot' ($S_{23}S_{f?}$) × 'Blanquerna' ($S_fS_{f?}$) a été étudiée parce que l'observation microscopique de la croissance des tubes polliniques après l'autopollinisation pendant plusieurs années a montré un comportement auto-incompatible inattendu chez la plupart des individus. Malgré que les zimogrammes des ribonucléases stylaires et les produits de PCR avec des primers spécifiques et primers consensus ont permis de distinguer les génotypes AI ou AC, l'expression de l'allèle* S_f *n'est pas toujours la même, comme le montre le phénotype AI chez beaucoup de plantes ayant un génotype AC, suggérant une possible expression différente de* S_f*. Après clonage et séquençage des SFB des deux parents, il sera possible de déterminer s'il existe des niveaux différents d'expression de protéine et d'étudier les gènes de F-box exprimés chez l'amandier.*

Mots-clés. *Amandier – Prunus – auto-(in)compatibilité – S-RNase.*

I – Introduction

Almond cultivars are mostly self-incompatible, thus needing cross pollination to set a crop. Self-incompatibility is a mechanism in flowering plants, which prevents fertilization by their own pollen (de Nettancourt, 2001). Thus, the almond breeding program at CITA (Centro de Investigación y Tecnología Agroalimentaria de Aragón) aims to obtain self-compatible and late-blooming cultivars which would provide the growers with the possibility of planting solid

orchards of single cultivars. Self-compatibility in breeding programmes mostly comes from 'Tuono', a cultivar from the Italian region of Apulia (Socias i Company, 2002). However, different sources of self-compatibility would be interesting in order to avoid inbreeding depression in the breeding progenies (Alonso and Socias i Company, 2005).

In almond, the incompatibility between pollen and pistil is of the gametophytic type and it is controlled by a single multiallelic *locus*, the *S*-locus (Crane and Lewis, 1942) that contains the genes controlling the pollen and pistil specificities. Stylar *S*-proteins have been identified as glycoproteins with ribonuclease activity (*S*-RNases). In almond, however, cultivars possessing the S_f allele, which is considered to confer self-compatibility (Socias i Company and Felipe, 1988), lacks ribonuclease activity (Boškovic *et al*., 1999). On the other hand, the F-box genes (SFB) were found to be good candidates for the *S* pollen factor in *Prunus* (Entani *et al*., 2003) and have been reported to be linked to the *S*-RNase gene. SFB has been characterized in almond (Ushijima *et al*., 2003), as well as in sweet cherry (Yamane *et al*., 2003b) and apricot (Romero *et al*., 2004).

The CITA breeding programme combines molecular techniques as well as the traditional approaches for selecting progenies. When looking to the pollen tube growth after self-pollination in the progeny of the cross 'Vivot' ($S_{23}S_{f?}$) × 'Blanquerna' ($S_fS_{f?}$), an unexpected self-incompatible phenotype was found in most of the seedlings. Thus, a combination of molecular techniques, such as the analysis of stylar proteins for *S*-RNase activity, as well as PCR amplification of *S*-RNases, was undertaken in order to ascertain the nature of this strange behaviour.

II – Materials and methods

1. Plant material

The plant material studied consisted of 90 seedlings from the cross 'Vivot' x 'Blanquerna' obtained from the CITA almond breeding programme in Zaragoza. The female parent 'Vivot' is a local Spanish cultivar from the island of Majorca with a genotype apparently S_{23} $S_{f/30}$, and 'Blanquerna', the male parent, is a release from this breeding programme obtained from an open pollination of 'Genco' and having the genotype S_f $S_{f/30}$).

2. Pollen tube growth

During three years, a minimum of 12 flowers buds at stage D (Felipe, 1977) were collected for each seedling, emasculated in the laboratory and placed in trays in contact with water. The pistils were pollinated with their own pollen and collected 96 h after pollination. Samples were immediately autoclaved and maintained at 4ºC. Microscopic observation was according to Socias i Company *et al.* (1976).

3. Fruit set after bagging

In both parents, a branch with a minimum of 100 flowers was bagged before bloom in the field in order to asses the level of self-compatibility by evaluating seed set in enclosed branches (Grasselly and Olivier, 1984). Three months after bagging the total number of fruits was counted and ranged according to Grasselly *et al*. (1981): (i) fruit sets lower than 0.5% of the initial bud number: self-incompatible; (ii) between 1% and 5%: low self-compatible; (iii) between 6% and 10%: self-compatible; and (iv) higher than 11%: highly self-compatible.

4. Protein analysis

Ribonuclease activity of seedlings and parents was determined by protein extraction from 30 styles and frozen at -80ºC until use. The stylar proteins were separated electrophoretically on poliacrylamide gels using Non-Equilibrium pH Gradient Electrofocusing (NEpHGE) according to Boškovíc *et al*. (1997).

5. Identification of S-RNase by PCR

Genomic DNA was extracted from young leaves using the procedure described by Doyle and Doyle (1987). Almond genomic DNA was PCR-amplified using specific (S_fF/S_fR and S_{23}F/S_{23}R) (Channuntapipat *et al*., 2003) and consensus primers to amplify from signal peptide to C5 region (PaConsIF/PaConsIIR and PaConsIF/EMPC5consRD) (Sonneveld *et al*., 2003; Ortega *et al*., 2006). The PCR products were separated on agarose gel and stained by ethidium bromide.

III – Results

1. Pollen tube growth

'Vivot' self-pollen tubes stopped their growth in the middle third of the style, as expected in a self-incompatible genotype, but 'Blanquerna' self-pollination showed a self-compatible behaviour. However, less than 25% of the progeny showed the arrival of the own pollen tubes at the ovary level. In most seedlings pollen tube growth was arrested in the middle third of the style as in 'Vivot', showing as a consequence self-incompatible phenotypes.

2. Fruit set after bagging

Fruit set after three months in the bagged branches was only 1.32 % for 'Vivot', whereas it was 20.8 % for 'Blanquerna', thus confirming the self-incompatibility of the mother parent and the self-incompatibility of the pollen parent.

3. Protein analysis

The presence of the S_f allele has been always related to the lack of ribonuclease activity, whereas the self-incompatibility alleles produce RNase activity. However, one band in 'Blanquerna' and two in 'Vivot' as well as in all the seedlings of their progeny were found, even for the genotypes with the S_f allele, where no RNase activity was expected (Fig. 1). These two bands followed two different patterns, identical to each parent, 55% to 'Blanquerna' and the rest to 'Vivot'.

4. Identification of S-RNase by PCR

The identification by consensus primers showed two different bands in 'Vivot', one corresponding to the putative S_f and the other to S_{23}. In 'Blanquerna' only one band was identified, corresponding to the S_f allele with the conserved primers amplifying from the signal peptide to the C5 region. In order to corroborate these results, PCR analysis was done using S_f and S_{23} specific primers. In 55% of the seedling, it was only possible to identify the S_f allele, assuming that these are homozygous SC. In the rest of the seedling, two alleles were detected, S_{23} an another with a very faint band presumably to the S_f. This distribution agrees with that obtained by NEpHGE.

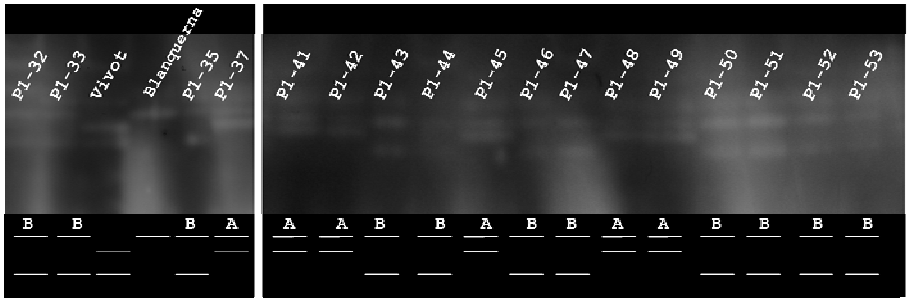

Fig.1. Zymogram of stylar ribonucleases in some seedlings of the progeny 'Blanquerna x Vivot' and in both parents.

IV – Discussion

'Vivot' and 'Blanquerna' alleles have been partially sequenced to verify their identity because allelic determination by PCR using specific and consensus primers was not apparently enough to ascertain the presence of either S_f or S_{30}. Partial sequence is neither enough to its determination, so we are continuing in order to get the amino acid sequence. 'Blanquerna' possesses at least one S_f allele, as confirmed by its self-compatible behaviour, both because of the high level of fruit set after bagging and by its pollen tube growth. However, even in the case of 'Blanquerna' possessing only one S_f allele, independently of the genotype of 'Vivot', at least 50% of the progeny should show a self-compatible behaviour following a Mendelian distribution, and this hypothesis is contrary to what has been observed during three consecutive years through microscopic observation, with only 24% of self-compatible seedlings.

The presence of two different bands in all individuals by NEpHGE analysis may be caused by the presence of a functional S_f -RNase in the pistil arresting the S_f-pollen tube growth. This S_f-RNase could be due to a functional gene expression contrary to what happens in the self-compatible cultivars, where a deficient expression is supposed, as no RNase activity is detected.

According to Ushijima *et al.* (1998) the activation of functional gene expression could be controlled by a region located further upstream of the promoter region. Hegedüs *et al.* (2006) have reported that the presence of modifier genes outside the *S* locus may contribute to the SC phenotype of peach and consequently it may influence the regulation of the transcript expression and activation of the S_f-RNase is *Prunus*.

Boškovic *et al*. (2007) have recently recognized a new allele, identified as S_{30}, in some cultivars from the Italian region of Apulia, the same where 'Tuono' originated. This new allele has been amplified with the S_f specific and consensus primers with a size of 1184 bp, the same size than S_f and has been shown to express ribonuclease activity, a characteristic of all active *S*-RNases. Although it has been reported that the Italian cultivar 'Fra Giulio Grande' (S_1S_{30}) could not be pollinated neither by 'Tuono' (S_1S_f) nor 'Falsa Barese' (S_1S_f), no reason was given for failed pollination. Thus, these authors suggested that S_{30} represents the wild-type progenitor allele from which S_f is derived. However, our observations indicate that the irregular results obtained in our progeny do not fit in the hypothesis of the presence of the new S_{30} allele in our progeny.

Further approaches are being undertaken in these genotypes to study the role of the *S*-RNases and SFBs codified by the S-locus and their expression, in order to clarify the mechanism of incompatibility in almond as well as in other *Prunus* species.

Acknowledgements

This research was conducted under the Spanish CICYT project AGL2007-65853-C02-02. A. Fernández-Martí acknowledges the receipt of a studentship co-funded by the Spanish "Ministerio de Educación y Ciencia" and the European Social Fund (FSE), under projects AGL 2004-06674-C02-01 and BES-2006-12621.

References

Alonso J.M. and Socias i Company R., 2005. Self-incompatibility expression in self-compatible almond genotypes may be due to inbreeding. In: *J. Amer. Soc. Hort. Sci*., 130, p. 865-869.

Boškovic R. and Tobutt K.R., 1996. Correlation of stylar ribonuclease zymograms with incompatibility alleles in sweet cherry. In: *Euphytica,* 90, p. 245-250.

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

Boškovic R., Tobutt K.R., Duval H., Battle I., Dicenta F. and Vargas F., 1999. A stylar ribonuclease assay to detect self-compatible seedlings in almond progenies. In: *Theor. Appl. Genet*., 99, p. 800-810.

Boškovic R., Tobutt K.R., Ortega E., Sutherland B.G. and Godini A., 2007. Self- (in) compatibility of the almonds *P. dulcis* and *P. webbii*: detection and cloning of 'wild-type S_f' and new self-compatibility alleles encoding inactive *S*-RNases. In: *Mol. Genet. Genomics,* 278, p. 265-676.

Channuntapipat C., Winthersohn M., Armes S.A., Batlle I., Arús P., Sedgley M. and Collins G., 2003. Identification of incompatibility genotypes in almond (*Prunus dulcis* Mill.) using specific primers based on the introns of the *S*-alleles. In: *Plant Breed.,* 122, p. 164-168.

Crane M.B. and Lewis D., 1942. Genetical studies in pear. III: Incompatibility and sterility. In: *J. Genet.,* 43, p. 31-43.

De Nettancourt D., 2001. *Incompatibility and incongruity in wild and cultivated plants*. Springer-Verlag, Berlin.

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

Entani T., Iwano M., Shiba H., Che F.S., Isogai A. and Takayama S., 2003. Comparative analysis of the self-incompatibility (S-) locus region of *Prunus mune*: identification of a pollen-expressed F-box gene with allelic diversity. In: *Genes Cells,* 8, p. 203-213.

Felipe A.J., 1977. El almendro: estados fenologicos. In: *Inf. Técn. Econ.Agrar*., 8(27), p. 8-9.

Grasselly C., Grossa Raynaud P., Olivier G. and Gall H., 1981. Transmission du caractere d'autocompatibilité chez l'amandier (*Amygdalus communis*). In: *Options Méditerranéennes,* Série Études, No. 1981-I, p. 71-75.

Hegedüs A., Szabó Z., Nyéki J., Halász J. and Pedryc A., 2006. Molecular analysis of *S*-haplotypes in peach, a self-compatible *Prunus* species. In: *J. Amer. Soc. Hort. Sci.,* 131, p. 738-743.

Ortega E., Boskovic R., Sargent D.J. and Tobutt K.R., 2006. Analysis of *S*-RNase alleles of almond (*Prunus dulcis*): characterization of new sequences, resolution of synonyms and evidence of intragenic recombination. In: *Mol. Gen. Genomics*, 276, p. 413-426.

Romero C., Vilanova S., Burgos L., Martínez-Calvo J., Vicente M., Llácer G. and Badenes M.L., 2004. Analysis of the *S*-locus structure in *Prunus armeniaca* L. Identification of *S*-haplotype specific *S-RNase* and F-box genes. In: *Plant Mol. Biol.*, 56, p. 145-157.

Socias i Company R., 2002. Latest advances in almond self-compatibility. In: *Acta Hort*., 591, p. 205-212.

Socias i Company R. and Felipe A.F., 1988. Self-compatibility in almond: transmission and recent advances in breeding. In: *Acta Hort,* 224, p. 307-317.

Socias i Company R., Kester D.E. and Bradley M.V., 1976. Effect of temperature and genotype on pollen tube grothw of some self-incompatible and self-compatible almond cultivars. In: *J. Amer. Soc. Hort. Sci*., 101, p. 490-493.

Sonneveld T., Tobutt K.F. and Robbins T.P., 2003. Allele-specific PCR detection of sweet cherry self-incompatibility (*S*) alleles S_1 to S_{16} using consensus and allele-specific primers. In: *Theor. Appl. Genet*., 107, p. 1059-1070.

Ushijima K., Sassa H., Tao R., Yamane H., Dandekar A.M., Gradziel T.M. and Hirano H., 1998. Cloning and characterization of cDNAs encoding *S*-RNases from almond (*Prunus dulcis*): Primary structural features and sequence diversity of *S*-RNases in Rosaceae. In: *Mol. Gen. Genet.,* 260, p. 261-280.

Ushijima K., Sassa H., Dandekar M.A., Gradziel T.M., Tao R. and Hirano H., 2003. Structural and transcriptional analysis of the self-incompatibility locus of almond: identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. In: *Plant Cell*, 15, p. 771-781.

Yamane H., Ikeda K., Ushijima K., Sassa H. and Tao R., 2003. A pollen-expressed gene for a novel protein with an F-motif that is very tightly linked to a gene for *S*-RNase in two species of cherry, *Prunus cerasus* and *P. avium*. In: *Plant Cell Physiol*., 44, p. 764-769.