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Short communication. Cloning and sequencing of partial genomic DNA fragments corresponding to the S_{11} and S_{12} alleles of the Spanish almond cultivar 'Marcona'

O. Kodad[1], A. Sánchez[2], N. Saibo[2], J. M. Alonso[1], M. M. Oliveira[2,3] and R. Socias i Company[1]*

[1] *Unidad de Fruticultura. Centro de Investigación y Tecnología Agroalimentaria de Aragón. Apdo. 727. 50080 Zaragoza. Spain*

[2] *Instituto de Tecnologia Química e Biológica (ITQB). Instituto de Biologia Experimental e Tecnológica (IBET). Quinta do Marqués, 2784-505 Oeiras. Portugal*

[3] *Universidade de Lisboa. Faculdade de Ciências. Dep. Biologia Vegetal. Campo Grande, 1749-016 Lisbon. Portugal*

Abstract

This paper reports the cloning and partial genomic DNA sequencing of two *S*-RNases (S_{11} and S_{12}) of almond. DNA from the Spanish almond cultivar Marcona, the most highly appreciated in Spain, was amplified by PCR using the primer pair AS1II and AmyC5R designed for the conserved regions of almond *S*-alleles. The cloned and sequenced S_{11} allele of cv. Marcona appears to be identical to that of cvs. Rumbeta and Bertina (which have been previously cloned and sequenced). This is the first time, however, that S_{12} has been sequenced in almond. The amplified and cloned S_{12} allele fragment possessed 1080 bp corresponding to the second intron and 506 bp corresponding to the second and third exons. The product of PCR-specific amplification showed that cv. Marcona and the Portuguese cultivar Pestaneta carry the same S_{12} allele. Knowledge of the sequences of these alleles will be helpful in the design of specific primers for S_{11} and S_{12}, and could be of use when employing antisense techniques in genetic engineering projects.

Additional key words: PCR, *Prunus amygdalus* Batsch, self-incompatibility, stylar ribonucleases.

Resumen

Nota corta. Clonación y secuenciación de fragmentos de ADN genómico correspondientes a los alelos S_{11} y S_{12} del cultivar español de almendro 'Marcona'

Este trabajo muestra la clonación y el análisis de secuencias de fragmentos de DNA genómico correspondientes a dos *S*-RNasas (S_{11} y S_{12}) de almendro. Estos fragmentos se amplificaron mediante PCR a partir de DNA del cultivar 'Marcona', el más apreciado en el mercado español, utilizando la pareja de cebadores AS1II y AmyC5R, diseñados en regiones conservadas de los alelos *S* de almendro. El alelo S_{11} fue clonado y secuenciado en 'Marcona', y es idéntico al clonado y secuenciado previamente en 'Rumbeta' y 'Bertina'. En cambio, es la primera vez que el alelo S_{12} se secuencia en almendro. El fragmento analizado de este alelo contiene el segundo intrón (1080 pb) y secuencias parciales del segundo y tercer exón (506 pb). La amplificación específica por PCR mostró que el cultivar español 'Marcona' y el portugués 'Pestaneta' poseen el mismo alelo S_{12}. La secuenciación de estos alelos permitirá, no sólo diseñar cebadores específicos para S_{11} y S_{12}, sino también aplicar posiblemente la tecnología anti-sentido en ingeniería genética.

Palabras clave adicionales: auto-incompatibilidad, PCR, *Prunus amygdalus* Batsch, ribonucleasas estilares.

Self-incompatibility is a mechanism developed by higher plants to avoid inbreeding. By increasing their genetic variability, it also helps them adapt to different environments (de Nettancourt, 2001). Almond (*Prunus amygdalus* Batsch) is mostly a self-incompatible species (Socias i Company, 1990); like other *Prunus* species it has a homomorphic, monofactorial and gametophytic-type self-incompatibility system (de Nettancourt, 2001).

* Corresponding author: rsocias@aragon.es
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J. M. Alonso and R. Socias i Company are members of the SECH.

Knowledge of the allelic composition of almond cultivars is important in orchard design; it helps to ensure cross-pollination and good crop yields. It is also important for choosing parental plants when performing crosses. Traditionally, the alleles carried have been determined by test crosses in the field (Crossa-Raynaud and Grasselly, 1985), a time-consuming technique, the results of which are strongly influenced by the environment. Recently, it has been shown that the self-incompatibility *S*-alleles of almond (Bošković *et al.*, 2003) code for specific glycoproteins in the style tissue, as in other Rosaceae (Sassa *et al.*, 1996). These possess ribonuclease activity that inhibits pollen tube growth.

The development of new molecular techniques, such as the use of genomic DNA in PCR analysis (Tamura *et al.*, 2000), has dramatically advanced the identification of *S*-alleles in almond. However, the primers developed by Tamura *et al.* (2000) do not always distinguish alleles with a similar number of nucleotides, such as S_5 and S_{25} (López *et al.*, 2004), or S_3 and S_f (Sánchez-Pérez *et al.*, 2004). Allele sequencing is therefore often advisable for identifying almond *S*-alleles.

The allelic composition of the Spanish almond cultivar Marcona ($S_{11}S_{12}$), the most highly appreciated in Spain, was established using stylar RNases by Bošković *et al.* (2003). However, the allelic sequences remained unknown. The aim of this work was to establish the DNA genomic sequence of the S_{11} and S_{12} alleles in this cultivar.

Cultivars whose alleles have previously been identified - cvs. Atocha ($S_{13}S_{22}$), Bertina (S_6S_{11}) (Bošković *et al.*, 2003), Desmayo Largueta (S_1S_{25}) (López *et al.*, 2004), Ferragnès (S_1S_3) (Crossa-Raynaud and Grasselly, 1985), and Pestaneta ($S_{12}S_{23}$) (Certal *et al.*, 2002) - were included in the present experiments as standards. All the material studied was provided by the almond germplasm collection of the *Centro de Investigación y Tecnología Agroalimentaria de Aragón* (CITA), in Zaragoza, Spain. This germplasm is maintained in the form of live plants. Young leaves were collected, frozen in liquid nitrogen and stored at –80ºC until use. Genomic DNA was extracted according to Gepts and Clegg (1989). The primers used were AS1II (forward) 5'-TATTTTCAATTTGTGCAACAATGG-3' and AmyC5R (reverse) 5'-CAAAATACCACTTCATGTAACAAC-3' from the conserved regions (C1-C5) of the *S*-alleles (Tamura *et al.*, 2000). PCR reactions and conditions were performed according to Sánchez-Pérez *et al.* (2004).

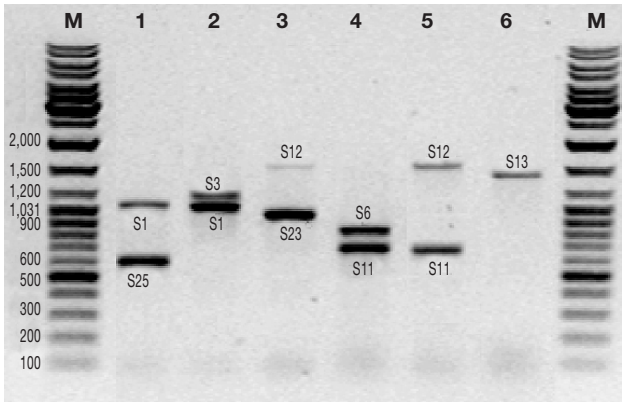

Figure 1. Agarose (1.5%) gel showing *S*-allele fragments amplified with the AS1II and AmyC5R in several almond cultivars. 1: Desmayo Largueta. 2: Ferragnès. 3: Pestaneta. 4: Bertina. 5: Marcona. 6: Atocha. M: 1 kb DNA Ladder (Fermentas).

The PCR reactions were very efficient in the identification of almost all the *S*-alleles of the cultivars reported in this study (S_1, S_3, S_6, S_{11}, S_{12}, S_{13}, S_{23} and S_{25}) (Fig. 1). The PCR-amplified fragments of genomic DNA of cv. Marcona were of about 700 bp for S_{11} and 1600 bp for S_{12}, in agreement with that reported by Sánchez-Pérez *et al.* (2004).

Genomic DNA was inserted into the 3.9 kb vector pCR®2.1 using the TA Cloning®Kit (Invitrogen) and confirmed both by restriction enzyme digestion with *Eco*RI and by PCR using the same primers. The positive clones were isolated and the plasmid DNAs purified using the QIAquick PCR Purification Kit (Qiagen). DNA sequence data were analysed using the BLAST (NCBI) program (Altschul *et al.*, 1997) and the Clustal W program (DNAStar Inc.). The nucleotide sequence data was deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers DQ437650 for S_{11} and DQ437651 for S_{12}.

Sequencing included the second and third exons of the S_{11} and S_{12} alleles of cv. Marcona as well as the second intron between these two exons (Fig 2). The partial nucleotide sequence for cv. Marcona S_{11} was determined to be of 686 bp after sequencing. The intron size, determined by matching the partial exon sequence with those of S_1, S_3, S_6 and S_{23} (Fig. 2), was 194 bp. The partial exon sequence of the S_{11}-gene amplified in this study was 100% identical to that reported for cvs. Bertina (GenBank AF487915) and Rumbeta (GenBank AF510415).

The S_{11} allele band amplified from cv. Marcona was of the same size as the S_{11} from cv. Bertina (Fig. 1), and after sequencing was found to show 100% homology

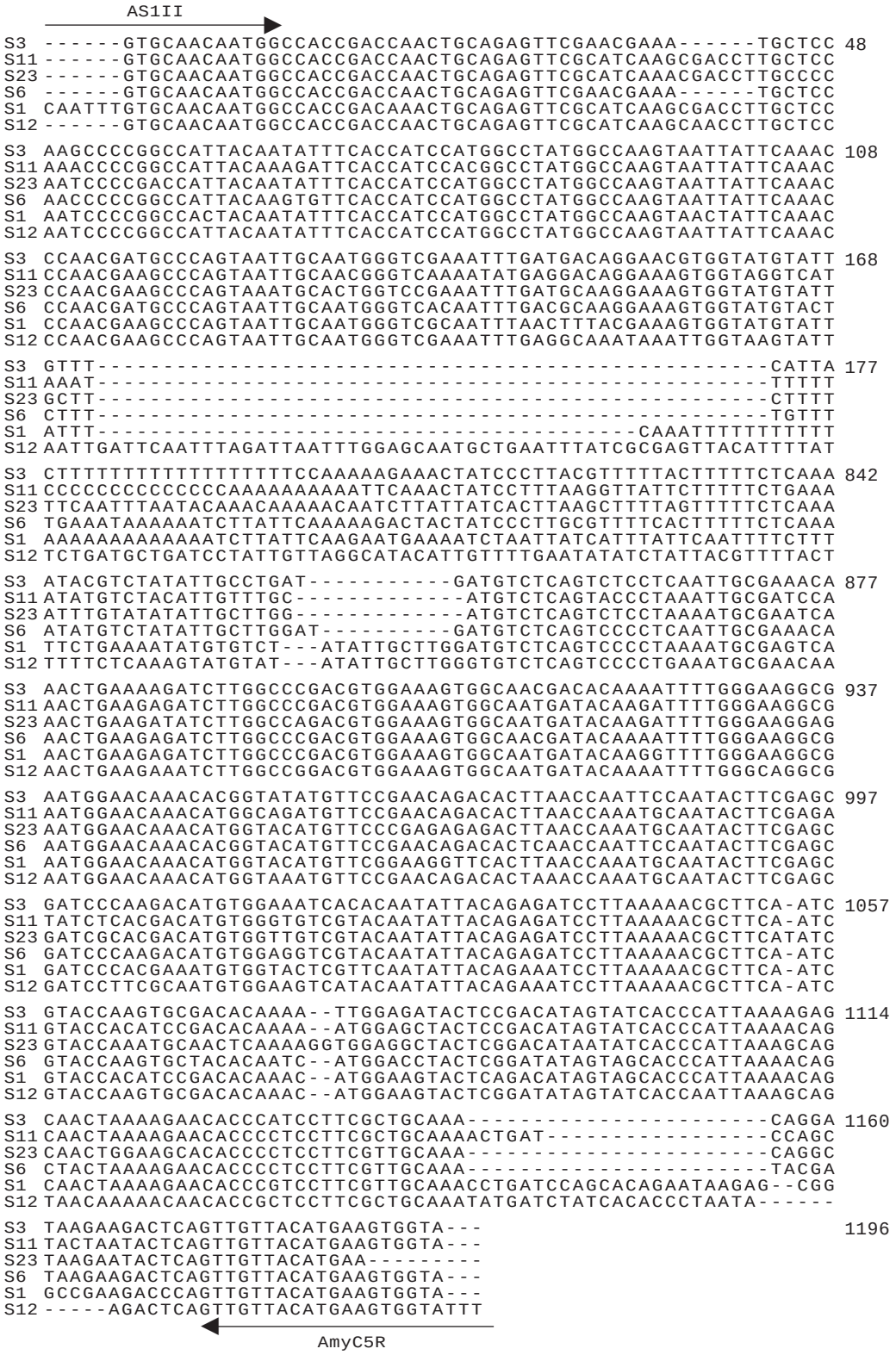

Figure 2. Sequence alignment of alleles S_1 and S_3 from cv. Ferragnès (acc. nos. AF 149039 and AF 510417, respectively), S_6 and S_{23} from cv. Ramillete (AF 510419 and AF 454002, respectively), and S_{11} and S_{12} from cv. Marcona (this work). The front exons start at 1-158 bp, the partial introns start at 159-871 bp, and back exons start at 872-1,196 bp. The AS1II and AMYC5R primer regions are underlined. Arrows indicate the primer orientation.

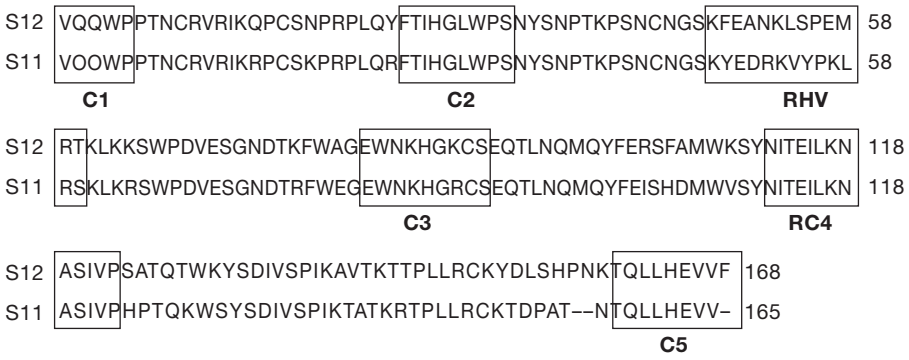

Figure 3. Deduced amino acid sequences of S_{11} and S_{12} RNases of cv. Marcona. The five conserved domains and the hypervariable region are boxed.

with S_{11} of cvs. Bertina and Rumbeta. These cultivars therefore share the same S_{11} allele, although its origin is unknown (Felipe, 2000). The presence of S_{11} in these cultivars, all of which originated in eastern Spain, suggests they have a common ancestor.

The S_{12} band amplified from the genomic DNA of cv. Marcona was of the same size as that of the S_{12} band of cv. Pestaneta (Fig. 1). Based on stylar ribonuclease analysis, Certal *et al.* (2002) assigned the $S_{12}S_{23}$ allele composition to cv. Pestaneta obtained from the collection belonging to the Direcção Regional de Agricultura de Trás-os-Montes (Portugal). Using the consensus primer pairs PaConsI-F and EM-PC1consR (developed at the East Malling Research Station, UK), Ortega *et al.* (2005) amplified a band from the genomic DNA of cv. Pestaneta that corresponded to the band size amplified in cv. Marcona. These results, together with those obtained herein, show that the S_{12} amplified in cv. Marcona appears to be the same as that in cv. Pestaneta.

The partial nucleotide sequence for cv. Marcona S_{12}, cloned and sequenced here for the first time, involved 1584 bp (Fig. 2). As with other almond *S*-RNases, this sequence included the second intron (1080 bp). The partial sequence of the second and third exons was about 504 bp long. When this sequence and those of S_1, S_3, S_6 and S_{23} were aligned (Fig. 2), the S_{11} exons showed 80, 85.5, 84.9 and 82.5% homology with S_1, S_3, S_6 and S_{23} respectively, while the S_{12} exons showed 85.3, 85.1, 86.5 and 83.3% homology respectively. The sequence of the intron/exon splice junction regions followed the GT/AG consensus sequence rule (Thangstad *et al.,* 1993); thus, the sequence adjacent to the splice junctions was highly conserved (Fig. 2).

The nucleotide composition of the partial exons, and of the second intron, for each *S*-allele showed a higher proportion of A+T nucleotides (69.35% for S_{11} and 64.26% for S_{12}) than C+G nucleotides. The partial sequence of the exons contained a higher proportion of C+G nucleotides (44.85% for S_{11} and 42.09% for S_{12}) than the introns (30.41% for S_{11} and 35.74% for S_{12}). The deduced amino-acid sequence of the S_{12} allele (Fig. 3) shows the expected ribonuclease T2 family conserved domain of Rosaceae (Sassa *et al.*, 1996).

Knowledge of the *S*-allele sequences of cv. Marcona will be helpful in the design of specific primers for S_{11} and S_{12}, and in the identification of allelic self-incompatibility groups in Spanish almond cultivars. It may also be useful for modifying the self-incompatible nature of this very important cultivar, for instance via the use of antisense technology in genetic engineering projects.

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