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Detection and inheritance of stylar ribonucleases associated with incompatibility alleles in apricot

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Abstract Stylar proteins were surveyed by non-equilibrium pH gradient electrofocusing to identify S-RNases associated with gametophytic self-incompatibility in nine apricot cultivars. RNase activities associated with the alleles of incompatibility S_1, S_2, S_5, and S_6 and with the allele of compatibility *Sc* were clearly identified. Two other bands that we considered related to the alleles S_3 and S_4 were unique to cultivars Sunglo and Harcot, respectively. Two generations of 17 seedlings from the cross Moniquí × Pepito and 38 from Gitano × Pepito were used to determine the inheritance of the S-RNases. Inheritance of these RNase bands followed the expected segregation ratios and the band combinations correlated perfectly with the known self-incompatibility status of the seedlings determined after self-pollination and observation of pollen tube growth. All evidence presented in this study strongly suggests that RNases are associated with gametophytic self-incompatibility of apricot and that RNases may be the *S*-gene products. This is the first report identifying S-RNases and describing the inheritance of these S-RNases in apricot.

Key words Apricot · Incompatibility · Inheritance · *Prunus armeniaca* · Ribonuclease

Introduction

Apricots have been classified into eight different eco-geographical groups. The cultivars grown in Europe, North America, South Africa and Australia belong to the European group. This group is the youngest and the least variable of the four most important groups. Most of the cultivars of this group are also considered self-compatible (Mehlenbacher et al. 1991). However, in the last 15 years many widespread commercial varieties have been described as self-incompatible (Lamb and Stiles 1983; Nyútjó et al. 1985; Glucina et al. 1988; Burgos et al. 1993; Burgos et al. 1997a).

Incompatibility in apricot is gametophytic and the trait is controlled by one gene with several alleles. Alleles for self-compatibility would allow pollen tubes to grow in any style and reach the ovules. Alleles for self-incompatibility arrest pollen tube growth if the same allele is present in the pollen grain and in the pistil (Burgos et al. 1997b).

Studies have been widely carried out on species of the Solanaceae, another family that presents a gametophytic system of incompatibility. Different *S* alleles have been identified and glycoproteins encoded by these alleles have been characterized as having RNase activity (McClure et al. 1989). The RNase activity of these proteins is implicated in the inhibition of pollen tube growth in incompatibility reactions and may involve degradation of ribosomal RNA (McClure et al. 1990).

S-RNases are responsible for the pistil's ability to recognize and reject self pollen (Lee et al. 1994; Murffet et al. 1994), although the basis of the allelic interactions between S-RNases and the pollen *S*-allele products is not yet understood. The S-RNases may be specifically taken up by incompatible pollen tubes. This specific uptake may involve domains on the surface of the protein that are very different in sequence between different S-RNase alleles (Matton et al. 1997). An alternative hypothesis is based on non-specific uptake of S-RNases into the pollen tube, followed by specific inactivation or other modification. In both cases, the RNase is thought to act as a cytotoxin and degrade the RNA essential for protein translation so that arrest of pollen-tube growth follows (Matton et al. 1994).

Within the Rosaceae studies are unfortunately less extensive, but a large amount of work has been done with

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Japanese pear, a species that also presents gametophytic incompatibility. A correlation has been established between known genotypes of Japanese pear and protein bands from electrophoresis of stylar extracts. These proteins have been characterized as glycoproteins with RNase activity (Sassa et al. 1992). Similar results have been found in apple (Sassa et al. 1994), where five *S*-alleles were also cloned and shown to encode RNases that were characterized in great detail (Broothaerts et al. 1995; Janssens et al. 1995). Electrophoretic separation and staining for RNase activity to reveal bands corresponding to *S* alleles could be a quicker method for genotyping cultivars than making a series of crosses and assessing pollen tube growth or fruit set.

It was therefore encouraging for us to know that evidence of RNases related to *S* alleles have been found in *Prunus* as well, in both sweet cherry (Boskovic and Tobutt 1996) and almond (Boskovic et al. 1997; Tao et al. 1997).

The aim of this paper is to demonstrate a relationship between protein bands with RNase activity found in stylar extracts from several apricot cultivars and the information available on the incompatibility genotype of those cultivars. Further, evidence is presented that the *S*-allele-associated RNases are inherited. These results may indicate that the RNases are the *S*-gene products in apricot.

Materials and methods

Plant material

Nine cultivars growing in the apricot collection at the Departamento de Mejora y Patología Vegetal (CEBAS-CSIC) in Murcia, Spain were used in this study. The cultivars Goldrich, Hargrand, Lambertin, Harcot and Sunglo produced in North American breeding programs and the Spanish cultivars Pepito, Gitano, Moniquí and Colorao were studied. In addition, seedlings from two crosses among the Spanish cultivars (Gitano × Pepito and Moniquí × Pepito) were included. All cultivars and seedlings (with the exception of Colorao, a male sterile cultivar) were tested for self-compatibility by following pollen tube growth in the style after self-pollination (Burgos et al. 1997b).

Protein extraction

Styles with stigmas uncontaminated with pollen were collected from flowers just before or soon after anthesis. They were transported to the laboratory in labeled plastic bags, emasculated, and the styles were then harvested. The excised styles were collected directly into eppendorf tubes in an ice bath and stored at –20°C. About 25–30 styles were crushed in liquid nitrogen and the frozen powder was suspended in 1 ml of extraction solution. The solution contained 30% (v/v) dimethyl sulphoxide, 10% (w/v) sucrose, 0.1% (w/v) sodium metabisulphite, 0.2% (v/v) Pharmalyte pH 3–10 and 0.5% (v/v) 10% Triton X-100. The slurry was centrifuged at –4°C for 30 min at 14 000 rpm and the supernatant was stored at –70°C.

Non-equilibrium pH gradient electrofocusing

The extracts were separated on vertical polyacrylamide gels (15 cm×21 cm×1 mm, consisting of 6% polyacrylamide with 10% sucrose and one or more Pharmalytes. Different ampholyte combinations and running times were tried but the main results presented here were obtained when gels contained 1.4 ml of Pharmalyte pH 5–8. The catalyte and analyte used were 0.1 M sodium hydroxide and 0.04 M DL-glutamic acid respectively. After prefocusing for 30 min at 100 V, 20 µl of each sample was loaded at the anodal end. The focusing run comprised 1 h at 130 V, 2 h at 260 V and 2 h at 350 V. The temperature was maintained at 4°C.

Staining

After electrophoresis gels were stained for ribonuclease activity, preincubated for 10 min at 37°C in 0.1 M sodium acetate buffer (pH 5.8) containing 0.1 M potassium chloride, and then incubated in fresh buffer with 0.2% (w/v) yeast RNA added for 20 min at 37°C. The gel was then postincubated with the preincubation buffer for 15–20 min at 37°C, fixed for 3 min in 7% (w/v) acetic acid, stained with 0.2% (w/v) toluidine blue in 0.5% (v/v) acetic acid for 1 min, and washed several times with tap water.

Results and discussion

The conditions used to run the samples, described in the Materials and methods section, showed only bands of RNase activity which we considered related to self-incompatibility alleles because they correlated with the available information on the cultivars' genotypes. Non-polymorphic bands such as those found in sweet cherry (Boskovic and Tobutt 1996) or almond (Tao et al. 1997) in this pH range were not detected in this study (Fig. 1).

The identification of *S*-allele-associated proteins for apricot cultivars is difficult at present because little information on their *S*-genotypes is available. However, it is likely that any proteins which correlate with cross-incompatibility relationships (or information on S-genotypes from inheritance or molecular studies) in apricot and which have characteristics similar to those of the S-glycoproteins of other *Prunus* species are involved in self-incompatibility in apricot. Hence, the presence of the S_1 and S_2 RNases in the cross-incompatible cultivars Lambertin, Goldrich and Hargrand, the S_2-RNase band, again, in Sunglo (the female parent of Goldrich) and the S_1-RNase band in Harcot (Fig. 1), point out the relationship between these bands of RNase activity and the alleles for incompatibility.

It still must be proved that S-RNases S_3 and S_4, present in Sunglo and Harcot respectively (Fig. 1), are actually related to alleles for self-incompatibility, since the only evidence we have is that they are unique to these cultivars among those studied. In both cases, this could be done by studying the segregation of these bands in seedlings from these cultivars.

While studying the inheritance of incompatibility in apricot we screened two large families with the same self-compatible male parent, Pepito, and two self-incompatible female parents, Gitano and Moniquí. Segregation of self-incompatibility in the two families was very different; Gitano × Pepito produced approximately 50% self-compatible seedlings, while the Moniquí × Pepito family produced very close to 100% (Burgos et al. 1997b). These results indicated a common allele in Moniquí and Pepito genotypes that is confirmed by the com-

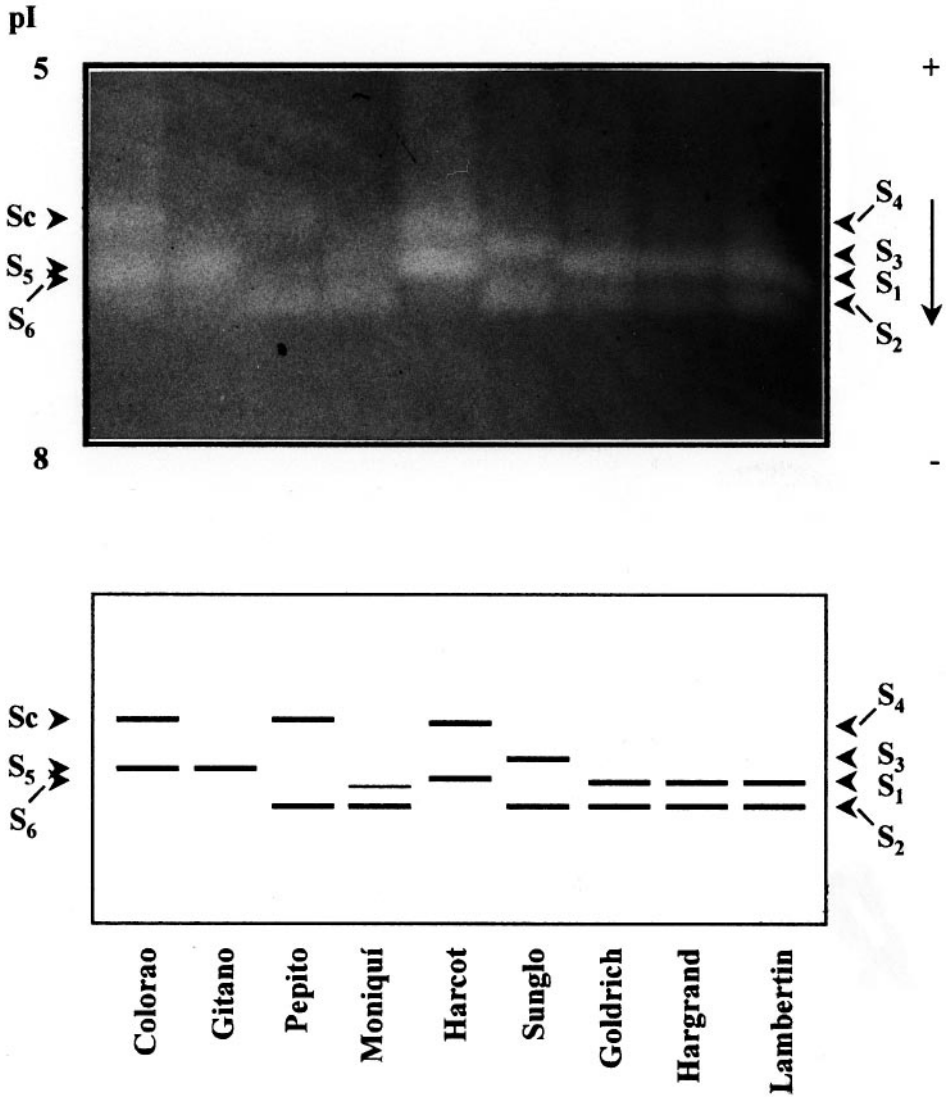

Fig. 1 Zymograms of apricot stylar ribonucleases separated on non-equilibrium pH gradient electrofocusing (NEpHGE) gel containing 4% ampholine pH 5–8 showing considerable polymorphism. Lambertin, Hargrand and Goldrich, three cross-incompatible cultivars assigned the genotype S_1S_2, (Egea and Burgos 1996) show two common bands of RNase activity. Sunglo, the female parent of Goldrich, presents one band related to the allele designated S_2. Harcot styles present a RNase related to the allele S_1 from Perfection. This cultivar is the male parent of Goldrich and also an ancestor of Harcot (I15–64 × (Phelps × Perfection)]. Sunglo and Harcot also present two other bands, unique to these cultivars, that may be related to alleles for self-incompatibility S_3 and S_4, respectively. The common band related to S_2 present in stylar extracts from Moniquí and Pepito confirms previous inheritance studies where the segregation of the seedlings for self-incompatibility indicated a common *S* allele in these two cultivars. Gitano presents only one band with RNase activity, designated S_5, and different from those of Pepito, as also expected from that study. There is another band related to the *Sc* allele for self-compatibility in Pepito and a band with very low activity just above S_2 in the Moniquí lane (S_6). Finally, Colorao, a male-sterile cultivar closely related to Pepito and Gitano has bands related to *Sc* and S_5

mon band related to S_2 whereas there would not be any common allele in Gitano and Pepito genotypes (Fig. 1).

The S_5 band of Gitano could be a combination of two different RNases as it happened in sweet cherry, depending on the electrophoretic conditions for specific combinations of RNases (Boskovic and Tobutt 1996). However, when seedlings from the Gitano × Pepito cross were studied, only one band was segregating in combination with those of Pepito, producing two distinct genotypes (also different from that of Gitano) with a missing band, Sc_ and S_2_ (Table 1).

Since Gitano is a self-incompatible cultivar, the lack of a second RNase could not be the result of a mutation affecting the expression of the stylar RNase activity, as in the Japanese pear cultivar Osa-Nijisseiki (Sassa et al. 1992). Recently it was demonstrated that the mutation in Osa-Nijisseiki is a deletion of the S-RNase gene which leads to non-functioning of the *S*-allele in the style but does not affect the function of the allele in pollen. This finding suggests that separate genes control style activity and pollen activity (Sassa et al. 1997).

The other RNase band that should be present in Gitano may have neutral or acidic pH, like that of Japanese pear cultivars Atago and Matsukaze which lacked the basic proteins except for a common band not related to any *S*-allele (Hiratsuka et al. 1995).

Colorao is one of the few male-sterile cultivars grown commercially in Spain. Male sterility in apricot is controlled by one recessive gene (Burgos and Ledbetter 1994) and since the cross Gitano × Pepito segregated 25% male-sterile seedlings (unpublished results), both cultivars should be heterozygous for male sterility. The three cultivars have many similar agronomic and fruit characteristics; in addition, Colorao and Pepito have the same restriction fragment length polymorphism (RFLP) phenotype, except for a single RFLP band out of the 48 polymorphic fragments scored (de Vicente et al. 1998). We hypothesized that Colorao and Pepito are closely related, which is in agreement with the results of the RNase study. Colorao would set fruit after self-pollination if it were not male-sterile since it presents the same

Table 1 Self-compatibility phenotype and proposed genotype for apricot seedlings. *SC* and *SI*, self-compatible and self-incompatible; *nt*, not tested; *ms*, male sterile

Seedling number	SC/SI phenotype	Proposed genotype	Seedling number	SC/SI phenotype	Proposed genotype
Moniqui×Pepito			Gitano×Pepito		
Z101-44	SC	ScS_6	Z111-02	SC	ScS_5
Z102-24	SC	ScS_2	Z111-28	nt	ScS_5
Z102-27	SC	ScS_2	Z112-10	SC	ScS_5
Z102-41	SC	ScS_2	Z112-35	SI	S_2S_5
Z102-55	SC	ScS_2	Z112-46	SC	ScS_5
Z103-50	SC	ScS_6	Z113-01	SC	ScS_5
Z103-61	SC	ScS_2	Z113-05	SI	S_2S_5
Z104-09	SC	ScS_2	Z113-08	SI	$S_{2\_}$
Z104-14	SC	ScS_2	Z113-13	SC	ScS_5
Z104-29	SC	ScS_6	Z113-33	ms	$Sc_\_$
Z104-56	SC	ScS_2	Z113-35	SC	ScS_5
Z105-02	SC	ScS_6	Z113-37	SC	ScS_5
Z105-08	SC	ScS_2	Z113-41	SC	$Sc_\_$
Z105-44	SC	ScS_2	Z113-51	SC	ScS_5
Z105-48	SC	ScS_6	Z113-53	ms	S_2S_5
Z106-09	SC	ScS_2	Z113-57	SI	S_2S_5
Z106-34	SC	ScS_2	Z114-13	SI	S_2S_5
			Z114-19	SC	ScS_5
Gitano×Pepito			Z114-23	ms	ScS_5
Z107-20	SI	S_2S_5	Z114-29	SC	ScS_5
Z107-36	SC	ScS_5	Z114-31	nt	$S_{2\_}$
Z107-43	SI	S_2S_5	Z114-35	ms	S_2S_5
Z108-10	SC	$Sc_\_$	Z114-37	nt	ScS_5
Z108-28	SI	$S_{2\_}$	Z114-45	SC	ScS_5
Z108-29	SI	S_2S_5	Z114-47	SI	S_2S_5
Z108-37	SC	$Sc_\_$	Z114-59	nt	$S_{2\_}$
Z108-38	SC	ScS_5	Z115-03	nt	ScS_5
Z109-28	SI	S_2S_5	Z115-05	SC	$Sc_\_$
Z110-54	SI	$S_{2\_}$			

RNase band that in Pepito is related to the allele for self-compatibility. Interestingly, the other Colorao band is the same as the S_5 band in the stylar extracts of Gitano (Fig. 1).

The correspondence we found between stylar ribonuclease bands and different information on *S*-alleles in *Prunus armeniaca* cultivars is in accordance with the findings of other authors in *Pyrus serotina* (Sassa et al. 1992), *Malus* × *domestica* (Sassa et al. 1994), *Prunus avium* (Boskovic and Tobutt 1996) and *Prunus dulcis* (Boskovic et al. 1997; Tao et al. 1997). Moreover, this study is the first to demonstrate the inheritance of these RNase bands in apricot with perfect correlation between the self-compatible/self-incompatible status of the seedlings and the RNase band combinations. Only one previous report showed evidence of the inheritance of RNases in almond (Ballester et al. 1998).

In the Moniquí × Pepito and Gitano × Pepito crosses, Pepito is the pollen parent and its self-compatible phenotype is the result of possessing the *Sc* allele, which makes it a half-compatible phenotype since 50% of the pollen would contain a functional S_2 allele making it incompatible with the stylar S_2 allele.

Segregation of the RNase bands correlated perfectly with information available on the self-compatibility status of these seedlings (Table 1). The Sc-RNase band is present in 100% of the seedlings from Moniquí × Pepito while the expected ratio for S_2 and S_5 (1:1) cannot be rejected at the 5% level of significance (χ^2=2.5, *df*=1).

In the family Gitano × Pepito individual S-RNases segregated in the expected ratio 1:1:1:1, which cannot be rejected at the 1% level of significance (χ^2=9.9, *df*=3). By considering the segregation of RNases, we could establish the genotype for five seedlings not tested before and also for four male-sterile seedlings whose genotype could not be determined by self-pollination.

The band related to the *Sc* allele in Pepito is present in all seedlings from the cross Moniquí × Pepito, which are all self-compatible. It is also present in all self-compatible seedlings from the cross Gitano × Pepito and absent in all self-incompatible seedlings from this cross (Fig. 2).

McClure et al. (1990) obtained results which support a model in which the self-incompatibility system in *Nicotiana alata* acts through cytotoxic action of the RNases activity of S-RNases. Although the nature of allelic specificity remains unclear, one hypothesis is that the *S*-allele specificity of interaction between pollen tube and styles resides in the mechanism by which the S-RNase gains access to the cytoplasmic compartment of the pollen tube (Matton et al. 1994). The self-compatible cultivar included in this study shows a band with strong RNase activity. Hence, if the cytotoxic mechanism of self-incompatibility described in *Nicotiana* is present in apricot, the S-RNase may have a mutation in the recognition domain that renders it unable to recognize the pollen component in self-incompatibility interactions or that may affect something in the activity of pollen grains carrying the *Sc* allele which permits pollen tubes to grow in Pepito pistils after self-pollination.

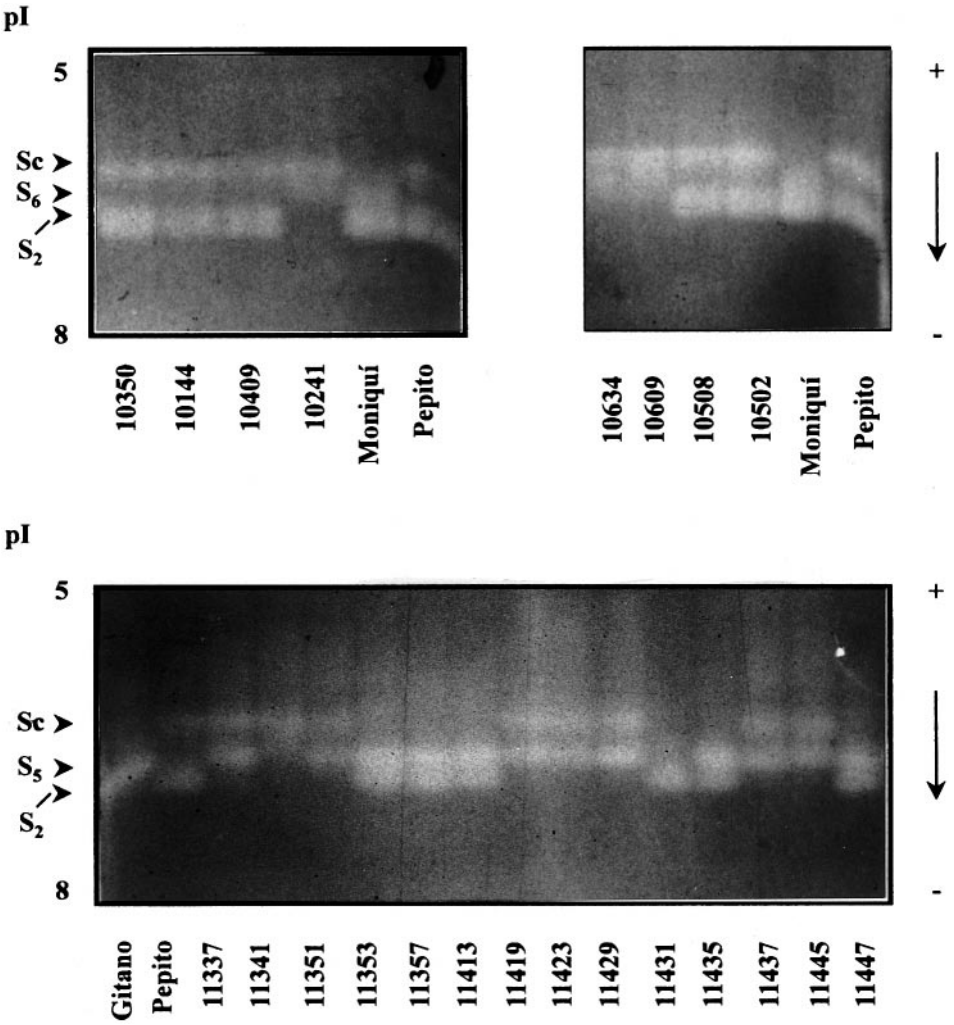

Fig. 2 Zymogram of apricot stylar ribonucleases separated on NEpHGE gel containing 4% ampholine pH 5–8. **A** Seedlings from the cross Moniquí × Pepito segregated in only two different combinations of RNase bands (ScS_2 and ScS_6), as might be expected from a semi-compatible cross. **B** Segregation of RNases in seedlings from the cross Gitano × Pepito produced four different combinations of bands of RNase activity (ScS_5, $Sc\_$, S_2S_5 and $S_2\_$) that correlate with the four possible genotypes for self-compatibiltiy that this cross would yield

In self-incompatible species with a gametophytic system, self-compatible cultivars originate after mutations of *S*-alleles or introgression from self-compatible species. The sweet cherry Stella and the Japanese pear Osa-Nijisseiki are known to have mutated *S*-alleles. However, the mutations are very different; while the mutation in Osa-Nijisseiki affects stylar activity and produces a band of very low RNase activity, as compared to the wild type in Nijisseiki (Sassa et al. 1992), the mutated allele in Stella affects pollen activity and the related band seems to migrate and has the same RNase activity as the wild type (Boskovic and Tobutt 1996).

In almond, the self-compatibility of several cultivars from Puglia (Italy) has been attributed to introgression from the self-compatible species *Prunus webbii* that grows in that region (Godini 1979). Boskovic et al. (1997) did not find a clear relationship between a ribonuclease band and self-compatibility, and a band, apparently similar to that related to a self-compatible allele, occurs in some of the self-incompatible cultivars studied.

It will be interesting to examine RNase bands in other self-compatible apricot cultivars to detect possible polymorphism in the self-compatible alleles.

The detection of incompatibility genotypes via stylar ribonucleases offers a simple way of elucidating incompatibility relationships between commercially important apricot cultivars and of recording the segregation of incompatibility in a progeny and thence of mapping the incompatibility locus. The segregation of stylar ribonuclease isozymes in the almond cross Ferragnes × Tuono allowed the incompatibility locus to be placed on the almond linkage map (Ballester et al. 1998).

RNases may be used in breeding to produce a larger number of self-compatible seedlings from controlled crosses. Knowledge of the incompatibility alleles in self-compatible cultivars and selections allows crosses that should generate only self-compatible seedlings (Burgos et al. 1997b). Thus, Pepito should yield exclusively self-compatible seedlings if crossed with cultivars in which one of the alleles is S_2, i.e. Z107-20, Z107-43, Z102-24, etc. Z102-24 will also generate 50% homozygous self-compatible cultivars when crossed with Pepito. The study of RNases in other cross-incompatible apricot cultivars, like the Hungarian óriás apricots (Nyéki and Szabó 1995) or related cultivars within a well-known family tree, can extend the number of

known alleles so that alleles from cultivars with unknown genotypes could also be determined. Traditionally, such work on incompatibility has involved a laborious series of crosses followed by assesment of pollen tube growth or fruit set.

In future work the *S*-allele-associated RNases found in this study should be characterized in detail. Finding a molecular marker specific to the *Sc* allele (or alleles) would allow earlier determination of seedling phenotype, resulting in important savings in time and labour during the breeding process. If an additional technique to identify the different alleles at an early stage of plant growth could be developed (Janssens et al. 1995) the method would greatly improve RNase determinations, and consequently, apricot breeding.

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