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A stylar ribonuclease assay to detect self-compatible seedlings in almond progenies

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Abstract Six almond progenies, each the product of a cross between a self-compatible and a self-incompatible parent, were analysed for stylar ribonucleases. Proteins were extracted and separated using non-equilibrium pH gradient electrofocusing (NEPHGE), and the gels were stained for ribonuclease activity. Most seedlings showed either two principal bands, interpreted as corresponding to two incompatibility alleles, or a single band. The seedlings were also bagged in the field at flowering time to determine fruit set after selfing, and some were also examined for the growth of pollen-tubes in selfed styles using UV fluorescence microscopy. With very few exceptions, those seedlings showing single-banded zymograms were found to be self-compatible according to field and microscope studies, and those with two bands were found to be self-incompatible. We conclude that the allele for self-compatibility in almond does not code for ribonuclease activity and that the ribonuclease isoenzyme assay is a convenient technique for predicting self-compatibility in segregating progenies. A novel band in two derivatives of 'Ferrastar' was ascribed to a new incompatibility allele, $S_{10.}$

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Introduction

Most cultivars of almond (*Prunus dulcis*) are more or less self-incompatible, needing pollination by a compatible cultivar to set an economic crop. Self-incompatibility and cross incompatibility in almond were attributed by Gagnard (1954) to a multi-allelic gametophytic locus, *S*, and supporting inheritance data have since come from other workers, e.g. Crossa Raynaud and Grasselly (1985).

Some self-compatible cultivars, able to crop in the absence of pollinators, have been identified. This desirable characteristic was attributed to the allele S_f (Grasselly and Olivier 1976), and Socias i Company and Felipe (1977) demonstrated the inheritance of this character by some seedlings of a self-compatible cultivar. Breeding for self-compatibility in almond was reviewed at length by Socias i Company (1990), and significant publications since then include a research paper on the inheritance of self-compatibility by Dicenta and Garcia (1993) and a recent review by Godini (1996). Self-compatibility is an objective of the almond breeding programmes at INRA Avignon, France, IRTA Mas Bové, Spain, and CEBAS Murcia, Spain. Self-compatibility, or rather self-fertility, is determined generally by bagging unopened flowers in the field and noting subsequent set or by monitoring pollen-tube growth in selfed styles by fluorescence microscopy.

Recently, extending work developed in cherry at HRI-East Malling (Bošković and Tobutt 1996), we have used non-equilibrium pH gradient electrofocusing (NEPHGE) and appropriate staining to reveal stylar ribonuclease bands in almond that correspond to the seven incompatibility alleles identified in almond cultivars and to two putative new alleles (Bošković et al. 1997). For the self-compatible cultivars, a band corresponding to one or other of the *S* alleles was observed, but evidence

for a second band that corresponded to S_f was equivocal. A band with a pI value of slightly less than 7.3 was seen in the self-compatible cultivars 'Falsa Barese', 'Filippo Ceo', 'Genco' and 'Tuono' and in five self-compatible cultivars or selections derived from 'Tuono'. However, self-incompatible seedlings of 'Tuono' were not studied for comparison; moreover, an apparently similar band was seen in some self-incompatible cultivars.

In addition, Tao et al. (1997) analysed almond stylar proteins by electrophoresis and staining for ribonuclease activity and detected bands corresponding to two of the known incompatibility alleles.

In the investigation presented here we extended our work to several progenies derived from 'Tuono' or 'Genco' that segregate for self-compatibility and scored these in the field. With some seedlings we also scored for pollen-tube growth. Our objectives were to clarify the ribonuclease phenotype corresponding to self-compatibility and to determine how the ribonuclease assay may be used to identify self-compatible seedlings.

Materials and Methods

Plant material

Six almond progenies were studied, three each from the breeding programmes of INRA Avignon and IRTA Mas Bové. One parent of each progeny was self-compatible. The crosses and the numbers of seedlings per progeny are given in Table 1. At the time of testing, seedlings were growing in the selection plots and were at least four years old.

Methods

Self-compatibility of the seedlings was assessed (1) by analysing stylar ribonuclease bands revealed by NEPHGE, (2) by measuring fruit set in the field after bagging unopened flowers and (3), for some seedlings of the three Avignon progenies, by observing pollen-tube growth by fluorescence microcopy after hand selfing. For some seedlings, tests by one or more methods were repeated. Seedlings not giving consistent results were rechecked. Seedlings setting very few fruit when open pollinated were considered as sterile and were not included in the analyses.

RNase analysis

For each seedling of the IRTA progenies, styles were collected from flowers at the balloon stage, wrapped in aluminium foil, maintained at –20° C and later sent on dry ice to HRI-East Malling. For each seedling of the INRA families, shoots with flower buds were mailed to HRI-East Malling where the styles were collected from the flowers at the balloon stage or when just opening. Styles of parent cultivars were also collected. The extraction of stylar proteins followed procedures described by Bošković and Tobutt (1996) and Bošković et al. (1997), as did the electrophoresis, NEPHGE, with some modifications. For progenies INRA 236, IRTA 1 and IRTA 8, the gels contained 4% Pharmalyte of pH 3–10 and 1.2% Pharmalyte of pH 6.7–7.7, and the runs were for 1 h at 150 V, 1 h at 300 V and 3 h at 400 V, which is the standard method of Bošković et al. (1997). However, for progenies INRA D263, INRA D251 and IRTA 7, the gels contained 4% Pharmalyte pH 5–8, and the runs were for 1 h at 130 V, 2 h at 260 V and 2 h at 350 V. Staining the gels for ribonuclease activity was in accord with the method described by Bošković and Tobutt (1996) and Bošković et al. (1997). The interpretative diagrams in Figs 1–4 show as solid lines the main bands we regard as being associated with incompatibility alleles. The bands represented by dotted lines appear to be either secondary bands associated with *S* alleles or other stylar ribonucleases not associated with incompatibility alleles. The pI value of the band considered to correspond to a new allele was established by reference to markers from the Pharmacia high pI calibration kit, pH 5–10.5, run under isoelectric focusing (IEF) conditions, i.e. on gels incorporating 4% Pharmalyte pH 3–10 and 1.2% Pharmalyte pH 6.7–7.7, run for 1 h at 150 V, 1 h at 300 V and 4 h at 450 V.

Fruit set

For the self-pollination test in the field, at least 100 closed flower buds per seedling were bagged using paper (IRTA) or mesh (INRA) to exclude bees and the remaining buds on those branches were removed. Bags were removed 2 weeks after bagging, and the percentage fruit set was recorded 60 days later. This test was often

Table 1 Almond progenies analysed for segregation of stylar ribonucleases and self-compatibility

Progeny no.	Parentage	Genotype	Number of seedlings
INRA			
D263	R1080[c] ([Ardechoise × IXL[b]] × Ferrastar) × Lauranne[a]	$S_1S_{10} \times S_3S_f$	66[d]
D236	R1089[c] (Ferragnes[a] × Ferraduel[a]) × R987[a] (Tardy Nonpareil[b] × Tuono[b])	$S_1S_4 \times S_8S_f$	33
D251	R987[a] (Tardy Nonpareil[b] × Tuono[b]) × R1051[c] (Ferrastar × Tardy Nonpareil[b])	$S_8S_f \times S_7S_{10}$	31
IRTA			
1	Lauranne[a] × Desmayo Largueta[b]	$S_3S_f \times S_1S_5$	38[e]
7	Genco[b] × Masbovera[a]	$S_1S_f \times S_1S_9$	42
8	Glorieta[a] × Lauranne[a]	$S_1S_5 \times S_3S_f$	39

[a] Parentage and *S* genotype given in Table 1 of Bošković et al. (1997)
[b] *S* genotype given in Table 1 of Bošković et al. (1997)
[c] *S* genotype not published previously
[d] RNase analysis indicated 1 seedling was from outcrossing
[e] RNase analysis indicated 7 seedlings were 'Lauranne' selfed

repeated; year to year variation could be great, and the figures given in the tables are the maximum values recorded, rather than the means. Seedlings with more than 4% fruit set were considered to be self-compatible.

Pollen-tube growth

With some seedlings of the three Avignon families, branches with at least 10 flower buds at the balloon stage were collected, brought into the laboratory and maintained in jars of water at about 20°C. When the flowers were open and the anthers were dehiscing, the stigmas were pollinated with a brush to transfer the pollen. Seventy-two hours after pollination, the pistils were fixed for histological examination as described by Dicenta and Garcia (1993) and sent to CEBAS Murcia. They were stored at 4°C until pollen-tube growth in the pistil was observed histologically according to procedures described by Dicenta and Garcia (1993). The position at which the pollen-tube growth was arrested in most of the pistils examined was scored as: 1, stigma; 2, upper style; 3, lower style; 4, ovary. Seedlings in which pollen-tubes reached the ovary in most styles were considered to be self-compatible.

Test crosses

To test some of our predictions of incompatibility genotypes in the French progenies, we made several test crosses at Avignon in 1997. In D263, 8 selections were pollinated by 'Ferragnes', known to be S_1S_3: these were seedlings 9, 14, 19, 55, 64 and 84, which showed ribonuclease bands corresponding to S_1S_3, and seedlings 1 and 6, which appeared as S_1S_f, with 30 flowers per cross. In D236, five crosses were made involving 6 selections, all of which showed the stylar ribonucleases corresponding to S_1S_8; seedlings 10, 17, 29, 39 and 59 were each pollinated by seeding 19, with about 40 flowers pollinated per cross. In D251, 4 selections appearing as S_7S_8, namely seedlings 5, 8, 15 and 27, were pollinated by 'Nonpareil', which is known to be S_7S_8 (Bošković et al. 1997), with 30 flowers per cross. Fruit set was recorded after six weeks.

Results

INRA D263 = R1080 × 'Lauranne'

An annotated zymogram for the parents and 8 seedlings is given in Fig. 1 and the ribonuclease, fruit set and pollen-tube growth data are shown in Table 2.

The zymogram of R1080 shows the band corresponding to the allele S_1 and a doublet, which we interpreted as a main band with a secondary product, which we attribute to S_{10}, an allele not found previously. The pI value of this band was determined as 8.60. The self-compatible parent 'Lauranne', reported to be S_3S_f, shows the expected band corresponding to the allele S_3 but, in place of the band that may or may not correspond to S_f, two bands close together; the separating conditions used for this progeny differed from those reported previously (Bošković et al. 1997), which showed a single band that might correspond to S_f.

The seedling zymograms were interpreted as comprising four different patterns. Some had two bands, corresponding to S_1 and S_3, or to S_3 and S_{10}, and some had only a single band, corresponding to either to S_1 or S_{10}. In addition, most had one or other of the two bands that may or may not correspond to S_f. These two bands were clearly non-allelic with S_3 and, as reported later, neither

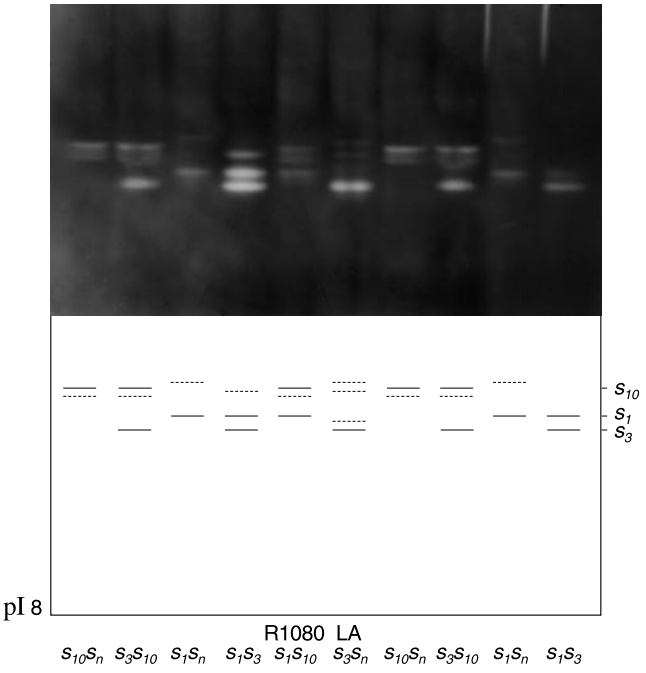

Fig 1 Segregation of stylar ribonucleases in almond family INRA D263, R1080 (interpreted as S_1S_{10}) × Lauranne' (S_3S_f), run under NEPHGE. Seedling genotypes are S_1S_3, S_3S_{10}, S_1S_{11}, and $S_{10}S_{11}$, where the null allele S_n indicates S_f; the latter two classes are self-compatible

correlated with self-compatibility; therefore they are not considered to correspond to alleles of the *S* locus. (In the remaining five progenies we will not refer to them). For convenience we will refer to these as "non-*S*" bands. The segregation observed was 17 S_1S_3, 10 S_3S_{10}, 17 S_1S_n and 21 $S_{10}S_n$, where S_n indicates the absence of a second band. This segregation corresponds to a 1:1:1:1 segregation ($\chi^2 = 3.86$, 3 df).

Of the 27 seedlings scored as S_1S_3 or S_3S_{10}, all set less than 4% fruit when selfed by bagging in the field whether or not they showed one or other of the two non-*S* bands; indeed they failed to set at all. All 17 of these seedlings that were tested for pollen-tube growth appeared to be self-incompatible. Of the 38 seedlings scored as S_1S_n or $S_{10}S_n$ all set at least 4% fruit after selfing, again irrespective of which, if any, of the non-*S* bands they showed. All 25 of these seedlings that were tested for pollen-tube growth appeared to be self-compatible.

Seedling 96 was scored as having the S_1 band and another band probably corresponding to an unknown *S* allele, indicated as S_x, presumably as a result of out-crossing. This set just 1% fruit after selfing.

The test crosses of 6 seedlings appearing to have the genotype S_1S_3 with pollen of 'Ferragnes', known to be S_1S_3, failed and thus confirmed the seedling genotypes. The test crosses of seedlings 1 and 6, which appeared to be S_1S_f, with 'Ferragnes' gave 6 and 13 fruits respectively from 30 pollinated flowers, confirming that the seedlings were not S_1S_3.

Table 2 Progeny INRA D263 = R1080 (S_1S_{10}) x 'Lauranne' (S_3S_f) – seedling data for stylar ribonucleases and self-compatibility

Seedling identity	Stylar ribonucleases alleles: + = S_1S_f or $S_{10}S_f$		Percentage fruit set after selfing: + = ≥ 4%		Pollen-tube growth after selfing: + = class 4, reaching ovary	
1	S_1S_f	+	5.0	+		
3	$S_{10}S_f$	+	12.0	+	4	+
5	S_3S_{10}	–	0.0	–	2	–
6	S_1S_f	+	12.0	+	4	+
7	$S_{10}S_f$	+	17.2	+	4	+
8	$S_{10}S_f$	+	15.0	+	4	+
9	S_1S_3	–	0.0	–	1	–
11	$S_{10}S_f$	+	35.0	+	4	+
12	S_3S_{10}	–	0.0	–	2	–
14	S_1S_3	–	0.0	–	2	–
16	S_1S_3	–	0.0	–		
17	$S_{10}S_f$	+	16.0	+	4	+
18	S_1S_f	+	30.0	+		
19	S_1S_3	–	0.0	–	2	–
21	$S_{10}S_f$	+	35.0	+		
22	S_3S_{10}	–	0.0	–	1	–
23	S_3S_{10}	–	0.0	–	1	–
24	S_1S_f	+	16.0	+		
25	$S_{10}S_f$	+	21.0	+	4	+
26	S_1S_f	+	35.0	+		
27	$S_{10}S_f$	+	5.0	+	4	+
28	$S_{10}S_f$	+	31.4	+		
29	S_1S_f	+	16.0	+	4	+
31	S_1S_3	–	0.0	–		
34	S_1S_f	+	14.0	+	4	+
35	S_1S_f	+	22.9	+	4	+
36	S_3S_{10}	–	0.0	–	1	–
37	$S_{10}S_f$	+	8.0	+		
38	S_1S_f	+	40.0	+		
39	S_1S_f	+	30.0	+		
40	S_1S_3	–	0.0	–		
41	$S_{10}S_f$	+	41.0	+		
42	$S_{10}S_f$	+	22.0	+	4	+
43	S_1S_f	+	12.0	+	4	+
44	S_1S_3	–	0.0	–		
46	S_1S_f	+	24.0	+	4	+
47	$S_{10}S_f$	+	34.0	+	4	+
48	$S_{10}S_f$	+	13.0	+	4	+
49	S_1S_3	–	0.0	–	2	–
50	S_1S_3	–	0.0	–		
54	S_1S_3	–	0.0	–	2	–
55	S_1S_3	–	0.0	–	2	–
57	S_3S_{10}	–	0.0	–	1	–
58	S_1S_f	+	5.0	+	4	+
59	S_1S_3	–	0.0	–	1	–
60	S_3S_{10}	–	0.0	–		
62	$S_{10}S_f$	+	20.0	+		
64	S_1S_3	–	0.0	–	1	–
65	$S_{10}S_f$	+	26.7	+		
66	S_3S_{10}	–	0.0	–	2	–
68	$S_{10}S_f$	+	14.0	+	4	+
69	$S_{10}S_f$	+	29.2	+		
70	$S_{10}S_f$	+	34.0	+	4	+
72	S_1S_f	+	19.0	+	4	+
73	$S_{10}S_f$	+	30.0	+	4	+
75	S_1S_3	–	0.0	–	2	–
76	S_3S_{10}	–	0.0	–	1	–
79	S_3S_{10}	–	0.0	–		
80	S_1S_3	–	0.0	–		
81	S_1S_f	+	16.4	+	4	+
84	S_1S_3	–	0.0	–		
85	$S_{10}S_f$	+	12.0	+	4	+
88	S_1S_f	+	22.5	+	4	+
89	S_1S_3	–	0.0	–		
92	S_1S_f	+	15.0	+	4	+
96	S_1S_x[a]	–	1.0	–		

[a] This seedling is presumably from open pollination

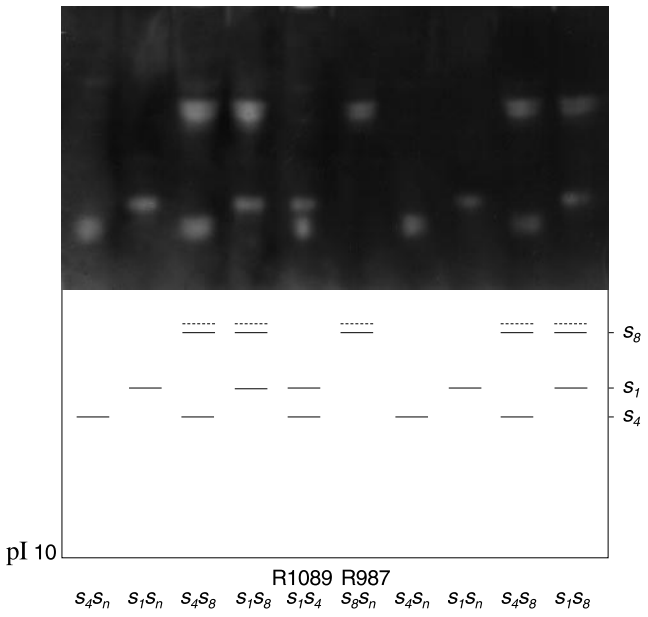

Fig 2 Segregation of stylar ribonucleases in almond family INRA D236, R1089 (interpreted as S_1S_4) × R987 (S_8S_f), run under NEPHGE. Seedling genotypes are S_1S_8, S_4S_8, S_1S_{11} and S_4S_n, where the null allele S_n indicates S_f; the latter two classes are self-compatible

INRA D236 = R1089 × R987

An annotated zymogram for the parents and for 4 seedlings repeated twice is given in Fig. 2 and the data for ribonucleases, fruit set and pollen-tube growth are given in Table 3.

The zymogram of R1089 shows bands corresponding to S_1 and S_4. This genotype is consistent with its parentage of 'Ferragnes', S_1S_3, × 'Ferraduel', S_1S_4. The zymogram for the self-compatible parent R987 reported as S_8S_f shows the band expected for S_8 and the associated secondary band.

The seedling zymograms show four different patterns of bands. Some have a band and a doublet, corresponding to S_1 and S_8, or to S_4 and S_8, and some have single bands corresponding to S_1 and to S_4. The segregation was 10 S_1S_8, 1 S_4S_8, 15 S_1S_n and 7 S_4S_n. This approximates very poorly to a 1:1:1:1 segregation (χ^2 = 12.45, 3 df); in this progeny the allele S_8 has been inherited less frequently than S_f, and S_4 less frequently than S_1. In fact, before our experiments, poor cropping seedlings of this progeny had been discarded, and this may have favoured the self-compatible seedlings.

All 11 seedlings scored as S_1S_8 or S_4S_8 set less than 4% fruit after bagging, and the 3 of these seedlings tested for pollen-tube growth appeared to be self-incompati-

Table 3 Progeny INRA D236 = R 1089 (S_1S_4) × R987 (S_8S_f) – seedling data for stylar ribonucleases and self compatibility

Seedling identity	Stylar ribonucleases alleles: + = S_1S_f or S_4S_f		Percentage fruit set after selfing: + = ≥ 4%		Pollen-tube growth after selfing: + = class 4, reaching ovary	
2	S_1S_f	+	4.6	+		
5	S_4S_8	–	0.0	–	1	–
7	S_1S_f	+	12.7	+		
10	S_1S_8	–	0.0	–		
11	S_4S_f	+	11.1	+		
13	S_1S_8	–	0.7	–		
14	S_4S_f	+	8.0	+	4	+
17	S_1S_8	–	0.0	–	2	–
19	S_1S_8	–	0.7	–		
20	S_1S_f	+	18.3	+		
21	S_1S_8	–	0.0	–		
22	S_1S_f	+	32.5	+		
23	S_1S_f	+	4.6	+		
26	S_4S_f	+	13.3	+		
27	S_1S_8	–	0.0	–	3	–
28	S_1S_f	+	7.0	+		
29	S_1S_8	–	0.0	–	1	–
32	S_4S_f	+	27.0	+		
33	S_1S_f	+	4.7	+		
37	S_1S_f	+	6.0	+		
39	S_1S_8	–	0.8	–	1	–
40	S_4S_f	+	7.0	+		
41	S_1S_f	+	4.4	+		
42	S_1S_f	+	7.7	+		
44	S_1S_f	+	10.0	+	4	+
49	S_4S_f	+	4.6	+		
51	S_1S_f	+	29.0	+		
52	S_1S_f	+	16.0	+	4	+
54	S_1S_f	+	9.2	+		
56	S_4S_f	+	9.3	+		
58	S_1S_8	–	0.0	–		
59	S_1S_8	–	0.0	–	1	–
60	S_1S_f	+	15.7	+		

Table 4 Progeny INRA D251 = R987 (S_8S_f) × R1051 (S_7S_{10}) – seedling data for stylar ribonucleases and self-compatibility

Seedling identity	Stylar ribonucleases alleles: + = S_7S_f or $S_{10}S_f$		Percentage fruit set after selfing: + = ≥ 4%		Pollen-tube growth after selfing: + = class 4 reaching ovary	
3	S_7S_f	+	2.0	–	1	–
4	S_7S_8	–	0.0	–	1	–
5	S_7S_8	–	0.0	–	4	+
6	S_7S_8	–	0.0	–	3	–
7	S_8S_{10}	–	0.0	–		
8	S_7S_8	–	0.0	–	1	–
9	$S_{10}S_f$	+	5.7	+		
10	S_8S_{10}	–	0.0	–	2	–
11	S_8S_{10}	–	0.0	–	1	–
12	$S_{10}S_f$	+	4.0	+	4	+
13	S_8S_{10}	–	0.0	–	1	–
14	S_7S_f	+	5.0	+	4	+
15	S_7S_8	–	0.0	–	1	–
16	$S_{10}S_f$	+	5.5	+		
17	S_7S_f	+	9.0	+		
18	S_7S_f	+	17.8	+	4	+
19	$S_{10}S_f$	+	10.0	+	4	+
20	S_7S_f	+	14.0	+	4	+
21	S_7S_f	+	4.0	+	4	+
22	S_7S_f	+	11.4	+		
23	S_7S_f	+	8.5	+	4	+
25	S_7S_f	+	25.0	+		
26	S_7S_f	+	12.0	+	4	+
27	S_7S_8	–	0.6	–	1	–
28	$S_{10}S_f$	+	12.9	+		
30	S_7S_f	+	7.0	+		
31	S_7S_f	+	4.0	+		
32	S_7S_f	+	12.5	+	4	+
33	S_8S_{10}	–	0.0	–	2	–
35	S_7S_f	+	4.0	+		
36	S_8S_{10}	–	0.0	–		

ble. All 22 scored as S_1S_n or S_4S_n set at least 4% fruit and the 6 of these tested for pollen-tube growth were scored as self-compatible.

The test crosses among six seedlings appearing to have the genotype S_1S_8 failed, indicating that these 6 seedlings all had the same genotype.

INRA D251 = R987 × R1051

The data for ribonucleases, fruit set and pollen-tube growth are given in Table 4.

R987, the self-compatible parent, reported to be S_8S_f, showed the double band expected for the allele S_8, as described in the previous section. R1051 showed the band for S_7, presumably derived from 'Tardy Nonpareil', and the band now ascribed to S_{10}, presumably derived from 'Ferrastar'.

The seedling patterns were of four different types, corresponding to the genotypes S_7S_8, S_8S_{10}, S_7S_n and $S_{10}S_n$ (the band corresponding to S_8 generally appeared as a doublet), with 6, 6, 14 and 5 seedlings respectively. This approximates to a 1:1:1:1 segregation ($\chi^2 = 6.81$, 3 df).

All 12 seedlings scored as S_7S_8 or S_8S_{10} set less than 4% fruit after bagging and, of the 10 of these tested for pollen-tube growth, all but one, seedling 5, appeared to be self-incompatible. Of the 19 seedlings scored as S_7S_n or $S_{10}S_n$, 18 set at least 4% fruit, the exception being seedling 3 which set 2% fruit. Of the 10 seedlings scored as S_7S_n or $S_{10}S_n$ and tested for pollen-tube growth, all but one, seedling 3 (again), appeared to be self-compatible. The test crosses of 5 seedlings appearing to have the genotype S_7S_8 with 'Nonpareil', known to be S_7S_8 failed, and thus confirmed the seedling genotype.

IRTA 1 = 'Lauranne' × 'Desmayo Largueta'

The data for ribonucleases and fruit set are given in Table 5.

'Lauranne', the self-compatible parent, reported to be S_3S_f, showed the S_3 band. 'Desmayo Largueta', reported to be S_1S_5, showed the bands expected for S_1 and for S_5.

The seedling patterns were of five different types; four corresponded to S_1S_3, S_3S_5, S_1S_n and S_5S_n, and a fifth, showing no principal bands, we interpreted as S_nS_n. For the first four types, the seedling numbers were 11, 6, 9 and 5, respectively. This segregation approximates to 1:1:1:1 ($\chi^2 = 2.94$, 3 df).

The fifth phenotype, with no bands, that we ascribe to S_nS_n may have resulted from the selfing of the 'Lauranne' parent after imperfect emasculation or by subsequent contamination of the unbagged flowers. It was seen in seedlings 2, 13, 21, 49, 265, 292 and 569. The other

Table 5 Progeny IRTA 1 'Lauranne' (S_3S_f) x 'Desmayo Largueta' (S_1S_5) – seedling data for stylar ribonucleases and self-compatibility

Seedling identity	Stylar ribonuclease alleles: + = S_1S_f, S_5S_f or S_fS_f		Percentage fruit set after selfing: + = ≥ 4%	
2	S_fS_f[a]	+	18.0	+
4	S_1S_3	–	0.0	–
6	S_1S_3	–	0.0	–
7	S_5S_f	+	12.3	+
11	S_3S_5	–	0.0	–
13	S_fS_f[a]	+	14.7	+
14	S_1S_3	–	0.0	–
16	S_1S_f	+	13.9	+
17	S_1S_f	+	22.4	+
21	S_fS_f[a]	+	10.0	+
22	S_1S_f	+	6.1	+
26	S_1S_3	–	0.0	–
27	S_1S_3	–	0.0	–
30	S_5S_f	+	28.4	+
33	S_5S_f	+	4.1	+
38	S_1S_3	–	0.7	–
39	S_3S_5	–	0.0	–
49	S_fS_f[a]	+	37.3	+
62	S_5S_f	+	10.9	+
65	S_1S_f	+	23.9	+
68	S_1S_f	+	8.9	+
81	S_5S_f	+	21.2	+
114	S_1S_f	+	21.9	+
116	S_1S_3	–	0.0	–
128	S_1S_3	–	0.0	–
151	S_3S_5	–	0.0	–
156	S_1S_f	+	10.0	+
218	S_1S_3	–	0.0	–
235	S_1S_3	–	0.0	–
236	S_3S_5	–	0.0	–
265	S_fS_f[a]	+	30.1	+
292	S_fS_f[a]	+	5.5	+
314	S_1S_f	+	72.2	+
333	S_1S_3	–	0.0	–
349	S_3S_5	–	0.0	–
569	S_fS_f[a]	+	46.0	+
578	S_3S_5	–	0.0	–
782	S_1S_f	+	17.5	+

[a] These 7 seedlings are presumably 'Lauranne' selfs

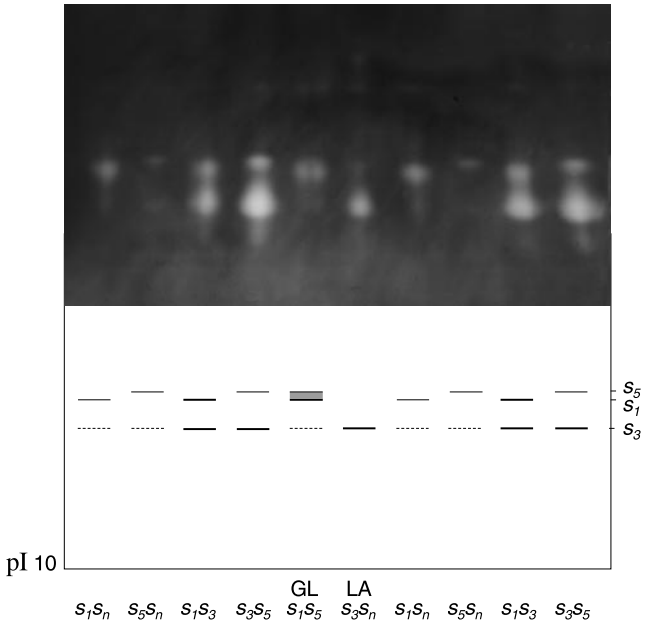

Fig 3 Segregation of stylar ribonucleases in almond family IRTA 8, 'Glorieta' (S_1S_5) × 'Lauranne' (S_3S_f), run under NEPHGE. Seedling genotypes are S_1S_3, S_3S_5, S_1S_n and S_5S_n, where the null allele S_n indicates S_f ; the latter two classes are self-compatible.

class that might result from such selfing, S_3S_n, was not seen.

All 17 seedlings scored as S_1S_3 or S_3S_5 set less than 4% fruit after bagging. The 14 seedlings scored as S_1S_n or S_5S_n set at least 4% fruit after bagging, as did the 7 seedlings scored as S_nS_n.

IRTA 8 = 'Glorieta' × 'Lauranne'

An annotated zymogram for the parents and for 8 seedlings is given in Fig. 3, and the data for ribonucleases and fruit set are given in Table 6. The zymogram for 'Glorieta', reported to be S_1S_5, shows the bands expected for S_1 and S_5 to be rather close together. The zymogram for the self-compatible parent, 'Lauranne', reported to be S_3S_f, shows the band expected for S_3. The seedling

Table 6 Progeny IRTA 8 'Glorieta' (S_1S_5) x 'Lauranne' (S_3S_f) – seedling data for stylar ribonucleases and self-compatibility

Seedling identity	Stylar ribonucleases alleles: + = S_1S_f or S_5S_f		Percentage fruit set after selfing: + = ≥ 4%	
51	S_5S_f	+	16.6	+
53	S_3S_5	–	0.0	–
54	S_5S_f	+	2.3	–
55	S_1S_3	–	0.8	–
67	S_3S_5	–	0.0	–
77	S_3S_5	–	0.0	–
140	S_3S_5	–	0.0	–
167	S_1S_f	+	18.2	+
170	S_3S_5	–	0.0	–
192	S_1S_f	+	6.5	+
214	S_1S_3	–	0.0	–
216	S_1S_f	+	17.6	+
226	S_5S_f	+	7.0	+
253	S_1S_f	+	10.5	+
262	S_1S_f	+	5.7	+
273	S_3S_5	–	0.0	–
284	S_1S_f	+	20.6	+
286	S_5S_f	+	13.7	+
306	S_1S_3	–	0.0	–
495	S_5S_f	+	13.8	+
553	S_5S_f	+	12.6	+
577	S_5S_f	+	16.2	+
649	S_3S_5	–	0.0	–
650	S_1S_3	–	0.0	–
653	S_1S_3	–	0.0	–
656	S_5S_f	+	10.9	+
658	S_1S_f	+	23.0	+
664	S_5S_f	+	5.2	+
665	S_5S_f	+	6.9	+
666	S_5S_f	+	8.0	+
668	S_1S_f	+	15.5	+
669	S_1S_3	–	0.0	–
670	S_1S_3	–	0.0	–
696	S_1S_f	+	26.1	+
699	S_1S_f	+	5.1	+
804	S_5S_f	+	29.6	+
807	S_5S_f	+	17.4	+
849	S_5S_f	+	19.2	+
890	S_5S_f	+	37.9	+
1021	S_5S_f	+	20.5	+
1027	S_3S_5	–	0.0	–
1031	S_3S_5	–	0.0	–

zymograms were of four types, corresponding to S_1S_3, S_3S_5, S_1S_n and S_5S_n, with the numbers of seedlings in each class being 7, 9, 10 and 16, respectively. This segregation approximates to 1:1:1:1 (χ^2 = 4.29, 3 df).

The 16 seedlings scored as S_1S_3 or S_3S_5 set less than 4% fruit after bagging, whereas 25 of the 26 seedlings scored as S_1S_n or S_5S_n set at least 4% fruit, the exception being seedling 54, which set 2.3% fruit.

IRTA 7 = 'Genco' × 'Masbovera'

An annotated zymogram for the parents and for 8 seedlings is given in Fig. 4, and the data for ribonucleases and fruit set are given in Table 7.

The zymogram for the self-compatible parent 'Genco', reported to be S_1S_f, shows the expected S_1 band and an additional more anodal band. That for 'Masbovera', reported to be S_1S_9, shows the bands expected for S_1 and S_9. As the parents have S_1 in common, the cross is semi-compatible.

The seedlings thus show two classes, S_1S_9 and S_9S_n, with 18 and 21 seedlings respectively. This segregation approximates to 1:1 (χ^2 = 0.23, 1 df). The additional band in 'Genco' is not seen in the seedlings.

The 18 seedlings scored as S_1S_9 set less than 4% fruit after bagging. Of the 21 seedlings scored as S_9S_n, 20 set at least 4% fruit, the exception being seedling 638, which set 2.6% fruit.

Discussion

In the work reported here, 249 seedlings from progenies segregating for self-compatibility were analysed for stylar ribonucleases and also for fruit set after bagging and, in some cases, for pollen-tube growth after selfing, the two customary ways of assessing self-compatibility. The

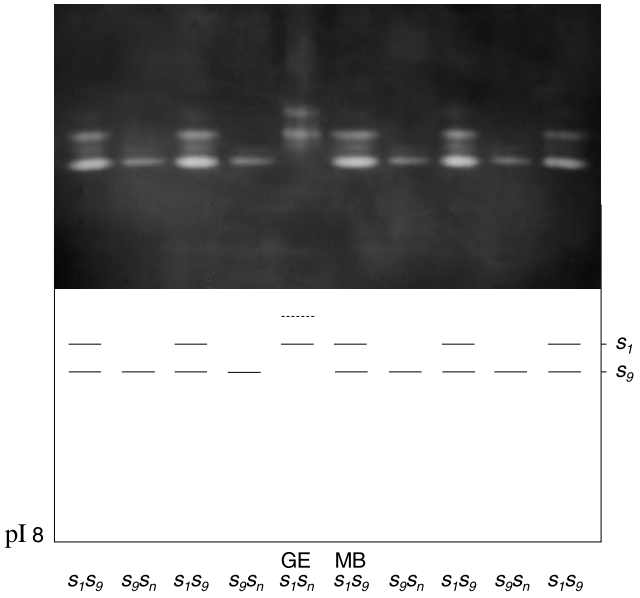

Fig 4 Segregation of stylar ribonucleases in almond family IRTA 7, 'Genco' (S_1S_f) × 'Masbovera' (S_1S_9), run under NEPHGE. Seedling genotypes are S_1S_9 amd S_9S_n, where the null allele S_n indicates S_f; the latter class is self-compatible. The segregation for just two classes is consistent with the parents sharing the S_1 allele.

ribonuclease assay demonstrated clearly that the five fully compatible crosses segregated for four phenotypes, whereas the semi-compatible cross segregated for only two. The 102 seedlings that showed bands corresponding to two *S* alleles set less than 4% fruit after bagging, and only 8 set any fruit and then no more than 1%; of the 30 tested for pollen-tube growth, 29 appeared to be self-incompatible. Of the 147 seedlings that showed bands corresponding to only one *S* allele or to none at all, 144 set at least 4% fruit, with the remaining three setting at least 2%; of the 41 tested for pollen-tube growth, 40 appeared to be self-compatible. Thus, there was excellent consistency of the ribonuclease genotype with the percentage

Table 7 Progeny IRTA 7 'Genco' (S_1S_f) x 'Masbovera' (S_1S_9) – seedling data for stylar ribonucleases and self-compatibility

Seedling identity	Stylar ribonucleases alleles: + = S_9S_f		Percentages fruit set after selfing: + = ≥ 4%	
85	S_9S_f	+	18.5	+
132	S_1S_9	–	0.0	–
155	S_1S_9	–	0.0	–
164	S_1S_9	–	0.0	–
165	S_9S_f	+	7.2	+
202	S_9S_f	+	8.7	+
208	S_9S_f	+	8.0	+
221	S_9S_f	+	23.3	+
255	S_1S_9	–	0.0	–
258	S_1S_9	–	0.0	–
285	S_1S_9	–	0.0	–
329	S_1S_9	–	0.0	–
347	S_9S_f	+	36.8	+
348	S_9S_f	+	8.9	+
477	S_9S_f	+	7.3	+
593	S_1S_9	–	0.0	–
595	S_9S_f	+	16.6	+
599	S_9S_f	+	16.2	+
600	S_1S_9	–	0.0	–
623	S_9S_f	+	13.7	+
629	S_9S_f	+	24.4	+
632	S_9S_f	+	16.9	+
636	S_1S_9	–	0.0	–
637	S_9S_f	+	39.1	+
638	S_9S_f	+	2.6	–
643	S_9S_f	+	32.3	+
645	S_9S_f	+	11.4	+
672	S_1S_9	–	0.7	–
676	S_1S_9	–	0.7	–
681	S_1S_9	–	0.0	–
729	S_9S_f	+	25.0	+
740	S_1S_9	–	0.0	–
785	S_1S_9	–	0.0	–
786	S_9S_f	+	19.0	+
791	S_9S_f	+	29.0	+
809	S_1S_9	–	0.0	–
853	S_9S_f	+	19.5	+
945	S_1S_9	–	0.0	–
974	S_1S_9	–	0.0	–

fruit set after selfing and with the growth of pollen-tubes after selfing.

This shows that self-compatibility in almond, attributed by Grasselly and Olivier (1976) to S_f, is associated with, and is detectable as, the absence of a stylar ribonuclease band that we ascribed earlier to S_n. Thus, the allele for self-compatibility in almond differs from that in cherry, S_4', which codes for a stylar ribonuclease (Bošković and Tobutt 1996). In Japanese pear, *Pyrus serotina*, Sassa et al. (1992) reported that the ribonuclease band corresponding to the S_4 allele in the self-compatible mutant 'Osa Nijisseiki' was much less intense than the S_4 band in the original 'Nijisseiki'. Later, Norioka et al. (1996) and Sassa et al. (1997) found that the S_4 ribonuclease gene transcript was not detectable in 'Osa Nijisseiki'. Furthermore, genomic DNA encoding S_4 ribonuclease could not be detected by Southern blotting, indicating a deletion in the stylar part of the S_4 allele as the cause of self-compatibility in this instance (Sassa et al., 1997). In the tomato species *Lycopersicon peruvianum*, Kowyama et al. (1994) showed that the self-compatibility allele S_c codes for a defective protein which has no ribonuclease activity. At present it is not known whether the inactive *S* allele, and thus self-compatibility, in almond is due to a deletion of the corresponding genomic fragment or to production of a defective protein. It would be instructive to stain gels of stylar extracts from the segregating progenies for protein rather than ribonuclease activity to see if a protein band cosegregates with self-compatibility. Detection of such a band would indicate that 2D-PAGE separation could be used to isolate the protein for sequencing, which could clarify the nature of the S_f allele.

The apparent absence of a ribonuclease band in self-compatible seedlings suggests that the mechanism of self-compatibility in almond is as follows. When a cultivar with genotype S_1S_f is selfed, the S_1 and S_f pollen-tubes encounter only S_1 ribonuclease in the style. The S_1 pollen is duly inactivated, but the S_f pollen-tube is not, and achieves fertilization.

Our results also show that the ribonuclease assay can be used to indicate with a high degree of success which seedlings in segregating progenies are self-compatible, i.e. those seedlings having just one band, derived from the self-incompatible parent. This assay could render unnecessary the routine bagging and pollination tests which are time-consuming during the busy season for almond breeders. In any case, year to year variation for these tests can be considerable, especially for the bagging test, so that poor set in one year alone may not be conclusive of self-incompatibility. Although flowering plants are needed for the analysis of stylar ribonucleases, seedlings just 2 or 3 years old may produce the 5 flowers sufficient for ribonuclease analysis. Thus, the assay can be carried out on relatively young seedlings as an early selection technique to discard those lacking S_f. The ribonuclease assay only indicates genetic self-compatibility and, to confirm that a promising selection is sufficiently self-fertile for commercial production, subsequent field tests would still be needed. In due course, DNA-based methods of predicting self-compatibility, for example using linked markers or allele-specific primers, may become available and, for breeders having access to DNA facilities, these would allow screening to be carried out with leaf material.

A few seedlings from the progeny IRTA 1, with the self-compatible cultivar 'Lauranne' as the female parent, appear to have resulted from selfing and had no bands at all; they were presumably S_fS_f. We know of no previous reports of almonds homozygous for S_f. If such homozygous types are used in crosses, all the seedlings should inherit S_f. Hitherto, the only way to produce progenies comprising solely self-compatible seedings was by making semi-compatible crosses of the kind $S_xS_y \times S_xS_f$ (Grasselly et al. 1985; Dicenta and Garcia 1993) as was pointed out many years ago in cherry (Williams and Brown 1960).

The source of self-compatibility, of S_f, may be the related species *P. webbii* which grows wild in the regions of Southern Italy from which 'Genco' and some others originate (Godini 1979). It would be interesting to analyse accessions of *P. webbii* from this region to see if self-compatibility in *P. webbii* is caused by the lack of active ribonuclease alleles.

Although this study has focused on self-compatibility, it has also detected a new self-incompatibility allele. On the basis of a novel stylar ribonuclease band in R1080 and R1051, we have proposed S_{10}. 'Ferrastar', which comes from a cross of 'Cristomorto' × 'Ardechoise', is a parent of both these selections and, when analysed for stylar ribonucleases, it was found to be S_2S_{10} (data not shown).

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