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TITLE:

Determination of Self- and Inter-(in)compatibility Relationships in Apricot Combining Hand-Pollination, Microscopy and Genetic Analyses

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KEYWORDS:

apricot, fluorescence microscopy, gametophytic self-incompatibility, pollen tube, pollination, *Prunus armeniaca, S*-alleles, *S*-genotype.

SUMMARY:

We present a methodology to establish the pollination requirements of apricot (*Prunus armeniaca* L.) cultivars combining the determination of self-(in)compatibility by fluorescence microscopy with the identification of the S-genotype by PCR analysis.

ABSTRACT:

Self-incompatibility in Rosaceae is determined by a Gametophytic Self-Incompatibility System (GSI) that is mainly controlled by the multiallelic locus *S.* In apricot, the determination of self- and inter-(in)compatibility relationships is increasingly important, since the release of an important number of new cultivars has resulted in the increase of cultivars with unknown pollination requirements. Here, we describe a methodology that combines the determination of self-(in)compatibility by hand-pollinations and microscopy with the identification of the *S*-genotype by PCR analysis. For self-(in)compatibility determination, flowers at balloon stage from each cultivar were collected in the field, hand-pollinated in the laboratory, fixed, and stained with aniline blue for the observation of pollen tube behavior under the fluorescence microscopy. For the establishment of incompatibility relationships between cultivars, DNA from each cultivar was extracted from young leaves and *S*-alleles were identified by PCR. This approach allows establishing incompatibility groups and elucidate incompatibility relationships between cultivars, which provides a valuable information to choose suitable pollinizers in the

design of new orchards and to select appropriate parents in breeding programs.

INTRODUCTION:

Self-incompatibility is a strategy of flowering plants to prevent self-pollination and promote outcrossing[1]. In Rosaceae, this mechanism is determined by a Gametophytic Self-Incompatibility System (GSI) that is mainly controlled by the multiallelic locus *S*[2]. In the style, the *RNase* gene encodes the *S*-stylar determinant, a RNase[3], while a F-box protein, which determines the *S*-pollen determinant, is codified by the *SFB* gene[4]. The self-incompatibility interaction takes place through the inhibition of pollen tube growth along the style preventing the fertilization of the ovule[5,6].

In apricot, a varietal renewal has taken place worldwide in the last two decades[7,8]. This introduction of an important number of new cultivars, from different public and private breeding programs, has resulted in the increase of apricot cultivars with unknown pollination requirements[8].

Different methodologies have been used to determine pollination requirements in apricot. In the field, self-(in)compatibility may be established by controlled pollinations in caged trees or in emasculated flowers and subsequently recording the percentage of fruit set[9–12]. In addition, controlled pollinations have been carried out in the laboratory by semi-in vivo culture of flowers and analysis of the pollen tube behavior under fluorescence microscopy[8,13–17]. Recently, molecular techniques, such as PCR analysis and sequencing, have allowed the characterization of incompatibility relationships based on the study of the *RNase* and *SFB* genes[18,19]. In apricot, thirty-three S-alleles have been reported (S_1 to S_{20}, S_{22} to S_{30}, S_{52}, S_{53}, S_v, S_x), including one allele related with self-compatibility (S_c)[12,18,20–24]. Up to now, 26 incompatibility groups have been stablished in this species according to the *S*-genotype[8,9,17,25–27]. Cultivars with the same *S*-alleles are inter-incompatible, whereas cultivars with at least one different *S*-allele and, consequently, allocated in different incompatible groups, are inter-compatible.

To define the pollination requirements of apricot cultivars, we describe a methodology that combines the determination of self-(in)compatibility by fluorescence microscopy with the identification of the *S*-genotype by PCR analysis in apricot cultivars. This approach allows establishing incompatibility groups and elucidate incompatibility relationships between cultivars.

PROTOCOL:

1. Self-(in)compatibility determination

1.1 Sample the flowers in the field. It is necessary to collect the flowers at balloon stage (**Figure 1A**), corresponding to stage 58 on the BBCH scale for apricot[28], to avoid unwanted previous pollination.

1.2. Self- and cross-pollinations in the laboratory

1.2.1. Remove the anthers of the flowers at balloon stage and place them on a piece of paper to dry at laboratory temperature.

1.2.2. After 24 h, sieve the pollen grains by using a fine mesh (0.26 mm) (**Figure 1B**).

1.2.3. Emasculate a group of 30 flowers at the same balloon stage for each self-pollination and cross-pollination and place the pistils on florist foam in water at laboratory temperature (**Figure 1C**).

1.2.4. Hand pollinate the pistils with the help of a paintbrush with pollen from flowers of the same cultivar 24 h after emasculation. In addition, pollinate another set of pistils of each cultivar with pollen from flowers of a compatible pollinizer as control (**Figure 1D**).

1.2.5. After 72 h, fix the pistils in a fixative solution of ethanol/acetic acid (3:1) for at least 24 h at 4 °C[29]. Then discard the fixative and add 75% ethanol ensuring that the samples are completely submerged in the solution. Samples can be conserved in this solution at 4 °C until use[8,17,30-32].

1.3. Evaluating pollen viability through in vitro pollen germination

1.3.1. To prepare the germination medium, weight 25 g of sucrose, 0.075 g of boric acid (H_3BO_3) and 0.075 g of calcium nitrate ($Ca(NO_3)_2$) [33].

1.3.2. Add the components of the medium in 250 mL of distilled water and dissolve completely.

1.3.3. Solidify the medium adding 2 g of agarose and mix by swirling.

1.3.4. Check the pH of the medium using a pH meter and adjust the value to 7.0 with NaOH or HCl solution.

1.3.5. Autoclave the mixture to sterilize the medium.

1.3.6. After autoclaving, cool down the medium and distribute it into Petri dishes in a sterile laminar flow hood.

1.3.7. Scatter the pollen grains of the same cultivars used for the controlled pollinations in the solidified pollen germination medium and observe them under the microscope after 24 h[6].

NOTE: To sterilize the laminar flow hood, clean the surface with 70% ethanol and switch on the UV lamp during 10 min.

1.3.7. Store the Petri dishes in a refrigerator at 4 °C until use.

1.4. Microscopy observations

1.4.1. Wash the pistils three times for 1 h with distilled water and leave them in 5% sodium sulphite at 4 °C. After 24 h, autoclave them at 1 kg/cm^2 during 10 min in sodium sulphite to soften the tissues[34].

1.4.2. Place the autoclaved pistils over a glass slide and, with the help of a scalpel, remove the trichomes around the ovary to get a better visualization of the pollen tubes. Then, squash the pistils with a cover glass.

1.4.3. Prepare 0.1% (v/v) aniline blue stain: mix 0.1 mL of aniline blue in 100 mL of 0.1 N potassium phosphate tribasic (K_3PO_4). Apply a drop of aniline blue over the preparations to stain callose depositions during pollen tube growth.

1.4.4. Observe the pollen tubes along the style by a microscope with UV epifluorescence using 340–380 bandpass and 425 longpass filters.

2. DNA extraction

2.1. Sample 2-3 leaves in the field in spring. It is recommended to sample the leaves at young stages since DNA obtained is of higher quality and lower levels of phenolic compounds compared to old leaves.

2.2. Extract Genomic DNA following the steps described in a commercially available kit (see **Table of Materials**).

2.3. Analyze the quantity and quality of DNA concentrations using UV-vis spectrophotometer (260 nm).

3. *S*-allele identification

3.1. Setting up of the PCR Reactions

3.1.1. Prepare a 50 ng/µL dilution in distilled water of each DNA extraction sample.

3.1.2. Thaw out the PCR reagents slowly and keep them on ice. Leave the DNA polymerase in the freezer until needed.

3.1.3. Prepare the amplification reactions using the different combinations of primers. Create the PCR reaction mix by combining the components in **Table 1**. Vortex the PCR reaction mix well and distribute the volume indicated for the different combinations of primers to each well of the PCR plate. Then, add 1 µL of the DNA dilution in each well.

3.1.4. Place the PCR plate in the thermocycler and run the corresponding PCR program shown

in **Table 1**.

3.2. Analyze the amplified fragments. There are mainly two different ways to analyze the PCR amplified fragments: capillary electrophoresis (CE) with fluorescent-labelled primers or as visualize amplicons of agarose gel electrophoresis with not-labelled primers.

3.2.1. Capillary Electrophoresis

3.2.1.1. To prepare the loading buffer, mix 35 µL of deionized formamide with 0.45 µL of labeled sizing standard. Vortex the reagent to mix well, and then dispense 35.5 µL into the well of the reader plate.

3.2.1.2. Add 1 µL of the PCR product into the well. In addition, add a drop of mineral oil to prevent water evaporation.

3.2.1.3. Prepare the separation plate adding separation buffer.

3.2.1.4. Use the commercial software included with the gene analyzer (see **Table of Materials**). Create a new sample plate and save the sample names for all wells on the plate.

3.2.1.5. Select the method of analysis. In this case, denature the samples at 90 °C for 120 s, inject at 2.0 kV for 30 s, and separate at 6.0 kV for 35 min.

3.2.1.6. Insert the two plates into the gene analyzer. Fill the capillary array with distilled water. Load the patented linear polyacrylamide (LPA) gel. Finally, click ***Run***.

3.**2.2. Gel Electrophoresis**

3.2.2.1. Prepare a 1% agarose gel adding 1.5 g of molecular biology grade agarose in 150 mL of 1x TAE (Tris-acetate-EDTA) electrophoresis running buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0). Dissolve the agarose by microwave heating for 2-3 min.

3.2.2.2. To visualize the DNA, add 4 µL of a nucleic acid stain (see Table of Materials) and mix gently.

3.2.2.3. Add a gel comb, with sufficient wells for ladders, controls and samples, into a gel tray. Then, pour slowly the mix into the middle of the gel tray and avoid bubbles.

3.2.2.4. Let the gel cool down for 30-45 min at room temperature until the gel has completely solidified. Introduce the gel in the electrophoresis chamber, remove the gel comb and fill the chamber with enough 1x TAE buffer to cover the gel.

NOTE: Check the placement of the gel. The wells should be placed close to the negative pole since negatively charged DNA migrates towards the cathode.

3.2.2.5. Add 5 µL of loading buffer (0.1% (v/v) bromophenol blue) to the PCR products and mix well.

3.2.2.6. To estimate the size of the bands, load 5 µL of DNA molecular weight ladder (see **Table of Materials**).

3.2.2.7. Load the samples into the additional wells of the gel.

3.2.2.8. Once all the samples and the DNA molecular weight ladder are loaded, run the gel at 90 V for 1-1.5 h, until the blue dye line is approximately at 75% the length of the gel.

3.2.2.9. Visualize the bands in a transilluminator for nucleic acids.

REPRESENTATIVE RESULTS:

Pollination studies in apricot require the use of flowers at the late balloon stage one day before anthesis (**Figure 1A**). This stage is considered the most favorable for both pollen and pistil collection, since floral structures are nearly mature, but anther dehiscence has not yet occurred. This prevents the interference of undesired pollen, not only of pollen from the same flower but also from other flowers, since the closed petals impede the arrival of insects carrying external pollen. The pollen grains are easily sieved through a fine mesh (**Figure 1B**) from dehisced anthers previously placed on a piece of paper for 24 h at room temperature or with slight extra heat. Likewise, pistils are obtained from flowers at balloon stage after the removing of petals, sepals and stamens with the help of tweezers or fingernails (**Figure 1C**). Pistils can be self- and cross-pollinated with a fine brush (**Figure 1D**).

The hermaphroditic flowers of apricot have five dark red sepals, five white petals (**Figure 1A**), a single pistil (**Figure 2A**) and 25-30 stamens. The pistil has three main structures: stigma, style and ovary. The ovary has two ovules, and the fertilization of at least one of them is required for fruit setting. During pollination, insects, mainly bees, transfer pollen grains to the stigma (**Figure 1A**), where they germinate (**Figure 2B**) within 24 h following pollination. A pollen tube is produced from each germinating pollen grain, which grows through the pistil structures to reach the ovary after 3–4 days and fertilize one of the two ovules after around 7 days. In self-incompatible cultivars in which the *S* allele of the pollen grain is the same as one of the two of the pistils, pollen tube stops growing at the upper style, preventing fertilization (**Figure 2C**). However, the pollen tubes from a compatible cultivar, with a different *S* allele, can grow through the style (**Figure 2D**), reach the ovary (**Figure 2E**) and fertilize one of the two ovules.

The analysis of in vitro pollen germination showed good pollen viability in all the cultivars analyzed here, since most pollen tubes were longer than the length of the pollen grain after 24 h in the culture medium. Germinated pollen grains were observed at the stigma surface (**Figure 2B)** in pistils from all pollinations, indicating adequate pollination (**Figure 3**).

To determine the self-(in)compatibility for each cultivar, pollen tube behavior in self- and cross-pollinations done in laboratory-controlled conditions was observed under fluorescence microscopy. Pollen tube growth was recorded along the style in all the pistils examined. Cultivars were considered as self-incompatible when pollen tube growth was arrested along the style in most self-pollinated pistils (**Figure 2C, Figure 3**) and self-compatible when at least one pollen tube reached the base of the style in most of the pistils examined (**Figure 2E, Figure 3**).

The study of the *S*-locus by PCR analysis allowed characterizing the *S*-genotype of each cultivar. Firstly, the *S*-alleles were identified by the amplification of the first *S-RNase* intron using the primers SRc-F/SRc-R (**Table 2**). The size of the amplified fragments was analyzed by capillary electrophoresis (**Figure 4A**) and was used to classify the genotypes analyzed in their corresponding incompatibility group (I.G.) (**Table 3**).

Some pairs of alleles, such as S_1 and S_7 or S_6 and S_9, showed similar fragment sizes for the first intron. Thus, the differentiation of these alleles was done by amplifying a region of the second intron of the *RNase* with the primers Pru-C2/PruC4R, SHLM1/SHLM2 and SHLM3/SHLM4 (**Table 2**). The PruC2/PruC4R primer combination was used to distinguish between S_6 and S_9. For S_6, a fragment of 1300 bp was amplified whereas a fragment of around 700 bp was observed for the S_9 allele (**Figure 4B, Table 3**). The specific primers SHLM1/SHLM2 and SHLM3/SHLM4 amplified a fragment of approximately 650 bp in the S_1 allele and 413 bp in the S_7 allele (**Figure 4C, Table 3**).

The primers AprFBC8-(F/R) that amplify the V2 and HVb variable regions of the *SFB* gene were used to distinguish S_c and S_8 alleles since both alleles show identical *RNase* sequence. The S_8 allele showed a PCR-fragment of approximately 150 bp whereas a 500 bp fragment corresponded to the S_c allele (**Figure 4D, Table 3**). Once the *S*-genotype was determined for all the cultivars, self-incompatible cultivars were assigned to their corresponding incompatibility groups based on their *S*-alleles (**Table 3**).

This approach requires determining the self-(in)compatibility of each cultivar by controlled self- and cross-pollinations in the laboratory (**Figure 5A**) concomitantly with the characterization of the *S*-genotype by genetic analysis (**Figure 5B**). As a result, the pollination requirements of each cultivar and the incompatibility relationships among apricot cultivars can be determined.

FIGURE AND TABLE LEGENDS:

Figure 1. Experimental set up for the determination of self-(in)compatibility in apricot. (**A**) Flowers at balloon stage (black arrows) in the field. (**B**) Sieve of pollen grains using a fine mesh. (**C**) Pistils placed on florist foam in water. (**D**) Hand-pollination of the pistils with the help of a paintbrush.

Figure 2. Diagrammatic representation of gametophytic incompatibility relationships in apricot flowers. (**A**) In Gametophytic Self-Incompatibility (GSI), both compatible and incompatible pollen grains germinate on the stigma. The pollen grain carries one of two *S*-alleles of the original genotype, in this case either S_1 or S_2. If the *S*-allele of the pollen grain

matches one of the two *S*-alleles of the pistil, in this case S_1S_3, pollen tube growth is inhibited in the upper one-third of the style. (**B**) Germination of pollen grains on the stigma surface. (**C**) Pollen tube arrested in the style indicating an incompatible behavior. (**D**) Pollen tubes growing along the style. (**E**) Pollen tubes at the base of the style indicating a compatible behavior. Scale bars, 100 µm.

Figure 3. Representative results of pollen germination and pollen tube growth through the style for self-compatible and self-incompatible cultivars after self-pollinations. Percentage of pistils with pollen grains germinating at the stigma surface, with pollen tubes in halfway the style, at the base of the style, and reaching the ovule.

Figure 4. PCR fragment amplification using five primer pair combinations for the identification of *S*-alleles. (**A**) Gene analyzer output for the SRc-(F/R) primers showing the size of the two amplified fragments of the *RNase* first intron region corresponding to the *S*-alleles. (**B**) PCR amplification using the primers PruC2/PruC4R for the identification of the S_6 and S_9 alleles. (**C**) PCR products obtained using the specific primers SHLM1 and SHLM2 for the differentiation of the S_1 allele and SHLM3 and SHLM4 to distinguish the S_7 allele. (**D**) PCR amplification with the AprFBC8-(F/R) primers for identifying S_c and S_8 alleles. M^I: 1 kb DNA Ladder. M^{II}: 100 bp DNA Ladder.

Figure 5. Scheme of the experimental design to elucidate the self- and inter-(in) compatibility relationships in apricot cultivars. (**A**) Workflow of self-(in)compatibility determination by controlled pollinations in the laboratory. (**B**) Workflow of the *S*-allele identification by molecular approaches.

Table 1. Reaction and cycling conditions for different primer combinations used in this protocol.

Table 2. Primers used in this protocol, sequence and reference for *S*-genotype characterization in *Prunus armeniaca*.

Table 3. *S*-genotyping of apricot cultivars with five primer pairs used in this protocol and incompatibility group assignment. The different polymerase chain reaction product sizes of *S*-alleles amplified using SRc-(F/R), PruC2/PruC4R, SHLM1/ SHLM2, SHLM3/SHLM4, and AprFBC8-(F/R) primers are shown in the table.

DISCUSSION:

Traditionally, most commercial apricot European cultivars were self-compatible[36]. Nevertheless, the use of North American self-incompatible cultivars as parents in breeding programs in the last decades has resulted in the release of an increasing number of new self-incompatible cultivars with unknown pollination requirements[7,8,37]. Thus, the determination of self- and inter-(in)compatibility relationships in apricot cultivars is increasingly important. This is accentuated in those areas where winter chilling is decreasing, since high year to year variations in the time of flowering are preventing the coincidence in flowering of cultivars and

their pollenizers in many cases, especially in cultivars with high chilling requirements[38]. The methodology described herein, combining hand-pollination, microscopy and genetic analyses has been very useful to determine the self(in)compatibility of each cultivar and to establish its potential pollinizer cultivars.

Pollination requirements can be determined through field-control experiments in orchard conditions[11,39]. However, the exposition to external factors including meteorological adverse conditions can cause pollination failure[10], which may result in erroneous diagnoses of self-incompatibility. The methodology described herein allows to evaluate self-(in)compatibility more accurately by microscopy observations of hand-pollinated flowers in laboratory-controlled conditions, avoiding environmental influence. Moreover, this approach allows analyzing a higher number of cultivars per year, since only a small number of flowers is required instead of several adult trees for each cultivar that are required in field experiments[40].

Incompatibility relationships can be established combining hand-pollinations and microscopy[14]. However, pollinations can only be performed for a short period during the flowering season in spring, and adult trees near the laboratory are needed, since the lifespan of the flowers collected is very short. Thus, the number of incompatibility relationships that can be analyzed by controlled hand-pollinations in each season is very low. The characterization of the genes encoded by the *S*-locus has enabled the development of PCR-based methods for *S*-allele genotyping[18,41]. This approach accelerates *S*-allele identification since it does not require flowers, and the experiments can be carried out with any vegetative tissue[42]. This extends the period during which plant material, usually young leaves, can be collected[43]. Furthermore, the leaves can be lyophilized or frozen, so that the analysis can be done at any time of the year, unlike pollinations that can only be done on fresh flowers during the flowering season[44]. An additional benefit is that leaves can be collected from young trees even before entering flowering age, facilitating the collection of samples and the early obtaining of results[45].

The genetic analysis allows a better differentiation of self-incompatibility alleles since it provides precise results of amplified fragment sizes[21,46]. To date, thirty-three *S*-alleles have been identified in apricot[12,18,20–24], which has allowed to establish 36 incompatibility groups based on *S*-genotype[8,9,17,25–27]. On the other hand, a drawback of this methodology is that different alleles in the same range size or mutations can be erroneously identified as the same allele. Thus, S_c and S_8 alleles are identical for the *RNase* sequence but a 358-bp insertion is found in the SFB gene of *Sc*[19]. Likewise, the first intron region of the alleles S_1 and S_7 are identical and are indistinguishable using the primers SRc-F/SRc-R. In addition, several homologies, such as S_6 and S_{52}[8] or S_{20} and S_{55}, and S_7, S_{13} (EF062341) and S_{46}[17], have been found because some of these alleles have been partially sequenced or by failures during PCR amplification and, consequently, further work is needed to distinguish them correctly.

PCR analysis and *S-RNase* sequencing are adequate for establishing incompatibility relationships through the identification of *S*-alleles and the allocation of cultivars in their corresponding Incompatibility Group [8,17,26,27]. However, this methodology has the limitation of preventing the determination of the self-(in)compatibility for particular apricot cultivars. Self-

compatibility (SC) has been associated to particular *S*-alleles in other *Prunus* species[47], as almond (S_f)[48,49] or sweet cherry (S_4')[50,51]. However, in apricot, the *Sc* allele, which has been associated to SC[21], can be erroneously identified as S_8, a self-incompatible allele[19,22], and possible mutations not linked to the *S* locus, as the *M*-locus [12,52], conferring SC have been identified. Recently, the *M*-locus has been genotyped using SSR markers[12]. Therefore, the genetic identification of SC for apricot genotypes needs further research and, in order to avoid mistakes due to factors not linked to the *S* locus, in this work the characterization of self-(in)compatibility has been determined also by phenotyping the behavior of the pollen tubes through the pistil of self-pollinated flowers.

The methodology described herein combining the determination of self-(in)compatibility by hand-pollinations in laboratory conditions with the subsequent observation of the behavior of pollen tubes in the pistil of controlled self-pollinations under the fluorescence microscopy and the identification of the *S*-genotype by PCR analysis allows establishing the pollination requirements of apricot cultivars. This provides a valuable information for growers and breeders, since it allows establishing the incompatibility relationships between cultivars to choose suitable pollinizers in the design of new orchards as well as to select appropriate parents to design new crosses in apricot breeding programs.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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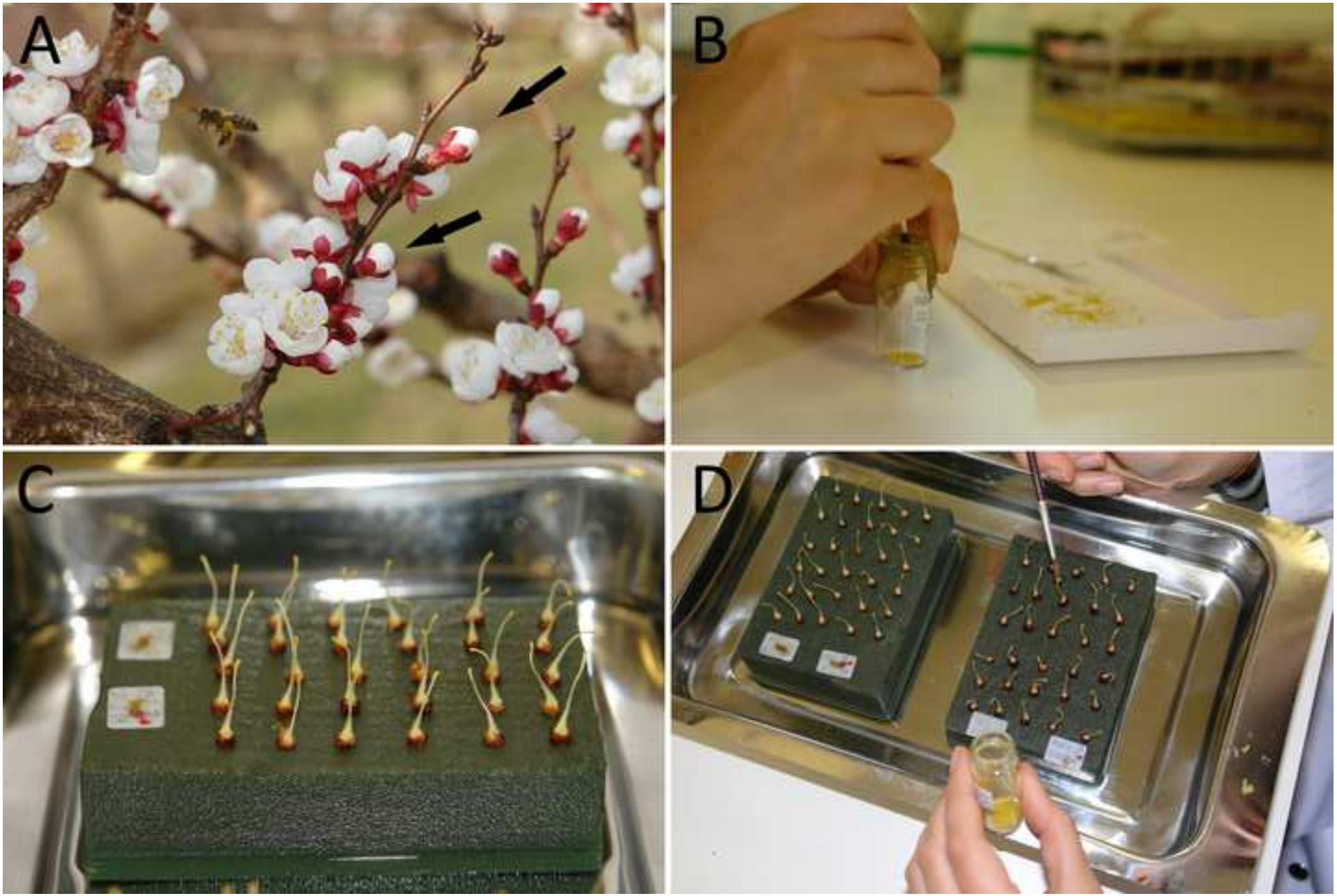

Figure 2

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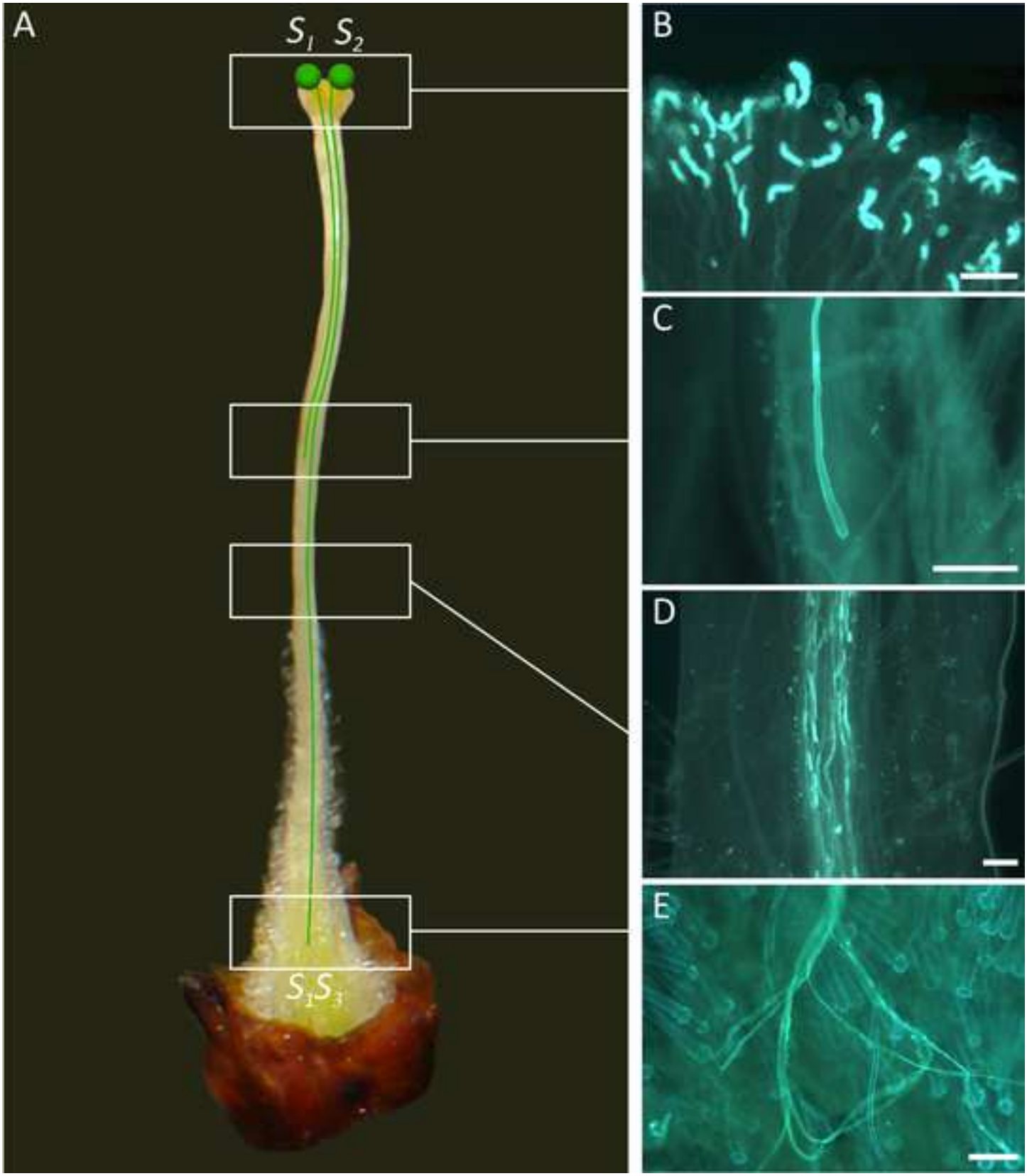


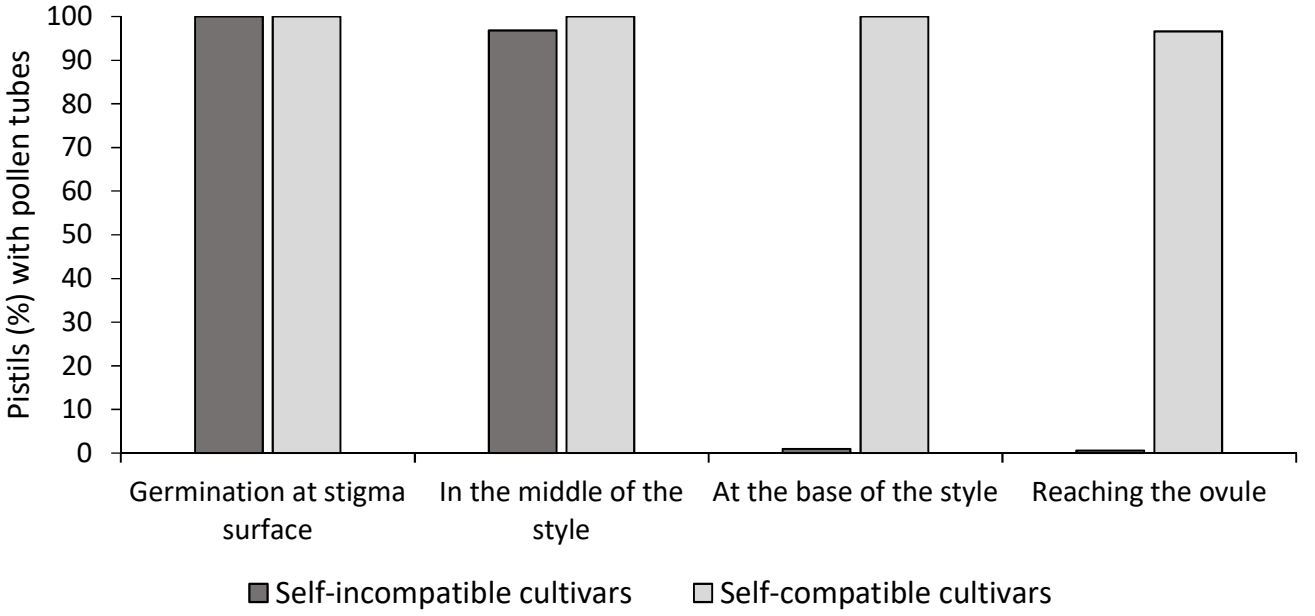
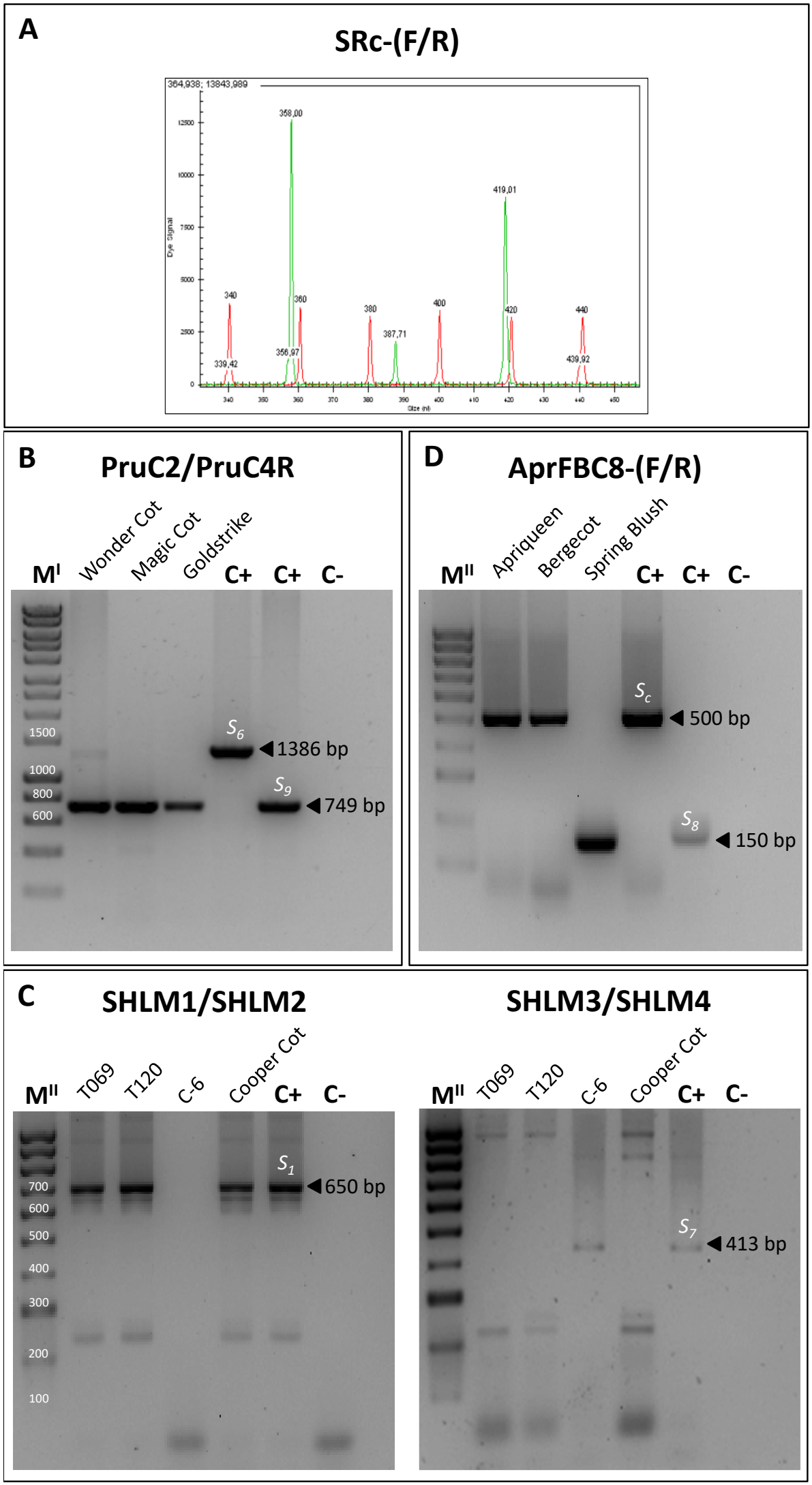

Pistils (%) with pollen tubes
100
90
80
70
60
50
40
30
20
10
0
Germination at stigma surface
In the middle of the style
At the base of the style
Reaching the ovule
Self-incompatible cultivars
Self-compatible cultivars

Figure 4



PLANT MATERIAL

A **Self-(in)compatibility determination**

B **Determination of incompatibility relationships**

Flowers

Leaves

PROTOCOL

♀ Emasculate flowers → Pistils in florist foam

♂ Remove the anthers → Sieve the pollen

Hand self-pollination

Microscopy observations

DNA extraction

PCRs

Capillary Electrophoresis

Gel Electrophoresis

S-genotyping

RESULTS

Self-compatible

Self-incompatible

Incompatibility groups (I.G)

Table

PCR Master Mix

Components	Final Concentration	15 µL reaction
10x NH_4 Reaction Buffer	10x	1.5 µL
50 mM $MgCl_2$ Solution	25 mM	1.2 µL
100 mM dNTP	2.5 mM	0.6 µL
Primer SRc-F	10 µM	0.6 µL
Primer SRc-R	10 µM	0.6 µL
500 U Taq DNA Polymerase	0.5 U	0.2 µL
H_2O		8.3 µL

Thermocycler conditions

Cycle Step	Temperature	Time	Cycles
Initial denaturation	94 °C	3 min	1
Denaturing	94 °C	1 min	35
Annealing	55 °C	1 min	
Extension	72 °C	3 min	
Final Extension	72 °C	5 min	1
	4 °C	hold	

Components	Final Concentration	25 µL reaction
10x PCR buffer	10x	2.5 µL
5x Q-solution	5x	5 µL
100 mM dNTP	2.5 mM	0.5 µL
Primer PruC2	10 µM	0.2 µL
Primer C4R	10 µM	0.2 µL
250 U Taq DNA Polymerase	10 U	0.13 µL
H_2O		15.5 µL

Cycle Step	Temperature	Time	Cycles
Initial denaturation	94 °C	2 min	1
Denaturing	94 °C	10 s	10
Annealing	55 °C	2 min	
Extension	68 °C	2 min	
Denaturing	94 °C	10 s	25
Annealing	58 °C	2 min	
Extension*	68 °C	2 min	
Final Extension	72 °C	5 min	1
	4 °C	hold	

* with 10 s added each cycle to the 68 %C extension step.

Components	Final Concentration	25 µL reaction
10x PCR buffer	10x	2.5 µL
5x Q-solution	5x	5 µL
100 mM dNTP	2.5 mM	0.5 µL
Primer SHLM1	10 µM	0.2 µL
Primer SHLM2	10 µM	0.2 µL
250 U Taq DNA Polymerase	10 U	0.13 µL
H_2O		15.5 µL

Cycle Step	Temperature	Time	Cycles
Initial denaturation	94 °C	2 min	1
Denaturing	94 °C	30 s	35
Annealing	62 °C	1.5 min	
Extension	72 °C	2 min	
Final Extension	72 °C	5 min	1
	4 °C	hold	

Components	Final Concentration	20 µL reaction
5x PCR Buffer	5x	4 µL
dNTP	2.5 mM	1.6 µL
Primer SHLM3	10 µM	1 µL
Primer SHLM4	10 µM	1 µL
100 U DNA Polymerase	5 U	0.2 µL
H_2O		12.4 µL

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 s	1
Denaturing	98 °C	10 s	35
Annealing	51 °C	30 s	
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	1
	4 °C	hold	

Components	Final Concentration	25 µL reaction
10x PCR buffer	10x	2.5 µL
100 mM dNTP	2.5 mM	2 µL
Primer FBC8-F	10 µM	1 µL
Primer FBC8-R	10 µM	1 µL
250 U Taq DNA Polymerase	10 U	0.125 µL
H_2O		17.4 µL

Cycle Step	Temperature	Time	Cycles
Initial denaturation	94 °C	2 min	1
Denaturing	94 °C	30 s	35
Annealing	55 °C	1.5 min	
Extension	72 °C	2 min	
Final Extension	72 °C	5 min	1
	4 °C	hold	

Table 2

Primers	Sequence	Reference
SRc-F	5'-CTCGCTTTCCTTGTTCTTGC-3'	18
SRc-R	5'-GGCCATTGTTGCACCCCTTG-3'	18
Pru-C2	5'-CTTTGGCCAAGTAATTATTCAAACC-3'	35
Pru-C4R	5'-GGATGTGGTACGATTGAAGCG-3'	35
SHLM1-F	5'-GGTGGAGGTGATAAGGTAGCC-3'	17
SHLM2-R	5'-GGCTGCATAAGGAAGCTGTAGG-3'	17
SHLM3-F	5'-TATATCTTACTCTTTGGC-3'	17
SHLM4-R	5'-CACTATGATAATGTGTATG-3'	17
AprFBC8-F	5'-CATGGAAAAAGCTGACTTATGG-3'	26
AprFBC8-R	5'-GCCTCTAATGTCATCTACTCTTAG-3'	26

Table 3

Cultivar	SRc-(F/R) (bp)	PruC2/PruC4R (bp)	SHLM1/SHLM2 (bp)	SHLM3/SHLM4 (bp)	AprFBC8-(F/R) (bp)	*S*- Genotype	Incompatibility group (I.G)
Wonder Cot[8]	420, 420	749, 1386				S_6S_9	VIII
Magic Cot[8]	334, 420	749				S_2S_9	XX
Goldstrike[8]	334, 420	749				S_2S_9	
T069[17]	334, 408		650			S_1S_2	I
T120[17]	334, 408		650			S_1S_2	
C-6	334, 408			413		S_2S_7	IV
Cooper Cot[8]	274, 408		650			S_1S_3	XVIII
Apriqueen	358, 358				500	S_cS_c	-
Bergecot[8]	334, 358				500	S_2S_c	-
Spring Blush[8]	274, 358				150	S_3S_8	XXI

Table of Materials

Click here to access/download;Table of Materials;Material table.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Agarose D1 Low EEO	Conda	8010.22	
BIOTAQ DNA Polymerase kit	Bioline	BIO-21060	
Bright field microscope	Leica Microsystems	DM2500	
CEQ System Software	Beckman Coulter		
DNeasy Plant Mini Kit	QIAGEN	69106	
dNTP Set, 4 x 25 µmol	Bioline	BIO-39025	
GenomeLab DNA Size Standard Kit - 400	Beckman Coulter	608098	
GenomeLab GeXP Genetic Analysis System	Beckman Coulter		
GenomeLab Separation Buffer	Beckman Coulter	608012	
GenomeLab Separation Gel LPA-1	Beckman Coulter	391438	
HyperLadder 100bp	Bioline	BIO-33029	
HyperLadder 1kb	Bioline	BIO-33025	
Image Analysis System	Leica Microsystems		
Molecular Imager VersaDoc MP 4000 system	Bio-Rad	170-8640	
NanoDrop One Spectrophotometer	Thermo Fisher Scientific	13-400-518	
pH-Meter BASIC 20	Crison		
Phusion High-Fidelity PCR Kit	Thermo Fisher Scientific	F553S	
Power Pack P 25 T	Biometra		
Primer Forward	Isogen Life Science		
Primer Reverse	Isogen Life Science		
Quantity One Software	Bio-Rad		
Stereoscopic microscope	Leica Microsystems	MZ-16	
Sub-Cell GT	Bio-Rad		
SYBR Safe DNA Gel Stain	Thermo Fisher Scientific	S33102	
T100 Thermal Cycler	Bio-Rad	1861096	
Taq DNA Polymerase	QIAGEN	201203	
Vertical Stand Autoclave	JP Selecta		

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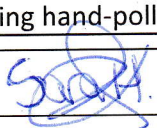
Signature:	
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Zaragoza, June 11th, 2019

Dear Editor,

We are pleased to enclose a revised version of our manuscript JoVE60241 "*Determination of self- and inter-(in)compatibility relationships in apricot combining hand-pollination, microscopy and genetic analyses*" (by S. Herrera, J. Lora, J.I. Hormaza and J. Rodrigo)

The paper has been revised giving careful consideration to the points raised by the editor and reviewers. We describe in detail below the changes made in this new version.

We do appreciate the comprehensive reviews that have clearly contributed to improve our paper over our original submission. We look forward to hearing from you on your final decision.

Yours sincerely,

Sara Herrera

Changes made addressing the comments of the handling editor and reviewers:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The document has been carefully revised.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited

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3. Please avoid long steps (more than 4 lines).

The step 3.2.1.4. has been divided into three, so two new ones (3.2.1.5. and 3.2.1.6.) have been included.

4. Step 1.1: Please write this step in the imperative tense.

The imperative tense has been corrected.

5. 1.3: Please do not highlight a step without highlighting any of the sub-steps.

The step 1.3. has been rewritten, including a new one (1.3.7.) that has been highlighted.

6. 3.2: Please write this step in the imperative tense.

The imperative tense has been corrected.

7. Please sort the items in alphabetical order according to the name of material/equipment.

The items have been alphabetically ordered in the Material table

8. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please rewrite lines 254-256, 269-273

Both paragraphs have been rewritten (lines 267-270, 283-289)

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Self-incompatibility trait and S-genotypes information of apricot cultivars are very important to introduce new cultivar and select cultivars in orchard production. This paper provide useful protocol to determine self-incompatibility phenotypes and S-genotypes in apricot.

Major Concerns:

I have one major concern. As the authors described in Discussion section, self-compatibility in apricot germplasm is controlled not only by S-locus but also by M-locus (reference 12). This protocol provides the method to determine S-alleles but not M-alleles. M-locus genotyping system is quite important especially during the selection of self-compatibility in apricot. Therefore, it would be better to include M-locus genotyping system if established. If it has not been established, the authors at least have to emphasize this point by showing self-compatibility determination scheme in figure 5.

The fact that self-compatibility may be controlled by other mutations not linked to the *S*-locus, as the *M*-locus, and the need of further research to accurately allow the genetic identification of self-compatibility in apricot have been stressed in Discussion (lines 448-450), including the *M*-locus genotyping performed by Muñoz-Sanz et al 2017. In fact, in order to avoid possible mistakes due to additional factors involved in self-incompatibility not linked to the *S* locus, we believe that the approach followed in this work combining phenotypic and molecular observations are appropriate. We have highlighted this in Discussion (lines 450-455). Thus, *S*-allele identification was performed to define the incompatibility relationships between cultivars rather than to determine *self-(in)compatibility*. To clarify this, Figure 5 has been modified, changing the heading "*S*- allele identification" to "Determination of incompatibility relationships"

Minor Concerns:

1. Line107, several different kinds of fixation solution were known. Are you really sure that this solution is the best? Some researchers use chloroform-based fixation solution.

Although different fixation solutions are available, including chloroform- and formaldehyde-based solutions, the fixative solution of ethanol/acetic acid (3:1) (Williams et al., 1999) used herein has proved been useful for fixing pistils after controlled pollinations and subsequent observation of pollen tubes under the microscope in different *Prunus* species, which have been cited in the protocol (lines 111-114).

2. Line 269, if possible, please show the picture containing ovule in Figure 2E.

As mentioned in section 1.4.2 (lines 149-151), pollen tube growth along the style was observed in squash preparations of the pistils. This technique allows to visualize the pollen tubes, but the rest of the tissues of the pistil, including the ovules, are crushed. Therefore, it was not possible to obtain images showing the ovules and, instead, we have included an image (Figure 2E) showing the pollen tubes at the base of the style and at the entrance of the ovary.

Thank you very much for the very constructive review of our manuscript.

Reviewer #2:

Manuscript Summary:
This is a great paper I think it is important for plant breeders and scientist working with self-incompatibility of Prunus species. I recommend acceptance of this manuscript.

Thank you very much for the very constructive review of our manuscript.

Reviewer #3:

Manuscript Summary:
The paper by Sara Herrera and colleagues is aimed to increase understanding of the histological and molecular processes for establishing the pollination requirements in apricot. The subject is very interesting and provides a valuable information to select cultivar combination in the design of new orchards. On the whole, the purpose of the MS is supported by a depth and good experimental design. I consider that the paper can be accepted for publication.

Thank you very much for the very constructive review of our manuscript.