Identification of Almond Self-Incompatibility and Self-Compatibility Sources Using Molecular Markers

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Objectives:

1. Develop PCR(polymerase chain reaction)-based molecular markers effective in rapidly and unequivocally distinguishing among the different self-incompatibility and self-compatibility genotypes currently utilized in the UCD almond breeding program.
2. Begin to test the utility of these markers to identify/characterize the self-compatibility type in advanced UCD almond breeding lines to allow evaluation of associated differences in the final expression of self-compatibility and yield potential for different self-compatibility genes including different combinations of self-compatibility genes.

Interpretive Summary:

Because California almond cultivars are self-sterile, cross-compatible pollenizer cultivars need to be interplanted with the main commercial cultivar (which is often Nonpareil). Cross-compatibility in most commercial cultivars, as well as self-compatibility being developed in new cultivars, is controlled by a single S-gene whose different forms (alleles) will determine final compatibility. Nonpareil flowers and pollen have the alleles S_7 and S_8, so that any pollen having either the S_7 or S_8 (either from Nonpareil or any other almond cultivar) will be cross-incompatible and so fail to fertilize for required seed set. Precise knowledge of the S-allele identity of current and future almond cultivars is thus required in order to ensure orchard plantings are cross-compatible and so fully productive. Because of its uniqueness, the S-alleles can also be used as a means of variety identification as well as paternity testing (i.e.

knowing which cultivar has been most effective in fertilizing your main cultivar). The PCR-based markers used in this project are particularly accurate for identifying individual S-alleles because they measure the S-gene directly. To do this, two fixed reference or 'primer' points on the S-gene DNA are developed near the beginning and end of the gene in order to measure the relative size of the intervening DNA in that particular allele. (For example, a rapid and easily automatable method to separate this report from others would be to count the number of individual letters between the '**Objectives:'** and '**Interpretive Summary'** headings above. It is very unlikely that any two different reports will have the exact same count). PCR is a precise and highly automatable procedure for measuring relative differences in allele size based on the number of nucleotides (i.e. genetic 'letters') present between the primers, which has already proven useful for the initial characterization of California almond cross-incompatibility groups. However, the previous PCR procedures used for almond (see references) are both tedious and prone to error. In this research, we have improved both accuracy and throughput by incorporating more precise molecular methods which have been recently developed. Preliminary testing against known S-genotypes confirms the accuracy of this new, more efficient approach. However, the Nonpareil S-alleles, S7, and S8, continue to pose difficulties in their precise discrimination even by this method. Because of the importance of Nonpareil both as a commercial cultivar and a major recurrent parent in the breeding programs, the precise and rapid determination of these S-alleles is highly desirable. A major part of the difficulty is the very large size of the PCR generated stands for S7 and S8, which approaches the functional limit of current molecular separation procedures. To overcome this obstacle, we are now developing new reference 'primers' which will encompass shorter lengths of the targeted S-alleles and so result in shorter PCR stands which should maintain a high level of accuracy but allow more consistent molecular determination. These improved procedures and reference primers should also allow better discrimination amongst the different self-compatibility alleles currently being developed by the breeding program (since these strands are also often exceptionally long using the old procedures). The ability to discriminate amongst these alleles will be crucial to the final deployment of the best S-allele combinations for maximum year-to-year self-compatibility in new cultivars.

Materials and Methods:

A set of 13 almond varieties was selected based on published data (Barckley et al. 2006, Sánchez-Pérez et al. 2004, Tamura et al. 2000) that, as a group contain a majority of the known alleles of the self-incompatible gene in almond (S-alleles). The set consists of current varieties, old heritage varieties and the self-compatible variety 'Tono' (**Table 1**). Total genomic DNA was extracted from dried leaves using a commercial extraction kit (DNeasy® 96 Plant Kit, Qiagen, Valencia, CA).

Relatively small portions of the self-incompatibility gene were amplified by Polymerase Chain Reaction (PCR) using standard protocols. These fragments were amplified using previously published primer sequences specific to various S-alleles (Sánchez-Pérez et al. 2004, Tamura et al. 2000). The length, the size in base-pairs (bp), which should be uniquely diagnostic for each S-allele, was determined by capillary electrophoresis on a Genetic Analyzer (ABI Prism 3100, Applied Biosystems) using various experimental protocols.

Results and Discussion:

Many of the S-allele specific fragments amplified using published primers are longer than the roughly 500 base-pairs (bp) maximum length that can be routinely detected by automated capillary electrophoresis with existing protocols. The focus of this small work was to increase the upper limit of the fragment length that could be detected, to make routine identification of S-alleles with lengths below this new limit and to begin developing a method to uniquely identify all other S-alleles.

Several combinations of previously published primers were tested for the ability to amplify specific fragments diagnostic for S-alleles, or groups of S-alleles (**Table 2**). The combination of the universal forward and universal reverse primers (AS1II and AmyC5R) consistently amplified S-allele fragment of the expected size ranges. The addition of the S_f specific primer (CEBASf) to the reaction mix with AS1II and AmyC5R) consistently amplified the expected portion of the self-fertile allele, if it was present in the sample, and did not interfere with amplification of the other alleles. Combining these primers into one reaction mix reduces time and cost of the analysis.

Unfortunately, the primers specific to the S-alleles of 'Nonpareil' (S_7 and S_8) did not work despite repeated attempts with an array of PCR protocols. Others have remarked on the lack of reliability of these primers. Redesigning these primers is now a pressing future goal.

Once amplified the length of the S-allele fragment was ascertained by capillary electrophoresis using a genetic analyzer; the length of the amplified fragment determines the S-allele genotype. Our particular goal was to more than double the length of allele detection from 500bp (base pairs) to over 1100bp; this would allow the identification of the very common allele S_1 from 'Mission', which is reported to be 1080bp. The genetic analyzer requires an internal size standard to calculate the length of the tested fragments. We tested a new product, 1200LIZ, from Applied BioSystems. Though this new size standard is ready to use "out of the box", the protocols for its use are not. In particular software modifications instructing the genetic analyzer to "look for" fragments twice the normal detection limit had to be established by trial and error.

We were able to detect the size standard all the way out to 1200bp. However detection at the upper limit was not consistent. Clear reproducible results were obtained up to roughly 1100bp. This includes the S_1 allele from Mission (**Table 3**). With our current protocols, we can routinely detect and identify all known S-alleles in almond with lengths of 1100bp or less with the exceptions of S_7 and S_8. As noted above, for these two alleles the issue is the initial amplification of the allele fragment. Clearly new primers must be developed specific to S_7 and S_8.

New primers or alternative methods also need to be developed for the S-alleles with fragment lengths beyond the new practical detection limit of 1100 bp. Several technical issues suggest that the 1200 bp range may be the upper limit possible for capillary electrophoresis. Appropriate size standard could be synthesized, perhaps by using the S-alleles themselves. However, detection of these extra long fragments would require extra long run times or the use

of significantly higher electrical current. These options increase shearing and denaturing of the DNA fragment, particular problems since the goal is to determine the full length of the intact fragment. More likely, the solution will involve designing primers.

Table 1. Almond varieties and corresponding self-incompatibility alleles with published and determined lengths in base-pairs.

Variety	S-allele[a]	S-allele[b]	Published size[e]
CARMEL	a, d	5, 8	600, 340
FARRANGES	b, na[c]	1, 3	1080, 1240
HARRIOT[d]	e, j	6, 14	850, 1300
JORDANOLO	c, j	7, 14	221, 1300
KAPAREIL	d, g	8, 13	340, 1360
MARCONA	na, na	11, 12	700, 1600
MISSION	a, b	5, 1	600, 1080
NONPAREIL	c, d	7, 8	221, 340
PADRE	b, h	1, 18	1080, 650
PEERLESS	b, e	1, 6	1080, 850
RUBY	b, e	1, 6	1080, 850
SWEETHEART	b, j	1, 14	1080, 1300
TUONO	b, f	1, f	1080, 450

[a] California nomenclature
[b] European nomenclature
[c] Not assigned
[d] Sample may be misidentified.
[e] The length of several alleles has been inferred from published results.

Table 2. Primer combinations used to amplify Self-incompatibility gene fragments diagnostic for specific S-alleles.

	Forward Primer	Reverse Primer	Target S-alleles
A	ASIII (universal forward)	AmyC5R (universal reverse)	All except S_f, S_7, S_8
B	ASIII + CEBASf (Sf specific forward)	AmyC5R	All except S_7, S_8
C	AIsc1 (S7 specific forward)	AmyC5R	S7
D	AIsd2 (S8 specific forward)	AmyC5R	S8

Table 3. Almond S-alleles that can now be consistently and reliably genotyped with capillary electrophoresis.

S-allele	Published allele size	Sample Size	Detected Size +/- Standard Deviation
S_f	470	4	480.65 +/- 0.31
S_5	600	6	597.65 +/- 0.46
S_{18}	650	4	643.76 +/- 0.42
S_{11}	700	4	702.34 +/- 0.5
S_6	850	7	855.55 +/- 0.55
Unknown[a]	unk	4	991.3 +/- 0.63
S_1	1080	26	1081.52 +/- 0.81

[a] Allele obtained from possibly misidentified 'Harriot'.

Research Effort Recent Publications:
None

References Cited:

Barckley, K., S. Uratus, T.M. Gradziel, A.M. Dandekar (2006) Multidimensional analysis of S-alleles from cross-incompatible groups of California almond cultivars. J. Amer. Soc. Hort. Sci. 131(5):632-636.
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