Multidimensional Analysis of S-alleles from Cross-incompatible Groups of California Almond Cultivars

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ABSTRACT. The California almond industry is the largest supplier of almonds [*Prunus dulcis* (Miller) D.A. Webb] in the United States and throughout the world. Self-incompatibility is a major issue in almond production as it greatly affects nut set. In this study, we determined full-length sequences for alleles S^a - Sⁱ, determined the genotypes of 44 California cultivars, and assigned the cultivars to cross-incompatibility groups (CIGs). Newly identified S-alleles led to an increase in the number of CIGs. A pairwise distance tree was constructed using the aligned amino acid sequences showing their similarity. Four pairs of alleles (S^c and S^e, S^g and S^h, S^d and Sⁱ, and S^b and S^f) showed high sequence similarity. Because of its simplicity, reproducibility, and ease of analysis, PCR is the preferred method for genotyping S-alleles.

According to the Almond Board of California (2004), nearly 470 million kg of almonds were harvested in California in 2004. One-third was consumed domestically, while 328 million kg of almonds were exported, making California the number one supplier of almonds worldwide.

One of the major issues surrounding the almond industry is a genetic phenomenon known as self-incompatibility (SI). This naturally occurring process is the reason growers need pollinizers. Without cross-compatible cultivars, almonds would not be able to set their edible seed. SI suppresses inbreeding and promotes out-crossing (de Nettancourt, 1977). Almond, a member of *Prunus* L., exhibits gametophytic SI (GSI) that operates from a single, multi-allelic locus (Dicenta and Garcia, 1993; Lewis and Crowe, 1954). In the Solanaceae, the pistil gene product is an S-RNase that consists of five conserved regions (C1-C5) and two hypervariable regions (HV_a and HV_b) (Ioerger et al., 1991). In almond, S-RNases contain five conserved regions, one hypervariable region, and two introns (Ortega et al., 2005; Ushijima et al., 1998). The structural properties of S-RNases have been useful in genotyping almond cultivars (Bošković et al., 1997, 1999, 2003; Channuntapipat et al., 2001, 2003; Ma and Oliveira, 2001; Ortega et al., 2005; Tamura et al., 2000; Ushijima et al., 1998). Although no definitive mechanism for SI has been described, SI has been reviewed in detail by McCubbin and Kao (2000), Silva and Goring (2001), Stone (2002), and Stone and Goring (2001).

The ability to produce a self-compatible and self-fruitful almond cultivar would eliminate the need for growers to incur the expense of pollinizers and pollinator bees. Until that option becomes widely available, we are assisting growers and nurseries to identify potential cross-compatible cultivars prior to planting,

thus reducing the cost of misplantings. This research is an extension of work by Kester et al. (1994) and Ushijima et al. (1998).

Materials and Methods

PLANT MATERIAL. Young almond leaves were collected and used to extract DNA from 44 cultivars (Table 1). Styles were collected during the popcorn stage of flower development and used for RNA extraction. The styles were frozen at -80 °C until needed. Samples were collected from cultivars at the Univ. of California orchards in Davis and Winters, Calif., and from growers throughout California.

DNA AND RNA ISOLATION. DNA was isolated from 100 mg of young almond leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, Calif.). Grinding of leaf tissue was accomplished using a TissueLyser (Qiagen). The tissue was weighed and placed in 1.5-mL tubes, frozen with liquid nitrogen and broken up into smaller pieces using a pointed spatula. A 3-mm tungsten carbide bead was placed into each tube along with the AP1 buffer and 3 µL RNase A. The tubes were placed in previously cooled blocks and shaken for 2 min at a frequency of 30 times/s. Total RNA was extracted from 200 mg of styles from 'Winters', 'Harriot', and 'UCD 25-75' using the RNeasy Plant Mini Kit (Qiagen).

cDNA SYNTHESIS. RNA was converted to cDNA using the Marathon cDNA Amplification Kit (Clontech, Mountain View, Calif.).

CLONING AND SEQUENCING OF DNA. Amplification of the S-alleles was accomplished using primers AS1II (5'TATTTCAATTTGTGCAACAATGG3'), AmyC5R (5'CAAAATACCACTTCATGTAACAAC3'), Als1 (5'CAGACACTTAATCAATTCCAG3') and Als2 (5'GTGTATCCTCAGTTGCGAACCA3') as described by Tamura et al. (2000). Polymerase chain reaction (PCR) was used to amplify S-alleles from genomic DNA using the program: 1 min 94 °C, 2 min 55 °C, and 2 min 65 °C, 20–25 cycles. The PCR products were cloned into a pCR2.1 TOPO vector (Invitrogen, Carlsbad, Calif.). DNA from positive clones was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, Calif) and sequenced by Davis Sequencing (Davis, Calif.). Sequence results were analyzed using Sequencher (Gene Codes Corp., Ann Arbor, Mich.) and MacDNAsis programs (Hitachi Software, San Bruno, Calif.).

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Table 1. California almond cultivars placed into cross-incompatibility groups (CIGs) based upon their S-alleles. The cultivars listed within the same row or CIG designation are incompatible pollinizers, but are compatible with cultivars listed in differing CIGs. Bolded cultivars indicate previously genotyped cultivars and the cultivars genotyped in this manuscript are unbolded. Genotypes were based on PCR of intron length polymorphisms. Successful cross-compatibility between CIGs has been confirmed by growers and nurseries throughout California. While the European alleles are not 100% homologous to the California alleles, the European genotypes listed have either been reported using the same cultivars or based on alignment of all the sequence data are the most similar to the California genotypes listed.

CIG	Genotype	Almond cultivars	European genotype
I	S ^c S ^d	Nonpareil , Tardy Nonpareil, Grace, West Steyn, UCD F8:7-180, Galaxy	S ₇ S ₈
II	S ^a S ^b	Mission	S ₅ S ₁
III	S ^a S ^c	Thompson , Sauret no.2, Mono, Wood Colony, Durango, Le Grand, Wassum	S ₅ S ₇
IV	S ^b S ^c	Merced , Ne Plus Ultra, Rosetta, Price cluster, Aldrich, Pearl, Jenette, Sano	S ₁ S ₇
V	S ^a S ^d	Carmel , Sauret no. 1, Livingston	S ₅ S ₈
VI	S ^b S ^d	Monterey , Butte, Dottie Won, Plateau, Avalon, UCD D3-25, UCD F8:7-179, Folsom, Blue Gum	S ₁ S ₈
VII	S ^a S ⁱ	Arbuckle	S ₅ S ₇
VIII	S ^b S ^e	Fritz, Ruby, Peerless	S ₁ S ₆
IX	S ^b S ^h	Padre	S ₁ S ₁₈
X	S ^b S ⁱ	UCD 13-1 (Winters), UCD 36-52	S ₁ S ₁₄
XI	S ^c S ^e	Tokyo	S ₇ S ₆
XII	S ^c S ^g	Milow	S ₇ S ₁₃
XIII	S ^c S ⁱ	Jordanolo	S ₇ S ₁₄
XIV	S ^d S ^e	Kochi, UCD F8:8-160	S ₈ S ₆
XV	S ^d S ^g	Solano, Sonora, Vesta, Kapareil, UCD F8:7-161	S ₈ S ₁₃
XVI	S ^d S ⁱ	UCD 25-75	S ₈ S ₇
XVII	S ^c S ⁱ	Harriot	S ₆ S ₁₄
XVIII	S ^a S ⁱ	Carrion	S ₅ S ₁₄
XIX	S ^d	Jeffries, UCD 3-6, Johlyn	S ₈
XX	S ^f	Tuono	S _f
XXI	S ^k	UCD 54P455 (peach)	---

References for alleles include Bošković et al., 1997; Channuntapipat et al., 2001, 2003; Ma and Oliveira et al., 2001.

5'-AND 3'-RACE. RACE was performed on S-alleles, S^a-Sⁱ, using the Marathon cDNA Amplification Kit (Clontech). The desired product was purified using Gene Clean (QBiogene, Irvine, Calif.) and cloned. Reverse primers of AS111 and AmyC5R (universal primers) were used for 3'- and 5'-RACE, respectively. Procedures for cloning, sequencing and analysis were as described above.

INTRON I AMPLIFICATION. Primers flanking intron I (see Table 2 for primer sequences) were designed from sequences obtained from 5'-RACE procedures. Intron I was amplified from DNA of young almond leaves using PCR (1 min 94 °C, 2 min 55 °C, and 2 min 65 °C, 25 cycles). The PCR product was TOPO cloned and sequenced as described above.

PROTEIN COMPARISONS. SRNase sequences were obtained from GenBank. Apple (*Malus xdomestica* Borkh.) sequences were from accession numbers AY187627, AY039702 and AY201748. European almond S alleles were represented by accession numbers AAF73756, AAG09286, AAF82613, AAG09287, AAK58577, AAK58579, AAK58580 corresponding to sequences from Ma and Oliviera (2001, 2002) for S₁ and S^c-S₁, respectively. California almond S alleles were from accession numbers (BAA95317, AAD51787, AAD51788, and AAD51789) corresponding to S^a-S^d, respectively (Tamura et al., 1999; Ushijima et al., 1998). For a list of accession numbers corresponding to sequences determined in this work refer to Table 3.

Four S-alleles, S^g-S₁, isolated from European cultivars were compared with California S-alleles S^a-S₁ and percent homolog calculated. Amino acid sequences were aligned using Clustal X (Thompson et al., 1997). Three amino acid sequences from apple were aligned with the almond S-alleles. The pairwise distance

Table 2. Oligonucleotide primers used for 5'-RACE amplification of full length cDNA encoding S-RNase alleles of the almond cultivars, 'Mission', 'Nonpareil', 'Peerless', 'Sonora', 'Sonoma', 'UCD 25-75', 'Winters', and 'Tuono'.

Alleles	Forward primers
S ^a	5'GTCTCTTCTTGTGTTTCACTA3'
S ^b /S ^f	5'CCTCTCCCATTCCTGTAGTTTC3'
S ^c	5'GTTGAAATCGTCACTCGCTTTCC3'
S ^d	5'GTGTTTCATTATGAGCAC3'
S ^e	5'CTCTCATTCTGTTGTAATTTCTTGTGTC3'
S ^g	5'GTATTCTCCTAGTATGGGGATGTTGAAATCGTC3'
S ^h	5'CTCATTCTGTTGTAATTTCTTGTGTC3'
S ⁱ	5'GCTTCTTCATGTGTTTCATTATGTGC3'
S ₁	5'GCAGGTGCGATGTTGAAATCGTCACTCGGTTTCC3'
Name	Reverse primer
RC1	5'CCATTGTTGCACAAATTGAAAATA3'

tree, based on the aligned amino acid sequences, using the neighbor joining method was constructed using PAUP* version 4.0 (Swofford, 2002). Bootstrap analysis of 1000 replicates was also completed. Additionally a pairwise distance tree was constructed with almond S-alleles determined by Ma and Oliviera (2002).

Results

GENETIC ANALYSIS OF S-ALLELES. Six S-alleles (S^c-S₁) were amplified by PCR, cloned, and sequenced. In addition, RACE was performed for all S-alleles (S^a-S₁) to determine sequences at the 5' and 3' ends. Large differences in the size of the two introns

Table 3. GenBank accession numbers and corresponding DNA sequences encoding individual S-RNase alleles from different almond cultivars.

Accession no.	Description
DQ150569	S ^a -RNase, Mission, gDNA
DQ133943	S ^b -RNase, intron I, gDNA
DQ133944	S ^c -RNase, intron I, gDNA
DQ133945	S ^d -RNase, intron I, gDNA
DQ156216	S ^c -RNase, Peerless, gDNA
DQ157873	S ^c -RNase, Peerless, mRNA
DQ156217	S ^f -RNase, Tuono, gDNA
DQ157874	S ^f -RNase, Tuono, mRNA
DQ156218	S ^g -RNase, Sonora, gDNA
DQ157875	S ^g -RNase, Sonora, mRNA
DQ156219	S ^h -RNase, Padre, gDNA
DQ157876	S ^h -RNase, Padre, mRNA
DQ059314	S ⁱ -RNase, UCD 25-75, Arbuckle, mRNA
DQ058401	S ⁱ -RNase, Arbuckle, gDNA
DQ058402	S ⁱ -RNase, UCD 25-75, gDNA
DQ059313	S ⁱ -RNase, Winters, Harriet, mRNA
DQ058403	S ⁱ -RNase, Winters, gDNA
DQ058404	S ⁱ -RNase, Winters, gDNA

were observed (Table 4). These differences in intron size allow characterization of S- alleles by PCR.

A comparison of amino acid sequences was performed (Fig. 1) to determine exon differences. It should be noted that S^k was not included in this analysis because it is derived from peach and is not a true almond allele. All the alleles maintained the overall structure of five conserved regions, two introns, and a hypervariable region, but showed individual differences, especially within the hypervariable region. More single amino acid changes seemed to occur in conserved region C4 than regions C1, 2, 3, or 5. Although there are still some unknown sequences at the 5' and 3' end of the alleles, the most important regions of the sequences are evident from the analysis.

CROSS-INCOMPATIBLE GROUPS (CIGs). The ability to identify S-alleles using PCR allows rapid genotyping of cultivars and their assignment to a CIG (Table 1). The European designations of the alleles are shown. Cultivars within a CIG are cross-incompatible and thus not suitable for use as pollinizers. Thus, Table 1 groups the cultivars in such a way as to help determine potential pollinizers based on the S-alleles. Any new almond cultivars can easily be tested and added to the chart.

PAIRWISE DISTANCE TREE. Using amino acid alignment, a pairwise distance tree was constructed (Fig. 2). The tree shows that the apple sequences are genetically different from the almond S-alleles. Analysis of the almond S-alleles indicates that the S^a allele was perhaps a progenitor S-allele from which all others were derived, as it is an outlier in Fig. 2. It is interesting to note that the Sⁱ allele is the only one that does not seem to be closely related to another S-allele. According to the bootstrap analysis, S^b of 'Mission' and S^f of 'Tuono' are the only pair that is highly similar. The other pairings did not derive very high bootstrap percentages and therefore may not be as strongly similar as the tree suggests.

Discussion

GENETIC ANALYSIS OF S-RNASES. We successfully identified 10 S-alleles (S^a-Sⁱ) in all known California almond cultivars. As shown by Tamura et al. (2000), a simple PCR confirms each

Table 4. List of intron length polymorphisms for S-alleles in almond. Intron lengths were determined by PCR, cloning and sequencing. Specific primers designed from 5'-RACE sequences were used to amplify the intron I region. Universal primers AS1II and Amy C54 (Ushijima et al., 1998) were used for amplification of intron II. Intron II lengths for S^a-S^d were previously reported by Ushijima et al. (1998).

	Intron I (bp)	Intron II (bp)	Cultivar
S ^a	807	84	Mission
S ^b	797	556	Mission
S ^c	275	1526	Nonpareil
S ^d	327	>3500	Nonpareil
S ^e	276	328	Peerless
S ^f	801	561	Tuono
S ^g	302	832	Sonora
S ^h	215	129	Padre
S ⁱ	317	710	UCD 25-75
S ^j	1075/806 ²	783	Winters

²Intron size has not been definitively determined.

allele based on intron size differences (Table 4). Each allele has two introns that vary in length, allowing for easy detection and confirmation. Additionally, all S-alleles have the same structural sequence motifs identified for the original four S-alleles (S^a, S^b, S^c, S^d) (Tamura et al., 2000; Ushijima et al., 1998). According to Matton et al. (1997), the HV region is required for allelic specificity and for the pollen rejection mechanism. The aligned amino acid sequences show the HV region, which is interrupted by intron II, as being highly variable among S-alleles. Therefore, amino acid changes within conserved regions should not have a negative effect on the ability to recognize "self" pollen. Sequences at the 5' end have been analyzed for almost all of the S-alleles, resulting in the identification of the signal peptide, an additional intron (intron I), and the start of the mature protein. The structure as identified by the amino acid comparisons remains similar among the 10 analyzed S-alleles (Fig 1). The S^f allele cloned in this study is derived from 'Tuono' and has an amino acid sequence identical to the S^b allele. While our data show that the exons are the same, the introns differ in size (Table 4), indicating that S^f differs from S^b. However, if the HV region is responsible for allele specificity, then S^f should be recognized as S^b as the HV regions are the same. The presence of only one allele, or one self-compatible allele, however, does indicate self-compatibility. In a self-compatible system, pollen is not recognized as "self," and therefore pollen tube growth is not terminated. The cultivar Tuono expresses the S^f allele, a self-compatible allele (Ma and Oliveira, 2001), which has previously been shown to have the genotype S₁S^f (Bošković et al., 1997; Sanchez-Perez et al., 2004). However, according to our data, 'Tuono' does not express an Sⁱ allele.

Historically, the European S-alleles are designated with numbers, while the California S-alleles are designated with letters. When the European alleles listed in GenBank were compared with the lettered California alleles, the exon sequences showed almost complete homology. The intron I size of European almond S-alleles was determined by Ortega et al. (2005). When compared with California S-alleles, the intron I lengths were found to be significantly different. Intron II lengths also differed (Channuntapipat et al., 2001; Tamura et al., 2000). Additionally, Ma and Oliveira (2002) identified S^g, S^h, Sⁱ, S^j, and S^k alleles in European almond cultivars. When the sequences were compared with the sequences from California almonds (data not shown), the majority of homology occurred within the conserved regions. The Sⁱ allele had the least amount of homology (67%) between the European and California almonds, while the Sⁱ allele had the most

Fig. 1. A multiple alignment of the S-RNase protein sequences obtained from different California almond cultivars. The S-RNase alleles are listed alphabetically in the left hand column. The aligned sequences were obtained from the following almond cultivars 'Mission' (S_a and S_b), 'Nonpareil' (S_c and S_d), 'Peerless' (S_e), 'Tuono' (S_f), 'Sonora' (S_g), 'Padre' (S_h), 'UCD 25-75' (S_i), 'Winters' (S_j). The amino acid sequences were aligned using the program Bioedit (Ibis Therapeutics, Carlsbad, Calif.), that uses Clustal W to align the sequences. Homology between alleles is shown by shading. The conserved areas are boxed and labeled C1-C5 and the hypervariable region is boxed and labeled HV. The signal peptide region occurs prior to the start of the mature protein and was determined based on 5'-RACE sequence identification. Intron I occurs just upstream of the mature protein and intron II intersects the HV region. The intron I sequences were determined from a specific PCR of the 5' region and the intron II sequences were determined using universal primers (Ushijima et al., 1998). The exact positions of the introns and the start of the mature protein were based on DNA sequence, start codon locations, and N-terminal sequence as described by Tao et al. (1997).

Fig. 2. Construction of a pairwise distance tree of California almond S-RNases based on an amino acid alignment with Clustal X. Almond cultivars from which the sequences were determined are the same as those previously mentioned in Fig. 1. The tree and bootstrap analysis were constructed using PAUP* version 4.0. Sequences from *Malus domestica* served as an out-group. Four pairs (S_c and S_d, S_e and S_f, S_g and S_h, and S_i and S_j) segregated out, indicating high homology between them relative to the other S-alleles. S_i was the only S-allele that was not closely linked with any other S-allele, while the S_a allele was perhaps a progenitor S-allele from which all others were derived, as it is an outlier.

homology (81%). Additionally, the S_f allele, cloned from 'Tuono' by Ma and Oliveira (2001) differs in sequence from the S_f allele isolated in this study using the same cultivar. Therefore, it can be said that the alleles found in European and Californian cultivars are similar but not identical.

CROSS-INCOMPATIBLE GROUPS (CIGs). This study added 15 CIGs to the six previously identified by Kester et al. (1994). The original CIGs were determined through cross-pollinations and zymograms (Kester et al., 1994). The genotypes of 'Ruby', 'Aldrich', and 'Pearl' (cultivars also analyzed in this study) were previously determined using zymograms (Bošković et al., 1997, 1999, 2003; Channuntapipat et al., 2001). Due to the difference in methods, discrepancies have arisen between previous genotype designations (Bošković et al., 1997, 1999, 2003; Channuntapipat et al., 2001; Kester et al., 1994) and the genotypes/CIGs assigned in this research. Previously the genotype designations as determined by Bošković et al. (1997, 1999, 2003), Channuntapipat et al. (2001), and Kester et al. (1994), were 'Ruby' (S₆S₁₆), 'Aldrich' (S₇S₁₇), and 'Pearl' (S₇S₁₆). The CIGs designated in this study ['Ruby' (S_bS_e), 'Aldrich' (S_bS_c), and 'Pearl' (S_bS_c)] were a product of a multidimensional analysis using PCR to genotype the almond cultivars, followed by sequencing of the alleles (cDNA and gDNA) for specific confirmation. Our CIGs are consistent with the observations at the Univ. of California almond breeding program (directed by T. Gradziel) and several individuals in the California almond industry. Intron I amplification was used for additional confirmation. The PCR and

sequencing method gave clear reproducible results. These methods have been used to determine S-alleles in such systems as sweet cherry (*Prunus avium* L.), sour cherry (*Prunus cerasus* L.), and apricot (*Prunus armeniaca* L.) (Hauck et al., 2002; Sonneveld et al., 2001; Tao et al., 2002; Wunsch and Hormaza, 2004). Because of its simplicity, reproducibility, and ease of analysis, PCR is the preferred method for genotyping S-alleles.

PAIRWISE DISTANCE TREE. Ushijima et al. (1998) compared S-RNases from different *Rosaceae* species in terms of their evolution. Our pairwise distance tree breaks down the larger picture by examining the relationships between individual S-alleles. Four pairs of alleles (S^c and S^e , S^g and S^h , S^d and S^i , and S^b and S^f) have very few nucleotide substitutions, signifying that they are closely related. Ma and Oliveira (2002) formulated a phylogenetic tree showing the evolutionary relationships between S-alleles in various *Rosaceae* species. The relationships between S-alleles as determined by Ma and Oliveira (2002) do not correlate with our results. Additionally, Ma and Oliveira (2002) added additional sequences from other species. When the almond S-allele sequences were aligned and put into a pairwise distance tree using the same method for constructing our tree, a different output resulted (data not shown). The tree differed from the published tree, which was expected, but it also differed from our pairwise distance tree. The differences between the European S-allele tree we constructed and the tree in this manuscript are partly due to the differences in sequences used. The pairwise distance tree constructed from our amino acid alignment was based on full-length sequences whereas the sequences listed by Ma and Oliveira (2002) are only partial sequences and are not 100% homologous with the sequences aligned in this manuscript. Due to these variances, different clustering of alleles was expected.

Conclusion

This research expanded the number of known S-alleles in California almond cultivars and also increased the number of CIGs. PCR-based genotyping of almond cultivars remains the preferred method to determine their cross-compatibility. Until future research can expand our knowledge of the self-incompatibility system, genotyping of almond cultivars is the ideal way to determine the compatibility of cultivars.

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