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Determination of almond S-alleles using PCR primers designed from their introns

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SUMMARY – Almond (*Prunus dulcis* Mill.) has numerous S-alleles and therefore many combinations of incompatibility groups. The identification of these groups is important for designing crossing matrices for breeding, selecting progeny and testing for hybridity. This work describes a novel molecular technique for the identification of S-alleles in almond using PCR primers designed from the sequences of the introns without the need for restriction enzyme digestion. Thirteen specific pairs of primers have been designed for the S_1 , S_2 , S_3 , S_4 , S_5 , S_6 , S_7 , S_8 , S_9 , S_{10} (putative), S_{11} , S_{23} , and S_I alleles. The S_{23} allele has been detected in some old South Australian almond selections, and is possibly derived from an early importation of the cultivar 'Ramillete' from Spain. This technique provides a quick and precise method for predicting incompatibility alleles from the genomic DNA of almond cultivars. The practical use of this new technique in the Australian almond breeding programme is presented here.

Key words: Rosaceae, *Prunus amygdalus*, progenies, self-incompatibility, S-allele sequences.

RESUME – "Détermination des allèles S de l'amandier en utilisant des amorces PCR conçues à partir d'introns". L'amandier (*Prunus dulcis* Mill.) a de nombreux allèles S et donc beaucoup de combinaisons des groupes d'incompatibilité. L'identification de ces groupes est importante pour concevoir des schémas de croisement pour multiplier, sélectionner la descendance et déterminer l'hybridité. Ce travail décrit une technique moléculaire novatrice pour l'identification des allèles S chez l'amandier à l'aide d'amorces PCR conçues à partir de séquences d'introns sans besoin de digestion d'enzymes de restriction. Treize paires spécifiques d'amorces ont été conçues pour les allèles S_1 , S_2 , S_3 , S_4 , S_5 , S_6 , S_7 , S_8 , S_9 , S_{10} (putatif), S_{11} , S_{23} , et S_I . L'allèle S_{23} a été détecté dans quelques vieilles sélections d'amandier d'Australie du Sud, et est probablement dérivé d'une importation antérieure du cultivar 'Ramillete' d'Espagne. Cette technique fournit une méthode rapide et précise pour prévoir des allèles d'incompatibilité de l'ADN génomique des cultivars d'amandier. L'utilisation pratique de cette nouvelle technique dans le programme australien d'amélioration de l'amandier est présentée ici.

Mots-clés : Rosaceae, *Prunus amygdalus*, descendance, auto-incompatibilité, séquences d'allèles S.

Introduction

The Australian almond breeding programme combines investigations of molecular techniques with the traditional approach of controlled hybridisation and selection. Molecular techniques include the development of markers linked to important traits such as self-incompatibility, and disease resistance to bacterial spot and anthracnose. Self-fertility has been assigned to the allele S_I (Grasselly and Olivier, 1976), and breeding for this trait is a major focus of the Australian almond breeding programme. According to Vargas *et al.* (1998) there are around 30 self-fertile almond cultivars worldwide, although none is widely cultivated. Within the breeding programme, 'Nonpareil' is the main parent because of its high kernel quality, although, as is the case with most other cultivars, it is self-incompatible and relies on cultivars of a different incompatibility group for cross-pollination. Knowledge of the S-genotypes of almond is important both for designing crosses for breeding and for selecting progeny. Cross-compatibility can be determined by the time consuming and laborious methods of fruit set or the observation of pollen tube growth in pistils. The primary aim of this study was to overcome these disadvantages by designing primers from the intron sequences of specific S RNases to aid in identification of self-incompatibility groups.

Materials and methods

Leaves of almond cultivars were obtained from Australia and the Middle East (Woolley *et al.*, 2000), and the Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Mas Bové, Spain. Many of these had only one or neither of the *S* allele identified. Genomic DNA was extracted from leaves using the technique of Mekuria *et al.* (1999) and the partial sequences of the *S*-alleles were amplified, cloned and sequenced using the techniques described in Channuntapipat *et al.* (2001). Primers were designed from the intron sequences for the *S*-alleles as reported by Channuntapipat *et al.* (2001, 2002, 2003). Sequences were aligned with Clustal X (Thompson *et al.*, 1997), and edited with BioEdit v. 4.8.1 (North Carolina State University; Hall, 1999).

Results

The partial sequences for the *S*₁, *S*₇, *S*₈, *S*_f (Channuntapipat *et al.*, 2001), *S*₂, *S*₉, *S*₁₀ (putative), *S*₂₃, (Channuntapipat *et al.*, 2002), *S*₃, *S*₄, *S*₆, and *S*₁₁ alleles from genomic DNA were 1072, 2019, 2823, 1205, 790, 1829, ~1200, 983, 1198, 918, 844, and 688 bp respectively (Fig. 1). Genbank accession numbers appear in Table 1. Figure 2 shows the homologous regions when these twelve partial sequences are aligned. The partial sequences of exons varied from 483 to 615 bp, and showed between 72.39 and 88.95% homology. There was very little homology between the introns. For each of the alleles examined, the sequences of the intron/exon splice junction regions followed the GT/AG consensus sequence rule (Thangstad *et al.*, 1993), and the sequences adjacent to the splice junction were highly conserved (Fig. 2).

The primers confirmed the known alleles from almond cultivars and identified some allele compositions previously unknown. Table 2 shows the results of testing all of the primers against the Australian cultivars and some European cultivars. The allele, *S*₂₃ was identified in seven Australian cultivars: 'Chellaston', 'Johnston's Prolific', 'Parkinson', 'Pethick's Wonder', 'Pierce', 'Somerton' and 'White Brandis'.

Table 1. Genbank accession numbers for *S*-alleles. Reproduced with permission from McComb J.A. (Ed.), 'Plant Breeding for the 11th Millennium'. Proceedings of the 12th Australasian Plant Breeding Conference, Perth W. Australia; (Australasian Plant Breeding Association Inc.), 2002

S-allele	Number	Isolated separately from
<i>S</i> ₁	AY061960	'Ferragnès'
<i>S</i> ₁	AF510421	'Ne Plus Ultra'
<i>S</i> ₂	AF454000	'Anxaneta'
<i>S</i> ₂	AF510416	'Cristomorto'
<i>S</i> ₃	AF490505	'Aï'
<i>S</i> ₃	AF510417	'Ferragnès'
<i>S</i> ₄	AF490506	'Aï'
<i>S</i> ₄	AF510418	'Ferraduel'
<i>S</i> ₆	AF487914	'Bertina'
<i>S</i> ₆	AF510419	'Ramillete'
<i>S</i> ₇	AF444787	'Nonpareil'
<i>S</i> ₇	AF510420	'Ne Plus Ultra'
<i>S</i> ₈	AF444788	'Nonpareil'
<i>S</i> ₉	AF454001	'Primorskiy'
<i>S</i> ₁₀	AF454003	'Gabaix'
<i>S</i> ₁₁	AF487915	'Bertina'
<i>S</i> ₁₁	AF510415	'Rumbeta'
<i>S</i> ₂₃	AF454002	'Ramillete'
<i>S</i> _f	AF444786	'IRTA 12-2'
<i>S</i> _f	AF510414	'Lauranne'

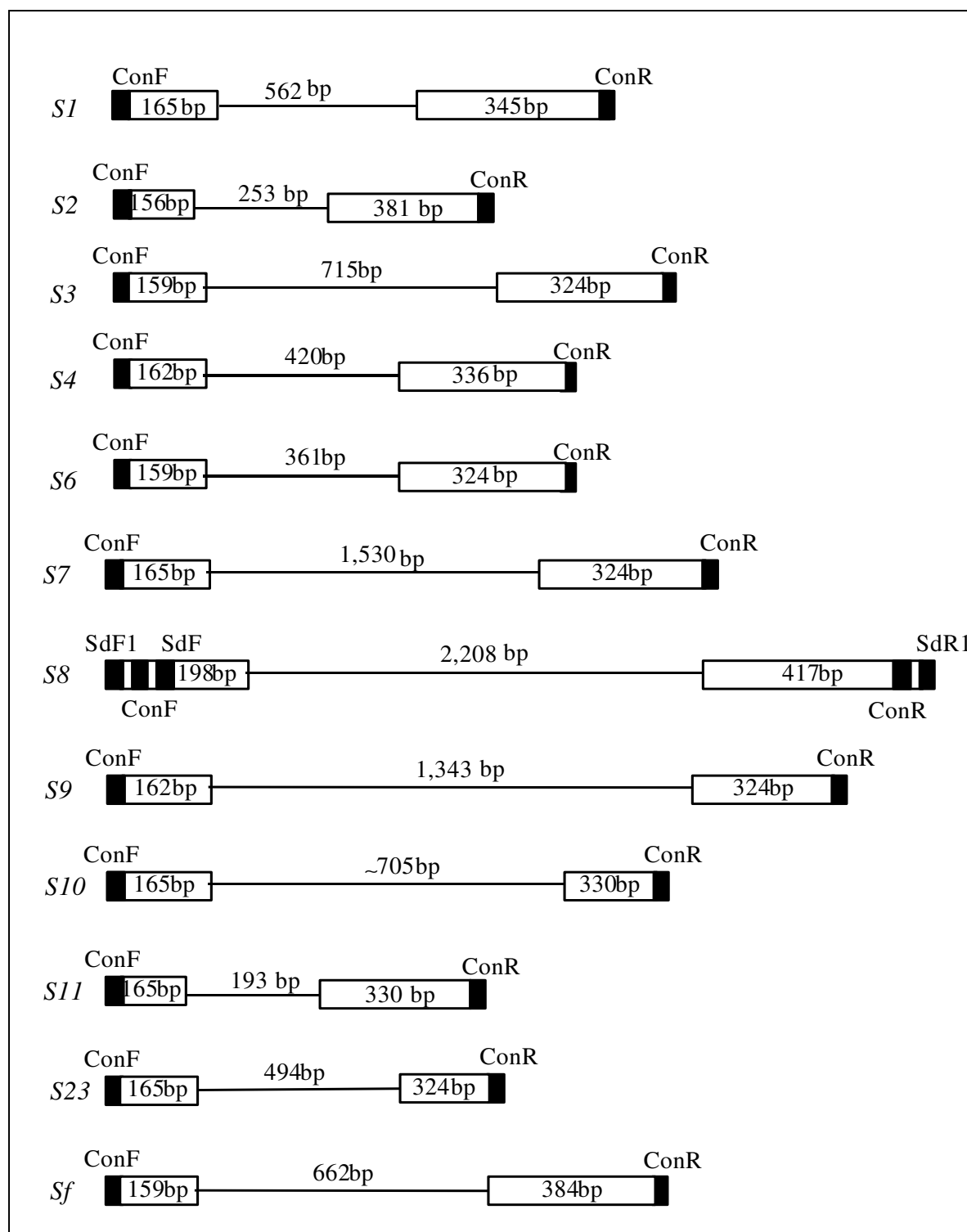


Fig. 1. Scheme of structures of PCR-amplified products of genomic DNAs and cDNAs of twelve *S*-alleles from almond, *S*₁, *S*₂, *S*₃, *S*₄, *S*₆, *S*₇, *S*₈, *S*₉, *S*₁₀, *S*₁₁, *S*₂₃, and *S*_f. Introns are represented by the bars between two boxes which are the exons. Reproduced with permission from McComb J.A. (ed.), "Plant Breeding for the 11th Millennium". Proceedings of the 12th Australasian Plant Breeding Conference, Perth W. Australia; (Australasian Plant Breeding Association Inc.), 2002.

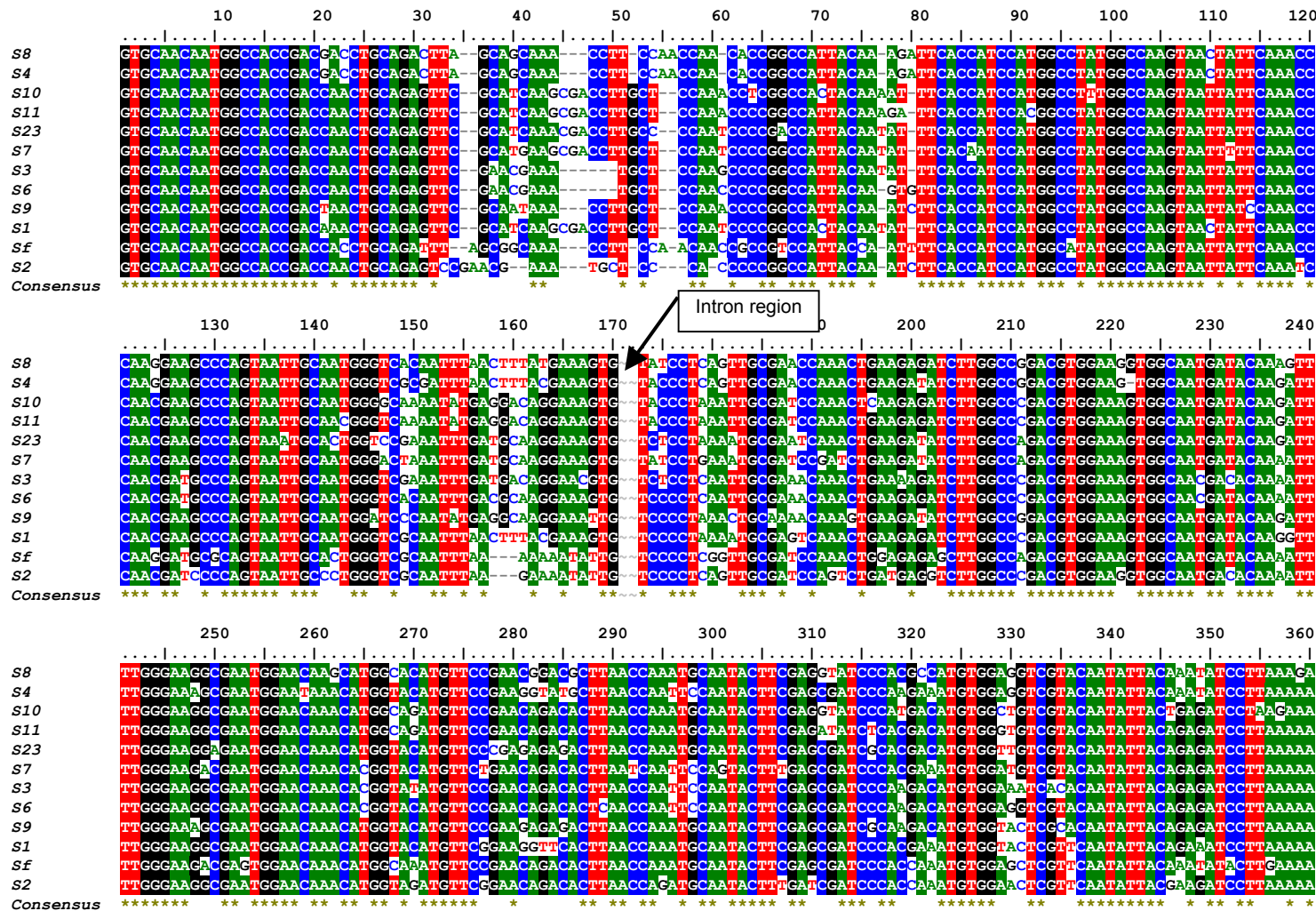


Fig. 2. Alignment of partial sequences of 12 *S*-alleles from almond, *Prunus dulcis*. Reproduced with permission from McComb J.A. (ed.), "Plant Breeding for the 11th Millennium". Proceedings of the 12th Australasian Plant Breeding Conference, Perth W. Australia; (Australasian Plant Breeding Association Inc.), 2002.

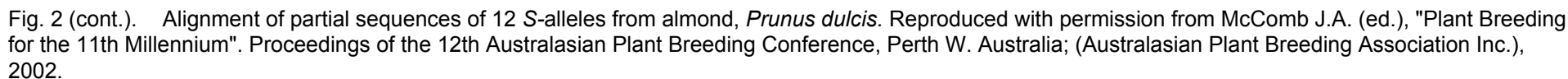


Table 2. S-allele identification of almond cultivars using specific primers

Cultivar	Reported alleles	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{23}	S_f
'Anxaneta'	S_2S_9	—	√	—	—	—	—	—	—	√	—	—	—	—
'Ferraduel'	S_1S_4	√	—	—	√	—	—	—	—	—	—	—	—	—
'Ferrastar'	S_2S_{10}	—	√	—	—	—	—	—	—	—	√	—	—	—
'Lauranne'	S_3S_f	—	—	√	—	—	—	—	—	—	—	—	—	√
'Ramillete'	S_6S_{23}	—	—	—	—	—	√	—	—	—	—	—	√	—
'Baxendale'	Unknown	—	—	—	—	√	—	√	—	—	—	—	—	—
'Chellaston'	Unknown	—	—	—	—	—	—	√	—	—	—	—	√	—
'Johnston's Prolific'	$S_{23}S_7$	—	—	—	—	—	—	—	—	—	—	—	√	—
'Keanes Seedling'	Unknown	—	—	—	—	—	—	√	—	—	—	—	—	—
'McKinlay's'	Unknown	—	—	—	—	—	—	√	√	—	—	—	—	—
'Parkinson'	Unknown	—	—	—	—	—	—	—	—	—	—	√	√	—
'Pethick Wonder'	Unknown	—	—	—	—	—	—	—	—	—	—	—	√	—
'Pierce'	$S_{23}S_7$	—	—	—	—	—	—	—	√	—	—	—	√	—
'Somerton'	Unknown	√	—	—	—	—	—	—	—	—	—	—	√	—
'Strout's Papershell'	Unknown	—	—	—	—	—	—	—	—	—	—	√	—	—
'White Brandis'	Unknown	—	—	—	—	—	√	—	—	—	—	—	√	—

Discussion

Determination of the sequences of S-RNase genes is useful for studying the system of gametophytic self-incompatibility in almonds. We have designed PCR primers from the sequences of the introns for 13 different S-alleles in almond. In particular the primer for S_f has been used to screen progeny from a self-incompatible x self-fertile cross to identify self-fertile seedlings. Many Australian cultivars are of unknown self-incompatibility groups and these primers have helped identify their S-alleles which is essential in breeding programs. All Australian cultivars originated from either European or American cultivars (Woolley *et al.*, 2000). The S_{23} allele was identified in many Australian cultivars. This allele was also identified in the Spanish cultivar 'Ramillete' (S_6S_{23}). We have been unable to determine the parents of 'Ramillete' at this stage, and therefore it is possible that 'Ramillete' or one of its parents was an early introduction to South Australia. In addition to their use in the identification of self-incompatibility groups, the sequences of the S-alleles determined in this study will be used to overcome self-incompatibility in the major commercial cultivars via transformation (Ainsley *et al.*, 2001). This study has produced a novel technique, which uses two specific primers, designed from the introns of each S allele to amplify genomic DNA without the need for restriction enzyme cutting. It is a simple and efficient method to determine the incompatibility groups of almond.

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