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Molecular identification of S-alleles associated with self-incompatibility in apple (*Malus* spp.) genotypes

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ABSTRACT

Gametophytic self-incompatibility, governed by the S-locus in apple (*Malus* spp.) plays a vital role for pollination and fruit set. The identification and cloning of RNases has enabled the use of molecular techniques to characterize S-genotypes in apple cultivars. To identify the S-alleles associated with self-incompatibility, allele specific primers were tested using PCR, evaluating eight apple genotypes. A total of 6 pollen incompatibility groups in apple genotypes were identified among eight accessions by PCR based S-allele typing analysis. Eight putative S-alleles (S1, S2, S7, S19, S21, S23, S24 and S26) were identified with S1S7, S1S23, S1S24, S2S26, S19S24 diploid and S1S21S24 triploid combinations that had not previously been identified from apple cultivars. The molecular allele typing system of S-genotypes based on PCR is a useful and rapid method for identifying new S-alleles and incompatibility groups in apple and the present results enabled the characterization of eight apple cultivars with respect to S-allele composition which is an important and preliminary step for pollination management and hybridization in apple breeding programmes.

Key words: Allele typing, Apple, Incompatibility, S-allele, Pollination management

Most of the cultivated apple (*Malus* spp.) varieties exhibit self-incompatibility and cross incompatibility mechanism which inhibits fertilization following self-pollination and cross pollination from similar S-allelic genotype. Self-incompatibility in apple is of gametophytic type and is controlled by a single multiallelic locus named the S-locus (Broothaerts *et al*. 2004). In the gametophytic SI system in apple one gene residing at the S-locus is known, i.e. the S-gene, which encodes a family of ribonucleases in the pistil. These S-RNases specifically interact with a component in the male partner, encoded by an as yet unknown gene residing at the same S-locus, and presumably acting as an inhibitor of all non-corresponding S-RNases (Golz *et al*. 2001). The recognition between the allelic products of the S-locus genes determines whether or not further pollen-tube growth is arrested in the style. The basic model for the RNase-mediated self-incompatibility system proposes that S-RNase is secreted from the style cells and enters the pollen tube. The RNase's cytotoxicity (degrading the pollen's RNA) halts pollen growth and fertilization is prevented. However, in cross-fertilization, the S-RNase is recognized by the SFB protein, leading to its degradation; as a result, fertilization occurs. The S-RNase gene is exceptional in that it comprises hundreds of alleles (genetic forms), tens of them per species. Thus, most cultivars differ in their content of S-RNase alleles and this variation enables their molecular typing. Full genetic compatibility occurs when the cultivars differ in both of their S-loci, semi-compatibility when they differ in one of their two S-loci, and full incompatibility when both cultivars carry the same S-genotype.

For self incompatibility studies methods like controlled pollination tests, pollen tube growth tests, stylar ribonuclease detection have been used but these methods are not reproducible and reliable. The controlled pollination and pollen tube growth tests are unable to distinguish compatible from incompatible genotypes due to interference by physiological and environmental factors (Tromp and Borsboom 1994). With the RNase tests, it is not clear when RNase activity is actually associated with Sallele products (Choi *et al*. 2002). Furthermore, in genetic enhancement programs, these methods are inefficient because they require trees that are flowering in order to collect the test material. Therefore, the use of molecular markers to detect self-incompatible genotypes at the early stages of selection would increase efficiency during individual selection (Broothaerts 2003, Badeness *et al.* 2000), taking to the final evaluation stages plants that fulfill the aims of the program and the method is reproducible with high level of efficiency.

In present study, S-allele specific primers were used to identify compatibility nature in eight apple genotypes

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Table 1 S-genotyping of apple cultivars determined by S-allele specific PCR analysis

Cultivar/Accession Number	S-genotype
Carla/EC-539446	S19S24
Carpendoo Bianco/EC-539447	S1S21S24
Contessa/EC-539449	S1S7
EC-539452	S19S24
Malus robusta	S1S23
Winter Commercial	S1S23
Benoni	S1S24
Jonica	S2S26

whose S-allele composition was unknown with the aim of using the knowledge of this finding to propose their possible use as parents in breeding programs and for pollination management strategies during the layout of an orchard.

MATERIALS AND METHODS

Fresh and tender leaves of eight apple genotypes (Table 1) were harvested during the growing season from field grown apple trees from the field gene bank of Central Institute of Temperate Horticulture, Rangreth, Srinagar, J & K, India. Collected leaves were processed immediately in liquid nitrogen. Approximately 0.2 g of fresh leaves was used for total genomic DNA extraction using DNA extraction kit (Genei). DNA purity and concentration was measured using nanodrop (Thermo).

Allele-specific PCR amplification was done using S-allele specific primers (Table 2) on thermal cycler (Takara, Japan), programmed as: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and finally 7 min at 72 °C, followed by cooling to 4 °C. Standard PCR conditions (in 20 ìl total volume) included 1 × PCR buffer (Fermentas), 1.25 mM of $MgCl_2$, 200 ìM dNTPs, 1 ìM of each primer, and 0.5 U of Taq DNA polymerase (Fermentas). Under these conditions, the amplification appeared sufficiently selective for the S-allele assayed, although some primer pairs could work at different annealing temperatures. Self-incompatibility-associated alleles were detected by analyzing the presence or absence of the band associated with S-alleles present in agarose gels (Sigma Aldrich). DNA ladder (Genei-100bp and 50bp) was used for measuring the exact loci size (Broothaerts 2003).

RESULTS AND DISCUSSION

Apple crop improvement through conventional breeding approaches depends on utilization of compatible partners during the hybridization. In apple inspite of vast studies on S-allele genotyping (Janssens *et al*. 1995, Verdoodt *et al*. 1998, Nerum *et al*. 2001, Sakurai *et al*. 1997, Matsumoto and Kitahara 2000), most of the cultivars still remain uncharacterized with respect to allelic composition. In the present study those cultivars were selected whose S-allele combination was not known hence were not fully exploited in our breeding programmes. The selected cultivars possess some traits of interest like early maturity (Benoni), scab tolerance (*Malus robusta*, Contessa and Carpendoo Bionca), blended (sourness and sweetness) fruit taste (Jonica) etc but their utilization in transfer of traits to other cultivars depends on the compatibility status. Therefore, the aim of present study was to decipher the allelic combinations of selected apple cultivars. PCR analysis was conducted using the S-allele consensus primer sets for determination of compatibility among eight selected apple cultivars. The S-allele composition of these selected apple cultivars was previously unknown. A total of seventeen primers (Broothaerts 2003) were used out of which only eight primer combinations (Table 2) gave amplification in selected apple cultivars. Among eight cultivars, seven cultivars, viz. *Malus robusta*, Winter Commercial, Benoni, Jonica, Contessa, EC-539452 and Carla were found diploid and one cultivar (Carpendoo Bionca) was found triploid in nature (Table 1 and Table 3). Eight putative S-alleles (S1, S2, S7, S19, S21, S23, S24 and S26) were identified among the selected cultivars. Seven cultivars were found diploid in nature with allelic composition of S1S7 (Contessa), S1S23 (*Malus robusta* and Winter Commercial), S1S24 (Benoni), S2S26 (Jonica), S19S24 (Carla and EC-539452) and one cultivar was found triploid with three alleles S1S21S24 (Carpendoo Bionca). The allelic composition of these cultivars was previously unknown; therefore present study will be used for utilization of these genetic resources for crop improvement programmes through hybridization. These studies also help in pollination management because orchard layout is done with proper distribution of compatible pollinizers. The cultivated apple is a functional diploid (2n=34) (Ryugo 1988) although it is frequently present as triploids (2n=3x=51). Triploids are not suitable as pollinizers because either their

Table 2 Primers used for S-allele identification of a germplasm collection of eight apple cultivars (Broothaerts 2003)

Loci name	Primer name	Sequence (5'-3')	Amplicon size (bp)
S1	MdS1Sp	TGTAAGGCACCGCCATATCATACCAACCTCAACCAATTCAGTCAATGA	700
S2	MdS2Sp	AACATGAATCGAAGTGAATTATTTATTGAGGTTTGGTTCCTTACCATG	450
S7	MdS7Sp	AGTAAATCAACCGTGGATGCTCAGTTACAATATCTACCTGTTTCCTGGG	300
S19	MdS19Sp	GCCTTCAAACAAGAATGGACCTCAATATCCACCAATGACCTGTT	500
S21	MdS21Sp	AAGTAATTGCCCGATAAGGAACATAAGTTTATGAAATGTTCTCCGCTGTA	250
S23	MdS23Sp	AAGAATACAACCATTACGCCTCAGCATTGTTGGTACTAATGCTTATGGCG	450
S24	MdS24Sp	ATGGCTCCTGTGCGTCTTCCCCGTCATCCGTGTATAGGGCAACT	400
S26	MdS26Sp	TCCATCAAACGTGACTTCTCATATCCTTCAGCATCCTGATTCG	450

Table 3 S-allele distribution and incompatibility grouping in apple genotypes

S-alleles	S1	S2	S7	S19	S21	S23	S24	S26
S1			EC-539449		EC-539447^{S24}	*M. robusta;* Winter Commercial	Benoni; EC-539447^{S21}	
S2								Jonica
S7	EC-539449							
S19							EC-539446; EC-539452	
S21	EC-539447^{S24}							
S23	*M. robusta;* Winter Commercial							
S24	Benoni; EC-539447^{S21}			EC-539452; EC-539452				
S26		Jonica						

pollen are not viable or may lead to fruit disorders. Pollen viability of triploid cultivars, such as "Jonagold" and "Mutsu," is low since triploids have 51 chromosomes and the chromosomes are unequally divided during meiosis (Matsumoto 2014). In our study we observed one triploid cultivar Carpendoo Bionca (S1S21S24). The utilization of this cultivar for breeding programmes will be restricted because it may lead to failure of fertilization or development of malformed fruits. Although, this cultivar possess some degree of scab tolerance but the transfer of such trait will be difficult. Among diploid cultivars, *Malus robusta* and Winter Commercial with S1S23 combination and EC-539452 and EC539446 with S19S24 combination are fully incompatible because they posses similar allelic composition, Contessa, Benoni, *Malus robusta* and Winter Commercial are partially compatible due to presence of common S1 allele, Benoni and Carla are partially compatible due to common S24 allele. Triploid cultivar Carpendoo Bionca will show different degrees of semicompatibility, because diploid pollen can have more than one *S*-allele. Carpendoo Bionca shares S1 allele with Contessa, *Malus robusta*, Winter Commercial and Benoni, and allele S24 with Benoni, Carla and EC-539452. Therefore different levels of incompatibility will be shown by Carpendoo Bionca with other diploid cultivars sharing one or two S-alleles with cultivar Carpendoo Bionca and more incompatibility chances are with cultivar Benoni (S1S24) because two alleles are common with this cultivar. Cultivar "Jonica" is fully compatible with all other cultivars with both its alleles "S2S26" different to other cultivars. Therefore, cultivars "Jonica" is the universal pollinizers for other cultivars and can be efficiently utilized for breeding programmes for transfer of its traits.

Successful fertilization following pollination between compatible pollen and ovule of self or other plant is an essential step during apple breeding and cultivation. The prior knowledge about the status of compatibility between cultivars to be planted in an orchard or crossed for hybridization programmes is key for the success. S-allele combinations that result in incompatibility, semi-compatibility and full-compatibility need to be ascertained before proceeding for any breeding programme in apple. The use of molecular markers for ascertaining such phenomenon makes the pre-selection possible and is promising in the self-compatibility breeding program.

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