Fruit Research

ARTICLE

https://doi.org/10.48130/FruRes-2021-0010
Fruit Research **2021**, 1: 10

A high-throughput *S-RN*ase genotyping method for apple

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Abstract

Knowledge of the genotypes for the self-incompatibility locus (*S*-locus) in apple varieties and in genotypes being used as parents is critical for breeding and commercial production. We present a high-throughput set of molecular markers for the identification of 13 common *S-RN*ase alleles (S_1, S_2, S_3, S_5, S_7, S_8, S_9, S_{10}, S_{20}, S_{23}, S_{24}, S_{25} and S_{28}). This set is composed of seven allele-specific quantitative PCR-based High-Resolution Melting assays and four multi-allelic SSR markers. Validation of these markers was performed using 86 apple accessions, including cultivars with known *S*-genotypes and recent commercial varieties arising from the Plant & Food Research (PFR) cultivar breeding programme. We also characterized the *S*-genotypes of 183 genotypes representing some of the most valuable parents within PFR's cultivar breeding programme. The results of this work demonstrate the practical usefulness of this marker set to provide accurate cross-compatibility information to optimise choice of pollenisers in commercial apple orchard design, and to identify compatible parents and guide parental selection when executing apple breeding programmes, to optimise fruit crop yield and quality.

Citation: López-Girona E, Bowatte DR, Smart MEM, Alvares S, Brancher TL, et al. 2021. A high-throughput *S-RN*ase genotyping method for apple. *Fruit Research* 1: 10 https://doi.org/10.48130/FruRes-2021-0010

INTRODUCTION

Apple (*Malus domestica* Borkh.) fruit are the final products of the fusion between two gametic nuclei: the male pollen grain and the female egg-cell. After the pollen grain is deposited onto the flower's stigma, it germinates and grows a pollen tube down the style to fertilize the egg-cell. The pollen can come from the same plant or from a different one. However, a broadly distributed, pre-zygotic and genetic mechanism called gametophytic self-incompatibility (GSI) prevents the self-fertilization of closely related individuals, promoting out-crossing and thereby maintaining genetic diversity. GSI is found in many angiosperms, including the *Solanaceae*, *Rosaceae*, and *Plantaginaceae* families[1,2].

Apple has a homomorphic GSI mechanism where inhibition of self-fertilization occurs through genetic or biochemical mechanisms that function regardless of flower morphology[3] operating in a reproductive system, which has two different and tightly linked components (*S*-genes). One is located in thepistilandtheotherisspecifictothepollen[1,4].Thepistilcomponent is an extracellular ribonuclease (*S-RN*ase) that inhibits self-pollen tube growth[5]. The pollen-specific component is controlled by multiple genes called *SFBB*s (i.e. *S*-locus F-box brothers) that interact with the *S-RN*ase in an allele-specific way[6]. Both components work in a collaborative manner to control the single, multi-genic and multi-allelic *S*-locus. Each *SFBB* interacts with *S-RN*ases, such that non-self-RNases are degraded allowing pollen tube growth[7] (Fig. 1). The selective pressures underlying this collaborative recognition mechanism generate a lower diversity of the *S*-pollen genes than is found on the *S*-pistil locus, which shows a higher degree of allelic polymorphism[8]. However, these multi-genic *S*-haplotypes are inherited as single segregating units keeping their functionality across generations[9,10].

Apple breeding relies on compatible and productive cross-pollination. A breeder needs information about the self-incompatibility genotypes (*S*-genotypes) of both parents to execute successful crosses and facilitate the selection of individuals carrying a combination of desirable traits. Traditionally, incompatibility was determined using time-consuming cross-pollination experiments, where successful fruit set was measured over many combinations of parents. Recently, less time consuming and more cost-effective molecular markers have been implemented to replace such field experiments, using either allele-specific markers amplifying a single *S-RN*ase allele[11–18] or markers based on restriction enzyme digestion of polymerase chain reaction (PCR) products (cleaved amplified polymorphic sequences CAPS or PCR-RFLP). Simple Sequence Repeat (SSR) markers have also been used for screening *S*-alleles[14,15,19]. CAPS and SSR markers have helped determine most of the *S*-genotypes for common commercial apple varieties. However, most of the existing assays involve the use of restriction enzymes after PCR reactions and the visualization of the products on agarose gels, making them very laborious and time-consuming when handling large numbers of samples. This can be problematic evidenced when new seedlings/selections, that are potential parents in a breeding programme, need to be checked for compatibility each season.

The objective of this study was to develop and validate a

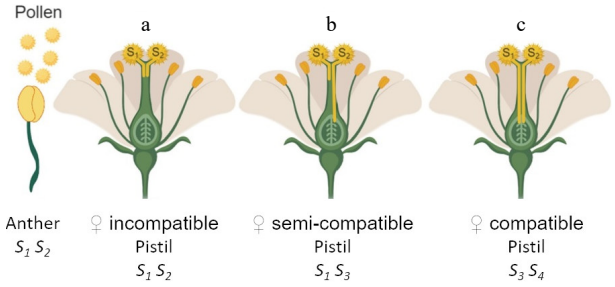

Fig. 1 Genetic control of gametophytic self-incompatibility (GSI) in *Malus*. The *S*-locus is composed of two tightly linked components, found in the pollen and pistil respectively. In GSI, the pollen self-incompatibility phenotype is controlled gametophytically, i.e., the genotype of the haploid pollen itself (gametophyte) determines its incompatibility type. For example, the pollen composition of a certain pollen donor plant is phenotypically half S_1 and half S_2. In the female parent, two alleles are co-dominant and both are expressed in the pistil. Pollen inhibition occurs when there is a match between the donor pollen *S*-haplotype and either of the two haplotypes present in the pistil, producing an incompatible reaction that inhibits the growth of the 'self' pollen tube growth. Three types of reactions can occur during a cross: (a) incompatible; neither of the two gametes will germinate, (b) semi-compatible; half the donor pollen will be inhibited and the other half will germinate and grow normally, (c) compatible; all pollen will germinate and grow normally.

high-throughput and practical method to identify 13 different apple *S-RN*ase alleles (S_1, S_2, S_3, S_5, S_7, S_8, S_9, S_{10}, S_{20}, S_{23}, S_{24}, S_{25} and S_{28}). Our method is composed of seven allele-specific High-Resolution Melting (HRM) and four multi-allelic SSR markers, which do not require post-PCR restriction enzyme digestions or agarose gels for allele-scoring analysis. We used as controls 70 out of a total of 86 commercial apple cultivars for which *S*-genotypes had already been reported, to validate the accuracy of our markers for identifying the correct *S*-alleles. We then demonstrate the usefulness of these assays by genotyping 183 genotypes representing some of the most valuable parents within the PFR breeding program.

RESULTS

A novel, rapid and high-throughput protocol for *S-RN*ase allele genotyping

The four developed SSR markers Myb110a1_PFR, Myb110a2_PFR, Myb110b_PFR and GSI_SSR_PFR amplify polymorphic PCR products associated with 13 different *S*-alleles (S_1, S_2, S_3, S_5, S_7, S_8, S_9, S_{10}, S_{20}, S_{23}, S_{24}, S_{25} and S_{28}) (Table 1). All four primer pairs amplify PCR products linked to the S_1, S_3, S_5, S_7, S_{24} and S_{25} *S*-alleles. Both Myb110b_PFR and GSI_SSR_PFR can be used to distinguish the S_{10} *S*-allele. Myb110b_PFR exclusively identifies S_2 and S_9, whilst GSI_SSR_PFR amplifies a PCR product linked to the S_{28} *S*-allele. All four primer pairs, except Myb110b_PFR, amplify PCR products linked to S_{20} and all four but Myb110a1_PFR can identify the S_{23} *S*-allele.

Additionally, we developed seven quantitative-PCR allele-specific markers. Six of these markers (S_1, S_2, S_5, S_9, S_{23} and S_{28}_apple_PFR) amplify specific single nucleotide polymorphisms (SNPs) identified by a pairwise alignment of the coding sequences of 25 *S-RN*ase alleles, previously published by De Franceschi et al.[20]. The seventh marker for S_8 was adapted to work with our HRM methodology, by modifying the forward primer from the previously published primers pairs (Larsen et al.[19]) (Fig. 2). When used on their own, without the SSR markers, these *S*-allele-specific qPCR markers can identify eight *S*-alleles in total. The *S*1_apple PFR marker can resolve the S_{20}, S_{24} and S_1 *S*-alleles as separate melting curves.

Validation of the S-*RN*ase allele genotyping method on a large set of apple commercial cultivars

The *S*-allele genotyping of 86 apple accessions (81 different cultivars, counting a red mutant sport of 'Fuji' and four 'Gala' mutant sports) – including 70 traditional varieties with previously reported *S*-alleles, five varieties with unknown *S*-alleles and ten newer cultivars arose from the PFR breeding programme – was undertaken using the new set of molecular markers. The *S*-genotypes of 59 of the 70 traditional apple varieties were in complete agreement with previous reports and the *S*-genotypes of their respective parents. For ten accessions, our results for one of the two *S*-alleles disagreed with those published previously (Supplemental Table S1): 'Abbondanza' had been reported as (S_3 S_5)[20], while (S_3 S_7) *S*-alleles were detected in the present study. 'Antonovka' was reported (S_8 S_{32})[20], while we detected S_8 and other allele sizes that could equally be linked to S_3, S_7 or S_{20}. 'Priscilla' was reported as (S_3 S_9)[21] or (S_9 S_{20})[22] or (S_7 S_{10})[20], while we detected (S_7 S_{28}) alleles. 'Ingrid Marie' was reported as (S_5 S_{43})[23], while our most likely observed *S*-genotype was (S_3 S_5), 'James Grieve' was reported as (S_5 S_8)[23], while we detected (S_5 S_{20}). 'Ben Davis' was reported as (S_5 S_{23}) while we detected (S_7 S_{23}); 'Jonathan' was reported previously as (S_7 S_9)[24–28]; however, we detected (S_9 S_{23}). 'Early Cortland' has been reported as (S_5 S_{28})[29,30]; however, we report it here as (S_1 S_{28})[29]. 'Yellow Transparent' has been reported as (S_1 S_5)[31,32], while we identified (S_1 S_7 S_9 S_{24}). Finally, 'McIntosh' was reported as (S_{10} S_{25})[11,23], while we found (S_2 S_{25}).

The new markers also resolved both *S*-alleles for three out of four traditional varieties with previously undetermined *S*-genotypes: 'Red Dougherty' (S_1 S_7), 'Pinkie' (S_2 S_3) and 'Merton Russet' (S_5 S_{24}). We identified only the S_{25} allele for 'Paulared'. The ten cultivars that have recently arisen from the PFR breeding programme were successfully typed for their *S*-alleles: 'Scired' (S_2 S_9), 'PremA093' (S_2 S_9), 'Scifresh' (S_2 S_{24}), 'PremA153' (S_2 S_{24}), 'PremA34' (S_2 S_3), 'Scilate' (S_5 S_9), 'PremA96' (S_5 S_9), 'PremA17' (S_5 S_{24}), 'PremA280' (S_5 S_{24}) and 'PremA129' (S_9 S_{24}). For the Canadian variety 'Sunrise' we identified *S*-alleles (S_3 S_{24}).

For a remaining set of five cultivars, just one *S*-allele could be determined: 'Hetlina' and 'Geheimrat Dr Oldenburg' were reported as (S_1 S_{16b})[12] and (S_3 S_{28})[20], respectively. The respective S_1 and S_3 *S*-alleles were detected in our study; however, we did not identify allele sizes that could be linked to either S_{16b} or S_{28} using the new markers. For 'Benoni', reported as (S_5 S_{11})[33], we detected the S_5 allele, as well as other marker alleles not linked to the expected S_{11}. 'Regent' was reported as (S_3 S_{10})[34]; we identified the S_{10} allele, but no S_3-linked alleles were observed. Instead, an allele linked to S_{25} was detected. Finally, 'Panenské České' was reported as (S_7

Table 1. Primer sequences of quantitative real-time PCR and SSR-based markers.

Marker name	Type		Primer sequences '5 - 3'	Physical location	Genbank locus	S-Rnase alleles and product sizes (bp)
S1_apple_PFR	HRM	Forward Reverse	ACAGGCCACTGGTGGA ATTGCGTATGGCATTTTCAAT	not found in reference genome Chr17:30844510–30844530	MG598487.1:1981–1996 MG598487.1:1998–2018	S_1, S_{20}, S_{24} (38)
S2_apple_PFR	HRM	Forward Reverse	TTGAACAAATATTATTCAATGGGGA CATCGTAACTATATATACCATCCGCGTA	Chr17:31240988–31240964 Chr17:31240964–31240943	MG598488.1:860–884 MG598488.1:886–913	S_2 (54)
S5_apple_PFR	HRM	Forward Reverse	AATTTATAAAACACGTGATCA GCTCCTATTGATCGATCAT	not found in reference genome not found in reference genome	MG598491.1:326–346 MG598491.1:350–370	S_5 (43)
S8_apple_PFR	HRM	Forward Reverse	TTCGATTATTTTCAATTTACGCTT ATTTAAGGTTGTTTCTTTGCAATAC	Chr17:31240889–31240871 not found in reference genome	MG598494.1 :1159–1182 MG598494.1 :1296–1320	S_8 (162)
S9_apple_PFR	HRM	Forward Reverse	GCTCAGGAAATGACCCAATATAC AATATTACCTTAGTAGAATTCATGGTTGT	not found in reference genome not found in reference genome	MG598495.1:1284–1306 MG598495.1:1315–1344	S_9 (61)
S23_apple_PFR	HRM	Forward Reverse	TTTATGGCCTTCAAACTGGAA CAGAAGATTGGGTCGGGT	not found in reference genome not found in reference genome	MG598501.1:1065–1085 MG598501.1:1089–1106	S_{23} (42)
S28_apple_PFR	HRM	Forward Reverse	TGCCTCGCTCTTGAACAAA CCCCGTAATTCCCATTGAATAATA	not found in reference genome not found in reference genome	MG598505.1:782–800 MG598505.1:805–828	S_{28} (47)
Myb110a1_PFR	SSR	Forward Reverse	TCTCCCTCATCCCAGAACA CGAGCCAAACAAAATTGGA	Chr17:32151473–32151491 Chr17:32151642–32151624		S_1 (166), S_3 (184), S_5 (180), S_7 (170), S_{20} (158), S_{24} (176), S_{25} (188)
Myb110a2_PFR	SSR	Forward Reverse	CTCTCCCTCATCCCAGAACA TCCTACTCGGCTCGACAATC	Chr17:32151472–32151491 Chr17:32151800–32151781		S_1 (325), S_3 (343), S_5 (339), S_7 (314), S_{20} (317), S_{23} (309), S_{24} (320), S_{25} (347)
Myb110b_PFR	SSR	Forward Reverse	CTTCGGGCTTATTTGGGTTT TTTGCCCCTTCAAAGATCAG	Chr17:32187809–32187790 Chr17:32187616–32187635		S_1 (202), S_2 (233), S_3 (214), S_5 (209), S_7 (191), S_9 (247), S_{10} (216), S_{23} (239), S_{24} (217), S_{25} (238)
GSI_SSR_PFR	SSR	Forward Reverse	GCCCCTTACATTCCTTTTCTTT CAATCTTGAGTTGTCGTTGGAG	Chr17:31704109–31704130 Chr17:31704430–31704409		S_1 (314), S_3 (338), S_5 (335), S_7 (324), S_{10} (328), S_{20} (317), S_{23} (322), S_{24} (217), S_{25} (329), S_{28} (352)

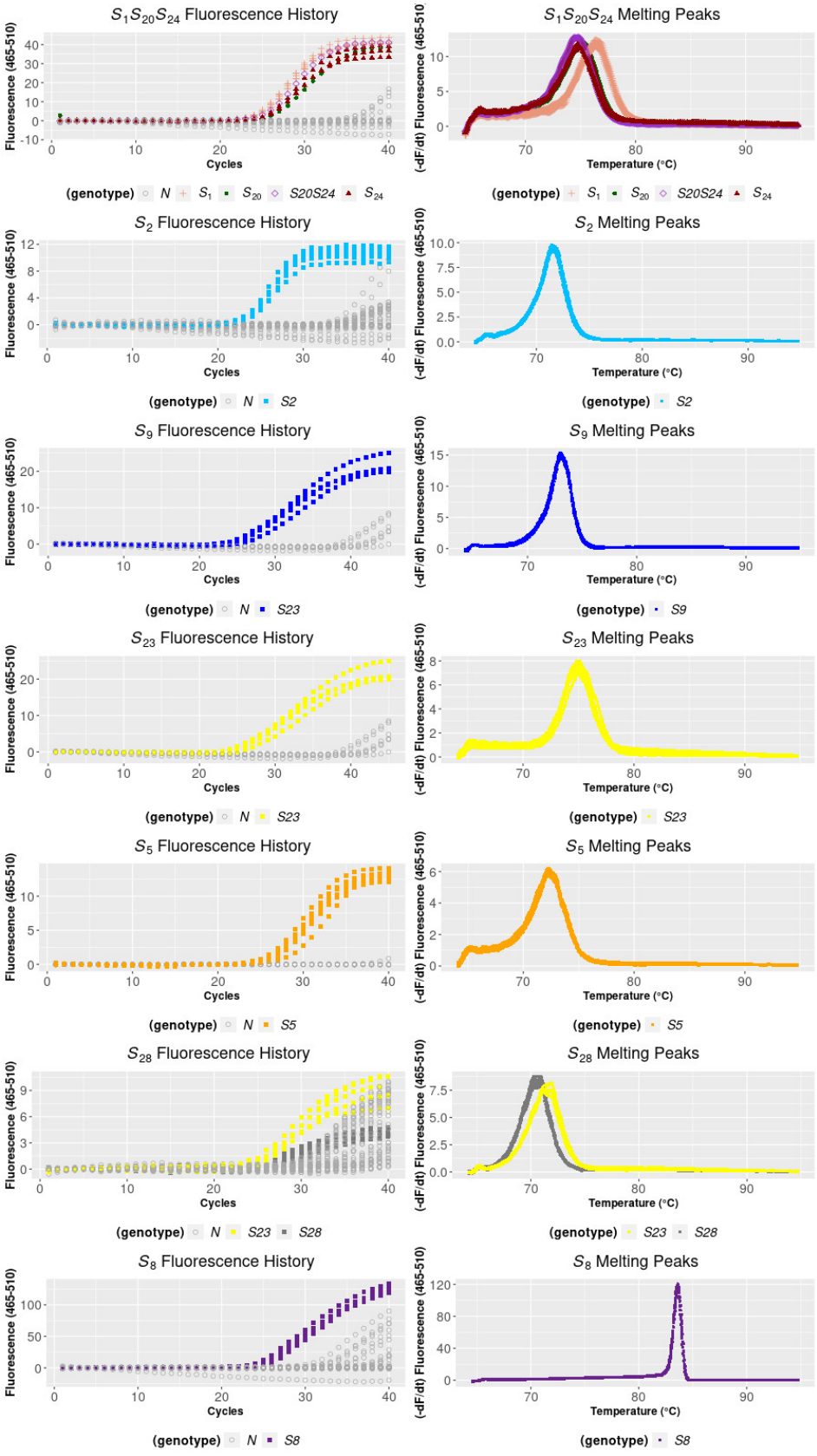

Fig. 2 High resolution melting (HRM) curve profiles of seven *S*-allele-specific markers. Amplification curves of real-time PCR marker assays (left panels), HRM difference plots, where the derivative fluorescence signal (dF/dT) is plotted as a function of temperature (right panels). Each colour represents a specific *S*-genotype as shown by the legends. Light grey represents samples that were not amplified in the real-time PCR.

S_{10})[20], but only the S_7 allele was detected using three SSR markers. Marker alleles linked to S_{10} were not found, rather the Myb110b marker detected alleles linked to S_3 and S_{24}.

Diversity of *S-RN*ase alleles in the PFR apple cultivar breeding programme

When the new markers were screened over 183 genotypes, including some of the most valuable parents within the PFR apple cultivar breeding programme, we were able to determine their *S*-genotypes. These genotypes are the seedlings of 76 biparental families (Supplemental Table S2). For 32 of these families (from a total of 132 selections), the *S*-genotypes of their parental pedigrees could be verified. The frequency of *S-RN*ases alleles found among this pool of genotypes is shown in Fig. 3. Among the 183 genotypes screened, S_2 was the most common *S*-allele, present in 21.3% of the samples, followed by S_3 (19.9%), S_{24} (18.6%), S_5 (17.5%), S_{23} (13.7%), S_9 (5.7%) and S_7 (0.8%). Rare *S*-alleles were S_{28} (0.5%), S_{25}, S_1 and S_{20} (0.3% each). Only 2.2% of the *S*-alleles could not be assigned. The most prevalent genotypes were: (S_3 S_{24}), (S_2 S_{24}), (S_2 S_5), (S_2 S_{23}), (S_3 S_{23}), (S_3 S_5) and (S_5 S_{23}) observed at frequencies of: 7.1, 7.1, 6.3, 5.2, 4.4, 4.1 and 3%, respectively. Other less prevalent genotypes were: (S_5 S_{24}), (S_5 S_9), (S_3 S_9), (S_2 S_3), (S_9 S_{24}), (S_2 S_9) and (S_{23} S_{24}), which were observed at frequencies of 2.2, 1.6, 1.4, 1.4, 1.4, 1.1 and 0.8%, respectively. The following rare genotypes were each found at a frequency of 0.3%: (S_1 S_3), (S_2 S_7), (S_5 S_7), (S_7 S_9), (S_{20} S_{28}) and (S_3 S_{28}). For 1.1% of the selections, just one allele was identified (S_3 ?) in 0.8% and (S_{23} ?) in 0.3%).

DISCUSSION

A high-throughput method to identify the *S*-genotypes of apples was developed and validated in this study. This will help to inform the selection of compatible parental combinations when designing a crossing programme. We present a new high-throughput marker set based on four multi-allelic SSR and seven allele-specific qPCR markers. The four SSR markers can identify 13 *S-RN*ase-alleles (S_1, S_2, S_3, S_5, S_7, S_8, S_9, S_{10}, S_{20}, S_{23}, S_{24}, S_{25} and S_{28}) and the seven HRM markers allow the identification of eight *S-RN*ase-alleles (S_1, S_2, S_5, S_8, S_9, S_{20}, S_{23} and S_{28}). The identification of the 13 *S*-alleles can be achieved economically and efficiently by employing three PCR reactions, using two (Myb110b_PFR and GSI_SSR_PFR) of the four SSR markers and the *S*8_apple_PFR marker (with the addition of an M13-tail on the 3'-end). These three markers can be multiplexed by using different fluorescent labels that can be simultaneously separated and scored on a capillary electrophoresis instrument.

Alternatively, for laboratories with access to a real-time qPCR system, as well as a capillary electrophoresis instrument, the seven qPCR allele-specific markers and one of the SSR markers (either Myb110b_PFR or GSI_SSR_PFR) will be sufficient to identify and resolve the whole set of 13 *S*-alleles.

The usefulness of the new markers was validated in over 59 of 70 well-established apple cultivars with known *S*-genotypes. Ten of the discrepancies with previous assays are supported by our results from parental pedigree allele analysis, acknowledging that some might be mistakes with labelling, or incorrect germplasm harvest: 'Priscilla' has been reported as (S_3 S_9)[21], (S_9 S_{20})[22] or (S_7 S_{10})[20], while we detected (S_7 S_{28}), with S_{28} as probably coming from 'Starking Delicious', which is reported as (S_9 S_{28})[29]. 'Ingrid Marie' was reported as (S_5 S_{43})[23], while our most likely observed *S*-genotype was (S_3 S_5), where S_5 is derived from 'Cox's Orange Pippin' (S_5 S_9). However, neither S_{43} nor S_3 has been reported in 'Cox's Pomona' *S*-genotype (S_1 S_{34})[19]. 'Early Cortland' has been reported as (S_5 S_{28})[29,30], which is consistent with its parentage: 'Cortland' (S_5 S_{25})[29] and 'Lodi' (S_1 S_{28})[30]; however, we reported it here as (S_1 S_{28})[29]. 'Abbondanza' was reported as (S_3 S_5)[20], while (S_3 S_7) *S*-alleles were detected here.

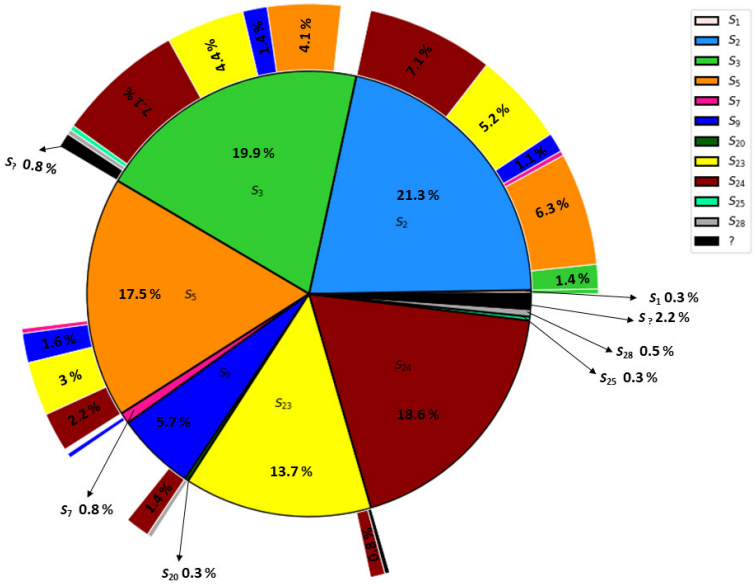

Fig. 3 Frequency of *S*-alleles and *S*-genotypes of the 183 apple advanced selections of the PFR's breeding programme. Inner plot shows the percentage frequency distribution of *S*-alleles from the total 366 alleles observed among the 183 genotypes tested. Outer plot represents the absolute frequency of each *S*-genotype. All outer slices not showing a percentage value in the figure represent 0.3% respectively.

'Antonovka' was reported (S_8 S_{32})[20], while we detected allele S_8; however, we observed different allele sizes for our SSR markers that we could link to either S_3, S_7 or S_{20}. There are different 'Antonovka' accessions[35], so it is probable that they have different *S*-genotypes. 'James Grieve' was reported as (S_5 S_8)[23], while we detected (S_5 S_{20}), although the Myb110b_PFR marker showed an additional 202 bp allele, which is linked to S_1 *S-RN*ase, but did not exhibit any other allele sizes linked to S_1 in any of the other SSR or qPCR markers. Then, 'Ben Davis' was reported as (S_5 S_{23}), while we detected (S_7 S_{23}), with the same allele sizes found and expected for 'Lady Williams' (S_7 S_{23}). 'Jonathan' was reported previously as (S_7 S_9)[24–28]; however, we characterised it as (S_9 S_{23}), but note that we do have molecular and phenotypic indicators suggesting this could be an incorrectly identified accession in the PFR germplasm (Vincent Bus, pers. comm.). Finally, 'Yellow Transparent' is reported as (S_1 S_5)[31,32], while we identified (S_1 S_7 S_9 S_{24}), which is consistent with this cultivar being a tetraploid sport mutant[36].

We demonstrated the usefulness of the markers by determining the *S*-genotypes of ten newer cultivars arising from the PFR breeding programme ('Scired', 'PremA093', 'Scifresh', 'PremA153', 'PremA34', 'Scilate', 'PremA96', 'PremA17', 'PremA280' and 'PremA129'). The *S*-genotype information for such new cultivars is valuable information for growers, enabling them to plant compatible pollenisers in commercial orchards. Despite the high diversity of *S-RN*ase alleles that have been characterized in *Malus* (at least 35 different *S*-alleles were found among cultivars in Matsumoto's database[29]), the common worldwide practise of using a relatively small pool of cultivars that combine premium fruit quality as well as resistance to pests and environmental stresses in breeding programmes leads to new cultivars with restricted allelic combinations. Among the 183 PFR apple genotypes tested here, a pool of only 11 *S*-alleles was found (S_1, S_2, S_3, S_5, S_7, S_9, S_{20}, S_{23}, S_{24}, S_{25}, and S_{28}). This is not surprising given that all the *S*-alleles of the main founders of PFR's cultivar breeding programme [namely 'Splendour' (S_2 S_9), 'Cox's Orange Pippin' (S_5 S_9), 'Red Delicious' (S_9 S_{28}), 'Golden Delicious' (S_2 S_3), 'Red Dougherty' (S_1 S_7), 'Worcester Pearmain' (S_2 S_{24}), 'Jonathan' (S_{23} S_9), 'Fuji' (S_1 S_9), 'Braeburn' (S_9 S_{14}), 'Granny Smith' (S_3 S_{23}), 'James Grieve' (S_5 S_{20}), 'Wagener' (S_3?), 'Cripp's Pink' (S_2 S_{23}) and 'Akane' (S_7 S_{24})[34]] have ten of these 11 *S*-alleles. However, it is possible that the wider parental pool also has undetected *S*-alleles beyond these 11, as some breeding parents, not represented in the 183 genotypes tested here, are also derived from minor founders.

The most frequently observed allele was S_2 (21.3%), which is one of the two alleles carried by 'Royal Gala' (S_2 S_5), a parent or grandparent in pedigrees of most of the PFR genotypes. For instance, 'Scired', 'Sciros', 'Scilate', and 'Sciray' were used as the pollen parents for many crosses and they all are progeny of a cross between 'Gala' (S_2 S_5) and 'Splendour' (S_2 S_9). 'Gala's' parentage is 'Golden Delicious' (S_2 S_3) and 'Kidd's Orange Red' (S_5 S_9), so 'Golden Delicious' is the source of this allele in 'Gala' or 'Royal Gala' crosses.

S_3 was the second most abundant allele (19.9%), being present in crosses of genotypes with 'Pinkie' in their parentage. 'Pinkie' likely inherited the allele from 'Granny Smith' (S_3 S_{23}), although we do not know the *S*-alleles carried by its other parent A679-2. Also, crosses produced using 'Fiesta' (S_3 S_5), have inherited the S_3 allele from 'Idared' (S_3 S_7), which has 'Wagener' (S_3 ?) as a parent.

Allele S_{24} was observed in 18.6% of the genotypes, those arising from crosses with 'Braeburn' (S_9 S_{24}) as one of the parents: 'Scifresh' is a progeny of the cross 'Braeburn' 'Royal Gala'; while 'PremA153' is derived from a 'Gala' × 'Braeburn' cross. 'PremA129' has 'Braeburn' as a grandparent, being a progeny of 'PremA280' × 'Scired', with 'PremA280' having 'Gala' and 'Braeburn' as parents. 'PremA17' (S_5 S_{24}) also has this allele, presumably from 'Braeburn': this cultivar was derived from a cross between genotypes A045R13T007 × A020R02T167, which unfortunately are no longer available in the orchard. Another source of this allele is 'Akane' (S_7 S_{24})[34], which inherited it from 'Worcester Pearmain' (S_2 S_{24}).

The origin of S_5 (17.5%) in our breeding programme is 'Cox's Orange Pippin' (S_5 S_9). Crosses that involve 'Fiesta' or 'James Grieve' have 'Cox's Orange Pippin' as a grandparent. It is also a great-grandparent in crosses that have 'Gala' as a parent and a great-great-grandparent in crosses that include cultivars such as 'Sweetie', 'PremA17' and 'PremA96' as parents.

The S_{23} allele is present (13.7%) in seedlings derived from our A068 family; however, we still need to confirm the source of the allele as we do not know the *S*-genotypes of grandparents. S_9 is found in 5.7% of seedlings derived from crosses where one of the parents was 'Scired', or alternatively with 'Splendour' as one of the grandparents or great-grandparents. Other important sources of the S_9 allele are 'Cox Orange Pippin' (S_5 S_9) and 'Braeburn' (S_9 S_{24}).

At the other end of the scale, the S_7 allele was only found in 4.8% progeny of crosses with 'Red Free' (S_3 S_7) as a parent or grandparent and in some crosses using 'Akane' (S_7 S_{24})[34], which has 'Jonathan' (S_7 S_9) as the likely parental source of this allele. The alleles S_{25} and S_{28} occur rarely in the PFR breeding programme. They probably come from 'McIntosh' (S_{10} S_{25}) and 'Delicious' (S_9 S_{28}), respectively; however, the pedigree of the few selections with these alleles is not complete: further information is needed to confirm this hypothesis.

According to Sheick et al.[18], 11 *S*-alleles (S_1, S_2, S_3, S_5, S_9, S_{10}, S_{20}, S_{23}, S_{24}, S_{25}, and S_{28}) are represented among the U.S. industry's most produced apples. These are predominantly coming from 'Red Delicious' (S_9 S_{28}), 'Gala' (S_2 S_5), 'Granny Smith' (S_3 S_{23}), 'Fuji' (S_1 S_9), 'Golden Delicious' (S_2 S_3), 'Honeycrisp' (S_2 S_{24}), 'McIntosh' (S_{10} S_{25}), 'Rome' (S_{20} S_{24}), 'Cripps Pink' (S_2 S_{23}), and 'Empire' (S_{10} S_{28}). These same 11 *S*-alleles but S_{10} are represented in the New Zealand PFR breeding programme. Instead of $S_{10,}$ we have S_7 included in our pool of *S*-alleles, which is represented in crosses having 'Jonathan' (S_7 S_9) and 'Red Free' (S_3 S_7) in their pedigree. Another recent study by Lays Brancher et al.[37] identified 11 *S*-alleles (S_1, S_2, S_3, S_5, S_7, S_9, S_{10}, S_{19}, S_{20}, S_{23}, and S_{24}) among 42 apple genotypes, including cultivars, advanced selections and accessions of the Apple Germplasm Bank of Epagri (Caçador, Santa Catarina, Brazil). The S_3 and S_5 alleles were most frequent (30.2% and 18.6%, respectively). The higher frequency of these alleles can be explained as 26 of the 42 accessions tested were direct or indirect descendants from the cultivars Imperatriz (S_3 S_5), Golden Delicious (S_2 S_3) and/or

Gala (S_2 S_5), which have served as the basis for the crosses of the Epagri Apple Breeding Program.

A Danish study by Larsen et al.[19] found 25 *S*-alleles (S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9, S_{10}, S_{11}, S_{16b}, S_{16c}, S_{20}, S_{21}, S_{23}, S_{24}, S_{25}, S_{26}, S_{28}, S_{31}, S_{33}, S_{34}, S_{36} and S_{40}) in 432 *Malus* accessions including a selection of *M. domestica* cultivars of mainly Danish origin (402 accessions), as well as a selection of other *Malus* species (30 accessions). Among the 402 Danish accessions the allele S_3 (28 %) was the most common followed by S_1 and S_7 (both 27 %). Previous studies[16,38] using cultivars from Northern Europe and the Carpathian basin found similar results where S_3 and S_7 were the two most common *S*-alleles.

Although selections used as parents in breeding programmes around the world are different due to consumer preferences, climate conditions, resistances to pest and diseases, etc., there is a common set of cultivars among worldwide breeding programmes[39]. These are 'Golden Delicious' (S_2 S_3), 'Braeburn' (S_9 S_{24}), 'Fuji' (S_1 S_9), 'Gala' (S_2 S_5), 'Granny Smith' (S_3 S_{23}), 'Idared' (S_3 S_7), 'Jonathan' (S_7 S_9) and 'Red Delicious' (S_9 S_{28}). The *S*-alleles carried by these cultivars are also the most common among the total 183 advanced selections tested within PFR's breeding programme. The S_3 allele is the most common *S*-allele worldwide as seen in the previous studies mentioned here and among other older studies including European, American and Japanese cultivars[12,23,40]. This low diversity of *S-RN*ase alleles highlights the need of introducing breeding cultivars with some of the less common *S*-alleles into breeding programmes to increase mate compatibility among parental selections.

METHODS

Plant material

Leaves from 86 apple cultivars were collected at PFR, Havelock North, New Zealand, and Washington State University, Pullman, WA, USA. Total genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method[41]. This DNA was used as a set for evaluating the new markers (Supplemental Table S1). Additionally, leaves from 183 apple genotypes, from 76 biparental families, were collected from trees in PFR's elite parental apple collections, to identify their *S*-genotypes.

SSR-based *S*-allele markers

Three new primer pairs were designed around two single sequence repeats (SSR) linked to the *Myb110a* and *Myb110b* genes[42] and named Myb110a1_PFR, Myb110a2_PFR and Myb110b_PFR, which are closely linked to the *S*-locus on apple chromosome 17. A fourth primer pair was designed for a SSR located within the *S*-locus (GSI_SSR_PFR) (Table 1)**.** Design of the primer pairs was based on the GDDH13v1.1[43] apple genome as a reference and employed using the Krait software[44]. The M13 sequence TGTAAAACGACGGCCAGT was added to the 5′ end of the forward primer to enable the use of Schuelke's[45] approach to fluorescent labelling. PCR was performed in a 15 µL reaction mixture containing 1.5 mM $MgCl_2$, 200 uM dNTPs, 13 nM of forward primer, 200 nM of reverse primer, 8.33 µL DNA-free water and 1× PCR Buffer (-$MgCl_2$) and 0.5 U of Platinum™ Taq DNA polymerase (Thermo Fisher Scientific, 10966034). The conditions of the touchdown PCR included an initial denaturing at 94 °C for 2 min, then five cycles (94 °C for 55 s, 65 °C for 55 s (decreased by 1 °C each cycle), 72 °C for 1 min and 39 s), then 35 cycles (94 °C for 55 s, 55 °C for 55 s and 72 °C for 1 min and 39 s) and a final extension at 72 °C for 10 min. The final amplicons were subjected to capillary electrophoresis using an ABI 3500 DNA sequence analyser (Applied Biosystems, Foster City, USA) and sized using GenScan™ 500 LIZ Size Standard (Applied Biosystems). SSR allele profiles were analysed using GeneMarker™[46] version 2.20 (SoftGenetics LLC®, State College, PA, USA, www.softgenetics.com).

Quantitative real-time PCR-based markers

SNPs were identified by performing a multiple sequencing progressive pairwise alignment[47] of the coding sequences of 25 *S-RNase* alleles previously published by De Franceschi et al.[20] in Geneious version 10.0.9 (https://www.geneious.com), with the following parameters: global alignment with free end gaps algorithm, 70% similarity cost matrix, gap open penalty of 11.9, gap extension penalty of 2 and 2 refinement iterations.

Seven *S-RN*ase allele specific primer pairs named *S*x_apple_PFR (x being: 1, 2, 5, 8, 9, 23 or 28 alleles) were designed to amplify a single product of 250 bp or less. These primer pairs can be used on a conventional PCR machine or by employing the High Resolution Melting (HRM) methodology[48] on a quantitative PCR instrument. The primer pairs for *S*8_apple_PFR marker were modified from the ones previously published by Larsen et al.[19].

Conventional PCR reactions were carried out in 15 µL volume containing 1× PCR buffer mix (Invitrogen), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 3 µM each primer, 0.1 U Platinum™ DNA polymerase (Thermo Fisher Scientific, 10966034) and 20 ng template DNA. Amplifications were carried out on a MasterCycler ProS thermocycler (Eppendorf). The conditions of the touchdown PCR included an initial denaturing at 95 °C for 5 min, then ten cycles (94 °C for 30 s, 60 °C for 30 s (decreasing 1 degree in each cycle) and 72 °C for 45 s), then forty cycles (94 °C for 30 s, 50 °C (for *S*5_apple_PFR primer pair) or 55 °C (for *S*1, 2, 9, 23, 28_apple_PFR primer pairs) for 30 s and 72 °C for 45 s) and a final extension at 72 °C for 5 min. PCR products were then visualized on a 2% agarose gel stained with RedSafe™ 20000x (ChemBio, UK) after 1 h of electrophoresis at 100 V.

Quantitative PCR reactions were performed in a total volume of 10 µL containing 20 ng of template DNA, 2.5 mM $MgCl_2$, 200 nM forward and reverse primers and 1× HRM master mix (Roche Applied Science). PCR and HRM were performed on a LightCycler® 480 (Roche Diagnostics). The PCR parameters were an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s. Following amplification, the samples were heated to 95 °C for 1 min and then cooled to 40 °C for 1 min. Melting curves were generated with continuous fluorescence acquisition during a final ramp from 65 °C to 95 °C at 4.8 °C/s, followed by a final cooling step of 40 °C for 30 s. The resultant fluorescence data were processed using the LightCycler® 480 software (version 1.5; Roche Applied Science). Primer sequences, fragment sizes and their respective associated *S-RN*ase alleles are shown in Table 1.

S-genotyping *Malus* cultivars and breeding seedlings

The four SSR-based markers were initially screened using a DNA set from 70 out of a total of 86 apple cultivars with known *S*-genotypes, based on previously reported CAPS or PCR-RFLP detection methods (as referenced in Supplemental Table S1). Following this, the seven HRM assays were screened over the same cultivars to validate the allele-specificity of each primer pair (Table 1).

Following the screening of the first DNA set, all 11 markers for *S*-genotype were further validated using 183 apple genotypes from the PFR breeding programmes. The *S*-alleles were confirmed by verifying the *S*-genotype composition within each family and by examining their pedigree composition up to the grandparent level. For the PFR breeding populations, a summary of the *S*-genotype composition of the tested seedlings within families was made.

Data availability

Raw data and R script for statistical analysis are available at link https://github.com/hrpelg/Rnotebook_Self-incompatibility

CONCLUSIONS

We demonstrated the efficiency of a set of markers for the *S*-locus in a *Malus domestica* germplasm set with known *S*-genotypes and we determined the *S*-genotypes of uncharacterized cultivars, with an emphasis on new commercial releases. We showed the *S*-genotyping efficacy of this method on a large sample of advanced apple genotypes from the PFR breeding programme, where *S*-genotypes were concordant with their parental pedigree.

This robust, reproducible, simple and cost-efficient *S-RN*ase-genotyping method is an alternative to the present molecular approaches. The existing molecular methods employ single allele specific markers per every single *S*-allele or use marker based restriction enzyme digestions of PCR products to distinguish among few *S*-alleles needing to be visualized on agarose gels. The flexibility of our method permits to know 13 different *S*-alleles by employing just three different PCR reactions in a laboratory provided with a capillary electrophoresis instrument. These three PCR reactions can be multiplexed in a single electrophoresis run by using three different fluorescent colours. Alternatively, if a qPCR instrument is also available, this can be done using seven different HRM-markers and a single SSR marker. The use of a qPCR instrument allows the analysis of 384 samples per run or the multiplexing of four markers per PCR for every 96 samples.

This method is provided to scientists, breeders and growers to select compatible pollenisers and to develop new cultivars. The benefits of knowing the *S*-alleles that each parental selection carries are: pollination success between compatible parental pollen and pistil, higher yields of orchards planted with compatible varieties and possible parentage identificationof unknown seedlings' descent due to undesired open pollination.

ACKNOWLEDGMENTS

We would like to gratefully thank Stijn Vanderzande for providing the US plant material and Susan E. Gardiner for helpful advice and review of the manuscript. This work was funded by Prevar Limited (https://prevar.co.nz).

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (http://www.maxapress.com/article/doi/10.48130/FruRes-2021-0010)

Dates

Received 6 August 2021; Accepted 8 October 2021; Published online 25 October 2021

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