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Molecular characterisation of apple accessions with respect to aminocyclopropane-1-carboxylic acid synthase gene (*ACS1*) polymorphism

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Abstract: The ARC apple gene bank collection was genotyped for the fruit expressed gene ACS1, in which a short-interspersed element (SINE) in the promoter is known, when homozygous, to correlate with the delayed ethylene production. Primers were designed amplifying products less than 500 bp and 224 cultivars of domestic apple were analysed, 169 not previously genotyped. Of these, 82 were *aa* (homozygous for the high ethylene allele at 202 bp), 73 were ab and 14 bb (homozygous for the low ethylene allele, with the SINE, at 339 bp). The difference between the allele sizes, 137 bp, observed in the current study is consistent with the indel of 138 bp originally described, but differs considerably from the indel of 166 bp reported in literature. In addition, 21 accessions of other Malus species were analysed. Only one, *M.* 'Golden Hornet', had the *b* allele, which suggests it may have been introgressed from *M. pumila*.

Keywords: apple; fruit quality; genotyping; storability; Malus sp.; marker-assisted selection

The domestic apple (*Malus pumila* Mill.) belongs to the sub-tribe Pyrinae of the rosaceous sub-family Spiraeoideae, together with the European pear (*Pyrus communis* L.) and other pome fruits (Potter et al. 2007). It is an important deciduous fruit crop in South Africa, which produces approximately 800 000 tonnes annually of which 40% is exported to northern hemisphere markets (HORTGRO 2016). The Agricultural Research Council (ARC) Infruitec-Nietvoorbij institute for Deciduous Fruit, Vines and Wine is breeding new cultivars for South African growers and an important objective is the extended storage potential necessary for supplying distant markets.

Apple fruits are climacteric in nature; their ripening is associated with the increased ethylene production and cellular respiration that continues after harvesting (Gorny, Kader 1997). The enzyme aminocyclopropane-1-carboxylic acid synthase (ACS) is a significant compound in the biochemical synthesis of ethylene as it is responsible for the conversion of S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC). This is generally accepted as a rate limiting factor in the ethylene production pathway and manipulating this step can influence the amount of ethylene produced which, in turn, affects the rate of ripening (Nybom et al. 2008; Dougherty et al. 2016).

Several pre- and post-harvest methods are utilised commercially to inhibit the ethylene production during storage. Chemicals known to inhibit pre-harvest ethylene production, naphthalene acetic acid (NAA) and aminoethoxyvinylglycine (AVG), or that block ethylene receptors post-harvest, 1-methylcyclopropane (1-MCP), contribute to

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prolonging the fruit storage ability (Schupp, Greene 2004; Yuan, Carbaugh 2007). Additionally, a controlled atmosphere in the storage chambers (Bulens et al. 2014) can be used as an alternative, but both methods present additional costs. The breeding of low ethylene producing cultivars can help counter this problem by slowing down the ripening process and reducing the post-harvest fruit losses.

As an outcrossing species, the apple genome is highly heterozygous. Several gene families are over-represented in the genome, including those related to the sorbitol transport, disease resistance, and antioxidant metabolism (Velasco et al. 2010). Apple cultivars also differ with respect to their fruit storage ability (Alston 1989). An allelic variation at the ACS1 locus with respect to the insertion of a short-interspersed element (SINE) of 162 bp in the promoter region, as well as a concomitant dele+tion of 24 bp, correlates with this trait (Sunako et al. 1999). Accessions that are homozygous for the SINE tend to show delayed ethylene production and enhanced storability. The ACS1-5 primers were designed to amplify the two alleles, denoted as *1* and *2*, to genotype seedlings as early as one week from germination (Sunako et al. 1999; Kumar et al. 2012). Alleles *a* and *b*, in the current study, correspond to alleles *1* and *2* of Sunako et al. (1999) for high and low ethylene production, respectively. Amplification products, reportedly of 489 bp (allele *1*) and 655 bp (allele *2*), can be distinguished when separated with agarose gel electrophoresis. However, this 166 bp difference between the two alleles is inconsistent with the events described in Sunako et al. (1999), which explain a net difference of only 138 bp; notably, this anomaly has not been pointed out in the literature.

The use of fluorescently labelled primers for distinguishing the *ACS1* alleles via an automated sequencer has not been reported. Although fluorescent sizing is costly, it can reveal variation in the product size not distinguishable with agarose gel electrophoresis, simultaneously providing scope for automated data capture and multiplexing with other markers. In the case of the multi-allelic *S* locus in *Prunus*, fluorescent primers amplifying across the introns, which show allele specific length polymorphism, have proved very informative (Vaughan et al. 2006).

The ARC Infruitec-Nietvoorbij maintains the South African collection of domestic apples and some related species, primarily as a gene bank for the breeding programme. The collection of 245 items, comprising 224 *M. pumila* primary cultivars and 21 representatives of other *Malus* species, has not been genotyped for *ACS1* previously. To enhance its value to breeders, the accessions are being genotyped for various horticulturally significant genes for which which functional variants have been sequenced and for which primers, such as *ACS1*, are available.

The purpose of the current study was to primarily determine the genotypes of the accessions in the ARC Infruitec-Nietvoorbij apple collection with respect to *ACS1* using an automated genotyping approach. The primers were designed for fluorescent sizing to detect any possible minor variations in allele size and investigate the possibilities for multiplexing. The information gained will enhance the characterisation of the accessions and provide information useful to the breeder designing crosses to target storability.

MATERIAL AND METHODS

Plant material. A total of 245 ARC apple accessions in the collections at Drostersnes (S34°4'29.028", E19°4'33.852") and Grabouw (S34°4'54.037", E19°0' 40.978") Research Farms in the Elgin Valley, Western Cape, South Africa, were selected for the *ACS1* genotyping (Supplementary Table 2). Included were 224 accessions of *M. pumila* cultivars, excluding clones and duplicates, and 21 representatives of the *Malus* species and hybrids. Tree locations are given as identifiers. Young expanding leaves were collected in the spring, weighed to approximately 0.3 g (± 0.1 g) and stored at –20 °C until further use. The samples were prepared in duplicate to allow for a repeat analysis.

DNA extraction. The genomic DNA was extracted following a modified version of the method reported by De la Rosa et al. (2002). A 1 mm stainless steel ball bearing was placed inside the tube containing the frozen leaf sample. Extraction reagents were added, the samples were mixed with the reagents, and ground thoroughly using a Tissuelyser II ball mill (Qiagen, Germany). The samples were incubated for 30 min at 65 °C and the ball bearings were removed. A two-part cleaning step using chloroform-isoamyl alcohol was conducted. The top aqueous phase was recovered and precipitated with cold isopropanol overnight. After precipitation, the samples were centrifuged and the pellet washed in 70% (v/v) ethanol, dried and re-suspended in a 1X TE buffer.

Primers. The primers were designed to generate products shorter than 500 bp. These were ACS1-Pr′F 5′-agc ata tgg acc agg gtg ggt c-3′ (annealing at position 1220-1241) and ACS1-Pr′R 5′-ggc gtt cac cat tac

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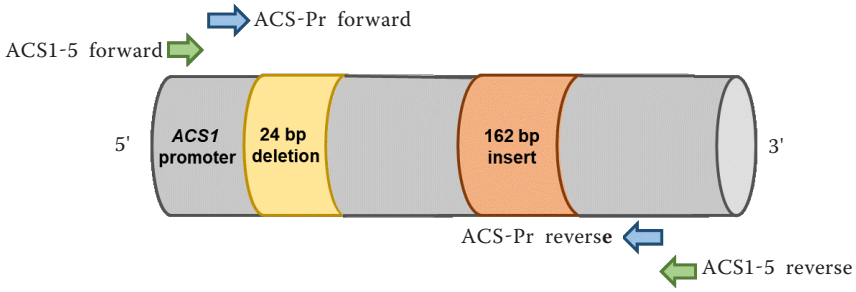

Figure 1. The structure of the promoter region of the *ACS1* gene in an apple (based on Sunako et al. 1999) showing the 162 bp SINE insertion and the concomitant 24 bp deletion that distinguish alleles *a* and *b*

Green horizontal arrows – represent the published ACS1-5 primers; blue horizontal arrows – indicate the newly designed ACS-Pr primers amplifying across the indel giving products differing by approximately 138 bp

ctg gca tata-3′ (annealing at position 1553-1530). They are based on the only sequence of the *ACS1* promoter region in the apple found on the National Center for Biotechnology Information (NCBI) website in 2014, AB010102.1, and flanked the indel (Figure 1) to give products of expected sizes of 198 bp (allele *a*) and 335 bp (allele *b*). Note that the sizes estimated via fluorescence detection can vary slightly, but the difference between the alleles remains constant. The forward primer was fluorescently labelled with photoinduced electron transfer (PET) (Applied Biosystems, South Africa).

In addition, the fluorescent versions of the ACS1-5 primers reported by Sunako et al. (1999) were used on a small set of cultivars to verify the reported product sizes, 489 bp (allele *1*) and 655 bp (allele *2*). The forward primer was labelled with Victoria (VIC) (Applied Biosystems). Five cultivars of *M. pumila,* 'Delicious', 'Golden Delicious', 'Granny Smith', 'Jersey Mac' and 'Jonagold' were analysed using the conditions as described for the ACS1-Pr primers.

***ACS1* genotyping.** Polymerase chain reactions (PCRs) were performed in a final volume of 12.5 µL containing 1.5 µL of the 100 ng template DNA, 6.25 µL of the 2X Multiplex PCR Master Mix (Qiagen), 1 µL of the *ACS1* primer mix and 3.75 µL of RNase-free MilliQ water. Amplification was carried out in a GeneAmp (Applied Biosystems) thermal cycler using the following conditions: an initial denaturation at 95 °C for 15 min, followed by 29 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and a final 30 min extension at 60 °C.

The PCR products were sized with capillary electrophoresis on a 3130 DNA capillary analyser (Applied Biosystems). The sizes of the amplified products were established in comparison with the internal size standard, GS500(-250)LIZ (Applied Biosystems) in the case of the ACS1-Pr primers and a GS1200LIZ standard in the case of the ACS1-5 primers, as the product size of allele *2* was outside the GS500(-250) LIZ range. The software GENEMAPPER 5.0™ (Applied Biosystems) was used to visualise the peaks and aid the allele scoring.

Accessions that exhibited single peaks on the GENEMAPPER traces were assumed to be homozygous; those with only the 202 bp product were designated *aa*, or *aaa* in the case of known triploids, and those with only the 339 bp peak were designated *bb*, or *bbb* in the known triploids. Heterozygous accessions with both the 202 bp and 339 bp peaks, were designated *ab* or, in the case of the known triploids, *ab-*. Occasional variants of the *a* allele were observed and designated with a subscript denoting the size, e.g., a_{204}.

ACS1-Pr multiplexing with apple microsatellite markers. To investigate the suitability of the ACS1-Pr primers for the multiplexing, they were used in a multiplex with three microsatellite markers known to give product sizes of 175 to 257 bp: CH04e05 (6-FAM), CH02c11 (NED) and CH02d08 (VIC) (Liebhard et al. 2002). Eight accessions for which the ACS1 genotypes have previously been published were analysed using the combination described by Fernández-Fernández (2010). They were five cultivars of *M. pumila*, 'Braeburn', 'Delicious', 'Golden Delicious', 'Granny Smith' and 'Jonagold' and two accessions of *Malus* species, *M. floribunda* and *M. robusta* 5.

RESULTS

Performance of fluorescent primers, *ACS1* genotyping and novel variants in apple. The redesigned primer, ACS1-Pr, successfully amplified the products in all the *Malus* samples tested giving either one or two products per item that were conveniently identified via an automated sequencer (Tables 1 and 2). In *M. pumila*, allele sizes of 202 bp

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Table 1. The *ACS1* genotypes of 59 *M. pumila* cultivars and the accessions of three *Malus* species or hybrids in the ARC collection genotyped with the fluorescently labelled primers, ACS1-Pr, and compared with the genotypes reported in the literature. The published genotypes *1/1*, *1/2* and *2/2* are equivalent to *aa*, *ab* and *bb*

Location	Accession name	Alleles	ARC	Reported	Reference‡
E1-15-18	Akane	339	*bb*	*2/2*	O'04
E1-7-3	Alkmene	339	*bb*	*2/2*	O'07
E1-9-10	Anna	202/339	*ab*	*1/2*	P'14
DN7-24-9	Antonovka Seedling	202	*aa*	*1/1, 1/2*	S'99, P'14
E1-11-1	Braeburn	202/339	*ab*	*1/2*	O'07, ZB'08
DN7-5-3	Cox's Orange Pippin	202/339	*ab**	*1/1*	S'99
E1-9-14	Delblush	202	*aa**	*2/2*	ZB'08
E1-18-8	Democrat	202	*aa*	*1/1*	Z'09
DN7-4-5	Elstar (Red)	339	*bb*	*2/2*	P'14
E1-9-9	Fuji Akufi	339	*bb*	*2/2*	All
E1-9-2	Gala	339	*bb*	*2/2*	O'04, ZB'08
DN7-16-9	Ginger Gold	202/339	*ab*	*1/2*	ZB'08
E1-7-20	Gloster	339	*bb*	*2/2*	Z'09
E1-16-12	Golden Delicious	202/339	*ab*	*1/2*	All
E1-11-13	Goldrush	202/339	*ab*	*1/2*	ZB'08, P'14
DN7-19.1	Granny Smith	202/339	*ab*	*1/1, 1/2*	O'04/Others
E1-7-9	Gravenstein (Red) †	202	*aaa*	*1/1*	P'14
E1-11-19	Himekami	339	*bb*	*2/2*	S'99, ZB'08
E1-1-13	Hokuto	202/339	*ab*	*1/2*	ZB'08
E1-1-2	Homei Tsugaru	202/339	*ab*	*1/2*	S'99, H'00, O'04
E1-13-16	Hunter Ontario	202/339	*ab**	*1/2, 2/2*	Z'09/P'14
DN7-5-10	Jersey Mac	202	*aa*	*1/1*	H'00, O'07
DN7-17-5	Jonagold†	202/339	*ab-*	*1/2*	H'00, O'04, M'14
E1-16-7	Jonathan	202/339	*ab*	*1/2*	S'99, H'00, O'04, M'14
E1-10-13	Jumbo Orin	202	*aa**	*1/2*	S'99, H'00, O'04, Z'09
E1-17-15	Kidd's Orange Red	202/339	*ab*	*1/2*	Z'09
E1-18-15	Klara	202	*aa*	*1/1*	Z'09
DN7-6-4	Lady Williams	202/339	*ab*	*1/2*	O'07
DN7-5-2	Liberty	202	*aa**	*1/2*	P'14
E1-13-6	Lord Lambourne	202/339	*ab*	*1/2*	P'14
E1-19-1	McIntosh	202	*aa*	*1/1*	S'99, H'00, O'04', P'14
E1-3-11	Melba	202	*aa*	*1/1*	Z'09
DN7-5-6	Melrose	339	*bb*	*2/2*	Z'09
DN7-1-1	Mollie's Delicious	202/339	*ab*	*1/2*	P'14
E1-10-17	Mutsu†	202/339	*ab*	*1/2*	S'99, H'00, O'04
DN7-4-8	Northern Spy	202	*aa*	*1/1*	S'99, Z'09
DN7-16-7	Pink Lady	202/339	*ab*	*1/2*	ZB'08
DN7-2-2	Pinova	339	*bb*	*2/2*	ZB'08
DN7-6-9	Prima	202/339	*ab*	*1/2*	Z'09
DN7-6-10	Priscilla	202/339	*ab*	*1/2*	P'14
E1-14-4	Red Delicious	202/339	*ab*	*1/2*	O'07
E1-9-16	Red Statesman	202	*aa*	*1/1*	O'07

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Table 1 to be continued. The *ACS1* genotypes of 59 *M. pumila* cultivars and the accessions of three *Malus* species or hybrids in the ARC collection genotyped with the fluorescently labelled primers, ACS1-Pr, and compared with the genotypes reported in the literature. The published genotypes *1/1*, *1/2* and *2/2* are equivalent to *aa*, *ab* and *bb*

Location	Accession name	Alleles	ARC	Reported	Reference‡
E1-9-16	Red Statesman	202	*aa*	*1/1*	O'07
E1-16-3	Redfree	202	*aa*	*1/1*	Z'09
E1-3-18	Reinette du Canada†	202	*aaa*	*1/1*	S'99
E1-8-5	Resista	202/339	*ab*	*1/2*	Z'09
E1-17-19	Rokewood	202	*aa*	*1/1*	O'07
E1-6-12	Rome Beauty	202/339	*ab**	*1/1*	S'99, O'07
DN7-17-10	Russian Seedling	202	*aa*	*1/1*	P'14
E1-19-9	Selena	202	*aa*	*1/1*	Z'09
E1-7-5	Senshu	339	*bb*	*2/2*	O'04, ZB'08
E1-2-6	Shampion	202/339	*ab*	*1/2*	Z'09
E1-18-4	Shizuka	202/339	*ab*	*1/2*	ZB'08
E1-5-14	Spartan	202	*aa*	*1/1*	Z'09
DN7-6-5	Splendour	339	*bb*	*2/2*	O'07, ZB'08
E1-10-5	Sundowner	202/339	*ab*	*1/2*	ZB'08
E1-2-2	Sunrise	202/339	*ab*	*1/2*	ZB'08, P'14
E1-5-2	Vista Bella	202	*aa*	*1/1*	H'00
E1-5-3	White Winter Pearmain	202/339	*ab*	*1/2*	S'99
E1-4-18	Zvonkove	202/339	*ab*	*1/2*	Z'09
Malus					
DN7-34-1	*M.* Aldenhamensis	202/204	aa_{204}	*1/1*	H'00
DN7-17-6	*M. floribunda* 821	202/204	aa_{204}	*1/1*	S'99
DN7-2-7	*M. spectabilis*	202	*aa*	*1/1*	S'99

**ACS1* genotype inconsistent with the published genotype; † triploid, ‡S'99 – Sunako et al. (1999); H'00 – Harada et al. (2000); O'04 – Oraguzie et al. (2004); O'07 – Oraguzie et al. (2007); ZB'08 – Zhu and Barritt (2008); Z'09 – Zoufalá et al. (2009); M'14 – Marić and Lukić (2014); P'14 – Peace (2014)

and 339 bp were observed for alleles *a* and *b*, respectively; i.e., differing by 137 bp, not 166 bp as previously reported.

For the 224 *M. pumila* cultivars, 102 accessions were homozygous for allele *a*, 100 were heterozygous *ab* and 22 were homozygous for allele *b*.

Novel variants for the *a* allele, namely 204, 205 and 206 bp, were, however, detected in some other *Malus* species. Only one of the 21 *Malus* species and hybrids was heterozygous for the *b* allele, while the remaining accessions were homozygous for allele *a*; no *bb* genotypes were observed. Interestingly, there were several variants of allele *a* in most of the *Malus* species and hybrids (Figure 2), with sizes of 204, 205 and 206 bp, which had not been detected in previous studies using agarose gel electrophoresis.

The *ACS1* genotypes of 59 *M. pumila* cultivars in the ARC gene bank were compared with the published *ACS1* genotypes (Table 1). For five of the 59 accessions, the *ACS1* genotypes were inconsistent, while the remaining 54 matched. No inconsistencies were found when comparing the genotypes of the three *Malus* species and hybrids with the reported genotypes.

The genotypes for the 187 accessions determined in the current study (Table 2) are reported for the first time; of these, 169 were *M. pumila* cultivars and 18 were accessions of the *Malus* species and hybrids. In the case of heterozygous triploids, it was not possible to distinguish whether the two peaks represented *aab* or *abb*.

Investigation of discrepancies with reported sizes. When the fluorescently labelled ACS1-5 primers (Sunako et al. 1999) were tested on five cultivars, the allele scores of 514 bp and 652 bp were consistently observed for the two alleles

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Table 2. Previously unreported *ACS1* genotypes of 225 apple accessions in the ARC collection determined with the fluorescently labelled ACS1-Pr primers (†triploid)

Location	Accession Name	*ACS1*	Location	Accession Name	*ACS1*
***M. pumila* cultivars**			E1-4-13	Elise	*ab*
DN7-15-5	Adina	*bb*	E1-12-13	Diva Gold	*ab*
E1-3-12	Adina (syn Frankad)	*bb*	DN7-4-4	Drakenstein	*aa*
E1-3-10	African Carmine	*ab*	E1-16-17	Dunn‘s Seedling	*aa*
E1-9-19	Alfmission	*ab*	E1-9-12	Earligold	*aa*
E1-14-11	Alsop‘s Beauty	*aa*	DN7-22-2	Edgewood	*ab*
DN7-16-1	Aport	*aa*	E1-15-11	Eikhoff	*aa*
E1-12-4	Arapkizi	*aa*	E1-4-13	Elise	*ab*
E1-17-11	Atties Favourite	*ab*	E1-8-19	Elsie Grant	*ab*
DN7-31-2	Austin	*aa*	E1-7-13	Florentina	*aa*
E1-13-10	Baujade†	*ab*	E1-19-5	Forum†	*ab-*
E1-12-12	Beaumont	*aa*	E1-4-11	Gavin	*bb*
E1-14-3	Belle de Boskoop†	*aaa*	E1-9-18	Gloire de Hollande	*aa*
E1-14-8	Belrene	*aa*	E1-9-20-	Goldsmith	*ab*
E1-13-11	Beni Osho	*aa*	E1-10-20-	Goosen	*bb*
DN7-4-7	Beverly Hills	*aa*	E1-9-8	Grand Richard	*aa*
DN7-27-1	Bittenfelder	*bb*	E1-11-15	Granearli	*ab*
E1-14-6	Blairmont	*ab*	E1-1-6	Greensleeves	*ab*
E1-13-21	Boiken	*ab*	E1-13-17	Harberts Reinette	*aa*
E1-17-18	Calville de Saint Souve	*ab*	DN7-24-8	Hofer Seedling	*bb*
E1-7-19	Canvade	*aa*	DN7-4-6	Hoplan X	*aa*
E1-17-16	Champion	*aa*	E1-17-20	Hoplan Y	*ab*
E1-18-14	Chantecler	*aa*	E1-14-10	Hops Late Red	*ab*
E1-17-10	Charden†	*ab*	E1-11-3	Howell	*bb*
E1-14-2	Climax	*aa*	DN7-33-2	Jester	*ab*
E1-17-5	Coast	*ab*	E1-15-5	Jonafree (=Co-op 22)	*ab*
E1-16-16	Commerce	*ab*	E1-16-2	Karmijn de Sonnaville†	*ab-*
E1-16-15	Co-op 19	*bb*	DN7-5-1	Kashawi	*aa*
E1-7-12	Co-op 20	*ab*	E1-13-19	King of Tomkins County	*ab*
E1-7-17	Coromandel Red	*ab*	E1-1-5	Kirks B	*aa*
E1-16-21	Crab A	*aa*	E1-16-11	Kogetso	*ab*
E1-9-4	Crab C	*aa*	E1-7-1	Koo	*aa*
E1-7-14	Criterion	*ab*	E1-15-14	Lakeside	*ab*
E1-16-18	Dakota	*ab*	E1-17-12	Langkloof	*aa*
E1-13-15	Dayton (=Co-op 21)	*aa*	E1-12-15	Laxton‘s Superb	*aa*
DN7-24-1	Dayton Seedling No6	*aa*	DN7-19-2	Le Vant	*ab*
DN7-18-4	Delkistar	*ab*	E1-10-6	Lemon†	*ab-*
E1-12-13	Diva Gold	*ab*	E1-10-19	Leyda	*aa*
DN7-4-4	Drakenstein	*aa*	E1-12-9	London Pippin	*bb*
E1-16-17	Dunn‘s Seedling	*aa*	E1-11-8	Longford	*aa*
DN7-21-6	Duquesa	*ab*	DN7-20-10	M1	*aa*
E1-9-12	Earligold	*aa*	DN7-20-11	M4	*aa*
DN7-22-2	Edgewood	*ab*	DN7-21-11	M7	*aa*
E1-15-11	Eikhoff	*aa*	DN7-2-11	M9	*aa*

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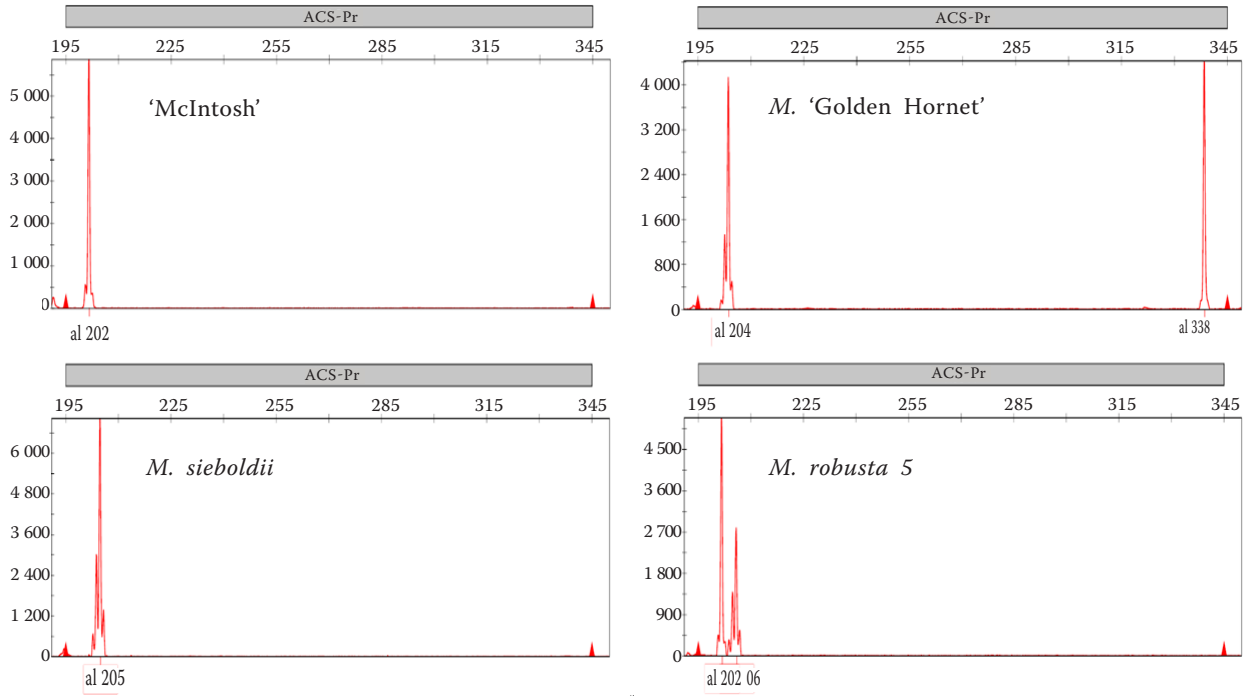

Figure 2. The products of the *ACS1* promoter in the *Malus* accessions *M.* 'Golden Hornet', *M. sieboldii* and *M. robusta* 5, compared to the *M. pumila* accession 'McIntosh' amplified with the ACS1-Pr primers and visualised with GENEMAPPER, showing the variants of allele *a*, of 204, 205, and 206 bp, rather than 202 bp. The accession *M.* 'Golden Hornet' is the only accession not belonging to *M. pumila* that has 'the' *b* allele

(Figure 3). These are different from the published allele sizes of 489 bp and 655 bp. Note that the difference in the sizes between the two alleles detected with the redesigned fluorescently labelled primers in the current study was 138 bp whereas the difference between the sizes of the two published alleles (489 and 655 bp) was 166 bp. The difference observed with the fluorescently labelled ACS1-5 primers is in close agreement with the difference observed in the current study with the ACS-Pr primers, namely 137 bp.

DISCUSSION

Fluorescently labelled markers. The redesigned fluorescently labelled ACS1-Pr primers proved informative in detecting the *ACS1* genotypes in the apples. Product sizes of 202 bp and 339 bp with a difference of 137 bp were detected. The difference was consistent with the indel phenomenon reported by Sunako et al. (1999), but not with the difference of 166 bp between the allele sizes reported in that study and at least five other subsequent studies, all which used agarose gel electrophoresis.

Variants of *a* allele. part from the 202 bp product, various *Malus* accessions showed a minor variation in length of the amplification product of allele *a*, which had not previously been reported and would not have been detectable using agarose gel electrophoresis. No variants were detected for allele *b*.

Discrepancies in reported sizes. The difference of 137 bp detected with the PET fluorescently labelled ACS1-Pr primers was essentially confirmed with the VIC fluorescently labelled version of the ACS1-5 primers designed by Sunako et al. (1999) which gave products of 514 bp and 652 bp, a difference of 138 bp. This differed markedly from the allele sizes of 489 bp and 655 bp (differing by 166 bp) previously reported by Sunako et al. (1999) using unlabelled ACS1-5 primers and several subsequent studies using the same primers (Harada et al. 2000; Oraguzie et al. 2004, 2007; Zhu, Barritt 2008; Zoufalá et al. 2009; Marić, Lukić 2014). The insertion and deletion phenomena explained by Sunako et al. (1999) account for a difference of 138 bp between the two alleles. Fluorescent detection can introduce a minor shift in the product length (Sutton et al. 2011), so the difference of 137 bp observed in the current study, rather than

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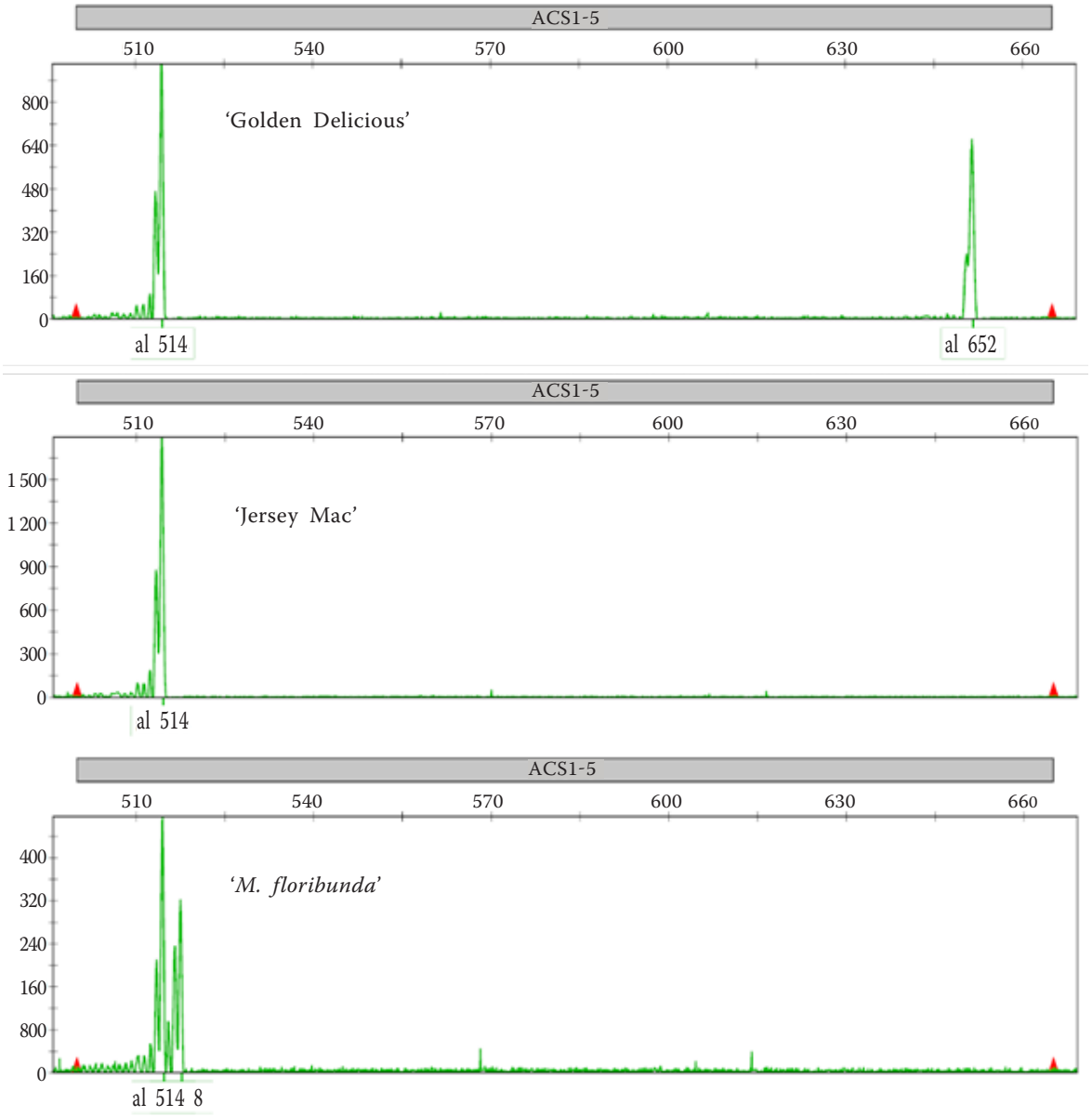

Figure 3. Products of ACS1 promoter in the cultivars 'Golden Delicious', 'Jersey Mac' and *M. floribunda* amplified with the fluorescently labelled version of the ACS1-5 primers (Sunako et al. 1999) and visualised with GENEMAPPER 5.0TM. Allele sizes of 514 bp (representing allele *a* or *1*) and 652 bp (allele *b* or *2*), differing by 138 bp were observed. Similar allele sizes were observed for 'Delicious' (*ab*), 'Granny Smith' (*ab*) and 'Jonathan' (*ab*). Additionally, variation of the a allele was detected in *M. floribunda*

the expected 138 bp, is not surprising. The apparent mis-sizing of the two alleles in all the previous studies may be attributed to a miscalculation of the indel phenomena in the initial paper, by particularly neglecting the 24 bp deletion associated with the 162 bp insertion, and perpetuated by the subsequent authors. Of course, the use of agarose gel electrophoresis might not allow for the detection of such errors when sizing the amplification products.

ARC *ACS1* genotypes. A total of 224 *M. pumila* cultivars were genotyped, but only 22 cultivars were observed to have the genotype *bb* associated with the low ethylene production. A collation of international apple accessions from the literature indicates that 59 out of 290 *M. pumila* accessions have the genotype *bb* (Supplementary Table 1).

The *ACS1* genotypes of 169 'primary' *M. pumila* cultivars in the ARC gene bank have not been re-

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ported previously. There were only 11 *bb* genotypes observed among them. Of 169 *M. pumila* accessions, 14 were triploid, four of which were homozygous for allele *a*. It was, however, not possible to distinguish the *aab* from the *abb* genotypes in the triploids by the differential peak sizes.

For the 21 accessions of the *Malus* species and hybrids analysed, 18 genotypes had not been reported previously. Variants of *a* were observed in 16 of the 21 accessions. A more detailed survey of other *Malus* species and hybrids may reveal how the length variation with respect to allele *a* occurred in relation to the speciation, and to establish if the variation exists in only certain sections of the genus *Malus*. The presence of allele *b* only in *M*. 'Golden Hornet', possibly a seedling of *M*. × *zumi* 'Calocarpa' × *M*. *prunifolia* 'Coccinea', suggests the involvement of a *M*. *pumila* parent in its pedigree – unless allele *b*, and the introduction of the SINE, predates the speciation of *M*. *pumila*. However, one accession each of *M*. *prunifolia*, *M*. *toringoides* and *M*. *yunnanesis* have also been reported to be heterozygous *ab* (Sunako et al. 1999).

Comparison of ARC genotypes with published *ACS1* genotypes. There are several possible explanations, such as mislabelling, why five of the 59 *M*. *pumila* accessions had *ACS1* genotypes inconsistent with the published genotypes. Three of the accessions, 'Cox's Orange Pippin', 'Liberty' and 'Rome Beauty', appeared to be true to type based on the comparison of the microsatellite fingerprints with those recorded for the UK national apple collection at Brogdale (Fernández-Fernández, personal communication). The cultivars 'Delblush' and 'Jumbo Orin' were not in the Brogdale collection, but both are reported to be seedlings of 'Golden Delicious' and did indeed share a common allele with that cultivar. There are at least three inconsistent reports in literature. In the case of 'Granny Smith', the genotype was reported as *1/1* by Oraguzie et al. (2004) but *1/2* in other studies (Sunako et al. 1999; Harada et al. 2000; Peace 2014). 'Hunter Ontario' was reported to be *1/2* by Zoufalá et al. (2009), whereas Peace (2014) reported it to be *2/2* while 'Cox Orange Pippin' was reported to be *1/1* by Sunako et al. (1999) but Marić and Lukić (2014) reported it to be *1/2*.

No inconsistencies were observed for the three *Malus* accessions when compared with the reported genotypes for similarly named accessions in other studies. However, in two cases, the variant allele a_{204} was detected in addition to a_{202}.

Genetic resources, breeding and multiplexing. Knowledge of the *ACS1* genotypes associated with the ripening will guide the choice of the parents for breeding low ethylene producing cultivars for improved storability that may not need treatment with ethylene inhibitors (Zhu, Barritt 2008). Intercrossing cultivars with *bb* genotypes would create progenies not segregating for the trait, but in cases where heterozygous *ab* parents are crossed with each other or with *bb* homozygotes, the progenies will segregate. The fluorescent primers can be used for marker-assisted selection to select the seedlings with the homozygous *bb* genotype soon after germination. However, although *ACS1* is an important factor influencing the fruit firmness before harvesting, other genes are also important in the ripening pathway and affect the overall storability of apple fruits, including 1-aminocylopropane-1-oxidase, *ACO1* (ethylene production), *ACS3* (*ACS1* accelerator), polygalacturonase, *PG* (post-harvest softening) and expansin, *Exp7* (fruit softening) (Costa et al. 2005; Wakasa et al. 2006; Costa et al. 2008; Wang et al. 2009; Nybom et al. 2012; Varanasi et al. 2013). Hence, it would be useful to the breeding programme to genotype the accessions for these genes too.

CONCLUSION

The fluorescently labelled ACS1-Pr primers were useful for assigning the *ACS1* genotypes to the accessions in the ARC apple gene bank collection, 187 which were genotyped for the first time, other *Malus* species included. Discrepancies in the reported allele sizes were highlighted and the correct sizes were proposed. Novel variants were detected for allele *a* lacking the SINE. The multiplexing potential of the ACS1-Pr primers was demonstrated. Breeding cultivars homozygous for the *b* allele will assist in addressing the storage challenge faced by South African fruit exporters and may help small-scale farmers who cannot afford sophisticated post-harvest facilities to store fruit longer. The evolutionary and functional significance of the variation in the *a* allele in *Malus* species and hybrids, and the occasional presence of the *b* allele in the accessions known not to derive from *M*. *pumila*, is not yet understood. Characterising germplasm collections for other 'known function' genes such as *ACO1* and *S*-incompatibility in addition to *ACS1* will provide further information needed by breeders to plan crosses and align their breeding programmes with industry needs.

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