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Molecular cloning and characterization of allele *d* – a newly identified allele of the 1-aminocyclopropane-1-carboxylate oxidase 1 (*ACO1*) gene in apple – Short Communication

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Abstract

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Apple is a climacteric fruit of economic importance and high nutritional value. Better understanding of the apple ripening process is needed to improve fruit quality and extend apple shelf life. This paper presents the results of molecular cloning and characterization of a newly identified allele of the ripening-specific *ACO1* gene from *Malus floribunda* 821. A polymorphism of the *ACO1* gene was detected using the polymerase chain reaction and *Bam*H1 and *Rsa*I enzymatic digestion. Whilst cloning and sequencing data confirmed the identity of allele *d*, it also revealed high sequence conservation (97.3 to 98.9%). In addition, a comparison of sequence data revealed some differences within the coding regions of the *ACO1* alleles identified so far.

Keywords: *Malus floribunda* 821; polymorphism of *ACO1* gene; ripening; ethylene

Ethylene, the gaseous plant hormone, plays a major role in many developmental processes, such as seed germination, fruit ripening, abscission and senescence (Abeles et al. 1992). The level of ethylene in plants is determined by the activity of two enzymes that catalyse the two final reaction steps in ethylene biosynthetic pathway: 1-aminocyclopropane-1-carboxylate (ACC) synthase (EC: 4.4.1.14; the *ACS* gene) and ACC oxidase (EC: 1.14.17.4; the *ACO* gene) (Yang 1985). The conversion of ACC to ethylene is catalysed by ACC oxidase.

In apple, *ACO* genes are encoded by a multigene family, and three isoforms have been reported so far, namely *ACO1*, *ACO2* and *ACO3* identified by Binnie and McManus (2009); all three gene isoforms are differentially expressed in fruit and leaf tissue. Of these *ACO* isoforms, *ACO1* is predominantly expressed in ripening apple fruit (Wakasa et al. 2006). The *ACO1* locus was mapped to LG10 in the apple genome (Costa et al. 2005).

Several alleles of the *ACO1* gene have been detected in apple: A and B (Castiglione et al. 1999), which correlate with alleles *a* and *b* (Marić et al. 2005; Marić, Lukić 2014) and alleles *ACO1-2* and *ACO1-1* (Costa et al. 2005); alleles *c* and *n* were reported by Marić et al. (2005) and Marić and Lukić (2014). Alleles *ACO1-1* and *ACO1-2* were identified by amplifying a fragment which encom-

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passes part of the third and the fourth exons, as well as the third intron which showed a large length polymorphism (Costa et al. 2005). Discrimination of *ACO1* alleles was based on the amplification of the entire gene and subsequent digestion with restriction enzymes, which allowed the identification of three (*a*, *b* and *c*) *ACO1* alleles (Marić et al. 2005; Marić, Lukić 2014).

This work studied the polymorphisms of the *ACO1* gene in apple by polymerase chain reaction (PCR) and restriction analysis, and characterized a novel allele of *ACO1* by cloning and sequencing. The *ACO1* alleles were denoted using the numbering system proposed by Marić and Lukić (2014).

MATERIAL AND METHODS

Plant material and extraction of genomic DNA. Three apple (*Malus* × *domestica* Borkh.) cultivars (Čadel, Starking and Bihorka) and *Malus floribunda* 821 were sampled from the collections of the Fruit Research Institute, Čačak, Republic of Serbia and East Malling Research, East Malling, United Kingdom, respectively. *Malus floribunda* 821 is a wild species carrying the most widely used *Vf* gene as a source of scab resistance; therefore, the majority of commercial apple scab resistant cultivars carry this gene. DNA was extracted from leaves according to Doyle and Doyle (1987).

Amplification of the *ACO1* gene and detection of an allelic polymorphism. The PCRs for the amplification of *ACO1* gene were performed according to Marić et al. (2005). Specific primers M11 and M12 used for amplifying *ACO1* were reported by Castiglione et al. (1999). Identification of the *ACO1* alleles was based on the digestion of the PCR product with *Bam*H1 and *Rsa*I (Thermo Scientific, Paisley, Scotland, UK) (Marić, Lukić 2014). Detection and visualization of the PCR products and DNA digested fragments of *ACO1* was reported in the study by Marić and Lukić (2014).

Cloning and sequencing of allele *d* of *ACO1* gene. The PCR product of *ACO1* from *Malus floribunda* 821 was purified and concentrated using the QIAquick PCR Purification Kit (Qiagen, GmbH, Hilden, Germany). After adding deoxyadenine to the ends, the PCR product was cloned into the pCR2.1 vector (Original TA Cloning® Kit, Invitrogen, Groningen, The Netherlands), whereupon it was transformed into One Shot^{TM} TOP10F´ competent *Escherichia coli* and screened by colony PCR with M13-F and M13-R primers. Allele variability was assessed by digestion with *Eco*RI and *Rsa*I. Plasmids from three positive colonies were extracted using a QIAprep Spin Miniprep kit and sequenced using M13 primers.

Sequence alignment and analysis. The sequence contigs were assembled and translated using the SeqMan and EditSeq programs (DNAStar, Madison, USA), respectively. The sequence of allele *d* of the *ACO1* gene from this manuscript was submitted to the NCBI GenBank database, for which the accession number KJ206336 was obtained. Nucleotide sequences and deduced amino acid sequences were aligned using the Clustal W method of the MegAlign program. The following published EMBL sequences for alleles *a*, *b* and *c* of *ACO1* in apple were included for comparison: AF030859, AJ001646, AY598767, AY598768, JQ675679, JQ675680, JQ675681, X98627 and Y14005.

RESULTS AND DISCUSSION

Determination of *ACO1* allelic constitutions of the assessed apple genotypes

The PCR product of the *ACO1* genomic fragment (Fig. 1) was approximately of 1,500 bp (cv. Starking and *Malus floribunda* 821) or 1,600 bp (cvs Čadel and Bihorka). The size of the PCR products is consistent with the amplified products of the

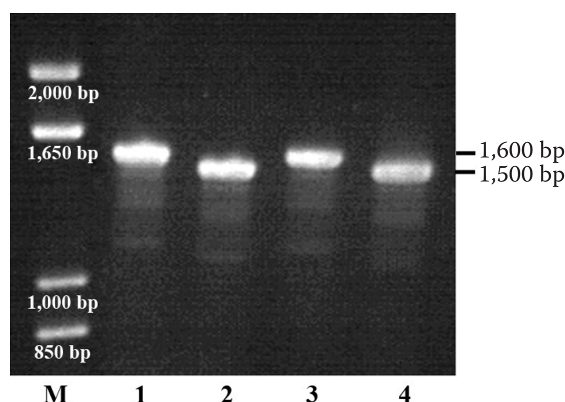

Fig. 1. PCR product of the amplification of the *ACO1* gene in assessed apple genotypes, separated on a 1.5% agarose gel and stained with ethidium bromide: cv. Čadel (lane 1), cv. Starking (lane 2), cv. Bihorka (lane 3), *Malus floribunda* 821 (lane 4); 1 kb Plus DNA ladder, Invitrogen (M)

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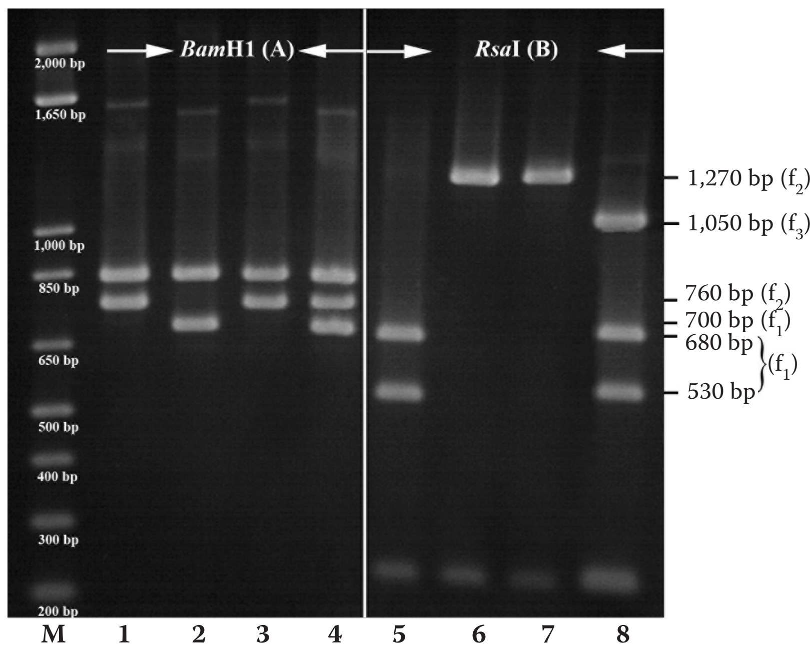

Fig. 2. DNA fragments of the *ACO1* gene obtained upon digestion with *Bam*H1(A) and *Rsa*I (B), separated on a 2% agarose gel and stained with ethidium bromide: cv. Čadel (lanes 1 and 5), cv. Starking (lanes 2 and 6), cv. Bihorka (lanes 3 and 7), *Malus floribunda* 821 (lanes 4 and 8); 1 kb Plus DNA ladder, Invitrogen (M). The relationship between restriction fragments (f_1, f_2 and f_3) and the *ACO1* allelic constitution is provided in Table 1

ACO1 gene obtained for different apple genotypes (Marić et al. 2005; Marić, Lukić 2014).

The polymorphism observed upon digestion of the PCR product with *Bam*H1 (two segregating fragments of 700 bp (f_1) and 760 bp (f_2)) and *Rsa*I (three segregating fragments of 530 and 680 bp (together considered as f_1 fragment), 1,270 bp (f_2) and 1,050 bp (f_3)) was interpreted as described by Marić and Lukić (2014). Upon comparison with published data, the f_3 fragment of 1,050 bp, obtained upon digestion with *Rsa*I, was identified only in *Malus floribunda* 821. This study confirmed three alleles (*a, b* and *c*) and revealed one novel allele (*d*) and four genotypes (*aa* for cv. Čadel, *bb* for cv. Starking, *cc* for cv. Bihorka and *dd* for *Malus floribunda* 821). The examples of banding patterns for *Bam*H1 and *Rsa*I are shown in Fig. 2, while the relationship between restriction fragments and the *ACO1* genotypes is presented in Table 1.

Table 1. Relationship between restriction fragments (f_1, f_2, and f_3), obtained upon digestion with *Bam*H1 and *Rsa*I restriction enzymes, and the allelic constitution of the *ACO1* gene

DNA fragment	*Bam*H1	*Rsa*I	Allelic constitution of *ACO1* gene
f_1		+	
f_2	+		*aa*
f_3			
f_1	+		
f_2		+	*bb*
f_3			
f_1			
f_2	+	+	*cc*
f_3			
f_1	+	+	
f_2	+		*dd*
f_3		+	

Cloning and sequencing of allele *d* of the *ACO1* gene

This study describes the full-length nucleotide sequence of 1,532 bp for allele *d* of the *ACO1* gene. The sequence of alelle *d* consists of four exons interrupted by three introns, covering a total of 902 and 630 bp, respectively. However, the sequencing data confirmed the unique identity of allele *d*, which was previously identified by digestion of the PCR product with restriction enzymes. All of the aforementioned results, including the location of the intron/exon junction, support the structure of the *ACO1* gene in apple (Marić, Lukić 2014).

The percentage identity between the full-length nucleotide sequence of alelle *d* and other EMBL-published sequences ranged from 97.4% (when compared to the sequence of allele *a*; AY598767) to 98.7% (when compared to the sequence of allele *b*; JQ675680), whereas the identity among the se-

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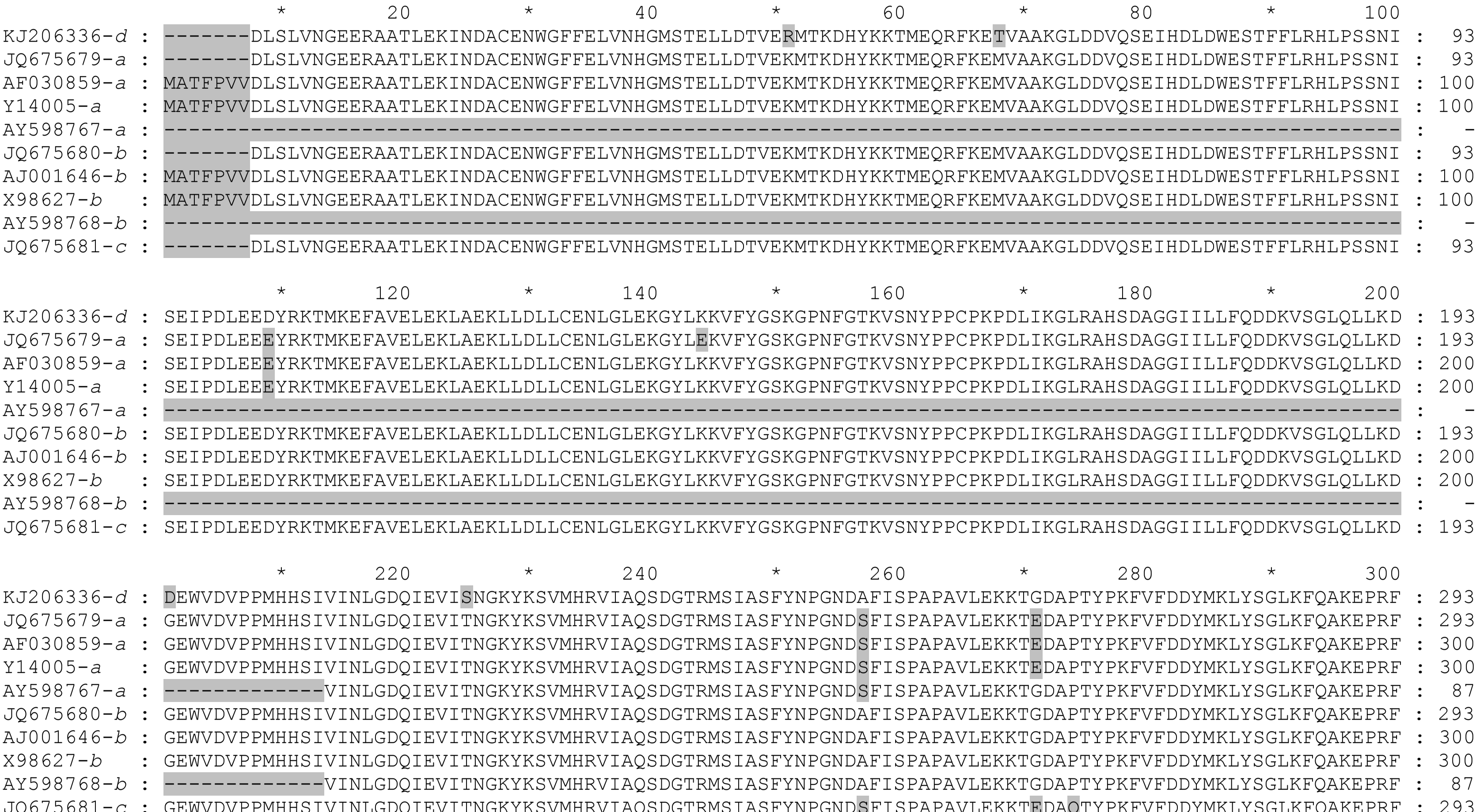

Fig. 3. Alignment of the deduced amino acid sequence of allele *d* from *Malus floribunda* 821 and published *ACO1* EMBL sequences: *ACO1d* – KJ206336; *ACO1a* – JQ675679; *ACO1a* – AF030859; *ACO1a* – Y14005; *ACO1a* – AY598767; *ACO1b* – JQ675680; *ACO1b* – AJ001646; *ACO1b* – X98627; *ACO1b* – AY598768; *ACO1c* – JQ675681; residues highlighted in grey indicate divergence in amino acid sequences or sequences which have not been analysed previously

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quences of allele *d* and alleles *a*, *b* and *c* from cvs Jonathan (*ab*) and Ducat (*cc*) reported by Marić and Lukić (2014) ranged from 98.3% (*d* and *a*; *d* and *c*) to 98.7% (*d* and *b*). A comparison of the introns among these alleles revealed that the sequences of the first intron are highly conserved. The highest variation in individual nucleotide replacements was observed in the sequences of the third intron (9 between *d* and *b*; 11 between *d* and *c*; 12 between *d* and *a*). In addition, the third intron of allele *d* from *Malus floribunda* 821 contained a 62 bp deletion. This deletion was previously identified in the third intron of allele *b*, but not in alleles *a* and *c* (Marić, Lukić 2014).

The comparison of the deduced amino acid sequence of allele *d* and the other published *ACO1* EMBL sequences (Fig. 3) revealed that the sequence identity ranged from 97.3% (when compared to the sequence of allele *a*; JQ675679) to 98.9% (when compared to the sequence of allele *b*; AY598768). Additionally, the sequence of allele *d* revealed differences in four, seven and eight amino acid residues in comparison with alleles *b*, *c*, and *a*, respectively (Marić, Lukić 2014). The amino acid sequences of these alleles indicate that *ACO1* in apple is highly conserved (the sequence identity is 97.3–99.0%).

The amino acid sequence of allele *d* revealed the presence of a Fe^{2+}-binding motif (His-177-X-Asp-179-X(54)-His-234), putative co-substrate hydrogen-binding residues (Arg-244-X-Ser-246), and both Lys-296 and Arg-299 residues in the C-terminal helix, all of which play a key role in the activity of the enzyme (Yoo et al. 2006).

In conclusion, this is the first report describing the molecular properties of the newly identified allele *d* of *ACO1* gene in apple. This polymorphism of the *ACO1* gene (four alleles) could provide further impetus to genotyping and identifying the apple germplasm. Current work is focused on the analysis of segregating progenies in order to elucidate the impact of the identified *ACO1* alleles on the storage ability of apple fruit.

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