

Results of highlighting genes associated with ethylene production in apple genotypes cultivated in Romania

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ABSTRACT

Molecular screening using SSLP and CAPS techniques enabled the genotyping of 40 apple cultivars developed at the main national research centres in Romania. All markers proved to be informative, successfully amplifying the target fragments previously reported in multiple studies. Genotyping with SSLP and CAPS techniques revealed a moderate but statistically significant level of genetic diversity and a high degree of allelic polymorphism. SSLP-based genotyping detected all expected allelotypes in the cultivars, whereas the CAPS technique identified all three expected allelotypes only when digested with the TaqI enzyme; digestion with BstNI did not yield all anticipated allelotypes. Pearson correlation analysis between allelotypes identified through SSLP and firmness loss after 30 days of storage showed no significant correlation for the bb/aa allelotype, which is associated with efficient ethylene limitation. However, a weak negative correlation was observed for the bb/ab allelotype, corresponding to a moderate ethylene production rate. The estimated inbreeding coefficient indicated a moderate reduction in observed heterozygosity for the MdACS1 marker, while a moderate heterozygote excess ($H_o > H_e$) was recorded for MdACO1 and MdACS3a markers. The study highlights a significant level of genetic diversity and provides valuable information for marker-assisted selection aimed at developing cultivars with superior postharvest quality.

Keywords: Firmness, CAPS technique, SSLP technique, Md-ACS1, MdACO1, MdACS3a, Apple species.

INTRODUCTION

Fruit ripening is a complex physiological process regulated by the so-called System II of ripening and mediated through mechanisms involving the plant hormone ethylene. In the context of apple breeding, understanding the genetic mechanisms that regulate ethylene concentration is essential, as producing high-quality fruits and extending storage life are major objectives. To date, seven genes encoding 1-aminocyclopropane-1-carboxylate synthase (ACS) have been identified in apple: MdACS1 (U89156), MdACS3a (AB243060), MdACS3b (AB243061), MdACS3c (AB243062), MdACS4, MdACSSA (AB034992), and MdACSSB (AB034993), among which only MdACS1 and MdACS3a are expressed in fruit tissues (Li *et al.*, 2013). Fruit firmness is physiologically influenced by ethylene concentration, and previous studies in apple have shown that ethylene biosynthesis is regulated by MdACS1, MdACO1, and MdACS3a. The ACS enzyme is encoded by MdACS1 and

MdACS3a, with MdACS1 playing a limiting role in the ethylene production pathway, while MdACS3a appears to mediate the transition from System I (basal ethylene production) to System II (the climacteric ethylene surge). Yue *et al.* (2020) reported that the transcription factor MdERF2, a member of the ERF family, restricts the accumulation of ACC (1-aminocyclopropane-1-carboxylic acid), thereby delaying the shift from System I to System II. Consequently, reduced expression of MdERF2 leads to upregulation of MdACS3a and MdACS1, accelerating fruit ripening and promoting firmness loss (Li *et al.*, 2017). MdACO1 encodes an ACC oxidase responsible for converting ACC into ethylene, and the molecular marker developed by Costa *et al.* (2005), also used in the present study, amplifies two DNA fragments of 525 bp and 587 bp a result confirmed by other studies (Shamshin *et al.*, 2018; Lyzhin, 2020). Expression of the System II gene MdACS1 correlates with ethylene production at the onset of fruit maturity: the MdACS1-2 allele (655 bp) is associated with low ethylene production and extended storage life, whereas MdACS1-1 (489 bp) corresponds to higher ethylene levels and shorter storage potential (Costa *et al.*, 2005; Sunako *et al.*, 1999). Heterozygous genotypes MdACS1-1/2 exhibit intermediate ethylene production compared with MdACS1-1/1 (higher) and MdACS1-2/2 (lower) (Wu *et al.*, 2021). MdACO1 acts independently of MdACS1 and has a less pronounced effect on storage capacity (Costa *et al.*, 2005; Oraguzie *et al.*, 2004). Allelic variation in the System I gene MdACS3a, expressed during fruit development (Costa *et al.*, 2005; Dougherty, 2019; Lundmark, 2019; Shamshin *et al.*, 2018), also plays a key role in ripening regulation. A G289V point mutation renders the ACC synthase enzyme non-functional (Wang *et al.*, 2009). Dougherty (2019) found that the G/T allelotype produces more ethylene than G/G, but less than MdACS1-1/1. Using the CAPS₈₇₀ marker, the T/T genotype showed a one-day delay in the ethylene peak compared with C/C and C/T, although ethylene levels and firmness loss identified through CAPS₈₆₆ and CAPS₈₇₀ markers were not consistently correlated. Overall, allelic effects of MdACS3a seem to limit the ethylene peak, whereas firmness loss is likely influenced by factors other than ethylene concentration. The aim of this study is to identify and highlight the genes associated with ethylene production in apple genotypes cultivated in Romania, in order to better understand the genetic variation underlying fruit ripening processes and storage capacity.

MATERIALS AND METHODS

Biological material. The biological material studied consists of 40 cultivar of apple, cultivar from the collections of the Research Institute for Fruit Growing Pitești - Maracineni ('Colonade', 'Colmar', 'Nicol', 'Rebra', 'Remus', 'Romus 3', 'Romus 4', 'Romus 5', 'Rustic', 'Doina', 'Alex', 'Aura', 'Bistrițean', 'Dany', 'Jonaprim', 'Salva', 'Starkprim', 'Precoce de Ardeal', 'Auriu de Cluj', 'Productiv de Cluj', 'Estival') și Research Station for Fruit Growing Voinești ('Cezar', 'Ciprian', 'Dacian', 'Delicios de Voinești', 'Discoprim', 'Frumos de Voinești', 'Generos', 'Inedit', 'Iris', 'Irisem', 'Luca', 'Pomona', 'Real', 'Redix', 'Remar', 'Revidar', 'Valery', 'Voinea', 'Voinicel').

PCR-SSLP and PCR-CAPS amplification. The PCR reaction mixture was optimised to a final volume of 15 µl, of which 11.5 µl was constituted of MyTaq™ Red Mix. For each primer pair (forward and reverse), 0.1 µl was added at a concentration of 0.1 mM, resulting in a final primer concentration of 0.666 µM per primer. DNA concentration was maintained within the range of 75–100 ng/µl, and 0.3 µl of ultrapure water was added to achieve the final reaction volume. Reaction conditions were identical across all primer pairs and optimised as follows: initial denaturation at 95 °C for 3 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2.5 min; followed by a final extension at 72 °C for 10 min.

Enzymatic restriction. Amplicons generated through amplification with the dCAPS (ACS3a) primer were digested with two restriction enzymes, BstNI and TaqI. The digestion reaction

was optimised to a final volume of 15 µl, comprising 10 µl of DNA, 2 µl of 10X Buffer R, 2 µl of BstNI or TaqI, and 1 µl of ultrapure water. The mixtures were incubated for 1 h at 37 °C for BstNI and at 65 °C for TaqI using a FastGene analyser.

Statistical analysis. The Pearson correlation coefficient was calculated using Minitab v.18, and the interpretation of the resulting values followed the criteria proposed by Colton (1974) (Table 1). The inbreeding coefficient was estimated according to Sewall Wright's concept (Wright, 1965), which defines it as the proportional reduction in observed heterozygosity relative to the level expected under Hardy-Weinberg equilibrium. This coefficient is expressed mathematically as $F_{is} = 1 - H_o/H_e$, where H_o represents the observed heterozygosity and $H_e = 2pq$ denotes the expected heterozygosity, with p and q being the allele frequencies in the population, and $p + q = 1$. Allelic polymorphism was evaluated using the Polymorphic Information Content (PIC) index, calculated using the formula $2f(1 - f)$, as described by Roldán-Ruiz *et al.* (2000) and Soengas *et al.* (2006). Genetic diversity was further quantified using the Shannon and Simpson indices, based on the formulas reported by Shannon (1948) and Simpson (1960). Thus, the Shannon index was computed as $\sum(n/N * \ln(n/N))$, and the Simpson index as $(\sum n(n - 1)) / (N(N - 1))$, where n denotes the number of alleles at a given locus and N the total number of alleles.

Table 1. Colton's rules (enounced in 1974)

Range of values	Level of differentiation
$-0,25 < r < 0,25$	Without correlation
$-0,5 < r < -0,25$ sau $0,25 < r < 0,5$	Poor correlation
$-0,75 < r < -0,5$ sau $0,5 < r < 0,75$	Moderate correlation
$r < -0,75$ sau $r > 0,75$	Strong correlation

RESULTS AND DISCUSSIONS

Genotyping of the cultivars using the MdACS1 marker identified 13 cultivars homozygous for the Md-ACS1-1 allele (489 bp), 8 cultivars ('Cezar', 'Discoprim', 'Luca', 'Productiv de Cluj', 'Romus 5', 'Salva', 'Remar', and 'Valery') homozygous for the Md-ACS1-2 allele (655 bp) and 19 heterozygous cultivars corresponding to the ab allelotype (Md-ACS1-1/2) (Table 2). Analysis of the Md-ACO1 marker revealed an additional amplification fragment (512 bp) alongside the previously known fragments (525 bp and 587 bp), as reported by Costa (2005). Our results corroborate those of Shamshin *et al.* (2018). So, 19 cultivars were identified as homozygous for the Md-ACO1-1 allele (525 bp), and 20 cultivars were heterozygous, corresponding to the ab allele (Md-ACO1-1/2). The homozygous Md-ACO1-2 state (bb allelotype, 587 bp) was not observed in any of the cultivars analysed. Additionally, the genetic profile of the 'Generos' cultivar did not conform to the observed allelotypic patterns, presenting a rare atypical profile that may indicate a potential mutation at this locus. (Table 2). In the case of the MdACS3a marker, digestion with BstNI resulted in 21 heterozygous cultivars corresponding to the G/T allelotype (MdACS3a/MdACS3a-G289V) and 19 homozygous cultivars corresponding to the G/G allelotype (MdACS3a/MdACS3a).

No cultivars were identified for the T/T allelotype (MdACS3a-G289V/G289V) among the total cultivars analysed (Fig. 1), and digestion with Taq1 enzyme, resulted in 23 heterozygous cultivars corresponding to the C/T allelotype (MdACS3a/Mdacs3a), 13 homozygous cultivars associated with the T/T allelotype (Mdacs3a/Mdacs3a), and 4 homozygous cultivars corresponding to the C/C allelotype (MdACS3a/MdACS3a) (Figure 2).

Table 2. The result of genotyping and highlighting alleotypes*

Nr. crt.	Cultivar	Highlighted gene				Genotyping	FD
		Md-ACS1	Md-ACO1				
		Alleles of ethylene biosynthesis genes				Allelotype	loss of firmness
		Md-ACS1-1 489 bp	Md-ACS1-2 655 bp	Md-ACO1-1 525 bp	Md-ACO1-2 587 bp	Md-ACS1 / Md-ACO1	Kgf/m ²
1	Alex	+	+	+	+	ab/ab	0,132
2	Ciprian	+	+	+	+	ab/ab	0,181
3	Dany	+	+	+	+	ab/ab	0,585
4	Delicios de Voinești	+	+	+	+	ab/ab	0,15
5	Frumos de Voinești	+	+	+	+	ab/ab	0,843
6	Irisem	+	+	+	+	ab/ab	1,17
7	Nicol	+	+	+	+	ab/ab	0,280
8	Redix	+	+	+	+	ab/ab	0,089
9	Rebra	+	+	+	+	ab/ab	0,122
10	Romus 4	+	+	+	+	ab/ab	0,324
11	Voinea	+	+	+	+	ab/ab	0,17
12	Voinicel	+	+	+	+	ab/ab	0,374
13	Colmar	+	-	+	-	aa/aa	0,11
14	Colonade	+	-	+	-	aa/aa	0,12
15	Dacian	+	-	+	-	aa/aa	0,43
16	Estival	+	-	+	-	aa/aa	0,428
17	Real	+	-	+	-	aa/aa	0,236
18	Remus	+	-	+	-	aa/aa	0,193
19	Romus3	+	-	+	-	aa/aa	0,324
20	Starkprim	+	-	+	-	aa/aa	0,504
21	Discoprim	-	+	+	-	bb/aa	0,377
22	Luca	-	+	+	-	bb/aa	0,888
23	Productiv de Cluj	-	+	+	-	bb/aa	0,275
24	Salva	-	+	+	-	bb/aa	0,560
25	Valery	-	+	+	-	bb/aa	0,21
26	Auriu de Cluj	+	-	+	+	aa/ab	0,367
27	Bistrițean	+	-	+	+	aa/ab	1,162
28	Doina	+	-	+	+	aa/ab	0,59
29	Jonaprim	+	-	+	+	aa/ab	0,39
30	Pomona	+	-	+	+	aa/ab	0,189
31	Aura	+	+	+	-	ab/aa	0,86
32	Iris	+	+	+	-	ab/aa	0,25
33	Inedit	+	+	+	-	ab/aa	0,300
34	Revidar	+	+	+	-	ab/aa	0,410
35	Precoce de Ardeal	+	+	+	-	ab/aa	0,60
36	Rustic	+	+	+	-	ab/aa	0,214
37	Remar	-	+	+	+	bb/ab	0,216
38	Romus5	-	+	+	+	bb/ab	0,213
39	Cezar	-	+	+	+	bb/ab	0,030
40	Generos	+	+	-	-	ab/0	0,435

* (bb/aa)= effective limitation of ethylene production; (bb/ab)= high ethylene level than bb/aa; (ab/aa)= high ethylene level than bb/ab; (ab/ab)= high ethylene level than ab/aa; (aa/aa)= high ethylene level than ab/ab; (aa/ab)= nivel de etilenă mai ridicat decât alelotipul aa/aa;

This distribution highlights the genetic diversity at the MdACS3a locus and suggests that heterozygous and homozygous allelotypes could be valuable for breeding programs targeting ethylene regulation and improved postharvest firmness.

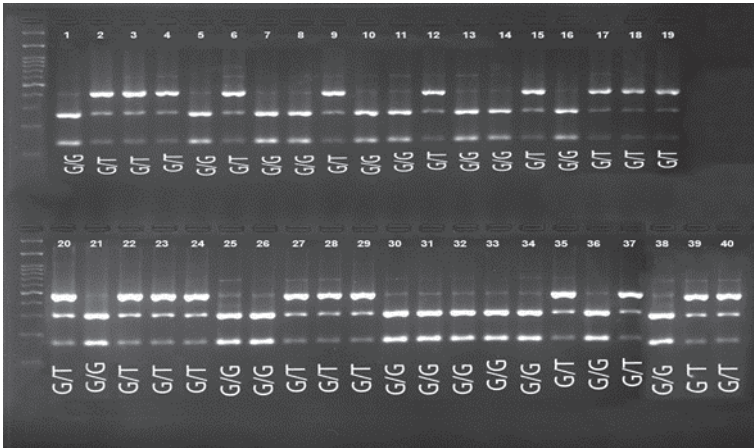


Figure 1. Electrophoretic profile for ACS3a marker with BstNI enzyme restriction.
1. 'Estival', 2. 'Rebra', 3. 'Bistrițean', 4. 'Aura', 5. 'Romus 3', 6. 'Dany', 7. 'Romus 5', 8. 'Productiv de Cluj', 9. 'Luca', 10. 'Ciprian', 11. 'Irisem', 12. 'Slava', 13. 'Jonaprim', 14. 'Rustic', 15. 'Precoce de Ardeal', 16. 'Iris', 17. 'Starkprim', 18. 'Auriu de Cluj', 19. 'Generos', 20. 'Colonade', 21. 'Nicol', 22. 'Colmar', 23. 'Delicios de Voinești', 24. 'Romus 4', 25. 'Remus', 26. 'Redix', 27. 'Alex', 28. 'Doina', 29. 'Voinicel', 30. 'Inedit', 31. 'Dacian', 32. 'Voinea', 33. 'Valery', 34. 'Real', 35. 'Discoprim', 36. 'Cezar', 37. 'Frumos de Voinești', 38. 'Pomona', 39. 'Revidar', 40. 'Remar'

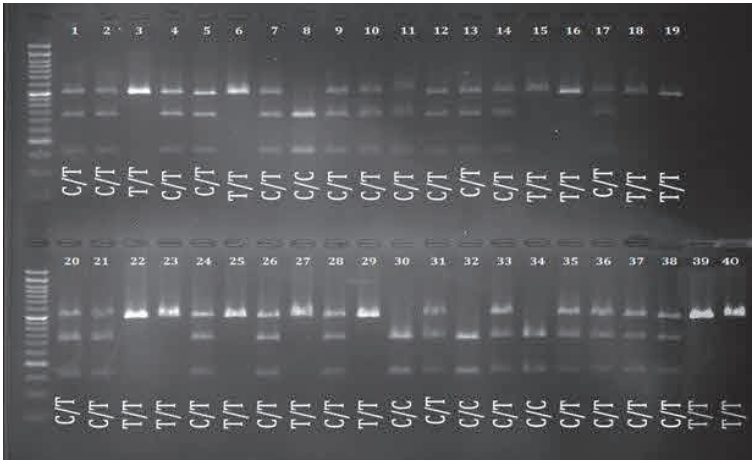


Figure 2. Electrophoretic profile for ACS3a marker with TaqI enzyme restriction.
1. 'Estival', 2. 'Rebra', 3. 'Bistrițean', 4. 'Aura', 5. 'Romus 3', 6. 'Dany', 7. 'Romus 5', 8. 'Productiv de Cluj', 9. 'Luca', 10. 'Ciprian', 11. 'Irisem', 12. 'Slava', 13. 'Jonaprim', 14. 'Rustic', 15. 'Precoce de Ardeal', 16. 'Iris', 17. 'Starkprim', 18. 'Auriu de Cluj', 19. 'Generos', 20. 'Colonade', 21. 'Nicol', 22. 'Colmar', 23. 'Delicios de Voinești', 24. 'Romus 4', 25. 'Remus', 26. 'Redix', 27. 'Alex', 28. 'Doina', 29. 'Voinicel', 30. 'Inedit', 31. 'Dacian', 32. 'Voinea', 33. 'Valery', 34. 'Real', 35. 'Discoprim', 36. 'Cezar', 37. 'Frumos de Voinești', 38. 'Pomona', 39. 'Revidar', 40. 'Remar'.

The inbreeding coefficient revealed a moderate reduction in observed heterozygosity for the MdACS1 marker and a moderate heterozygote excess ($H_o > H_e$) for the MdACO1 and MdACS3a markers (Table 3).

Table 3. Genetic diversity and inbreeding parameters for each marker

Marker	q	p	He	Ho	Fis
MdACS1	0,4375	0,5625	0,4921	0,475	0,034
MdACO1	0,2564	0,7435	0,3812	0,5128	-0,3452
MdACS3a (BstNI)	0,2625	0,7375	0,3871	0,525	-0,3562
MdACS3a (TaqII)	0,6125	0,3875	0,4746	0,575	-0,2115

Allelic polymorphism displayed a high information content (0.34125), surpassing the moderately informative threshold, while genetic diversity showed a moderately informative value (Table 4).

Table 4. Statistical analysis of allelic polymorphism and genetic diversity

PIC value>0,25 (moderately informative)	Indicele Shannon H > 3 (high genetic diversity)	Indicele Simpson (D) D=0 (infinite diversity) D=1 (lack of diversity)	Simpson diversity index (1-D), value € [0,1]
0,34125	2,0884191	0,1279803	0,8720197

Correlation analysis conducted using Minitab 18, in accordance with Colton (1974) criteria, between allelotypes identified through the SSLP technique and pulp firmness loss after 30 days of storage indicated a non-significant Pearson correlation coefficient (Table 5). Nevertheless, a weak negative correlation was observed for the *bb/ab* allelotype, suggesting that the presence of this allelotype contributes to reduced firmness loss. The homozygous *bb* state, corresponding to the *MdACS1* gene is associated with effective limitation of ethylene production, whereas the heterozygous *ab* state, corresponding to the *MdACO1* gene, is linked to an intermediate ethylene production level.

Table 5. Pearson correlation analysis between fruit firmness loss and identified allelotypes after 30 days of storage

Allelotip	bb/aa	bb/ab	ab/aa	aa/aa	aa/ab	ab/ab	ab/0
Pearson Index	0,110	-0,239	0,087	-0,163	0,217	-0,034	0,031
P-Value	0,500	0,138	0,595	0,314	0,179	0,835	0,851

Experimentally determined firmness values after 30 days of storage for the *bb/ab* allelotype showed that only three cultivars ('Discoprim', 'Productiv de Cluj', and 'Valery') exhibited good firmness retention, although they did not show the lowest overall firmness loss. As expected, the *bb/aa* allelotype should have conferred a more efficient reduction in ethylene production, compared to the *bb/ab* allelotype.

Nevertheless, genetic determinism alone does not fully account for the association between allelotype and firmness retention, suggesting that additional factors—climatic, agronomic, physiological, chemical, regulatory protein-related, and other genetic influences—also contribute to this trait.

Previous studies have shown that fruit firmness is a physico-structural characteristic influenced by physiological processes (Mangas *et al.*, 1992; Costa *et al.*, 2016; Ornelas-Paz *et al.*, 2018; Gwanpua *et al.*, 2015; 2016; Eshtiaghi and Kuldiloke, 20).

Consequently, the genotyping results obtained through the CAPS technique proved useful for studying polymorphism and genetic diversity (inbreeding coefficient, PIC, Shannon, and Simpson indices).

CONCLUSIONS

Apple fruit storability is largely governed by genetic factors but is also modulated by physiological influences, as no significant correlation was observed between allelotypes of System I ripening genes (*MdACS1* and *MdACO1*) and postharvest firmness loss. Analysis using molecular markers associated with ethylene biosynthesis demonstrated a notable

level of genetic diversity among the evaluated apple cultivars, reflected in a moderate excess of heterozygotes and in the values of Shannon and Simpson indices. The identification of allelotypes linked to firmness retention provides a solid foundation for marker-assisted selection, supporting the development of cultivars with enhanced postharvest quality.

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