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GENETICAL POLYMORPHISM OF ACC SYNTHASE AND ACC OXIDASE IN APPLE SELECTIONS BRED IN ČAČAK

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The work on breeding new apple cultivars, of improved quality and longer storage life has been going on for a long time at the Fruit and Grape Research Centre in Čačak. As a result nine promising apple selections, that show the range of fruit storage capabality (J/1/7, J/1/20, J/2/12, J/2/14, J/11/31, J/54/53/59, J/60/7/63, Šumatovka 1 O.P. and Šumatovka 2 O.P.), were singled out. Fruit ripening is genetically programmed, complex physiological process with the important role of plant hormone ethylene. Allelic polymorphism of the genes encoding ACC synthase and ACC oxidase, enzymes on ethylene biosynthetic pathway, was studied in promising apple selections and compared to their storage life. Polymorphism was detected by the polymerase chain reaction (PCR method) and restriction analysis with 6 restriction enzymes. Two alleles of the gene encoding ACC synthase (*ACS1-1* and *ACS1-2*), three alleles of the ACC oxidase gene (*a*, *b* and *n*) were identified and a positive test for early seedling selection, the fruits of which will be characterized by long storage life, was indicated.

Key words: apple, ethylene, ACC synthase, ACC oxidase, polymorphism, fruit ripening

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INTRODUCTION

The apple is a perennial, cross-pollinated, polyploidy, heterozygous fruit species with a long juvenile period. The work on breeding new apple cultivars has long been under way at the Fruit and Grape Research Centre in Čačak and as a result of systematic work two cultivars have been released and a number of promising selections have been singled out. In addition to the improvement of resistance to diseases and pests, high quality fruit with long storage life is the main objective of the program.

Apple fruit ripening is genetically programmed, complex physiological process with the important role of plant hormone ethylene. Apple is a typical climacteric fruit, whose ripening is associated with an upsurge in the rate of respiration and ethylene production (KNEE, 1993) and it is generally accepted that ethylene is the key regulator of apple ripening (THEOLOGIS, 1992). The suppression of ethylene biosynthesis and its action are the primary mechanisms by which controlled atmospheres extend the storage life of apples (LAU *et al.*, 1986; GORNY and KADER, 1996; 1997). Ethylene is synthesized from S-adenosyl-L-methionine (SAM) via a short pathway catalysed by two enzymes: 1-aminocyclopropane-1-carboxylate (ACC) synthase and 1-aminocyclopropane-1-carboxylate (ACC) oxidase (YANG and HOFFMAN, 1984). The conversion of SAM to ACC is the first step in the ethylene biosyntetic pathway and it is generally considered to be the rate-limiting step (LAU *et al.*, 1986). So far, SUNAKO *et al*. (1999) identified two alleles of gene encoding ACC synthase (*ACS1* gene). New allele *ACS1-2*, which possesses retroposon-like insertion (SINE) of 162 bp in the promotor region, has a very low transcription activity as compared to original allele (*ACS1-1*), and it has been speculated that this allele contributes to a improved storage life of the fruits of some apple cultivars. HARADA *et al*. (2000) and ORAGUZIE *et al*. (2004) have also reported that homozygosity of *ACS1-2* results in low ethylene production rate in the fruit of some apple cultivars.

For ACC oxidase, the analysis of the restriction products showed evidence of the existence of at least two allelic forms (A and B) of this gene that are assumed to affect the ethylene production rate in different apple species and cultivars (CASTIGLIONE *et al.*, 1999).

The aim of this work was to identify allelic forms of ACC synthase and ACC oxidase genes in promissing apple selections and to test the correlation between their allelic constitution for these genes and the storage capability of their fruits.

MATERIAL AND METHODS

Nine promising apple selections derived from the crosses Granny Smith x Golden Delicious (J/1/7, J/1/20, J/2/12, J/2/14, J/60/7/63), Prima x Melrose (J/11/31), Cox's Orange Pippin O.P. (J/54/53/59) and Šumatovka O.P. (Šumatovka

1 O.P. and Šumatovka 2 O.P) were analysed. The fruits of the assessed selections are characterized by differing storage capability. Apple selections J/1/7, J/1/20, J/2/12, J/11/31, J/54/53/59, J/60/7/63 are characterized by the long storage life of their fruits, selections J/2/14 and Šumatovka 1 O.P. by moderate storage capability, whereas selection Šumatovka 2 O.P is characterized by poor storage capability.

Genomic DNA was isolated from young leaves using CTAB mini prep method described by DOYLE and DOYLE (1987).

The reaction mixture (50 µl) for PCR amplification of the fragment of ACC synthase gene contained about 50 ng genomic DNA, 0.2 µM of each primer, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 1 x PCR reaction buffer and 2.5 U Taq DNA polymerase. The primers: ACS1-5'F AGAGAGATGCC-ATTTTTGTTCGTAC (861-887) and ACS1-5'R CCTACAAACTTGCGTG-GGGATTATAAGTGT (1379-1350) were used (SUNAKO *et al*., 1999). PCR conditions for amplification of ACC synthase alleles were as follows: 94^0C for 3 min, followed by 35 cycles of 94^0C for 1 min, 58^0C for 1 min and 72^0C for 2.5 min, with a final 10-min extension step at 72^0C.

The reaction for PCR amplification of the fragment of ACC oxidase gene was carried out in a 50 µl volume with 50 ng genomic DNA, 0.1 µM of each primer, 0.2 mM of each dNTP, 1 x PCR reaction buffer, 1.5 mM MgC_2 and 0.625 U Taq DNA polymerase. The primers, specific for ACC oxidase, M11 (GACTTGAGCCTTGTCAATG) and M12 (GATTCCTTGGCCTTCATAGC) were chosen on the basis of the apple fruit cDNA sequence stored in the EMBL data library (CASTIGLIONE *et al*., 1999). PCR conditiones were: 92^0C for 3 min, followed by 5 cycles of 92^0C for 1 min, 65^0C for 1 min and 72^0C for 1 min 30 sec; 35 cycles of 92^0C for 1 min, 60^0C for 1 min and 72^0C for 1 min 30 sec with a final 10-min extension step at 72^0C.

The amplified DNA products were analysed on 1.5% agarose gel. Electrophoresis lasted for 2 h at 70 volts/cm. Upon it, the gels were stained in ethidium bromide. As a ladder, 1 Kb plus DNA (GibcoBRL®, Life Technologies) was used.

PCR product of ACC oxidase gene was digested with 6 restriction enzymes of Type II: *Eco* RI, *Eco* RV, *Hind* III, *Bam* H1, *Pst* I and *Rsa* I, and digestion was done as follows: 15 µl PCR product was mixed with 15 µl of the mixture containing restriction enzyme (10 U/µl), buffer (10 x dissolved) and distilled water. The mixture was incubated for 12 h at 37^oC. The results of digestion were analyzed on 2% agarose gel. Electrophoresis lasted for 2 h at 70 volts/cm.

RESULTS AND DISCUSSION

Amplification of the fragment of the gene encoding ACC synthase. - The amplification of the *ACS1* gene fragment using ACS1-5'F and ACS1-5'R primers resulted in two DNA fragments of 490 and 640 bp. Fragment of 490 bp corresponds to allele *ACS1-1*, while fragment of 640 bp corresponds to allele *ACS1-2* (Fig. 1).

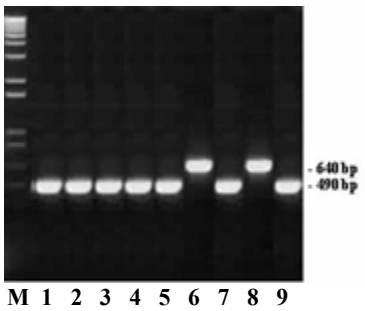

Fig. 1. PCR products of the amplified fragments of ACS1 gene obtained with primers ACS1-5'F and ACS1-5'R in 9 apple selections: 1- J/1/7, 2- J/1/20, 3- J/2/12, 4- J/2/14, 5- J/60/7/63, 6- J/11/31, 7- Šumatovka 1 O.P., 8- J/54/53/59, 9- Šumatovka 2 O.P; analysed on 1.5 % agarose gel and stained in ethidium bromide; M- 1Kb plus DNA ladder

Out of 9 promising selections and 6 parental cultivars studied, 8 were homozygous for *ACS1-1*, 4 heterozygous and 3 homozygous for *ACS1-2* (Tab. 1).

Table 1. Allelic constitution of ACS1 gene for the assessed apple selections and parental cultivars

ACS1-1/ACS1-1	ACS1-1/ACS1-2	ACS1-2/ACS1-2
	Granny Smith Golden Delicious	
J/1/7 J/1/20 J/2/12 J/2/14 J/60/7/63		
	Prima	Melrose J/11/31
	Cox's Orange Pippin	
		J/54/53/59
Šumatovka Šumatovka 1 O. P. Šumatovka 2 O. P.		

Fruits of the selections, as well as parental cultivars, homozygous for *ACS1-2* (J/11/31, J/54/53/59, Melrose) were characterized by a long-term storage, they can be stored until the end of the March in the cold store at 1-3^{0}C. No selection or parental cultivar, the fruits of which are charcterized by short storage life, has *ACS1-2/ACS1-2* allelic constitution.

These results supports the findings of SUNAKO *et al*. (1999), HARADA *et al*. (2000) and ORAGUZIE *et al*. (2004), who pointed out that homozygosity for *ACS1-2* could be attributed to a good storage potential, typical examples from their work were cvs Fuji and Ralls Janet.

They provide further evidence for the correlation between allelic constitution *ACS1-2/ACS1-2* and good storage capability of apple cultivars and indicate a reliable positive test for chosing parental combinations and for the early seedling selection. However, this test is unreliable for prescreening for poor storage capability as the selections J/1/7, J/1/20, J/2/12, J/60/7/63 are characterized by good storage potential (they can be stored in cold store at 1-3^{0}C until mid-March) even though their allelic constitution for *ACS1* gene is *ACS1-1/ACS1-1* rather than *ACS1-2/ACS1-2*. This indicate that fruit ripening is complex physiological process, in which other factors such as ones involved in ethylene perception have an important role.

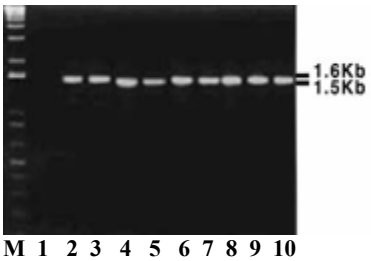

Fig. 2. DNA fragments obtained by PCR using M8 and M9 primers specific for the fragment of ACC oxidase gene in 1 apple cultivar and 9 selections: 1- Prima, 2- J/2/14, 3- J/1/20, 4- J/54/53/59, 5- J/11/31, 6- Šumatovka 1 O.P., 7- Šumatovka 2 O.P., 8- J/1/7, 9-J/2/12, 10- J/60/7/63; analysed on 1.5 % agarose gel and stained in ethidium bromide; M-1Kb plus DNA ladder

Amplification and restriction analysis of the fragment of the gene encoding ACC oxidase. - The amplification of the fragment of the gene encoding ACC oxidase using M11 and M12 primers in all the selections and parental cultivars studied, resulted in two DNA fragments of 1500 and 1600 bp, and absence of amplification in cv Prima (Fig. 2).

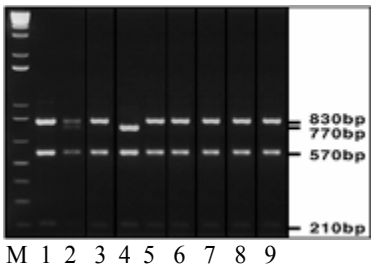

Fig. 3. DNA fragments (f1 and f2) upon digestion of PCR product of the amplified fragment of ACC oxidase gene with Hind III in 9 apple selections: 1- J/2/14, 2- J/54/53/59, 3- J/1/20, 4- J/11/31, 5- J/60/7/63, 6- J/2/12, 7- J/1/7, 8- Šumatovka 1 O.P., 9- Šumatovka 2 O.P; analysed on 2 % agarose gel and stained in ethidium bromide; M- 1Kb plus DNA ladder

Upon digestion of PCR product of the ACC oxidase gene, variation was obtained with all 6 restriction enzymes, but it was observed that *Eco* RI, *Eco* RV, *Hind* III, *Bam* H1 and *Pst* I revealed the same pattern of polymorphism. Therefore, only the patterns obtained by digestion of PCR product with restriction enzymes *Hind* III and *Rsa* I (Fig. 3 and 4) are presented in the paper.

In all analysed selections and parental cultivars, digestion with *Hind* III resulted in common fragments of 210 bp and 570 bp and polymorphic fragments of 770 bp (f_1) and 830 bp (f_2) (Fig. 3).

Digestion of PCR product ACC oxidase gene with restriction enzyme *Rsa* I revealed in all analysed selections and parental cultivars common fragment of 230 bp. In addition, polymorphic fragments of 530 bp and 680 bp (together considered as fragment f_1) and 1270 bp (f_2) were also obtained (Fig. 4).

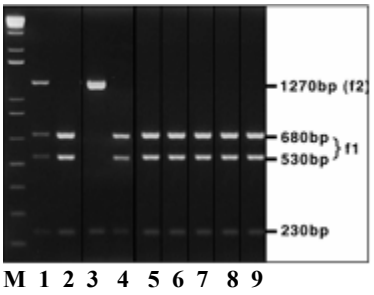

Fig. 4. DNA fragments (f1 and f2) upon digestion of PCR product of the amplified fragment of ACC oxidase gene with Rsa I in 9 apple selections: 1- J/54/53/59, 2- J/1/7, 3-J/11/31, 4-Šumatovka 1 O.P., 5- Šumatovka 2 O.P., 6- J/1/20, 7- J/2/12, 8- J/2/14, 9-J/60/7/63; analysed on 2 % agarose gel and stained in ethidium bromide; M-1Kb plus DNA ladder

Comparison of polymorphism observed upon restriction with *Hind* III and *Rsa* I enzymes resulted in identification of three alleles of the ACC oxidase gene: *a*, *b* and *n*. Alleles *a* and *b* identified in the paper correlate with alleles A and B reported by CASTIGLIONE *et al*. (1999). The new allele (*n*) was identified in selection J/11/31 (*bn*) and its parental cultivar Prima (the absence of amplification), and denoted as 'null' allele.

Restriction analysis of PCR product of the amplified fragment of ACC oxidase gene in all the assessed selections and their parental cultivars exhibited 4 allelic constitutions: *aa*, *nn*, *ab* i *bn*. The DNA fragments obtained upon digestion with the two restriction enzymes and deduced allelic constitutions are reconciled as follows:

Hind III	*Rsa* I	Allelic constitution
f_2 /	f_1 /	*aa*
/	/	*nn*
f_1f_2	f_1f_2	*ab*
f_1 /	f_2 /	*bn*

Out of 15 evaluated selections and parental cultivars, 11 have *aa* allelic constitution, 1 *nn*, 2 *ab* and 1 *bn* (Tab. 2).

Table 2. The results of digestion of PCR product of the amplified fragment of the gene encoding ACC oxidase with Hind III and Rsa I restriction enzymes and corresponding allelic constitution in the assessed apple selections and parental cultivars

Selection/cultivar	*Hind* III	*Rsa* I	Allelic constitution
Granny Smith	f_2 /	f_1 /	*aa*
Golden Delicious	f_2 /	f_1 /	*aa*
J/1/7	f_2 /	f_1 /	*aa*
J/1/20	f_2 /	f_1 /	*aa*
J/2/12	f_2 /	f_1 /	*aa*
J/2/14	f_2 /	f_1 /	*aa*
J/60/7/63	f_2 /	f_1 /	*aa*
Prima	/	/	*nn*
Melrose	f_1f_2	f_1f_2	*ab*
J/11/31	f_1 /	f_2 /	*bn*
Cox's Orange Pippin	f_2 /	f_1 /	*aa*
J/54/53/59	f_1f_2	f_1f_2	*ab*
Šumatovka	f_2 /	f_1 /	*aa*
Šumatovka 1 O.P.	f_2 /	f_1 /	*aa*
Šumatovka 2 O.P.	f_2 /	f_1 /	*aa*

Preliminary results obtained by CASTIGLIONE *et al*. (1999), indicate that allele B of ACC oxidase gene might be correlated with a long storage capability. Selections J/11/31 and J/54/53/59, the fruits of which are characterized by long shelf life, have allelic constitution *bn* and *ab* for ACC oxidase gene but this could not be considered as further support for the positive role of allele *b* as these two selections are homozygous for *ACS1-2* alelle.

In cv Prima the fruits of which have short storage life, a new allele *n* of ACC oxidase gene was identified. Since the pedigree of cv Prima includes accession of another species, *Malus floribunda* 821, it is possible that non-homologous ACC oxidase or alternative senescence pathway exists.

CONCLUSION

Based on the analysis of allelic polymorphism of the genes encoding ACC synthase and ACC oxidase and its comparison with the fruit storage capability of 9 apple selections, bred at the Fruit and Grape Research Centre in Čačak, and 6 parental cultivars, the following could be summarized:

- Two alleles of the ACC synthase gene (*ACS1* gene) were identified: *ACS1-1* and *ACS1-2*;
- All the assessed apple genotypes – selections and cultivars, may be classified into three groups that are homozygous or heterozygous for the allelic forms of *ACS1* gene;
- Fruits of the selections and parental cultivars homozygous for *ACS1-2* have without exception good storage capability of their fruits;

- A positive test for early selection of the seedlings having fruits with good storage capability was indicated;
- Three alleles of the gene encoding ACC oxidase: *a, b* and *n*, were identified in the assessed selections and parental cultivars.
- A new allele *n,* denoted as 'null' allele, was not reported before;
- Four allelic constitutions of the ACC oxidase gene – *aa, nn, ab* and *bn*, were determined in all the evaluated selections and parental cultivars;
- Further validation of the proposed prescreening test and the role of ACC oxidase allele *b* should be carried out by progeny analysis. Such study should also include the analysis of the factors implicated in ethylene perception.

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GENETIČKI POLIMORFIZAM ACC SINTAZE I ACC OKSIDAZE KOD SELEKCIJA JABUKE STVORENIH U ČAČKU

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I z v o d

Na programu stvaranja novih sorti jabuke, poboljšanog kvaliteta i dužine čuvanja ploda, radi se već dugi niz godina u Centru za voćarstvo i vinogradarstvo u Čačku. Kao rezultat kontinuiranog i sistematskog rada izdvojeno je 9 selekcija jabuke čiji se plodovi odlikuju različitom trajašnošću u skladištu (J/2/14, J/1/20, J/60/7/63, J/2/12, J/1/7, J/11/31, J/54/53/59, Šumatovka 1 O.P. i Šumatovka 2 O.P.). Dozrevanje ploda je genetički programiran, kompleksan fiziološki proces u kome važnu ulogu ima biljni hormon etilen. Alelni polimorfizam gena koji kodiraju ACC sintazu i ACC oksidazu, enzime na putanji biosinteze etilena, proučavan je kod izdvojenih selekcija jabuke i poređen sa trajašnošću njihovih plodova u skladištu. Polimorfizam je detektovan lančanom reakcijom polimeraze (PCR metodom) i restrikcionom analizom sa 6 restrikcionih enzima. Identifikovana su dva alela gena koji kodira ACC sintazu (*ACS1-1* i *ACS1-2*), tri alela gena koji kodira ACC oksidazu (*a*, *b* i *n*) i dobijen je pozitivan test za ranu selekciju sejanaca, čiji će se plodovi odlikovati dobrom trajašnošću u skladištu.

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