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THE POLYMORPHISM OF *ETR1* GENE IN AUTOCHTHONOUS APPLE CULTIVARS

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Ethylene is a plant hormone, which plays an important role in the ripening of climacteric fruits such as the apple. We studied allelic polymorphism of the *ETR1* gene, encoding ethylene receptor, in 23 autochthonous apple cultivars. The polymorphism was revealed by combining the gene specific PCR and restriction of PCR product. Four alleles of the *ETR1* gene (*a*, *b*, *c* and *d*) were detected, and their possible association with the fruit storage ability examined.

Key word: apple, ethylene, *ETR1* gene, polymorphism, fruit ripening.

INTRODUCTION

Ethylene is the simplest signaling molecule with a hormone-like function identified in plants (ALONSO and STEPANOVA, 2004). It plays an important role in

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the ripening of climacteric fruits such as the apple (THEOLOGIES, 1992; KNEE, 1993). Ethylene is necessary for fruit ripening, however its high production affects negatively shelf life and quality of apple fruits.

So far in the apple, only the allelic polymorphism of the genes encoding the enzymes involved in the ethylene biosynthetic pathway have been studied, i.e. ACC synthase (*Md-ACS1* gene) and ACC oxidase (*Md-ACO1* gene).

Two alleles of the *Md-ACS1* gene (*ACS1-1* and *ACS1-2*) have been identified. It has been suggested that allele *ACS1-2*, which possesses retroposon-like insertion, contributes to improved storage life of fruit (SUNAKO *et al*. 1999; HARADA *et al*., 2000; ORAGUZIE *et al*., 2004; COSTA *et al*., 2005; MARIĆ *et al*., 2005b). Five alleles of the *Md-ACO1* gene (*a*, *b*, *c*, *d* and *n*) have been identified. Two of them were reported by CASTIGLIONE *et al*. (1999) and COSTA *et al*. (2005), and three by MARIĆ *et al*. (2005a; 2005b).

Ethylene perception and signal transduction have been extensively studied in the model plant *Arabidopsis thaliana*, and identification and functional analysis of the corresponding genes in other plant species uncovered a high degree of conservation of this signaling cascade in the plant kingdom (ALONSO and STEPANOVA, 2004). Five different genes associated with ethylene perception have been identified in *Arabidopsis* (CHANG *et al*., 1993; HUA *et al*. 1998; SAKAI *et al*. 1998). These genes are subdivided into two groups, based on the differences in predicted amino acid sequence, i.e. the *ETR1* (*ETR1* and *ERS1* genes) and *ETR2* (*ETR2, ERS2* and *EIN4* genes) subfamilies (HUA *et al*., 1998). BASSETT *et al*. (2002) have isolated and characterized a peach (*Prunus persica* [L.] Batsch.) homologue of the gene encoding the ethylene receptor, *Pp-ETR1*. EL-SHARKAWY *et al*. (2003) have reported three ethylene receptor genes (*Pc-ETR1a*, *Pc-ERS1a* and *Pc-ETR5*) and *Pc-CTR1* gene in pear (*Pyrus communis* L.).

LEE *et al*. (1998) constructed cDNA library from cortical tissue of apple cultivar 'Granny Smith' and isolated the clone of 2.4 Kb that revealed a similarity with other *ETR1* genes of *Arabidopsis,* and concluded that it was an apple *ETR1* homologue. The aim of this work was to study allelic polymorphism of the *ETR1* gene in autochthonous apple cultivars and test a possible coincidence of particular allelic combinations with the good storage performance of their fruits.

MATERIAL AND METHODS

Twenty-three autochthonous apple cultivars from the Fruit Collection of Fruit Research Institute have been analyzed. The cultivars 'Bošnjanka', 'Budimka', 'Jabuka', 'Kablarka' (Kablar), 'Muslimka', 'Pozna Kolačara', 'Strekinja', 'Šarenika', 'Šumatovka', 'Valjnika' and 'Zelenika' are characterized by long storage life of fruits, while 'Kamenica 1', 'Opaljenik 1', 'Sirogojno 1', 'Sirogojno 2' and 'Zejtinka' have a moderate storage capability. 'Babovača', 'Kablarka (Trepča)', 'Kraljica', 'Petrovača', 'Pop Miladin', 'Samoniklaja' and 'Sinlija' exhibit poor storage ability.

Genomic DNA was isolated from young leaves using the CTAB mini prep method (DOYLE and DOYLE, 1987). The primers, used to amplify genomic fragment encoding ETR1 receptor, were designed on the basis of the *MdETR1* cDNA clone (AF032448, LEE *et al.*, 1998), using the DNAStar-Primer Select program. They were: ETR1-F (5'-CTAGTCAGCCCGTCGTCTCCTCTC-3') and ETR1-R (5'-AAGTTAGCGTTGCCAGTTTACACA-3'). The reaction was carried out in a 50 µl volume with 100 ng of genomic DNA, 1 x PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer and 2.5 U of *Taq* DNA polymerase (Qiagen). The amplification program consisted of initial denaturation at 94^0C for 1 min, followed by 10 cycles of 94^0C for 10 sec, 63^0C for 1 min and 68^0C for 4 min and 25 cycles of 94^0C for 10 sec, 63^0C for 1 min and 68^0C for 4 min + 10 sec per cycle, with a final 10 min extension step at 68^0C. PCR amplification was carried out in an MJ Research PTC-200 thermocycler, and the PCR amplified products were separated on 1% agarose gels. Electrophoresis lasted for 2 h at 80 volts/cm, whereupon the gels were stained in ethidium bromide. As a ladder, 1 Kb plus DNA (GibcoBRL®, Life Technologies) was used.

PCR product of *Md-ETR1* gene was digested with three different restriction enzymes of Type II: *Rsa*I, *Alu*I and *Hinf*I (Boehringer Mannheim). The reaction was performed for about 12 hours in the final volume of 30 µl containing 26,7 µl of PCR product, 3 µl of buffer and 0,2 µl of restriction enzyme (10 U/µl). The resulting products were separated on 2% agarose gel. Electrophoresis lasted for 2 h at 70 volts/cm.

RESULTS AND DISCUSSION

Amplification of the genomic fragment of *ETR1* gene. In all autochthonous apple cultivars monomorphic PCR product of about 5 Kb was amplified (Fig. 1).

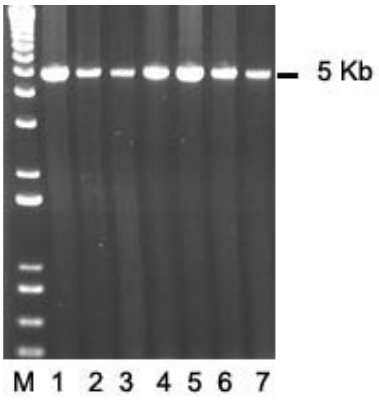

Fig.1 1.5 Kb PCR product, corresponding to *ETR1* gene, amplified in 7 autochthonous apple cultivars: 1- 'Budimka', 2- 'Šumatovka', 3- 'Petrovača', 4- 'Pozna Kolačara', 5- 'Kablarka (Kablar)', 6- 'Muslimka', 7- 'Kraljica'; M- 1Kb plus DNA ladder

Restriction analysis of the genomic fragment of *ETR1* gene – To reveal polymorphism the PCR product was digested with three restriction enzymes (*Rsa*I, *Alu*I and *Hinf*I).

Digestion with *Rsa*I resulted in several common fragments and a polymorphic fragment of 890 bp (Fig. 2).

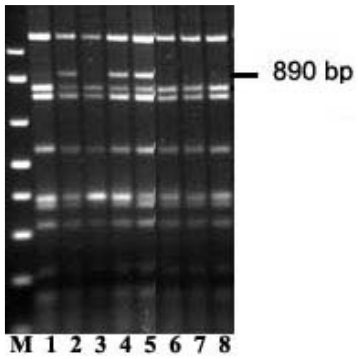

Fig. 2. DNA fragments obtained upon digestion, of the PCR product corresponding to *ETR1* gene, with *Rsa*I in 8 autochthonous apple cultivars: 1- 'Šumatovka', 2- 'Jabuka (Valjevo)', 3- 'Budimka', 4- 'Pozna kolačara', 5- 'Kablarka (Kablar)', 6- 'Muslimka', 8- 'Petrovača'; M- 1Kb plus DNA ladder

Digestion with *Alu*I resulted in a polymorfic band of 850 bp and several common fragments (Fig. 3).

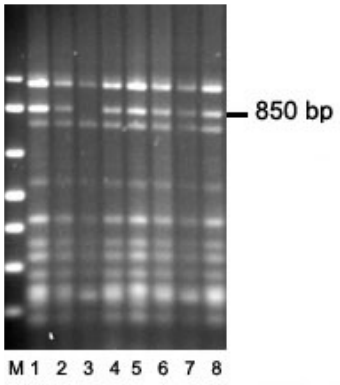

Fig. 3. DNA fragments obtained upon digestion, of the PCR product corresponding to the *ETR1* gene, with *Alu*I in 8 autochthonous apple cultivars: 1- 'Kablarka (Kablar)', 2- 'Pozna Kolačara'; 3- 'Šumatovka'; 4- 'Jabuka (Valjevo)', 5- 'Babovača', 6- 'Bošnjanka', 7- 'Kraljica', 8- 'Strekinja'; M- 1 Kb plus DNA ladder

Digestion with *Hinf*I revealed polymorphic fragments of 1130 bp and several monomorphic bands (Fig. 4).

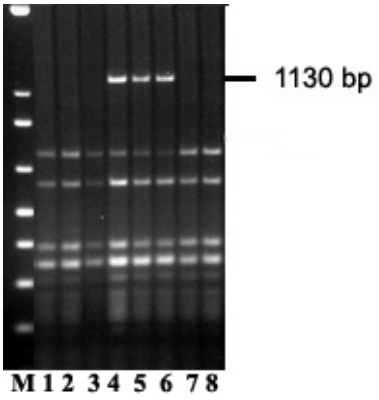

Fig. 4. DNA fragments obtained upon digestion, of the PCR product corresponding to *ETR1* gene, with *Hinf*I in 8 autochthonous apple cultivars: 1- 'Šumatovka', 2- 'Petrovača', 3- 'Jabuka (Valjevo)', 4- 'Babovača', 5- 'Bošnjanka', 6- 'Strekinja', 7- 'Budimka', 8- 'Kablarka (Kablar)'; M- 1 Kb plus DNA ladder

Based on restriction patterns, four alleles were deduced (*a*, *b*, *c*, *d*). The DNA fragments obtained upon digestion and deduced alleles are reconciled as follows:

*Rsa*I	*Alu*I	*Hinf*I	Allele
890 bp	850 bp	1130 bp	
-	-	-	*a*
+	+	-	*b*
-	+	-	*c*
-	+	+	*d*

In this work, the alleles of the *ETR1* gene in apple have been identified for the first time.

Allelic constitutions of the assessed autochthonous apple cultivars for the *ETR1* gene are presented in Table 1.

Table 1. Allelic constitutions of autochthonous apple cultivars for ETR1 gene

Allelic constitution of *ETR1* gene	Autochthonous apple cultivar
aa	'Budimka', 'Muslimka', 'Opaljenik 1', 'Petrovača', 'Pop Miladin', 'Sinlija', 'Šumatovka', 'Tip (2) Sirogojno', 'Valjnika', 'Zejtinka', 'Zelenika'
b,*a*/*c*	'Jabuka (Valjevo)', 'Kablarka (Kablar)', 'Pozna Kolačara', 'Samoniklaja'
c,*a*/*c*	'Kablarka (Trepča)', 'Kraljica', 'Tip 1 (Sirogojno)'
d,*a*/*d*	'Babovača', 'Bošnjanka', 'Kamenica 1', 'Šarenika', 'Strekinja'

Out of 23 evaluated autochthonous apple cultivars, 11 were scored as *aa*; in 4 apple cultivars the allele *b* was identified, and according to the phenotype the second allele could be *a* or *c* resulting in allelic constitutions of *ab* or *bc*; in 3 cultivars, the allele *c* was identified while the second allele could be *a* or *c* resulting in an allelic constitution of *cc* or *ac*; in 5 cultivars, the allele *d* was identified, the second allele could be *a* or *d* resulting in an allelic constitution of *dd* or *ad*. The allele *d* was identified only in the autochthonous cultivars, i.e. cvs 'Babovača', 'Bošnjanka', 'Kamenica 1', 'Šarenika' and 'Strekinja' but not in commercially grown apple cultivars (data not shown). Cloning and sequencing of the DNA fragments corresponding to the alleles *a*, *b*, *c* and *d* (work in progress) will provide their further characterisation and allow development of accurate method for cultivar genotyping.

When these provisional *ETR1* phenotypes were compared with the fruit storage ability of these cultivars no obvious correlation was observed. Within each phenotype there were cultivars with good and poor storage ability. However, it should be noted that the incomplete resolution of the *ETR1* phenotypes and lack of homozygous for the alleles *b*, *c* and *d* made such comparison unreliable. Proper test should include a progeny analysis based on accurate genotyping for *ETR1* gene and the genes involved in ethylene synthesis as well as phenotypic assessment of fruit storage ability.

CONCLUSION

The results, of analysis of the allelic polymorphism of the *ETR1* gene and its comparison with fruit storage life of 23 autochthonous apple cultivars from the Fruit Collection of Fruit Research Institute, could be outlined as follows:

- Primers based on the *MdETR1* cDNA clone were designed, and the PCR conditions for the amplification of genomic fragment encoding the ETR1 receptor were developed;
- Four alleles of the *ETR1* gene (*a*, *b*, *c* and *d*) were identified;

- The allele *d* has been presented only in autochthonous material;
- The following allelic constitutions of the *ETR1* gene have been determined: *aa*, *b*,*a*/*c*, *c*,*a*/*c* and *d*,*a*/*d*;
- Obvious correlation between the allelic constitution of the *ETR1* gene of the autochthonous apple cultivars and shelf life of their fruits was not observed;
- The impact of the identified *ETR1* alleles on the storage ability of apple fruits is currently studied using seregating apple progeny.

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REFERENCES

ALONSO, J.M. and A.N. STEPANOVA (2004): The ethylene signaling pathway. Science, *306*, 1513-1515.

BASSETT, C., T. ARTLIP and A. CALLAHAN (2002): Characterization of the peach homologue of the ethylene receptor, *PpETR1*, reveals some unusual features regarding transcript processing. Planta, *215*, 679-688.

CASTIGLIONE, S., B. PIROLA, F. SALA, M. VENTURA, M. PANCALDI and S. SANSAVINI (1999): Molecular studies of ACC synthase and ACC oxidase genes in apple. Acta Horticulturae, *484*, 305-309.

CHANG, C., S.F. KWOK, A.B. BLEECKER and E.M. MEYEROWITZ (1993): *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science, *262*, 539-544.

COSTA, F., S. STELLA, W.E. VAN de WEG, W. GUERRA, M. CECCHINEL, J. DALLAVIA, B. KOLLER and S. SANSAVINI (2005): Role of the genes *Md-ACO1* and *Md-ACS1* in ethylene production and shelf life of apple (*Malus domestica* Borkh). Euphytica, *141*, 181-190.

DOYLE, J.J. and L.J. DOYLE (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical bulletin, *19*, 11-15.

EL-SHARKAWY, I., B. JONES, G.Z. LI, M.J. LELIÈVRE, C.J. PECH and A. LATCHÉ (2003): Isolation and characterization of four ethylene perception elements and their expression during ripening in pears (*Pyrus communis* L.) with/without cold requirement. Journal of Experimental Botany, *54*, *387*, 1615-1625.

HARADA, T., T. SUNAKO, Y. WAKASA, J. SOEJIMA, T. SATOH and M. HIIZEKI (2000): An allele of the 1-aminocyclopropane-1-carboxylte synthase gene (*Md-ACS1*) accounts for the low level of ethylene production in climacteric fruts of some apple cultivars. Theor. Apple. Genet., *101*, 742-746.

HUA, J, H. SAKAI, S. NOURIZADEH, Q.G. CHEN, A.B. BLEECKER, J.R. ECKER and E.M. MEYEROWITZ (1998): EIN4 and ERS2 are members of the putative ethylene-receptor gene family in *Arabidopsis*. Plant Cell, *10*, 1321-1332.

KNEE, M. (1993): Pome fruits. In: Seymour G.B., J.E. Taylor and G.A. Tucker (eds). Biochemistry of fruit ripening. Chapman & Hall, London, 325-346.

LEE, S., G. ROSS and R. GARDNER (1998): An apple (*Malus domestica* L. Borkh cv Granny Smith) homologue of the ethylene receptor gene ETR1 (Accession No. AF032448). (PGR98-125). Plant Physiol., *117*, 1126.

MARIĆ, S., R. BOŠKOVIĆ, Ž. TEŠOVIĆ and M. LUKIĆ (2005a): Genetički polimorfizam ACC sintaze i ACC oksidaze kod autohtonih sorti jabuke. Jugoslovensko voćarstvo, *150*, 139-148.

MARIĆ, S., R. BOŠKOVIĆ, Ž. TEŠOVIĆ and M. LUKIĆ (2005b): Genetical polymorphism of ACC synthase and ACC oxidase in apple selections bred in Čačak. Genetika, *37*, *3*, 225-235.

ORAGUZIE, C.N., H. IWANAMI, J. SOEJIMA, T. HARADA and A. HALL (2004): Inheritance of the *Md-ACS1* gene and its relationship to fruit softening in apple (*Malus* x *domestica* Borkh.). Theor. Apple. Genet., *108*: 1526-1533.

SAKAI, H., J. HUA, Q.G. CHEN, C. CHANG, L.J. MEDRANO, A.B. BLEECKER and E.M. MEYEROWITZ (1998): *ETR2* is an *ETR1*-like gene involved in the ethylene signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. USA, *95*, 5812-5817.

SUNAKO, S., W. SAKURABA, M. SENDA, S. AKADA, R. ISHIKAWA, M. NIIZEKI and T. HARADA (1999): An allele of the ripening-specific 1-aminocyclopropane-1-carboxylate synthase gene (*ACS1*) in apple fruit with a long storage life. Plant Physiol., *119*, 1297-1303.

THEOLOGIS, A. (1992): One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell, 70, 181-184.

POLIMORFIZAM *ETR1* GENA KOD AUTOHTONIH SORTI JABUKE

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Etilen je biljni hormon koji ima važnu ulogu u dozrevanju klimakteričnih plodova, uključujući jabuku. Do sada su ispitivani i okarakterisani geni uključeni u proces biosinteze etilena. U ovom radu proučavan je alelni polimorfizama *ETR1* gena, koji kodira receptor za etilen kod jabuke. Polimorfizam je analiziran lančanom reakcijom polimeraze (PCR metodom) i restrikcionom analizom. Istraživanjem su obuhvaćene 23 autohtone sorte jabuke, locirane u_kolekcionom zasadu Instituta za voćarstvo, čiji se plodovi odlikuju različitom trajašnošću u skladištu. U analiziranom materijalu detektovana su četiri alela *ETR1* gena (*a*, *b*, *c* i *d*) i testirana je veza između identifikovanih alela i dužine čuvanja plodova u skladištu.

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