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DETERMINATION OF *ETR1* GENOTYPES IN PROMISING APPLE SELECTIONS DEVELOPED AT FRUIT RESEARCH INSTITUTE – ČAČAK

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Ethylene, as the simplest signaling molecule with hormone-like function, regulates a broad spectrum of different processes in plants, including ripening. It is perceived by a receptor family divided into two subfamilies (I and II). In apple, *ETR1* gene encodes one of ethylene receptors – ETR1 receptor which is a member of subfamily I. *ETR1* genotypes were determined for six promising apple selections bred at Fruit Research Institute in Čačak [J/54/53/59 (‘Cox's Orange Pippin’ O.P.), J/1/7, J/1/20, J/2/14 and J/60/7/63 (‘Granny Smith’ × ‘Golden Delicious’), and J/2/50 (‘Idared’ O.P.)] and four commercially important parental cultivars (‘Cox's Orange Pippin’, ‘Golden Delicious’, ‘Granny Smith’ and ‘Idared’). Polymorphism of *ETR1* gene was detected by restriction analysis of PCR amplified product with two restriction enzymes (*Rsa*I and *Alu*I). Three alleles (*a, b* and *c*) and four allelic constitutions of *ETR1* gene (*aa*, *ac*, *b,a/c* and *c,a/c*) were detected. This study has confirmed that *ETR1* gene is inherited in Mendelian fashion and showed that polymorphism of *ETR1* gene can aid cultivar and selection genotyping. Based on allelic constitution of genes involved in ethylene biosynthesis and perception, and on the major biological and agronomic traits, J/54/53/59 has been singled out as elite apple selection.

Key words: *ETR1 gene, apple, selection, ethylene*

INTRODUCTION

The gaseous phytohormone ethylene mediates a wide spectrum of developmental and physiological processes, including germination, growth, senescence, ripening, and responses to biotic and abiotic stress, throughout the plant life cycle (CHEN *et al*., 2010). The fact that

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ethylene plays important role in ripening of climacteric fruits has led many scientists to study genes involved in ethylene biosynthesis and perception.

In apple, particular attention has been paid to the study of allelic polymorphism, inheritance and mapping of genes encoding the enzymes in the ethylene biosynthetic pathway, i.e. *ACS1* gene (ACC synthase) and *ACO1* gene (ACC oxidase) (MARIĆ *et al*., 2005a, 2005b; COSTA *et al*., 2005, ZHU and BARRITT, 2008; FERNÁNDEZ-FERNÁNDEZ *et al*., 2008; MARIĆ *et al*., 2009a). Regarding the ethylene perception, most of the work at the molecular level has been conducted in *Arabidopsis* and five ethylene receptors divided into two subfamilies, i.e. *ETR1*, *ERS1*, *ETR2*, *ERS2* and *EIN4* have been identified (CHEN *et al*., 2005). First apple *ETR1* homologue of 2.4 Kb was isolated by LEE *et al*. (1998). Progress in the discovery of allelic polymorphism in apple *ETR1* gene was made by amplification of genomic fragment and restriction analysis with different restriction enzymes (MARIĆ *et al*., 2007). Five new alleles, viz. *a*, *b*, *c*, *d* and *e* were found by using such methods (MARIĆ *et al*., 2009a). MARIĆ *et al*. (2010) reported that in recent period the research was focused on identifying functional markers and markers used for *ACS1*, *ACO1* and *ETR1* genes belong to this category of markers.

The work on breeding new apple cultivars at Fruit Research Institute in Čačak (FRI) has resulted in the release of two cultivars – 'Čačanska Pozna' ('Starking' × 'Jonathan') and 'Čadel' ('Golden Delicious' × 'Jonathan'), and a number of promising selections which have been singled out (LUKIĆ *et al*., 2012). This breeding programme has always had a goal of developing superior apple cultivars with enhanced quality, genetic resistance to diseases and extended storage life (low ethylene producing genotypes). Recently, within the apple breeding programme at FRI, we have started with application of functional markers with the aim to improve efficiency by enabling early selection for adult traits and simultaneous selection for multiple traits.

Thus, the purpose of this study was to identify the *ETR1* alleles genotypes of six promising apple selections and four commercially important parental cultivars. The assessed promising selections were singled out within the apple breeding programme at FRI and some of them deserve to be included in the release procedure.

MATERIALS AND METHODS

Plant material

Ten apple genotypes, including parents and their derivatives, were sampled from the Fruit Collection of Fruit Research Institute in Čačak. Analysed promising apple selections derived from the following crosses: 'Cox's Orange Pippin' O.P. (selection J/54/53/59), 'Granny Smith' × 'Golden Delicious' (selections: J/1/7, J/1/20, J/2/14 and J/60/7/63) and 'Idared' O.P. (selection J/2/50).

Genomic DNA extraction and PCR amplification of ETR1 gene

The total genomic DNA was extracted from young leaves using the CTAB mini prep method described by DOYLE and DOYLE (1987). Extracted DNA was diluted in water (Sigma) and RNA was eliminated by the addition of RNase A (Invitrogen). PCR procedure and primer set (ETR1-F and ETR1-R) for the amplification of genomic fragment of *ETR1* gene were identical to those described by MARIĆ *et al*. (2007). The PCR reaction was carried out in final mix of 50 µl containing 100 ng of template DNA, 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 0.2 µM of each primer (forward and reverse) and 2.5 U of *Taq* DNA polymerase (Qiagen). PCR amplification was carried out in *TPersonal*

Biometra thermo cycler and primers gave satisfactory amplification under the following conditions: 1 min at 94°C, 10 cycles of 94°C for 10 sec, 63°C for 1 min and 68°C for 4 min, and 25 cycles of 94°C for 10 sec, 63°C for 1 min and 68°C for 4 min + 10 sec per cycle, with a 10 min final extension step at 68°C. The amplification products were separated in 1% agarose gel (70 V for 3−4 h), visualised by ethidium bromide staining and sized by comparison with a 1 Kb Plus DNA ladder (Invitrogen).

Restriction analysis of PCR product of ETR1 gene and detection of allelic polymorphism

To reveal allelic polymorphism of the *ETR1* gene, PCR product was digested with *Rsa*I, *Alu*I and *Hinf*I (Fermentas, Termo Scientific) as follows: 26.8 µl PCR product was incubated for 12 h at 37°C with 3 µl of buffer (10 × dissolved) and 0.2 µl of restriction enzyme *Rsa*I/*Alu*I/*Hinf*I (10 U/µl). After the digestion, 20 µl of the restriction fragments were separated in 2% agarose gel at 70 V for 4 h.

RESULTS AND DISCUSSION

Amplification of genomic fragment of ETR1 gene

The use of primers ETR1-F and ETR1-R (MARIĆ *et al*., 2007) allowed the amplification of *ETR1* genomic fragment which was approximately of 5,000 bp (Figure 1). In all promising apple selections bred at FRI and their parents, monomorphic PCR product was amplified. The size of the amplified products is in agreement with PCR products of *ETR1* gene obtained for autochthonous (MARIĆ *et al*., 2007) and standard apple cultivars (MARIĆ *et al*., 2009a).

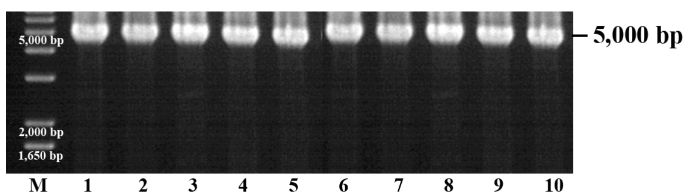

Figure 1. Monomorphic PCR product (5,000 bp) of *ETR1* gene amplified in 10 apple genotypes: 1- ‘Cox's Orange Pippin’, 2- ‘Granny Smith’, 3- ‘Idared’, 4- ‘Golden Delicious’, 5- J/54/53/59, 6- J/1/7, 7- J/1/20, 8- J/2/14, 9- J/60/7/63, 10- J/2/50; M- 1 Kb plus DNA ladder (Invitrogen)

ETR1 genotypes of parental cultivars and derived promising selections

The polymorphism observed upon digestion of the PCR product with *Rsa*I (two segregating fragments of 800 bp and 890 bp) and *Alu*I (one polymorphic fragment of 850 bp) restriction enzymes was interpreted as described in MARIĆ *et al*. (2007; 2009a) and revealed three alleles (*a, b* and *c*, presented in Table 1) and four genotypes (*aa*, *ac*, *b,a/c* and *c,a/c*, presented in Table 2). The examples of banding patterns for *Rsa*I and *Alu*I enzymes are shown in Figure 2 and Figure 3, respectively. Although the polymorphic fragments in the assessed apple genotypes were not observed upon digestion with *Hinf*I, the restriction analysis with this enzyme was necessary because *ETR1* genotype of parental cultivar ‘Granny Smith’ was unknown. According to MARIĆ *et al*. (2009a), digestion with *Hinf*I is required to identify allele *d* (fragment of 1,130 bp) and allele *e* (fragment of 800 bp) of *ETR1* gene. Figure 4 represents the banding pattern obtained upon digestion with *Hinf*I restriction enzyme.

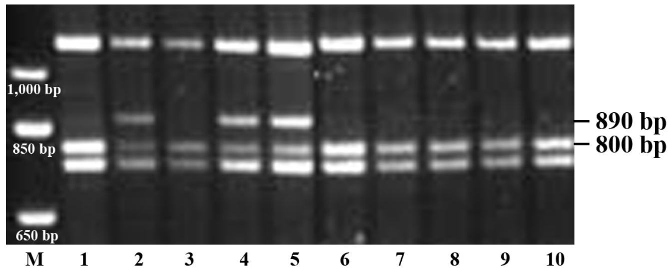

Figure 2. Polymorphic DNA fragments (800 bp and 890 bp) obtained upon digestion of the PCR product of *ETR1* gene with *Rsa*I in 10 apple genotypes: 1- ‘Golden Delicious’, 2- ‘Cox's Orange Pippin’, 3- ‘Granny Smith’, 4- ‘Idared’, 5- J/54/53/59, 6- J/1/7, 7- J/1/20, 8- J/2/14, 9- J/60/7/63, 10- J/2/50; M- 1 Kb plus DNA ladder (Invitrogen)

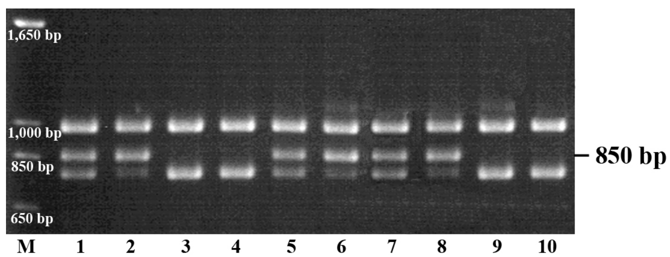

Figure 3. Polymorphic DNA fragment (850 bp) obtained upon digestion of the PCR product of *ETR1* gene with *Alu*I in 10 apple genotypes: 1- ‘Cox's Orange Pippin’; 2- ‘Golden Delicious’, 3- J/1/7, 4- J/1/20, 5- J/54/53/59, 6- ‘Granny Smith’, 7- ‘Idared’, 8- J/2/50, 9- J/2/14, 10-J/60/7/63; M- 1 Kb plus DNA ladder (Invitrogen)

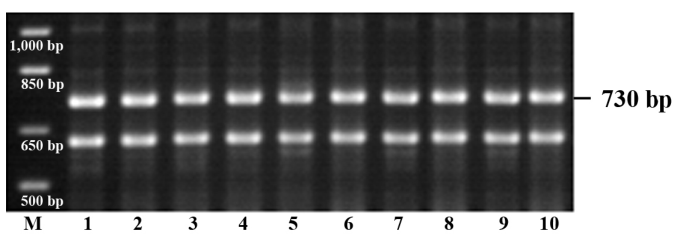

Figure 4. Monomorphic DNA fragments obtained upon digestion of the PCR product of *ETR1* gene with *Hinf*I in 10 apple genotypes: 1- ‘Cox's Orange Pippin’, 2- ‘Granny Smith’, 3- ‘Idared’, 4- ‘Golden Delicious’, 5- J/54/53/59, 6- J/1/7, 7- J/1/20, 8- J/2/14, 9- J/60/7/63, 10- J/2/50; M- 1 Kb plus DNA ladder (Invitrogen)

Out of ten evaluated apple genotypes, four promising selections were scored as *aa*; two cultivars were scored as *ac*; in two apple cultivars and one promising selection 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 one selection the allele *c* was identified while the second allele could be *a* or *c* resulting in an allelic constitution of *cc* or *ac*.

Table 1. The DNA fragments obtained upon digestion of PCR product of ETR1 gene with three restriction enzyme (RsaI, AluI and HinfI) and deduced ETR1 alleles

*Rsa*I		*Alu*I	*Hinf*I		Allele
800 bp	890 bp	850 bp	800 bp	1130 bp	
+	−	−	−	−	*a*
−	+	+	−	−	*b*
+	−	+	−	−	*c*

Table 2. ETR1 genotypes of the parental cultivars and derived promising apple selections

Cultivar / Selection	Cross	*Rsa*I		*Alu*I	*Hinf*I		*ETR1* genotype
		800 bp	890 bp	850 bp	800 bp	1130 bp	
'Cox's Orange Pippin'		+	+	+	-	-	*b,a/c*
J/54/53/59	'Cox's Orange Pippin' O.P.	+	+	+	-	-	*b,a/c*
'Granny Smith'		+	-	+	-	-	*ac*
'Golden Delicious'		+	-	+	-	-	*ac*
J/1/7	'Granny Smith' × 'Golden Delicious'	+	-	-	-	-	*aa*
J/1/20	'Granny Smith' × 'Golden Delicious'	+	-	-	-	-	*aa*
J/2/14	'Granny Smith' × 'Golden Delicious'	+	-	-	-	-	*aa*
J/60/7/63	'Granny Smith' × 'Golden Delicious'	+	-	-	-	-	*aa*
'Idared'		+	+	+	-	-	*b,a/c*
J/2/50	'Idared' O.P.	+	-	+	-	-	*c,a/c*

ETR1 genotypes for parental cultivars 'Cox's Orange Pippin', 'Golden Delicious' and 'Idared' were reported by MARIĆ *et al.* (2009a), whereas this is the first report of *ETR1* genotypes for commercially important parental cultivar 'Granny Smith' and promising selections derived from the studied crosses at FRI. The *ETR1* genotypes of all six promising selections accorded with parental genotypes (Table 2) and these results confirm that *ETR1* gene is inherited in Mendelian fashion.

Our results also revealed that the allelic constitution of *ETR1* gene in parental cultivars 'Golden Delicious' and 'Granny Smith' is *ac*. Namely, based on restriction analysis reported in the paper of MARIĆ *et al*. (2009a), in the cultivars ‘Golden Delicious’ and ‘Granny Smith’ the allele *c* was identified while the second allele could be *a* or *c* resulting in *cc* or *ac* genotypes. The final verification of *ETR1* genotypes of these two parental cultivars can be confirmed after the analysis of the segregating progenies or selections released from the cross of these two parents. Since the *ETR1* allelic genotypes of all four promising selections raised from the ‘Granny Smith’ × ‘Golden Delicious’ cross are *aa* and inheritance of *ETR1* gene is in Mendelian fashion (MARIĆ *et al*., 2009b), our results revealed that *ac* is the allelic constitutions of *ETR1* gene in ‘Golden Delicious’ and ‘Granny Smith’. For the final determination of *ETR1* genotypes of parental cultivars ‘Cox's Orange Pippin’ and ‘Idared’, it is necessary to analyse selections raised from the known crosses, not only selections singled out from the open pollinated progenies. Furthermore, our survey of *ETR1* allelic constitution for genotypes, which has not been published so far, expands the list of apple genotypes that can be used as parents in breeding of low ethylene producing genotypes.

ETR1 as functional marker

In recent years, functional markers, namely DNA sequences putatively involved in the expression of certain traits, have become very important. Literature provides more data on studies of genes involved in ethylene biosynthesis as well as data on utilization of both *ACS1* and *ACO1* functional markers for selecting the progeny at the seedling stage with low ethylene production, firm fruit and long storage potential (ZHU and BARRITT, 2008). Namely, the allele *ACS1-2* is considered to contribute to the low level of ethylene production at ripening stage of some apple genotypes (SUNAKO *et al*., 1999; HARADA *et al*., 2000; COSTA *et al*., 2005; MARIĆ *et al*., 2005b, ZHU and BARRITT, 2008). Positive role of allele *b* of *ACO1* gene in ripening of apple fruit was indicated by MARIĆ *et al*. (2005b), whereas CASTIGLIONE *et al*. (1999) reported that allelic forms of *ACO1* may correlate with a wide range of ethylene production (allele B may control low ethylene production).

Markers used in this study for *ETR1* gene also belong to the category of functional markers, but in the literature there is no research data about the association between *ETR1* allelotype and low ethylene production and/or long storage life of apple genotypes. Namely, MARIĆ *et al*. (2007) tried to examine possible correlation between the *ETR1* genotypes of the autochthonous apple cultivars and shelf life of their fruits. However, incomplete resolution of the *ETR1* genotypes and the lack of homozygous for the alleles *b*, *c* and *d* made such comparison unreliable. Our further studies (the work in progress) indicate the following association, i.e. allele *a* − high ethylene production and allele *b* − low ethylene production, but the linkage between *ACS1* and *ETR1* genes (MARIĆ *et al*., 2009a) need to be considered for the validation of the role of the identified alleles.

Our results provide new information on the allelic constitution of *ETR1* gene in promising apple selections bred at FRI. Nevertheless, results from this and previous studies indicate that out of six analysed promising selections, one can be singled out as elite selection. Based on allelic constitution of genes involved in ethylene biosynthesis and perception, i.e. *ACS1* genotype (*ACS1-2/2;* MARIĆ *et al*., 2005b*), ACO1* genotype (*ab*; MARIĆ *et al*., 2005b) and *ETR1* genotype (*b,a/c*), as well as on the major biological and agronomic properties, selection J/54/53/59 (‘Cox's Orange Pippin’ O.P.) deserves to be introduced into the release procedure and production, but also in breeding programme as a parent. The major biological and agronomic properties of this selection are as follows: moderate vigour; medium large fruit (150 g) of green ground colour with intense red blush; flesh is firm, greenish-white, subacid in flavour; ripens in the first decade of October; fruits have very good shelf life and can be kept in cold storage until June of the following year; no symptoms of scab and mildew occur in field conditions.

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DETERMINACIJA *ETR1* GENOTIPA PERSPEKTIVNIH SELEKCIJA JABUKE STVORENIH U INSTITUTU ZA VOĆARSTVO – ČAČAK

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Izvod

Etilen je najjednostavniji signalni molekul sa hormonskom funkcijom koji reguliše veliki broj različitih procesa tokom rasta i razvića biljaka, uključujući i dozrevanje ploda. Za percepciju etilena je odgovorna familija receptora koja se sastoji od dve potfamilije (potfamilija I i II). ETR1 receptor je jedan od receptora za etilen kod jabuke, pripada potfamiliji I i kodiran je *ETR1* genom. Alelna konstitucija *ETR1* gena je određena kod šest perspektivnih selekcija jabuke stvorenih u Institutu za voćarstvo – Čačak [J/54/53/59 (‘Cox's Orange Pippin’ O.P.), J/1/7, J/1/20, J/2/14 i J/60/7/63 (‘Granny Smith’ × ‘Golden Delicious’), i J/2/50 (‘Idared’ O.P.)] i četiri komercijalno značajne sorte (‘Cox's Orange Pippin’, ‘Golden Delicious’, ‘Granny Smith’ i ‘Idared’). Polimorfizam je detektovan nakon digestije PCR-om amplifikovanog genomskog fragmenta *ETR1* gena sa dva restrikciona enzima – *Rsa*I i *Alu*I. Na bazi restrikcione analize identifikovana su tri alela (*a*, *b* i *c*) i četiri alelne konstitucije *ETR1* gena (*aa*, *ac*, *b,a/c* i *c,a/c*). Istraživanja u ovom radu su potvrdila da se *ETR1* gen nasleđuje u skladu sa Mendelovim zakonima i pokazala da se polimorfizam *ETR1* gena može koristiti za genotipizaciju sorti i selekcija jabuke. Na osnovu alelne konstitucije gena uključenih u sintezu i percepciju etilena, kao i na osnovu bioloških i agronomskih osobina ispitivanih genotipova, selekcija J/54/53/59 se može izdvojiti kao elitna selekcija jabuke .

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