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ASSOCIATION OF ALLELE VARIANTS OF GENE *Rf* WITH RED SKIN FRUIT COLOR OF APPLE CULTIVARS OF UKRAINIAN BREEDING

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Aim. To estimate the possibilities of using the promising apple cultivars, created by the Institute of Horticulture (IH) NAAS, in the breeding programs according to the availability of valuable allele variants of gene *Rf*, related to red color of apple fruit. **Methods.** Polymerase chain reaction, electrophoresis in agarose gel, the evaluation of phenotype manifestation of the color trait of apple skin according to the grading system. **Results.** 17 genotypes of apple cultivars of different genetic origin, including 12 cultivars, bred by IH NAAS, were identified by allele variants of gene *Rf*. The comparison was made between the genetic analysis results and visual observations of the phenotype manifestation of fruit skin color of different apple cultivars. **Conclusions.** Apple cultivars Amulet, Perlyna Kyieva and Edera, which are homozygous carriers of dominant alleles *A*¹ and *A*² of gene *Rf* are recommended as basic forms in breeding programs. It was demonstrated that exact prediction of apple skin color was possible only in case of the available homozygous genotype by both alleles of gene *Rf*.

Keywords: apple, breeding genes of apple skin color, molecular markers, scab resistance.

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INTRODUCTION

The common practice while creating new apple cultivars is to concentrate the spectrum of economically valuable trait for marker-assisted selection (MAS) mainly on the resistance to scab, taste qualities, flesh density, columnar habitus and skin color [1–4].

Apple is one of the most relevant fruit crop in Ukraine, the consumption of apple fruit by the population is prioritized among all the fruit and small-fruit crops. The prominent place in the Register of cultivars of Ukraine is taken by apple cultivars, created by the Institute of Horticulture (IH) NAAS and its network. Our studies have proven that these cultivars are valuable genetic material, which is insufficiently characterized from the standpoint of relevant allele variants of genes, responsible for economically valuable traits. Their identification will expand the possibilities of involving the mentioned cultivars to MAS programs, in particular, by fruit skin color.

The fruit of Ukrainian apple cultivars have different colors – from green color, like that of Papirovka cultivar, to dark red, like that of cultivars Rubinove Duky or Amulet. Fruit skin of many cultivars is of uneven color, it has stripes or “blush”, which may be manifested differently even among fruit of the same cultivar. Apple fruit color is not only a remarkable trait of the cultivar, but also a relevant criterion, affecting the consumer’s choice. In particular, buyers often prefer cultivars with red fruit color, which is associated with better ripeness and thus better taste and aroma [5]. Red color of apple fruit is mainly determined by the presence of secondary metabolites – anthocyanins [6, 7]. The intensity of red color of fruit skin is directly dependent on the accumulation degree of these natural antioxidants, which varies in a wide range and changes depending on the ripeness degree and environmental conditions. The ripest fruit, well lit by the sun during their growth, have the richest color and thus higher content of anthocyanins in their skin compared to not ripe, insufficiently lit fruit of the same cultivar [8]. It is noteworthy that in

addition to anthocyanins, carotenoids and chlorophyll participate in coloring apple fruit as well [9].

High content of anthocyanins is also considered to be valuable for human health due to antioxidant features of these compounds [10, 11]. Thus, a trait of red apple skin got transformed into a marker of the health-improving property of apple fruit in the breeding programs.

MAS by skin color was launched after the identification of RAPD-marker, associated with gene *Rf*. Cultivars may be divided into "predominantly red" and "non-red" by its use. Thus, the first breeding programs of creating new apple cultivars by this trait used the mentioned marker [12]. It was assumed that the trait of red apple skin was determined by the availability of dominant alleles of gene *Rf* – A^1 and A^2 (each of them separately and both together), yellow-green color was conditioned by the expression of the homozygous recessive genotype by one or two alleles of gene *Rf*.

Later dCAPS-marker, associated with single nucleotide polymorphism (SNP) in the promotor of gene *MdMYB1*, was created [13]. Its application allowed discriminating 136 seedlings from crossing of cultivars Golden Delicious and Crisp Pink [1]. However, a number of works noted that the availability of allele *MdMYB-1* in the genome of apple cultivars cannot guarantee the occurrence of this trait, instead it indicates only a higher probability of the manifestation of red color of fruit skin [14, 15].

The aim of this work was to investigate the possibility of introducing promising apple cultivars, breeding by IH NAAS, by the availability of valuable allele variants of gene *Rf*, associated with the color of apple fruit skin, into breeding programs in Ukraine.

MATERIALS AND METHODS

The shoots of apple cultivars and hybrid forms, selected in the experimental orchards of the Department of breeding of fruit and small-fruit crops, IH NAAS, were used as initial material. DNA was extracted from fresh young leaves using modified CTAB (hexadecyltrimethylammonium bromide) method.

Universal primers for this gene were used to identify allele variants of gene *Rf*, responsible for apple skin color [12].

Nucleotide sequences in the direction 5'→3':

Forward – GACAGGTTACGGTCCACTGCT;

Reverse – ACGTAAGGTCAAAGATTCAGATC.

The reaction mixture for PCR of 20 µl contained 40 ng DNA; 75 mM tris-HCl (pH 8.8 at the temperature of

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M

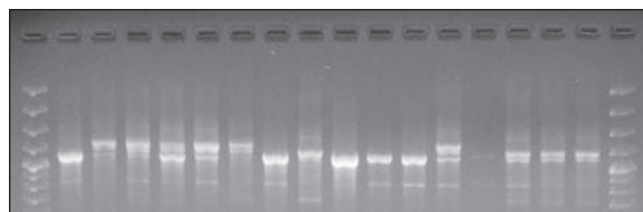


Fig. 1. The electrophoregram of amplification products of genomic DNA of apple cultivars and hybrid forms with universal primers to the gene of red apple skin, *Rf*: 1 – Teremok; 2 – Kateryna; 3 – Skifske zoloto; 4 – Gala; 5 – Idared; 6 – Golden Delicious; 7–16 – hybrid forms

25 °C); 20 mM $(\text{NH}_4)_2\text{SO}_4$; 0.01% Tween20; 0.2 mM dNTP; 200 mmol of each primer; 1 unit of *Taq*-polymerase.

The amplification reaction was conducted in three stages: I – 1 cycle: 94 °C, 2 min; II – 40 cycles: 94 °C, 30 s; 47 °C, 1 min; 72 °C, 1 min; III – 1 cycle: 72 °C, 7 min.

The fragments obtained were separated in 1.5 % agarose gel in tris-borate buffer at the voltage of 5 V/cm for 1 h. The gels were registered by photography after staining with ethidium bromide. 100 bp DNA Ladder Plus (Fermentas, "Lithuania") was used as a molecular weight marker (Fig. 1).

The amplification with these primers allows obtaining four markers. The availability of dominant allele A^1 is determined by the marker of 1160 bp, and that of a recessive allele a^1 – 1230 bp. The availability of dominant allele A^2 is determined by the marker of 1180 bp, and that of a recessive allele a^2 – 1320 bp (Table 1). Markers of 1160, 1180, and 1230 bp are known for their high homology. The differences between them consist in a small insertion-deletion and one nucleotide replacement.

RESULTS AND DISCUSSION

The availability of red fruit skin color of some degree was observed in 13 out of 17 apple cultivars in the collection. Fruit color was evaluated visually by its availability and intensity degree of red color. Five groups of cultivars were distinguished (Fig. 2): cultivars of

Table 1. Markers, defining apple tree genotype by gene *Rf*

Allele	Length, bp	Phenotype manifestation
A^1	1160	Red skin
a^1	1230	Yellow skin
A^2	1180	Red skin
a^2	1320	Yellow skin

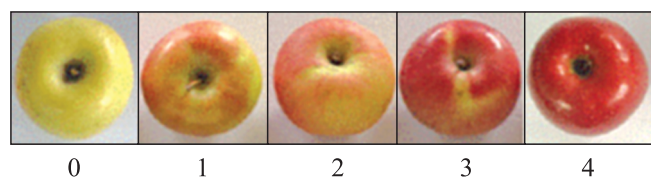


Fig. 2. The categories of phenotype manifestation of the feature of red color of apple fruit (0–4 – score) [12]

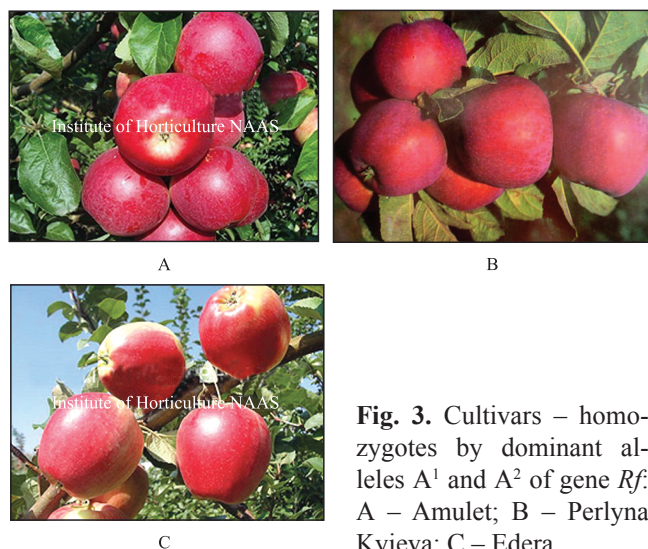


Fig. 3. Cultivars – homozygotes by dominant alleles A^1 and A^2 of gene Rf : A – Amulet; B – Perlyna Kyieva; C – Edera

dark red and purple color (4 points), with red color (3 points), with medium color degree (2 points), with poor color (1 point) and cultivars with no red color at all (0 points). Cultivars Papirovka, Golden Delicious, Antonivka and Mutsu were referred to the last group. The control cultivars were the ones, the genotypes of which by gene Rf are already known, in particular, Golden Delicious, Idared and Gala [12].

The analysis using universal primers to gene Rf demonstrated the carriers of homozygous genotype by dominant alleles A^1 and A^2 to be cultivars Perlyna Kyieva, Edera and Amulet (Table 2). Thus, among all the studied cultivars the latter are the most promising ones to be used as basic forms in breeding schemes of creating cultivars with red color of fruit skin. It is noteworthy that phenotype manifestations of the mentioned trait in three mentioned cultivars are expressed differently, in particular, cultivars Amulet and Perlyna Kyieva may be scored 4 points, whereas Edera – 3 points (Fig. 3).

Another cultivar with high potential for breeding by this trait is Harant with the genotype $A^1a^1A^2A^2$ (Fig. 4). In addition, homozygous carriers of dominant alleles are five other cultivars with red skin, including Rubinove Duky, Radohost, and Askolda with the same

Table 2. Genotypes of apple cultivars of Ukrainian and international selection by genes Rf

Cultivar	Fruit color, score	Origin	Genotype by gene Rf
Teremok	Red, 4	25/2-D (Aport Alexander × Jonathan) × Grive Rouge	A^1A^1
Radohost	Red, 4	Jonathan × Greensleeves	A^2A^2
Kateryna	Yellow-red, 2	Golden Delicious × McIntosh Wjczik	$a^1a^1A^2a^2$
Skifske zoloto	Green with light “bloom”, 1	SR-0523 [Red Melba × (Wolf River × <i>Malus atrosanguinea</i> -804) × Honey-gold	$a^1a^1A^2a^2$
Rubinove Duky	Red, 4	Jonathan × Aport Alexander	A^2A^2
Papirovka	Green, 0	Baltic old selection cultivar	a^1a^1
Askolda	Red-green, 3	25/2-D (Aport Alexander × Jonathan) × Kidd’s Orange Red	A^2A^2
Garant	Red, 2	Idared × -2034	$A^1a^1A^2A^2$
Ornament	Red, 4	22/22 D (Jonathan × Aport Alexander) × Starkrimson	A^1A^1
Edera	Red, 4	Rubin × Priscilla	$A^1A^1A^2A^2$
Perlyna Kyieva	Red, 4	Rubin × Priscilla	$A^1A^1A^2A^2$
Amulet	Red, 3	Rubin × Priscilla	$A^1A^1A^2A^2$
Antonivka	Green, 0	Russian old selection cultivar	$a^1a^1A^2A^2$
Mutsu	Green-yellow, 0	Golden Delicious × Indo	$a^1a^1a^2a^2$
Gala	Red, 3	Kidd’s Orange Red × Golden Delicious	$A^1a^1a^2a^2$
Idared	Red, 3	Wagener prize × Jonathan	$a^1a^1A^2a^2$
Golden Delicious	Green-yellow, 0	Seedling	$a^1a^1a^2a^2$

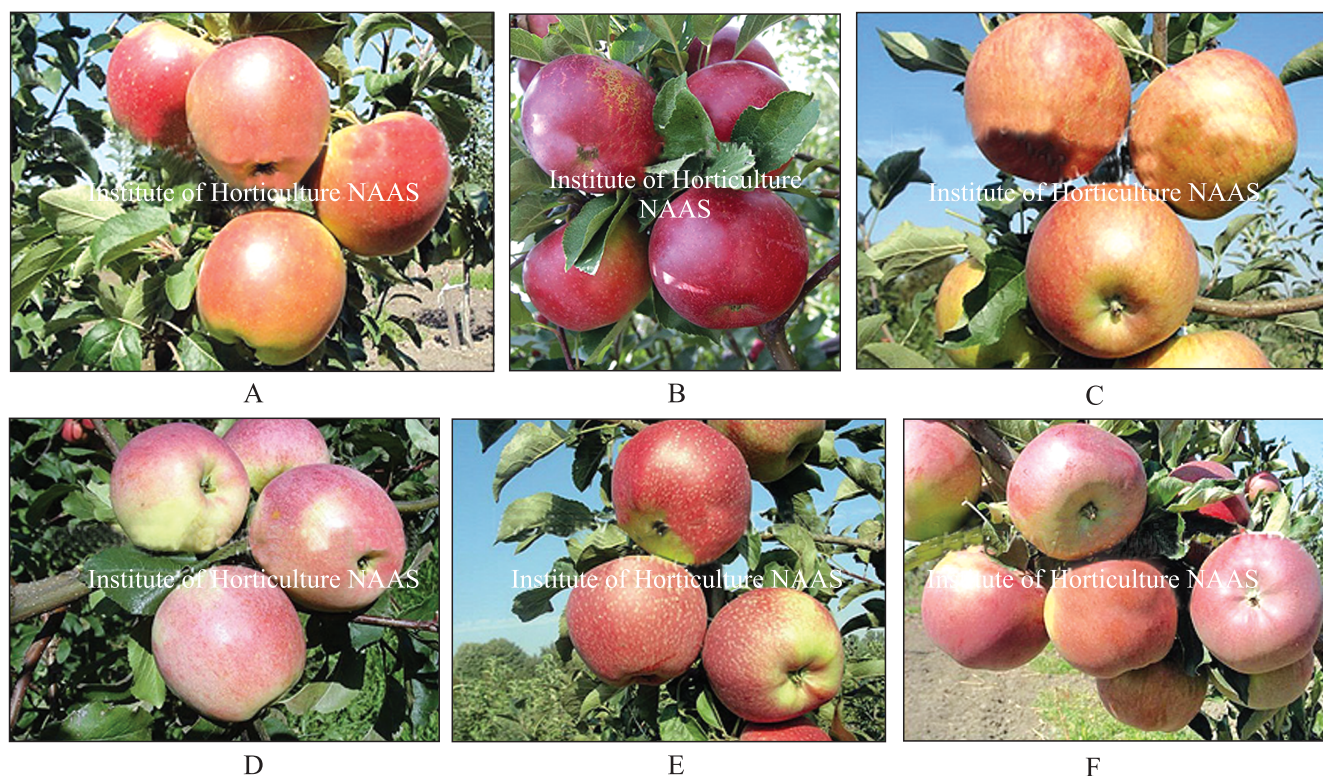


Fig. 4. Cultivars – homozygotes by one of the dominant alleles A^1 and A^2 of gene *Rf*: A – Harant; B – Rubinove Duky; C – Radohost; D – Teremok; E – Ornament; F – Askolda



Fig. 5. Phenotype of cultivars with genotype $a^1a^1 A^2a^2$: A – Kateryna; B – Skifske zoloto; C – Idared

genotypes of A^2A^2 , though phenotypically they are considerably different in their colors.

Cultivars Kateryna and Skifske zoloto were found to be equal in their genotype – $a^1a^1 A^2a^2$. The phenotypic manifestation is similar for them (Fig. 5, A, B). At the same time Idared, which has the same genotype by these markers, is remarkable for much more intense color of its skin (Fig. 5, C). This specificity may be explained by the fact that it is the carrier of another allele variant, associated with red skin, namely, dCAPS-marker, associated with SNP in the promotor of gene *MdMYB1* [14]. A similar genotype by gene *MdMYB1* is

observed for cultivars Amulet and Perlyna Kyieva [14]. All the mentioned cultivars are remarkable for their intense red color, and may undoubtedly score 4 points.

All the cultivars with green skin, analyzed in the presented work (Fig. 6), had homozygous genotype by the recessive allele a^1 . Cultivars Golden Delicious and Mutsu had the same genotype by two alleles – $a^1a^1 A^2a^2$, and Papirovka – genotype, determined solely by one allele – a^1a^1 . The genotype of Antonivka cultivar ($a^1a^1 A^2A^2$) (Fig. 6, A) with green fruit was found to be rather close to genotypes of cultivars Kateryna and Skifske zoloto, which would score 2 points for fruit



Fig. 6. Cultivars with green skin: A – Antonivka; B – Mutsu; C – Papirovka; D – Golden Delicious

color. Thus, the genotype which has both recessive and dominant alleles of gene *Rf*, does not ensure precise prognosis of skin color at the juvenile stage. This requires the identification of other genes, determining skin color of apple fruit.

Therefore, high reliability prognosis of apple skin color is possible only in case of the availability of a homozygous genotype by dominant alleles A^1 and A^2 of gene *Rf*. In other cases the matter may be only about tendencies in possible phenotype manifestation of the trait. It is also evident that another gene, for instance, *MdMYB1*, may influence the intensity of apple fruit color by the presence of homozygous genotype by allele a^1 and the heterozygous genotype – by allele A^2 , which was found while comparing the results, obtained by other authors.

CONCLUSIONS

The promising cultivars in terms of the gene of red skin color of apple fruit were identified, in particular, carriers of homozygous genotypes by dominant alleles A^1 and A^2 : Perlyna Kyeva, Edera and Amulet.

Cultivars Rubinove Duky, Ornament and Teremok, which are homozygotes by one of dominant alleles (A^1 or A^2) have high potential of being involved in breeding programs by fruit color.

The prediction of apple fruit skin color is possible only in case of the available homozygous genotype by both alleles of gene *Rf*.

The plants with heterozygous genotypes by gene *Rf* require additional observations and studies.

Асоціація алельних варіантів гена *Rf* з червоним забарвленням шкірки плодів у сортів яблуні української селекції

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Мета. Оцінити можливості залучення до селекційних програм перспективних сортів яблуні селекції Інституту

садівництва (ИС) НААН за наявністю цінних алельних варіантів гена *Rf*, пов'язаного з червоним кольором шкірки плодів яблуні. **Методи.** Полімеразна ланцюгова реакція, електрофорез в агарозному гелі, оцінка фенотипового прояву ознаки кольору шкірки яблука за бальною шкалою. **Результати.** Визначено генотипи 17 сортів яблунь різного генетичного походження, у тому числі 12 сортів селекції ИС НААН, за алельними варіантами гена *Rf*. Здійснено порівняння результатів генетичного аналізу і візуальних спостережень за фенотиповим проявом ознаки кольору плодів різних сортів яблуні. **Висновки.** У селекційних програмах як вихідні форми рекомендовано використовувати сорти яблуні Амулет, Перлина Києва і Едера, котрі є гомозиготними носіями домінантних алелів A^1 і A^2 гена *Rf*. Показано, що точне прогнозування кольору шкірки яблука можливе лише за наявності гомозиготного генотипу за обома алелями гена *Rf*.

Ключові слова: яблуня, гени забарвлення шкірки яблука, стійкість проти парші, селекція, молекулярно-генетичні маркери.

Ассоциация алельных вариантов гена *Rf* с красным цветом кожицы плодов у сортов яблони украинской селекции

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Цель. Оценить возможности привлечения в селекционные программы перспективных сортов яблони селекции Института садоводства (ИС) НААН в соответствии с присутствием в их генотипе ценных алельных вариантов гена *Rf*, связанного с красным цветом кожуры плодов яблони. **Методы.** Полимеразная цепная реакция, электрофорез в агарозном геле, оценка фенотипического проявления признака цвета кожуры яблока по балльной шкале. **Результаты.** Определены генотипы 17 сортов яблони разного генетического происхождения, в том числе 12 сортов селекции ИС НААН по алельным вариантам гена *Rf*. Проведено сравнение результатов генетического анализа и визуальных наблюдений за

фенотипическим проявлением признака цвета кожуры плодов различных сортов. **Выводы.** В селекционных программах как выходны́е формы рекомендуется использовать сорта Амулет, Перлы́на Киева и Э́дера, которые являются гомозиготными носителями доминантных аллелей A1 и A2 гена *Rf*. Показано, что точное прогнозирование цвета кожуры яблок возможно только при наличии гомозиготного генотипа по обоим аллелям гена *Rf*.

Ключевые слова: яблоня, гены окраски кожицы яблока, стойкость против парши, селекция, молекулярно-генетические маркеры.

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