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Analysis of strawberry genetic collection (*Fragaria* L.) for *Rca2* and *Rpf1* genes with molecular markers

I.V. Luk'yanchuk ✉, A.S. Lyzhin, I.I. Kozlova

I.V. Michurin Federal Scientific Centre, Michurinsk, Tambov region, Russia

Strawberry (*Fragaria* × *ananassa* Duch.) varieties are susceptible to many fungal diseases. Identification of forms, carrying resistance genes, is an important stage in breeding programs leading to resistant varieties. The use of molecular markers allows to determine with high reliability the presence of the necessary genes in the genome and to identify promising forms. Some of the common strawberry's diseases, causing significant damage to strawberry plantations, are anthracnose (*Colletotrichum acutatum* Simmonds) and red stele root rot (*Phytophthora fragariae* var. *fragariae* Hickman). Dominant *Rca2* gene is involved in monogenic resistance to *C. acutatum* pathogenicity group 2. *Rpf1*, *Rpf2*, *Rpf3* genes are determined in monogenic resistance to red stele root rot. The purpose of this study was molecular genetic testing genotypes of genus *Fragaria* L. to identify carriers of *Rca2* allele anthracnose resistance and *Rpf1* allele red stele root rot resistance. The objects of study were the wild species of the genus *Fragaria* L. and strawberry varieties (*Fragaria* × *ananassa* Duch.) of different ecological and geographic origin. To assess allelic state *Rca2* anthracnose resistance gene the dominant SCAR marker STS-Rca2_240 was used, was linked to the resistance gene *Rca2* with a genetic distance of 2.8 cM. *Rpf1* gene red stele root rot resistance was identified with the dominant SCAR marker R1A, was linked to the resistance gene *Rpf1* with a genetic distance of 3.0 cM. The resistant allele of the marker STS-Rca2_240 was identified in the Laetitia variety (*Rca2Rca2* or *Rca2rca2* genotype), which allows us to recommend it as a promising source in breeding for anthracnose resistance. The other studied forms have homozygous recessive state of the marker STS-Rca2_240 (putative genotype *rca2rca2*). The resistant allele of the marker SCAR-R1A in the varieties and wild species of strawberry under study is absent, which presumably indicates their homozygous recessive genotype of *Rpf1* gene (*rpf1rpf1*).

Key words: strawberry; molecular markers; resistance; anthracnose; red stele root rot; *Rca2* and *Rpf1* genes.

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✉ e-mail: irina.lk2011@yandex.ru

Анализ генетической коллекции земляники (*Fragaria* L.) по генам *Rca2* и *Rpf1* с использованием молекулярных маркеров

И.В. Лукъянчук ✉, А.С. Лыжин, И.И. Козлова

Федеральный научный центр им. И.В. Мичурина, Мичуринск, Тамбовская область, Россия

Сорта земляники садовой (*Fragaria* × *ananassa* Duch.) восприимчивы ко многим грибным заболеваниям. Идентификация форм, несущих гены устойчивости, является важным этапом селекционных программ по созданию устойчивых сортов. Использование молекулярных маркеров позволяет с высокой надежностью определить присутствие в геноме необходимых генов и идентифицировать перспективные формы. К числу распространенных заболеваний земляники, наносящих значительный ущерб насаждениям, относятся антракнозная гниль (*Colletotrichum acutatum* Simmonds) и фитофторозное увядание (*Phytophthora fragariae* var. *fragariae* Hickman). Моногенная устойчивость к *C. acutatum* второй группы патогенности контролируется доминантным геном *Rca2*. Моногенная устойчивость земляники к фитофторозной корневой гнили детерминирована несколькими олигогенами – *Rpf1*, *Rpf2*, *Rpf3*. Целью настоящего исследования было молекулярно-генетическое тестирование генотипов рода *Fragaria* L. для идентификации носителей аллелей *Rca2* устойчивости к антракнозу и *Rpf1* устойчивости к фитофторозной корневой гнили. Объектами исследования являлись дикорастущие виды рода *Fragaria* L. и сорта земляники ананасной (*Fragaria* × *ananassa* Duch.) различного эколого-географического происхождения. Для оценки аллельного состояния гена *Rca2* устойчивости к антракнозу использовали доминантный SCAR-маркер STS-Rca2_240, локализованный на расстоянии 2.8 cM от гена. Для выявления гена *Rpf1* устойчивости к фитофторозной корневой гнили использовали доминантный SCAR-маркер R1A, находящийся на расстоянии 3.0 cM от гена. Доминантный аллель маркера STS-Rca2_240 идентифицирован у сорта Laetitia (генотип *Rca2Rca2* или *Rca2rca2*), что позволяет рекомендовать его в качестве перспективного источника устойчивости к антракнозу для селекции. Остальные изученные формы характеризуются рецессивным гомозиготным состоянием маркера STS-Rca2_240 (предполагаемый генотип *rca2rca2*). Доминантный аллель маркера SCAR-R1A у изучаемых сортов и дикорастущих видов земляники не выявлен, что предположительно свидетельствует об их рецессивном гомозиготном генотипе по гену *Rpf1* (*rpf1rpf1*).

Ключевые слова: земляника; молекулярные маркеры; устойчивость; антракноз; фитофтороз; гены *Rca2*; *Rpf1*.

Strawberry (*Fragaria* × *ananassa* Duch.) is the most popular and economically important berry crop (Hummer, Hancock, 2009). The commercial plantations of strawberry are cultivated in 75 countries and it account for over 2/3 of the world berries production (FAOStat, 2018). At the same time, the strawberry is susceptible to many diseases and the mass development of them can lead to the 100 % death of the crop (Folta, Davis, 2006).

Anthracnose (*Colletotrichum acutatum* Simmonds) and red stele root rot (*Phytophthora fragariae* var. *fragariae* Hickman) are among the most harmful strawberry diseases causing significant damage to strawberry plantations in Europe and America (Smith, 2008; Newton et al., 2010). The most of commercial strawberry varieties of foreign breeding are susceptible to anthracnose and red stele root rot (Van de Weg, 1997a; Denoyes-Rothan et al., 2005). For the last years the pathogens of anthracnose and red stele root rot being tested in strawberry plantings of Russia and besides *Phytophthora fragariae* var. *fragariae* is included in the list of Quarantine pests, weeds and plant diseases in Russia (Aleksandrov et al., 2007; Golovin, 2014; Dudchenko et al., 2015).

Anthracnose affects the aboveground part of strawberry: fruits, flowers, petioles, leaves, stolons, causing a significant suppression of the plant, and sometimes there can be the death of plant. Pathogen can be located on a plant for a long time in latent state, this fact significantly complicates the reliable identification and protective measures (Leandro et al., 2001). The losses of marketable yield of strawberries because of anthracnose may reach 80 % (Dudchenko et al., 2015).

P. fragariae var. *fragariae* affect the root system, inhibiting the growth, causing withering and afterwards death of the plants. It is difficult to diagnose the pathogen visually because of similarity of symptoms of infection and stress factors (de los Santos et al., 2004; Aleksandrov et al., 2007). The occurrence of pathogen can be observed mainly after using of infected planting material, and the oospores can persist in soil in the absence of host plant according to the different data from 3 to 17 years (Duncan, Cowan, 1980ж Szkuta, 2006).

In connection with it, the development of new highly resistant to pathogens strawberry varieties is an important breeding task. The cultivation of varieties with genetic resistance to diseases will increase the profitability of plantations and as well as positively effect the ecological state of strawberry agrocenoses due to reduce to pesticide load (Korbin, 2011).

To improve the strawberry assortment it is very important: to deepen the genetic research, to reveal the patterns of loci inheriting economically important trait and to identify the donors. One of the promising trends to raise the efficiency of strawberry genetic studies became the use of contemporary techniques of molecular genetic analysis of genome applying DNA markers. At present time the molecular markers are wide applied in the analysis of genetic diversity, mapping, and strawberry variety genotype identification. But molecular markers are less involved in strawberry breeding (Whitaker, 2011).

The purpose of current study was to carry out molecular genetic testing of genus *Fragaria* L. genotypes. It was necessary to identify carrier of *Rca2* anthracnose resistance gene and *Rpf1* red stele root rot resistance gene.

Materials and methods

Biological material was represented by wild species of the genus *Fragaria* L.: *F. orientalis* Los., *F. moschata* Duch., *F. ovalis* Rydb., *F. virginiana* Rydb. ssp. *platypetala* and strawberry varieties (*Fragaria* × *ananassa* Duch.) of different ecological and geographical origin from genetic collection of the FSSI "I.V. Michurin Federal Scientific Centre (FSC)", such as Lastochka, Privlekatelnaya, Urozhainaya CGL, Feyerverk, Flora and Yarkaya breeding by FSSI "I.V. Michurin FSC" (Russia), Baron Solemacher (Germany), Elsanta, Korona, Kimberly, Vicoda, Vima Tarda, Vima Xima, Vima Zanta (Netherlands), Florence (England), Kent (Canada), Laetitia (Italy), Red Gauntlet (Scotland).

To assess allelic state *Rca2* anthracnose resistance gene was used the dominant SCAR marker STS-Rca2_240. The SCAR marker STS-Rca2_240 was multiplexed with the microsatellite marker EMFv020 used as the positive PCR control (Lerceteau-Kohler et al., 2005).

The *Rpf1* red stele root rot resistance gene was identified with the dominant marker SCAR-R1A (Haymes et al., 2000).

Primer sequences and product length are reported in Table 1. Used in this study primers were synthesized by Syntol (Russia).

Total genomic DNA was extracted from the fresh leaves using the Diversity Arrays Technology P/L (DArT) protocol. However, the use of the basic protocol did not allow obtaining DNA extract with the degree of purity necessary for PCR analysis because despite of the high concentration of DNA in solution, inhibition of test PCR was observed. To reduce the content of inhibitory impurities in the DNA solution, an additional double purification of DNA with 5M NaCl with reprecipitation with 70 % ethanol included the following steps was used. 100 µl of 5M NaCl and 400 µl of 70 % ethanol were added to the DNA solution; mix centrifuged at 13,000 rpm for 5 min; the supernatant was removed; the precipitate was washed twice with 80 % ethanol, dried at room temperature and dissolved in deionized water. As a result of these steps, DNA extract with necessary of concentration and purity for PCR reaction was obtained.

PCR reactions were performed in 15 µl final volume containing: 20 ng of genomic DNA, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 U of Taq DNA polymerase (0.8 U for multiplex PCR STS-Rca2_240 + EMFv020) and 1.5 µl of PCR-buffer (+$(NH_4)_2SO_4$, –KCl). All components produced by Thermo Fisher Scientific.

The amplification was performed in T100 Thermal Cycler (BioRad). PCR conditions for the multiplex PCR reaction (STS-Rca2_240 and EMFv020) were as described by Lerceteau-Kohler et al. (2005) as follow: 3 min denaturation at 95 °C, 35 cycles of 50 s at 95 °C, 50 s at 64 °C, and 1 min at 72 °C, and a final extension step of 5 min at 72 °C.

PCR conditions for the SCAR-R1A marker were as described by Haymes et al. (2000) as follow: 3 min denaturation at 94 °C, 25 cycles of 30 s at 94 °C, 45 s at 60 °C, and 1 min at 72 °C, and a final extension step of 7 min at 72 °C.

The amplification products were separated on a 2 % agarose gel and visualized by ethidium bromide staining. GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific) was used as a molecular weight marker.

Table 1. Primer sequences and product length in base pairs of the molecular markers used in the study

Gene	Marker	Primer sequence (5′→3′)	Product length (bp)
Rca2	STS-Rca2_240	F 5′-GCCACGTCACTAGTCAAATTCAA-3′ R 5′-TCATGGACAGTGGTCTCAGC-3′	240
	EMFv020 (контроль ПЦР)	F 5′-CAGGCGCCAACGGCGTGCTCTTGT-3′ R 5′-CAGCGCCGCCAGCTCATCCCTAGG-3′	~170
Rpf1	SCAR-R1A	F 5′-TGCATCATTAATGTAGAAGTCTTT-3′ R 5′-TGATGCGACATACAAAAATATTAG-3′	285

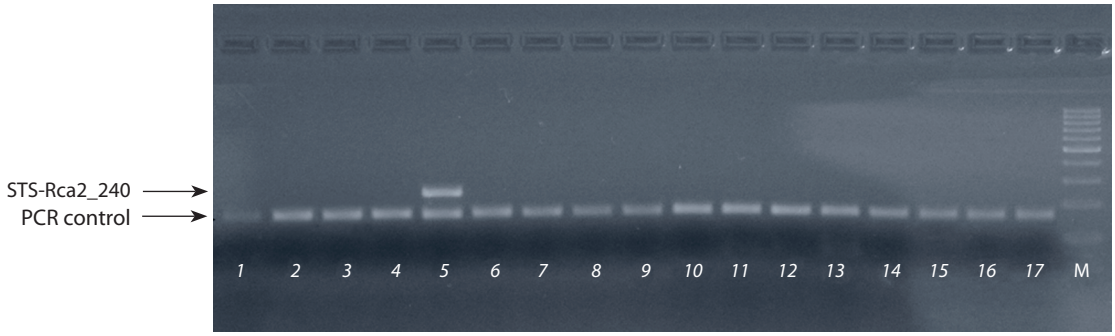

Fig. 1. Electrophoresis profile of marker STS-Rca2_240 at strawberry genotypes.

1 – Elsanta, *2* – Kent, *3* – Red Gauntlet, *4* – Florence, *5* – Laetitia, *6* – Vima Tarda, *7* – Urozhainaya CGL, *8* – Vicoda, *9* – Feyerverk, *10* – Privlekatelnaya, *11* – Kimberly, *12* – Vima Zanta, *13* – Korona, *14* – Yarkaya, *15* – Flora, *16* – Lastochka, *17* – Vima Xima, M – DNA ladder.

Results and discussion

Fragaria × *ananassa* Duch. is difficult object for molecular genetic analysis. The high ploidy level (8*x*), the combination of different basic genomes in the genotype, complex gene interactions, and polygenic control of traits make it difficult to study the genetics of strawberry. Disease resistance in strawberry in most cases is also controlled quantitatively.

Currently, however, monogenic resistance factors have been identified for some pathogens (*C. acutatum*, *P. fragariae* var. *fragariae*) (Van de Weg, 1997a, b; Lerceteau-Kohler et al., 2002) and this allows effective screening of resistant genotypes using molecular markers.

Anthracnose resistance in strawberry is controlled by quantitative and monogenic factors. Resistance to *C. acutatum,* pathogenicity group 1 is quantitative (Denoyes-Rothan et al., 2004). Single dominant gene, *Rca2*, controls the resistance to *C. acutatum,* pathogenicity group 2 (Lerceteau-Kohler et al., 2002).

The SCAR marker STS-Rca2_240 was linked to the resistance gene *Rca2* with genetic distance of 2.8 cM. The PCR product associated with the resistance *Rca2* allele has 240 bp. The linked inheritance of the marker alleles and the *Rca2* gene allows, based on the presence or absence of the marker alleles, to identify the allelic state of the *Rca2* gene (Lerceteau-Kohler et al., 2005).

In studied collection of strawberry, the resistant allele of the marker STS-Rca2_240 was identified in the Laetitia variety (*Rca2Rca2* or *Rca2rca2* genotype). The other studied forms have homozygous recessive state of the marker STS-Rca2_240 linked to *Rca2* resistance gene (putative genotype *rca2rca2*) (Fig. 1, Table 2).

The absence of a dominant allele of the STS-Rca2_240 marker in the anthracnose resistant variety Vicoda can be presumably due to two factors: the recombination that occurred in the variety (or its parental forms) between the *Rca2* resistance gene and the marker STS-Rca2_240 (Lerceteau-Kohler et al. (2005) noted similar recombination for the US292 genotype and its parent form Arking.) or the presence of other genetic resistance factors.

The recessive state SCAR marker STS-Rca2_240 in species *F. virginiana* ssp. *platipetala* and strawberry variety Elsanta confirmed in literature data (Njuguna, 2010; Sturzeanu et al., 2016). It should be noted that, according to W. Njuguna (2010), the dominant allele of the STS-Rca2_240 marker linked to the *Rca2* gene is present in the Red Gauntlet variety, while, according to our research, in this variety the *Rca2* gene is presumably represented by the recessive allele. Such a discrepancy in the results may be presumably due to an identification error and requires additional research. However, according to the results of artificial infection, the Red Gauntlet variety is susceptible to *C. acutatum* (Simpson et al., 1994), which indirectly confirms our data.

Monogenic resistance to red stele root rot of strawberry determined several genes. W.E. Van de Weg analyzing the interaction model of strawberry varieties and races of *P. fragariae* var. *fragariae* identified 5 resistance factors (R1–R5), corresponding to specific pathogen avirulence gene (Avr1–Avr5) (Van de Weg, 1997b). The main role in the formation of strawberry red stele root rot resistance belongs to three genes – *Rpf1*, *Rpf2*, *Rpf3* (Whitaker, 2011). *Rpf1* gene ensuring resistance of strawberry genotypes to 16 races of *P. fragariae* var. *fragariae* (Sasnauskas et al., 2007).

Table 2. Presence (1) or absence (0) PCR products of the indicated markers, linked to c *Rca2* anthracnose resistance gene and *Rpf1* red stele root rot resistance gene in different strawberry varieties

Species/variety	*Rca2*	*Rpf1*	Putative genotype
	STS-Rca2_240	SCAR-R1A	
F. orientalis Los.	0	0	*rca2rca2rpf1rpf1*
F. moschata Duch.	0	0	
F. ovalis Rydb.	0	0	
F. virginiana Rydb. ssp. *platypetala*	0	0	
Baron Solemacher (*F. vesca* L.)	0	0	
Lastochka (922-67 × Privlekatelnaya)	0	0	
Privlekatelnaya (Rubinovy kulon × Allbritton)	0	0	
Urozhainaya CGL (Zenga Zengana × Redcoat)	0	0	
Feyerverk (Zenga Zengana × Redcoat)	0	0	
Flora (Zenga Zengana × Redcoat)	0	0	
Yarkaya (Zenga Zengana × Redcoat)	0	0	
Elsanta (Gorella × Holiday)	0	0	
Florence (Tioga × (Red Gauntlet × (Wiltguard × Gorella))) × (Providence × Providence)	0	0	
Kent ((Red Gauntlet × Tioga) × Raritan)	0	0	
Kimberly (Gorella × Chandler)	0	0	
Korona (Tamella × Induka)	0	0	
Laetitia (Chance seedling)	1	0	*Rca2rca2rpf1rpf1*
Red Gauntlet ((NJ New Jersey 1051 × Climax) × (Climax × 1051))	0	0	*rca2rca2rpf1rpf1*
Vicoda (Chance seedling)	0	0	
Vima Tarda (Vima Zanta × Vicoda)	0	0	
Vima Xima (Chance seedling)	0	0	
Vima Zanta (Elsanta × Korona)	0	0	

Fig. 2. Electrophoresis profile of marker SCAR-R1A at strawberry genotypes.

K – Tristar (control), *1* – Elsanta, *2* – Kent, *3* – Red Gauntlet, *4* – Florence, *5* – Laetitia, *6* – Vima Tarda, *7* – Urozhainaya CGL, *8* – Vicoda, *9* – Feyerverk, *10* – Privlekatelnaya, *11* – Kimberly, *12* – Vima Zanta, *13* – Korona, *14* – Yarkaya, *15* – Flora, *16* – Lastochka, *17* – Vima Xima, M – DNA ladder.

The marker SCAR-R1A was linked to the resistance gene *Rpf1* with genetic distance of 3.0 cM. The PCR product associated with the resistance *Rpf1* allele has 285 bp. In genotypes with a homozygous recessive state of the gene *Rpf1* (*rpf1rpf1*), this product is not amplified (Haymes et al., 2000). The molecular analyzes performed with SCAR-R1A marker showed the absent PCR product of 285 bp in studied strawberry varieties (Fig. 2), which presumably indicates their homozygous recessive genotype of *Rpf1* gene – *rpf1rpf1* (see Table 2).

The recessive state SCAR-R1A marker in the Elsanta and Kent varieties is confirmed in literature data (Haymes et al., 2000; Sturzeanu et al., 2016). According to the data of W. Njuguna (2010), the populations of *F. virginiana* ssp. *platipetala* of various growing sites also have not the dominant allele SCAR-R1 marker linked to the *Rpf1* gene.

Thus, according to the results of molecular genetic analysis, the studied strawberry genotypes were identified as susceptible to red stele root rot by assessing the allelic state of the *Rpf1* gene. The Laetitia variety is characterized to anthracnose resistance by assessing the allelic state of the *Rca2* gene, which allows us to recommend it as a promising source in breeding for resistance to *C. acutatum*, pathogenicity group 2.

Conflict of interest

The authors declare no conflict of interest.

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ORCID ID

I.V. Luk'yanchuk orcid.org/0000-0003-1626-840X

A.S. Lyzhin orcid.org/0000-0001-9770-8731

I.I. Kozlova orcid.org/0000-0003-4150-3037