

Assessment of fire blight resistance in apple clonal rootstocks using molecular markers

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Оценка устойчивости клоновых подвоев яблони к бактериальному ожогу с использованием молекулярных маркеров

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Background. Clonal apple rootstocks are one of the main components of intensive gardening. The degree of rootstock damage by fire blight affects the resistance of the variety-rootstock combination. The paper presents a study on marking quantitative trait loci (QTL) of resistance to fire blight *Erwinia amylovora* in clonal apple rootstock. **Materials and methods.** A collection of 20 rootstock forms was analyzed. For the study, SCAR markers GE-8019, AE10-375 and microsatellite marker CH-F7-FB1 were used. **Results.** Polymorphism was observed for all three markers, and their various combinations in one genotype were revealed. It was previously noted that genotypes that carry all three markers were more resistant than those that lack them. The presence of all three markers was observed only in forms 62-396 (B10), 16-1 and 2-9-102. The other genotypes did not have the GE-8019 marker. The AE10-375 marker was identified in eight clonal rootstocks. Microsatellite marker CH-F7-FB1 was present in all tested rootstocks. However, polymorphism was detected there. Most genotypes had a 174 bp fragment, but a 210 bp fragment was identified in two of the 20 forms. Clonal rootstock 70-20-21 proved heterozygous for this marker. The analyzed collection also included samples that had only the microsatellite marker: G16, Malysh Budagovskogo, Paradizka Budagovskogo (B9), 54-118 (B118), 57-491, 70-20-20 (B119), 70-20-21, 71-7-22, 76-3-6, 83-1-15, 87-7-12, and 2-12-10. The study of rootstock forms on the basis of resistance to metabolites of the fire blight pathogen was carried out under laboratory conditions using the *E. amylovora* culture filtrate *in vitro* on leaf explants. Most of the studied genotypes had different combinations of markers. However, the experiments showed that forms 62-396 and 14-1 with two out of three markers (AE10-375 and CH-F7-FB1) phenotypically manifested the trait of resistance to metabolites of *E. amylovora*.

Key words: apple, fire blight, *Erwinia amylovora*, QTL, marker-assisted selection.

Актуальность. Клоновые подвои яблони – один из основных компонентов интенсивного садоводства. Степень поражения подвоя бактериальным ожогом влияет на устойчивость сорто-подвойной комбинации. Представлены исследования по маркированию локусов количественных признаков (QTL) устойчивости клоновых подвоев яблони к бактериальному ожогу плодовых культур (возбудитель *Erwinia amylovora* (Burrill.) Winslow et al.). **Материалы и методы.** Проведен анализ коллекций из 20 форм подвоев. Для исследования были использованы SCAR-маркеры GE-8019 и AE10-375, а также микросателлитный маркер CH-F7-FB1. **Результаты.** Отмечен полиморфизм по всем трем маркерам, выявлены различные их сочетания в одном генотипе. Ранее отмечалось, что генотипы, которые несут все три маркера, более устойчивы, чем те, у которых они отсутствуют. Наличие всех трех маркеров отмечено только у форм 62-396 (B10), 16-1 и 2-9-12. У остальных генотипов не выявлен маркер GE-8019. Маркер AE10-375 идентифицирован у восьми клоновых подвоев. Микросателлитный маркер CH-F7-FB1 присутствует у всех исследуемых подвоев. Однако здесь отмечен полиморфизм. У большинства генотипов присутствует фрагмент 174 пн, но у двух из 20 форм выявлен фрагмент 210 пн. Клоновый подвой 70-20-21 является гетерозиготным по этому локусу. В анализируемой коллекции также отмечены образцы, имеющие только микросателлитный маркер: 70-20-21, G16, 2-12-10, 83-1-15, 54-118 (B118), «Малыш Будаговского», 71-7-22, 57-491, «Парадизка Будаговского» (B9), 70-20-20 (B119), 76-3-6, 87-7-12. Изучение подвойных форм по признаку устойчивости к метаболитам возбудителя бактериального ожога проводили в лабораторных условиях с использованием культурального фильтрата *E. amylovora* на листовых эксплантах *in vitro*. У большинства изученных генотипов отмечены различные сочетания маркеров. Проведенные эксперименты показали, что у исследуемых форм с двумя маркерами из трех (AE10-375 и CH-F7-FB1) фенотипически проявлялся признак устойчивости к метаболитам *E. amylovora*.

Ключевые слова: яблоня, *Erwinia amylovora*, QTL, маркер-опосредованная селекция.

Introduction

In the Russian Federation, as in many countries of the world, the most economically significant fruit plant is the apple tree, the areas under which are expanding every year. The fire blight of fruit crops causes significant damage to commercial apple-tree orchards and stoolbeds. The main strategy for controlling the disease remains the destruction of infected trees, treatment with cupriferous and other pesticides, and, in some countries, with antibiotic solutions. However, the widespread use of such drugs causes the emergence of new bacteria races that are resistant to their effects (Emeriewen et al., 2018). The top-priority and environmentally friendly key focus area is the cultivation of resistant apple varieties and rootstocks.

It has been found that most resistant genotypes are concentrated among wild apple trees. High resistance is observed in two species – *Malus × robusta* (Carr.) Rehder 5 (QTL identified on chromosome 3) and *M. fusca* (Raf.) C.K. Schneid. (QTL identified on chromosome 10). The phenotypic trait manifestation, depending on the presence of QTL in these species, is 80% and 60%, respectively. However, the manifestation of high resistance is characteristic in relation to certain local strains of the pathogen (Peil et al., 2007; Emeriewen et al., 2018). Low susceptibility to the disease was also observed in other species: *M. baccata* (L.) Borkh. (Peil et al., 2014), *M. × robusta* var. *persicifolia* and *M. sieversii* (Ledeb.) M. Roem. (Fazio et al., 2013).

The causative agent of fire blight is the gram-negative phytopathogenic enterobacterium *Erwinia amylovora* (Burrill.) Winslow et al., which has a type III secretion (T3SS) that delivers effector proteins (PA11) to the host organism. The T3SS is encoded by a cluster of hypersensitive response and pathogenicity genes (called *hrp* genes) which control the ability of the pathogen to cause disease in susceptible host plants and induce a hypersensitive response (HR) in both resistant and non-resistant plants (Khan et al., 2012).

The genome studies of the domestic apple tree (*Malus domestica* Borkh.) have not identified individual genes that control monogenic resistance. However, the presence of saturated genetic maps has allowed identification of a number of quantitative trait loci (QTL) associated with resistance to fire blight (Maliepaard et al., 1998; Liebhard et al., 2002, 2003; Peil et al., 2008; Khan et al., 2007; Baldo et al., 2010; Wöhner et al., 2014; Kost, 2016). Molecular markers were found for most QTLs which were used to genotype the collections. Significant associations between the traits and markers indicate that these markers are located adjacent to the QTL (Khan et al., 2012).

The FBF7 (Fire blight Fiesta chromosome 7) QTL associated with resistance to fire blight was identified on chromosome 7 in cv. 'Fiesta'. Its correlation with the phenotypic manifestation of the trait varied in the range of 34.3–46.6% (Calenge et al., 2005).

Similar results were obtained from the analysis of seedlings derived from the crossing of cvs. 'Fiesta' and 'Discovery'. In that study, the QTL was also identified on the chromosome 7 (linkage group 7), and the level of phenotypic variability was consistent with previous studies: 37.5–38.6% (Khan et al., 2007).

On the basis of the summarized data it was found that the QTL of fire blight resistance, called "FBF7" (Fire Blight Fiesta chromosome 7), is located on the seventh chromosome. Two dominant SCAR markers were developed to identify this QTL. The AE10-375 and GE-8019 markers flank the region of chromosome 7 where the QTL is located. Besides,

the additional microsatellite marker CH-F7-Fb1 linked to the AE10-375 marker was produced to accurately identify the resistance locus. These markers can be successfully used for marker-assisted apple selection (Khan et al., 2007). However, most studies focused on resistance assessment and screening of apple varieties (Calenge et al., 2005; Khan et al., 2007; Peil et al., 2014; Baumgartner et al., 2015). Rootstocks are an integral part of the scion–rootstock combination and play a significant role in the development of a disease-resistant apple plant. Therefore, the choice of the rootstock and knowledge of the degree of its susceptibility to the disease are one of the important factors preventing the spread of infection.

The attack of fire blight in orchards with trees on dwarf rootstocks is especially dangerous due to high planting density and intensive spreading of the disease (Jensen et al., 2012). In addition, many dwarf apple rootstocks are susceptible to the pathogen. In areas significantly susceptible to the disease, there are recommendations against certain variety–rootstock combinations (Wilcox, 1994).

A number of studies were carried out to study fire blight resistance in apple rootstocks and their combinations with varieties (Russo et al., 2008; Jensen et al., 2012; Kviklys, 2012). A significant part of the most common clonal rootstocks (M9, M26, P series, Ottawa series) were found to be susceptible to the disease. This applied to both an individual rootstock plant and a scion–rootstock combination (Cline et al., 2001; Kviklys, 2012; Wilcox, 2014).

Only limited attention has been paid to rootstocks of Russian breeding. There are almost no domestic studies on this topic, including molecular diagnostics and collection screening. Of all the diversity of the existing assortment of Russian clonal rootstocks, the B9 (Paradizka Budagovskogo) remains the most studied. Its resistance to fire blight is assessed ambiguously in the published sources. When infected *in vitro*, the B9 rootstock shows high susceptibility to the pathogen. However, in the field and in variety–rootstock combinations, it demonstrates significant resistance, which increases with plant age (Norelli et al., 2003; Russo et al., 2008).

The aim of this work was to mark the QTL of fire blight resistance in clonal apple rootstocks in order to assess their resistance to pathogen metabolites under *in vitro* conditions, and to identify the genes for this valuable trait.

Materials and methods

The work was carried out at the facilities of Michurinsk State Agrarian University and the All-Russian Plant Quarantine Center (VNIKR).

The biological material of the study was the forms of clonal apple rootstocks from the collection of Michurinsk State Agrarian University. A total of 20 genotypes were analyzed. For DNA isolation, young healthy apple-tree leaves were taken from the apical part of the shoot, one sample for each form. Cv. 'Remo' was used as a positive reference, the presence of all markers in this variety being determined in the original work. The DNA extraction was carried out using the Quick-DNA Plant/Seed Miniprep Kit (DNA extraction kit) (Zymo Research, USA) according to the manufacturer's protocol. The amplification was performed in a SimpliAmp device manufactured by Applied Biosystems (USA). The reaction mixture for PCR with a volume of 15 µl contained: 20 ng DNA, 1.5 mM dNTP, 2.5 mM MgSO₄, 10 pM of each primer, 1 U Taq polymerase and 10× standard PCR buffer (Thermo Fisher Scientific, UK). Quantitative trait loci

(QTLs) for fire blight resistance were identified using three molecular markers (Table 1): two SCAR markers, AE10-375, GE-8019 and one SSR marker CH-F7-FB1 (Khan et al., 2007).

bility of this QTL on contrasting forms with varying degrees of resistance which was tested by inoculation with the pathogen. It was found that genotypes with all three mark-

Table 1. The sequences of primer pairs used in the work (Khan et al., 2007)

Таблица 1. Последовательности праймерных пар, использованных в работе (Khan et al., 2007)

Name / Название	Sequence (5'-3') Последовательность (5'-3')	Annealing temperature, °C / Температура отжига, °C	Amplified fragment size, bp / Размер амплифицируемых фрагментов, пн
AE10-375	F CTAAGCGCACGTTCTCC R CTGAAGCGCATCATTTCTGATAG	55	375
GE-8019	F TTGAGACCGATTTTCGTGTG R TCTCTCCCAGAGCTTCATTGT	55	397
CH-F7-Fb1	F AGCCAGATCACATGTTTTTCATC R ACAACGGCCACCAGTTTATC	60	174, 210

After the amplification, the samples were separated by electrophoresis on a 2% agarose gel, then analyzed under ultraviolet light and photographed using a digital camera.

The study of fire blight resistance in apple rootstocks was based on an estimation of the effect of *E. amylovora* metabolites on the host plant using the culture filtrate of strains VNIKR VRE16 and VNIKR TE1 isolated in Voronezh and Tambov Provinces of the Russian Federation, respectively, as a selection agent. Bacterial cultures were incubated in Chapek's liquid nutrient medium for a month, followed by sterilization by passing through a membrane filter (Millipore 0.22 µm, France). To determine the nature of the effect of the bacterial culture fluid filtrate on the host plant, the leaves of the *in vitro* microplants of apple rootstocks 54-118, 62-396 and 14-1 were placed on the surface of the Murashige-Skoog (MS) nutrient medium (Murashige, Skoog, 2006) containing bacterial metabolites, in accordance with the sterility standards. Each variant of the experiment included 21 explants. Medium variants with 5%, 10% and 20% concentrations of the bacterial culture fluid filtrate were used in the experiment. The explants were incubated for 4 weeks at 24°C under a 16-hour photoperiod. The results of the experiment were recorded one month after its initiation (De Castro et al., 2016 Pinheiro, 2016, Akomolafe et al., 2019; Iwamoto et al., 2019, Maggini et al., 2019).

Plant tissue damage according to the degree of resistance to the bacterium metabolites was assessed using a five-point scale: 0 – no damage; 1 – very weak lesion (chlorotic or necrotic spots are sparse); 2 – weak lesion (less than 10% of the leaf surface is occupied by necrosis or up to 25% by chlorosis); 3 – medium lesion (from 11 to 25% necrosis or from 26 to 50% chlorosis); 4 – severe lesion (from 26 to 50% – necrosis, more than 50% chlorosis); 5 – very severe damage (more than 50% necrosis) (Sedov, Ogoltsova, 1999).

Results and discussion

The collection accessions of apple clonal rootstocks were analyzed using SCAR markers GE-8019 and AE10-375 developed on the basis of AFLP and RAPD markers flanking the QTL sequence of fire blight resistance on chromosome 7 of cv. 'Fiesta', as well as the microsatellite marker CH-F7-FB1 linked to the AE10-375 marker (Khan et al., 2007). The authors of the original publication analyzed the action sta-

ers were more resistant than those that lacked them. The reproducibility of the obtained results makes it possible to use these markers for the selection of gene sources and the breeding of new resistant genotypes by marker-assisted selection techniques.

Screening the collection of apple clonal rootstocks from Michurinsk State Agrarian University using GE-8019, AE10-375 and CH-F7-FB1 markers succeeded in obtaining clear reproducible results (Table 2).

The presence of the FBF7 QTL in an apple genotype is most reliably characterized by the detection of at least two markers, GE-8019 and AE10-375. They flank the genomic region where the QTL is located. The CH-F7-FB1 marker is linked to the AE10-375 locus and confirms the accuracy of locus identification. The presence of all three markers suggests a high expectation of fire blight resistance (Khan et al., 2007).

Analyzing the obtained data made it possible to identify various combinations of the studied markers. The presence of all three markers was observed only in forms 62-396 (B10), 16-1 and 2-9-102. The other genotypes did not have the GE-8019 marker. The second flanking marker AE10-375 was identified in eight clonal rootstocks. The microsatellite marker CH-F7-FB1 was present in all studied rootstocks. However, polymorphism was revealed at this locus. Most genotypes had a 174 bp fragment, but two out of the 20 forms had a 210 bp fragment. The clonal rootstock 70-20-21 was heterozygous at this locus, because it had both fragments amplified. The analyzed collection also contained accessions with only the microsatellite marker: 70-20-21, G16, 2-12-10, 83-1-15, 54-118 (B118), Malysh Budagovskogo, 71-7-22, 57-491, Paradizka Budagovskogo (B9), 70-20-20 (B119), 76-3-6, 87-7-12.

The degree of fire blight resistance in apple clonal rootstocks of Russian breeding has not been studied thoroughly enough. Evaluation under field conditions or with artificial infection was done only for certain forms of rootstocks (Norelli et al., 2003). There is no information about the resistance of apple clonal rootstocks from the collection of Michurinsk State Agrarian University.

To assess the manifestation degree for the trait of resistance to *E. amylovora* in apple clonal rootstocks developed at Michurinsk State Agrarian University, preliminary studies were carried out under laboratory conditions using

Table 2 Results of the FBF7 QTL analysis of clonal apple rootstocks**Таблица 2. Результаты анализа FBF7 QTL у клоновых подвоев яблони**

Genotype Генотип	FBF7 QTL markers (fragment size, bp) / Маркеры FBF7 QTL (размер фрагмента, пн)			
	GE-8019 397 bp (пн)	AE10-375 375 bp (пн)	CH-F7-FB1	
			174 bp (пн)	210 bp (пн)
87-7-12	-	-	+	-
76-3-6	-	-	+	-
70-20-20 (B119)	-	-	+	-
Paradizka Budagovskogo (B9)	-	-	+	-
57-491	-	-	+	-
71-7-22	-	-	+	-
Malysh Budagovskogo	-	-	+	-
54-118 (B118)	-	-	+	-
62-396 (B10) *	+	+	+	-
83-1-15	-	-	+	-
2-12-10	-	-	+	-
2-15-2 **	-	+	+	-
3-4-7 **	-	+	+	-
14-1	-	+	-	+
4-6-5 **	-	+	+	-
2-9-102 *	+	+	+	-
<i>Malus sieboldii</i> (Regel) Rehder **	-	+	+	-
16-1 *	+	+	+	-
G16	-	-	+	-
70-20-21 **	-	-	+	+

Note / Примечание: «-» – the absence of a marker / отсутствие маркера;

«+» – the presence of a marker / наличие маркера;

* – the accession has three markers / наличие у образца трех маркеров;

** – the accession has two markers / наличие у образца двух маркеров

metabolites of the causative agent of this disease. The main goal of this work was to optimize the differentiating metabolite concentrations, which would be useful in future studies to rank apple genotypes according to the degree of their resistance to *E. amylovora*. Leaf explants of the *in vitro* culture were used as model objects of the clonal rootstock forms for which molecular analysis was performed.

For this work, samples were taken with different combinations of the studied molecular markers: 54-118 had only the microsatellite marker, 62-396 had all three markers, 14-1 had the microsatellite marker and the AE10-375 SCAR marker.

As a result of the study, it was found that it is advisable to use the 20% concentration of *E. amylovora* metabolites, since the studied forms in this variant of the experiment showed the greatest differences. In other variants of the experiment, the differences were within the error of the mean (Figure).

Since the absence of pathogen cells does not ensure the action of type III bacterial secretion, in the context of this experiment we should speak about the effect of nonspecific toxins.

Among the studied genotypes, the 54-118 rootstock proved to be unstable at different metabolite contents. On a medium with the 20% concentration of bacterial metabolites, its leaf explant damage had a high score, more than thrice exceeding the reference value.

Forms 62-396 and 14-1 on media with the same content of bacteria culture filtrate demonstrated slight differences from the reference.

The data obtained are not final. Further experiments are required, with an increased number of samples, on media with the 20% concentration of bacterial metabolites.

It should be noted that studies of other authors showed similar results. The analysis of 31 Hungarian apple varieties

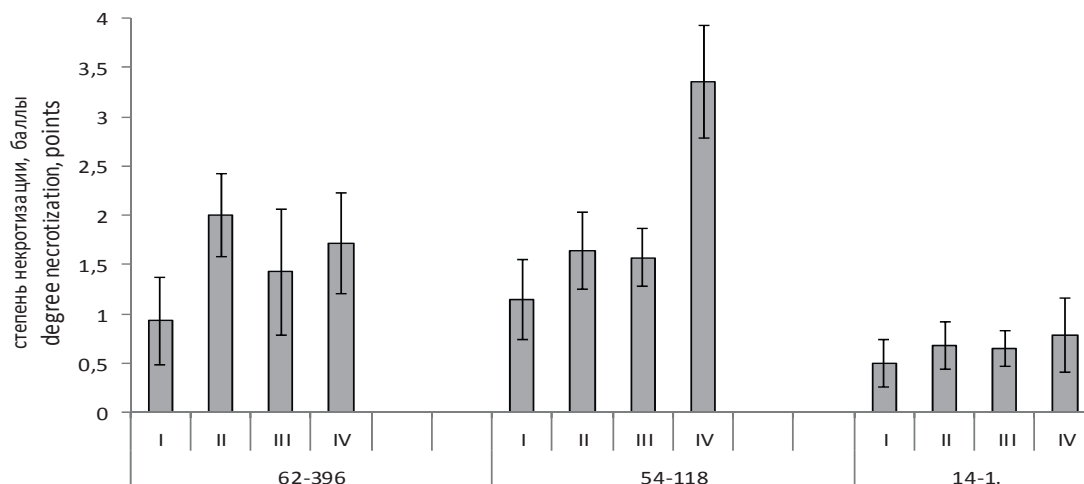


Figure. The degree of necrotization in leaf explants of apple rootstock forms on media with different contents of *Erwinia amylovora* (Burrill.) Winslow et al. metabolites:

I – reference; II – 5% metabolite concentration; III – 10% metabolite concentration; IV – 20% metabolite concentration

Рисунок. Степень некротизации листовых эксплантов подвойных форм яблони на средах с различным содержанием метаболитов *Erwinia amylovora* (Burrill.) Winslow et al.:

I – контроль, II – концентрация метаболитов 5%; III – концентрация метаболитов 10%; IV – концентрация метаболитов 20%

made it possible to establish the presence of the marker AE10-375 in most varieties, and GE-8019 in only half of the genotypes.

The AE10-375 marker was also found in 22 hybrids out of 32 ones obtained from crosses of two homozygous forms. Testing plants with QTL markers under artificial infection showed no clear correlation between the marker and the resistance character (Tóth et al, 2012). A different combination of markers GE-8019 and AE10-375 was observed in the analysis of 31 apple cultivars developed in Kazakhstan. Both markers were present only in two of them (Omasheva et al., 2016).

As reported by the authors of the original publication, the genes themselves can be damaged even in the presence of two SCAR markers, due to the large size of the quantitative resistance locus. Conversely, the absence of a marker does not necessarily indicate gene damage or absence. In addition, possible influence of the environment on the expression of quantitative resistance genes has been indicated. It is also possible that the presence of resistance is determined by effects of other QTLs.

The present research is preliminary and requires additions and extensions to the experiment in order to get a deeper insight into the resistance of apple clonal rootstocks to fire blight.

Conclusion

Thus, the results of molecular analysis and plant susceptibility to metabolites of the fire blight pathogen of fruit crops were compared. There was no clear relationship between the number of the present markers and the degree of plant tissue necrosis in the tested forms. However, the studies had shown that the presence of the AE10-375 SCAR marker and CH-F7-FB1 microsatellite in forms 62-396 and 14-1 provided the phenotypic manifestation of the resistance to *Erwinia amylovora* metabolites.

The studies were carried out in the framework of the State Task of the Ministry of Agriculture of the Russian Federation for 2020 on the topic: "Selection of winter-hardy dwarf apple clonal rootstocks using molecular markers and somatic tissue culture in vitro" (No. AAAA-A20-120011690041-9) at the Center for Collective Use "Crop breeding and production technology, storage and processing of food products for functional and therapeutic purposes" of Michurinsk State Agrarian University.

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