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DNA-SCREENING OF STRAWBERRY CULTIVARS AND HYBRIDS (*FRAGARIA ANANASSA* DUCH.) FOR RESISTANCE TO FUNGAL DISEASES

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Abstract

Disease resistance is one of the most important tendencies of breeding programs to improve the assortment of strawberries (*Fragaria ananassa* Duch.) all over the world. A trial was conducted using molecular SCAR markers to make it possible to identify genotypes carrying the genes of resistance to anthracnose black rot and Phytophthora root rot, to involve the selected genotypes in further crosses to obtain cultivars with complex resistance to pathogens. The trial was comprised of 29 cultivars of strawberries (*F. ananassa* Duch.) of various ecological and geographical origins, as well as 3 hybrid families from the VNIISPK collection. As a result of the DNA analysis of strawberry cultivars from the VNIISPK collection, the SCAR marker STS-Rca2_240, closely linked to the *Rca2* gene (determining the resistance of strawberries to anthracnose black rot, to the races of *Colletotrichum acutatum* Simmonds of the 2nd group of pathogenicity) was detected in 3 cultivars of Rosinka, Siria and Malwina and in 136 hybrids from the «Alba × Rosinka» and «Rosinka × Darselect» families. The marker of the dominant allele of the *Rpf1* (determining the resistance of strawberries to blight root rot, to the races of *Phytophthora fragariae* Hickman) SCAR-R1A gene was not found in any of the cultivars selected for analysis. However, the presence of the SCAR-R1A marker was found in the hybrid family «Rannya Plotnaya × Govorovskaya», which indicated its presence in one of the parents, DNA analysis of the parent cultivars was not carried out. The selected cultivars and hybrids with genetically determined resistance to anthracnose black rot (*Rca2*) and phytophthora root rot (*Rpf1*) are valuable and prospective for involvement in breeding for complex resistance to fungal diseases.

Key words: Strawberry, Anthracnose black rot; Phytophthora root rot; DNA-markers; Genes *Rca2*; *Rpf1*.

Introduction

Strawberry (*Fragaria ananassa*) with a chromosomal configuration of 2n = 8x = 56 represents one of the most significant and economically lucrative berry crops globally. Its yield, however, faces detrimental impacts from various infectious diseases, with fungal diseases taking precedence as the foremost threat. Consequently, the selection of genotypes exhibiting comprehensive resistance to these pathogens stands as a paramount objective within strawberry genetic enhancement programs worldwide. One effective approach facilitating such selection involves the identification of resistance-determining genes and the subsequent utilization of closely linked DNA markers. The application of DNA-based methodologies serves to expedite and streamline the identification of stable genotypes and to enhance the efficiency of the breeding process. Among the spectrum of strawberry diseases, strawberry anthracnose (*Colletotrichum acutatum* Simmonds) emerges as a relatively recent but already widespread global menace. This disease can lead to yield losses of up to 80%, with the potential for a loss exceeding 33% in mother plantings (Govorova & Govorov, 2015). Notably, anthracnose has held the status of a quarantine disease within the Eurasian Economic Community since 2016 (Tsvetkova & Kuznetsova, 2020).

Currently, a significant number of *C. acutatum* isolates, which belong to two pathogenicity groups, have been identified (Denoyes & Baudry, 1995). Resistance to *C. acutatum* of the first group of pathogenicity is polygenic, while resistance to *C. acutatum* Simmonds of the second group of pathogenicity is monogenic, controlled by the dominant *Rca2* gene (Demoyes-Rothanet *et al*., 2004; Lerceteau-Köhler *et al*., 2003). Data have been published on population mapping from the crossing of the resistant Capitola cultivar and the susceptible Pajaro using AFLP markers. According to the results, 4 markers were linked to the *Rca2* gene, and two of them were converted into SCAR markers (STS-Rca2_240 and STS-Rca2_417). The STS-Rca2_240 marker is localized at a distance of 2.8 cM from the *Rca2* gene, the STS-Rca2_417 marker is at 0.6 cM (Lerceteau-Kohler *et al*., 2005). These markers are currently used to assess the allelic state of *Rca2* (Luk'yanchuk *et al*., 2018; Lyzhin *et al*., 2019a,b; Lyzhin & Luk'yanchuk, 2021; Lyzhin & Luk'yanchuk, 2022a,b; Bezlepkina *et al*., 2022).

The Phytophthora (late blight) of strawberries is caused by two species of pseudo fungi of the *Phytophthora* genus. The first one is *Phytophthora fragariae* Hickman which causes late blight root rot or so–called "redness of the central cylinder of the root". The second one is *Phytophthora cactorum* Lebert & Cohn which causes the disease of phytophthora coriaceous rot (Govorova & Govorov, 2015). In European breeding programs, resistance to root rot is mainly determined by the presence of the *Rpf1, Rpf2,* and *Rpf3* genes. *Rpf1* controls resistance to at least 16 *P. fragariae* races (Nickerson & Murray, 1993; Sasnauskas *et al*., 2007). One of the DNA markers, that was used to assess the allelic state of the *Rpf1* gene, was SCAR-R1A (corresponded to the dominant allele of the gene) (Haymes *et al*., 1997; 2000). Domestic researchers also used the SCAR-R1A marker to determine the dominant allele of the *Rpf1* gene (Lyzhin *et al*., 2019a; Lyzhin & Luk'yanchuk, 2020; Bezlepkina *et al*., 2022).

Other genes conferring resistance to root rot in strawberries have received limited research attention. Notably, *Rpf2* is known to be present in cultivars such as "Climax," "Redgauntlet," "Siletz," and "Sparkle" (van de Weg, 1997). However, to the best of our knowledge, it has not been comprehensively mapped to date. *Rpf3* and *Rpf6*, found in the resistant cultivar "Yalova4," were successfully mapped using AFLP (Amplified Fragment Length Polymorphism) analysis, with their respective associations to markers E26M59H and E39M51B being established (Haymes et al., 1998; Hokanson & Maas, 2010). Nevertheless, the translation of these polymorphic AFLP fragments into practical molecular screening markers has not been pursued thus far (Khrabrov, 2019).

In the pursuit of enhancing strawberry (*Fragaria ananassa* Duch.) breeding programs, this study addresses a critical knowledge gap by focusing on the DNA-screening of strawberry cultivars and hybrids for resistance to fungal diseases. The novelty of this research lies in its comprehensive exploration of previously under-studied promising cultivars and hybrid forms for resistance genes, such as *Rca2* and *Rpf1*, as well as in the isolation of rare and very important genotypes for breeding with resistance to the most harmful fungal diseases. Our hypothesis posits that the genetic characterization along with identification of these resistance genes, will significantly expedite the selection of disease-resistant genotypes, ultimately contributing to the development of more vital and productive strawberry cultivars, which is important for sustainable strawberry production and reducing the economic costs of its cultivation worldwide.

Method and Materials

Twenty-nine strawberry (*F. ananassa* Duch.) cultivars of various ecological and geographical origin and 3 hybrid families from the VNIISPK collection were selected as objects of the study (Table 1).

Table 1. Strawberry cultivars (*F. ananassa* Duch.) involved in the analysis.

Name of the cultivar	Country of origin	Parental plants
Elianny(k)	Netherlands	-
Bereginya	Russia	Solovushka x Induka
Kokinskaya Rannya	Russia	Catskill x Rannya Makheraukha
Nelli	Russia	Elsanta x Marmolada
Rosinka	Russia	(Kokinskaya Rannya x Surpriz Olimpiade) x Vityaz
Studencheskaya	Russia	FB3 (*F.* × *ananassa* Duch. × *F. moschata* Duch.)
Tsaritsa	Russia	Venta x Redgauntlet
Alba	Italy	Albion x Cal.97.85.6
Asia	Italy	-
Civ 64	Italy	-
Clery	Italy	Sweet Charlie x Onebor
Darselect	France	Parker x Elsanta
Dezy	France	-
Frida	Norway	-
Gala Civ	Italy	-
Honeoye	USA	Vibrant x Holiday
Irma	Italy	Marmolala x (Eddy x Don).
Jive	Holland	-
Jonsok	Norway	SengaSengana x Valentine
Malling Centenary	United Kingdom	-
Malling Pandora	United Kingdom	-
Marmolada	Italy	Gorella x Nr 15
Malwina	Germany	-
Onda	Italy	By selecting seedlings of Marmolala
Rubino Civ	Italy	-
Rumba	Netherlands	-
Sara	Norway	-
Siria	Italy	-
Vima Kimberly	Netherlands	Chandler x Gorella
Vivaldi	Netherlands	-

Studied hybrid families of strawberries:
1. Alba × Rosinka-124 hybrids; 2. Rosinka × Darselect-139 hybrids; 3. Rannya Plotnaya × Govorovskaya-58 hybrids

DNA isolation and amplification: Genomic DNA for analysis was isolated from young strawberry leaves by CTAB (Doyle & Doyle, 1990) with Porebski modifications (Porebsky *et al*., 1997) to isolate DNA from plant tissue with a high content of polysaccharides and phenolics.

Amplification was carried out in a 25 ml reaction mixture containing a 1x PCR buffer solution, 100 µl of nucleotides, 0.1 µl of direct primer, 0.1 µl of reverse primer, and 1 unit. Taq DNA polymerase (a set of reagents for PCR with Taq polymerase HotStart produced by Dia-m) and 100 ng of DNA in a T100 amplifier (BioRad). Amplification protocol for STS-Rca2_240 marker and SSR marker for DNA isolation control EMFv020: predenaturation at 95°C for 3 min followed by 35 cycles of 95°C for 50 sec, 64°C–50 sec, 72°C for 1 min and post extension at 72°C for 5 min. Amplification protocol for SCAR-R1A marker: predenaturation: 94°C–3 min followed by 25 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 1 min and a 7 min post extension at 72°C.

Sequences of primers:

1. STS-Rca2_240 For: 5′-GCC ACG TCA CTA GTC AAA TTC AA-3′; STS-Rca2_240 Rev: 5′-TCA TGG ACA GTG GTC TCA GC-3′
2. SCAR-R1A For: 5′-TGC ATC ATT AAT GTA GAA GTC TTT-3′ SCAR-R1A Rev: 5′-TGA TGC GAC ATA CAA AAA TAT TAG-3′
3. EMFv020 For: 5′-CAG GCG CCA ACG GCG TGC TCT TGT-3′ EMFv020 Rev: 5′- CAG CGC CGC CAG CTC ATC CCT AGG-3′

The separation of the amplification products was carried out in 1.0% agarose gel in a 1×TBE buffer. The size of the resulting PCR products was determined visually by comparing it with the Step50Long DNA molecular weight marker (Biolabmix).

Results

To control the quality of the isolated DNA, all samples were tested by PCR with primers of the SSR marker EMFv020 (Hadonou *et al*., 2004), used as a positive PCR control (Lerceteau-Köhler *et al*., 2005, Luk'yanchuk *et al*., 2018).

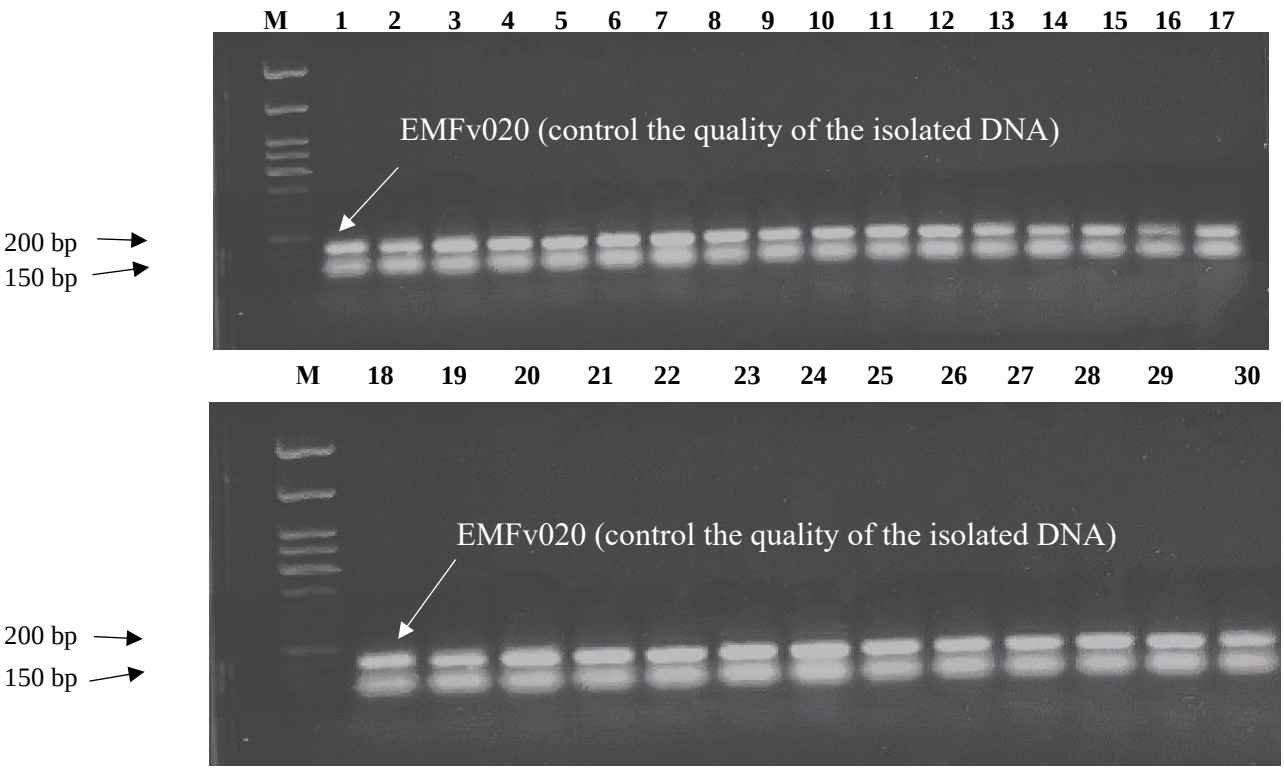
The expected size of the product during the amplification with primers EMFv020 was about 170 bp, the results of the electrophoresis are shown in Fig. 1.

In all analyzed samples, the target product of the marker EMFv020≈170 bp was identified in the genotype, which indicated the suitability of DNA for further analysis.

When screening for the presence of the marker STS-Rca2_240 (target product 240 bp) in the selected genotypes, the Elianny cultivar was selected as a positive control based on literature data. (Lyzhin *et al*., 2019a) (Fig. 2).

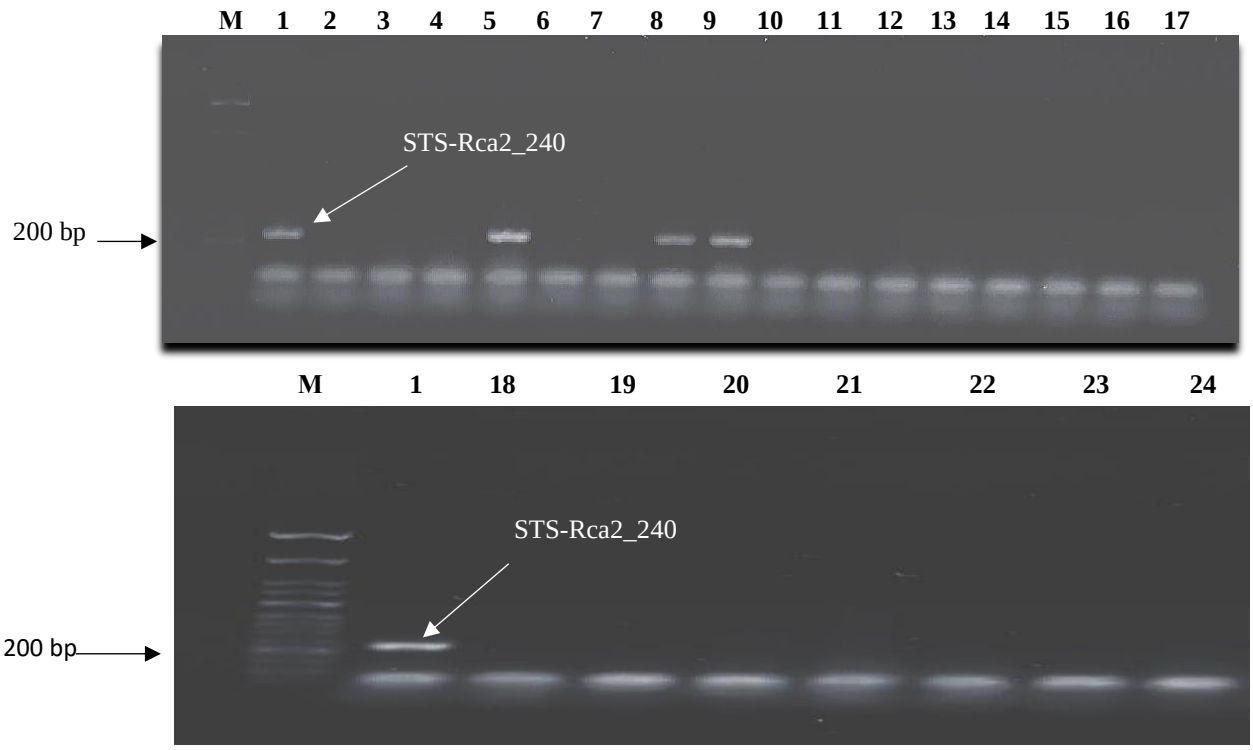
SCAR marker STS-Rca2_240, associated with the resistance to anthracnose rot (to *C. acutatum Simmonds* races of the 2nd group of pathogeneicity), was detected in Rosinka, Siria and Malwina.

Studencheskaya, Vima Kimberly, Tsaritsa, Kokinskaya Rannya, Honeyoye and Malling Pandora were analyzed earlier (Lyzhin & Luk'yanchuk, 2019; 2022a; Khrabrov *et al*., 2021), therefore, these genotypes were excluded from the analysis, the marker STS-Rca2_240 was present only in Malling Pandora. The data of the screening for the presence of the marker STS-Rca2_240 in strawberry cultivars are presented in (Table 2).



M–DNA molecular weight marker Step50Long (Biolabmix), 1 – Elianny(k), 2 – Frida, 3 – Clery, 4 –Rubino Civ, 5 – Rosinka, 6 – Gala Civ, 7 –Darselect, 8 –Siria, 9 –Malwina, 10 – Malling Pandora, 11 –Alba, 12 – Civ 64, 13 –Irma, 14 – Studencheskaya, 15 – Dezy, 16 – Vivaldi, 17 –Onda, 18 – Bereginya, 19 –Kokinskaya Rannya, 20 – Nelli, 21 – Asia, 22 – Tsaritsa, 23 – Honeoye, 24 –Vima Kimberly, 25 –Rumba, 26 – Sara, 27 – Malling Centenary, 28– Marmolada, 29 – Jive, 30 – Jonsok.

Fig. 1. Electrophoretic profile of the EMFv020 marker of strawberry cultivars.



M – DNA molecular weight marker Step50Long (Biolabmix), 1 – Elianny(к), 2 – Frida, 3 – Clery, 4 – Rubino Civ, 5 – Rosinka, 6 – Gala Civ, 7 – Darselect, 8 – Siria, 9 – Malwina, 10 – Nelli, 11 – Alba, 12 – Civ 64, 13 – Irma, 14 – Jonsok, 15 – Dezy, 16 – Vivaldi, 17 – Onda. 18– Bereginya, 19 – Malling Centenary, 20 – Asia, 21– Rumba, 22 – Sara, 23 – Marmolada, 24 –Jive.

Fig. 2. Electrophoretic profile of the marker STS-Rca2 240 in strawberry cultivars.

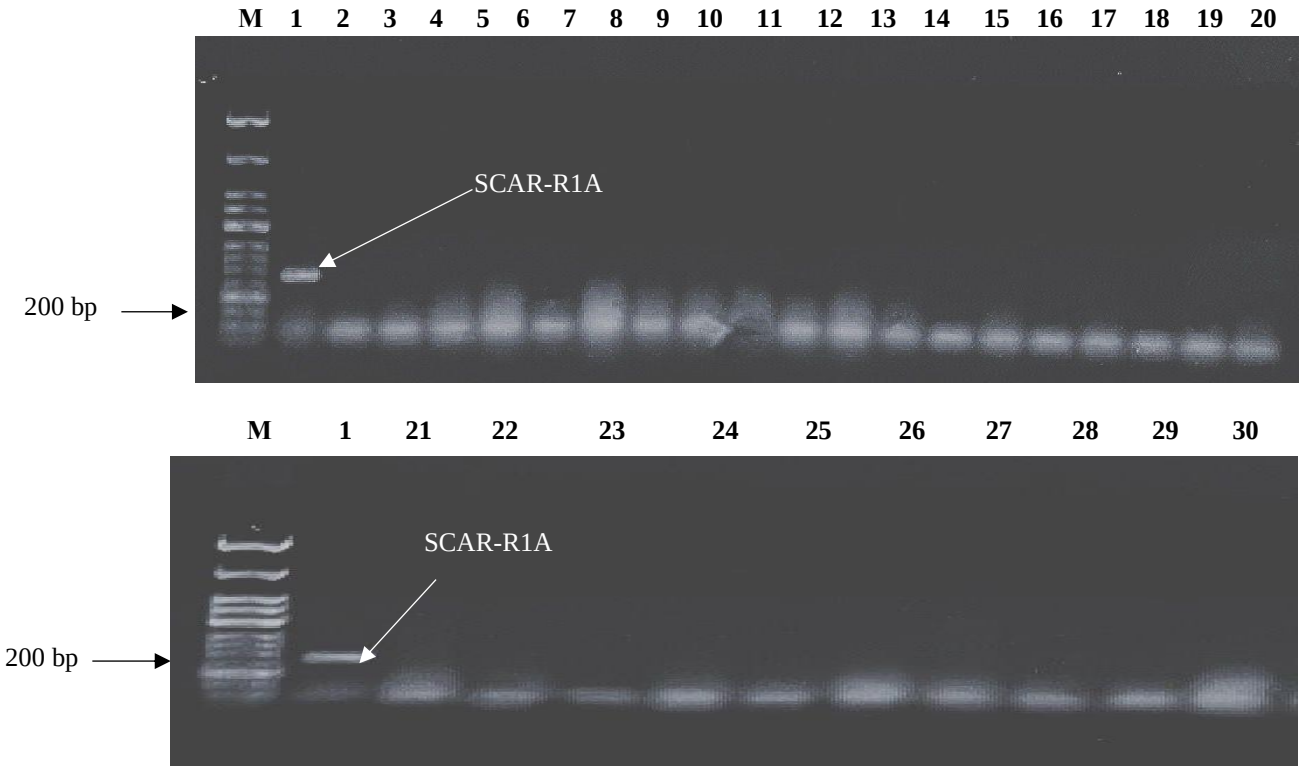
Table 2. Results of strawberry cultivars screening by marker STS-Rca2_240.

No.	Cultivar, genotype	Presence of STS-Rca2_240 marker
1.	Elianny(к)	+
2.	Frida	–
3.	Clery	–
4.	Rubino Civ	–
5.	Rosinka	+
6.	Gala Civ	–
7.	Darselect	–
8.	Siria	+
9.	Malwina	+
10.	Civ 64	–
11.	Irma	–
12.	Dezy	–
13.	Vivaldi	–
14.	Marmolada	–
15.	Sara	–
16.	Asia	–
17.	Onda	–
18.	Nelli	–
19.	Malling Centenary	–
20.	Rumba	–
21.	Jive	–
22.	Alba	–
23.	Jonsok	–
24.	Bereginya	–

Note: The symbols indicate the presence (+) or absence (–) of the STS-Rca2_240 marker

The analysis of 2 hybrid families was carried out, one of the parents of which was Rosinka, in which, as a result of the analysis, the presence of the DNA marker STS-Rca2_240 was revealed. The parent cultivars Alba and Darselect had no marker STS-Rca2_240. The DNA marker STS-Rca2_240 was identified in 97 hybrids out of 124 in the "Alba × Rosinka" family, and in 39 samples out of 139 in the "Rosinka × Darselect" family. The families "Alba × Rosinka" and "Rosinka × Darselect" were analyzed only for the presence of the marker STS-Rca2_240, since the parents had no marker SCAR-R1A.

A DNA analysis of the strawberry family was also carried out, in which the cultivars of Govorova G.F. breeding for immunity to fungal diseases were used as parents (Rannya Plotnaya and Govorovskaya). These parent cultivars are not currently in the collection, so they were not involved in our analysis of the cultivars, but they can potentially be complex donors of resistance to fungal diseases. The family "Rannya Plotnaya × Govorovskaya" was analyzed for the presence of both STS-Rca2_240 and SCAR-R1A markers. According to the screening results, the marker STS-Rca2_240 was absent in all 58 hybrids of the family, which indicated the absence of this marker in the parent cultivars as well. The marker SCAR-R1A (target product 285 bp) was identified in the family "Rannya Plotnaya x Govorovskaya" in 4 hybrids: 11(12), 11(47), 11(48), 11(57). This suggests the presence of this marker in Rannya Plotnaya or Govorovskaya. Additional analysis of parent cultivars is required. The DNA of the isolated genotype 11(12) was used as a positive control during screening of 29 strawberry cultivars from the VNIISPK collection (Fig. 3).



M – DNA molecular weight marker Step50Long (Biolabmix), 1 – 11(12) (κ), 2 – Frida, 3 – Clery, 4 – Rubino Civ, 5 Rosinka, 6 – Gala Civ, 7 – Darselect, 8 – Siria, 9 – Malwina, 10 – Malling Pandora, 11 –Alba, 12 – Civ 64, 13 – Irma, 14 – Studencheskaya, 15 – Dezy, 16 – Vivaldi, 17 – Onda,18– Nelli, 19 – Malling Centenary, 20 – Rumba, 21 – Jive, 22 – Asia, 23– Honeyoye, 24 – Sara, 25 – Marmolada, 26 – Jonsok, 27 – Vima Kimberly, 28 – Tsaritsa, 29 – Kokinskaya Rannya, 30 – Bereginya.

Fig. 3. Electrophoretic profile of the marker SCAR-R1A in strawberry cultivars.

The SCAR-R1A marker associated with resistance to late blight root rot was not found in any of the analyzed strawberry cultivars from the VNIISPK collection.

As a result of the analysis, Rosinka, Siria, Malwina were selected, as well as 136 hybrids from the families "Alba × Rosinka " and " Rosinka × Darselect" having the marker STS-Rca2_240. These genotypes are promising for breeding for resistance to anthracnose (to *C. acutatum* Simmonds races of the 2nd group of pathogenicity).

In 4 hybrids, 11(12), 11(47), 11(48) and 11(57) from the family "Rannya Plotnaya × Govorovskaya", the presence of the SCAR-R1A marker in the genome was found. These genotypes are perspectives for breeding for resistance to phytophthora root rot.

Discussion

The integration of molecular markers into breeding programs offers the advantage of swiftly and precisely identifying genetic determinants. This enables the selection of stable genotypes with promising traits as potential breeding parents, potentially expediting breeding timelines by one to two years. The STS-Rca2_240 marker is widely adopted for the identification of genotypes harboring the anthracnose black rot (Rca2) resistance gene in various strawberry collections, both internationally and among Russian researchers. In a study by Lerceteau-Köhler and colleagues (Lerceteau-Köhler *et al*., 2005), a diagnostic fragment of the STS-Rca2_240 marker was detected in 13 out of 43 examined cultivars. Additionally, in research conducted by Njuguna (2010), among 112 analyzed cultivars, the marker was found in 36 cultivars. Furthermore, A. S. Lyzhin and co-authors conducted an assessment of the allelic status of the *Rca2* anthracnose resistance gene in 60 Russian and foreign cultivars. Through PCR analysis, the STS-Rca2_240 marker was identified in several foreign cultivars, including Elianny, Troubadour, Laetitia, Roxana, Albion, Selva, as well as in two domestic cultivars Sudarushka and Borovitskaya (Luk'yanchuk *et al*., 2018; Lyzhin *et al*., 2019b; 2022a,b). During the screening of the VIR strawberry collection maintained at the Maikop Experimental Station, a diagnostic fragment of 240 bp of the Rca2_240 marker was detected in 22 cultivars out of 83 domestic and 52 foreign cultivars. Of the 17 cultivars of the Maikop breeding, the marker was identified in Maikopskaya Rannya, Peryt, and Shapsugskaya (Khrabrov *et al.*, 2021).

The SCAR-R1 marker is a critical tool utilized for the identification of genotypes harboring the gene responsible for resistance to late blight root rot, designated as *Rpf1*. As reported by Njuguna (2010), a diagnostic fragment of 285 base pairs was detected exclusively in a mere 22 out of the 158 examined cultivars. Similarly, among the 133 cultivars subjected to analysis by K.M. Haymes and colleagues (Haymes *et al*., 2000), the presence of the SCAR-R1A marker was confirmed solely in 24 samples. Domestically, researchers have also employed the SCAR-R1A marker for the determination of the dominant allele of the *Rpf1* gene.

Notably, in an investigation conducted by Lyzhin and co-authors (Lyzhin *et al*., 2019a; Lyzhin & Luk'yanchuk, 2020), encompassing the scrutiny of 48 strawberry genotypes originating from diverse ecological and geographical backgrounds, the diagnostic marker fragment was identified in only one of the examined samples, specifically, the Bylinnaya cultivar. These findings collectively underscore the infrequent occurrence of the markers STS-Rca2_240 and SCAR-R1A. The selected cultivars and hybrids, which exhibit genetically determined resistance to anthracnose black rot (*Rca2*) and phytophthora root rot (*Rpf1*), hold substantial promise and value for inclusion in breeding programs aimed at augmenting resistance to fungal diseases in the field of strawberry cultivation.

Conclusion

In conclusion, our DNA analysis of strawberries from the VNIISPK collection revealed the presence of the SCAR marker STS-Rca2_240, closely associated with the *Rca2* gene responsible for conferring resistance to anthracnose black rot caused by *C. acutatum* Simmonds, particularly to the 2nd group of pathogenicity races. This marker was identified in the strawberry cultivars Rosinka, Siria, and Malwina, as well as in 136 hybrids derived from the "Alba × Rosinka" and "Rosinka × Darselect" families. However, it is noteworthy that the dominant allele of the *Rpf1* gene, which imparts resistance to late blight root rot caused by *P. fragariae* Hickman, denoted by the SCAR marker SCAR-R1A, was absent in all the analyzed strawberry cultivars. Furthermore, the presence of the SCAR-R1A DNA marker in the hybrid family "Rannya Plotnaya × Govorovskaya," identified in hybrids 11(12), 11(47), 11(48), and 11(57), strongly suggests its presence in one of the parental cultivars. However, it is essential to note that DNA analysis of the parent cultivars has yet to be conducted to confirm this.

These findings hold significant importance in the realm of molecular genetic analysis of the strawberry genome and the breeding of strawberry cultivars with enhanced resistance to fungal diseases. They provide valuable insights for future research and the development of strawberry varieties with comprehensive disease resistance profiles.

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