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Validation of microsatellite markers to identify Pl_6, Pl_8 and Pl_{arg} genes that control resistance to *Plasmopara halstedii* in sunflower

Svetlana Alekseevna Ramazanova*, Evgeny Vitalievich Badyanov, Saida Zaurbievna Guchetl

V.S. Pustovoit All-Russian Research Institute of Oil Crops, 17 Filatova str., Krasnodar, 350038, Russia

** Corresponding author's E-mail: ramazanov1969@mail.ru*

ABSTRACT

Downy mildew caused by the oomycete *Plasmopara halstedii* (Farl.) Berl. et de Toni is one of the most harmful sunflower diseases. Among the various measures to control it, the most economical is the development of resistant genotypes. At present, Pl_6, Pl_8, and Pl_{arg} loci are promising for use in breeding, providing resistance to all known *P. halstedii* races. Microsatellite markers (SSR) help to control the transfer of genes that control resistance in breeding material. However, validation of the marker is needed to prove its reliability in gene detection. There was studied the polymorphism of 9 microsatellite loci in 196 sunflower lines with different resistance to downy mildew. The ORS328 microsatellite locus was chosen as a marker of the Pl_6 gene. Amplified fragment with 271 bp allows identifying genotypes resistant to the race 330. The lines that are the sources of the Pl_8 gene did not differ from the others in the allelic composition of the ORS781 locus. Among the analyzed breeding samples, no polymorphism was revealed at this locus. To identify the Pl_{arg} gene, SSR markers ORS662 and ORS509 were selected. The analysis of 12 samples of the F2 generation from RHA-419 × I_3BC_2 (VK585 × VK195) crossing at these loci showed that both markers are inherited codominantly. The studied DNA markers can be used in marker-assisted selection (MAS) of sunflower for resistance to downy mildew pathogen.

Key words: SSRs, MAS, Plasmopara halstedii, Resistance, Sunflower.
Article type: Research Article.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the second economically important oil crop (after soybean) cultivated in Russia and the first in terms of crop acreage. Over the past ten years, the sunflower crop acreage has increased by 20% and in 2019 reached 8.6 million hectares for the first time (Agriculture in Russia 2019). However, the yield of this crop is greatly affected by many limiting factors, among which various diseases are the most significant. One of the most harmful among them is downy mildew caused by the oomycete *Plasmopara halstedii* (Farl.) Berl. et de Toni. The oospores of the pathogen are keeping the infectious matter in conditions unfavorable for its development. They can remain viable in the soil for 8 years. The infection source is plant residues, fallen seeds, seeds. In spring, oospores emerge and form a mycelium penetrating plants. The mycelium is germinated by zoosporangiophores producing zoosporangia that affect roots and young seedlings, causing severe growth inhibition, leaf discoloration, and a decrease in resistance to other pathogens. Heavy precipitation contributes to the intensive development of the disease. In cold and humid years, yield losses can reach 50-95 % (Molinero-Ruiz *et al.* 2003; Markell *et al.* 2016). The use of various agricultural methods and seed treatment with fungicides were the most common measures to control this disease. However, oomycete has acquired resistance to a wide range of fungicides, which limits the effectiveness of chemicals and raises the issues about the economic viability of their use. Agrotechnical techniques are effective only during the early stages of development and are more of an auxiliary measure, and do not provide 100% guarantee (Albourie & Tourvieille 1998; Gulya *et al.* 1999). There

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are several reports about using microsattelite markers in plant and animal genetics (Shirangi *et al.* 2011; Gharibkhani *et al.* 2014; Norouzi & Samiei 2015). The most economical strategy for pathogen-induced yield loss prevention is to find resistance genes and use them in sunflower breeding. Currently, about 50 of *P. halstedii* pathotypes have been described in the world (Imerovski *et al.* 2014; Solodenko 2018), and 36 main resistance genes have been identified in sunflower, located in five linkage groups (LG 1, 2, 4, 8 and 13) and labelled as *Pl* (Qi *et al.* 2016b). Unfortunately, many of these genes were overcome in the field due to the rapid adaptation of *P. halstedii*, as well as due to the use of individual *Pl* as the only major gene in large areas (Imerovski *et al.* 2014; Qi *et al.* 2017). Therefore, it is necessary to use different sources of resistance genes and to develop varieties with combinations of two or more effective *Pl* genes. The identification and development of highly productive diagnostic DNA markers related to Pl genes will contribute to marker-assisted selection (MAS), especially for selection of entire gene stacks. Currently, the Pl_6, Pl_8, and Pl_{arg} loci are promising for use in breeding, which together provide resistance to all known *P. halstedii* races (Imerovski *et al.* 2014). Therefore, the goals of this work were the search and approbation of molecular markers for the clusters of these genes and the development of a system of markers on their basis with its subsequent use in breeding programs for the development of sunflower lines with complex resistance to different races of downy mildew pathogen.

MATERIALS AND METHODS

196 sunflower samples were used in the study, including both the lines and samples of the breeding of V.S. Pustovoit All-Russian Research Institute of Oil Crops and the sunflower resistance differentiating lines included in the standard international test kit for the identification of *P. halstedii* races (Iwebor *et al.* 2018) and 12 samples of the F2 generation from the cross combination RHA419 × I_3BC_2 (VK585 × VK195), provided by the staff of the laboratory of genetics of V.S. Pustovoit All-Russian Research Institute of Oil Crops. 3-7 day old sunflower seedlings were used for DNA isolation. DNA extraction was carried out by the method based on cell lysis with a buffer containing CTAB (cetyltrimethylammonium bromide), deproteinization with chloroform, and DNA precipitation with isopropanol (Saghai-Maroof *et al.* 1984). The concentration of DNA in the obtained preparations was determined visually by the intensity of the sample luminescence of 10 μl in ultraviolet light and in 1% agarose gel with the addition of 2 μl of ethidium bromide. The electrophoresis of DNA preparations was carried out at a voltage of 100 V for 60 min. For PCR analysis, there were used nine pairs of primers developed for marking the Pl_6, Pl_8, and Pl_{arg} loci (Table 1; Solodenko 2018; Şahin *et al.* 2018). The polymerase chain reaction was carried out in the reaction mixture (25 μl) according to the standard protocol. The reactions were carried out in an S1000TM thermal cycler (BioRad, USA). Temperature-time regimes were described earlier (Ramazanova *et al.* 2020). The electrophoresis of amplification products was performed in 8% polyacrylamide gel (PAAG) in the vertical electrophoresis chamber VE-20 (Helikon, Russia). The BIO-PRINT digital video documentation system (Vilber Lourmat, France) was used to visualize and document the results of electrophoresis.

Table 1. Nucleotide sequences of the primers used to identify Pl_6, Pl_8 and Pl_{arg} loci.

Gene	Name	Nucleotide sequence	Product size (bp)	Sequence identifier in NCBI
Pl_{arg}	ORS662	CGGGTTGGATATGGAGTCAA CCTTTACAAACGAAGCACAATTC	314	BV006121
	ORS371	GGTGCCTTCTCTTCCTTGTG CACACCACCAAACATCAACC	254	BV006649
	ORS509	CAACGAAAAGACAGAATCGAAA CCGGGAATTTTACAAGGTGA	207	BV006029
	ORS610	AGGAAGCGAAACGAGGAAGT TTGTGACCTTCTCCCTGCTC	144	BV006705
	ORS605	CGCGTGATGTGACGATTATT ACGGAGCAAAGTTTCGAGGT	196	BV006087
	ORS1182	TCTTCTGATTGTAAGCGGTGTTC TGTCATGTTCTCTACCGAGCTTT	174	BV006542
	ORS716	GAACTAACCGCCATCCAAGA CCCCACAACCCATAGCCTAA	395	BV006728
Pl_6	ORS328	GACCTGTAGGCCAATATGAGACTT TTATACCGGTGTTGTATCGTATCC	271	BV005925
Pl_8	ORS781	GTCAACCCATGACCCAAACC GATGTGGAGGAGAGAGGGTGT	410	BV006197

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RESULTS AND DISCUSSION

Nine molecular markers of genes that control sunflower resistance to downy mildew were examined in the present study. The work on gene labeling was carried out on the lines and breeding samples of V.S. Pustovoit All-Russian Research Institute of Oil Crops, which contrastingly differ in resistance to the pathogen of sunflower downy mildew. To mark the *Pl*$_6$ locus, three microsatellite markers ORS1043, ORS37, and ORS166 were studied in the previous studies, mapped in the linkage group LG8 (Ramazanova *et al.* 2020). However, no polymorphic fractions were found between resistant and susceptible sunflower samples, just as the HA-335 line, into which this gene was introgressed from the wild species *H. annuus*, did not differ from the others. A similar study with the same primers was carried out by Solodenko (2018), and it did not obtain DNA fragments characteristic of the HA-335 line (Solodenko 2018).

To mark the *Pl*$_6$ locus, there was used SSR primer ORS328, mapped in the same linkage group. In this study, we studied the molecular genetic polymorphism of this locus in 196 sunflower lines and breeding samples of V.S. Pustovoit All-Russian Research Institute of Oil Crops. Figure 1 shows an electrophoregram of the DNA amplification products of breeding samples and lines with different resistance to *P. halstedii* with this primer. Figure 1 (A) shows that all 12 samples of the HA-335 line had a 271 bp fragment. Validation of this marker on 77 breeding samples with different resistances suggests that it is suitable for marking the *Pl*$_6$ locus. For example, Figure 1 (B, C) shows the results of PCR analysis. The samples on the tracks 10, 11, 13 (Fig. 1B) and 3, 4, 12 (Fig. 1C), demonstrate the fragment with the length of 271 bp, the same as in the HA-335 line used as an internal control on the tracks 1 (Fig. 1B and C).

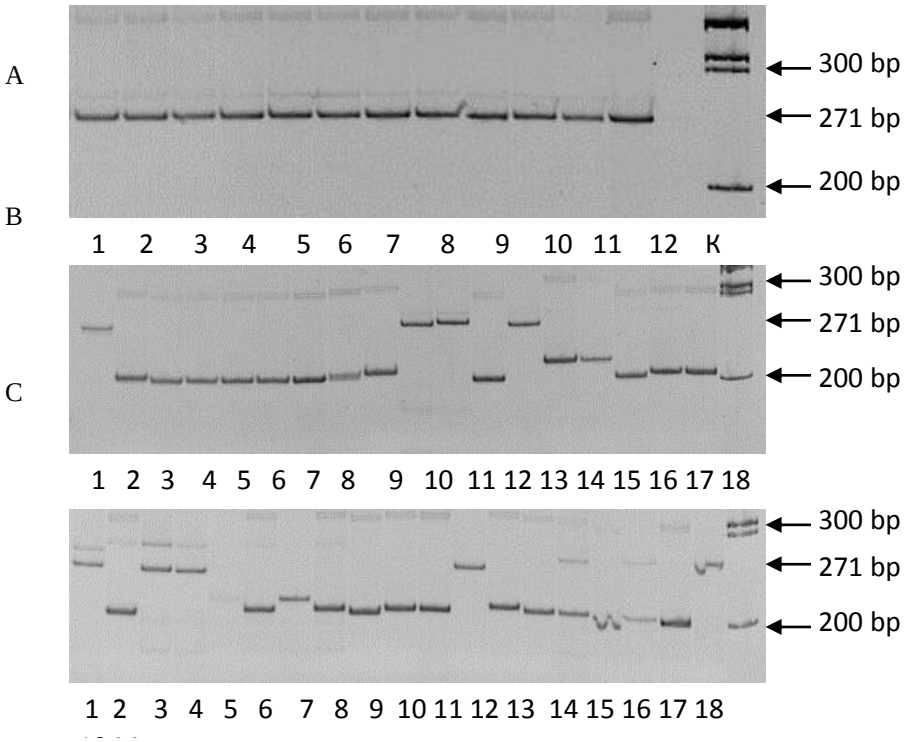

Fig. 1. Electropherogram of DNA amplification products of sunflower lines with the primer ORS328. (A) Tracks: the line 1–12 HA-335; (B) tracks: 1- the line HA-335, 2-18 breeding samples; (C) tracks: 1 - the line HA-335, the breeding samples 2-19; K - negative control; M - molecular weight marker 100 bp. The arrows indicate DNA marker fragments (orig.).

Earlier phytopathological evaluation has shown that these specimens are resistant to the race 330 and susceptible to the races 730, 710 and 334. The work on the search for molecular markers of the *Pl*$_8$ gene is continued. The ORS781 microsatellite locus was used for its identification. It is known from the literature that the amplified 410 bp fragment distinguishes the lines 803-1 and QHP-1, which are the carriers of the *Pl*$_8$ and *Pl*$_5$ genes, from all other lines - resistance differentiators (Solodenko 2018).

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However, in our study, these lines did not differ in the allelic composition of the ORS781 locus, and no polymorphism was revealed at this locus among the analyzed breeding samples. These observations additionally stress the need for careful validation of known markers on different collections of sunflower genotypes.

Also, the work continues on the marking of the Pl_{arg} gene, which is introgressed into the cultivated sunflower from the wild specimen of *H. argophyllus* 1575 and is still very effective against all known *P. halstedii* races for two decades (Vear *et al.* 2008a). Seven known microsatellite loci were used for its labeling (Table 1). After the analysis of DNA lines and breeding samples of sunflower, no polymorphism was detected at five loci: ORS371, ORS716, ORS1182, ORS605, and ORS610. Polymorphic DNA fragments were detected only at two loci ORS509 and ORS662. There were identified only two polymorphic DNA fragments at ORS662 locus with the length of approximately 314 and 245 base pairs. The RHA-419 line containing the Pl_{arg} gene was characterized by a 314 bp fragment. However, this fragment was also found in resistance differentiator lines PSC8, HA-335, 803-1, PM-17, DM-2, and RHA-265, as well as in some breeding samples. This DNA fragment is equally represented in genotypes with different resistance to downy mildew and does not allow to differentiate them reliably.

The ORS509 microsatellite locus was previously used to mark the Pl_{arg} gene and was identified as promising for this gene marking. Testing showed that a 207 bp fragment, characteristic of the RHA-419 line, was not detected in the studied samples of sunflower breeding by V.S. Pustovoit All-Russian Research Institute of Oil Crops. In the present study, its study was continued as the marker of the Pl_{arg} gene. The number of analyzed samples was increased, but the fragment 207 bp has not been identified in lines and breeding samples. Besides, there were analyzed 12 samples of the F_2 generation from RHA-419 × I_3BC_2 (VK585 × VK195) crossing at the ORS509 and ORS662 loci. As can be seen from Figure 2, there are three types of samples even in such a small sample. These are the plants carrying only one parental allele, maternal or paternal, and the alleles of both parents (heterozygous).

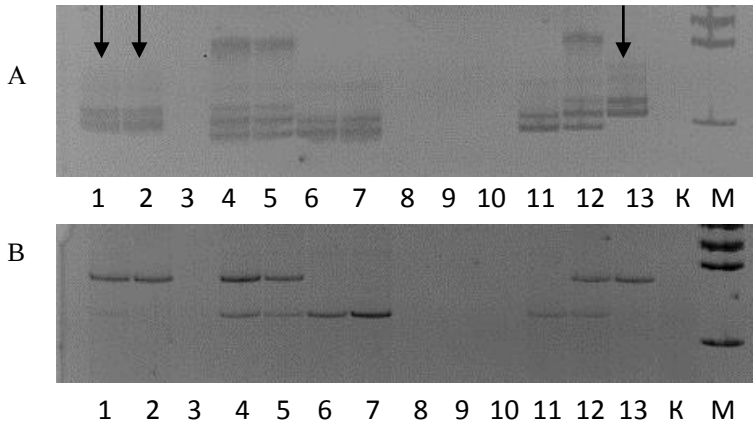

Fig. 2. Electropherogram of the sunflower DNA sample amplification products of the F2 generation from crossing RHA-419 × I_3BC_2 (VK585xVK195), (A) at the ORS509 locus, (B) at the ORS662 locus. Tracks: 1 - RHA-419; 2-13 - the samples of sunflower from the collection of V.S. Pustovoit All-Russian Research Institute of Oil Crops; K - negative control; M - molecular weight marker of 100 bp. The arrows indicate the DNA fragments of 207 bp. (orig.).

The fragments with the length of 207 bp were detected in the samples on the tracks 2 and 13, as in the parental line RHA-419 (Fig. 2A, track 1). The samples in tracks 6, 7 and 11 showed the fragments inherent in the second parent, and heterozygous samples with DNA fragments from both parents were found in the tracks 4, 5 and 12. The same result was obtained for two primers. Therefore, we assume that ORS662 can be used to identify the Pl_{arg} gene in combination with other markers.

CONCLUSION

Specific alleles of SSR markers were revealed in sunflower lines, which are resistance donors of Pl_6 and Pl_{arg} genes. Validation of these molecular markers on the collection of lines and breeding samples of V.S. Pustovoit All-Russian Research Institute of Oil Crops showed that three of them ORS328 (locus Pl_6), ORS509, and ORS662 (the locus Pl_{arg}) made it possible to identify these genes.

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The studied DNA markers may be of particular interest in marker-assisted selection of sunflower for resistance to the downy mildew pathogen.

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