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Original article

DNA Marker for the *HaOr7* Gene Conferring Resistance to the G_{RU} race of Broomrape (*Orobanche Cumana* Wallr.) in Sunflower.

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Abstract

Sunflower broomrape (Orobanche cumana Wallr.) is an obligate parasite known for significantly reducing sunflower yield. The most widespread and virulent race of broomrape in the Russian Federation is the G race. The most ecologically and economically effective strategy for its control involves breeding genetically resistant sunflower varieties and hybrids. The use of DNA markers in the selection of resistant plants significantly enhances the efficiency of the breeding process. Two genetic markers, RORS1 for the HaOr7 gene, and the wild-type allele marker, SORS9, were designed to detect the presence or absence of a specific DNA fragment. A codominant marker system for multiplex PCR was developed from these markers. An analysis of 57 plants derived from the progeny of two heterozygous F1 hybrids revealed that the frequencies of phenotypic segregation aligned fully with the marker system, following the 3:1 model. All resistant plants exhibited amplification of the RORS1 marker, indicating the presence of the HaOr7 gene, while susceptible plants lacked this marker. Association analysis was conducted using the marker system, screening 90 susceptible and resistant sunflower lines and hybrids of different origins. Susceptible lines and hybrids were characterized by the amplification of the SORS9 marker only, whereas resistant lines exhibited amplification of the RORS1 marker only. Additionally, screening of resistant hybrids from eight different breeding companies cultivated in the Russian Federation revealed that all hybrids resistant to G and G+ races of broomrape carried the HaOr7 gene in a heterozygous state. This novel marker system provides a critical tool for breeders, enabling faster and more accurate selection of resistant sunflower genotypes.

Keywords: DNA marker, broomrape resistance, marker-assisted selection.

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INTRODUCTION

One of the biotic factors that limit the yield of sunflower (*Helianthus annuus L.*) is the obligate parasite, sunflower broomrape (*Orobanche cumana Wallr.*). Growing sunflower in fields with heavy infestations can result in complete yield loss. The use of resistant sunflower hybrids is an environmentally friendly and effective method of controlling this parasite, a practice that has been widely employed in sunflower breeding from the beginning of sunflower breeding in the USSR to the present day. Broomrape is found in various sunflower-growing regions worldwide, including France, Spain, China, the Black Sea region, and parts of Africa (Fernández-Martínez et al., 2015). Broomrape populations are classified into races based on their virulence, defined as the ability to overcome key resistance genes. Currently, eight known races (A through H) have been identified globally. Antonova et al. (2022) studied the race composition of broomrape in 25 fields across six regions of the Russian Federation (Samara, Orenburg, Voronezh, Belgorod, Stavropol, and Krasnodar regions) and found that the G race prevails in most fields, while in some fields, the less virulent E and F races are present.

The current method of selecting sunflower genotypes for resistance to broomrape relies solely on phenotypic evaluation on an infectious background (Antonova et al., 2015). However, this process is labor-intensive and lacks the throughput required for large-scale breeding programs. The use of molecular markers facilitates efficient selection of genotypes carrying resistance genes during introgression into other breeding materials without the need for mass phytopathological analysis. Marker systems for accelerated sunflower breeding are widely used in many countries to streamline the selection process and improve breeding efficiency (Davar et al., 2010; Dimitrijević et al., 2017, 2018; Rauf et al., 2020).

In efforts to identify molecular markers for sunflower resistance to broomrape, several studies have aimed to locate sunflower resistance genes to different races of broomrape. For example, Lu et al. (2000) and Tang et al. (2003) mapped the race-specific gene Or_5, which confers resistance to race E, to the telomeric region of chromosome 3, 7.5 cM downstream of the ORS1036 marker. Imerovski et al. (2016) located the resistance gene to races above F, $or_{ab\text{-}vl\text{-}8}$, on the same chromosome. Despite their close proximity, the $or_{ab\text{-}vl\text{-}8}$ and Or_5 genes are distinct. Additionally, Imerovski et al. (2019) mapped 23 significant quantitative trait loci (QTL) for resistance in the sunflower genome, with major QTL *or3.1* and *or3.2* located on chromosome 3. The only broomrape resistance gene that has been cloned and sequenced to date, *HaOr7*, which confers resistance to race F_{GV}, is located on chromosome 7 (Duriez et al., 2019). Some reports suggest that this gene may also confer resistance to races higher than F (Martín-Sanz et al., 2020). Another resistance gene to race G, Or_{Deb2}, introgressed into cultivated sunflower from *H. debilis* subsp. *tardiflorus*, is located in the upper half of chromosome 4 between the proprietary SNP markers DHAI000240 and DHAI007796 (Velasco et al., 2012; Martín-Sanz et al., 2016; Gao et al., 2018; Fernández-Aparicio, 2022). It has also been reported that post-

haustorial resistance to races F and G of *O. cumana*, termed "system II resistance," is controlled by a single partially dominant Or_{SII} gene (Hassan et al., 2008; Martín-Sanz et al., 2020).

Despite these advancements, the location of resistance genes to broomrape races in sunflower cultivated in the Russian Federation remain unknown. Previous research by the authors excluded the location of the gene conferring resistance to race G of sunflower broomrape in resistant VNIIMK lines in close proximity to the $or_{ab\text{-}vl\text{-}8}$ gene on chromosome 3 (Guchetl et al., 2021). As the G_{RU} race of broomrape predominates in sunflower fields in the Russian Federation, it is relevant to search for DNA markers associated with sunflower resistance to this race for marker-assisted selection. Thus, the aim of this study is to develop DNA markers for the *HaOr7* gene and investigate their association with sunflower resistance to the G_{RU} race of broomrape.

MATERIALS AND METHODS

The research material included 63 resistant and susceptible lines and hybrids from the breeding program at VNIIMK, along with 2 differential lines for race F of broomrape – LC1093 and P96. Additionally, 25 resistant and susceptible commercial hybrids from 7 other breeding companies were studied (Fig. 1). Furthermore, an additional 57 sunflower plants of the F_2 generation were obtained by the genetic laboratory at VNIIMK from the progeny of two self-pollinated F_1 hybrids. Among these, 37 F_2 plants were obtained through self-pollination of a plant from the commercial hybrid SY Chester (Syngenta, Switzerland), and 20 plants from the F_1 cross combination VK680 × RG, where VK680 is a susceptible breeding line of VNIIMK, and RG is a donor line resistant to race G_{RU} of broomrape

Phytopathological evaluation of F_2 plants was conducted according Panchenko's method (1974). Broomrape seeds were collected from the fields of Bokovsky and Morozovsky districts in the Rostov region. The race identification, using well-established differential lines (Record 1–3 (C), S-1358 (D), P-1380 (E), LC1093 and P96 (F)), indicated that the seeds belonged to race G_{RU}. The collected broomrape seeds were stored under frozen conditions. To establish an infectious background in the greenhouse, broomrape seeds thoroughly mixed into boxes containing a soil-sand mixture at a rate of 200 mg per 1 kg of mixture, ensuring an even distribution throughout the substrate. The F_2 sunflower plants were cultivated for 30 days at a temperature of 25–27 °C under a 16-hour photoperiod. The plants were uprooted 25 days after the emergence of sprouts, and the broomrape specimens on their roots were counted. The variety VNIIMK 8883, susceptible to modern races of *O. cumana*, served as a susceptible control.

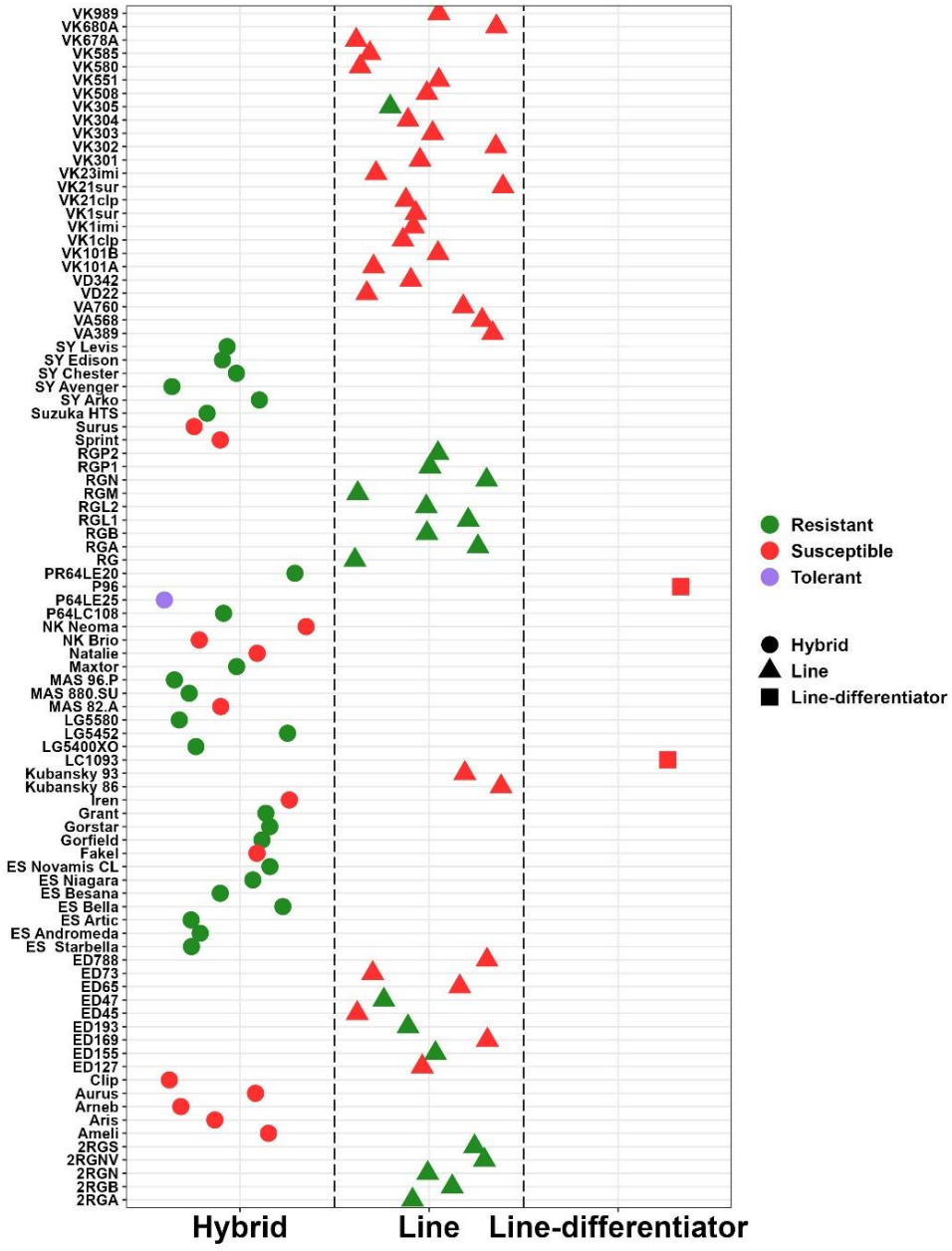

Fig. 1. Diversity of sunflower genotypes used in the research

Nucleotide sequences from the RefSeq (Reference Sequence) and GenBank® genetic databases (MN219479, MN219480, MF374791, and MF374792), which include regions associated with the *HaOr7* gene (Duriez et al. 2019), were used for DNA marker development. Primer design was performed using the online resource Primer-BLAST (Ye et al. 2012), with in silico verification of annealing specificity using the sunflower reference genome HanXRQr2.0-SUNRISE (Badouin et al. 2017). In total, four allele-specific markers were developed. UGENE software (Unipro, Russia) was used for nucleotide data analysis (Okonechnikov et al. 2012).

DNA extraction and purification from plant material was initially performed using the CTAB method with some modifications (Boom 1990) and the Diamond DNA Plant kit (Altaybiotech, Russia) for the preliminary validation of markers. For high-throughput screening of a larger number of plants, the MagnoPrime® FITO kit (NextBio, Russia) was employed using the Auto-pure 96 automated nucleic acid extraction and purification system (Allsheng, China).

The polymerase chain reaction (PCR) was performed using 25 µl of the following reaction mixture 2.5 µl of 10× PCR-buffer-B for Taq DNA polymerase; 0.2 mM of each deoxyribonucleoside triphosphate (dNTP); 10 pmol of each primer; 10-30 ng of template DNA; and 1 U of SynTaq DNA polymerase (Syntol, Russia). PCR was performed in a MiniAmp™ Nucleic Acid Amplifier (Thermo Fisher Scientific, USA). Amplification conditions: initial denaturation - 3 min at 94 °C, then 35 cycles: denaturation at 94 °C - 30 sec, annealing at 60 °C for 40 sec, extension- 40 sec at 72 °C, final extension - 5 min.

Electrophoresis of the amplification products was performed in agarose gel (1.5% agarose, 1×SB-buffer) using the SE.2 horizontal electrophoresis chamber (Helicon, Russia) for 1 hour at a current of 50-58 mA and a voltage of 80-100 V. The PCR products were stained with ethidium bromide. The results of electrophoresis were documented using a GenoSens 2200 gel documentation video system (Clinx, China). The size of DNA fragments was determined using Image Lab Software (Bio-Rad, USA) relative to a DNA ladder of 100-1000 bp (Syntol, Russia).

Mathematical analysis of phenotypic and genotypic segregation results was conducted utilizing the standard stats package for the R software environment, version 4.2.3 (R Core Team 2023), with the Chi-square test of correspondence of actual values with theoretically expected values in monohybrid crosses. Data were visualized using the ggplot2 package for R (Wickham 2016).

RESULTS AND DISCUSSION

In the first stage of the analysis of the association of markers with the resistance trait, 20 sunflower genotypes were used: one resistant commercial hybrid, 9 resistant and 10 susceptible lines of the VNIIMK breeding program (Table 1). The analysis involved evaluating the markers for stable amplification ability, lack of non-specific hybridization with the DNA template, and amplification of non-target PCR products, and their association with the trait. During the analysis, of the four allele-specific markers, one showed a lack of amplification and was subsequently discarded.

Table 1. PCR results with developed DNA markers

Phenotype	Sample name	Presence/absence of PCR product		
		RORS1	SORS1	SORS2
Resistant	SY Chester	+	+	+
	RGP1	+	-	-
	RGB	+	-	-
	RGP2	+	-	-
	2RGNV	+	-	-
	RGA	+	-	-
	RGL1	+	-	-
	RG	+	-	-
	RGN	+	-	-
	RGM	+	-	-
	RGL2	+	-	-
Susceptible	VK680	-	+	+
	Kub86	-	+	+
	VA760	-	+	+
	Kub93	-	+	+
	VK585	-	+	+
	VK508	-	+	+
	VK551	-	+	+
	VK580	-	+	+
	VK678	-	+	+

The marker SORS2 showed an association with the trait in the genotypes studied but was deemed unsuitable for further use due to the amplification of a non-specific PCR product when analyzing resistant lines. The SORS1 marker exhibited no amplification products in all resistant genotypes except the F_1 hybrid, which showed the presence of an amplified DNA fragment, as did all

susceptible lines. The RORS1 marker demonstrated the presence of a PCR product in all resistant sunflower genotypes and its absence in susceptible genotypes (Table 1). The lengths of the amplified fragments for the SORS1 marker were as expected, approximately 322 bp for SORS1 and 168 bp for RORS1, allowing their efficient separation in an agarose gel.

The primer pairs were tested *in silico* for nearly equal annealing temperature and the absence of primer dimers, enabling the development of a marker system for multiplex PCR. This system was tested on 29 sunflower genotypes (Fig. 2).

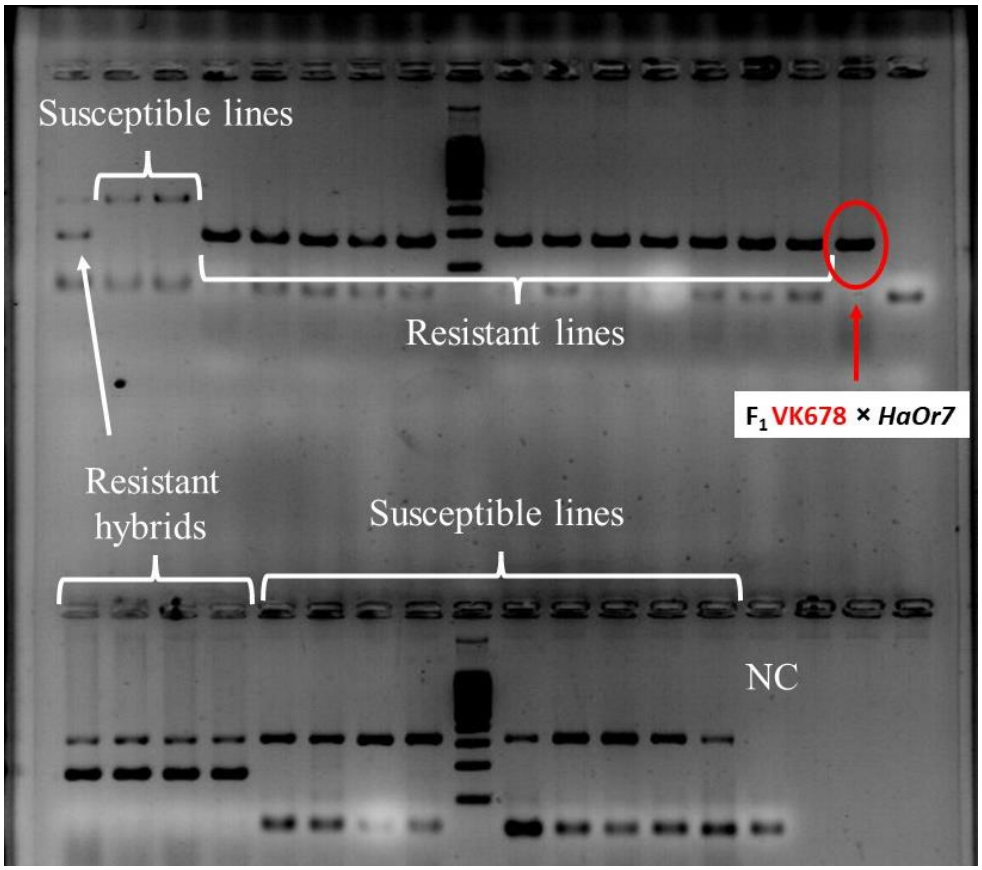

Fig. 2. Gel electrophoresis of DNA fragments amplified by multiplex PCR with RORS1/SORS1 markers. NC - negative control

The RORS1 and SORS1 marker system developed for multiplex PCR showed codominant inheritance and full association with the target trait in the sample studied. An exception was observed in the hybrid of the susceptible line VK678 and the resistant line RGM (Fig. 2, No. 16), where the PCR product characteristic of the susceptible allele was absent, and only the product for the resistant allele, inherited from the paternal line, was present in the gel electrophoresis.

Given the absence of the PCR product for the susceptible allele in the hybrid between VK678 and RGM, the pure line VK678 and its hybrids were added for further analysis. These included the hybrids F_1 (VK678 × RGB) and F_1 (VK678 × RGP2). The hybrid combination F_1 (VK680 × RGP1)

served as a positive control, as the issue with the absence of the PCR product was not observed in hybrids with the VK680 line.

Since only multiplex PCR had been tested, it was necessary to verify whether the disappearance of the SORS1 marker amplification could be reproduced in both multiplex and singleplex PCR. Therefore, two separate PCRs were performed: an RORS1/SORS1 multiplex and a singleplex with the SORS1 marker (Fig. 3).

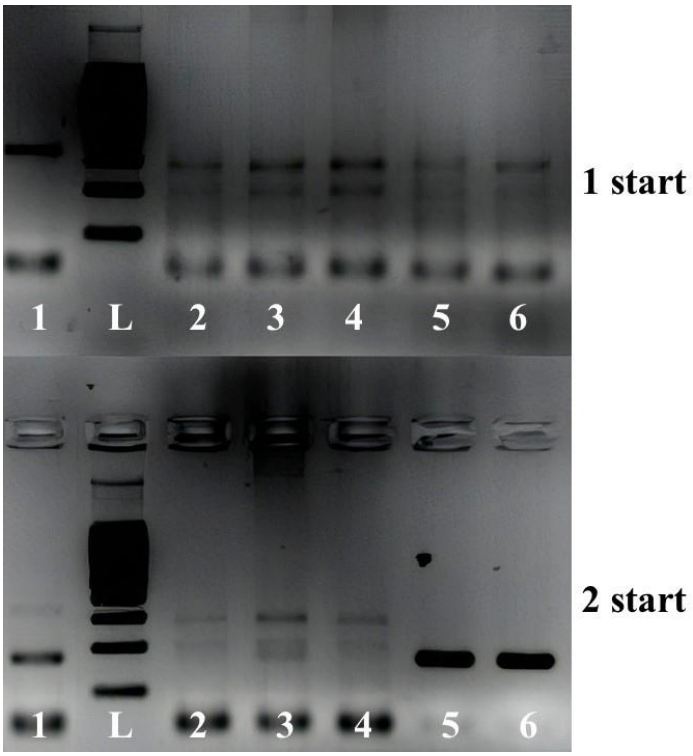

Fig. 3. Gel electrophoresis of DNA fragments amplified by: 1 start – singleplex with SORS1 marker; 2 start – multiplex with RORS1/SORS1 markers;

Lanes: 1 – F_1 (VK680 × RGP1); L – ladder; 2-4 – VK678; 5 - F_1 (VK678 × RGB); 6 – F_1 (VK678 × RGP1)

In both reactions, the control F_1 hybrid combination (VK680 × RGP1) produced the expected results: a PCR product of approximately 322 bp was obtained with the SORS1 marker, and two PCR products of approximately 168 bp and 322 bp were obtained in the RORS1/SORS1 multiplex reaction. In line VK678, the use of the SORS1 marker resulted in the amplification of two non-specific products of ≈ 300 and 200 bp. This amplification of non-specific products was observed in both single and multiplex PCR in the VK678 line. In the F_1 (VK678 × RGB) and F_1 (VK678 × RGP2) hybrids, singleplex PCR resulted in the amplification of two non-specific products, but multiplex PCR amplified specific DNA fragments of ≈ 168 bp, corresponding to the RORS1 marker, which is characteristic of the resistant parental lines RGB and RGP2, with no non-specific PCR products detected. Therefore, the SORS1 marker's unsuitability for evaluating the VK678 line suggests that the

entire RORS1/SORS1 multiplex system may be unreliable for broader use, as similar issues could arise with other lines.

To address this limitation, seven new allele-specific markers of the wild-type allele (wt) were developed *in silico* as an alternative to the SORS1 marker, while ensuring compatibility with the RORS1 marker for multiplex PCR. The new markers were tested on 30 sunflower lines and hybrids of the VNIIMK breeding, including VK678. Consequently, the SORS9 marker was selected, demonstrating consistent and reliable amplification of a DNA fragment with a predicted *in silico* size of approximately 217 bp in susceptible genotypes, without any non-specific PCR products in reactions with resistant lines. The effectiveness of multiplex PCR with RORS1/SORS9 markers was assessed on 13 lines and hybrids of the VNIIMK breeding (Fig. 4).

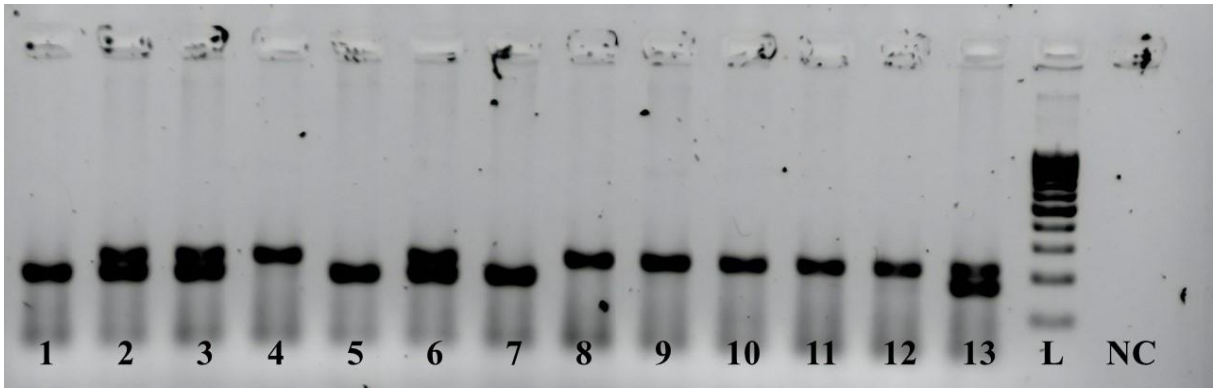

Fig. 4. Gel electrophoresis of the amplified DNA fragments obtained by multiplex PCR with RORS1/SORS9 markers. Lanes: 1 – RGM; 2 – F1 (VK680 × RGM); 3 – F1 (VK680 × RGP1); 4 – VK678; 5 –RGB; 6 – F1 (VK678 × RGP1); 7 – RG; 8 – VK304; 9 – VK551 =; 10 – VA389; 11 – VA568; 12 – VK508; 13 – F1 (VK678 × RGM); L - ladder; NC - negative control

The results of the analysis of 13 sunflower genotypes by multiplex PCR with RORS1/SORS9 markers demonstrated a perfect match between the expected and obtained PCR products, with the RORS1 product at approximately 168 bp and the SORS9 product at approximately 217 bp. Hybrids resulting from crosses between susceptible and resistant sunflower plants showed the presence of both fragments. Table 2 presents the characteristics of the RORS1 and SORS9 markers.

Table 2. Characteristics of the developed markers RORS1 and SORS9

Marker name	Primer name	Nucleotide sequence 5'-3'	Length of an amplified DNA fragment, bp
RORS1 (HaOr7)	RORS1 F	ACCATCTTTGCTTCCTATGGCA	168
	RORS1 R	CGTCACCCTTCTATGTCGCT	
SORS9 (wt)	SORS9 F	AGTGCATTCGCAATCTTCGC	217
	SORS9 R	TGCCATCCTCATCATTAAGGG	

The developed marker system was also tested on a small sample of progeny from two hybrids in the F_2 generation. PCR analysis was conducted at an early stage of plant growth to ensure objectivity, preceding phytopathological analysis (Table 3).

Table 3. Results of segregation for the marker system of the F_2 generation plants

Hybrid combination	Plants, total, pcs.	Theoretically expected segregation	Observed segregation	df	χ^2	P
F_2 SY Chester	37	3:1	28:9	1	0.01	0.92
F_2 SY Chester	37	1:2:1	12:16:9	2	1.16	0.56
F_2 VK680 × RG	20	3:1	14:6	1	0.27	0.61
F_2 VK680 × RG	20	1:2:1	4:10:6	2	0.40	0.82

For the hybrids studied, the Chi-squared test confirmed that the observed segregations in the F_2 generation matched theoretical expectations. In the SY Chester F_2 population, the probability levels were 0.92 for the 3:1 segregation model and 0.56 for the 1:2:1 model, while for the VK680 × RG F_2 population, the probability levels were 0.61 and 0.82, respectively. Both populations were suitable for further phenotypic analysis.

Each analyzed plant was labeled and a phytopathological analysis for resistance to the G_{RU} race of broomrape was performed (Table 4).

Table 4. Results of phenotypic segregation of F_2 generation plants

Hybrid combination	Plants, total, pcs.	Theoretically expected segregation	Observed segregation	df	χ^2	P
F_2 SY Chester	37	3:1	28:9	1	0.01	0.92
F_2 VK680 × RG	20	3:1	14:6	1	0.27	0.61

The number of plants in the resistant and susceptible groups was equal for both the presence and absence of the *HaOr7* gene, as determined by the DNA marker. Thus, the phenotypic segregation mirrored the segregation pattern observed with the marker system according to the 3:1 model, with plants carrying the *HaOr7* gene comprising the dominant group.

To establish the relationship between the presence of the *HaOr7* gene and the resistance of sunflower plants to the G_{RU} race of broomrape, data on the genotype obtained and the infection degree, expressed as the number of broomrape tubercles on each plant, were entered into two independent datasets and compared using the ggplot2 package (Fig. 5).

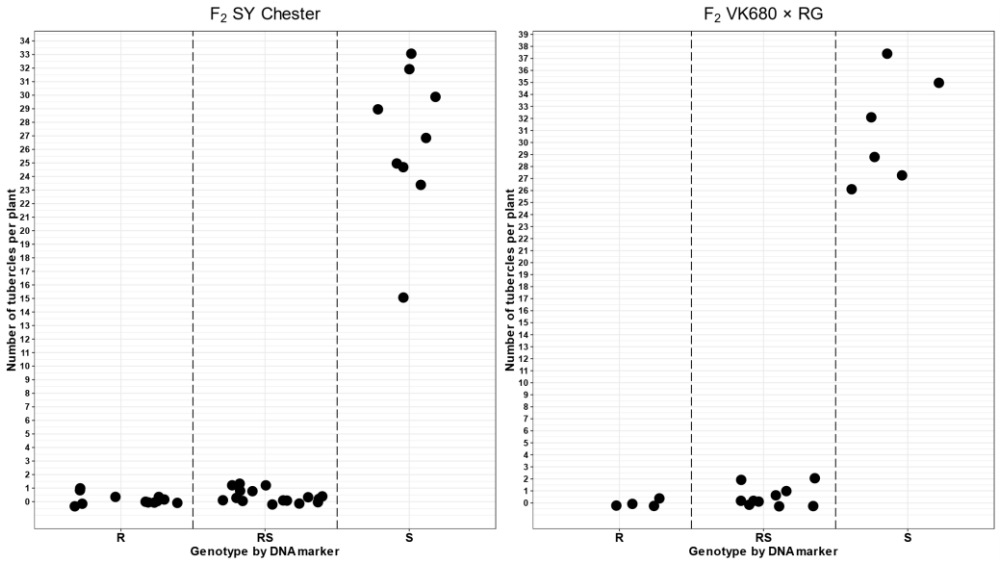

Fig. 5. The degree of broomrape infection in sunflower plants with different allelic states of the *HaOr7* gene. R - homozygote *HaOr7*, RS - heterozygote, S - homozygote of a wild type.

Plants carrying the *HaOr7* gene, including heterozygous plants, exhibited resistance to the G_{RU} race of broomrape, whereas all homozygous plants for the wild-type allele were susceptible. This, along with previous data, suggests that the dominant *HaOr7* gene confers resistance to the G_{RU} race of broomrape though further confirmation is required with larger sample sizes. For this purpose, 90 sunflower genotypes were screened using a marker system (Fig. 6-7).

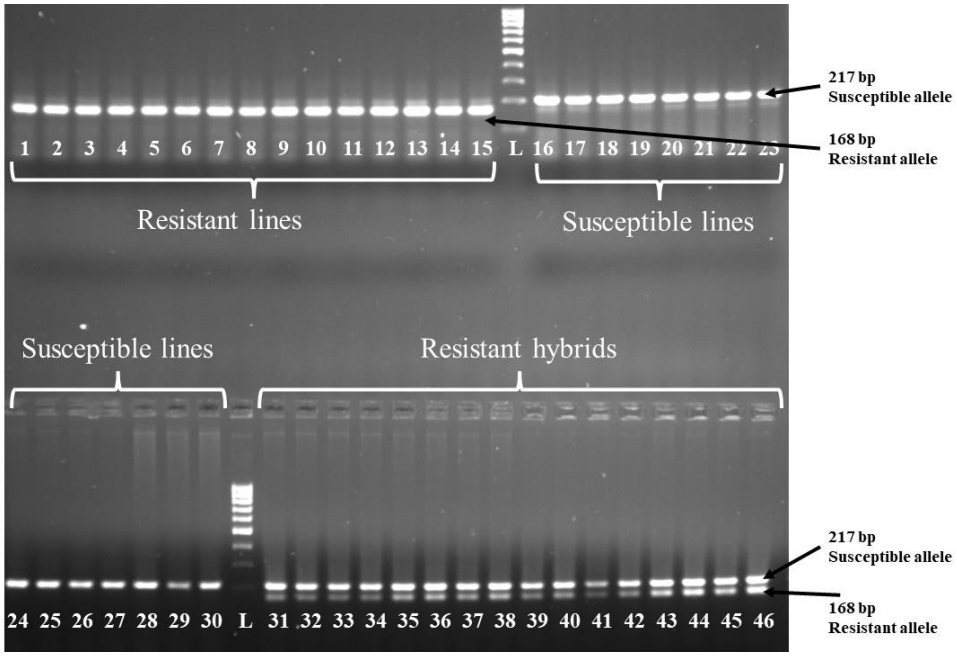

Fig. 6. Analysis of various sunflower genotypes using the RORS1/SORS9 marker system

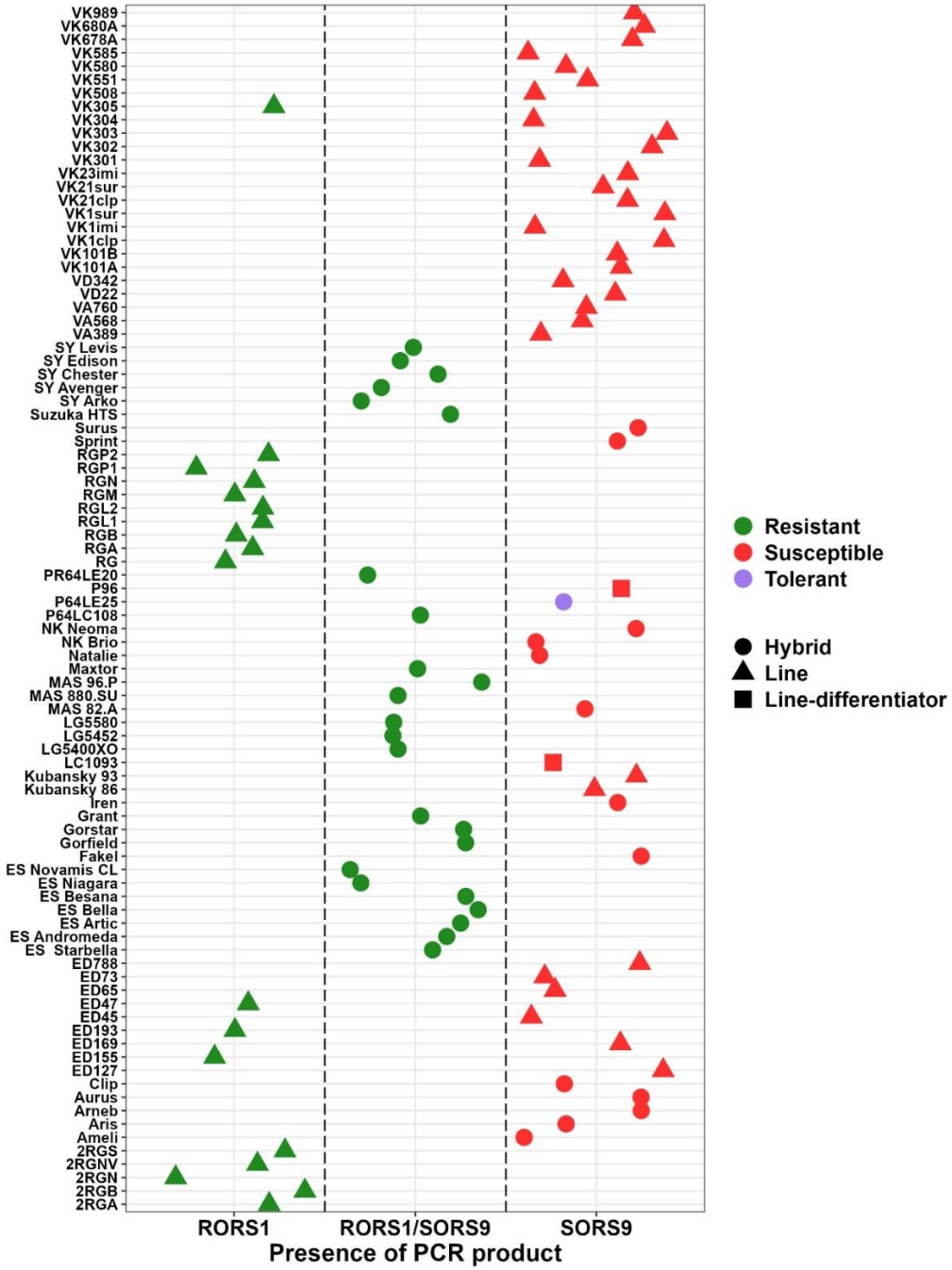

Fig. 7. Results of the analysis of the association of the RORS1/SORS9 marker system with sunflower resistance to G_{RU} race of broomrape

The screening revealed that sunflower lines resistant to the G_{RU} race of broomrape were characterized by the presence of the RORS1 PCR product, indicating the presence of the *HaOr7* gene, and the absence of the SORS9 marker, indicating the absence of the wild-type allele (wt). All susceptible lines and hybrids of the VNIIMK breeding program showed only the presence of the specific PCR product of the SORS9 marker, and were therefore homozygous for the wild-type allele.

Two differential lines to race F, LC1093 and P96, also showed the absence of the *HaOr7* gene, consistent with Duriez *et al.* (2019). The resistant hybrids were characterized by the presence of both DNA fragments of the RORS1 and SORS9 markers. Thus, all commercial hybrids reported as resistant to broomrape races G and G+ carried the *HaOr7* gene in a heterozygous state. Conversely, hybrids such as MAC 82 A, NK Brio and NK Neoma which are resistant to races A-E and the broomrape-tolerant hybrid P64LE25 showed the absence of the *HaOr7* gene.

The screening results suggest that *HaOr7* is currently the most prevalent or perhaps the sole vertical resistance gene employed in sunflower breeding to counteract the G_{RU} race of broomrape. However, this situation poses a considerable risk of broomrape evolving resistance, potentially giving rise to new pathogenic races. In an alternative approach to *HaOr7*, the P64LE25 hybrid utilizes the Or_{SII} gene, known for conferring non-race-specific tolerance to broomrape. This tolerance confers a resistant phenotype, particularly under conditions of low broomrape infestation.

To address the potential emergence of new broomrape races, and in line with efforts to find and integrate resistance genes from wild relatives, the strategy of pyramiding already known genes is crucial (Seiler and Jan, 2014; Seiler, 2019; Chabaud et al., 2022). Considering the latest findings that attribute the origin of new broomrape races to point mutations (Fernández-Melero et al., 2023), the combination of multiple resistance mechanisms within a single genotype significantly diminishes the likelihood and rate of new race development. For instance, according to the originator, the hybrid P64LC108 carries both *HaOr7* and Or_{SII} genes, providing enhanced resistance and stands out as the most broomrape-resistant hybrid from this company. The use of DNA markers greatly streamlines the breeding process, enabling more efficient development of heterozygous hybrids through marker-assisted selection. Additionally, this facilitates the pyramiding of resistance genes in the development of lines and homozygous hybrids, which is often challenging or unfeasible when relying solely on phenotypic selection.

CONCLUSION

Reliable allele-specific markers, RORS1 and SORS9, were developed, along with the first codominant DNA marker system for multiplex PCR to detect the allelic state of the *HaOr7* gene in sunflower. The marker system was validated on small samples of two segregating F_2 populations, demonstrating complete correspondence between the allelic states detected by the markers (RORS1 and SORS9) and the observed phenotypic resistance. The analysis of 90 sunflower genotypes confirmed the marker system's ability to distinguish resistant and susceptible lines and hybrids to G_{RU} race of broomrape. Thus, the developed DNA marker system is a valuable tool for marker-assisted selection in sunflower breeding.

Our findings suggest that the resistance gene to the G_{RU} race of broomrape in sunflowers cultivated in the Russian Federation is identical to the *HaOr7* gene, known for conferring resistance to the F_{GV} race of broomrape. Previous reports by Martín-Sanz et al. (2020) indicated that this gene can also confer resistance to races higher than F, a finding confirmed using the marker system we developed. Screening of 25 sunflower hybrids from seven breeding companies in the Russian Federation demonstrated that all hybrids resistant to races G and G+ carry the *HaOr7* gene.

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