The search for the location of the sunflower resistance gene to race G of broomrape on the linkage map in the lines of breeding of VNIIMK

S Z Guchetl¹², D L Savichenko¹ and S A Ramazanova¹

¹ V.S. Pustovoit All-Russian Research Institute of Oil Crops, 17 Filatova street, Krasnodar 350038, Russia
² E-mail: saida.guchetl@mail.ru

Abstract. The parasitic plant Orobanche cumana Wallr. is one of the main biotic limiting factors for obtaining high yields of sunflower. Since plant resistance to different broomrape races in different genotypes can be controlled by different genes, to ensure the evaluation of breeding material using DNA markers (MAS), it is necessary to localize the resistance gene for each of the sources. The aim of the research is to find the location of the Or⁷ gene, which controls the resistance to one of the most virulent broomrape races, on the sunflower linkage map and to determine the DNA markers cosegregating with it. For research we used the lines of breeding of V.S. Pustovoit of All-Russian Research Institute of Oil Crops (VNIIMK) resistant and susceptible to race G of broomrape. We used SSR-primers ORS 683, ORS 1040, ORS 1112, and ORS 202 for the PCR analysis. We excluded the location of the gene in the upper part of LG3 in the immediate proximity to the microsatellite loci ORS 683, ORS 1040, ORS 1112. Based on the studied literary sources and the reference genome of the sunflower HanXRQr2.0-SUNRISE, we compiled a partial physical map of LG3 and determined the area for further search for the localization of the Or⁷ gene and DNA markers cosegregating with it.

1. Introduction
Sunflower (Helianthus annuus L.) is the fourth oil crop in the world by cultivation area and the leading one in Russia. The parasitic plant Orobanche cumana Wallr. is one of the main biotic limiting factors for obtaining high yields of sunflower. Broomrape is widespread in Europe and Asia, especially in Central and Eastern Europe [1]. In the absence of resistance in cultivated hybrids, the damage caused by this parasite can amount to over 50 % of a harvest. Currently, at least eight races have been reported, represented by the letters A to H [2], in order of increasing virulence.

The most efficient and environmentally safe method of controlling broomrape is the cultivation of resistant varieties and hybrids of sunflower. The development of resistant genotypes includes finding and using sources of resistance in the breeding process, as well as providing accurate and efficient procedures of material evaluation. Considering that resistance genes are quickly overcome with the emergence of a more aggressive race of the parasite, it is necessary to carry out the process of introducing and combining of resistance genes on a continuous basis [3, 4].

Plant resistance to different races of broomrape in different genotypes can be controlled by different genes: one dominant gene [5], one or two recessive genes, and two partially dominant genes [4, 6]. This indicates the presence of various sources of resistance due to the origin of a genetic material. Thus, to provide a genetic evaluation of breeding material using DNA markers (marker-assisted selection), it is
necessary to determine the location of the resistance gene for each of the sources. Five QTLs (quantitative trait loci) that provide resistance to race E and six QTLs that provide resistance to race F were found in seven different chromosomes [5]. The race-specific gene that controls resistance to race E is Ors. It is mapped on the telomeric region of chromosome 3, being 7.5 cM above the ORS1036 marker [7]. Later, in the same chromosome was found a gene for resistance to the race higher than F, which was preliminary indicated as $o_{T_{ab-s}}$ and localized 1.5 cM below the ORS683 marker and 19.6 cM below the ORS1036 marker [4]. Despite the close location to the Ors gene, it has been proven that these resistance genes are different. Later, I. Imerovski et al. [8] mapped from 2 to 23 significant QTLs in the sunflower genome. The two main QTLs were located on chromosome 3. These QTLs were indicated as $or3.1$ and $or3.2$. QTL $or3.1$ is located in the upper part of the chromosome – the region of localization of the Ors gene, while QTL $or3.1$ was identified in the lower region of the same chromosome. It was confirmed in four different crossings using different sources of broomrape resistance. It was established that in all crossings resistance QTL were found in chromosome 3. Also, one gene of resistance to race F of O. cumana has been described and mapped on chromosome 7 [9].

At V.S. Pustovoit All-Russian Research Institute of Oil Crops (VNIIMK), there were developed sunflower lines of various origin, resistant to race G of broomrape. We found that the resistance gene in these lines is controlled by one gene with incomplete dominance [10]. These genotypes represent a valuable source for gene transfer using DNA markers (MAS). The use of these genes in breeding will allow to produce safe and high-quality food products using modern genetic methods. Therefore, the aim of this research is to find the location of the Ors gene on the linkage map of sunflower and the selection of microsatellite loci cosegregating with it.

2. Materials and methods

The research material was the source of resistance to race G of broomrape RGP1 and VK 678, susceptible to this race; they both were developed at VNIIMK. We crossed sunflower plants with contrasting alleles in the field to receive a heterozygous generation F1. Then, we carried out the self-pollination of F1 plants to obtain F2 progeny. We tested plants in a greenhouse for resistance and susceptibility to broomrape on an infectious background of seeds of broomrape of race G, using the method of early diagnosis [10].

We extracted sunflower DNA from the top leaves of young sprouts of vegetative plants. We carried out the DNA extraction using a modified method of M. Saghai-Maroof et al. [11]. We homogenized a sample weight of 0.2 g of plant tissue using a Speed Mill plus homogenizer (Analytic Jena, Germany). We determined the concentration of DNA in the resulting preparation by the intensity of the luminescence of a 10 μl sample in ultraviolet light after electrophoresis in 1 % agarose gel containing ethidium bromide.

To carry out the polymerase chain reaction, we used 25 μl of the reaction mixture of the following composition: 67 mM tris-HCl, pH8.8; 16.6 mM of ammonium sulfate; 1.5-3 mM of MgCl2; 0.01 % of Tween 20; 0.2 mM of deoxyribonucleoside phosphates; 10 pM of primers; 10 ng of template DNA and 1 unit of recombinant thermostable DNA polymerase (SibEnzyme, Russia). We used a S1000™ thermal cycler (BioRad, USA) for amplification. The amplification conditions were: initial denaturation at 96 °C for 2 min, then 30 cycles at temperature-time mode: denaturation at 94 °C for 30 sec, annealing at 60 °C for 40 sec, elongation at 70 °C for 1 min, final elongation for 2 min. We used the following SSR primers for the PCR analysis: ORS 683, ORS 1040, ORS 1112 n ORS 202 [4, 7].

We carried out the electrophoresis of the amplification products in polyacrylamide gel (8 %, 1xTBE) using a VE-20 chamber for vertical electrophoresis (Helicon, Russia) for 2.5-3 hours at a current strength of 40-50 mA and a voltage of 200-230 V. We determined the size of DNA fragments using the BIOPRINT gel-documentation digital video system and Bio-Capture software (Vilber Lourmat, France) with respect to the length marker of DNA fragments GeneRuler 100 bp DNA Ladder Thermo Scientific (SibEnzyme, Russia). We carried out the mathematical processing of the segregation results using $\chi^2$-test for the correspondence of the actual segregations to the theoretically expected ones in mono- and dihybrid crossings [12].
3. Results and discussion
Since, according to the results of the literature data, the researchers localized most of the genes for resistance to different races of broomrape in LG 3 [4, 7, 8, 13], we searched for DNA markers cosegregating with the Or7 gene in the upper part of this chromosome. To establish the localization of the gene that controls resistance to race G of broomrape, I. Imerovski et al. compiled a partial SSR map of LG 3 of sunflower, containing the locus or_{ab-vl-8} of resistance to the broomrape race higher than F [4]. On its basis, we carried out the selection of genetic markers for the Or7 gene [14]. Within the framework of the study, the SSR loci used in the work were compared with the assembly of the reference genome of the sunflower HanXRQr2.0-SUNRISE. Also, according to the results of a study using BSA-seq, I. Imerovski et al. identified the potential candidate resistance genes - HanXRQChr03g0076321 and HanXRQChr03g0065841 [8]. Based on these data, we have compiled a physical map of the location of loci in LG 3 (Fig. 1).

![Figure 1](image_url)

**Figure 1.** The location of SSR loci, presumably linked to the Or7 gene and candidate genes in LG 3 of *H. annuus*.

The locations of loci on the physical map were divided into 2 conditional groups, which coincided with the previously compiled molecular genetic map [4]. In the upper part of LG3, there is a group of loci located above the supposed location of the or_{ab-vl-8} gene on the molecular genetic map, and in the lower part, a group located below this gene. According to the molecular genetic map of LG3 of sunflower, the resistance gene to the broomrape race higher than F is localized in the upper part of the chromosome [4]. The closest marker ORS683 is 1.5 cM higher than the resistance gene.

To establish the localization of the Or7 gene in the lines of breeding of VNIIMK, we selected the sunflower lines RGP1, resistant to race G of broomrape, and VK 678, susceptible to this race. These lines had four marker microsatellite loci with contrasting allelic variants (Table 1). These are loci ORS202, ORS 1040, ORS 1112, ORS 683 and all of the, are located above the supposed location of the or_{ab-vl-8} gene on the SSR and the physical map of sunflower (Fig. 1).

**Table 1.** The differences in DNA loci in sunflower lines resistant and susceptible to race G of broomrape.

| Line  | Gene   | ORS683 | ORS1112 | ORS202 | ORS1040 |
|-------|--------|--------|---------|--------|---------|
| VK 678| or_{50r5}| 364*   | 347     | 309    | 192     |
| RGP1  | Or5Or5 | 400    | 375     | 333    | 200     |
We tested the sunflower seeds of F₁ obtained after crossing of parental lines (VK 678 × RGP1) for hybridity by DNA loci. We identified two amplified DNA fractions in the codominant loci, and one in the dominant ones (Table 2).

**Table 2.** The allelic conditions of SSR loci of sunflower DNA in hybrid F₁ (VK678× RGP1), obtained from parental lines resistant and susceptible to race G of broomrape.

| Hybrid F₁ | SSR loci | ORS 683 | ORS 1112 | ORS 202 | ORS 1040 |
|-----------|----------|---------|----------|---------|----------|
| VK 678×RGP1 | ORS 683 | 343/400 | 347/375 | 309/333 | 181/200 |

After receiving the F₂ progeny, the segregation analysis for the resistance of sunflower to race G of broomrape for this crossing combination showed that the actually observed segregation corresponded to the theoretically expected 3:1 model with monogenic inheritance of the trait. Therefore, the combination is suitable for further analysis (Table 3).

**Table 3.** The inheritance of the trait of sunflower resistance to race G of broomrape in F₂ when crossing lines VK678 and RGP1.

| Origin | Total number of plants, pcs. | Expected ratio | χ² | df | P |
|--------|-------------------------------|----------------|-----|----|---|
| VK 678 x RGP1 | 107                          | 3:1            | 0.24| 1  | 0.7-0.8 |

It is known that the sunflower resistance to race G of broomrape is provided by the action of one dominant Or₇ gene [10]. Our research has also confirmed this type of inheritance in this crossing combination.

As Tables 2 and 3 show, all primers detected polymorphic amplification products of DNA for the selected crossing combination. We carried out the genetic analysis of the segregation results for all four DNA loci. The loci ORS 683 and ORS 1112 are codominant, and in the second generation the segregation corresponds to the 1:2:1 model, while in the dominant loci ORS 1040 and ORS 202 it corresponds to the 3:1 model. But the actual segregations corresponded to the theoretically expected ones only in loci ORS 683, ORS 1040, ORS 1112 (Table 4).

**Table 4.** The inheritance of DNA SSR loci ORS 683, ORS 1040, ORS 1112 and ORS 202 when crossing lines VK 678 and RGP1.

| Locus   | Expected ratio | χ² | df | P   |
|---------|----------------|-----|----|-----|
| ORS 683 | 1:2:1           | 1.16| 2  | 0.56|
| ORS 1112| 1:2:1           | 2.37| 2  | 0.67|
| ORS 1040| 3:1            | 3.72| 1  | 0.05|
| ORS 202 | 3:1            | 59.00| 1 | <0.01|

χ²-test for these loci confirmed the correspondence of the actual segregations to the theoretically expected ones. We observed a segregation distortion in ORS 202 locus (\(χ²\) is 59.00). For this reason, we left only ORS 683, ORS 1040, ORS 1112 loci for further calculations to exclude the results distortion of the analysis of independent gene inheritance. We carried out the analysis for independent inheritance of the Or₇ gene with these three loci (Table 5).

**Table 5.** χ² values between the Or₇ resistance gene to race E of broomrape and DNA loci in F₂ progeny.

| Loci pairs | \(χ²_A\) | \(χ²_B\) | \(χ²_L\) | P₀ |
|------------|---------|---------|---------|----|
| Or₇–ORS683 | 0.24    | 1.16    | 7.78    | 0.18|
Table 5 presents data on the evaluation of independent inheritance of the Or7 gene with three DNA loci, where three values of $\chi^2$ are noted: $\chi^2_A$ characterizes deviations in segregation by the first locus, $\chi^2_b$ – by the second locus, $\chi^2_1$ – deviations from the independent segregation of loci A and B. The probability of the null hypothesis of independent inheritance ($P_L$) between the analyzed genes is also shown. The theoretical segregation with the dominance of one of the traits and codominance of the other in the F2 progeny should correspond to the 3:6:3:1:2:1 model (for gene pairs Or7 – ORS 683 and ORS 1112), with the dominance of both traits – the 9:3:3:1 model (for gene pairs Or7 – ORS 1040). The test for independent inheritance did not confirm the linkage of the Or7 loci with the DNA loci ORS 683, ORS 1040, ORS 1112. That means that the gene that controls resistance to race G of broomrape in the collection of VNIIMK is not identical to the $or_{ab-vi-s}$ gene described by Serbian scientists, and most likely is not in the upper part of LG3. Nevertheless, based on the literature data, we assume that it is localized on the same chromosome, below the region in which we made the search. The proposed candidate genes are also located in the region below the ORS 683 marker [8]. In this regard, we will continue work to search for the location of the Or7 gene and DNA loci linked to it in the lower regions of LG3. The DNA loci associated with the resistance gene will be further used to mark the gene in programs for the transfer of valuable breeding traits among sunflower lines. This will facilitate and advance the development of new sunflower genotypes with specified parameters of productivity and complex resistance to broomrape.

4. Conclusion
As a result of the research, we carried out the search for the localization of the resistance gene to the race G of broomrape on the linkage map of sunflower in breeding material of VNIIMK. We excluded the location of the gene in the upper part of LG3 in the region of close proximity to the microsatellite loci ORS 683, ORS 1040, ORS 1112.

On the basis of the studied literary sources and reference genome of the sunflower HanXRQr2.0-SUNRISE, we compiled a partial physical map of LG3, which allows more precise determination of the area of further search for the localization of the Or7 gene and DNA markers cosegregating with it.

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