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Validation of Microsatellite Markers of *Pl* Resistance Genes to Downy Mildew of Sunflower

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Abstract: Simple sequence repeats (SSR) polymorphism of 34 microsatellite loci (LG1, 8 and 13) was studied in lines carrying the downy mildew resistance genes *Pl* and lines with no *Pl*. The microsatellite loci ORS328 and ORS781 were selected as markers for genes *Pl6* and *Pl8* in lines HA 335 and QHP-1, respectively. Markers were identified for gene Pl_{ARG} in RHA 419 and some accessions of *H. argophyllus*. The SSR markers ORS509, ORS605, ORS610, ORS1182 and ORS1039 were proven to reliably identify the parental line carrying Pl_{ARG} gene, control and select the heterozygous F_1 hybrids and identify homozygous genotypes in F_2 generations. Obtained results indicate the necessity of validation of the markers in various germplasm pools and breeding collections. The SSR markers that are tightly linked to Pl_6, Pl_8, Pl_{ARG} would be useful in the sunflower breeding. Pl_{ARG} homozygous F_2 segregants, developed and identified with marker assisted selection in this study, are recommended for further breeding as a new source of genetically determined resistance to downy mildew.

Keywords: *Helianthus*, *Plasmopara halstedii*, resistance, *Pl* genes, SSR markers

Introduction

One of the main requirements to sunflower hybrids is resistance to the pathogen *Plasmopara halstedii* (Farl.) Berl.& de Toni which is causing downy mildew. The sunflower breeding for resistance to the disease is a complicated task due to the large amount of pathogen races and their significant variability. There are currently more than 30 pathotypes of downy mildew discovered in the cultivation areas of sunflower. Races 330, 700, 703, 710, 730 and 770 are the most harmful among others (Gulya, 2007; Jocić *et al*., 2010). An eco-friendly and economically advantageous

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way to prevent crop losses is a breeding and cultivation of the varieties and hybrids with genetically determined resistance to this pathogen. Resistance to different races of *P. halstedii* is controlled by several *Pl* genes grouped in some clusters. Genes Pl_1, Pl_2, Pl_6, Pl_7 were located on linkage group (LG) 8 of the genetic map that have been developed by using simple sequences repeat (SSR) markers (Gegil *et al.*, 2001). The genes cluster *Pl5/Pl8* was mapped on LG 13 (Radwan *et al.*, 2003). Genes Pl_{13} and Pl_{ARG} both are located on LG 1 but not linked (Mulpuri *et al.*, 2009; Duble *et al.*, 2004). The most effective *Pl* genes have been identified and introduced into the cultivated sunflower from wild *Helianthus* L. species. Pl_6 was introgressed from wild *H. annuus* ecotypes, Pl_5 from *H. tuberosus*, Pl_7 from *H. praecox*, while *Pl8* and Pl_{ARG} both originated from *H. argophyllus* (Jocić *et al.*, 2010).

South Ukraine population of *P. halstedii* includes race 730 (Solodenko *et al.*, 2013). Race 730 is one of the most aggressive pathotypes, resistance against which is determined by genes Pl_6, Pl_7, Pl_8, Pl_{13} and Pl_{ARG}. Some donors of effective *Pl* are known. Lines HA 335 and HA 336 are carriers of gene Pl_6; lines HA 337, HA 338, HA 339 contain Pl_7 in their genomes; RHA 340, 803–1, QHP 1 carry Pl_8; HA-R4 and HA-R5 were shown that their resistance to *P. halstedii*is mediated by Pl_{13}; ARG1575-2, 79ARGMTP, RHA 419 are characterized by presence of Pl_{ARG} (Wieckhorst *et al.*, 2010). These lines are derived either from the South American wild population of *H. annuus* or from crossing of cultivated sunflower with other species of *Helianthus* genera and are widely used by breeders from different countries to create resistant lines and hybrids adapted to the growing conditions of certain regions.

Identification of certain *Pl* genes in the genotype of variety or line allows the most objective estimation of their potential resistance to *P. halstedii*. DNA markers that are closely linked to the genes are required for development of marker assisted selection (MAS) in the process of creating new high-yielding sunflower genotypes. DNA markers are necessary for identification of certain alleles of *Pl* genes in sunflower genotypes at any stage of cultivation. Moreover, usage of DNA markers allows to reduce the amount of processed breeding material as well as time needed for development of new lines and hybrids.

SSR markers are the most commonly used in MAS projects because of their widely distribution in the genomes of plants, high levels of polymorphism and co-dominant type of inheritance (Gupta and Varshney, 2000). More than 2000 SSR markers have been developed for sunflower (Paniego *et al.*, 2002; Tang *et al.*, 2002; Yu *et al.*, 2003; Heesacker *et al.*, 2008; Talia *et al.*, 2010). It was shown the usefulness of this type markers for genetic mapping and identifying of certain genes, including resistance genes such as the rust resistance genes *R4, R5, R13* (Qi *et al.*, 2015), broomrape resistance gene *Or5* (Tang *et al.*, 2003), as well as loci associated with the resistance to white rot (Davar *et al.*, 2010). Some

SSR markers were identified for *Pl* genes (Duble *et al*., 2004; Mulpuri *et al*., 2009; Wieckhorst *et al*., 2010).

The aim of this study was to search for and validate SSR markers of *Pl* genes in a wide range of sunflower lines, F_1 and F_2 generations and marker assisted selection of the initial material for further breeding.

Materials and Methods

The following lines of sunflower were used in this study: HA288, RHA 265, RHA 274, DM-2, PM-13, PM-17, 803-I, QHP-1, FT 226 (analogue QHP-1), HA-R4, HA-R5, HA 335, RHA 419. These lines are included in the standard set serving for identification of *P. halstedii* races. Accessions of *Helianthus argophyllus* L. from the wild sunflower species collections of Plant Breeding and Genetics Institute (PBGI, Odessa, Ukraine) and Institute of Oilseed Crops (IOC, Zaporozhye, Ukraine) were investigated as well as susceptible inbred lines Od 108A, OC 1019B, OC 1029B, F_1 hybrids and F_2 segregating populations from the crosses (RHA 419 x OC 1019B) and (RHA 419 x OC 1029B). The stability of all investigated lines was confirmed by the observations over several years. Seed and plant material for study was kindly provided by Dr Boris Varenyuk, Plant Breeding and Genetics Institute.

DNA isolation from seedlings and leaves samples was done according a CTAB protocol (Doyle and Doyle, 1987). PCR mix in 20 µl consisted of 1 x reaction buffer (Fermentas), 0.2 mM of each dNTP, 0.2 µM of each primers, 20 ng DNA and 1 unit Tag polymerase (Fermentas). PCR reactions were performed using "Tercik" thermocycler under following protocol: 1 min at 92 °C, 30 cycles of 1 min at 92 °C, 30 sec at 60 °C, 30 sec at 72 °C, with final extension for 3 min at 72 °C. The amplification products were visualized by electrophoresis on polyacrylamide gels followed by silver staining. Evaluation of the band sizes was done by using software Gel Analyzer 2010a. Chi-square test was used to test if the observed ratio of plants in F_2 populations, carrying particular marker alleles, correspond the Mendelian ratios (Ajaala, 1984). The research was carried out in 2015–2017.

Results and Discussion

Polymorphism of 34 microsatellite loci of the public sunflower linkage map (Tang *et al*., 2002), which have been localized within 10 cM from *Pl* genes

clusters on LG 1, 8 and 13, was studied in sunflower inbred lines, carrying or not the *Pl* genes.

Microsatellite loci of LG 8 ORS1043, ORS166, ORS37 were shown to be effective markers for analysis of inheritance of Pl_6 in F_3 populations, in which introgressive line HA 336 was the source of resistance gene (Panković *et al*., 2007). Pl_6 gene was discovered in wild *H. annuus* populations and was introgressed into lines HA 336 and HA 335 (Miller and Gulya, 1991). According to our data, line HA 335 was similar to others based on allelic composition of ORS1043, ORS37 and ORS166. Analysis of PCR products showed polymorphism, which was not related to genes *Pl1, Pl2* and *Pl6*. Instead, the lines HA 335, 803-I, HA-R4, HA-R5, QHP-1, RHA 419 turned out to be polymorphic at ORS328 locus. Our studies of the downy mildew races composition in the South Ukraine population indicate that the lines carrying Pl_6 gene could be considered as sources of reliable resistance in breeding crossing programs. If HA 335 will be used for *Pl6* introgression, *ORS328* allelic composition would allow to unambiguously identify the hybrid and backcrosses plants carrying donor genetic material.

A total of the 14 SSR loci were used to search for the markers of Pl_8 gene. A particular attention was paid to ORS316, which was reported to clearly detect invariable resistant genotypes among descendants of *Pl8* donor line RHA 340 (Abratti *et al*., 2004). In our study, lines 803-I and QHP-1 carrying Pl_8 were not differ from other lines based on allelic composition of ORS316. This observation additionally stresses the necessity of careful validation of known markers in various germplasm pools and breeding collections. The polymorphism detected in 803-I and QHP-1 lines at microsatellite loci of LG13 was not associated with genes *Pl8* or *Pl5*. Instead, QHP-1 specific allele (410 bp) of ORS78 (Figure 1) might

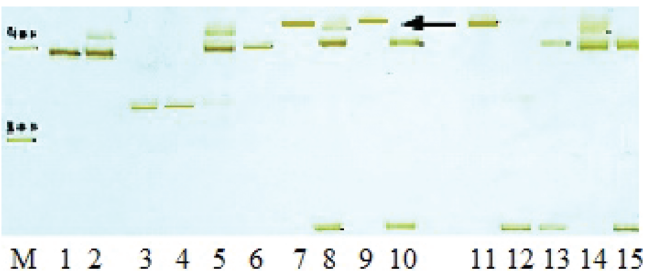

Figure 1: SSR ORS781 profile obtained for: 1 – RHA 265, 2 – RHA 274, 3 – DM 2, 4 – PM 13, 5 – PM 17, 6 – 803-I, 7 – HA-R4, 8 – HA-R5, 9 – QHP-1, 10 – HA 335, 11 – RHA 419, 12 – FT 226, 13 – HA 228, 14 – Od 108A, 15 – OC 1029B. M – molecular marker Ladder 100 (bands 300 bp and 400 bp). Arrow marks band 410 bp.

be useful for MAS among F_2 resistant plants, considering the common usage of this line as a *Pl8* donor.

We investigated 16 microsatellites loci, mapped on LG1 of sunflower genetic map, in order to identify the markers of Pl_{ARG} gene. Pl_{ARG} is mediating resistance to all known races of *P. halstedii*. Pl_{ARG} have been introgressed to cultivated sunflower from silverleaf sunflower *H. argophyllus* (Seiler *et al.*, 1991). RHA 419 is one of the lines carriers of Pl_{ARG} resistance gene (Miller *et al.*, 2002). Alleles of markers ORS509, ORS605, ORS610, ORS675, ORS1039, ORS1182 allowed to differentiate RHA 419 as well as the accessions of *Helianthus argophyllus* from all other investigated genotypes (Table). RHA 419 and *H. argophyllus* have the same alleles of ORS509 (207 bp) and ORS1182 (165 bp). Three alleles were identified at ORS605 locus: one allele is characteristic of *H. argophyllus* accessions, other allele is detected in RHA 419, and another one is revealed in all other studied lines. Six alleles were identified at ORS1039 (Figure 2), allele 220 bp is specific for only *H. argophyllus* accessions, allele 190 bp is specific to RHA 419. "Null"-allele was detected at ORS610 in *H. argophyllus* accessions and allele (130bp), which differentiates RHA 419 from other studied genotypes (Figure 3). SSR ORS675 profile of all investigated lines contained amplification fragment 240 bp, however RHA 419 and *H. argophyllus* characterized by presence of additional band 220 bp.

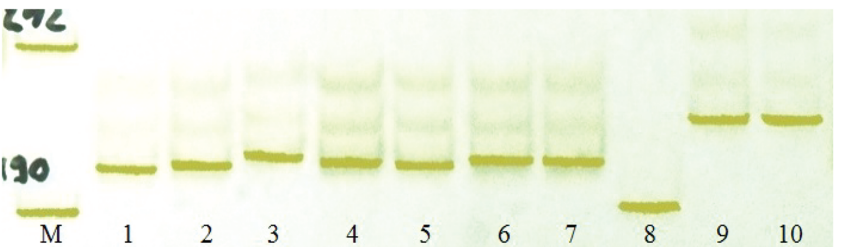

Figure 2: SSR ORS1039 profile obtained for: 1 – HA 228; 2 – QHP-1; 3 – FT 226; 4 – HA 335; 5 – Od 108A; 6 – OC 1019B; 7 – OC 1029B; 8 – RHA 419; 9 – *H. argophyllus* accession (PBGI); 10 – *H. argophyllus* accession (IOC); M – molecular marker pUC 19/MspI (bands 190 bp and 242 bp).

Wild relatives are commonly used in breeding programs as donors of resistance genes to various abiotic stresses and biotic pathogenes (Miller and Seiler, 2003; Jan *et al.*, 2002; Jan *et al.*, 2004b). *H. argophyllus* and line RHA 419 are involved in breeding for developing new resistant genotypes of sunflower. Presence of specific DNA-markers in F_1 and F_2 populations,

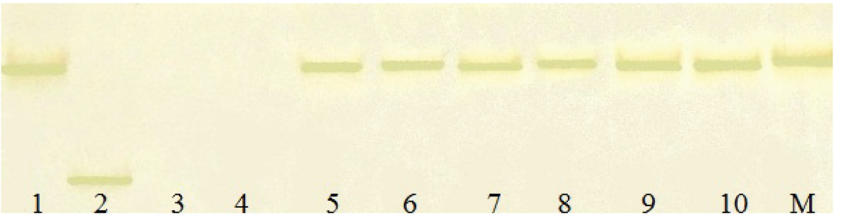

Figure 3: SSR ORS610 profile obtained for: 1 – HA 228; 2 – RHA 419; 3 – *H. argophyllus* accession (PBGI); 4 – *H. argophyllus* accession (IOC); 5 – QHP-1; 6 – FT 226; 7 – HA 335; 8 – Od 108A; 9 – OC 1019B; 10 – OC 1029B; M – molecular marker pUC 19/MspI (band 147 bp).

developed from parental *H. argophyllus* or RHA 419, would allow to identify the genotypes, carrying the Pl_{ARG} determined resistance. Wieckhorst *et al.* (2010) showed a close linkage between Pl_{ARG} and microsatellites ORS716, ORS509, ORS1128, ORS543. Mapping was done on segregating populations from the cross HA342 x ARG1575-2. According to our data, some microsatellite markers of Pl_{ARG} specific to ARG1575-2 genotype could not be used for MAS introgressions for which RHA 419 served as donor of Pl_{ARG}. Based on allelic composition of ORS716 and ORS543, RHA 419 did not differ from other studied lines (Table 1). There was no polymorphism detected in ORS1128 locus among the investigated samples.

Table 1: Genotypes of the studied lines and accessions of *H. Argophyllus* based on the alleles (bp) of microsatellite loci from LG1.

Lines	**Loci**							
	ORS 509	**ORS 605**	**ORS 610**	**ORS 675**	**ORS 1039**	**ORS 1182**	**ORS 716**	**ORS 543**
HA 288	200	202	147	240	201	170	300	244
QHP-I	200	202	147	240	203	170	335	263
FT 226	200	202	147	240	207	170	335	263
HA 335	190	202	147	240	205	170	335	250
RHA 419	207	197	130	220,240	190	165	300	260
Od 108A	200	202	147	240	205	170	335	260
OC 1019B	200	202	147	240	205	170	335	260
OC 1029B	200	202	147	240	205	170	300	263
H. argophyllus accession (PBGI)	207	190	X[1]	220,240	220	165	315	250
H. argophyllus accession (IOC)	207	190	X[1]	220,240	220	165	315	250

[1]No fragment detected.

In our study, we detected alleles of the microsatellite markers, specific for *H. argophyllus*: 190 bp (ORS605), 220 bp (ORS1039), 315 bp (ORS716). For RHA 419 we detected: 197 bp (ORS605), 130 bp (ORS610), 190 bp (ORS1039). Alleles 207 bp (ORS509), 165 bp (ORS1182) and 220 bp (ORS675) allow to identify a portion of LG1, originated from *H. argophyllus* or RHA 419 line.

As the next step, we designed the scheme for introgression of the effective resistance genes against *Plasmopara halstedii*. Line RHA 419 was selected as a donor of Pl_{ARG} gene, and inbred lines OC 1019B and OC 1029B were selected as recurrent genotypes. 1019B and OC 1029B lines are the best restorer-of-fertility inbred lines from PBGI breeding.They were shown to carry alleles, differentiating these lines from Pl_{ARG} donor RHA 419: 197 bp (ORS605), 130 bp (ORS610), 190 bp (ORS1039), 207bp (ORS509), 165 bp (ORS1182).

ORS610 and ORS1039 were utilized for hybridity evaluation of F_1 from the crosses (RHA 419 x OC 1029B) and (RHA 419 x OC 1019B). These SSR are linked to Pl_{ARG} at a genetic distance of 2.1 and 0.3 cM, respectively. Profiling of 16 "potentially hybrid" F_1 genotypes from the cross (RHA 419 x OC 1029B) with ORS610 and ORS1039 markers showed that all of 16 plants were heterozygous in both loci. At the same time, among 16 "potentially hybrid" F_1 plants from the cross (RHA 419 x OC 1019B) only 11 plants had the alleles of both parent lines, other plants were descendants of self-pollination.

30 plants of the F_2 populations (RHA 419 x OC 1019B) and 30 plants of the F_2 populations (RHA 419 x OC 1029B) were obtained from self-pollination of F_1 hybrid plants. All of obtained F_2 plants were genotyped with SSR markers ORS610 and ORS1039. Based on the presence/absence of particular ORS610 or ORS1039 alleles, the F_2 individuals divided into three classes. The first class consisted of plants carrying RHA 419 alleles. The second class included plants carrying OC 1019B or OC 1029B alleles. There were plants carrying alleles from both parents (heterozygous) in third class. Usually SSR markers show the Mendelian mode of inheritance. In F_2 population (RHA 419 x OC 1029B) the marker alleles segregation from both ORS610 and ORS1039 loci fits 1:2:1 ratio ($\chi^2 = 2.19$ for $\chi^2_{0.05}$ (df 2) = 5.99). In the F_2 population (RHA 419 x OC 1019) it was observed the deviation from the expected 1:2:1 Mendelian segregation ratio ($\chi^2 = 7.56$ for $\chi^2_{0.05}$ (df 2) = 5.99) mainly due to a significant lack of plants homozygous for RHA 419 alleles. Such results may be explained by the deficient number of obtained F_2 segregants.

Validated SSR markers that are tightly linked to Pl_6, Pl_8, Pl_{ARG} will be involved in following project of *Pl* genes pyramiding in hybrids with durable resistance to downy mildew.

Conclusion

We have identified alleles of SSR markers, specific for genotypes of the lines, which are known donors of resistance gene *Pl*. Specifically, line HA 335 as donor of gene *Pl6* can be identified with marker *ORS328*; line QHP-1 as donor of gene *Pl8* can be identified with marker *ORS781*. SSR markers ORS509, ORS605, ORS610, ORS1182, ORS1039 were validated to identify line RHA 419, which is known as carrier of resistance gene Pl_{ARG}. Profiling F_1 generation using specified markers allowed to select Pl_{ARG} heterozygous genotypes for producing segregating population F_2. MAS in F_2 allowed identification of Pl_{ARG} homozygous, which were recommended as a source for sunflower breeding that aimed on the development new inbred lines with genetically determined broad-spectrum resistance to downy mildew.

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