SSR Markers Suitable for Marker Assisted Selection in Sunflower for Downy Mildew Resistance

Abstract: The effectiveness of Pl genes is known to be resistant to downy mildew (DM) disease affected by fungus Plasmopara halstedii in sunflower. In this study phenotypic analysis was performed using inoculation tests and genotypic analysis were carried out with three DM resistance genes Plarg, Pl13 and Pl8. A total of 69 simple sequence repeat markers and 241 F2 individuals derived from a cross of RHA-419 (R) x P6LC (S), RHA-419 (R) x CL (S), RHA-419 (R) x OL (S), RHA419 (R) x 9758R (S), HA-R5 (R) x P6LC (S) and HA89 (R) x P6LC (S) parental lines were used to identify resistant hybrids in sunflower. Results of SSR analysis using markers linked with downy mildew resistance genes (Plarg, Pl8 and Pl13) and downy mildew inoculation tests were evaluated together and ORS716 (for Plarg and Pl13), HA4011 (for Pl8) markers showed positive correlation with their phenotypic results. These results suggest that these markers are associated with DM resistance and they can be used successfully in marker-assisted selection for sunflower breeding programs specific for downy mildew resistance.

Keywords: Helianthus annuus L., downy mildew, Plarg, Pl13, Pl8, simple sequence repeats.

1 Introduction

The sunflower (Helianthus annuus L.) is known as the most important crop for the oil industry. Sunflower downy mildew caused by the obligate biotroph Plasmopara halstedii (Farl.) Berl. & de Toni is regarded to be a very damaging leaf tissue disease and has spread to all the countries where sunflower production has been made. Downy mildew (DM) can induce yield loss up to 80% in sunflower production [1]. Pl (Pl1–Pl17, Pl21 and Plarg) downy mildew resistance genes discovered to date in sunflowers and for the source of the Pl genes, wild Helianthus annual species can be followed [2]. These Pl genes that are very effective against P. halstedii races have been mapped in different linkage groups of sunflower: The Pl1/Pl6 locus on linkage group LG8 [3, 4]; the Pl5/Pl8 and Pl21 loci on LG1 [5, 6, 7]; the Plarg locus on LG1 [8]; the Pl13, Pl14 and Pl16 loci on LG1 [8-12].

The main target of sunflower breeding programs is to improve of downy mildew resistance. However, emerging strains of P. halstedii challenge global sunflower production and this has resulted in susceptibility to certain downy mildew strains in many commercial hybrids [13]. Therefore, new hybrids of sunflower are sought that are resistant to DM. The most effective measure of controlling downy mildew is the use of available resistant hybrids. Determination of the resistance genes location in sunflower genome and facilitation of marker assisted introgression for elite germplasm have been supplied with genetic mapping of downy mildew resistance genes [11].

Molecular markers are crucial for understanding genome organization and provide important advantages in the means of development of new lines [14] and determination of differentiation between initial germplasm [15]. The development of molecular markers in sunflower is at an advanced level and different types of markers have been developed for marker-assisted selection (MAS) over the years. There are numerous different molecular markers available which can be used in sunflower
breeding [16]. Pérez-Vich and Berry [17] described three different generations of markers in sunflower research: Firstly, anonymous deoxyribonucleic acid (DNA) markers like RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and genomic SSR (Simple Sequence Repeat) markers were developed [18-24]. Usage of several molecular markers combined with numerous linkage maps makes it possible to develop a hybrid line that provides required properties [25]. There are several linkage maps available to use for marker assisted selection programmes. Researchers completed the first linkage map of sunflower in 2002 [24] and this was improved by another research group in 2003 through usage of new recombinant inbred lines population with SSR markers [26]. Another research group accomplished the genetic mapping of the fertility restoration gene by using SSR and TRAP (Targeted Region Amplified Polymorphism) markers [27]. Molecular markers related to different downy mildew resistance genes have been identified by bulk segregant analysis methods [28]. Mapping studies completed by RFLP and RAPD markers for identification of Pl1 [29], STS (Sequence Tagged Site) markers for identification of Pl5/Pl8 cluster [6], and SSR markers for identification of Pl6 and Pl13 locus. The Pl13 could be a useful source of resistance to the four major races of downy mildew and can be successfully transferred to different genetic backgrounds [30]. The identified markers closely linked to downy mildew resistance are expected to greatly enhance the efficiency of breeding using MAS [9]. Another study showed that Plarg loci provide resistance all known Plasmopara halstedii races [29].

Novel sources of resistance genes and suitable sequence specific molecular markers need to be found to keep up with the new pathogenic strains. More studies will be needed to quantitatively demonstrate resistance to downy mildew. MAS could in this way be used for detecting both major and minor genes and would bring us closer to achieving sustainable resistance to Plasmopara halstedii. Two DM resistance genes, Plarg and Pl8, are highly effective against P. halstedii races in the USA [31]. The objective of this study is to determine resistant sunflower lines for downy mildew using Plarg, Pl13 and Pl8 genes which gain resistance to downy mildew. SSRs were employed for screening resistant and susceptible parental lines and their F2 populations. Results of this study will permit an early selection of downy mildew resistant genotypes without inoculation and symptom detection as well as providing important knowledge for the development of new sunflower lines which are region specific.

2 Experimental Procedures

2.1 Plant Materials

DM-resistant parents RHA-419 (restorer oilseed sunflower which has Plarg resistance gene for downy mildew released by the USDA-ARS and the North Dakota Agricultural Experiment Station, Fargo, ND, USA), HA-R5 (restorer oilseed sunflower which has Pl13 resistance gene for downy mildew released by the USDA-ARS and the North Dakota Agricultural Experiment Station, Fargo, ND, USA), HA89 (oil seed sunflower which is susceptible for downy mildew released by the USDA-ARS and the North Dakota Agricultural Experiment Station, Fargo, ND, USA), DM-susceptible parents P6LC (resistant cultivar to IMI, Orobanche cumana, and downy mildew, Pl6 or Pl8), CL (IMI resistant and high oleic cultivar), 9758R (restorer oilseed sunflower which is susceptible for downy mildew released by Trakya Agricultural Research Institute, Edirne, TURKEY), OL (IMI resistant and high oleic cultivar) and 241 F populations from the cross of the DM-resistant and susceptible parents (Table 1) were used for screening for resistance to downy mildew.

The young leaf tissues of the Helianthus annuus L. species provided by Republic of Turkey Ministry of Agriculture and Livestock General Directorate of Agricultural Research and Policy, Trakya Agricultural Research Institute, Edirne, Turkey, have been used as plant material.

Table 1. Pl genes, parental lines and number of F2 individuals used in this study.

| Gene | Parental Lines | No. of F2 |
|------|----------------|-----------|
| Pl13 (LG1) | RHA-419 x P6LC | 39 |
| Pl1 (LG1) | RHA-419 x CL | 23 |
| Pl13 (LG1) | RHA-419 x OL | 26 |
| Pl13 (LG1) | RHA-419 x 9758R | 102 |
| Pl1 (LG1) | HA-R5 x P6LC | 30 |
| Pl13 (LG13) | HA89 x P6LC | 23 |
| RHA-419: Resistant | 9758R: Susceptible |
| HA89: Resistant | OL: Susceptible |
| HA-R5: Resistant | P6LC: Susceptible |
| | CL: Susceptible |

2.2 Downy mildew inoculation and phenotyping

Phenotypic screening for downy mildew was performed with inoculation tests in 10 replicates. Spores of
different races of *P. halstedii* were cultured on the appropriate susceptible sunflower variety, and then a sporangium suspension was prepared with spores. Spore concentration was adjusted to 30,000 sporangia/ml. For sunflower seed disinfection, seeds were soaked in 1% NaClO suspension for 3 minutes, then were washed in distilled water, sunflower seeds were put in the growth chamber (24-28°C) to germinate until 2-5 mm. rootless were formed. Germinated seeds were incubated in the sporangium suspension for 4 hours at 18°C. Inoculated seedlings were planted in plastic flats or pots filled with a sand/perlite mixture (3:2, v/v). The growing condition was optimum at 24°C temperature and a 12/14 hr photoperiod illuminated with warm-white, high pressure mercury lamps (HGLM-400, Tungsram) to provide the plants with a light intensity of about 12,000 lux. After 8-10 days, when the first true leaves were formed, seedlings were placed in 100% humid, 16-17°C growth chamber for 48 hours. From each plant three segments of about 1 cm were excised, one from each from the lower hypocotyl, the upper hypocotyl and the lower epicotyl. These were washed thoroughly with sterile distilled water, placed in Petri dishes lined with sterile moist filter paper and incubated at 18°C for 48 h in the dark to induce sporulation. Subsequently, white mildew spores could be seen under the cotyledon leaves of sensitive plants. Each plant infection level was assessed as a cotyledon/leaf surface covered with zoosporangiophores using a scale ranging from 0-3 [32] where 0: no sporulation, 1: sparse sporulation, 2: less than 50% of cotyledon/leaf area covered, and 3: more than 50% of cotyledon/leaf surface covered with zoosporangiophores.

### 2.3 DNA extraction and SSR Analysis

Leaf samples were harvested at seedling stage for DNA isolation and SSR analysis. 50-100 mg of plant young leaf tissues were homogenized by using a RetchMM400 mixer mill with liquid nitrogen and genomic DNA of the plants was isolated according to the CTAB method [33], the DNA quantity and quality were determined using a Qubit® 2.0 fluorometer.

A total of 69 SSR markers (12 markers for *Plarg*, 20 markers for *Pl13*, 37 markers for *Pl8*) were screened which showed linkage to the downy mildew resistance gene to identify polymorphisms between the parents (Table 2).

The PCR reagents mixed in a master mix 2X solution, consisting of dH2O, 2X Taq buffer, 2.5 mM MgCl2, 5 mM dNTP mix, Taq Polymerase 0.054 U/µl. Then master mix 2X solution is diluted into master mix X solution with water and primer addition to final volume of 23 µl per sample. The final reaction tube content has been calibrated as 1X Taq Buffer, 2.5 mM MgCl2, 2.5 mM dNTP, 0.8 mM primer, 0.027 U/µl Taq polymerase and 4 ng/µl genomic DNA. The PCR amplification profile included a hot start at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 59-62°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 10 min. Amplified products were run on 2% agarose gel. DNA isolation and SSR studies were performed in 3 replicates.

**Ethical approval:** The conducted research is not related to either human or animals use.

### 3 Results

Results of the downy mildew resistance test for phenotyping of the 241 F2 plants derived from RHA-419 x P6LC, RHA-419 x CL, RHA-419 x OL, RHA419 x 9758R, HA-R5 x P6LC, HA89 x P6LC parental lines were determined as homozygous resistant/susceptible and heterozygous samples numerically. These results were shown with genotyping data obtained using polymorphic markers together (Table 3). Parental (RHA419-resistant x 9758R-susceptible) polymorphism was determined by 2 SSRs (*ORS610, ORS716*) and different combinations of crosses (RHA419-resistant x P6LC-susceptible; RHA419-resistant x CL-susceptible; RHA419-resistant x OL-susceptible) were resulted with polymorphic pattern by 1 SSR (*ORS716*) out of 12 SSRs for *Plarg* gene. Polymorphism was determined between HARS (resistant) and P6LC (susceptible) parental lines by 7 SSRs (*ORS822, ORS803, ORS728, ORS716, HA4090, HA77, ORS1008*) out of 20 SSRs for *Pl13* gene. Amplification with five SSRs (*ORS707, ORS730, ORS215, ORS316, and HA4011*) out of 37 SSRs produced polymorphic pattern between HA89 (resistant) and P6LC (susceptible) for *Pl8* gene. As a result, fourteen SSR markers out of 69 were selected for screening of F2 individuals belong the crosses mentioned above regarding the genetic linkage to Plasmopara resistance genes namely *Plarg, Pl13* and *Pl8*. For this purpose, SSR analysis of 241 F2 plants derived from the cross of RHA-419 x P6LC, RHA-419 x CL, RHA-419 x OL, RHA419 x 9758R, HA-R5 x P6LC, HA89 x P6LC was conducted using fourteen polymorphic SSR markers. SSR results of *ORS716 (Plarg)* for RHA-419 x OL cross and their 26 F2; *ORS716 (Pl13)* for HA-R5 x P6LC cross and their 30 F2; *HA4011 (Pl8)* for HA89 x P6LC cross and their 23 F2 together with their phenotyping results were shown in Figure 1.
### Table 2. The primer sequences of the SSR markers linked to the sunflower downy mildew resistance genes Plarg, Pl13 and Pl8.

| Gene | SSR Marker | Forward Primer (5'-3') | Reverse Primer (5'-3') | Gene | SSR Marker | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|------|------------|------------------------|------------------------|------|------------|------------------------|------------------------|
| Pl$_{13}$ | HT324 | ggC CAC CAC AAC AAT AAT C | ATC AgA ATA TTC AAT AAT C | ORS500 | ACT CTT ggAT TgA AAg CtC C | CgC ACT gCC TTA AAC CTC C |
|      | HT446 | CgT ATT gTC TAT gTg Tgg TgT Tg | AAT CAA Tgg gAA gCT gAA TTT CTT | ORS879 | CTT CgT gTT TgA TgA TTT | gAA CTT CCC TTT gTT gTC ATA C |
|      | HT722 | CgT ATT gTC TAT gTg Tgg TgT Tg | AAT CAA Tgg gAA gCT gAA TTT | ORS1277 | AgT gCT AAT CTT gAA gAg CAC CT | TTT CgA CTg AgAT TgA TgA g |
|      | ORS5371 | CAC ACC ACC AAC CAT CAA CgA | ggT gCT CTC TCC TCC TgT | ORS1244 | CAC ACC AAC CAT CAA CgA | CAC ACC AAC CAT CAA CgA |
|      | ORS5503 | AAC AAC AAC AAC gCA Act | TgA ACC TTT AAA CTT gCT AATC A | ORS5956 | gAT gAg gCC CTT CTT gCT AAT | TgC gAT TAT TCT gAg AAg gTA C |
|      | ORS5509 | CAA CgG AAA AAC gAg AGC AAC | gGC gGA ATT TTA CAA gAg CAA | ORS707 | gCA gTC AAT CgT TaG gAg CAA | gCT gAA gCT gAA gAC AgA TCC |
|      | ORS5543 | CAC gTC ATT gTC TAT AAT gCA Act | gGC gAG gAG gAG gGC gTC gTTC gTg TgT CTTT gTT CTT TTT | ORS7530 | CTT TgT gAg gTT AAT gGC gTC gT |
|      | ORS5610 | AgA AAg CgE AAC AAT gAg gAT gT | TgT gTA CCT TCT CTC TgC Tg | ORS5976 | AAA TTA CAA CTT CCA CAC CTT ATT | CTT TgT TAT TgA gAg CACT AAT CA |
|      | ORS5716 | CCC CCC AAC CCC TAga CTT A | gAA CTA ACC gCC AAC TTA A | ORS215 | CCT TgT CgT AAT gAA gAg CAA | TgT TCA CCA gCA gTT gAg |
|      | ORS5959 | Cgg CTA ACC gAT AAC AAT C | CTT CgT CTT gCC AAT CATT CTT T | ORS317 | gAg CTA TgT CTT AAT TTT gCT | TTT gAg AAg TgT gAg gTA C |
|      | ORS5118-1 | ggC gAT AAT AGA TgC gAC ACT C | TCT gTT CCA CAC CTT TTT CTT Ag | ORS224 | AAA CAA AgC gCT gAA gAA ACT | TgAg ACT AAC TAC CAg AAg CAA C |
|      | ORS5118-2 | TCT TCT gAT TgT gAg CgG TgT gT | gAg CTA TCT CgT CTT CTT gAg CTT | ORS5179 | AAA Cgg gAA gCA gAg AAg AAg gAA gAA | gAg CTA gAg Cg CgC gAg AAg C |
|      | ORS5822 | AAA CAA ACC TTT ggA cGA AAc CCC | gAg CTA CgT gCT gCA gCT | ORS536 | gAA ATg AAg gAg gAg CTT ACC g | gAg gAg AAg AAg gAg AAg gAg |
|      | ORS5598 | ATA gCT CgT gAC gAg gTg ATAg g | CCA AAT gTg AAg Tgg gAg AA | ORS871 | gAT gAg gAg gAg gAg gAg gAg gAg | gCT AAC CCA gCC CCC AAA AA |
|      | ORS5222 | AAT TgAg gCT TAT AAG TgAg gTg A | AgT CgT gCg AAT TAA CCA CTA Cg | ORS1056 | gAg gTT AAT CAg TCA gTg CTT CTA | gAg gTT gAg gTT gTT gTA CTT gTT T |
|      | ORS5474 | gTg TgC gAg gTT AAT gCT TgT gT | gAg ACC TTT gAg gTT CTT | ORS5995 | CAT gCT CTT TgT gAg gAA CAA | TgT TAg TAg CAg AAg CAA AgT |
|      | ORS6050 | Acg gAg cAg AAT TgC gAg gT | CgC gTg TAg TgA CgA CAA TTA T | ORS551 | Cgg gTT gCg ATg TgA gAg ATg TA | TgAg CTA gAg TAAT gAA gCA |
|      | ORS5462 | Agg CTA cCA AAc ggT CTT CAC A | AgC TAg gAg gAg CgC CTT gC T | ORS5103 | CTT TgT CAg TAg gAgg AgA gAg Tg | CgA cTA ATT TAg AAg CCA gAA gTA |
|      | ORS5803 | Ccc gG CcC AAT gAA gGA gT | TTT gCT CAA ACC AAT CTT TTT TTT C | ORS7597 | CTT CCC CAC ATT cCT CTT | TCC gAgg AAg gTA CgA gCA A |
|      | ORS5718 | AgT CAA cAC CcG AAT CAA g | CAC TTT AgC gCC ACC AAA CC | ORS191 | gAg gCT gTT gAg ATg gCtt gT | gAg gCT gAg gCT gAg gCT gAg |
|      | ORS5965 | CAC TTT gAg gAg gAA cCA ACC CCC | TtT CgT ATg gAg TgA TgC CTA C | ORS5581 | ACT TTA TgAT TTT CCA gCA gAC | CTT gAg TTA AAg gCC gAA |
|      | ORS728 | CCA ACC CCT gAA TgA TAC TgT gAg CAA | CTT cTA gCg CAC ACC CAg CAA T | ORS5630 | gCA CcG gCC gAg TTg ATgA gT | gTg CAg gAT gAT gAT gAg CAg |
|      | ORS6662 | CcT TTA cCA AAc Agg AAc cAC cTA A | CgG gTT gAA TAT gAA gT CTA C | ORS5316 | gAg gTT AAg TgA gCT TgC gTg CTA | Tgg CgT gTT CAg TAg AAg CAg |
|      | ORS6607 | CcG cTA AAg gAA Agg gAg AgA | ATC TgA cAg gCA AAg gTA C | ORS4011 | gAg gTT TTT CTA gAT gTg CTT | CTT TgC TAg TAg gAA gTA CAg |
|      | ORS7516 | Ccc cAC AAc CcA TgC CTT A | gAA CTA ACC gCC gAA CAA CAA gT | ORS7597 | gAg gTT aAg CAg TgC CCA gCg C | CTT gAg CAg TAA gTA AAg CCA |
|      | ORS5970 | gTc TTA gAg AgT gAg AAT gTA TgT gT | TgT gTA TTT AAT CAg gAg CAA Tg | ORS317 | TAg TTA gAg gTT gAgAg gTT gAg | TgAg TTT gAg gTA gGg gGA AAA |
|      | ORS5625 | Ccc TTT gAg gAg gAA gTA gAg TgT gT | gCT cCT CgT gCg gTT CTA C | ORS2958 | CTT CCA TgT gCT gCA gCA gC | CTT gAg gAA gCA gAA gCA gAA |
|      | ORS5552 | CCA Tcc CTT CTT CTT CTT TTT | tcC CcC Agg AAc CAC CAA | ORS5179 | CTA gAT TgA TgA gAg CAA TTA | CTT CTA gAg CgA TgC CCA |
|      | HA4090 | gCc AGT ATg gTTC gTT CgC | TgT gGg gAT gAA gAg CAA | ORS4011 | gAg gTT TTT CTA gAT gTg CTT | CTT TgC TAg TAg gAA gTA CAg |
|      | HA77 | TgT gAg CAg TgC CAC CcC CcC | gTT gGg gAT gAA gAg CAA | ORS316 | gAg gTT AAg TgA gCT TgC gTg CTA | Tgg CgT gTT CAg TAg AAg CAg |
|      | ORS365 | CgA ggC AAA ggg TgT CTA A | gAA gAg gAg gAG gAA TgT CTT | ORS317 | TAg TTA ACC ATg gCT gAA gAC gCT g | gTT gAA gAA TAAT gTT gCg CTC gT |
|      | ORS1008 | CgAg gGc gGC CTT gTc gAT gTg TgT | gAT cAC cCT CAC TTA CAA CCA CcC | ORS7597 | gAg gTT AAg ATg TgA TgA gAg gTT C | gAg gTT AAg ATg TgA TgA |
|      | ORS5673 | gAg gTg TCC TCA CcG TCC TTA | TgT gAg CAg TCC CTT ACC TTA | ORS7597 | gAg gTT AAg ATg TgA TgA gAg gTT C | gAg gTT AAg ATg TgA TgA |
|      | ORS534 | gCA gCg AAA TAg gAA AAA Cg | TTT AAA ATT gC TTT CTT CcC | ORS7597 | gAg gTT AAg ATg TgA TgA gAg gTT C | gAg gTT AAg ATg TgA TgA |

The table provides primer sequences for SSR markers linked to sunflower downy mildew resistance genes Pl$_{13}$, Pl$_{14}$, and Pl$_{15}$. The forward and reverse primers are listed for each SSR marker. The primer sequences are crucial for identifying and locating the resistance genes in sunflower plants, which is essential for developing resistance to downy mildew, a fungal disease that严重影响s sunflower production.
Table 3. Genotypic results of polymorphic markers and phenotypic results of inoculation tests.

| Gene          | Parental Lines            | Polymorphic Markers | Genotypic Results | Phenotypic Results |
|---------------|---------------------------|---------------------|-------------------|--------------------|
| \( P_{10} \) (LG1) | RHA-419 x 9758R (102 F₂)  | ORS610              | R:62 S:27 H:1     | R:63 S:10 H:29    |
|               |                           | ORS716              | R:65 S:23         | R:63 S:10 H:29    |
|               |                           | RHA-419 x CL (23 F₂) | R:5 S:6 H:7      | R:3 S:2 H:17     |
|               |                           | RHA-419 x OL (26 F₂) | R:7 S:7 H:12     | R:4 S:5 H:16     |
|               |                           | RHA-419 x P64LC53 (39 F₂) | R:3 S:27 H:7  | R:4 S:5 H:25   |
| \( P_{15} \) (LG1) | HA-R5 x P6LC (30 F₂)      | ORS822              | R:6 S:24          | R:12 S:3 H:9     |
|               |                           | ORS803              | R:13 S:15         |                    |
|               |                           | ORS728              | R:18 S:12         |                    |
|               |                           | ORS716              | R:27 S:3          |                    |
|               |                           | HA4090              | R:6 S:22          |                    |
|               |                           | HA77                | R:28 S:2          |                    |
|               |                           | ORS1008             | R:8 S:21          |                    |
| \( P_{18} \) (LG13) | HA89 x P6LC (23 F₂)      | ORS707              | R:1 S:22          | R:9 S:3 H:9      |
|               |                           | ORS730              | R:9 S:14          |                    |
|               |                           | ORS215              | R:0 S:23          |                    |
|               |                           | ORS316              | R:10 S:13         |                    |
|               |                           | HA4011              | R:17 S:6          |                    |

R: Resistant
S: Susceptible
H: Heterozygous

Figure 1. PCR amplifications of SSR markers and phenotypic results (a) \( ORS716 \) (\( P_{10} \) - RHA-419 x OL and 26 F₂) (b) \( ORS716 \) (\( P_{15} \) - HA-R5 x P6LC and 30 F₂) (c) \( HA4011 \) (\( P_{18} \) - HA89 x P6LC and 23 F₂) M: 100 bp.
3.1 Correlation of Genotypic and Phenotypic Evaluation

When the results of genotyping and phenotyping were evaluated together, ORS716 (for Plarg and Pl13), HA4011 (for Pl8) markers showed positive correlation with their phenotypic results (Figure 1). For Plarg resistance in breeding population of RHA419 (resistant) x OL (susceptible) cross, one F2 individual (#93) was scored as resistant and two F2 individuals (#82, #85) were scored as susceptible by both phenotypic and genotypic evaluation while 7 individuals (#83, #86, #100, #101, #102, #103, #108) were scored as heterozygous both phenotypically and genotypically. Phenotypically nine heterozygous F2 individuals were segregated as 4 resistant (#88, #95, #98, #106) and 5 susceptible individuals (#79, #89, #91, #97, #104) by ORS716 marker (Figure 1a). For Pl13 resistance in breeding population of HA-R5 (resistant) x P6LC (susceptible) cross, eleven F2 individuals (#114, #115, #116, #117, #119, #120, #128, #130, #132, #135, #144) were scored as resistant by both phenotypic and genotypic evaluation while eight F2 individuals (#127, #133, #134, #136, #139, #143, #146, #147) were heterozygous phenotypically but they were scored as resistant by genotypic data. In the frame of these results, ORS716 SSR marker was found very effective for both Plarg and Pl13 genes to identify the downy mildew resistance in sunflower (Figure 1b). For Pl8 resistance in breeding population of HA89 (resistant) x P6LC (susceptible) cross, seven F2 individuals (#279, #281, #284, #288, #292, #293, #302) were scored as resistant by both phenotypic and genotypic evaluation while six F2 individuals (#283, #285, #287, #296, #300, #301) were heterozygous phenotypically but they were scored as resistant by genotypic data by HA4011 marker. The one individual (#282) was scored as susceptible by both phenotypic scoring and HA4011 marker (Figure 1c).

4 Discussion

Downy mildew is an important disease which causes serious yield losses in sunflower cultivation both in Turkey and worldwide. The most promising and powerful solution for resistance to downy mildew is the development of new sunflower lines that show genetic resistance to Plasmopara halstedii races.

Development of new lines by conventional breeding is a question of time and money. Therefore, integration of marker assisted selection to conventional breeding applications is essential. By establishment of a relationship between genes providing resistance to an important trait like resistance to downy mildew, sensitivity and reliability of the breeding programs can be increased. Marker assisted selection methods make breeders able to determine resistance/sensitivity of the plant in early stages of the cultivation and also allows the testing of more than one plant in a shorter period of time.

Classical genetic analysis by phenotyping segregating populations elucidated that Plarg is unlinked to the previous known major resistance loci Pl1, Pl2, Pl5, Pl6, Pl7 and Pl8 which are mainly used in breeding material [34, 35]. There are markers for several Pl genes, including Pl8, Pl13 and Plarg, which are still effective against all strains of P. halstedii [5,6,8-11,13].

Dufle et al. [8], studied 180 SSRs and they found 66 polymorphic markers between Arg1575-2xCmsHA342. Twelve polymorphic SSRs linked to the Plasmopara resistance gene Plarg were identified with the analysis of these 66 SSRs. These SSRs mapped on the same linkage group (LG1), as based on the map of Yu et al. [26], spanning a maximum distance of 9.3 cM. Two fertility restorer lines RHA-419 and RHA-420 were registered by Miller et al. [36] (derived from the cross RHA-373×Arg1575-2) which expressing resistance against Plasmopara races 300, 700, 730, and 770. Combination of Plarg with other known Pl resistance loci should provide a multigenic resistance for sunflower cultivars against new plasmopara epidemics. Radwan et al. [37] reported the association between Pl8 resistance system and hypersensitive response in the hypocotyl. Also, they observed a hypersensitive response for Plarg. Therefore, strategies have to be worked out to conserve the broad function of the resistance gene Plarg. Till now, Pl8 and Plarg confer resistance against all downy mildew races but Pl6 provides resistance to the races 304 and 314 [38]. A couple of studies were discussed how to extend the durability of Pl loci. Combination of monogenic Pl loci and quantitative resistance against downy mildew were proposed by Sakr [39], Tourvieille de Labrouhe et al. [40] and Vear et al. [41]. McDonald and Linde [42] recommended a pyramiding major resistance gene in hybrid cultivars or breeding cultivar mixtures including genotypes with diversified major resistance genes.

More information of the biochemistry and functional basis of resistance was needed for the implementation of these strategies. Mulpuri et al. [9], screened 116 F2 belong to HA-R5 x HA-821 sunflower parent combination using 500 SSR markers. They reported that 42.6% polymorphism determined between HA-R5 and HA-821 from 213 polymorphic bands. Using these polymorphic primers, they showed the association with the downy mildew resistance phenotype on S- and R- bulks and F, population
with identification of 7 SSR markers, including 1 marker from LG10 (ORS1008) and 6 markers from LG1 (ORS965-1, ORS965-2, ORS959, ORS71, ORS605, ORS716). ORS1008 and ORS965 markers were found close to the Pl13 locus comparing with the other markers. Similar to this study ORS1008 marker of Pl13 gene showed polymorphism between HA-R5 x P6LC. 30 F2 from this parental combination were screened using ORS1008 marker and showed 26.6% resistance. Qi et al. [43], identified 361 polymorphic markers from 849 SSR markers using HA-234. [43], identified 361 combination were screened using HA-458 marker and ORS1008 and HA-458 parents include a total of 17 linkage groups (LGs). Their BSA revealed the polymorphism that was

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