Research Article

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

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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 F_2 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.

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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 LG13 [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 F_2 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_2 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 F_2 individuals used in this study.

Gene	Parental Lines	No. of F2
Pl_{arg} **(LG1)**	RHA-419 x P6LC	39
Pl_{arg} **(LG1)**	RHA-419 x CL	23
Pl_{arg} **(LG1)**	RHA-419 x OL	26
Pl_{arg} **(LG1)**	RHA-419 x 9758R	102
Pl_{13} **(LG1)**	HA-R5 x P6LC	30
PL_8 **(LG13)**	HA89 x P6LC	23

RHA-419: Resistant
HA89: Resistant
HA-R5: Resistant
9758R: Susceptible
OL: Susceptible
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 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 dH_2O, 2X Taq buffer, 5 mM $MgCl_2$, 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 $MgCl_2$, 2,5 mM dNTP, 0,8 mM primer, 0,027 U/µl Taq polimerase 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 F_2 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 HAR5 (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 F_2 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 F_2 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 was conducted using fourteen polymorphic SSR markers. SSR results of *ORS716* (*Plarg*) for RHA-419 x OL cross and their 26 F_2; *ORS716* (*Pl13*) for HA-R5 x P6LC cross and their 30 F_2; *HA4011* (*Pl8*) for HA89 x P6LC cross and their 23 F_2 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')	ReversePrimer (5'-3')	Gene	SSR Marker	Forward Primer (5'-3')	ReversePrimer (5'-3')
Pl_{arg}	HT324	ggC CAC CAC AAC AAC ATA AAT C	ATC AgA ATA TCC AAC gCA gCA A	Pl_{8}	*ORS200*	ACT CTT gAT TgA ATg ATg CTC C	CgC ACT gCC TTA AAC CCT C
	HT446	CgT ATT gTC TAT ggT Cgg TgT TT	AAT CAA Tgg gAA gCT ggA TTT C		*ORS879*	CTC Cgg TTg CTg TTg ATg TCT	gAA CCT CCC TTT gTC TgC ATA TC
	HT722	CgT ATT gTC TAT ggT Cgg TgT TT	AAT CAA Tgg gAA gCT ggA TTT		*ORS1277*	ATg gCT AAC ATT CAA gAg CAC CT	TTg CgA TAT AgC gAT TTA TgT gA
	ORS371	CAC ACC ACC AAA CAT CAA CC	ggT gCC TTC TCT TCC TTg Tg		*ORS244*	Agg TgA ATC AAC gAg TgA ATg g	CAC CAC CAC CgC CgT CTC
	ORS503	AAC CAC ACA ACA CAg gCA AgA	TgA ACC TTC AAA CTT gCA ATC A		*ORS596*	CAT gAg ggC ATT CTT gTC ATT	TgC gAT TAT TCT Agg AAg gTC A
	ORS509	CAA CgA AAA gAC AgA ATC gAA A	CCg ggA ATT TTA CAA ggT gA		*ORS707*	gCA gTC AAT TCg TAg CAT Cg	gCT gAA gCT gAA gAC AgA TCC
	ORS543	CCA AgT TTC AgT TAC AAT CCA TgA	gggT CAT TAg gAg TTT ggg ATC A		*ORS730*	CAT gTT Agg TTC AAg ggC ATT T	gCC AAC TgA TgC AAA TCT gA
	ORS610	Agg AAg CgA AAC gAg gAA gT	TTg TgA CCT TCT CCC TgC TC		*ORS976*	AAA TTA CAA CCT CCA CAC CTT AT	CTT TTg TAT TCA AgC ACT AAT CA
	ORS716	CCC CAC AAC CCA TAg CCT AA	gAA CTA ACC gCC ATC CAA gA		*ORS215*	CCT CTg CTg ATT gAA Tgg ATT g	Tgg TTC TCA CCA gCA gTT TAg g
	ORS959	CCg CTA AgT ATA AAC CgC CTA TT	CgT CCT CTT CgC ATC AAT CTT AT		*ORS317*	ggT CgT ATg CTT AAT TCT TTC TCT	TTT ggC AgT TTg gTg gCT TA
	ORS1182-1	ggC gAT AAT AgA TgC gAC ACT C	TCT gTG CCA TAC CAC TTT ATT Cg		*ORS224*	AAC CAA AgC gCT gAA gAA ATC	Tgg ACT AAC TAC CAg AAg CTA C
	ORS1182-2	TCT TCT gAT TgT AAg Cgg TgT TC	TgT CAT gTT CTC TAC CgA gCT TT		*ORS1179*	AAA Cgg gAA gCA AgA ATA gAA CA	gAT TCg gAg CTg TTA ggA ggT Ag
Pl_{13}	*ORS822*	AAA CAA ACC TTT ggA CgA AAC TC	TgC CAT CTg TCA TCA gCT AC		*ORS536*	gAA ATA ggA ggg gAT CTT ACC g	gCg gAg AgA AAg ACg AAg Ag
	ORS598	ATA gTC CCT gAC gTg gAT gg	CCA AAT gTg Agg Tgg gAg AA		*ORS781*	gAT gTg gAg gAg AgA ggg TgT	gTC AAC CCA TgA CCC AAA CC
	ORS222	AAT TgA gCT TCA ATT Tgg Tgg A	ATC CgT gCg AAT TAA CCA TCA g		*ORS1056*	ggT gAA ATC TAg TCA TgT gCC TTA	gTg gTg ggT TTA ATg gTC TTT gT
	ORS474	gTg CTC ggg ATT gAT TCT gT	TgC ACC TTT gTT Tgg ATC TTC		*ORS995*	CAT gCT TTC TAg gAT ggT CAg TT	TgT ATg Tgg Agg CCA ACA AgT AT
	ORS605	ACg gAg CAA AgT TTC gAg gT	CgC gTg ATg TgA CgA TTA TT		*ORS511*	Cgg gTT gCg AgT AAC Agg TA	Tgg CTC AgA TTA AgT TCA CAC Ag
	ORS462	AAg CTA ACA AAC ggT CTT CAC A	ACA TTg ATT CTC gCg gTT CT		*ORS1030*	CCT TTg ATg TAg TTA Agg AAg TTg Tg	CgA TCA ATT TAT ATg ACC gAA TTA CC
	ORS803	ACC gCC gCA TAT AAA ggA gT	TTC CCA CCC ATC TTC ATC TC		*ORS799*	ACT CCC TCC CAT TCT CgT CT	TCC AgC AAg TCA gCA ACA AC
	ORS718	ATg CAA CAC CCg AAT CAA Ag	CAC TTT ACg CAC ACC AAA CC		*ORS191*	ACT gCg TTT gTg ATT ACT ggT g	CAT gCA CTg AAg ACA TAC ACC C
	ORS965	CAC TTT ACg CAC ACC AAA CC	TTg gAT TAC CTT ggA TAg TCA gC		*ORS581*	ATC TTA Tgg TCC gCA CAA gC	TCT CgT ATA ACg TgC CCT gA
	ORS728	CCA ACC TCT gAA TgA TAC TTg TgA C	CTC CAT AgC AAC CAC CTg AAA		*ORS630*	gCA CgA CCC ggA TAT gTA AC	TgT gCT gAg gAT gAT ATg CAg
	ORS662	CCT TTA CAA ACg AAg CAC AAT TC	Cgg gTT ggA TAT ggA gTC AA		*ORS316*	gAg ATT TgA gCT TCg TgT TgC	Tgg CgT CTT CAT AgC ATC Ag
	ORS675	Cgg CTA AgA gAA Agg gAg AgA	ATC TgA AAT Cgg ACA AgA TTC A		*HA4011*	ACT TCT ACC CTC CCC TTC TT	CTg TAC ACg TgC TgC TTT Ag
	ORS716	CCC CAC AAC CCA TAg CCT AA	gAA CTA ACC gCC ATC CAA gA		*HA1626*	gAT gTT ACA CgT TAg CAA Cg	gAA CTC AgC CTA AAA gTC
	ORS970	gTC ATA Tgg ATC ATg AAA ATg TTA gTg	TTg TgA TTT AAC TAT CAg gTg ACA Tg		*HA3417*	TAA TTg ATT ggg ggT AAA Tg	TAT gAT TTg gTg TgC TCA gA
	ORS425	CCC TTg gTC ATg TgT TgT gT	gCT CTC TCT CTT Cgg gTT CAV		*HA2598*	TTC TCA TgT gCT CAA AgA Tg	CCT gAA CCC TTT TgT TTC TT
	ORS552	CCA TCC CTT CCC TCT CTT TC	gTg gCT ggT ATC TCA TCA CC		*HA3330*	ggC TgA gTA AAT gCC AAA TAC gg	ggT TgT TgA TTA CAA gCT CTC C
	HA4090	gCC ATg ATT ggC TAA ggT TCg	TgC gTT ACC gAC AAC ACA Agg		*HA4208*	CCC gCA ATT gAA TAC gCg ACA TC	CAT CTC gTT gCC CgT TAA CTA TC
	HA77	TgT AAT CTg TAT CAC TTC CAC C	gTT gTT CTg TTA ggT CgT TCC g		*HANT8R1*	gCC CAA AAT TgA AAg AAA ggT gTg	ggC gAA ATT ggT TCC CgT gAg TCg
	ORS365	CgA ggC AAA ggg TgT CTA AA	gAA gCg AAg gAA Tgg Tgg TT		*HANT8R3*	TAg TTA ACC ATg gCT gAA ACC gCT g	TTT gAA AgA TAA gTT CgC CTC TCg
	ORS1008	CAT gAg ggC ATT CTT gTC ATT T	gAT CAC CTT CAC TAT CCA CAA CC		*HANT8R4*	ggT TTg AAg ATA TgT CgA gTT ggg	CCC AAC TCg ACA TAT CTT CAA ACC
Pl_{8}	*ORS673*	ggC ATg TTC TCA CCg TTC AT	Tgg TgC TAC TCC ATC CTT gA		*HANT8R5*	TAg TTA ACC ATg gCT gAA ACC gCT g	CCC CAT ATT gAC AAA gAg TTg Agg
	ORS534	gCA gCg AAA TAg gAA AAA Cg	TCC AAA CTC TCT CCC CCT CT		*HANT8R6*	TAg TTA ACC ATg gCT gAA ACC gCT g	CgT CTC Tgg TAg ATC gTT CAC CTT
	ORS1009	CCA TTT ACC CTT TAC ACg gAT CT	TAT gTA Tgg TCg CCA ATT TAC CA				

Table 3. Genotypic results of polymorphic markers and phenotypic results of inoculation tests.

Gene	Parental Lines	Polymorphic Markers	Genotypic Results	Phenotypic Results
Pl_{arg} **(LG1)**	RHA419 x 9758R (102 F_2)	*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 F_2)	*ORS716*	**R:**5 **S:**6 **H:**7	**R:**3 **S:**2 **H:**17
	RHA-419 x OL (26 F_2)		**R:**7 **S:**7 **H:**12	**R:**4 **S:**5 **H:**16
	RHA-419 x P64LC53 (39 F_2)		**R:**3 **S:**27 **H:**7	**R:**4 **S:**5 **H:**25
Pl_{13} **(LG1)**	HA-R5 x P6LC (30 F_2)	*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	
Pl_8 **(LG13)**	HA89 x P6LC (23 F_2)	*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

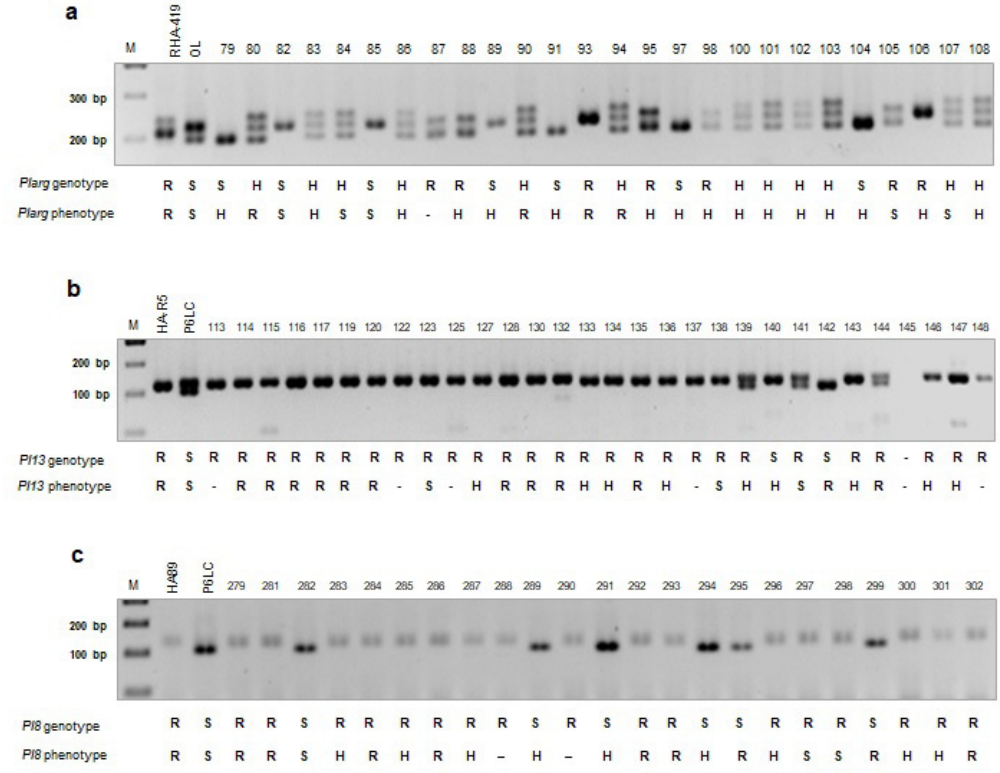

Figure 1. PCR amplifications of SSR markers and phenotypic results **(a)** *ORS716* (*Plarg* - RHA-419 x OlL and 26 F_2) **(b)** *ORS716* (*Pl13* - HA-R5 x P6LC and 30 F_2) **(c)** *HA4011* (*Pl8* - HA89 x P6LC and 23 F_2) **M:** 100 bp.

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 F_2 individual (#93) was scored as resistant and two F_2 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 F_2 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 F_2 individuals (#114, #115, #116, #117, #119, #120, #128, #130, #132, #135, # 144) were scored as resistant by both phenotypic and genotypic evaluation while eight F_2 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 F_2 individuals (#279, #281, #284, #288, #292, #293, #302) were scored as resistant by both phenotypic and genotypic evaluation while six F_2 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].

Dußle *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 F_2 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_2 population

with identification of 7 SSR markers, including 1 marker from LG10 (*ORS1008*) and 6 markers from LG1 (*ORS965-1, ORS965-2, ORS959, ORS371, 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 F_2 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 and HA-458 parents include a total of 17 linkage groups (LGs). Their BSA revealed the polymorphism that was produced by *ORS1197* and *ORS963* SSR markers on LG4 of sunflower genomes between the S- and R- bulks and they reported that all homozygous susceptible plants in S-bulk revealed the HA-234 allele, but 10 homozygous resistant plants in R-bulk showed the HA-458 allele. Qi *et al*. [2], studied with a new dominant downy mildew resistance gene (*Pl18*) transferred from wild *Helianthus argophyllus* (PI494573) into cultivated sunflower was mapped to LG2 of the sunflower genome by BSA using 869 SSR markers and they showed the validation of the resistance single gene inheritance with phenotyping 142 $BC1F_{2:3}$ families derived from the cross of HA89 and *H. agophyllus*. The *Plarg* gene gives resistance in effect to the *P. halstedii* strains detected up to now in the USA and France and *Pl8 gene* gives resistance (98%) to the *P. halstedii* isolates [13, 44, 45]. However, *P. halstedii* races that appear new in North America and France have overcome various DM resistance genes commonly used in sunflowers, such as *Pl6* (from *H. annuus)* and *Pl7* (from *H. praecox)*, although they were published simultaneously as *Pl8* [13, 44]. The *P. halstedii* populations are much active and constantly alter the virulence system, thus continuing the search and experiment of the downy mildew resistance genes introgression from *H. argophyllus* and other wild species.

In this study SSR markers were employed to identify resistance linked with genes *Plarg*, *Pl8* and *Pl13* in 241 F_2 progeny from the cross RHA-419 x P6LC, RHA-419 x CL, RHA-419 x OL, RHA419 x 9758R, HA-R5 x P6LC combination of resistant and susceptible parent lines. As a result of screening the markers on sunflower crosses, 14 SSRs produced polymorphic pattern between resistant and susceptible parents. Two markers (*ORS716* and *HA4011*) showed high similarity results with resistance test results when compared with others markers used in this study. This correlation between genotypic and phenotypic results revealed that these markers could be very useful for marker-assisted selection studies focused on downy mildew resistance in sunflowers.

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