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Association of SSR markers with partial resistance to *Sclerotinia sclerotiorum* isolates in sunflower (*Helianthus annuus* L.)

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Abstract

Basal stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most important diseases of sunflower in the world. The objective of present study was to estimate the number of markers and genomic region associated with basal stem rot resistance in sunflower. The genetic variability among 15 sunflower genotypes from different countries of origin was evaluated using 38 simple sequence repeat (SSR) markers. 88 markers were generated at 38 SSR loci and the mean for the number of allele per locus was 2.32. The UPGMA clustering algorithm based on Jaccard's coefficient of similarity classified the studied genotypes into three distinct groups. Marker-trait associations were studied for disease susceptibility of 15 sunflower genotypes against seven *S. sclerotiorum* isolates through one way ANOVA using a set of 88 SSR markers. Using ANOVA method fifteen putative SSR markers were detected for partial resistance against isolates. Two out of 15 SSR markers was common between some isolates whereas the others were specific for each isolate. ORS 1265 was common for SSU35 and SSU87isolates, and ORS 149 was common for SSU53 and SSU55 isolates. Regarding isolate-specific and isolate-nonspecific markers detected for partial resistance, it is evident that both genetic effects control partial resistance to *S. sclerotiorum* isolates. This confirms the need to consider different isolates in the basal stem rot resistance breeding programs.

Keywords: Association analysis, isolate-specific partial resistance, isolate-nonspecific partial resistance, phenotype-marker association, simple sequence repeat, single-marker analysis.
Abbreviations*:* AFLP- amplified fragments length polymorphism; ANOVA- analysis of variance; GLM- general linear model; MAS- marker-assisted selection; SSR- simple sequence repeat; QTLs- quantitative trait loci; SRAP- Sequence-related amplified polymorphism; UPGMA- un-weighted pair-group method using arithmetic average.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important annual species grown worldwide mostly as a source of vegetable oil. *Sclerotinia sclerotiorum* (Lib.) de Bary, which causes white rot and wilt, is a common and widespread pathogen of sunflower. Originally identified on sunflower in 1861, the fungus has been reported from all sunflower growing regions of the world (Gulya et al., 1997). The necrotrophic pathogen exhibits little host specificity and has a host range that includes more than 400, primarily dicotyledonous plant species (Boland and Hall, 1994). *S. sclerotiorum* can attack all parts of the plant. Sclerotinia can reproduce asexually by the production of a resting stage, sclerotia, which can undergo mycelial germination and attack the roots and basal stems of sunflower plants. However, sclerotinia can also reproduce sexually producing apothecia, which release ascospores directly into the atmosphere and these can attack susceptible aerial plant organs (Ekins et al., 2002). Soil and climatic conditions in production areas influence the plant part most attacked (Tourvieille de Labrouhe et al., 1992). In Iran, infections of the sunflower basal stem are considered a potential threat to the entire crop. The inoculums can survive in the soil for several years. Yield reductions depend on the growth stage at which plants are infected. General yield losses are reported to vary from 1 to 20%, but may reach up to 100% (Gulya et al., 1997). Rapid drying of the leaves and development of lesions on the tap roots and basal portion of the stem provoke plants to die within a few days after the onset of wilting (Dorrell and Huang, 1978). Chemical control of *S. sclerotiorum* is difficult and uneconomical (Mestries et al., 1998), genetic control appears to be of great value. Comparable to other crops, such as soybean (Kim and Diers, 2000), no source of complete resistance to *S. sclerotiorum* is available in cultivated sunflower. However, reports of identification of sunflower genotypes with moderate or low susceptibility are common in literature (Gulya et al., 1997; Davar et al., 2010). Resistance to *S. sclerotiorum* is polygenic and under additive control (Vear and Tourvielle, 1984; Castano et al., 1992; Davar et al., 2010), so that breeding programs have to combine favorable genes from different sources. Utilization of molecular markers to aid breeders in selecting genotypes with desirable traits through marker-assisted selection (MAS) has proved to be very effective in plant species. However, the success of MAS largely depends on the extent of genetic linkage

Table1. SSR markers detected for partial resistant to *Sclerotinia sclerotiorum* in sunflower inbred lines using single-marker analysis

Isolate	QTL	MS	F	Pr > F	$\bar{X}_0$	$\bar{X}_1$	R^2
SSKH2	ORS 988	1252.59	12.49	0.0037	93.08	73.70	0.49
	ORS 899	755.97	5.46	0.0361	73.52	87.75	0.30
	ORS 920	719.52	5.09	0.0419	70.37	85.06	0.28
SSKH26	ORS 996	614.85	4.68	0.0497	94.17	78.16	0.26
	ha4136	617.80	4.71	0.0490	83.88	65.00	0.27
	ORS822	614.85	4.68	0.0497	78.16	94.17	0.26
	ha2682	617.80	4.71	0.0490	83.88	65.00	0.27
	ha1604	795.37	6.78	0.0219	76.97	93.44	0.34
SSU35	ha3639	1073.37	6.49	0.0243	69.13	50.00	0.33
	ORS 822	1680.00	14.14	0.0024	52.71	73.93	0.52
	ORS 1265	884.97	4.92	0.0450	60.19	79.39	0.27
	ha3878	1073.37	6.49	0.0243	50.00	69.13	0.33
SSU53	ORS 149	1012.21	4.97	0.0498	61.27	94.50	0.33
SSU55	ORS 149	1089.61	14.16	0.0024	65.83	100.00	0.52
SSU73	ORS 880	1253.46	9.87	0.0078	61.44	82.11	0.43
SSU87	ORS 1265	1260.42	8.08	0.0138	73.19	96.11	0.38

For each isolate the first two letters refer to *Sclerotinia sclerotiorum* Lib. de Bary. The third and fourth letters show the abbreviated name of the locations where the isolates were collected. KH: Khoy; U: Urmia. The locations were ~200 km apart. QTL: Quantitative trait loci; $F=\frac{MS_{\text{Between marker classes (absence vs presence)}}}{MS_{\text{Between lines within marker classes}}}$; $\bar{X}_0$: Mean disease severity of individuals within marker class '0'; $\bar{X}_1$: Mean disease severity of individuals within marker class '1'; R^2: Percent of phenotypic variation explained by each marker.

between markers and quantitative trait loci (QTL) (Virk et al., 1996). Mapping of QTLs is carried out with materials obtained from systematic breeding populations such as F_2 or recombinant inbred lines. Identification of markers associated with important traits in a group of genotypes through ANOVA or regression analysis offers an alternative mean, as has been used in several plants species (Virk et al., 1996; Baldini et al., 2002, 2004; Darvishzadeh et al., 2008; Selvaraj et al., 2011). In numerous studied DNA markers associated with different traits were identified. Baldini et al. (2002, 2004) used single-marker regression and identified several AFLP and SSR markers associated with basal stem resistance to *S. sclerotiorum* in sunflower. In globe artichoke putative SRAP (Sequence-related amplified polymorphism) markers linked to two important agronomic traits, head color and precocity of production, were detected (Martin et al., 2008). The objectives of the present study were to evaluate the genetic variability and to identify putative SSR markers associated with partial resistance to *S. sclerotiorum* isolates using single-marker analysis. The SSR markers associated with partial resistance to different isolates could be used in pyramiding polygenes in sunflower disease breeding programs.

Results and discussion

Genetic diversity of 15 sunflower genotypes was assessed by using 38 microsatellite (SSR) markers. In the current study, the mean number of allele per locus is 2.32, which is close to that obtained by Hvarleva et al. (2007). It is much lower than the mean number of allele per locus reported in other studies for inbred lines and hybrids (Tang and Knapp, 2003; Yu et al., 2002). The lower value obtained in this study may be due to the low number of markers analyzed in comparison with previous studies. For example, Tang and Knapp (2003) used 122 microsatellite marker loci for genotyping 9 elite confectionery and oilseed sunflower inbred lines and 3.5 allele per locus averagely reported. The un-weighted pair-group method using arithmetic average (UPGMA) clustering algorithm based on a Jaccard's coefficient of similarity classified the studied genotypes into three distinct groups (Figure 1). This is implying that there is high level of genetic variation among studied sunflower genotypes. Using single-marker analysis, putative SSR markers associated with partial resistance to *S. sclerotiorum* isolates were identified (Table 1 and 2). ANOVA method revealed that 3, 5, 5, 1, 1, 1 and 1 SSR markers associate with partial resistance to *S. sclerotiorum* isolates SSKH2, SSKH26, SSU35, SSU53, SSU55, SSU73 and SSU87, respectively (Table 1). The phenotypic variance explained by each marker (R^2) ranged from 26 to 52% (Table 1). The markers ORS988, ORS899 and ORS920 were identified to associate with partial resistance to SSKH2 isolate. Five SSR markers were detected for SSKH26 isolate. Five markers were identified for SSU35 isolate, among these markers; ORS1265 marker was common with the SSU87 isolate. These two isolates (SSU38 and SSU87) come from the same region 'Urmia". Similarly, ORS149 marker was associated with partial resistance to SSU53 and SSU55 isolates. One marker was associated with SSU73 isolate. Two out of 15 SSR markers was common between some isolates whereas the others were specific for each isolate. ORS 1265 was common for SSU35 and SSU87isolates, and ORS 149 was common for SSU53 and SSU55 isolates (Table1 and 2). Common marker for isolates seems to be more important as it gives a constitutive perform-

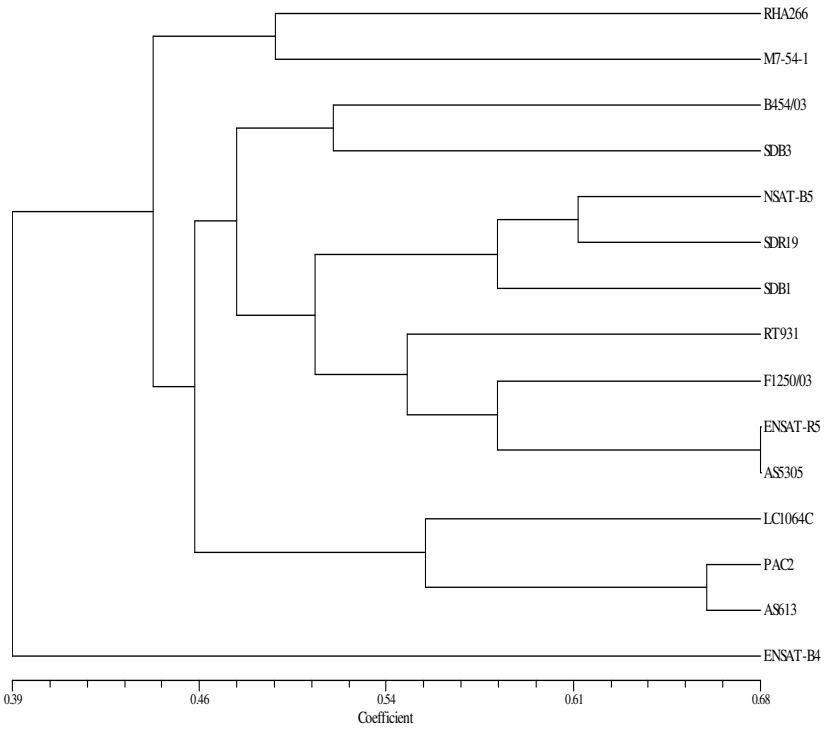

Fig 1. Dendrogram of 15 sunflower genotypes generated by the UPGMA clustering method based on a Jaccard's coefficient of similarity matrix.

ance for the traits without being affected by different isolates. However, the number of specific markers for each isolate and their effect (R^2) was much more than common markers, which explain the significant 'genotype-isolate' interaction. Result of the present investigation substantiates the usefulness of applying statistical approach for identifying one or a few most relevant marker(s) associated with specific *S. sclerotiorum* resistance through screening SSR markers. Molecular markers linked with QTL/major genes for traits of interest are being routinely developed in many crops utilizing materials derived from planned crosses such as F_2, RIL, DH populations, etc. Hopefully, some of these markers will be used for MAS in sunflower future breeding programs. However, non-availability of mapping populations and substantial time consuming of developing such populations are sometimes major limitations in the identification of molecular markers for specific traits (Gupta et al., 2005). To overcome these limitations, and as an alternative to planned populations, molecular markers for traits of interest have been detected through association studies conducted using germplasm collections (Gupta et al., 2005). Such association studies have been sparingly conducted in crop plants, involving the use of germplasm collections for the identification of molecular markers in cereal species such as rice (Virk et al., 1996; Selvaraj et al., 2011), barley (Pakniyat et al., 1997), wheat (Roy et al., 2006) as well as in sunflower (Yu et al., 2002; Baldini et al., 2002, 2004; Poormohammad Kiani et al., 2008, Darvishzadeh et al., 2008). Baldini et al. (2002, 2004) using single-marker analysis of AFLP and SSR data detected a combination of markers in association with *S. sclerotiorum* resistance in sunflower. Miller et al. (2004) identified one TRAP marker possibly associated with Sclerotinia susceptibility in the F_2 population. In conclusion, the present result, offers an approach for identifying a set of DNA markers projecting significant association with *S. sclerotiorum* resistance. Fifteen SSR markers were detected for partial resistance against seven *S. sclerotiorum* isolates. Two out of 15 SSR markers was common between some isolates whereas the others were specific for each isolate. Regarding isolate-specific and isolate-nonspecific markers detected for partial resistance, it is evident that both genetic effects control partial resistance to *S. sclerotiorum* isolates. This confirms the need to consider different isolates in the basal stem rot resistance breeding programs.

Materials and methods

Plant material and phenotypic data

The disease reaction data of fifteen sunflower genotypes from different countries of origin (Table 2) against seven *S. sclerotiorum* isolates were used in present study. Detail information on the levels of susceptibility of genotypes to *S. sclerotiorum* isolates is available elsewhere (Davar et al., 2011). Fifteen sunflower genotypes were selected among 184 genotypes on the basis of their susceptibility to *Phoma macdonaldii* and *S. sclerotiorum* isolates (Darvishzadeh et al. 2007, 2010a, Davar et al., 2011).

Molecular experiments

Genomic DNA of genotypes was extracted from the leaves of 4-weeks-old seedlings using the method described by Dellaporta et al. (1983). Genomic DNA was re-suspended in 100µl TE (10mM Tris pH 7.0, 1mM EDTA pH 8.0). The concentration of each DNA sample was determined spectrophotometrically at 260 nm (BioPhotometer 6131; Eppendorf, Hamburg, Germany). The quality of the DNA was checked by running 1µl DNA in 0.8% (w/v) agarose gels

Table2. Sunflower lines with their observed susceptibility values to *Sclerotinia sclerotiorum* isolates and their SSR markers associated with partial resistance.

Sunflower genotype	Origin	*Sclerotinia sclerotiorum* isolate							
			SSU53		SSU55		SSU73		SSU87
		DS	ORS 149	DS	ORS 149	DS	ORS 880	DS	ORS 1265
RHA266	USA	53.00	-	100.00	+	54.17	-	95.83	-
ENSAT-B4	France	51.67	-	57.50	-	61.25	-	54.00	-
B45403	Hungary	65.75	-	68.33	-	72.00	-	72.20	-
ENSAT-B5	France	61.60	-	73.33	-	80.00	+	73.33	-
RT931	France	66.67	.	73.33	-	65.83	+	100.00	+
F125003	Hungary	65.00	-	75.00	-	81.67	+	97.00	+
LC1064C	France	35.75	-	75.00	-	84.17	+	51.60	-
PAC2	France	50.20	-	58.33	-	62.00	+	65.00	-
ENSAT-R5	France	70.40	-	60.00	-	98.00	+	80.00	-
SDB1	USA	60.20	.	55.00	-	83.20	+	91.33	+
M7-54-1	France	55.60	-	56.00	-	58.33	-	72.50	-
SDR19	USA	87.50	-	74.50	-	98.33	+	76.83	-
AS613	France	77.50	-	70.00	-	85.00	+	86.20	-
AS5304	France	61.60	.	52.00	-	70.83	+	62.50	-
SDB3	USA	94.50	+	73.33	-	94.17	+	88.33	-

DS: disease severity; (+): presence of each marker; (-): absence of each marker.

Table2 (Continue)

Sunflower genotype	*Sclerotinia sclerotiorum* isolate														
		SSKH2				SSKH26						SSS45			
	DS	ORS 988	ORS 899	ORS 920	DS	ORS 996	ha4136	ORS822	ha2682	ha1604	DS	ha3639	ORS 822	ORS 1265	ha3878
RHA266	100.00	-	+	+	84.17	+	-	-	-	-	93.00	-	+	-	+
ENSAT-B4	78.75	-	+	+	90.00	-	-	+	-	+	46.67	-	+	-	+
B45403	85.00	+	-	+	85.83	+	-	-	-	-	88.67	+	-	-	-
ENSAT-B5	99.17	-	+	+	100.00	+	-	-	-	+	69.60	-	-	-	+
RT931	87.50	-	-	+	65.00	+	+	-	+	-	65.17	-	+	+	+
F125003	72.50	+	-	-	91.25	+	-	-	-	+	100.00	-	+	+	+
LC1064C	58.00	+	-	-	82.50	+	-	-	-	-	54.00	-	-	-	+
PAC2	63.83	+	+	+	71.67	+	-	-	-	-	51.60	+	-	-	-
ENSAT-R5	78.00	+	-	-	89.17	+	-	-	-	-	68.00	-	+	-	+
SDB1	63.83	+	-	+	100.00	-	-	+	-	-	81.50	-	+	+	+
M7-54-1	68.33	+	-	-	65.00	+	+	-	+	-	35.00	+	-	-	-
SDR19	86.67	+	+	+	92.50	-	-	+	-	+	58.25	-	+	-	+
AS613	100.00	-	+	+	70.00	+	-	-	-	-	86.00	+	-	-	-
AS5304	75.00	+	-	-	61.67	+	-	-	-	-	49.17	-	+	-	+
SDB3	85.83	+	+	+	71.67	+	-	-	-	-	86.00	-	-	-	+

DS: disease severity; (+): the presence of each marker; (-): the absence of each marker.

in 0.5X TBE buffer (45mM Tris base, 45mM boric acid, 1mM EDTA pH 8.0). DNA samples that gave a smear in the gel were discarded. Thirty eight microsatellite markers out of 339 'ORS' SSR markers from the SSR database (Tang et al. 2002) and 180 'HA' SSR markers developed by INTA (Paniego et al. 2002) were used for DNA fingerprinting (Table 3). The choice of SSR markers was based on clarity of produced bands and their genetic locations in order to give a uniform coverage of the sunflower genome (Poormohammad Kiani et al., 2007; Tang et al., 2002). Each PCR amplifications was performed in 20µl solution containing 2.5µM of each SSR primer, 0.4 Units *Taq* DNA polymerase (Life Technologies), 100µM of each dNTP (Promega), 2µl 10X PCR buffer, 2.5 mM MgCl2 (Promega), 0.20µl of stabilizer (1% W-1 (v/v), Life Technologies), ddH2O and 25 ng template DNA in a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany) (Darvishzadeh et al., 2010b). Touchdown PCR was used to amplify all the SSRs investigated as follows: 95ºC for 3 min, 1 cycle of 94ºC for 30s, 64ºC annealing for 30s, 72ºC for 45s, followed by 10 cycles with a decrease in annealing temperature of 1ºC per cycle, followed by 33 cycles of 94ºC for 30 s, 54ºC for 30 s and 72ºC for 45 s. A final extension was done for 20 min at 72ºC. The reaction products were then mixed with an equal volume of formamide dye [98% (v/v) formamide, 10 mM EDTA, bromophenol blue and xylene cyanol] and resolved in a 3% (w/v) agarose gel in 0.5X TBE,

Table3. Primer sequences and linkage groups of the 38 SSR loci applied to study genetic diversity of 15 sunflower genotypes.

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Linkage group	Number of alleles	Reference
ORS 149	GCTCTCTATCTCCCTTGACTCG	TGCTCTAAGATCTCAGGCGTGC	LG1	3	Tang et al. 2002
ORS 160	TCCCTTCCTTTCATCGTCTGCT	TGGCAATTTGCCAAGGACC	LG8	2	Tang et al. 2002
ORS 16	GAGGAAATAAATCTCCGATTCA	GCAAGGACTGCAATTTAGGG	LG12	2	Tang et al. 2002
ha3878	TTTGTTTAGCATCATCATCATC	GAGACCCTAACCATAACATGA	LG8	2	Poormohammad Kiani et al 2007
ha3513	TGACCCATTCAACTTCTTAA	TCATGGTTCCTGATGAGAAT	LG8	3	Poormohammad Kiani et al 2007
ha2505	GTGTCATGACTCGGT	GGACAATGTGATTGC	-	3	-
ha1604	GCAAATGCACTAAAGGCCCC	CCCTACTCAAACCTTACCTC	LG9	3	Poormohammad Kiani et al 2007
ORS 880	AAGTAGCTTTGCTTTCCTTCGTC	CGAAACGCGGATTATTGTCTTAT	-	2	-
ORS 928	CATGGTTATTTTGGTTTGGGTTT	GCTATTATCATGTCCTTGTCCTTTT	LG7	2	Tang et al. 2002
ha2682	CACAATCGTTTCTTTCCAAAA	ACCCATATGCCCACTCATAA	LG5	3	Poormohammad Kiani et al 2007
ORS 920	CGTTGGACGAAGAACTTGATTT	ACTTCCGTTTGTTCCGAGCTT	LG16	2	Tang et al. 2002
ha3555	GATATCTCTCATAAGTGCCG	GGTCTTGTGATGACGAA	LG12	3	Tang et al. 2002
ORS 58	TGTACCAAGGGTCGTTGTCA	CGACCCCGAGTTTTGTTG	-	3	-
ORS 154	GCACCTTTGGTGAGGAGATA	TGCATCAGTAGCTATTGTCTAT	LG8	2	Tang et al. 2002
ORS 1068	AATTTGTCGACGGTGACGATAG	TTTTGTCATTTCATTACCCAAGG	LG4	4	Tang et al. 2002
ORS 1265	GGGTTTAGCAAATAATAGGCACA	ACCCTTGGAGTTTAGGGATCA	LG9	2	Tang et al. 2002
ha4142	GAGTCGACATTTTCGGAAATCG	CTTCATCTTCTGACACCCAAC	LG3	2	Poormohammad Kiani et al 2007
ha3651	GGAATTATCCATTGTAGGTTTGG	GGATGATTGATTAATTGAGGG	-	2	-
ha4149	CAAAAACCTCTCTCCGTTGGC	GACTCCAAAGTCCACCAAATC	-	2	-
ha2879	CATACCGTTCTTGTTC	CAACCTCCTAGGTCA	-	2	-
ha4057	AAACCCTTCCGACTTATCTC	TAAAGAGAGAGCCCAACAAG	LG3	2	Poormohammad Kiani et al 2007
ha3638	GACATAATCACTAGTTGTTGGTGC	CTCCTCCCACCTCAACAATTTC	LG9	2	Poormohammad Kiani et al 2007
ha3639	GCAACATGCAGTTCCTAATCAAAC	TCACCGAACTTCAATATCACCAC	LG12	2	Poormohammad Kiani et al 2007
ha3691	GAATGAAGCATGTGGAAGGCGG	GTGGAGGTGATGATGGTATGAG	LG10	2	Poormohammad Kiani et al 2007
ha4136	CCTATTCCTGATAATTCACTAAGC	GGTAGCATGCTTACATTAAGATG	LG5	3	Poormohammad Kiani et al 2007
ORS 423	TCATATGGAGGGATCTGTTGG	AAGCAACCATAATGCATCAGAA	LG2	2	Tang et al. 2002
ORS 718	CACTTTACGCACACCAAACC	ATGCAACACCCGAATCAAAG	LG3	2	Poormohammad Kiani et al. 2007
ORS 844	ACGATGCAAAGAATATACTGCAC	CATGTTTAATAGGTTTTAATTCTAGGG	LG9	2	Tang et al. 2002
ORS 878	TGCAAGGTATCCATATTCCACAA	TATACGCACCGGAAAGAAAGTC	LG10	2	Tang et al. 2002
ORS 613	GTAAACCCTAGGTCAATTTGCAG	ATCTCCGGAAAACATTCTCG	LG10	2	Tang et al. 2002
ORS 988	TTGATTTGGTGAAAGTGTGAAGC	CGAACATTATTTACATCGCTTTGTC	LG17	2	Tang et al. 2002
ORS 899	GCCACGTATAACTGACTATGACCA	CGAATACAGACTCGATAAACGACA	LG16	2	Tang et al. 2002
ORS 996	CGGTGAGAATAACCTCGGAAGA	ATCAGTCCTTCAACGCCATTAGT	LG16	2	Tang et al. 2002
ORS 1088	ACTATCGAACCTCCCTCCAAAC	GGATTTCTTTCATCTTTGTGGTG	LG10	2	Tang et al. 2002
ORS 488	CCCATTCACTCCTGTTTCCA	CTCCGGTGAGGATTTGGATT	LG3	3	Tang et al. 2002
ORS 598	CCAAATGTGAGGTGGGAGAA	ATAGTCCCTGACGTGGATGG	LG1	3	Tang et al. 2002
ORS 822	CAATGCCATCTGTCATCAGCTAC	AAACAAACCTTTGGACGAAACTC	LG1	2	Tang et al. 2002
ORS 331	TGAAGAAGGGTTGTTGATTACAAG	GCATTGGGTTCACCATTTCT	LG7	2	Tang et al. 2002

stained with ethidium bromide (1.0 (gml-1) and photographed under UV light.

Single-marker analysis

The PCR amplification products were scored for the presence (1) or absence (0) of each marker band across all 15 genotypes and the data used to construct a binary data matrix (Mohammadi, 2006). A standard analysis of variance (one way ANOVA) was used to evaluate mean differences among sunflower lines defined by each SSR marker locus for each isolate, separately, in order to establish phenotype-marker association as suggested by Beckmann and Soller, (1986) and Edwards et al. (1987) using the general linear model (GLM) procedure of the Statistical Analysis System (SAS) program (SAS Institute Inc.). A significant F-test ($P \leq 0.05$) indicated segregation of marker locus genotypic classes with disease susceptibility. Significance at the $P = 0.05$ level was considered suggestive of a quantitative trait loci (QTL) at the marker locus (Vijayan et al., 2006).

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