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GENETIC ANALYSIS OF PARTIAL RESISTANCE TO BASAL STEM ROT (*Sclerotinia sclerotiorum*) IN SUNFLOWER

Masoumeh AMOUZADEH[1], Reza DARVISHZADEH[*,1], Parham HADDADI[2], Babak ABDOLLAHI MANDOULAKANI[1], Younes REZAEE DANESH[3]

[1]Department of Plant Breeding and Biotechnology, Urmia University, Urmia, Iran.
[2]INRA, Institute Jean-Pierre Bourgin (IJPB), Versailles Cedex France.
[3]Departeman of Plant Protection, Urmia University, Urmia, Iran.

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Basal stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the major diseases of sunflower (*Helianthus annuus* L.) in the world. Quantitative trait loci (QTLs) implicated in partial resistance to basal stem rot disease were identified using 99 recombinant inbred lines (RILs) from the cross between sunflower parental lines PAC2 and RHA266. The study was undertaken in a completely randomized design with three replications under controlled conditions. The RILs and their parental lines were inoculated with a moderately aggressive isolate of *S. sclerotiorum* (SSKH41). Resistance to disease was evaluated by measuring the percentage of necrosis area three days after inoculation. QTLs were mapped using an updated high-density SSR and SNP linkage map. ANOVA showed significant differences among sunflower lines for resistance to basal stem rot ($P \leq 0.05$). The frequency distribution of lines for susceptibility to disease showed a continuous pattern. Composite interval mapping analysis revealed 5 QTLs for percentage of necrotic area, localized on linkage groups 1, 3, 8, 10 and 17. The sign of additive effect was positive in 5 QTLs, suggesting that the additive allele for partial resistance to basal stem rot came from the paternal line (RHA266). The phenotypic variance explained by QTLs (R^2) ranged from 0.5 to 3.16%. Identified genes (HUCL02246_1, GST and POD), and SSR markers (ORS338, and SSL3) encompassing the QTLs for partial resistance to basal stem rot could be good candidates for marker assisted selection.

Key words: Basal stem rot, *Helianthus annuus* L., heritability, partial resistance, QTL mapping.

Corresponding author: Dr. R. Darvishzadeh, Department of Plant Breeding and Biotechnology, Urmia University, Urmia, Iran. E-mail: r.darvishzadeh@urmia.ac.ir. Phone: + 98 441 2972785. Fax: + 98 441 2779558

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the major oilseed crops grown worldwide. Commercially available sunflower varieties contain 39 to 49% oil in the seed which in 88% of the fatty acids are unsaturated. Basal stem rot caused by *Sclerotinia sclerotiorum* (Lib.) is one of the most important diseases of sunflower. It has been reported from all sunflower-growing regions in the world. The fungus is pathogenic to more than 480 plant species including oilseed crops (BOLAND and HALL, 1994). Under favorable climatic conditions for fungus growth such as high humidity and mild temperature, yield losses can reach up to 100% (SACKSTON, 1992). The fungus attacks most parts of the plant, including root, stem, leaf, terminal bud, and capitulum, at any developmental stage (PARTS *et al.,* 2007). Rapid drying of the leaves and development of lesions on the tap roots and basal portion of the stem causes plant death within a few days after the onset of wilting (DORRELL and HUANG, 1978). *Sclerotinia* wilt may occur anytime from the seedling to maturity stage.

Considering the wide host range and longevity of sclerotia, *S. sclerotiorum* is one of the most difficult pathogen to control. Chemical control is difficult, uneconomical and harmful to the environment. Genetic control is therefore of considerable importance, the aim being to select genotypes with high resistance to all forms of *S. sclerotiorum* found in regions in which sunflower is cultivated. No fully resistant sunflower genotype has been identified so far (HAHN, 2002), but in terms of susceptibility to disease, there are high variability, so production and identification of resistant varieties is possible.

Agriculturally important traits such as yield, quality and disease resistance are controlled by several genes and are known as quantitative traits (ABDI *et al.,* 2012; DAVAR *et al.,* 2010; HADDADI *et al.,* 2010a, b; HADDADI *et al.,* 2011; HADDADI *et al.,* 2012). Previous studies have shown that the inheritance of resistance to *S. sclerotiorum* in sunflower was generally found to be quantitative for all forms of infection (root, stalk and head) with different genes controlling the resistance in different organs (CASTAÑO *et al.,* 2002; DAVAR *et al.,* 2010; VAN BECELAERE and MILLER, 2004). Several QTL mapping studies were published up to date (BERT *et al.,* 2002; BERT *et al.,* 2004; DAVAR *et al.,* 2010; MESTRIES *et al.,* 1998; MICIC *et al.,* 2005a, b).Genetic analysis of quantitative trait loci (QTL) and mapping genes controlling trait can facilitate selection for that traits. The goal of the present research is to identify QTLs involved in genetic variation of partial resistance to basal stem rot in recombinant inbred lines (RILs) of sunflower using an integrated and high density genetic linkage map based on simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers.

MATERIALS AND METHODS

Plant material and experimental conditions

A population of 99 RILs derived from the cross between sunflower parental lines PAC2 and RHA266 through single-seed descent method was used in this study. RILs population was kindly provided by the National Institute of Agronomy Research (INRA, France). Paternal line (RHA266) was obtained from the cross between *H. annuus* and the wild genotype Peredovik by the United States Department of Agriculture (USDA). The maternal line (PAC2) was obtained from the crosses between *H. petiolaris* and HA61 by INRA, France (POORMOHAMMAD KIANI *et al*., 2007). Seeds of RILs and their two parental lines were planted in plastic containers filled with post grond H (www.klasmann–deilmann.com). The experiment was conducted in a completely randomized design with three replications. Each replicate consists of 6 plants. Plants

were grown in a growth chamber under controlled conditions [12h photoperiod and 25±2°C/18±2°C light/dark temperature, with a light intensity of 200μEm^{-2}s^{-1}, under 65% relative humidity] until growth stage of V6-V8 (sunflower plant with at least six to eight leaves) (SCHNEITER and MILLER, 1981). Moderately aggressive fungi isolate 'SSKH41' was selected for the QTL mapping program. This isolate came from a Sclerotinia-infected sunflower sample collected from Khoy region (Iran). Fungi isolate was cultured onto potato dextrose agar (PDA) (42gL^{-1}, pH 6) medium and grown in the dark at room temperature (25±1˚C). In the stage V6-V8 mycelium disks of isolate (3 mm diameter) were cut from the growing edge of the colony (2 days old on PDA) and were placed on the basal stem and covered by *Parafilm* for 48h (DAVAR *et al.,* 2010). *Parafilm* retains moisture for fungus growth. The percentage of necrotic area on 1 cm of the stem base and all around was measured visually three days after inoculation.

Statistical Analysis

Analysis of variance (ANOVA) of disease severity data were performed using the general linear model (GLM) procedure in the SAS software version 9.1 (SAS Institute Inc.). The function 'FREQ' of SPSS software (SPSS/PC-17, SPSS Inc) was used to analyze the frequency distribution of RILs and their parents for partial resistance to *S. sclerotiorum*.

Map construction and QTL mapping
SSR mapping

The genomic DNA of 123 RILs and their parents (PAC2 and RHA266) were extracted according to the method of extraction and purification presented by POREBSKI *et al.* (1997). We used Picogreen fluorescent stain (Quanti-iT^{TM} Picogreen®, Invitrogen) to quantify DNA concentration with the BioTek FL600 Fluorescence Microplate Reader. One hundred and fourteen SSRs were tested. Polymerase chain reaction (PCR) was done using a forward primer with a nucleotide extension at its 5'-end, identical to the sequence of an M13 sequencing primer and fluorescently (6-FAM, NED, VIC and PET) labelled Ml3-reverse standard sequencing primers. PCR products were diluted with ultrapure water (2µl of each PCR product in 20µl water) and 2µl of diluted PCR products mixed with 7.94µl Formamide HiDi^{TM} and 0.06µl GS^{TM} 500 LIZ^{TM} size standard (Applied Biosystems). After denaturing at 94°C for 5 min, we used sequencer ABI3730 and fragments were sized using the GeneMapper® software version 4 (Applied Biosystems). Chi-square-tests were performed for segregation distortion of each locus. All new SSRs are mapped to our previous map (POORMOHAMMAD KIANI *et al.,* 2007) by Carthagène (DE GIVRY *et al.,* 2005) and Mapmaker (LANDER *et al.,* 1987).

Candidate genes (CGs) mapping

Some important tocopherol and phytosterol pathway-related genes, enzymatic antioxidant-related genes, drought-responsive genes and *Arabidopsis* Sec14 homologue genes were selected and introduced into our map. Respective sequence data for CGs coding for these proteins were obtained from The *Arabidopsis* Information Resource (www.arabidopsis.org). In order to seek the *Helianthus* homolog sequences to the *Arabidopsis* genes, we used the Compositae expressed sequence tag (EST) assembly clusters, available at the *Helianthus*-devoted bioinformatics portal Heliagene (www.heliagene.org). The *Helianthus* EST clusters

presenting the reciprocal blast with the highest score and lowest E value with regarding to the original *Arabidopsis* genes were chosen for our studies. All primers were designed by MATLAB. Forward primers were tailed at 5' with M13-Fwd tail (5'CACGACGTTGTAAAACGAC3') and reverse primers were tailed at 5' with M13-Rev tail (5'ACAGGAAACAGCTATGAC3'). Between 2 to 4 various primer combinations per each candidate gene were tested on agarose gel. The PCR program was: 4 min at 94°C followed by 35 cycles; 30 s at 94°C, 30 s at 55°C or 58°C, 1 min at 72°C and final extension of 5 min at 72°C. One PCR fragment per gene was sequenced using M13-Fwd and M13-Rev primers. After sequencing; SNP-PHAGE (SNP discovery Pipeline with additional features for identification of common haplotypes within a sequence tagged site (Haplotype Analysis) and GenBank (-dbSNP) submissions), through the website at http://www.heliagene.org/, was applied for analyzing sequence traces from both parents to identify SNPs. Several types of markers such as dominant, co-dominant, HRM (high resolution melting), InDel (short insertions and deletions) and SNP-based cleaved amplified polymorphic sequences (CAPS) markers are developed for genotyping of the studied candidate genes.

QTL mapping

QTL mapping was performed by composite interval mapping (CIM) in Windows QTL Cartographer Version 2.5 (BASTEN *et al.,* 2002). The genome was scanned at 2cM intervals with a window size of 15cM and up to 15 background markers were used as a cofactors in the CIM analysis identified by the program Srmapqtl (model 6). A LOD threshold of 2.5 resulting from 1000 permutations was used for identifying significant QTLs. Additive effects of the detected QTLs were estimated with Zmapqtl program. The percentage of phenotypic variance (R^2) explained by the QTLs was estimated at the peak of curve by Windows QTL Cartographer. Map Chart 2.2 was used to draw a graphical presentation of linkage groups and map position of QTLs (VOORRIPS, 2002).

RESULTS

Three days after artificial inoculation, the majority of plants showed *S. sclerotiorum* infection symptoms on the basal stem. Analysis of variance showed significant differences among the sunflower genotypes for susceptibility to basal stem rot ($P \leq 0.05$) (Table 1). The disease severity of the RILs ranged from 45 to 99.11% (Figure 1). C100 showed high partial resistance to disease, while LR64 was highly susceptible (Table 1). Differences between the mean of RILs (X_{RIL}) and the mean of their parents (X_P) were not significant ($\bar{X}_{RILs}$-$\bar{X}_P$=-1.38). The difference between mean of the best RIL (X_{BRIL}) and the mean of the paternal lines (X_P) was significant ($\bar{X}_{BRIL}$-$\bar{X}_P$=32.07) (Table 1). The frequency distribution of the RILs and their parents for partial resistance to basal stem rot showed a continuous pattern (Figure 1). Some RILs showed a lower disease severity than their parents, while some others showed a higher disease severity.

Linkage map and QTL analysis

Of 114 SSR primer pairs tested, 32 SSR primers produced clear polymorphisms between the parental lines which segregated in a Mendelian manner. These new SSR markers together with candidate genes identified with the code HuCL were assigned to the previously reported linkage map (POORMOHAMMAD KIANI *et al.,* 2007). New SSR can be found in linkage

groups 1, 2, 3, 5, 6, 8, 10, 11, 13, 14, 15 and 17 and candidate genes (HuCL) in linkage group 1, 2, 8, 11, 14, 15, 16 and 17. The updated map consisted of 210 SSR markers and 11 genes placed in 17 linkage groups. Linkage groups were designated with 1 to 17 according to the reference linkage map of sunflower (TANG *et al.,* 2002) (Figure 2). The total map length is 1,653.1 cM with a mean density of 1 marker per 7.44 cM. The number of markers per linkage group ranged from 5 to 26. Linkage group 14 is the largest in term of cM size (197.6 cM; Figure 2) and linkage group 4 (32 cM; Figure 2) is the smallest.

Table 1. Analysis of variance and genetic parameters for partial resistance to Sclerotinia sclerotiorum in sunflower recombinant inbred lines (RILs) under controlled conditions.

Analysis of variance			
Source of variation	Degree of freedom	Mean of square	Significant
Genotype	98	366.42	*
Residual	207	295.18	
Genetic parameters			
Item	$\bar{X}$PNA[a]	Item[b]	$\bar{X}$PNA
PAC2(P_1)	64.99	Example of RILs:	
RHA266(P_2)	89.164		
P_1-P_2	$-24.194^{n.s}$	C100 (partial resistant to isolate)	45
$\bar{X}_P=(P_1+P_2)/2$	77.07	C38a (partial resistant to isolate)	45.55
$\bar{X}_{RILs}$	78.45	C128(partial resistant to isolate)	48.47
$\bar{X}_{RILs}-\bar{X}_P$	1.38^{ns}		
BRIL $-\bar{X}_P$	-32.07^{*}	C39 (susceptible to isolate)	98.54
$LSD = \sqrt{\frac{2MSE}{r}} \times t$	27.49	LR64 (susceptible to isolate)	99.11

[a]PNA: percentage of necrotic area. [b]$\bar{X}_{RILs}$: mean of all recombinant inbred lines ($\bar{X}_{RILs}$); $\bar{X}_P$: mean of the parents; BRIL: the best RIL. LSD $_{0.05}$, least significant difference calculated using $t_{0.05}$ and the error mean square of experiment; h^2, board-sense heritability. ns: non significant. *: significant at 0.05 probability level.

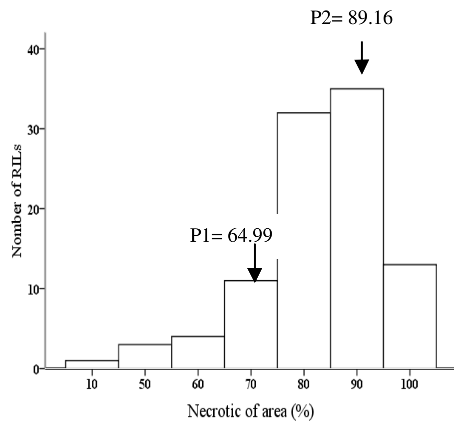


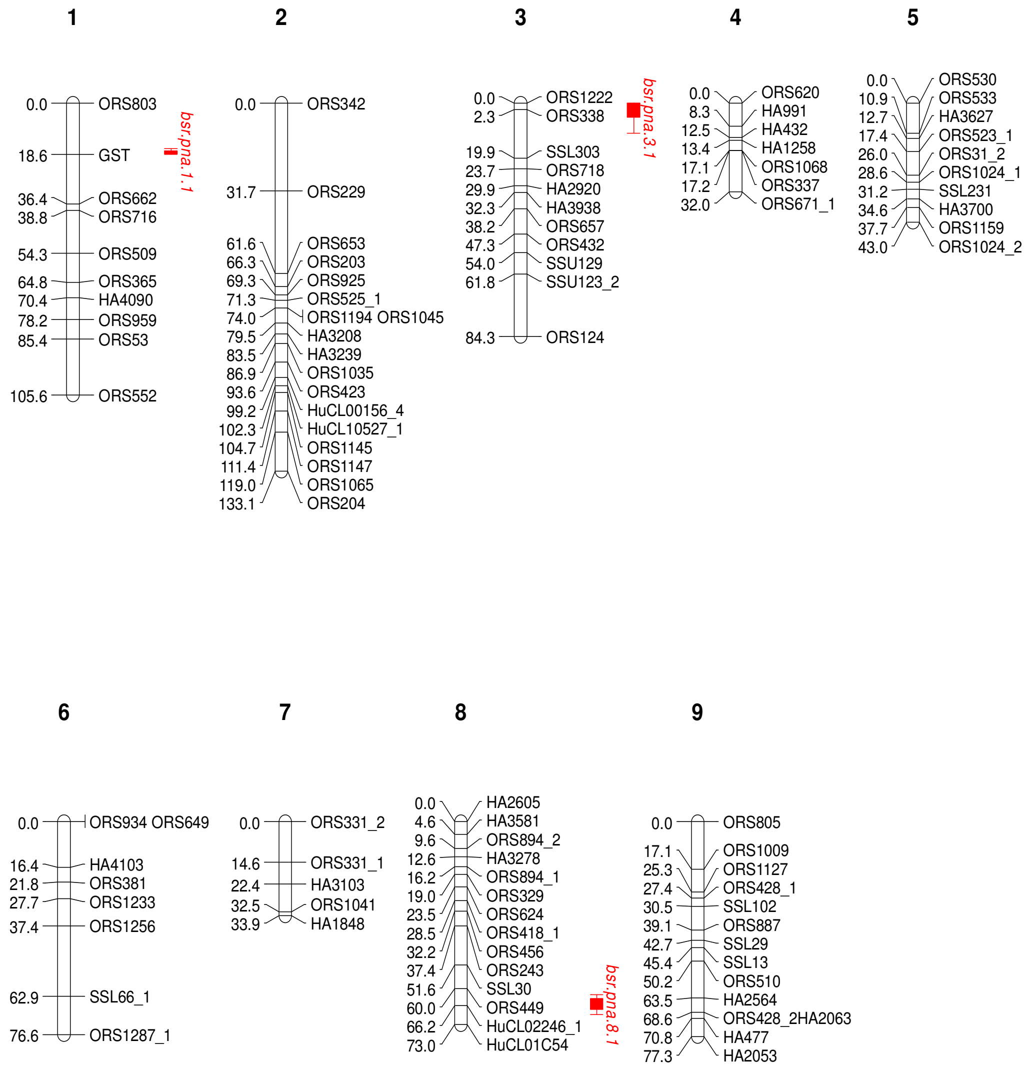
Figure 1. Frequency distribution of sunflower recombinant inbred lines (RILs) and their parents for partial resistance to *Sclerotinia sclerotiorum*. Arrows show the phenotypic value of the parental lines (P1=PAC2 and P2=RHA266).

Five QTLs were detected for partial resistance to *S. sclerotiorum* on linkage groups 1, 3, 8, 10 and 17. The map position of QTLs peaks, the percentage of phenotypic variance explained by each QTL (R^2) and the estimate of QTL effects based on a composite interval mapping are summarized in Table 2 and Figure 2. The phenotypic variance explained by identified QTLs (R^2) ranged from 0.5 to 3.16% (Table 2). The most important QTL was found on linkage group 17 at 99.81 cM and explained 3.16% of the phenotypic variance. The sign of additive effects was positive in identified QTLs. We have identified QTL for resistance to basal stem rot on linkage group 1, which was linked to Glutathione S-transferase gene (GST).

Table 2. Map position and effect of QTLs detected in RILs population for partial resistance to Sclerotinia sclerotiorum

QTL	Marker	LG	Position[a] (cM)	LOD[b]	Additive effect	R^2
Bsr.P.N.A.1.1	GST	1	18.01	3.03	0.0023	1.3
Bsr.P.N.A.3.1	ORS338	3	2.01	3.59	0.0213	2.97
Bsr.P.N.A.8.1	HUCL02246_1	8	65.61	3.01	0.0092	0.5
Bsr.P.N.A.10.1	SSL3	10	81.01	3.34	0.0084	0.83
Bsr.P.N.A.17.1	POD	17	99.81	2.72	0.0079	3.16

[a]Expressed in Kosambi, from the top of linkage group (LG). [b]LOD: likelihood that the effect occurs by linkage/ likelihood that the effect occurs by chance. A negative effect indicates that the resistance allele comes from the maternal line (PAC2). A positive effect indicates that the resistance allele comes from the paternal line (RHA266). R^2: Percentage of individual phenotypic variance explained, value determined by Win QTL Cartographer Version 2.5.


1
0.0 ORS803
18.6 GST
36.4 ORS662
38.8 ORS716
54.3 ORS509
64.8 ORS365
70.4 HA4090
78.2 ORS959
85.4 ORS53
105.6 ORS552
bsr.pna.1.1
2
0.0 ORS342
31.7 ORS229
61.6 ORS653
66.3 ORS203
69.3 ORS925
71.3 ORS525_1
74.0 ORS1194 ORS1045
79.5 HA3208
83.5 HA3239
86.9 ORS1035
93.6 ORS423
99.2 HuCL00156_4
102.3 HuCL10527_1
104.7 ORS1145
111.4 ORS1147
119.0 ORS1065
133.1 ORS204
3
0.0 ORS1222
2.3 ORS338
19.9 SSL303
23.7 ORS718
29.9 HA2920
32.3 HA3938
38.2 ORS657
47.3 ORS432
54.0 SSU129
61.8 SSU123_2
84.3 ORS124
bsr.pna.3.1
4
0.0 ORS620
8.3 HA991
12.5 HA432
13.4 HA1258
17.1 ORS1068
17.2 ORS337
32.0 ORS671_1
5
0.0 ORS530
10.9 ORS533
12.7 HA3627
17.4 ORS523_1
26.0 ORS31_2
28.6 ORS1024_1
31.2 SSL231
34.6 HA3700
37.7 ORS1159
43.0 ORS1024_2
6
0.0 ORS934 ORS649
16.4 HA4103
21.8 ORS381
27.7 ORS1233
37.4 ORS1256
62.9 SSL66_1
76.6 ORS1287_1
7
0.0 ORS331_2
14.6 ORS331_1
22.4 HA3103
32.5 ORS1041
33.9 HA1848
8
0.0 HA2605
4.6 HA3581
9.6 ORS894_2
12.6 HA3278
16.2 ORS894_1
19.0 ORS329
23.5 ORS624
28.5 ORS418_1
32.2 ORS456
37.4 ORS243
51.6 SSL30
60.0 ORS449
66.2 HuCL02246_1
73.0 HuCL01C54
bsr.pna.8.1
9
0.0 ORS805
17.1 ORS1009
25.3 ORS1127
27.4 ORS428_1
30.5 SSL102
39.1 ORS887
42.7 SSL29
45.4 SSL13
50.2 ORS510
63.5 HA2564
68.6 ORS428_2HA2063
70.8 HA477
77.3 HA2053

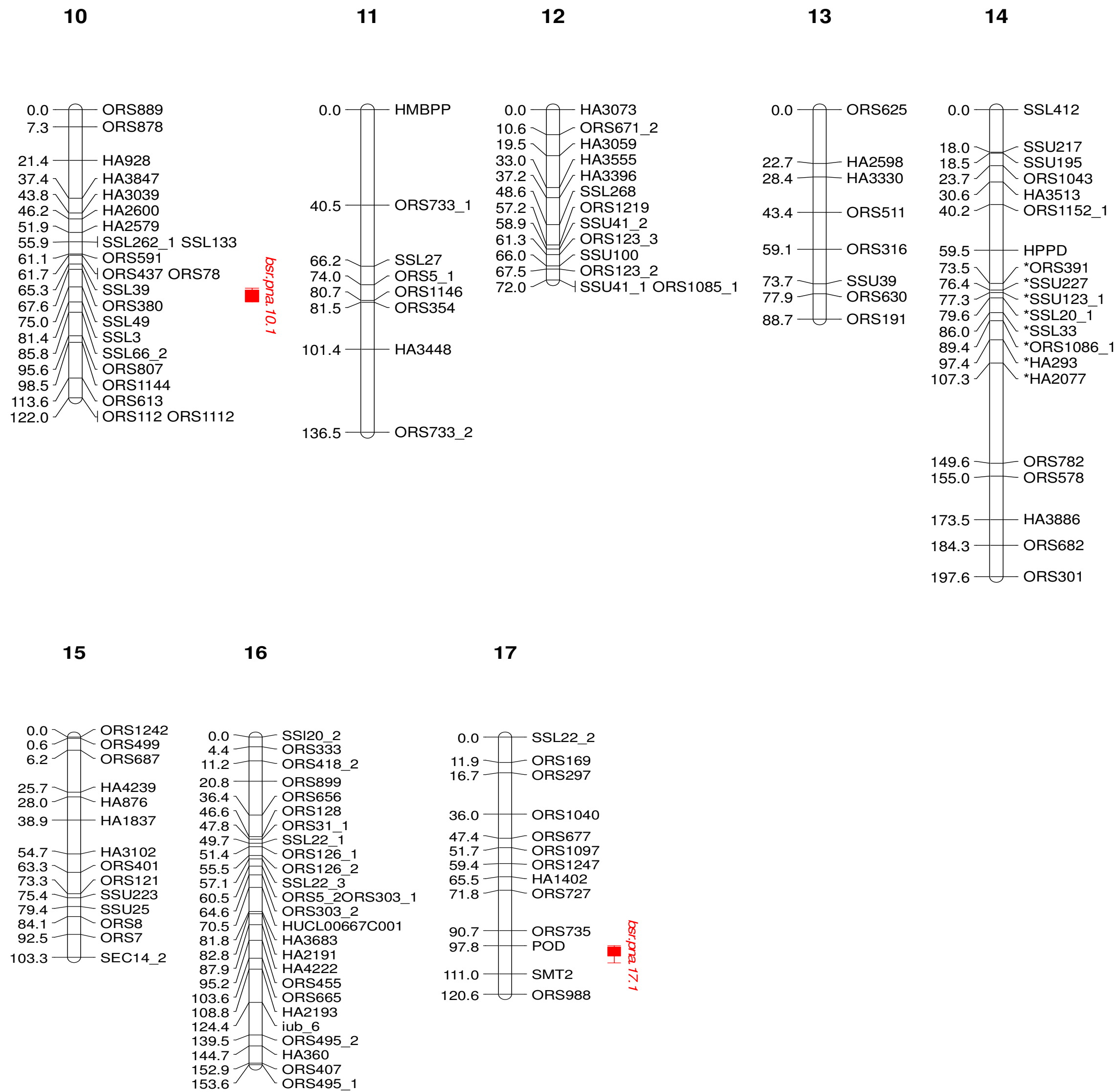


Figure 2. Molecular genetic linkage map of sunflower. The positions of QTLs for partial resistance to *Sclerotinia sclerotiorum* are shown on the right side of the linkage groups.

DISCUSSION

The RILs population presented a wide range of genetic variation for partial resistance to *S. sclerotiorum* (Table 1), indicating a potential for enhancing resistance to basal stem rot in sunflower. This result is in agreement with the findings of MICIC *et al.* (2005a, b) and DAVAR *et al.* (2010) in sunflower. Non-significant differences between the means of the RILs and the mean of the parents show that the RILs used in this study are representative of possible genotypic combinations of the cross 'PAC2 × RHA266' (Table 1). This finding was consistent with the previous report presented by DAVAR *et al.* (2010). Frequency distribution of RILs and their parents showed continuous patterns for partial resistance to disease, suggesting that resistance is controlled by a polygenic system. Polygenic inheritance of resistance to *S. sclerotiorum* in sunflower has previously been reported by MESTRIES *et al.* (1998), MICIC *et al.* (2005a, b), and DAVAR *et al.* (2010). Some RILs showed a lower disease severity than their parents, while some others showed higher disease severity that is an evidence for transgressive segregation for partial resistance in this cross. Transgressive segregation has previously been reported by MICIC *et al.* (2005a, b), and DAVAR *et al.* (2010) for partial resistance to *S. sclerotiorum*, as well as by RACHID AL-CHAARANI *et al.* (2002), BERT *et al.* (2004) and DARVISHZADEH *et al.* (2007) for partial resistance to phoma black stem disease in sunflower. This suggests that each parent possesses different resistance or susceptibility alleles.

Five genomic regions containing putative QTLs for partial resistance to basal stem rot were identified on linkage groups 1, 3, 8, 10 and 17. The percentages of phenotypic variance explained by QTLs (R^2) were small and ranged from 0.5 to 3.16%. The data confirm the hypothesis that a large number of genes with small effects are involved in resistance to basal stem rot. The sign of additive effect was positive in 5 QTLs, suggesting that the additive alleles for partial resistance to basal stem rot come from the paternal line (RHA266).

Several studies have been undertaken for mapping QTLs controlling partial resistance to *S. sclerotiorum* in sunflower (BERT *et al.,* 2002; DAVAR *et al.,* 2010; MESTRIES *et al.,* 1998; MICIC *et al.,* 2004; MICIC *et al.,* 2005a, b; RÖNICKE *et al.,* 2005). MESTRIES *et al.* (1998) identified 3 QTLs for partial resistance to head rot, explaining 12.3 to 17.5% of phenotypic variance. RÖNICKE *et al.* (2005) detected 5 QTLs for resistance to head rot, explaining 10.6 to 17.1% of phenotypic variance. However, the lack of SSR markers and common linkage group nomenclature in their map make it difficult to compare the location of the QTLs detected in the present study with those identified by MESTRIES *et al.* (1998) and RÖNICKE *et al.* (2005).

BERT *et al.* (2002) identified 3 QTLs for the mycelium extension on the leaves on the LGs 1, 9, 13. One QTL identified in the present study, and one QTL reported by Bert *et al.* (2002) both were located on linkage group 1. MICIC *et al.* (2004) identified 7 QTLs conferring partial resistance to stem lesions on the LGs 2, 3, 4, 6, 8, 15 and 16. In their study, the partial R^2s were 6% or smaller, but the effect of QTL detected on the LG8 was substantial and explained 36.7% of the phenotypic variance. In another study MICIC *et al.* (2005a) identified 2 QTLs for basal stem rot on the LGs 8 and 16. Two out of 5 QTLs detected in the present study, and 2 out of 7 QTLs reported by MICIC *et al.* (2004) were located on the same linkage groups (LGs 3 and 8). MICIC *et al.* (2005b) identified 3 QTLs for partial resistance to stem lesions on the LGs 4, 10 and 17. Two QTLs identified herein and 2 QTLs reported by MICIC *et al.* (2005b) were located on the same LGs, 10 and 17. DAVAR *et al.* (2010) detected 7 QTLs for partial resistance to

basal stem rot on the LGs 1, 2, 4, 6, 8, 14 and 17, which coincided with LGs 1, 8 and 17 in our study.

We have identified the region for partial resistance to basal stem rot on the LG 1, which was linked to the Glutathione S-transferase gene (GST). Glutathione S-transferases are multifunctional proteins involved in diverse intracellular events such as primary and secondary metabolisms, stress metabolism, herbicide detoxification and plant protection against ozone damages, heavy metals and xenobiotics (MOHSENZADEH *et al.,* 2011).

CONCLUSION

We have identified 5 QTLs for partial resistance to basal stem rot on the different linkage groups. LG1 and LG8 are good candidates for further analyses to develop molecular markers for resistance against *S. sclerotiorum*. Since QTLs conferring resistance to *S. sclerotiorum* have already been identified on these two linkage groups (LG1 and LG8) in various independent studies. These findings also provide a framework for marker-assisted selection of complex disease resistance and the positional cloning of partial resistance genes.

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GENETIČKA ANALIZA PARCIJALNE REZISTENTNOSTI NA CRVENILO OSNOVE STABLA *(SCLEROTINIA SCLEROTIORUM)* KOD SUNCOKRETA

Masoumeh AMOUZADEH[1], Reza DARVISHZADEH[*,1], Parham HADDADI[2], Babak ABDOLLAHI MANDOULAKANI[1], Younes REZAEE DANESH[3]

[1] Odelenje za oplemenjivanje biljaka i Biotehnolohiju, Urmia Univerzitet, Urmia, Iran
[2]INRA, Institut (IJPB), 78026, Versailles Cedex France.
[3] Odelenje za zaštitu biljaka, Urmia Univerzitet, Urmia, Iran

Izvod

Izvršena je identifikacija QTLs uključenih u kontrolu parcijalne rezistentnosti osnove biljke na crvenilo korišćenjem 99 rekombinovanih samooplodnih linija(RLs) iz ukrštanja roditelskih linija suncokreta PAC2 I RHA 266. Ispitivanja su vršena u kompletnom random blok sistemu, u tri ponavljanja u kontrolisanim uslovima a inokulacija je vršena sa umereno agresivnim izolatom *S. sclerotiorum* (SSKH41). Tri dana posle inokulacije vršeno je merenje površine nekroze. QTLs su mapirani korišćenjem SSR i SNP mape ukopčanosti visoke gustine . ANOVA analiza je pokazala značajne razlike u rezistentnosti između linija suncokreta ($P \leq 0.05$). Analizom složenosti intervala mapiranja je utvrđeno 5 QTLs za procenat nekrotičnog tkiva u grupama ukopčanosti 1,3,8,10 I 17. Znakovi aditivnog efekta u 5 QTls sugerišu da aditivni aleli za parcijalnu rezistentnost potiču iz roditeljske linije RHA 266. Fenotipska varijansa objašnjena sa QTLs (R^2) je u intervalu od 0.5 – 3.16 Identifikovani geni (HUCL02246_1, GST i POD), i SSR markeri (ORS338, i SSL3) ukazuju da QTLs za parcijalnu rezistentnost mogu biti dobri kandidati za selekciju korišćenjem markera (MAS).

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