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Research Papers

Single sequence repeat markers associated with partial resistance in sunflower to *Phoma macdonaldii*

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Summary. Phoma black stem of sunflower, caused by *Phoma macdonaldii*, occurs in many countries. The objective of the present study was to estimate the number of markers and genomic regions in sunflower associating with Phoma black stem resistance. Genetic variability among 32 sunflower genotypes, including recombinant inbred lines and their parents, M7 mutant lines developed by gamma irradiation, and some genotypes from different countries of origin, was evaluated using simple sequence repeat (SSR) markers. Eighty-eight markers were generated at 38 SSR loci, with a mean number of alleles per locus of 2.32. Using susceptibility data of 32 sunflower genotypes against seven *P. macdonaldii* isolates (Darvishzadeh *et al.*, 2007), one to four markers were associated with each of seven different *P. macdonaldii* isolates. To reduce the probability of false positives, a sequential Bonferroni-experiment-wise *P*-value was used for each marker trait association tested. The identified markers showed a promising trend, although they did not pass the more stringent bar of statistical significance, and should be studied further.

Key words: Association analysis, isolate-specific partial resistance, isolate-nonspecific partial resistance, phenotype-marker association, single-marker analysis.

Abbreviations used in the text: AFLP, amplified fragments length polymorphism; ANOVA, analysis of variance; PBS, Phoma black stem; QTL, quantitative trait loci; RAPD, random amplified polymorphic DNA; RIL, recombinant inbred line; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; SRAP, sequence-related amplified polymorphism; SSD, single-seed descent; SSR, simple sequence repeat.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important annual species grown worldwide as a source of vegetable oil and protein. It also serves as a model for genomic studies on the family Asteraceae (Paniego *et al*., 1999). Phoma black stem, caused by the necrotrophic fungus *Phoma macdonaldii* Boerema (teleomorph *Leptosphaeria lindquistii* Frezzi), is one of the most important diseases of sunflower (Debaeke and Pérès, 2003). This disease is characterized by black spots that appear on plant stems at the base of leaf petioles and then spread along stems. Yield losses are due to early plant senescence and are considered to be moderate, ranging from 0.2 to 0.7 t ha^{-1} (Debaeke and Pérès, 2003).

Genetic variability for partial resistance to Phoma black stem in sunflower was reported in both field (Pérès *et al.*, 1994) and controlled conditions (Roustaee *et al*., 2000a; Rachid Al-Chaarani *et al.*, 2002; Bert *et al.*, 2004; Darvishzadeh *et al.*, 2010a). Significant

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differences in pathogenicity exist among different *P. macdonaldii* isolates on the same host genetic material (Roustaee *et al.*, 2000b; Darvishzadeh *et al.*, 2007). Identification of new sources of resistance, pyramiding of major genes, and accumulation of polygenes are considered to be effective methods for preventing yield losses due to Phoma black stem.

During the last two decades, DNA-based molecular markers have been extensively used for a variety of purposes in many animal and plant systems. The advent of DNA markers, such as restriction fragment length polymorphism (RFLP) (Botstein *et al.*, 1980), random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), simple sequence repeats (SSRs) (Rafalski and Tingey, 1993), amplified fragments length polymorphism (AFLP) (Vos *et al.*, 1995), as well as single nucleotide polymorphism (SNP) (Haff and Smirnov, 1997) has facilitated mapping of agriculturally important QTLs in plants. SSR markers, due to their high polymorphism, random distribution and co-dominant Mendelian inheritance, are the most reliable markers for genetic studies in sunflower (Paniego *et al.*, 2002; Tang *et al.*, 2002; Yu *et al.*, 2002; Tang and Knapp, 2003; Zhang *et al.*, 2005; Darvishzadeh *et al.*, 2010b).

Mapping of QTLs is carried out with plant material obtained from systematic breeding populations such as F2, or recombinant inbred lines (RILs). Identification of markers associated with important traits in a group of genotypes through ANOVA or regression analysis offers an alternative method, 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; Darvishzadeh, 2012).

Many DNA markers associated with different traits have been identified using ANOVA and regression methods. Baldini *et al.* (2002, 2004) used single-marker regression and ANOVA methods and identified several AFLP and SSR markers associated with basal stem resistance in sunflower to *Sclerotinia sclerotiorum*. In globe artichoke, putative sequence-related amplified polymorphism (SRAP) markers were detected that linked to two important agronomic traits, head colour and precocity of production (Martin *et al.*, 2008). Babiker *et al.* (2009) identified microsatellite markers for stem rust resistance in F2 populations of wheat.

The objective of present study was to identify the putative SSR markers associated with partial resistance in sunflower to Phoma black stem, using single-marker analysis. The SSR markers associated with partial resistance to different pathogen isolates could be used in pyramiding polygenes in sunflower disease resistance breeding programs.

Materials and methods

Plant material and phenotypic data

Disease reaction data of 32 sunflower genotypes from different countries of origin (Table 1) against seven *P. macdonaldii* isolates were used in this study. Detailed information on the levels of susceptibility of genotypes to *P. macdonaldii* isolates is available elsewhere (Darvishzadeh *et al.*, 2007, 2010a).

Molecular experiments

Genomic DNA of 32 sunflower genotypes was extracted from the leaves of 2-week-old seedlings using the method described by Dellaporta *et al.* (1983). Genomic DNA was re-suspended in 100 μL TE (10 mM Tris pH 7.0, 1 mM 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 5 μL of DNA in 0.8% (w/v) gels in 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0). DNA samples that gave a smear in the gel were rejected.

Thirty eight microsatellite markers (Table 2) were used for DNA fingerprinting. The choice of SSR loci was based on their known genetic locations to obtain near-uniform coverage of the sunflower genome (Tang *et al.*, 2002; Poormohammad Kiani *et al.*, 2007). Each PCR amplification was performed in 20 μL containing 2.5 μM of each SSR primer, 0.4 Units Taq DNA polymerase (Life Technologies, Foster City, CA, USA), 100 μM of each dNTP (Promega, Madison, WI, USA), 2 μL 10× PCR buffer, 2.5 mM $MgCl_2$ (Promega), 0.20 μL of stabilizer (1% W-1 (v/v), Life Technologies), ddH2O and 25 ng template DNA using a Gene Amp PCR System 9700 Thermocycler (PerkinElmer Applied Biosystems, Foster City, CA, USA). Touchdown PCR was used to amplify all the SSRs investigated as follows: 95°C for 3 min, one cycle of 94°C for 30s, 64°C annealing for 30s, 72°C for 45s, followed by ten cycles with a decrease in annealing temperature of 1°C per cycle, followed by 33 cycles at 94°C for 30 s, 54°C for 30 s and 72°C for 45

Table 1. Sunflower lines and accessions, and their country of origin, used in experiments for identification of SSR markers associated with partial resistance to *Phoma macdonaldii* isolates.

Genotype No.	Sunflower line	Type[a]	Origin	Disease severity score of *Phoma macdonaldii* isolates (Darvishzadeh *et al.*, 2007)						
				MA6	MA7	MP10	MP11	MP3	MP6	MP8
C01	C81	RIL	France	7.16	7.63	6.99	6.94	5.83	6.21	6.85
C02	C43	RIL	France	3.76	4.8	5.43	3.23	6.95	4.07	1.88
C03	C79	RIL	France	2.23	5.68	3.37	4.01	4.03	6.14	6.51
C04	LR64	RIL	France	3.6	7.13	5.25	3.61	5.95	6.72	5.43
C05	RHA266	BL	USA	7.62	6.96	5.83	6.33	7.19	6.83	7.33
C06	PAC2	BL	France	7.62	7.5	7.31	7.56	7.38	7.51	7.31
C07	M6-54-1	M	France	4.6	6.19	7.06	3.65	7.88	5.97	4.53
C08	M6-133-2	M	France	6.48	6.57	6.26	5.16	7.6	7.45	6.62
C09	M6-85-3	M	France	6.66	6.8	7.53	6.13	7.57	7.34	7.72
C10	M6-894-2	M	France	3.14	3.58	3.95	3.92	7.7	2.2	5.58
C11	AS613	BL	France	2.4	5.43	1.06	6.76	3.59	3.13	0.99
C12	RHA274	BL	USA	3.24	5.39	5.68	5.14	3.7	4.66	4.36
C13	SDR18	BL	USA	7.07	5.28	5.55	3.66	2.3	7.16	1.6
C14	SDR19	BL	USA	2.88	5.01	3.73	5.97	5.83	2.13	2.15
C15	SDB1	BL	USA	0.82	6.15	2.9	3.55	2.67	3.78	2.21
C16	SDB3	BL	USA	1.76	1.76	0.77	3.5	2.52	1.58	1.56
C17	F651/1	BL	Hungary	1.56	3.08	4.03	3.35	6.99	6.14	3.89
C18	F1250/03	BL	Hungary	6.8	7.35	7.44	7.14	7.59	6.13	6.43
C19	B454/03	BL	Hungary	4.99	6.17	6.63	5.9	7.6	6.88	2.15
C20	ENSAT-B5	BL	France	1.74	4.82	7.19	3.64	4.31	1.87	1.48
C21	H565R	BL	France	4.02	5.29	4.89	4.24	4.48	3.1	4.32
C22	ENSAT-R5	BL	France	5.27	4.77	6.52	4.28	3.71	5.44	5.9
C23	H543R	BL	France	7.4	4.66	7.16	6.36	7.1	7.4	5.44
C24	RT931	BL	France	1.21	4.26	1.18	6.83	6.12	2.93	6.43
C25	AS5305	BL	France	2.4	2.58	1.34	2.49	2.48	2.93	1.92
C26	ENSAT-R4	BL	France	6.58	7.22	3.58	3.55	6.4	7.2	1.97
C27	ENSAT-B4	BL	France	4.19	6.15	6.83	4.34	8.15	4.7	4.74
C28	LC1064C	BL	France	4.37	3.71	6.48	4.05	4.98	5.71	4.15
C29	1151 North Dakota	W	USA	5.42	3.06	7.64	2.83	2.75	5.75	5.48
C30	510 Kansas	W	USA	3.51	4.81	4.13	7.58	7.58	4.25	3.5
C31	665 Iowa	W	USA	4.32	6.88	4.85	1.98	2.96	3	3.79
C32	1016 Nebraska	W	USA	2.22	3.73	4.24	4.35	5.02	4.14	3.27

[a]BL, breeder's line; RIL, recombinant inbred line; M, gamma-irradiation induced mutant line; W, wild type.

Table 2. Primer sequences and linkage groups of the 38 SSR loci applied to 32 sunflower genotypes.

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Linkage group	No. 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

(*Continued*)

Table 2. *Continues.*

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Linkage group	No. of alleles	Reference
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	ACTATCGAACCTCCCCTCCAAAC	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

s. A final extension was done for 20 min at 72°C. 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 0.5× TBE, stained with ethidium bromide (1.0 μg mL^{-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 32 genotypes, and the data were used to construct a binary data matrix. A standard analysis of variance (one way ANOVA) was used to evaluate mean differences among the two classes of sunflower genotypes defined by each SSR allele for each isolate, separately, in order to establish phenotype-marker association, as suggested by Beckmann and Soller (1986) and Edwards *et al*. (1987). To reduce the probability of false positives, a sequential Bonferroni-experiment-wise *P*-value was used for each marker trait association tested (Sokol and Rolf, 1981).

Results and discussion

Genotypes studied in this study differed considerably in partial resistance to black stem (Table 1). The most resistant genotypes to all *P. macdonaldii* isolates were two American lines, SDR19 and SDR18 (Table 1). The inbred sunflower lines B454/03, ENSAT-B5 and LC1064C showed high susceptibility to all isolates tested (Table 1). Other genotypes showed intermediate responses across the *P. macdonaldii* isolates. The results provide strong indications of the existence of specificity between *P. macdonaldii* isolates and sunflower genotypes for partial resistance to Phoma black stem. Detail information on the levels of susceptibility of genotypes to *P. macdonaldii* isolates is available in Darvishzadeh *et al.* (2007, 2010a).

Eighty eight alleles were generated at 38 SSR loci. Number of alleles per locus ranged from two to four, with an average of 2.32. Using ANOVA, three markers were identified to be associated with partial resistance to isolate MP8, four to isolate MA6, four to isolate MP11, one to isolate MP3, three to isolate MP6, four to isolate MP10 and three to isolate MA7 (Table 3). By applying sequential Bonferroni tests, none of the results passed that bar; all p-values were greater (Table 3).

Table 3. SSR markers detected for partial resistant to *Phoma macdonaldii* isolates in sunflower using single-marker analysis.

Isolate	SSR marker	MS	F	*P* >F[a]	Adjusted *P* value		0	1	R^2
MP8	ha3639	35.27	11.74	0.0019	0.000568182	ns	2.84	5.12	0.29
	ha3638	28.66	8.43	0.0068	0.000568182	ns	5.24	3.35	0.22
	ORS 822	18.63	4.99	0.0331	0.000568182	ns	5.35	3.74	0.14
MA6	ORS 928	23.78	6.35	0.0173	0.000574713	ns	5.56	3.70	0.17
	ORS 844	32.56	9.43	0.0045	0.000568182	ns	3.21	5.23	0.24
	ha3638	28.89	8.08	0.0080	0.000574713	ns	5.23	3.33	0.21
	ha4057	37.69	11.49	0.0020	0.000568182	ns	6.16	3.66	0.28
MP11	ORS 58	11.76	5.36	0.0277	0.000568182	ns	4.91	2.40	0.15
	ha2682	12.06	5.52	0.0256	0.000568182	ns	4.98	3.13	0.16
	ORS 149	12.77	5.29	0.0301	0.000568182	ns	5.08	3.31	0.17
	ORS 1068	10.25	4.43	0.0440	0.000568182	ns	3.59	5.05	0.13
MP3	ORS 58	15.22	4.37	0.0451	0.000574713	ns	5.71	2.86	0.13
MP6	ORS 928	17.77	5.69	0.0236	0.000581395	ns	6.12	4.51	0.16
	ORS 844	16.98	5.39	0.0273	0.000574713	ns	4.24	5.70	0.15
	ORS 988	26.28	8.90	0.0060	0.000568182	ns	4.10	6.01	0.25
MP10	ORS 928	16.56	4.33	0.0461	0.000574713	ns	4.53	6.02	0.13
	ha3638	17.86	4.73	0.0377	0.000574713	ns	4.18	5.70	0.14
	ha2879	19.25	5.16	0.0305	0.000568182	ns	5.77	4.21	0.15
	ORS 598	20.95	6.20	0.0190	0.000568182	ns	4.65	6.43	0.18
MA7	ORS 928	15.45	7.90	0.0086	0.000568182	ns	6.36	4.86	0.21
	ha3878	10.32	4.85	0.0355	0.000568182	ns	6.06	4.88	0.14
	ORS 822	14.98	7.60	0.0098	0.000568182	ns	6.15	4.76	0.2

[a] *P*-values are considered significant (*), if *P*-value<Bonferroni corrected P-value. R^2 is the percent of individual phenotypic variance explained by each marker.

Beside the efficient and reliable characterization of genetic diversity, molecular markers are helpful tools to complement traditional breeding procedures, giving improvement of cultivars through marker assisted selection (MAS). As a prerequisite, identifying tightly linked markers is important for the successful deployment of molecular marker technology within a breeding programme. MAS does directly select for a target trait, but for a marker tightly linked with the target trait. The less the distance between the marker locus and the target trait, the lower is the probability for recombination between these two loci, making the marker a more reliable indicator of the trait in question.

Association analysis based on elite lines and breeding material provides a particularly useful tool to detect loci for traits. The choice of germplasm is a key factor determining the resolution of association analysis. The germplasm selected should theoretically include all the genetic variation of a specific species. Diverse germplasm includes more extensive historical recombination and provides a high level of resolution (Wang *et al.*, 2008). The low number of alleles detected per locus in the present study, in comparison to other studies (e.g. Yu *et al.*, 2002; Tang and Knapp, 2003), may have affected our association analysis results. Differences in numbers of alleles could be due to differences in number of genotypes studied, their genetic background, and number of markers used, as well as techniques applied to detect polymorphism. For example, Tang and Knapp (2003) used 122 microsatellite marker loci for genotyping nine elite confectionery and oilseed sunflower in-

bred lines, and 3.5 alleles per locus were identified. Another possible cause of difference in results could be the matrix used to resolve PCR products. The present study used agarose instead polyacrylamide, so sensitivity in resolving bands could have been less than achieved with other media.

In conclusion, the present study offers an approach for identifying DNA markers projecting significant association with resistance in sunflower germplasm to *P. macdonaldii* isolates. The identified markers showed promise and did not pass the more stringent bar of statistical significance, and should therefore be studied further.

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