Identifying Quantitative Trait Loci for Resistance to *Sclerotinia* Head Rot in Two USDA Sunflower Germplasms

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ABSTRACT

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Sclerotinia head rot is a major disease of sunflower in the world, and quantitative trait loci (QTL) mapping could facilitate understanding of the genetic basis of head rot resistance and breeding in sunflower. One hundred twenty-three $F_{2,3}$ and $F_{2,4}$ families from a cross between HA 441 and RHA 439 were studied. The mapping population was evaluated for disease resistance in three field experiments in a randomized complete block design with two replicates. Disease incidence (DI) and disease sever-

ity (DS) were assessed. A genetic map with 180 target region amplification polymorphism, 32 simple sequence repeats, 11 insertion-deletion, and 2 morphological markers was constructed. Nine DI and seven DS QTL were identified with each QTL explaining 8.4 to 34.5% of phenotypic variance, suggesting the polygenic basis of the resistance to head rot. Five of these QTL were identified in more than one experiment, and each QTL explained more than 12.9% of phenotypic variance. These QTL could be useful in sunflower breeding. Although a positive correlation existed between the two disease indices, most of the respective QTL were located in different chromosomal regions, suggesting a different genetic basis for the two indices.

Sunflower (*Helianthus annuus* L.) is one of the staple oilseed crops of the world. The ubiquitous *Sclerotinia sclerotiorum* (Lib.) de Bary attacks all parts of the sunflower plant and causes three distinct diseases: head rot, basal stalk rot, and mid-stalk rot. It is one of the most important pathogens worldwide. In the northern Great Plains of the United States, head rot has been the greatest threat to sunflower production. The incidence of head rot can increase drastically under a high rainfall condition during the flowering stage. In 1999, more than 80% of the sunflower fields in eastern North Dakota had high incidences of head rot causing an estimated 70 million dollar loss (5). Breeding for resistant hybrids is an important approach to reduce the damage from *S. sclerotiorum* since chemical control is difficult and not feasible economically (14).

Progress in breeding for head rot resistance is limited due to the lack of effective sources of resistance. Breeding relies on identification and incorporation of partial resistance from diverse genotypes. Evaluation of head rot resistance in the field is difficult since environmental factors play a significant role in disease development. However, molecular markers tightly linked to head rot resistance genes can be employed to assist the selection for resistance genes during variety development. The advent and development of molecular markers and genetic maps have facilitated understanding of the genetic basis of head rot resistance. About a dozen linkage maps have been published using different molecular markers, including restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA

(RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR) in sunflower (4,20). In addition, Lai et al. (9) added 243 markers derived from expressed sequence tags (EST) to the sunflower SSR map. Mestries et al. (14) identified three quantitative trait loci (QTL) for head rot resistance, each explaining 12.3 to 17.5% of phenotypic variance. Measured by percentage attack and the number of days between infection and the appearance of symptoms (latency index) in two populations, Bert et al. (1,2) identified 20 QTL for head rot resistance. Röncke et al. (18) detected five QTL governing head rot resistance, and each explained 10.6 to 17.1% of the total phenotypic variance.

Recently, a polymerase chain reaction (PCR)-based target region amplification polymorphism (TRAP) marker technique was developed to take advantage of the annotated EST as a reference to generate polymorphic markers (6). This marker technique reveals high levels of polymorphisms in various crops (15,22), and over 400 TRAP markers have been added to the public sunflower linkage map (8). This study reports on the construction of a linkage map primarily using TRAP in an F_2 sunflower population and the result of QTL mapping for resistance to head rot in two U.S. Department of Agriculture (USDA)-released sunflower germplasms.

MATERIALS AND METHODS

Plant materials and disease evaluation. Two oilseed-type sunflower breeding lines, HA 441 (PI 639164) and RHA 439 (PI 639162), with resistance to head rot, were recently released by the USDA-ARS and the North Dakota Agricultural Experimental Station (16). HA 441 is a cytoplasmic male sterility maintainer line and RHA 439 is a restorer line. One hundred and twenty-three $F_{2,3}$ and $F_{2,4}$ families from a cross between HA 441 and RHA 439, as well as the two parents, were used in field inoculation

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tests in the summer of 2006 and 2007. Seeds of the $F_{2:3}$ families were harvested from 123 selfed F_2 individuals, and the seed of the $F_{2:4}$ families were the bulk of the seed from six randomly selfed individuals in each $F_{2:3}$ family. The $F_{2:3}$ and $F_{2:4}$ families (20 to 25 plants each) were planted at two field locations (Carrington and Fargo, ND) in a randomized complete block design with two replicates. At the flowering stage, approximately 12 heads in each plot were inoculated with a suspension of 5,000 ascospores per milliliter of water and 5 ml per head. Ascospores were applied directly to the face of individual heads with a hand spray bottle. A lab-grown strain of sclerotia (WM 274), which originally came as ascospores from Nebraska in 1999, was used throughout the study due to its consistent ability to form apothecia. Ascospores were produced with our in-house procedure: sclerotia were produced on cornmeal/sand (20:1, vol/vol). Uniform sized sclerotia were selected for conditioning with three repeated freeze (24 h at -20°C)/thaw (24 h at 22°C) cycles. The conditioned sclerotia were surface sterilized and were placed in petri dishes filled with moistened, sterile silica sand. The dishes were placed under fluorescent illumination (half "cool white" bulbs and half "near UV" bulbs) with a 12-h photoperiod, held at 15 to 17°C . Once stipes began to appear, those germinated sclerotia were transferred to water agar plates until the apothecia matured. Ascospore release was triggered by momentarily lifting the lid. The lids with adhering ascospores were removed daily, and stored at -80°C until needed.

To create an effective microenvironment for disease development, a misting system was installed and misted for 5 min after inoculation for 5 min every half-hour for 3 weeks. Five weeks after the inoculation, disease was scored on individual heads using a scale of 0 through 5 (0 = no symptom; 1 = 1 to 20% rot; 2 = 21 to 40% rot; 3 = 41 to 60% rot; 4 = 61 to 80% rot; and 5 = 81 to 100% rot). Disease resistance was evaluated by disease incidence (DI), calculated as the percentage of plants infected within a row, and disease severity (DS), measured by the average disease score of the infected plants within each plot. The test in Fargo was severely damaged by a storm in 2006. Thus, the data from three tests, Carrington in 2006 (test 1), Carrington in 2007 (test 2), and Fargo in 2007 (test 3) were used for QTL analysis in this study.

DNA preparation. Total genomic DNA was isolated from 50 mg (fresh weight) young leaf tissue sampled from 15 plants of each $F_{2:3}$ family using the Qiagen DNeasy 96 Plant Kit (QIAGEN,

Valencia, CA), following the manufacturer's instruction. DNA concentrations were determined with a DU7400 spectrophotometer (Beckman Coulter) and adjusted to 10 ng/ μl for PCR amplification.

Genotyping. The TRAP assays followed the procedures described by Hu and Vick (6) except for the change of the annealing temperature in the first five cycles from 35 to 40°C and in the last 30 cycles from 50 to 53°C . The fixed primers were designed against the expressed sequence tag (EST) sequences in the Compositae Genomics Initiative database, and selected cloned genes in the database of the National Center for Biotechnology Information. A total of 29 fixed primers designed against the EST primarily related to the conserved regions of leucine rich repeat (LRR), nucleotide binding site (NBS), kinase, and telomere were used to generate TRAP markers for map construction. We designed nine arbitrary primers labeled with either IR (infrared) 700 or IR 800 dye for TRAP analysis (6). Sequences of these primers are given in Table 1. The nomenclature of TRAP markers followed the rule of the code of fixed primer + the code of labeled primer + fragment size. In addition, two morphological markers, anthocyanin pigmentation in hypocotyls (*Hc*) and branching (*B*), were visually evaluated at seedling emergence and flowering stages, respectively.

To assign the linkage groups to previously published SSR maps, 220 SSR and insertion-deletion (INDEL) mapped markers distributed throughout the genome (23) were screened for polymorphisms between the two parents. Of these, 45 were polymorphic and were genotyped in the F_2 population. SSR and INDEL marker assays were conducted following the procedures described by Tang et al. (20).

Data analysis, map construction and QTL analysis. Analysis of variance (ANOVA) and correlation analysis were performed by the procedures of generalized linear modeling (GLM) and correlation (CORR) using the SAS program (19). The program Mapmaker/EXP 3.0 (11) was used to construct the genetic map (with the Kosambi map function). The means of the data from the two replications were used to detect QTL using the software of Mapmaker/QTL 1.1 (10,12).

RESULTS AND DISCUSSION

Phenotypic variance among parents and the $F_{2:3}$ and $F_{2:4}$ populations. The phenotypic differences between the parents as

TABLE 1. Source expressed sequence tags identity (EST ID) and accession number and sequence information of some of the fixed and arbitrary primers used for genotyping

Code	EST ID or GenBank accession no.	Sequences 5'-3'
Fixed primers		
T02	QHA10B18	GTTTGCCTTTAAGAACCG
T05	QHA11D14	ATACCCACCCGTCACAC
T08	QHA11I24	CGGTATAGCCTGTTTGTG
T10	QHA12P24	CTCCAGTCTGACCCGTTG
T12	QHA13J07	GGCAAGTCATATGCACGA
T17	QHA20I01	CCGAGTTGGTATGCTTGT
T29	QHB14G14	CTCGATAACATCCTCCCA
T30	QHB14G14	AATCTCAAGGACAAAAGG
T36	QHB18I19	CTGCCAAGTGAAAACGCT
T47	QHF15O11	CTGGAGCCAAGACATCTG
T61	HAU91339	ACCATTCAATATCTCATTG
T74	QHL12D20	CGAGAAAGCACATTCGTTA
T75	QHL12D20	CCCGTATGAATCCAAGAGTA
T83	QHK11F05	GAACCAAACTGGGTATGTA
T123	AT2G44990	ACGTTATGAGCCCATGAAGA
Arbitrary primers		
R03	TRAP03(IR-700)	CGTAGCGCGTCAATTATG
R19	SA12(IR-700)	TTCTAGGTAATCCAACAACA
R20	SA14(IR-700)	TTACCTTGGTCATACAACATT
R21	SA4(IR-700)	TTCTTCTCCCTGGACACAAA
R13	TRAP13(IR-800)	GCGCGATGATAAATTATC
R23	GA5(IR-800)	GGAACCAAAACACATGAAGA

well as the variance in the populations are summarized in Table 2. HA 441 had higher DI and DS than RHA 439 in test 1. Results, however, were reversed in test 2 and test 3, with a significantly higher DI in RHA 439 than in HA 441. These results suggested the two parents responded differently to the pathogen under different environments. The mean DI was highest in test 3, and lowest in test 2, while the mean DS did not fluctuate much in all three tests. Transgressive segregation was observed for DI and DS in the populations. The values of skewness and kurtosis for the traits were less than or close to 1.0 except for DS in test 1. This indicated the traits generally fit to a normal distribution (Table 2).

ANOVA of the data from different tests revealed that variance due to genotypic differences was highly significant for both traits. Variances from tests, block, and genotype by test for DI, and variance from genotype by test for DS were also significant (data not shown). This indicated that environments had significant influence on the traits, and similar results were also observed in previous studies (18). DI was more sensitive to environments than DS in this study. This may be explained by considering that disease incidence might be related to disease avoidance/or escape, which is more complex than disease development.

Correlations between DI and DS. Positive and significant correlations were detected between DI and DS in different tests. The coefficients were highest between DI and DS in the same tests (0.57 to 0.64), intermediate for DI across tests (0.40 to 0.49), and lowest between DI and DS in the different tests (0.19 to 0.32).

Polymorphism and map construction. For SSR markers, the polymorphism was low between the two parents, only 45 of the 220 (20.5%) primer pairs screened were polymorphic. Fifty-eight primer combinations in 29 TRAP reactions amplified 263 polymorphic markers. On average each primer combination generated 4.5 polymorphic bands with a range of 1 to 13. Of the 263 polymorphic markers, 19 were not linked to other markers and 64 markers were not used to construct the genetic map due to their clustering in certain regions on the chromosomes. The current genetic map for detecting head rot resistance QTL contains 180 TRAP, 32 SSR, 11 INDEL, and 2 morphological markers. This map has 19 linkage groups which span 1797.6 cM with an average distance of 8.0 cM between adjacent markers (Fig. 1). The SSR and INDEL markers were distributed on 16 linkage groups, and these markers were assigned to 16 of the 17 linkage groups of the public sunflower SSR map (23), the exception being linkage group 14. The morphological marker *B* was mapped to the same chromosomal region on linkage group 10 as described by Tang et al. (21). The other morphological marker, *Hc*, mapped to linkage group 6, and this locus was different from another locus (*T*) controlling anthocyanin pigment located on linkage group 11 (17).

QTL mapping. Nine QTL were detected for DI in the three tests and each explained 9.6 to 26.4% of phenotypic variance (Table 3). However, only *QDi4* was found in all three tests and two others were present in two tests. *QDi4* explained 14.8 to

26.4% of phenotypic variance in the three tests. HA 441 was the source of six of nine DI resistance QTL.

Seven DS resistance QTL were identified in three tests, each explaining 8.4 to 34.5% of the phenotypic variance. Two QTL, *QDs2* and *QDs5*, were detected in two of the three tests. HA 441 and RHA 439 contributed four and three resistance QTL, respectively, for decreasing DS.

Previous studies indicated that head rot resistance was conferred by multiple genes in sunflower (1,2,14,18). The results in this study also support the polygenic nature of resistance. Both parents in this study contributed head rot resistance QTL to their progenies. These results were expected since the two parents showed partial resistance to head rot (16), and previous studies also reported similar findings (1). It is possible, therefore, to develop germplasm lines with higher levels of resistance by pyramiding these resistance QTL from different genotypes.

Although a positive correlation existed between DI and DS in this study, only 6 of the 16 resistance QTL were located in the same chromosomal regions, suggesting a different genetic basis for the two disease indices. Thus, breeding lines with higher levels of resistance to head rot could be developed by pyramiding these QTL governing different disease indices. Moreover, the mean values of DI and DS decreased from 75.1 to 35.3%, and 4.4 to 3.7 with the increasing number of QTL for DI or DS, respectively. Moreover, the mean values of DI and DS in the families that possessed all 5 QTL were 33.9% and 3.6, respectively (Table 4). Thus, pyramiding resistance QTL for DI and DS could increase the resistance level of breeding lines and cultivars.

QTL congruence and comparison with previous reports. Resistance QTL for DI and DS were found in 13 chromosomal regions of 10 linkage groups (Fig. 1). Three intervals (*T123R20_380* to *T47R03_180* in the linkage group 2, *T29R03_850* to *T08R03_810* in the linkage group 9, and *T61R23_360* to *T36R03_670* in the linkage group 12) harbored resistance QTL for DI and DS. Moreover, DI and DS resistance QTL were consistently found in the *T61R23_360* – *T36R03_670* interval.

It is difficult to compare the resistance QTL detected in this study with previous results due to different molecular markers being employed. However, three resistance QTL detected in this study can be associated with previous QTL through nearby SSR or morphological markers. Röncke et al. (18) identified a head lesion length (similar to DS) resistance QTL close to the SSR marker ORS613 in linkage group 10. This study defined a resistance QTL for DS in the same chromosomal region. Near the locus harboring the branching gene (*b₁*), Bert et al. (1) identified two QTL (percentage attack on capitulum and latency index, similar to DI and DS, respectively) and Mestries et al. (14) detected one (capitulum index, similar to DS) QTL for head rot resistance. In this study, a resistance QTL for DI was detected in that chromosomal region in two tests.

The advantages of TRAP markers. In this study, a linkage map was constructed, mainly employing TRAP markers, for culti-

TABLE 2. Measurement of disease incidence and severity values and population parameters for 123 F_{2:3} and F_{2:4} families from a cross between sunflower (*Helianthus annuus* L.) lines HA 441 and RHA 439 in three field experiments

Traits ^a	HA 441	RHA 439	Population				
			Range	Mean	SD	Skewness	Kurtosis
Disease incidence (%)							
Test 1	66.7	26.7	0.0–94.4	43.3	23.5	0.3	–0.6
Test 2	6.5	68.8 ^b	0.0–90.6	31.3	17.6	0.5	0.1
Test 3	33.3	85.0 ^b	0.0–100.0	56.7	24.1	0.0	–0.7
Disease severity							
Test 1	4.2	4.2	0.0–5.0	2.6	1.1	–1.5	2.4
Test 2	2.5	4.5	0.0–5.0	4.0	1.0	–1.2	1.5
Test 3	3.4	4.9	0.0–5.0	3.9	1.1	–1.1	0.7

^a Test 1 = study at Carrington, ND, in 2006; Test 2 = study at Carrington, ND, in 2007; Test 3 = study at Fargo, ND, in 2007.

^b Difference is significant at the *P* = 0.01 level between the two parents.

Fig. 1. The location of quantitative trait loci (QTL) for head rot resistance detected in the three tests (test 1, Carrington, ND, in 2006; test 2, Carrington, ND, in 2007; and test 3, Fargo, ND, in 2007). The map is constructed from the HA 441/RHA 439 $F_{2.3}$ population. Sixteen simple sequence repeats (SSR)-anchored linkage groups are numbered the same as in the public sunflower map and three groups with only target region amplification polymorphism markers are named A to C.

TABLE 3. Quantitative trait loci (QTL) for head rot incidence and severity for F_{2:3} and F_{2:4} families from a cross between sunflower (*Helianthus annuus* L.) breeding lines HA 441 and RHA 439 using interval mapping

Traits ^a	QTL	Linkage groups	Marker interval	LOD	R ² % ^b	Resistant parent ^c
Disease incidence						
Test 1	<i>QDi1</i>	02	T123R20-380 – T47R03-180	3.4	13.9	H
	<i>QDi2</i>	07	T123R20-220 – T10R23-508	3.0	11.2	H
	<i>QDi3</i>	10	T36R03-390 – T05R21-610	3.7	14.3	R
	<i>QDi4</i>	12	T61R23-360 – T36R03-670	5.1	26.4	R
Test 2	<i>QDi5</i>	08	ORS70 – R03R13-430	5.2	17.9	R
	<i>QDi6</i>	09	T12R21-450 – T17R13-720	2.7	9.6	H
	<i>QDi3</i>	10	T36R03-390 – T05R21-610	3.0	12.9	R
	<i>QDi4</i>	12	T61R23-360 – T36R03-670	4.4	23.5	R
	<i>QDi7</i>	17	ORS1245 – T83R19-580	4.2	17.1	H
	<i>QDi1</i>	02	T123R20-380 – T47R03-180	5.3	18.5	H
Test 3	<i>QDi8</i>	03	ORS1036 – T74R21-155	2.8	11.1	H
	<i>QDi9</i>	09	T29R03-850 – T08R03-340	3.5	16.4	H
	<i>QDi4</i>	12	T61R23-360 – T36R03-670	3.7	14.8	R
Disease severity						
Test 1	<i>QDs1</i>	02	T123R20-380 – T47R03-180	2.9	12.8	H
	<i>QDs2</i>	04	T08R13-528 – T02R23-225	9.6	24.9	H
	<i>QDs3</i>	10	T36R13-460 – ORS749	11.8	34.5	R
Test 2	<i>QDs2</i>	04	T08R13-528 – T02R23-225	4.1	24.4	H
	<i>QDs4</i>	08	ZVG34 – T30R23-330	2.4	8.4	R
	<i>QDs5</i>	12	T61R23-360 – T36R03-670	4.1	22.0	R
	<i>QDs6</i>	13	T75R20-380 – ZVG61	3.5	23.9	H
	<i>QDs7</i>	09	T29R03-850 – T08R03-340	4.2	19.1	H
Test 3	<i>QDs5</i>	12	T61R23-360 – T36R03-670	3.3	23.6	R

^a Test 1 = study at Carrington, ND, in 2006; Test 2 = study at Carrington, ND, in 2007; Test 3 = study at Fargo, ND, in 2007.

^b Amount of phenotypic variance (%) explained by the QTL.

^c The QTL from HA 441(H) or RHA 439 (R) of the locus had a positive effect on this trait.

TABLE 4. Number of F_{2:3}/F_{2:4} families from a cross between sunflower (*Helianthus annuus* L.) breeding lines HA 441 and RHA 439, and their range and mean disease incidence and disease severity values evaluated in the three field tests that had different number of resistance quantitative trait loci (QTL) detected in more than one test

Traits ^a	No. of QTL	No. of families	DI		DS	
			Range ^b	Mean ^b	Range ^b	Mean ^b
DI	0	4	61.7–94.1	75.1	4.5–4.9	4.7
	1	16	42.8–84.2	61.3	3.2–4.8	4.3
	2	58	9.9–78.1	43.1	2.1–4.9	4.0
	3	44	9.6–54.3	35.3	2.3–4.9	3.8
DS	0	12	31.0–78.1	55.8	3.9–4.9	4.4
	1	60	14.7–84.2	45.7	2.7–4.9	4.2
	2	50	9.6–94.1	37.8	0.8–4.7	3.7
DI & DS	5	28	11.7–54.3	33.9	2.3–4.7	3.6

^a DI and DS are disease incidence and disease severity, respectively.

^b Mean and range are the average of the three tests (Carrington, ND, in 2006; Carrington, ND, in 2007; Fargo, ND, in 2007).

vated sunflower. Each PCR reaction generated 9 polymorphic TRAP markers on average in the present mapping population, whereas only 20.5% of the polymorphisms were detected between the two parents with SSR markers. Therefore, TRAP is an efficient molecular marker technique for the construction of a linkage map, as suggested in previous studies (6,7). TRAP markers have been used in linkage map construction in other plant species (3,13,15). Moreover, TRAP takes the advantage of the annotated EST information to generate markers around the target sequence (6). The information of the EST flanking the QTL might provide useful information to facilitate gene cloning. For example, the TRAP markers flanking the two major QTL, *QDi4* and *QDs2*, were designed against EST related to NBS (QHB18I19) and kinase (QHA11I24 and QHA10B18), respectively. These TRAP markers might be helpful for a better understanding and cloning of the resistance QTL in future studies.

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