SEARCHING FOR DNA MARKERS ASSOCIATED WITH *SCLEROTINIA* TOLERANCE IN CULTIVATED SUNFLOWER

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Abstract

Using the in-house-developed target region amplification polymorphism (TRAP) technique, we are aggressively searching for DNA markers associated with *Sclerotinia* tolerance in sunflower. The F2 population of 190 plants used for the current study was derived from a three-way cross of HA412/SD//Romania *Phomopsis* resistant B-line and segregated for *Sclerotinia* tolerance and plant height. The fixed primers for TRAP were designed against annotated ESTs (expressed sequence tags) homologous to identified plant disease resistance gene components such as kinase, leucine rich repeats (LRR), and nucleotide binding sites (NBS). The fixed primers in combination with fluorescently labeled arbitrary primers were used to amplify DNA fragments from genomic DNA. On average, each PCR reaction amplified 50 scorable fragments with length ranging from 50 to 900 base pairs. The number of polymorphic fragments varied from 1 to 12 per primer combination. Up to now, one hundred and two markers were generated in this population. Seventy-nine of these markers segregated in a ratio of 3:1, and thirteen segregated in a ratio of 1:1. This was expected because the population had three parental lines. One marker associated with *Sclerotinia* susceptibility was generated in this population. This marker sized 411 bp and amplified by the primer combination E10J20b and Ga5 was present in 15 of the 18 diseased plants and segregated in a ratio of 3:1 in the population.

Introduction

The *Sclerotinia* diseases are some of the most important diseases of cultivated sunflower (*Helianthus annuus* L.) in all sunflower-growing regions of the world. *Sclerotinia sclerotiorum* (Lib.) de Bary is today considered one of the most damaging pathogens of sunflower (Gulya et al., 1997) because it can attack several plant parts, causing wilt or stem/head rot. *Sclerotinia* head rot is considered a major disease in Europe, Argentina, and the United States. Severe attacks can cause up to 100% yield losses (Sackston, 1992).

Resistance to *Sclerotinia* has a complex inheritance pattern. No complete resistance source to *S. sclerotiorum* has been identified to date. The USDA-Agricultural Research Service Sunflower Research Unit has released several cultivated sunflower lines that showed field tolerance to the disease (Miller and Gulya, 1999). Previous research has shown that wild ancestors of cultivated sunflower are a source of genes for tolerance and resistance to several sunflower pathogens, and some wild species of sunflower were identified to be completely tolerant to *Sclerotinia* (Rashid and Seiler, 2001).

The development of molecular markers makes it possible to investigate the inheritance of complex traits and to locate genetic factors underlying these quantitative traits. TRAP, a PCR-based marker technique, uses ESTs as reference to generate polymorphic markers around targeted candidate gene sequences (Hu and Vick, 2003). The objective of this study was to generate DNA markers associated with *Sclerotinia* disease tolerance. These markers will be used to assist selection of the resistance in breeding and to pyramid resistance genes from different sources into elite breeding lines.

Materials and Methods

Plant Materials. The F2 population of 190 plants segregating for *Sclerotinia* tolerance and plant height was used. This population was derived from a three-way cross of HA412/SD//RHA409 and was evaluated for *Sclerotinia* tolerance and plant height at the Carrington Research and Extension Center in the 2003 growing season. HA412 was derived by crossing HA821 with selections from four lines that had some tolerance to *Sclerotinia* head rot. SD is maintainer oilseed inbred line which was selected for *Sclerotinia* head rot resistance. RHA409 is a restorer oilseed line which has excellent tolerance to *Sclerotinia* stalk rot, but does not have tolerance to head rot. The three-way cross was made in order to pyramid the partial tolerance genes from different sources. The distribution of plant height of the population is shown in Figure 1. *Sclerotinia* pathogen inoculation was conducted on August 15, 2003, with our in-house procedure. Each plant was inoculated once with Sclerotinia spores and was misted for five weeks. The disease symptoms were evaluated on a scale of 0 to 5 based on the percentage of head infection observed (Table 1) 5 weeks after inoculation. The disease symptoms were not fully expressed due to interference of an early frost. Only 18 plants showed *Sclerotinia* head rot in the population and these plants were used to confirm marker-trait association.

DNA Preparation. Total genomic DNA was isolated from 30 mg (wet weight) young leaf tissue of sunflower using the Qiagen DNeasy® 96 Plant Kit (QIAGEN, Valencia, CA, Cat # 69181)*, following the manufacturer's instructions with modifications. To elute the DNA, 1× TE Buffer (pH 8.0) was used instead of the provided Buffer AE. DNA concentration was determined with a DU7400 spectrophotometer (Beckman Coulter)* and

adjusted to 30-50 ng/µl for PCR amplification. The DNA samples were stored at 4C for short term use.

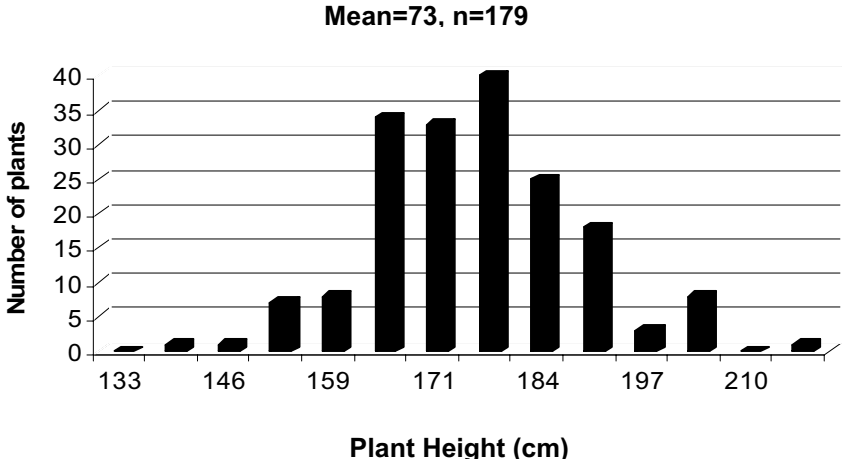

Figure 1. Distribution of plant height in the F2 population.

TRAP Analysis. TRAP was conducted following the procedures described by Hu and Vick, 2003. The fixed primers were designed against the EST sequences in the Compositae Genomics Initiative database (http://cgpdb.usdavis.edu). From the database, we chose ESTs homologous to a leucine rich repeat (LRR) region or a nucleotide binding site (NBS), two conserved major components of most plant disease resistance genes. The arbitrary primers were designed to contain an AT- or GC-rich core that anneals to introns or exons within the targeted gene, respectively and labeled with either IR (Infrared) 700 or IR 800 dye. Some of the fixed primers and random primers used in this study are listed in Table 2.

Table 1. Disease assessing criterion for sunflower head rot.

Head rot score	Disease rating scale
0	no symptoms
1	0-12.5% of head showing symptoms
2	12.5-25% of head showing symptoms
3	25-50% of head showing symptoms
4	50-100% of head showing symptoms
5	100% of head showing symptoms

PCR was conducted with a final reaction volume of 15 µl in 96-well microtiter plates in a GenAmper 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following components: 2 µl of the 30 to 50 ng/µl DNA sample, 1.5 µl of 10×reaction buffer (Qiagen), 1.5 µl of 25 mM MgCl2, 1 µl of 5 mM dNTPs, 3 pmol each of 700- and 800-IR dye labeled

random primers, 10 nmol of the fixed primer, and 1.5 units of *Taq* DNA polymerase (Qiagen). The PCR was performed by initially denaturing template DNA at 94C for 2 min; then five cycles at 94C for 45 s, 35C for 45 s and 72C for 1 min; followed by 35 cycles at 94C for 45 s, 50C for 45 s and 72C for 1 min; then a final extension step at 72C for 7 min.

Following the amplification reactions the PCR products were mixed with 7 µl of 5×loading dye (containing 0.313 M Tris-HCl pH 6.8 at 25C, 10% SDS, 0.05% bromophenol blue, and 50% glycerol). A 0.8-µl aliquot of each sample was loaded onto a 6.5% polyacrylamide sequencing gel in a Li-Cor* Global DNA Sequencer using an eight-channel Hamilton* syringe (Hamilton Company Reno, NV*). IRD-labeled molecular markers (Li-Cor) were loaded in two lanes as a standard. The electrophoresis was conducted at 1500 volts for 3.5 h. The amplified product was analyzed with a Li-Cor Global DNA sequencer with SAGA program to score the amplified fragments.

Table 2. Some of the fixed and random primers used in this study.

A: Fixed Primers	**Sequences (5' to 3')**	**Homology by Blast Search**
QHB22D05a QHB22D05b	CCGGAGAGTTCTATCGCT GAAGCTTCACAGGGAGTT	putative LRR receptor protein kinase; protein id: At2g23950.1 [*Arabidopsis thaliana* L.]
QHE10J20a QHE10J20b	CTTCAGCAGTGTCTCTCC GTTATGGTGAAGGCAACG	cytosolic glutamine synthetase [*Helianthus annuus* L.]
QHA20I01a QHA20I01b	CCGAGTTGGTATGCTTGT AGCTCTGGAAACCGTCTG	LRR receptor-like protein kinase [*Nicotiana tabacum* L.]
QHG17L12a QHG17L12b	TACCTTATGCCTTCGGGT ACAATTCGTCTCAAGCGG	putative disease resistant protein RGA2 [*Solanum bulbocastanum* Dunal]
QHB18I19a QHB18I19b	CGTTTATTTCCTCGCCTC CTGCCAAGTGAAAACGCT	putative disease resistance protein (NBS-LRR class); protein id: At5g47280.1 [*Arabidopsis thaliana*]
QHB6G05a QHB6G05b	TGGATTTTCACCAGCGTC GAAATTAACGGGGTTGGA	NBS-LRR protein [*Solanum acaule* Bitter]
B: Arbitrary Primers	**Sequences (5' to 3')**	**Labeled with:**
TRAP03	CGTAGCGCGTCAATTATG	700 Infrared dye
TRAP13	GCGCGATGATAAATTATC	800 Infrared dye
TRAP06	GCTGACGTAGTAATTCCA	700 Infrared dye
TRAP16	TGCGTAGTAGATGCGCGC	800 Infrared dye
Sa4	TTCTTCTTCCCTGGACACAAA	700 Infrared dye
Ga5	GGAACCAAACACATGAAGA	800 Infrared dye

Results and Discussion

The TRAP procedure can generate a large number of polymorphic markers in sunflower (Hu and Vick, 2003). In this research, each PCR reaction amplified about 50 scorable fragments with length ranging from 50 to 900 bp among the 190 plants in the F2 population. A total of 32 PCR reactions amplified 102 polymorphic markers from this population and the number of polymorphic fragments varied from 1 to 12 per primer combination.

Of the 102 polymorphic markers, 79 polymorphic markers segregated in the expected Mendelian ratios (3:1), and 13 segregated 1:1. Figure 2 shows some of the polymorphic

markers produced by primer combination G17L12a and Trap13 in 64 F2 plants. These markers all segregated in a ratio of 3:1 in the 102 plants.

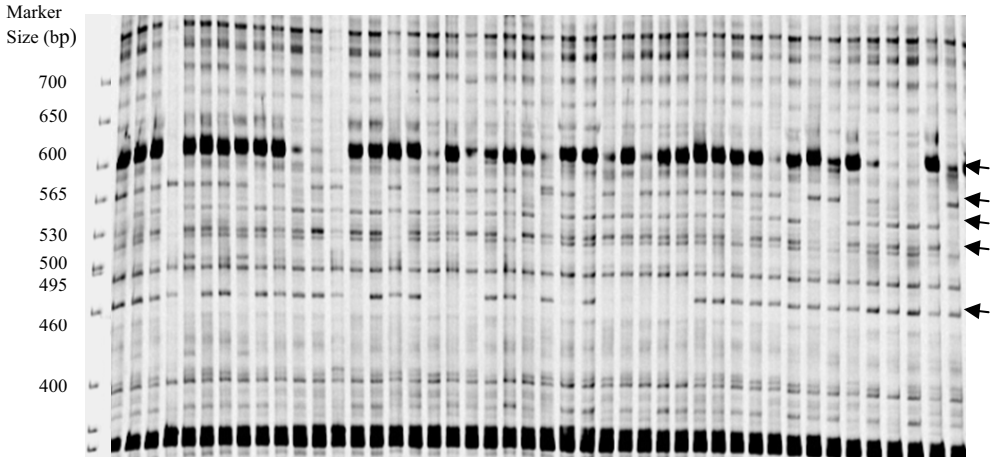

Figure 2. The amplification profile produced by primer combination G17L12a and Trap13 in 64 F2 segregating plants. The polymorphic markers (arrows) segregated in the expected Mendelian ratios (3:1) in the whole population.

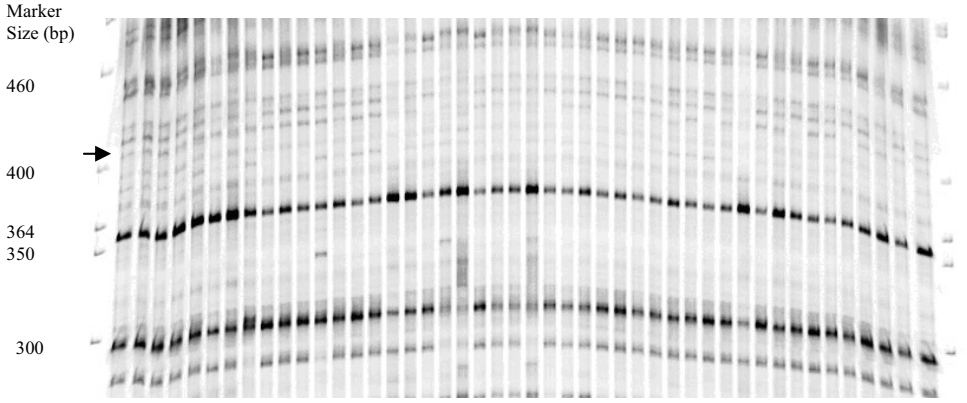

Figure 3. A TRAP marker possibly associated with *Sclerotinia* head rot susceptibility in the F2 population. Forty-six plants (Lane 1-46) were used. Plants 1-18 showed head rot symptoms (disease score 1-5) and plants 19-46 no symptoms (disease score 0). The TRAP marker E10J20b-Ga5-410 (arrow) is present in 15 of the 18 diseased plants and segregating among the rest of the plants in the population, which suggests an association with head rot susceptibility.

We identified one TRAP marker possibly associated with *Sclerotinia* head rot susceptibility in the F2 population. This marker, sized 411 bp and amplified by primer combination E10J20b and Ga5, was present in 15 of the 18 diseased plants, while segregating

in a ratio of 3:1 in the whole population (Figure 3). Since resistance to *Sclerotinia* is controlled by multiple genes or QTLs, this marker could be linked to one of the genes or located in one of the QTL regions. This marker can be used to assist selection resistance and to accelerate the process of developing sunflower germplasm tolerant to *Sclerotinia*.

As mentioned above, about 13% of the molecular markers do not have the expected Mendelian ratios of 3:1 (at $\alpha = 0.05$). This was expected because the population we used was derived from three parental lines.

With the polymorphic markers identified it is possible to construct a genetic linkage map using Mapmaker software v3.0 (Lander et al., 1987). The genetic map will be useful to sunflower breeders for the improvement of sunflower and the development of new varieties. This study will also provide basic information to the sunflower geneticists who are attempting to clone genes of sunflower. The next step that we need to do is to find more polymorphic markers and to construct a molecular genetic linkage map for sunflower.

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