TRAP MARKERS FOR A SUNFLOWER DOWNY MILDEW RESISTANCE GENE FROM A NEW *HELIANTHUS ANNUUS* SOURCE, PI 468435

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Abstract

This paper reports the development of DNA markers associated with a downy mildew resistance gene from a new source, PI 468435, a wide *Helianthus annuus* L. population collected from Idaho, USA. We used the newly developed TRAP marker technique and segregating populations (F2 and BC1) derived from the cross between HA 434 and PI 468435. Downy mildew susceptibility of individual seedlings in the segregating populations was determined by both the presence of sporulation on the cotyledons after conventional seed immersion inoculation and the presence of DM-specific fragments amplified from the DNA extracted from the cotyledon of the inoculated seedlings prior to sporulation. Segregating data suggested that PI 468435 carries a dominant DM resistance gene. One fragment of 257 base pairs showed intensity variation associated with the DM resistance phenotype in both F2 and BC1 populations. This fragment was amplified by a TRAP primer designed against a sunflower EST sequence, QHB18F12, which is homologous to the disease resistance gene RPS2, cloned from *Arabidopsis*, in combination with an arbitrary primer, TRAP03. The same primer combination did not detect a similar variation in the segregating populations containing the *Pl6* (from HA 335) and *Pl8* (from HA 340) genes. The preliminary results imply that the DM resistance gene in PI 468435 was different from both the *Pl6* and *Pl8* genes.

Introduction

Downy mildew (DM) caused by the obligate parasite *Plasmopara halstedii* (Farl.) Berl. and de Toni, is one of the most important diseases of sunflower in North America (Gulya et al., 1997) and in all the sunflower growing regions except Australia (Roeckel-Drevet et al., 2003). Downy mildew affects sunflower plants at all growth stages. Affected young seedlings appear dwarfed and have chlorosis on the leaves, and the capitula of affected plants

produce fewer seeds. When the disease incidence is high and the climate conditions favor disease development, substantial yield losses can result from DM infection.

Only two races of DM, races 100 and 300, were identified in the 1970s (Zimmer, 1974; Sackston, 1978) and two dominant genes, *Pl1* and *Pl2*, confer complete resistance to those races. After the successful incorporation of the resistance genes into modern sunflower hybrids to combat DM, new races overcoming the resistance genes arose frequently during the past two decades (Gulya et al., 1991; Molinero-Ruiz et al., 2003). The continued search for effective resistance genes has uncovered 11 *Pl* genes that confer resistance to one or all of the identified DM races (Miller and Gulya, 1991; Vear et al., 1997; Molinero-Ruiz et al., 2003). Two of the DM resistance genes, *Pl6* from *Helianthus annuus* L. and *Pl8* from *H. argophyllus* Torrey and Gray, have been incorporated into two USDA released germplasm lines, HA 335 and HA340, respectively (Miller and Gulya, 1991). Linkage analysis showed that these two genes are linked with other DM resistance genes in two different linkage groups of the RFLP composite map (Gentzbittel et al., 1999), the *Pl1*/*Pl2*/*Pl6* complex in linkage group 1 and the *Pl5*/*Pl8* complex on linkage group 6 (Roeckel-Drevet et al., 1996; Bert et al., 2001). Further molecular studies revealed that the *Pl1*/*Pl2*/*Pl6* complex belongs to the TIR-NBS-LRR resistance gene group, while the *Pl5*/*Pl8* complex belongs to the non-TIR-NBS-LRR resistance gene group; many PCR markers linked to each of the two loci have been developed (Bouzidi et al., 2002; Radwan et al., 2003; Slabaugh et al., 2003; Radwan et al., 2004). It is still not clear whether these loci are single resistance genes conferring non-race-specific resistance or clustered genes or multiple alleles giving resistance to individual races following the gene-for-gene hypothesis.

The use of DNA-based markers in crop plant breeding is an increasing component of the application of molecular biology to agriculture. The development of DNA markers associated with DM resistance in sunflower followed the same trends as in other crop species. The first generation of PCR-based markers associated with disease resistance loci were generated with primers of random sequences such as RAPD (Brahm et al. 2000). The second generation of PCR-based markers for disease resistance were the resistance gene candidate (RGC) and resistance gene analog (RGA), which were amplified with degenerate oligonucleotide primers designed against the conserved motif sequences shared by many plant disease resistance genes (Gentzbittel et al. 1998; Bouzidi et al. 2002; Radwan et al. 2003; 2004). Target region amplification polymorphism (TRAP) represents a novel approach to developing DNA markers associated with traits of interest. TRAP harnesses the rich expressed sequence tag (EST) resources of individual crop species and utilizes bioinformatics tools and the annotated EST sequence information to generate polymorphic markers around targeted candidate gene sequences (Hu and Vick, 2003). In this paper we present the progress in application of the TRAP technique to identify markers associated with a new DM resistance source derived from PI 468435, a wild *H. annuus* population collected from Idaho, USA.

Materials and Methods

From a preliminary screening for DM resistance among the wild *H. annuus* accessions in the USDA sunflower germplasm collection, PI 468435 collected from Idaho, USA, was found segregating for resistance to DM races 770 and 733. A resistant plant was crossed to HA 434 to generate two segregating populations, F2 (HA 434/PI 468435) and BC1 (HA 434//HA 434/PI 468435), for the current study.

For the DM resistance assay, mixed DM race 770 was used following the whole seedling immersion procedure (Miller and Gulya, 1991). Namely, germinated seeds with radicles of 1 to 2 cm in length were immersed in inoculum suspension containing 20,000 to 30,000 sporangia/ml for 3 hours. The inoculated seedlings were planted in flats containing 1 to 1 (v/v) mixture of perlite and sand and kept in the greenhouse for 12 to 14 days with temperature between 20 and 25C and a 16-h photoperiod. These flats were then transferred to a chamber with 100% relative humidity for 16 hours at 18C. Susceptibility phenotyping was assessed by the presence of sporulation on the first true leaf and/or on the cotyledons.

For DNA marker development, total DNA samples were prepared from half a cotyledon taken from each of the inoculated seedlings prior to high humidity incubation. DNA was extracted with a DNeasy 96 Plant Kit (QIAGEN*, Valencia, CA), following the manufacturer's instructions. The concentrations of DNA were determined with a DU7400 spectrophotometer (Beckman Coulter*) and adjusted to 30 to 50 ng/µl for PCR reactions. PCR products were run in the Li-Cor Global DNA sequencer (Li-Cor Biosciences*, Lincoln,

Table 1. Partial list of the primer sequences used in the current study. The names of the fixed primers correspond to the Sequence ID in the Sunflower EST Database.

A: Fixed Primers	**Sequences (5' to 3')**	**Homology by BLAST search**
QHA20I01a QHA20I01b	CCGAGTTGGTATGCTTGT AGCTCTGGAAACCGTCTG	gb|AAF66615.1| LRR receptor-like protein kinase [*Nicotiana tabacum* L.]
QHB6G05a QHB6G05b	TGGATTTTCACCAGCGTC GAAATTAACGGGGTTGGA	emb|CAB56299.1| NBS-LRR protein [*Solanum acaule* Bitter]
QHB18F12a QHB18F12b	GCTTCAGAGCATTGAAGT TCTTCAGTTTGGATAGGC	gb|AAK38117.1|AF368301_1 disease resistance protein RPS2 [*Arabidopsis thaliana* L.]
QHB18I19a QHB18I19b	CGTTTATTTCCTCGCCTC CTGCCAAGTGAAAACGCT	ref|NP_199539.1| putative disease resistance protein (NBS-LRR class); protein id: At5g47280.1 [*Arabidopsis thaliana*]
QHB22D05a QHB22D05b	CCGGAGAGTTCTATCGCT GAAGCTTCACAGGGAGTT	ref|NP_179973.1| putative LRR receptor protein kinase; protein id: At2g23950.1 [*Arabidopsis thaliana*]
B: Arbitrary Primers	**Sequences (5' to 3')**	**Labeled with:**
TRAP03	CGTAGCGCGTCAATTATG	700 Infrared dye
TRAP04	CGTAGTGATCGAATTCTG	700 Infrared dye
TRAP06	GCTGACGTAGTAATTCCA	700 Infrared dye
TRAP13	GCGCGATGATAAATTATC	800 Infrared dye
TRAP14	GTCGTACGTAGAATTCCT	800 Infrared dye
TRAP16	TGCGTAGTAGATGCGCGC	800 Infrared dye

NE) using a 6.5% polyacrylamide sequencing gel at 1500 volts for 3.5 h, and the images were collected by the SAGA software. Eighteen fixed primers were designed against 11 sunflower EST (expressed sequence tag) sequences in the Compositae Genomics Database (http://compgenomics.ucdavis.edu), and six IR-dye-labeled arbitrary primers were used to generate DNA markers following the TRAP protocol of Hu and Vick (2003). Seven ESTs used in designing the fixed primers have homology to the conserved regions, such as NBS (nucleotide binding site), and LRR (leucine-rich repeat) domains, of many plant resistance genes (Table 1). All these primers were designed with the web-based PCR primer designing program "Primer 3" http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi (Rozen and Skaletsky, 2000).

Results

The Inheritance of the DM Resistance. Forty-eight germinating F2 seeds were planted into sand after DM spore immersion with only 39 seedlings emerging. Among these, 29 were scored as resistant, seven as susceptible and three were questionable, based on the sporulation on the cotyledons. One hundred and eleven amplified fragments were classified as DM-specific because they were persistently present in nine susceptible seedlings (Figure 1, upper panel). The DM fungal origin of these fragments was confirmed by TRAP amplification of the DNA samples extracted from pure DM fungal spores. Among these nine seedlings, six were from the seven susceptible seedlings and three were the questionable seedlings. However, TRAP failed to amplify DM-specific fragments from one of the seedlings scored as susceptible. Among the 24 seedlings in the BC1 population, nine were scored as resistant and 15 as susceptible. No DM-specific fragment was amplified from the nine resistant seedlings. For the 15 susceptible seedlings, the DM-specific fragments were present in only 11 seedlings. Because the sporulation on the seedlings was obvious, we counted them as susceptible for genetic analysis. Thus, there were 28 resistant and 11 susceptible seedlings in the F2 population. This segregation fit the expected three to one ratio of monogenic regulation (Chi-square value = 0.213, $p > 0.50$). The 9R and 15S segregation in the BC1 generation confirmed the one dominant gene ratio of one to one (Chi-square value = 1.50, $p > 0.20$).

TRAP Markers Associated with DM Resistance. Thirty-six primer combinations in 18 sets of PCR reactions amplified 65 polymorphic markers from the DNA samples extracted from 39 inoculated seedlings of the F2 population. Sixty-two markers segregated in the expected 3:1 ratio. All but one of the markers was independent from the DM resistance phenotype. One marker, a 257 base-pair fragment amplified by the primer combination, QHB18F12b and TRAP03 showed intensity variation was associated with DM resistance in both the F2 and BC1 populations. This fragment showed much stronger intensity in lanes amplified from the resistant seedlings than in that from the susceptible seedling (Figure 1, upper panel). A similar amplification pattern supporting the marker-trait association was obtained in the BC1 population comprising 24 seedlings. Because many DM-specific fragments were amplified in the same PCR reaction, the intensity variation could be a result of competition between sunflower and DM DNA. To eliminate this possibility, we carried out an experiment of amplifying DNA extracted from the 24 seedlings from the same F2 and BC1 populations that were not inoculated with DM fungus. The variation in intensity of the 257 bp fragment was reproduced. Progeny tests on selected seedlings are being conducted to verify this marker-trait association. The same primer combination (QHB18F12b and

TRAP03) did not detect the same variation from the segregating populations containing the *Pl6* (HA 434/ HA 335 F2) and *Pl8* (HA 434/ HA 340 F2) genes, suggesting that the DM resistance gene in PI 468435 was different from both *Pl6* and *Pl8* genes.

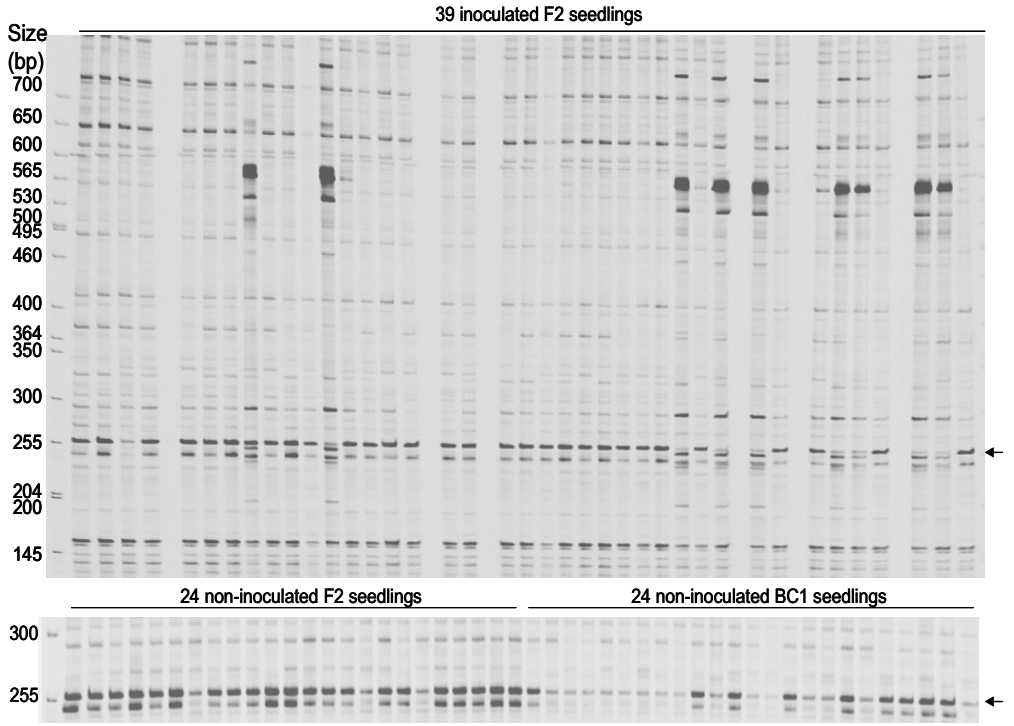

Figure 1. TRAP profiles amplified by a primer combination of QHB18F12b and TRAP03 showing DM-specific fragments, the intensity variation of the 257 bp fragment (arrow) and its association with DM resistance. Upper panel: Amplified with DNA isolated from 39 inoculated F2 seedlings, seven DM-specific fragments are consistently present in nine samples. The intensity variation of the 257 bp fragment (arrow) seems associated with DM resistance. Lower panel: Amplified with DNA samples prepared from non-inoculated seedlings, the 257 bp fragment exhibits similar intensity variation.

Discussion

Relationship between the PCR-Detected Presence of Downy Mildew and the Susceptible Phenotype in the Segregating Population. We reported that the TRAP technique is capable of detecting the presence of downy mildew and genotyping the host plants in the same PCR reaction (Hu et al., 2004). In the current study, TRAP detected the presence of DM in three seedlings scored as questionable in the F2 population. However, it failed to amplify the DM-specific fragments in five out of 24 seedlings that developed susceptible symptoms in the two segregating populations. A total of 111 DM-specific fragments were amplified with 36 primer combinations, with an average of 3.1 and a range of 0 to 9 per primer combination. None of these fragments was amplified from two seedlings, #3 of the F2 population and seedling #17 of the BC1 population, which were scored as susceptible. This

could be a result of a situation in which the DM population was very low in these seedlings at the time of tissue sampling for DNA preparation, and TRAP was not sensitive enough to amplify the DM-specific fragment.

Identifying Markers Associated with Resistance. One of our research activities is to look for new DM resistance sources and to incorporate them into cultivated sunflower. Molecular markers associated with disease resistance are useful in accelerating breeding programs for incorporating disease resistance. In sunflower, markers associated with the DM resistance gene *Pl2* were developed with the RAPD (random amplified polymorphic DNA) technique (Brahm et al. 2000). The markers linked to *Pl6* or *Pl5/Pl8* loci were amplified with degenerate primers based on the available sequence information on the cloned and characterized disease resistance genes from other plant species (Gentzbittel et al., 1998; Bouzidi et al., 2002, and Radwan et al., 2003, 2004). In this paper, we report a different approach to identify markers with disease resistance genes, that is, application of the TRAP protocol (Hu and Vick, 2003) that uses annotated sunflower EST-derived primers. It is interesting that the primer which produced the polymorphic fragment was designed from a sunflower EST that has homology with the disease resistance gene RPS2, cloned from the model plant *Arabidopsis*. Since the TRAP primer combination (QHB18F12b and TRAP03) did not detect the same variation from the segregating populations containing the *Pl6* and *Pl8* genes, it seems safe to conclude that the wild *H. annuus* population PI 468435 possesses a DM resistance gene that is different from both the *Pl6* and *Pl8* genes. Although a detailed genetic analysis in a larger population is needed to map this marker precisely, this marker can be used in a marker-assisted program to transfer the DM resistance gene from the wild *H. annuus* population PI 468435 into elite sunflower lines.

Acknowledgements

The authors thank Angelia Hogness, Dale Rehder, and Scott Radi for their technical assistance.

*****Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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