Research Article

Monitoring Three *Plasmopara halstedii* Resistance Genes in Iranian Sunflower Inbred Lines

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Background: Downy mildew caused by *Plasmopara halstedii* is a devastating disease in sunflower worldwide. Several dominant resistance genes designated as *Pl* have been identified and linked molecular markers have been demonstrated. However, no information on theresistance genes is available forIranian lines.
Objectives: The presence of three map-based molecular markers previously proved to be linked to different resistance genes were evaluated in sunflower inbred lines.
Materials and Methods: Using PCR-based and CAPS molecular markers, 26 sunflower inbred lines with different responses to *P. halstedii* race 100 were used to detect the presence of three resistance loci including Pl_1, Pl_6 and Pl_{13} within the lines.
Results: Molecular marker linked to Pl_{13} was present in some of the sunflower lines but was not correlated with the phenotypic reaction of the lines to race 100. Despite the use of three markers linked to Pl_6, PCR failed to amplify any corresponding product. This data may suggest that none of the genotypes possessed Pl_6 locus. Pl_1-linked cleaved amplified polymorphic sequences (CAPS) were present in several resistance lines and effectively differentiated susceptible and resistant sunflower lines.
Conclusions: Applicability of molecular markers in breeding programs revisited in disease management.

Keywords: CAPS; Downy mildew; Molecular marker; PCR; Pl_1 locus

1. Background

Downy mildew of sunflower caused by an oomycete, *Plasmopara halstedii* (Farl.) Berl. etde Toni, is a widespread destructive plant disease worldwide (1). Yield losses under conductive environmental conditions can be high and reported to be up to 50% (2). The disease first was reported in USA in 1888 (3) and subsequently was observed in Europe in 1960 (4). The rapid spread of the disease during the last decades in Europe has led to a serious concern, threatening sunflower production (4). The causal agent of the disease was subjected to quarantine regulation in European Union since 1992 (4). Many approaches have been used to minimize the effects of the pathogen, including the use of fungicides and use of resistant genotypes. Under favorable conditions, application of systemic fungicides has been a common practice to control the disease. Nevertheless, extensive fungicide application impose high selection pressure on the pathogen populations leading to the emergence of fungicide resistance strains that eventually compromise effective disease control (5, 6). Therefore, employment of resistant genotypes is by far the most cost-effective and environmentally-safe strategy to control the disease. Rapid spread and high population dynamic of causal agent of the disease (4) highlights the need that sunflower producing countries equip their sunflower germplasm with different resistance genes.To this aim, screening of sunflower genotypes for resistance to the disease is required prior to their release in high risk growing regions.

Existence of physiologic races in *P. halstedii* is evident in many reports. Similar to many plant-pathogen interactions, sunflower-*P. halstedii* interaction follows the gene-for-gene model (7). Up to now, at least 35 races from different parts of the world have been reported (8). Nomenclature of the races is based on their reactions to a set of differential lines, which are lines possessing different resistance genes and show differential responses to specific races (9). To

date, a number of dominant genes designated as *Pl*, have been proven to confer complete resistance to the pathogen in sunflower cultivars or their wild relatives (10). Pl_1 was the first resistance gene identified in sunflower that provides resistancetorace100 (11). Several otherresistance genes have been identified and introduced to sunflower cultivars conferring resistance against different physiological races (12, 13, 14). Pl_2confers resistance to race 300 (15). Pl_3 and Pl_4 were designated independently, but it was shown that they are equal to Pl_1 and Pl_2, respectively (11, 16). Pl_5 shows resistance to race 700 (17). Pl_6, Pl_7, and Pl_8 confer resistanceagainst races 100, 300, 310, 330, and 700 (18, 19). Resistance to races 310, 330, and 300 is conferred by Pl_9, Pl_{10}, and Pl_{11}, respectively (19). It has been found that Pl_{12} provides resistance against races100, 300, and 700 (12). $Pl_{13}$was also identified as a locus conferring resistance to races300, 700, 730, and 770 (20). The recent identified resistance gene, Pl_{14}, confers resistance to race 730 (14). Although, several lines of evidence shows that *Pl* genes might have the coiled coils-nucleotide binding site-leucine-rich repeat (CC-NBS-LRR) signature, so far none of the *Pl* genes are cloned (21, 13, 22). Nevertheless, tightly linked molecular markers to a few *Pl* loci have been previously reported (23). For instance, toll/interleukin1 receptors-NBS-LRR classes of plant resistance genes were used to develop markers that are linked to Pl_6 locus. Furthermore, CAPS markers linked to Pl_6 was also developed (24). Two full length sequences belonging to CC-NBS-LRRclasses of plant resistance genes were cloned and their subsequent primers were designed to tag Pl_5/Pl_8 region (13). CAPS molecular marker linked to Pl_1 locus was identified through cloning of candidate resistance genes belonging to NBS superfamily of genes (25). These markers have been successfully used by many research groups as a fast and reliable technique to screen sunflower genotypes.

Despite the fact that downy mildew is the major sunflower disease in Iran, limited information is available on the presence of physiological races as well as the presence or absence of resistance genes within cultivated sunflower genotypes. Recently, *P. halstedii* race 100 was identified as the dominant race infecting sunflower in Iran (26). The responses of several sunflower inbred lines to this race were also evaluated and several resistant genotypes were identified (26). The race identification and germplasm screening to *P. halstedii* have been performed using whole seedling immersion in zoospore suspension. Although the method is sensitive and accurate, it is cost effective, time-consuming and requires high experience to perform the experiment. Moreover, screening of F_2 segregation populations using inoculation assays would eliminate some susceptible individuals with other useful traits. These limitations encourage breeders to take the advantages of molecular markers linked to disease resistance genes. In this study, we used previously published molecular markers linked to Pl_1, Pl_6 and Pl_{13} to screen the available sunflower genotypes in Iran.

2. Materials and Methods

2.1. Assessment of Sunflowere Resistance to P. halstedii

Sunflower inbred lines (26 lines), were provided by Oil Seed Department, Seed and Plant Improvement Institute (SPII; Table 1).They are highly inbred lines,

Table 1. Name and responses of the sunflower lines to *P. halstdii*race 100 used in this study

No.	Line name	Response to *P. halstdii**	Line type
1	SUN21	resistant	restorer line
2	SUN25	resistant	restorer line
3	SUN27	resistant	restorer line
4	SUN 31	resistant	restorer line
5	SUN36	resistant	restorer line
6	SUN37	resistant	restorer line
7	SUN38	resistant	restorer line
8	SUN41	resistant	restorer line
9	SUN45	resistant	restorer line
10	SUN47	resistant	restorer line
11	SUN48	resistant	restorer line
12	SUN20	susceptible	B-line**
13	SUN23	susceptible	B-line
14	SUN28	susceptible	B-line
15	SUN30	susceptible	B-line
16	SUN32	susceptible	B-line
17	SUN33	susceptible	B-line
18	SUN39	susceptible	B-line
19	SUN44	susceptible	B-line
20	SUN100	susceptible	B-line
21	SUN102	susceptible	B-line
22	SUN104	susceptible	B-line
23	SUN124	susceptible	B-line
24	SUN128	susceptible	B-line
25	SUN130	susceptible	B-line
26	SUN139	susceptible	B-line

*Their responses have been previously evaluated using whole seedling immersion (Rahmanpour, personal communication)
**B-line stands for maintainer line

which have been produced by at least 20 rounds of selfing. The genotypes were previously evaluated for resistance to *P. halstedii* race 100 using whole seedling immersion method (Personal communication with Rahmanpur (27). Germinated seeds (2-3 days seedlings) were immersed in pathogen zoospore suspension.The seedlings were cultivated in humid and dark green house and the plantlets were evaluatedfor the resistance after two weeks (Table 1).

2.2. Molecular Marker Analyses

The oldest leaf at five-leaf stage was cut andsnap-frozen in liquid nitrogen. Genomic DNA was extracted according to previously described procedure (28). Integrity of the extracted DNA was checked using 1% (w/v) agarose gel electrophoresis. PCR reactions were performed using primer pairs for Pl_6, Pl_{13} and Pl_1 (Table 2). PCR programs and ingredients were according to the earlier reports (20, 23, 25). Briefly, PCR reactions were performed in 20 µL containing 30 ng sunflower genomic DNA, 2 µL 10×PCR buffer, 0.5 mM dNTPs, 0.5 µM of each primer, 3.75 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (CinnaGen, Iran). The PCR conditions were 95ºC for 4 min, followed by 35 cycles of 94ºC for 30s, 52ºC for 30s and 72ºC for 30 to 90s (according to the expected fragment size), plus a final extension at 72ºC for 3 min. Cleaved amplified polymorphic sequences (CAPS) analysis was performed according to previously described method (25) using restriction enzyme *Tsp509*I (Fermentas, London). DNA fragments were separated on 2% (w/v) agarose gel, stained with ethidium bromide and visualized using a UV trans-illuminator. All the above steps were repeated twice.

Table2. Polymorphism and PIC values of ISSR and RAPD markers used for genetic diversity of 34 *Pyrenophera tritici-repentis* isolates

Name	Forward (5´ to 3´)	Reverse (5´ to 3´)	Linked to	Reference
Hap1	GGTAATGGCTGTTGAATTTATGGAGC	AGCATGATCCGGCTAGAGCCTTCTA	Pl6	(21)
Hap2	GTCTACTACATGGTTTCCGTTTTC	TGCTTCTTCCTTCTATCTCACTC	Pl6	(21)
Hap3	GTTTGTGGATCATCTCTATGCG	TGCTTCTTCCTTCTATCTCACTC	Pl6	(21)
STS10D6	AACTACGACCCACAAAAGGACAAG	TTAGACCAGGGCCCAACAAAC	Pl13	(18)
4W2	ATGCGGAAATCTCTCACC	GACAGCCTCGTCTTGTGA	Pl1	(23)

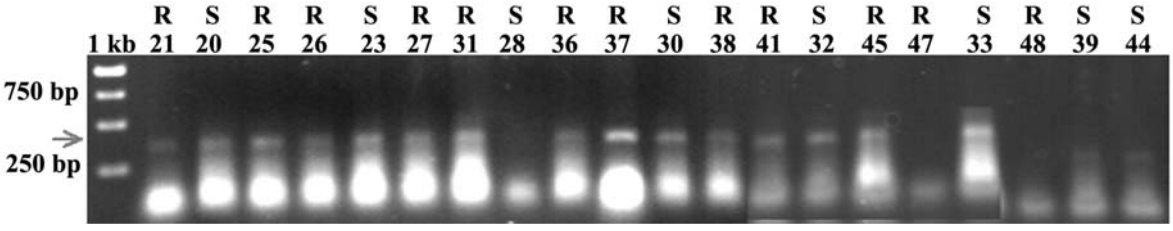

Figure 1. ThePCR profile of Pl_{13}-linked marker (indicated by arrow) using the DNA of 20 sunflower inbred lines as template in PCR reaction.The genotype codes and their reactions to *P. halstedii*race 100 are presented on the top of PCR panel. R and S indicate resistant and susceptible lines, respectively

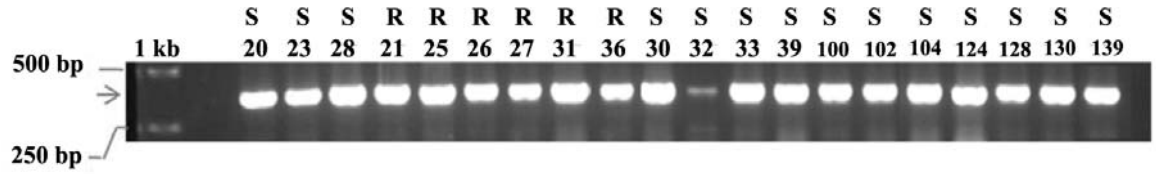

Figure 2. The PCR products of Pl_1-linked locus, with the expected size of 370 bp as indicated by arrow, using the DNA of 20 sunflower inbred lines as template in PCR reaction.The genotype codes and their reactions to *P. halstedii*race 100 are presented on the top of PCR panel. R and S indicate resistant and susceptible lines, respectively

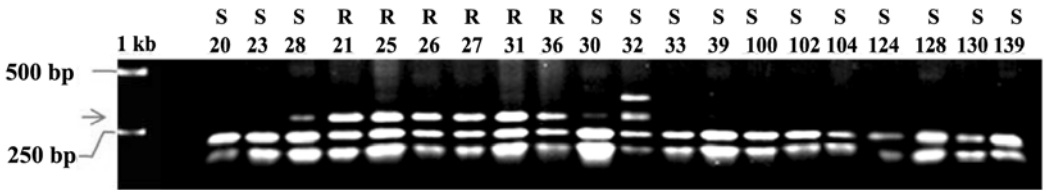

Figure 3. The cleavage pattern of PCR products presented in Figure 3. The products were digested using *Tsp509*I. The arrow indicates a band representing 276 bp resistance marker as described previously (23)

3. Results

DNAs from sunflower inbred lines with different responses to *P. halstedii* race 100 were used as template in PCR reactions using primer pair STS10D6 previously reported to serve as tightly linked to locus Pl_{13}. A PCR fragment ranging in size from 250 to 500 bp was amplified in almost all lines (n=20; Figure 1). The DNA fragment was absent in one susceptible and two resistant lines. Thus, this marker could not dissect the resistant and susceptible lines as differentiated by race 100. Primer pairs Hap1, Hap2, and Hap3 were used to amplify markers linked to Pl_6. No DNA fragments (data are not shown) with the expected sizes ranging from 500 to 3000 bp were amplified as reported (23). This mayindicate that none of the genotypes possessed Pl_6 locus. PCR amplification using primer pair 4W2, used to amplify marker linked to Pl_1, had resulted in a band of 370 bp in size (25). As expected, the band was monomorph among all tested lines (Figure 2). PCR reactions were treated with restriction enzyme *Tsp509*I and separated on a 1.5% (w/v) agarose gel (Figure 3). Digestion of PCR products resulted in two monomorphic bands below 250 bp for all sunflower lines regardless of their reactions to *P. halstedii*. In addition to these two bands, all six resistant lines contained a sharp band with the size of 276 bp as reported previously (25). Only two exceptions were observed among all lines, including the susceptible line #28 that had a banding pattern similar to resistant lines and line #32 that had an extra band with the approximate size of 370 bp.

4. Discussion

Employing molecular markers, either gene-based or map-based markers, efficiently accelerated breeding (29). Molecular markers have been widely used to monitor resistant genes in many species (30, 31, 32, 33, 34, 35) and most importantly in the early generation testing (36, 37). In this study, the presence of molecular markers linked to three *Pl* loci was investigated using PCR-based method. DNAs from sunflower inbred lines with different responses to *P. halstedii* race 100 were used as template in PCR reactions using primer pair STS10D6 previously reported to serve as tightly linked to Pl_{13} locus. Results showed that Iranian genotypes possess markers linked to Pl_{13} that can serve as a resistance gene against races 300, 700, 730, and 770 in the future (20). As expected there was no correlation between the phenotypic reactions of these genotypes to *P. halstedii* race 100 and the presence of this marker in the genotypes. However our results revealed that these genotypes still could be sources of resistance to other races other than race 100.

Pl_6 was another locus that was investigated using three different markers. No PCR amplification product was observed in any of the genotypes tested in this study. It is reported that all of these markers are STS-based markers and, therefore, lack of these markers in Iranian lines might be a real reflection of lacking Pl_6 resistance gene. Thus, to avoid the possible outbreak of the disease under Iranian growing conditions, breeding efforts must be made to incorporate Pl_6 resistance gene into Iranian sunflower lines.

The primer pair 4W2 was used to amplify the marker linked to Pl_1 locus. The amplified band with the approximate size of 370 bp appeared to be monomorph among all the tested lines. Nevertheless, single-stranded conformational polymorphism (SSCP) analysis has been used to determine the possible complexity of the resulting band (25). The results showed that 4W2 primers bind to multiple sites in the NBS regions in susceptible and resistant lines leading to amplification of a single band with approximate size of 370 bp that consists of several different DNA amplicons (25). They showed that treating the PCR products with *Tsp509*I would lead to three bands (88, 93, and 188 bp) in susceptible lines while it generates four bands (88, 93, 188, and 276 bp) in resistant lines (25). In our experiment, we observed two monomorphic bands in all sunflower lines. The lowest band was con-

sisted of two DNA fragments (88 and 93 bp) that were not separated due to approximate similar sizes and the second band with the size of 188 bp. In resistant lines, a 276bp band was observed that was indicator of resistant lines. Line #32 had an extra band similar to undigested band (370 bp), which may represent partial digestion, a phenomenon observed when the digestion inhibitors are presented in the reactions. Another exception was line #28 (susceptible line) that had a banding pattern similar to resistant lines. Because the same result was obtained when the experiment repeated, we concluded that maybe line, #28, incorrectly been scored during phenotypic evaluation and might have a resistance response to *P. halstedii* race 100. Alternatively, it is possible that the marker used to differentiate the resistant and susceptible lines is not completely linked to Pl_1 locus and thus some rare exceptions can be present in marker-assisted selection process. Overall, presence or absence of the band with size about 276 bp in sunflower lines might be used to differentiate the resistant and susceptiblelines to *P. halstedii* race 100.

In this study we used molecular markers to track loci conferring resistance to *P. halstedii*, and compared with the phenotypic reaction of sunflower lines to race 100. Our data showed that molecular markers can be efficiently used in screening and breeding programs. Good correlation between the presence of markers linked to resistance loci and their phenotypic reactions to pathogen justify implementation of these markers in marker-assisted selection programs. Our data suggest that application of molecular markers can facilitate breeding programs towards disease management.

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