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Original Article

Identification of Downy Mildew Resistance Loci in Sunflower Germplasm

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Abstract

Downy mildew caused by *Plasmopara halstedii* is one of the most economically important fungal diseases on sunflower (*Helianthus annuus*). To date, several downy mildew resistance genes called *Pl* genes have been reported on sunflower genetic map. Previous findings have confirmed that Iranian sunflower germplasms are harbouring *Pl* resistance genes that may be used to control downy mildew. In the current study, there were investigated the Pl_5 and Pl_{16} downy mildew resistance genes in 51 inbred lines of Iranian sunflower, using PCR-based method. Fifteen differential lines carrying Pl_5 and Pl_{16} downy mildew resistance genes were used as positive control. DNAs from 51 sunflower inbred lines were used in PCR reactions using primer pair RS1008 and Hap3 previously reported to serve as tightly linked to Pl_{16} and P_5 loci, respectively. The PCR results confirmed the presence of two Pl_{16} and Pl_5 bands with the size of about 280 and 1,580 bp, respectively, in differential lines. The results indicated that 1 inbred line out of 51 was found to carry Pl_5 gene and 10 lines were found to carry Pl_{16} gene across the studied Iranian sunflower genotypes. These findings may be used to assist breeders for conservation and selection of downy mildew resistant sunflower genotypes.

Keywords: differential lines, downy mildew, molecular markers, *Plasmopara halstedii*, resistance genes, sunflower

Introduction

Sunflower (*Helianthus annuus* L.), a diploid species ($2n = 2x = 34$), is the fourth most important oilseed crop in the world, with an annual production of 41.4 million tons of seed in 2014 (Faostat, 2014). Sunflower produces a healthy oil rich in unsaturated fatty acids as well as high vitamin E content (Gascuel *et al*., 2016).

Downy mildew is one of the major fungal diseases in most sunflower producing areas of the world (Gascuel *et al*., 2015). *Plasmopara halstedii*, a seed, air and soil-borne pathogen is the causal agent of the disease. It is assumed to be originated from central region of the North American continent (Leppik, 1966). The pathogen survives for up to 10 years in soil or on plants residues as sexual, thick-walled oospores. It can finally produce visible, characteristic downy hyphae on the leaves of host the plants (Agrios, 1988). Downy mildew damage may vary according to region, year, environmental conditions, cultivar and planting date. The systemic infection of downy mildew in sunflower may range from traces to 50% or even more up to 95% (Sackston, 1981) which cause up to 80% yield loss (Molinero-Ruiz *et al*., 2003). Control of downy mildew in sunflower production involves a combination of crop rotation, fungicide treatments and the use of downy mildew-resistant genotypes, which has been found through germplasms screening (Rahim *et al*., 2002; Gulya, 2005; Hulke *et al*., 2010). Breeding sunflower genotypes to resist downy mildew is a sustainable strategy to increase crop yield and reduce the use of fungicides.

To date, thirty six pathotypes of *P. halstedii* have been recognized (Gascuel *et al*., 2016). These pathotypes are often defined by an international nomenclature system, based on differential virulence profiles on a set of sunflower inbred lines containing different resistance gene called *Pl* (Tourvieille de Labrouhe *et al*., 2012; Gascuel *et al*., 2015). Modern sunflower genotypes carry one or more dominant *Pl* resistance genes. So far, more than 20 *Pl* gene (Pl_1 to Pl_{21}, Pl_{Arg}, Pl_{PMI3}), conferring resistance to at least one pathotypes of *P. halstedii*, have been discovered in sunflower and wild species and 13 of them (Pl_1, Pl_2, Pl_5-Pl_8, Pl_{13}-Pl_{17}, Pl_{21}, and Pl_{Arg}) have been mapped in sunflower and assigned to six main clusters localized on five different linkage groups (LGs) (LG1, 2, 4, 8, and 13) (Mouzeyar *et al*., 1995; Roeckel-Drevet *et al*., 1996; Vear *et al*., 1997; Molinero-Ruiz *et al*., 2003; Yu *et al*., 2003; Mulpuri *et al*., 2009; de Romano *et al*., 2010; Bachlava *et al*., 2011; Liu *et al*., 2012; Qi *et al*., 2015), but none has been cloned so far (Gascuel *et al*., 2015; Qi *et al*., 2015, 2016). Most of the *Pl* genes have originated from wild *Helianthus* annual species, for example, the Pl_1, Pl_2, and Pl_{13} originated from a Canadian

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line 953-102-1-1, which is a selection involving wild *H. annuus* (Fick and Zimmer, 1974; Vear *et al*., 2008). The Pl_5 originated from *H. tuberosus* (Vranceanu *et al*., 1981), Pl_6 was derived from wild non-cultivated *H. annuus* and Pl_7 from *H. praecox* Englem and Gray (Miller and Gulya, 1991). Two *Pl* genes, Pl_8 and Pl_{Arg}, were also derived from *H. argophyllus* Torrey and Gray (Seiler, 1991). The use of gene-for-gene hypothesis (Flor, 1955) is the most common breeding approach for resistance to downy mildew in sunflower. Thus, discovery of resistance loci and genes is one of the best strategies for increasing resistance against downy mildew in sunflower. Incorporating of the resistance genes into sunflower genotypes could help to mitigate the threat posed by downy mildew races. This can be obtained only once the identity and locus of a *Pl* gene is determined.

Therefore, molecular mapping of the resistance genes is necessary to conclude whether its resistance gene is different from known *Pl* genes. Thus, the objective of this study was to identify downy mildew resistance genes in 51 Iranian sunflower inbred lines.

Materials and Methods

Plant materials and growing conditions

Fifteen differential lines, carrying resistance genes against downy mildew, as positive control were kindly supplied by Dr Denis Tourvieille de Labrouhe, French National Institute for Agricultural Research, Department of Plant Health and Environment. The lines are listed in Table 1. In addition, 51 Iranian sunflower inbred lines (Table 2) were obtained from Department of Oilseeds, Seed and Plant Improvement Institute, Karaj, Iran.

Sunflower seeds were surface-sterilized by soaking in 10% sodium hypochlorite for 10 min and 70% ethanol for 4 min and then rinsed with sterile distilled water. A total of 66 seeds were sown in plastic pots and placed in controlled environmental room at 28/20 °C (day/night) with 70% relative humidity and a 12 h-photoperiod (light intensity of 350 μmol photons m^{-2} s^{-1}) until four leaf stage.

DNA extraction and PCR conditions

Leaf samples were collected from the plants by detaching the leaves and freezing them in liquid nitrogen. Genomic DNA was isolated from the lyophilized tissues using the CTAB method (Li *et al*., 2007). The integrity of DNA samples were examined on 1% (w/v) agarose gel.

The PCR amplifications were conducted following Liu *et al*. (2012) in a 15 μl PCR reaction mixture for differential lines and 20 μl for inbred lines. Briefly, PCR reactions contained 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 primer pairs of RS1008 and Hap3, previously reported to serve as tightly linked to Pl_{16} and P_5 loci, respectively, were used in this study (Table 3). According to Liu *et al*.(2012), D_7 differential line carries Pl_{16} resistance gene, which is a ~280 bp band, so the Pl_{16} was first amplified in D1, D7 and D15 differential lines using the primer pair ORS1008 and then, PCR amplifications were performed to detect Pl_{16} in all inbred lines. In this study the D6 (803-1) differential line was used instead of YSQ differential line which carries Pl_5 resistance gene (a ~1580 bp band) (Radwan *et al*., 2004). The PCR program was set as follows: 4 min at 95 °C for pre-denaturation followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60.1 °C for 45 s, extension at 72 °C for 70 min, and a final extension at 72 °C for 10 min.

Similarly, the Pl_5 resistance gene was first amplified in D1, D6 and D15 differential lines using the primer pair Hap3 and then PCR amplifications were performed to detect Pl_5 in all inbred lines. The PCR program was set as follows: 4 min at 95 °C for pre-denaturation followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55.3 °C for 45 s, extension at 72 °C for 70 min, and a final extension at 72 °C for 10 min.

The PCR products were separated on a 1% (w/v) agarose gel, at 100 W for 30 min (1× TAE). The gels were analysed after being stained with ethidium bromide and imaged with a Gel-Doc (Uvitec, UK).

Table 1. Sunflower differential lines used as positive control to identify Pl_5 and Pl_{16} resistance genes

D1 NEW- GB	D6- 803-1	D11- PSC8
D2- RHA 265	D7- HAR4	D12- XA
D3- RHA 274	D8 NEW- QHP2	D13- PSS2 RM
D4- PMI3	D9- HA335	D14- VAQ
D5- PM17	D10- Y7Q	D15- RHA 419

Table 2. Sunflower inbred lines used to identify Pl_5 and Pl_{16} resistance genes

Number	Inbred line	Number	Inbred line	Number	Inbred line
1	M-225	18	A-130	35	RF-81- 65
2	A-19	19	RN1-73	36	AF-80- 436
3	M-263	20	A-350/2	37	RF-81-131/1
4	A-19	21	RN1-33	38	AF-81 222/2
5	M-289	22	A-156/1	39	R-1031
6	A-112	23	R-1031	40	M- 89XR 1031
7	AF-81-58	24	A-586/1	41	F1
8	A-112	25	R-14	42	AF-81-112
9	R-14	26	A-67	43	AF-81-112
10	AF-81-78	27	R-43	44	AF-81-136
11	RN1-41	28	A-350/1/1	45	AF-81-136
12	A-19	29	RN1-41	46	AF-81-136
13	R-14	30	A-1052/1	47	AF-81-136
14	A-19	31	R-14	48	RF-82-054
15	R-232	32	A-67	49	RF-82-053
16	A-19	33	AF-81-65	50	BF-82-028
17	R-1031	34	AF-80-429	51	RF-82-029/2

M: Mutant line; A: Male sterile line; B: Maintenance line; R: Restorer line

Table 3. The primer sequences of ORS1008 and Hap3 primer pairs used in the study

Primers	Sequence	Tm (°C)
ORS1008	Forward: 5´-GATCAC CTT CAC TAT CCA CAA CC-3´	62.9
	Reverse: 5´-CAT GAG GGC ATT CTT GTC ATT T-3´	58.4
Hap3	Forward: 5´-TAG TTA ACC ATG GCT GAA ACC GCT G-3´	65.8
	Reverse: 5´-TTT GAA AGA TAA GTT CGC CTC TCG-3´	61.8

Results

DNAs from sunflower differential lines were used as positive control in PCR reactions using RS1008 and Hap3 pair primers. The RS1008 and Hap3 pair primers have been previously reported to serve as tightly linked to locus Pl_{16} and Pl_5, respectively.

According to the results, a PCR fragment ranging in size from 250 to 280 bp related to Pl_{16} was amplified in D7 and D15 differential lines (Fig. 1). The DNA fragment was absent in D1 differential line (Fig. 1). Thus, this line could not dissect the resistant and susceptible lines.

Primer pairs Hap3 was used to amplify fragment linked to Pl_5. No DNA fragment with the expected sizes ranging from 1,500 to 1,580 bp was amplified in D1 differential line genomic DNA (Fig. 2), whereas PCR amplification using primer pair Hap3 used to amplify fragment linked to Pl_5, had resulted in a band from D6 and D15 differential lines genomic DNA (Fig. 2). These results may indicate that D1 differential line does not possess Pl_5 or Pl_{16} genes.

PCR amplifications of Pl_{16} gene using RS1008 pair primers showed bands ranging from 250 to 280 bp in 10 out of 51 inbred lines which means these lines carry Pl_{16} gene (Fig. 3). The Pl_5 gene was present in only one of the 51 inbred lines i.e. A-19. According to data presented in Table 2, five inbred lines numbered as 2, 4, 12, 14 and 16, were found to be A-19 as the PCR results in terms of Pl_{16} were quite similar amongst these inbred lines. However, Pl_5 PCR results indicated that the band was observed only in A-19 and not in other inbred lines (Fig. 4). In other words, Pl_5 band was not repetitive in all lines related to A-19. This finding might be due to distance between marker and gene as the further apart between two loci is the more likely crossing over will occur between them. It has been reported that there is 0.3 cM (centimorgan) distance between Pl_{16} and RS1008 primer (Liu *et al*., 2012) whereas distance between Pl_5 gene and Hap3 primer has found to be 4.8 cM (Radwan *et al*., 2004). This may increase recombination between Pl_5 and Hap3 loci and reduce repetition.

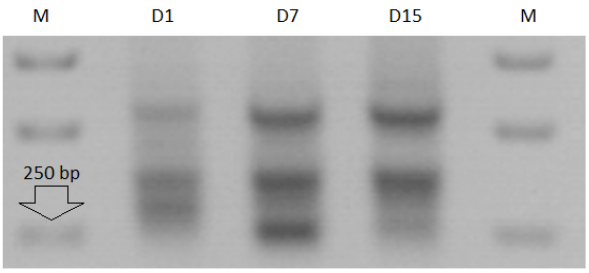

Fig. 1. PCR amplification of Pl_{16} with the expected size of 250 bp (as indicated by arrow) using ORS1008 primer pairs in D1, D7 and D15 differential lines. The D7 and D15 differential lines showed the expected band. M: 1Kb marker

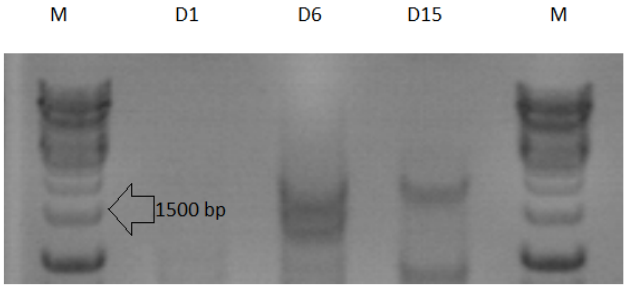

Fig. 2. PCR amplification of Pl_5 with the expected size of 1,500 bp (as indicated by arrow) using Hap3 primer pairs in D1, D6 and D15 differential lines. The D6 and D15 differential lines showed the expected band. M: 1Kb marker

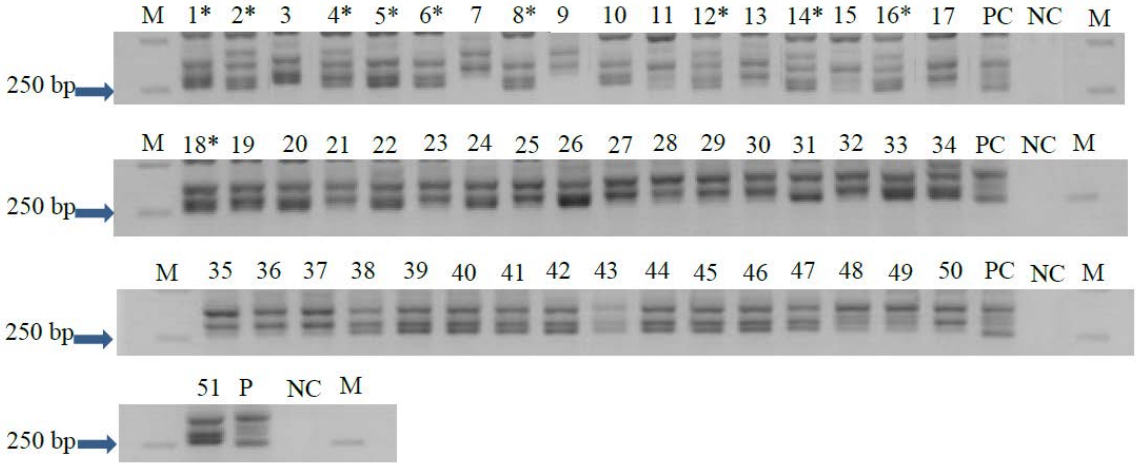

Fig. 3. PCR amplification of Pl_{16} with the expected size of 250 bp (as indicated by arrow) using ORS1008 primer pairs in 51 inbred lines. The 1, 2, 4, 5, 6, 8, 12, 14, 16 and 18 inbred lines (as indicated by asterisk) showed the expected band. M: 1Kb marker, PC: positive control, NC: negative control

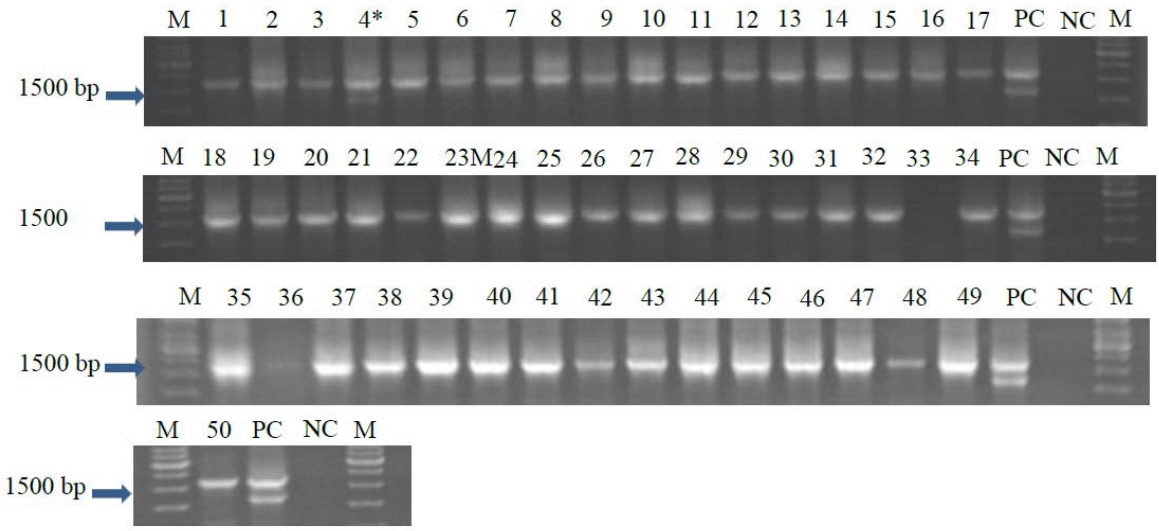

Fig. 4. PCR amplification of Pl_5 with the expected size of 1,500 bp (as indicated by arrow) using Hap3 primer pairs in 51 inbred lines. Only the number 4 inbred lines (as indicated by asterisk) showed the expected band. M: 1Kb marker, PC: positive control, NC: negative control

Discussion

Using molecular markers efficiently accelerated crop breeding (Collard and Mackill, 2008). The use of molecular markers to investigate resistance genes has been reported in several studies (Gordon *et al*., 2007; Bipinraj *et al*., 2011; Kim *et al*., 2011). To date, several molecular markers associated with downy mildew resistance genes have been identified and mapped on the chromosomes (Mouzeyar *et al*., 1995). For instance, RFLP and RAPD markers have been used to map Pl_1 locus (Gascuel *et al*., 2015). Moreover, to identify Pl_{13} resistance gene, SSR markers related to Pl_{13} locus have been used (Mulpuri *et al*., 2009). STS marker was used to identify Pl_5 and Pl_8 (Radwan *et al*., 2004). In addition, SSR (ORS1008) marker was reported to detect Pl_{16} locus (Liu *et al*., 2012). Genetic studies have confirmed that there are significant correlations between some resistance genes, for example, Pl_2 and Pl_4 (Sackston, 1981), Pl_2 and Pl_1 (Mouzeyar *et al*., 1995) and Pl_1 and Pl_{16} (Roeckel-Devert *et al*., 1996). The first report of Pl_2 was published by Zimmer and Kinman (1972) and Fick and Zimmer (1974), who showed that both HA61 and RHA274 harboured a single dominant gene Pl_2 giving resistance to race 300. Based on the gene for gene hypothesis, Gulya *et al*. (1991), suggested that RHA274 carried not only Pl_2, giving resistance to race 300 but also Pl_9, giving resistance to race 310. However, they did not study genetic segregation patterns for the two genes. Molinero-Ruiz *et al*. (2003) observed segregations indicating a single gene for resistance to race 310 in RHA274 and RHA325, which they confirmed as Pl_9, but made no comparison with Pl_2. These authors also reported (Molinero-Ruiz *et al*., 2002) that both RHA274 and HA61 carried 2 complementary genes for resistance (Pl_w and Pl_x) to race 330(SP) giving F2 segregations of 13R: 3S, meaning that the double recessive was resistant. Rahim *et al*., (2002) reported segregations indicating 2 independent genes in RHA274 giving resistance to races 100 and 300 and since they considered that for each race, different genes are involved, they concluded that RHA274 carries *Pl1* and Pl_{11} giving resistance to race 100 and Pl_2 and Pl_{12} giving resistance to race 300.

Conclusions

Overall, presence or absence of the band with size about 280 and 1,580 bp in sunflower lines might be used to differentiate the resistant and susceptible lines to downy mildew pathogen. The results obtained in the current study showed that molecular markers can be efficiently used in screening and breeding programs and the data suggest that application of molecular markers can facilitate breeding programs towards disease management.

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