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ORIGINAL PAPER

Molecular mapping of the Pl_{16} downy mildew resistance gene from HA-R4 to facilitate marker-assisted selection in sunflower

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Abstract The major genes controlling sunflower downy mildew resistance have been designated as *Pl* genes. Ten of the more than 20 *Pl* genes reported have been mapped. In this study, we report the molecular mapping of gene Pl_{16} in a sunflower downy mildew differential line, HA-R4. It was mapped on the lower end of linkage group (LG) 1 of the sunflower reference map, with 12 markers covering a distance of 78.9 cM. One dominant simple sequence repeat (SSR) marker, ORS1008, co-segregated with Pl_{16}, and another co-dominant expressed sequence tag (EST)-SSR marker, HT636, was located 0.3 cM proximal to the Pl_{16} gene. The HT636 marker was also closely linked to the Pl_{13} gene in another sunflower differential line, HA-R5. Thus the Pl_{16} and Pl_{13} genes were mapped to a similar position on LG 1 that is different from the previously reported Pl_{14} gene. When the co-segregating and tightly linked markers for the Pl_{16} gene were applied to other germplasms or hybrids, a unique band pattern for the ORS1008 marker was detected in HA-R4 and HA-R5 and their F_1 hybrids. This is the first report to provide two tightly linked markers for both the Pl_{16} and Pl_{13} genes, which will facilitate marker-assisted selection in sunflower resistance breeding, and provide a basis for the cloning of these genes.

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Introduction

Downy mildew caused by the fungus *Plasmopara halstedii* (Farl.) Berl. & de Toni is one of the more destructive diseases of cultivated sunflower (*Helianthus annuus* L.). The fungus infects via the roots of seedlings to initiate a systemic infection, as well as airborne sporangia causing local foliar lesions. Chemical seed treatments and genetic resistance are the two major methods used to control this disease. The major dominant resistance genes for this disease have been designated as *Pl* genes. With the evolution of the fungus, many new virulent *P. halstedii* races have emerged that overcome the known resistance genes. To date, at least 37 races of *P. halstedii* have been reported worldwide (Gulya 2007; T. Gulya, personal communication). Of concern are those new races which overcome *Pl* genes used in commercial hybrids. For example, the Pl_2 and Pl_6 genes have been overcome by eight races in France (Delmotte et al. 2008). A new virulent race which overcomes the Pl_6 resistance gene, designated as 734, was identified in the US in 2009, and by 2011 another six races were identified which overcome the Pl_6 gene (unpublished data, T. Gulya). North America, the center of origin of both sunflower and *P. halstedii*, has the greatest number of races reported, 28 to date, with Europe not far behind at 21 races. Race diversity is far less in other sunflower producing areas, with ten races reported in Africa, five in Argentina, and only three races in India and China. Prior to the identification of races attacking the Pl_6 gene, four races (700, 710, 730, and 770) were predominant worldwide (Gulya 2007). In the US prior to 2009, races 730 and 770 comprised 73% of over 350 samples

tested. By 2011, these two races still make up the majority of the US fungal population, but the eight races which overcome the *Pl*$_6$ gene comprise 14% of samples tested between 2009 and 2011. Therefore, the development of germplasms with diverse disease resistance genes is critical. Resistance genes to downy mildew have been detected in wild and cultivated sunflower germplasms with many employed in sunflower breeding. At least 23 downy mildew resistance genes have been identified (*Pl*$_{1–15}$, *Plv*, *Plw*, *Plx-z*, *Mw*, *Mx* and *Pl*$_{Arg}$) (Bachlava et al. 2011; de Romano et al. 2010; Rahim et al. 2002; reviewed by Mulpuri et al. 2009). However, only the *Pl*$_8$, *Pl*$_{Arg}$ and *Pl*$_{15}$ genes are resistant to all *P. halstedii* races so far.

Ten *Pl* genes have been assigned to linkage maps using molecular markers (Bachlava et al. 2011; Bert et al. 2001; Bouzidi et al. 2002; Brahm et al. 2000; de Romano et al. 2010; Dußle et al. 2004; Gedil et al. 2001; Gentzbittel et al. 1998; Mouzeyar et al. 1995; Mulpuri et al. 2009; Panković et al. 2007; Radwan et al. 2003, 2004; Roeckel-Drevet et al. 1996; Vear et al. 1997). Similar to many disease resistance (*R*) genes in other plants, the *Pl* genes in sunflower also occur in clusters on the linkage groups. Two major *Pl* gene clusters are located on linkage groups (LGs) 8 and 13 of the public simple sequence repeat (SSR) or microsatellite sunflower map, respectively. One cluster containing *Pl*$_1$, *Pl*$_2$, *Pl*$_6$, *Pl*$_7$ and *Pl*$_{15}$ was mapped to LG 8 of the SSR linkage map of Yu et al. (2003) (Bert et al. 2001; Bouzidi et al. 2002; Gentzbittel et al. 1998; Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997; Radwan et al. 2008; Slabaugh et al. 2003; de Romano et al. 2010). A second major cluster containing *Pl*$_5$ and *Pl*$_8$ was located on LG 13 of the SSR linkage map of Yu et al. (2003) (Bert et al. 2001; Radwan et al. 2004). LG 1 of the SSR linkage map of Tang et al. (2003) was also reported to contain *Pl* genes, including *Pl*$_{Arg}$ from *H. argophyllus* ARG-1575-2, and *Pl*$_{13}$ from HA-R5, as well as *Pl*$_{14}$ from 29004, a proprietary line of Advanta Semillas S.A.I.C (Bachlava et al. 2011; Dußle et al. 2004; Radwan et al. 2004; Mulpuri et al. 2009). However, the *Pl*$_{Arg}$ gene was assigned to a different location on this linkage group than the *Pl*$_{13}$ and *Pl*$_{14}$ genes, with *Pl*$_{Arg}$ at the upper end and *Pl*$_{13}$ with *Pl*$_{14}$ at the lower end. It is not clear whether the *Pl* genes on LG 1 are singletons or clusters for resistance to different downy mildew races, because there were no recombinants observed in the mapping populations after disease resistance screening, even after fine mapping of *Pl*$_{Arg}$ (Mulpuri et al. 2009; Wieckhorst et al. 2010).

Closely or tightly linked markers for the *Pl* genes have been developed. Several random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) markers were closely linked to the *Pl*$_2$ locus, at a genetic distance of about 2 cM between *Pl*$_2$ and RAPD markers OPAA14$_{750}$ and OPAC20831, and an AFLP marker E35M48-3 (Brahm et al. 2000). One SSR marker, ORS1043, co-segregated with the *Pl*$_1$ gene, and another marker, ORS166, was closely linked at a distance of 3.4 cM (Slabaugh et al. 2003). The ORS166 marker was also linked with the *Pl*$_{15}$ locus at a distance of 3.4 cM (de Romano et al. 2010). Sequencing analysis of 13 STS markers, having a genetic distance of 0.0–1.4 cM to the *Pl*$_6$ locus, proved the existence of conserved resistance genes belonging to the toll-interleukin1 receptor-nucleotide binding site-leucine rich repeat (TIR-NBS-LRR) class of plant resistance genes (Bouzidi et al. 2002). Panković et al. (2007) developed two co-dominant cleaved amplified polymorphic sequence (CAPS) markers co-segregating with the *Pl*$_6$ gene. So far, the closest marker to the *Pl*$_{13}$ gene is a dominant SSR marker, ORS1008, at a genetic distance of 0.9 cM (Mulpuri et al. 2009). Recently, the co-segregating SSR or expressed sequence tag (EST)-SSR markers were reported for the *Pl*$_{Arg}$ gene, and NBS-LRR-encoding RGCs have been identified flanking the *Pl*$_{Arg}$, *Pl*$_8$ and *Pl*$_{14}$ genes (Wieckhorst et al. 2010; Bachlava et al. 2011). These markers will facilitate the marker-assisted selection (MAS) and pyramiding of resistance genes. Pyramiding the major genes through MAS is considered to be one of the important methods to enhance disease resistance in crops (Michelmore 2003; McHale et al. 2009).

The objective of this study was to map the downy mildew resistance gene in USDA line HA-R4, herein designated as *Pl*$_{16}$. An F_2 population, derived from an HA-R4 (resistant) × HA 821 (susceptible) cross, and SSR and EST-SSR markers were used. The application of the tightly linked markers for sunflower breeding will be discussed.

Materials and methods

Plant materials

An F_2 population with 169 individuals derived from an HA-R4 × HA 821 cross was used for mapping the downy mildew resistance gene *Pl*$_{16}$ of HA-R4. HA-R4 derived from the Argentine line, 'Saenz Pena 74-1-2', is a composite of 25-45 S3 plants that trace to a single S2 plant. Saenz Pena 74-1-2, in turn, is a selection from the Argentine open-pollinated variety Cabure INTA. This variety has a complex breeding history including Russian and Romanian open-pollinated varieties plus wild annual and perennial sunflowers including *H. petiolaris*, *H. angustifolius* and *H. maximiliani* (Molestina 1988). When released in 1985, it was resistant to all known rust races (*Puccinia helianthi* Schwein), Verticillium wilt (*Verticillium dahliae* Klebahn) and downy mildew races. When HA-R4 was tested in 2009 and 2010, it was resistant to the predominant races and all new virulent races overcoming the *Pl*$_6$ gene (Gulya 1985; Gulya

et al. 2011; T. Gulya, personal communication). HA 821 is susceptible to all known races of downy mildew (Roath et al. 1986). In 1997, CMS HA 821 plants were pollinated with pollen collected from each F_2 plant to obtain testcross progenies.

The F_2 population with 116 individuals derived from the cross of HA-R5 × HA 821 was also used for the analysis of closely linked markers identified for the *Pl*$_{16}$ gene in HA-R4. HA-R5 is a germplasm derived from another Argentine open-pollinated variety, 'Guayacan', from INTA, which is resistant to four races of rust, 21 downy mildew races, and Verticillium wilt (Gulya 1985; T. Gulya, personal communication). In total, 38 germplasms or hybrids were used for the analysis of tightly linked markers, including eight lines resistant to race 734 (HA-R4, HA-R5, TX 16, RHA 294, RHA 340, RHA 419, RHA 428, and HA 458), eighteen lines susceptible to race 734 (RHA 274, HA 821, HA 89, HA 410, HA 441, HA 234, CMS HA 89-3149, Conf Scl R5, HA 372, HA 852, RHA 271, RHA 280, RHA 858, P 21, Hopi Dye, Peredovik, Luch, and Armarvir 3497), three F_1s (HA-R4 × HA 821, HA-R5 × HA 821, CMS HA 89-3149 × RHA 280), six wild perennial *Helianthus* species accessions (*H. maximiliani*, PI 586892; *H. giganteus*, PI 547182; *H. grosseserratus*, PI 613793; *H.californicus* 2376; *H. schweinitzii* 2405; *H. nuttallii* 102), a new CMS 514A with *H. tuberosus* cytoplasm, and two individuals derived from CMS 514A crossed by an amphiploid of *H.angustifolius* × P 21. The resistance genes in seven differential lines for downy mildew race determination, as well as RHA 340 and RHA 419, are shown in Table 1.

Phenotype determination of the F_2 individuals to downy mildew races

The testcross progenies were used to determine phenotypes of the F_2 individuals. For each testcross family, 10–40 seedlings were disease tested using three *P. halstedii* races, 300, 770, and 734, in 2009–2010. Zoosporangia were increased according to the method of Mulpuri et al. (2009), after the cryogenically stored (in liquid nitrogen) spores were heat shocked at 60°C for 30 s. The methods for germination, inoculation and sporulation were followed according to Mulpuri et al. (2009). Each inoculation treatment was conducted on HA-R4 and the testcross parent CMS HA 821, as well as the other eight differential lines (HA 89, RHA 265, RHA 274, DM-2, PM 17, 803-1, HA-R5, and HA 335). Seedlings with visible fungal sporulation on the cotyledons and true leaves were scored as susceptible. The F_2 individuals were categorized to be homozygous resistant, heterozygous resistant or homozygous susceptible phenotypes according to the ratios of the resistant to susceptible plants within their respective testcross families.

DNA extraction and PCR analysis

Genomic DNA was extracted from lyophilized leaf samples, according to the protocol of the Qiagen DNAeasy 96 Plant Kit (Qiagen, Valencia, CA, USA). The concentration of DNA was quantified with a Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology®, Cambridge, UK) and diluted to 20 ng/µl for PCR amplification.

The PCR amplification was conducted following Liu et al. (2011), containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.27 µM for each of the forward and reverse primers, 40 ng of DNA, and 1 U *Taq* DNA polymerase (Qiagen) in a 15 µl PCR reaction mixture. PCR amplifications were performed using the "touchdown" profile (Feng and Jan 2008; Lai et al. 2005) in an MJ Research (Watertown, MA, USA) single or Bio-Rad (Hercules, CA, USA) single or dual 96-well thermal cycler. The PCR products were separated on a 6.5% nondenaturing polyacrylamide gel at 60 W for 1.0 h (0.5× TBE), or a denaturing polyacrylamide gel at 60 W for 2.0 h (1× TBE) after a prerun at 60 W for 1.0 h, on a CBP Scientific gel electrophoresis system. After being stained with GelRed nucleic acid gel stain (Biotium Inc., CA, USA), the gels were scanned with a Typhoon 9410 variable mode imager (Molecular Dynamics Inc., CA, USA) with the genotypes manually analyzed.

Table 1 The *Pl* genes contained in seven differential lines plus RHA 340 and RHA 419

Material	*Pl* gene	References
HA 89	*Mw*, *Mx*, *Pl*$_z$	Molinero-Ruiz et al. (2002)
RHA 265	*Pl*$_1$	Rahim et al. (2002)
RHA 274	*Pl*$_1$, *Pl*$_2$, *Pl*$_9$, *Pl*$_{10}$, *Pl*$_{11}$, *Pl*$_{12}$	Gulya et al. (1991); Rahim et al. (2002); Molinero-Ruiz et al. (2003)
DM-2	*Pl*$_5$, *Pl*$_{11}$, *Pl*$_{12}$	Rahim et al. (2002)
HA-R4	*Pl*$_{16}$	This study.
HA-R5	*Pl*$_{13}$	Mulpuri et al. (2009)
HA 335	*Pl*$_6$	Miller and Gulya (1991); Bouzidi et al. (2002)
RHA 340	*Pl*$_8$	Miller et al. (1988); Miller and Gulya (1991)
RHA 419	*Pl*$_{Arg}$	Miller et al. 2002; Wieckhorst et al. (2010)

Polymorphic primer screening

The bulked segregant analysis (Michelmore et al. 1991) was employed for polymorphism screening. Resistant or susceptible bulks were created using equal quantities of DNA from 10 homozygous resistant or susceptible F_2 individuals as tested against each of the three *P. halstedii* races (300, 770, and 734). Two hundred and forty-six pairs of SSR primers mapped to the 17 sunflower linkage groups from the Compositae database (http://compositdb.ucdavis.edu) were used for polymorphism screening for the two bulks. The primers that generated reproducible polymorphism with fewer than 50% recombinants of each bulk were selected for F_2 genotyping. An additional set of SSR and EST-SSR primers on the candidate LG 1 from 23 maps in the Sunflower CMap Database (http://sunflower.uga.edu/cgi-bin/cmap/map search) were used for polymorphism detection between the two parents. Polymorphic markers were used for genotyping of the mapping population after confirmation.

Statistical analysis and linkage map construction

Chi-square test was used for the deviation analyses of the disease reaction phenotype of the F_2 population for the three *P. halstedii* races and their marker loci by comparison with the expected Mendelian ratios. The linkage analysis was conducted with MAPMAKER/Exp version 3.0b program (Whitehead Institute, Cambridge, MA, USA) (Lander et al. 1987) from the data of the phenotypes and molecular genotypes, using the Kosambi mapping function (Kosambi 1944). Markers were assigned to linkage groups by the "group" command, with a minimum LOD score of 4.0 and a maximum recombination frequency of 0.50. Additional markers were assigned to the linkage group by the "try" command. After arrangement and confirmation of the order of the markers by the "compare" and "ripple" commands, the linkage map was generated using Mapdraw V2.1 (Liu and Meng 2003). The error detection option was off during the map construction.

Results

Segregation of the resistant to susceptible plants in the population

For the three *P. halstedii* races tested in this study (races 300, 770, and 734), the percentage of noninfected plants for both the susceptible differentials and the susceptible parent was 4.7, 5.4, and 4.2%, respectively. All F_1 hybrid plants were resistance to all three races. No recombinants were observed during the phenotype test for the entire F_2 population containing 169 individuals for the three races. In total, the testcross progeny had 40 homozygous resistant, 87 heterozygous resistant, and 42 homozygous susceptible individuals. Chi-square test of the phenotypes of the F_2 population was consistent with a Mendelian monogenic segregation ratio of 1:2:1 ($\chi^2 = 0.20$, $P = 0.907$) (Table 2). These results indicated that a single gene or a tightly linked cluster of genes in HA-R4 controlled resistance to the three *P. halstedii* races.

Polymorphic marker screening between the bulks

Among the 246 SSR primers screened between the resistant and susceptible bulks, six primers produced weak bands or no product (2.4%). Seven primers detected polymorphism

Table 2 Segregation of the downy mildew resistance (Pl_{16}) locus, six SSR (ORS) markers, and six EST-SSR (HT) markers linked to the Pl_{16} gene in an F_2 population derived from the cross of HA-R4 × HA 821

Trait or marker	Number of F_2 plants	Number[a]				Ratio tested	χ^2	P
		A	H	B	D			
Pl_{16}	169[b]	40	87	42		1:2:1	0.20	0.907
HT421	169	30	91	48		1:2:1	4.83	0.089
HT974	169	30	91	48		1:2:1	4.83	0.089
HT353	169	30	89	50		1:2:1	5.21	0.074
ORS965	165			51	114	1:3	3.07	0.080
HT1018	169	30	88	51		1:2:1	5.51	0.064
ORS716	169	30	91	48		1:2:1	4.83	0.089
ORS837	169	32	88	49		1:2:1	3.71	0.156
HT784	169	32	83	54		1:2:1	5.78	0.056
ORS53	169	33	87	49		1:2:1	3.18	0.204
ORS552	169	33	85	51		1:2:1	3.84	0.147
HT636	169	40	88	41		1:2:1	0.30	0.860
ORS1008	169			42	127	1:3	0.00	1.000

[a] A, HA-R4 (*PlPl*); H, heterozygous (*Plpl*); B, HA 821 (*plpl*); D, *PlPl* or *Plpl*

[b] For downy mildew races 770 and 734, testcross progenies for two plants were not screened because of limited seeds. The phenotypes to race 300 were used for them

between the two bulks (2.8%), including three from LG 1 (ORS53, ORS552, and ORS837), two from LG 10 (ORS908 and ORS1008), one from LG 8 (ORS243), and one from LG 15 (ORS344). Using the ten-plant resistant or susceptible bulks, one dominant marker tightly linked to the resistance genes was identified, which is a ~280 bp band from HA-R4 amplified by the primer pair ORS1008. After the analysis of the F_2 mapping population using ORS1008, no recombinants were detected between this marker and the phenotypes. Therefore, it co-segregated with the *Pl*$_{16}$ gene for the three *P. halstedii* races. Representative results on a nondenaturing or denaturing polyacrylamide gel is shown in Fig. 1a and Supplemental Fig. 1a, respectively.

Linkage group determination

The ORS1008 marker is located on LG 10 of the SSR map (Tang et al. 2003), and also was assigned to LG 1 of the Western Sunflower *H. anomalus*, Desert Sunflower *H. deserticola* (Lai et al. 2005), and another differential line HA-R5 (Mulpuri et al. 2009). It is also located on LG 7 and LG 8 of other *Helianthus* species (the Sunflower CMap Database, http://sunflower.uga.edu/cgi-bin/cmap/map search). After screening with 28 markers from LG 1, and 13 SSR markers from LG 10, an additional 29 SSR and 26 EST-SSR markers on LG 1 from 23 maps in the Sunflower CMap Database and 32 markers from LG 10 were used to further determine the linkage group of the *Pl*$_{16}$ gene. As a result, seven SSR and eight EST-SSR markers from LG 1, and 17 markers from LG 10 showed polymorphism between the parents.

After confirmation with the individuals constituting each bulk, these polymorphic markers were analyzed in the mapping population. One co-dominant marker produced by an EST-SSR primer pair HT636 from LG 1 was identified to be tightly linked to the *Pl*$_{16}$ gene. On a nondenaturing polyacrylamide gel, in addition to a ~460 bp band shared in the three F_2 phenotypes, HT636 amplified a ~510 bp band in the susceptible parent, HA 821, and homozygous susceptible F_2 plants, a faint band of the same molecular size in the F_1s and heterozygous resistant F_2 plants, and a null band in the resistant parent, HA-R4, and the homozygous resistant F_2 plants (representative PCR results shown in Supplemental Fig. 1b). The band patterns on a denaturing polyacrylamide gel confirmed the co-dominant condition of the HT636 marker (representative results shown in Fig. 1b). A lower band was detected for HA-R4 and the homozygous resistant F_2 plants, an upper band for HA 821 and homozygous susceptible F_2 plants, with both bands co-dominant in the F_1 and heterozygous resistant F_2 plants. One recombinant between the HT636 marker and the phenotype of the population was observed. In total, 12 markers (6 SSR and 6 EST-SSR markers) from LG 1 were linked to the *Pl*$_{16}$ gene. None of the Chi-square tests of the genotypes of the 169 F_2 individuals with all these markers significantly deviated from the 1:2:1 or 1:3 Mendelian segregation ratio (Table 2). Therefore, the location of this gene on LG 1 was confirmed.

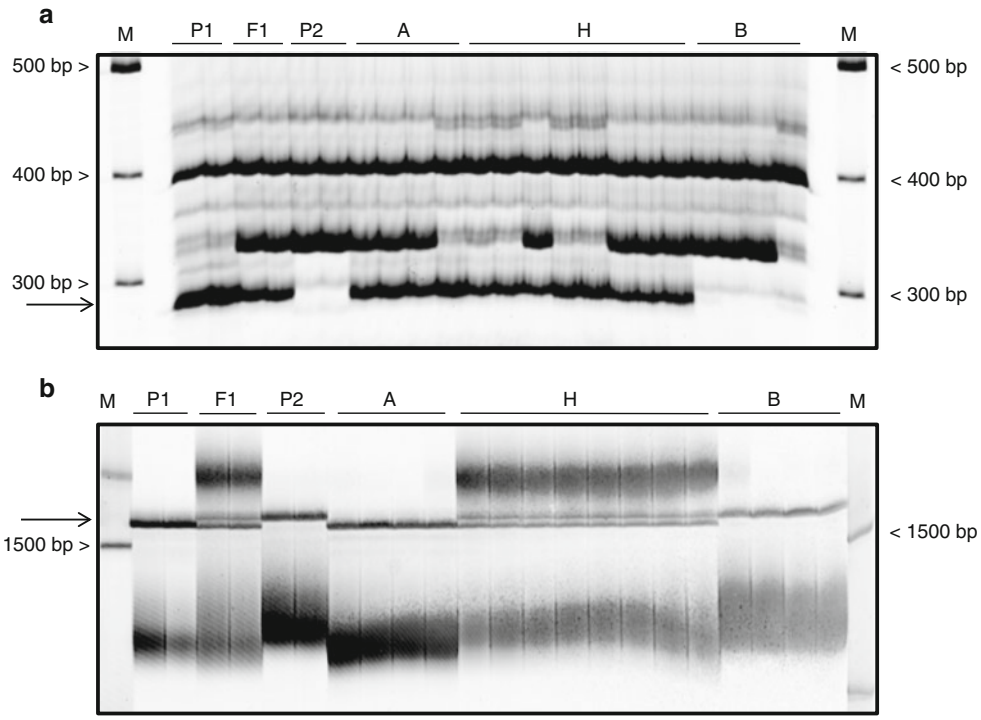

Fig. 1 Representative genotyping of the F_2 individuals derived from the cross of HA-R4 × HA 821 with both parents (P1—HA-R4, P2—HA 821) and F_1s using the ORS1008 marker on a nondenaturing polyacrylamide gel (**a**) and the HT636 marker on a denaturing polyacrylamide gel (**b**), respectively. A, H, and B represent the homozygous resistant, heterozygous resistant and homozygous susceptible F_2 individuals to downy mildew, respectively. The *arrows* indicate the markers. *M* indicates a 100 bp plus ladder Gelpilot (Qiagen)

Linkage group construction and comparison to other reference maps

After genotyping the entire mapping population, a linkage group including the 12 markers (6 SSR and 6 EST-SSR markers), and the *Pl*$_{16}$ gene was constructed using MAPMAKER/Exp version 3.0b, covering a genetic distance of 78.9 cM (Fig. 2a). Ten of the markers were co-dominant, but ORS965 and ORS1008 were dominant markers. The order of the upper part of the linkage group is similar to LG 1 of the reference maps of Radwan et al. (2008) and RHA 280 × RHA 801_RIL (in press) in the Sunflower CMap Database, together with a common marker HT636 at the lower ends of the three maps, which confirmed the accuracy of the mapping. The *Pl*$_{16}$ gene was mapped at the lower end of the linkage group, co-segregating with the ORS1008 marker and HT636 tightly linked at a distance of 0.3 cM proximal to it. Another two markers, ORS552 and ORS53, were linked at a genetic distance of 21.5 and 38.3 cM, respectively.

The ORS1008 marker is common between the LG 1 of the maps of HA-R4 × HA 821 (Fig. 2a) and HA-R5 × HA 821 (Fig. 2b). In order to further compare the two linkage groups where the *Pl*$_{13}$ and *Pl*$_{16}$ genes were mapped, the two

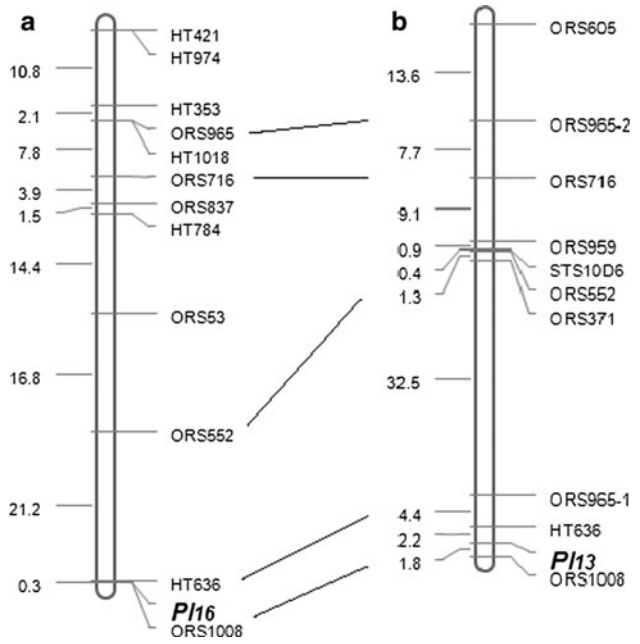

Fig. 2 The position of the downy mildew resistance gene *Pl*$_{16}$ on LG 1 of the sunflower map. **a** Mapping result of the *Pl*$_{16}$ locus on LG 1, including six SSR (ORS), six EST-SSR (HT) linked markers and the *Pl*$_{16}$ locus, based on the analysis of 169 F_2 individuals derived from the cross of HA-R4 × HA 821. **b** The position of the *Pl*$_{13}$ gene on LG 1 based on the linkage analysis in an F_2 population derived from HA-R5 × HA 821, with the map covering a distance of 74.0 cM (Mulpuri et al. 2009; this study). Shared markers are indicated by *lines* between the maps. The distances are given in centimorgan (cM) at the *left* side of the maps

markers ORS552 and HT636 were also applied to the F_2 mapping population derived from HA-R5 × HA 821. A similar order of the three markers was noticed for the *Pl*$_{13}$ gene and the *Pl*$_{16}$ gene, with ORS552 located 40.4 cM above the *Pl*$_{13}$ gene, ORS1008 1.8 cM below *Pl*$_{13}$, and HT636 2.2 cM between ORS552 and ORS1008. The HT636 marker is also tightly linked to the *Pl*$_{13}$ gene, and therefore, it can be used to discriminate the homozygous resistant, heterozygous resistant and homozygous susceptible plants in the progenies derived from HA-R5 in combination with the ORS1008 marker.

Application of the closely linked markers to other materials

In order to associate the two markers to the *Pl*$_{13}$ and *Pl*$_{16}$ genes on more germplasms, eight lines resistant to downy mildew race 734 and eighteen race 734 susceptible lines, as well as some other germplasms were examined. For the ORS1008 marker, at least 15 different patterns were observed on a nondenaturing polyacrylamide gel (Fig. 3a). The resistant ~280 bp marker amplified by ORS1008 was detected only in HA-R4 (sample 1 and 40), HA-R5 (sample 2) and the F_1s (samples 4, 5 and 41). This marker was observed even more clearly on a denaturing polyacrylamide gel (Fig. 3b). For the HT636 marker, at least 14 different band patterns were evident on both the nondenaturing and denaturing polyacrylamide gels (Fig. 3c, d). The specific patterns amplified in HA-R4 and HA-R5 by HT636 were observed in two susceptible lines, HA 372 (sample 18), and P 21 (sample 22), on both the nondenaturing and denaturing gels (Fig. 3c, d). The co-dominant band pattern was observed only in the F_1s of HA-R4 × HA 821 and HA-R5 × HA 821 among the materials tested using HT636, with similar patterns detected in TX 16 (sample 11), RHA 340 (sample 12), and HA 89 (sample 15) on a denaturing gel, but not on a nondenaturing gel. Six susceptible lines, Conf Scl R5 (sample 10), RHA 274 (sample 14), HA 441 (sample 17), RHA 858 (sample 21), Peredovik (sample 31), and Armarvir 3497 (sample 39), as well as three other lines, including G08/621, G09/2645, and G09/2643 (samples 26-28), had the same band pattern with the HT636 primer as HA 821 (sample 3 and 42). Furthermore, the resistant line, RHA 419 (sample 13), also amplified the same pattern as HA 821 using this primer. The three resistant lines, RHA 294, HA 458, and RHA 428 (sample 6–8), and another five samples, including CMS HA 89-3149, RHA 280, and their F_1 hybrid, Hopi Dye and Luch (sample 23–25, 30, 32) have a similar pattern that differs from HA 821 for this locus, indicating that the resistance genes in these germplasms are different than those in HA-R4 and HA-R5. Also, each of the six wild species had a different banding pattern than cultivated sunflower for both of the markers, which suggests a genetic diversity in the wild perennial *Helianthus* species at

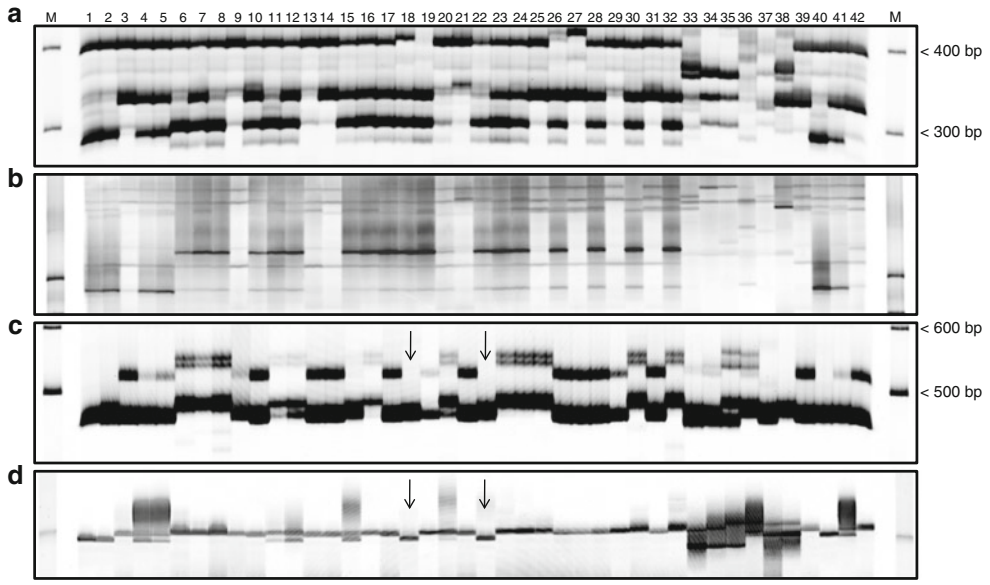

Fig. 3 Amplification results of 38 sunflower lines with the primers ORS1008 (**a** and **b**) and HT636 (**c** and **d**). The band patterns on a nondenaturing polyacrylamide gel (**a** and **c**). The band patterns on a denaturing polyacrylamide gel (**b** and **d**). Samples 1 and 40: HA-R4; 2: HA-R5; 3 and 42: HA 821; 4 and 41: F_1 of HA-R4 × HA 821; 5: F_1 of HA-R5 × HA 821; 6: RHA 294; 7: HA 458; 8: RHA 428; 9 and 29: HA 234; 10: Conf Scl R5; 11: TX 16; 12: RHA 340;13: RHA 419; 14: RHA 274; 15: HA 89; 16: HA 410; 17: HA 441; 18: HA 372; 19: HA 852; 20: RHA 271; 21: RHA 858; 22: P 21; 23: CMS HA 89-3149; 24: F_1 of CMS HA 89-3149 × RHA 280; 25: RHA 280; 26: G08/621; 27: G09/2645; 28: G09/2643; 30: Hopi Dye; 31: Peredovik; 32: Luch; 33: *H. maximiliani*, PI 586892; 34: *H. giganteus*, PI 547182; 35: *H. grosseserratus*, PI 613793; 36: *H. californicus* 2376; 37: *H. schweinitzii* 2405; 38: *H. nuttallii* 102; 39: Armarvir 3497. *Arrows* in **c** and **d** indicate that HA 372 and P 21 amplified a band with the same size as HA-R4 and HA-R5. *M* indicates a 100 bp plus ladder Gelpilot (Qiagen)

this locus (Fig. 3a, d). Therefore, the PCR patterns amplified by the primer ORS1008 appear to be specific to the downy mildew resistance genes in HA-R4 and HA-R5, whereas HT636 could be used for diversity analysis.

Discussion

The downy mildew resistance gene of HA-R4 was mapped on LG 1 of the sunflower SSR map

In this study, the downy mildew resistance gene of USDA-released HA-R4, Pl_{16}, was mapped to the lower end of LG 1 of the public sunflower map (Tang et al. 2003), with one dominant marker, ORS1008, co-segregating with it, and one co-dominant marker, HT636, linked at a distance of 0.3 cM. So far, this is the fourth *Pl* gene mapped on LG 1. The first one was the Pl_{Arg} locus in the sunflower germplasm ARG-1575-2, which confers resistance to all currently identified downy mildew races reported (Dußle et al. 2004; Wieckhorst et al. 2010). This gene was mapped to the upper part of LG 1 based on the map of Yu et al. (2003), with two SSR, two EST-SSR, and three RGC markers co-segregating with it in an F_2 progeny derived from the cross between (CMS) HA 342 and ARG-1575-2. Fine mapping of this gene anchored two RGC-containing BAC contigs to this region (Wieckhorst et al. 2010). The compressed recombination was detected in LG 1 by comparing the map with other reference maps, and a significant segregation distortion was observed for the phenotype evaluation (Dußle et al. 2004).

The second gene was the Pl_{13} gene from HA-R5, which was mapped to the lower end of LG 1, with two dominant SSR markers ORS1008 and ORS965-1 closely linked at a genetic distance of 0.9 and 5.8 cM, respectively (Mulpuri et al. 2009). Interestingly, the segregation ratio of the phenotypes in the mapping population derived from HA-R5 × HA 821 did not exhibit significant distortion as did Pl_{Arg}.

The third gene was the Pl_{14} gene from 29004, which was a line derived from HA-R4 (Bachlava et al. 2011). The Pl_{14} gene was located between RGC188 (2.6 cM) and RGC203 (1.6 cM), with the HT636 marker at a distance of 5.8 cM distal to it. The segregation of the two RGCs and the phenotypes all showed significant deviation from the expected ratios in the F_2 mapping population derived from IMISUM-1 × 29004, which does not fit the expected monogenic 1:2:1 ratio. As for the present study, the deviation of the segregation of the phenotypes and the markers was not evident, indicating that a single gene controls resistance to the different *P. halstedii* races in HA-R4. Because the population is limited in size, the existence of a tightly linked

cluster controlling the resistance to downy mildew in HA-R4 cannot be ruled out. Moreover, considering the breeding history of HA-R4, we can conclude that HA-R4 is a material with complex composition. Therefore, it is possible that diverse *Pl* genes exist in HA-R4.

According to these results, both the *Pl* genes in HA-R4 and HA-R5 were mapped to a similar position at the lower end of LG 1 of the sunflower SSR map, and any evidence of segregation deviation of the phenotypes and markers was lacking (Mulpuri et al. 2009; Tang et al. 2003). The Pl_{14} gene confers type II resistance to downy mildew (Radwan et al. 2011), while no spores were observed on the cotyledons of HA-R4 and HA-R5 with the *P. halstedii* races used, i.e., it confers type I resistance to downy mildew (Mulpuri et al. 2009; this study). Thus, Pl_{13} and Pl_{16} are likely different than the other two *Pl* genes mapped on LG 1, and different than the *Pl* genes on LG 8 and LG 13. PCR results of the ORS1008 primer revealed a common marker for HA-R4, HA-R5, and the F_1 hybrids (by crossing them with HA 821, respectively), in the 38 germplasms and hybrids examined, providing support for the uniqueness of the Pl_{13} and Pl_{16} genes. In an analysis of 37 *P. halstedii* races from different countries, HA-R4 and HA-R5 had different reactions to six races (Gulya et al. 2011). HA-R4 was susceptible to a race 731 in Canada, and resistant to three races (502, 702, and 722) in Canada, and two races (732 and 772) in North America, whereas HA-R5 was resistant to race 731, and susceptible to the other five races (Gulya et al. 2011). Therefore, the downy mildew resistance genes in HA-R4 and HA-R5 are not the same, and thus it is reasonable to designate the downy mildew resistance gene in HA-R4 as Pl_{16}. Allelism tests among different *Pl* genes are needed to determine the relationship between Pl_{16} and the other *Pl* genes.

Molecular mapping provides two useful markers for tracking the downy mildew resistance genes derived from HA-R4 and HA-R5

Both HA-R4 and HA-R5 have been used as differential lines for *P. halstedii* race determination. They are resistant to at least 20 races, including the newly emerged 'hot' race 734 (Gulya et al. 2011; T. Gulya, personal communication). They are also resistant to different rust races, and Verticillium wilt (Gulya 1985). Therefore, they are good sources for pyramiding genes in sunflower breeding. Molecular markers closely linked to the resistance genes will speed the breeding process. In this study, we identified one dominant SSR marker, ORS1008, co-segregating with the Pl_{16} gene, as well as a co-dominant EST-SSR marker, HT636, linked to it at a genetic distance of 0.3 cM. The ORS1008 marker was previously reported as the closest marker linked to the Pl_{13} gene at a distance of 0.9 cM. The co-dominant HT636 marker also was linked to this gene at a distance of 2.2 cM. By comparison of the PCR patterns to other materials tested, ORS1008 was identified as a specific marker in HA-R4 and HA-R5 and their F_1 hybrids, while the HT636 marker has specificity to a lesser degree. Since both the markers, ORS1008 and HT636, co-segregated or were tightly linked to the Pl_{13} or Pl_{16} gene, these two markers will be useful for tracking downy mildew resistance genes derived from HA-R4 and HA-R5, and for the identification and selection of homozygous or heterozygous resistant/susceptible phenotypes to downy mildew. This will increase the efficiency of selection, as compared to the use of dominant markers only. This is the first report to provide two tightly linked dominant and co-dominant markers for both the Pl_{16} and Pl_{13} genes.

The co-segregating and tightly linked markers will provide a basis for map-based cloning of the Pl_{13} and Pl_{16} genes

Similar to soybean (Bhattacharyya et al. 2005; Kanazin et al. 1996; Yang et al. 2010; Zhang et al. 2011), lettuce (Meyers et al. 1998a, b; McHale et al. 2009), *Arabidopsis* (Holub 2001; Meyers et al. 2003), rice (Zhou et al. 2004; Yang et al. 2006; Gu and Guo 2007), peanut (Ratnaparkhe et al. 2011), and potato (Bakker et al. 2011), many recognition-dependent *R* genes in sunflower also encode NBS-LRR protein homologs. These genes were found to be duplicated, evolutionarily diverse, and arranged in the genome as large multi-gene. According to the domain in the homologs, NBS-LRR was divided into two main subclasses based on the CC or TIR domain at the N terminus (Meyers et al. 2003). Both sub-classes of NBS-LRR genes have been reported in sunflower. Gentzbittel et al. (1998) mapped restriction fragment length polymorphisms (RFLPs) for an RGC (Ha-NBS-R3), belonging to the TIR-NBS-LRR group, in three mapping populations where the Pl_6 gene cluster is located (Vear et al. 1997). The conserved resistance genes contained at the Pl_6 locus encode proteins belonging to the TIR-NBS-LRR class, as well as a cDNA PLFOR48 from a downy mildew resistant sunflower line, RHA 266 (Bouzidi et al. 2002; Hewezi et al. 2006).

Now, many disease resistance gene analogs (RGAs) or RGCs have been cloned according to the sequence similarity to NBS-LRR encoding genes and other *R* genes from many plants including sunflower (Bachlava et al. 2011; Danilova et al. 2007; Gedil et al. 2001; Plocik et al. 2004; Radwan et al. 2003, 2004, 2005, 2008; Slabaugh et al. 2003). Many of them were co-located with the previously mapped *R* genes (Radwan et al. 2008; Bachlava et al. 2011). The non-TIR-NBS-LRR RGAs are reported to be clustered and linked to the resistance Pl_5/Pl_8 locus on the SSR map of LG 13 (Yu et al. 2003) (Radwan et al. 2004, 2008). Slabaugh et al. (2003) developed a cluster of

HaRGC1-related markers from a TIR-NBS-LRR RGC, Ha-4W2, (Gedil et al. 2001) using degenerate primers, and mapped the 24 HaRGC1 loci to a 2–4 cM segment of LG 8, which was located in the Pl_1–Pl_2–Pl_6 region. They also identified the RGC markers co-segregating with the *Pl* genes as the candidates for the Pl_1 and Pl_2 structural genes by haplotype analysis. Radwan et al. (2008) identified 630 NBS-LRR homologs from common and wild sunflower species, developed DNA markers from 196 unique NBS-LRR sequences, and mapped 167 NBS-LRR loci in 44 clusters or singletons. Bachlava et al. (2011) analyzed the sequences of the bacterial artificial chromosome (BAC) clones proximal to the Pl_8 and R_{Adv} genes on LG 13 and the Pl_{14} gene on LG1, and identified seven novel non-TIR-NBS-LRR RGCs. Recently, the full-length cDNA of the RGC151, co-segregating with the Pl_{Arg} gene (resistance type I), and that of RGC203, tightly linked to the Pl_{14} gene (resistance type II), were cloned and sequenced. The results suggested that RGC151 belongs to the TIR-NBS-LRR subclass whereas RGC203 belongs to the coiled-coil (CC)-NBS-LRR subclass (Radwan et al. 2011).

In this study, the Pl_{13} and Pl_{16} genes were assigned to a similar position at the lower end of the LG 1 of the public sunflower SSR map (Tang et al. 2003), where a cluster of RGCs have been mapped previously (Radwan et al. 2008; Mulpuri et al. 2009; Wieckhorst et al. 2010; Bachlava et al. 2011). The single-locus HT636 marker is a common EST-SSR marker close to this RGC cluster on LG 1 of the reference maps, and tightly linked to the Pl_{16} or Pl_{13} gene mapped in the F_2 populations derived from HA-R4 × HA 821 and HA-R5 × HA 821, respectively (Fig. 2). The HT636 primer was located at the coding sequence of a putative basic leucine zipper domain (bZIP) DNA-binding protein [*Capsicum chinense*] (GenBank: AF127797) (Heesacker et al. 2008), which belongs to a family of transcription factors engaged in protein dimerization (O'Shea et al. 1989; Carrillo et al. 2010). The co-location of the HT636 marker with the Pl_{13} or Pl_{16} gene and RGCs suggests that these two *Pl* genes, and even the HT636 itself, maybe belong to an RGC cluster located on this part of LG 1.

Map-based cloning and the function study of these genes will indicate which class of *R* genes these genes belong to, and whether a single gene or a cluster of genes control resistance to downy mildew in HA-R4 and HA-R5. The two co-segregating or tightly linked markers identified in the present study will provide a molecular basis for targeting the region of interest and map-based cloning of these two *Pl* genes. Fine mapping with more molecular markers, such as single-nucleotide polymorphisms (SNPs) and RGC markers, will help saturate the linkage map in these regions. Also, several BAC libraries have been constructed for different sunflower lines, including RHA 325 with the downy mildew resistance gene Pl_2 (Özdemir et al. 2004), YDQ resistant to all downy mildew races (Bouzidi et al. 2006), and HA 89 resistant to race 330 (Feng et al. 2006), which will provide a platform for cloning of other *Pl* genes, and thus help to understand the mechanism for downy mildew disease resistance and host–pathogen interaction for sunflower.

Also, clusters harboring resistance genes to rust and downy mildew in sunflower include the Pl_1 and HaRGC1 *R* gene clusters on LG 8, and Pl_8, R_{Adv} and R_4 on LG 13 (Bachlava et al. 2011; Qi et al. 2011; Slabaugh et al. 2003). Besides downy mildew, HA-R4 and HA-R5 are also resistant to different rust races, as well as to Verticillium wilt. It is not known if the rust resistance genes of HA-R4 and HA-R5 are located on LG 13 as is the R_4 allele in HA-R3 (Miller et al. 1988; Qi et al. 2011), or whether the cluster containing the resistance genes to downy mildew (Pl_{13} and Pl_{16}) on LG 1 will also contain genes conferring resistance to other diseases, such as Verticillium wilt. Further phenotype and genotype analyses of different rust races and other pathogens will be conducted to answer these questions for HA-R4, as well as HA-R5.

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