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ORIGINAL ARTICLE

Genetics and mapping of a novel downy mildew resistance gene, *Pl*$_{18}$, introgressed from wild *Helianthus argophyllus* into cultivated sunflower (*Helianthus annuus* L.)

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Abstract
Key message **A novel downy mildew resistance gene, *Pl*$_{18}$, was introgressed from wild *Helianthus argophyllus* into cultivated sunflower and genetically mapped to linkage group 2 of the sunflower genome. The new germplasm, HA-DM1, carrying *Pl*$_{18}$ has been released to the public.**

Abstract Sunflower downy mildew (DM) is considered to be the most destructive foliar disease that has spread to every major sunflower-growing country of the world, except Australia. A new dominant downy mildew resistance gene (*Pl*$_{18}$) transferred from wild *Helianthus argophyllus* (PI 494573) into cultivated sunflower was mapped to linkage group (LG) 2 of the sunflower genome using bulked segregant analysis with 869 simple sequence repeat (SSR) markers. Phenotyping 142 $BC_1F_{2:3}$ families derived from the cross of HA 89 and *H. argophyllus* confirmed the single gene inheritance of resistance. Since no other *Pl* gene has been mapped to LG2, this gene was novel and designated as *Pl*$_{18}$. SSR markers CRT214 and ORS203 flanked *Pl*$_{18}$ at a genetic distance of 1.1 and 0.4 cM, respectively. Forty-six single nucleotide polymorphism (SNP) markers that cover the *Pl*$_{18}$ region were surveyed for saturation mapping of the region. Six co-segregating SNP markers were 1.2 cM distal to *Pl*$_{18}$, and another four co-segregating SNP markers were 0.9 cM proximal to *Pl*$_{18}$. The new BC_2F_4-derived germplasm, HA-DM1, carrying *Pl*$_{18}$ has been released to the public. This new line is highly resistant to all *Plasmopara halstedii* races identified in the USA providing breeders with an effective new source of resistance against downy mildew in sunflower. The molecular markers that were developed will be especially useful in marker-assisted selection and pyramiding of *Pl* resistance genes because of their close proximity to the gene and the availability of high-throughput SNP detection assays.

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Introduction

Cultivated sunflower (*Helianthus annuus* L.) is a diploid species ($2n = 2x = 34$), and is one of the few major food crops of the world that originated from North America (Rieseberg and Seiler 1990; Harter et al. 2004; Blackman et al. 2011). There are 53 wild species in the genus *Helianthus*, including 14 annual and 39 perennial (Moyers and Rieseberg 2013; Marek et al. 2014; Seiler and Jan 2014). Wild *Helianthus* annual species are diploid, with the same chromosome number ($2n = 2x = 34$) as cultivated sunflower, whereas wild *Helianthus* perennial species include 29 diploids ($2n = 2x = 34$), 4 tetraploids ($2n = 4x = 68$), and 6 hexaploids ($2n = 6x = 102$). Cultivated sunflower has a narrow genetic base because of its recent origin, domestication, and breeding. However, the wild sunflower species in North America have adapted to a wide range of environments during their spread and possess considerable genetic variability that could be used for sunflower improvement

against biotic and abiotic stresses. Many resistance genes for several major diseases, including rust, downy mildew, Verticillium wilt, Sclerotinia, Phomopsis stem canker, and Phoma black stem, have been reported in the wild sunflower species (for review see Seiler 2010). Introgressive hybridization with wild species is widely used to broaden the genetic base of cultivated sunflower.

Plasmopara halstedii (Farl.) Berl. et de Toni, the causal agent of sunflower downy mildew is assumed to have originated in the central portion of the North American continent, as did its sunflower host (Leppik 1966). Sunflower downy mildew is considered to be the most destructive foliar disease and has spread to every major sunflower-growing country of the world, except Australia. The disease can cause up to an 80 % yield loss in sunflower production (Molinero-Ruiz et al. 2003). The defense against this pathogen has been the use of the seed fungicide metalaxyl. Downy mildew has become an increasing problem in North America and Europe with the appearance of metalaxyl-resistant strains of *P. halstedii*, with no single fungicide seed treatment found to replace metalaxyl (Albourie et al. 1998; Gulya et al. 1999; Gulya 2001, 2002). The other available defense is host plant resistance, which has been found through germplasm screening (Miller and Gulya 1987, 1988, 1991; Seiler 1991; Rahim et al. 2002; Gulya 2005; Hulke et al. 2010).

Nineteen downy mildew resistance genes (*R* genes), denoted as *Pl* (Pl_1–Pl_{17}, Pl_{21}, and Pl_{Arg}), have been discovered to date in sunflowers and its wild species. Thirteen of these genes (Pl_1, Pl_2, Pl_5–Pl_8, Pl_{13}–Pl_{17}, Pl_{21}, and Pl_{Arg}) were assigned to specific linkage groups (LGs) of the sunflower genome (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. 2015b). The origin of most of the *Pl* genes can be traced to wild *Helianthus* annual species. Pl_6 and Pl_{17} were derived from wild *H. annuus* L. (Miller and Gulya 1991; Hulke et al. 2010; Qi et al. 2015b), and Pl_1, Pl_2, and Pl_{13} originated from a Canadian line 953-102-1-1, which is a selection involving wild *H. annuus* (Fick and Zimmer 1974; Vear et al. 2008). Pl_5 originated from *Helianthus tuberosus* L. (a perennial species) (Vrânceanu et al. 1981) and Pl_7 from *Helianthus praecox* Englem. and Gray (Miller and Gulya 1991). Two *Pl* genes, Pl_8 and Pl_{Arg}, were derived from *H. argophyllus* Torrey and Gray (Miller and Gulya 1988; Seiler 1991).

Race-specific *Pl* resistance genes have played a major role in the fight against downy mildew by successfully deploying resistance genes in sunflower hybrids since 1978 (Vrânceanu et al. 1981; Miller and Gulya 1988). However, global sunflower production is continually challenged by newly emerging physiological races of *P. halstedii*. In the 1970s only two races, 100 and 300, were reported in North America and Europe (Zimmer 1974), whereas in 2006, 36 *P. halstedii* races have been identified around the world (Gulya 2007; Gascuel et al. 2014). Five North American (NA) races and eight new races of *P. halstedii* from France have been shown to be virulent against Pl_6 and Pl_7 in the 2000s. Pl_6 and Pl_7 have been broadly deployed in the major sunflower-growing areas of the world, especially in North America and Europe (Tourvieille de Labrouhe et al. 2000; Gulya 2007). This has resulted in many commercial hybrids becoming susceptible to certain downy mildew races (Gulya et al. 2011). Therefore, there is a need for a diversity of resistance genes to avoid selection pressure on one gene used too widely, and the search for new sources of resistance to downy mildew is also necessary to provide sunflower growers with new resistant hybrids.

Helianthus argophyllus is a wild diploid annual species ($2n = 2x = 34$) found mainly in the southern part of Texas, USA (Rogers et al. 1982) and has been an important source of disease resistance genes for sunflower improvement. Resistance to rust (genes R_{adv} and R_5) (Bachlava et al. 2011; Qi et al. 2012), downy mildew (Pl_8 and Pl_{Arg}) (Miller and Gulya 1991; Seiler 1991; Dußle et al. 2004), and Sclerotinia (Qi et al. 2013) has been transferred from *H. argophyllus* into cultivated sunflower. Gulya (2005) evaluated 13 additional accessions of *H. argophyllus* for resistance to infection with a mixture of races with a summed virulence equivalent to a theoretical 777 race and found that five accessions had >90 % of individual plants immune to 777 downy mildew mixture, and one accession had all immune plants. Here, we report the introgression of a novel downy mildew gene, Pl_{18}, from *H. argophyllus* accession PI 494573 into cultivated sunflower and the mapping this gene to LG2 of the sunflower genome, which is the first *Pl* gene located on this linkage group to date.

Materials and methods

Plant materials and mapping population

The *H. argophyllus* accession PI 494573 was originally collected in 1984 from Texas, USA, and found to be resistant to new races of downy mildew (Gulya 2005). The initial cross was made between a nuclear male-sterile (NMS) HA 89 with PI 494573 in 2009 and the F_1 was backcrossed twice to the inbred maintainer line HA 89. HA 89 (PI 599773) is a selection from the high-oil Russian cultivar VNIMK 8931 (PI 262517) released by the USDA and the Texas Agricultural Experiment Station in 1971. The NMS HA 89 was produced by chemically induced mutation of HA 89 using streptomycin and possessing a single recessive male-sterile gene, *ms9* (Jan and Rutger 1988; Chen et al. 2006).

Helianthus argophyllus is an open-pollinated species with high heterozygosity. The F_1 plants of the cross of NMS HA 89/PI 494573 were evaluated for downy mildew resistance in a greenhouse in 2010, and resistant plants were backcrossed to HA 89. The second backcross population was made by crossing selected resistant BC_1 plants with HA 89. The homozygous resistant introgression lines were selected from the BC_2F_2 population and advanced to BC_2F_3. Three sunflower genotypes, Cargill 272, a sunflower hybrid developed by the Cargill Company (Minneapolis, MN, USA) susceptible to downy mildew; an inbred line HA 335 (PI 518773) carrying the gene *Pl₆*, which was susceptible to new races, such as 734; and an inbred line RHA 340 (PI 518778) harboring the *Pl₈* resistant gene to most of downy mildew races, were used as controls in the downy mildew resistance tests of the BC_2F_3.

The BC_1F_2 population used for mapping the resistance genes was developed from a single-resistant BC_1F_1 plant. A total of 240 BC_1F_2 plants were grown in the greenhouse in 2011, and 169 $BC_1F_{2:3}$ plants were harvested.

Evaluation of downy mildew resistance

Four *P. halstedii* races, 730, 734, 770, and 774, were chosen to test seedlings of the BC_2F_3 homozygous introgression lines for resistance to downy mildew. Races 734 and 770 were identified as new virulent races in North America in 2010 that overcome the Pl_6 and Pl_7 genes, whereas 730 was one of the predominant races in North America and Europe (Rashid et al. 2006; Gulya 2007; Gulya et al. 2011). Downy mildew spores of each race collected from the field in 2012 were increased on susceptible sunflower lines and tested on nine differential lines for race verification (Gulya, personal communication). Race 734 was collected in 2009 and selected to inoculate seedlings of the backcross populations, and the BC_1F_3 mapping population.

Downy mildew resistance was evaluated in the greenhouse trials using the whole seedling immersion method described by Gulya et al. (1991). Briefly, seeds were surface sterilized with a 20 % bleach solution (~1 % sodium hypochlorite) for 10 min, rinsed well with tap water, evenly spaced on germination paper, and incubated in a germinator in the dark at 22–24 °C for 3 days. Freshly produced *P. halstedii* spores were used to inoculate 3-day-old seedlings by immersion for 3–5 h in suspensions of $2–4 \times 10^4$ zoosporangia at 18 °C. Inoculated seedlings were grown in a sterilized mixture of sand and perlite (2:3, *v*/*v*) in the greenhouse (24 ± 3 °C, 16 h photoperiod) for 10–12 days. The plants were placed in a chamber maintained at 18 °C and 100 % relative humidity overnight to induce sporulation and then returned to the greenhouse. A plant was considered susceptible (S) if sporulation was observed on cotyledons and true leaves and was considered resistant (R) if no sporulation was observed.

Following the cross of NMS HA 89 × PI 494573, the F_1 progeny were screened in the greenhouse for resistance to *P. halstedii* race 734, as described above. This process was repeated for the BC_1 through BC_2F_2 generations. The homozygous BC_2F_3 plants selected from self-pollinated resistant BC_2F_2 were tested with four races, 730, 734, 770, and 774.

For downy mildew tests of the BC_1F_3 population, 40 seeds of each 142 $BC_1F_{2:3}$ families were germinated at 22–24 °C in a growth chamber and 30 seedlings of each family were inoculated with downy mildew race 734 in March, 2012. The F_3 families were classified as homozygous resistant if none of the seedlings had sporulation, segregating if some seedlings (about one-quarter in a F_3 family) had sporulation on the cotyledons and true leaves, and homozygous susceptible if all seedlings had sporulation on cotyledons and true leaves.

DNA extraction and PCR conditions

Leaf tissue was collected from the parental lines, HA 89 and PI 494573, and 142 BC_1F_2 plants. Tissue samples were also collected from eight homozygous BC_2F_3 families with six plant samples mixed for each family. Genomic DNA was isolated from the lyophilized tissues using the DNeasy 96 plant kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA), and the quantity and quality of DNA were determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Initially, a total of 869 simple sequence repeat (SSR) markers were used to screen the parents, HA 89 and PI 494573. To ensure complete genome coverage, markers were chosen based on their distribution throughout the linkage maps of sunflower (Tang et al. 2002, 2003; Yu et al. 2003). Polymerase chain reaction (PCR) for SSR primers was performed on a Peltier thermocycler (Bio-Rad Lab, Hercules, CA, USA) with a touchdown program as described by Qi et al. (2011). The PCR products were diluted 20- to 120-fold before analysis depending on the yield of the PCR products. The PCR-amplified fragments were separated according to size with an IR2 4200/4300 DNA Analyzer (Li-COR, Lincoln, NE, USA). All of the fragment sizes included 19 bp of the M13 fluorescent tag.

Location of *Pl* gene and linkage map

Linkage of an SSR marker to downy mildew resistance in the BC_1F_2 population was initially identified through bulked segregant analysis (BSA, Michelmore et al. 1991). Aliquots of DNA from 10 homozygous resistant and 10

homozygous susceptible BC_1F_2 plants were combined into resistant (R) and susceptible (S) bulks, respectively. The SSR markers that were polymorphic between two parents were used to test the two bulks. The markers that showed a polymorphic pattern between the R and S bulks were considered to be potentially linked to the resistance gene and were further evaluated in the BC_1F_2 population.

The Chi-square (χ^2) test was used to assess goodness-of-fit to the expected segregation ratio for downy mildew resistance and each marker in the mapping populations. JoinMap 4.1 was used for linkage analyses and map construction with a regression mapping algorithm and Kosambi mapping function (Van Ooijen 2006). A minimum likelihood of odds (LOD) $\geq$3.0 and a maximum distance of $\leq$50 centimorgans (cM) were used to test linkage among markers.

Map saturation with SNP markers

After the *Pl* resistance gene was located on LG2 of the sunflower genome using SSR markers, 46 single nucleotide polymorphism (SNP) markers that might cover the *Pl* gene region in LG2 were selected, 25 from the SNP map developed by the National Sunflower SNP Consortium (hereafter referred to as NSA SNPs, Table S1, Talukder et al. 2014) and 21 from the Bower's SNP map (hereafter referred to as SFW SNPs, Table S2, Bowers et al. 2012). These SNP markers were screened for polymorphism between the two parents to identify markers more closely linked to the gene.

Genotyping of the parental lines and the BC_1F_2 population with NSA SNPs was conducted by BioDiagnostics, Inc. (River Falls, WI, USA), where the NSA SNPs were developed (Pegadaraju et al. 2013). Genotyping of the SFW SNPs was performed using a newly developed technique of converting the SNPs into length polymorphism markers in our lab. Briefly, for each SNP, two-tailed forward allele-specific primers (AS-primers F1 and F2) and one common reverse primer were designed. Two alternate permutation of artificial mismatches are introduced at 3rd and 4th base from 3′ end each in either of AS-primers according to A and T → C, G → A, and C → T. An additional 5-base oligonucleotide (5′-ATGAC-3′) was inserted between the tail and the allele-specific sequences in the AS-primer F2 to produce a length difference between the two alleles after amplification (Table 1). A universal priming-element-adjustable primer (PEA-primer 5-ATAGCTGG-Sp9-GCAACAGGAACCAGCTATGAC -3) with an attached fluorescence tag at the 5′ terminus was used in each PCR reaction. PCR amplifications were performed in 15 µl containing 0.8 M Betaine, 0.04 % BSA, 2 mM $MgCl_2$, 100 µM dNTP each, 0.2 µM common reverse primer, 0.2 µM universal fluorescence-labeled PEA-primer, and 0.04 µM each of AS-primers (the tail sequences being identical to PEA-primer), 1 × PCR buffer, 1 U of Taq polymerase (Bioline, Randolph, MA, USA), and 10–20 ng of genomic DNA. PCR was conducted with initial denaturation at 94 °C for 3 min, followed by 6 cycles in which the annealing temperature was decreased by 1 °C for each cycle starting at 94 °C for 20 s, and then 56 °C for 30 s, followed by an additional 36 cycles of 2-step PCR protocol: 94 °C for 20 s, 62 °C for 30 s. The PCR products were diluted 40–240 times and size segregated in an IR^2 4300/4200 DNA Analyzer with denaturing polyacrylamide gel electrophoresis (LI-COR, Lincoln, NE, USA).

Results

Introgression of downy mildew resistance from *H. argophyllus* into cultivated sunflower

All crosses and backcrosses, as well as downy mildew screenings were conducted in the greenhouse under controlled conditions. The cross of NMS HA 89 with *H. argophyllus* PI 494573 yielded 45 seeds from the 2,375 pollinated florets, with a seed set of 1.9 % in the spring 2009. Fifteen F_1 seedlings were tested for resistance to race 734, and six resistant plants were selected to backcross to HA 89. In BC_1, 24 seedlings were inoculated with race 734, 13 were resistant and 11 susceptible, fitting a 1:1 ratio ($\chi^2 = 0.17$, $df = 1$, $P = 0.68$). A subsequent backcross was conducted to obtain the BC_2 generation in the fall, 2010. The BC_2F_1 seed was planted for resistance testing to race 734 in the greenhouse in the spring, 2011, and the selected resistant plants were self-pollinated to produce the BC_2F_2 generation. After screening the 120 BC_2F_2 seedlings with race 734, 25 resistant BC_2F_2 plants were advanced to the BC_2F_3 generation in the winter, 2011. The 20–30 seedlings of each 25 BC_2F_3 families were inoculated with race 734 in August, 2012, and eight families were found to be homozygous for downy mildew resistance (Table 2).

Downy mildew resistance in a homozygous BC_2F_3 line, 11-261-16, was further evaluated with four races, 730, 734, 770, and 774, along with their recurrent parent HA 89, and the susceptible and resistant checks. As expected, HA 89 and Cargill 272 were susceptible to all races, whereas, the BC_2F_3 line and RHA 340 were resistant to all races tested, indicating that the downy mildew resistance from *H. argophyllus* was successfully transferred into cultivated sunflower (Table 3). No segregation was observed in 184 BC_2F_3 plants tested, revealing the homozygous nature of the selected BC_2F_3 family.

Table 1 The primer sequences of the linkage group 2 SNPs mapped in this study

SNP name	SNP primer	Primer sequence (5–3′)
SFW00725	SFW00725F1a	GCAACAGGAACCAGCTATGACGATAAAGGTGATGGAAGATGTCCA
	SFW00725F2a	GCAACAGGAACCAGCTATGAC*ATGAC*GATAAAGGTGATGGAAGATGCTCG
	SFW00725R1	ATGAAAGACCACTGAATTTGGCA
SFW00880	SFW00880F1	GCAACAGGAACCAGCTATGACCGTCTTGAAACCCATTGACCGG
	SFW00880F2	GCAACAGGAACCAGCTATGAC*ATGAC*CGTCTTGAAACCCATTGATTGA
	SFW00880R	TGTGTTTCAAAACCTGCAAA
SFW03013	SFW03013F1a	GCAACAGGAACCAGCTATGACGCAAAGGATGTTGCTGACTCT
	SFW03013F2a	GCAACAGGAACCAGCTATGAC*ATGAC*GCAAAGGATGTTGCTGATCCC
	SFW03013R1	CAAGGCAGACTTGTCGGATAAA
SFW03060	SFW03060F1a	GCAACAGGAACCAGCTATGACCAAATGGGTTCTCTGATCATACTCTT
	SFW03060F2a	GCAACAGGAACCAGCTATGAC*ATGAC*CAAATGGGTTCTCTGATCATACCTTC
	SFW03060R1	GATCAAGAACTGAAGATGAAGTGTT
SFW03883	SFW03883F1	GCAACAGGAACCAGCTATGACCTGGCTGCAGAGGCATTTCT
	SFW03883F2	GCAACAGGAACCAGCTATGAC*ATGAC*CTGGCTGCAGAGGCATCCCG
	SFW03883R	GGTTCTACTTGTGCCATTTC
SFW04107	SFW04107F1	GCAACAGGAACCAGCTATGACGGTTCACGGGCCCACAAGTT
	SFW04107F2	GCAACAGGAACCAGCTATGAC*ATGAC*GGTTCACGGGCCCACAGATC
	SFW04107R	GGTTGCGTTTCAAAAATCCT
SFW04370	SFW04370F1	GCAACAGGAACCAGCTATGACGATTTTGTCCCTTTGTTATTACTA
	SFW04370F2	GCAACAGGAACCAGCTATGAC*ATGAC*GATTTTGTCCCTTTGTTATTGTTG
	SFW04370R	TTTGGGGATTTTGAGAGGAG
SFW04623	SFW04623F1	GCAACAGGAACCAGCTATGACTAATGTTCTCGGGTACGCAGCG
	SFW04623F2	GCAACAGGAACCAGCTATGAC*ATGAC*TAATGTTCTCGGGTACGCGACT
	SFW04623R	GAAAAGCTTGTACCAACCAC
SFW06076	SFW06076F1	GCAACAGGAACCAGCTATGACCTGCTGTTGGTGAGGATGACCT
	SFW06076F2	GCAACAGGAACCAGCTATGAC*ATGAC*CTGCTGTTGGTGAGGATGGTCC
	SFW06076R	GAACGCCCCTTAAAATCTCT

The tail sequence is underlined and the additional five-base oligonucleotide insertion in AS-primer F2 is italic

Table 2 Homozygous resistant tests of eight BC_2F_3 families with downy mildew race 734

Plant no.	Materials	No. of seeds for germination	No. of seedling inoculated	DM score	
				S	*R*
	HA335 (*Pl_6*)	40	25	25	
	HA 89*	40	30	30	
11-261-4	HA89//(NMS HA89 × *H. argophyllus* PI 494573) BC_2F_3	50	30		30
11-261-8		50	21		21
11-261-14		40	20		20
11-261-15		50	24		24
11-261-16		50	30		30
11-264-1		50	30		30
11-264-2		50	30		30
11-264-4		50	25		25

S susceptible, *R* resistant

* Susceptible recurrent parent

Table 3 Downy mildew resistant tests of homozygous BC_2F_3 with four downy mildew races

Line	No. seedlings tested	DM spore		DM score	
		Race	ID#	*S*	*R*
Cargill 272	12	730	16	12	
HA 89	9			9	
RHA 340	16				16
11-261-16/BC_2F_3	20				20
Cargill 272	9	734	117	9	
HA 89	10			10	
RHA 340	22				22
11-261-16/BC_2F_3	20				20
Cargill 272	10	734	120	10	
HA 89	12			12	
RHA 340	19				19
11-261-16/BC_2F_3	26				26
Cargill 272	12	770	58	12	
HA 89	8			8	
RHA 340	19				17
11-261-16/BC_2F_3	22				10
Cargill 272	11	774	41	11	
HA 89	9			9	
RHA 340	19				19
11-261-16/BC_2F_3	22				22
Cargill 272	5	774	46	5	
HA 89	9			9	
RHA 340	33				33
11-261-16/BC_2F_3	36				36
Cargill 272	8	774	47	8	
HA 89	12			12	
RHA 340	25				25
11-261-16/BC_2F_3	20				20
Cargill 272	12	774	130	12	
HA 89	11			11	
RHA 340	20				20
11-261-16/BC_2F_3	13				13
Cargill 272	12	774	131	12	
HA 89	12			12	
RHA 340	23				23
11-261-16/BC_2F_3	17				17

Cargill 272 and RHA 340 susceptible and resistant checks, *HA 89* susceptible recurrent parent, *11-261-16* homozygous BC_2F_3 family, *S* susceptible, *R* resistant

Genetic mapping of the downy mildew resistance gene

Thirty seedlings each of the 142 BC_1F_3 families were inoculated with *P. halstedii* race 734. These families were categorized as 34 resistant, 66 segregating, and 42 susceptible according to the disease evaluation data. On the basis of a Chi-square contingency test, the observed ratio of 34:66:42 did not significantly differ from the 1:2:1 ratio ($\chi^2 = 1.06$, $df = 2$, $P = 0.59$), supporting segregation of a single locus. Thus, resistance to race 734 in the mapping population was conferred by a single gene.

A set of 869 SSR markers covering the sunflower genome were used to screen the parents, HA 89 and PI 494573. Of these, 427 (49.14 %) SSR primers that were distributed across 17 linkage groups amplified polymorphic fragments between the two parents, with the number ranging from 15 for LG12 to 38 for LG3. Bulked segregant analysis was conducted in S- and R-bulks with 281 SSR markers polymorphic between the parents. The results revealed that seven SSR markers, ORS127, ORS203, ORS963, ORS1011, ORS1073, ORS1211, and CRT214, from LG2 were positive for BSA in the S- and R-bulks. These SSRs, along with 20 additional polymorphic SSR markers from LG2, were selected to genotype the 142 BC_1F_2 individuals to confirm marker–trait association.

The resulting genetic map was 4.4 cM in length, including 19 SSR loci (12 dominant, 7 co-dominant) and one *Pl* gene, with an average genetic distance of 0.22 cM per locus (Fig. 1a). No segregation distortion was observed in the population, and all markers were segregated as expected, with a 1:2:1 (co-dominant markers) or 3:1 (dominant markers) ratio. The resistance gene was placed in an interval of 1.5 cM and flanked by SSR loci ORS203 0.4 cM proximal to the gene and CRT214 1.1 cM distal to the gene, respectively. Both ORS203 and CRT214 are co-dominant markers. As no other *Pl* gene has been mapped to LG2, the *Pl* gene introgressed from *H. argophyllus* PI 494573 is a novel gene and designated as Pl_{18}.

Marker enrichment

To identify additional markers in the Pl_{18} region, a total of 46 SNP markers were selected from two SNP genetic maps based on the position of common SSR markers (Bowers et al. 2012; Talukder et al. 2014). The 25 NSA and 21 SFW SNPs covered a region of 1.1 and 4.8 cM on the original SNP maps, respectively (Table S1 and S2). Genotyping of 25 NSA SNPs in the BC_1F_2 population and their parents was conducted by BioDiagnostics, Inc. (River Falls, WI, USA). Four SNPs, NSA_004665, NSA_001247, NSA_005362, and NSA_003261, were placed on the linkage map covering the Pl_{18} region, and two SNPs, NSA_005362, and NSA_003261, were 0.9 cM proximal to Pl_{18} (Fig. 1b).

Twenty-one SFW SNPs were first screened between the two parents, HA 89 and PI 494573. Ten were polymorphic and subsequently used to genotype the BC_1F_2 population. No SNP markers were placed on the interval between CRT214 and ORS203, which flanked Pl_{18}. However, six co-segregating SFW SNP markers were 0.1 cM distal to

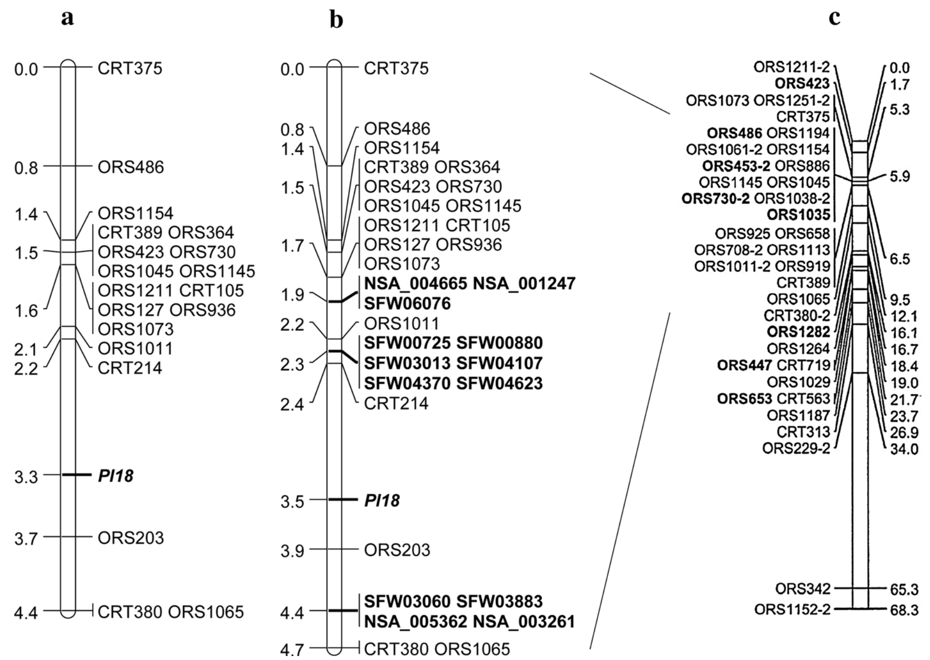

Fig. 1 Genetic maps of linkage group (LG) 2. **a** LG2 SSR map of Pl_{18}, **b** LG2 combined map of Pl_{18}, **c** LG2 public SSR map (Tang et al. 2003)

CRT214 and 1.2 cM distal to Pl_{18}, and another two co-segregating SFW SNP markers were 0.9 cM proximal to Pl_{18}. These SNP markers delineated Pl_{18} to an interval of 2.1 cM compared to 1.5 cM interval of two SSR markers flanking Pl_{18} (Fig. 2b).

Comparison of the physical position of Pl_{18} and the rust resistance gene R_5 in LG2

Different from many previously mapped downy mildew and rust *R* genes that are clustered in LGs 1, 8, and 13 of the sunflower genome, Pl_{18} and R_5 are the only downy mildew and rust *R* gene mapped to LG2. The rust *R* gene R_5 also originated from *H. argophyllus* (Qi et al. 2012). To compare their physical position, the sequences of 10 SNPs surrounding Pl_{18} and three SNPs flanking R_5 (Qi et al. 2015c) were aligned against the sunflower whole genome sequence of HA412 v1.1.bronze.20141015 pseudomolecules available at http://sunflowergenome.org/early_access/repository/main/pseudomolecules/.

Of the 10 SNPs associated with Pl_{18}, two, NSA_003261 and NSA_005362, aligned to LG5, and another eight aligned to LG2, spanning a physical length of 38.3 Mb at the position of 99.0–137.3 Mb (Table 4). Three SNPs flanking R_5 all aligned to LG2 at a region of 166.8–178.7 Mb. The R_5 location on LG2 was approximately 37.8 Mb distant from the Pl_{18} region, indicating that the two genes are not in a cluster. Six co-segregating SNP markers 1.2 cM distal to Pl_{18} spanned a physical length of 30.0 Mb, whereas the two co-segregating SNPs 0.9 cM proximal to Pl_{18} spanned a physical length of 8.3 Mb. The two SNPs, SFW003013

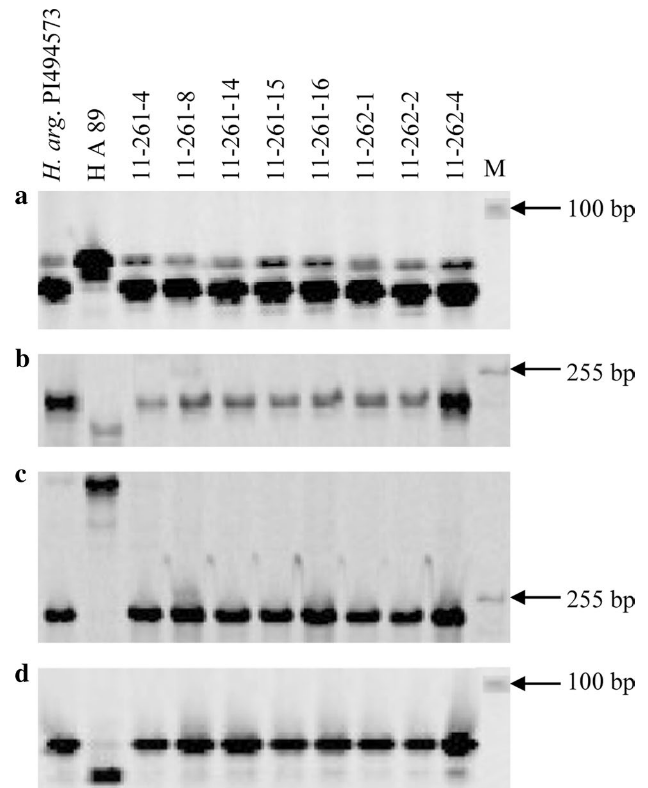

Fig. 2 PCR amplification of SSR and SNP primers linked to Pl_{18} in two parental lines (*H. arg*. PI 494573 and HA 89) and eight homozygous BC_2F_3 families (11-261-4 to 11-262-4). SNP markers SFW04370 (**a**) and SFW03883 (**d**); SSR markers CRT214 (**b**) and ORS203 (**c**). All BC_2F_3 families show *H. arg*. PI 494573 allele. The molecular weight marker *M* is an IRDye-labeled DNA ladder (LI-COR)

Table 4 Comparison of physical positions between the downy mildew *R* gene Pl_{18} and the rust *R* gene R_5 on linkage group 2

Genetic position (cM)	Query					Target			
	Name	Size (bp)	Start (bp)	End (bp)	Stain	Name	Size (bp)	Start (bp)	End (bp)
2.3	SFW00880	120	0	120	+	Ha2	209013747	98999420	98999671
2.3	SFW04370	120	0	120	+	Ha2	209013747	116294250	116294372
2.3	SFW04107	120	0	120	+	Ha2	209013747	119737479	119737599
2.3	SFW04623	120	0	120	−	Ha2	209013747	122163167	122163287
2.3	SFW00725	120	0	120	−	Ha2	209013747	123366663	123366783
2.3	SFW03013	85	0	85	−	Ha2	209013747	128982062	128982147
3.5	Pl_{18}								
4.4	SFW03060	120	0	120	−	Ha2	209013747	129082451	129082571
4.4	SFW03883	97	0	97	+	Ha2	209013747	137321748	137321845
14.4*	NSA_001605	440	0	440	+	Ha2	209013747	166787192	166787632
15.1*	R_5								
16.1*	NSA_000267	293	0	293	+	Ha2	209013747	171796564	171796857
14.9*	SFW03654	120	0	120	−	Ha2	209013747	178691912	178692033

* Map position was taken from Qi et al. (2015a)

and SFW03060, closest to the downy mildew *R* gene delimited Pl_{18} at an interval of 0.1 Mb with a recombination rate of 0.05 Mb/cM (Table 4).

Marker validation in the selected homozygous resistant BC_2F_3 families

Eight homozygous resistant BC_2F_3 families that were selected from downy mildew tests and their parental lines HA 89 and PI 494573 were screened with the 10 DNA markers (two SSRs and eight SNPs) linked to Pl_{18} developed in this study. All of the BC_2F_3 families consistently showed the PI 494573 alleles at these marker loci, indicating association of these markers with the downy mildew resistance trait introgressed from *H. argophyllus* (Table S3; Fig. 2).

Discussion

Downy mildew resistance derived from the wild species *H. argophyllus* accession PI 494573 has been successfully introgressed into cultivated sunflower in the present study. Genetic studies confirmed that the resistance was controlled by a single gene, Pl_{18}, which was mapped to LG2 of the sunflower genome. Both SSR and SNP markers identified closely linked to Pl_{18} delineated this gene to an interval of 1.5 cM. Homozygous resistant BC_2F_3 families derived from the cross between HA 89 and *H. argophyllus* were selected through a downy mildew screening and marker validation. As a result, the BC_2F_3-derived germplasm line HA-DM1 was developed and released to the public in 2015. HA-DM1 is also resistant to all *P. halstedii* races identified in the USA to date (Gilley et al. 2015). This new germplasm will provide breeders with an effective new source of resistance against *P. halstedii* in sunflower. The DNA markers that we developed will be especially useful in marker-assisted selection and pyramiding of downy mildew resistance genes because of their close proximity to the gene and the availability of efficient SNP marker detection assays.

Thirteen *Pl* genes were previously mapped to the sunflower genome, four (Pl_{13}, Pl_{14}, Pl_{16}, and Pl_{ARG}) in LG1 (Dußle et al. 2004; Mulpuri et al. 2009; Wieckhorst et al. 2010; Bachlava et al. 2011; Liu et al. 2012), one (Pl_{17}) in LG4 (Qi et al. 2015b), five in LG8 (Pl_1, Pl_2, Pl_6, Pl_7, and Pl_{15}) (Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Brahm et al. 2000; Gedil et al. 2001; Bouzidi et al. 2002; Slabaugh et al. 2003; Radwan et al. 2008; de Romano et al. 2010; Franchel et al. 2013), and three in LG13 (Pl_5, Pl_8, Pl_{21}) (Bert et al. 2001; Radwan et al. 2003, 2004; Bachlava et al. 2011; Vincourt et al. 2012). Pl_{18} is the only *Pl* gene mapped to LG2. The origins of these *Pl* genes were mainly traced to wild *H. annuus* and also to other *Helianthus* species, such as *H. argophyllus*, *H. praecox*, and *H. tuberosus* (Vear et al. 2008). In contrast to the *Pl* genes that clustered on LGs 1, 8, and 13, three *Pl* genes, Pl_{Arg}, Pl_{18}, and Pl_8, derived from *H. argophyllus* are located on three different LGs: 1, 2, and 13. Pl_{Arg} in LG1 is far from a *Pl* gene cluster (Pl_{13}, Pl_{14}, and Pl_{16}) in this linkage group. Pl_8 was reported to be in a gene cluster on LG13, with Pl_5 and Pl_{21}, whereas Pl_{18} is, so far, the only gene located on LG2. The sunflower germplasms carrying Pl_8 (RHA 340) and Pl_{Arg} (Arg1575-2) were released in 1988 and 1991, respectively (Miller and

Gulya 1988; Seiler 1991). The gene Pl_{Arg} still effectively confers resistance to all of the *P. halstedii* races identified so far in the USA and France, and Pl_8 confers resistance to 98 % of the *P. halstedii* isolates (Gulya et al. 2011; Gascuel et al. 2014; Gilley et al. 2015). However, newly emerged races of *P. halstedii* in North America and France have overcome several DM resistance genes widely used in sunflower, such as Pl_6 and Pl_7 derived from *H. annuus* and *H. praecox*, respectively, although they were released at the same time as Pl_8 (Gulya et al. 2011; Gascuel et al. 2014). *P. halstedii* populations are very dynamic and continually change virulence structure (Viranyi et al. 2015), therefore, continual search and introgression of the new downy mildew resistance genes from *H. argophyllus* and other wild species are underway.

Despite the diverse *Pl* genes discovered in *H. argophyllus*, this species has a fairly limited geographic distribution in the USA. Out of 51 *H. argophyllus* accessions that have been deposited in the Germplasm Resources Information Network (GRIN), 40 were collected in the USA, including 37 in Texas, where they are native to the sandy soils of the southern Texas coastal plain; one in Florida; and two in North Carolina (http://www.ars-grin.gov/cgi-bin/npgs/html/tax_search.pl; Rogers et al. 1982). The resistant donors of Pl_8 and Pl_{18} (PI 435629 and PI 494573, respectively) were all collected in Texas. Although the resistant donor of Pl_{Arg} (PI 468648) originated from Florida, it is believed that the accession likely escaped from cultivation (Rogers et al. 1982). It was also reported that downy mildew resistance is most frequent in wild *H. annuus* originating in south central USA, specifically in Texas (Gulya 2005). These raise two interesting questions: how do host plants acquire different resistance genes in a narrowed geographic area, and how does the host–pathogen co-evolve in natural populations. Unfortunately, no downy mildew pathogen samples have been collected from Texas, which would be an interesting topic for future investigation (Gulya 2007; Gulya et al. 2011).

In the initial SSR mapping, two parents, HA 89 and *H. argophyllus* PI 494573, were screened with 55 SSR markers that covered the entire LG2 from 869 selected SSRs; however, polymorphic markers were only found in a certain region. The LG2 genetic map constructed in the present study consisted of 32 marker loci (19 SSRs and 13 SNPs) with the Pl_{18} gene spanning a genetic distance of 4.7 cM, with an average density of 0.14 cM per locus. This region represents a linkage block of the introgressed segment from *H. argophyllus* in LG2 and is highly polymorphic. Suppressed recombination was observed between markers CRT375 and CRT214 distal to Pl_{18}, where 24 markers spanned a genetic distance of 2.4 cM, with an average density of 0.1 cM per locus compared to the mean density of 0.28 cM per locus in the sunflower SNP map (Talukder et al. 2014). This suppressed recombination may not be relative to the introgressed alien segment because the same suppressed recombination in this region was observed in the public SSR map developed from a recombinant inbred line population derived from the cross of two inbred lines RHA 208 and RHA 801, where 19 markers cluster in a region between 5.3 and 6.5 cM, with an average of 0.06 cM per locus (Fig. 1c; Tang et al. 2003). The LG2 chromosome is a metacentric/submetacentric chromosome (Feng et al. 2013). The sunflower genome sequence assembled on LG2 is approximately 209.0 Mb in length (Table 4), while six SNPs that are 0.1 cM distal to CRT214 are located at a position between 99.0 and 129.0 Mb (Table 4), indicating that the region between CRT375 and CRT214 with suppressed recombination in LG2 is likely close to the centromere. It is known that the specific centromeric structure and associated heterochromatin suppresses recombination since it has also been observed in other crops, such as wheat, barley, maize, and tomato (Tanksley et al. 1992; Gill et al. 1996a, b; Faris et al. 2000; Künzel et al. 2000; Anderson et al. 2003; The Tomato Genome Consortium 2012).

In contrast to the above suppressed recombination region, the region covering Pl_{18} seems to have a higher level of recombination, with an average density of 0.5 cM per locus between CRT214 and ORS203; two SSRs flanking Pl_{18} (Fig. 1a); and an almost one-time increase in recombination rate compared to the mean density of 0.28 cM per locus at a genome level (Talukder et al. 2014). Furthermore, the two SNP markers, SFW03013 and SFW03060, physically delimited Pl_{18} to an interval of 0.1 Mb with a recombination rate of 0.05 Mb/cM compared to 2.4 Mb/cM at the whole genome level (Qi et al. 2015b), indicating that it is a recombination hot spot. This will allow for a possible recombination between the marker and Pl_{18} and will reduce linkage drag that often accompanies genes derived from wild species (Young and Tanksley 1989) when transferring Pl_{18} into an elite line by backcrossing and marker-assisted selection. In addition, Pl_{18} is located in a region approximately 37.8 Mb away from a rust *R* gene, R_5, in LG2 (Table 4). It is also possible to recombine these two genes into a single line at the coupling phase, providing resistance to both downy mildew and rust in sunflower production. Flanking markers are very useful in constructing compound genes, which are now available for both Pl_{18} and R_5 (Qi et al. 2012, 2015c) and will facilitate this breeding approach.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments were performed in compliance with the current laws of the USA.

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