Theor Appl Genet (2017) 130:1519–1529
DOI 10.1007/s00122-017-2906-4

ORIGINAL ARTICLE

Genotyping-by-sequencing targeting of a novel downy mildew resistance gene Pl_{20} from wild *Helianthus argophyllus* for sunflower (*Helianthus annuus* L.)

G. J. Ma[1] · S. G. Markell[1] · Q. J. Song[2] · L. L. Qi[3]

Received: 28 February 2017 / Accepted: 7 April 2017 / Published online: 21 April 2017

Abstract

Key message **Genotyping-by-sequencing revealed a new downy mildew resistance gene, Pl_{20}, from wild *Helianthus argophyllus* located on linkage group 8 of the sunflower genome and closely linked to SNP markers that facilitate the marker-assisted selection of resistance genes.**

Abstract Downy mildew (DM), caused by *Plasmopara halstedii*, is one of the most devastating and yield-limiting diseases of sunflower. Downy mildew resistance identified in wild *Helianthus argophyllus* accession PI 494578 was determined to be effective against the predominant and virulent races of *P. halstedii* occurring in the United States. The evaluation of 114 $BC_1F_{2:3}$ families derived from the cross between HA 89 and PI 494578 against *P. halstedii* race 734 revealed that single dominant gene controls downy mildew resistance in the population. Genotyping-by-sequencing analysis conducted in the BC_1F_2 population indicated that the DM resistance gene derived from wild *H. argophyllus* PI 494578 is located on the upper end of the linkage group (LG) 8 of the sunflower genome, as was determined single nucleotide polymorphism (SNP) markers associated with DM resistance. Analysis of 11 additional SNP markers previously mapped to this region revealed that the resistance gene, named Pl_{20}, co-segregated with four markers, SFW02745, SFW09076, S8_11272025, and S8_11272046, and is flanked by SFW04358 and S8_100385559 at an interval of 1.8 cM. The newly discovered *P. halstedii* resistance gene has been introgressed from wild species into cultivated sunflower to provide a novel gene with DM resistance. The homozygous resistant individuals were selected from BC_2F_2 progenies with the use of markers linked to the Pl_{20} gene, and these lines should benefit the sunflower community for *Helianthus* improvement.

Communicated by Volker Hah.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-017-2906-4) contains supplementary material, which is available to authorized users.

✉ L. L. Qi
lili.qi@ars.usda.gov

[1] Department of Plant Pathology, North Dakota State University, Fargo, ND 58108, USA

[2] Soybean Genomics and Improvement Lab, USDA-Agricultural Research Service, Beltsville, MD 20705-2350, USA

[3] Red River Valley Agricultural Research Center, USDA-Agricultural Research Service, 1605 Albrecht Blvd N, Fargo, ND 58102-2765, USA

Introduction

Cultivated sunflower (*Helianthus annuus* L.), native to temperate North America, is one of the major global oilseed crops. Most currently used sunflower hybrids were derived from a germplasm pool intensely selected during the nineteenth century for high oil content (Baute et al. 2015). This effort narrowed the genetic base of cultivated sunflower against other biotic and/or abiotic stresses. However, the genus *Helianthus* comprises 14 annual and 39 perennial species (Schilling 2006; Stebbins et al. 2013), among which wild sunflower species have long been a valuable resource of genetic variability (for review, see Seiler et al. 2017). It has been reported that wild sunflower species harbor resistance (*R*) genes against downy mildew, rust, Verticillium wilt, Phomopsis stem canker, Phoma black stem, and diseases caused by *Sclerotinia sclerotiorum* (Gulya 2005; Seiler 2010). The identification of disease resistance

genes from the crop wild relatives and their incorporation into cultivated sunflower have resulted in an effective strategy for providing durable sunflower disease resistance.

Sunflower downy mildew (DM), caused by pathogen *Plasmopara halstedii* (Farl.) Berl. et de Toni, occurs in all sunflower-growing regions throughout the world, except for Australia and New Zealand (http://www.cabi.org/isc/datasheet/41911; Constantinescu and Thines 2010). The pathogen can overwinter for many years as a thick-walled oospore in the soil. Under favorable environmental conditions, oospores produce zoosporangia, which release motile zoospores that infect germinating seedlings and cause a systemic infection that kills most infected plants (Humann et al. 2016; Markell et al. 2016). If infected seedlings survive the vegetative stages they will remain stunted and produce erect heads with few or no seeds. In wet and cool years, not only will infections reduce sunflower yield, but also affect the seed quality (Molinero-Ruiz et al. 2003). Management tools for DM include the destruction of volunteer hosts, fungicides applied as seed treatments, and planting DM-resistant hybrids; the latter being the most economically and environmentally-friendly tool (Humann et al. 2016).

The control of DM resistance in sunflower through dominant major genes, designated *Pl*, was first demonstrated in the early 1970s (Vrânceanu and Stoenescu 1970; Zimmer and Kinman 1972). The DM resistance in sunflower and DM virulence in pathogens are consistent with the gene-for-gene hypothesis (Flor 1955), contributing to the use of DM race-specific resistance as the main breeding strategy against downy mildew disease in sunflower. More than twenty *Pl* genes have currently been reported in cultivated and wild sunflowers, i.e., Pl_1–Pl_{19}, Pl_{21}, and Pl_{Arg} (Table S1, Zimmer and Kinman 1972; Miller and Gulya 1991; Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997, 2008; Bert et al. 2001; Rahim et al. 2002; Molinero-Ruiz et al. 2003; Radwan et al. 2003; Vear et al. 2008; Mulpuri et al. 2009; de Romano et al. 2010; Bachlava et al. 2011; Liu et al. 2012; Qi et al. 2015, 2016a; Zhang et al. 2017). Among these resistance genes, Pl_{13}, Pl_{14}, Pl_{16}, and Pl_{Arg} are localized on linkage group (LG) 1 of the sunflower genome; Pl_1, Pl_2, Pl_6, Pl_7, and Pl_{15} are localized on LG8; Pl_5, Pl_8 and Pl_{21} are localized on LG13; Pl_{17} and Pl_{19} are localized on LG4; and Pl_{18} is localized on LG2. Most of these *Pl* genes were derived from wild *H. annuus* species, while some genes were derived from other wild *Helianthus* species (Vear et al. 2008). Pl_5 originated from *H. tuberosus* L. (Vrânceanu et al. 1981), whereas Pl_7 originated from *H. praecox* Englem. and Gray (Miller and Gulya 1991), and Pl_8, Pl_{Arg}, and Pl_{18} were derived from *H. argophyllus* Torrey and Gray (Miller and Gulya 1988; Seiler 1991; Qi et al. 2016a).

However, new *P. halstedii* races frequently emerge and can render deployed resistance genes ineffective (Markell et al. 2016). To date, at least 24 *P. halstedii* races have been identified in Europe and 40 *P. halstedii* races have been identified in North America (Gulya et al. 2011; Gascuel et al. 2015; Viranyi et al. 2015), and virulence to many of the resistance genes deployed exists widely in sunflower production regions (Markell et al. 2016). The USDA-released inbred lines carrying Pl_6 and Pl_7 (Miller and Gulya 1988, 1991) have been extensively used to develop DM-resistant hybrids in sunflower production worldwide. In 2000, virulence to the widely-used resistance gene Pl_6 gene (race 304) was first identified in France (de Labrouhe et al. 2000; Viranyi et al. 2015). Between 2000 and 2008, six more races conferring virulence to Pl_6 were identified in France, namely races 307, 314, 334, 704, 707, and 714 (Ahmed et al. 2012). The first race conferring virulence to Pl_6 in the United States (U.S.) was reported in 2009 (race 734), and by 2010, five *P. halstedii* races (races 314, 704, 714, 734, and 774) conferring virulence to both Pl_6 and Pl_7 were identified in the country (Gulya et al. 2010, 2011). These newly identified races of *P. halstedii* pose a serious threat to sunflower production, and substantial economic loss is possible when commercial lines possess only the Pl_6 or Pl_7 single DM resistance gene. In 2014–2015, Gilley et al. (2016) tested 185 *P. halstedii* isolates collected from the U.S. sunflower production region of North Dakota, South Dakota, Minnesota, and Nebraska against 16 sunflower lines carrying 11 downy mildew *R*-genes; Pl_1, Pl_2, Pl_5, Pl_6, Pl_{13}, Pl_{15}-Pl_{18}, Pl_{21}, and Pl_{Arg}. Only lines carrying Pl_{Arg}, Pl_{15}, Pl_{17}, Pl_{18} genes, and the lines RHA 468 and TX 16R [with an unknown gene(s)] were resistant to all isolates tested. With few effective resistance genes remaining, expanding and diversifying the DM resistance gene pool is needed to protect cultivated sunflower with DM resistance, thereby solidifying the basis of DM resistance breeding efforts.

Helianthus argophyllus ($2n = 2x = 34$), a wild diploid annual species, primarily occurs in beach environments or sandy habitats in Texas, U.S. (Rogers et al. 1982; Gulya 2005). This species is an important donor of different disease resistance genes in sunflower, such as rust resistance genes R_{adv} and R_5 (Bachlava et al. 2011; Qi et al. 2012), DM resistance genes Pl_8, Pl_{Arg}, and Pl_{18} (Miller and Gulya 1991; Qi et al. 2016a), and *Sclerotinia* resistance (Qi et al. 2016b). In an investigation of the DM resistance of *H. argophyllus*, Gulya (2005) inoculated 13 *H. argophyllus* accessions with a mixture of *P. halstedii* races with an aggregate pathogen phenotype of race 777. Of the 13 *H. argophyllus* accessions, six including the accession PI 494578 were highly resistant to DM, in which more than 90% of the tested individuals showed resistance.

Genotyping-by-sequencing (GBS) was designed and performed based on next-generation sequencing (NGS) for the efficient genotyping of a large number of samples. The simultaneous discovery and genotyping of thousands of SNPs can be achieved at 96- to 384-plex levels per flow channel on NGS sequencers, such as the Illumina HiSeq instrument. The genomic regions with low copy number are targeted and enriched using methylation-sensitive restriction enzyme(s) that efficiently reduce the genome complexity (Elshire et al. 2011). As an efficient and cost-effective method, GBS technology generates an enormous number of SNP markers, making it feasible to tag gene(s) from the genome in a timely manner. Here, we report the incorporation of a new DM resistance gene, Pl_{20}, from wild *H. argophyllus* accession PI 494578 into cultivated sunflower and the molecular mapping of this novel gene to LG8 of the sunflower genome using a genotyping-by-sequencing approach.

Materials and methods

Plant materials and mapping population

H. argophyllus accession PI 494578 was collected at Premont, Texas, U.S. in 1984 (GRIN 2017). This accession was later characterized as highly resistant to DM with approximately 97% of tested plants showing resistance to a mixture of *P. halstedii* races (aggregate phenotyping of *P. halstedii* race 777) (Gulya 2005). The high oil Russian cultivar VNIMK 8931 (PI 262517) was a parent of HA 89 (PI 599773) susceptible to DM infection, that was released by the USDA and Texas Agricultural Experiment Station in 1971. The nuclear male sterile (NMS) HA 89 was produced through the chemically induced mutation of HA 89 using streptomycin, and this line possesses a single recessive nuclear male-sterile gene, *ms9* (Jan and Rutger 1988; Chen et al. 2006). Cargill 270, a sunflower hybrid developed by the Cargill Company (Minneapolis, MN, USA), was used as susceptible control, and HA-DM1 (harboring DM *R*-gene Pl_{18}) (Qi et al. 2016a) was used as a resistant control in the downy mildew resistance tests of the BC_1F_3 of NMS HA 89 × *H. argophyllus* PI 494578.

The initial cross was made between NMS HA 89 and *H. argophyllus* PI 494578 in 2013. The open-pollinated and highly heterozygous nature of *H. argophyllus* made it necessary to test DM reaction of F_1 hybrids. After screening against *P. halstedii* race 734, the resistant F_1 individuals were selected, and the pollen was mixed to pollinate HA 89 and generate the BC_1 generation. Similarly, DM-resistant BC_1 individuals were selected to perform another round of crosses with HA 89 to generate the BC_2 generation. The selected resistant BC_2F_1 plants were further advanced to BC_2F_2. Homozygous resistant introgression lines were selected from the BC_2F_2 population and advanced to BC_2F_3.

The BC_1F_2 population used for mapping the DM resistance gene from PI 494578 was developed from two resistant BC_1F_1 plants. A total of 207 BC_1F_2 plants were grown in the greenhouse in 2014, and 118 $BC_1F_{2:3}$ plants were harvested; 114 of these plants had sufficient seeds for further phenotypic analysis.

Downy mildew inoculation and phenotyping

The whole seedling immersion method according to Gulya et al. (1991a) and Qi et al. (2015) was used to test the reaction to downy mildew for sunflower seedlings. *P. halstedii* race 734, which overcomes the Pl_6 (residing in HA 335) and Pl_7 (residing in HA 337) resistance genes, was used to test the F_1 to BC_2F_2 generations (Gulya et al. 2011). Further, six *P. halstedii* races, 314, 700, 710, 714, 734, and 774, were used to test the seedlings of the homozygous resistant BC_1F_3 families. The *P. halstedii* races 314, 700, 710, 714, and 774 were the most prevalent races observed in 2014 and 2015 in North and South Dakota, U.S. (Gilley et al. 2016). Sunflower lines Cargill 270 and HA 89 were used as susceptible controls, and HA-DM1 was used as a resistant control. A plant was considered susceptible (*S*) if sporulation was observed on cotyledons and true leaves and resistant (R) if no sporulation was observed.

For DM tests of the BC_1F_3 mapping population, 40 seeds from each of the 114 $BC_1F_{2:3}$ families were germinated at 22–24 °C in a germinator, and at least 30 seedlings were inoculated with isolate of *P. halstedii* race 734 in October, 2015. 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.

To select homozygous BC_2F_2 plants, 300 BC_2F_2 seedlings from the cross of NMS HA 89/PI 494578 were inoculated with *P. halstedii* race 734 on May, 2016. Ten days after inoculation, 191 resistant seedlings were identified, and 186 plants were transferred into 36-cell plastic flats (one seed per cell measuring 4.6 × 5.4 cm) for the selection of homozygous resistant plants using the SNP markers developed in the present study.

DNA extraction and genotyping-by-sequencing (GBS) analysis

Leaf tissue was collected from the parental lines, HA 89 and PI 494578, and 114 BC_1F_2 plants. Tissue samples were also collected from DM resistant BC_2F_2 plants of NMS HA

89 × PI 494578. Genomic DNA was isolated from the lyophilized tissues using the DNeasy 96 plant kit according to 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).

After approval for DNA quality, 50 µl of genomic DNA from each of the 114 BC_1F_2 and two parental lines was submitted to the Biotechnology Resource Center (BRC) Genomic Diversity Facility at Cornell University for genotyping-by-sequencing according to Elshire et al. (2011, http://www.biotech.cornell.edu/brc/genomic-diversity-facility/services). The GBS library was constructed according to Elshire et al. (2011), with some modifications. Briefly, at the 96-plex level, the genomic DNA was digested with the restriction enzyme *Eco*T221, a six base cutter (ATGCAT), followed by ligation with a barcode adaptor and a common Illumina sequencing adaptor. After PCR amplification, the library was sequenced on the Illumina HiSeq 2000/2500 (100 bp, single-end reads). The FASTQ files from sequencing were pipeline-analyzed with the use of TASSEL-GBS discovery pipeline version 3.0.166 (http://tassel.bitbucket.org/TasselArchived.html) (Bradbury et al. 2007) to screen the barcoded (tagged) sequence reads of good quality. These sequence reads were aligned using the Burrow–Wheelers Alignment tool (BWA) version 0.7.8-r455 (Li and Durbin 2009) to the sunflower reference genome HA412.v1.0.bronze.20140814.fasta.gz (http://sunflowergenome.org/early_access/repository/main/pseudomolecules/). The TASSEL-GBS quantitative SNP caller was used for SNP calling after conversion into a 'TagsOnPhysicalMap' (TOPM) file. Identical SNPs were merged using the 'MergeDuplicateSNPs', and SNPs monomorphic between the two parents were removed. The sequences flanking SNP sites of LG8 were extracted for further use (Table S2). The chromosomes were renamed in a manner compatible with the GBS pipeline, where S1–S17 represents linkage groups 1–17 of the sunflower genome, and S18 represents those that were not assigned to any specific linkage group. The SNPs were named using a prefix of S1–S17 followed by a number representing the physical position of the SNP on the genome.

Linkage mapping

Software JoinMap 4.1 was used to perform linkage analysis (Van Ooijen 2006). The Chi square test was used to assess goodness-of-fit to the expected 1:2:1 segregation ratio of the markers using the 'locus genotype frequencies' feature of the JoinMap. Linkage maps were constructed using a regression mapping algorithm and the Kosambi mapping function (Van Ooijen 2006). A minimum likelihood of odds (LOD) ≥3.0 and a maximum distance of ≤50 centimorgans (cM) were used to test linkage among markers. The SNP markers resulting from GBS analysis were, respectively, classified into 17 LGs, and markers belonging to each of the 17 LGs were separately analyzed using DM phenotypic data to detect linkage.

Map saturation and conversion of SNP into PCR-based length polymorphism marker

To saturate the DM *R*-gene region, a total of 59 SNP markers potentially surrounding the region on LG8 were selected upon comparison with publically available genetic maps. Twelve SNPs were selected from the SNP map developed by the National Sunflower Association SNP Consortium (SNP prefixed with NSA, Table S3, Talukder et al. 2014), and 47 SNPs were selected from the published SNP map (SNP prefixed with SFW, Table S3, Bowers et al. 2012). Additionally, four SSR markers, ORS166, ORS185, ORS830, and CRT169, linked to the DM *R*-gene cluster in LG8 were included in the present study (Slabaugh et al. 2003). Four GBS generated SNPs flanking the novel DM *R*-gene from *H. argophyllus* PI 494578 and 59 selected SNPs were converted into PCR-based length polymorphism markers according to Long et al. (2016), with some modifications. Briefly, three primers were designed for each SNP, two-tailed forward allele-specific primers (AS-primer, F1 and F2) and a common reverse primer (Table 1). Two alternate permutations of artificial mismatches were introduced at the 3rd and 4th bases from the 3′ end in either of the AS-primers according to A/T → C, G → A, and C → T. An additional 5-base oligonucleotide (5′-ATGAC-3′) was inserted between the tail and allele-specific sequences of AS-primer F2 to generate length polymorphism between two alleles during amplification. A fluorescence labeled universal priming-element-adjustable primer (PEA-primer 5′-GCAACAGGAACCAGCTATGAC-3′) was applied in each polymerase chain reaction (PCR). The PCR amplifications were performed in a 15-µl reaction volume containing 1 × PCR buffer, 0.8 M betaine, 0.04% BSA, 2 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM universal fluorescence-labeled PEA-primer, 0.2 µM common reverse primer, 0.04 µM each of AS-primers (F1 and F2), 1 U of Taq polymerase (Bioline, Randolph, MA, USA), and 10–20 ng of genomic DNA. The PCR amplifications were performed using a two-step protocol under the following conditions: initial denaturation of the template DNA at 94 °C for 3 min, 6 cycles at 94 °C for 20 s, and annealing at 55 °C for 30 s (1 °C decrease in each cycle) followed by another 33 cycles at 94 °C for 20 s and 60 °C for 30 s. The PCR products were diluted 40 times and visualized using an IR2 4200/4300 DNA Analyzer with denaturing polyacrylamide gel electrophoresis (LI-COR, Lincoln, NE, USA). The SSR markers were genotyped according to Qi et al. (2011).

Table 1 Primer sequences of the SNPs mapped in the present study

SNP name	SNP primer	Primer sequence (5′–3′)
S8_7763647	S8_7763647F1	GCAACAGGAACCAGCTATGACGCATTCATATTTTTACTCAAATACTTAAAACAAG
	S8_7763647F2	GCAACAGGAACCAGCTATGAC*ATGAC*GCATTCATATTTTTACTCAAATACTTAAAAACAC
	S8_7763647R	GAGGCCAGGAACGAGATAAGA
S8_11272025	S8_11272025F1	GCAACAGGAACCAGCTATGACCGATGAACAAGCTTTTCAAATTCATACTCA
	S8_11272025F2	GCAACAGGAACCAGCTATGAC*ATGAC*CGATGAACAAGCTTTTCAAATTCATAACCG
	S8_11272025R	TCGCTTCATCCCACTTTCTCT
S8_11272046	S8_11272046F1	GCAACAGGAACCAGCTATGACATCAACGTCTAATGAAGGAGATCAG
	S8_11272046F2	GCAACAGGAACCAGCTATGAC*ATGAC*ATCAACGTCTAATGAAGGAGACTAA
	S8_11272046R	TCGCTTCATCCCACTTTCTCT
S8_100385559	S8_100385559F1	GCAACAGGAACCAGCTATGACCAGTCGGTTTTGACACCTTTC
	S8_100385559F2	GCAACAGGAACCAGCTATGAC*ATGAC*CAGTCGGTTTTGACACCCCTA
	S8_100385559R	GCATTGGCTTGAAGAAATCA
SFW01920	SFW01920F1	GCAACAGGAACCAGCTATGACCATAAGCCTTCTCCACCTCCCG
	SFW01920F2	GCAACAGGAACCAGCTATGAC*ATGAC*CATAAGCCTTCTCCACCTTTCT
	SFW01920R	AGAAACCGGAAGAAATCCG
SFW09076	SFW09076F1	GCAACAGGAACCAGCTATGACGGTGAGATCGGGTTCGACAGT
	SFW09076F2	GCAACAGGAACCAGCTATGAC*ATGAC*GGTGAGATCGGGTTCGATGGC
	SFW09076R	TCAGCTGTGAAAGTGAAAGT
SFW04358	SFW04358F1	GCAACAGGAACCAGCTATGACCAGTCATTGCCATATGTGGTGCCTT
	SFW04358F2	GCAACAGGAACCAGCTATGAC*ATGAC*CAGTCATTGCCATATGTGGTGTTTC
	SFW04358R	ACGCGTAGTATAACCACATC
SFW02745	SFW02745F1	GCAACAGGAACCAGCTATGACCCTATGGCTGCAAGTGCTTAATT
	SFW02745F2	GCAACAGGAACCAGCTATGAC*ATGAC*CCTATGGCTGCAAGTGCTTGGTC
	SFW02745R	CCCAAATCAAAAGTCAGCTT
SFW01496	SFW01496F1	GCAACAGGAACCAGCTATGACGGGCGTTTTTGGTCGAAGAGCAA
	SFW01496F2	GCAACAGGAACCAGCTATGAC*ATGAC*GGGCGTTTTTGGTCGAAGAATAG
	SFW01496R	TCTGAATCTTCAAAACCTTCTT
SFW09117	SFW09117F1	GCAACAGGAACCAGCTATGACCAGTGTAGACATTGCTACGAAACTT
	SFW09117F2	GCAACAGGAACCAGCTATGAC*ATGAC*CAGTGTAGACATTGCTACGAAGTTC
	SFW09117R	TAACATACGGCACTAACGAG
SFW02168	SFW02168F1	GCAACAGGAACCAGCTATGACCCCAGTTGACCAAACAAATTTTATC
	SFW02168F2	GCAACAGGAACCAGCTATGAC*ATGAC*CCCAGTTGACCAAACAAATTTCGTT
	SFW02168R	AGAGATTGTGATTTGGAGAAAG
NSA_005795	NSA_005795F1	GCAACAGGAACCAGCTATGACGAGTTGTGACCGCATACGCAG
	NSA_005795F2	GCAACAGGAACCAGCTATGAC*ATGAC*GAGTTGTGACCGCATACATAA
	NSA_005795R	TTGTCGGTTAGTTTTGTTTCA

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

Results

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

DM resistance from wild sunflower species *H. argophyllus* was introgressed into cultivated oilseed sunflower through crosses and backcrosses. *H. argophyllus* PI 494578 was initially crossed with NMS HA 89 in January, 2013. The F_1 plants were segregating for DM resistance because of the open-pollinated nature of wild species. A total of 38 F_1 plants were tested for resistance against *P. halstedii* race 734. Twenty F_1 plants were resistant, and 18 plants were susceptible. The selected resistant F_1 hybrids were used as male parents in the first backcross to HA 89 to create BC_1 in November, 2013. Similarly, the selected resistant BC_1 plants from the DM tests were again used as male parents to backcross with the recurrent parent HA 89 in June, 2014. The BC_2F_1 seeds were planted in greenhouse for DM testing

with race 734, and the resistant seedlings were selected and self-pollinated for BC_2F_2 in March, 2015 for further selection of homozygously resistant plants.

Genetic mapping of downy mildew resistance from PI 494578

A total of 118 $BC_1F_{2:3}$ families were harvested in 2014, and four of these plants did not germinate well in 2015. Thirty seedlings each from the 114 $BC_1F_{2:3}$ families were inoculated with *P. halstedii* race 734 in a greenhouse in October, 2015. The DM reaction data suggested that 27 families were homozygous susceptible, 57 families were segregating, and 30 families were homozygous resistant. The observed ratio of 27:57:30 did not significantly differ from the theoretical 1:2:1 ratio ($\chi^2 = 0.16$, $df = 2$, $P = 0.9231$) according to Chi square test, suggesting that the resistance to *P. halstedii* race 734 in this mapping population was conferred by a single dominant gene.

A total of 118,577 SNP markers were generated from GBS, which were not evenly distributed among the 17 LGs of the sunflower genome (Table S4). A total of 18,504 (15.6%) SNPs were polymorphic between the two parents, HA 89 and *H. argophyllus* PI 494578. These polymorphic SNP markers were subjected to Chi square tests, and those showing significant segregation distortion from the expected 1:2:1 ratio were excluded, leaving a total of 336 SNPs, ranging from 0 SNP on LGs 6, 7, and 16 to 191 SNPs on LG8, for further linkage analysis (Table S4). The SNPs from each of the 17 LGs were subsequently analyzed using the DM phenotypic data, and the results indicated that the DM resistance from PI 494578 was associated with the SNP markers on LG8. The LG8 genetic map was constructed with 187 SNP markers encompassing 79.4 cM of genetic distance (Table S5). SNP markers were evenly distributed in LG8, except for a large marker cluster with 53 SNPs in an interval at the position of 61.9–63.9 cM. The DM *R*-gene derived from PI 494578 was located on the upper end of LG8 and co-segregated with marker S8_11272046. Since no other *Pl* gene derived from *H. argophyllus* has been mapped to LG8, the *Pl* gene introgressed from *H. argophyllus* PI 494578 is a novel DM resistance gene, designated Pl_{20}.

Saturation mapping of the Pl_{20} region

A total of 59 SNP and four SSR markers most likely in the Pl_{20} gene region on LG8 were screened for polymorphisms between the two parents, HA 89 and PI 494578 (Table S3). Fifteen SNPs and three SSRs were polymorphic, respectively. In addition, we converted four GBS SNPs, S8_7763647, S8_11272025, S8_11272046, and S8_100385559, flanking Pl_{20} into PCR-based length polymorphism markers (Table 1). All the tested markers produced co-dominant polymorphic amplicons between the parents, except for one NSA SNP marker, NSA_005795, which produced a dominant amplicon in HA 89 rather than in PI 494578. The BC_1F_2 population derived from HA 89 and PI 494578 was used for genotyping with these polymorphic markers and the PCR-based GBS SNPs. Eight SNPs were mapped to a region surrounding the Pl_{20} gene region, whereas none of the SSR markers was mapped in this population. The segregation of PCR-based GBS SNPs in the population was consistent with the GBS genotyping data, except for one BC_1F_2 individual (14-207a-18) differing between PCR-based and GBS genotype for marker S8_100385559 (Fig. 1). As expected, the Pl_{20} gene co-segregated with four markers, SFW02745, SFW09076, S8_11272025, and S8_11272046, and was placed in an interval of 1.8 cM, with 0.1 cM distance from marker SFW04358, and 1.7 cM distance from S8_100385559 (Fig. 2).

Physical location of Pl_{20} and DM *R*-gene cluster on LG8

The Pl_{20} gene is the sixth DM *R*-gene mapped on LG8, close to the DM *R*-gene cluster on this LG, which includes Pl_1, Pl_2, Pl_6, Pl_7, and Pl_{15} (de Romano et al. 2010; Gascuel et al. 2015). A BLAST search against HA412.v1.1.bronze.20141015 database revealed that SSR marker ORS166 is tightly linked to the DM *R*-gene cluster, and the TIR3F1R2 marker within candidate Pl_6 gene was upstream of Pl_{20} (Table 2, Slabaugh et al. 2003; de Romano et al. 2010). Although ORS166 did not map to the region surrounding the Pl_{20} gene in the present study, this locus was physically close to Pl_{20}. ORS166 and TIR3F1R2 are located at 10,687,506 and 10,939,101 bp on LG8, respectively, whereas the Pl_{20} gene is located in the region from 11,271,845 to 13,781,094 bp on LG8, proximal to the DM gene cluster (Table 2). The genetic position of GBS SNP_100385559 was confirmed when it was converted to a PCR-based marker in the population; however, the physical position of this SNP conflicted with its genetic position (Fig. 1; Table 2).

DM resistance spectrum of Pl_{20} in cultivated sunflower background

Downy mildew resistance in a homozygous resistant $BC_1F_{2:3}$ line, 14-207-58, along with the recurrent parent HA 89, as well as susceptible (Cargill 270) and resistant (HA-DM1) checks was further evaluated for resistance against additional *P. halstedii* races, 314, 700, 710, 714, and 774 and race 734. These selected *P. halstedii* races represent the most predominant and virulent races

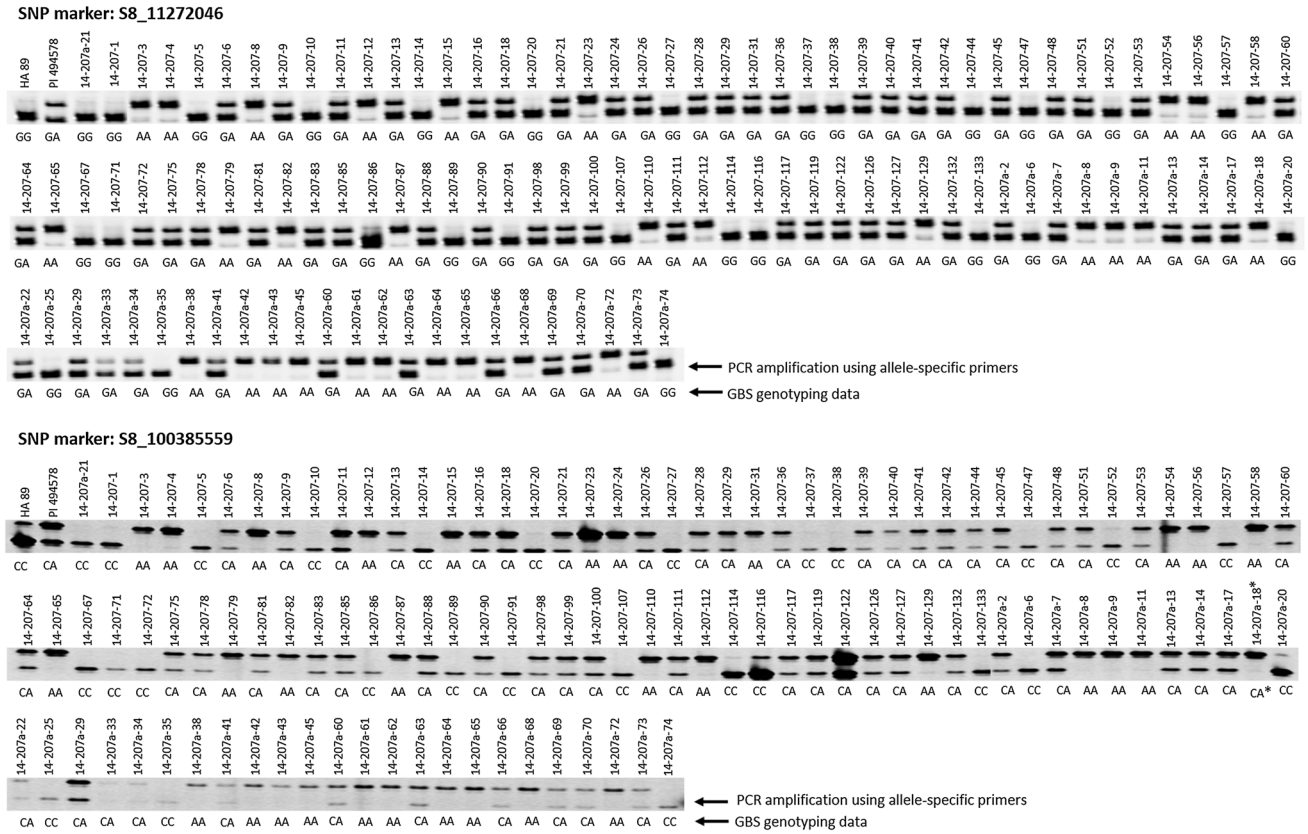

Fig. 1 The segregation of PCR-based and GBS genotyping data of SNP markers S8_11272046 and S8_100385559 in the BC_1F_2 population and parents HA 89 and PI 494578. (*Asterisk*) The genotype of the PCR-based SNP was different from that of GBS data

currently identified in North America (Gulya et al. 2011; Gilley et al. 2016). As expected, HA 89 and Cargill 270 were susceptible to all six races tested, and abundant white sporulation was observed on the leaf surfaces of the seedlings. In contrast, the homozygous resistant line 14-207-58 carrying the Pl_{20} gene and HA-DM1 harboring the Pl_{18} gene were immune to all *P. halstedii* races tested, with no sporulation on cotyledons or true leaves (Table 3).

Selection of homozygous resistant introgression lines from BC_2F_2 populations

Three hundred BC_2F_2 seedlings from NMS HA 89/PI 494578 were inoculated with *P. halstedii* race 734. One hundred and ninety-one resistant seedlings were identified, and 186 *R*-plants were transferred to 36-cell plastic flats for the selection of homozygous resistant plants. Three SNP markers, SFW04358, S8_11272025, and S8_100385559, linked to the Pl_{20} gene, were used to genotype the selected resistant BC_2F_2 individuals, together with their parents, HA 89 and PI 494578. A total of 62 plants were identified as homozygous resistant, showing identical PI 494578 marker alleles from the 186 plants tested, and 32 of these plants were advanced to the BC_2F_3 generation.

Discussion

Many resistance genes in plants are organized into gene clusters along the chromosomes, widely observed in sunflower *Pl* genes (Islam and Shepherd 1991; Jones et al. 1993; Song et al. 1997; Meyers et al. 1998; Huang et al. 2003). Two (Pl_{17} and Pl_{19}), three (Pl_5, Pl_8 and Pl_{21}), three (Pl_{13}, Pl_{14}, and Pl_{16}), and five (Pl_1, Pl_2, Pl_6, Pl_7 and Pl_{15}) *Pl* genes were, respectively, clustered in LGs 4, 13, 1, and 8 of the sunflower genome, as reported in previous studies (Table S1). Among these genes, Pl_1, Pl_2, Pl_6, Pl_7, and Pl_{15} and the rust resistance gene R_1 are clustered in the upper end of LG8, which is one of the largest gene clusters in sunflower. In the present study, we also mapped a novel DM *R*-gene, Pl_{20}, to this cluster. The SSR marker, ORS166, which has been linked to the Pl_{15} and Pl_1–Pl_2–Pl_6–Pl_7 cluster, was not mapped in the Pl_{20} population, but rather mapped to a physically close location with

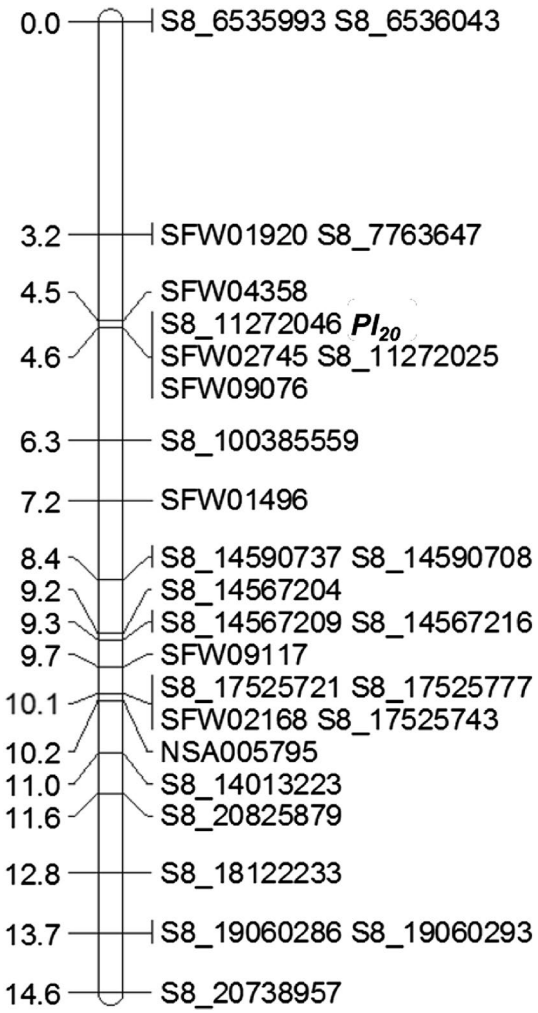

Fig. 2 Part of genetic map of sunflower linkage group 8, showing the Pl_{20} position on the map

the Pl_{20} gene (Table 2, Slabaugh et al. 2003; de Romano et al. 2010).

Although Pl_{20} was mapped to the DM *R*-gene cluster in LG8, this gene has a different origin and DM resistance specificity from other DM *R*-genes in this cluster. The origin of Pl_1 and Pl_2 can be traced to a Canadian line 953 involving wild *H. annuus* (Zimmer and Kinman 1972; Vear et al. 2008). Pl_6 and Pl_7 originated from wild *H. annuus* and *H. praecox*, respectively (Miller and Gulya 1988, 1991), and Pl_{15} was identified from an Argentinian restorer inbred line RNID (de Romano et al. 2010), whereas Pl_{20} was derived from *H. argophyllus*. Additionally, Pl_1 and Pl_2 are no longer effective against downy mildew infection, and Pl_6 and Pl_7 are susceptible to virulent *P. halstedii* races, whereas Pl_{20} is resistant to all *P. halstedii* races tested in the present study, which are the predominant and virulent races currently identified in North America (Gilley et al. 2016). Altogether, Pl_{20} is a novel and effective DM resistance gene located on the DM *R*-gene cluster on LG8.

Table 2 Genetic and physical positions of molecular markers flanking the Pl_{20} gene and markers linked to the Pl_6/Pl_7 cluster in linkage group 8 (LG8); the physical length of LG8 is 192,129,815 bp

Marker id	Map position (cM)	HA412.v1.1.bronze. 20141015 pseudomolecules	
		Start	End
S8_7763647	3.2	7,763,446	7,763,847
SFW01920	3.2	8,626,528	8,626,648
SFW09076	4.6	9,317,680	9,317,800
SFW04358	4.5	10,072,537	10,072,657
SFW02745	4.6	10,452,253	10,452,373
ORS166	–	10,687,506	10,687,817
TIR3F1R2[a]	–	10,939,101	10,939,438
S8_11272025	4.6	11,271,824	11,272,225
S8_11272046	4.6	11,271,845	11,272,246
Pl_{20}	4.6		
S8_100385559	6.3	100,385,358	100,385,759
SFW01496	7.2	13,781,004	13,781,094
SFW09117	9.7	14,906,477	14,906,577
SFW02168	10.1	16,209,353	16,209,473
NSA_005795	10.2	16,221,544	16,221,872

[a] Primers within candidate Pl_6 gene (Franchel et al. 2013)

Linkage group 8 was reported to harbor the largest nucleotide-binding site and leucine-rich repeats (NBS-LRR) in sunflower (Radwan et al. 2008). A total of 54 NBS-LRR loci were discovered on LG8, and three subclusters were detected in the 36-cM region. The *Pl* gene cluster in LG8 was associated with a TIR (toll-interleukin receptor)-NBS-LRR subcluster (Bouzidi et al. 2006; Radwan et al. 2008; Franchel et al. 2013) within the first and largest subcluster comprising TIR members exclusively in LG8.

Helianthus argophyllus is a wild annual species primarily located in the U.S. Among the 94 *H. argophyllus* accessions deposited in the Germplasm Resources Information Network (GRIN), 78 accessions were located in the U.S., 11 accessions were located in Australia, two accessions were located in former Soviet Union, two accessions were located in Mozambique, and one accession was of unknown origin (http://www.ars-grin.gov/cgi-bin/npgs/html/tax_search.pl). The state of Texas is the main reservoir containing *H. argophyllus* in the U.S., including a total of 75 accessions, with only one accession observed in Florida (PI 468651) which is the donor of the Pl_{Arg} gene and two accessions observed in North Carolina (PI 664729 and PI 664730). Unlike the clustered DM *R*-genes from wild *H. annuus*, the DM *R*-genes from *H. argophyllus* are

Table 3 Downy mildew resistance tests of homozygous BC_1F_3 family (14-207-58) with six *P. halstedii* races

Line	Downy mildew races											
	314		700		710		714		734		774	
	S	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*
Cargill 270	27	0	25	0	21	0	21	0	26	0	23	0
HA 89	15	0	16	0	16	0	17	0	17	0	22	0
HA-DM1	0	15	0	17	0	14	0	14	0	14	0	9
14-207-58	0	35	0	33	0	34	0	34	0	33	0	40

widely distributed across the sunflower genome. The four *Pl* genes, Pl_{Arg}, Pl_8, Pl_{18}, and Pl_{20}, discovered in *H. argophyllus,* were separately located on LGs, 1, 13, 2, and 8, and all but Pl_{Arg} were originally collected from Texas. It remains unknown how the host-pathogen co-evolved to develop diversified DM *R*-genes in *H. argophyllus* in a narrowed geographic area (Qi et al. 2016a).

GBS holds a number of interesting points that can benefit both scientists and breeding communities, such as time-, labor-, and cost-efficiency. An enormous number of SNPs can be produced in a relatively short time compared to other marker systems that function as an effective tool in such applications as population genetics, quantitative trait locus discovery, genome-wide association study, and high-resolution mapping. However, there are some inevitable and inherent limitations when conducting GBS analysis, particularly when dealing with the F_2 population. Theoretically, 50% of the SNP sites called are expected in a heterozygous condition in the F_2 population. Higher sequencing coverage, more accurate calling, and more sophisticated bioinformatics are required to call heterozygotes (Hyma et al. 2015). In the present study, one of the mapping parents, *H. argophyllus* PI 494578, is a wild sunflower species with an open-pollinated nature, making this species highly heterozygous between alleles, further complicating heterozygote calling. In the mapping population in the present study, a high degree of segregation distortion was observed, in which only 0.26% of SNPs fit the expected Mendelian ratio (Table S4). The number of F_2 individuals carrying heterozygous genotypes was significantly reduced, with a corresponding excess of HA 89 homozygous genotypes. In some LGs, all the generated SNPs showed segregation distortion, such as those on LGs 6, 7, and 16; however, LG8 harbored 56.8% (191/336) of SNPs with no segregation distortion (Table S4). After GBS analysis of the localized Pl_{20} gene on LG8, we further 'post-processed' and validated the linkage, i.e., converting the GBS-generated SNPs into PCR-based length polymorphism. The PCR-based genotyping data for closely linked GBS markers S8_11272025, S8_11272046, and S8_100385559 precisely match those from the GBS analysis for each of the 114 BC_1F_2 individuals, except of one individual (Fig. 2), further confirming the linkage relationship of Pl_{20} with these markers.

Downy mildew resistance from wild sunflower species can be introgressed into cultivated sunflowers to provide resistance to many/or all currently known races of the pathogen. In the present study, a new DM *R*-gene, Pl_{20}, was introgressed into cultivated sunflowers, which could further be used for *R*-gene pyramiding. It has previously been reported that *P. halstedii* pathogens co-evolve with host resistance genes, leading to the ineffectiveness of existing downy mildew *R*-genes (Gascuel et al. 2015; Viranyi et al. 2015; Gilley et al. 2016). The strategies typically utilized in sunflower production are to first continuously search for new resistance, and second, conduct gene pyramiding in which multiple resistance genes are combined into a single genotype. Reaction to *P. halstedii* infection showed that the Pl_{20} gene is effectively resistant to the most predominant and virulent races currently identified in North America. This novel *R*-gene has not yet been incorporated into any commercial lines and provides a very high level of resistance (immunity) to the disease. An ongoing *R*-gene pyramiding project is underway to provide multiple resistances to cultivated sunflower against downy mildew infection.

Author contribution statement Conceived and designed the experiments: LLQ, GJM. Performed the experiments: GJM, LLQ, SGM. Analyzed data: GJM, QJS, LLQ. Wrote the paper: GJM, LLQ, Commented on the manuscript before submission: SGM, QJS.

Acknowledgements The authors thank Dr. Loren Rieseberg for providing access to the Sunflower Genome Data Repository. They also thank Drs. Gerald Seiler and Gongjun Shi for critical review of the manuscript, Angelia Hogness for technical assistance, and Michelle Gilley for *P. halstedii* isolates. This project was supported by the USDA-AMS Specialty Crop Block Grant Program 15-SCBGP-ND-0026 and the USDA-ARS CRIS Project No. 3060-21000-039-00D. Mention of trade names or commercial products in this report is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. The USDA is an equal opportunity provider and employer.

Compliance with ethical standards

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

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

References

Ahmed S, de Labrouhe DT, Delmotte F (2012) Emerging virulence arising from hybridization facilitated by multiple introductions of the sunflower downy mildew pathogen *Plasmopara halstedii*. Fungal Genet Biol 49:847–855

Bachlava E, Radwan OE, Abratti G, Tang S, Gao W, Heesacker AF, Bazzalo ME, Zambelli A, Leon AJ, Knapp SJ (2011) Downy mildew (Pl_8 and Pl_{14}) and rust (R_{Adv}) resistance genes reside in close proximity to tandemly duplicated clusters of non-TIR-like NBS-LRR-encoding genes on sunflower chromosomes 1 and 13. Theor Appl Genet 122:1211–1221

Baute GJ, Kane NC, Grassa CJ, Lai Z, Rieseberg LH (2015) Genome scans reveal candidate domestication and improvement genes in cultivated sunflower, as well as post-domestication introgression with wild relatives. New Phytol 206:830–838

Bert PF, de Labrouhe TD, Philippon J, Mouzeyar S, Jouan I, Nicolas P, Vear F (2001) Identification of a second linkage group carrying genes controlling resistance to downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus* L.). Theor Appl Genet 103:992–997

Bouzidi MF, Franchel J, Tao Q, Stormo K, Mraz A, Nicolas P, Mouzeyar S (2006) A sunflower BAC library suitable for PCR screening and physical mapping of targeted genomic regions. Theor Appl Genet 113:81–89

Bowers JE, Bachlava E, Brunick RL, Rieseberg LH, Knapp SJ, Burke JM (2012) Development of a 10,000 locus genetic map of the sunflower genome based on multiple crosses. G3 Genes Genom Genet 2:721–729

Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES (2007) TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633–2635

Chen J, Hu J, Jan CC (2006) Molecular mapping of a nuclear male sterility gene in sunflower (*Helianthus annuus* L.) using TRAP and SSR markers. Theor Appl Genet 113:122–127

Constantinescu O, Thines M (2010) *Plasmopara halstedii* is absent from Australia and New Zealand. Pol Bot J 55:293–298

de Labrouhe DT, Pilorgé E, Nicola P, Vear F (2000) Le mildiou du tournesol. INRA and CETIOM, Paris

de Romano AB, Romano C, Bulos M, Altieri E, Sala C (2010) A new gene for resistance to downy mildew in sunflower. In: Proceedings of Int Symposium "Sunflower breeding on resistance to diseases", Krasnodar, Russia, June 23–24, 2010

Dußle CM, Hahn V, Knapp SJ, Bauer E (2004) Pl_{Arg} from *Helianthus argophyllus* is unlinked to other known downy mildew resistance genes in sunflower. Theor Appl Genet 109:1083–1086

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 6:e19379

Flor HH (1955) Host-parasite interaction in flax rust: its genetics and other implications. Phytopathology 45:680–685

Franchel J, Bouzidi MF, Bronner G, Vear F, Nicolas P, Mouzeyar S (2013) Positional cloning of a candidate gene for resistance to the sunflower downy mildew, *Plasmopara halstedii* race 300. Theor Appl Genet 126:359–367

Gascuel Q, Martinez Y, Boniface MC, Vear F, Pichon M, Godiard L (2015) The sunflower downy mildew pathogen *Plasmopara halstedii*. Mol Plant Pathol 16:109–122

Gedil MA, Slabaugh MB, Berry S, Segers B, Peleman J, Michelmore R, Miller JF, Gulya T, Knapp SJ (2001) Candidate disease resistance genes in sunflower cloned using conserved nucleotide binding site motifs: genetic mapping and linkage to downy mildew resistance gene Pl_1. Genome 44:205–212

Gilley M, Misar C, Gulya T, Markell S (2016) Prevalence and virulence of *Plasmopara halstedii* (downy mildew) in sunflowers. In: Proceeding 38th Sunflower Research Forum. Fargo ND. http://www.sunflowernsa.com/uploads/research/1277/Prevalence.Downey_Gilley.etal_2016.rev.pdf. Accessed 18 Apr 2017

GRIN (2017) Germplasm Resources Information Network. United States Department of Agriculture, Agricultural Research Service, Beltsville, MD. http://www.ars-grin.gov/. Accessed 18 Apr 2017

Gulya TJ (1985) Registration of five disease-resistant sunflower germplasms. Crop Sci 25:719–720

Gulya TJ (2005) Evaluation of wild annual *Helianthus* species for resistance to downy mildew and Sclerotinia stalk rot. In: Proceeding 27th Sunflower Research Forum. Fargo ND, 12–13 Jan 2005. http://www.sunflowernsa.com/uploads/research/265/GulyaWildHelianthus_studies_05.pdf. Accessed 18 Apr 2017

Gulya TJ, Miller JF, Viranyi F, Sackston WE (1991a) Proposed internationally standardized methods for race identification of *Plasmopara halstedii*. Helia 14:11–20

Gulya TJ, Sackston WE, Viranyi F, Masirevic S, Rashid KY (1991b) New races of the sunflower downy mildew pathogen (*Plasmopara halstedii*) In Europe and North and South America. J Phytopathol 132:303–311

Gulya TJ, Due B, Hutter M (2010) New virulent races of downy mildew: distribution, status of DM resistant hybrids, and USDA sources of resistance to races that overcome the Pl_6 gene. In: Proceedings of 32th Sunflower Research Forum. Fargo ND, January 12–13, 2010. http://www.sunflowernsa.com/uploads/16/gulya1_virulentraces_10.pdf. Accessed 18 Apr 2017

Gulya TJ, Markell S, McMullen M, Harveson B, Osborne L (2011) New virulent races of downy mildew: distribution, status of DM resistant hybrids, and USDA sources of resistance. In: Proceedings of 33th Sunflower Research Forum. Fargo ND, January 12–13, 2011. http://www.sunflowernsa.com/uploads/17/gulya_virulentracesdownymildew.pdf. Accessed 18 Apr 2017

Huang L, Brooks SA, Fellers JP, Gill BS (2003) Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploidy genome of bread wheat. Genetics 164:655–664

Hulke BS, Miller JF, Gulya TJ, Vick BA (2010) Registration of the oilseed sunflower genetic stocks HA 458, HA 459, and HA 460 possessing genes for resistance to downy mildew. J Plant Regist 4:93–97

Humann RH, Gulya TJ, Markell SG (2016) Downy Mildew. In: Harveson RM, Markell SG, Block CC, Gulya TJ (eds) Compendium of Sunflower Diseases and Pests. American Phytopathology Press, St. Paul, pp 18–20

Hyma KE, Barba P, Wang M, Londo JP, Acharya CB, Mitchell SE, Sun Q, Reisch B, Cadle-Davidson L (2015) Heterozygous mapping strategy (HetMappS) for high resolution genotyping-by-sequencing markers: a case study in grapevine. PLoS One 10:e0134880

Islam MR, Shepherd KW (1991) Present status of genetics of rust resistance in flax. Euphytica 55:255–267

Jan CC, Rutger JN (1988) Mitomycin C- and streptomycin-induced male sterility in cultivated sunflower. Crop Sci 28:792–795

Jones DA, Dickinson MJ, Balint-Kurti PJ, Dixon MS, Jones JDG (1993) Two complex resistance loci revealed in tomato by classical and RFLP mapping of the *Cf-2*, *Cf-4*, *Cf-5*, and *Cf9* genes for resistance to *Cladosporium fulvum*. Mol Plant Microbe Interact 6:348–357

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25:1754–1760

Liu Z, Gulya TJ, Seiler GJ, Vick BA, Jan CC (2012) Molecular mapping of the Pl_{16} downy mildew resistance gene from HA-R4 to facilitate marker-assisted selection in sunflower. Theor Appl Genet 125:121–131

Long YM, Chao WS, Ma GJ, Xu SS, Qi LL (2016) An innovative SNP genotyping method adapting multiple platforms and throughputs. Theor Appl Genet. doi:10.1007/s00122-016-2838-4

Markell SG, Humann RH, Gilley M, Gulya TJ (2016) Downy mildew pathogen. In: Harveson RM, Markell SG, Block CC, Gulya TJ (eds) Compendium of sunflower diseases and pests. American Phytopathology Press, St. Paul, pp 15–117

Meyers BC, Chin DB, Shen KA, Sivaramakrishnan S, Lavelle DO, Zhang Z, Michelmore RW (1998) The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell 10:1817–1832

Miller JF, Gulya TJ (1987) Inheritance of resistance to race 3 downy mildew in sunflower. Crop Sci 27:210–212

Miller JF, Gulya TJ (1988) Registration of six downy mildew resistant sunflower germplasm lines. Crop Sci 28:1040–1041

Miller JF, Gulya TJ (1991) Inheritance of resistance to race 4 of downy mildew derived from interspecific crosses in sunflower. Crop Sci 31:40–43

Molinero-Ruiz ML, Melero-Vara JM, Dominguez J (2003) Inheritance of resistance to two races of sunflower downy mildew (*Plasmopara halstedii*) in two *Helianthus annuus* L. lines. Euphytica 131:47–51

Mouzeyar S, Roeckel-Drevet P, Gentzbittel L, Philippon J, de Labrouhe DT, Vear F, Nicolas P (1995) RFLP and RAPD mapping of the sunflower Pl_1 locus for resistance to *Plasmopara halstedii* race 1. Theor Appl Genet 91:733–737

Mulpuri S, Liu Z, Feng J, Gulya TJ, Jan CC (2009) Inheritance and molecular mapping of a downy mildew resistance gene, Pl_{13} in cultivated sunflower (*Helianthus annuus* L.). Theor Appl Genet 119:795–803

Qi LL, Gulya TJ, Seiler GJ, Hulke BS, Vick BA (2011) Identification of resistance to new virulent races of rust in sunflowers and validation of DNA markers in the gene pool. Phytopathol 101:241–249

Qi LL, Gulya TJ, Hulke BS, Vick BA (2012) Chromosome location, DNA markers and rust resistance of the sunflower gene R_5. Mol Breeding 30:745–756

Qi LL, Long YM, Jan CC, Ma GJ, Gulya TJ (2015) Pl_{17} is a novel gene independent of known downy mildew resistance genes in the cultivated sunflower (*Helianthus annuus* L.). Theor Appl Genet 128:757–767

Qi LL, Foley ME, Cai XW, Gulya TJ (2016a) Genetics and mapping of a novel downy mildew resistance gene, Pl_{18}, introgressed from wild *Helianthus argophyllus* into cultivated sunflower (*Helianthus annuus* L.). Theor Appl Genet 129:741–752

Qi LL, Long YM, Talukder ZI, Seiler GJ, Block CC, Gulya TJ (2016b) Genotyping-by-sequencing uncovers the introgression alien segments associated with Sclerotinia basal stalk rot resistance from wild species—I. *Helianthus argophyllus* and *H. petiolaris*. Front Genet 7:219

Radwan O, Bouzidi MF, Vear F, Philippon J, de Labrouhe TD, Nicolas P, Mouzeyar S (2003) Identification of non-TIR-NBS-LRR markers linked to the Pl_5/Pl_8 locus for resistance to downy mildew in sunflower. Theor Appl Genet 106:1438–1446

Radwan O, Gandhi S, Heesacker A, Whitaker B, Taylor C, Plocik A, Kesseli R, Kozik A, Michelmore RW, Knapp SJ (2008) Genetic diversity and genomic distribution of homologs encoding NBS-LRR disease resistance proteins in sunflower. Mol Genet Genom 280:111–125

Rahim M, Jan CC, Gulya TJ (2002) Inheritance of resistance to sunflower downy mildew races 1, 2 and 3 in cultivated sunflower. Plant Breed 121:57–60

Roeckel-Drevet P, Gagne G, Mouzeyar S, Gentzbittel L, Philippon J, Nicolas P, de Labrouhe DT, Vear F (1996) Colocation of downy mildew (*Plasmopara halstedii*) resistance genes in sunflower (*Helianthus annuus* L.). Euphytica 91:225–228

Rogers CE, Thompson TE, Seiler GJ (1982) Sunflower species of the United States. National Sunflower Association, Bismarck, pp 4–22

Schilling EE (2006) In: Flora of North America Editorial Committee (eds) Flora of North America North of Mexico. Oxford University Press, New York, Oxford. *Helianthus* 21:141–169

Seiler GJ (1991) Registration of 13 downy mildew tolerant interspecific sunflower germplasm lines derived from wild annual species. Crop Sci 31:1714–1716

Seiler GJ (2010) Utilization of wild *Helianthus* species in breed for disease resistance. In: Proceedings of the International Symposium "Sunflower Breeding on Resistance to Diseases", Krasnodar, Russia. International Sunflower Association, Paris, France. June 23–24, 2010, pp 37–51

Seiler GJ, Qi LL, Marek LF (2017) Utilization of sunflower crop wild relatives for cultivated sunflower improvement. Crop Sci. doi:10.2135/cropsci2016.10.0856

Slabaugh MB, Yu JK, Tang SX, Heesacker A, Hu X, Lu GH, Bidney D, Han F, Knapp SJ (2003) Haplotyping and mapping a large cluster of downy mildew resistance gene candidates in sunflower using multilocus intron fragment length polymorphisms. Plant Biotechnol J 1:167–185

Song WY, Pi LY, Wang GL, Gardner J, Holsten T, Ronald PC (1997) Evolution of the rice *Xa21* disease resistance gene family. Plant Cell 9:1279–1287

Stebbins JC, Winchell CJ, Constable JVH (2013) *Helianthus winteri* (Asteraceae), a new perennial species from the southern Sierra Nevada foothills, California. Aliso 31:19–24

Talukder ZI, Gong L, Hulke BS, Pegadaraju V, Song QJ, Schultz Q, Qi LL (2014) A high-density SNP map of sunflower derived from RAD-sequencing facilitating fine-mapping of the rust resistance gene R_{12}. PLoS One 9:e98628

Van Ooijen JW (2006) JoinMap® 4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma BV, Wageningen, Netherlands. http://www.kyazma.com. Accessed 4 Jan 2016

Vear F (1974) Studies on resistance to downy mildew in sunflower (*Helianthus annuus* L.). In: Proceedings of 6th International Sunflower Conference, 22–24 July, 1974, Bucharest, Romania, pp 297–302

Vear F, Leclercq P (1971) Deux nouvea ux genes resistance au mildiou du tournesol. Ann Amelior Plantes 21:251–255

Vear F, Gentzbittel L, Philippon J, Mouzeyar S, Mestries E, Roeckel-Drevet P, de Labroube DT, Nicolas P (1997) The genetics of resistance to five races of downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus* L.). Theor Appl Genet 95:584–589

Vear F, Seriveys H, Petit A, Serre F, Boudon JP, Roche S, Walser P, de Labrouhe DT (2008) Origins of major genes for downy mildew resistance in sunflower. In: Proceedings of 17th International Sunflower Conference Cordoba, Spain, 2008, pp 125–130

Viranyi F, Gulya TJ, Tourieille DL (2015) Recent changes in the pathogenic variability of *Plasmopara halstedii* (sunflower downy mildew) populations from different continents. Helia 38:149–162

Vrânceanu V, Stoenescu F (1970) Immunity to sunflower downy mildew due to a single dominant gene. Probl Agric 22:34–40

Vrânceanu VL, Pirvu N, Stoenescu FM (1981) New sunflower downy mildew resistance genes and their management. Helia 4:23–27

Zhang ZW, Ma GJ, Zhao J, Markell SG, Qi LL (2017) Discovery and introgression of the wild sunflower-derived novel downy mildew resistance gene Pl_{19} in confection sunflower (*Helianthus annuus* L.). Theor Appl Genet 130:29–39

Zimmer DE, Kinman ML (1972) Downy mildew resistance in cultivated sunflower and its inheritance. Crop Sci 12:749–751