



Article

High-Density Mapping and Candidate Gene Analysis of *Pl*₁₈ and *Pl*₂₀ in Sunflower by Whole-Genome Resequencing

Guojia Ma ¹, Qijian Song ², Xuehui Li ¹ and Lili Qi ^{3,*}

¹ Department of Plant Sciences, North Dakota State University, Fargo, ND 58108, USA; guojia.ma@ndsu.edu (G.M.); Xuehui.li@ndsu.edu (X.L.)

² USDA-Agricultural Research Service, Soybean Genomics and Improvement Laboratory, Beltsville, MD 20705-2350, USA; qijian.song@usda.gov

³ USDA-Agricultural Research Service, Edward T. Schafer Agricultural Research Center, Fargo, ND 58102-2765, USA

* Correspondence: lili.qi@usda.gov; Tel.: +1-701-239-1351

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Abstract: Downy mildew (DM) is one of the severe biotic threats to sunflower production worldwide. The inciting pathogen, *Plasmopara halstedii*, could overwinter in the field for years, creating a persistent threat to sunflower. The dominant genes *Pl*₁₈ and *Pl*₂₀ conferring resistance to known DM races have been previously mapped to 1.5 and 1.8 cM intervals on sunflower chromosomes 2 and 8, respectively. Utilizing a whole-genome resequencing strategy combined with reference sequence-based chromosome walking and high-density mapping in the present study, *Pl*₁₈ was placed in a 0.7 cM interval on chromosome 2. A candidate gene HanXRQChr02g0048181 for *Pl*₁₈ was identified from the XRQ reference genome and predicted to encode a protein with typical NLR domains for disease resistance. The *Pl*₂₀ gene was placed in a 0.2 cM interval on chromosome 8. The putative gene with the NLR domain for *Pl*₂₀, HanXRQChr08g0210051, was identified within the *Pl*₂₀ interval. SNP markers closely linked to *Pl*₁₈ and *Pl*₂₀ were evaluated with 96 diverse sunflower lines, and a total of 13 diagnostic markers for *Pl*₁₈ and four for *Pl*₂₀ were identified. These markers will facilitate to transfer these new genes to elite sunflower lines and to pyramid these genes with broad-spectrum DM resistance in sunflower breeding.

Keywords: sunflower; downy mildew; disease resistance; whole-genome resequencing; fine mapping; SNP markers

1. Introduction

Downy mildew (DM) is a devastating sunflower disease throughout the world, particularly in Europe and North America [1,2]. It is incited by the oomycete pathogen *Plasmopara halstedii* (Farl.) Berlese & de Toni, which could overwinter and persist in the soil for 5–10 years. Cool and moist soil favors downy mildew epidemics in sunflower fields. Although sunflower is the field crop that was infected by this DM fungus, other susceptible plants of weeds in the Compositae family, such as marsh elder, could function as reservoirs for this soil-borne fungus (<https://www.ag.ndsu.edu/extensionentomology/recent-publications-main/publications/A-1331-sunflower-production-field-guide>). DM infection is found mostly in the Northern Great Plains within the U.S., and the disease infected approximately 16% of sunflower fields in 2015 [3]. Substantial yield loss is expected upon DM infection, as severely infected plants will not proceed to growth at the seedling stage with few exceptions in which infected plants could still grow to maturity but not produce viable seeds.

Seed treatment would help DM management to some extent; however, it increases costs with potential harm to the environment. The utilization of resistant hybrids is the first choice to mitigate the negative effect of DM disease. The DM resistance present in sunflower is predominantly identified as a single dominant gene, designated *Pl*, and a total of 36 *Pl* genes, *Pl*₁–*Pl*₃₅, and *Pl*_{Arg}, have been reported in sunflower and its wild relatives so far (Supplementary Table S1). Because of the emergence of new *P. halstedii* pathotypes when DM resistance genes (*R* genes) are widely used in sunflower production, most DM *R* genes are no longer effectively resistant to *P. halstedii* infection [4]. Discovering and integrating new resistance genes is essential to ensure commercial hybrids to remain resistant to ever-changing pathogens.

Sunflower wild species represent a large gene pool for agronomically important traits including male sterile cytoplasm/male fertility restorers, disease resistance, abiotic tolerance, and herbicide resistance, which are widely explored in sunflower breeding [5]. Most DM *R* genes can be traced to their wild origins, mainly from wild *Helianthus annuus* and *Helianthus argophyllus* (Supplementary Table S1). The two DM *R* genes, *Pl*₁₈ and *Pl*₂₀, were identified from *H. argophyllus* accessions of PI 494573 and PI 494578 originally collected in 1984 from Texas, U.S., respectively, with a broad spectrum of resistance to new races of *P. halstedii* [1,6,7]. Molecular mapping has placed *Pl*₁₈ in a 1.5 cM interval flanked by SSR markers, CRT214 and ORS203, on chromosome 2 of the sunflower genome [7]. The breeding project successfully introgressed *Pl*₁₈ from sunflower wild species into cultivated sunflower. The oilseed sunflower germplasm HA-DM1 carrying *Pl*₁₈ was released to the public and was highly resistant to all *P. halstedii* races identified in the U.S. [4,8].

Biparental mapping efforts placed *Pl*₂₀ in a 1.8 cM interval on the upper end of sunflower chromosome 8 within a gene cluster harboring the five DM *R* genes, *Pl*₁, *Pl*₂, *Pl*₆, *Pl*₇, and *Pl*₁₅, and the two rust *R* genes, *R*₁ and *R*₁₅ [1,9–12]. HA-DM7 is an oilseed maintainer line introgressed with *Pl*₂₀ released in 2019. The flanking markers of *Pl*₁₈ and *Pl*₂₀ were physically positioned *Pl*₁₈ and *Pl*₂₀ within intervals of 780 kb and 1 Mb on chromosomes 2 and 8 in the XRQ genome, respectively. Traditional biparental mapping can chromosomally locate genes efficiently; however, the relatively large gene interval could still impair its use in breeding programs, as recombination events would be inevitable within the defined intervals. Diagnostic molecular markers resulting from high-resolution mapping would be helpful to integrate new resistance gene through marker-assisted selection (MAS) in an accurate manner.

Currently, two sunflower lines, HA412-HO and XRQ, have been whole-genome sequenced, assembled and annotated and are publicly available, each utilizing different sequencing techniques (<https://www.heliagene.org/>). The HA412-HO whole-genome sequence was assembled based on Illumina HiSeq and Roche 454 s-generation sequencing (SGS) data with short reads, while the XRQ whole-genome sequence was assembled based on PacBio RS II data with an average read length of 10.3 kb (N50 of 13.7 kb) [13]. The two reference genome sequences provide alternative tools to study marker-trait associations, to accelerate the fine-scale genetic mapping of important sunflower genes, and to identify diagnostic markers near or even within genes of interest. With the rapid progress and innovation of sequencing technologies with reduced cost, whole-genome sequencing (WGS) has become the most global approach to enable high-resolution trait mapping, candidate gene discovery, and breeding applications in plants. In this study, we performed WGS of two sunflower lines, HA-DM1 and HA-DM7, which carry the DM *R* genes *Pl*₁₈ and *Pl*₂₀, respectively. Sequence information was used to develop SNP markers closely linked and diagnostic to *Pl*₁₈ and *Pl*₂₀ and to identify gene candidates associated with resistance to DM.

2. Results

2.1. Saturation Mapping of *Pl*₁₈

The DM *R* gene *Pl*₁₈ was previously placed in an interval of 1.5 cM genetic distance flanked by the SSR markers CRT214 and ORS203 on chromosome 2 [7]. Neither SSR marker can be aligned to the

reference genomes, and their physical positions on the genome are unknown. Alternatively, two SNP markers, SFW03013 closely linked to CRT214 at 0.1 cM and SFW03060 closely linked to ORS203 at 0.5 cM, were selected, delimiting Pl_{18} to a physical interval of 780,432 bp between 128,511,770–129,292,202 bp in the XRQ genome and 100,508 bp between 128,982,063–129,082,571 bp in the HA412-HO genome (Table 1, Figure 1a). A total of 150 SNPs were selected based on SNPs/InDels between HA-DM1 carrying Pl_{18} and the two reference genomes in the targeted region of chromosome 2. Forty-nine were selected from the HA412-HO genome, and 101 were selected from the XRQ genome. Forty-three SNP markers showed polymorphisms between HA 89 and HA-DM1 and were used to genotype the initial 142 BC₁F₂ individuals. Thirty-one SNP markers were mapped around Pl_{18} , and SNP C2_128652042 was the only marker mapped to the Pl_{18} interval between SSRs CRT214 and ORS203, which is 0.3 cM distal to Pl_{18} (Figure 1b). A large marker cluster with 26 co-segregating SNPs was proximal to ORS203 at a 0.7 cM genetic distance.

Table 1. Genetic and physical positions of markers linked to Pl_{18} on the saturation map of sunflower chromosome 2.

Marker	No. Recombination	Genetic Distance (cM)	Physical Position on XRQ Assembly		Physical Position on HA412-HO Assembly	
			Start	End	Start	End
S2_128980821		0.0	128,510,328	128,510,728	128,980,621	128,981,021
S2_128982087	0	0.0	128,511,594	128,511,994	128,981,887	128,982,287
SFW03013	0	0.0	128,511,854	128,511,770	128,982,147	128,982,063
S2_128982628	0	0.0	128,512,135	128,512,535	128,982,428	128,982,828
C2_128624983	0	0.0	128,624,783	128,625,183	132,735,799	132,736,199
C2_128640408	0	0.0	128,640,208	128,640,608	132,741,177	132,741,577
CRT214	1	0.4	NA	NA	NA	NA
C2_128652042	2	0.7	128,651,842	128,652,242	NA	NA
Pl_{18}	1	0.3	-	-	-	-
ORS203	1	0.4	NA	NA	NA	NA
C2_128652376	2	0.7	128,652,176	128,652,576	132,753,709	132,753,885
C2_128663385	0	0.7	128,663,185	128,663,585	97,034,302	97,033,902
C2_128673440	0	0.7	128,673,240	128,673,640	97,024,972	97,024,572
C2_128676979	0	0.7	128,676,779	128,677,179	97,021,432	97,021,031
C2_128842104	0	0.7	128,841,904	128,842,304	129,433,726	129,434,126
C2_128842607	0	0.7	128,842,407	128,842,807	129,434,224	129,434,624
C2_128843016	0	0.7	128,842,816	128,843,216	129,434,633	129,435,033
C2_128843240	0	0.7	128,843,040	128,843,440	129,434,857	129,435,257
C2_128844350	0	0.7	128,844,150	128,844,550	129,435,967	129,436,367
C2_128847542	0	0.7	128,847,342	128,847,742	129,646,291	129,646,691
C2_128869892	0	0.7	128,869,692	128,870,092	129,451,579	129,451,979
C2_128870626	0	0.7	128,870,426	128,870,826	129,452,313	129,452,713
C2_128872074	0	0.7	128,871,874	128,872,274	129,670,656	129,670,912
C2_128880353	0	0.7	128,880,153	128,880,553	129,545,140	129,545,540
C2_128880801	0	0.7	128,881,001	128,881,401	129,461,631	129,462,020
C2_128888340	0	0.7	128,888,140	128,888,540	129,468,821	129,469,221
C2_128889755	0	0.7	128,889,555	128,889,955	129,470,227	129,470,627
C2_128916238	0	0.7	128,916,038	128,916,438	129,497,380	129,497,780
C2_128917327	0	0.7	128,917,127	128,917,527	129,498,469	129,498,869
C2_128920653	0	0.7	128,920,453	128,920,853	129,501,790	129,502,189
C2_128922069	0	0.7	128,921,869	128,922,269	129,503,206	129,503,605
C2_128925784	0	0.7	128,925,584	128,925,984	129,507,004	129,507,404
C2_128926640	0	0.7	128,926,440	128,926,840	129,507,860	129,508,260
S2_129074800	0	0.7	129,300,154	129,299,754	129,074,600	129,074,800
S2_129077858	0	0.7	129,297,096	129,296,696	129,077,658	129,078,058
SFW03060	0	0.7	129,292,083	129,292,202	129,082,571	129,082,452

The diagnostic SNP marker for Pl_{18} is shown in bold. NA: not available.

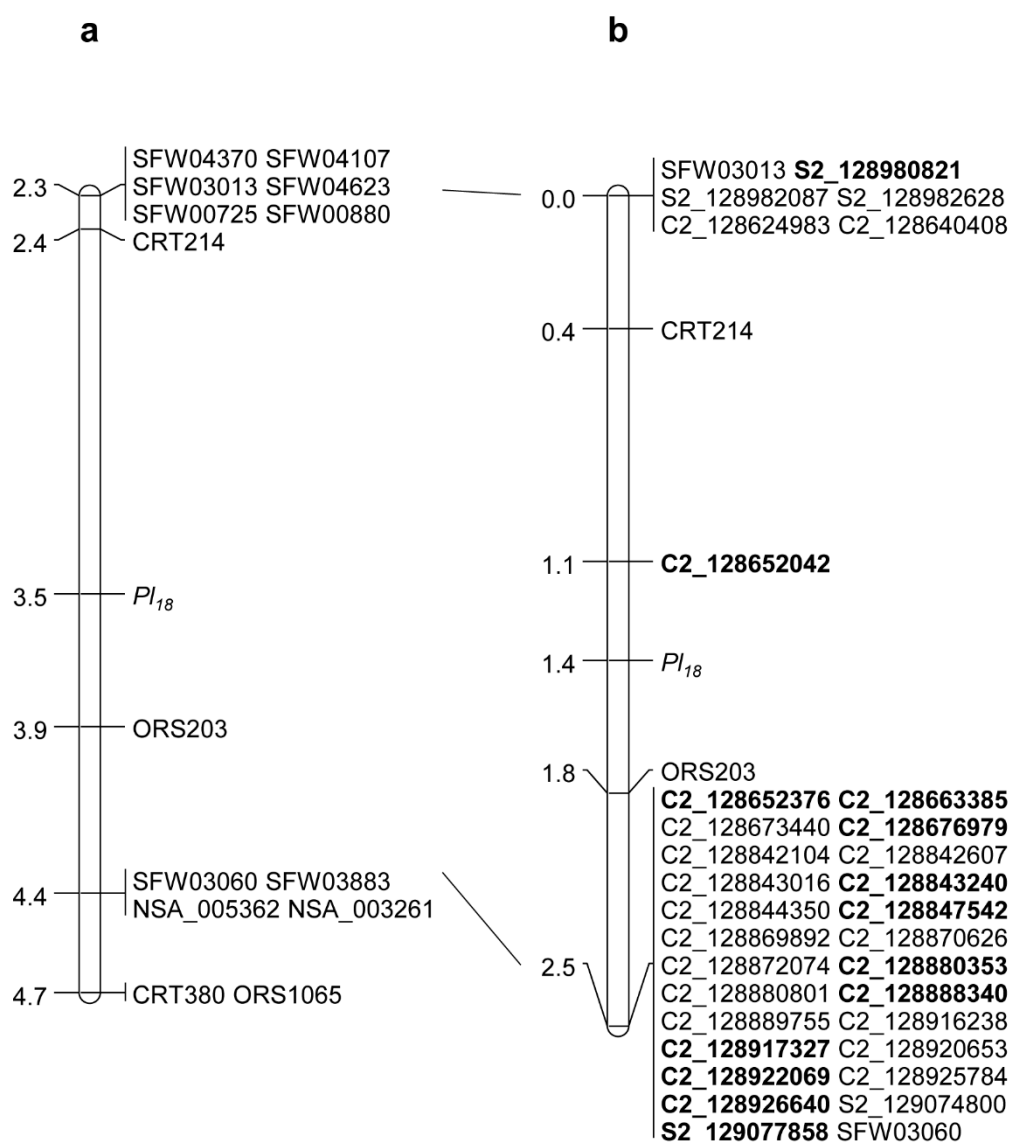


Figure 1. *Pl*₁₈ genetic maps. (a) *Pl*₁₈ basic map [7]; (b) *Pl*₁₈ saturation map. The diagnostic markers for *Pl*₁₈ are shown in bold.

Among 31 SNP markers mapped to the *Pl*₁₈ interval between SNP markers SFW03013 and SFW03060, only four, S2_128980821, S2_128982087, S2_129074800, and S2_129077858, were from the HA412-HO genome, and their physical positions on both HA412-HO and XRQ assemblies were in agreement with their genetic positions (Table 1, Figure 1b). However, no SNPs designed from the 93 kb region between S2_128982087 and S2_129074800 were mapped. The remaining 27 mapped SNPs were all from the XRQ genome (Figure 1b). All of the markers were physically in accordance with their genetic positions on the XRQ assembly; however, these SNPs did not align to the target region between 128,982,147 and 129,082,571 bp delimited by SFW03013 and SFW03060 in the HA412-HO assembly (Table 1). Among 27 mapped SNPs derived from a 302 kb region between C2_128624983 and C2_128926640 of XRQ, three aligned to 132.74–132.75 kb positions of HA412-HO, two to 97.02–97.03 kb positions, 21 to 129.43–129.67 kb positions, and one did not align to chromosome 2 (Table 1). The results suggest that the 93 kb region between S2_128982087 and S2_129074800 in HA412-HO may not be assembled correctly, which may also explain why no SNPs designed from this region were mapped.

2.2. Saturation and Fine Mapping of Pl_{20}

Pl_{20} was previously placed within the 2.5 Mb interval of 11,271,845–13,781,094 bp on chromosome 8 of the HA412-HO reference genome flanked by SNP markers S8_11272046 and SFW01496 [1]. A total of 244 SNP markers were selected from the SNP/InDel calling of whole-genome sequencing of HA-DM7 with HA412-HO and XRQ on chromosome 8; 84 from the HA412-HO genome covering a region of 447 kb (12,254,876–12,701,559 bp), and 160 from the XRQ genome covering a region of approximately 1.0 Mb (7,890,010–8,906,527 bp). The 244 SNP markers potentially surrounding Pl_{20} were tested between parents HA 89 and HA-DM7, and 25 showed polymorphism, including 2 from HA412-HO and 23 from XRQ. These 25 polymorphic markers were further genotyped with 114 BC₁F₂ individuals of the original population, and all were mapped around Pl_{20} (Figure 2b). A marker cluster with 26 SNPs co-segregated with Pl_{20} , and SNP marker C8_8639656 was proximal to Pl_{20} at a 0.88 cM genetic distance.

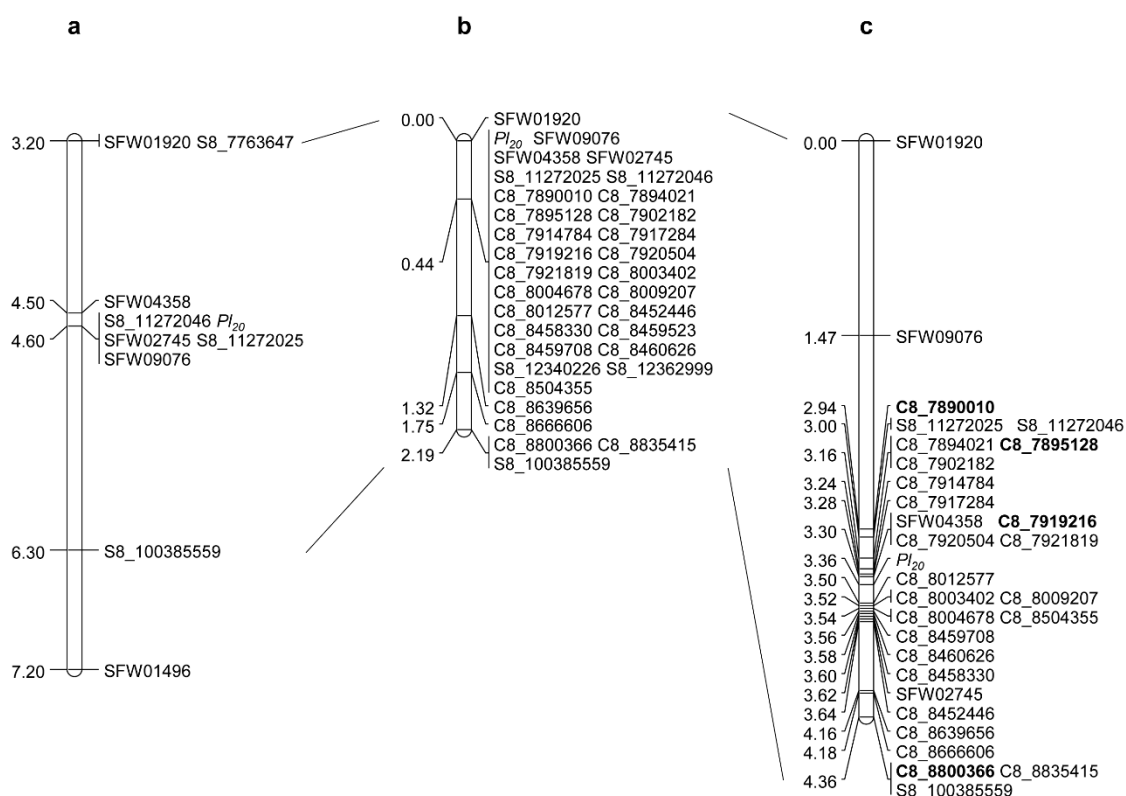


Figure 2. Pl_{20} genetic maps. (a) Pl_{20} basic map [1]; (b) Pl_{20} saturation map; (c) Pl_{20} fine map. The diagnostic markers for Pl_{20} are shown in bold.

To dissect the above marker cluster and to develop a high-density map of Pl_{20} , a large population with the 2485 BC₁F₃ individuals selected from the BC₁F₃ families heterozygous for Pl_{20} was genotyped using two flanking SNP markers, SFW01920 and S8_100385559. A total of 214 BC₁F₃ recombinants were identified in the target region delimited by the two markers and advanced to the next generation for DM testing of the recombinant families.

Twenty-two co-dominant SNP markers in the saturation map were selected to genotype the 214 recombinants identified from the above large population. Additionally, seven previously mapped SNP markers, SFW01920, SFW09076, S8_11272025, S8_11272046, SFW04358, SFW02745, and S8_100385559, within the Pl_{20} region were also included in the fine mapping [1]. The combined phenotype and marker data of the recombinants positioned Pl_{20} to a 0.20 cM interval, flanked by SNP markers C8_7921819 (0.06 cM) and C8_8012577 (0.14 cM) (Figure 2c). This genetic region corresponds to a 91.2 kb segment in the XRQ assembly (Table 2).

Table 2. Genetic and physical positions of markers linked to *Pl*₂₀ on the fine map of sunflower chromosome 8.

Marker	No. Recombination	Genetic Distance (cM)	Physical Position on XRQ Assembly (bp)	Physical Position on HA412-HO Assembly (bp)
SFW01920 [†]		0.00	855,100–855,219	8,626,529–8,626,648
SFW09076 [†]	73	1.47	6,259,434–6,259,553	9,317,681–9,317,800
C8_7890010	73	1.47	7,889,810–7,890,210	11,328,554–11,328,954
S8_11272025 [†]	3	0.06	7,890,621–7,891,021	11,271,825–11,272,225
S8_11272046 [†]	0	0.00	7,890,600–7,891,000	11,271,846–11,272,246
C8_7894021	8	0.16	7,893,821–7,894,221	11,268,522–11,268,922
C8_7895128	0	0.00	7,894,928–7,895,328	11,323,742–11,324,142
C8_7902182	0	0.00	7,901,982–7,902,382	11,262,375–11,262,775
C8_7914784	4	0.08	7,914,584–7,914,984	11,251,806–11,252,206
C8_7917284	2	0.04	7,917,084–7,917,484	11,249,306–11,249,706
SFW04358 [†]	1	0.02	6,438,630–6,438,749	10,072,538–10,072,657
C8_7919216	0	0.00	7,919,016–7,919,416	11,247,370–11,247,773
C8_7920504	0	0.00	7,920,304–7,920,704	11,246,082–11,246,482
C8_7921819	0	0.00	7,921,619–7,922,019	11,244,767–11,245,167
<i>Pl</i> ₂₀	3	0.06	–	–
C8_8012577	7	0.14	8,012,377–8,012,777	9,689,417–9,689,678
C8_8003402	1	0.02	8,003,202–8,003,602	9,680,535–9,680,935
C8_8009207	0	0.00	8,009,007–8,009,407	10,853,046–10,853,441
C8_8004678	1	0.02	8,004,478–8,004,878	9,681,811–9,682,211
C8_8504355	0	0.00	8,504,155–8,504,555	10,905,758–10,906,158
C8_8459708	1	0.02	8,459,508–8,459,908	12,109,436–12,109,838
C8_8460626	1	0.02	8,460,426–8,460,826	12,226,272–12,226,515
C8_8458330	1	0.02	8,458,130–8,458,530	12,108,058–12,108,458
SFW02745 [†]	1	0.02	8,456,520–8,456,639	11,614,201–11,614,082
C8_8452446	1	0.02	8,452,246–8,452,646	11,620,100–11,620,414
C8_8639656	26	0.52	8,639,456–8,639,856	12,827,866–12,828,204
C8_8666606	1	0.02	8,666,406–8,666,806	12,796,693–12,797,102
C8_8800366	9	0.18	8,800,166–8,800,566	13,920,131–13,920,531
C8_8835415	0	0.00	8,835,215–8,835,615	13,960,633–13,961,033
S8_100385559 [†]	0	0.00	8,907,619–8,908,019	100,385,359–100,385,759

[†] Mapped previously in Ma et al. 2017 [1]. The diagnostic SNP marker for *Pl*₂₀ is shown in bold.

2.3. Identification of Candidate Genes for *Pl*₁₈ and *Pl*₂₀

In the high-density map, all of the newly developed SNP markers were mapped around *Pl*₁₈ and physically located in a 780 kb region between 128,511,770 and 129,292,202 bp on chromosome 2 of the XRQ assembly (Table 1). These SNP markers are genetically and physically consistent with the position in the XRQ genome, and thus the 780 kb genomic sequences in the target region were analyzed from the XRQ database (<https://www.heliogene.org/HanXRQ-SUNRISE/>). Seven highly confident genes were found in the target region, and one putative gene, HanXRQChr02g0048181, was predicted to code a powdery mildew resistance protein with the typical disease resistance gene domain of nucleotide binding and leucine-rich repeat (NLR) (Table 3). This gene is located from 128,920,787 to 128,926,787 bp along chromosome 2 with a length of 6 kb. Its genetic and physical positions, as well as its functional domains and predicted functions, support it as a candidate gene for *Pl*₁₈.

Table 3. Predicted genes in the intervals of Pl_{18} and Pl_{20} from the XRQ annotation.

Genes	Definition	Physical Position	Length (bp)
For Pl_{18}			
HanXRQChr02g0048101	Probable mog1/PsbP/DUF1795-like photosystem II reaction center PsbP family protein	128,508,926...128,514,561	5636
HanXRQChr02g0048111	Putative glycoside hydrolase family 17; Glycoside hydrolase superfamily	128,551,551...128,553,503	1953
HanXRQChr02g0048131	Putative alcohol dehydrogenase superfamily, zinc-type; L-threonine 3-dehydrogenase; NAD(P)-binding domain	128,639,137...128,639,993	857
HanXRQChr02g0048141	Putative alcohol dehydrogenase superfamily, zinc-type; GroES-like	128,642,764...128,642,967	204
HanXRQChr02g0048151	Putative alcohol dehydrogenase superfamily, zinc-type; GroES-like	128,651,738...128,652,649	912
HanXRQChr02g0048171	Putative NAC domain	128,841,438...128,843,598	2161
HanXRQChr02g0048181	Putative NB-ARC; Powdery mildew resistance protein, RPW8 domain; P-loop containing nucleoside triphosphate hydrolase; Leucine-rich repeat domain, L domain-like	128,920,787...128,926,787	6001
For Pl_{20}			
HanXRQChr08g0210011	Probable GYF domain-containing protein	7,910,378...7,917,771	7394
HanXRQChr08g0210051	Putative NB-ARC; Toll-like receptor; P-loop containing nucleoside triphosphate hydrolase; Leucine-rich repeat domain, L domain-like	8,010,685...8,035,718	25,034
HanXRQChr08g0210081	Putative tify domain; CO/COL/TOC1, conserved site	8,273,535...8,277,186	3652
HanXRQChr08g0210111	Putative bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain	8,306,497...8,309,225	2729
HanXRQChr08g0210131	Probable CONTAINS InterPro DOMAIN/s: WW/Rsp5/WWP (InterPro:IPR001202)	8,325,909...8,327,735	1827
HanXRQChr08g0210141	Probable B-box type zinc finger protein with CCT domain	8,398,470...8,403,825	5356
HanXRQChr08g0210151	Putative leucine-rich repeat-containing N-terminal, plant-type; Leucine-rich repeat domain, L domain-like	8,408,566...8,411,305	2740

Similarly, an approximately 610 kb genomic sequence (7,894,928–8,504,555 bp) between SNP markers C8_7895128 and C8_8504355 of chromosome 8 was extracted from the XRQ database for annotation, and seven genes in this region were identified. Out of seven genes, HanXRQChr08g0210051 has a typical NLR domain, and HanXRQChr08g0210151 has a leucine-rich repeat domain (Table 3). HanXRQChr08g0210051 was located from 8,010,685 to 8,035,718 bp on chromosome 8 with a length of 25 kb, which falls to the *Pl*₂₀ gene interval between SNP markers C8_7921819 and C8_8012577 and could be a candidate gene for *Pl*₂₀ (Table 3). HanXRQChr08g0210151 was located from 8,408,566 to 8,411,305 bp on chromosome 8 with a length of 2.7 kb (Table 3).

2.4. Development of Diagnostic Markers for *Pl*₁₈ and *Pl*₂₀

The 31 new SNP markers mapped to *Pl*₁₈ in the saturation map were first tested in six sunflower lines, including four resistant lines, HA 458 (*Pl*₁₇), HA-DM1 (*Pl*₁₈), RHA 340 (*Pl*₈), and RHA 464 (*Pl*_{Arg}), and two susceptible lines, HA 89 and CONFSCLB1. Twenty-two of them showed a unique PCR pattern in HA-DM1 in contrast to the other three resistant and two susceptible lines and were further genotyped in an evaluation panel with 96 selected sunflower lines to determine their specificity in the sunflower population and to assess their potential in MAS for *Pl*₁₈ (Supplementary Table S2). Thirteen of the 22 SNP markers could differentiate *Pl*₁₈ from other reported *Pl* genes, including *Pl*_{Arg}, *Pl*₁–*Pl*₃, *Pl*₆–*Pl*₁₃, *Pl*₁₅–*Pl*₂₁, *Pl*₃₃, and *Pl*₃₄, in the selected sunflower lines (Table 1; Figure 3a). *Pl*₁₈-introgressed lines, HA-DM1 and HA-DM4, show unique *Pl*₁₈ marker alleles, differentiating them from other sunflower lines (Figure 3a).

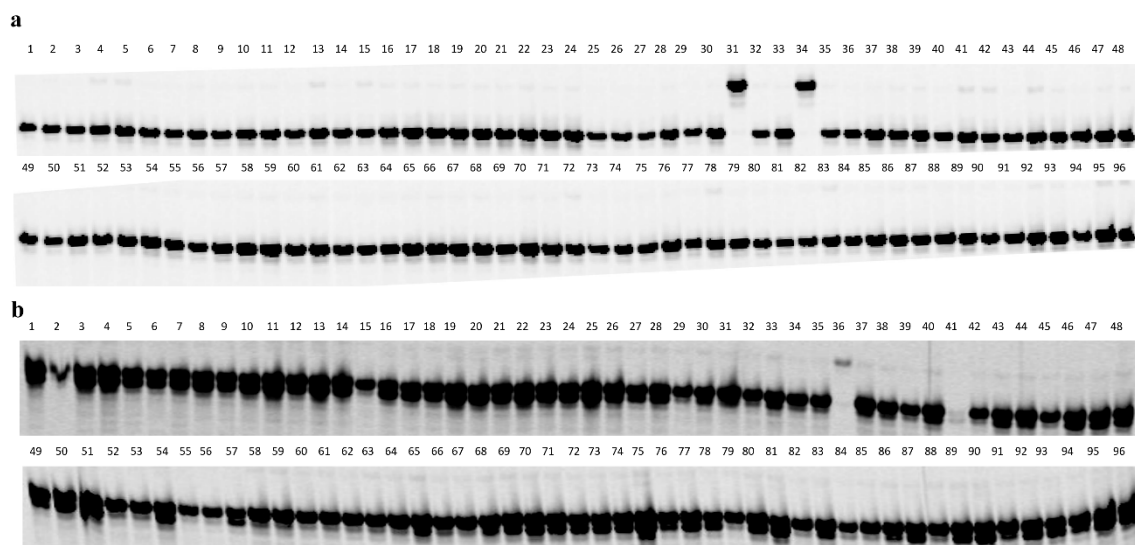


Figure 3. The polymerase chain reaction (PCR) amplification pattern of 96 selected sunflower lines with *Pl*₁₈ and *Pl*₂₀ diagnostic single nucleotide polymorphism (SNP) markers. The names and pedigrees of 96 selected sunflower lines (lanes) are listed in Supplementary Table S2. (a) PCR amplification pattern with SNP marker C2_128922069 diagnostic for *Pl*₁₈. Lane 31: HA-DM1, and lane 34: HA-DM4, both have the *Pl*₁₈ gene and show the *Pl*₁₈ marker allele. (b) PCR amplification pattern with SNP marker C8_7895128 diagnostic for *Pl*₂₀. Lane 36: HA-DM7 with the *Pl*₂₀ gene.

Eight SNP markers fine mapped around *Pl*₂₀ were selected to test for specificity in the evaluation panel of 96 selected sunflower lines, and four showed unique patterns in HA-DM7 (*Pl*₂₀-introgressed line), in contrast to others without the *Pl*₂₀ gene (Table 2; Figure 3b). These *Pl*₁₈ and *Pl*₂₀ diagnostic markers identified are of great importance and usefulness to assist selection for both genes in sunflower breeding programs.

3. Discussion

Similar to other disease resistance genes in crops, DM *R* genes were found mostly in clusters along sunflower chromosomes 1, 4, 8, and 13. Out of 29 mapped DM *R* genes, eight in chromosome 1 included sub-cluster I of *Pl*_{Arg}, *Pl*₂₃, *Pl*₂₄ and *Pl*₃₅, and sub-cluster II of *Pl*₁₃, *Pl*₁₄, *Pl*₁₆ and *Pl*₂₅, six each in chromosome 4 (*Pl*₁₇, *Pl*₁₉, *Pl*_{27–29}, and *Pl*₃₃) and 8 (*Pl*₁, *Pl*₂, *Pl*₆, *Pl*₇, *Pl*₁₅, and *Pl*₂₀), and seven (*Pl*₅, *Pl*₈, *Pl*₂₁, *Pl*₂₂, *Pl*₃₁, *Pl*₃₂, and *Pl*₃₄) in chromosome 13 (Supplementary Table S1). Most of the clusters are characterized as having NLR motifs, which are the major class of disease-resistant genes found in flowering plants [14–17]. In the present study, the predicted candidate genes for both *Pl*₁₈ and *Pl*₂₀ are NLR-type genes, which are strongly indicative of disease-resistant genes, as found in chickpea [18], soybean [19], maize [20], wheat [21], and rice [22].

Genomic regions encompassing NLR clusters are very likely attributed to duplications in which chromosome doubling is presumed to occur during sunflower evolution [13]. Three models have been proposed for duplicated genes, i.e., pseudogenized (loss of regulatory sub-function), sub-functionalized (partitioning of the function between daughter copies) and/or neo-functionalized (functional diversification) [23]. With a fair chance that some of the NLR-involved genes within the clusters were pseudogenized after duplication and during the interaction with pathogens, it is possible that the genes conferring resistance might not be present in the reference genome of XRQ, even though the typical NLR motifs were present. To validate their candidacy, the candidate genes predicted from the reference genome need to be landed to the resistance donor lines, followed by functional characterization. Because of the short reads from the Illumina whole-genome sequencing and high level of repetitive sequences in the sunflower genome, it is difficult to assemble a scaffold covering the entire gene sequence from the sequenced donor line because most of the contigs (81%) and scaffolds (86%) assembled in a previous study ranged between 100 and 500 bp, and only 6% of contigs and 8% of scaffolds were over 1 kb, leaving a large number of gaps [24]. The physical localization of each *Pl*₁₈ and *Pl*₂₀ to a region less than 100 kb on chromosomes 2 and 8, respectively, in the present study represents a significant step toward the final cloning and functional characteristics of these *R* loci. PacBio long-read target region sequencing provides a powerful tool to capture these two genomic regions harboring the candidate genes. This technology combined with analysis of ethyl methanesulfonate (EMS)-induced mutants will allow us to distinguish among the possibilities and to uncover the genetic and molecular basis of DM disease resistance in sunflower.

Unlike most DM *R* genes located on clusters in sunflower chromosomes 1, 4, 8, and 13 mentioned above, only two genes, *Pl*₁₈ and *Pl*₂₆, were mapped to sunflower chromosome 2 [25]. As a result of the limited mapping resolution and lack of recombination in the region, *Pl*₂₆ was placed in a relatively larger interval of 114 Mb physically on XRQ (26,000,000–140,000,000 bp), while *Pl*₁₈ was located within the 128,640,208–129,297,096 bp interval of chromosome 2. *Pl*₁₈ originated from *H. argophyllum* accession PI 494573 collected from Texas, U.S., while *Pl*₂₆ originated from *H. annuus* HAS103. Although *Pl*₁₈ falls within the large region encompassing *Pl*₂₆ on chromosome 2, their different origins suggest that they are different resistance genes. Further fine mapping of *Pl*₂₆ would elucidate the genetic relationship of the two genes.

Chromosome 8 of sunflower represents the largest and most important NLR cluster, including 54 NLR loci [26]. The DM *R* gene cluster was located in the first and largest sub-cluster containing *Pl*₁, *Pl*₂, *Pl*₆, *Pl*₇, *Pl*₁₅ and *Pl*₂₀ and two rust *R* genes *R*₁ and *R*₁₅ [1]. *Pl*₂₀ originated from *H. argophyllum* is different from other *Pl* genes in the cluster in which *Pl*₁, *Pl*₂ and *Pl*₆ were from wild *H. annuus*, *Pl*₇ was from *H. praecox*, and *Pl*₁₅ was identified from an Argentinian restorer inbred line [1,27–31]. *Pl*₂₀ was immune to all *P. halstedii* races identified in North America, including those predominant and virulent races; however, the remaining *Pl* genes, except *Pl*₁₅ in the cluster, have already been overcome by some or all of the identified *P. halstedii* races. In the current study, four SNP markers, C8_7890010, C8_7895128, C8_7919216, and C8_8800366, showed unique PCR patterns in HA-DM7 (*Pl*₂₀-introgressed line), distinguishing it from other *Pl* genes in the cluster. All the findings suggest

that *Pl*₂₀ is a novel and most effective DM *R* gene that serves as a powerful resistance resource for durable DM control in sunflower.

MAS has been extensively used in modern plant breeding, especially for traits controlled by single genes. The success of MAS is influenced by the relationship between the markers and the genes of interest, and it is important that the recombination frequency between the target gene and the marker is as low as possible. Our high-resolution genetic maps and the diagnostic markers for *Pl*₁₈ and *Pl*₂₀ developed will be useful tools facilitating the transfer of these new genes to elite sunflower lines in breeding programs.

4. Materials and Methods

4.1. Mapping Populations and Evaluation Panel

The *Pl*₁₈ F₂ initial mapping population was created from a cross of nuclear male sterile (NMS) HA 89 × *H. argophyllum* accession PI 494573, and F₁ was backcrossed with normal HA 89, including 142 BC₁F₂ individuals [7]. Similarly, the *Pl*₂₀ F₂ initial mapping population was developed from a cross of NMS HA 89 × *H. argophyllum* accession PI 494578, including 114 BC₁F₂ individuals [7]. The *H. argophyllum* accessions, PI 494573 and PI 494578, were found to be resistant to new races of *P. halstedii*, while HA 89 is susceptible to all *P. halstedii* races [6]. The germplasms HA-DM1 with *Pl*₁₈ and HA-DM7 with *Pl*₂₀ were developed and released in 2015 and 2019, respectively [8], and were each used as the *Pl*₁₈ and *Pl*₂₀ donor lines for whole-genome resequencing for high-density mapping of both *R* genes.

For fine mapping of *Pl*₂₀, recombinants were screened from 2,485 BC₁F₃ individuals selected from the previously characterized BC₁F_{2.3} families heterozygous for *Pl*₂₀. Each selected heterozygous F₃ family equates to a segregating F₂ population for the *Pl*₂₀ gene.

The specificity of diagnostic *Pl*₁₈ and *Pl*₂₀ SNP markers was tested in the sunflower evaluation panel, consisting of 96 sunflower inbred lines of diverse origins. This panel includes 24 and 17 lines with different DM and rust *R* genes, respectively (Supplementary Table S2).

4.2. SNP Marker Development from Whole-Genome Resequencing

HA-DM1 (*Pl*₁₈) and HA-DM7 (*Pl*₂₀) were sequenced at the whole-genome level separately by CD Genomics Inc. using the Illumina HiSeq sequencing platform. According to the protocols, genomic DNA of HA-DM1 and HA-DM7 was first checked for quality to ensure that the level of contamination and degradation was low enough to meet their requirements. The quality genomic DNA was sheared with the use of an S/E210 focused ultrasonicator (Covaris, Woburn, MA, USA) for library construction. Qualified libraries for either *Pl*₁₈ or *Pl*₂₀ were pooled and subjected to sequencing at 40 × genome coverage. The raw reads containing adaptors, reads with >1% ambiguous bases, and reads with low quality (greater than 50% bases less than 15 Q score) were removed and excluded for further analysis. The clean reads were aligned to the two reference genomes of XRQ (<https://www.heliagene.org/HanXRQ-SUNRISE/>) and HA412-HO (<https://www.heliagene.org/HA412.v1.1.bronze.20141015/>), respectively. After filtering of low-quality reads, a total of 1,166,680,112 (99.09%) HA-DM1 reads and 1,023,555,572 (98.74%) HA-DM7 reads were mapped to the references XRQ and HA412-HO, respectively. All SNPs and InDels were identified using the mapped reads. The SNP markers were named with prefix C2, S2, C8 or S8 followed by a number representing the physical position of the SNPs along either chromosome 2 or 8 of each reference genome assembly. C2 and C8 represent the SNPs from chromosomes 2 and 8 of the XRQ reference genome, while prefixes S2 and S8 represent the SNPs from chromosomes 2 and 8 of the HA412-HO reference genome.

4.3. Genotyping of PCR-Based SNP Markers and Linkage Analysis

Polymerase chain reaction (PCR)-based SNP primers were designed with the Primer 3 program, and specific mismatches and length polymorphisms for SNP primers were created (Supplementary Table S3) as described by Qi et al. [32] and Long et al. [33] based on SNP flanking

sequences (Supplementary Tables S4 and S5). PCR for SNPs was conducted as described by Ma et al. [34], and amplicons were separately scored on a 6.5% polyacrylamide gel using an IR2 4300/4200 DNA analyzer (LI-COR, Lincoln, NE, USA).

The chi-square (χ^2) test was performed on genotyping data of each marker to test for goodness-of-fit to the Mendelian segregation ratio, i.e., 1:3 for dominant markers and 1:2:1 for co-dominant markers. Upon the exclusion of those unfitted, markers fitting Mendelian ratios were linkage analyzed with either *Pl*₁₈ or *Pl*₂₀ phenotyping data using JoinMap 4.1 software in which a regression mapping algorithm and Kosambi's mapping function were selected [35]. The cutoffs of linkage analysis among markers were set at a likelihood of odds (LOD) \geq 3.0 and maximum genetic distance \leq 50 centimorgans (cM).

4.4. Phenotypic Evaluation of Recombinants

*Pl*₂₀ recombinants identified with the respective flanking markers were tested for DM resistance using the *P. halstedii* isolate of race 734, together with their introgressed line HA-DM7 and susceptible parent HA 89, using the whole seedling immersion method as described by Gulya et al. [36] and Qi et al. [32]. Briefly, approximately 40 seeds from each recombinant family were germinated and inoculated with the *P. halstedii* isolate of race 734 after 2–3 days, and at least 30 seedlings for each recombinant family were evaluated. Susceptible seedlings showed sporulation on cotyledons and true leaves, and resistant seedlings showed no sporulation. The genotype of each recombinant was determined as homozygous susceptible if all seedlings in the recombinant family showed sporulation on cotyledons and true leaves, homozygous resistant if none of the seedlings exhibited sporulation, and segregating if some seedlings showed sporulation on cotyledons and true leaves while some showed no sporulation.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1422-0067/21/24/9571/s1>, Table S1. Originating species of designated downy mildew resistance genes; Table S2. Evaluation panel of 96 sunflower lines used to determine the specificity of *Pl*₁₈ and *Pl*₂₀ markers; Table S3. Primer sequences of SNP markers mapped in the present study; Table S4. Sequences of SNP markers mapped around *Pl*₁₈ in the present study (The diagnostic SNP markers for *Pl*₁₈ are shown in bold); Table S5. Sequences of SNP markers mapped around *Pl*₂₀ in the present study (The diagnostic SNP markers for *Pl*₂₀ are shown in bold). Reference [37–59] are cited in supplementary materials part.

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