MOLECULAR MAPPING OF THE DISEASE RESISTANCE GENE AND ITS IMPACT ON SUNFLOWER BREEDING

Lili QI[1], Guojia MA[2], Zahirul TALUKDER[3], Gerald SEILER[1], Brent HULKE[1], Chao-Chien JAN[1], Tom GULYA[1]

[1]USDA- Agricultural Research Service, Northern Crop Science Laboratory, 1605 Albrecht Blvd N, Fargo, ND, 58102-2765 USA, lili.qi@ars.usda.gov; Gerald.Seiler@ars.usda.gov; brent.hulke@ars.usda.gov; ChaoChien.Jan@ars.usda.gov; tjgulya@gmail.com

[2]Department of Plant Pathology, North Dakota State University, Fargo, ND, 58102 USA, guojia.ma@ndsu.edu

[3]Department of Plant Sciences, North Dakota State University, Fargo, ND, 58102 USA, Zahirul.Talukder@ars.usda.gov

ABSTRACT

Rust, downy mildew (DM), and Sclerotinia diseases are the major yield limiting factors in global sunflower production. The use of resistant hybrids, where available, is the most efficient measure of controlling these diseases. Development of DNA markers linked to the resistance genes will facilitate molecular breeding of disease-resistant hybrids in sunflower.

We have molecularly mapped seven rust *R*-gene loci, R_2, R_4, R_5, R_{11}, R_{12}, R_{13a}, and R_{13b} to linkage groups (LG) of the sunflower genome, developed both simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers linked to these *R*-genes, and used them in marker-assisted gene pyramiding in sunflower.

Two new DM *R*-genes, Pl_{17} and Pl_{18}, were mapped to LGs 4 and 2 of the sunflower genome, respectively, different from all known DM *R*-genes previously mapped to LGs 1, 8, and 13. Pl_{18} was recently transferred from *H. argophyllus* into cultivated sunflower. We also identified diagnostic SNP markers linked to the DM *R*-genes, Pl_{Arg} and Pl_8.

Quantitative trait loci (QTL) for *Sclerotinia* basal stalk rot resistance were identified in a sunflower recombinant inbred line population derived from the cross HA 441/RHA 439 using genotyping-by-sequencing approach. A total of six QTL were identified, one each on linkage groups (LGs) 4, 9, 10, 11, 16 and 17, each explaining between 6 and 29% of the observed phenotypic variance in the RIL population. The QTL on LGs 10 and 17 were detected in multiple environments with very high LOD values (5.49-12.01), while the remaining QTL were detected in single environment. A combined analysis with integrated phenotypic data across environments also detected the QTL on LGs 10 and 17, each explaining 32 and 20%, respectively of the phenotypic variation for the trait.

Key words: Sunflower, disease, resistance gene, genetic mapping, quantitative trait loci

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the fifth most important oilseed crop in the world and provides about 13 % of the world's edible oil. In the United States, the majority of sunflower produced is the oil-type, whereas 10-20% of production is confection, a high value seed product used primarily in human diets as a snack. Rust, downy mildew (DM), and Sclerotinia diseases are the major yield limiting factors in sunflower production of North

America and the world. The development of disease resistant sunflowers is a major goal of sunflower breeders because this effort may increase yield stability.

Rust and downy mildew, caused by the fungus, *Puccinia helianthi* Schwein. and the oomycete *Plasmopara halstedii* (Farl.) Berl. et de Toni, respectively, are serious sunflower diseases in the world. Genetic studies of resistance to rust and downy mildew in sunflower have indicated that the sources of resistance to both diseases are usually controlled by single dominant genes. The rust and DM resistance genes (*R* genes) have frequently been deployed singly in sunflower production, and as a consequence, the commercial life of resistant hybrids is quickly challenged as new *P. helianthi* and *P. halstedii* races with increased virulence evolve (Gulya and Markell 2009; Gulya et al. 2011; Moreno et al. 2012; Viranyi et al. 2015). Effective breeding strategies are needed to avoid rapid breaking down of resistance mediated by race-specific genes in sunflower. Gene pyramiding, which aims to combine different *R*-genes from multiple parents into a single genotype is considered an effective strategy to increase the durability of disease resistance (Singh et al. 2001; Mago et al. 2011). However, phenotype-based gene pyramiding cannot track the accumulation of resistance genes, especially when different *R*-gene cannot be distinguished using pathogen bioassays. Thus, mapping rust and DM *R* genes and developing of robust DNA markers that are closely linked to the specific genes are required to facilitate this breeding approach and add precision to selection.

Sclerotinia sclerotiorum (Lib.) de Bary, a necrotrophic fungus, causes three distinctly different diseases on sunflower, basal stalk rot (BSR) or wilt, mid-stalk rot (MSR), and head rot (HR). Unlike other hosts, BSR or wilt symptom starts from root infection resulting from myceliogenic germination of sclerotia. MSR commonly begins as a leaf infection, while HR infection begins on capitula; both symptoms are incited by airborne ascospores released from carpogenic germination of sclerotia. The MSR is not as commonly observed in the United States as the BSR and HR, and the latter two are serious problem in sunflower-growing areas of the humid temperate, as well as tropical and sub- tropical regions of the world. As the mode of infection for the two important sunflower diseases caused by *S. sclerotiorum* varies, the underlying genetics of resistance for the two diseases also appears to be different, effectively doubling the effort needed to combat the pathogens (Talukder et al., 2014a). The BSR resistance is genetically complex and conditioned by multiple genes, each having a small effect (Talukder et al., 2014b). Little is known about the quantitative trait loci (QTL) for resistance to Sclerotinia BSR in sunflower. Here, we reported development of a genetic map of sunflower using SNP markers generated using genotyping-by-sequencing (GBS) approach and identify QTL associated with BSR resistance in a high resolution genetic location of the cultivated sunflower genome.

MATERIALS AND METHODS

Mapping populations

Populations for mapping of the rust and DM *R*-genes are listed in Table 1. A population of 106 F_7 RILs for QTL mapping of Sclerotinia BSR was developed by single seed descent from a cross between sunflower inbred lines, HA 441 (PI 639164) and RHA 439 (PI 639162). Greenhouse and field screening trials at multiple locations in North Dakota, South Dakota, and Minnesota from 2008 to 2014 revealed that HA 441 and RHA 439 were moderately to highly tolerant to BSR.

Population for rust resistance gene pyramiding in confection sunflower

The confection line HA-R6 carrying a rust *R*-gene R_{13a} was crossed to a BC_3F_2-derived line "12-105" with the pedigree CONFSCLB1*4/HA-R2 (R_5) and a BC_4F_2-derived line '12-55' with the pedigree CONFSCLB1*5/MC29 (R_2) in a greenhouse in 2012, respectively. CONFSCLB1 is a narrow-based maintainer line composite of the confection sunflower that is susceptible to rust. The two F_2 populations were developed to pyramid R_{13a} with R_2 and R_5, respectively, and select homozygous double-resistant plants carrying the *R*-genes from both parents.

Table 1. Susceptible and resistant parents used for development of the mapping populations.

Susceptible parent	Resistant parent	Combination	No. of individuals	F_2 *R*-gene
HA 89	MC29 (USDA)	HA 89/MC29 F_2	120	R_2
HA 89	HA-R3	HA 89/HA-R3 F_2	94	R_4
HA 89	HA-R2	HA 89/HA-R2 F_2	118	R_5
HA 89	*H. annuus* PI 613748	HA 89/PI 613748 BC_1F_2	192	R_{11}
HA 89	RHA 464	HA 89/RHA 464 F_2	140	R_{12}
HA 89	HA-R6	HA 89/HA-R6 F_2	140	R_{13a}
HA 89	RHA 397	HA 89/RHA 397 F_2	140	R_{13b}
HA 89	RHA 464	HA 89/RHA 464 F_2	140	Pl_{Arg}
HA 434	RHA 340	HA 89/RHA 340 F_2	130	Pl_8
HA 234	HA 458	HA 89/HA 458 F_2	186	Pl_{17}
HA 89	*H. argophyllus* PI 494573	HA 89/PI 494573 BC_1F_2	142	Pl_{18}

Plant disease inoculation and score

For rust phenotyping, a North America (NA) rust race 336 was used to inoculate all F_2 and F_3 populations (20 plants for each F_3 family) as described by Qi et al. (2011). Rust evaluations were made at 12–14 d post inoculation to allow full development of symptoms evaluated using both pustule size or infection type (IT) (Yang et al. 1986) and percentage of leaf area covered with pustules (severity) on all inoculated leaves (Gulya et al. 1990). IT 0, 1, and 2 combined with pustule coverage of 0 to 0.5% were classified as resistant, while IT 3 and 4 with pustule coverage more than 0.5% were considered susceptible.

For DM phenotyping, the whole seedling immersion method described by Gulya et al. (1991) was applied for all F_3 population tests (30 seeds for each F_3 family) using the NA DM race 734, which is a new, virulent race identified in the US in 2010 (Gulya et al. 2011). 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.

All 106 RILs from the cross between HA 441 and RHA 439 along with the parents were evaluated for BSR resistance at five environments (locations and/or years) in North Dakota and Minnesota. Field trials were conducted at Carrington, ND in 2012 and 2014, at

Crookston, MN in 2012 and 2013, and at Grandin, ND in 2014. All field screening trials were conducted using a randomized complete block design (RCBD). The 2012 and 2013 field trials were conducted with two replications, while the 2014 trials had four replications. Fields were artificially inoculated at the V-6 growth stage following the method proposed by Gulya et al. (2008) depositing 90 gm of *S. sclerotiorum* mycelia grown on proso millet in furrows of 5 to 7 cm depth on one side next to the row. Disease incidence (DI) was expressed as the percent of plants showing wilting and/or a basal stem rot lesion. An analysis of variance (ANOVA) of DI of the RIL population was performed across all five environments using PROC MIXED in SAS 9.3 (SAS Institute, 2011).

Genetic mapping of disease resistance genes and QTL

For genetic mapping of the rust and DM *R*-genes, simple sequence repeat (SSR) markers that were previously mapped were used first to screen the parents. Bulked segregant analysis (BSA, Michelmore et al. 1991) was performed with those SSRs showing polymorphism between resistant and susceptible parents. SSR markers showing associations with the resistance bulk in BSA were genotyped on F_2 individuals to confirm the marker-trait associations. JoinMap 4.1 was used for linkage analyses and map construction with a regression mapping algorithm and Kosambi's mapping function (Van Ooijen 2006). The Chi-Square (χ^2) test was used to assess goodness-of-fit to the expected segregation ratio for each marker. Newly developed single nucleotide polymorphism (SNP) markers were used to further saturate the region where the *R*-gene resides.

Genotyping-by-sequencing (GBS) using the NGS technology was used for simultaneous discovery and genotyping of SNP markers for the HA 441/RHA 439 RIL population. Genomic DNA from each of the 106 RILs and two parental lines were sent to the Biotechnology Resource Center (BRC) Genomic Diversity Facility at Cornell University for GBS as described by Elshire et al. (2011) and at http://www.biotech.cornell.edu/brc/genomic-diversity-facility/services. A total of 1,236 SNP markers were used for linkage analysis using JoinMap 4.1 (Van Ooijen 2006). The SNPs were named with a prefix of S1 to S17 based on the draft sunflower genome assembly that corresponds to the 17 sunflower linkage groups (LGs), followed by a number representing the physical position of the SNP on the genome. However, there are some SNPs with a prefix of S18, which were discovered in scaffolds yet to be assigned a physical position in the genome. Four-hundred bp nucleotide sequences flanking the SNP position were retrieved from the draft sunflower reference genome, HA412.v1.0.bronze.20140814.fasta.gz. Quantitative trait loci analysis was conducted each environment separately, and also with integrated data across environments. The composite interval mapping (CIM) (Zeng, 1994) as implemented in WinQTL cartographer version 2.5 (Wang et al., 2005) was used to detect QTL. The output of the QTL analysis was verified with PLABQTL version 1.2 (Utz and Melchinger, 2006).

RESULTS AND DISCUSSIONS

Molecular mapping of the rust *R*-genes

Since 2011, we have molecularly mapped seven rust *R*-gene loci, R_2, R_4, R_5, R_{11}, R_{12}, R_{13a}, and R_{13b} to four LGs of the sunflower genome; R_5 in LG2, R_{12} to LG11, R_4, R_{11}, R_{13a} and R_{13b} in LG13, and R_2 in LG14 (Table 2). The results were summarized as follows.

R_5: On the initial SSR map, the R_5 gene from HA-R2 was located on LG2, flanked by two SSR markers, ORS1197 and ORS653 at 3.3 and 1.8 cM of genetic distance, respectively

(Qi et al. 2012a). After screening the 67 LG2 SNP markers, two SNPs, SFW03654 and NSA_000267, were found flanking R_5 at a genetic distance of 0.6 cM and 1.2 cM, respectively. This flanking narrowed the genetic interval containing R_5 from 5.1 to 1.8 cM in length (Qi et al. 2015b).

R_{12}: Virulence phenotypes of seedlings for the F_2 population and $F_{2:3}$ families suggested that a single dominant gene confers rust resistance in RHA 464, and this gene was designated as R_{12}. Bulked segregant analysis identified 10 LG11 SSR markers polymorphic between resistant- and susceptible-bulks. In subsequent genetic mapping, two markers, CRT275 and ZVG53 delimited R_{12} in an interval of 10.6 cM (Gong et al. 2013a). Later, the same F_2 population was used to assign SNP markers to the genetic map. When rust phenotypic data were integrated with SNP marker data, seven linked SNP markers were identified, five on one side (NSA_000064, NSA_003320, NSA_003426, NSA_004155, NSA_008884), and two on the other side (NSA_001570, and NSA_001392), defining an interval less than 2.3 cM surrounding the previously mapped R_{12} gene in LG11 (Talukder et al. 2014c).

R_4, R_{11}, R_{13a}, and R_{13b}: The four rust *R*-gene loci were all mapped to the lower end of LG13. R_{11} from RfANN-1742 is mapped distal to SSR marker ORS316, a common marker among maps related to *R*-gene cluster in the lower end of LG13. This gene is closely linked to a restorer gene *Rf5* at a genetic distance of 1.6 cM, and shared a common marker, ORS728, which was mapped 1.3 cM proximal to *Rf5* and 0.3 cM distal to R_{11} (*Rf5*/ORS728/R_{11}). Two additional SSRs were linked to *Rf5* and R_{11}: ORS995 was 4.5 cM distal to *Rf5* and ORS45 was 1.0 cM proximal to R_{11} (Qi et al. 2012b).

Three rust *R*-genes, R_4 from HA-R3, R_{13a} from HA-R6, and R_{13b} from RHA 397, were all mapped proximal to ORS316 (Qi et al. 2011; Gong et al. 2013b). The allelic analysis indicated that that R_4 and R_{13a} are two distinct rust resistance genes, but very closely linked, whereas, R_{13a} and R_{13b} are the same rust *R*-gene (Gong et al. 2013b; Qi et al. 2015b). The SSR and SNP markers linked to R_4, R_{13a}, and R_{13b} are listed on Table 2.

***R_2*:** Based on phenotypic assessments and SSR marker analyses of the 117 F_2 individuals derived from a cross of HA 89 with MC29 (carrying R_2), R_2 was mapped to LG14 of the sunflower, and not to the previously reported location on LG9. The closest SSR marker HT567 was located at 4.3 cM distal to R_2. Furthermore, 36 selected SNP markers from LG14 were used to saturate the R_2 region. Two SNP markers, NSA_002316 and SFW01272, flanked R_2 at a genetic distance of 2.8 and 1.8 cM, respectively. Of the three closely linked markers, SFW00211 amplified an allele specific for the presence of R_2 in a marker validation set of 46 breeding lines, and SFW01272 was also shown to be diagnostic for R_2 (Qi et al. 2015a).

Molecular mapping of the DM *R*-genes

***Pl_{17}*:** DM resistance in HA 458 has been shown to be effective against all virulent races of *P. halstedii* currently identified in the United States. To determine the chromosomal location of this resistance, 186 $F_{2:3}$ families derived from a cross of HA 458 with HA 234 were phenotyped for their resistance to race 734 of *P. halstedii*. The segregation ratio of the population supported that the resistance was controlled by a single dominant gene, named as Pl_{17}. Bulked segregant analysis using 849 SSR markers located Pl_{17} to LG4, which is the first DM gene discovered in this linkage group. An F_2 population of 186 individuals was screened with polymorphic SSR and SNP primers from LG4. Two flanking markers, SNP SFW04052 and SSR ORS963, delineated Pl_{17} in an interval of 3.0 cM (Qi et al. 2015c). A search for the

physical location of flanking markers in sunflower genome sequences revealed that the *Pl17* region has a recombination frequency of 0.59 Mb/cM, which is a 4-fold higher recombination rate relative to the genomic average. This region can be considered amenable to molecular manipulation for further map-based cloning of *Pl17*.

***Pl18*:** A new dominant DM resistance gene (*Pl18*) transferred from wild *Helianthus argophyllus* (PI 494573) into cultivated sunflower was mapped to LG2 of the sunflower genome using bulked segregant analysis with 869 SSR markers. Since no other *Pl* gene has been mapped to LG2, this gene was novel and designated as *Pl18*. SSR markers CRT214 and ORS203 flanked *Pl18* at a genetic distance of 1.1 and 0.4 cM, respectively. Forty-six SNP markers that cover the *Pl18* region were surveyed for saturation mapping of the region. Six co-segregating SNP markers were 1.2 cM distal to *Pl18*, and another four co-segregating SNP markers were 0.9 cM proximal to *Pl18* (Qi et al. 2016a). The new BC_2F_4-derived germplasm, HA-DM1, carrying *Pl18* has been released to the public. This new line is highly resistant to all *P. halstedii* races identified in the US providing breeders with an effective new source of resistance against downy mildew in sunflower.

Table 2. List of DNA markers closely linked to the rust and downy mildew resistance genes.

Gene donor	Gene	LG	Linked marker	Map position (cM)	Reference
HA-R2	*R5*	2	NSA_001605 SFW03654 ***R5*** NSA_000267	14.4 14.9 15.5 16.7	Qi et al. 2012a, 2015b
RHA464	*R12*	11	NSA03320, NSA_003426, NSA_004155 ***R12*** NSA_001392, NSA_001570	44.6 45.4 46.8	Gong et al. 2013a Talukder et al. 2014C
HA-R3	*R4*	13	ORS316, ZVG61, SFW05240, SFW05630, SFW06095, SFW08283 ***R4*** SFW01497, SFW05453, SFW08875	3.5 4.1 4.8	Qi et al. 2011, 2015a
HA-R9	*R11*	13	ORS728, ***R11*** ORS45	6.1 7.1 9.1	Qi et al. 2012b
HA-R6	*R13a*	13	ORS316, ZVG61, SFW05832, SFW08188 *R13a* SFW05743	3.4 3.8 4	Gong et al. 2013b Qi et al. 2015b
RHA397	*R13b*	13	ORS316, ZVG61, SFW05832, SFW08188 ***R13b*** SFW00757	5.9 6.8 6.8	Gong et al. 2013b Qi et al. 2015b
MC29 (USDA)	*R2*	14	HT567	42.4	Qi et al. 2015a

			SFW00211	43.8	
			R₂	46.7	
			SFW01272	48.5	
RHA 464	*Pl_Arg*	1	NSA_002131, NSA_002798, NSA_008037, NSA_007595	29.7	Qi et al. unpublished data
			Pl_Arg	29.7	
			NSA_001835	30.0	
			NSA_006530	30.5	
H.argophyllus PI494573	*Pl18*	2	SFW03013	2.3	Qi et al. 2016a
			CRT214	2.4	
			Pl18	3.5	
			ORS203	3.9	
			SFW03060	4.4	
HA458	*Pl17*	4	SFW04052	14.3	Qi et al. 2015c
			Pl17	16.4	
			ORS963	17.2	
			SFW08268	18.2	
RHA 340	*Pl8*	13	NSA_000423, SFW01497, SFW08875	1.2	Qi et al. 2016b
			Pl8	1.7	
			SFW06597	2.6	
			NSA_002220, NSA_002251	3.0	

Development of diagnostic SNP markers linked to DM *R*-genes, *Pl_Arg* and *Pl8*

Pl_Arg and *Pl8* both originating from the wild *H. argophyllus* were previously mapped to LGs1 and 13, respectively (Dußle et al. 2004; Bachlava et al. 2011). An F_2 population of 140 individuals from the cross of HA 89 and RHA 464 harboring *Pl_Arg* and *R12* was previously used as a mapping population to map SNP markers in the sunflower genome of the National Sunflower Association (NSA) SNP Consortium project (Talukder et al. 2014c). DM phenotypic data from $F_{2:3}$ families of this population were integrated with SNP marker data. Seventy-eight co-segregating SNP markers were 0.01 cM distal to *Pl_Arg*, and SNP marker NSA_001835 was 0.31 cM proximal to *Pl_Arg*. Genotyping of 80 SNP markers flanking *Pl_Arg* in the 548 collected sunflower lines discovered diagnostic SNP markers for selection of *Pl_Arg* in most of the sunflower backgrounds (Table 2).

A total of 30 seeds from each of 120 F_3 families derived from the cross of HA 434 and RHA 340 (*Pl8*) were inoculated with NA DM race 734 and tested for their resistance in the greenhouse. Forty-one SNP markers were selected from the lower end of LG13 of two SNP maps, which covered the *Pl8* region (Bowers et al. 2012; Talukder et al. 2014c). Nine SNP markers that showed polymorphism between the parents were genotyped in the F_2 population. Three co-segregating SNPs were 0.4 cM distal to *Pl8* and SNP SFW06579 was 1.3 cM proximal to *Pl8* (Table 2, Qi et al. 2016b). Three SNPs, NSA_00423, NSA_02220, and NSA_02251 were used to genotype the 548 sunflower lines. More than 85% of sunflower lines did not share the resistant alleles of both NSA_00423 and NSA_02220 with RHA 340. These two SNPs can be potentially used as diagnostic markers for the selection of *Pl8* in sunflower breeding programs.

QTL mapping of Sclerotinia basal stock rot (BSR) resistance

Quantitative trait loci (QTL) for BSR resistance were identified in a sunflower recombinant inbred line (RIL) population derived from the cross HA 441/RHA 439 (Talukder et al. 2016). The genotyping-by-sequencing (GBS) approach was adapted to discover SNP markers and simultaneously genotyping the RIL population. A genetic linkage map was developed comprising of 1,053 SNP markers on 17 LGs spanning 1,401.36 cM. The RILs were tested in five environments (locations and/or years) for resistance to BSR. Due to the presence of significant genotype × environment interactions, QTL analyses were first performed for each of the five environments separately, followed by a combined QTL analysis using mean disease incidence across environments. A total of six QTL were identified in all five environments, one each on LGs 4, 9, 10, 11, 16 and 17 (Table 3).The QTL on LG10, *Qbsr-10.1* was detected at every environment between 57.9 and 66.5 cM genomic positions with LOD values ranging from 5.5-12.0. This QTL alone explaining 17-29% of the phenotypic variations across five environments. The QTL on LG17, *Qbsr-17.1*was detected in three of the five environments with LOD values ranging from 3.1-7.0. The remaining four QTL, *Qbsr-4.1*, *Qbsr-9.1*, *Qbsr-11.1* and *Qbsr-16.1* were detected in only one environment on LGs 4, 9, 11 and 16, respectively. Each of these QTL explains between 6 and 11% of the phenotypic variation in their respective environment. In the combined analysis with mean BSR disease incidence across environments, the *Qbsr-10.1* and *Qbsr-17.1* were detected with high LOD values each accounting for 32% and 20% of the phenotypic variation, respectively. Alleles conferring increased resistance were contributed by both parents (Table 3).

Table 3. Significant quantitative trait loci (QTL) for *Sclerotinia* basal stalk rot resistance identified in the HA 441/RHA 439 RIL sunflower population in five individual environments

QTL name	Linkage group	Peak QTL position (cM)	Flanking markers (cM position)		$R^{2\dagger}$	1-LOD‡ interval
			Left	Right		
Qbsr-4.1	4	32.0	S4_147688288 (29.1)	S4_135190076 (33.1)	6.4	6.5
Qbsr-9.1	9	45.0	S9_153762438 (45.0)	S9_158145790 (46.6)	9.3	1.5
Qbsr-10.1	10	66.5	S10_288646223 (66.5)	S10_281294015 (67.5)	31.6	1.6
Qbsr-11.1	11	83.2	S14_148877201 (83.2)	S14_148877253 (83.4)	7.7	3.5
Qbsr-16.1	16	87.3	S16_157591485 (87.3)	S16_137964301 (87.9)	10.5	10.4
Qbsr-17.1	17	23.9	SFW02170 (23.7)	S17_228661362 (24.0)	20.2	4.2

†Percentage of phenotypic variance explained by the QTL in the population.
‡LOD, logarithm of odds.
SNP markers nearest to the QTL pick position are underlined

Marker-assisted gene pyramiding in confection sunflower

The DNA markers linked to the genes R_2 and R_{13a} were used to screen 524 F_2 individuals from a cross of a confection R_2 line and HA-R6 carrying R_{13a}. Eleven homozygous double-resistant F_2 plants with the gene combination of R_2 and R_{13a} were obtained. Similarly, a total of 368 F_2 plants from the cross between confection R_5 with HA-R6 were screened by DNA markers linked to the R_5 and R_{13a}. Twelve F_2 plants were identified to be homozygous for a combination of R_5 and R_{13a}. These double-resistant lines will be extremely useful in confection sunflower, where few rust *R*-genes are available, risking evolution of new virulence phenotypes and further disease epidemics. The germplasms HA-R12 carrying R_2 and R_{13a} and HA-R13 harboring R_5 and R_{13a} have been released to the public (Ma et al. 2016).

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