

Molecular mapping of R_{11} , a novel rust resistance gene in sunflower (*Helianthus annuus* L.)

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ABSTRACT

- Sunflower production in North America has recently been threatened by the evolution of new virulent pathotypes of sunflower rust caused by the fungus *Puccinia helianthi* Schwein. Rf ANN-1742, an 'HA 89' backcross restorer line derived from wild annual sunflower (*Helianthus annuus* L), was identified as resistant to North America rust races 336 (the predominant race over three years) and 777 (the most virulent race). The aim of this study was to elucidate the inheritance of rust resistance and identify the chromosome location of the underlying gene in Rf ANN-1742.
- The F_2 and $F_{2:3}$ populations derived from a heterozygous rust resistant plant, 09-519-1, selected from Rf ANN-1742 were inoculated with North America rust race 336. A set of 723 mapped SSR markers of sunflower was used to screen the polymorphism between HA 89 and the resistant plant. The bulked segregant analysis was performed to identify the genomic region harboring the rust resistance gene.
- Chi-squared analysis of rust response segregation in F_2 and F_3 populations revealed that a dominant gene governs rust resistance in the line Rf ANN-1742 and was designated as R_{11} . Bulked segregant analysis subsequently located R_{11} on linkage group (LG) 13 of the public sunflower genetic map. Based on the SSR analyses of 192 F_2 individuals, R_{11} was mapped to the lower end of LG13 and flanked by ORS728 and ORS45, with a genetic distance of 0.3 cM and 1.0 cM, respectively.
- R_{11} is genetically independent from the rust R -genes R_1 , R_2 , and R_5 , but may be closely linked to the rust R -gene R_{adv} derived from wild *H. argophyllus*, forming a large rust R -gene cluster of $R_{adv}/R_{11}/R_4$ in the lower end of LG13. Any combination of the genes R_2 , R_4 , and R_5 with R_{11} would give resistance to a majority of rust races and provide protection against the spread of new rust races. The molecular markers closely linked to the R_{11} gene developed in this study should make this task more feasible, and will allow breeders to effectively select disease-resistant progeny in early segregating generations.

Key words: Gene R_{11} - mapping- rust resistance- simple sequence repeat- sunflower

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INTRODUCTION

Rust, caused by the fungus *Puccinia helianthi* Schwein., is a disease of sunflower that can cause significant losses in both yield and seed quality on susceptible hybrids when conditions are favorable for disease development. In the northern Great Plains area of North America, sunflower production has recently been threatened by the evolution of new virulent pathotypes of sunflower rust. Thirty-nine North American (NA) rust races were identified in 2008 with races 334 and 336 being dominant (Gulya and Markell, 2009). A newly evolved NA rust race 777 was collected in Texas and Kansas, and was able to infect all nine differentials (7350, MC 90, MC 29, P386, HA-R1, HA-R2, HA-R3, HAR-4, and HAR5) (Gulya and Viranyi, 1994). The majority of commercial hybrids are susceptible to the new predominant and virulent races (Gulya and Markell, 2009). Presently, race 777 remains at a low frequency in the rust populations, but can potentially cause serious epidemics if it becomes prevalent in the sunflower growing areas in North America. The limited durability of single *R*-genes made it necessary to continue the discovery and introgression of new *R*-genes.

The germplasm line Rf ANN-1742 was derived from a BC₁F₂ population by crossing cms HA 89 with a wild *H. annuus* accession, PI 613748, which originated from Hinton, Oklahoma, US (Seiler and Jan, 1997). Recent evaluation of rust resistance identified the Rf ANN-1742 line as resistant to the predominant and virulent races, 336 and 777, respectively (Qi et al., 2011a). Utilization of this novel source along with some defeated genes in commercial sunflower hybrids in North America will significantly reduce the occurrence of new pathotypes and consequent yield losses due to the disease.

Molecular mapping of rust resistance genes has been carried out to accelerate the introgression of these genes into elite cultivars and *R*-gene pyramiding in sunflower. The rust resistance genes, *R*₁ and *R*₂, were mapped to linkage groups (LGs) 8 and 9 of sunflower, respectively (Lawson et al., 1998, 2011; Yu et al., 2003). Both *R*_{adv} and *R*₄ genes were mapped to LG13 (Lawson et al., 1998; Yu et al., 2003; Bachlava et al., 2011; Qi et al., 2011b), whereas, the *R*₅ gene was mapped to LG2 (Qi et al., 2011c). Here we report genetic mapping of a novel rust resistance gene, *R*₁₁, in LG13.

MATERIALS AND METHODS

Plant materials and mapping population

The Rf ANN-1742 line released as a fertility restorer in 1997 showed resistance to virulent rust races tested in a heterozygous condition (Seiler and Jan, 1997; Qi et al., 2011a). A resistant plant, 09-519-1, was selected from Rf ANN-1742 and self-pollinated. A progeny test from the selfed plant for reaction to rust indicated that 09-519-1 was still heterozygous for the rust resistance gene and fertility restoration, and could serve as an F₁ hybrid. An F₂ population of 207 plants derived from 09-519-1 was planted in the greenhouse in May 2010. Seeds from each F₂ plant were harvested separately to develop F₃ families. A total of 146 rust resistant/fertile F_{2,3} families were progeny-tested to determine homozygous and heterozygous resistant plants. The genotype of the individual F₂ plants was assigned based on the F₃ family test.

Evaluation of rust resistance

A total of 207 F₂ plants along with parental line HA 89 were inoculated with race 336 of *P. helianthi* at the four-leaf seedlings stage using the procedure previously described (Gulya and Masirevic, 1996; Qi et al., 2011b). Infection type (IT), described by Yang et al. (1986), combined with percentage of leaf area covered with pustules (severity), described by Gulya et al. (1990), were assessed 12-14 days post inoculation. Infection type 0, 1, and 2 combined with pustule coverage of 0 to 0.5% were classified as resistant, and IT 3 and 4 with pustule coverage more than 0.5% were considered susceptible.

After scoring for rust infection, the 207 F₂ plants were transferred to 2-gallon pots for fertility evaluation, and grown to obtain F₃ seeds in the greenhouse. A total of 146 resistant/fertile F₃ families were subjected to progeny-test. Twenty plants of each F₃ family were inoculated with race 336 at the four-leaf stage in October 2010 and April 2011, respectively. The F₃ families were classified as homozygous resistant if all seedlings had low IT and severity or segregating if seedlings varied for low and high IT and severity.

DNA extraction and PCR conditions

Genomic DNA was isolated from young leaves of the parents and F₂ individuals using the Qiagen DNeasy 96 plant kit (Qiagen, Valencia, CA). The quantity and the quality of DNA were determined with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Polymerase chain reaction (PCR) was conducted on a Peltier thermocycler (Bio-Rad Lab, Hercules, CA, USA) with a touchdown program as described by Qi et al. (2011a). PCR products were diluted 20- to 120-fold before analysis. SSR fragments were size separated by using an IR2 4300 DNA Analyzer (Li-COR, Lincoln, Nebraska).

Bulked segregant analysis (BSA) and genetic mapping

For marker screening of parents, we selected an F₃ plant (10-275-2) homozygous for both rust resistance and fertility restoration as the resistant parent versus susceptible parent HA 89. Two bulks of DNA were made. DNAs of the 10 homozygous resistant F₂ plants, based on the F₃ progeny-test, were pooled in an equal amount to create a resistant (R) bulk. Likewise, DNAs of the 10 homozygous susceptible F₂ plants were pooled to produce a susceptible (S) bulk. Genomic DNAs of HA 89 and 10-275-2 were first screened with a set of 723 sunflower SSR primers to detect polymorphic markers between parents. The bulked segregant analysis (Michelmore et al., 1991) was performed with polymorphic SSRs to determine the chromosome region containing the *R₁₁* gene. A total of 192 F₂ plants were used for *R₁₁* mapping.

The phenotype and SSR data were combined for linkage analysis. Marker order and map distance were estimated using MapMaker software for the Macintosh with default parameters of LOD =3.0. The recombination fractions were transformed by the Kosambi mapping function to estimate the map distance.

RESULTS

Inheritance of the rust resistance gene R₁₁

The inbred line HA 89 was highly susceptible to rust race 336 with an IT 4 and more than 20% of the leaves covered with pustules, whereas the selected homozygous plant 10-275 from an F₂ population was resistant, showing localized necrosis at infection sites with an IT 2 and 0.1-0.5% of the leaves covered with pustules. The 207 F₂ individuals segregated at a ratio of 158R:49S which did not differ significantly from the expected 3:1 ratio ($\chi^2=0.13$, $df=1$, $P=0.718$). These results support the previous hypothesis that a dominant gene governs rust resistance in the line Rf ANN-1742 (Qi et al., 2011a), and this gene has been designated as *R₁₁*.

Forty-nine rust susceptible F₂ plants were also completely male-sterile, whereas, only three of 158 rust resistant F₂ plants were male-sterile (155MF:52MS), indicating that the rust resistance gene is closely linked to the restorer gene in the coupling phase in this population. Fifty-two male-sterile plants did not produce any seeds. Rust phenotyping of 146 resistant/fertile F_{2,3} families (20 plants per family) showed that the F₂ population had 45 homozygous resistant and 101 heterozygous resistant plants. A Chi-square test indicated that this fits a 1RR: 2Rr segregation ratio ($\chi^2=0.303$, $df=1$, $P=0.657$), which would be expected for a single gene trait segregating 1 homozygous resistant: 2 heterozygous resistant: 1 homozygous susceptible. The results confirm that the gene *R₁₁* fits a single gene model (Seiler and Jan, 1994; Qi et al., 2011a).

Bulked segregant analysis

Out of 723 SSR primer pairs used, 73 showed polymorphism between the susceptible HA 89 and resistant 10-275-2, an average of ~10% polymorphism. Fifteen of 17 linkage groups of sunflower had at least some polymorphic marker coverage, the lone exceptions being LGs 4 and 15. The minimum number of polymorphic SSRs on a linkage group was one (LGs 1, 5, and 6) and maximum number was 24 (LG11). The bulked segregant analysis (Michelmore et al., 1991) was performed to identify the genomic region harboring the rust resistance gene. The parents, two bulks, and two F₂ individual plants selected, 10-149-45 homozygous susceptible and 10-149-68 homozygous resistant, were screened with 50 polymorphic SSRs from LGs 2 (8 SSRs), 9 (10 SSRs), 11 (24 SSRs), and 13 (8 SSRs). Only SSR primers from LG13 generated polymorphic DNA fragments between the two bulks, indicating the location of the gene *R₁₁* in LG13. Of the eight polymorphic SSRs of LG13, six showed the HA 89 allele in the S-bulk and

susceptible F_2 plants, whereas, the R-bulk shared the same PCR pattern as the resistant line 10-275-2 and the resistant F_2 plant selected (Fig. 1).

Surprisingly, out of 24 polymorphic SSRs in LG11, 22 showed no HA 89 allele, the recurrent parent used to develop the Rf ANN-1742 mapping population. Both the susceptible F_2 plant and the S-bulk had the same allele as the resistant plants and the R-bulk (Fig. 1). These markers detected a ~ 60 cM segment of donor DNA that showed no evidence of recombination in this region. Similarly, no HA 89 allele was detected in the F_2 population with 10 polymorphic SSR markers from LG9. These markers are located on the upper end encompassing a ~38 cM segment of wild DNA introgression. Of eight polymorphic SSRs of LG2, only three detected the HA 89 allele in the population. These markers were positioned to the lower end of LG2, a region of higher recombination (Fig. 1). The results indicated that the chromosome segments introduced from wild annual species were transferred as coherent linkage blocks on LGs 2, 9, and 11 in the cultivated sunflower background, and recombination appears to be suppressed in these regions.

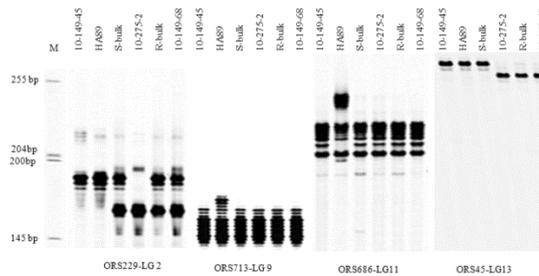


Fig 1. PCR pattern of parental and bulked DNA samples with SSR primers of ORS229, ORS713, ORS686, and ORS45. ORS229 detected HA 89 allele in the F_2 population, but did not show polymorphism between two bulks, whereas, ORS713 and ORS686 did not detect HA 89 allele in the population. ORS45 detected polymorphism between bulks. 10-149-45: homozygous susceptible F_2 plant; S-bulk from the homozygous susceptible F_2 plants; 10-275-2: $F_{2,3}$ homozygous resistant plant; R-bulk from the homozygous resistant F_2 plants; 10-149-68: homozygous resistant F_2 plant. M: 50 -700-bp DNA ladder (Li-COR. Inc. Lincoln, Nebraska, US). PCR products were diluted 20-fold and were size-separated in an IR2 4300 DNA Analyzer (Li-COR, Lincoln, Nebraska). The PCR fragment size amplified by SSR primers included a 19-bp M13 tail primer.

Genetic mapping of the gene R_{11}

Eight polymorphic SSRs in LG13 were assayed across the mapping population of 192 F_2 progenies to confirm linkage with R_{11} . Recombination mapping showed that these eight polymorphic markers (5 co-dominant and three dominant) along with the R_{11} locus were all located in the lower end of LG13 encompassing a genetic distance of 17.1 cM (Fig. 2). The marker order was found to be in good agreement with those of Tang et al. (2003) and Qi et al. (2011b). The rust resistance gene R_{11} was found to be closely linked to markers ORS728 and ORS45 at a genetic distance of 0.3 cM and 1.0 cM, respectively. ORS728 was previously mapped to two LGs, 1 and 13 (Tang et al., 2003; Yu et al., 2003). The primers amplified two fragments in both HA 89 and 10-275-2. The top fragment showed no polymorphism between the two lines, whereas the bottom one was polymorphic, generating a co-dominant marker linked to the R_{11} gene.

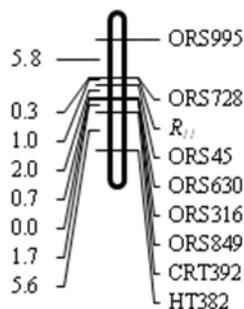


Fig. 2 Map position of the gene R_{11}

DISCUSSION

In this study, we mapped a novel rust resistance gene, R_{11} , derived from wild *H. annuus* to LG13 in sunflower. Out of 68 markers in LG13 screened in the study, only eight SSRs detected wild donor alleles in the population. All of them were mapped to the lower end of LG13, indicating that an alien chromosome segment carrying the R_{11} gene represents a coherent linkage block in the cultivated sunflower background. Two co-dominant SSR markers, ORS728 and ORS45, flank R_{11} at 0.3 and 1.0 cM of genetic distances, respectively, and are well suited for marker-assisted selection.

The lower end of sunflower LG13 was reported to harbor the second largest cluster of nucleotide binding site-leucine-rich repeat (NBS-LRR) encoded by plant R -genes identified in sunflower, and is considered as a large R -gene cluster due to the extensive number of R -genes mapped to this chromosome segment (Lawson et al., 1998; Bert et al., 2001; Yu et al., 2003; Radwan et al., 2008; Qi et al., 2011b). This region harbors downy mildew R -gene Pl_5/Pl_8 and rust R -genes R_{adv} , R_{11} , and R_4 . Tracing the origin of three rust R -genes indicated that they were derived from diverse sources (Gulya 1985; Miller and Gulya, 1988; Seiler and Jan, 1997). Rust tests reveal that each gene encodes different rust resistance specificities. R_{adv} is resistant to race 700 (NA race 4, Miller and Gulya, 1988), but is not effective against the new virulent races of 336 and 777 (Qi et al., 2011a). Tests with race 777 distinguished R_4 and R_{11} ; the first being susceptible, the second resistant, but both these genes are resistant to race 336 (Gulya and Markell, 2009; Qi et al., 2011a).

In spite of the location of three rust R -genes on the lower end of LG13, there are no markers in common. The markers, ZVG61 and ORS581, are closely linked to the R_4 gene, but did not show polymorphism in the Rf ANN-1742 line, and were mapped to the distal end of LG13 (Qi et al., 2011b). The polymorphic fragment of SCX20₆₀₀ linked to $R_{adv}/P2$ was also not present in the Rf ANN-1742 line. Overall, by combining pedigree information, resistance specificity, and molecular mapping, our data suggests that R_{11} is closely linked to R_{adv} forming a large rust R -gene cluster of $R_{adv}/R_{11}/R_4$ in the lower end of LG13, although we cannot exclude the possibility that R_{11} is an allele of R_{adv} .

It has become abundantly clear that cultivars with single genes for resistance are of limited value because race-specific R -genes can obviously be overcome by new pathotypes relatively rapidly. Until now, six of nine sunflower rust resistance genes were genetically mapped to LGs 2 (R_5), 8 (R_1), 9 (R_2), and 13 ($R_{adv}/R_{11}/R_4$), providing an opportunity to combine more rust genes in an inbred line (Lawson et al. 1998, 2011; Yu et al. 2003; Bachlava et al., 2011; Qi et al., 2011b, 2011c). The genes R_2 , R_4 , and R_5 have been thoroughly studied for their reaction to 300 NA rust isolates in the years 2007-2008 (Gulya and Markell, 2009). Any combination of these genes with R_{11} would give resistance to a majority of rust races and provide protection against the spread of new rust pathotypes. The fact that rust resistance is conferred by single genes will also facilitate pyramiding them together with other R -genes. For example, $Pl_8/R_{adv}+R_{11}$ would give resistance to all NA downy mildew races and the most virulent rust races. The molecular markers closely linked to the different resistance genes should make this task more feasible, and will allow breeders to effectively select disease-resistant progeny in early segregating generations.

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