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ORIGINAL PAPER

Molecular tagging of a novel rust resistance gene R_{12} in sunflower (*Helianthus annuus* L.)

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Abstract Sunflower production in North America has recently suffered economic losses in yield and seed quality from sunflower rust (*Puccinia helianthi* Schwein.) because of the increasing incidence and lack of resistance to new rust races. RHA 464, a newly released sunflower male fertility restorer line, is resistant to both of the most predominant and most virulent rust races identified in the Northern Great Plains of the USA. The gene conditioning rust resistance in RHA 464 originated from wild *Helianthus annuus* L., but has not been molecularly marked or determined to be independent from other rust loci. The objectives of this study are to identify molecular markers linked to the rust resistance gene and to investigate the allelism of this gene with the unmapped rust resistance genes present in HA-R6, HA-R8 and RHA 397. 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 ten markers polymorphic between resistant and susceptible bulks. In subsequent genetic mapping, the ten markers covered 33.4 cM of genetic distance on linkage group 11 of sunflower. A co-dominant marker CRT275-11 is the closest marker distal to R_{12} with a genetic distance of 1.0 cM, while ZVG53, a dominant marker linked in the repulsion phase, is proximal to R_{12} with a genetic distance of 9.6 cM. The allelism test demonstrated that R_{12} is not allelic to the rust resistance genes in HA-R6, HA-R8 and RHA 397, and it is also not linked to any previously mapped rust resistance genes. Discovery of the R_{12} novel rust resistance locus in sunflower and associated markers will potentially support the molecular marker-assisted introgression and pyramiding of R_{12} into sunflower breeding lines.

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Introduction

Sunflower rust, incited by *Puccinia helianthi* Schwein., has recently emerged as one of the most serious diseases of sunflower (*Helianthus annuus*) in North America. The pathogen is a macrocyclic and autoecious basidiomycete capable of infecting commercial and volunteer *H. annuus* and other wild *Helianthus* species. Prior to 2008, the occurrence of urediniospores did not typically occur until the late reproductive growth stages of commercial sunflowers and the pycnial and aecial stages of the pathogen were rarely observed. As a result of late disease onset, economic losses to the disease were rare. In late June and July in 2008, aecia occurrence was widespread across the North Dakota and Minnesota sunflower growing region (Markell et al. 2009). The early occurrence of the pathogen led to economic loss in the forms of yield and quality reductions and increased fungicide inputs. Similarly, the occurrence of aecia demonstrated widespread sexual recombination of the pathogen.

Cultivation of resistant hybrids is the preferred management strategy for sunflower rust due to low input cost, reduced labor, and no negative environmental impact. However, the majority of commercial hybrids are

susceptible to the newly emerged predominant and virulent rust races and, until recent epidemics, only limited effort has been made to breed for rust resistance (Gulya 2006; Gulya and Markell 2009; Qi et al. 2011a). Several genes conferring rust resistance, e.g., R_1–R_5, R_{10}, R_{11}, Pu_6, and R_{adv}, have been identified in sunflower (Putt and Sackston 1963; Miah and Sackston 1970; Miller et al. 1988; Yang et al. 1989; Goulter 1990; Lambrides and Miller 1994; Lawson et al. 1998; Bachlava et al. 2011; Qi et al. 2011b, 2012a, b). However, only R_{11} provides resistance to North America (NA) rust races 336 and 777, the predominant and most virulent phenotypes in the Northern Great Plains, respectively (Gulya and Markell 2009; Qi et al. 2011a, 2012b). All of the remaining *R*-genes have been overcome by new virulent races such as race 777. Pyramiding multiple resistance genes from diverse sources into a single host genotype will reduce the possibility of resistance ineffectiveness resulting from pathotype evolution. However, the selection of individuals carrying several resistant genes is limited by classical breeding. Identification of molecular markers closely linked to resistance genes will facilitate genotype selection for rust gene pyramiding.

Experimental tests of 104 sunflowers previously released and collectively labeled as rust resistant revealed that only 13 lines were resistant to rust races 336 and 777, while the remaining 91 lines were all susceptible to race 777 (Qi et al. 2011a). Among the 13 resistant lines, 9 were released by the USDA-Agricultural Research Service (USDA-ARS). Four of them, HA-R6, HA-R8, RHA 397, and RHA 464 (Miller and Gulya 1997, 2001; Hulke et al. 2010), are inbred lines, and five, PH3, PH4, PH5, TX16R, and Rf ANN-1742 (Seiler and Jan 1997; Jan et al. 2004; Jan and Gulya 2006), are germplasm lines in which rust resistance is segregating, with the exception of PH3, which does not appear to segregate. Three entries were commercial hybrids from Australia (Hysun 36, Hysun 37, Hysun 47), while PI 650362 was an old breeding line from France. These lines provide a diverse source of resistance genes, but none of them are currently mapped, except the resistance gene R_{11} in Rf ANN-1742 (Qi et al. 2012b).

RHA 464 was released as a male fertility restorer line resistant to both races 336 and 777 (Hulke et al. 2010; Qi et al. 2011a). The gene conditioning rust resistance in RHA 464 is a single dominant locus derived from PI 413047, a wild *H. annuus* collected near Slough Bridge, California, USA in 1974 (http://www.ars-grin.gov). RHA 464 also carries the downy mildew [*Plasmopara halstedii* (Farl.) Berl. et de Toni] resistance gene Pl_{ARG} derived from wild *Helianthus argophyllus* Torrey & A. Gray which was mapped to sunflower linkage group (LG) 1 (Dußle et al. 2004; Hulke et al. 2010). Donor sources that carry resistance to more than one disease are favored by sunflower breeders. However, the rust resistance gene in RHA 464 has not been molecularly marked and little is known about the allelic relationships of this gene with other rust loci, limiting the development of optimal breeding strategies. The objectives of this study are to identify molecular markers linked to the rust resistance gene in RHA 464 and to investigate the allelic relationships of this gene with other unmapped rust resistance genes in the inbred lines HA-R6, HA-R8, and RHA 397. This work will potentially support the molecular marker-assisted introgression and pyramiding of rust resistance gene into sunflower breeding lines.

Materials and methods

Plant materials

An F_2 mapping population consisting of 141 individuals was derived from a single F_1 head of the cross HA 89/RHA 464. HA 89 is considered universally susceptible to all *P. helianthi* races. F_2 individuals were self-pollinated and F_3 seeds were harvested from each F_2 plant. A total of 141 $F_{2:3}$ lines were developed for phenotypic testing to confirm the genotypes of F_2 individual plants.

Four previously identified rust-resistant inbred lines, HA-R6, HA-R8, RHA 397, and RHA 464, were crossed in a half-diallel scheme to test for allelism. The rust resistance genes in lines, HA-R6, HA-R8, and RHA 397, were derived from different sources (Miller and Gulya 1997, 2001). The line PI 650362, introduced from France, was the donor of the rust resistance gene in HA-R6. A landrace collected from the Havasupai Indian Reservation, Arizona (PI 432512), was the rust resistance source of HA-R8. The rust resistance in RHA 397 was derived from the line RO-20-10-3-3-2 that was obtained from a germplasm exchange of USDA with the Republic of South Africa. The hybrid nature of F_1s from the half-diallel cross combinations was confirmed by molecular marker analysis (Fig. 1), and F_1s were self-pollinated to produce F_2 seeds. Six F_2 populations were developed for phenotypic testing.

Rust inoculation and disease ratings

To phenotype the mapping population, and six half-diallel populations, plants were inoculated at the four-leaf stage with *P. helianthi* urediniospores of race 336 in the greenhouse. HA 89 and RHA 464 were used as susceptible and resistant controls. The inoculation method and the evaluation of rust resistance followed the procedure described by Qi et al. (2011a, b), based on the method developed by Gulya and Masirevic (1996). Urediniospores stored in liquid nitrogen were heat shocked for 1 min at 45 °C in a water bath just before inoculation. Spores were then suspended in SOLTROL 170 isoparaffin (Chevron Phillips

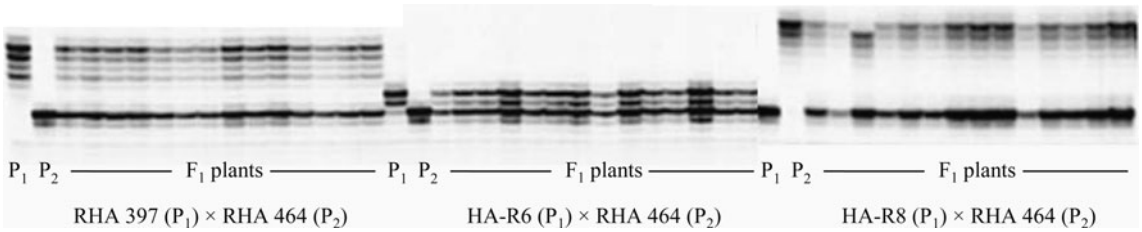

Fig. 1 Hybrid nature of F_1s was confirmed by SSR marker CRT84. In each cross combination, 14 F_1 plants were tested using this marker. All F_1 plants showed one allele inherited from each parent

Chemical Co., The Woodlands, TX, USA) to a final concentration of $1.5-3.0 \times 10^6$ spores/ml (about 5−10 mg spores/10 ml). The spore suspension was atomized onto the upper surface of all leaves with compressed air. Inoculated seedlings were incubated in an automated mist chamber to provide continuous leaf wetness for 16–20 h at 20 °C in the dark. The plants were then grown in a greenhouse at 22 ± 2 °C with a photoperiod of 16-h light and 8-h dark, using sodium vapor lamps for supplemental illumination. Plants were evaluated 10–14 days after inoculation for the phenotypic rust reaction.

The rust evaluation combined infection type (IT) and severity (pustule coverage). Categories of IT type were as follows: 0 = immune, no uredia and no hypersentive flecks; 1 = highly resistant, presence of hypersensitive flecks or lesions, or pustules smaller than 0.2 mm in diameter with or without chlorotic haloes; 2 = resistant, pustules smaller than 0.4 mm; 3 = susceptible, pustules 0.4–0.6 mm in diameter; 4 = highly susceptible, pustules larger than 0.6 mm (Yang et al. 1986). Pustule coverage was visually assessed using computer-generated diagrams. Pustule coverage of 0–0.5 % was classified as resistant, and larger than 0.5 % was classified as susceptible (Gulya et al. 1990). The final phenotype of rust reaction in each plant was determined by the combination of IT category and pustule coverage. IT 0, 1, and 2 along with pustule coverage of 0–0.5 % were rated as resistant, while IT 3 and 4 along with pustule coverage larger than 0.5 % were rated as susceptible. HA 89 and all susceptible plants in the segregating populations of F_2 and F_3 showed a fully susceptible reaction with IT of 4 and more than 20 % of the leaf area covered with pustules, clearly distinguishable from resistant plants which show small pustules (IT 2) and 0.1–0.5 % of the leaf area covered with pustules.

DNA extraction and genetic mapping

DNA was isolated from lyophilized young leaves using the Qiagen DNeasy 96 plant kit and DNA concentration was quantified on a NanoDrop 2000 Spectrophotometer (Qiagen, Valencia, CA; Thermo Fisher Scientific, Wilmington, DE). The total volume of the PCR reaction mixture was 10 µl and contained 10−20 ng DNA, 2 mM dNTPs, 0.6 × PVP (polyvinylpyrrolidone), 0.5 units *Taq* DNA polymerase, 2 mM $MgCl_2$ and 1 × reaction buffer (Bioline, Randolph, MA, USA), 0.04 µM forward primer with an M13 tail added to its 5′ end (5′-CACGACGTTGTAAAACGAC-3′), 0.2 µM reverse primer, and 0.2 µM IRD 700/800-labeled M13 tail. Touchdown PCR was performed as follows: denaturation at 95 °C for 2 min, followed by seven cycles of 45 s at 94 °C, 45 s at 68 °C (decreasing each cycle by 2 °C), and of 60 s at 72 °C. Products were subsequently amplified for 30 cycles at 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. PCR products were separated using an IR^2 4300/4200 DNA Analyzer (LI-COR, Lincoln, NE, USA).

A total of 870 published SSR and InDel markers (Burke et al. 2002; Tang et al. 2002, 2003; Yu et al. 2003; Heesacker et al. 2008) were screened for polymorphism between the two parents, HA 89 and RHA 464. The polymorphic markers were further screened between rust-resistant and susceptible bulks to identify markers potentially linked to the rust resistance. The two bulks consisted of DNA from ten randomly selected homozygous resistant/susceptible plants in equimolar amounts.

Segregation of selected marker loci in 141 F_2 plants was calculated using a Chi-square test ($P = 0.05$). Markers that showed highly significant segregation distortion from the expected 1:2:1 (co-dominant) or 3:1 (dominant) ratio were excluded from map construction. JoinMap® version 4.1 (Stam 1993) was used for linkage analysis and map calculations. Marker order and genetic distance were calculated using a regression mapping algorithm with the following parameters: Kosambi mapping function, Rec = 0.4, LOD = 1.0, Jump = 5.

Results

Allelic relationships among the rust resistance genes in RHA 464, HA-R6, HA-R8, and RHA 397

Among six F_2 populations from the half-diallel crosses, segregation for rust resistance was observed in five F_2

populations (Table 1). No susceptible plants were detected among 220 plants in the cross HA-R6/RHA 397, indicating that the gene in HA-R6 was tightly linked to the resistance gene in RHA 397 or the two genes were allelic. In the F_2 population from HA-R8/RHA 464, the observed segregation ratio (resistant plants vs. suceptible plants, R:S) matched the expected ratio 15R:1S ($P = 0.986$). In the populations of HA-R6/RHA 464 and RHA 397/RHA 464, the susceptible plants occurred in higher than expected proportions; however, the Chi-square test indicated the observed ratios still fit the expected 15R:1S ratio ($P = 0.160$ and 0.340, respectively). The 15R:1S segregation ratio suggested that R_{12} was not allelic to any of the rust genes in HA-R6, HA-R8, and RHA 397, nor was R_{12} linked to any of them. The segregation ratios of rust resistance in both the F_2 populations of HA-R6/HA-R8 and RHA 397/HA-R8 fit 15R:1S ratios as expected, indicating that the resistance gene in HA-R8 was independent of the *R*-genes in HA-R6, RHA 397, and RHA 464 (Table 1).

Genetic analysis of rust resistance in RHA 464

F_1, F_2, and $F_{2:3}$ families were inoculated with urediniospores of the same *P. helianthi* isolate used above. HA 89 was highly susceptible (infection type 4) and showed 20–40 % of leaf-pustule coverage, whereas RHA 464 was highly resistant (IT 1) with necrosis and 0–0.1 % of pustule coverage on the leaves. All F_1 plants were rust resistant with IT 2 and 0.1–0.5 % of leaf-pustule coverage. The F_2 population fit a 3:1 ratio (103 resistant: 38 susceptible; $\chi^2_{(3:1)} = 0.116$, $P = 0.734$). $F_{2:3}$ families were phenotyped to determine the rust resistance genotype of each F_2 plant. Out of the 141 $F_{2:3}$ families, 36 were homozygous resistant, 66 were heterozygous, and 38 were homozygous susceptible. One family did not develop enough seeds for rust inoculation. The segregation ratio fit the expected 1:2:1 ratio of F_2 genotypes ($\chi^2_{(1:2:1)} = 0.510$, $P = 0.770$) consistent with a single dominant gene which was designated as R_{12}.

Identification of molecular markers linked to R_{12}

Among 870 SSR and InDel markers screened for polymorphism between the two parents, 398 markers were polymorphic. Bulked segregant analysis (BSA; Michelmore et al. 1991) was performed using these polymorphic markers. Eight markers (CRT275, ORS210, ORS583, ORS748, ORS1146, ORS1214, ORS1227, and ZVG53), which mapped to LG11 on the published maps (Yu et al. 2003; Del Moral et al. 2012), were polymorphic between R- and S-bulks. Two markers, CRT169 and ORS1118, which previously mapped to LG8 and LG10, respectively, showed polymorphism between the two bulks as well. These ten polymorphic markers and ten additional markers previously mapped in LG11 showing polymorphism between two parents were genotyped for the 141 F_2 individuals. Chi-square analysis showed all markers fit a typical Mendelian segregation ratio of a single gene in an F_2 population (1:2:1 or 3:1). Fifteen LG11 markers were associated with R_{12} and spanned a genetic distance of 77.6 cM (Fig. 2). Six of them (ORS557, ORS583, ORS666, ORS1214, CRT169, and ZVG53) were dominant markers. Seven of these markers were common between HA 89/RHA 464 F_2 population and the RHA 280/RHA 801 RIL population in the Sunflower CMap Database and were mapped in the same order in LG11. R_{12} was flanked by two markers, a co-dominant SSR marker CRT275-11 and a dominant InDel marker ZVG53. The closest marker, CRT275-11, was distal to R_{12} at a genetic distance of 1.0 cM; the other, ZVG53, was linked to R_{12} in repulsion phase at a genetic distance of 9.6 cM (Fig. 2).

Discussion

In this study, we mapped the rust resistance gene R_{12} to LG11, which is the only disease resistance gene mapped to LG11 of the sunflower genome thus far. Additionally, the allelism test demonstrated that R_{12} was not allelic to the rust resistance genes present in the three lines carrying unknown resistance loci, HA-R6, HA-R8, and RHA 397,

Table 1 Segregation of rust resistance in six F_2 populations derived from half-diallel crosses

Cross combinations	Observed segregation		$\chi^2_{15:1}$	*P* value
	Resistant	Susceptible		
HA-R6 × RHA 397	220	0	14.667	0.000
HA-R8 × RHA 464	194	13	0.000	0.986
HA-R6 × RHA 464	229	21	1.972	0.160
RHA 397 × RHA 464	227	19	0.912	0.340
HA-R6 × HA-R8	196	12	0.082	0.775
RHA 397 × HA-R8	234	20	1.143	0.789

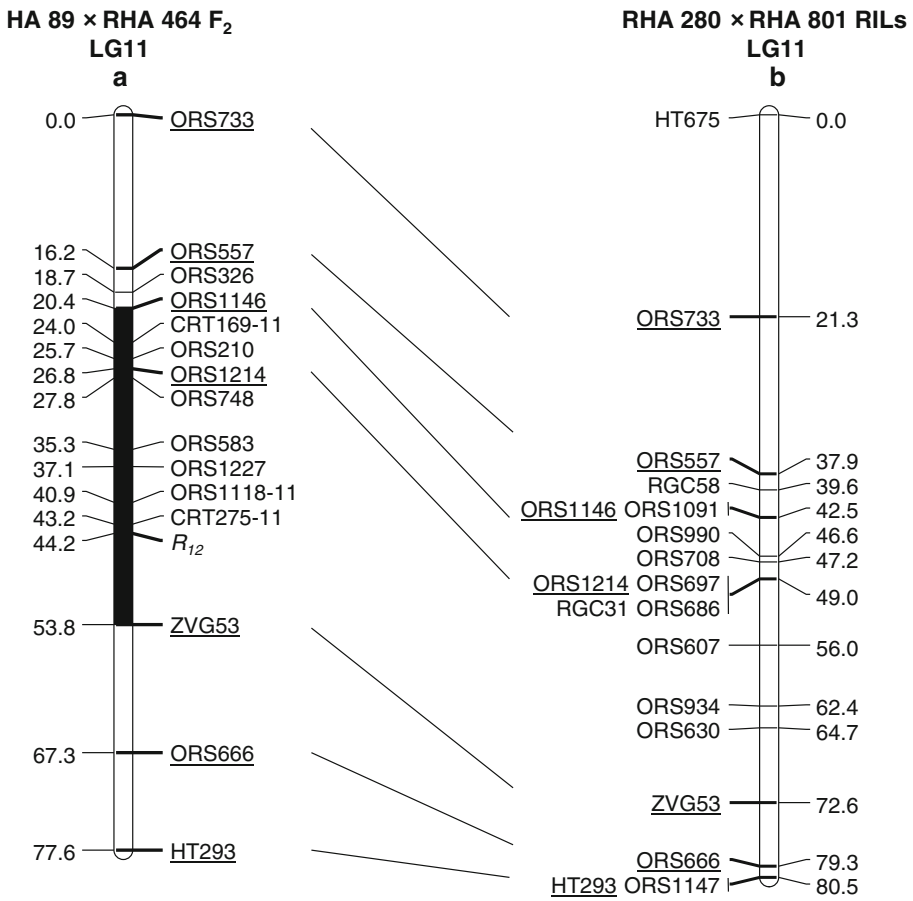

Fig. 2 R_{12} in RHA 464 was mapped to LG11. **a** Ten polymorphic markers between two bulks reside in a region spanning 33.4 cM of genetic distance (*marked in black bar*). It starts from ORS1146 at 20.4 cM to ZVG53 at 53.8 cM. **b** LG11 of the public SSR map (Tang et al. 2003). Seven common markers between two maps are aligned and underlined

indicating that R_{12} is a novel rust resistance locus in sunflower. We identified a rust-resistant related chromosome region spanning 33.4 cM of genetic distance in LG11, which contained ten markers showing polymorphism between the two bulks (Fig. 2). Notably, nine markers were distal to R_{12}, and only one marker, ZVG53, was proximal to the gene (9.6 cM from R_{12}). CRT275, a multiple locus marker previously mapped to LG7 and LG11, respectively (Tang et al. 2003; Del Moral et al. 2012), was the closest marker to R_{12} (1.0 cM). This co-dominant marker (CRT275-11) can clearly distinguish homozygous resistant from heterozygous resistant and homozygous susceptible plants. The combination of the two flanking markers, CRT275-11 and ZVG53, will provide reliable selection of rust-resistant genotypes in most cases.

Seven rust resistance genes were mapped to five linkage groups of the sunflower genome so far, LG2 (R_5), LG8 (R_1), LG9 (R_2), LG11 (R_{12}), and LG13 (R_4, R_{11}, and R_{adv}) (Lawson et al. 1998, 2011; Yu et al. 2003; Bachlava et al. 2011; Qi et al. 2011b; 2012a, b). Unlike sunflower downy mildew resistance genes (designated as *Pl*), which are clustered in three linkage groups of the sunflower genome (Pl_{13}, Pl_{14}, Pl_{16} on LG1; Pl_1, Pl_2, Pl_6, Pl_7, Pl_{15} on LG8, and Pl_5, Pl_8, on LG13; Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997; Gentzbittel et al. 1998; Bert et al. 2001; Gedil et al. 2001; Bouzidi et al. 2002; Radwan et al. 2003, 2004; Pankovic et al. 2007; Mulpuri et al. 2009; de Romano et al. 2010; Bachlava et al. 2011; Liu et al. 2012), it appears that the sunflower rust *R*-genes are more randomly distributed in the sunflower genome. One notable exception seems to be LG13, in which three rust *R*-genes, R_{adv}, R_{11}, and R_4, are clustered (Yu et al. 2003; Qi et al. 2011b; 2012b). The allelism test in the present study revealed that the rust resistance genes in HA-R6 and RHA 397 may be closely linked to each other and ongoing molecular mapping indicates that both of them are also located on LG13, close to R_4 locus (Gong et al. unpublished data).

RHA 464 also has resistance to downy mildew, which is conferred by a single dominant gene Pl_{ARG} located on the upper end of LG1, far from the other DM *R*-gene cluster on LG1 (Dußle et al. 2004; Hulke et al. 2010; Wieckhorst et al. 2010). This gene provides resistance to all known races of downy mildew, an economically important disease of sunflower that is re-emerging in the Northern Great Plains as new races virulent to commonly utilized resistance genes are being found (Gulya et al. 2011). These genes could be combined with other rust or downy mildew

R-genes to reduce the probability of either pathogen overcoming the resistance genes. Although sunflower rust *R*-genes, R_2 in CM 29 and R_4 in HA-R3, are overcome by rust race 777, they remain effective against 85–90 % of the NA rust isolates (Gulya and Markell 2009; Qi et al. 2011a; Friskop et al. 2012). Additionally, R_4 is also resistant to Argentina's most predominant rust race 700 and many others known to occur in the Argentine sunflower production areas (Moreno et al. 2012). Pyramiding of R_2 or R_4 with Pl_{ARG} and R_{12} from RHA 464 is underway in both oil and confectionery sunflower backgrounds. The identification of molecular markers tightly linked to R_{12} in the present study should expedite its deployment for *R*-gene pyramiding and thereby improve breeding efficiency.

Molecular mapping of R_{12} gene to LG11 lays the foundation for map-based cloning of the R_{12} gene. Currently, a high-density sunflower genetic map with 10,080 SNP loci is publicly available (Bachlava et al. 2012; Bowers et al. 2012). Genetic mapping of an additional 8,000 sunflower SNPs is in progress and, when completed, their utilization will greatly increase the marker density of current sunflower genetic maps (Venkatramana et al. 2012). Additionally, a fully sequenced sunflower genome is expected in the near future (Kane et al. 2011). Both these resources will facilitate fine mapping of the R_{12} gene and eventually lead to cloning of the gene.

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