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ORIGINAL PAPER

Genetic mapping of rust resistance genes in confection sunflower line HA-R6 and oilseed line RHA 397

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Abstract Few widely effective resistance sources to sunflower rust, incited by *Puccinia helianthi* Schwein., have been identified in confection sunflower (*Helianthus annuus* L.). The USDA inbred line HA-R6 is one of the few confection sunflower lines resistant to rust. A previous allelism test indicated that rust resistance genes in HA-R6 and RHA 397, an oilseed-type restorer line, are either allelic or closely linked; however, neither have been characterized nor molecularly mapped. The objectives of this study are (1) to locate the rust resistance genes in HA-R6 and RHA 397 on a molecular map, (2) to develop closely linked molecular markers for rust resistance diagnostics, and (3) to determine the resistance spectrum of two lines when compared with other rust-resistant lines. Two populations of 140 $F_{2:3}$ families each from the crosses of HA 89, as susceptible parent, with HA-R6 and RHA 397 were inoculated with race 336 of *P. helianthi* in the greenhouse. The resistance genes (*R*-genes) in HA-R6 and RHA 397 were molecularly mapped to the lower end of linkage group 13, which encompasses a large *R*-gene cluster, and were designated as R_{13a} and R_{13b}, respectively. In the initial maps, SSR (simple sequence repeat) and InDel (insertion and deletion) markers revealed 2.8 and 8.2 cM flanking regions for R_{13a} and R_{13b}, respectively, linked with a common marker set of four co-segregating markers, ORS191, ORS316, ORS581, and ZVG61, in the distal side and one marker ORS464 in the proximal side. To identify new markers closer to the genes, sunflower RGC (resistance gene candidate) markers linked to the downy mildew *R*-gene Pl_8 and located at the same region as R_{13a} and R_{13b} were selected to screen the two F_2 populations. The RGC markers RGC15/16 and a newly developed marker SUN14 designed from a BAC contig anchored by RGC251 further narrowed down the region flanking R_{13a} and R_{13b} to 1.1 and 0.1 cM, respectively. Both R_{13a} and R_{13b} are highly effective against all rust races tested so far. Our newly developed molecular markers will facilitate breeding efforts to pyramid the R_{13} genes with other rust *R*-genes and accelerate the development of rust-resistant sunflower hybrids in both confection and oilseed sunflowers.

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Introduction

Sunflower (*Helianthus annuus* L.) is widely grown in the world, predominantly as an oilseed crop with some germplasm selected as confection types, intended for direct human consumption. Of approximately 1 million hectare of sunflowers that are grown annually in the United States (US), 80–90 % are the oilseed type, and only 10–20 % are the confection type (National Sunflower Association 2011). Sunflowers in the US are a high-risk crop because of potential losses from diseases, insects, birds, and weeds. Confection sunflower is more vulnerable to biotic and abiotic stresses than oilseed sunflower. This is partially due to the majority of research focusing on the larger oilseed sunflower acreage base, and the relatively limited research on the confection sunflowers heavily concentrated on seed size. Oilseed sunflower germplasm is more genetically

diverse and is distinct from confection sunflower germplasm, as demonstrated by genetic distance data (Cheres and Knapp 1998).

Sunflower rust, cause by the biotrophic fungus, *Puccinia helianthi* Schwein., is one of the major yield limiting factors in sunflower production in countries such as the US, Argentina and Australia. In an annual field survey coordinated by North Dakota State University Cooperative Extension Service and the US National Sunflower Association, sunflower rust is frequently found from 60 to 77 % of surveyed fields. Yield losses to the disease can occur in a range of environments and climatic conditions, from hot and dry climates where sunflowers are grown under pivot irrigation, such as in the US Central Great Plains (Gulya 2006; Kandel 2008, 2012) to cooler and wetter climates where sunflowers are grown in dry-land production systems such as in North and South Dakota (Markell et al. 2009; Friskop et al. 2011). Sunflowers with severe rust infection can have a significantly reduced yield (smaller head size, fewer achenes, lighter test weight, and decreased oil content) as compared to uninfected plants (Fick and Zimmer 1975; Shtienberg and Zohar 1992; Markell et al. 2009). In addition, the quality of the confection seeds in severely infected fields may not meet grading standards established by the industry and desired by consumers, exacerbating the economic loss to sunflower producers. Management of the disease in confection sunflowers with fungicide applications is possible, but the high cost of chemicals and hiring an aerial applicator make multiple fungicide applications unpalatable, if not prohibitive. Developing genetically resistant hybrids is the preferred approach for disease management considering its effectiveness, low cost, and sustainability. Currently, however, there are no confection hybrids with resistance to both North American (NA) rust races 336 and 777, the predominant and the most virulent races, respectively, in the US (Gulya 2006; Gulya and Markell 2009).

To identify possible sources of resistance, 104 public sunflower breeding lines, previously described as rust resistant at their time of release, were tested for resistance to NA rust races 336 and 777 (Qi et al. 2011a). The results revealed 13 lines resistant to both races, among which the breeding line HA-R6 is the only confection sunflower with high levels of resistance. Previously, 10 rust resistance genes, R_1–R_5, R_{10}–R_{12}, P_{u6}, and R_{adv}, were documented 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; Radwan 2010; Bachlava et al. 2011; Qi et al. 2012b; Gong et al. 2013). None of these genes, however, are present in public confection germplasm. To reduce the risk of disease epidemics and selection of pathotypes with novel virulence caused using single resistance gene in confection sunflower hybrids, we have recently transferred three rust resistance genes, R_2, R_4, and R_5, into confection sunflower from oilseed germplasm by means of the backcross breeding method (Gong et al. unpublished data). Three rust *R*-genes, R_2 in the line MC 29, R_4 in HA-R3, and R_5 in HA-R2, were reported to provide resistance to more than 85 % the NA rust isolates tested during 2007–2009, but were moderately susceptible to NA race 777 (Gulya and Markell 2009; Qi et al. 2011a; Friskop et al. 2012). R_2, R_4, and R_5 were molecularly mapped to linkage groups (LGs) 9, 13, and 2 of sunflower, respectively, providing diverse sources to pyramid together to attain durable resistance against rust in confection sunflower (Lawson et al. 2011; Qi et al. 2011b, 2012a). Efficient pyramiding requires accurate selection on all involved genes, which is difficult and time consuming using classical breeding approaches. However, diagnostic molecular markers closely linked to rust genes can improve the reliability and speed of selection in a gene pyramiding program.

Two USDA released lines, HA-R6 (confection) and RHA 397 (oilseed), contain rust resistance genes that have not been previously characterized (Miller and Gulya 1997, 2001). An allelism test indicated that the rust resistance genes in HA-R6 and RHA 397 were either allelic or closely linked, but independent from the gene R_{12} in RHA 464 and *R*-gene present in HA-R8 (Gong et al. 2013). Linkage group of these rust genes also has not been determined. The objectives of this study were (1) to locate the rust genes in these two lines on a molecular map, (2) to develop closely linked molecular markers for rust resistance diagnostics, and (3) to determine the resistance spectrum of the two lines when compared with other rust resistance lines.

Materials and methods

Plant materials

Two F_2 mapping populations were developed for this study. HA 89 was used as a common susceptible parent (female) for both populations, and HA-R6 and RHA 397 were used as rust-resistant parents (male). HA-R6 is a selection from the cross HA 323/PI 650362. PI 650362 is an accession from France and is the rust *R*-gene donor for HA-R6. HA-R6 was released as a confection sunflower resistant to rust race 777 with lodging resistance and single-headed plant type (Miller and Gulya 2001). RHA 397 is a male fertility restorer line of oilseed sunflower derived from the cross RHA 274/RO-20-10-3-3-2. RO-20-10-3-3-2 was obtained from a germplasm exchange with Republic of South Africa and is reported to be the *R*-gene donor for RHA 397 (Miller and Gulya 1997). In each population, 140 F_2 individuals were self-pollinated and F_3 seeds were

harvested from each F_2 plant; accordingly, 140 $F_{2:3}$ families were developed. Each $F_{2:3}$ family was tested for the segregation of rust resistance to confirm the genotypes of rust resistance in each F_2 individual.

Thirteen lines putatively harboring different *R*-genes were chosen to observe virulence/avirulence parents when tested with ten rust races (Table 1). Universal susceptible HA 89, a highly susceptible oilseed maintainer line, has been used as a common susceptible parent in several rust mapping projects, was also included. Among the 13 lines, seven of them; HA-R6, HA-R8, RHA 397, RHA 464, Rf ANN-1742, TX16R, and PH3, were reported to have resistance to both NA rust races 336 and 777, whereas the remaining six lines, MC 90, MC 29 (USDA), MC 29 (Australia), HA-R2, HA-R3, and RHA 340 were either susceptible to race 777 or to both races 336 and 777 (Miller and Gulya 2001, Hulke et al. 2010; Qi et al. 2011a). The line MC 29 (Australia), was reported to carry the rust *R*-genes R_2 and R_{10}, conferring resistance to NA races 1, 3, and 6 using previously defined virulence nomenclature (Lambrides and Miller 1994).

Assessment of rust resistance

To map the gene(s) conferring rust resistance in HA-R6 and RHA 397, rust race 336 was used to inoculate the F_1 and $F_{2:3}$ families (20 plants from each family), together with parents HA 89, HA-R6 and RHA 397. Resistance phenotyping of the F_2-derived F_3 families of HA-R6 and RHA 397 was conducted in the fall of 2011 and in the spring of 2012, respectively. Seedlings were inoculated at the four-leaf stage in the greenhouse. Inoculation and virulence phenotyping followed the procedure described by Qi et al. (2011a, b) and the method used by Gulya and Masirevic (1996). Plants were evaluated 12–14 days after inoculation for the phenotypes of rust reaction.

Ten single-pustule derived rust races, collected from US sunflower fields and representing a cross section of rust diversity, including the most predominant (336) and virulent (777) races, were used to generate virulence phenotypes on the 13 lines with putatively identified resistance genes. Each race was inoculated to eight individual plants per sunflower line, for a total of 80 inoculated plants per line (Table 1). The spectrum of resistance in HA-R6 and RHA 397 was assessed by comparing the virulence phenotypes of 10 different races on the 13 lines with putatively identified genes.

The rust evaluation combined infection type (IT) and severity (pustule coverage). Categories of IT type were as follows: 0, immune, no uredia and no hypersensitive 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 as aides. Pustule coverage of 0–0.5 % was classified as resistant,

Table 1 Results of resistance trials of HA-R6, RHA 397 and 12 other sunflower lines to ten rust races in the present study

Lines	Resistance genes	Linkage groups	Rust races										
			300	304	334	336	337	376	377	734	736	776	777[b]
HA 89	–	–	S	S	S	S	S	S	S	S	S	S	S
MC 90	R_1	8	S	S	S	S	S	S	S	S	S	S	S
MC 29 (USDA)	R_2	9	R	R	R	R	R	R	R	R	R	R	S
MC 29 (Australia)	$R_2 + R_{10}$	9 and unknown	R	R	R	R	R	R	R	R	R	R	S
HA-R2	R_5	2	R	R	R	R	R	R	R	R	S	R	S
HA-R3	R_4	13	R	MR	R	R	S	R	R	R	R	R	S
HA-R6	R_{13a}	13	R	R	R	R	R	R	R	R	R	R	R
HA-R8	Unknown	Unknown	R	Seg.	Seg.	R	R	R	Seg.	R	Seg.	R	R
Rf ANN-1742[a]	R_{11}	13	R	R	MR	R	R	R	R	R	MR	R	R
RHA 340	R_{adv}	13	R	S	S	S	S	S	S	S	S	S	S
RHA 397	R_{13b}	13	R	R	R	R	R	R	R	R	R	R	R
RHA 464	R_{12}	11	R	R	R	R	R	R	R	R	R	R	R
PH3	Unknown	Unknown	R	R	R	R	R	R	R	R	R	R	R
TX16R	Unknown	Unknown	R	R	R	R	R	R	R	R	R	R	R

R represents rust resistance, *MR* represents moderate resistance, *S* represents rust susceptibility, *Seg.* represents a line segregating for resistance

[a] Selected homozygous plants from heterozygous Rf ANN-1742 was used in the present study

[b] Data for rust race 777 were taken from Qi et al. (2011a)

while pustule coverage 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. In the case that the susceptible check showed an IT of 4 and more than 40 % of leaf-pustule coverage, plants with IT 2–3 and pustule coverage 0.5–1 % were rated as moderately resistant.

Genotyping and genetic mapping

Genomic DNA was isolated from lyophilized fresh 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, USA). Polymerase chain reaction (PCR) was performed with a touchdown program as described by Gong et al. (2013). The total volume of the PCR reaction mixture was 10 µl, and contained 10–20 ng DNA, 2 mM dNTPs, 0.6× polyvinylpyrrolidone (PVP), 0.5 U 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. PCR products were separated using an IR^2 4300/4200 DNA Analyzer (LI-COR, Lincoln, NE, USA).

In each F_2 population, two bulks (resistant and susceptible) were built for bulked segregant analysis (BSA) (Michelmore et al. 1991). The two bulks consisted of DNA from ten randomly selected homozygous resistant or susceptible F_2 plants in equimolar amounts. Initially, a total of 870 published SSR markers were screened for polymorphism among the three parental lines HA 89, HA-R6, and RHA 397 (Burke et al. 2002; Tang et al. 2002, 2003; Yu et al. 2003; Heesacker et al. 2008). Then, the polymorphic markers were further screened between rust resistant and susceptible bulks to identify markers potentially associated with rust resistance gene(s). Those identified markers associated with resistance in HA-R6 and RHA 397 were further genotyped by analysis of 140 F_2 individuals in each population, respectively. Segregation of each locus was calculated using a Chi-square test ($P = 0.05$). The markers that showed highly significant segregation distortion ($P < 0.001$) 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 determined using a regression mapping algorithm with LOD score of 3.0, and Kosambi's mapping function.

Map saturation around the *R*-genes

To further narrow down the region harboring the *R*-genes, five resistance gene candidate (RGC) markers around the region of interest (Bachlava et al. 2011; Radwan et al. 2008) and six HA SSR markers previously mapped to the linkage group 13 (Talia et al. 2010) were selected in the present study (Table 2). In addition, a 125.6-kb BAC clone (Genebank accession HQ222361) which corresponded to NBS-LRR locus RGC251 closely linked to downy mildew *R*-gene Pl_8 (Bachlava et al. 2011), was used to design five new primer pairs (Table 3). Two primer pairs, SUN11 and SUN12, were developed from sequences in the two ends of the BAC clone. SUN13, SUN14, and SUN15 were developed from SSR-containing sequences within the BAC. In total, 16 additional primer pairs were screened in parents for polymorphism. Polymorphic markers were subsequently genotyped in the two F_2 populations and genetically mapped.

Results

Genetic analysis of rust resistance in HA-R6 and RHA 397

Virulence phenotyping with the *P. helianthi* race 336 indicated that HA 89 was highly susceptible, expressing an

Table 2 RGC primers tested in two F_2 populations in this study

Primer name	Gene bank accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Region amplified	Marker type
RGC15/16	AF017751	TAACGATGCTTCCCAGAAGG	ACCAACTCCACCCATACCAA	CC	SSCP
RGC30	AY153854	AACGATACGCGAGTGAAGGG	CCCTACTTCCAGTAGCACCA	NBS	SSCP
RGC250	AY490793	TGAAAGTGTGCTGAGAAGTCTGGT	CCAATGTTATCCTCATCCTCCTCT	LRR	SSCP
RGC258	AF528544	CTCAAGGCATGGGTTTGTGTATC	AGAATGAAATGGACGCACTAGGTT	NBS	SSCP
RGC260	AF528552	AAGCAAGTCAAGGATCGGTTTG	CCACACATCATCTAACACCAAGAG	NBS	INDEL

CC coiled-coil, *LRR* leucine-rich repeat, *NBS* nucleotide-binding site, *SSCP* single-strand conformation polymorphism, *INDEL* insertion–deletion

Table 3 Primer sequences, annealing temperature, fragment sizes, SSR motifs and positions in the BAC clone HQ222361 anchored by RGC251

Primer name	Gene bank accession	Forward primer (5′-3′)	Reverse primer (5′-3′)	Anneal temp (°C)	Size (bp)	SSR motif	Position in the BAC
SUN11	HQ222361	CGAAGGATTCGACAGATGGT	GGGAGTTTGTTGGTGCACTT	60	308	–	Left end
SUN12	HQ222361	AATTGACCGTAGGCGATCTG	CCATTGTGGTCTTGGGTCTT	60	324	–	Right end
SUN13	HQ222361	GGGTCTGGTTGAATTGTTGG	GTGGCCAATTAAACCCATTTC	60	218	(AT)7	Start from 11,611 bp
SUN14	HQ222361	CCATGTAGGCGTCAACGTAA	GACTTCGTGTAGCCGGTCTC	59	145	(AC)5(AT)6(TA)6(TG)9	Start from 56,722 bp
SUN15	HQ222361	TGCAGCAGATGATGTTCCTC	GGCAATCCAGTTCAAGTTCC	60	132	(AGG)5	Start from 26,020 bp

IT of 4 and 20–40 % of leaf-pustule coverage, whereas HA-R6 and RHA 397 were highly resistant to this race, expressing an IT of 1 with necrosis and 0–0.1 % of pustule coverage. All F_1 plants were rust resistant with an IT of 2 and 0.1–0.5 % of leaf-pustule coverage. In the F_3 family tests, susceptible plants expressed a reaction similar to that of HA 89, clearly distinguishing them from resistant plants which expressed a reaction of IT of 1–2 and 0.1–0.5 % of leaf-pustule coverage. Of the 140 $F_{2:3}$ families from the cross of HA 89/HA-R6, 36 were homozygous resistant, 71 were heterozygous, and 33 were homozygous susceptible. Out of the 140 $F_{2:3}$ families from the cross of HA 89/RHA 397, 24 were homozygous resistant, 78 were heterozygous, and 37 were homozygous susceptible. The segregation ratio in both populations fit the expected 1:2:1 ratio of F_2 genotypes ($\chi^2 = 0.16$, $P = 0.92$ in HA-R6; $\chi^2 = 4.51$, $P = 0.11$ in RHA 397), suggesting that a single dominant gene confers rust resistance in HA-R6 and RHA 397. We temporarily designated the rust resistance gene in HA-R6 as R_{13a} and in RHA 397 as R_{13b}.

Identification of molecular markers linked to R_{13a} and R_{13b}

Among 870 previously mapped sunflower SSR and InDel markers screened, 473 showed polymorphism between HA 89 and HA-R6. These polymorphic markers were used to perform BSA. Twenty-one markers in LG13, and one marker (CRT391) previously mapped on LG1, showed polymorphism between R- and S-bulks. All 22 markers were genotyped in the F_2 population. Chi-square test indicated that these markers fit a typical Mendelian segregation ratio (1:2:1 or 3:1). A LG13 genetic map was constructed with 22 SSR/InDel markers covering a genetic length of 28.0 cM. The marker order in this map is in agreement with the public SSR map (Fig. 1, Tang et al. 2003). R_{13a} was mapped to the lower end of LG13. A co-segregating marker cluster, which included three dominant markers ORS45, ORS191, and ORS581, and two co-dominant markers ZVG61 and ORS316, was the closest locus distal to R_{13a} at a genetic distance of 0.4 cM (Fig. 1). ORS464 (dominant) and HT382 (co-dominant) were mapped at the proximal end of R_{13a} at a genetic distance of 2.4 and 4.7 cM, respectively.

Similarly, the same set of 870 molecular markers was used to screen polymorphism between HA 89 and RHA 397. BSA with 417 polymorphic markers revealed that 12 markers mapped on LG13, one marker (CRT391) previously mapped on LG1, and one marker (CRT306) previously mapped on LG4, showed polymorphism between R- and S-bulks. Subsequent genotyping of the F_2 population mapped these polymorphic markers to a region spanning a genetic length of 29.2 cM (Fig. 1). No segregation distortion was observed on these markers. R_{13b} was mapped

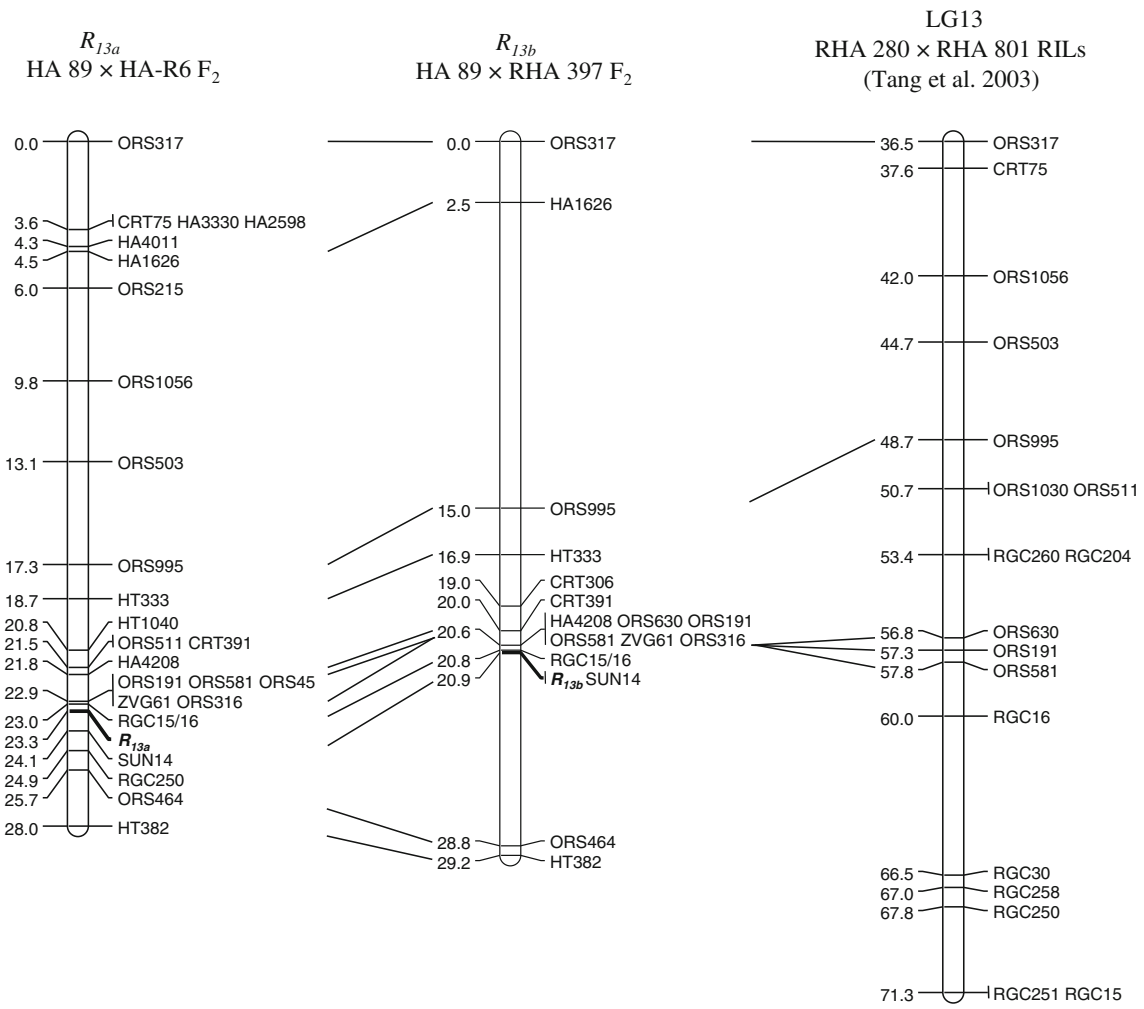

Fig. 1 Genetic maps of R_{13a} in HA-R6 and R_{13b} in RHA 397 and the public genetic map of linkage group 13. Common markers among linkage groups are aligned

on LG13 at the same position as R_{13a} linked with the same co-segregated marker cluster as R_{13a} at a genetic distance of 0.3 cM. Two SSR markers, ORS464 (dominant) and HT382 (co-dominant), were mapped proximal to R_{13b} at a genetic distance of 7.9 and 8.3 cM, respectively. When comparing gel patterns of all common markers starting from ORS995 to HT382 on the R_{13a} and R_{13b} maps, no apparent differences in the PCR fragment sizes were observed between HA-R6 and RHA 397, indicating they share the same alleles in all loci.

Map saturation around the *R*-gene

Among 16 additional markers tested, only RGC15/16 and SUN14 were polymorphic in both sets of parents, whereas RGC250 was polymorphic between HA 89 and HA-R6, and HA4208 was polymorphic between HA 89 and RHA 397. RGC15/16 is a co-dominant marker, whereas RGC250 and SUN14 are dominant markers in the coupling phase where amplified fragment was present in rust-resistant lines and absent in HA 89. In the saturated genetic map of R_{13a}, the genetic distance around R_{13a} was further narrowed to 1.1 cM, R_{13a} was flanked by RGC15/16 and SUN14 at a genetic distance of 0.3 and 0.8 cM, respectively, and RGC250 was placed 0.8 cM toward SUN14 (Fig. 1). RGC15/16 was mapped 0.1 cM distal to R_{13b} and SUN14 co-segregated with R_{13b} in the saturated genetic map of R_{13b}.

Spectrum of rust resistance in HA-R6 and RHA 397

All ten races were virulent on the universal susceptible line HA 89, whereas HA-R6 and RHA 397 were resistant to all races tested, as were sunflower lines Rf ANN-1742, RHA 464, PH3, TX16, and HA-R8 (Table 1). Segregation of the rust resistance to races 304, 334, 377 and 736 was observed in HA-R8, indicating that HA-R8 is not a pure line. Both MC 29 (USDA), carrying the R_2 gene, and MC 29

(Australia), possessing both R_2 and R_{10}, were resistant to all tested races, whereas HA-R3, containing R_4, and HA-R2, containing R_5, were susceptible to races 337 and 736, respectively. MC 90, carrying the R_1 gene, was susceptible to all races tested. RHA 340, carrying the gene R_{adv}, was only resistant to race 300.

Discussion

In the current study, the rust resistance gene R_{13a}, in the confection sunflower line HA-R6, was mapped to LG13, flanked by the same molecular markers at the same position as the gene R_{13b}, in the oilseed sunflower line RHA 397. Recombination ratios differ only slightly between the two mapping populations in this region, making it difficult to determine if the two alleles are at the same locus by map position alone (Fig. 1). Further, both resistant lines retain a shared set of the PCR patterns in all common markers. The rust races used in this study could not differentiate the rust resistance genes found in HA-R6 and RHA 397 because both lines are resistant to all races tested (Table 1). The genomic proximity of both alleles was determined in a different study, where 220 F_2 plants from the cross HA-R6/RHA 397 were observed for segregation in resistance to race 336. No susceptible plants were found, indicating the resistance alleles in the two lines are located at the same chromosome position (Gong et al. 2013). Taking into consideration all of the above, it is most likely that HA-R6 and RHA 397 carry the same rust *R*-gene, and thus R_{13a} and R_{13b} are allelic.

To date, eight rust resistance genes were molecularly mapped on the sunflower genome and characterized, which confer a broad spectrum of resistance to rust (Table 1, Gulya and Markell 2009). Four of them are positioned on linkage groups 2 (R_5), 8 (R_1), 9 (R_2), 11 (R_{12}), whereas the remaining four are all located on the lower end of LG13 (R_{adv}, R_4, R_{11}, and R_{13}) (Lawson et al. 1998, 2011; Yu et al. 2003; Bachlava et al. 2011; Qi et al. 2011b, 2012a, b). Among four rust *R*-genes in LGs, 2, 8, 9, and 11, only R_1 in LG8 is located in a *R*-gene rich region which harbors downy mildew *R*-genes, Pl_1, Pl_2, Pl_6, Pl_7, and Pl_{15} (Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997; de Romano et al. 2010). These rust *R*-genes also show differential specificities to rust (Table 1). The shift of rust pathogen populations makes the R_1 gene no longer effective, and the new rust race 777 is virulent to R_2 and R_5, whereas R_{12} is effective against this and all other rust races identified so far in the US (Hulke et al. 2010).

The sunflower rust *R*-gene cluster in LG13 is similar to the multi-gene clusters in other crops (Islam and Shepherd 1991; Meyers et al. 1998, 2003; Hulbert et al. 2001; Richly et al. 2002; Wei et al. 1999, 2002; Baumgarten et al. 2003). This region encompasses four rust *R*-genes (R_4, R_{adv}, R_{11}, and R_{13}), and three downy mildew *R*-genes (Pl_5, Pl_8, and Pl_{21}) (Lawson et al. 1998; Bert et al. 2001; Yu et al. 2003; Radwan et al. 2003, 2008; Qi et al. 2011b; Vincourt et al. 2012). Based on map position, the seven *R*-genes could be further divided into two sub-clusters; sub-cluster I includes R_{adv} and R_{11} distal to SSR marker OSR316, a common marker among maps related to *R*-genes, and sub-cluster II consists of five genes, R_4, R_{13}, Pl_5, Pl_8, and Pl_{21}, proximal to ORS316 (Fig. 2). The four rust *R*-genes could also be distinguished by their different patterns of resistance and susceptibility to isolates of rust (Table 1).

In sub-cluster I, near the lower end of LG13, the gene R_{adv} in RHA 340 and R_{11} in Rf ANN-1742 were derived from *Helianthus argophyllus* and *H. annuus*, respectively, and are positioned distal to ORS316 at the genetic distances of 3.0 and 3.7 cM in two mapping populations (Fig. 2a, Bachlava et al. 2011; Qi et al. 2012b). However, these two genes encode specific recognition to rust infection. When infected by 10 rust races, R_{adv} was found to provide resistance to race 300 only, whereas R_{11} conferred resistance to all races (Table 1). Interestingly, the sub-cluster I also harbors two male fertility restoration genes, *Rf1* and *Rf5*, and R_{11} was tightly linked to *Rf5* at a genetic distance of 1.6 cM (Gentzbittel et al. 1995; Horn et al. 2003; Yue et al. 2010; Qi et al. 2012b).

In sub-cluster II, two rust *R*-genes, R_4 in HA-R3 and R_{13}, were placed in the same region linked to the common markers of ORS316, ORS581, and ZVG61 (Fig. 2a). The alleles amplified from R_4 and R_{13} for the common markers ORS316 and ZVG61 could be clearly discriminated from fragment sizes (data not shown). The patterns of resistance and susceptibility to the rust races distinguish R_4 and R_{13} (Table 1, Gulya and Markell 2009; Qi et al. 2011a). However, we cannot rule out the possibility that R_{13} may be an allele of the R_4 locus. The alleles of the R_4 locus were reported present in three lines, HA-R1, HA-R4, and HA-R5, by classical allelic and marker validation analyses (Miller et al. 1988; Qi et al. 2011b) and four Argentinean breeding lines, P386, Cabure Precoz, B648, and PNR1, by BSA analysis (Bulos et al. 2012). In addition, based on marker association analysis, Sendall et al. (2006) proposed a total of 21 alleles at R_4 locus. Clarification as to whether R_4 and R_{13} are functionally distinct alleles encoding different resistance specificities at a single locus, similar to the *L* locus of flax (Ellis et al. 1999, 2007), the *RPP13* locus of Arabidopsis (Rose et al. 2004), and the *Pm3* locus of wheat (Srichumpa et al. 2005), will depend on being able to clone and sequence these genes to determine their precise structure.

Interestingly, the *R*-gene cluster in the lower end of LG13 also corresponds to a large NBS-LRR cluster identified in LG13 (Fig. 2b), which is the second largest NBS-

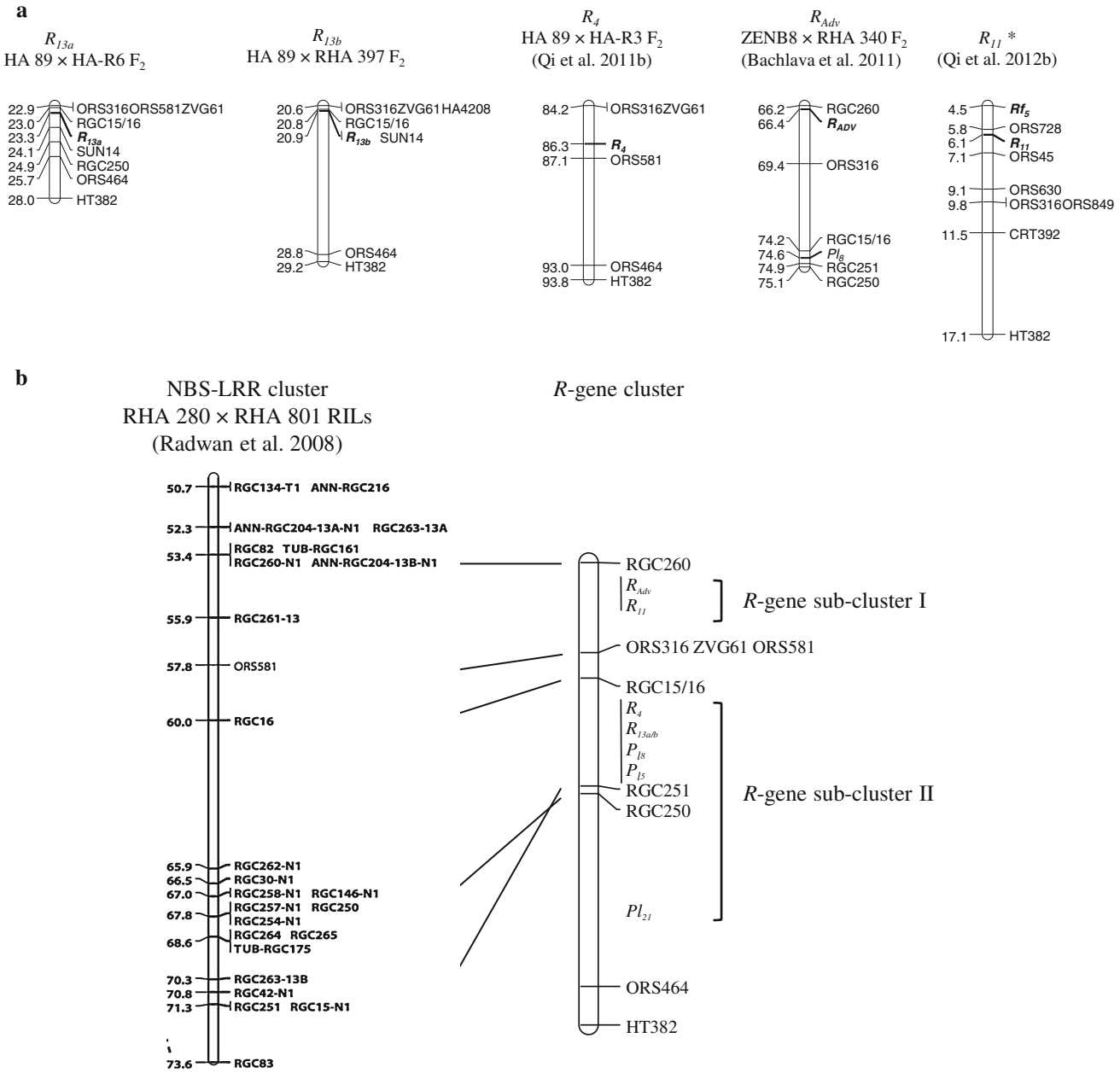

Fig. 2 Genetic maps of rust *R*-genes, R_{adv}, R_4, R_{11}, R_{13a} and R_{13b}, and downy mildew *R*-genes, Pl_5, Pl_8, and Pl_{21} on linkage group 13. **a** Chromosome locations of rust *R*-genes, R_{adv}, R_4, R_{11}, R_{13a} and R_{13b}, on linkage group 13. R_{adv} and R_{11} were distal to ORS316, while R_4, R_{13a}, and R_{13b} were proximal to this marker. **b** The genetic maps showed possible location of this *R*-gene cluster within the large cluster of NBS-LRR RGCs. *R_{11} mapping population was developed from a single plant heterozygous for both rust and male fertility selected from the line Rf ANN-1742 (Qi et al. 2012b)

LRR cluster in the sunflower genome (Radwan et al. 2008). Bachlava et al. (2011) reported a NBS-LRR-encoding RGC marker, RGC260, was mapped to 0.2 cM distal to the R_{adv} locus, and two RGCs, RGC15/16 and RGC251, flanked downy mildew *R*-gene Pl_8 at 0.3 and 0.4 cM, respectively. We tested RGC260, RGC15/16, five primer pairs designed from a BAC contig anchored by RGC251, and three additional RGC markers, RGC30, RGC250 and RGC258, residing in the Pl_8 region in our two mapping populations (Tables 2, 3). No polymorphism of RGC260 associated with R_{adv} was detected in two populations. However, the RGC15/16 marker, as well as SUN14 marker designed from the RGC251 BAC contig, was mapped close to R_{13} locus in two populations (Fig. 1). These results indicated that Pl_8 gene is closely linked to the R_{13} locus in sub-cluster II. The downy mildew *R*-gene Pl_5 was reported to be located at the same cluster as Pl_8, whereas, Pl_{21} was mapped 8.0 cM proximal to Pl_5/Pl_8 in LG13 (Radwan et al.

2004; Vincourt et al. 2012). It is assumed that in the sub-cluster II of the LG13 distal end, two rust *R*-genes, R_4, and R_{13}, and two downy mildew *R*-genes, Pl_5 and Pl_8, are tightly linked forming a complex *R*-gene cluster in sunflower. Further work is needed to determine whether such a diversified *R*-gene cluster formed from intragenic unequal crossing-over or gene conversion between allelic sequences, or functional gene diversification (Michelmore and Meyers 1998; Ellis et al. 2000; Leister 2004; Krattinger et al. 2011).

Frequent pathogen evolution can hasten the defeat of commercially available resistance genes. The rust resistance in HA-R6 and RHA 397 has been effective against a wide range of rust races currently present in the US. However, a new Argentinean rust race coded as 7001 was reported to be virulent to HA-R6 (Moreno et al. 2012). To extend the effectiveness of resistance genes, pyramiding genes conferring resistance to different races of the pathogen within the same genotype will reduce vulnerability of the US sunflower crop to rust, especially in confection sunflower where few rust *R*-genes are available. Currently, three rust *R*-genes, R_2, R_4, and R_5, have be transferred from oil sunflower to confection by backcrossing (Gong et al. unpublished data). Three F_2 populations were then developed by crossing the lines carrying R_2, R_4, and R_5 with the confection line harboring R_{13a} to develop double-resistant lines in confection sunflower. To select simultaneously for double-resistant plants with resistance genes from each parent, it is necessary to use two isolates of rust, one that is virulent to the gene(s) of each parent. However, since R_{13} is, and hopefully will remain, resistant to all known NA rust races, our newly developed molecular markers would facilitate breeding efforts to pyramid the R_{13} gene with other *R*-genes.

Population size for selection of the double-resistant types is dependent on gene location. For genes located on different chromosomes, double homozygotes could be selected from a small F_2 population. We obtained 13 homozygous individuals for the gene combination of $R_5 + R_{13a}$ from 368 F_2 plants with the aide of molecular markers. However, if *R*-genes are located on the same chromosome or chromosomal arm, the frequencies in which the double-resistant types are recovered vary dependent on the genetic distance between the genes. Screening of a large F_2 population (>1,000 plants) from the cross between R_4 and R_{13a} is underway given the understanding that R_4 and R_{13a}, if not allelic, are tightly linked. Similar strategies were used to pyramid rust and downy mildew *R*-genes in oil sunflower. We made the cross between RHA 464 harboring the rust R_{12} gene (LG11) and downy mildew Pl_{ARG} gene (LG1), with the line RHA 397 carrying R_{13b}. SSR markers linked to R_{12} (Gong et al. 2013), newly developed SNP (single nucleotide polymorphism) markers linked to Pl_{ARG} (Qi et al. unpublished data), and markers linked to R_{13b} will accelerate the selection of resistance pyramids in a segregating population. These lines should be very useful in breeding programs to develop commercial sunflower hybrids with high-level durable resistance to rust and downy mildew.

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