Theor Appl Genet (2011) 123:351–358
DOI 10.1007/s00122-011-1588-6

ORIGINAL PAPER

Molecular mapping of the rust resistance gene R_4 to a large NBS-LRR cluster on linkage group 13 of sunflower

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Received: 8 December 2010 / Accepted: 29 March 2011 / Published online: 11 April 2011

Abstract Rust is a serious fungal disease in the sunflower growing areas worldwide with increasing importance in North America in recent years. Several genes conferring resistance to rust have been identified in sunflower, but few of them have been genetically mapped and linked to molecular markers. The rust resistance gene R_4 in the germplasm line HA-R3 was derived from an Argentinean open-pollinated variety and is still one of most effective genes. The objectives of this study were to determine the chromosome location of the R_4 gene and the allelic relationship of R_4 with the R_{adv} rust resistance gene. A total of 63 DNA markers previously mapped to linkage group (LG) 13 were used to screen for polymorphisms between two parental lines HA 89 and HA-R3. A genetic map of LG 13 was constructed with 21 markers, resulting in a total map length of 93.8 cM and an average distance of 4.5 cM between markers. Two markers, ZVG61 and ORS581, flanked the R_4 gene at 2.1 and 0.8 cM, respectively, and were located on the lower end of LG 13 within a large NBS-LRR cluster identified previously. The PCR pattern generated by primer pair ZVG61 was unique in the HA-R3 line, compared to lines HA-R1, HA-R4, and HA-R5, which carry other R_4 alleles. A SCAR marker linked to the rust resistance gene R_{adv} mapped to LG 13 at 13.9 cM from the R_4 locus, indicating that R_{adv} is not an allele of the R_4 locus. The markers tightly linked to the R_4 gene will facilitate gene pyramiding for rust resistance breeding of sunflower.

Communicated by A. Bervillé.

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Introduction

Rust, caused by the fungus *Puccinia helianthi* Schwein., is one of the most serious diseases of sunflower (*Helianthus annuus* L.) worldwide. The rapid changes that occur in the virulence characteristics of populations raise a continuous threat to the effectiveness of existing rust-resistant inbred lines and hybrids (Gulya and Markell 2009; Qi et al. 2011). Hence, there is an urgent need for strategies to develop inbred lines with durable resistance to the disease. The concept of combining resistance genes, i.e. incorporating multiple resistance genes (*R*-genes) into a single cultivar, to achieve greater durability is referred to as 'gene pyramiding' or 'gene stacking'. This pyramiding approach is envisaged to make it more difficult for the pathogen to overcome these multiple resistances as, presumably, mutations in multiple pathogen genes will be required. However, the selection of genotypes with gene combinations is difficult by conventional methods. Mapping rust resistance genes and developing robust molecular markers will facilitate this breeding approach and add precision to selection.

Several genes conferring resistance to rust have been identified in sunflower including R_1, R_2, R_3, R_4, R_5, P_{u6}, and R_{adv} (Putt and Sackston 1963; Miah and Sackston 1970; Miller et al. 1988; Yang et al. 1989; Goulter 1990; Lawson et al. 1998). In addition to the already named rust resistance genes, several inbred lines and interspecific germplasm lines were reported to have resistance to

different rust races of sunflower, especially to races 336, the predominant races in North America, and 777, the most virulent race in North America. These lines can be used as new rust resistance gene sources (Quresh et al. 1993; Gulya et al. 2000; Jan et al. 2004; Jan and Gulya 2006; Hulke et al. 2010; Qi et al. 2011). Lawson et al. (1998) developed two sequence characterized amplified region (SCAR) markers that co-segregated with rust resistance genes R_1 and R_{adv}, respectively. Yu et al. (2003) mapped SCAR markers $SCT06_{950}$ associated with the R_1 gene and $SCX20_{600}$ associated with the R_{adv} gene on the linkage groups (LGs) 8 and 13, respectively. However, Lawson et al. (2010) reported the mapping of $SCT06_{950}$ on LG9, 17 cM away from the R_2 rust resistance gene locus. The R_1 gene is no longer effective against the predominant rust races and the R_{adv} gene is present only in the proprietary line P2 developed by Pioneer Seeds (Pioneer Hi-Bred Australia) and is unavailable for use in public breeding programs (Lawson et al. 1998; Qi et al. 2011).

Rust resistance gene R_4 in the germplasm line HA-R3 was derived from the Argentinean open-pollinated variety 'Charata INTA' (Gulya 1985). The origin of its rust resistance gene can be traced to wild annual *Helianthus* species. Charata INTA was selected from the cross between Russian lines and wild sunflower species, including *H. annuus*, *H. argophyllus*, and *H. petiolaris* (de Romano and Vazquez 2003). Miller et al. (1988) reported that rust resistance genes in three germplasm lines, HA-R1, HA-R4, and HA-R5, which were also selected from Argentinean open-pollinated varieties (Gulya 1985), were allelic to the R_4 locus. These *R* genes express resistance to different sunflower rust isolates; therefore, each encodes different resistance specificity. The line HA-R3 confers resistance to 88% of 300 rust isolates tested in the US in the years 2007 and 2008, but it is moderately susceptible to the new virulent race 777 (Rashid 2006; Gulya and Markell 2009; Qi et al. 2011). It is possible to combine this gene with other resistance genes to attain a wide spectrum of resistance using molecular marker-assisted selection.

Many of the plant disease resistance genes that have been cloned encode proteins with a putative nucleotide binding site and leucine-rich repeats (NBS-LRR resistance genes) (Bent et al. 1994; Hammond-Kosack and Jones 1997; Hulbert et al. 2001; Huang et al. 2003; Yahiaoui et al. 2004). Previous research has identified 149 and 480 NBS-LRR genes in *Arabidopsis* and rice genomes, respectively (Meyers et al. 2003; Zhou et al. 2004; Yang et al. 2007). Radwan et al. (2008) identified 630 NBS-LRR homologs in the sunflower genome and mapped 167 NBS-LRR loci throughout the sunflower genome in 44 clusters or single locus. Of 44 clusters, the LG 8 cluster is the largest with 54 NBS-LRR loci in three subclusters. The second largest NBS-LRR cluster was found on LG 13 with 27 NBS-LRR loci distributed on the lower end of LG 13 which harbors downy mildew (Pl_5 and Pl_8) and rust (R_{adv}) resistance genes (Lawson et al. 1998; Radwan et al. 2003, 2008; Slabaugh et al. 2003; Yu et al. 2003). Sendall et al. (2006) also reported that the R_4 gene was located on LG 13, but no molecular marker associated with the R_4 gene and its position were reported. In the study presented here, we mapped the R_4 gene to a large NBS-LRR cluster on LG 13 of sunflower. The R_4 locus was located in the interval of 0.8–2.9 cM delimited by the INDEL marker ZVG61 and SSR marker ORS581.

Materials and methods

Plant materials

The mapping population used in this study was derived from a cross of HA 89 with HA-R3. HA 89 is a sunflower inbred line used as a susceptible parent and HA-R3 served as a resistant parent that carries the rust resistance gene R_4 (Miller et al. 1988). The population consisted of 120 F_2 plants derived from a single F_1 plant. A progeny test of 118 $F_{2:3}$ families was performed in order to confirm the phenotype and assign the genotype of the F_2 plants. Three germplasm lines, HA-R1, HA-R4, and HA-R5 previously reported as carrying the R_4 alleles (Miller et al. 1988), were chosen to identify allele-specific microsatellite patterns.

Plant growth and inoculations

Seeds were planted in 36-cell plastic flats (each cell 4.6 cm × 5.4 cm) filled with Sunshine SB 100B potting mixture (SunGro Horticulture, Bellevue, WA). The greenhouse was maintained at 24°C day/20°C night with a 16-h photoperiod and sodium vapor lighting. Plants were fertilized weekly with a water soluble 15-16-17 analysis fertilizer and sprayed weekly to runoff with B-Nine (daminozide; Chemtura USA, Middlebury, CT) growth regulator at 0.5% w/v to maintain compact growth. For phenotypic analysis of rust resistance, a total of 120 F_2 plants and 118 $F_{2:3}$ families (20 plants from each family, a total of 2,360 F_3 individuals), along with two parental lines and F_1 plants, were inoculated with *P. helianthi* spores of race 336 at the four-leaf stage (about 3 weeks after planting) using the procedure described by Gulya and Masirevic (1996). Race 336 was collected originally from cultivated plants in North Dakota in 2009, and is the predominant race in North America, and was increased from a single pustule. Urediniospores were collected from greenhouse-grown Mycogen hybrid 7350 plants with the aid of a cyclone collector (Trevet et al. 1951) and stored at 4°C or liquid nitrogen until needed. Spores were suspended in

SOLTROL 170 isoparaffin (Chevron Phillips Chemical Co., The Woodlands, TX) at 5–10 mg spores/10 ml which was equivalent to 1.5–3.0 × 10^6 spores/ml. The spore suspension was atomized onto all leaf surfaces with compressed air. After allowing the SOLTROL 170 to evaporate for 15 min, the plants were incubated in sealed chambers equipped with automated ultrasonic humidifiers to provide continuous leaf wetness within a room maintained at 18–20°C in the dark for 16–24 h. Plants were then returned to the greenhouse and maintained under the conditions mentioned above.

Rust evaluation

Rust pustules started to appear in 7–10 days, and evaluations were made at 12–14 days post inoculation to allow full development of symptoms. Rust evaluations were made using both pustule size or infection type (IT) and percentage of leaf area covered with pustules (severity) on all inoculated leaves, as cited in previous papers (Qi et al. 2011). A modified Sackston's numerical rating system (1962) described by Yang et al. (1986) was used to categorize infection type. Infection categories 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. Reactions 0, 1, and 2 were classified as resistant, while reactions 3 and 4 were rated as susceptible. This categorization is similar to that observed for cereal rust (Stakman et al. 1962), but the sunflower/*Puccinia helianthi* pathosystem does have different symptomotology. Pustule coverage was visually assessed using the computer generated diagrams of Gulya et al. (1990), showing pustule coverage from 0.1 to 40%. Pustule coverage of 0–0.5% was classified as resistant, along with IT of 0–2. Susceptible parent HA 89 and susceptible plants in F_2 and F_3 populations always had IT 3 or 4 pustules with 10–20% or more pustule coverage, whereas resistant plants gave IT 1 or 2 pustules with 0.1–0.5% pustule coverage.

DNA marker analysis

Total DNA was extracted from seedlings of parental lines and individual F_2 plants using the Qiagen DNeasy 96 plant kit with a modified protocol described by Horne et al. (2004) and DNA concentration was quantified on a NanoDrop 2000 Spectrophotometer (Qiagen, Valencia, CA; Thermo Fisher Scientific, Wilmington, DE). Sendall et al. (2006) reported that the rust resistance gene R_4 was located on LG 13 in sunflower. A total of 59 simple sequence repeat (SSR) markers, three INDEL markers, and one SCAR marker, $SCX20_{600}$, that were previously mapped to LG 13 were used to screen HA 89 and HA-R3 (Lawson et al. 1998; Burke et al. 2002; Tang et al. 2002, 2003; Yu et al. 2003). Polymerase chain reaction (PCR) in a 15-µl volume contained 2 mM $MgCl_2$, 200–250 µM of each dNTP, 0.02–0.06 µM forward primer with an M13 tail (CACG ACGTTGTAAAACGAC) at the 5′ end, 0.1–0.3 µM reverse primer, 0.1–0.3 µM fluorescently labeled M13 primer, 1× PCR buffer, 0.5 units *Taq* polymerase (Bioline, Randolph, MA, USA), and 10–20 ng of genomic DNA. The PCR reactions were performed in a Peltier thermocycler (Bio-Rad Lab, Hercules, CA, USA) with a touchdown program described by Qi et al. (2011). PCR products were diluted 10- to 40-fold before analysis. SSR fragments were size separated by using an IR2 4300 DNA Analyzer (Li-COR, Lincoln, Nebraska).

The PCR conditions for SCAR marker SCX20 were previously described in Lawson et al. (1998). A multiplex PCR procedure was applied to the SSR primer ORS581, in which the SSR primer CRT504 was used as an internal control for the reactions. A 25-µl PCR mixture contained 1.25 µM of each primer of ORS581 and CRT504, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 1× PCR buffer, 1 unit *Taq* polymerase (Bioline, Randolph, MA, USA), and 20 ng of DNA template. The reaction was incubated at 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C with a final extension at 72°C for 20 min. The PCR products were separated in 2.0% agarose gels and visualized under UV light.

Genetic mapping of the R_4 gene

The genetic linkage map of LG 13 with the R_4 gene was constructed using the population of 118 F_2 plants derived from the cross HA 89 with HA-R3. This population segregating for the R_4 resistance locus was used to estimate genetic linkage between the resistance locus and closely linked DNA markers. The mapping data were analyzed with the computer program Mapmaker V2.0 for Macintosh (Lander et al. 1987) using default parameters of LOD = 3.0 and the Kosambi mapping function (Kosambi 1944). Goodness-of-fit to a 1:2:1 segregation ratio of F_2 genotypes for rust reaction from the F_3 families was tested by means of a chi-square analysis.

Results

Reaction of parents, F_2 population, and $F_{2:3}$ progenies to rust infection

The segregating F_2 population was inoculated with the *P. helianthi* spores of race 336, which is avirulent on plants

containing the gene R_4. The rust resistance genotype of each F_2 plant was determined by testing their F_3 progenies. HA 89 showed a fully susceptible reaction with infection type 4 and 10–20% of the leaves covered with pustules, whereas HA-R3 was highly resistant to rust race 336 with necrotic or small pustules (IT 1) and 0–0.1% of the leaves covered with pustules. Heterozygous F_1 plants gave an intermediate infection type (IT 2) and 0.1–0.5% of the leaves covered with pustules. Therefore, rust symptoms in resistant plants were clearly distinct from those in susceptible plants. Resistant and susceptible plants segregated in the F_2 population in a 3:1 ratio (94 resistant and 26 susceptible). Of the 118 $F_{2:3}$ families selected for molecular mapping, 31 were homozygous resistant (RR), 62 were heterozygous (Rr) segregating for resistance and susceptibility, and 25 were homozygous susceptible (rr) (Fig. 2b). The scoring results fit the expected 1:2:1 ratio of F_2 genotypes ($\chi^2 = 0.9153$, $df = 2$, $0.75 > P > 0.50$), indicating that a single dominant gene in the HA-R3 line controls the rust resistance.

Molecular mapping of the rust resistance gene R_4

A total of 59 SSR markers, three INDELs, and one SCAR marker that previously mapped to LG 13 were selected (Lawson et al. 1998; Burke et al. 2002; Tang et al. 2002, 2003; Yu et al. 2003). Of those, 52 markers were mapped only to LG 13, 10 to two LGs, and one to three LGs. Twenty-five SSR markers (40%) were polymorphic between HA 89 and HA-R3. Among the 25 polymorphic markers, 21 were mapped to LG 13 in the present study. The genetic map of LG 13 consisted of 21 markers spanning a total of 93.8 cM for an average distance of 4.5 cM between markers. Similar to the previous LG 13 linkage map, the SSR markers were concentrated on the upper and lower ends of LG 13 with a sizable gap between marker ZVG59 and ORS317 (Fig. 1a, Tang et al. 2002, 2003). Fifteen markers concentrated on the lower end of LG13 spanned 21.4 cM in genetic length, averaging a distance of 1.4 cM between markers (Fig. 1a).

The R_4 gene showed linkage with the INDEL marker ZVG61 and the SSR marker ORS581. ZVG61 is a

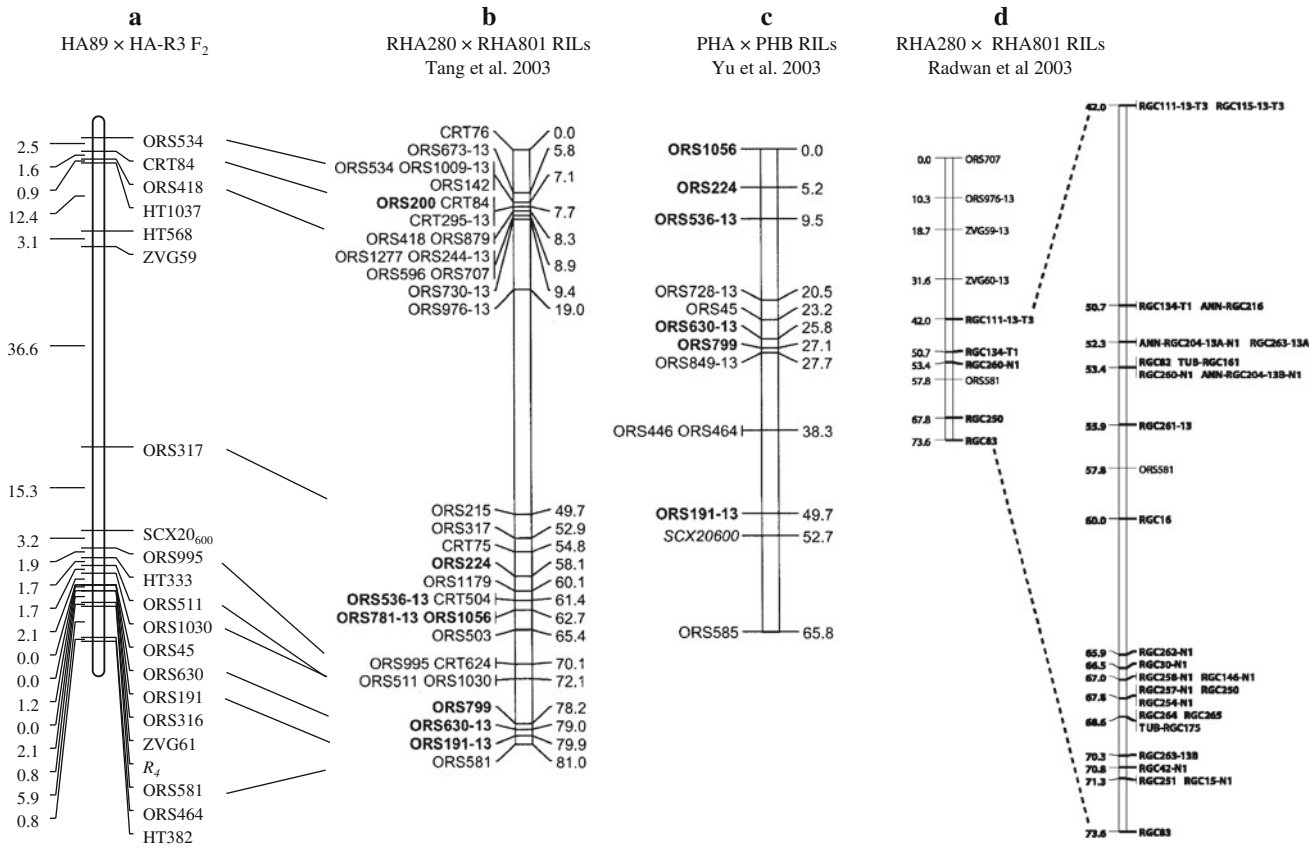

Fig. 1 Genetic linkage map of sunflower linkage group (LG) 13. **a** LG 13 genetic map, showing the position of the R_4 locus and SCX20 marker. **b** A dense public genetic linkage map of LG 13. **c** LG 13 genetic map, showing the position of the SCX20 marker. **d** LG 13 genetic map, showing the position of 27 NBS-LRR loci on the lower end of LG 13. SSR marker ORS581 was located on the NBS-LRR cluster. Common SSR markers were aligned between **a** and **b**

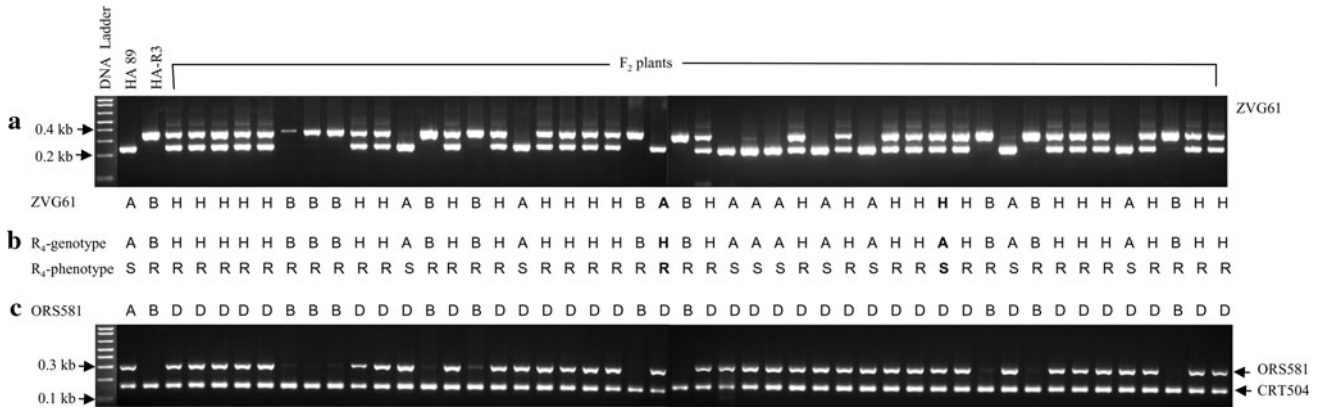

Fig. 2 PCR patterns of markers ZVG61 (**a**) and ORS581 plus CRT504 (**c**) and R_4 gene phenotype (rust score) and R_4 genotype (**b**) in an F_2 segregating population (showing 46 of 118 F_2 plants from *lane 2* to *48*). SSR primer CRT504 was used in the multiplex PCR reaction with ORS581 as an internal control to rule out the possibility that the missing ORS581 fragment is caused by PCR error. PCR pattern of ORS581 primers for the heterozygous F_2 plants is the same as that of HA 89. F_2 genotypes for the R_4 gene were determined by $F_{2:3}$ progeny test. The symbol *A* represents homozygous HA 89 (genotype AA); *B* homozygous HA-R3 (genotype BB); *H* heterozygous (genotype AB); *D* either homozygous HA 89 (AA) or heterozygous (AB). *S* susceptible, *R* resistant. The *bold capital* indicates the recombination between ZVG61 and the R_4 gene. The PCR fragment size amplified by ZVG61, ORS581, and CRT504 includes a 19-bp M13 tail primer. The molecular weight marker is a 1-kb plus DNA ladder (Invitrogen, Carlsbad, CA, USA). The PCR products were separated in 2.0% agarose gels

codominant marker that mapped 2.1 cM distal to the R_4 gene (Fig. 1a). A polymorphic fragment in HA-R3 that was amplified by ZVG61 primers was 336 bp in length compared to a 220-bp fragment in HA 89 (Fig. 2a). The SSR marker ORS316 co-segregated with ZVG61, providing another codominant marker for the R_4 gene. ORS581 is a dominant-repulsion marker that mapped 0.8 cM proximal to the R_4 gene. A 308-bp fragment amplified with ORS581 primers was present in HA 89, but absent in HA-R3 (Fig. 2c). In a multiplex PCR using SSR marker CRT504 as an internal control in the F_2 population, each sample displayed the presence of the CRT504 band demonstrating that the DNA was suitable for amplification and ruling out any possible false negative of the ORS581 marker due to PCR failure (Fig. 2c).

The SCAR marker $SCX20_{600}$ was previously reported to co-segregate with the rust resistance gene R_{adv}, which is present in the proprietary line P2 and was mapped to LG 13 (Lawson et al. 1998; Yu et al. 2003). We tested this marker on HA 89 and HA-R3. The $SCX20_{600}$ primers amplified a 600-bp fragment that was present in HA-R3, but absent in HA 89 (Fig. 3c). The screening of the $SCX20_{600}$ marker in the F_2 population from the cross of HA 89 with HA-R3 indicated that this marker is approximately 13.9 cM from the R_4 gene locus (Fig. 1a).

The two molecular markers linked to the R_4 gene, ZVG61 and ORS581, were tested in three germplasm lines, HA-R1, HA-R4, and HA-R5 that possess resistance genes which are allelic to R_4 (Miller et al. 1988). The three lines show the same PCR pattern as HA-R3 with ORS581 primers, i.e. they lack the 308-bp fragment which is present in HA 89. In contrast, they share the same PCR pattern as HA 89 with primers of ZVG61 (Fig. 3).

Discussion

The rust resistance in germplasm line HA-R3 was thought to be controlled by a single dominant gene by means of classic genetic analysis of F_2 and BC_1F_1 progenies derived from the cross of the susceptible line S37388 with HA-R3 (Miller et al. 1988). The rust test results of $F_{2:3}$ families segregating in an approximate ratio of 1:2:1 in the present study supported the hypothesis of a single dominant gene. This gene was mapped to LG 13 within a large NBS-LRR cluster, which is the first step for further research toward cloning the resistance gene through the map-based cloning method. The tightly linked markers will facilitate efficient resistance breeding in sunflower.

PCR-based SSR markers currently are among the most widely used marker systems and often the best choice because of their high information content, ease of genotyping, and codominant nature. Codominant markers are preferable to dominant markers due to the larger information content. A codominant molecular marker allows unequivocal distinction of homozygous and heterozygous genotypes on an electrophoresis gel. It is particularly useful for marker-assisted selection in breeding programs. However, a high proportion of dominant SSR markers was found in the present study. Of 21 polymorphic markers (18 SSRs, two INDELs, and one SCAR marker) mapped to LG 13, nine SSR markers (50%), ORS534, ORS418,

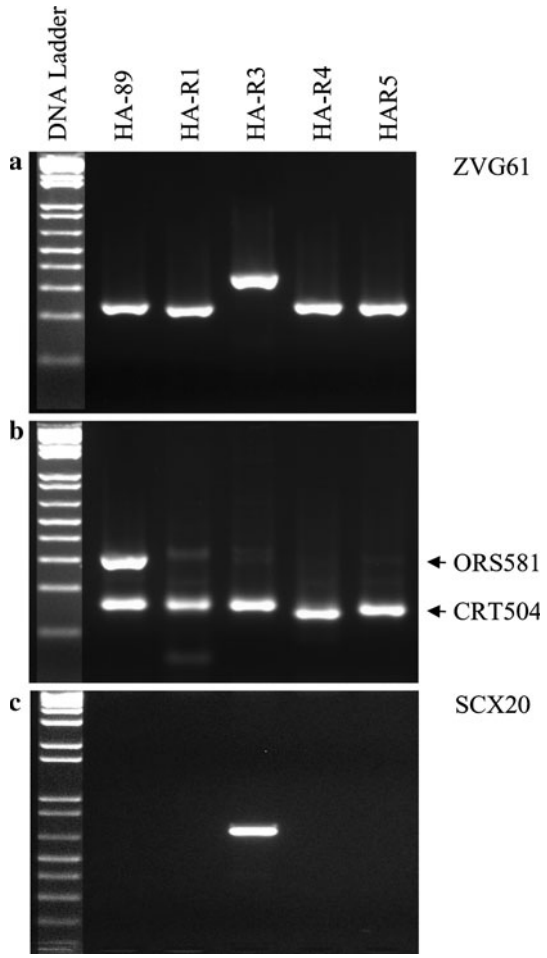

Fig. 3 PCR patterns of markers ZVG61 (**a**) and ORS581 plus CRT504 (**b**), and $SCX20_{600}$ (**c**) in the lines HA 89, HA-R1, HA-R3, HA-R4, and HA-R5. ZVG61 shows a unique PCR pattern in HA-R3 different from that in HA-R1, HA-R4, and HA-R5, whereas HA-R1, HA-R4, and HA-R5 share the same PCR pattern as HA-R3 with ORS581, missing a 308-bp fragment. In the multiplex PCR reaction with ORS581, a fragment (~154 bp) amplified by CRT504 primers was present in all lines. $SCX20_{600}$ only amplified a ~600-bp fragment in HA-R3. The PCR fragment size amplified by ZVG61, ORS581, and CRT504 includes a 19-bp M13 tail primer. The PCR products were separated in 2.0% agarose gels

HT1037, HT568, ORS511, ORS630, ORS191, ORS581, and ORS464, showed dominant nature. In these cases SSRs were analyzed on the basis of the presence or absence of a band on an electrophoresis gel. As a result, dominant homozygous individuals are not distinguished from heterozygous individuals, resulting in a considerable loss of information. There are two phases of dominant markers based on the amplified fragment presence or absence in a target chromosome. In a dominant-coupling phase, a fragment amplified from a given pair of primers is present in the target chromosome, whereas in a dominant-repulsion phase there is an opposite situation. In the present study, two markers, ZVG61 and ORS581, flanked the rust resistance gene R_4 within a 2.9-cM interval. As a dominant-repulsion marker, ORS581 can only detect plants homozygous for the R_4 gene as revealed by the lack of a PCR product, whereas heterozygous plants show the same PCR pattern as the susceptible parent (Fig. 2c). Combining the use of the codominant marker ZVG61 will allow an efficient selection in the disease resistance breeding in sunflower.

Clusters of genes conferring resistance to plant diseases in the host chromosomes have been identified in diverse plant species (Islam and Shepherd 1991; Jones et al. 1993; Song et al. 1997; Salmeron et al. 1996; Ellis et al. 1997; Meyers et al. 1998; Michelmore and Meyers 1998; Richter and Ronald 2000; Wei et al. 2002; Huang et al. 2003). Genes within a cluster can be allelic or closely linked as, for example, the flax rust resistance genes at the L and M loci. At the flax L locus, a single gene has 13 alleles encoding different rust resistance specificities, whereas a series of tightly linked genes with related coding sequences separated by unique regions were found at the M locus (Islam and Mayo 1990; Anderson et al. 1997; Ellis et al. 1997, 2007). The R_4 alleles have been characterized in sunflower germplasm lines HA-R1, HA-R3, HA-R4, and HA-R5 by means of classical allelic tests (Miller et al. 1988). HA-R1, HA-R3 and HA-R4 all have similar origins but differ in their reaction to rust. All were derived from an Argentinean interspecific pool with Russian open-pollinated varieties crossed with *H. annuus*, *H. argophyllus*, and *H. petiolaris* (Gulya 1985; de Romano and Vazquez 2003). HA-R4 (also referred to AMES 18925) was also found to be highly resistant to a Spanish isolate of *P. helianthi* (CO97) (Prats et al. 2007). HA-R5 was derived from a selection of the cultivar Guayacan INTA, whose rust resistance can be traced to the undomesticated form of *H. annuus* (de Romano and Vazquez 2003). When these lines were tested with markers ZVG61 and ORS581 linked to the R_4 gene, the same amplification pattern was found for the four lines with primer pair ORS581, all of which were absent of a 308-bp fragment. However, the amplification pattern with primer pair ZVG61 was unique in HA-R3, distinguishing the R_4 allele in HA-R3 from those in HA-R1, HA-R4, and HA-R5 (Fig. 3). Such a marker is of interest because the R_4 allele in HA-R3 is the most effective one in North America.

The SCAR marker $SCX20_{600}$ is linked to the rust resistance gene R_{adv} in the proprietary line P2 (Lawson et al. 1998). Yu et al. (2003) mapped $SCX20_{600}$ to LG 13 closely linked to SSR marker ORS191 using a RIL population derived from the cross of two proprietary inbred lines PHA and PHB developed by Pioneer Hi-Bred International Inc. (Johnston, Iowa). Sendall et al. (2006) reported that the R_{adv} gene is an allele to the R_4 locus based on linkage association data, and two additional SSR

markers, ORS781 (0.0 cM) and ORS995 (1.3 cM), are linked to this gene. In the present study we mapped $SCX20_{600}$ distal to ORS995 with a 3.2 cM genetic distance on LG 13 (Fig. 1a). The marker $SCX20_{600}$ has a genetic distance of 13.9 cM from the R_4 locus, which does not support the conclusion that the R_{adv} gene is an allele of the R_4 locus. The discrepancy between the mapping position of $SCX20_{600}$ in our map and Yu's map is probably due to the different segregating population used for mapping. However, the marker order in our map is identical to the RHA 280 × RHA 801 map, a dense public genetic linkage map in sunflower (Fig. 1b, Tang et al. 2003).

Interestingly, both rust resistance genes R_4 and R_{adv} are present within a large NBS-LRR cluster corresponding to disease resistance identified in LG 13 (Radwan et al. 2008). Two RGCs (resistance gene candidates), RGC261-13 and RGC16, flank SSR marker ORS581, the closest marker linked to the R_4 gene, with 1.9 and 2.2 cM of genetic distance, respectively (Fig. 1d). Fine-mapping of the R_4 gene region with different DNA markers, especially newly developed SNP (single-nucleotide polymorphism) markers, is underway. Cloning of the gene(s) will contribute to better understanding the allelic relationships in this complex locus.

Acknowledgments We thank Drs. Justin Faris and Chao-Chien Jan for critical review of the manuscript, and Angelia Hogness and Megan Ramsett for excellent technical assistance both in the lab and greenhouse. This project was supported by the USDA-ARS CRIS Project No. 5442-21000-034-00D.

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