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ORIGINAL PAPER

Genetics and mapping of the R_{11} gene conferring resistance to recently emerged rust races, tightly linked to male fertility restoration, in sunflower (*Helianthus annuus* L.)

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Abstract Sunflower oil is one of the major sources of edible oil. As the second largest hybrid crop in the world, hybrid sunflowers are developed by using the PET1 cytoplasmic male sterility system that contributes to a 20 % yield advantage over the open-pollinated varieties. However, 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 the newly emerged rust races. The aim of this study was to elucidate the inheritance of rust resistance and male fertility restoration and identify the chromosome location of the underlying genes in Rf ANN-1742. Chi-squared analysis of the segregation of rust response and male fertility in F_2 and F_3 populations revealed that both traits are controlled by single dominant genes, and that the rust resistance gene is closely linked to the restorer gene in the coupling phase. The two genes were designated as R_{11} and *Rf5*, respectively. A set of 723 mapped SSR markers of sunflower was used to screen the polymorphism between HA 89 and the resistant plant. Bulked segregant analysis subsequently located R_{11} on linkage group (LG) 13 of sunflower. Based on the SSR analyses of 192 F_2 individuals, R_{11} and *Rf5* both mapped to the lower end of LG13 at a genetic distance of 1.6 cM, and shared a common marker, ORS728, which was mapped 1.3 cM proximal to *Rf5* and 0.3 cM distal to R_{11} (*Rf5*/ORS728/R_{11}). Two additional SSRs were linked to *Rf5* and R_{11}: ORS995 was 4.5 cM distal to *Rf5* and ORS45 was 1.0 cM proximal to R_{11}. The advantage of such an introduced alien segment harboring two genes is its large phenotypic effect and simple inheritance, thereby facilitating their rapid deployment in sunflower breeding programs. Suppressed recombination was observed in LGs 2, 9, and 11 as it was evident that no recombination occurred in the introgressed regions of LGs 2, 9, and 11 detected by 5, 9, and 22 SSR markers, 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 *Helianthus argophyllus*, forming a large rust *R*-gene cluster of R_{adv}/R_{11}/R_4 in the lower end of LG13. The relationship of *Rf5* with *Rf1* is discussed based on the marker association analysis.

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Introduction

Sunflower oil provides about 13 % of the world's edible oil. The high proportion of polyunsaturated fatty acids renders sunflower oil as a popular source of essential fatty acids in the diet. The discovery of cytoplasmic male sterility (CMS) and the gene for male fertility restoration in the early 1970s allowed for the production of hybrid sunflower, demonstrating a 20 % yield advantage over the open-pollinated varieties (Leclercq 1969; Kinman 1970). The first PET1-type CMS derived from *Helianthus petiolaris* subsp. *petiolaris* Nutt. and the restorer gene *Rf1* have

been extensively utilized in the commercial seed production of hybrid sunflower worldwide, which raises questions about the potential threat of genetic vulnerability of sunflower hybrids. Developing several CMS/Rf systems and elucidating their molecular mechanisms will broaden the genetic diversity of sunflower hybrids. In addition to *Rf1*, the restorer genes *Msc1* and *Rf3* that was recently reported in RHA 340 and RHA 280 lines also are able to restore CMS PET1 (Gentzbittel et al. 1999; Jan and Vick 2007; Abratti et al. 2008; Liu et al. 2011), whereas the *Rf4* gene is specific for male fertility restoration of CMS GIG2, a system different from CMS PET1 (Feng and Jan 2008).

The germplasm line Rf ANN-1742 was released as a male fertility restorer in 1997. However, genetics of male fertility restoration in this line has not been investigated (Seiler and Jan 1997). Recent evaluation of rust resistance identified the Rf ANN-1742 line as resistant to the newly emerged rust races of sunflower (Qi et al. 2011a).

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 in 1995 (Miller and Gulya 2001), 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; Rashid 2006; Gulya and Markell 2009). The majority of commercial hybrids are susceptible to the new predominant and virulent races (Gulya 2006; Gulya and Markell 2009). Presently, race 777 remains at 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. Rf ANN-1742 is an important source of resistance to sunflower rust race 777, and utilization of this novel source along with other resistance genes in commercial sunflower hybrids in North America will significantly reduce the occurrence of new pathotypes and consequent yield losses due to the disease.

The sunflower rust pathosystem follows the classical gene-for-gene concept. Genetic studies of resistance to rust have indicated that nearly all sources of rust resistance in sunflower are controlled by single, usually dominant, genes. Currently, eight genes, R_1–R_5, R_{10}, P_{u6}, and R_{adv}, have been postulated to confer resistance to different rust races, and a novel gene, R_{11}, is described for the first time in the present study (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). New virulent races are able to overcome the rust resistance genes R_1, R_3, P_{u6}, and R_{adv}. The genes R_2 in line MC 29, R_4 in HA-R3, and R_5 in HA-R2 remain resistant to the predominant race 336, but all are susceptible to the most virulent race 777 (Rashid 2006; Gulya and Markell 2009; Qi et al. 2011a). The gene R_{10} was reported as a second resistance gene present in an Australia selection of MC 29 (AUS) that harbors the R_2 gene (Lambrides and Miller 1994). Line MC 29 (AUS) was also susceptible to race 777 (Qi unpublished data). However, the gene R_{11} in line Rf ANN-1742 was recently identified as resistant to the predominant and virulent races, 336 and 777, respectively (Qi et al. 2011a).

Molecular mapping of both rust resistance genes and male fertility restorer 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; Slabaugh et al. 2003; Yu et al. 2003). Both R_{adv} and R_4 genes were mapped to LG13 (Lawson et al. 1998; Yu et al. 2003; Radwan 2010; Bachlava et al. 2011; Qi et al. 2011b), whereas the R_5 gene was mapped to LG2 (Qi et al. 2011c). The male fertility restorer gene *Rf1* was also mapped to LG13 (Gentzbittel et al. 1995; Berry et al. 1997; Horn et al. 2003; Yu et al. 2003; Kusterer et al. 2005; Yue et al. 2010), whereas the *Msc1* and *Rf3* restorer genes were mapped to LG7 (Mazeyrat et al. 1998; Gentzbittel et al. 1999; Abratti et al. 2008; Liu et al. 2011) and *Rf4* was mapped to LG3, respectively (Feng and Jan 2008). Here, we report the genetic mapping of a novel rust resistance gene, R_{11}, tightly linked to a restorer gene, *Rf5*, in LG13.

Materials and methods

Plant materials and mapping population

The Rf ANN-1742 line was derived from a BC_1F_2 population by crossing cms HA 89 with a wild *Helianthus annuus* accession, PI 613748, which originated from Hinton, Oklahoma, US. This line was released as a CMS male fertility restorer which segregated for rust resistance (Seiler and Jan 1997; Qi et al. 2011a). One resistant plant, 09-519-1, was self-pollinated and a progeny test indicated that it was heterozygous for both rust resistance and male fertility restoration, and thus that it could serve as the equivalent of an F_1 for these characters. The F_2 population from this plant was sown in the greenhouse in 2010 and seeds from each plant harvested separately to provide F_3

Table 1 List of sunflower inbred lines used in the present study

Line	Pedigree*	Response to CMS	Restorer gene	Type
HA 89	VNIIMK 8931 Sel	M		Oil
Rf ANN-1742	cms HA 89 *2/PI 613748	R	*Rf5*	Oil
HA 291	INRA 6501 Sel	M		Oil
HA 850	High oil population	M		Oil
HA 342	HA 89 *2/pervenets	M		Oil
HA 323	Sundak selection (midge resistant)	M		Confectionery
HA 350	HA 292*2/pervenets high oleic	M		Confectionery
RHA 265	2* Peredovik/953-102-1-1-41 = T66006-2-1-3-1	R	*Rf1*	Oil
RHA 274	CMS PI343765/HA119//HA62-4-5/2/T66006-2	R	*Rf1*	Oil
RHA 348	RHA 274 *2/pervenets high oleic	R	*Rf1*	Oil
RHA 340	HA 89 *3/*H. argophyllus* 415	R	*Rf3****	Oil
RHA 365	SELECT	R		Oil
RHA 374	ARG-R43	R		Oil
RHA 386	82 ROM. R-LINE BULK	R		Oil
RHA 400	AUSTRALIA 85 R-LINE POP	R		Oil
RHA 408	ROMANIA R-LINE SCL POP-1	R		Oil
RHA 801	Derived from a restorer composite	R		Oil
RHA 417	RHA 801/NS-RF POP 3 (SUNBURST)	R		Oil
RHA 439	RHA 377/AS 3211	R		Oil
RHA 280	Sundak Sel	R	*Rf3****	Confectionery
RHA 282	Boneta Giant Manchurian/Mennonite RR	R		Confectionery
RHA 293	Commander/Mennonite RR	R		Confectionery
RHA 325	R811-3	R		Confectionery
Rf GIG2**	CMS GIG2/*H. maximiliani* amphiploid	R	*Rf4*	Oil

M maintainer, *R* restorer

*The pedigree information was taken from http://www.ag.ndsu.nodak.edu/aginfo/seedstock/varieties/VH-SUNF.htm

**The pedigree information was taken from Feng and Jan (2008)

***The restorer genes in RHA 340 and RHA 280 were both named *Rf3* (Abratti et al. 2008; Liu et al. 2011)

families. Progeny tests of 146 rust resistant/fertile $F_{2:3}$ families for rust resistance and male fertility restoration indicated the genotypes of their F_2 plants.

Six sunflower maintainer lines and 18 male fertility restorer lines were used to validate DNA markers linked to the restorer genes (Table 1). Among the 18 restorer lines, RHA 265, RHA 274, and RHA 348 were known to carry the *Rf1* gene (Kinman 1970; Korell et al. 1992). Both RHA 340 and RHA 280 were reported to carry the restorer gene *Rf3* (Abratti et al. 2008; Liu et al. 2011), and Rf GIG2 and Rf ANN-1742 lines harbor *Rf4* and *Rf5*, respectively (Feng and Jan 2008). The remaining 11 lines were selected from diverse sources, and the *Rf* genes in these lines are unknown (Korell et al. 1992).

Evaluation of rust resistance

Sunflower rust race 336 was collected originally from cultivated plants in North Dakota in 2009, and is the predominant race in North America (Gulya and Markell 2009). A total of 207 F_2 seeds and 20 seeds of parental line HA 89 were planted in 36-cell plastic flats (one seed per cell of 4.6 cm × 5.4 cm) filled with Sunshine SB 100B potting mixture (SunGro Horticulture, Bellevue, WA) in May 2010. The F_2 plants and HA 89 plants were inoculated with race 336 of *P. helianthi*. Urediniospores from a liquid N_2 tank were heat shocked at 45 °C for 1 min before use. Spores were then suspended in SOLTROL 170 isoparaffin (Chevron Phillips Chemical Co., The Woodlands, TX) at 5–10 mg spores/10 ml and sprayed onto four-leaf stage seedlings using the procedure previously described (Gulya and Masirevic 1996; Qi et al. 2011b). After inoculation, seedling plants were allowed to dry for 15–30 min and then incubated in a dew chamber equipped with automated ultrasonic humidifiers to provide continuous leaf wetness, and held 16–20 h at 20 °C in the dark. Seedlings were placed on a greenhouse bench maintained at 22 ± 2 °C with a photoperiod of 16 h after incubation. 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 0–0.5 % 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 F_2 plants (49 rust susceptible and 158 rust resistant plants) were transferred to 2-gallon pots for male fertility evaluation in the greenhouse. Forty-nine rust susceptible plants and 3 of 158 rust resistant plants were found to be completely male sterile and did not produce any seeds. The remaining 155 F_2 rust resistant plants were grown to obtain F_3 seeds. A total of 146 resistant/fertile F_3 families were subjected to progeny test. Twenty seeds of each F_3 family were planted in 36-cell plastic flats in October 2010 and April 2011, respectively, and were inoculated with race 336 at the four-leaf stage. The F_3 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.

Evaluation of male fertility restoration

The F_2 and F_3 plants were visually scored for the presence or absence of pollen. Plants that produced anthers and shed pollen were considered fertile, whereas those without anthers or pollen were considered sterile. The F_2 population was evaluated for male fertility and sterility in the greenhouse in May 2010. One hundred and forty-six fertile $F_{2:3}$ families were grown in rows of 30 plants sown in the field in June 2011. Evaluation of male fertility was conducted at the flowering stage. The results of the F_3 family test were used to infer the genotypes of F_2 plants for the restorer gene. A Chi-squared (χ^2) analysis was performed to verify whether the observed ratios of segregation for rust resistance and male fertility in F_2 and F_3 populations fit expected models.

DNA extraction and PCR conditions

Genomic DNA was isolated from young leaves of the parents and F_2 individuals using the Qiagen DNeasy 96 plant kit with a modified protocol described by Horne et al. (2004) (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). The PCR reaction mixture (15 µl) contained 1× *Taq* DNA polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.02 µM forward primer with an M13 tail (CACGACGTTGTAAAACGAC) at the 5′ end, 0.1 µM reverse primer, 0.1 µM fluorescently labeled M13 primer, 0.6× PVP (polyvinylpyrrolidone), 0.5 units *Taq* polymerase (Bioline, Randolph, MA, USA), and 10–20 ng of genomic DNA. 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). The PCR conditions for SCAR markers SCX20 (Lawson et al. 1998), HRG01 and HRG02 (Horn et al. 2003), and STS marker STS115 (Yue et al. 2010) were previously described.

Bulked segregant analysis (BSA) and genetic mapping

For marker screening of parents, we selected an F_3 plant (10-275-2) homozygous for both rust resistance and fertility restoration as the resistant parent versus susceptible parent HA 89. DNAs of the ten homozygous resistant F_2 plants, based on the F_3 progeny test, were pooled in an equal amount to create a resistant (R) bulk. Likewise, DNAs of the 10 homozygous susceptible F_2 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 BSA (Michelmore et al. 1991) was performed with polymorphic SSRs to determine the chromosome region containing the R_{11} gene. A total of 192 F_2 plants were used for R_{11} and *Rf5* 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 (Lander et al. 1987). The recombination fractions were transformed by the Kosambi mapping function to estimate the map distance (Kosambi 1944).

Results

Inheritance of the rust resistance gene R_{11} and male fertility restorer gene *Rf5*

The inbred line HA 89 was highly susceptible to rust race 336, a predominant race in North America, with IT 4 and more than 20 % of the leaves covered with pustules, whereas the selected homozygous plant 10-275 was resistant, showing localized necrosis at infection sites with an IT 2 and 0.1–0.5 % of the leaves covered with pustules (Fig. 1). The 207 F_2 individuals segregated at a ratio of 158R:49S, which did not differ significantly from the expected 3:1 ratio ($\chi^2 = 0.13$, $df = 1$, $P = 0.718$), indicating a dominant gene governs rust resistance in the line Rf ANN-1742, and this gene is designated as R_{11}.

Forty-nine rust susceptible F_2 plants were also completely male sterile, whereas only 3 of 158 rust resistant F_2

Fig. 1 Seedling rust response of HA 89 and 10-275 genotypes against *P. helianthi* race 336, showing infection type (IT) and severity 12 days after inoculation. 10-275 is an $F_{2:3}$ homozygous resistant plant selected from Rf ANN-1742

plants were male sterile (155MF:52MS), indicating that the rust resistance and male-fertility restoration were closely linked in the coupling phase in this population. Fifty-two male-sterile plants did not produce any seeds. Segregation of male-fertile and male-sterile plants fit an expected 3:1 ratio ($\chi^2 = 0.0008$, $df = 1$, $P = 0.977$), indicating that one dominant gene segregated in the F_2 population and is responsible for male fertility restoration of the male-sterile PET1 cytoplasm. This gene was named *Rf5*.

Rust phenotyping of 146 resistant/fertile $F_{2:3}$ families (20 plants per family) showed that the F_2 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 F_3 family data for fertility from 146 fertile $F_{2:3}$ families (30 plants per family) evaluated in the field were also consistent with a segregation of 1:2. Forty-seven F_3 families were nonsegregating, whereas 99 were segregating ($\chi^2 = 0.040$, $df = 1$, $P = 0.841$). The results confirm that both genes, *Rf5* and R_{11}, fit a single gene model (Seiler and Jan 1994; Qi et al. 2011a).

Table 2 Summary of polymorphic SSR markers between HA 89 and 10-275-2

LG	No. SSR tested	No. polymorphic SSR
1	39	1 (2.6)[a]
2	60	7 (11.7)
3	48	3 (6.3)
4	40	0 (0.0)
5	42	1 (2.4)
6	21	1 (4.8)
7	34	3 (8.8)
8	33	3 (9.1)
9	73	10 (13.7)
10	44	2 (4.5)
11	46	24 (52.2)
12	25	2 (8.0)
13	68	8 (11.8)
14	31	3 (9.7)
15	41	0 (0.0)
16	43	2 (4.7)
17	36	2 (5.6)
Total	723	72 (10.0)

[a] The number in parentheses represents the percentage of polymorphic SSR markers

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 1 (LGs 1, 5, and 6) and maximum number was 24 (LG11) (Table 2).

The parents, two bulks, and two F_2 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 conferring resistance to rust 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. 2).

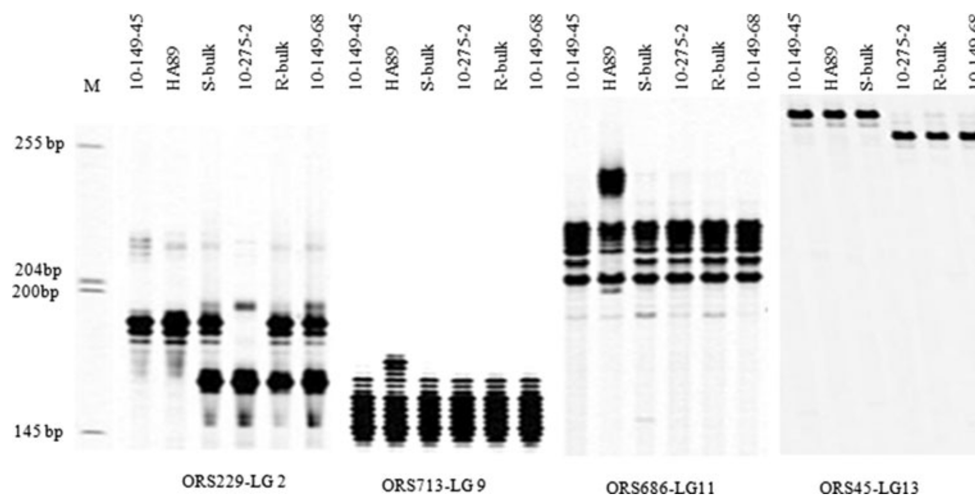

Fig. 2 PCR pattern of parental and bulked DNA samples with SSR primers of ORS229, ORS713, ORS686, and ORS45. ORS229 detected HA 89 allele in the F_2 population, but did not show polymorphism between two bulks, whereas ORS713 and ORS686 did not detect HA 89 allele in the population. ORS45 detected polymorphism between bulks. 10-149-45: homozygous susceptible F_2 plant; HA 89: susceptible parent; S-bulk from the 10 homozygous susceptible F_2 plants; 10-275-2: $F_{2:3}$ homozygous resistant plant; R-bulk from the 10 homozygous resistant F_2 plants; 10-149-68: homozygous resistant F_2 plant. *M* 50- to 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

Surprisingly, out of 24 polymorphic SSRs in LG11, 22 showed no HA 89, a recurrent parent used to develop Rf ANN-1742, allele present in the 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. 2). These markers detected a 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 ten polymorphic SSR markers from LG9. Nine of these ten markers are located on the upper end based on the public SSR genetic map (Fig. 3, Tang et al. 2003). Of eight polymorphic SSRs of LG2, only three, CRT313, ORS229, and ORS342, detected HA 89 allele in the population. These markers were positioned to the lower end of LG2, a region of higher recombination (Fig. 3).

Genetic mapping of the genes R_{11} and *Rf5*

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} and *Rf5* loci were all located in the lower end of LG13 encompassing a genetic distance of 17.1 cM, an average of 1.94 cM per locus (Fig. 4a). The marker order was found to be in good agreement with those of Tang et al. (2003) and Qi et al. (2011b) (Fig. 4a, c, d). The rust resistance gene R_{11} was found to be closely linked to the restorer gene *Rf5*. The interval between *Rf5* and R_{11} covered $\sim$1.6 cM of genetic distance. Both genes were closely linked to the marker ORS728, which is 1.3 cM proximal to *Rf5* and 0.3 cM distal to R_{11} (*Rf5*/ORS728/R_{11}) (Fig. 4a). ORS728 was previously mapped to two LGs, 1 and 13 (Tang et al. 2002; 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 *Rf5* and R_{11} genes, respectively (Fig. 5c). Two additional SSR markers were linked to *Rf5* and R_{11}; ORS995 was 4.5 cM distal to *Rf5* and ORS45 was 1.0 cM proximal to R_{11} (Fig. 4a).

Validation of DNA markers linked to *Rf* genes

Three DNA markers, including STS marker STS115 and two SCAR markers HRG01 and HRG02, linked to the *Rf1* gene (Horn et al. 2003; Yue et al. 2010), along with ORS728, a marker closely linked to *Rf5*, were tested in 6 maintainer lines and 18 restorer lines. No PCR product with primers of the three *Rf1* markers was observed in all maintainer lines and four restorer lines, RHA 340, RHA 280, Rf GIG2, and 10-275-2 (a selection of Rf ANN-1742), each harboring different *Rf* gene from *Rf1*. In contrast, the polymorphic fragments of the *Rf1* markers were present in 14 other restorer lines, suggesting these lines may all have the *Rf1* gene (Fig. 5; Table 3). The ORS728 primers amplified a unique fragment only from 10-275-2, but not from any other lines tested (Fig. 5).

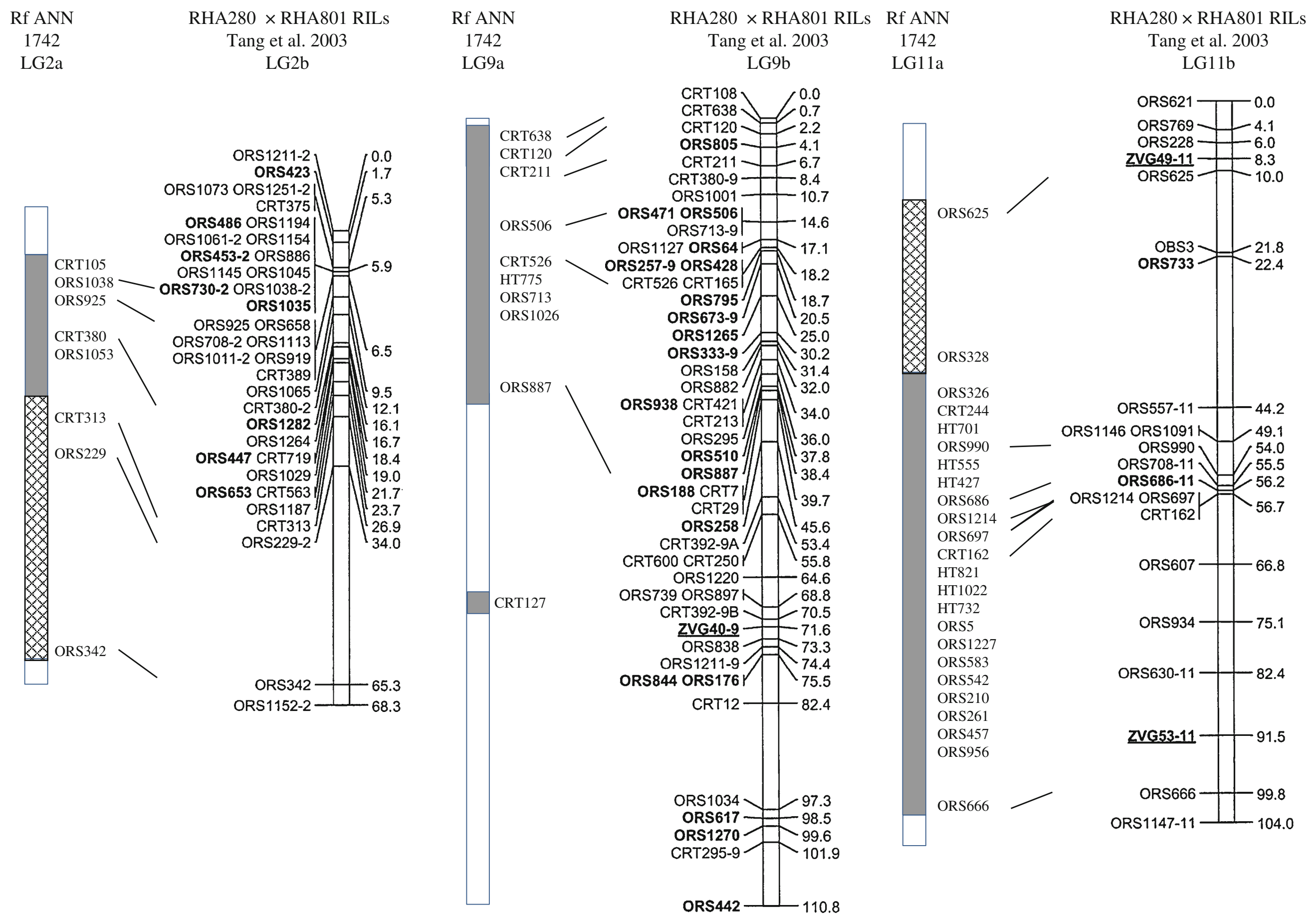

Fig. 3 Diagram of inferred suppression of recombination in linkage groups 2, 9, and 11 in Rf ANN-1742. HA 89 segments are *white*, wild donor segments are *gray*, and segments possessing recombination events are crisscross. Genetic maps of linkage groups 2, 9, and 11 were taken from Tang et al. (2003) as a cross reference of marker order, and common SSR markers were aligned between *a* and *b*. Possible order of other markers on LGs 2, 9, and 11 of Rf ANN-1742 were referenced to Burke et al. (2002), Yu et al. (2003), and the sunflower CMap database (http://sunflower.uga.edu/cmap/)

Discussion

In this study, we mapped two genes derived from wild *H. annuus*, a rust resistance gene R_{11} and a male restorer gene *Rf5*, to LG13 in sunflower. The two genes are tightly linked with a genetic distance of 1.6 cM in the coupling phase. To our knowledge, this is the first male fertility restorer gene which is closely linked to a rust resistance gene in sunflower. The advantage of such an introduced alien segment harboring both *Rf5* and R_{11} is its large phenotypic effect and simple inheritance, thereby facilitating their rapid deployment in sunflower breeding programs. It will be also of special interest for studying gene evolution and structure in this region in the future.

Out of 68 markers in LG13 screened in the study, only 8 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 *Rf5* and R_{11} genes 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 marker ORS728 is also closely linked to the *Rf5* gene at 1.3 cM, providing an additional selection for male fertility restoration.

Plant resistance genes that tend to cluster in genomes have been reported in diverse plant species (Saxena and Hooker 1968, 1974; Islam and Shepherd 1991; Jones et al. 1993; Song et al. 1997; Salmeron et al. 1996; Ellis et al. 1997; Michelmore and Meyers 1998; Richter and Ronald 2000; Hulbert et al. 2001; Wei et al. 1999, 2002). 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 that harbors downy mildew *R*-gene Pl_5/Pl_8 and rust *R*-genes R_{adv}, R_{11}, and R_4 (Lawson et al. 1998; Bert et al. 2001; Yu et al. 2003; Radwan et al. 2003, 2008; Radwan 2010; Qi et al. 2011b). Tracing the origin of three rust *R*-genes indicated that they were derived from diverse

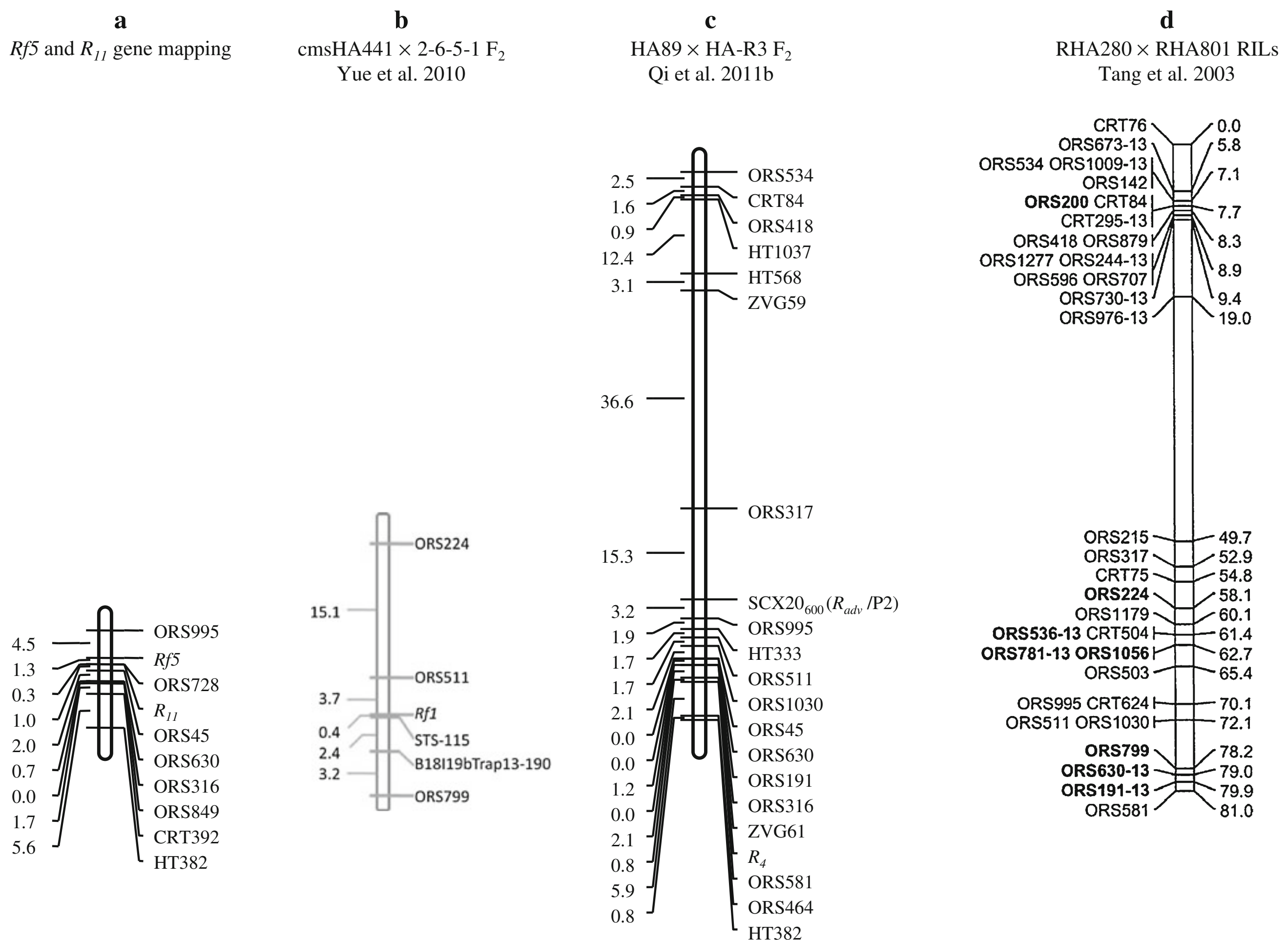

Fig. 4 Genetic maps of sunflower linkage group (LG) 13. **a** *Rf5* and R_{11} map. **b** *Rf1* map. **c** linkage group 13 genetic map, showing the positions of SCAR marker SCX20 and the R_4 locus. **d** public genetic map of linkage group 13 (Tang et al. 2003)

sources. R_4 originated from an interspecific pool of crosses between Russian varieties and wild sunflower species, including *H. annuus*, *Helianthus argophyllus*, and *H. petiolaris* (Gulya 1985; De Romano and Vázquez 2003). R_{adv} in lines P2 and RHA 340 was derived from wild *H. argophyllus* (Kong personal communication; Miller and Gulya 1988). R_{11} was transferred from wild *H. annuus* into an HA 89 background (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 (Rashid 2006; 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 (Fig. 4). 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 (Fig. 4c). The polymorphic fragment of $SCX20_{600}$ linked to R_{adv}/P2 was also not present in the Rf ANN-1742 line. Sendall et al. (2006) reported that R_{adv}/P2 was also linked to SSR markers ORS995 at 1.3 cM and ORS45 at 8.0 cM. Qi et al. (2011b) mapped $SCX20_{600}$ (R_{adv}/P2) to LG13 at 13.9 cM from the R_4 locus and 3.2 cM distal to ORS995 (Fig. 4c). In the present study, R_{11} was mapped at a position 6.4 cM proximal to ORS995 and 1.1 cM distal to ORS45 (Fig. 4a). Overall, by combining pedigree information, resistance specificity, and molecular mapping, our data suggested 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}.

Interestingly, the lower end of sunflower LG13 also harbors both *Rf1* and male fertility restoration from Rf ANN-1742, denoted *Rf5*, the latter together with R_{11} locus that confers resistance to rust (Gentzbittel et al. 1995; Berry et al. 1997; Horn et al. 2003; Yu et al. 2003; Kusterer

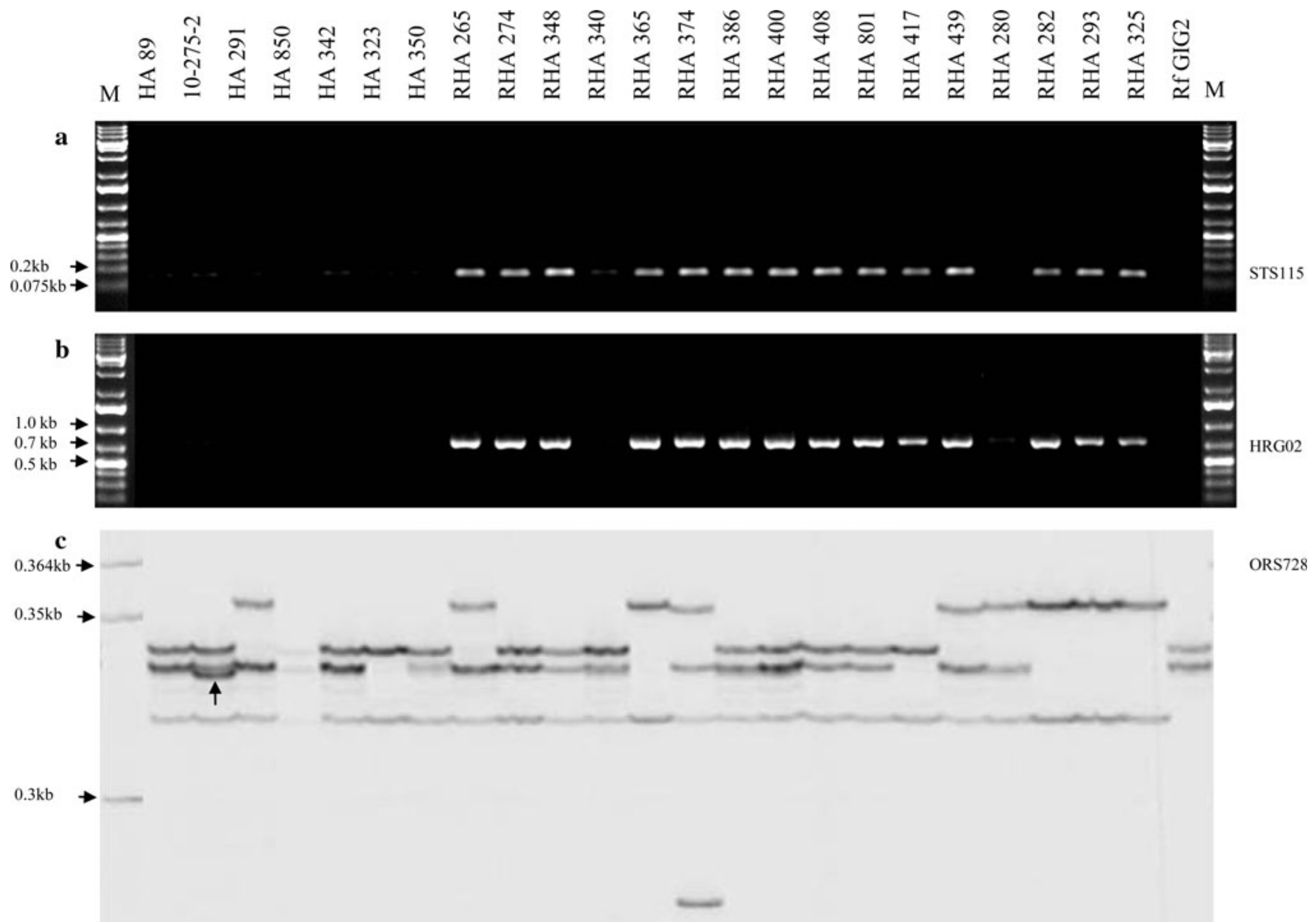

Fig. 5 Detection of *Rf* loci with the PCR-based markers, STS115, HRG02, and ORS728. The *Rf1* markers, STS115 (**a**) and HRG02 (**b**), were present in 14 restorer lines in which it was assumed that all carried the *Rf1* gene, whereas they were absent in 6 maintainer lines and 4 restorer lines harboring different restorer genes, 10-275-2 (*Rf5*), RHA 340 (*Rf3*), RHA 280 (*Rf3*), and RfGIG2 (*Rf4*). The *Rf5* marker, ORS728-specific fragment (**c**), is only present in 10-275-2 possessing the *Rf5* gene and absent in all maintainer and restorer lines. *Arrow* points to the ORS728-polymorphic fragment in the 10-275-2 plant. PCR products of primers STS115 and HRG02 were separated on a 1.5 % agarose gel, and the molecular weight marker is from GeneRuler 1 kb Plus DNA Ladder (Fermentas Inc. Maryland, US). The PCR product of ORS728 was diluted 80-fold and was size separated in an IR2 4300 DNA Analyzer (Li-COR, Lincoln, Nebraska), and the molecular weight marker is a 50- to 700-bp DNA ladder (Li-COR. Inc. Lincoln, Nebraska, US)

et al. 2005; Yue et al. 2010). We were not able to precisely position *Rf1* and *Rf5* relative to one another on the map because the polymorphic fragments that were amplified by three markers HRG01, HRG02, and STS115 linked to *Rf1* were not present in our mapping population. Meanwhile, the ORS728-specific fragment linked to *Rf5* was also absent in all the restorer lines carrying the *Rf1* gene, indicating that the *Rf5* gene may not be an allele at the *Rf1* locus (Fig. 5). In the map of Yue et al. (2010), the closest SSR marker linked to Rf_1 is ORS511, about 4 cM distal to this gene (Fig. 4b). Although ORS511 is not mapped in the present study, the marker falls in the region between ORS995 and ORS45 where *Rf5* resides (Fig. 4). Thus, we propose that *Rf5* may be closely linked to *Rf1*. Allelic tests of these two genes and its ability to restore different sunflower CMS lines of the *Rf5* gene are under investigation.

Suppressed recombination was observed in LGs 2, 9, and 11. The introgressed chromosome segments in these three linkage groups are mostly inherited as large blocks of chromatin, and no recombination occurred in specific regions of LG2 (span 5 SSR markers), 9 (9 SSR markers), and 11 (22 SSR markers), respectively. During the development of Rf ANN-1742, selection was performed against male fertility. Therefore, retention of large blocks of introgressed segments in LGs 2, 9, and 11 could be due to gamete selection, indicating that the chromosomes carrying wild donor segments may be preferentially transmitted in progeny. Variation of chromosome structure between cultivated and wild *H. annuus* was not detected (Heiser 1954; Chandler et al. 1986; Burke et al. 2002, 2004). Therefore, suppressed recombination observed in LGs 2, 9, and 11 could be due to low levels of homology between wild and

Table 3 The results of DNA markers linked to the restorer genes tested in maintainer and restorer lines of sunflower

Lines	Restoration genes	DNA markers			
		STS115-*Rf1*	HRG01-*Rf1*	HRG02-*Rf1*	ORS728-*Rf5*
HA 89		–	–	–	–
10-275-2[a]	*Rf5*	–	–	–	+
HA 291		–	–	–	–
HA 850		–	–	–	–
HA 342		–	–	–	–
HA 323		–	–	–	–
HA 350		–	–	–	–
RHA 265	*Rf1*	+	+	+	–
RHA 274	*Rf1*	+	+	+	–
RHA 348	*Rf1*	+	+	+	–
RHA 340	*Rf3*[b]	–	–	–	–
RHA 365		+	+	+	–
RHA 374		+	+	+	–
RHA 386		+	+	+	–
RHA 400		+	+	+	–
RHA 408		+	+	+	–
RHA 801		+	+	+	–
RHA 417		+	+	+	–
RHA 439		+	+	+	–
RHA 280	*Rf3*[b]	–	–	–	–
RHA 282		+	+	+	–
RHA 293		+	+	+	–
RHA 325		+	+	+	–
Rf GIG2	*Rf4*	–	–	–	–

plus sign polymorphic fragment present, *minus sign* polymorphic fragment absent

[a] Homozygous selection of Rf ANN-1742

[b] The restorer genes in RHA 340 and RHA 280 were both named *Rf3* (Abratti et al. 2008; Liu et al. 2011)

cultivated sunflower chromosomes. DNA markers that detected the large blocks of introgressed segments in the present study can be used to select genotypes without wild donor chromatins in LGs 2, 9, and 11 in breeding programs, consequently reducing the introgressed chromosome segments from wild *H. annuus*.

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; Slabaugh et al. 2003; Yu et al. 2003; Qi et al. 2011b, c). 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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