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ORIGINAL PAPER

Relocation of a rust resistance gene R_2 and its marker-assisted gene pyramiding in confection sunflower (*Helianthus annuus* L.)

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Abstract

Key message **The rust resistance gene R_2 was reassigned to linkage group 14 of the sunflower genome. DNA markers linked to R_2 were identified and used for marker-assisted gene pyramiding in a confection type genetic background.**

Abstract Due to the frequent evolution of new pathogen races, sunflower rust is a recurring threat to sunflower production worldwide. The inbred line Morden Cross 29 (MC29) carries the rust resistance gene, R_2, conferring resistance to numerous races of rust fungus in the US, Canada, and Australia, and can be used as a broad-spectrum resistance resource. Based on phenotypic assessments and SSR marker analyses on the 117 F_2 individuals derived from a cross of HA 89 with MC29 (USDA), R_2 was mapped to linkage group (LG) 14 of the sunflower, and not to the previously reported location on LG9. The closest SSR marker HT567 was located at 4.3 cM distal to R_2. Furthermore, 36 selected SNP markers from LG14 were used to saturate the R_2 region. Two SNP markers, NSA_002316 and SFW01272, flanked R_2 at a genetic distance of 2.8 and 1.8 cM, respectively. Of the three closely linked markers, SFW00211 amplified an allele specific for the presence of R_2 in a marker validation set of 46 breeding lines, and SFW01272 was also shown to be diagnostic for R_2. These newly developed markers, together with the previously identified markers linked to the gene R_{13a}, were used to screen 524 F_2 individuals from a cross of a confection R_2 line and HA-R6 carrying R_{13a}. Eleven homozygous double-resistant F_2 plants with the gene combination of R_2 and R_{13a} were obtained. This double-resistant line will be extremely useful in confection sunflower, where few rust *R* genes are available, risking evolution of new virulence phenotypes and further disease epidemics.

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Introduction

Sunflower seed is the fourth largest source of vegetable oil worldwide, following soybean, palm, and canola (rapeseed). The 2012 United States (US) sunflower crop totaled nearly 2.8 billion pounds and was valued at nearly $727.8 million (NASS 2013). While the majority of sunflower produced in the US is oil-type, 10–20 % of production is confection, high-value seed product used primarily in human diets as a snack. Confection seed quality includes many quantitatively inherited parameters, such as seed stripe pattern, seed size (length, width, and screen test size), kernel weight, and freedom from insect and disease damage (Hulke and Kleingartner 2014).

Rust (caused by *Puccinia helianthi* Schwein.) is a major yield-limiting disease of sunflower in many sunflower producing countries. The pathogen is macrocyclic and autoecious, able to overwinter in harsh climates and readily infects many

wild *Helianthus* relatives and cultivated volunteers (Gulya et al. 1997; Markell et al. 2009). These factors contribute to a high frequency of sexual recombination that can quickly and frequently lead to evolution of new virulence pathotypes. In North America, 38 *P. helianthi* races were identified in 2008 with races 334 and 336 being predominant, and rust incidence has continued to increase from 17 % in 2002 to over 70 % of surveyed fields in recent years (Gulya and Markell 2009; Kandel 2014). A significant change in the composition of races was reported recently in Argentina (Moreno et al. 2012). Among 17 *P. helianthi* isolates collected in Argentina from 2004 to 2011, race 700 was the predominant, and the remaining isolates also conferred virulence to each differential in the first triplet, making them 700 groups of races. No isolate belonging to the 100, 300, or 500 group of races (which do not confer virulence on all three differentials in the first triplet) was identified, but they were prevalent before 1985 (Antonelli 1985). In Australia, only one pathotype of *P. helianthi,* Aus 1, was reported before 1983 (Kong et al. 1999), but after a dramatic increase in number of races, 77 pathotypes were recently identified (Sendall et al. 2006). In all cases, the emergence of novel pathogen virulence through either sexual or asexual reproduction has rendered most of the commercial hybrids susceptible to rust, which could lead to epidemics when weather conditions are conductive to disease development (Kong et al. 1999; Gulya 2006; Gulya and Markell 2009; Markell et al. 2009; Qi et al. 2011a; Moreno et al. 2012). The frequent pathogen evolution makes discovery of novel rust *R* genes and development of DNA markers closely linked to the previously identified genes important for resistance breeding efforts and, ultimately, long-term management of sunflower rust.

Currently, 13 gene loci for resistance to rust (R_1–R_5, R_{10}–R_{12}, R_{13a}, R_{13b}, R_{14}, P_{u6}, and R_{adv}) have been described in sunflower, with R_{13a} being the only rust *R* gene present in confection 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. 2013a, b; Jan et al. 2014). Due to the high virulence variability of *P. helianthi* isolates, R_1 in MC90, R_3 in PhRR3, and R_{adv} in RHA340 already have been overcome in North America, but the remaining genes still effectively convey resistance to the pathogen (Gulya and Markell 2009; Qi et al. 2011a; Gong et al. 2013b). To facilitate marker-assisted selection and gene pyramiding in sunflower rust breeding programs, six *R* genes (R_4, R_5, R_{11}–R_{14}) have recently been assigned to linkage groups (LG) of the sunflower genome, and molecular markers linked to the respective genes have been developed (Qi et al. 2011b, 2012a, b; Gong et al. 2013a, b; Jan et al. 2014).

The gene R_2, originally from accession 953–88 (a wild annual sunflower growing near Renner, Texas), was first reported by Putt and Sackston in (1963). Inheritance studies of Morden Cross 29 (MC29) carrying R_2 indicated that MC29 is resistant to North America (NA) *P. helianthi* races 1 and 3 (respectively corresponding to NA *P. helianthi* races 100 and 500 of the current triplet coding system) and the resistance is controlled by a dominant gene (Putt and Sackston 1963; Miah and Sackston 1970). Lambrides and Miller (1994) reported that an Australian selection of MC29 [hereafter referred to as MC29 (AUS)] is resistant to NA race 6, whereas MC29 maintained in the Sunflower Research Unit of USDA-ARS, Fargo, North Dakota (ND) [hereafter referred to as MC29 (USDA)] is susceptible to NA race 6. MC29 (AUS) is reported to carry an additional gene named R_{10}, which is independent and non-allelic to R_2 (Lambrides and Miller 1994). Despite being identified approximately 50 years ago, the resistance in MC29 (USDA) and MC29 (AUS) is still widely effective on multiple continents. MC29 (USDA) provided resistance to approximately 90 % of 300 *Puccinia helianthi* isolates collected between 2007 and 2008 (Gulya and Markell 2009), and 108 ND isolates collected in 2011 (Friskop et al. 2012). MC29 (AUS) provided resistance to all known Australian rust races (Sendall et al. 2006).

R_2 was previously mapped to LG9 of the sunflower genome and co-segregated with an SSR marker ORS333 (Lawson et al. 2011). However, during a study aimed at transferring the R_2 gene from oil sunflower to confection sunflower, marker-assisted selection was conducted in the BC_4F_2 population using ORS333, but no marker-trait association was found, leading to further investigation of mapping of this gene. Here, we describe the relocation of the resistance gene R_2 to LG14, identification of SSR and SNP markers linked to this gene, and use of the linked markers in marker-assisted gene pyramiding.

Materials and methods

Plant materials

For molecular mapping of the gene R_2, the F_2 and F_2-derived F_3 populations were developed from a cross of HA 89 and MC29 (USDA). HA 89 (PI 599773) is a selection from the high-oil Russian cultivar 'VNIMK 8931 (PI 262517)' and was released by USDA and the Texas Agricultural Experiment Station in 1971. HA 89 is universally susceptible to all known races of *P. helianthi*. MC29 (USDA) is an oil-type Canadian line and carries the rust resistance gene R_2 (Putt and Sackston 1963; Lambrides and Miller 1994).

An F_2 population for rust *R* gene pyramiding was developed from the cross of HA-R6 and a BC_4F_2-derived line '12–55' of the pedigree CONFSCLB1*5/MC29 (AUS).

HA-R6 (PI 607509) is a maintainer line of confection sunflower and was released by the USDA-ARS Sunflower Research Unit and the North Dakota Agricultural Experiment Station in 2001 (Miller and Gulya 2001). HA-R6 is the only public confection sunflower line resistant to all known NA *P. helianthi* races identified so far and its resistance gene, R_{13a}, was mapped to LG13 of sunflower (Qi et al. 2011a; Gong et al. 2013b). CONFSCLB1, susceptible to rust, is a narrow-based maintainer line composite of confection sunflower and was released by the USDA-ARS Sunflower Research Unit and the North Dakota Agricultural Experiment Station in 2006. MC29 (AUS) is an Australian selection resistant to most of NA races of *P. helianthi* and carries the rust *R* genes, R_2 and R_{10} (Lambrides and Miller 1994; Gong et al. 2013b). An initial cross was made between the recurrent parent CONFSCLB1 and donor MC29 (AUS) to transfer rust resistance from oilseed to confection sunflower in 2009.

A set of 46 diverse sunflower inbred lines including 11 rust resistant and 35 susceptible lines were used for marker validation (Table 1).

Rust resistance evaluation

Phenotypic screening for rust resistance was performed with artificial inoculation tests using the procedure described by Qi et al. (2011a). Inoculation of the F_2 and F_3 mapping populations was conducted at the four-leaf stage in the greenhouse in August 2012 and April 2013, respectively. A total of 204 F_2 plants along with their parents, HA 89 and MC29 (USDA), were inoculated with NA *P. helianthi* race 336, a predominant race in North America (Gulya and Markell 2009). One-hundred seventeen F_2-derived F_3 families (20 plants from each F_3 family) were screened to confirm the resistance phenotype and genotype of F_2 plants. Plants were evaluated for their infection types (ITs) 12–14 days after inoculation based on the 0–4 scale of Yang et al. (1986) and the percentage of leaf area covered with pustules (severity) of Gulya et al. (1990). IT 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. A Chi square (χ^2, $p > 0.05$) test was performed on the phenotypic dataset to determine if the rust resistance segregation fits expected ratios (3:1 for F_2 and 1:2:1 for F_3 family).

The BC_1F_1 progeny from the cross of CONFSCLB1 and MC29 (AUS) was screened in the greenhouse for resistance to *P. helianthi* race 336 as described above. The next backcross generation was made by crossing selected resistant BC_1 plants with the recurrent parents. This process was repeated for the BC_2 through BC_4 generations. In the BC_4F_2 generation, a resistant plant, '12–55', was selected to cross with HA-R6 to produce an F_2 population for gene pyramiding in confection sunflower.

DNA extraction and PCR conditions

Leaf tissue was collected from the parents and F_2 plants at the four-leaf stage and freeze dried. Total DNA was extracted from the lyophilized tissue samples using the DNeasy 96 plant kit (Qiagen, Valencia, CA, USA) and DNA concentration was quantified on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

For simple sequence repeat (SSR) markers, polymerase chain reaction (PCR) of 15-µl contained 2 mM $MgCl_2$, 200–250 µM of each dNTP, 0.02–0.06 µM forward primer with an M13 tail (CACGACGTTGTAAAACGAC) 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. PCRs were performed in a Peltier thermocycler (Bio-Rad Lab, Hercules, CA, USA) with a touchdown program described by Qi et al. (2011a). PCR products were diluted 40- to 160-fold before analysis. SSR fragments were size separated using an IR2 4300/4200 DNA Analyzer (LI-COR, Lincoln, NE, USA).

Marker analysis

Simple sequence repeat markers were used for initial mapping studies. Lawson et al. (2011) reported that the rust resistance gene R_2 was located on LG9 in sunflower and co-segregated with SSR marker ORS333. When ORS333 was used to screen a BC_4F_2 population derived from CONFSCLB1*5/MC29 (AUS), no marker-trait association was found. Tang et al. (2002) reported that ORS333 represents two loci; one in LG9 and another in LG14. A total of 34 SSR markers from LG14 were used to screen polymorphism among HA 89, MC29 (USDA), and MC29 (AUS). Subsequently, polymorphic SSR markers were evaluated in the HA 89/MC29 (USDA) F_2 population to map the R_2 gene on LG14.

A total of 36 single-nucleotide polymorphism (SNP) markers, which possibly cover the R_2 region on LG14, were selected to saturate the genetic linkage map. Out of 36 SNPs, 23 were selected from the SNP map developed by the National Sunflower SNP Consortium (hereafter referred to as the NSA SNPs, Table S1, Talukder et al. 2014) and 13 were selected from the first published SNP map (hereafter referred to as the SFW SNPs, Table S2, Bowers et al. 2012). Twenty-three NSA SNPs and 13 SFW SNPs cover a region of 10.2 and 4.9 cM on LG14 on their respective maps (Tables S1 and S2).

Table 1 Sunflower lines selected for marker validation

Line	Pedigree	Rust phenotype[a]	Type	References
CONFSCLB1	HA 411/ROM PH//HA 442/3/HA 441/4/CONF/5/CONF	S	CB	–
CONFSCLR5	RO 12-13//RHA 274/Dobritch/3/PSC 8/4/CONF/5/CONF	S	CR	–
HA 89	VNIIMK 8931 Sel	S	OB	–
MC 29 (USDA)	Morden cross 29 selection	R	–	Putt and Sackston (1963)
MC 29 (AUS)	Morden cross 29 selection	R	–	Lambrides and Miller (1994)
MC 90	–	S	–	–
TX16R	TX16/HA89//TX16/HA89	R	OB	Jan and Gulya (2006)
HA-R1	Pergamino 71/538 selection	S	OB	Gulya (1985)
HA-R2	Impira INTA selection	R	OB	Gulya (1985)
HA-R3	Charata selection	R	OB	Gulya (1985)
HA-R4	Saenz Pena 74-1-2 selection	S	OB	Gulya (1985)
HA-R5	Guayacan INTA selection	S	OB	Gulya (1985)
HA-R6	HA 323/Ames 3234 (PI 650362)	R	CB	Miller and Gulya (2001)
HA-R7	RHA 324/Ames 3234 (PI 650362)	S	CR	Miller and Gulya (2001)
HA-R8	RHA 377/PI 432512	R	OR	Miller and Gulya (2001)
HA-R9	Selection Rf-ANN 1742	R	OR	Qi and Seiler 2013
P-386	Selection from Charata INTA	S	OB	Lambrides (1992)
Ph3	HA 89//PI 413038/HA 89*2	R	OB	Jan et al. (2004)
HA 61	953-88-3/Armavirski 3497	S	OB	–
HA 290	4*P-21-VR1/HA60	S	OB	Fick et al. (1979a)
RHA 265	2* Peredovik/953-102-1-1-41	S	OR	–
RHA 274	CMS PI343765/HA119//HA62-4-5/2/T66006-2	S	OR	Fick et al. (1975)
RHA 279	CMS PI343765/HA119//HA62-4-5/2/T66006-2-2-11-3-2	S	OR	–
RHA 298	CMS HA89/RHA273	S	OR	Fick et al. (1979b)
RHA 854	RHA 273 selection	S	OR	Roath et al. (1987)
RHA 855	CMS HA 89/RHA 273	S	OR	Roath et al. (1987)
RHA 856	RHA 299/Sorem HT 58	S	OR	Roath et al. (1987)
RHA 857	S310/RHA 297	S	OR	Roath et al. (1987)
RHA 858	P1161/RHA 298	S	OR	Roath et al. (1987)
RHA 859	NSH 43/RHA 299	S	OR	Roath et al. (1987)
RHA 344	RHA 274 *2/Pervenets high oleic	S	OR	Miller et al. (1987)
RHA 345	RHA 274 *2/Pervenets high oleic	S	OR	Miller et al. (1987)
RHA 340	HA 89 *3/H. argophyllus 415	S	OR	Miller and Gulya (1988)
RHA 397	RHA 274/RO-20-10-3-3-2	R	OR	Miller and Gulya (1997)
RHA 464	RHA 418/RHA 419/3/RHA 801//RHA 365/PI 413047	R	OR	Hulke et al. (2010a)
RHA 280	Sundak Sel	S	CR	Fick et al. (1974)
HA 353	HA 292*2/Pervenets high oleic	S	CB	Miller et al. (1987)
HA 285	Mennonite RR-18-1 selection	S	CB	Fick et al. 1974
HA 286	Israel selection	S	CB	Fick et al. (1974)
HA 287	MG4/Mennonite RR//Mingren/Commander	S	CB	Fick et al. 1974
HA 288	Mennonite RR selection	S	CB	Fick et al. (1974)
HA 292	3*Commander/Mennonite RR	S	CB	Fick et al. (1979b)
HA 234	2*Smena//HA6/HA8	S	OB	–
HA 458	HA 434*4/PI 468435	S[b]	OB	Hulke et al. (2010b)
RHA 428	RHA 801//RHA 365/PI 413157	S[b]	OR	Miller et al. (2002)
RHA 468	RHA 428/RHA 426//RO 12-13/3/RHA 274/PRS 5 (IMI R, Scl)	S[b]	OR	–

OB oil-B line, *OR* oil-R line, *CB* confection-B line, *CR* confection-R line, *S* susceptible, *R* resistant

[a] Virulence phenotype to *P. helianthi* race 336 as presented in Qi et al. (2011a)

[b] Present study

Table 2 Primer sequences of the SFW SNPs mapped in this study

SNP name	SNP primer	Primer sequence (5′-3′)[a]	Tm (Ta)
SFW00211	SFW00211F1	GCAACAGGAACCAGCTATGACGGTGTTGAATGATGCTTCC-3	56.3
	SFW00211F2	GCAACAGGAACCAGCTATGAC*ATGAC*GGTGTTGAATGATGCCCCA-3	55.5
	SFW00211R	CTTAGCCCAGATCACTGGACC-3	59.9
SFW00944	SFW00944F1a	GCAACAGGAACCAGCTATGACCGTCTTCTGTTTCTTCATCA-3	55.8
	SFW00944F2a	GCAACAGGAACCAGCTATGAC*ATGAC*CGTCTTCTGTTTCTTCGCCG-3	56.9
	SFW00944Ra	ACTGAGAAGATACATGGTTCTGATG-3	58.4
SFW01272	SFW01272F1a	GCAACAGGAACCAGCTATGACGCAACGTCAGCAATTTC-3	54.2
	SFW01272F2a	GCAACAGGAACCAGCTATGAC*ATGAC*GCAACGTCAGCAACCTT-3	53.6
	SFW01272Ra	GGCGAACGAAAACGGACATC-3	60.2
SFW02060	SFW02060F1a	GCAACAGGAACCAGCTATGACACTCATGGCTGCCCGC-3	57.5
	SFW02060F2a	GCAACAGGAACCAGCTATGAC*ATGAC*ACTCATGGCTGCATGT-3	55.7
	SFW02060Ra	TGCTTGCTGCTGTAGTGAAGA-3	59.9
SFW03772	SFW03772F1a	GCAACAGGAACCAGCTATGACCGAATAGTTTAGCGTCCTAG-3	56.1
	SFW03772F2a	GCAACAGGAACCAGCTATGAC*ATGAC*CGAATAGTTTAGCGTCTCAA-3	55.6
	SFW03772Ra	GCAAGGCACTGTCACTACTC-3	58.6

[a] The tail sequence is underlined and the additional five-base oligonucleotide insertion in AS-primer F2 is italicized

SNP genotyping

Genotyping of parental lines and F_2 population with NSA SNPs was conducted in BioDiagnostics Inc. (River Falls, WI, USA) where the NSA SNPs were developed (Pegadaraju et al. 2013). Genotyping of the SFW SNP was performed using a strategy of converting SNPs into length polymorphic markers. For each SNP, three primers were designed, two-tailed forward allele-specific primers (AS-primers F1 and 2), and one common reverse primer (Table 2). An additional 5-base oligonucleotide (5′-ATGAC-3′) was inserted between tail and allele-specific sequences in AS-primer F2 to produce length polymorphism between two alleles after amplification. A universal priming-element-adjustable primer (PEA-primer 5-ATAGCTGG-Sp9-GCAACAGGAACCAGCTATGAC-3) attached with fluorescence at 5′ terminus was used in each PCR.

In general, PCR amplifications were performed in 15 µl containing 0.8 M Betaine, 0.04 % BSA, 2 mM $MgCl_2$, 100 µM dNTP each, 0.2 µM common reverse primer, 0.2 µM universal fluorescence-labeled PEA-primer, and 0.04 µM each of AS-primers (the tail sequences being identical to PEA-primer), 1 × PCR buffer, 1 U of Taq polymerase (Bioline, Randolph, MA, USA), and 20 ng of genomic DNA. PCR was performed with initial denaturation at 94 °C for 3 min, followed by 8 cycles in which the annealing temperature was decreased by 1 °C for each cycle starting at 94 °C for 20 s, and then 55 °C for 30 s, followed by an additional 32 cycles of 2-step PCR protocol: 94 °C for 20 s, 60 °C for 30 s. The PCR products were diluted 40–240 times and size segregated in an IR^2 4300/4200 DNA Analyzer with denaturing polyacrylamide gel electrophoresis (LI-COR, Lincoln, NE, USA).

Genetic linkage map

The Chi square test ($p > 0.05$) was used to assess goodness-of-fit to the expected segregation ratio (1:2:1 co-dominant or 3:1 dominant) for each marker. JoinMap 4.1 was used for linkage analyses and map construction using a regression mapping algorithm with LOD score 3.0 and Kosambi's mapping function (Van Ooijen 2006).

Results

Phenotypic assessments

Distinct reactions were observed between the parental lines and their F_2 progenies when inoculated with NA *P. helianthi* race 336. HA 89 was highly susceptible with an IT of 4 and 30–40 % of the leaves covered with pustules, whereas MC29 (USDA) was highly resistant with necrotic or small pustules (IT 1) and 0–0.1 % of the leaves covered with pustules. The 204 F_2 individuals segregated at a ratio of 149R:55S which fits to an expected 3R:1S ratio ($\chi^2 = 0.4182$, $P = 0.52$). Phenotyping of the 117 F_3 families showed that the F_2 population had 22 homozygous resistant, 66 heterozygous resistant, and 29 homozygous susceptible plants, fitting a 1:2:1 ratio ($\chi^2 = 2.7605$, $P = 0.25$). Genetic segregation confirmed that resistance to rust in this population is due to a single dominant gene (R_2) originating from the resistant line MC29 (USDA).

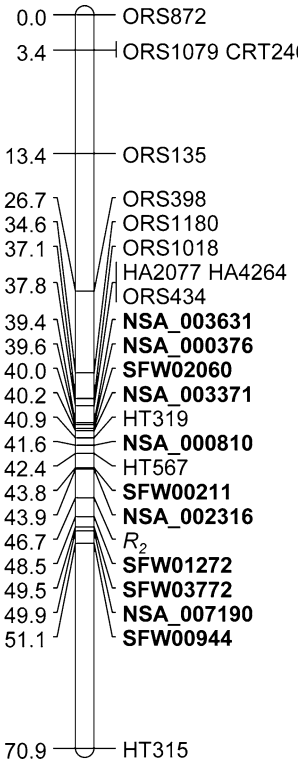

Fig. 1 Genetic map of sunflower linkage group 14, showing the R_2 position. SNP markers are in *boldface*

Genetic mapping of the rust resistance gene R_2

Thirty-four SSR markers previously mapped to LG14 of sunflower were screened between two parents, HA 89 and MC29 (USDA), as well as MC29 (AUS). Out of 34 markers, 17 (50 %) were polymorphic, including ORS333, which was previously reported to co-segregate with R_2 (Lawson et al. 2011). Both MC29 (USDA) and MC29 (AUS) shared common polymorphic markers with the same PCR patterns. All the polymorphic SSRs were assayed across the mapping population of 117 F_2 individuals and showed segregation in the expected 1:2:1 (co-dominant) or 3:1 (dominant) ratio. Thirteen polymorphic SSRs (all co-dominant) were placed in the LG14 genetic map, showing linkage to R_2, whereas ORS333 was not mapped to LG14 in this population. Resistance gene R_2 was positioned 4.3 cM distal to the closest marker HT567 and was 23.2 cM proximal to the marker HT315 (Fig. 1).

To further saturate the R_2 region, 36 SNP markers were applied to test for polymorphism between the parental lines, HA 89 and MC29 (USDA), and 11 were polymorphic (30.6 %). The eleven SNPs encompassed a region of 11.7 cM around R_2 in the F_2 population (Fig. 1). The resulting map placed R_2 to a 4.6 cM interval between two SNPs, NSA_002316 and SFW01272, with a genetic distance of 2.8 and 1.8 cM, respectively (Fig. 1). The map created by the F_2 population agrees well with previously published SSR and SNP maps (Table S1 and S2, Tang et al. 2002; Bowers et al. 2012; Talukder et al. 2014).

Marker validation

To investigate the possibility that SNP markers developed in this study could be used as diagnostic markers for R_2, eleven LG14 SNP markers including six NSA SNPs (NSA_003631, NSA_000810, NSA_00376, NSA_003371, NSA_002316, and NSA_007190) and five SFW SNPs (SFW02026, SFW00211, SFW01272, SFW03772, and SFW00944), were used to test five parental lines, namely HA 89, CONFSCLB1, MC29 (USDA), MC29 (AUS), and HA-R6 (Table 3). As expected, MC29 (USDA) and MC29 (AUS) share the same allele of all markers, indicating that the region carrying R_2 on LG14 in both MC29 lines likely originated from the same source. Genotypes of eleven SNPs could be classified into three groups based on parental line genotypes. Group I had two SNPs which gave an HA 89 allele different from the other four lines, CONFSCLB1, MC29 (USDA), MC29 (AUS) and HA-R6 (Table 3). Five SNPs in group II shared an allele between MC29 and HA-R6, whereas the remaining four SNPs in group III showed a unique allele for MC29 (Table 3).

The diagnostic value of the four closely linked markers to R_2, HT567, SFW00211, NSA_002316, and SFW01272, was further evaluated in 46 inbred lines with different genetic backgrounds (Table 1). As indicated above, the MC29 allele of the SNP marker NSA_002316 was not unique to R_2, and the resistant allele was shared by 18 lines of the 46 lines tested (Table 4). However, both the SSR marker HT567 and SNP marker SFW00211 displayed a specific allele diagnostic for the rust resistance gene R_2. No line with the same PCR pattern for HT567 and SFW00211 as MC29 among this set of lines was identified (Table 4; Fig. 2a). Out of the 46 lines genotyped with SNP marker SFW01272, only two lines, HA-R2 and HA-R8, shared a similar PCR pattern to MC29 (Table 4; Fig. 2b). These lines are also rust resistant but HA-R2 possesses a different locus from R_2 (Qi et al. 2012a) and the identity of the *R* locus in HA-R8 is unknown. Because of the rarity of the resistant allele, SFW01272 can be also used as a diagnostic marker for the selection of R_2 in the most genetic backgrounds.

Marker-assisted gene pyramiding of R_2 and R_{13a}

Initially, two SSR markers, HT567 linked to R_2 and ORS316 linked to R_{13a}, were used to screen an F_2 population from the cross of a resistant BC_4F_2 plant carrying R_2 with HA-R6 carrying R_{13a}. Out of 524 F_2 progenies screened, 11 plants (2.1 %) were found to be homozygous for the gene combination of R_2 and R_{13a}. Seven of the 11

Table 3 Allele detection of SNP markers linked to the resistance gene R_2 among parental lines

Line	Rust *R*-gene	Group I		Group II					Group III			
		NSA_003631	NSA_002316	NSA_000810	NSA_007190	SFW02060	SFW03772	SFW00944	NSA_000376	NSA_003371	SFW00211	SFW01272
HA 89	–	GG[a]	GG	AA	AA	–	–	–	AA	AA	–	–
CONFSCLB1	–	AA	AA	AA	AA	–	–	–	AA	AA	–	–
MC29 (USDA)	R_2	AA	AA	CC	GG	+	+	+	GG	CC	+	+
MC29 (AUS)	$R_2 + R_{10}$	AA	AA	CC	GG	+	+	+	GG	CC	+	+
HA-R6	R_{13a}	AA	AA	CC	GG	+	+	+	AA	AA	–	–

+, MC29 allele; –, Not MC29 allele

[a] Showing NSA SNP genotype

plants had good fertility and were further tested with the four markers, two LG14 SNP markers, SFW00211 and SFW01272, and two LG13 SSR markers, ZVG61, which co-segregated with R_{13a}, and HT382, which is proximal to R_{13a} at a genetic distance of 4.7 cM (Gong et al. 2013b). All plants consistently showed homozygous alleles of the selected markers linked to R_2 and R_{13a}, respectively (Table 5; Fig. 3). The F_4 progeny derived from homozygous double-resistant F_3 families was immune to rust infection tested by races 336 and 777 (data not shown).

Discussion

Resistance gene R_2 from MC29 (USDA) was reassigned to LG14 of the sunflower genome in the present study. SSR and SNP markers on LG14 were associated with the rust resistance in MC29 (USDA) supporting this relocation. Of eight previously mapped rust *R* genes, R_1 was mapped to LG8 (Yu et al. 2003), R_5 to LG2 (Qi et al. 2012a), R_{12} and R_{14} to LG11 (Gong et al. 2013a, Jan et al. 2014), and the remaining four (R_{adv}, R_4, R_{11}, and R_{13}) to LG13 (Bachlava et al. 2011; Qi et al. 2012a, b; Gong et al. 2013b). R_2 is the first rust resistance gene located on LG14 of the sunflower genome. Despite being one of the first discovered rust *R* genes in 1963 (Putt and Sackston 1963), R_2 is still effective against all *P. helianthi* isolates tested in Australia (Sendall et al. 2006) and most NA *P. helianthi* races identified in the US and Canada (Gulya and Markell 2009; Qi et al. 2011a; Gong et al. 2013b).

However, since pathogens frequently overcome single-gene host-resistance over time due to the emergence of new pathogen races (Kong et al. 1999; Gulya 2006; Sendall et al. 2006; Gulya and Markell 2009), the best strategy for preservation of resistance genes and long-term disease management is diversified resistance. Gene pyramiding, which combines a series of resistance genes identified in different parents into a single genotype, is a practical approach to achieving multiple and durable resistance (Singh et al. 2001; Servin et al. 2004; Werner et al. 2005; Joshi and Nayak 2010; Mago et al. 2011). The gene R_2 can easily be pyramided with any rust *R* genes located on different sunflower chromosomes if marker-assisted breeding resources are available. It has been a challenge in many cases to pyramid resistance genes by conventional approaches, because pathogen races that discriminate among resistance genes may be not available. Currently, no *P. helianthi* race has been detected that can distinguish resistance conditioned by the rust *R* genes R_{11}, R_{12}, R_{13a}, and R_{13b} in the US, making it difficult to combine these genes with R_2 based on virulence phenotyping (Miller and Gulya 2001; Hulke et al. 2010a; Qi et al. 2011a, Gong et al. 2013b). The molecular markers linked to R_2, as shown here, are helpful for

Table 4 Allele detection of markers linked to R_2 on 46 sunflower inbred lines with or without known rust resistance gene (s)

Line	Reaction to race 336	Resistance gene	Linkage group	Marker pattern			
				NSA002316	HT567	SFW00211	SFW01272
CONFSCLB1	S			AA*	−	−	−
CONFSCLR5	S			AA	−	−	−
HA 89	S			GG	−	−	−
MC 29 (USDA)	R	R_2	14	AA	+	+	+
MC 29 (AUS)	R	$R_2 + R_{10}$	14 and unknown	AA	+	+	+
MC 90	S	R_1	8	na	−	−	−
TX16R	R	Unknown		na	−	−	−
HA-R1	S			GG	−	−	−
HA-R2	R	R_5	2	AA	−	−	+
HA-R3	R	R_4	13	GG	−	−	−
HA-R4	S			AA	−	−	−
HA-R5	S			AA	−	−	−
HA-R6	R	R_{13a}	13	AA	−	−	−
HA-R7	S			AA	−	−	−
HA-R8	R	Unknown	Unknown	AA	−	−	+
HA-R9	R	R_{11}	13	GG	−	−	−
P-386	S			na	−	−	−
PH3	R	R_{14}	11	na	−	−	−
HA 61	S			GG	−	−	−
HA 290	S			GG	−	−	−
RHA 265	S			AA	−	−	−
RHA 274	S			GG	−	−	−
RHA 279	S			GG	−	−	−
RHA 298	S			GG	−	−	−
RHA 854	S			GG	−	−	−
RHA 855	S			GG	−	−	−
RHA 856	S			GG	−	−	−
RHA 857	S			AA	−	−	−
RHA 858	S			AA	−	−	−
RHA 859	S			GG	−	−	−
RHA 344	S			GG	−	−	−
RHA 345	S			GG	−	−	−
RHA 340	S	R_{adv}	13	GG	−	−	−
RHA 397	R	R_{13b}	13	GG	−	−	−
RHA 464	R	R_{12}	11	AA	−	−	−
RHA 280	S			AA	−	−	−
HA 353	S			GG	−	−	−
HA 285	S			GG	−	−	−
HA 286	S			AA	−	−	−
HA 287	S			GG	−	−	−
HA 288	S			AA	−	−	−
HA 292	S			AA	−	−	−
HA 234	S			AA	−	−	−
HA 458	S			GG	−	−	−
RHA 428	S			AA	−	−	−
RHA 468	S			GG	−	−	−

na not available

+, MC29 allele; −, Not MC29 allele

* Showing NSA SNP genotype

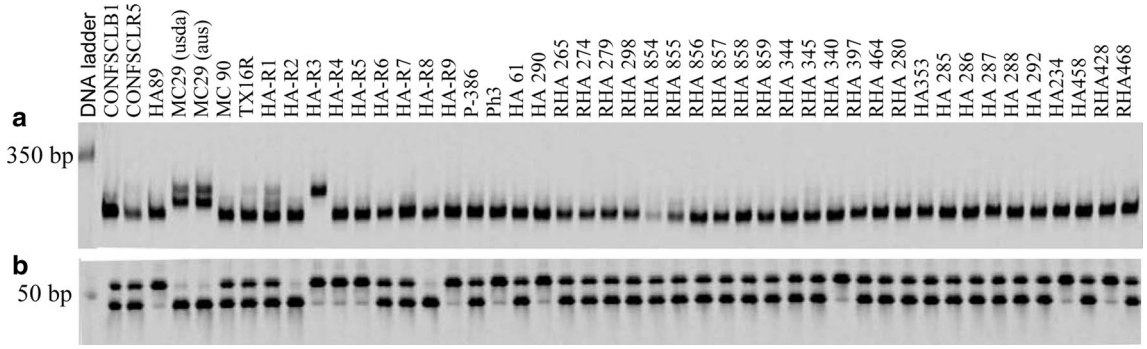

Fig. 2 PCR amplifications of markers HT567 (**a**) and SFW01272 (**b**) in a set of the 46 sunflower lines. The DNA ladder is a 50- to 700-bp molecular weight marker

Table 5 Summary of marker tests of the double-resistant lines with the gene combination of R_2 and R_{13a}

Line	Rust R-gene	LG14 markers linked to R_2			LG13 markers linked to R_{13a}		
		HT567	SFW00211	SFW01272	ORS316	ZVG61	HT382
HA 89	–	–	–	–	–	–	–
CONFSCLB1	–	–	–	–	–	–	–
MC29 (USDA)	R_2	+	+	+	–	–	–
MC29 (AUS)	$R_2 + R_{10}$	+	+	+	–	–	–
HA-R6	R_{13a}	–	–	–	‡	‡	‡
12-891-1	$R_2 + R_{13a}$	+	+	+	‡	‡	‡
12-891-2	$R_2 + R_{13a}$	+	+	+	‡	‡	‡
12-891-3	$R_2 + R_{13a}$	+	+	+	‡	‡	‡
12-891-5	$R_2 + R_{13a}$	+	+	+	‡	‡	‡
12-891-9	$R_2 + R_{13a}$	+	+	+	‡	‡	‡
12-893b-23	$R_2 + R_{13a}$	+	+	+	‡	‡	‡
12-894b-24	$R_2 + R_{13a}$	+	+	+	‡	‡	‡

+, MC29 allele for SSR and the SFW SNPs; ‡ HA-R6 allele; –, Neither CM29 nor HA-R6 allele

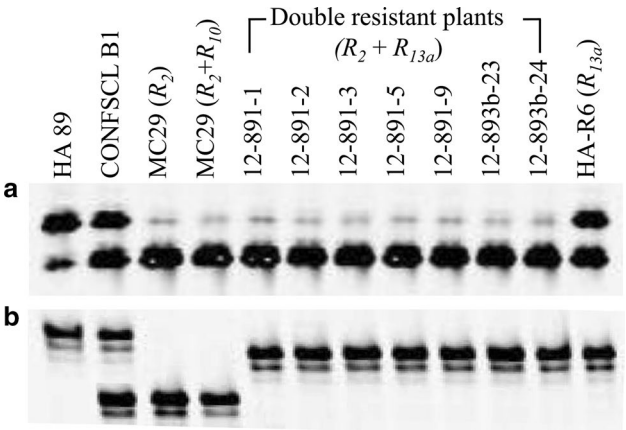

Fig. 3 PCR amplification of markers SFW00211-R_2 (**a**) and ORS316-R_{13a} (**b**) in parental lines and seven double-resistant F_2 plants. The double-resistant F_2 plants show CM29 (R_2) and HA-R6 (R_{13a}) alleles in **a** and **b**, respectively

marker-assisted gene pyramiding. Using these markers, the corresponding resistance genotypes of individuals can be assessed easily in a segregating population.

In recent years, SNP markers have emerged as powerful tools for plant molecular genetics and breeding because of their high genomic abundance, co-dominant inheritance, and potential for high-throughput automation. The increasing availability of SNP markers in sunflower provides a potentially valuable source for marker enrichment (Bachlava et al. 2012; Bowers et al. 2012; Pegadaraju et al. 2013; Talukder et al. 2014). In our initial SSR map, the gene R_2 was mapped to an interval of 28.5 cM on LG14 (Fig. 1). SSR marker HT567 is distal to R_2 at a genetic distance of 4.3 cM, whereas HT315 is 24.2 cM proximal of R_2. Subsequent SNP mapping delimited R_2 to the 4.6 cM interval on LG14 (Fig. 1). Two SNP markers, NSA_002316 and SFW01272, flanked R_2 at a genetic distance of 2.8

and 1.8 cM, respectively. The detection of additional SNP markers allows us to increase the resolution in mapping R_2, and reduces the likelihood of recombination between markers and the target loci during the breeding process. However, the NSA_002316 allele is shared by many susceptible lines (Table 4), indicating its limited diagnostic value. In contrast, the SNP marker SFW00211 adjacent to NSA_002316 amplified a fragment that is diagnostic for the gene R_2 in our set of genotypes. Thus, it is a good candidate for marker-assisted selection. To some extent, the SNP marker SFW01272 was also shown to be diagnostic for R_2, which is the closest marker linked to R_2. The combined use of two flanking markers may lead to reduction of the selection of false positives in breeding programs.

Marker-assisted gene pyramiding for disease resistance has been reported in rice (Huang et al. 1997; Hittalmani et al. 2000; Singh et al. 2001), wheat (Kloppers and Pretorious 1997; Liu et al. 2000; Samsampour et al. 2009; Mago et al. 2011), barley (Werner et al. 2005), and soybean (Shi et al. 2009). However, resistance gene pyramiding using marker-assisted selection has not yet been reported in sunflower. We report here the application of DNA markers for gene pyramiding of R_2 and R_{13a} in sunflower for rust resistance. These two genes are located on different sunflower chromosomes (Gong et al. 2013b), and double-resistant homozygotes could be selected from a relatively small F_2 population. Although in practice, the window of detection for a given recombinant in a segregating population is less than the theoretical limit, we were able to get 11 (2.1 %) double homozygotes from 524 F_2 plants screened by the DNA markers linked to these genes. Pyramids with the genes R_2 and R_{13a} will be extremely useful in confection sunflower where few rust *R*-genes are available, thus reducing producer's reliance on fungicides for control of rust and reducing the potential of a disease epidemic from the use of single-gene resistance (Qi et al. 2011a). As a hybrid crop, this line can be used as one parent to cross with another rust resistant line to produce F_1 hybrids with multiple genes to further broaden resistance.

Lambrides and Miller (1994) proposed that the Australian selection of MC29 carries two rust resistance genes, R_2 and R_{10}, whereas MC29 (USDA) only carries R_2. The DNA markers linked to R_2 developed in the present study revealed that the gene R_2 in both MC29 lines may originate from a common ancestor. In a follow-up experiment to our current work, MC29 (AUS) was used as a resistant donor to transfer rust resistance from oil-type to confection sunflower, and virulence phenotyping was conducted in each generation during backcrossing. At the BC_4F_2 generation, two resistant plants were tested with the DNA markers herein described, which are linked to R_2. One plant showed the association with R_2 markers, but the other resistant plant did not have the R_2 marker alleles (Qi et al. unpublished data), indicating that the resistance in these plants is controlled by the gene different from R_2 (perhaps R_{10}) which supports the previous report. Molecular mapping of R_{10} is underway to further elucidate its inheritance.

Author contribution statement Conceived and designed the experiments: LLQ BSH. Performed the experiments: LLQ GJM YML. Analyzed data: LLQ GJM YML. Contributed reagents/materials/analysis tools: LLQ BSH. Wrote the paper: LLQ BSH SJM.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments were performed in compliment with the current laws of USA.

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