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ORIGINAL PAPER

A transgressive segregation factor (*RKN2*) in *Gossypium barbadense* for nematode resistance clusters with gene *rkn1* in *G. hirsutum*

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Abstract Host plant resistance is an important strategy for managing root-knot nematode (*Meloidogyne incognita*) in cotton (*Gossypium* L.). Here we report evidence for enhanced resistance in interspecific crosses resulting from transgressive segregation of clustered gene loci. Recently, a major gene, *rkn1*, on chromosome 11 for resistance to *M. incognita* in cv. Acala NemX was identified using an intraspecific *G. hirsutum* cross with susceptible cv. Acala SJ-2. Using interspecific crosses of Acala NemX × susceptible *G. barbadense* cv. Pima S-7, F_1, F_2, $F_{2:3}$, backcross, and testcross Acala NemX × F_1 (Pima S-7 × SJ-2), parental entries and populations were inoculated in greenhouse tests with *M. incognita*. Genetic analyses based on nematode-induced root galling and nematode egg production on roots, and molecular marker analysis of the segregating interspecific populations revealed that gene *rkn1* interacted with a gene (designated as *RKN2*) in susceptible Pima S-7 to produce a highly resistant phenotype. *RKN2* did not confer resistance in Pima S-7, but when combined with *rkn1* (genotype *Aa* or *aa*), high levels of resistance were produced in the F_1 and segregating F_2, F_3, and BC_1F_1 populations. One SSR marker MUCS088 was identified tightly linked to *RKN2* within 4.4 cM in a NemX × F_1 (Pima S-7 × SJ-2) testcross population. Using mapped SSR markers and interspecific segregating populations, MUCS088 linked to the transgressive gene from the susceptible parent and was located in the vicinity of *rkn1* on chromosome 11. Diverse genome analyses among A and D genome diploid and tetraploid cottons revealed that marker MUCS088 (165 and 167 bp) is derived from *G. arboreum*, A_2 diploid genome. These results demonstrated that a highly susceptible parent contributed to nematode resistance via transgressive segregation. Derived highly resistant lines can be used as improved resistance sources in cotton breeding, and MUCS088 can be used to monitor *RKN2* introgression in diverse populations. The close genomic location of the transgressive resistance determinants provides an important model system for studying transgressive segregation and epistasis in plants.

Keywords Cotton · *Gossypium barbadense* · *Gossypium hirsutum* · Mapping · *Meloidogyne incognita* · Resistance gene cluster · Root-knot nematodes · Transgressive segregation

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Introduction

Novel sources and enhanced levels of pathogen resistance are desirable for genetic improvement of crop plants. One source of enhanced resistance is that generated through transgressive segregation. Transgressive segregation is the phenomenon in which segregating hybrids exhibit phenotypes that are extreme or novel relative to the parental lines

(Rieseberg et al. 2003). Transgressive segregants for numerous traits have been reported, and can be derived from both intraspecific and interspecific crosses (Rieseberg et al. 2003; Bell and Travis 2005). Here we report on a system of transgressive segregation and epistasis in cotton for resistance to parasitic nematodes, in which the genetic determinants of enhanced resistance are clustered in the same genomic region.

Transgressive segregation appears to be quite common in allotetraploid cotton (*Gossypium hirsutum* L.), although the genetic basis of the generated novel phenotypes is generally not known. Transgressive segregation is one of the major selection sources in cotton for enhanced resistance to root-knot nematode (*Meloidogyne incognita* Kofoid and White (Chitwood)), an important pest of cotton (Goodell and Montez 1994). Transgressive segregation also has been observed in other pathogen-cotton systems, such as for Verticillium wilt resistance in an F_2 (resistant *G. barbadense* Pima S-7 × susceptible Acala 44) (Bolek et al. 2005), and for Fusarium wilt resistance in the F_2 or F_3 (resistant Pima S-7 × susceptible Acala SJ-2 or susceptible Acala NemX) (Wang and Roberts 2006a). A susceptible parent was found to contribute to bacterial blight resistance in the BC_4F_4 generation within an intraspecific cross of cotton (Bayles et al. 2005). Transgressive segregation for resistance also has been reported in other plant-pathogen systems, such as soybean–soybean cyst nematodes (Winter et al. 2007), soybean—*Rhizoctonia* root and hypocotyl rot (Zhao et al. 2005), *Arabidopsis—Leptosphaeria maculans*, blackleg disease (Staal et al. 2006), wheat—stripe rust (Zhang et al. 2001; Imtiaz et al. 2003a, b; Navabi et al. 2004), and barley—*Pyrenophora teres* (Cherif and Harrabi 1993).

Recently, we conducted genetic and molecular analysis of the *M. incognita* resistance in Acala NemX through an intraspecific *G. hirsutum* cross with closely related susceptible cv. Acala SJ-2 using different segregating populations including F_1, F_2, $F_{2:3}$, BC_1F_1 and $F_{2:7}$ recombinant inbred lines (RIL) (Wang et al. 2006a, b; Wang and Roberts 2006b). A single major gene (*rkn1*) that determined the resistance was identified in Acala NemX (Wang et al. 2006b), and two PCR-based markers, CIR316 SSR (Wang et al. 2006a) and GHACC1 CAPS (Wang and Roberts 2006b) tightly linked to the *rkn1* locus were identified on linkage group A03, which was recently assigned to chromosome 11 (Frelichowski et al. 2006; Wang et al. 2006c). The *rkn1* gene is effective in suppressing both nematode induced root-galling and nematode reproduction on cotton roots. The heterozygous F_1 did not differ from the susceptible parent Acala SJ-2 with root galling reactions, and had slightly suppressed nematode egg production, indicating an incomplete recessive behavior. Moreover, we observed transgressive segregation for nematode resistance in some $F_{2:7}$ RI homozygous resistant lines, indicating susceptible genotype Acala SJ-2 contributed to the level of resistance (Wang et al. 2006b).

Several other sources of nematode resistance have been bred into advanced cotton lines, including *G. hirsutum* Auburn 623 RNR, Auburn 634 RNR and their derived M-lines (Shepherd 1974, 1982; Shepherd et al. 1988, 1996), and N-lines (Hyer and Jorgenson 1984). Among these, Auburn 623 RNR and some N-lines (N6074, N9281, N9308 and N9311) were reported to be transgressive segregants for *M. incognita* resistance (Shepherd 1974; Hyer et al. 1979; Hyer and Jorgenson 1984). McPherson et al. (1995) postulated that highly resistant Auburn 623 RNR might carry two genes with one coming from each parent, Clevewilt 6-3-5 and Mexico Wild Jack Jones. Clevewilt 6-1, a moderately resistant *G. hirsutum* germplasm, may contain one recessive gene conferring *M. incognita* resistance in crosses with Stoneville 213 (Bezawada et al. 2003). Genetic analysis of the M-line, M-315 RNR for *M. incognita* resistance revealed a two-gene model of one dominant and one additive gene (McPherson et al. 2004). Shen et al. (2006) reported that one major dominant gene in M-120 RNR associated with SSR marker CIR316 was localized in chromosome 11 and one minor gene on chromosome 7 when crossed with *G. barbadense* Pima S-6. Through SSR marker analysis in one cross within *G. hirsutum* of resistant (from Auburn 634) × susceptible near-isolines, Ynturi et al. (2006) identified two independent genes, one additive and dominant on chromosome 14 and the other additive on chromosome 11. Turcotte et al. (1963) reported that two recessive genes determined *M. incognita* resistance based on susceptible F_1 and segregating F_2 in a study of crosses of root-knot-resistant *G. darwinii* (originally classified as *G. barbadense* race *darwinii*) × susceptible *G. barbadense* cv. Pima S-1 and Pima S-2 breeding stocks.

Pima cottons (*G. barbadense* L.) are good hosts for *M. incognita* (Robinson et al. 1999), and in preliminary studies with interspecific Pima S-7 × Acala NemX progenies, we observed some genotypes with levels of nematode resistance higher than the resistant parent indicating strong transgressive segregation and epistasis effects on resistance. To further characterize the transgressive segregation for resistance in interspecific crosses of susceptible *G. barbadense* cv. Pima S-7 × Acala NemX, F_1, F_2, $F_{2:3}$, backcross, and testcross NemX × F_1 (Pima S-7 × SJ-2), the following specific objectives were set for this study: (1) to characterize how the *rkn1* gene behaves in the interspecific crosses of susceptible Pima S-7 × Acala NemX, and (2) to identify, map, and trace the origin of the gene (*RKN2*) in susceptible Pima S-7 contributing to the transgressive resistance, as compared to the SSR marker CIR316 linked to *rkn1* in this interspecific cross.

Materials and methods

Plant materials and crosses

Plant genotypes used in this study were susceptible *G. hirsutum* cv. Acala SJ-2 (USDA-ARS), resistant *G. hirsutum* cv. Acala NemX (Bayer Corp., formerly by CPCSD; Oakley 1995), and susceptible *G. barbadense* Pima S-7 (USDA-ARS). The F_1 populations were developed from crosses between Acala NemX, Acala SJ-2, and Pima S-7 in all combinations, including reciprocal crosses. The F_1 (Pima S-7 × NemX) was selfed to produce F_2 and $F_{2:3}$ populations. Pima S-7 and Acala NemX were backcrossed to F_1 (Pima S-7 × NemX) and F_1 (NemX × Pima S-7) plants to produce corresponding BC_1F_1 populations. One testcross was made between Acala NemX and F_1 (Pima S-7 × SJ-2). Different tetraploid and diploid cottons that were used to investigate the origin of the marker (allele/PCR product) linked to the transgressive resistance gene *RKN2* are presented in Table 1. Ninety-six individuals of the interspecific Pima S-7 × Acala NemX F_2 population (Pop 1, $N = 121$) were used for mapping. The intraspecific Acala NemX × Acala SJ-2 mapping population (Pop 2), which comprised 96 F_2 individuals previously reported by Wang et al. (2006b) was also included for mapping analysis.

Nematode resistance screening

Cotton populations were evaluated for nematode resistance under controlled conditions in a greenhouse using the methods of Wang et al. (2006b). Three-week-old seedlings were inoculated with approximately 50,000 eggs of *M. incognita* race 3 (isolate Project 77, from a cotton field in California). Air temperatures in the greenhouse were maintained between 28 and 35°C during the day and 24°C at night. The F_1 populations, 179 F_2 individuals (Test I), 64 $F_{2:3}$ families and 52 testcross Acala NemX × F_1 (Pima S-7 × NemX) plants were phenotyped 60 days after inoculation. Each $F_{2:3}$ family represented by 13–20 plants per family was screened for resistance. In order to collect enough $F_{2:3}$ (Pima S-7 × NemX) and BC_1F_2 seeds, 169 F_2 (Test II), 90 BC_1F_1 NemX × F_1 (Pima S-7 × NemX), and 114 Pima S-7 × F_1 (Pima S-7 × NemX) plants were phenotyped for resistance reaction 240 days after inoculation. A 0–10 root-gall rating scale (Bridge and Page 1980), ranging from no galling (0) to severe galling reaction (10), was used to evaluate resistance reaction to nematodes (Wang et al. 2006b). Cotton resistance was also evaluated by the numbers of nematode eggs per gram of fresh root. Eggs were extracted from the roots in NaOCl (Hussey and Barker 1973). Because large numbers of plants were evaluated and testing encompassed different generations or populations, the phenotyping experiments were done in different tests with each incorporating parental entries as checks. Plants were classified as resistant or susceptible based on comparisons with the susceptible and resistant parent phenotypes in each test. The mean and standard deviation (SD) for galling index and eggs per gram of root for each parent were used to determine the threshold for resistance in each test (Wang et al. 2006b).

In F_2 populations, non-segregating resistant genotypes were identified when all individual plants in a $F_{2:3}$ family were resistant, non-segregating susceptible genotypes when all individual $F_{2:3}$ plants were susceptible, and segregating resistant or susceptible genotypes when plants in a $F_{2:3}$ family exhibited these traits in different ratios.

Table 1 The cotton entries tested for the origin of root-knot resistance genes

	Germplasm	Species
Tetraploid	NemX (Bayer Corp.), TM1, MD51ne, SJ-2, USC-1 (Upland Susceptible Check 1), USC-2 (Upland Susceptible Check 2), FBCX-2a, FBCX-2b, and N901 (USDA-ARS)	*Gossypium hirsutum* (AD_1)
	Pima S-7, 89590, 3–79 (USDA-ARS), DP744 (Delta and Pine Land Co), and Phytogen 800 (Phytogen Co).	*G. barbadense* (AD_2)
	NM24016 (NMSU)	Stable interspecific inbred line between *G. barbadense* and *G. hirsutum*
Diploid	A_1: 9, 017, 018, 018-b, 023, 024, 030, 098	*G. herbaceum*
	A_2: 019, 020, 027, 030, 031, 041, 72, 076, 079, 082, 083, 084, 086, 087, 100, 101, 113, 144, 159, 190, 194, 241	*G. arboreum*
	D_1: 23, 35	*G. thurberi*
	D_2	*G. harknessii*
	D_4: US04, US017	*G. aridum*
	D_5	*G. raimondii*
	D_8: 1, 6	*G. trilobum*

Marker analysis

The protocols of DNA extraction and amplification of SSR markers and their resolution on agarose and polyacrylamide gels were as reported in Wang et al. (2006a). One hundred and fifty-two SSR markers polymorphic between *G. hirsutum* and *G. barbadense* were used to identify linkage with gene *RKN2* in the testcross population Acala NemX × F_1 (Pima S-7 × SJ-2) based on bulked segregant analysis (BSA) (Michelmore et al. 1991). These markers were used out of 384 previously screened in the regions of marker availability (Wang et al. 2006a). The above markers were used throughout this study in different analyses and populations. Primers were synthesized by IDT (IDT, Coralville, IA, USA). The reaction was performed in 10 µl volume containing 20 ng DNA, 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 0.1 µM forward primer, 0.1 µM reverse primer, and 0.5 U *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), followed by electrophoresis in agarose gels. For polyacrylamide gel analysis, a tailed SSR protocol was used and forward primers were synthesized with an M13 forward sequence on the 5′-end. The 0.025 µM IRD-labeled M13 primer (5′-CACGACGTTGTAAAACGAC-3′, LI-COR, Lincoln, NE, USA) was included in the PCR reaction. PCR was performed with a "touchdown" program (further described in Wang et al. 2006a). To separate the amplified products agarose gel (3% SFR Invitrogen) was used with 0.5 µg/ml of ethidium bromide followed by staining for electrophoresis in 1× TBE buffer at 80 V for 4–5 h. The bands were visualized by illumination with ultraviolet light. To obtain better resolution for some primers, denatured DNA fragments were resolved in 25-cm gels (0.25 mm spacer thickness) containing 8% Long Ranger^{TM} polyacrylamide gel (Cambrex, Rockland, ME, USA). Electrophoresis and detection were performed on a two-dye, model 4000 LI-COR IR^2 automated sequencer.

Amplified fragment length polymorphism (AFLP) was generated using the protocol of IRDyeTM Fluorescent AFLP kit for large plant genome analysis (LI-COR, Lincoln, NE, USA). Further information on the AFLP analysis and on the conversion of an AFLP marker (E-AAG/M-CCG primer combination) to a dominant CAPS marker (designated GHACC1) is described by Wang and Roberts (2006b).

Two resistant bulks and two susceptible bulks were made, each bulk containing eight individual plants. The markers with putative linkage to the *RKN2* gene within the bulks were then tested with the whole testcross population. Subsequently, 600 more mapped SSR markers (about 65% genome coverage) were screened to check for additional marker linkage and for localization of *RKN2*. The marker (MUCS088) linked to *RKN2* was used to test F_2 (Pima S-7 × NemX) plants. Identification of *rkn1* with SSR marker CIR316a was performed with 121 F_2 (Pima S-7 × NemX) plants.

Data analysis

Phenotypic data were subjected to one-way ANOVA. Fisher's Protected LSD test was used to compare the treatment means. Data for nematode egg production were transformed to $\log_{10}(x + 1)$ for analysis. The data for resistance segregation were tested for goodness-of-fit to predicted Mendelian inheritance ratios by Chi-square test.

Genetic linkage analysis and linkage group/chromosome 11 construction

The following procedures were utilized to construct the linkage groups for Pop 1 and Pop 2. Informative bands were scored as present (+) or absent (−) for a dominant marker (expected genotypic ratios 3:1) and if alleles from both parents were identified then the marker was scored as co-dominant (expected genotypic ratios 1:2:1). The JoinMap^R version 3.0 (Van Ooijen and Voorrips 2001) computer program was used to test for Chi-square goodness-of fit for expected versus observed genotypic ratios and to develop the final positional groups/chromosome 11. Recombination values supported by LOD scores between 3 and 10 were used for generating the map for each population, with the Kosambi map function and a maximum distance of 40 centiMorgans (cM) to determine linkage between two markers. In addition, JoinMap^R was used to assemble the three linkage groups, using the recombination estimation from the different populations with common markers and testing for heterogeneity in order to join the two groups. Recombination values supported by LOD scores between 3 and 10 were used to develop the joint-linkage group for chromosome 11.

Results

Phenotype of parents and F_1

Pima S-7, Acala NemX and Acala SJ-2 differed from each other for galling index and egg production ($P < 0.05$) in each test. The three parents were included in each test. The resistant parent Acala NemX had lower ($P < 0.05$) galling index (GI) (GI, 1.8, Fig. 1a) and supported fewer ($P < 0.05$) numbers of nematode eggs per gram root (EGR) (EGR, 518, Fig. 1b) than the two susceptible parent genotypes Acala SJ-2 (GI, 5.6 and EGR, 12431) and Pima S-7 (GI, 5.5 and EGR, 14040). The F_1 (Pima S-7 × NemX) had a lower ($P < 0.05$) galling index (GI, 0.7, Fig. 1a) and fewer

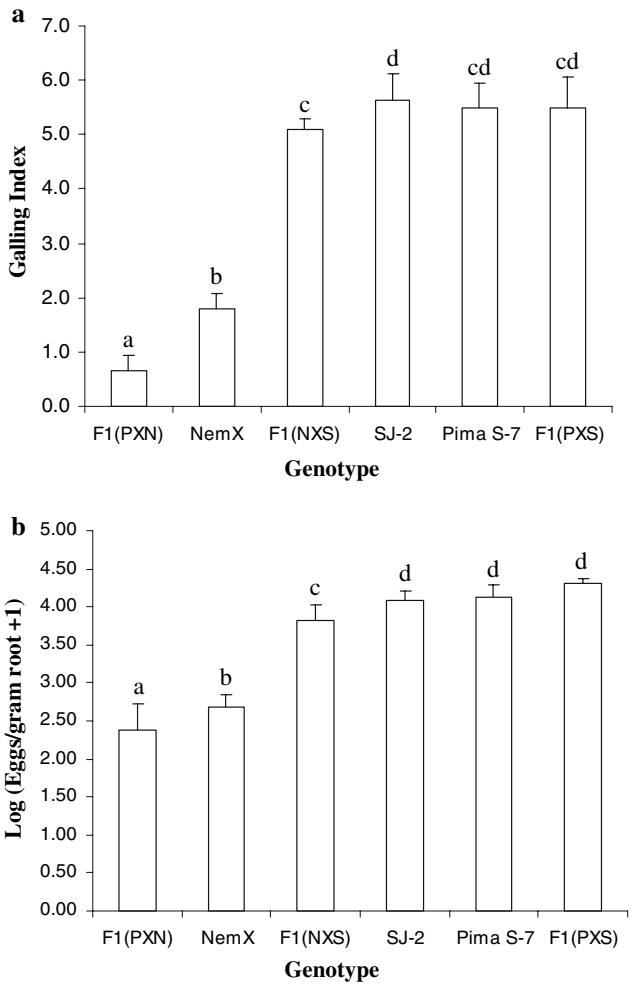

Fig. 1 Root-galling (**a**) and egg production (**b**) of *Meloidogyne incognita* on susceptible [Acala SJ-2 (S), Pima S-7 (P)] and resistant [Acala NemX (N)] cotton cultivars and their F_1. Data were collected 60 days after inoculation. The *error bars* show standard deviation. Genotypes with same letter do not differ according to Fisher's protected LSD test ($P < 0.05$). $\text{Log}_{10}(x + 1)$ transformed data were used for analysis of eggs per gram of root. Galling index: 0–10 scale; *0* no galling, and *10* severe galling

($P < 0.05$) numbers of nematode eggs (EGR, 291, Fig. 1b) than the resistant parent Acala NemX. However, the F_1 (NemX × SJ-2) had higher ($P < 0.05$) galling indices (GI, 5.1, Fig. 1a) and greater ($P < 0.05$) numbers of nematode eggs (EGR, 7252, Fig. 1b) than the resistant parent Acala NemX, but a lower galling index and numbers of nematode eggs than Acala SJ-2. The F_1 (Pima S-7 × SJ-2) with galling index 5.5 and 20840 nematode eggs per gram root did not differ from the two susceptible parents Acala SJ-2 and Pima S-7 based on galling index (Fig. 1a) and egg production (Fig. 1b). In previous experiments (Wang et al. 2006a, b) with the cross Acala NemX × SJ-2, the F_1 plants were susceptible, and genetic analysis of segregating populations revealed a pattern for an incompletely recessive gene (*rkn1*) for resistance in Acala NemX. However, the F_1 (Pima S-7 × NemX) was highly resistant in the current test, indicating susceptible Pima S-7 must contribute to the resistance in the F_1.

Analysis of F_2, $F_{2:3}$ (Pima S-7 × NemX) and backcross populations

Considering one major recessive gene *rkn1* (genotype *aa*) in Acala NemX confers root-knot nematode resistance when crossed with Acala SJ-2 (*AA*) (Wang et al. 2006b), we hypothesized that one factor (designated *RKN2*, *BB*) from Pima S-7 that does not confer resistance in Pima S-7 promotes resistance when combined with *rkn1* in the homozygous (*aa*) or heterozygous (*Aa*) condition to produce higher resistance. Higher resistant and higher susceptible plants than the resistant and susceptible parents, respectively, were observed in the F_2, $F_{2:3}$ families, and BC_1F_1 populations. The phenotypic distributions of 179 F_2 (Test I) for root galling index, total eggs and eggs per gram root are shown in Fig. 2a–c, respectively. Higher resistance in the F_1 (Pima S-7 × NemX) than in the resistant parent Acala NemX and susceptibility of the F_1 (NemX × SJ-2) were observed consistently in this test (Fig. 2). Seventeen out of 64 $F_{2:3}$ families had higher mean galling indices than Acala NemX (mean GI, 1.6) and 378 out of 924 plants from 64 $F_{2:3}$ families had a low galling index of from 0 to 1, indicating a transgressive effect in this population (Fig. 3). Plants from the 64 $F_{2:3}$ families that showed lower and higher scores for GI than the parents, indicated the presence of at least one minor modifying gene for this transgressive segregation effect in some families. Differences detected ($P < 0.05$) between outlying plants from these families, and resistant and susceptible parents indicated that approximately 41% of the plants from the $F_{2:3}$ (Pima S-7 × NemX) families were transgressive segregants for resistance (Fig. 3). This observation indicated that selection for root-knot resistance could be accomplished in a progeny as early as the F_2 generation and/or advanced generations if one of the contributing parents used to develop this population carries at least one allele each of the Acala NemX resistance gene *rkn1* and susceptible Pima gene *RKN2* (Figs. 1, 3). The 41% frequency of the 'ultra-resistant' progeny, being lower than the 56% expected if *rkn1* and *RKN2* were independent, also indicated that the two genes are linked.

Backcross populations were phenotyped 240 days after inoculation due to collecting BC_1F_2 seeds. Mean galling indices of the parents were 2.9 (range 2–3.5) for Acala NemX and 6.0 for Pima S-7 (range 5–7). Mean eggs per gram root was 417 (range 60–1,000) for Acala NemX and 3777 (range 2,300–7,000) for Pima S-7. In the backcross population to resistant Acala NemX, 75 plants had egg numbers ≤1,000 and 15 plants >1,000. In the backcross population to susceptible parent Pima S-7, 47 plants had

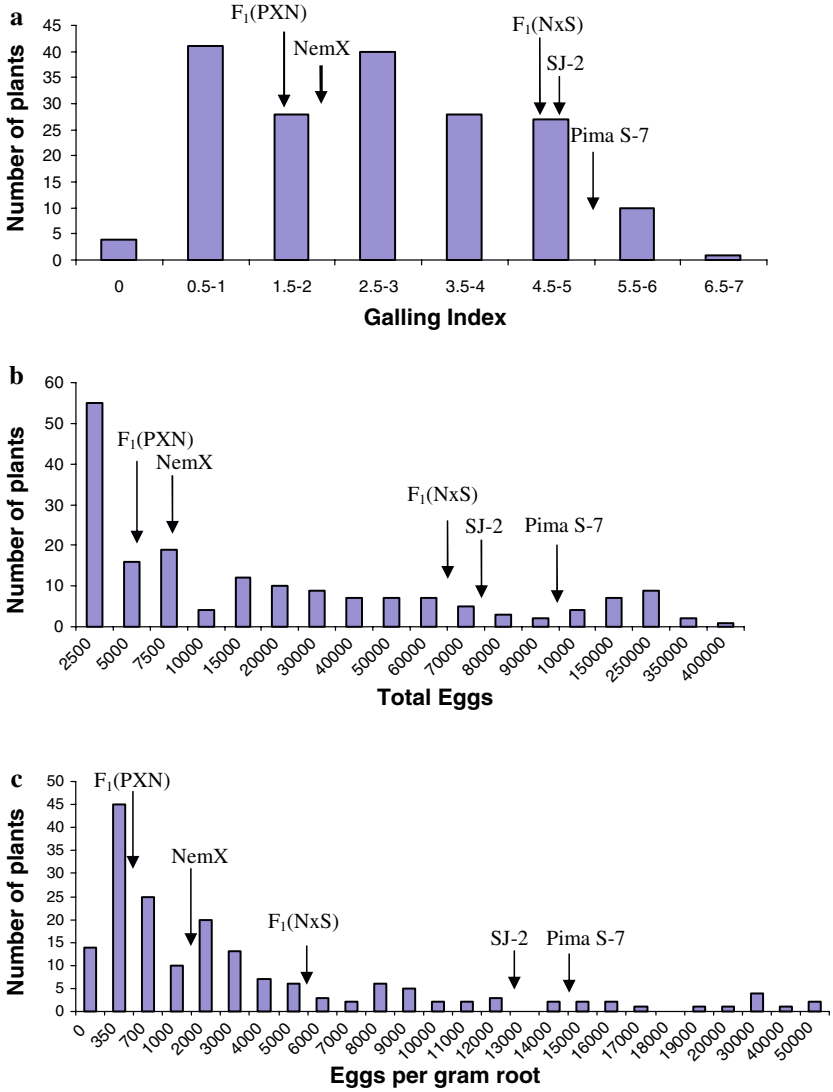

Fig. 2 Distribution of galling index (**a**), total eggs (**b**) and eggs per gram root (**c**) in the F_2 (Pima S-7 × Acala NemX) population. Data were collected 60 days after inoculation. Galling index: 0–10 scale; *0* no galling, and *10* severe galling. The *arrows* point to the phenotypic reaction in Pima S-7 (P), Acala NemX (N) and F_1 (P × N) plus Acala SJ-2 (S) and F_1 (N × S)

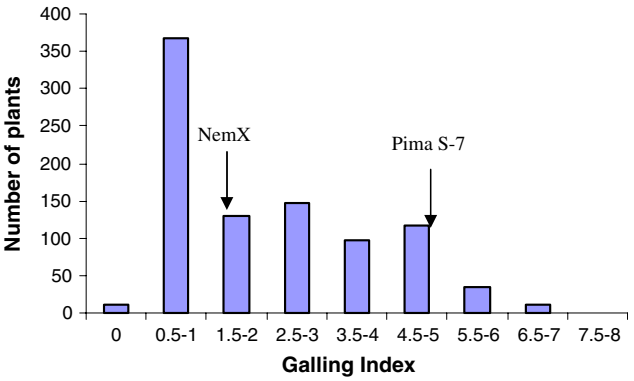

Fig. 3 Distribution of mean Galling Index in the 64 $F_{2:3}$ families. Data were collected 60 days after inoculation. Galling index: 0–10 scale; *0* no galling, and *10* severe galling. The *arrows* point to the parental positions

egg numbers ≤1,000 and 67 plants >1,000. The range of galling indices in the population of Acala NemX × F_1 was 1–6.5, and 1–8 in Pima S-7 × F_1. These resistance phenotype levels further confirm that one gene from Pima S-7 contributed to transgressive segregation for resistance when combined with at least one allele of *rkn1* in Acala NemX.

Analysis of the testcross Acala NemX × F_1 (Pima S-7 × SJ-2)

To confirm that susceptible Pima S-7 contributes to the nematode resistance, the testcross Acala NemX × F_1 (Pima S-7 × SJ-2) was made and phenotyped for resistance reaction to *M. incognita*. Root galling index had a high correlation ($r = 0.7947$) with egg production in the testcross population (Fig. 4), confirming the results from the parent and F_1 phenotype reactions (Fig. 1a, b). Two classes of individuals within the testcross population were clearly distinguished based on galling index and nematode egg production (Fig. 4). The Chi-square test ($P = 0.405$) showed the segregation of the 52 individual testcross plants fit closely a 1:1 (29R:23S) ratio between resistance (R) and

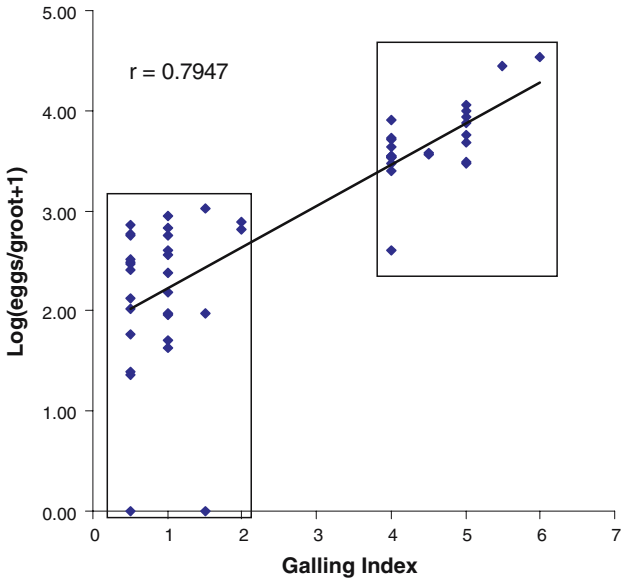

Fig. 4 The relationship of galling index and egg production in the segregating testcross population of Acala NemX × F_1 (Pima S-7 × SJ-2). $Log_{10}(x + 1)$ transformed data were used for analysis of eggs per gram of root. Data were collected 60 days after inoculation. Galling index: 0–10 scale; *0* no galling, and *10* severe galling. *Boxes* identify the two phenotype classes (R and S) in the testcross

susceptibility (S) (Fig. 4). The average of galling index and egg numbers per gram root of the resistant class was 0.9 and 329, respectively, and 4.5 and 7250, respectively, for the susceptible class. As expected, the phenotype of the 29 resistant plants was similar to the F_1 (Pima S-7 × NemX), and that of the 23 susceptible plants similar to the F_1 (NemX × SJ-2). These results further confirmed that Pima S-7 contributed to the level of nematode resistance when crossed with Acala NemX.

Identification and origin of SSR markers linked to *RKN2* in Pima S-7

Approximately 384 microsatellite or SSR makers that gave genome coverage on average of 20 cM in the regions of marker availability (Nguyen et al. 2004; Park et al. 2005; Frelichowski et al. 2006; Han et al. 2006) were used. Some of these markers were previously assigned to cotton chromosomes by hypoaneuploid deficiency analysis (http://www.mainlab.clemson.edu/cgi-bin/cmd/ViewMarkers.cgi). One hundred and fifty-two microsatellite markers were tested using BSA for potential association with nematode resistance in the testcross population Acala NemX × F_1 (Pima S-7 × SJ-2). One marker (MUCS088) was identified and then used to screen the individual testcross plants. The amplification patterns of the testcross Acala NemX × F_1 (Pima S-7 × SJ-2) population with the MUCS088 marker revealed a close linkage to gene *RKN2* in Pima S-7 (Fig. 5). Four out of 50 testcross plants showed recombination between MUCS088 (165 and 167 bp) and *RKN2*. The observed segregation ratio (84 present:28 absent) for the dominant MUCS088 marker fit exactly an expected segregation ratio of 3:1 ($\chi^2 = 0$, $P = 1.00$) in the F_2 (Pima S-7 × NemX) population. The F_2 (Pima S-7 × NemX) plants with marker MUCS088 were distributed across the complete range of galling index from 1 to 8. Of 36 F_2 plants with the galling index range of 6–8, 32 plants had the dominant MUCS088 marker profile from Pima S-7. However, in the testcross Acala NemX × F_1 (Pima S-7 × SJ-2), linkage analysis showed that the estimated distance between MUCS088 and *RKN2* was 4.4 cM (LOD score = 9). The primer sequence for the SSR marker MUCS088 is as follows: 5′-AAATTTGCAGCTCCTTC AG-3′ and 3′-AAATGTGTGTGAAGAGCAAAGC-5′.

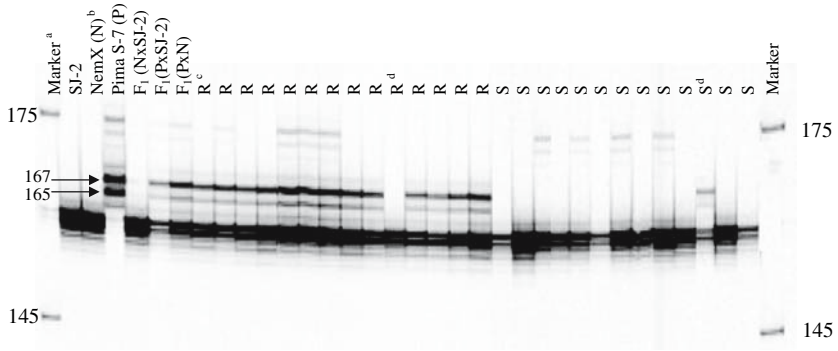

Fig. 5 Images of amplification products with marker MUCS088 in the segregating population Acala NemX × F_1 (Pima S-7 × SJ-2) electrophoresed on polyacrylamide gel with model 4000 LI-COR IR^2 automated sequencer. [a]Marker: 50–350 bp size standard (LI-COR); [b]N, NemX; P, Pima S-7; F_1 (PXN), F_1 (Pima S-7 × NemX), and so on. [c]R, resistant; S, susceptible, all based on phenotype. [d]R, recombinant line with resistant phenotype (R) and susceptible marker profile or susceptible phenotype (S) and resistant marker profile. The *arrows* point to the marker positions, one of 165 and 167 bp from Pima S-7. Note all allelic sizes include the M13 primer tail

Genetic mapping for linkage of MUCS088

Subsequently, 600 more SSR markers mapped previously on several cotton chromosomes were screened to check for additional marker linkage and for localization of *RKN2* to a specific cotton chromosome. Because, originally, MUCS088 was mapped to chromosome 17 (Park et al. 2005) several other chromosomes, including 17, were heavily targeted with SSR markers to find linkage for MUCS088. However, after revisiting markers mapped to chromosome 11, MUCS088 was found to be linked to BNL 1231 with cM distance of 16.9 and to CIR316 with a 3.2 distance in the interspecific F_2 Pop 1 (Pima S-7 × NemX) with a LOD 4.0. This linkage group representing chromosome 11 comprised 5 SSR markers with a total distance of 42.3 cM, in which four of the five SSR markers are known to be part of chromosome 11 (Fig. 6).

A second mapping intraspecific F_2 population, Pop 2, was used to localize *RKN2* relative to *rkn1* in tetraploid *G. hirsutum* genome of chromosome 11. A linkage group representing chromosome 11 which comprised seven molecular markers—two SSRs (Wang et al. 2006a), four ALFP and one CAP (Wang and Roberts 2006b)—was constructed from Pop 2 (NemX × SJ 2) with a total length of 46.2 cM. As expected, the CIR316 and CAP (GHACC1) markers were found to be closely linked to gene *rkn1* as previously reported (Wang et al. 2006a; Wang and Roberts 2006b; Fig. 6).

The joint-linkage group representing chromosome 11 developed from Pop 1 and Pop 2 comprised 12 loci, including *rkn1* and *RKN2* and covered 67 cM cotton chromosome 11 based on the JoinMap^R computer program (Van Ooijen and Voorrips 2001). The merging of the two groups from Pop 1 and Pop 2 was possible because of the two common markers between the two populations, CIR316 and BNL1231. The placement of *RKN2* was based on the test-cross population Acala NemX × F_1 (Pima S-7 × SJ-2) linkage analysis that showed an estimated distance between MUCS088 and *RKN2* of 4.4 cM (LOD score = 9). It is possible that the *rkn1* and *RKN2* resistance genes are only about 8.9 cM distance apart in the tetraploid cotton *G. hirsutum* chromosome 11 and this chromosome carries important resistance genes not only for root-knot nematode, but for other related diseases (Fig. 6).

In order to further investigate the origin of the MUCS088 allele linked to the *RKN2* gene for root-knot resistance, 51 diverse cotton germplasm lines were fingerprinted with this marker. This germplasm included two tetraploid species (AD_1 and AD_2), seven diploids (two from the A genome and five from the D genome), and one stable interspecific inbred line (Table 1). Fifteen of the 22 *G. arboreum* (A_2) entries (019, 027, 030, 031, 041, 72, 076, 079, 082, 083, 084, 086, 087, 113, and 194) and four *G. barbadense* (Table 1) entries contained the same size bands (165 and 167 bp) as Pima S-7 (Fig. 7), indicating that MUCS088 might have an A_2 (*G. arboreum*) genome origin, and that its introgression may

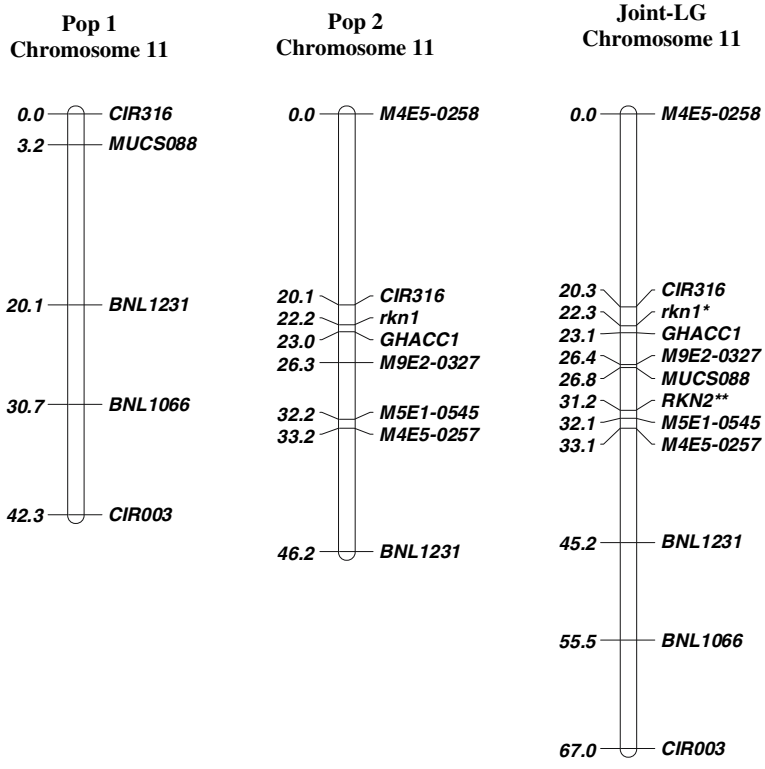

Fig. 6 Linkage groups and Joint-group representing cotton chromosome 11 showing the distance and position relationships between SSR markers and the nematode resistance genes *rkn1** and *RKN2***. F_2 interspecific (Pima S-7 × NemX) mapping population (Pop 1) and F_2 intraspecific (NemX × SJ-2) mapping population (Pop 2). The JoinMap^R was used to assemble the two groups for Pop 1 (LOD = 4.0) and Pop 2 (LOD = 6.0) and the joint-linkage group (Joint-LG) representing chromosome 11

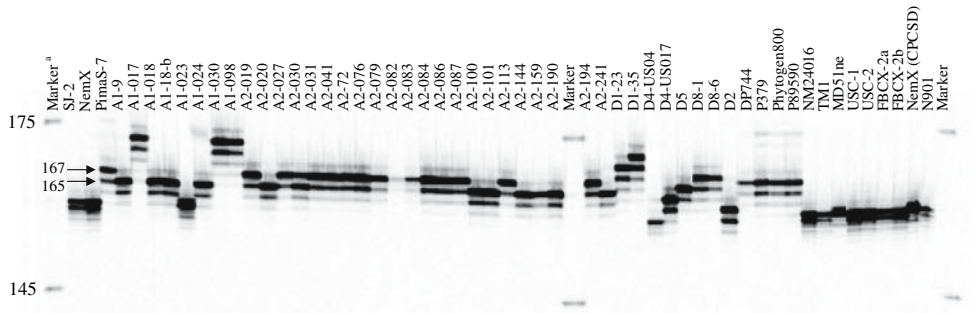

Fig. 7 Image of amplification products with MUCS088 marker in cotton germplasm electrophoresed on polyacrylamide gel with model 4000 LI-COR IR^2 automated sequencer. [a]Marker, 50–350 bp sizing standard. The *arrows* point to the marker positions, 165 and 167 bp from Pima S-7. Note all allelic sizes include the M13 primer tail

come from artificial hybridization (triple hybrid) and selection of the resistant parent source or during cotton genome evolution. One interspecific inbred (NM24016) and eight *G. hirsutum* lines had the same MUCS088 amplification pattern as Acala SJ-2 and Acala NemX in our study (Fig. 7).

Association of *rkn1* marker CIR316 with nematode resistance in F_2, F_3 and testcross populations

The CIR316a (221 bp) marker linked to the *rkn1* gene in Acala NemX (Wang et al. 2006a) was screened in 110 individual F_2 (Pima S-7 × NemX) plants. The observed segregation ratio (81 present:29 absent) for the dominant CIR316a marker fit an expected segregation ratio of 3:1 (82 present:28 absent) ($\chi^2 = 0.1111$, $P = 0.73888$). Based on the genotypes in the F_2, the plants with genotype *aa* or *Aa* should contain the marker, whereas plants with genotype *AA* should not contain the marker. Within the 29 plants with the absence of the CIR316 resistance linked allele, 25 plants were highly susceptible with a galling index ranging from 6 to 8, and 4 plants had a galling index ranging from 1 to 4. Seventy-seven of 81 plants with the presence of the CIR316 allele had a galling index ranging from 1 to 6, and 4 plants had a galling index 6.5–7.

Among 44 F_2 plants with postulated genotypes based on phenotypic screening of the $F_{2:3}$ families, 28 out of 32 plants with genotype *aa* or *Aa* were positive for the CIR316 marker–allele, and 11 out of 12 plants with genotype *AA* lacked CIR316. These data further indicated that the *rkn1* gene in Acala NemX was associated with the resistance in the interspecific cross Pima S-7 × Acala NemX, and that susceptible Pima S-7 also contributed to the high level of resistance.

Discussion

Genetic and molecular analysis of progenies from the cross Pima S-7 × Acala NemX and the testcross Acala NemX × F_1 (Pima S-7 × SJ-2) revealed that one gene, designated here as *RKN2*, in susceptible Pima S-7 conferred a high level of nematode resistance when combined with gene *rkn1* from Acala NemX. Plants found in the F_1, F_2, F_3 and BC_1F_1 progenies with higher resistance than resistant parent Acala NemX indicated that transgressive segregation for nematode resistance occurred in the interspecific crosses. This gene, *RKN2*, was mapped within the vicinity of *rkn1* on chromosome 11 (Fig. 6). This is the first report providing evidence based on both traditional and molecular genetic level analyses that a susceptible parent can contribute genetically to increase nematode resistance levels, having a major effect on the resistance phenotype expression and mapping to the same region as major nematode resistance genes.

Transgressive segregation has been observed not only in cotton-pathogen resistance (Bolek et al. 2005; Bayles et al. 2005; Wang and Roberts 2006a; this study) and other plant-pathogen resistance systems (Cherif and Harrabi 1993; Zhang et al. 2001; Imtiaz et al. 2003a, b; Navabi et al. 2004; Zhao et al. 2005; Staal et al. 2006; Winter et al. 2007), but also for other traits, such as fiber quality (Percy and Turcotte 1988; Kohel et al. 2001; Jenkins et al. 2007) and yield (Jenkins et al. 2006) in cotton, agronomic traits in tomato (de Vicente and Tanksley 1993), and protein content in rice (Hu et al. 2004; Watanabe et al. 2006). Thus, transgressive segregation for pathogen resistance and other traits is quite common in plants. Rieseberg et al. (1999, 2003) reported that transgressive segregation occurs frequently in segregating plant and animal populations based on numerous studies of many traits. This is an expected consequence of the genetic architecture of differentiated populations or species. However, in most cases the genetic basis of the effect has not been defined; the transgressive factor(s) from the opposing parent genotype (e.g., susceptible, lower fiber quality, etc.) are either not characterized genetically, are due to small effect QTL or minor genes that interact or have additive effects on the

target phenotype (e.g., pathogen resistance, high fiber quality, etc.), or are genes that confer a measurable level of resistance in the combining parent (Staal et al. 2006). However, here we describe a major effect gene that has no measurable impact on phenotype in the highly susceptible parent but interacts strongly with an R gene to enhance resistance. Applying the method of El Attari et al. (1998) for calculation of genetic gain (GG) for resistance in F_1 or TC_1 populations, in which the principal R gene, *rkn1*, is in the heterozygous condition, indicated GG in resistance over the best parent (Acala NemX) to be at least 100% ($P < 0.05$) based on root galling phenotype. The GG when *RKN2* is combined with homozygous *rkn1* could be even greater, since some F_2, F_3 and BC_1F_1 (Nem X × F_1) individuals had this genotype (*aaB-*) and extremely low galling scores were attained including some with Galling Index (GI) = 0. However, we did not distinguish those specific individuals with genotype *aaB-* from others with genotype *AaB-* in these populations. Analysis of stripe rust resistance in wheat using F_1-derived double haploid lines revealed transgressive segregation with an unidentified gene being contributed by the susceptible parent in combination with two genes in a moderately resistant parent, in which GG of 68–95% was reported (Imtiaz et al. 2003b).

The microsatellite marker MUCS088 is a complex sequence repeat marker derived from consensus cotton fiber EST sequence. Initially, the MUCS088 marker was reported to be mapped on chromosome 17 with around 50 cM linkage distance to the next marker (Park et al. 2005). To confirm the location of MUCS088, additional markers (Nguyen et al. 2004; Lacape et al. 2003; Han et al. 2006) distributed on chromosomes 12 and 17 were used to screen individual plants of testcross Acala NemX × F_1 (Pima S-7 × SJ-2), however, no linkage with MUCS088 was found. In addition, another 600 SSR markers with known cotton genome locations were screened to identify the linkage relationship of MUCS088. Finally, we found MUCS088 linked to BNL231 in the F_2 (Pima S-7 × NemX) mapping population after revisiting markers from chromosome 11. MUCS088 was also mapped to the same region as *rkn1* on chromosome 11 (Fig. 6). This linkage association and location for markers MUCS088 and CIR 316 was also confirmed after re-evaluation of the scores from MUCS088 and re-running of these markers on the RIL TM1 × 3-79 mapping population (Frelichowski et al. 2006; Yu and Kohel 2001; M. Ulloa, personal communication).

Fifteen of 22 *G. arboreum*, A_2, lines showing the same amplification pattern as Pima S-7 and its absence in the other diploid genome types suggested that MUCS088 in tetraploid *G. barbadense* might be introgressed from a diploid cotton A_2 genome (Fig. 7). The four additional *G. barbadense* cotton entries that have the same amplification pattern as Pima S-7 indicated a common origin of MUCS088 in *G. barbadense* derived from the A_2 genome. A different locus amplified in the tested A and D diploid genomes (Fig. 7) suggested a pattern of variation developed during cotton genome evolution or artificial hybridization (triple hybrid) and selection of the resistant parent source.

The F_2 (Pima S-7 × NemX) plants with marker MUCS088 were distributed throughout the range of galling index 1–8, as expected due to the requirement of co-presence with *rkn1* for resistance expression. Its presence in both highly susceptible and highly resistant genotypes indicated that MUCS088 cannot be used alone to identify resistance. However, with combined presence of the dominant MUCS088 and co-dominant CIR316 markers, we could readily distinguish highly resistant genotypes (such as *aabb* and *aaB-*) or moderately susceptible and susceptible genotypes (such as *Aabb* or *AAB-*). These results were confirmed in the individual F_2 plant tests with genotypes determined based on phenotypic tests of derived F_3 families. The CIR316c marker, which was reported before (Wang et al. 2006a; Wang and Roberts 2006b), was closely associated with susceptible plants in the testcross population NemX × F_2 (Pima S-7 × SJ-2). Collectively, these results indicated that CIR316 and MUCS088 alleles will be valuable tools for marker-assisted selection (MAS) in cotton. Further, it is possible that the *rkn1* plus *RKN2* resistance is more durable than *rkn1* alone, and this should be tested with *M. incognita* isolates that show some virulence to *rkn1* (Ogallo et al. 1997).

In summary, the *rkn1* gene in Acala NemX was associated with the resistance in the interspecific cross Pima S-7 × Acala NemX, and one gene (designated as *RKN2*) in Pima S-7 contributed significantly to the resistance based on genetic and marker analysis. The *RKN2* (*BB*) gene did not confer resistance in susceptible Pima S-7, but when combined with *rkn1* (genotype *Aa* or *aa*) it contributed high levels of resistance in the F_1 and segregating F_2, F_3 and BC_1F_1. The marker MUCS088 tightly linked to *RKN2* confirmed that a susceptible parent contributed to nematode resistance resulting in transgressive segregation. Furthermore, the marker MUCS088 was mapped to the resistance gene cluster on chromosome 11 containing gene *rkn1* (Fig. 6). The test of 51 cotton entries suggested *RKN2* is derived from the *G. arboreum*, A_2, diploid genome (Fig. 7). The highly resistant lines and the combined resistance gene markers can be used for improved resistance sources in nematode breeding programs in cotton. The close genomic location of these two genes that determine transgressive segregation and epistasis effects provides an important genetic model for studying the transgressive segregation process in plants.

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