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Development of AFLP and derived CAPS markers for root-knot nematode resistance in cotton

Congli Wang · Philip A. Roberts

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Abstract Resistance to root-knot nematode (*Meloidogyne incognita*) is determined by a single major gene *rkn1* in *Gossypium hirsutum* Acala NemX cotton. Bulked segregant analysis (BSA) combined with amplified fragment length polymorphism (AFLP) was used to identify molecular markers linked to *rkn1*. DNA pools from homozygous susceptible (S) and resistant (R) bulks of an $F_{2:3}$ originating from the intraspecific cross NemX × SJ-2 were screened with 128 *Eco*R1/*Mse*1 primer combinations. Putative AFLP markers were then screened with 60 $F_{2:7}$ RIL plants and four AFLP markers were found linked to *rkn1*. The linkage of AFLP markers to *rkn1* was also confirmed in a F_2 population. The closest AFLP marker was converted to a cleaved amplified polymorphic sequence (CAPS) marker (designated GHACC1) by aligning the sequences from both susceptible and resistant parents. GHACC1 linkage to *rkn1* was confirmed in the F_2 (1R:3S), $F_{2:7}$ RIL (1R:1S) and the backcross population SJ-2 × F_1 (NemX × SJ-2) (1 heterozygous: 1 homozygous). The four AFLP markers, GHACC1 plus two SSR markers (CIR316 and BNL1231) linked to *rkn1* from previous work were mapped to intervals of 2.6–14.2 cM from the *rkn1* locus, and the genomic region around *rkn1* was spanned to about 28.2 cM in the $F_{2:7}$ population. The PCR-based GHACC1 and CIR316 markers were tested on 21 nematode resistant and susceptible cotton breeding lines and cultivars. GHACC1 was suitable for nematode resistance screening within *G. hirsutum*, but not *G. barbadense*, whereas CIR316 was useful in both species, indicating their potential for utilization in marker-assisted selection.

Keywords Bulked segregant analysis · *Gossypium hirsutum* · High-resolution genetic mapping · Marker-assisted selection · *Meloidogyne incognita* · Resistance gene *rkn1* · Root-knot nematodes

Abbreviations

AFLP	Amplified fragment length polymorphism
CAPS	Cleaved amplified polymorphic sequence
MAS	Marker-assisted selection
RIL	Recombinant inbred line
STS	Sequence tag site

Introduction

The southern root-knot nematode *Meloidogyne incognita* Kofoid and White (Chitwood) is one of

C. Wang · P. A. Roberts (✉)
Department of Nematology, University of California , Riverside, CA 92521-0415, USA
e-mail: philip.roberts@ucr.edu

the most economically important pests of crops (Sasser 1977), and causes significant economic losses to cotton (*Gossypium hirsutum* L.) (Goodell and Montez 1994). The root-knot-nematode-Fusarium wilt disease complex results in severe yield loss in some growing regions (Jeffers and Roberts 1993; Abawi and Chen 1998). Nematicides are becoming more restricted in their use for nematode control in cotton, and increased efforts are being made to develop elite cotton cultivars possessing effective nematode resistance.

The first commercial *G. hirsutum* cultivar with high resistance to root-knot nematode, Acala NemX, was released for use in California in 1995 (Oakley 1995; Ogallo et al. 1997). A major gene (*rkn1*) in Acala NemX was identified as the primary determinant of resistance based on genetic analysis of the intraspecific cross of Acala NemX × Acala SJ-2, and was mapped to cotton linkage group A03 based on microsatellite (SSR) analysis of intraspecific and interspecific cotton crosses (Wang et al. 2006). One SSR marker CIR316a tightly linked to *rkn1* was found (Wang et al. 2006). Based on co-dominant marker correlations, susceptible plants could be categorized further into heterozygous and homozygous classes with lower and higher susceptible phenotype, respectively. The identification of molecular markers linked to resistance genes is an essential step towards both marker-assisted selection (MAS) and map-based gene cloning. MAS increases selection efficiency not only by allowing for earlier selection but also reducing plant population size used during selection without phenotypic testing. Phenotyping cotton plants for *M. incognita* resistance is time consuming, expensive, and requires technical expertize. Molecular markers will enable confirmation of the genetic control of root-knot nematode resistance including the number and uniqueness of the genes involved. Comparison of potentially diverse resistance sources including the many advanced cotton breeding lines with high levels of nematode resistance, such as *G. hirsutum* Auburn 623 RNR, Auburn 634 RNR and their derived M-lines (Shepherd 1974, 1982; Shepherd et al. 1988, 1989), and N-lines (Hyer and Jorgenson 1984), will aid the identification of novel genes that could then be pyramided in one genotype or deployed individually. Such strategies could increase the durability of resistance because *M. incognita* is known to become virulent through selection pressure from some resistance genes including in cotton (Ogallo et al. 1997; Roberts et al. 1998).

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique that visualizes DNA restriction fragments by PCR amplification. Compared with other molecular markers (RAPD, RFLP), many AFLP markers can be observed at a single time. Bulked segregant analysis (BSA) is a powerful technique for identifying markers linked to resistance genes (Michelmore et al. 1991). The objective of the present study was to identify AFLP markers linked to nematode resistance gene *rkn1* in Acala NemX. To facilitate marker-assisted selection, one closely linked AFLP marker was converted to a CAPS marker. The derived CAPS marker and an SSR marker CIR316 were used to screen a set of root-knot nematode resistant and susceptible cotton breeding lines for polymorphism.

Materials and methods

Plant materials and nematode resistance screening

Plant genotypes used in the marker development were *M. incognita* susceptible *G. hirsutum* cv. Acala SJ-2 and resistant *G. hirsutum* cv. Acala NemX. The populations used for molecular analysis were developed from the intraspecific cross between Acala NemX and Acala SJ-2 and included 2 resistant and susceptible bulks from an $F_{2:3}$, 60 $F_{2:7}$ recombinant inbred lines (RIL), 96 individual F_2 plants from a separate cross of the same parental lines, and 48 plants of BC_1F_1 (SJ-2 × F_1). These populations were evaluated for resistance to a *M. incognita* race 3 isolate under controlled conditions in a greenhouse as described previously (Wang et al. 2006). Briefly, plants were evaluated for root-galling reaction on a 0–10 root-gall rating scale (Bridge and Page 1980), and by the numbers of nematode eggs per gram fresh root extracted in NaOCl (Hussey and Barker 1973). Plants were classified as resistant (R), susceptible

(S) or heterozygous (H) based on comparisons with the susceptible and resistant parent phenotypes in each test. The F_2 was classified as R, H and S, the BC_1 (SJ-2 × F_1) as H and S, and the $F_{2:7}$ population as R and S (Wang et al. 2006).

AFLP screening

Fresh or frozen (–80°C) young cotton leaves under liquid nitrogen were ground to a fine powder using a mortar and pestle, and genomic DNA was extracted using the DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA). Two DNA pools were made, including seven homozygous resistant plants and eight homozygous susceptible plants from an $F_{2:3}$ (NemX × SJ-2) population based on phenotype screening of $F_{2:3}$ (NemX × SJ-2) families. The two bulks and the two parents were screened for markers showing polymorphisms between the four samples. AFLP markers with putative linkage to resistance gene *rkn1* were confirmed by screening the individuals within the bulks. The confirmed markers were analyzed further and mapped using additional populations segregating for *rkn1*, including the $F_{2:7}$ RIL and F_2.

AFLP were generated using the protocol of IRDye™ Fluorescent AFLP® Kit for large plant genome analysis (LI-COR, Lincoln, NE, USA) with modifications. The half volumes of the protocol described were used for restriction and ligation of genomic DNA and pre-amplification of the diluted template DNA. Selective amplification reactions were performed with 128 primer combinations including eight IRDye-labeled *Eco*R1 primer plus 3 nucleotides (AAC, AAG, ACA, ACT, ACC, ACG, AGC and AGG) (LI-COR, Lincoln, NE, USA) and 16 unlabeled *Mse*1 primer plus 3 nucleotides (CNN, LI-COR). Amplification was performed in a MasterCycler® Gradient (Eppendorf, Hamburg, Germany).

The denatured selective amplification products were resolved in 25-cm gels (0.25 mm spacer thickness) containing 8% polyacrylamide gel (50% Long Ranger™ solution, Cambrex, Rockland, ME, USA), 5.6 M Urea (Amersham Biosciences, Freiburg, Germany) and 0.8 × TBE buffer (LI-COR). Electrophoresis and detection were performed on a two-dye, model 4000 LI-COR IR^2 automated sequencer (Global edition, LI-COR, Lincoln, NE, USA). The gel images collected automatically were analyzed with Saga^{MX} automated analysis software (LI-COR).

Conversion of an AFLP marker to a dominant CAPS marker

AFLP fragments containing the targeted AFLP marker were excised from silver-stained polyacrylamide gel (Bassam et al. 1991) and extracted with crush and soak solution (0.5 M ammonium acetate, 0.1% sodium dodecyl sulfate, 0.1 mM EDTA) (http://axon.med.harvard.edu/~cepko/protocol/mike/D5.html). The recovered DNA was amplified with the initial AFLP primers and purified in 2% agarose gel. The bands from the agarose gel were extracted with QIAquick Gel Extraction Kit (QIAGEN, Alameda, CA, USA). The purified DNA was then cloned into pGEM-T vector (Promega, Madison, WI, USA) following the protocol of the manufacturer and transformed into *Escherichia coli* DH5α competent cells. The presence of the insert in the plasmid was checked by PCR with the universal primers M13 (Forward and Reverse). The reaction was performed in 10 μl volume containing half of the picked clone, 1 × PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs (Roche Diagnostics GmbH, Germany), 0.1 μM forward primer, 0.1 μM reverse primer, and 0.5 U *Taq* polymerase (Invitrogen). PCR was performed at initial denaturing temperature of 94°C for 2 min and then 30 cycles of 94°C for 1 min, 55°C for 45 s and 72°C for 1 min, and the final extension at 72°C for 10 min. The electrophoresis was performed in 1.5% agarose gel stained with 0.5 μg ml^{-1} ethidium bromide in 1 × TBE buffer. Eight confirmed clones with insert were re-cultured and purified with QIAprep® Miniprep kit (QIAGEN), and inserts were sequenced in both reverse and forward reaction at the UCR Genomics Institute Core Instrumentation Facility (Univ. of Calif., Riverside, CA, USA).

Sequence-tagged site (STS) primers were designed using vector NTI suite 6.0 (Informax Inc., Bethesda, Maryland, USA) and synthesized by Integrated DNA Technologies DT (IDT, IA, USA). One STS marker amplified a 300 bp segment contained within the original E-AAG/M-CCG-327 bp AFLP fragment. The forward

sequence of the STS marker was 5′-AAGTTT AGTCAACTTCTAAA (STS1-FWD), and reverse sequence 5′-CCGGTGGGTTATTGCC TGAC (STS1-REV). These primers amplified a 300-bp fragment from both susceptible Acala SJ-2 and resistant Acala NemX. The reaction and PCR conditions were the same as described above for checking the presence of the inserts, except for the primers.

One CAPS marker was developed by digesting the STS PCR products with restriction endonuclease *Nla* III (New England Biolabs, Beverly, MA, USA). To digest the STS amplified products, a total 15 μl volume composed of 10 U of restriction enzyme and 1 × NBE buffer 4 with 1 × bovine serum albumin based on manufacturer's recommendations was added to the 10 μl of STS amplification reaction and incubated at 37°C for 16 h overnight. Restriction digested PCR products were resolved by electrophoresis on 3% (w/v) agarose gels stained with ethidium bromide in 1 × TBE buffer and photographed.

Segregation and linkage analysis

The data for resistance phenotype and marker segregation ratios were tested for goodness-of-fit to predicted Mendelian inheritance ratios by the Chi-square test ($P = 0.05$). The linkage analysis of the AFLP, CAPS, and SSR markers previously obtained (Wang et al. 2006), and the root-knot nematode resistance locus *rkn1* was performed with the software JoinMap® 3.0 (Van Ooijen and Voorrips 2001). The linkage groups with all markers or loci were intact at a LOD score 5. The recombination fractions were converted into centiMorgans (cM) based on Kosambi's mapping function (Kosambi 1944).

CAPS and SSR marker screening of cotton germplasm

The identified CAPS marker and one SSR marker (CIR316a) tightly linked to resistance gene *rkn1* were used to screen another 21 nematode resistant and susceptible cotton breeding lines and cultivars (Table 1). The PCR products and assays for the CAPS marker were the same as those described above. SSR marker protocol was provided in Wang et al. (2006).

Results

AFLP markers linked to *rkn1*

In the $F_{2:3}$ (NemX × SJ-2), 64 polymorphic markers showed putative linkage to *rkn1* based on analysis of the homozygous resistant and homozygous susceptible bulks plus two parents with 128 *Eco*R1/*Mse*1 primer combinations. After screening within the bulks, 32 markers were identified with potential linkage to *rkn1*. Seventeen of these 32 markers were screened with 60 $F_{2:7}$ (NemX × SJ-2) RIL, and 2 coupling-phase markers (E-AAG/M-CCG-327 and E-ACG/M-CAT-258) and two repulsion-phase markers (E-AAC/M-CTA-545 and E-ACG/M-CAT-257) were found to be linked to the resistance gene. Four markers showed bimodal distribution with 1:1 ratio in the RIL population. Three out of 60 individual $F_{2:7}$ lines showed recombination between marker E-AAG/M-CCG-327 and *rkn1*. The amplification patterns of the $F_{2:7}$ segregating population with the E-AAG/M-CCG-327 AFLP marker closely linked to *rkn1* are shown in Fig. 1. The screened 17 putative markers linked to *rkn1* were amplified from 14 *Eco*R1/*Mse*1 primer combinations. A total of 1,057 bands were amplified with the 14 primer combinations, of which 51 were polymorphic (51/1057; 4.8%) between Acala SJ-2 and Acala NemX.

Conversion of E-AAG/M-CCG-327 AFLP marker to CAPS marker

In order to develop high throughput markers for use in marker-assisted selection, the dominant marker E-AAG/M-CCG-327 was cloned and sequenced. Four out of eight clones showed one 300-bp sequence (S1) and the other four clones showed one 295-bp sequence (S2).

Sequence analysis of the cloned 300 bp marker (S1) and comparison with entries in the NCBI database with Blast search revealed that about 80-bp-length-sequence was conserved with GTP-binding protein in *Arabidopsis thaliana*

Table 1 Cotton genotypes tested with dominant markers GHACC1, CIR316a (221 bp, amplified from NemX), and CIR316c (210 bp amplified from SJ-2) and their known *Meloidogyne incognita* resistance phenotype

Genotypes	Phenotype [g]	GHACC1 300 bp	CIR316a 221 bp	CIR316c 210 bp
Gossypium barbadense				
Pima S-2	S[a]	+[h]	–[i]	–
Pima S-3	?[b]	+	–	–
Pima S-4	?[b]	+	–	–
Pima S-7	S[a]	+	–	–
TX 110	S[c]	+	–	–
Tanguis	?[b]	+	–	–
Gossypium hirsutum				
Wild Mexico Jack Jones	MR[d]	+	–	+
Clevewilt 6	MR[d]	+	+	–
Auburn 623 RNR	R[d]	+	+	+
Auburn 634 RNR	R[a, d]	+	+	–
Auburn 56	MR[e]	–	–	–
M-75	R[d]	–	–	+
M-78	MR[d]	–	–	+
M-120	R[d]	+	+	–
M-188	MR[d]	–	–	+
M-315	R[a, d]	+	+	–
LA RN 4-4	R[d]	+	+	–
LA RN 1032	R[d]	+	+	–
Coker 100	MS[f]	–	–	+
Coker 307-6	?[b]	–	–	+
Acala 442	S[f]	–	–	+
Acala NemX	R[a]	+	+	–
Acala SJ-2	S[a]	–	–	+

[a]Our test; [b]No report, likely susceptible; [c]Starr and Roberts (2004); [d]Robinson et al. (2001); [e]Shepherd (1974); [f]Robinson et al. (1999); [g]S, susceptible; ? phenotype unknown; MS, moderately susceptible; MR, moderately resistant; R, resistant; [h]+ Band present; [i]– Band absent

genomic DNA. Based on S1 sequence, sequence tag site (STS) primers, STS1-FWD and STS1-REV, were designed to amplify PCR products in the two parents, susceptible Acala SJ-2 and resistant Acala NemX. However, these primers amplified a 300-bp fragment in both parents.

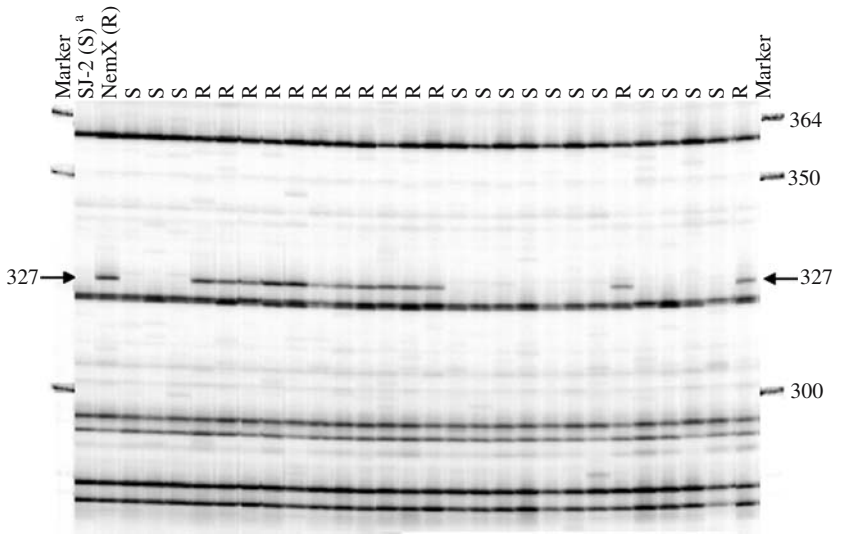

Fig. 1 Image of selective amplification products with E-AAG/M-CCG primer combination in $F_{2:7}$ (NemX × SJ-2) RIL population electrophoresed on polyacrylamide gel with model 4000 LI-COR IR^2 automated sequencer. [a]SJ-2, susceptible parent; NemX, resistant parent; S, susceptible based on phenotype; R, resistant based on phenotype. The arrows point to the marker positions, 327 bp marker from NemX

Purified DNA from the fragment in both parents was directly sequenced and aligned, revealing a few nucleotide substitutions between the two parents. One restriction enzyme site *Nla* III (CATG′) was identified at nucleotide 190 bp to produce one dominant CAPS marker, which was designated as GHACC1 (*Gossypium hirsutum* AFLP Converted CAPS). DNA from susceptible Acala SJ-2 was fully digested with the restriction enzyme *Nla* III to produce cleavage products of two bands (190 and 110 bp) and partially digested in resistant Acala NemX to produce three bands (non-cleaved product: 300 bp; cleavage products: 190 bp and 110 bp) (Fig. 2). PCR analysis of DNA from 60 $F_{2:7}$ (NemX × SJ-2) RIL revealed that the AFLP marker E-AAG/M-CCG-327 and CAPS marker GHACC1 completely co-segregated (Fig. 3). Three of 60 RIL showed recombination between GHACC1 and *rkn1*. The amplification patterns of the $F_{2:7}$ segregating population with GHACC1 linked to *rkn1* are shown in Fig. 2. The distance of GHACC1 and the co-segregating E-AAG/M-CCG-327 marker from *rkn1* was 2.6 cM in the $F_{2:7}$ population (Fig. 3). The linkage map (Fig. 3) was combined with SSR markers that we had identified previously (Wang et al. 2006) as linked to *rkn1*. The *rkn1* gene was mapped to cotton linkage group A03 (Wang et al. 2006), so the four AFLP markers and the converted CAPS marker also were mapped to linkage group A03.

Sequence analysis of the cloned 295 bp marker (S2) and comparison with entries in the Genbank database revealed about 30 bp sequence was conserved with *Medicago truncatula* clone mth2-7f22 or *Arabidopsis thaliana* putative CCR4-associated factor 1. STS2-FWD and STS2-REV were designed based on sequence S2. These primers amplified a 295-bp fragment in both parents. Purified PCR products from both parents were cloned into vectors and sequenced. The four clones of NemX had similar sequences with a few nucleotide substitutions (data not shown). However, four clones of SJ-2 had the same sequence, and the sequence was the same as one of four clones of NemX. Therefore, the 295 bp marker was not informative for *rkn1*.

Confirmation of AFLP and CAPS markers linked to resistance in F_2 and BC_1F_1

In the F_2, the E-AAG/M-CCG-327 AFLP marker was linked to *rkn1* with an estimated distance of 4.1 cM (2.6 cM in $F_{2:7}$ population). To confirm the dominant GHACC1 marker linkage, the F_2 and backcross population SJ-2 × F_1 were used. In the F_2, homozygous susceptible plants showed two bands on agarose gel, whereas heterozygous susceptible and homozygous resistant plants showed three bands with the GHACC1 marker. Linkage analysis confirmed the 4 AFLP markers and GHACC1 were associated with nematode resistance in the F_2 population (data not shown). The genotype of F_2 plants with GHACC1 was the same as that observed with the original AFLP marker, except that three plants showing the AFLP band had the homozygous susceptible pattern for GHACC1, indicating E-AAG/M-CCG-327 marker and GHACC1 were not completely co-segregant. The distance was 0.8 cM between GHACC1 and *rkn1* in F_2. Only heterozygous (three bands) and homozygous susceptible (two bands) genotypes for GHACC1 were observed on agarose gels in the backcross of SJ-2 × F_1 and one of 48 plants was recombinant between the marker and the nematode resistance phenotype.

GHACC1 CAPS and CIR316 SSR marker screening of cotton germplasm

To determine the potential of the GHACC1 CAPS and CIR316 SSR markers for indirect selection of nematode resistance, 15 *G. hirsutum* and 6 *G. barbadense* cotton germplasm lines, either with nematode resistance or used in nematode resistance breeding programs, were screened with both markers. The amplified patterns are shown in Figs. 4, 5. The root-knot nematode resistance phenotype and the molecular marker profiles are given in Table 1. In all the *G. barbadense* cotton lines GHACC1 amplified the same size bands as NemX (Fig. 4), and no 221 and 210 bp size bands were amplified for CIR316 (Fig. 5). Within *G. hirsutum*, the same pattern of marker distribution was found with both the GHACC1 and CIR316a markers, except in Wild Mexico Jack Jones (Table 1). These two markers

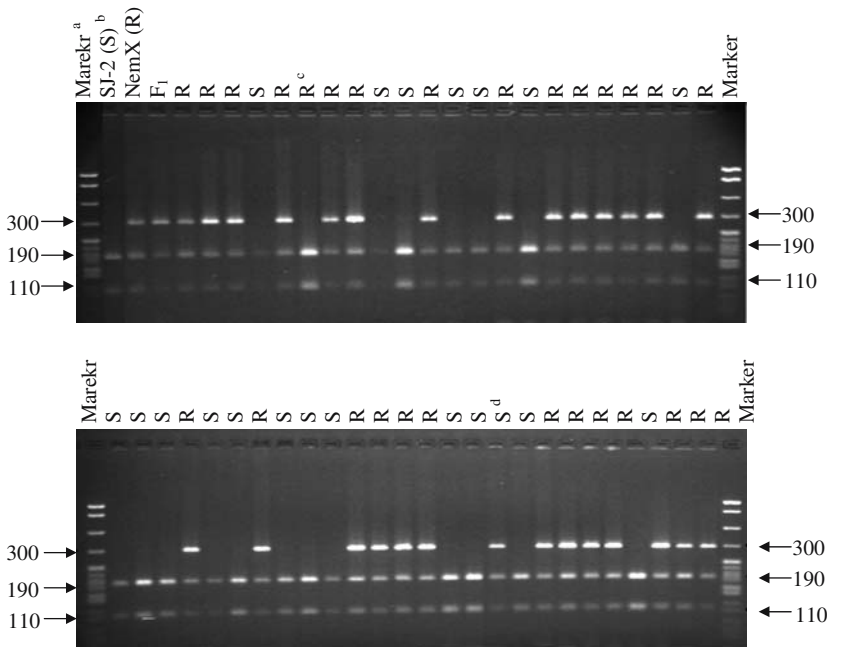

Fig. 2 Images of amplification products with the dominant CAPS marker GHACC1 in the $F_{2:7}$ (NemX × SJ-2) RIL population electrophoresed on agrose gel. [a]Marker, 100 bp size pBR322 *Msp*I marker (New England Biolabs, Ipswich, MA, USA). [b]SJ-2, susceptible parent; NemX, resistant parent; S, susceptible; R, resistant, all based on phenotype. [c]R, recombinant line with resistant phenotype (R) and susceptible parent CAPS marker profile. [d]S, recombinant line with susceptible phenotype (S) and resistant parent CAPS marker profile. The arrows point to the marker positions, NemX pattern including three bands (300, 190 and 110 bp) and SJ-2 pattern including two bands (190 and 110 bp)

were associated with eight of the known resistant lines and not with any of the known or likely (based on pedigree) susceptible *G. hirsutum* lines. Both the 221 bp size coupling-phase marker (CIR316a) amplified from resistant Acala NemX and the 210 bp size repulsion-phase marker (CIR316c) amplified from susceptible Acala SJ-2 were associated with the *rkn1* locus (Wang et al. 2006). The *G. hirsutum* germplasm lines showed either CIR316a marker or CIR316c marker, except Auburn 623 with both bands and Auburn 56 without both bands, indicating CIR316a and CIR316c may be associated with resistance and susceptibility, respectively (Table 1).

Discussion

Using bulked segregant analysis with AFLP marker technology on different segregating populations from intraspecific *G. hirsutum* crosses of SJ-2 × NemX, we identified four AFLP markers and one converted CAPS marker linked to gene *rkn1* in Acala NemX. These markers mapped to linkage group A03 based on previous work (Wang et al. 2006). Combining the four AFLP markers and one CAPS marker with two SSR markers linked to *rkn1* gene from a related study (Wang et al. 2006), the genomic region around *rkn1* was spanned to about 28.2 cM (Fig. 3).

AFLP markers

We found low polymorphism (4.8%) with 14 *Eco*R1/ *Mse*1 AFLP primer combinations in the intraspecific populations derived from the cross of *G. hirsutum* Acala NemX and Acala SJ-2. Four out of 17 AFLP markers putatively linked to *rkn1* based on bulked segregant analysis were found to be closely linked to the *rkn1* resistance gene. Previous work reported 6.3% polymorphism between Acala NemX and Acala SJ-2 with SSR markers and two out of 18 polymorphic markers were found to be associated with the *rkn1* locus in

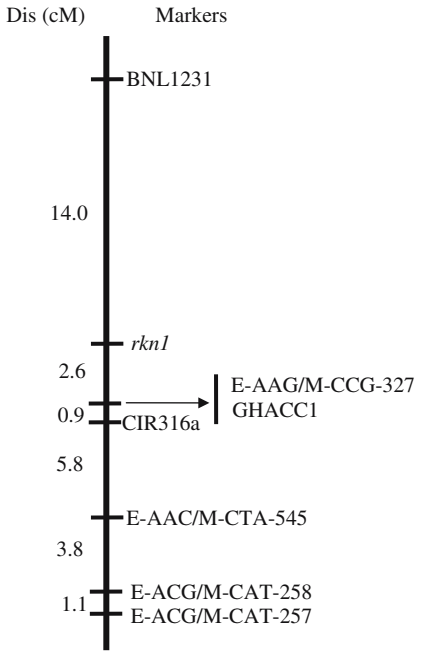

Fig. 3 Location of the resistance gene *rkn1* relative to four AFLP, one CAPS (GHACC1) and two SSR markers (CIR316a and BNL1231) in an $F_{2:7}$ (NemX × SJ-2) segregating population. Distances are reported in Kosambi cM

Acala NemX using the same cross (Wang et al. 2006). It is not unexpected that Acala NemX and Acala SJ-2 have low polymorphism because Acala NemX was derived from the inbred line of Acala SJ-2 with selection based on nematode resistance. The results from this study further indicated that a significant portion of the minor difference between the two cultivars may be associated with nematode resistance.

Two sequences (S1 and S2) were obtained from the E-AAG/M-CCG-327 bp band, and from the S1 sequence we were able to develop one CAPS marker closely linked to *rkn1*. The S2 sequence was not informative because Acala SJ-2 had the same sequence as Acala NemX after amplification with STS2 primer pair. Obtaining two sequences from the same band may indicate co-migrating AFLP fragments. The phenomenon of co-migrating AFLP fragments at the same position in the gel was also reported by Mechanda et al. (2004). Based on this assertion, S2 may be a false positive band that co-migrates with S1, which could explain why the original AFLP marker did not co-segregate with the CAPS marker in three F_2 plants whose genotypes were not homozygous.

CAPS marker

The CAPS marker GHACC1 behaved as a dominant marker. However, there was no *Nla* III restriction enzyme site (CATG, Fig. 6) in the S1 sequence from which GHACC1 was derived. After PCR with STS1 primers in Acala NemX, two sequences were found, only one of which had an *Nla* III restriction enzyme site. As a result, one sequence with the *Nla* III restriction enzyme site was cut, forming two bands (190 and 110 bp), and the other was uncut (300 bp). The susceptible SJ-2 profile contained only the sequence with the *Nla*

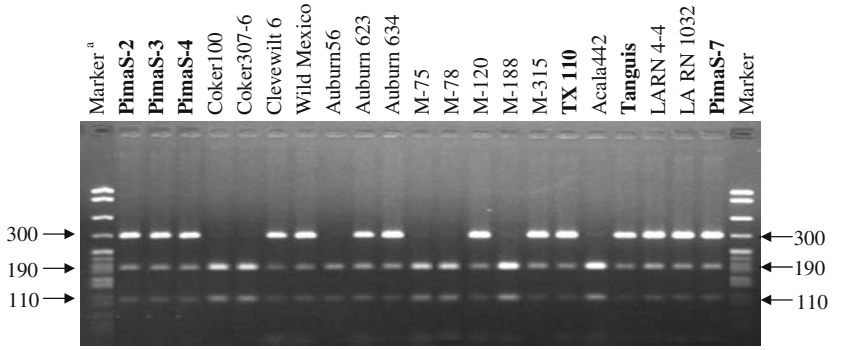

Fig. 4 Image of amplification products with dominant CAPS marker GHACC1 in cotton germplasm lines electrophoresed on agrose gel. [a]Marker, 100 bp size pBR322 *Msp*I marker (New England Biolabs). The arrows point to the marker positions, NemX pattern including three bands (300, 190 and 110 bp) and SJ-2 pattern including two bands (190 and 110 bp). All *G. barbadense* germplasm lines are with bold fonts

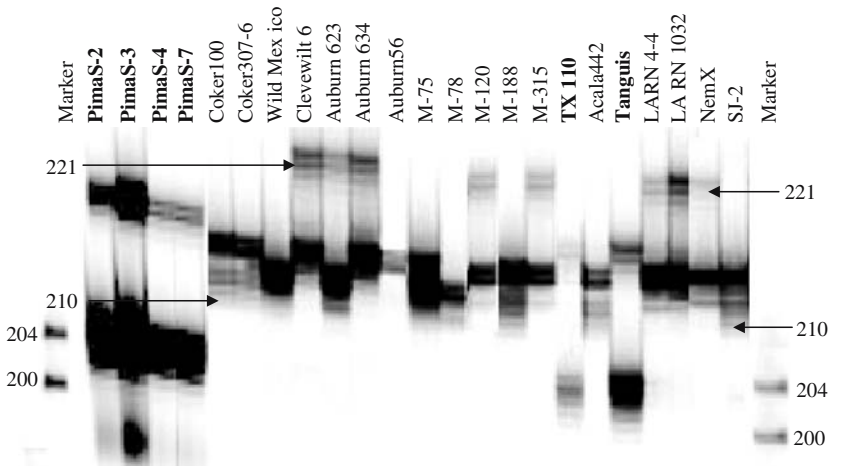

Fig. 5 Image of amplification products with CIR316 marker in cotton germplasm electrophoresed on polyacrylamide gel with model 4000 LI-COR IR^2 automated sequencer. The arrows point to the marker positions, one of 221 bp from NemX and one of 210 bp from SJ-2. Note all allelic sizes include the M13 primer tail. All *G. barbadense* germplasm lines are with bold fonts

III restriction enzyme site, thereby producing two bands. Two sequences with one nucleotide substitution in Acala NemX with STS1 primers showed that different fragments migrated to the same position in the agarose gel. The two homologous sequences may be derived from the same chromosomal region due to a single nucleotide mutation or from different regions of genome, such as homologous chromosomes or linkage groups. Thirteen pairs of homologous chromosomes or linkage groups have been identified in allotetraploid cotton (Nguyen et al. 2004; Rong et al. 2004). In our study, one nucleotide substitution between Acala NemX and Acala SJ-2 was identified that produces polymorphism useful in distinguishing resistant and susceptible genotypes. Caranta et al. (1999) reported that an AFLP marker was successfully converted into a co-dominant CAPS marker for potyvirus *Pvr4* resistance in pepper. Successful conversion of AFLP to CAPS provides another way for resolving failure of conversion of AFLP to STS markers (Prins et al. 2001, von Stackelberg et al. 2003).

Presence of the CAPS and SSR markers in 23 genotypes

The root-knot nematode resistance phenotypes of 19 cotton germplasm lines are provided in Table 1, together with Pima S-3, Pima S-4, Tanguis and Coker 307, whose phenotypes are

S1 176 bp ---- 220 bp (from AFLP band E-AAG/M-CCG-327 in Acala NemX)

TAGACGCACG ATCGAGCCCT C**CGTG**TAATC CAGCGCAGAA TTTCATCAAG

NemX (from PCR product)

TAGACGCACG ATCGAGCCCT C**C[A]TG**TAATC CAGCGCAGAA TTTCATCAAG

TAGACGCACG ATCGAGCCCT C**CGTG**TAATC CAGCGCAGAA TTTCATCAAG

SJ-2 (from PCR product)

TAGACGCACG ATCGAGCCCT C**C[A]TG**TAATC CAGCGCAGAA TTTCATCAAG

Fig. 6 An alignment of a portion of the AFLP marker E-AAG/M-CCG-327 sequence from 176 to 220 bp and sequences amplified by the STS1-FWD and STS1-REV primers from both root-knot nematode resistant Acala NemX and susceptible Acala SJ-2 cotton. Nucleotides in bold indicate the *Nla* III restriction site, with the single nucleotide polymorphism indicated by boxes

not known. Robinson et al. (1999) reported that Pima S-5, Pima S-6 and Pima S-7 were susceptible to root-knot nematode and that Pima S-2 was moderately resistant to root-knot nematode. However, in our tests, Pima S-2 was susceptible to root-knot nematode and had only slightly less infection than susceptible Pima S-7. Turcotte et al. (1963) reported that Pima S-1 and Pima S-2 were both susceptible to root-knot nematodes. Tanguis is a very old *G. barbadense* variety and was involved in the development of Pima S-1 (Feaster and Turcotte 1965) and Pima S-3 (Young et al. 1976). However, its root-knot nematode resistance status has not been reported. Most Coker lines except Coker 100W were reported to be susceptible to root-knot nematodes (Robinson et al. 1999). Based on these breeding histories, the four genotypes with unknown phenotype are likely to be susceptible to root-knot nematodes.

The GHACC1 and CIR316 markers tightly linked to *rkn1* in Acala NemX were accurate in predicting phenotype in the population of *G. hirsutum* NemX × SJ-2. Moreover, the other 15 *G. hirsutum* cotton genotypes showed polymorphism with the two markers. However, the GHACC1 marker amplified the same size bands as Acala NemX in all *G. barbadense* germplasm lines (Table 1), indicating that GHACC1 is not suitable for MAS in *G. barbadense* cotton but could be useful for indirect selection of resistance in *G. hirsutum* cotton. The GHACC1 marker was derived from the E-AAG/M-CCG-327 AFLP marker, and the E-AAG/M-CCG-327 bp band as found in Acala NemX was also amplified in Pima S-7 (data not shown). The PCR product after amplification with primer STS1 was sequenced, and two sequences, one with (C**A**TG) and one without (CGTG) *Nla* III restriction enzyme site, were identified to be similar to those in Acala NemX (Fig. 6), except for a few nucleotide substitutions. These results explained why susceptible Pima S-7 showed the same three bands as Acala NemX with GHACC1. Molecular patterns of other *G. barbadense* genotypes with GHACC1 were the same as that of Pima S-7, indicating that recombination may have occurred between GHACC1 and *rkn1* prior to divergence between *G. barbadense* and *G. hirsutum*.

CIR316 is a co-dominant marker linked to *rkn1* in the population NemX × SJ-2, with CIR316a (221 bp) amplified from Acala NemX and CIR316c (210 bp) from Acala SJ-2 (Wang et al. 2006). The CIR316a marker was not present in any of the known susceptible and likely susceptible genotypes (Table 1), indicating that CIR316a has potential utility for MAS with *G. hirsutum* and for introgressing resistance alleles from *G. hirsutum* into *G. barbadense* breeding backgrounds. In order to validate the utility of CIR316a for use with *G. barbadense*, the cross between Pima S-7 and NemX was made and the derived segregating population F_2 was tested (Wang et al. 2006). The primary resistance phenotype test suggested that CIR316a may be useful for MAS in this population (data not shown).

Considering the origin of the resistance source within *G. hirsutum*, Auburn 623 RNR was derived from the cross of Clevewilt 6 and Wild Mexico Jack Jones (Shepherd 1974). The cross between Auburn 623 RNR and Auburn 56 was used to develop Auburn 634 RNR, a highly resistant genotype from which the M-line series was derived with different levels of resistance to root-knot nematodes (Shepherd 1982; Shepherd et al. 1988, 1989; Robinson et al. 1999). The origin of the root-knot nematode resistance in LA RN 4-4 and LA RN 1032 was reported to be Clevewilt 6 (Robinson et al. 1999). However, the origin of the resistance in Acala NemX conferred by incompletely recessive resistance gene *rkn1* is not known. Bezawada et al. (2003) reported that one recessive gene conferred moderate resistance in Clevewilt 6. As shown in Table 1, the same molecular patterns were amplified with both GHACC1 and CIR316 markers among resistant cotton genotypes Acala NemX, Clevewilt 6, Auburn 623 RNR, Auburn 634 RNR, M-120, M-315, LA RN 4-4 and LA RN 1032, with the exception of Auburn 623 RNR with the CIR316c marker. These results suggested that Acala NemX may have the same resistance source as Clevewilt 6, and that other lines derived from Clevewilt 6 may carry the same gene as Clevewilt 6. In order to confirm these resistance relationships, crosses from Acala NemX and Acala SJ-2 with Clevewilt 6 and other resistant lines have been made and segregating populations are being developed for

resistance phenotype and marker screening. Recessive gene(s) can be masked in the presence of a dominant gene. McPherson et al. (2004) reported that M-315 carried one dominant gene and one additive gene for resistance. The GHACC1 and CIR316 markers should be useful to test this population to confirm whether M-315 carries the recessive *rkn1* gene. McPherson et al. (1995) reported that M-188 and M-78 with moderate resistance might have different resistance genes to those found in M-315, and M-75 may have the same genes as M-315 based on phenotypic tests. Our results showed that M-75, M-78 and M-188 have the same kind of marker profiles as Acala SJ-2 and they were different from M-315 with both markers, suggesting M-75, M-78 and M-188 lines may not carry the *rkn1* gene and that they have different resistance genes from M-315. In addition, we found that Wild Mexico Jack Jones had the same marker profiles as Acala SJ-2 with CIR316 and different profiles with GHACC1 (Table 1), indicating recombination between the two markers in this genotype. Further study with molecular markers linked to the resistance genes will help to clarify these resistance gene relationships and expedite marker-assisted selection for root-knot nematode resistance.

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