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BREEDING AND GENETICS

SSR Marker(s) Associated with Root Knot Nematode Resistance Gene(s) in Cotton

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ABSTRACT

Root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] is a major pest on cotton (*Gossypium hirsutum* L.) across the U. S. Cotton Belt. DNA markers that enable marker-assisted selection for root-knot nematode resistance gene(s) will foster development of root-knot nematode-resistant cultivars. The objectives of this research were 1) to identify DNA markers associated with the resistance to root-knot nematodes in upland cotton, and 2) to determine the mode of inheritance of the root-knot nematode resistance gene(s). The moderately resistant line, Clevewilt 6-1, and the susceptible cultivar, Stoneville 213, were crossed. The resulting F_1 and F_2 populations and both parents were genotyped using 120 simple sequence repeat (SSR) primer pairs, providing 16 polymorphic markers. Gall indices, which were the best measure of resistance, were used to determine that the population fit a 3:1 Mendelian segregation ratio (susceptible: resistant). Phenotypic and marker data indicate that Clevewilt 6-1 was likely the source of the recessive gene for resistance to galling. The molecular marker BNL 1421 explained 8% of the variation in gall index in the segregating F_2 population. Mapmaker analysis indicated that BNL 1421 and BNL 1669 were linked with a distance of 15.4 cM. Both of these markers showed distorted segregation. The small effect of BNL 1421 on resistance could be due to the weak linkage of the marker with the root-knot nematode resistant trait or to false linkage, because they segregated abnormally.

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The root knot nematode (RKN), a sedentary endoparasite, forms galls or knots on the roots of plants and causes yield losses on many crops throughout the world, including upland cotton. Yield losses in cotton attributed to nematodes in 1999 across the U. S. Cotton Belt were 582,702 bales (about $175 million), approximately 5.65% of the total U. S. cotton production (Blasingame and Patel, 2000). Decreases in fiber length, and fiber quality are also associated with nematode infection (Smith et al., 1991). Root-knot nematodes are not only destructive alone (Brodoe et al., 1960; Minton and Minton, 1966), but also increase the incidence and severity of Fusarium wilt [*Fusarium oxysporum* Schlectend:Fr f. sp. *vasinfectum* (Atk.) Snyd. & Hans.] (Smith, 1941; Martin et al., 1956; Minton and Minton, 1966) and seedling diseases in cotton (Brodie and Cooper, 1964; Cauquil and Shepherd, 1970).

Genetic resistance is an environmentally safe method of pest management. The primary limitation to developing cotton germplasm with resistance to RKN is the paucity of information available about resistance gene(s). Molecular markers linked to RKN-resistance gene(s) would provide the option to select by genotype rather than by phenotype. An accurate and detailed map of the RKN gene(s) associated with DNA markers will be a valuable tool to expedite the breeding process by identifying resistant lines. A DNA marker linked closely to a gene of interest might also provide a tool for positional cloning of the gene of interest.

The objectives of this research were 1) to elucidate the genetics of RKN resistance in Clevewilt 6-1 using an F_2 population developed from the cross of Clevewilt 6-1, a moderately resistant line, and Stoneville (ST) 213, a susceptible upland cotton line, and 2) to search for DNA marker(s) for RKN resistance genes based on segregation in an F_2 population.

MATERIALS AND METHODS

Clevewilt 6-1, a moderately resistant upland cotton line, and Stoneville (ST) 213, a susceptible

upland cotton cultivar, were crossed in the field at the Plant Science Research Center, Mississippi State University, MS in 1998. The F_1 plants were grown at the cotton winter nursery in Mexico during the winter of 1998, and were selfed to develop an F_2 population. The individual plants of the F_2 population were used to study the genetic inheritance of RKN resistance and SSR markers.

Screening methods for root-knot nematode resistance. The experiments to screen for RKN resistance were conducted in a greenhouse using a modification of methods from Shepherd (1979). To obtain eggs for inoculation, six-week-old infested cotton roots were treated with 20% solution of sodium hypochlorite and shaken gently at 3000 rpm for 3 min, following the method of Hussey and Baker (1973).

The greenhouse pots and soil beds were filled with sandy loam soil fumigated with methyl-bromide before planting. Six centimeter-deep holes were dug in the soil bed, and 8.9 cm x 7.6 cm (diameter x depth) pots filled with soil placed in the holes. The soil in each pot was inoculated with approximately 5000 nematode eggs. Seeds of Clevewilt 6-1(20), Stoneville 213 (20), F_1 (20), and F_2 plants (91) were sown individually into pots. The pots were covered with brown plastic paper for 7 d until the nematode eggs hatched.

The plants were harvested 40 d after sowing. The plants were cut at cotyledonary node to separate the roots, and the roots were washed with water to remove free soil. The roots were stained with a Phloxine B solution for 15 min to enhance visualization of RKN egg masses and then placed in water for 10 min to remove the excess stain. Root galling on plants was rated using the 1 to 5 index of Shepherd (1974), where 1 = plants with no galls or very light galling, 2 = plants with light galling, 3 = plants with moderate galling, 4 = plants with heavy galling, and 5 = plants with very heavy galling.

Plants with a gall index of 1 or 2 were considered resistant plants and plants with a gall index from 3 to 5 were considered susceptible plants. The roots were rated for root gall index by three different people in these experiments.

DNA extraction. Leaf samples were obtained from individual plants of the parents, F_1 and F_2 populations, when the plants were at the four- to six-leaf stage and stored at –80ºC in a freezer until freeze-dried using the method of Saha et al. (1997). The DNA was extracted from the freeze-dried leaf tissues by using the method of Khan et al. (1997).

Simple sequence repeat (SSR) marker. Fluorescent-labeled 5' and un-labeled 3' SSR primer pairs were purchased from Research Genetics, Huntsville, AL. The primers were labeled with 6-FAM (6-carboxyfluorescein), HEX (4,7,2',4',5',7',-hexachloro-carboxyfluorescein), or NED (7',8'-benzo-5-fluoro-2', 4 ‘,7’’,-trichloro-5-carboxyfluorescein), respectively. Gene Amp PCR reagent kits (PE Applied Biosystems, Foster City, CA) containing Ampli Taq DNA polymerase, dNTPs, $MgCl_2$, and 10X buffer were used. PCR was conducted using 1X PCR buffer, 0.1 mM SSR primer pair, 2.5 mM $MgCl_2$, 0.25 Units Ampli Taq Gold DNA polymerase, and 50 ng template DNA sample. The PCR was conducted according to the method of Gutierrez et al. (2002). A negative control without DNA template was used in each run to confirm the results. The procedure was repeated three times.

Capillary electrophoresis. The amplified DNA markers were analyzed using an automated capillary electrophoresis system ABI 310 genetic analyzer (PE Applied Biosystems, Foster City, CA) having GeneScan analysis software (Perkin Elmer Corp., Norwalk, CT) following the method of Gutierrez et al. (2002). To avoid the problem of background noise, a DNA marker was considered to be a valid data point if peak height on the Y-axis was more than 50 arbitrary units and the peaks showed at least 1 bp difference from the closest marker peak on the X-axis.

Analysis of variance to identify association of SSR markers with resistance traits. A one-way analysis of variance was used to determine the association of the DNA markers with the resistance trait. The hypothesis was that the mean gall index of plants with and without the marker would be equal, indicating no linkage. If the hypothesis was rejected, the mean gall index was not the same when the marker was present and when the marker was absent, thus the trait was linked with the marker. For dominant markers, there was one degree of freedom in the analysis that did not allow for separation of the dominance and additive effects. For the co-dominant markers, there were two degrees of freedom that could be used for two linear contrasts to measure the significance of the dominance and additive effects. The coefficient of determination (r^2) was calculated to measure the extent of the variation in the resistance trait that could be attributed to association with the marker.

MapMaker for linkage analysis. The MapMaker (Lander et al., 1987) program was used to construct a linkage map of the SSR Markers and the RKN resistant trait based on individual F_2 plant analysis. Linkage was significant if the LOD score was equal to or greater than 3.0.

Chi-square analysis. Chi-square (X^2) analysis for the phenotypic ratio was calculated by using the formula,

$$X^2 = (O-E)^2 / E,$$

where for O is an observed value, and E is expected value. Given there are just two genetic classes for RKN resistance, the data featured just one degree of freedom, so each Chi-square value was considered significant ($P \leq 0.05$) if its value was greater than 3.84. The mode of genetic inheritance was studied using the method of Karaca et al. (2002).

RESULTS

Root gall indices. Cotton root systems infected with RKN galls turn pink when stained with Phloxine B (Fig. 1). Gall indices of the susceptible parent ST 213, the resistant parent Clevewilt 6-1, and the F_1 and F_2 plants from the cross of Clevewilt 6-1with ST 213 are reported in Table 1. Fourteen F_1 plants were susceptible (gall rating >3) based on gall index, statistically supporting the model of a recessive resistant gene controlling gall index.

The root gall data on 91 individual F_2 plants were analyzed to determine the inheritance of RKN resistance. In F_2 plants, there were 14, 6, 5, 13, and 53 plants in gall index classes 1, 2, 3, 4, and 5, respectively. The frequency distribution of the plants was skewed. Considering the 1 and 2 gall index class as resistant and index classes 3, 4, and 5 as susceptible, the observed segregation was 71:20 and the Chi-square was 0.44, relative to an expected segregation ratio of 3:1 (susceptible : resistant plants). The results are congruent with the hypothesis that a single recessive gene controls this RKN resistance trait.

Molecular analysis of SSR markers. DNA was extracted from 80 of the 91 F_2 plants classified individually for nematode infection. Since three of the samples did not amplify in the PCR reactions, DNA from 77 individual plants was subsequently used for molecular analysis.

One hundred twenty primers were used to detect polymorphisms between the parents. A total of 16 primers yielded 16 polymorphic markers (Table 2). The percentage of plants with a polymorphic primer was 13%.

Chi-square analysis showed 6 of the 16 markers segregated as expected for a monogenic Mendelian trait in the F_2 population (Table 3). BNL 1421 was polymorphic in size between the resistant parent (190 bp) and susceptible parent (231 bp) with co-dominant inheritance. BNL 1669 was polymorphic with a fragment size of 190 in the resistant parent and 232 in the susceptible parent, respectively. Mapmaker analysis indicated that BNL 1421 and BNL 1669 were linked with a distance of 15.4 cM. BNL 1421 was significant ($F = 3.01$) with an R^2 of 8.3% for the variability of the gall index for the resistant trait. Both of the SSR markers exhibited distorted segregation.

DISCUSSION

Root-knot nematode infestation of cotton is a insidious problem because: 1) nematode infections do not produce distinct symptoms on the above-ground portion of the plant; 2) the nematodes may interact with other pathogens forming a disease complex, making disease identification more difficult; 3) nematodes are not visible without a microscope; 4) nematode distribution within a field can be sporadic, ranging from high levels in one area to none in other parts of the same field, which requires special care for

Table 1. Gall indices for Stoneville 213 and Clevewilt 6-1 and the resulting F_1 and F_2 populations

Line	Number of plants in each gall classification[z]					Total number of plants
	1	2	3	4	5	
Stoneville 213	0	2	5	0	8	15
Clevewilt 6-1	0	8	5	6	0	19
F_1	2	1	0	1	13	17
F_2	14	6	5	13	53	91

[z] Root galling on plants was rated on a scale of 1 to 5, where 1 = plants with no galls or very light galling, 2 = plants with light galling, 3 = plants with moderate galling, 4 = plants with heavy galling, and 5 = plants with very heavy galling.

Table 2. Polymorphic SSR markers used in the linkage analysis

Primer	Clevewilt 6-1 DNA fragment size (bp)	Stoneville 213 DNA fragment size (bp)	Inheritance
BNL 4030	113	109	Co-dominant
BNL 1672	104, 133	104	Dominant
BNL 1231	195	190	Co-dominant
BNL 673	176	176, 192	Dominant
BNL 1669	190	232	Co-dominant
BNL 3502A	0	154	Dominant
BNL 3502B	204	220	Co-dominant
BNL 2590	182, 186, 191	182, 186, 188	Co-dominant
BNL 3968	95	95, 97	Dominant
BNL 119	212	222	Co-dominant
BNL 1227	156, 173	156, 185	Co-dominant
BNL 3948	230, 232	230	Dominant
BNL 2449	140	158	Co-dominant
BNL 3994	93, 123	95, 123	Co-dominant
BNL 2646	119	142	Co-dominant
BNL 1421	190	231	Co-dominant

Table 3. Analysis of variance for gall index distributed between resistant and susceptible plants

Primer[y]	Number of Plants	r^2	F value[z]
BNL 4030	70	0.056	1.99
BNL 1672**	64	0.000	0.01
BNL 1231	59	0.045	1.33
BNL 673	61	0.001	0.03
BNL 1669	66	0.068	2.30
BNL 3502A**	65	0.003	0.22
BNL 3502B	61	0.065	2.02
BNL 2590	67	0.004	0.14
BNL 3968**	57	0.007	0.43
BNL 119	66	0.036	1.23
BNL 1227**	61	0.056	1.75
BNL 3948	60	0.022	1.29
BNL 2449	60	0.025	0.73
BNL 3994**	66	0.042	1.37
BNL 2646**	72	0.005	0.18
BNL 1421	69	0.083	3.01*

[y] Primers marked with ** segregated in normal Mendelian fashion.

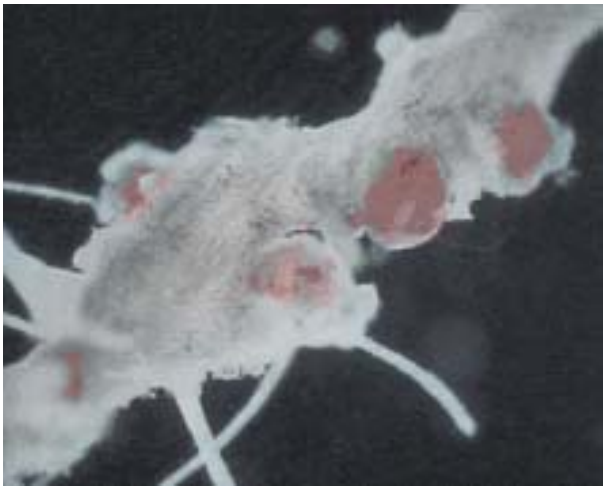
[z] * indicates the value is significant at $P \leq 0.05$.

sample collection, and 5) management of nematodes requires identification of the correct nematode species, which is a very costly, labor intensive and time consuming process. A reliable and accurate method of screening that eliminates the difficulties presented by field evaluation in nurseries is needed for use in breeding programs. DNA markers associated with one or more nematode resistance genes could provide such a tool to the breeder, and expedite breeding by enabling selection based on the genotype rather than on the phenotype.

Gall index. One of the difficulties in designing a strategy to breed cultivars with resistance to RKN is the screening technique. Segregation of gall index in our study among F_2 progeny showed that the F_2 plants segregated as expected for the Mendelian ratio of 3:1, and according to the single recessive gene model for the resistant trait. Although no currently grown commercial cotton cultivar is highly resistant to RKN, there is a long history of breeding for resistance to RKN (McPherson et al., 1995; Jenkins et al., 1993, 1995; Robinson et al., 1997; Shepherd, 1974a, b; Shepherd et al., 1988). The cotton cultivar, Nem X, was selected for resistance to RKN based on the gall index of plants (Garber and Oakley, 1996). Zhou (1999) reported that root galling and RKN reproduction were lowest on M-315, the highest on 'Deltapine

90', and intermediate and variable on 'Acala NemX' and 'Stoneville LA 887'. His results showed the presence of a single recessive gene for RKN resistance in the cotton cultivars NemX and Stoneville LA 887 and two major genes in the cotton line M-240. He reported that the same single recessive gene controls resistance to RKN in NemX and Stoneville LA 887. Robinson et al. (1997) showed that the rapid way to combine high levels of RKN resistance with higher yield and superior fiber properties was to use the F_2 population. They observed that M249 RNR lines had the fewest wilted plants due to RKN, and F_2 hybrids were not significantly different from M249 RNR. McPherson et al. (1995) suggested that M78RNR and M249 RNR upland cotton lines might have different resistance genes. Turcotte et al. (1963) observed that in crosses of RKN resistance and susceptible *G. barbadense* L. breeding stocks, the F_1 was similar in resistance to the susceptible parent and that, in the F_2, two recessive genes conditioned resistance. Shepherd (1974b) designated A623 and A61 with a high level of resistance to RKN. In this study, Clevewilt 6-1 showed a moderate level of resistance to RKN. Although we did not find any significant relation of SSR markers with the RKN resistance trait, the results demonstrated that BNL 1421 marker can explain about 8% variation in resistance based on gall index. Linkage analysis showed that BNL 1421 and BNL 1669 were linked with a distance of 15.4 cM and both of these markers exhibited distorted segregation. The small effect of BNL 1421 could be due to the weak linkage of the marker with the RKN resistant trait or to false linkage, because they segregated abnormally.

Figure 1. Galls (red structures) formed by root-knot nematode infection of a cotton root.



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