Introgression of resistance to reniform nematode (*Rotylenchulus reniformis*) into upland cotton (*Gossypium hirsutum*) from *Gossypium arboreum* and a *G. hirsutum/Gossypium aridum* bridging line

Erik J. Sacks, A. Forest Robinson

**ABSTRACT**

*Gossypium hirsutum* L. is the predominant cotton of commerce and all cultivars of this species are susceptible to the reniform nematode, *Rotylenchulus reniformis*. To introgress resistance to *R. reniformis* into the tetraploid 2*(AD)* *G. hirsutum*, a resistant diploid A2-genome *Gossypium arboreum* accession (A2-190) was crossed with a hexaploid 2*(AD1)*D4 bridging line (G 371) to obtain a tetraploid triple-species hybrid. The three-species hybrid was back-crossed to *G. hirsutum* and a population of 277 BC1 individuals was produced. The BC1s and controls were assayed in growth chambers for resistance to *R. reniformis*. Fortuitously, the hexaploid bridging line G 371 was also found to be resistant to *R. reniformis*. The BC1 segregated 3:1, resistant:susceptible, indicating that resistance was conferred by dominant genes at two different loci, with each originating from a distinct germplasm source. This study demonstrated that it is possible to introgress and pyramid genes for resistance to *R. reniformis* in *G. hirsutum*. Published by Elsevier B.V.

1. Introduction

In 2007, 26 × 10^9 kg of cotton were produced worldwide on 33 × 10^8 ha (http://www.fas.usda.gov/cotton/circular/Current.htm). China, India and the U.S. were the top three producers. *Gossypium hirsutum* L. (upland cotton) is one of four independently domesticated cotton species but it accounts for about 90% of world cotton production (Gillham et al., 1995). Thus, efforts to improve upland cotton can have a broad impact.

The reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira, is an obligate plant parasite that feeds on roots; it has a broad host range and it is found in tropical and subtropical areas throughout the world (Robinson et al., 1997). In the U.S., *R. reniformis* has been expanding its geographic range in U.S. cotton growing areas and has caused increased economic losses to cotton growers (Blasingame, 2006; Robinson, 2007). No sources of host-plant resistance have been found in the cultivated upland cotton germplasm, despite extensive evaluations of more than 2000 accessions (Robinson et al., 1999; Usery et al., 2005; Weaver et al., 2007). To date, control of reniform nematode in upland cotton has been limited to crop rotations and application of nematicides. However, resistance to *R. reniformis* has been reported in other *Gossypium* species, including *Gossypium arboreum* L., *Gossypium barbadense* L., *Gossypium herbaceum* L., and *Gossypium longicalyx* J.B. Hutch. & B.J.S. Lee (Carter, 1981; Robinson et al., 2004; Stewart and Robbins, 1995; Yik and Birchfield, 1984). Thus, introgression of host-plant resistance into upland cotton is a long-term goal being pursued by many laboratories (Robinson et al., 2008).

Two of the domesticated cotton species, *G. hirsutum* and *G. barbadense*, are indigenous to the Americas, and are tetraploids with a 2*(AD) (2n = 4x = 52) genomic constitution. The other two domesticated cotton species, the Asian *G. arboreum* and the African *G. herbaceum*, are A-genome diploids. The 13 or so species of D-genome diploids are indigenous to the Americas but none have been domesticated due to their limited fiber production. No A-genome species have been found in the New World, so how an A-genome and D-genome species combined to give rise to the New World tetraploids remains a mystery. However, the initial speciation event for the 2*(AD) tetraploids was an evolutionary genetic bottleneck (Wendel and Cronn, 2003).

Given the evolutionary history of *G. hirsutum*, the A-genome diploids represent an important source of genes that could be used to develop improved upland cotton cultivars. Introgression of genes from the A-genome diploid species into upland cotton is hampered by post-zygotic breeding barriers in addition to the...
differences in chromosome number (Altman, 1988; Beasley, 1940; Gerstel, 1954; Stewart, 1995; Stewart and Hsu, 1978). Despite breeding barriers, there are examples of genes for disease resistance and fiber quality being successfully introgressed from diploid Gossypium species into G. hirsutum and G. barbadense (Blank, 1971; Knight, 1948; Fryxell, 1976; Stewart, 1995). Recently, Robinson et al. (2007) and Konan et al. (2007) documented the introgression of a gene for resistance to R. reniformis from the F-genome diploid, G. longicalyx, into upland cotton. In the current study, we investigated the introgression of resistance to R. reniformis from a G. arboreum accession into upland cotton by using a 2(ADD) hexaploid G. hirsutum/Gossypium aridum bridging line, that in a fortuitous coincidence was also found to be resistant.

2. Materials and methods

2.1. Plant material

The G. hirsutum cultivars Deltapine 16 and MD51ne were used as susceptible controls in nematode assays and as recipient parents in crosses to introgress resistance genes. Deltapine 16 is an obsolete upland cultivar that has been used as a susceptible control in previous studies of reniform nematode resistance in cotton (Carter, 1981; Robinson et al., 1999; Yik and Birchfield, 1984), and it is in the pedigree of many modern cultivars adapted to the Mississippi Delta. MD51ne was derived from Deltapine 16, and has improved fiber qualities and lint yield (Meredith, 1993). G. barbadense, GB-713, has been reported to be highly resistant to reniform nematodes (Robinson et al., 2004) and was included in the nematode resistance assays as a resistant control. To test previous reports of resistance in G. arboreum A2-019, A2-087, A2-100, A2-113, A2-190, A2-194 and G. herbaceum A1-24 (Carter, 1981; Stewart and Robbins, 1995; Yik and Birchfield, 1984), nematode resistance assays were conducted on these accessions; similarly, G. arboreum A2-082 and A2-101 were also tested to validate previous reports of susceptibility. All of the G. hirsutum, G. barbadense, G. arboreum, and G. herbaceum accessions used in this study were highly inbred and reproduced true to type. To further ensure genetic uniformity for the G. arboreum, G. barbadense and G. herbaceum accessions, the seeds used in this study were obtained by self-pollinating single representative plants. Seeds of the above accessions can be obtained from the USDA National Plant Germplasm System (http://www.ars-grin.gov/npgs/).

A 2((AD1)D4) hexaploid S1, named G 371 [G. hirsutum cultivar NC8/G. aridum (Rose & Standley) Skovsted accession G 248; Maréchal, 1983], was kindly provided as cuttings by Wendel (Iowa State University, Ames, IA). We used the G 371 S1 as a bridging line

---

**Fig. 1.** Pedigree, genomic constitution, and resistance to reniform nematodes for the cotton genotypes and populations in this study.
to obtain a triple-species hybrid with A2–190 (G. arboreum/G. hirsutum/G. aridum; AA(2(ADD))→AADD; Fig. 1). The theory behind the diploid/hexaploid crossing strategy was described previously by Brown and Menzel (1950), Stewart (1995) and Sacks and Robinson (2008). By crossing an A-genome diploid with the 2(ADD) hexaploid, the resulting progeny were expected to have the same tetraploid genomic constitution as G. hirsutum and thus be crossable with G. hirsutum. The G 371 S1 was also self-pollinated to obtain S2 progeny that were assayed for nematode resistance.

Previously we described the development and initial characterization of the A2–190/G 371 F1 (Sacks and Robinson, 2008). In summary, the A2–190/G 371 F1 was obtained as a mature seed from a ripe fruit. The A2–190/G 371 F1 inherited purple petals and short-day flower-initiation from the G. aridum parent (in contrast to the yellow petals and day neutral habit of the G. arboreum parent). Crossing the A2–190/G 371 F1 to G. hirsutum (Deltapine 16 and MD51ne) was considered analogous to backcrossing because half of the A1’s genome was from G. hirsutum. The BC1s segregated for petal color and petal spot. The A2–190/G 371 F1 had viable pollen but produced few F2 seed. In contrast to the F2 generation, BC1 seed was readily obtained by crossing the F2s as either the male or female to G. hirsutum, and the BC1 progenies were typically vigorous, self-fertile and crossable with G. hirsutum (Sacks and Robinson, 2008). A total of 277 A2–190/G 371 BC1/G. hirsutum BC1 individuals were assayed with nematodes, of which 30 were derived from Deltapine 16 and 247 from MD51ne.

2.2. Nematode resistance assays

The assay protocol was modified from Robinson et al. (1999). Six assays were conducted: five (Expts. 1, 2, 3, 5, and 6) in a growth chamber in Stoneville, MS and one (Expt. 4) in a growth chamber in College Station, TX. The chamber in Stoneville was maintained at a constant 28 ± 1 °C, with 12 h of light per day provided by an equal mixture of 400 W metal halide and sodium vapor lamps. The College Station chamber was set to 30 °C during the day, 26 °C at night, with 14 h of light per day provided by a mixture of fluorescent and incandescent lamps. Light intensity varied with distance between the top of the growing plant canopy and the lamps but was no less than 383 μmol photons m−2 s−1. Seed were scarified just prior to planting by nicking the seed coat with a nail clipper. Plants were grown in 500-mL pots containing drainage holes. The potting mix used in Stoneville was autoclaved Metro-Mix 200 (Sun Gro Horticulture, Bellevue, WA), which contained vermiculite, peat moss, perlite and dolomitic limestone. In College Station, the potting mix was a 6:1 mixture of fine sand (<400 μm diameter) and vermiculite, with 5 kg g−1 pelletized limestone. Each pot in Stoneville was topdressed at planting with 5 mL of time-release fertilizer (Osmocote 14-14-14; The Scotts Miracle-Gro Company, Marysville, OH). In College Station, each pot was fertilized weekly with 50 mL containing 100 mg of dissolved nutrients (15N:16P:17K:1.08Mg:0.2Fe:0.1Zn). All pots were watered daily.

A population of R. reniformis originally from Baton Rouge, LA was used in all assays. Pots were inoculated with 8–14 veriform R. reniformis ml−1 potting mix. Inoculations were made 14 days after planting for Expts. 1–4 and at planting for Expts. 5 and 6. Inoculated pots without plants were included as controls in all assays except for Expt. 4. Eight to nine weeks after inoculation, three cores of potting mix from each pot were taken with a brass soil corer (0.8 cm inner diameter and 15 cm long). Active, veriform stages were extracted by Baermann funnel and counted. The water extract and dried potting mix samples were weighed. Bulk density of the Metro-Mix 200 was ~0.34 g ml−1 and the sand–vermiculite mixture was ~1.98 g ml−1. Thus for each pot, the final population density of R. reniformis was calculated as the number of veriform nematodes ml−1 potting mix. Estimates of nematode reproduction were obtained by subtracting the average number of nematodes that were extracted from the control pots that lacked plants, in each experiment, from the final population density of each pot with a plant. Within each experiment, nematode reproduction was standardized as a percentage of the average value for the susceptible G. hirsutum control.

3. Results and discussion

3.1. Nematode reproduction

Growing conditions were conducive to vigorous and uniform plant growth. As expected, the susceptible G. hirsutum controls supported high levels of nematode reproduction and the resistant control GB-713 supported only low levels of nematode reproduction (Table 1 and Fig. 2). In Metro-Mix 200, the average nematode reproduction for all inoculated Deltapine 16 pots in Expts. 1 and 2 was the same as the average nematode reproduction on MD51ne in Expts. 3, 5 and 6 (1096 nematodes ml−1; Table 1). Thus, approximately 100-fold nematode reproduction was observed on the susceptible G. hirsutum controls grown in Metro-Mix 200. Nematode reproduction on Deltapine 16 grown in sand (Expt.4) was about half of that observed for those grown in Metro-Mix 200 (Table 1), a result that was consistent with prior unpublished observations that peat moss-based potting mixes can support higher levels of R. reniformis reproduction than sand-based mixes but the former may be more prone to population crashes over time.

Yik and Birchfield (1984) defined high resistance to R. reniformis in cotton as ≤10% the reproduction on Deltapine 16. Average nematode reproduction for GB-713 was only 8 ± 1% (n = 46) of the susceptible G. hirsutum controls, and this was consistent with the 8.5% (n = 169) reported by Robinson et al. (2007). Moreover, this consistency among the standardized estimates of nematode reproduction was found regardless of whether the potting mix was sand (Expt. 4 and the Robinson et al., 2007 study) or Metro-Mix 200 (Expts. 3, 5 and 6). Thus, one conclusion of this study is that a peat moss-based potting mix is an effective medium for conducting assays of R. reniformis reproduction and resistance, and it has the advantage of being light-weight and inexpensive to transport.

Resistance and susceptibility previously reported for the G. arboreum and G. herbaceum accessions were confirmed (Carter, 1981; Stewart and Robbins, 1995; Yik and Birchfield, 1984; Table 1). Nematode reproduction on A2–190 averaged 6 ± 1% of the G. hirsutum controls, which was similar to the resistance observed for GB-713.

The G 371 hexaploid was chosen as a parent solely for its value as a bridging line between the A-genome diploid resistance gene donors and the susceptible 2(AD1) G. hirsutum cultivars. Prior to this study, we had no information about how G 371 would react to a challenge by R. reniformis. All of the 27 G 371 S2 individuals challenged by R. reniformis were resistant (Fig. 2), indicating that the G 371 S1 was resistant. Nematode reproduction for the G 371 S2 averaged 10 ± 1% of the G. hirsutum controls (Table 1). To our knowledge, this is the first report of resistance to R. reniformis in a 2(ADD) hexaploid cotton. Thus, this result further validates the method of using hexaploid bridging lines to introgress desirable traits from diploid donor species into tetraploid upland cotton.

3.2. Inheritance of resistance

The source of resistance in G 371 could not be determined unambiguously because we were unable to obtain its G. hirsutum and G. aridum parents. However, of the thousands of wild and domesticated G. hirsutum accessions assayed for resistance to R. reniformis, none with high levels of resistance, such as those observed in G 371, have been found, and no resistance at all has
been found in *G. hirsutum* cultivars (Robinson et al., 1999; Usery et al., 2005; Weaver et al., 2007). The *G. hirsutum* parent of G 371, NC8, is an old cultivar from Africa and not a wild accession from the species center of diversity in the Americas. Molecular marker studies have confirmed that genetic diversity in *G. hirsutum* cultivars is low, a result of bottlenecks during domestication (Iqbal et al., 2001; Vafaie-Tabar et al., 2004; Wendel et al., 1992). Thus, it is unlikely that NC8 is the source of the resistance in G 371. To our knowledge, only two accessions of *G. aridum*, an undomesticated genetically diverse American species, have been evaluated for resistance to *R. reniformis* (Yik and Birchfield, 1984). Although these *G. aridum* accessions were not resistant to *R. reniformis*, some highly resistant accessions of other D-genome diploid species were found (Yik and Birchfield, 1984). Though not conclusive, the above data suggest it is more likely that the resistance in G 371 originated from the D-genome *G. aridum* parent than from the *G. hirsutum* cultivar NC8, and such a deduction leads to the hypothesis that the triple-species hybrid between A2-190 and G 371 incorporates unlinked loci for resistance to *R. reniformis* from the A and D genomes, respectively.

The BC$_1$ segregated for resistance and susceptibility but the distribution was skewed towards the resistant phenotype (Fig. 2), indicating dominant gene action. If resistance in the A$_2$-190 and G 371 S$_1$ parents had been conferred by dominant alleles at the same locus, then the F$_1$ would have been homozygous resistant and all of the BC$_1$ progeny should have been heterozygous resistant, but this was not observed. Thus, the presence of many highly susceptible BC$_1$ progeny (Fig. 2) indicated that the resistance in each of the two parent lines was conferred by different loci. Though others have identified resistance to *R. reniformis* in different *Gossypium* species and accessions, to our knowledge this is the first report that resistance to *R. reniformis* from two different cotton germplasm sources is conferred by different loci. This is a welcome finding because it indicates that pyramiding genes for resistance to *R. reniformis* in upland cotton is feasible, and we would expect such a strategy to play an important role in ensuring that durable resistance is deployed in the field.

If resistance in the A$_2$-190 and G 371 S$_1$ parents had been conferred by dominant alleles at two unlinked loci, then the F$_1$ would have been heterozygous resistant at the two loci and the BC$_1$ should...
have segregated 3:1 resistant:susceptible. Testing this hypothesis was complicated by the blurring of distinct classes by environmental variation, a feature that is typical of nematode resistance assays. For example, the normal distributions observed for the homozygous susceptible *G. hirsutum* parents represent the extent of environmental variation in susceptible genotypes for *R. reniformis* reproduction, and include one outlier (escape) out of 60 pots (Fig. 2).

In addition, Avila et al. (2005) and Laffoe (2005) observed partial dominance for *R. reniformis* resistance in intraspecific *G. arboreum* F2 populations derived from resistant A2-190 or A2-19 crossed with a susceptible *G. arboreum* parent. Partial dominance could further blur the distinction between classes.

Ignoring the single outlier in the *G. hirsutum* distribution, there was still an overlap with the A2-190 distribution at the bin representing 40% nematode reproduction of the *G. hirsutum* average (Fig. 2). If we assigned half of the 40% bin BC1 pots each to the resistant and susceptible classes, then the overall distribution was not significantly different from a 3:1 ratio (*p* = 0.1030; out of 277 progeny, 196 resistant individuals were observed and 208 were expected, and 81 susceptible individuals were observed and 69 were expected). Thus, assuming a degree of partial dominance, it appears that the resistance in the BC1 was conferred by two independent loci. Furthermore, we predict that the resistance locus from the G 371 parent is located in the D-genome contributed by *G. aridum*. Though our knowledge about the genomic locations of nematode resistance genes in cotton is currently limited, it is interesting to consider that the loci for resistance to *R. reniformis* in the A-genome of *G. arboreum* and the D-genome of *G. aridum* might be homeologues. For example, given that Robinson et al. (2007) observed that they introgressed the resistance to *R. reniformis* from the F-genome (*G. longicalyx*) into chromosome 11 of the A-genome, we might hypothesize that chromosome 11’s D-genome homeologue, chromosome 21, would contain the resistance gene from *G. aridum*. Future studies, using molecular markers, would permit further testing of these conclusions and hypotheses.

Subsequently, we have used the G 371 S1 bridging line to also obtain triple-species hybrids with A2-113 and A2-100, and have backcrossed these to *G. hirsutum* (Sacks and Robinson, 2008). In addition, most of the A2-190/G 371 BC1 genotypes that were assayed with nematodes were subsequently selfed and backcrossed to produce BC1S1 and BC2 seed. A preliminary report by Avila et al. (2006) indicates that they introgressed resistance from A2-194 into *G. hirsutum* (BC2) via a colchicine-derived chimeric (diploid and tetraploid) hybrid with the D-genome diploid *G. trilobum* (Moc. & Sess. ex DC.) Skov.: resistance was conferred by a single partially dominant gene. It will need to be determined if the resistance genes introgressed from A2-113, A2-100 and A2-194 represent different alleles or loci from those derived from A2-190. Similarly, the relationship among the introgressed resistance genes described in this study and the resistance gene introgressed from *G. longicalyx* (Robinson et al., 2007) will need to be investigated if the goal of pyramiding genes from different sources is to be achieved fully. By introgressing and pyramiding genes for resistance to *R. reniformis* from two germplasm sources into a tetraploid, fertile, and predominantly upland cotton genetic background, this study demonstrated that a key initial step towards developing resistant upland cotton cultivars has been achieved.

**Disclaimer**

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

**Acknowledgements**

We thank Mr. Hans Hinrichsen for technical assistance. We also thank Drs. Osman Gutierrez and Jack McCarty for helpful suggestions on how to improve the manuscript.
References