Introgression in tetraploid cotton of the resistance to reniform nematode *Rotylenchulus reniformis* Lind. and Oliveira from the *Gossypium hirsutum* L. x *G. longicalyx* Hutch and Lee x *G. thurberi* Tod trispecific hybrid

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ABSTRACT

*To develop upland cotton plants resistant to reniform nematode (*Rotylenchulus reniformis *Lind. and Oliveira), a three-species hybrid including* Gossypium hirsutum *L. (2n = 4 x = 52, $(AD)_1$ genome) was created using G.* longicalyx *Hutch and Lee as the donor parent (2n = 2x = 26, F_1 genome) and G.* thurberi *Tod. (2n = 2x = 26, D_1 genome) as the bridge species. The morphology and the reniform nematode resistance of the parents and thirty selfed progeny of a 2x[G.* hirsutum *x G.* longicalyx*] x G.* thurberi *(HLT) trispecific hybrid were assessed. G.* longicalyx*, the G.* hirsutum *x G.* longicalyx *hexaploid and the 30 selfed progeny of the HLT hybrid were very resistant to reniform nematode, unlike G.* hirsutum *which was very susceptible to it. Cytogenetic studies showed that HLT hybrid and its direct progeny have 52 chromosomes as the main cotton cultivated species does. The HLT hybrid has good pollen fertility and a very high level of chromosome pairing at metaphase I. It is self-fertile but presents important incompatibility barriers when crossed as female parent with G.* hirsutum*. The prospects to develop upland cotton commercial cultivars resistant to reniform nematodes from the available interspecific genetic stocks are discussed.*

Introduction

The reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) is widely distributed in many tropical and subtropical regions and in warmer parts of the temperate zone of the world (Ayala and Ramirez, 1964; Robinson, 1999; Ferris, 2002). It has been reported in tropical and sub-tropical West and Central Africa, central and south America, Southeast Asia, the Caribbean, Mexico, Japan, the Middle East, South Pacific, Italy, Spain, China and the Far East. *Rotylenchulus reniformis* is present in most cotton-producing areas causing serious problems for cotton production (Robinson, 1999). The deleterious effects of this parasite on cotton are, on the one hand, delay of plant growth and development, and, on the other hand, reduction in lint yield, boll size, lint percent, seed index, and fiber micronaire value (Cook *et al*., 1997; Robinson, 1999). In addition, the reniform nematode is important for increasing the incidence and severity of Fusarium wilt on cotton (Kinloch and Rich, 2001). In the regions infested by this nematode, cotton losses range from 15 to 75% according to the levels of infestation and the meteorological conditions (Yik and Birchfield, 1984; Robinson, 1999; McLean and Weaver, 2002). The standard strategies for managing *R. reniformis* in cotton include nematicide applications and rotation with non-host crops. Unfortunately, *R. reniformis* has a very wide host range (Ajala and Ramirez, 1964), often rendering crop rotation an ineffective method of control. The use of nematicides is uneconomical and for various reasons not always efficient and sometimes dangerous for environment. Therefore, growing nematode-resistant cultivars of cotton appears to be the most economic method of management and the most sustainable environmentally. Unfortunately, cotton cultivars with adequate resistance to *R. reniformis* are not yet available (Robinson and *al*., 1999; Kinloch and Rich, 2001; McLean and Weaver, 2002). Among the 46 diploid species of the genus *Gossypium*, there is one however, *G. longicalyx* Hutch. & Lee (F genome), that seems to be immune to this nematode (Yik and Birchfield, 1984). The introgression of this useful trait from *G. longicalyx* into cultivated tetraploid cotton *G. hirsutum* L. (genome A_hD_h) is the main objective of our work. We present here the results we have obtained regarding the development of fertile tetraploid cotton genotypes resistant to *R. reniformis*.

Experimental procedure

Production of plant material, and morphological and cytological observations

According to the pseudophyletic introgression method (Mergeai, 2003), the hexaploid *G. hirsutum* x *G. longicalyx* was crossed to *G. thurberi* to create a trispecific hybrid 2x[*G. hirsutum* x *G. longicalyx*] x *G. thurberi* (designated the HLT hybrid). This hybrid was self-pollinated and backcrossed to *G. hirsutum* (Figure 1). Morphological observations were made on the HLT hybrid and on its selfed progeny. To determine chromosome number and meiotic chromosome associations, HLT flower buds and selfed progeny were fixed in Carnoy's solution (95% ethanol-chloroform-glacial acetic acid, 6:3:1 v:v:v) for 72 h and stored at 4 °C in 70 % ethanol until their evaluation. On a slide, microsporocytes were squashed and stained in a drop of 1.5% acetocarmine solution enriched in iron. The slide, recovered with a cover slip, was heated a few seconds over a flame to improve chromosome staining. After being well flattened, cells were examined for meiotic configurations with a Nikon Eclipse E800 microscope equipped with a JVC KY-F 58E camera.

Pollen fertility was also determined. For each plant, about 1000 pollen grains were dipped in 1.5% acetocarmine solution for 30 min, and pollen fertility was estimated under a stereomicroscope as the percentage of stained pollen; only large, bright and red grains were considered fertile.

Resistance to R. reniformis

Resistance to *R. reniformis* of HLT parents (*G. hirsutum*, *G. longicalyx*, *G. thurberi* and the hexaploid *G. hirsutum* x *G. longicalyx*) and thirty selfed progeny of the HLT hybrid were assessed in two successive experiments. The first experiment (test of the parental lines) was conducted in a growth chamber programmed for 12-hour light ($120–140.10^{-6}$ E/m².s) and 12-hour dark with day and night temperatures of 28 °C and 26 °C, respectively. Air humidity was about 55%-60%. The second experiment (test of the 30 selfed progeny of the HLT hybrid) was carried out during spring and summer in the tropical greenhouses of the Gembloux Agricultural University where growing conditions were not controlled.

Thirty day old plants planted in 5 litre pots filled with a 3:2:1 (v:v:v) mixture of compost, sand and peat were challenged with reniform nematode eggs. The planting medium in each pot was infested with 6000 *R. reniformis* eggs by injecting the appropriate nematode egg suspension 2 to 3 cm deep at four points 2 cm from the stem. Sixty days after inoculation the soil was removed by soaking the roots in water and entire root systems were gently harvested. Roots with nematode egg masses were blotted dry with absorbing paper and weighed. Eggs were extracted with 10 % NaOCl (sodium hypochloride) followed by gradient centrifugation in a kaolin-$MgSO_4$ solution with a specific gravity of 1.18. Eggs were counted using two 15 ml aliquot and the number of eggs per gram of root was determined for each plant. The host status was assessed following the scale proposed by Yik and Birchfield (1984) where relative plant resistance is based on egg production per gram of root expressed as a percentage of egg production per gram on *G. hirsutum* control plants within the test. This scale contains the following classes of infestation: 0% = immune, 1-10% = highly resistant, 11-25% = resistant, 26-40% = moderately resistant, 41-60% = moderately susceptible, 61-100% = susceptible (equivalent to control plant), and above 100% = very susceptible.

Results

Production of plant material and morphological observations

The HLT hybrid has a slender habit and is self-fertile. All the self-pollinated capsules harvested from HLT were smaller than those of *G. hirsutum* with two to eight seeds per capsule. These 74 seeds carried the same dirty-white lint of the HLT hybrid but the linters were three types of colour: 40 had green linters, 28 had pale-green linters, and 6 had brown linters. Of 33 HLT selfed progeny seedlings observed, seven had distinctly distorted cotyledons. The cotyledons were united along the petioles or for the entire length of the leaves giving the impression of a unique leaf. These distortions seem, however, to be minor and they had no obvious detrimental consequences on the development of the plants.

HLT selfed progeny also had a slender habit with vegetative branches shorter than 40 cm length. The capsules were also smaller than *G. hirsutum* capsules. Two types of plants were observed: plants with rather small, embossed leaves and plants with large and normal leaves. Both types of plants were 152 to 236 cm tall with 12 to 34 nodes on the main stem. The number of fruit bearing nodes ranged from five to 19 and the time from sowing to flowering varied between 103 and 133 days. The *G. hirsutum* control plants were much shorter (70 to 91 cm) with only eight to 12 nodes. The number of fruit bearing nodes on the control plants varied from five to 10 and the time from sowing to flowering was 64 to 84 days. These results are summarized in Table 1. The HLT hybrid and its selfed progeny seem to be photoperiodic. The flowering of these plants only started in end of September when the day length in Gembloux was close to 12 hours. Backcrosses of the HLT hybrid to *G. hirsutum* gave a very low success rate. On 57 backcrosses performed, only one seed was obtained.

Pollen fertility and, chromosomes number and association

Pollen fertilities for the *G. hirsutum* control line and the HLT hybrid were 98% and 96%, respectively, whereas twenty HLT selfed progeny had pollen fertilities that ranged from 9.50% to 96.7%. There is a distinct segregation for pollen fertility of HLT selfed progeny but we noted that more than the half of the analysed plants had pollen fertilities greater than 70%.

Chromosome counts showed that both HLT hybrids and its selfed progeny have 52 chromosomes. Univalents, bivalents and multivalents were observed. The mean frequency of these associations is summarized in Table 2. The high percentage of bivalents indicates a high level of chromosome homology between the *G. longicalyx* and the *G. hirsutum* A-subgenome chromosomes, which is consistent with the high self-fertility observed in these hybrids. These results are also similar to those Brown and Menzel (1950) observed in a 2X (*G. hirsutum* x *G. herbaceum*) x *G. armourianum* trispecific hybrid

Resistance to R. reniformis

Among the parent genotypes of HLT tested in growth chamber, *G. hirsutum* had the greatest number of eggs per gram root (14 690 eggs.g^{-1}) and *G. thurberi* had the second highest egg count (8 570 eggs.g^{-1}). In contrast, the number of eggs per gram root of *G. longicalyx* and the hexaploid were very low (186 and 288 eggs.g^{-1}, respectively). In these conditions, *G. thurberi* ranked as moderately susceptible (58% eggs.g^{-1}) whereas *G. longicalyx* (1.27% eggs.g^{-1}) and the hexaploid (1.96% eggs.g^{-1}) ranked as very resistant (Table 3.) The 30 HLT selfed progeny were also very resistant to *R. reniformis* with a percentage of eggs per gram of root varying between 0 and 9.34% in greenhouse conditions (Table 4).

Discussion and Conclusions

The pseudophyletic method resulted in the successful synthesis of the trispecific HLT hybrid. This hybrid is self-fertile with a good level of pollen fertility (96%). Morphological observations made on the HLT plant hybrid and its selfed progeny identified some traits that are not desirable for a commercial variety: excessive height, delayed flowering, very small capsules, and coarse discolored fiber. To get rid of all these traits coming from the wild donor and bridge species, a program of backcrosses with the recipient species *G. hirsutum* is essential. The first backcrosses of the HLT hybrid (as female) to *G. hirsutum* (var. gazuncho) only realized one seed from 57 backcrosses. This very low success rate is probably due to the presence of incompatibility barriers between HLT hybrids and the male parent, *G. hirsutum*. Cytogenetic data show 52 chromosomes for both HLT and its selfed progeny as for the main cotton cultivated species. They indicate also a low frequency of univalents and multivalents and a high number of bivalents (about 24II). This level of pairing is one of the highest observed so far in trispecific synthetic allotetraploid hybrids. It confirms the close "relationship" of *G. longicalyx* to the A-genome species.

Results of the evaluation of resistance to *R. reniformis* of the HLT hybrid parents show that *G. hirsutum* is susceptible (100% eggs.g^{-1}) and *G. thurberi* is moderately susceptible (58% eggs.g^{-1}) whereas *G. longicalyx* (1.27% eggs.g^{-1}) and the hexaploid (1.96% eggs.g^{-1}) are very resistant. These results confirm the high resistance of *G. longicalyx* to reniform nematode (*R. reniformis*). They also confirm the expression of this trait in the *G. hirsutum* x *G. longicalyx* hexaploid. The assessment of 30 selfed progeny of the HLT hybrid revealed the high level of resistance to *R. reniformis* in all of them. There was practically no segregation among them for this trait, which contrasted with the segregation noticed for the morphological traits. With its high rates of chromosome pairing, its good level of self-fertility, and its high resistance to reniform nematodes, the HLT hybrid seems to be an ideal donor germplasm from which reniform nematode resistance can be introgressed into cultivated cotton. The final challenge will be overcoming the incompatibility barriers that exist between the HLT hybrid and *G. hirsutum*.

Acknowledgments

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Figure 1. *Development and breeding strategy for the HLT (2x[G.* hirsutum *x G.* longicalyx*] x G.* thurberi*) trispecific hybrid.*

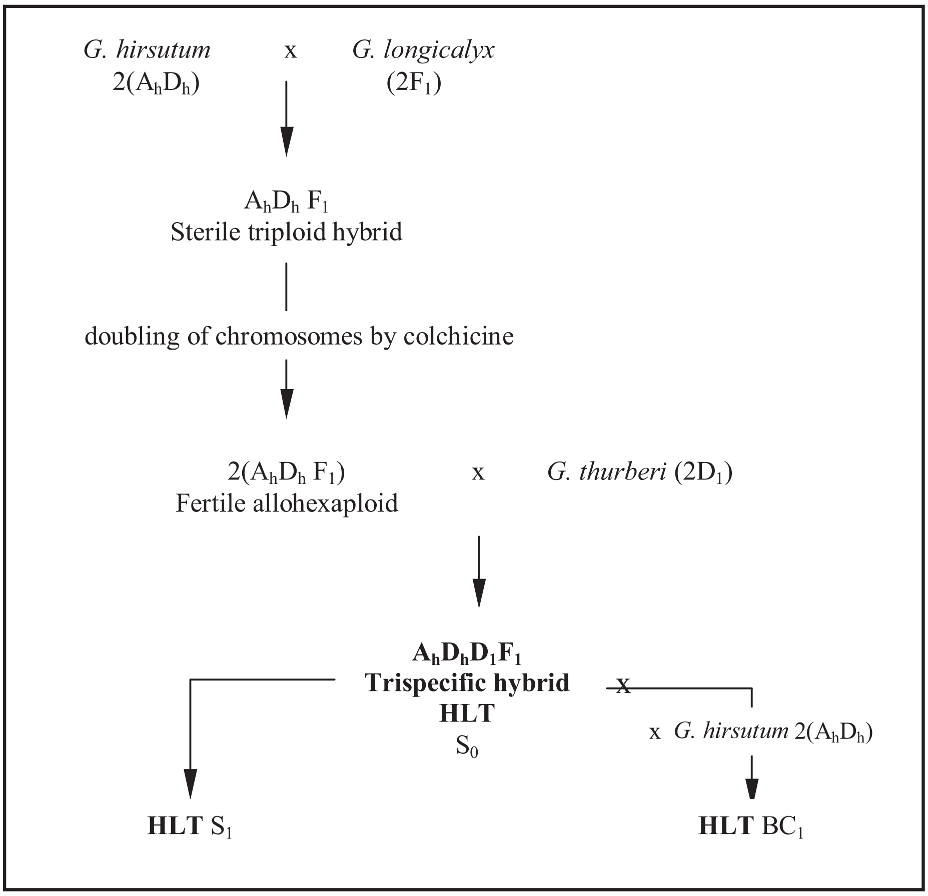

Figure 2. *Chromosome configurations at metaphase I of meiosis: a) cell of HLT with 26 bivalents; b) 26 bivalents in a cell of HLT self-progeny; c) 2 univalents (arrow) and 25 bivalents in a cell of HLT self-progeny.*

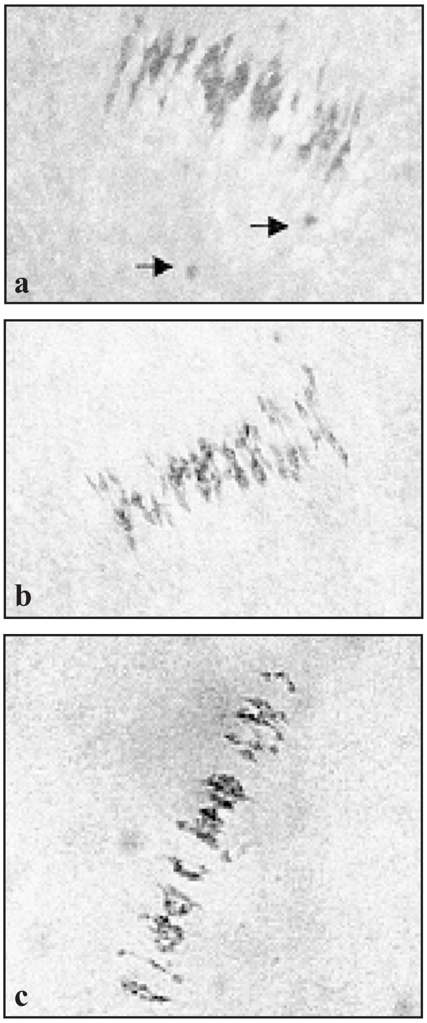

Table 1. Descriptive parameters of HLT selfed progeny plants.

Genotype	Descriptive parameters	Flowering cycle (days)	Main stem nodes	Fruit bearing nodes	Plant height (cm)
25 plants of HLT selfed progeny	Average	116	25	13	192
	Min - Max	103-133	12-34	5-19	152-236
	Standard deviation	7.91	5.11	3.80	23.49
5 control *G. hirsutum* plants	Average	76	11	9	82
	Min - Max	64-84	8-12	5-10	70-91
	Standard deviation	8.50	1.52	2.07	8.35

Table 2. Result of cytogenetic analysis on HLT hybrid and its selfed progeny.

Genotype	Descriptive parameters	Chromosome configuration						No. chromosomes	No. cells observed
		I	II	III	IV	V	VI		
HLT	Mean number/cell	0.66	24.76	0.47	0.047	0	0.047		
	Max – Min	0 - 4	20 - 26	0 - 2	0 - 1	0 - 0	0 - 1	52	27
HLT selfed progeny	Mean number/cell	0.85	24.29	0.62	0.038	0.038	0.038		
	Max – Min	0 -3	18 - 26	0 - 4	0 - 1	0 - 1	0 - 1	52	21

Table 3. Results of the assessment in growth chamber of the resistance to Rotylenchulus reniformis *of parent genotypes of the HLT hybrid.*

Genotypes	Eggs produced/g roots	Egg productions per gram root (%)	Host status
G. hirsutum (C2)	14 690	100 %	susceptible
G. thurberi	8 570	58 %	Moderately susceptible
G. longicalyx	186	1.27 %	Highly resistant
Hexaploid	288	1.96 %	Highly resistant

Table 4. Results of the evaluation in greenhouse of the resistance to R. reniformis *of 30 plants belonging to the HLT selfed progeny.*

Descriptive parameters	Eggs produced per plant	Egg produced per gram of root (%)	Host status
Max - min	0 – 1 334	0 – 9.34*	
Standard deviation	336	2.4	Highly resistant

* Data calculated considering a mean infestation in cotton control plants of 207 eggs per gram of root.