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Full Length Research Paper

Cross compatibility of cultivars of *Gossypium hirsutum* L. and feral *Gossypium barbadense* L. in the Southern Highlands of Tanzania

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Cotton is the second most important cash crop contributing about 15% to the annual foreign earning in Tanzania and is purely from *Gossypium hirsutum* L. cultivars. *Gossypium barbadense* L., a textile source in other parts of the world occurs as a feral perennial of ornamental and medicinal value in home gardens. *G. barbadense* L. is a natural host of the red bollworm, a destructive pest to cotton. The Southern Highlands (SH) of Tanzania have been quarantined from cotton production to control spread of the red bollworm to other growing areas. Transgenic cotton expressing the delta-endotoxin genes from *Bacillus thuringiensis* (Bt) offers an alternative control to the pest and reduced dependence on insecticide. Gene flow between wild or valued feral relatives and transgenic crops is a biosafety concern should transgene escape result in resistance development and contamination of germplasm. Potential gene flow between feral *G. barbadense* (including accessions Gb_1 and Gb_2) from the SH and *G. hirsutum* cultivars was assessed using controlled hybridization. The crosses produced fertile F1 but intraspecific seeds from *G. barbadense* did not germinate. *G. barbadense* is more likely to receive than donate genes implying development of pest resistance if introgressed filial generations express the Bt product.

Key words: Hybridization, gene flow, morphological markers, feral cotton, bollworm, *Bacillus thuringiensis* (Bt) cotton.

INTRODUCTION

Cotton is the world's most important natural textile fibre and a significant oilseed crop that provides income to approximately 100 million families whereas 150 countries are involved in cotton import and export. The National Cotton Council of America reports cotton's economic impact is approximately $500 billion per year worldwide with China being the largest producer and consumer of raw cotton, followed by United States and others. Over 95% of the global cotton production is *Gossypium hirsutum* L., and about 2% is from *Gossypium barbadense* L. and both are tetraploid species with AD-genome (Brubaker and Wendel, 1994; Bi et al., 1998, 1999). In Tanzania, cotton is the second most important cash crop after coffee contributing about 15% to the country's foreign earnings per annum. It provides employment to about 40% of the approximately 38 million Tanzanian population where most of cotton production activities are done by women as men generally tend to migrate to urban in search for jobs (Baffes, 2005; Lukonge et al., 2008).

Cotton production is constrained by insect pests and

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their management imposes significant cost including environmental problems such as toxicity and development of resistance (Andow and Zwahlen, 2006). Of the insect pests, bollworms are of paramount economic importance (Bennett et al., 2005a, b) which have been until recently controlled by the use of pesticides. Alternative pesticides (which are considered destructive to the environment and of risk to human and animal health) include the use of biotechnology in the form of genetic engineering. In cotton, genetic engineering has enabled the development of *G. hirsutum* L. cultivars expressing the delta-endotoxin genes from *Bacillus thuringiensis* (Bt) that kill members of the order Lepidoptera. Bt is also available as a synthetic spray and is considered relatively safer than other pesticides as it persists moderately in the environment largely degrading on exposure (Whalon and McGaughey, 1998). The spraying of synthetic Bt requires appropriate timing and planning to be effective and minimise resistance build up which is generally caused by low dosage on the inappropriate pest growth phase. The genetically modified alternative Bt cotton is effective on growth phases of the pest that infest the crop, reducing the use of pesticides and thus presenting a sustainable Integrated Pest Management (IPM) system for cotton production (Berberich et al., 1996; Bertz et al., 2000).

However, production of Bt cotton poses biosafety concerns including potential impacts on non-target species and resistance evolution and gene flow (Andow and Zwahlen, 2006). These concerns vary with eco-geographic conditions and cannot be extrapolated to other areas thus it is generally recommended that site specific assessments are conducted prior to introduction of a genetically modified crop.

In Tanzania, a field survey conducted in the Southern Highlands revealed the presence of feral *G. barbadense* (a tetraploid with AD-genome) and data mining from two herbaria and cotton research stations recorded two other diploid wild cottons namely the *Gossypium longicalyx* (F-genome) and *Gossypioides kirkii* (Shilla et al, in press). The diploid species are not known to cross with cultivated cotton. Feral *G. barbadense* is grown as an ornamental in the Southern Highlands (SH) of Tanzania where cotton production has been quarantined since 1968 due to the infestation of the Red bollworm and acts as a natural host to the bollworm.

Ram et al. (2008) showed that hybridization within the genus G*ossypium* is rare as the genus is generally self-pollinating. However, it was observed that hybridization between compatible species is generally facilitated via insect mediated pollination. According to Brubaker et al. (1999), feral *G. barbadense* and *G. hirsutum* (both of the AD-genome) potentially hybridise and this implies a potential risk of unregulated transgene escape from genetically modified Bt cotton to feral cotton if produced within the same area. Consequences of unregulated gene flow may result in resistance build-up and/or contamination of valued germplasm. The United States though the second largest producer of Bt cotton, restricts its production in areas where feral and wild relatives *G. tormentosum* grow such as Hawaii and Southern Florida (Showalter et al., 2009). Likewise in Australia, commercial production of Bollgard II cotton (containing 2 *Bt* genes) and Bollgard II/Roundup Ready cotton has been restricted to south of latitude 22° to prevent gene flow to native cotton in northern Australia (Mayer and Stirling, 2004). These restrictions set precedence on the need for gene flow studies to assess not just the potential for transgene escape in a specified eco-geographic area but to establish the consequences of the escape. Armstrong et al. (2005) recommend the use of evidence on interspecific reproductive compatibility derived from experimental crosses as an important component of risk assessment, particularly where data from field observations are unavailable. Risk assessment is a science based tool employed to support decision-making on the biosafety of transgenic crops and their products deployed at any particular point.

The present study assessed potential hybridisation between cultivated non-transgenic *G. hirsutum* and feral *G. barbadense* under controlled conditions so as to establish the potential for gene flow. The data derived intends to postulate potential for bollworm resistance build up against the Bt toxin, should Bt cotton be deployed as a means of bollworm control in Tanzania.

MATERIALS AND METHODS

The experiment was set up at ARI-Ilonga during the 2006 to 2007 seasons. Seeds from two morphologically distinct feral *G. barbadense* populations collected in Southern Highlands; from Hanga-A village in Ruvuma region and Magamba village in Chunya district, in Mbeya region were used in the experiments. The one group of feral seeds, Gb_1, gave plants with very sparse fuzzy but fused seeds while the group of seeds, Gb_2 gave plants with fused seeds with a greenish fuzz.

Prior to the crossing experiments, Gb_1 and Gb_2 seeds were germinated to establish synchrony with cultivars as the period between germination to flowering was not known. It was observed that feral cotton takes longer to mature from germination, thus Gb_1 and Gb_2 seeds were sown prior (approximately 20 days) to the cultivars so as to synchronize flowering time (Table 1). Two *G. hirsutum* cultivars namely Ilonga-85 (IL 85) and Mkombozi (MK) both developed at Ilonga Agricultural Research Institute (ARI-Ilonga) were used in the experiment.

The experiment was divided into eight blocks (A-H). Each of the blocks A, B, C and D was planted at a spacing of 50 cm × 90 cm with feral Gb_1, cultivar MK, cultivar IL85 and feral Gb_2, respectively. Block E and F were planted with the cultivars IL85 and MK respectively as controls. Block, G was maintained to test the seed viability of the F1 generation and block H was for replanting. The planting densities ranged from 42 to 64 based on available seed (Table 1). The experiment was set off season thus it depended solely on irrigation. To test the F1 generation seed viability, sixteen plants were raised from seeds for every cross pattern.

Crossing techniques

The crosses were done in a bidirectional manner; cultivar and feral

Table 1. Plant density, sowing date and number of days to first boll opening for feral and cultivated cotton cultivars.

Trial	Seed source	Total plant	Total rows/ block	Sowing date	Days to germination	Days to flowering	Days to 1st boll open
Block A	Gb_1	64	4	02.05.2006	7	97	155
Block B	MK	64	4	23.05.2006	5	126	124
Block C	IL85	64	4	15.072006	4	72	120
Block D	*Gb*$_2$	42	4	18.07.2006	5	98	152
Block E	IL85	64	4	04.09.2006	5	67	100
Block F	MK	64	4	17.11.2006	3	52	126
Block G	F1-seed viable test	64	4	02.02.2007	6	130	185
Block H	Seed gap replanting			18.02.2007	7	91	165

plants were crossed reciprocally to each other. Both intraspecific and interspecific crosses were performed simultaneously by the same individuals for all the crosses. Crossing techniques were according to Brubaker et al. (1999), Ahuja et al. (2007) and Konan et al. (2007). Emasculation was done a day before pollination between 16:00 and 18:00 hours and before the anthers dehisced. The gynoecia were then covered with drinking straw sealed at the top. Flowers to be used as pollen donors were tied at the top with a thread a day prior to the crosses. On crossing, day anthers were sun-dried for ten to twenty minutes and hand pollination done between 09:00 and 11:00 hours and appropriately labelled to indicate crosses making sure that the pollen grains stuck to the emasculated flowers. The pollinated gynoecia were covered with a drinking straw, which helped to keep the stigma moist and avoid unintended pollination. Generally a single pollen donor was used to pollinate three to four female flowers of the same plant. Total number of flowers pollinated per plant, the number of male plants used per flower and female plant, and total number of male plants per female accession were variable for every cross. This is because feral cotton had long and uneven flowering pattern hence it was difficult to maintain the same number of crossing for every plant. Between about 50 and 130 days after crossing, depending on the *Gossypium* species, successful crosses were recorded as those plants where fruits and seeds developed.

Concomitant to the collection of crossing data, four plants from each parental group were randomly selected and the morphological data scored which were used to differentiate individual plants. The crossing data comprised of successful crosses; these are pollinated flowers which developed into fruits and unsuccessful crosses were the reverse and the latter were expressed into percentage (Table 2). Morphological descriptors were scored as shown in Table 3 and later converted into binary form prior to analysis in R- program.

Data analysis

Principal component analysis (PCA) using the R-2.9.2-software (www.r-project.org) programme was done to assess the variations among parents and their hybrids based on morphological data. To test if the different crossings patterns were equally successful (as percentage developed bolls/flowers pollinated), a pair-wise Chi-square (χ^2) test was used to determine the significance of the crossing results on the Graph Pad InStat Version 3.0.

RESULTS

The results from the crossing data ranged from highly successful to almost unsuccessful, with uneven variation in crossing results. For instance Gb_1 produced successful crosses with both MK and IL85 when used as both a pollen donor and recipient. Gb_2 produced less successful crosses with both MK and IL85 when used as a pollen donor and as recipient (Figure 1 and Table 2). The crossing success (presence of fruit and seed) indicates that the two *Gossypium* species are sexually compatible. Contrary to the interspecific crosses, the intraspecific crosses for both feral *G. barbadense* (2.3 and 0.0%) and the *G. hirsutum* (11.40 and 9.0%) cultivars showed low crossing percentage (Figure 1 and Table 2) despite cotton being a self-pollinating crop. When the pair-wise $\chi 2$ test was performed on the percentage crosses (either fruit or seed or both) the test for independence was 234.59 and $\chi 2$ test for trend was 157.4 both registering as significant ($P < 0.0001$).

Furthermore, it was observed that the higher the crossing percentage the higher the maximum, minimum and mean number of seeds per boll. The variations of mean number of seeds per boll from different crosses are indicated in Figure 1. MK x Gb_2 and IL85 x Gb_2 crosses despite exhibiting a low crossing success (25 and 34.2% respectively) had a considerably high number of seeds per boll suggesting that the interspecific cultivar crosses produce more seeds.

F_1 seed viability test

F_1 seeds from successful crosses of Gb_2 x MK, IL85 x MK and Gb_2 x IL85 showed low germination rates most of which did not develop into plants with the exception of IL85 x MK. The Gb_2 x Gb_1 seeds did not germinate despite repeated efforts and different germination techniques including scarifying, germination in Petri dishes and direct sowing in the soil. The Gb_1 x Gb_2 cross gave bolls with empty embryos.

Morphological characterization

The principal component analysis (PCA) performed suggests that 88% of the clustering was accounted for by

Table 2. Rate of success (%), seed range and variance among different crosses.

Pollen donor (♂)	Seed plant (♀)	Crossed flowers	Successful crosses	Crossed plants	Mean pollen donors/maternal	Number of seeds per cross (Max. – Min)	Variance	Mean(Max., Min.) seeds per boll	Percentage successful crosses
	MK	73	56	36	12	7	7.6	24(17, 24)	76.70
*Gb*1	IL85	72	58	48	16	13	30.9	30(17, 30)	80.60
	*Gb*2	216	5	53	18	9	75.6	10(8, 17)	2.30
	IL85	193	66	55	18	4	2.9	29(21, 25)	34.20
*Gb*2	MK	196	49	51	17	5	6.3	23(28, 33)	25.00
	*Gb*1	60	0	13	6	0	0	0	0.00
	*Gb*1	47	33	26	9	8	11.0	24(17, 25)	70.20
MK	*Gb*2	54	3	23	8	4	4.9	9(7, 11)	5.60
	IL85	141	16	36	12	10	25.5	18(32, 42)	11.40
	*Gb*1	40	27	34	11	6	7.6	18(18,24)	67.50
IL85	*Gb*2	49	2	21	7	0	0	0	4.10
	MK	156	14	40	16	11	25.7	13(17, 28)	9.00

Table 3. Morphological characterisation of feral G.barbadense, G. hirsutum and their hybrids.

Parameter	IL	MK	*Gb*1	*Gb*2	*Gb1*	IL85♂	IL85	MK♂	IL85	*Gb1*♂	IL85	*Gb2*♂
Petal colour	Light yellow	Cream	Deep yellow	Light yellow	Light yellow	Cream	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow
*Petal spot	Absent	Absent	Present	Present	Present	Absent	Absent	Present	Present	Present	Present	Present
Pollen colour	Cream	Pale yellow	Pale yellow	Deep yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Deep yellow	Deep yellow	Deep yellow
Boll shape	Oval	Oval	Conical	Conical	Conical	Oval	Oval	Conical	Conical	Conical	Conical	Conical
Boll colour	Light green	Light green	Light green	Deep green	Deep green	Light green	Light green	Light green	Light green	Light green	Light green	Light green
Boll surface	Smooth	Smooth	Pitted	Pitted	Pitted	Smooth	Smooth	Pitted	Pitted	Pitted	Pitted	Pitted
Seed fuzz	Fuzzy	Fuzzy	Sparse	Fuzzy	Naked	Fuzzy	Fuzzy	Fuzzy	Fuzzy	Fuzzy	Fuzzy	Fuzzy

Table 3. Contd.

Fuzz colour	Grey	Grey	Greyish	Green	Naked	Brown	Green	Green	Brown	Brown	Green	Green
Seed nature	Single	Single	Fused	Fused	Single	Single	Single	Single	Single	Single	Single	Single
Leaf hair	Long	Long	Glabrous	Glabrous	Short	Short	Short	Short	Short	Short	Short	Short
Leaf colour	Light green	Light green	Deep green	Deep green	Deep green	Light green	Light green	Deep green	Deep green	Deep green	Deep green	Deep green

the morphological markers employed. The most important morphological markers included the purple petal spots, yellow petal, conical and pitted boll, kidney (fused) seeds, broad dark green and 3 to 7 lobed leaves and yellow pollen. The PCA revealed that intraspecific hybrids clustered close to the cultivars suggesting that they are more similar to *G. hirsutum* than *G. barbadense*. Furthermore a clear separation was observed between the two parents groups and the interspecific hybrids (Figure 2).

DISCUSSION

In Tanzania, cotton is grown by small holder farmers on less than 2 ha with different cultivars on the same plot. A study by Shilla et al. (2007) to assess distribution and diversity of feral *G. barbadense* in the Southern Highlands showed that there was a clear variation within feral accessions based on morphology characters suggesting *in vivo* interspecific crossing and/ or environmental adaptation.

Crossing compatibility

The study findings show that feral *G. barbadense* in Tanzania is sexually compatible to cultivated *G. hirsutum* as evidenced by the high interspecific crossing as compared to intraspecific crosses that produced viable F1 hybrids. Our findings are in accordance with results from Brubaker et al. (1999) and He et al. (2008) whose studies showed that cross-pollination within *G. hirsutum* and between *G. hirsutum* and feral populations of cotton species is possible and that fertile progeny are produced when *G. hirsutum* is pollinated with *G. barbadense*. The results imply that *Gossypium* species with the same genome may hybridize with little crossing barriers as seen within and between tetraploid cotton species of the AD genome. Brubaker et al. (1999) suggests that where hybridization is rare or does not occur then there may be strong pre- and post-zygotic barriers and/ or sterile offspring for chance crossings. The value of the crossings where successful enables transfer of desired traits from one species to another. For example Zhang et al. (2005) showed that Acala cotton germplasm (cultivated in the USA) is actually an interspecific hybrid of *G. hirsutum* and *G. barbadense.* Acala cotton exhibits tolerance to *Verticillium* wilt a character derived from *G. barbadense.*

Feral *G. barbadense* accessions performed better as pollen donors, as Gb_1 gave higher crossing successes compared to *G. hirsutum*. These observations complement findings by Brubaker et al. (1999) who showed *G. barbadense* and *G. hirsutum* hybridisation to occur in both directions, but hybridization of *G. barbadense* to *G. hirsutum* is more common.

The low success of intraspecific crosses recorded in this study could be explained using the arguments of Ram et al. (2008), who postulated pre-zygotic barriers at both stigmatic and stylar level to be responsible for the failure of seed set in many crosses of cotton.

F1 seed viability

The non-viable seeds from intraspecific feral accessions and less viable seeds from crosses of Gb_2 x MK, and Gb_2 x IL85 that germinated but did not develop into full plants can be explained by poor pollen viability though this was not tested. Brubaker et al. (1999) argue that the receptivity of the plant to the pollen and its germination through the pollen tube and pre-zygotic and post-zygotic barriers are possible causes for the low germination success.

Morphological discrimination

Morphologically hybrids resulting from feral *G. barbadense L.* and *G. hirsutum* L. cultivars exhibited characteristics from both parents. The selected morphological markers proved effective

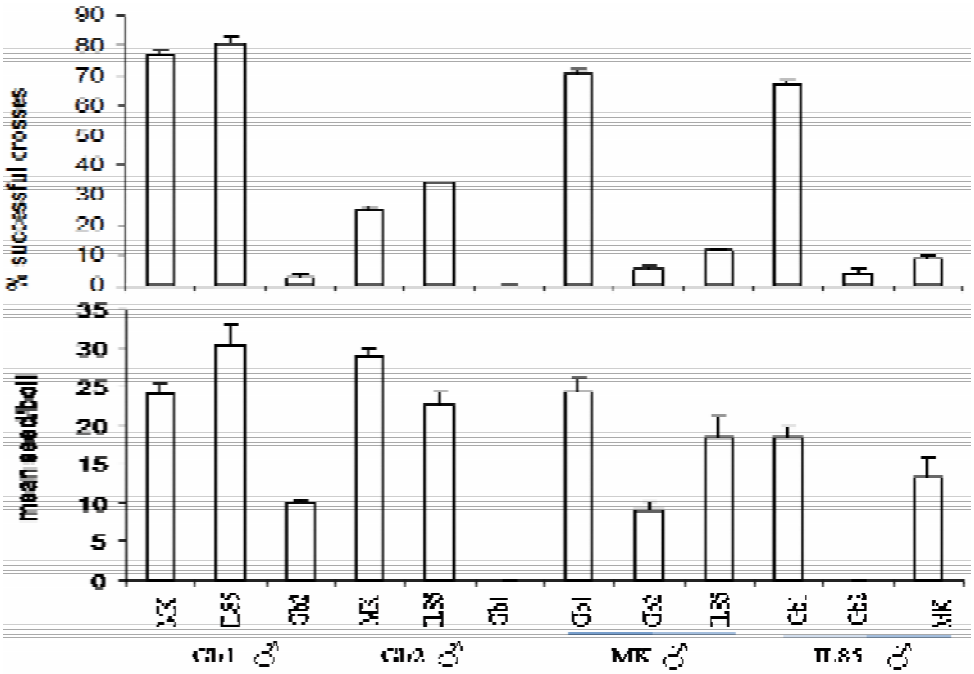


Figure 1. Percentage successful crosses and means number of seeds per boll from different crosses.

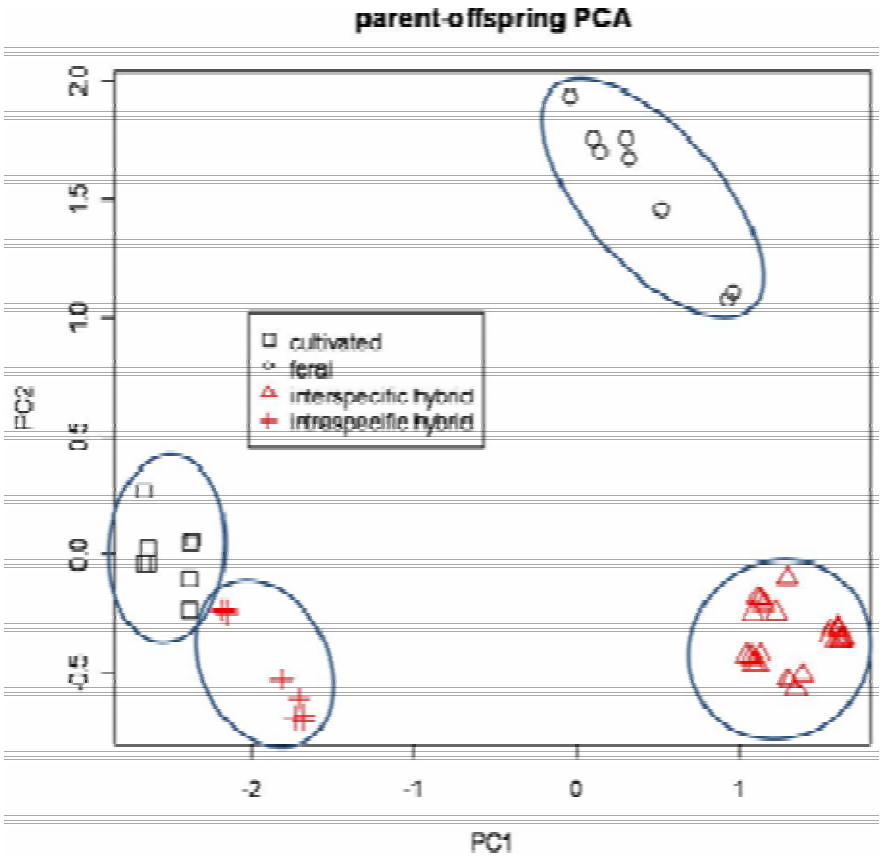


Figure 2. Discrimination of cultivars, feral *G. barbadense* and their F1 hybrids by PCA ordination based on morphological markers.

In discriminating the F1 offspring from the parents as demonstrated by the PCA ordination.

Barriers for *in vivo* hybridisation

The observed hybridisation between *G. hirsutum* L. and feral *G. barbadense* L. suggests that should Bt cotton be produced in areas where *G. barbadense* L. grows in Tanzania, *G. barbadense* L. may be exposed to transgenes. However, the likelihood of gene flow between these species is inferred from a controlled experiment and *in vivo* conditions may be different. Controlled experiments do not necessarily depict what might occur in nature (Armstrong et al., 2005). Stewart et al. (2003) and Baack and Rieseberg (2007) argue that, whereas hybridization indicates gene flow, this does not necessarily lead to introgression whereby there is successful integration of the novel gene into chromosomes of the recipient species. Introgression of transgenes is considered contamination of wild germplasm and an undesired consequence particularly where the effects of contamination are not established. Backcross studies between the successful F1 and their parents would facilitate an understanding on the fitness of hybrids generating information on the potential fitness of transgenic hybrids where the *G. hirsutum* L. cultivar would be Bt cotton. Where hybrids have fitness advantage over their parents, then they can persist to future generations. For Bt cotton this implies a possibility of the recipient plants to express the Bt toxin at unknown levels. As feral cotton is rarely destroyed outside of the production season these plants act as hosts for the Lepidopterans. Should the transgene have escaped into the feral plants and is being expressed at levels lower than needed to destroy the pests, the pests could develop resistance to the toxin, defeating the purpose of the technology. Furthermore, a parallel study in 2006 (unpublished) which assessed the feral *G. barbadense* L. diversity and distribution in SH observed that feral cotton is very sparsely distributed and did not occur in populations but rather scattered in small areas. Thus, exposure of *G. barbadense* to plantations of cultivated cotton would be very low and if transgenes did escape into the feral cotton the impacts would be limited to a few plants. Another natural barrier for *in vivo* hybridisation between the two species is the differences in germination to flowering times; feral cotton had long flowering period compared to the cultivars. Hence sowing of the cultivars if done outside the growing period of feral cotton will ensure the two species do not hybridise.

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