2005 Beltwide Cotton Conferences, New Orleans, Louisiana - January 4 - 7, 2005

IMPROVEMENT OF COTTON VIA GENETIC MANIPULATION OF THE CHLOROPLAST GENOME

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Abstract

In 2004, the U.S. farmers planted about 76% GM cotton, developed through nuclear genetic transformation, but the global acreage of GM cotton is only 11%. In the U.S., GM cotton is planted only in seven states and is not planted in several southern states and Hawaii because of the presence of out-crossing relatives. Also low level of Bt expression raises concerns about development of resistance against this biopesticide. To resolve these issues, we have established an alternative approach of the genetic modification of cotton via the chloroplast genetic engineering. Chloroplast transformation technology has the potential to solve most of the problems associated with nuclear genetic engineering such as transgene containment, low levels of expression, position effect, gene silencing, presence of non-essential foreign DNA, etc. The successful transformation of the cotton chloroplast genome with important agronomic traits should address the concern about transgene escape, insects developing resistance, inadequate insect control and promote the public acceptance of an environmentally friendly approach for genetic modification of cotton.

Introduction

Cotton (*Gossypium hirsutum* L.) is a most important fiber crop of the world. The US accounts for 21% of the total world fiber production and is the leading exporter in the global trade of raw cotton. The annual value of cotton production at the farm level is about $6.1 billion and annual business revenue stimulated by cotton in the U.S. economy exceeds $120 billion, making cotton America's number one value-added crop. Nuclear GM cotton has the potential to hybridize with Hawaiian cotton, *G. tomentosum*, and feral populations of *G. hirsutum* in the Florida Keys, and of *G. hirsutum* / *G. barbadense* in the U.S. Virgin Islands and Puerto Rico. Therefore, commercial planting of Bt-cotton has been restricted in these areas. Similarly, GM cotton is now planted only in the regions of the world where there are no wild relatives in order to avoid potential outcross with related species. Dispersal of pollen from transgenic cotton plants to surrounding non-transgenic plants has been reported in the Australia (Llewellyn and Fitt, 1996) and USA (Umbeck et al., 1991). Transgene escape could be avoided via chloroplast genetic engineering because of maternal inheritance of transgenes in cotton.

Low expression of Bt toxins in GM cotton (produced via the nuclear genome modification) might result in an increased risk of pests developing Bt resistance (Daniell, 2000). There are also reports that Bt-cotton failed to control *Heliothus armigera* in Australia and cotton bollworm on at least 20,000 acres in Texas and also herbicide resistant transgenic lines suffered a similar failure (Hilder and Boulter, 1999). Moreover, Bt cotton is not fully protected from the insect-pest attack and it needs repeated pesticide applications on crop fields to minimize the yield loss (epa.gov/scipoly/sap/2000/october/brad7_cotton_final.pdf). Among the potential environmental concerns, transgene containment, insects developing resistance and impact on non-target insects are of paramount importance. These studies suggest the need for higher levels of *cry* gene expression. Previously, *cry2Aa2* gene engineered via the chloroplast genome has been shown to kill insects that developed resistance (up to 40,000-fold) to insecticidal proteins (Kota et al., 1999). In another study expression of the *Cry*IIa5 in transgenic chloroplasts provided a complete protection against the larvae of *Helicoverpa armigera* irrespective of its development stage (Reddy et al., 2002). DeCosa et al. (2001) expressed very high levels of CRY protein accumulation (up to 46.1 % total leaf protein) that killed cotton bollworm and beet armyworm, which are highly tolerant or resistant to Bt toxins.

Chloroplasts genetic engineering approach offers a number of attractive advantages over nuclear genetic engineering, such as a high-level of transgene expression (Daniell et al., 2004a-c; Viitanen et al., 2004; Dhingra et al., 2004; Chebolu and Daniell, 2004; Watson et al., 2004), multi-gene engineering in a single transformation event (DeCosa et al., 2001; Ruiz et al., 2003), and transgene containment via maternal inheritance (Daniell et al., 1998; Daniell, 2002; Daniell et al., 2004a). Other major advantages with chloroplast transformation include the lack of gene silencing (Lee *et al.,* 2003; DeCosa *et al.,* 2001), position effect due to site specific transgene integration (Daniell et al., 2004) and lack of pleiotropic effects due to sub-cellular compartmentalization of transgene products (Daniell et al., 2001; Lee *et al.,* 2003). However, chloroplast transformation (Table 1) has been so far quite efficient

mainly in tobacco (Daniell et al., 2001; Fernanadez San Millan et al., 2003). Here we present the first report of stable and reproducible chloroplast transformation of cotton using the selectable markers (*aph*A-6 and *npt*II) capable of expression in different plastid types in the light or dark.

Table 1. Agronomic traits engineered via the chloroplast genome in plants.

Trait	**Transgene**	**Promoter 5'/3' UTR's**	**Transgene Integration site**	**Laboratory/ References**
Insect resistance	*Cry*1A (c)	Prrn *rbc*L/T*rps*16	*trn*V/*rps*12/7	McBride / McBride *et al.* (1995)
Herbicide resistance	*Aro*A	Prrn ggagg/T*psb*A	*rbc*L/*acc*D	Daniell/ Daniell *et al.* (1998)
Insect resistance	*Cry*2A*a*2	Prrn ggagg (native)/T*psb*A	*rbc*L/*acc*D	Daniell / Kota *et al.* (1999)
Herbicide resistance	*bar*	Prrn *rbc*L/psbA	*rbc*L/*acc*D	Day/ Iamtham & Day (2000)
Insect resistance	*Cry*2A*a*2 operon	Prrn native 5' UTR's/T*psb*A	*trn*I/*trn*A	Daniell / DeCosa *et al.* (2001)
Disease resistance	*MSI*-99	Prrn ggagg/T*psb*A	*trn*I/*trn*A	Daniell/ DeGray *et al.* (2001)
Drought tolerance	*tps*	Prrn ggagg/T*psb*A	*trn*I/*trn*A	Daniell / Lee *et al.* (2003)
Phytoremediation	*mer*A[a]/*mer*B[b]	Prrn ggagg[a,b]/T*psb*A	*trn*I/*trn*A	Daniell / Ruiz *et al.* (2003)
Salt tolerance	*badh*	Prrn-F ggagg/*rps*16	*16S-trn*I/*trn*A-23S	Daniell / Kumar *et al.* (2004a)

a and *b* refer to genes and their respective regulatory sequences

Materials and Methods

Biolistics is the most widely used and effective method for transforming chloroplasts (Daniell, 1997). Gold particles coated with chloroplast-specific vector DNA (Figure 1A) were bombarded on cotton calli powered by a helium gene-gun (Figure 1B) to facilitate integration of the transgenes into the specified region (*16S*/*trnI – trnA*/*23S*) of the chloroplast genome into the non-green cotton cell cultures (Figure 1C). Transgenic cell cultures were selected and multiplied on MST1 medium supplemented with 50-100 mg/l kanamycin and converted into somatic embryos and plantlets on MST0 medium (MS salts, B5 vitamins) containing potassium nitrate (1.9 g/l) (Figure 1D). PCR-confirmed chloroplast transgenic plantlets (Figure 1E) were transferred to a growth chamber for flowering and seed setting (Figure 2) and Southern blot analysis as described (Daniell et al., 2004b, Kumar and Daniell 2004, Kumar et al., 2004a,b). For analysis of transgene inheritance in cotton, emasculated non-transgenic cotton plants were pollinated with pollen derived from chloroplast transgenic cotton plants. Seeds of T1 transgenic lines derived from crosses+ non -transgenic x > transgenic as well as selfed transgenic lines were germinated on ½ MS basal medium supplemented with 50-mg/l kanamycin (Figure 3).

Results and Discussion

Several transgenic cell lines were recovered when non-green cell cultures were bombarded with double barrel vector pDD-*Gh-aphA6/nptII* (Figure 1A) and using a single gene single selection plastid vector pKD-*Gh-aph*A-6. Integration of *aph*A*6-nptII* and *aph*A*6* genes into the cotton chloroplast genome was confirmed by PCR using different sets of primers. Transgenic cotton plants confirmed with Southern blots were grown in the growth chamber along with non-transgenic plants, under similar growth conditions. Growth of chloroplast transgenic lines, onset of flowering, floral parts, boll formation and seed setting were similar to the untransformed cotton plants (Figure 2). Emasculated flowers of non-transgenic cotton were pollinated with pollen derived from chloroplast transgenic lines. Seedlings from F1 crosses (non-transgenic+ x > transgenic chloroplast) failed to germinate on the kanamycin selection medium, whereas selfed transgenic seeds germinated well, produced normal roots and shoots,

thereby confirming resistance to kanamycin (Figure 3). This demonstrates that there is no paternal inheritance in cotton and that the chloroplast transgenic trait is inherited maternally.

This study reports the first and reproducible process for generating cotton plastid transgenic lines from transformed calli via somatic embryogenesis. Successful plastid transformation with high frequency is demonstrated in a recalcitrant crop using a species-specific chloroplast vector. The higher transformation efficiency observed in cotton might be due to the use of long homologous flanking sequences that contain one of the chloroplast origins of replication; one possibility is that this could offer large number of templates within plastids for integration (Guda et al., 2000). This report of chloroplast transformation has opened new opportunities for genetic engineering of cotton for higher expression of transgenes and overcome potential ecological and environmental concerns.

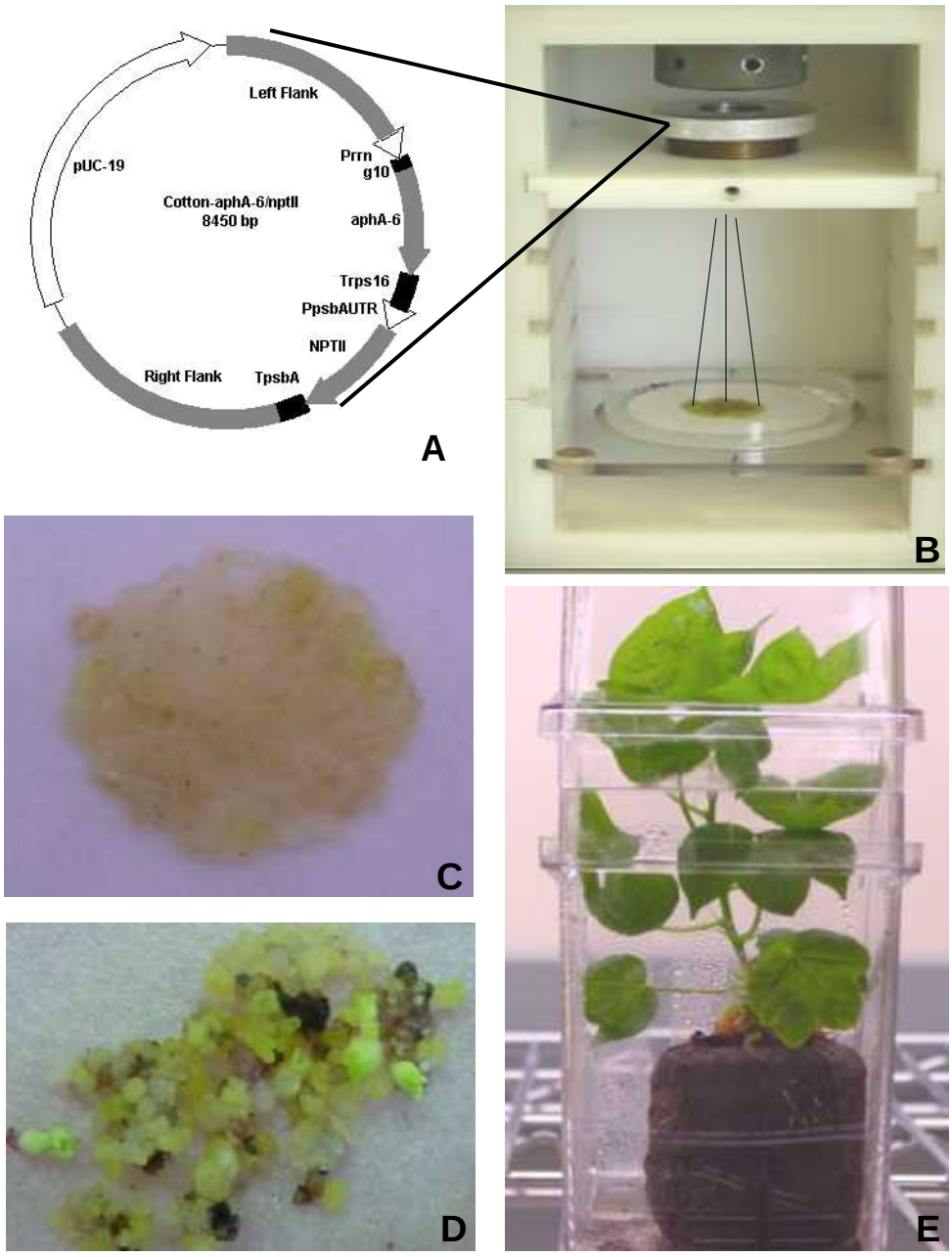

Figure 1 (A-E). Genetic transformation of cotton chloroplasts. **(A)** Species-specific cotton chloroplast vector. **(B)** Bombardment of cotton cell cultures with chloroplast vector coated on gold-particles via a helium gene-gun. **(C)** Non-green bombarded cell cultures of cotton. **(D)** Selection of transformed embryogenic cotton cultures on kanamycin medium. **(E)** Transgenic cotton plant growing under *in-vitro* conditions

Figure 2. Mature transgenic and non-transgenic cotton plants with their reproductive parts

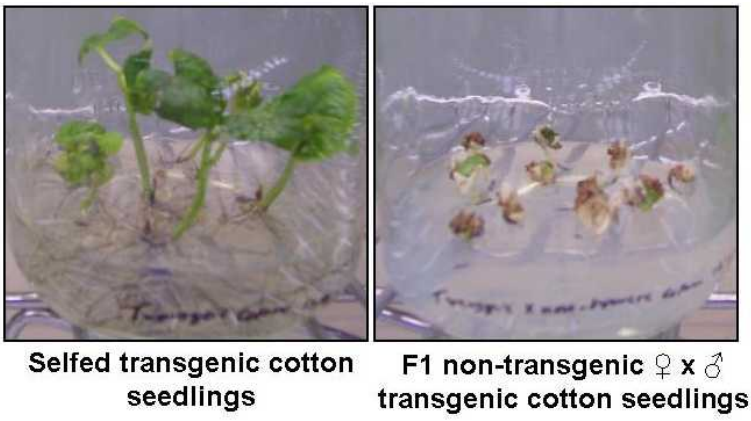

Figure 3. Growth of selfed transgenic and F1 non-transgenic+ x> transgenic seedlings on the antibiotic selection medium

Acknowledgments

Investigations reported in this article were supported in part by funding from NIH R 01 GM63879 and USDA 3611-21000-017-00D grants to HD.

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