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Enhanced Tissue-Specific Expression of the Herbicide Resistance *bar* Gene in Transgenic Cotton (*Gossypium hirsutum* L cv. Coker 310FR) Using the Arabidopsis *rbcS atsl*A Promoter

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Abstract

A highly regenerating cotton (*Gossypium hirsutum* L.) cultivar, Coker 310FR, was used to generate transgenic plants expressing the herbicide resistance gene, *bar*, encoding phosphinothricin acetyltransferase (PAT), under the transcriptional control of the ribulose-1, 5-bisphosphate carboxylase (Rubisco) small subunit (rbcS) *atslA* gene promoter from *Arabidopsis thaliana*. Expression levels of the rbcS *atsl*A-*bar* transgenes were compared to *bar* transgenes under the control of the high level constitutive promoter from the Cauliflower Mosaic Virus *35S* gene containing a dual enhancer region (*2xE CaMV 35S*). Significantly higher levels of *bar* mRNA, PAT protein and enzymatic activity, and enhanced levels of resistance to the herbicide Basta were observed in transgenic plants expressing *bar* under the *rbcS atslA* promoter compared to the *2xE CaMV 35S* promoter. Transgenic plants containing *2xE CaMV 35S-bar* transgenes tolerated the maximum herbicide (Basta) application up to 200 mg l^{-1} PPT whereas rbcS *atslA-bar* transgenic plants were capable of detoxifying Basta up to 400 mg l^{-1} PPT. These findings indicate that the *rbcS atslA* promoter may be useful for higher expression of transgenes in developing tissues of cotton for improving it further through genetic engineering.

Key words: Bialaphos, Cauliflower Mosaic Virus *35S* promoter, GM crops, phosphinothricin resistance, Rubisco, Transgenic cotton.

Abbreviations

BSA, bovine serum albumin; DTT, dithiothreitol; GUS, β-glucuronidase; MS, Murashige and Skoog; neomycin phosphotransferase II, NPTII; PMSF, phenylmethyl sulphonyl fluoride; Rubisco, ribulose-1, 5-bisphosphate carboxylase

Introduction

Cotton (*Gossypium hirsutum* L.) belongs to the Malvaceae family and is one of the world's most important commercial crops, with over 180 million people depending on it for their livelihood (Benedic and Altman, 2001). Cotton is grown in over 90 countries, with an estimated 32.6 million hectares planted annually (FAO, 1993). Cotton production worldwide is limited by a variety of biotic and abiotic factors, among which insect infestation and associated damage and disease are particularly devastating. The greatest impact is felt in developing nations where use of pesticides is limited by availability and cost.

Among the major pest are a large number of difficult-to-control insects that include *Pectinophora gossypiella* (pink bollworm), *Earias vittella* (spotted bollworm), *Tetranychus* spp. (spider mites), *Heliothis armigera* (American bollworm), *Spodoptera litura* (beet armyworm) and *Amrasca biguttula* (Jassids). These insects predominately affect developing leaf and floral tissues of the plant, causing substantial yield losses. The control of these insect pests has become a major issue, as they have become resistant to a large number of pesticides that were previously very effective. Approximately 45% of the pesticides produced worldwide are used on cotton (Kidd, 1994). In the world today more than 235 weed biotypes have developed resistance to one or more herbicides and Oerke *et al.* (1999) estimated the global annual pre-harvest losses in 8 major crops including cotton approximately 34.9% of their potential production. Of the 34.9%, 13.8%

was due to insect pests, 11.6% was due to diseases, and 9.5% was due to weeds. Thus, engineering cotton with higher expression of insect-resistant and herbicide-resistant traits would be an economically important approach.

A major limitation for the genetic engineering of cotton is the inefficiency of the existing transformation technology to integrate genes and subsequent plant regeneration due to the lack of a good regeneration system. Several investigators have worked extensively on plant regeneration through somatic embryogenesis; however, the genotype dependent embryogenic response and low frequency of somatic embryos production from the genetically transformed tissues has been major concerns for regenerating transgenic cotton. So far, most of the genetic transformation in cotton has been achieved using one of the "Coker" cultivars (i.e., Coker 100S, 201, 208, 304, 310, 312, 315, 4360 and 5110), which have proven to be the most reliable in terms of *in vitro* regeneration. However, all these cultivars vary in their embryogenic response due to genotype specificity (Trolinder and Chen, 1989; Kumar *et al.,* 1998), which severely impact the efficacy of genetic transformation. Therefore, use of a fully regenerating line would potentially help in rapid improvement of cotton through high frequency of genetic transformation (Kumar *et al.,* 1998; Chaudhary *et al.,* 2003).

Currently, the most widely used promoter for the constitutive high level expression of transgenes in monocot and dicot plants, including cotton, is that derived from the 35S gene promoter of Cauliflower Mosaic Virus (CaMV 35S) (Benfy and Chua, 1990; Holtorf *et al.,* 1995; Mitsuhara *et al.,* 1996). The CaMV 35S promoter has been well characterized (Benfy and Chua, 1990). There has been only limited effort towards the development of transcriptional expression systems optimized for expressing foreign genes in transgenic cotton (Sunilkumar *et al.,* 2002; Emani *et al.,* 2003). Clearly, to develop transgenic cotton with specialized agronomic traits, which express mainly in leaf and developing tissues of cotton different constitutive and tissue-specific promoters will be required (Rinehart *et al.,* 1996). One strategy for improving and regulating the expression of a foreign gene in transgenic plants is the use of promoter sequences that not only provide high levels of expression, but also show precise temporal and spatial regulation in specific plant parts. The Rubisco holoenzyme constitutes up to 50% of the soluble protein in green plant leaves. It consists of a chloroplast-encoded large subunit polypeptide (rbcL) and a nuclear-encoded small subunit polypeptide (rbcS), the expression of which have been extensively studied in many monocotyledons and dicotyledonous species (Dean *et al.,* 1989). It is well documented that the rbcS subunit is encoded by a multigene family in most vascular plant species and that different members of the rbcS gene family in a particular plant species have different levels of light- and tissue-specific expression (Sugita and Gruissem, 1987; Khoudi *et al.,* 1997). The rbcS gene family of *Arabidopsis thaliana* consists of four members, of which the *rbcS atslA* gene appears to be the most highly expressed (Krebbers *et al.* 1988). In fact, previous studies have shown that the rbcS *atslA* promoter is particularly useful for conferring light- and tissue-specific patterns of expression on foreign genes (De Almeida *et al.,* 1989; Arguello-Astorga and Herrera-Estrella, 1998; Martinez-Hernandez *et al.,* 2002).

As part of an ongoing program aimed at developing better transformation, selection, and transgene expression characteristics for cotton, we report here, a highly efficient genetic transformation system for cotton, and present a comparative analysis of the expression of the *bar* resistance gene under the control of the *Arabidopsis rbcS atslA* gene promoter and a promoter derived from the Cauliflower Mosaic Virus 35S gene (*CaMV 35S*) containing a dual enhancer sequence (termed the *2xE CaMV35S* promoter). By comparing *bar* transcript levels, levels of PPT protein and enzyme activity, and levels of Basta herbicide resistance in transgenic cotton plants, this study demonstrates the utility of the *rbcS atslA* promoter for creating agronomically-important transgenic cotton.

Material and Methods

Vector construction

Binary vectors pGSFR780A (**Fig. 1A**) and pGSFR780B (**Fig. 1B**) were used for this study. Binary vector pGSFR780A was kindly provided by Professor Deepak Pental (UDSC, New Delhi), constructed by the Plant Genetic Systems group, Belgium (Deblaere *et al.,* 1987). Plasmid pGSFR780B was constructed by amplifying promoter ats1A from plasmid pGS1401 (De Almeida, *et al.,* 1989) using primers Syn-Far-ats1A (5´GAATTCAGGCCTAAATTTATTATG3´) and Syn-rev-ats1A (5´GGATCCATCTTTGGAGTGGTCGGAG-3´) to generate a PCR product of about 1.6 kb (PSSU *Arabidopsis atslA* promoter; accession number X14565). The 1.6 kb fragment digested with *Stu*I and *Bam*HI was inserted at the place of *2xE CaMV 35S* promoter by digesting a plasmid pGSFR780A with *Stu*I and *Bam*HI to yield an exact fusion between the promoter and the initiation

codon of bar. DNA sequences at initiation and stop codons were confirmed using ABI 310 DNA sequencer (Applied Biosystems, USA).

Agrobacterium-mediated genetic transformation and selection of transgenic plants

The *bar* and *npt*II gene cassettes flanked by the T-DNA borders of binary vectors (pGSFR780A and pGSFR780B) were transferred into *Agrobacterium tumefaciens* strain (GV3101), following standard molecular methods. Hypocotyl explants (five days old) of *Gossypium hirsutum* cv. Coker 310FR, a fully regenerating line for embryogenesis (Kumar *et al.,* 1998), were co-cultivated with the disarmed *Agrobacterium* strain. Explants were immersed for 2 min. in MST1 liquid medium (MS salts, B5 vitamins; Murashige and Skoog, 1962; Gamborg *et al.,* 1968; pH 5.3) supplemented with 0.1 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} kinetin (Trolinder and Goodin, 1988) at 0.3 OD (conc. of *Agrobacterium* strain). Infected explants were transferred in the dark on solid medium MST1 (Kumar *et al.,* 1998) without antibiotic selection. After two days, explants were washed with MST1 liquid medium containing 500 mg l^{-1} carbenicillin and selected on MST1 solid medium supplemented with 10-20 μg ml^{-1} Basta (phosphinothricin herbicides containing active ingredient glufosinate ammonium) or 50 μg ml^{-1} kanamycin (conferred resistance to *nptII* gene for neomycin phosphotransferase) along with 400-500 μg ml^{-1} carbenicillin (bacteriostatic agent).

For induction of somatic embryos, transgenic calli were transferred to basal MST2 medium (MS salts with B5 vitamins and 1.9 g/l KNO_3) with or without any antibiotic selection. Transgenic plantlets generated from embryogenic cultures on the germination media MSG, MSG1 and MSG2 (Kumar and Pental, 1998), were transferred to the greenhouse for flowering and seed set.

PAT activity in transgenic plants

Phosphinothricin acetyltransferase (PAT) activity was tested in the leaf tissues of transformed plants with the help of thin layer chromatography (TLC). Standard technique for the enzyme assay of PAT was used as described by De Block *et al.* (1987). Crude extracts from transgenic leaf tissues were isolated using protein extraction buffer (50 mM Tris-HCl of 7.5 pH, 2 mM Na-EDTA, 0.15 mg ml^{-1} PMSF, 0.15 mg ml^{-1} Leupeptin, 0.15 mg ml^{-1} BSA, 0.15 mg ml^{-1} DTT). Homogenized samples (100 mg in 100 μl extraction buffer) were centrifuged at 10,000 g for 10 min at 4℃ and total soluble protein was estimated by Bio-Rad Protein Assay (Bio-Rad). For loading the protein samples on TLC plates, a mixture was prepared with 13 μl diluted leaf protein extract (final conc. adjusted to 0.1 mg ml^{-1}), 0.8 μl PPT (phosphinothricin) and 1.3 μl ^{14}C-labeled acetyl-coenzyme A. This reaction mixture was incubated for 30 min at 37℃ and centrifuged for 1 min at 10,000 g. Samples (6 μl/lane) were spotted on TLC silica-gel plate. Ascending chromatography was carried out in a glass tank saturated with buffer containing a mixture of 1-propanol and ammonium hydroxide (25% NH_3 aqueous) in the ratio of 3:2. Once solvent front reached near to the upper side of the TLC plate (in about 3h), the plate was removed, air-dried and visualized by autoradiography and acetylated PPT was quantified from autoradiograms by a scanner (Gel Doc 2000, Bio-Rad) using ^{14}C PPT dilution series as a standard.

Northern analysis of transgenic plants

Total RNA was isolated from leaves of transgenic plants using RNAzol kit (Invitrogen). Total RNA (10 μg) from each sample was loaded on 1.2% agarose gel after 5 min incubation at 75℃. RNA transferred to nitrocellulose membrane was hybridized with DNA probe (bar fragment eluted from plasmid pGSFR780A, digested with BglII and BamHI) labeled with ^{32}P-dCTP following random priming method (Vendor's method, Amersham) and membrane was exposed to X-ray film at -70℃.

Western analysis of transgenic plants

Crude protein from transgenic leaf material (100 mg) was isolated using 100 μl of 2x extraction buffer (0.12 M Tris-HCl of 6.8 pH, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 10% 2-Mercaptoethanol). Samples boiled for 5 min were centrifuged at 10,000g for 5 min and loaded (50 μg protein per slot) on 12% SDS-poly-acrylamide gels, in mini-gel apparatus (Bio-Rad). Gel was run at 75V for 45 min (till samples crossed the 6% stacking gel) then at 150V for one hour. Protein from gel was transferred to a nitrocellulose membrane at 65 V in 3 hours. Using 5% Carnation milk prepared in 1xPBS buffer (8.9 g NaCl, 0.023 g KCl, 0.144 g Na_2HPO_4, 0.144 g KH_2PO_4 in 100 ml volume and pH 7.4) membrane was blocked for 1 hour and exposed to primary antibodies of rabbit polyclonal antiserum against phosphinothricin acetyltransferase (PAT) for another 1 hour. After three washing with 1xPBS for 5 min each, membrane was incubated with secondary antibodies of goat anti-rabbit, as described (De Almedia *et al.,* 1989). Hybridized protein signals were detected on X-ray film using ECL procedure (Amersham Pharmacia Biotech, USA).

Basta resistance in transgenic plants

For testing Basta resistance in greenhouse, untransformed control plants and transformed F1 plants (*ats1*A-bar and 2xE CaMV 35S-*bar*) were sprayed with a commercial herbicide Basta containing 200 g/l (200,000 PPM) glufosinate ammonium as an aqueous concentrate or aqueous solution. All transgenic and non-transgenic control plants were sprayed (until leaves were wet) twice with 200 mg l^{-1} PPT (40 ml/m^2) after two weeks interval. Transgenic plants were further tested for increased Basta resistance by consecutive application of 400 mg l^{-1} PPT (80 ml/m^2) and 600 mg l^{-1} (120 ml/m^2) after two weeks interval.

Results

Plant transformation and regeneration

To study the comparative expression of *bar* under the control of rbcS ats1A and *2xE CaMV 35S* promoters, two constructs were used as shown in Fig. 1. Constructs pGSFR780A and pGSFR780B carrying *bar* gene under different promoters, flanked by the T-DNA border repeats were mobilized in *Agrobacterium* strain GV3101. Hypocotyl explants (4-5 mm size) excised from five-day-old seedlings of cotton cultivar Coker 310FR were transformed with the help of *Agrobacterium*. Transgenic calli (as an independent transformation events from hypocotyl explants) were selected on MST1 medium containing Basta or kanamycin. Using 10-20 μg/ml Basta, only a small amount of transgenic callus was induced from explants selected on MST1 medium, after four months of initial culture. This callus could not proliferate further into friable or embryogenic callus when it was subcultured to fresh medium even at low concentrations of Basta (5 μg/ml). In contrast, transgenic calli selected on MST1 medium supplemented with 50 μg/ml kanamycin was successfully converted into somatic embryos upon transferred to basal MST2 medium (devoid of kanamycin) and somatic embryos were easily converted into plantlets on MSG, MSG1 and MSG2 media (Kumar and Pental, 1998). The concentration of carbenicillin (400-500 μg/ml) used for selecting the transgenic callus was reduced to 250 μg ml^{-1} for the efficient conversion of embryos into plantlets. A total 18 transgenic plantlets were recovered from 120 hypocotyl explants infected with *Agrobacterium* (i.e. 8 plants with pGSFR780A and 10 plants with pGSFR780B construct). Transgenic plantlets were confirmed by PCR using *bar* gene specific internal primers were transferred to soil and F1 seeds were collected from selfed-crossed plants. The seed of transgenic plants germinated on 1/2MSB medium supplemented with 50 mg l^{-1} kanamycin were segregated into Mendelian fashion (3:1). For molecular characterization, transgenic plants (carrying *rbcS ats1A-bar* and *2xE CaMV 35 S-bar* transgenes) showing equal morphological growth in the greenhouse were tested for PAT enzyme activity, RNA and protein analysis.

Comparison of PAT activity in leaves regulated by rbcS ats1A and 2xE CaMV 35S promoter.

Activity of phosphinothricin acetyltransferase (PAT) encoded by the bar gene was compared in the crude leaf extract of transgenic plants. The leaf extract of *ats1A-bar* transgenic plants (I-V) showed higher PAT activities in comparison to *2xE CaMV 35S-bar* transgenic plants (1-5) (**Fig. 2**). The acetylated PPT quantified in the leaf tissues of different transgenic plants demonstrated two-fold

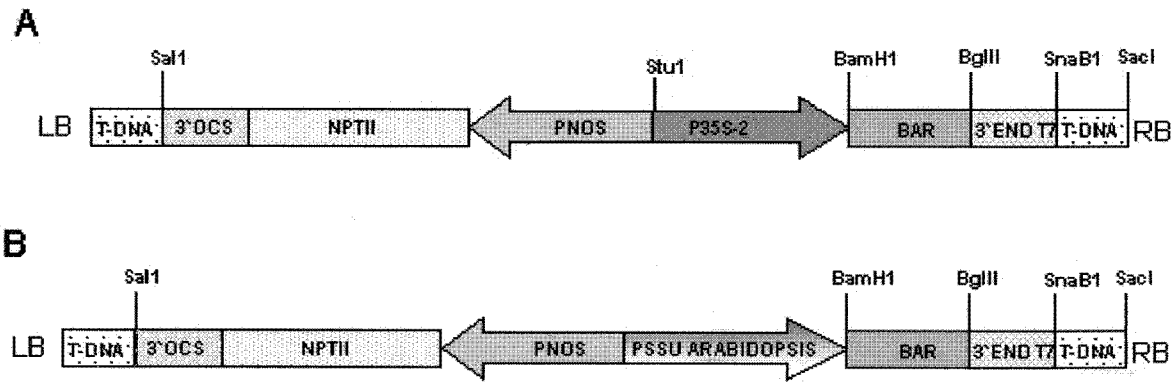

Fig. 1 Physical map of binary vectors pGSFR780A and pGSFR780B, harbored into Agrobacterium strain GV3101 for genetic transformation of *Gossypium hirsutum* cv Coker 310FR. (**A**) Vector pGSFR780A carries the *bar* gene expressed under the regulation of *2xE CaMV 35S* promoter. (**B**) Vector pGSFR780B carries the *bar* gene under the control of the *rbcS ats1A* promoter. Transgenic plants were generated using *nptII* gene as selection marker derived by *nos* promoter in both the constructs.

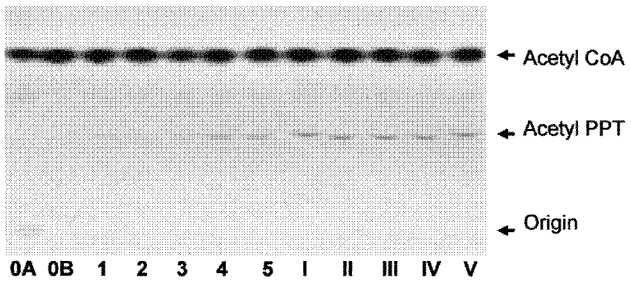

Fig. 2 Detection of phosphinothricin acetyltransferase (PAT) activity in transgenic cotton leaf tissues. Protein was extracted from transgenic cotton leaf tissues, incubated with ^{14}C-acetyl CoA as described in the Materials and Methods, and crude protein extracts were diluted to adjust the final concentration of protein to 0.1 mg ml^{-1}. Aliquots were spotted on TLC silica-gel plate for chromatographic separation reaction. ^{14}C-ascetyl CoA substrate and labeled PPT reaction products were visualized by autoradiography. Lanes **I-V** are from plants expressing the rbcS *ats*1A-bar constructs (vector pGSFR780B); Lanes **1-5** are from plants expressing the *2xE CaMV 35S-bar* constructs (vector pGSFR780A) ; Lanes **0A** (0.2 mg ml^{-1}) and **0B** (0.1 mg ml^{-1}) are leaf extracts from control untransformed cotton plants.

higher PAT activity in the *ats1A-bar* transgenic plants in comparison to *2xE CaMV 35S-bar* transgenic plants. The *ats1A-bar* transgenic plants (I, II, III, IV, V) yield PAT activity ~4.16, 3.76, 3.83, 4.03, 4.02 nmol acetyl-PPT/min mg^{-1} protein respectively when compared to *2xE CaMV 35S-bar* transgenic plants (1, 2, 3, 4, 5), about 1.86, 1.82, 2.01, 2.98, 2.43 nmol acetyl-PPT/min mg^{-1} protein respectively. No PAT activity was detected in the untransformed control leaf extracts (**Fig. 2**).

Expression of rbcS ats1A-bar and 2xE CaMV 35S-bar at the RNA level

Northern analysis was performed (on the same transgenic plants tested for PAT assay) to determine the relative levels of PAT mRNA in the different plants. Total RNA was isolated from the leaves of transgenic plants, separated by electrophoresis on 1.2% agarose gels, transferred to nitrocellulose membranes, and hybridized with a radioactively-labeled probe capable of detecting the *bar* mRNA. As shown in Fig. 3, the levels of PAT-encoding transcripts found in transgenic plants encoding the *rbcS ats1A-bar* constructs (i.e., **Fig. 3**, plants I-V) showed about two-fold higher steady-state levels of PAT-encoding transcripts in comparison to the plants expressing the *2xE CaMV 35S-bar* transgenes (i.e., **Fig. 3**, plants 1-5.) In general, the levels

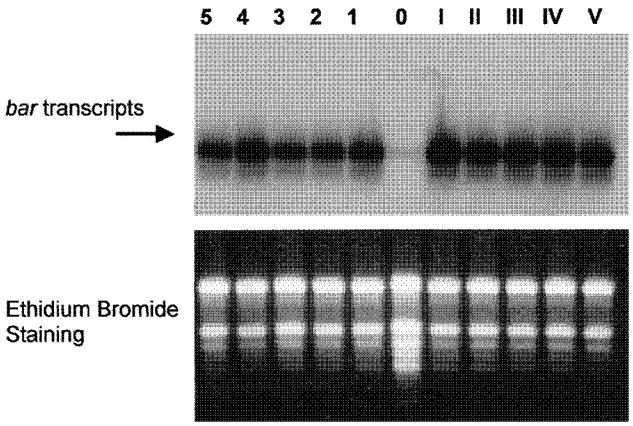

Fig. 3 Northern blot analysis of *bar* gene expression under the control of the *2xE CaMV 35S* and rbcS *ats1A* promoters. Total RNA was isolated from transgenic cotton plants expressing the the *2xE CaMV 35S-bar* transgene (Lanes 1, 2, 3, 4, 5) and rbcS ats1A-bar transgene (lanes I, II, III, IV, V). The RNA was separated by agarose gel electrophoresis, blotted to nitrocellulose and hybridized with a 550 bp fragment of the *bar* gene labeled with ^{32}P-dCTP. The arrow indicates the bar transcript. The lower panel shows an ethidium bromide staining of the agarose gel to demonstrate that equivalent amounts of RNA were loaded in each lane. Lane 0 is total RNA isolated from an untransformed control cotton plant.

of PAT transcript observed in the various transgenic plants paralleled the levels of PAT enzymatic activity observed

Expression of rbcS ats1A-bar and 2xE CaMV 35S-bar at the protein level

Western blot analyses was conducted on transgenic plants with each construct (pGSFR780A and pGSFR780B) to determine if the difference in the expression pattern at the PAT activity and RNA levels also reflected at the protein level. Protein extracted from different transgenic leaf material was loaded on gel in equal amount and blot was hybridized with polyclonal antiserum against phosphinothricin acetyltransferase (PAT). The average amount of the PAT protein was obtained approximately doubled in transgenic plants numbered I-III expressing the *rbcS ats1A-bar* compared to the *2xE CaMV 35S-bar* transgenic plants numbered 1-3 (**Fig. 4**).

Basta resistance of in rbcS ats1A-bar and 2xE CaMV 35S-bar transgenic plants

Five untransformed control plants and 20 transformed cotton plants with each constructs

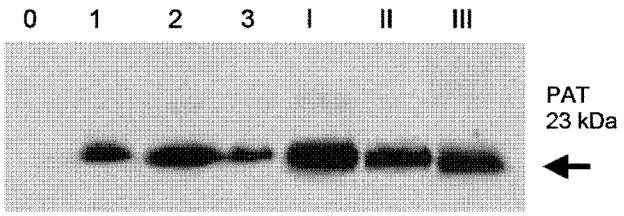

Fig. 4 Western blot analysis on transgenic cotton plants carrying *ats1A-bar* and *2xE CaMV 35S-bar* transgenes. Total protein from transformed and untransformed plants was hybridized with polyclonal antiserum, recognizing the 23 kDa PAT protein and visualized using horseradish peroxidase conjugated secondary antibody. Lanes I, II, III expressed PAT in *rbcS ats1A-bar* transgenic plants. Lane 1, 2, 3 expressed PAT in *2xE CaMV 35S-bar* transgenic plants. Lane **0** showed no hybridization signal in a untransformed control plant.

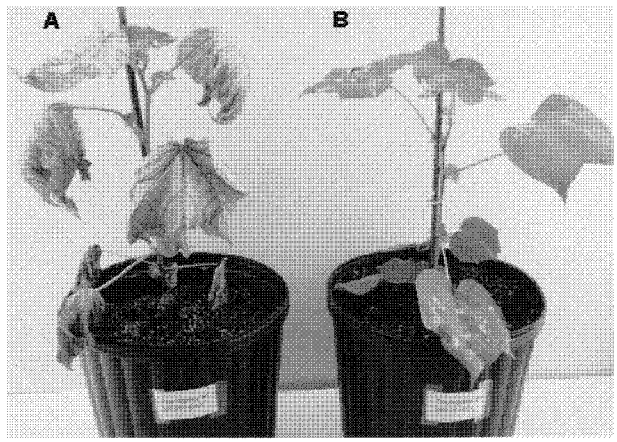

Fig. 5 Resistance response of transgenic cotton plants against Basta. Transgenic cotton plants were sprayed with 400 mg l^{-1} PPT as described in the Materials and Methods. (**A**) Representative transgenic plant expressing *2xE CaMV 35S-bar* transgene (**B**) Representative transgenic plant expressing *rbcS ats1A-bar* transgene.

pGSFR780A and pGSFR780B were tested for phosphinothricin resistance by spraying with 200, 400 and 600 mg l^{-1} PPT, respectively. In the first set of experiments, transgenic plants sprayed twice with a 200 mg l^{-1} PPT solution at two weeks intervals showed no visible effects on plant growth or no significant damage to leaves in either the *rbcS ats1A-bar* or *2xE CaMV 35S-bar* expressing transgenic plants. In another set of experiments, transgenic plants expressing the *2xE CaMV 35S-bar* construct showed complete necrosis of the leaves, 7-days after a single spray of 400 mg l^{-1} PPT. The leaves present on all of the plants senesced, fell off, and all of the plants died within 3-4 weeks of the second spray (**Fig. 5A**). Transgenic plants expressing the *rbcS ats1A-bar* sprayed twice with 400 mg l^{-1} PPT showed some browning and necrotic areas on leaves, but the plants continued to grow and produced new leaves (**Fig. 5B**). However, when the *rbcS ats1A-bar* expressing plants were sprayed with 600 mg l^{-1} PPT to test the maximal level of herbicide tolerance, none of the plant was able to survived. Untransformed control plants showed complete browning and necrosis of leaves and died within two weeks after the first Basta treatment (200 mg l^{-1} PPT).

Discussion

Transgenic plants with *rbcS ats1A-bar* and *2xE CaMV 35S-bar* cassettes were produced via hypocotyl explants with the help of *Agrobacterium* strain GV3101. A high frequency of transgenic plants (15%) was obtained using *G. hirsutum* cv. Coker 310FR, an embryogenic line that was developed to produce 100% in vitro regeneration through somatic embryogenesis (Kumar *et al.*, 1998). Previously, transformation frequency in cotton has been shown very poor using Coker cultivars (Umbeck *et al.*, 1987; Lyon *et al.*, 1993; Finer and McMullen, 1990; Rajasekaran *et al.*, 1996), as regeneration via somatic embryogenesis is highly genotype specific and it vary from seed to seed (Trolinder and Chen, 1989). Albeit callus induced from highly embryogenic cultivars does not produce somatic embryos from the entire surface, only a few sectors induced the embryogenesis. This is the main factor in cotton that dramatically reduced the frequency of recovering transgenic somatic embryos from transgenic callus. However, Coker 310FR, which is purified through six generations of selection, yield 100% embryogenesis from the entire transgenic callus surface to yield a high frequency of transgenic somatic embryos.

Our attempts for recovering transgenic cotton plants using bar gene as a selectable marker were futile as small amount of transgenic compact calli was produced after 4 months from hypocotyl explants of Coker 310FR, selected on MST1 medium containing 10-20 mg l^{-1} Basta. However, this callus could not differentiate further into friable callus or embryogenesis due to the degenerating effect of Basta on cotton cell cultures, even if Basta was decreased to 5 mg l^{-1} Therefore, all the transgenic plants were generated using kanamycin as a selecting agent.

In the comparative studies of bar gene expression driven by the *Arabidopsis rbcS ats1A* and *2xE*

CaMV 35S promoters, plants expressing the *rbcS atslA-bar* constructs showed consistently higher steady-state levels of PAT mRNA, higher PAT protein levels, and higher PAT enzymatic activity compared to transgenic plants expressing the *2xE CaMV 35S-bar* constructs (**Figs. 2, 3, and 4**). These results are similar to a previous study carried out using transgenic tomato plants, where expression of a maize sucrose-phosphate synthase (SPS) gene was compared using an *rbcS* gene promoter and *CaMV 35S* promoter (Laporte *et al.,* 2001). The *rbcS* promoter gave approximately 3-fold greater total extractable SPS activity compared to plants expressing SPS under the control of the *CaMV 35S* promoter (Laporte *et al.,* 2001). Using a coffee *RBCS1* promoter-*uidA* translational fusion, Marraccini, *et al.* (2003) demonstrated that this promoter function as a leaf-specific and light-regulated promoter in transgenic tobacco plants. No GUS expression was detected in the roots of transgenic tobacco plants grown in the greenhouse and also illuminated roots of the same plants during growth *in vitro*. These data suggest that the approximately 1-kb coffee *RBCS1* promoter sequence contained all the *cis*-elements required for developmental and light-mediated control of gene expression. These findings were consistent with the work of De Almeida *et al.* (1989) who demonstrated tissue-specific expression of *rbcS atslA* transgenes in transgenic tobacco. These investigators also found that transgene expression was enhanced when the sequences encoding the Rubisco small subunit chloroplast transit peptide (*TP*) were amino-terminally fused in frame with the transgene coding region. In their study, De Almeida *et al.* (1989) showed that higher levels of bar gene expression were achieved in leaf tissues of transgenic tobacco plants expressing *rbcS atslA-TP-bar* transgenes compared to atslA-bar transgenes. This might be due to the higher stability of an mRNA molecule, which includes the transit peptide encoding sequence, or a higher rate of translation of those transcripts, which may in turn stabilize the RNAs. Also, it may be due to the presence of a eukaryotic sequence between the initiation codon and the prokaryotic *bar* gene. Tissue specificity may vary from plant to plant due to transgene integration at different chromosomal positions in nuclear transformation. Out of 18 transgenic plants (eight plants with construct pGSFR780A and 10 plants with construct pGSFR780B), only five transgenic plants with each construct resembling each other in morphological growth that were used for PAT and Northern analysis to make an appropriate comparison.

Among individual transgenic cotton plants expressing the *2xE CaMV 35S-bar* gene, transgenic plant number 4 showed highest *bar* transcript levels (**Fig. 3**). However, the average expression level of these plants was less than that observed in plants I-V expressing the *rbcs atslA-bar* transgene (**Fig. 3**). Similar variations were noticed in transgenic plants at protein expression levels (**Figs. 2** and **4**) and also reported by other workers (De Almeida *et al.,* 1989, Nagy *et al.,* 1985; An, 1986; Kay, *et al.,* 1987; Gidoni *et al.,* 1988). Therefore, organ specificity of heterologous gene expression can vary between independent transgenic plants and variation in tissue specificity may depend on the chromosomal environment in which a gene integrates.

Tissue-specific expression of transgenes in cotton might play a key role in plant protection against the insects and pests that attack leaf tissues. It may be desirable to express resistance genes at a defined expression level in specific parts of plants. This may be achieved via the use of tissue, organ specific or inducible promoters (Hoeven, 1994). Earlier, many field trials of cotton carrying modified *Bt* genes directed by a *CaMV 35S* promoter have been carried out. These trials, however, have not led to full success in the field. Bt-cotton reportedly failed to control *Heliothines armigera* in Australia (Hilder and Boulter, 1999). In 1996, two million acres of the US cotton belt were planted with *Bt*-transgenic cotton for the control of pink bollworm, tobacco budworm and cotton bollworm. The crop failed to control cotton bollworm on at least 20,000 acres in Texas. Possible causes include inadequate expression levels of Bt or low-level expression of transgenes that perhaps induced resistance in insects (Kaiser, 1996). The following year's cotton crop using Monsanto's herbicide resistant transgenics suffered a similar failure (Hilder and Boulter, 1999). Thus, there is continuing need to increase the expression level of transgenes in developing tissues of plants for efficient protection of cotton that could effectively block the resurgence of insect resistance. Despite the fact that the *CaMV 35S* promoter is a constitutive promoter and has been widely used to drive transgene expression in different crops, *CaMV* 35S promoter driven GUS expression in cotton at the initial stages of development when the plant needs more protection has been found to be very low (Sunilkumar *et al.,* 2003). Previously, the *bar* gene from *Streptomyces hygroscopicus* was inserted into the commercial varieties of cotton, DP50, Pima S6 and Coker 312 under the control of the *CaMV* 35S promoter fused to the AMV 5' leader sequence. Herbicide (Basta) tolerance up to 150 mg l^{-1} was demonstrated in greenhouse trials (Keller *et al.,* 1997). These results are in agreement with our

findings that plants expressing the *2xE CaMV 35S-bar* transgene tolerated Basta up to 200 mg l^{-1}. By comparison, plants expressing the *rbcS ats1A-bar* transgenes showed Basta tolerance up to 400 mg l^{-1} (**Fig. 5**). This is the highest level of tolerance to the herbicide Basta reported in the literature for transgenic cotton expressing the bar gene. Since transgenic plants expressing *bar* are known to survive in the field following spray treatments of Basta up to 200-300 mg l^{-1}, use of the rbcS ats1A promoter should facilitate using herbicide (Basta) on transgenic cotton in the future. Thus, our results clearly demonstrate that the *Arabidopsis rbcS atslA* promoter works more effectively in cotton than the *2xE CaMV 35S* promoter. This observation is consistent with the finding of Song, *et al.* (2000) who compared these two promoters to drive GUS expression in transgenic cotton.

Based on our findings, the *rbcS ats1A* promoter is among the best choices for high level tissue-specific expression of foreign genes in (green) photosynthetic tissues of cotton. It may also be among the best choices for general high levels expression of agronomically important disease and pest resistance traits useful in the production of improved transgenic cotton.

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References

An, G., 1986. Development of plant promoter expression vectors and their use for analysis of differential activity of the nopaline synthase promoter in transformed tobacco cells. Plant Physiol., **81**: 86-91.

Arguello-Astorga, G., Herrera-Estrella, L., 1998. Evolution of light-regulated plant promoters. Annu. Rev. Plant Physiol. Plant Mol. Biol., **49**: 525-555.

Benedict JH, Altman DW (2001) Commercialization of transgenic cotton expressing insecticidal crystal protein. In: Jenkins JN, Saha S (eds.) Genetic improvement of cotton. USDA- ARS, pp. 136-201 Oxford & IBH, New Delhi.

Benfy, P. N., Chua, N.-H., 1990. The Cauliflower mosaic virus promoter: Combinational regulation of transcription in plants. Science, **250**: 959-956.

Chaudhary, B., Kumar, S., Prasad, K. V. S. K., Oinam, G. S., Burma, P. K., Pental, D., 2003. Slow desiccation leads to high-frequency shoot recovery from transformed somatic embryos of cotton (*Gossypium hirsutum* L. cv. Coker 310FR). Plant Cell Rep., **21**: 955-960.

Dean, C., Pichersky, E., Dunsmuir, P., 1989. Structure, evolution, and regulation of RbcS genes in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol., **40**: 415-439.

De Almeida, E. R. P., Gossele, V., Muller, C. G., Dockx, J., Reynaerts, A., Botterman, J., Krebbers, E., Timko, M. P., 1989. Transgenic expression of two marker genes under the control of an Arabidopsis rbcS promoter: Sequence encoding the Rubisco peptide increase expression levels. Mol. Gen. Genet., **218**: 78-86.

Deblaere, R., Reynaerts, A., Hofte, H., Hemaisteen, J.P., Leemans, J., Van Montagu, M., 1987. Vectors for cloning in plant cells. Methods Enzymol., **153**: 277-292.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Movva, N. R., Thompson, C., Van Montagu, M., Leemans, J., 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J., **6**: 2513-2518.

Emani, C., Garcia, J. M., Lopata-Finch, E., Pozo, M. J., Uribe, P., Kim, D. J., Sunilkumar, G., Cook, D. R., Kenerley, C. M., Rathore, K. S., 2003. Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from *Trichoderma virens*. Plant Biotechnol. Journal, **1**: 321-336.

FAO 1993. Commodity Review and Outlook, FAO, Rome, pp: 118-123.

Finer, J. J., McMullen, M. D., 1990. Transformation of cotton *Gossypium hirsutum* L. *via* particle bombardment. Plant Cell Rep., **8**: 586-589.

Gamborg, O. L., Miller, R. A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., **50**: 151-158.

Gidoni, D., Bond-Nutter, D., Brosio, P., Jones, J., Bedbrook, J., Dunsmuir, P., 1988. Coordinated expression between two photosynthetic petunia genes in transgenic plants. Mol. Gen. Genet., **211**: 507-514.

Hilder, V. A., Boulter, D., 1999. Genetic engineering of crop plants for insect resistance-A critical review. Crop Protection, **18**: 177-191.

Hoeven, C. V. D., Dietz, A., Landsmann, J., 1994. Expression of phosphinothricin acetlyltransferase from the root specific par promoter in transgenic tobacco plants is sufficient for herbicide tolerance. Plant Cell Rep., **14**: 165-170.

Holtorf, S., Apel, K., Bohlman, H., 1995. Comparision of different constitutive and inducible promoters for the over expression of transgenes in Arabidopsis thaliana. Plant Mol. Biol., **29**: 637-646

Kaiser, J., 1996. Pest overwhelm Bt cotton crop. Nature, **273**: 423.

Kay, R., Chan, A., Daly, M., McPherson, J., 1987. Duplication of CAMV 35S promoter sequences creates a strong enhancer for plant genes. Science, **236**: 1299-1302.

Keller, G., Spatola, L., Mccabe, D., Martinell, B., Swain, M., John, M.E., 1997. Transgenic cotton resistant to herbicide bialaphos. Transgenic Research, **6**: 385-392

Kidd, G., 1994. Analyzing the opportunities in U.S. cotton.

Bio/Technology, **12**: 22-23.

Khoudi, H., Vezina, L. P., Mercier, J., Castonguay, Y., Allard, G., Laberge, S., 1997. An alfalfa rubisco small subunit homologue shares cis-acting elements with the regulatory sequences of the rbcS-3A gene from pea. Gene, **197**: 343-351.

Krebbers, E., Scurinck, J., Herdies, L., Cashmore, A.R., Timko, M.P., 1988. Four genes in two diverged subfamilies encode the ribulose-1,5-bisphosphate carboxylase small subunit polypeptides of *Arabidopsis thaliana*. Plant Mol. Biol., **11**: 745-759.

Kumar, S., Pental, D., 1998. Regeneration of Indian cotton variety MCU-5 through somatic embryogenesis. Curr. Sci., **74**: 538-540.

Kumar, S., Sharma, P., Pental, D., 1998) A genetic approach to in vitro regeneration of non-regenerating cotton (*Gossypium hirsutum* L.) cultivars. Plant Cell Rep., **18**: 59-63.

Laporte, M. M., Galagan, J. A., Prasch, A. L., Vanderveer, P. J., Hanson, D. T., Shewmaker, C. K., Sharkey, T. D., 2001. Promoter strength and tissue specificity effects on growth of tomato plants transformed with maize sucrose-phosphate synthase. Planta, **212**: 817-822.

Lyon, B. R., Cousins, Y. L., Llewllyn, D. J., Dennis, E. S., 1993. Cotton plant transformed with bacterial degradation genes are protected from accidental spray drift damage by the herbicide 2,4 - dichlorophenoxyacetic acid. Transgenic Res., **2**: 162-169.

Marraccini, P., Cuurjault, C., Caillet, V., Lausanne, F., Lepage, B., Rogers, W. J., Tessereau, S., Deshayes, A., 2003. Rubisco small subunit of *Coffea arabica*: cDNA sequence, gene cloning and promoter analysis in transgenic tobacco plants. Plant Physiol. and Biochem., **41**: 17-25.

Martinez-Hernandez, A., Lopez-Ochoa, L., Arguello-Astorga, G., Herrera-Estrella, L., 2002. Functional properties and regulatory complexity of a minimal RBCS light-responsive unit activated by phytochrome, cryptochrome, and plastid signals. Plant Physiol., **128**: 1223-1233

Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., Gotoh, Y., Katayose, Y., Nakamura, S., Honkura, R., Nishimiya, S., Ueno, K., Mochizuki, A., Tanimoto, H., Tsugawa, H., Otsuki, Y., Ohashi, Y., 1996. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol., **37**: 49-59.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant., **15**: 474-497.

Nagy, F., Morelli, G., Fraley, R. T., Rogers, S. G., Chua, N. H., 1985. Photoregulated expression of a pea rbcs gene in leaves of transgenic plants. EMBO J., **4**: 3063-3068.

Oerke, E. C., Dehne, H. W., Schonbeck, F., Weber, A., 1999. Crop Production and Crop Protection - Estimated Losses In Major Food and Cash Crops. Elsevier Science, Amsterdam, Netherlands. ISBN: 0444820957, pp 802.

Rinehart, J. A., Petersen, M. W., John, M. E., 1996. Tissue-specific and developmental regulation of cotton gene FbL2A. Demonstration of promoter activity in transgenic plants. Plant Physiol., **112**: 1331-1341.

Rajasekaran, K., Grula, J. W., Hudspeth, R.L., Pofelis, S., Anderson, D. M., 1996. Herbicide-resistant Acala and Coker cottons transformed with a native gene encoding mutant forms of acetohydroxyacid synthase. Mol. Breed. **2**: 307-319.

Song, O., Heinen, J. L., Burns, T. H., Allen, R. D., 2000. Expression of two tissue-specific promoters in transgenic cotton plants. The J. Cotton Sci., **4**: 217-223.

Sunilkumar, G., Mohr, L., Lopata-Finch, E., Emani, C., Rathore, K.S., 2002. Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP Plant Mol. Biol., **50**: 463-474

Sugita, M., Gruissem, W., 1987. Developmental, organ-specific, and light-dependent expression of the tomato ribulose-1,5-bisphosphate carboxylase small subunit gene family. Proc. Natl. Acad. Sci. USA, **84**: 7104-7108.

Trolinder, N. L., Goodin, J.R., 1988. Somatic embryogenesis in cotton (Gossypium) I. Effects of source of explant and hormone regime. Plant Cell Tissue Organ Cult., **12**: 178-181.

Trolinder, N. L., Chen, X. X., 1989. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep., **8**: 133-136.

Umbeck, P., Johnson, G., Barton, K., Swain, W., 1987. Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Biotechnol. **5**: 263-266.