Agrobacterium-mediated transformation of *Cry1C*, *Cry2A* and *Cry9C* genes into *Gossypium hirsutum* and plant regeneration

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Abstract

Three constructs harbouring novel *Bacillus thuringiensis* genes (*Cry1C, Cry2A, Cry9C*) and *bar* gene were transformed into four upland cotton cultivars, Ekangmian10, Emian22, Coker201 and YZ1 *via Agrobacterium*-mediated transformation. With the *bar* gene as a selectable marker, about 84.8 % of resistant calli have been confirmed positive by polymerase chain reaction (PCR) tests, and totally 50 transgenic plants were regenerated. The insertions were verified by means of Southern blotting. Bioassay showed 80 % of the transgenic plantlets generated resistance to both herbicide and insect. We optimized conditions for improving the transformation efficiency. A modified *in vitro* shoot-tip grafting technique was introduced to help entire transplantation. This result showed that *bar* gene can replace antibiotic marker genes (ex. *np*t II gene) used in cotton transformation.

Additional key words: *Agrobacterium tumefaciens, bar* gene, cotton, novel *Bt* genes.

Introduction

Several genes derived from *Bacillus thuringiensis* (*Bt*) have been successfully incorporated into the genomes of commercial crops such as cotton, maize, potato and rice (Shu *et al*. 2000, Nester *et al*. 2002). *Bt*-transgenic crops have considerable advantages both for the environment and for farm-worker safety, but the prevalence of *Bt* crops has raised concerns that these gains will be short-lived due to the possible emergence of *Bt*-resistant pests. Insects can evolve resistance when exposed to the same *Bt* protein under constant selection pressure (Tabashnik *et al*. 1990, Meng *et al*. 2004). Insect resistance may become the largest impediment to the successful deployment of *Bt* crops. One effective way of crops containing insecticidal proteins that provide resistance to insect pests was to develop novel *Bt* genes.

By using modern transgenic technology, *Bt* genes transferred and expressed in cotton will enhance the resistance ability against pests noticeably. Of the transformation protocols available, due to its better efficiency, *Agrobacterium*-mediated transformation with embryogenic callus as explant is currently the top choice for most cotton biotechnology laboratories in the world (Agrawal *et al*. 1997, Leelavathi *et al*. 2004, Jin *et al*. 2005). The *bar* gene, which encodes phosphinothricin acetyltransferase (PAT) that acetylates L-phosphino-thricin (L-L-PPT) as the active ingredient of the herbicide *Glufosinate* (Thompson *et al*. 1987) can be used in herbicide-resistant crop breeding and screening procedure for transformants as a selectable gene. The transformation practices of *bar* gene have achieved successes in a variety of plants (De Block *et al*. 1987, 1989, Cao *et al*. 1992, Keller *et al*. 1997, Popelka *et al*. 2003, Zeng *et al*. 2004, Manickavasagam *et al*. 2004).

In this study, we report the successful transformation of three novel *Bt* genes and *bar* gene into several elite cotton cultivars *via Agrobacterium*-mediated trans-formation and the establishment of a new, simple and reliable transformation and regeneration system based on *bar* gene as a selectable marker.

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Materials and methods

Plants and cultivation: Four upland cotton (*Gossypium hirsutum* L.) cultivars, Coker 201 (C201), YZ1, Ekangmian 10 (EK10), Emian 22 (EM22), were chosen for transformation. Their seeds were surface disinfected by 0.1 % (m/v) aqueous $HgCl_2$ solution and washed four times with sterile water. Hypocotyls were excised from the seedlings which developed from aseptic seeds and cut into 5 - 6 mm segments. Callus induction was carried out on MSB_1 medium, MS (Murashige and Skoog 1962) inorganic salts and B_5 vitamins (Gamborg *et al.* 1968) supplemented with 3 % (m/v) glucose, 0.25 % (m/v) *Phytagel*, 1.0 mg dm^{-3} indolebutyric acid (IBA), and 0.5 mg dm^{-3} kinetin. Embryogenic calli (EC) with high proliferation tendency were obtained after three to seven rounds (2 weeks a round) of subculture according to their genotypes. Then high-quality EC were pre-cultured on MSB (MS salts and B_5 vitamins) medium supplemented with 3 % (m/v) glucose and 0.25 % (m/v) *Phytagel*, 0.5 mg dm^{-3} IBA, 0.15 mg dm^{-3} kinetin, 1.0 g dm^{-3} glutamine, and 0.5 mg dm^{-3} asparagine for 15 d.

***Agrobacterium* strain and plasmid:** The *Agrobacterium tumefaciens* strains EHA105 harboring modified plasmid pCAMIA1300 were kindly offered by Prof. Lin Yongjun, College of Life Science and Technology, Huazhong Agricultural University. The *Agrobacterium* strain carries one of the three novel *Bt* genes, *Cry1C* (Lin *et al.* 2004a)*, Cry2A* (Lin *et al.* 2004b) and *Cry9C* (Fig. 1*A,B,C*), and
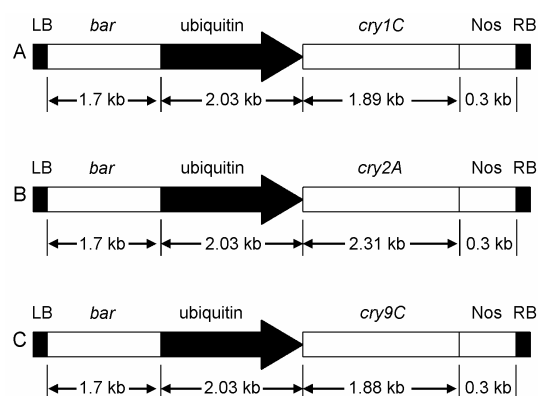

Fig. 1. T-DNA region of the expression vector for transformation. C*ry1A, cry2A, cry9c* genes were controlled by a maize ubiquitin promoter and nos-terminator. The *bar* gene was used as a selective gene under the control of CaMV 35S promoter and CaMV 35S polyA tail. LB: T-DNA left border repeat, RB: T-DNA right border repeat.

bar gene as a selectable marker in the T-DNA region of their own plasmid. *Agrobacterium* was grown in LB (tryptone 5 g dm^{-3}, NaCl 10 g dm^{-3}, yeast extraction 5 g dm^{-3}) liquid medium on a shaker (200 rpm) overnight at 28 °C. The cells were harvested by centrifugation (1380 *g*, 2 min) and further re-suspended in MGL (tryptone 5 g dm^{-3}, NaCl 5 g dm^{-3}, $MgSO_4 \cdot 7 H_2O$ 0.1 g dm^{-3},

KH_2PO_4 0.25 g dm^{-3}, and glycine 1.0 g cm^{-3}, pH 5.8) liquid medium at a density of 5×10^6 - 5×10^7 cells dm^{-3} (A_{600} = 0.05 - 0.5) for callus infection. EC were suspended in the *Agrobacterium* solution for 20 min, and then dried on sterilized filter papers under the air flow of clean bench. The dry calli were then co-cultured in the dark environment on MSB_1 medium at 17 - 23 °C for 48 h. Acetosyringone (AS) was added in either co-cultivation MSB_1 medium or liquid MGL medium.

Selection and plant regeneration: To determine a lethal concentration of L-L-PPT to EC, high-quality EC pre-cultured on MSB medium for 15 d were inoculated on MSB_1 medium supplemented with 0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 mg dm^{-3} L-PPT respectively. The tolerances of calli to L-L-PPT were observed 14 d after application. The chlorotic and dead calli were considered sensitive. The experiment was replicated three times including 60 - 70 calli per treatment. According to the optimal selection pressure decided above, the co-cultured calli were transferred onto selection medium (MSB_1 containing 5 - 20 mg dm^{-3} L-L-PPT and 500 mg dm^{-3} cefotaxime), and subcultured every 2 weeks for 3 - 4 times. After selection, L-L-PPT-resistant calli formed and were moved to MSB_2 medium [MS basal salts (KNO_3 doubled but NH_4NO_3 removed), B_5 vitamins, 3 % (m/v) glucose, 1.0 g dm^{-3} glutamine, 0.5 g dm^{-3} asparagine, 0.5 mg dm^{-3} IBA, 0.15 mg dm^{-3} kinetin and 0.25 % (m/v) *Phytagel*, pH 5.8] for embryo maturity and plant regeneration. Abnormal embryos at different stages were collected and rectified by slow desiccating on 1/3 MSB medium by silica gel Petri plate or 1/3 MSB medium supplemented with 50 mg dm^{-3} active carbon. An *in vitro* grafting technique developed previously in our laboratory to recover transgenic plants was adopted (Jin *et al.* 2005).

PCR and Southern blot analysis: Genomic DNA was extracted from L-PPT-resistant callus according to Paterson *et al.* (1993). For PCR amplification tests, the primers used were:
5' - TTCTACTGGGGGAGGACATCG - 3',
5' -CGGTATCTTTGGGTGATTGG- 3' for *Cry1C*;
5' - CGTGTCAATGCTGACCTGAT - 3',
5' - GATGCGGACAGGATGTAGT - 3' for *Cry2A*;
5' - ACCAGTTCCTGCTGAACACC - 3',
5'-TACAGGCGCACGTCGTAGTA -3' for *Cry9C*.
PCR amplification program consisted of denaturation at 94 °C, 2 min; annealing at 55 °C, 1 min; extension at 72 °C, 1 min, 30 cycles; and final extension at 72° C, 10 min. For Southern analysis, genomic DNA (20 μg) from transgenic plants with *Cry9C* gene were digested by *Hind*III and probed by *Cry9C* gene. All standard procedures follow the description of Sambrook *et al.* (1989).

Herbicide resistance and insect bioassay of transgenic plants: Expression of *bar* gene was detected by

inoculating transgenic and non-transgenic plantlets on MS medium supplemented with 50 mg dm^{-3} L-PPT. The insect bioassay of transgenic cotton plants was modified according to Wu *et al.* (2005). Fully expanded young leaves were detached and placed into Petri plates 9 cm in diameter with a piece of wet filter paper at the bottom. Non-transgenic parent plants were used as controls. Each leaf was inoculated with eight first-instar cotton bollworm (*Helicoverpa armigera*) larvae. Five days later, the amount of leaf consumed was photographed.

Results

Optimization of the parameters in transformation: Before transformation, the optimal L-PPT concentration for selection was decided by L-PPT-sensitive test. The results showed that different genotypes demanded different screening pressure. L-PPT at concentration of 5, 10, 10, and 20 mg dm^{-3} were regarded as optimal during resistant calli screening for EM22, C201, YZ1 and EK10, respectively. The optimal screening concentration of L-PPT was judged by killing more than 90 % mini-mass calli tested.

Table 1. Effect of acetosyringone (AS) concentration in the co-cultivation and bacterium proliferation medium on transformation efficiency.

AS [mg dm^{-3}] co-cultivation	proliferation	Number of calli tested	Number of resistant calli	Resistant calli [%]
0	0	30	4	13.3
0	50	32	5	15.6
50	0	30	5	16.7
50	50	30	5	16.7

Several factors during the co-cultivation stage were proved to be crucial in achieving an effective *Agrobacterium*-mediated transformation. Acetosyringone (AS), a phenolic compound in inducing the initiation of *Vir* genes of Ti plasmid, is regarded helpful to T-DNA transfer in a wide range of crops. In our study, we added AS to MGL liquid medium or co-cultivation solid medium respectively (Table 1). As the result, we adopted two ways of introducing AS into co-cultivation environments. Both methods can improve the transformation efficiency to a similar extent. Adding AS both to bacterium proliferation and co-cultivation media can not improve transformation to a higher level (Table 1). Furthermore, the *Agrobacterium* strain EHA105 has strong tendency in infecting the recipient material of cotton, which suggests a lower concentration of strain EHA105 is appropriate in the process of infection. The concentration of the *Agrobacterium* solution more than A$_{600}$ = 0.5 can easily cause overgrowth of bacterium, and a relatively low concentration such as A$_{600}$ = 0.1 is necessary to keep a high transformation frequency and a low overgrowth rate of bacterium (Table 2). In addition, the temperature for co-cultivation was adjusted. Three temperature points, 17, 19, and 23 °C were compared (Table 3) and the results revealed that temperature at 19 °C was the best co-culture temperature for transformation, which was in accordance with our previous report (Jin *et al.* 2005).

Table 2. Effect of *Agrobacterium* concentration on percentage of calli with overgrowth of *Agrobacterium* and resistant calli. Means followed by the same letters (a, b, c or A, B) within a column do not differ significantly at $P \leq 0.05$ or $P \leq 0.01$.

A$_{600}$	Number of calli tested	Calli with overgrowth [%]	Resistant calli [%]
0.05	56	3.9 ± 0.2c	8.5 ± 0.8A
0.10	78	7.1 ± 1.2c	12.3 ± 1.2AB
0.20	50	24.0 ± 2.2b	16.0 ± 1.3B
0.40	50	23.8 ± 2.3b	14.3 ± 2.1B
0.50	29	92.0 ± 3.1a	0

Table 3. Effect of co-cultivation temperature on percentage of calli with overgrowth of *Agrobacterium* and resistant calli. Means followed by the same letters (a, b, c or A, B) within a column do not differ significantly at $P \leq 0.05$ or $P \leq 0.01$.

Temperature [°C]	Number of calli tested	Calli with overgrowth [%]	Resistant calli [%]
17	53	8.0 ± 0.3b	2.0 ± 0B
19	100	13.0 ± 1.6a	8.0 ± 1.5B
23	48	6.0 ± 0.2b	56.0 ± 4.5A

Characterization and identification of insertion events: The putative resistant calli were tested by PCR amplification with corresponding primers for *Cry1C, Cry2A* and *Cry9C* genes. The overall value of PCR positive frequency was 84.8 %, which displayed L-PPT can be used as an effective selection factor to screen out EC transformants carrying *bar* gene (Table 4). At the same time, the results varied among different cultivars and different *Bt* genes. PCR positive frequency of YZ1 as explant is the highest (92.6 %) (Table 4, Fig. 2), C201 and E22 follow subsequently, and the lowest EK10 is 80.5 %. The fact that the genotype correlates with the PCR positive frequency shows that during transformation stage genotype of EC may influence the transformation efficiency due to their different adaptability to specific transformation protocol.

DNA from transformed plants of YZ1 with *Cry9C* insertion were extracted and digested by *Hind*Ⅲ and

Table 4. Number and percentage (in parentheses) of PCR-positive calli of different transgenic clones.

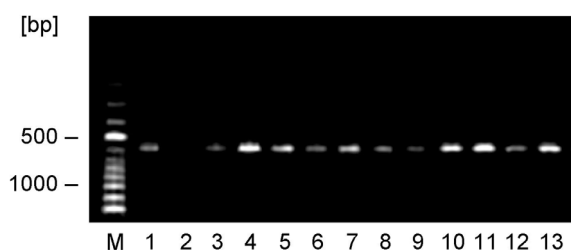| Cultivar	*Cry1C* calli tested	PCR-positive	*Cry2A* calli tested	PCR-positive	*Cry9C* calli tested	PCR-positive	Total
EK10	12	9 (75.0 %)	15	14 (93.3 %)	9	6 (66.7 %)	80.5 %
E22	12	10 (83.3 %)	7	7 (100.0 %)	6	5 (83.3 %)	88.0 %
C201	10	6 (60.0 %)	11	11 (100.0 %)	16	13 (81.3 %)	81.1 %
YZ1	9	7 (77.8 %)	8	8 (100.0 %)	10	10 (100.0 %)	92.6 %
Total	43	32 (74.4 %)	41	40 (97.6 %)	41	34 (82.9 %)	84.8 %

Fig. 2. PCR detection of part of resistant calli (YZ1×*Cry9C*). M - marker, 1 - plasmid, 2 - untransformed cotton as negative control, 3 to 13 - transformants.

then hybridized with *Cry9C* probe. Since the T-DNA has a single *Hind* Ⅲ site, the number of hybridizing bands indicated the insert copy numbers of *Cry9C* genes. The copy numbers of the transgenic plants (Fig. 3, *lane 5 - 8*) ranged from 1 to 2, while resistant calli (Fig. 3, *lane 3,4*) have up to 7 - 8 copies of insertion fragment which proves their chimeric constitution deriving from multiple transformed cells. Southern blotting analysis also indicated T-DNA insertion was randomly integrated in the cotton genome.

Herbicide resistance and insect bioassay of transgenic plants: Non-transformed and transformed plantlets were cultured in the same MSB medium supplemented with 50 mg dm^{-3} L-PPT. The non-transformed perished gradually from leaves along vascular bundle to root within the subsequent 2 weeks, while the transgenic
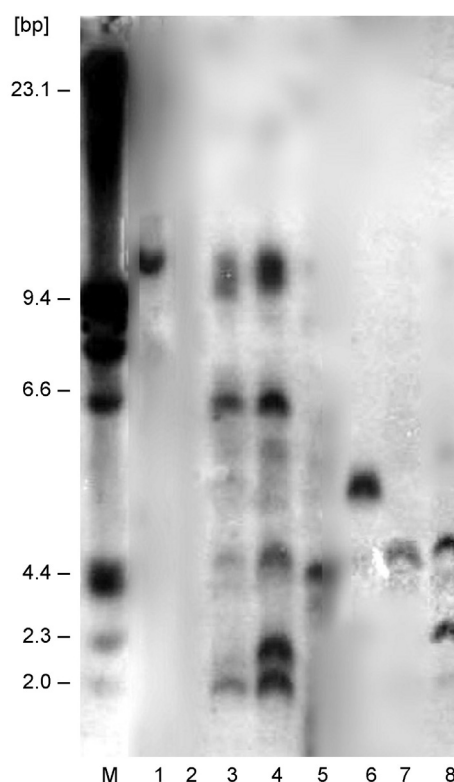

Fig. 3. Southern blotting of transgenic calli and plantlets YZ1×*Cry9C*. M - marker, 1 - plasmid, 2 - non-transformed plant as control, 3 to 4 - resistant calli, 5 to 8 - transgenic plantlets.

Fig. 4. Herbicide resistance and insect bioassay of transgenic plants. *A* - Detection of L-PPT resistance of C201 × *Cry9C* [control (N) and the transformant (R)]. *B* and *C* - Insect bioassay of transgenic plant (E22 × *Cry2A*) leaves expressing *Bt* protein [leaves from transformants (*B*) and the non-transgenic control (*C*)].

plantlet remained intact in the same flask (Fig. 4*A*). This result showed the *bar* gene made transgenic plantlets express resistance to L-PPT. The leaves of transgenic plantlets were collected and fed to first-instar cotton bollworm in bioassay experiment. Transformants of EM22 with *Cry2A* were tested, the result displayed that the insect resistance of transgenic plant was higher than a non-transformed control according to the amount of leaf consumed by the insects. The control leaf was stripped seriously by the bollworm (Fig. 4*C*), but the transgenic leaf remained intact (Fig. 4*B*).

A high-efficient plant regeneration system: An effective transformation system started with obtaining vigorously growing EC by subculturing EC on the medium MSB1 for 2 weeks, then co-cultured with *Agrobacterium* solution and stored for 40 h at 19 °C in the dark chamber. After 3 - 4 rounds of selection on the medium with L-PPT, resistant colonies emerged at the end of this stage (Fig. 5*A*). The resistant calli were moved to medium MSB2 for embryo maturity (Fig. 5*B*) and embryo germination. Slow desiccation created by silica gel and low salt was found effective in rectifying the abnormal embryos to some extent (Fig. 5*C*) (Chaudhary *et al.* 2003). Since most plantlets (60 - 80 %) were accompanied with abnormal root growth, a modified *in vitro* grafting technique was adopted to help those transgenic seedlings build strong root. The grafts stay in the rooting medium for root development over 2 - 3 weeks. Then they were transferred to water vessels for acclimation and further root growth (Fig. 5*D*), and then to pots with autoclaved soil in a greenhouse for adaptation to environment (Fig. 5*E*). Over 2 - 3 weeks, the plantlets were moved and planted to field finally (Fig. 5*F*) (Jin *et al.* 2005).
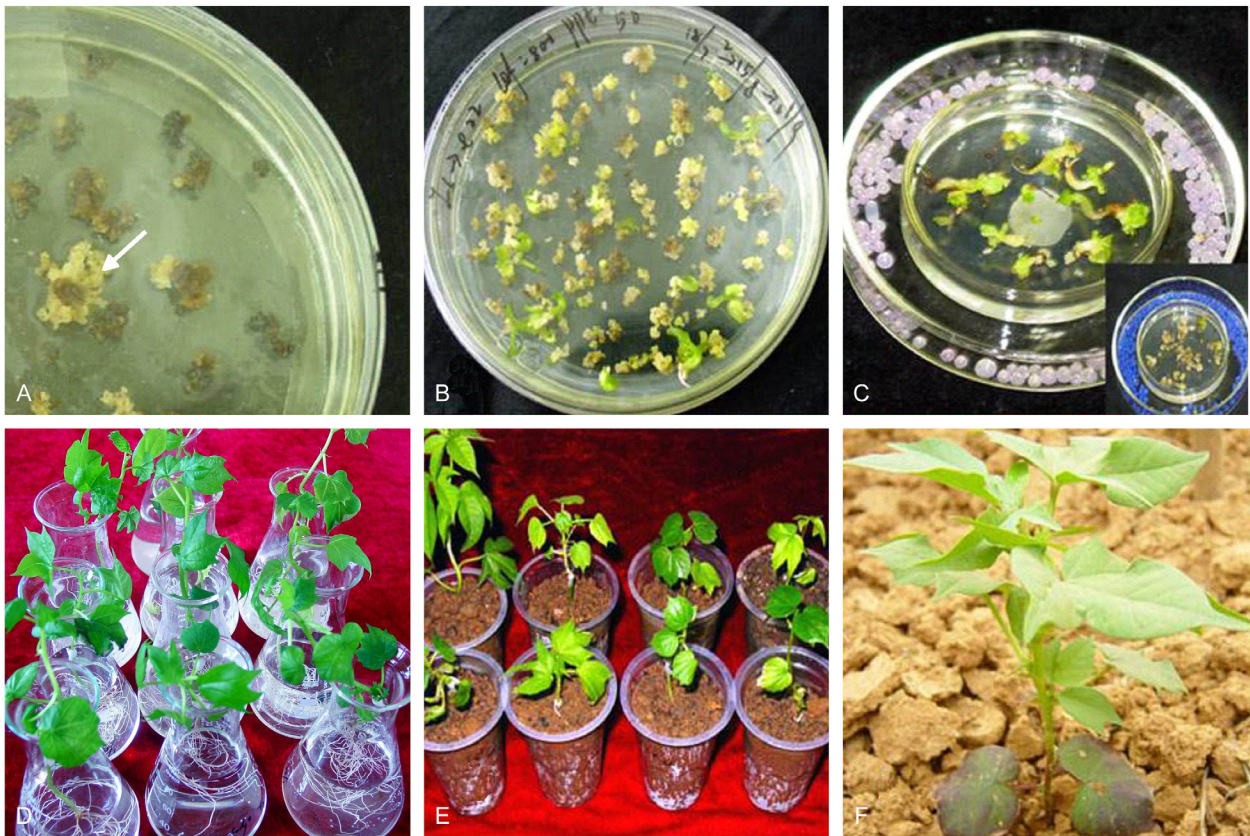

Fig. 5. Transformation, regeneration and transplantation. *A* - Resistant calli (*arrow indicated*) on the selection medium; *B* - differentiating resistant calli; *C* - rectification of abnormal embryos under the slow desiccation created by silica gel; *D* - acclimation in water after *in vitro* grafting; *E* - transgenic plants in small pots at greenhouse; *F* - transgenic plant grown in field.

Discussion

Once the embryogenic line was established, EC can be routinely subcultured and used for transformations. The procedure is economical, rapid, and only requires approximate half duration of *Agrobacterium*-mediated transformation with hypocotyls or cotyledonary leaves as explants (Jin *et al.* 2005). The elite cotton cultivars, such as YZ1, E22 and EK10, were also transformed successfully together with the model cultivar C201,

which implies the period of transgenic breeding through backcross could be saved by transforming elite cultivars directly. However, chromosome variation of cotton embryogenic callus is expected to rise with the increase of the regeneration cycle (Zheng *et al.* 1991). In our study, we found extended selection and subculture can lead to severe incurable abnormality of regenerated embryos (data not shown). Multiply methods should be taken to minimize the potential risks (Skirvin *et al.* 1994).

For the purpose of observing expression patterns of transfomants and obtaining the seeds of next generation, transgenic plantlets have to be transferred to field. Commonly, cotton demands lengthy recovery period after transplantation, and transgenic regenerated plantlets always have root system with poor quality and stagnant growth (Liu *et al.* 2004). Grafting is required to help them build vigorously growing root for transplanting into field.

In our research, the *bar* gene seems a useful selectable gene for highly efficient transformation system in cotton transformation. The routinely used kanamycin resistant *npt-II* gene and hygromycin resistant *hpt* gene easily arose biosafety concerns and could be replaced by *bar* gene. For transgenic *Bt* cottons containing *bar* gene, the resistance both to pest and herbicide were input to cotton genome, which make cotton production much less laborious. As the result, biotechnology for cotton with both herbicide and insect resistance will be highly promising in the future cotton production.

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A new book on photosynthesis is always very welcome. I find it surprising that such an important complex of processes has been treated in relatively few books only. Of course, there are numerous volumes dealing with very detailed information on photosynthesis at individual levels of structures and processes. However, overviewing summaries of photosynthesis covering it from the level of thylakoid to canopy are rare. Hence, this new volume is most valuable. Unfortunately, it is in French. Although I personally like this language, it will be this title's handicap for the majority of scientific community worldwide.

The book starts with a preface by the authors briefly explaining the content of the individual chapters. The first part (67 pages) is devoted to an overview of the photosynthetic processes (light energy, pigments, reaction centres, principles of oxidoreduction processes) including adequate information on the history of the most important discoveries. Part II (110 pages, its title failed to be included into the content) describes in much detail classification of the photosynthetic organisms, leaf and chloroplast structure, oxygenic photosynthesis, electron transfer on the thylakoids, ATP synthesis, photosynthetic efficiency, distribution of radiation energy between the two photosystems and photosynthesis of algae and cyanobacteria. Part III (30 pages) explains anoxygenic photosynthesis of both bacteria and phototrophes. The large part IV (86 pages) deals with carbon metabolism and includes also principles of nitrogen and sulphur assimilation. Furthermore, carbohydrate synthesis and export as well as C4 and CAM metabolisms are explained. This part terminates with brief notions on the CO_2 assimilation of organisms not equipped with rubisco (*Chlorobiaceae* and *Chloroflexus*). The final part V covers the effects of the environment on photosynthesis: gas exchange of the leaves and canopies, the effect of water availability, irradiance, oxygen, nitrogen and CO_2 concentration. Finally, some agronomic and global aspects of photosynthesis are also mentioned. The annex contains an overview of the herbicides inhibiting photosynthesis. Valuable glossary (13 pages) defines or explains the most important terms frequent in the photosynthetic literature.

The description of the content clearly indicates that this book covers the majority of aspects related to photosynthesis. This is its most valuable feature. Obviously, not all parts have been dealt with the same profundity. Structures and processes at the level of chloroplasts and thylakoids are precisely and adequately described. However the ecophysiological aspects should be taken only as a preliminary introduction. For example, for unknown reasons the effects of an enhanced CO_2 concentration have been illustrated by a detailed and not well-founded description of an experiment carried out in 1994 although much more has been known and reviewed in many recent publications.

More citations should have been included. For example, the chapter on global carbon sources and sinks contains no one citation although several numbers and one figure illustrate quantitative fluxes. Similarly, results by O. Warburg are rightly mentioned (p. 176, 234) with no reference to his publications. The important remark on the ATP synthase of the bacterium *Propiogenium modestum* (p. 162) would deserve the indication of the source of such information. Finally, several figures have been included with no information on their authorship. And my last objection relates to the literature: from more than 300 cited references, only 10, 6 and 1 were published in the years 2003, 2004 and 2005, respectively, although the book was published in 2006 and photosynthesis research continuously flourishes.

My discordant remarks should not negate the many positive values of this book. According to its authors, it is intended to be used by students and teachers of agronomy, pharmacy, and biology. I would recommend it also to many researchers who need an easy to survey text on photosynthesis in its broad contexts. And let me terminate with my personal wish: I would be pleased to get a new revised edition in English. I am sure that it would be welcome by many from the photosynthetic research community.

L. NÁTR (*Praha*)