Kasetsart J. (Nat. Sci.) 33 : 149 - 156 (1997)

Agrobacterium Mediated Transformation of Sri Sumrong 60, a Thai Cotton Variety

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ABSTRACT

Techniques were developed for the induction of embryogenic callus, as well as the transformation and regeneration of Sri Sumrong 60, a Thai cotton variety. Somatic embryogenesis was obtained by culturing hypocotyl explants on MS based medium containing zeatin and NAA and subculturing on an hormone-free medium. This method yielded 23-50% embryogenic callus induction. Transformation of Sri Sumrong 60 with a synthetic *cryIA(b)* gene was achieved by using *Agrobacterium tumefaciens* strain, with the addition of 100 mM acetosyringone to the medium during inoculation and co-cultivation steps. PCR amplification was used to verify the presence of *cryIA(b)* in regenerated plants, thus suggesting the successful transformation of the Thai cotton variety Sri Sumrong 60.

Key words : *Bacillus thuringiensis*, *Agrobacterium tumefaciens*, cotton, somatic embryogenesis, *cryIA(b)*.

INTRODUCTION

Cotton production in Thailand has decreased by nearly 50% over the last fifteen years. It is estimated that cotton growing area has been reduced from 160,000 ha in 1980 to only 80,000 ha in 1994 (Castella and Trebuil, 1996). The resistance of cotton pests to chemical pesticides is believed to be responsible for this phenomenon. The cotton bollworm, *Helicoverpa armigera* (Hubner) the key pest in Thai cotton cropping has developed resistance to various chemical insecticides. One strategy to circumvent this problem is the transfer to cotton plants of genes encoding *Bacillus thuringiensis* (B.t.) endotoxins which have been identified as toxic to this insect.

Cotton transformation was made possible after the establishment of the first protocol of cotton somatic embryogenesis (Davidonis and Hamilton, 1983 ; Trolinder and Goodin, 1987). The successful transformation and production of fertile transgenic plants have been reported since then (Firoozabady *et al.*, 1987 ; Umbeck *et al.*, 1987 ; Finer and McMullen, 1990 ; Pannetier *et al.*, 1993). The *cryIA(b)* synthetic gene has already been transferred to American variety Coker 310 (Perlak *et al.*, 1990). It has been shown that cotton somatic embryogenesis and genetic transformation are genotype-dependent (Trolinder and Xhixian, 1989). And, even though the Australian variety

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Siokra 1-3 has been transformed (Cousins *et al.*, 1991), the success of regeneration and transformation is limited to the Coker varieties. Backcrossing from the transformed variety in order to transfer the target gene to the economically important varieties has been practiced but it is still a time-consuming process.

The objectives of this study were to improve the procedure for cotton somatic embryogenesis from the one previously reported (Kuhapituktum *et al.*, 1995) and to transfer B.t. toxin gene directly to Sri Sumrong 60, a Thai variety via *Agrobacterium tumefaciens*.

MATERIAL AND METHODS

Seed germination

Seeds of Thai cotton variety Sri Sumrong 60 were obtained from the Suwanwajokasikit Research Station, Kasetsart University. Seeds were surface sterilized by dipping twice in 20 % Clorox (5% w/w sodium hypochlorite) and 2 drops of Tween per 100 ml of distilled water for 20 min. They were then rinsed with sterile distilled water and kept in water for 1 to 2 hours. Seed coats were removed and seeds were germinated on a medium consisting of half-strength MS inorganic salts (Murashige and Skoog, 1962), vitamins from Morel and Whetmore (1951), 1% sucrose and 0.65% agar. The pH was adjusted to 5.8 before autoclaving.

Somatic embryogenesis

Hypocotyls were excised from 7 to 8-day-old seedlings, cut into 1 cm long explants and plated on callus induction medium (MS salts, Morel and Whetmore vitamins, 2% glucose, 0.3% Phytagel (Sigma), 1 mg/l zeatin, 0.1 mg/l naphtalene acetic acid(NAA)) and incubated at 27°C, 16 hour photoperiod. The explants were subcultured every 4 weeks. To improve the somatic embryogenesis process, 24 conditions corresponding to different zeatin/NAA ratios were tested. After one month, calli derived from each treatment were transferred onto the same medium, or to a medium containing the same growth regulators with an inverted ratio, or on an hormone-free medium. Embryogenic calli were transferred onto regeneration medium (MSO) containing standard MS salts, Morel and Whetmore vitamins, 3% sucrose and 0.3 % Phytagel.

Plasmid constructs

Plasmids pKY-*cryIA(b)* derived from pKYLX71-35S^2 (Maïti *et al.*, 1993) was introduced into *Agrobacterium tumefaciens* strain C58 : pGV2260 (Deblaere *et al.*, 1985). The pKY-*cryIA(b)* vector carries a synthetic *cryIA(b)* gene (Sardana *et al.*, 1996) under the control of the CaMV35S promoter with a doubled enhancer and the 3'Rbsc terminator. The *nptII* gene under the control of *nos* promoter and terminator is also present for kanamycin selection of transformed cells.

Gene transfer

Overnight culture of *A. tumefaciens* strain containing pKY-*cryIA(b)* was used for inoculation. Based on the method used by Pannetier *et al.* (1993) with Coker varieties, Sri Sumrong 60 fragments of one week-old hypocotyls were inoculated by dipping the explants in a 50 or 100 fold dilution of the bacteria for 40 min. supplemented with acetosyringone at 100 mM. The explants were blotted dry on sterile filter paper and plated on culture medium without antibiotics. After 2 days of co-culture, explants were transferred to the same medium supplemented with 50 mg/l kanamycin and 500 mg/l cefotaxim. The explants were subcultured onto the same medium every 2 weeks.

Embryogenic calli were separated from the explants and transferred onto MSO medium. Germinated embryos were transferred to a tube containing Sorbarod plug (Ilacon, England) weted

with MSO liquid medium. Recovered plantlets were transplanted to soil in a greenhouse.

DNA extraction and PCR analysis

DNA was extracted from callus and leaf material using a modified CTAB extraction method (Bi *et al.*, 1996). PCR amplification (100 ml reaction) was performed using primers designed to amplify a 1323 bp fragment of the synthetic *cryIA(b)* gene. Relative to the sequence of *cryIA(b)* given in Perlak *et al.* (1990) the upstream primer (5' GGT CCA TCT CAA TGG G 3') is located between nucleotides 211 and 226, while the downstream primer (5' TGC TAA TCT GGC CAG G3') is located between nucleotides 1534 and 1519. Twenty ml of each PCR-amplified sample was used for electrophoresis in 0.8 % agarose gel.

Table 1 Effect of growth regulators on somatic embryogenesis of Thai cotton variety Sri Sumrong 60 cultured on basic MS medium for 3 months.

Plating medium zeatin : NAA (mg/L)	Transferring medium zeatin : NAA (mg/L)	Embryogenic calli (%)
1 : 0.01	1 : 0.01	-
	0.01 : 1	-
	0 : 0	33.33
1 : 0.05	1 : 0.05	-
	0.05 : 1	-
	0 : 0	23.52
1 : 0.1	1 : 0.1	-
	0.1 : 1	-
	0 : 0	4.54
2 : 0	2 : 0	7.14
	0 : 2	3.70
	0 : 0	5.88
2 : 0.01	2 : 0.01	-
	0.01 : 2	-
	0 : 0	41.66
2 : 0.05	2 : 0.05	-
	0.05 : 2	-
	0 : 0	50.00

RESULTS AND DISCUSSION

Callus induction and somatic embryogenesis

Callogenesis was observed in all hypocotyl explants when plated on callus induction medium containing different combinations of zeatin and NAA (data not shown). Callus embryogenesis was observed only on six combinations, 45 days after transferring. A high percentage of embryogenic callus induction, ranging from 23 to 50 %, was obtained after transfer on hormone-free medium (Table 1). This suggests that growth regulators are not needed for embryogenic callus production for Sri Sumrong 60 in contrast to what was reported by Firoozabady and De Boer (1993) for other cotton varieties. Using this protocol, greater number of embryogenic calli were obtained within 3 to 4 months plating compared to 6 to 8 months as previously reported (Kuhapituktum *et al.*, 1995). These embryogenic calli give rise to both developing somatic embryos and proliferating embryogenic calli, suggesting that embryogenic cotton calli are apparently habituated as observed by Trolinder and Goodin (1987).

cryIA(b) gene transformation and plant regeneration

The selection of transformed cells was achieved on a selective medium containing 50 mg/l kanamycin and 500 mg/l cefotaxim. Transformed cells survived and developed calli while non-transformed cells died. These have recovered up to 24 % of resistant calli (relative to the number of explants plated) after subculturing explants on antibiotic containing medium for 2-3 months.

Embryogenic calli were obtained from eleven independent antibiotic resistant calli. Embryos of different types were observed after transferring embryogenic calli onto MSO medium. However, the number of plantlets regenerated from these embryogenic calli was low and plants were abnormal. Normal plantlets were recovered mainly from tulip-shaped embryos as described by Voo *et al.* (1991) and cultured on germination medium. Eighteen normal plantlets were obtained from 7 independently transformed embryogenic calli. After transfer to potting medium for 2 months, these plants flowered and produced bolls indicating that they were fertile (Figure 1).

Molecular analysis of transformed calli and plants

DNA isolated from recovered plants derived from kanamycin-resistant calli were subjected to PCR analysis to verify the presence of synthetic *cryIA(b)*. From the tested 18 plants derived from 7 independently transformed lines, only 17 plants corresponding to 6 lines was observed to contain *cryIA(b)* specific amplification products. The result indicated that they were transformed (Figure 2).

CONCLUSION

The process of somatic embryogenesis of the Thai cotton variety, Sri Sumrong 60 was improved. Explants giving rise to embryogenic calli were obtained, in shorter time and better percentage. *Agrobacterium*-mediated gene transfer technique was also established for the transformation of Sri Sumrong 60.

The synthetic *cryIA(b)* gene encoding the *Bacillus thuringiensis* endotoxin was successfully transferred to this cotton variety. However, the resistance of transgenic Sri Sumrong 60 plants to the attack of the cotton bollworm, *Helicoverpa armigera* will have to be further investigated.

ACKNOWLEDGEMENTS

This work is a collaborative program between Annual Crop Department of the Centre de

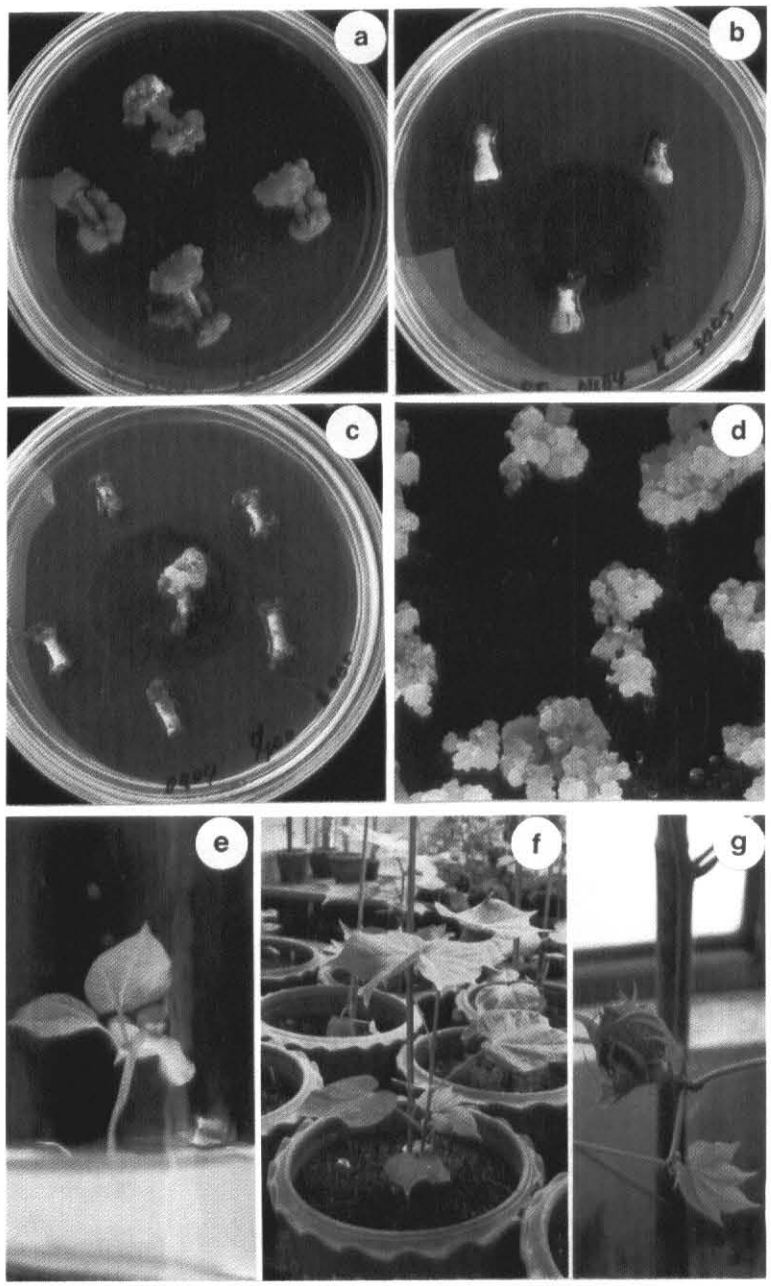

Figure 1 *cryIA(b)* gene transfer in Sri Sumrong 60.

a. Control, non-transformed explants on MS medium.
b. Non-transformed explants on medium containing antibiotics.
c. Resistant calli.
d. Embryogenic calli derived from resistant calli.
e. Regenerated plant.
f. Plants grown in potting medium in the greenhouse.
g. A cotton boll of transgenic plant.

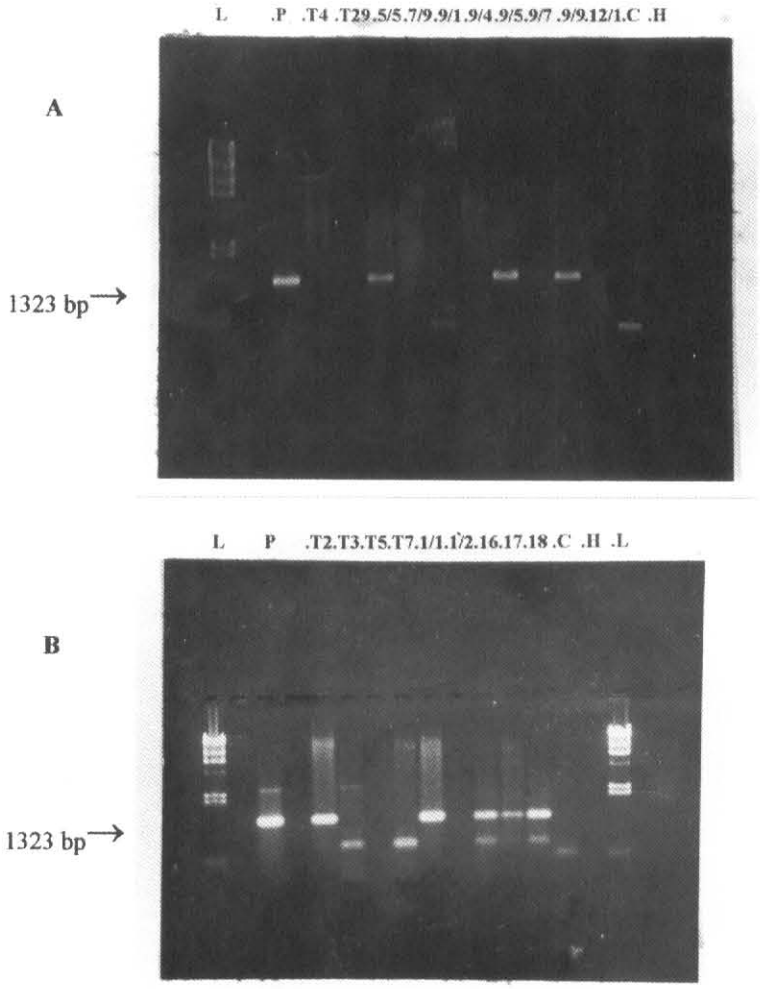

Figure 2 PCR-amplification of DNA isolated from transformed cotton.
A. and B. Regenerated plants.
T2, T3, T4, T5, T7, T29, 16, 17 and 18, correspond to different plants obtained from the same line T.
1/1 and 1/2 are obtained from the line 1.
5/5, 7/9 and 12/1 are plants corresponding to the independent lines 5, 7 and 12 respectively.
9/1, 9/4, 9/5, 9/7 and 9/9, correspond to plants obtained from the line 9.
L = Ladder, Lambda/*HindIII*. P = plasmid pKY-*cryIA(b)*. C = non-transformed plant.
Arrow indicated the 1323 bp fragment of synthetic gene *cryIA(b)*.

Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD-CA) in France and Plant Genetic Engineering Unit (PGEU) of Kasetsart University, Kamphaengsaen, Thailand. The authors thank I. Altossar for providing *cryIA(b)* synthetic gene, and M. Giband for constructing pKY-*cryIA(b)* and for valuable scientific advice.

LITERATURE CITED

Bi, I.V., L. Harvengt, A. Chandelier, G. Mergeai, and P. DuJardin. 1996. Improved RAPD amplification of recalcitrant plant DNA by the use of activated charcoal during DNA extraction. Plant Breeding. 115 : 205-206.

Castella, J.C. and G. Trébuil. 1996. La production cotonnière en Thailande. Histoire et leçons d'une crise. Agriculture et développement. 10 : 18-35.

Cousins, Y.L., B.R. Lyon, and D.J. Llewellyn. 1991. Transformation of an Australian cotton cultivar : Prospects for cotton improvement through genetic engineering. Aust. J. Plant Physiol. 18 : 481-494.

Davidonis, G.H. and R.H. Hamilton. 1983. Plant regeneration from callus tissue of *Gossypium hirsutum* L. Plant Sc. Letters. 32 : 89-93.

Deblaere, R., B.Bytebier, H. De Greve, F. Deboeck, M. Van Montagu, and J. Leemans. 1985. Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer. Nucleic Acids Res. 13 : 4777-4788.

Finer, J.J. and M.D. McMullen. 1990. Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep. 8 : 568-589.

Firoozabady, E., D. De Boer, D. Merlo, E. Halk, L. Amerson, K. Rashka, and E. Murray. 1987. Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. Plant Mol. Biol. 10 : 105-116.

Firoozabady, E. and D. De Boer. 1993. Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). In Vitro Cell Dev. Biol. 29 :166-173.

Kuhapituktum, R., A. Vermeulen, and S. Attathom. 1995. Regeneration and transformation of Thai cotton variety, pp. 47-53. *In* S. Mongkolsuk, S. Loprasert, P. Srifah (eds.), Proceedings of a Conference on Biotechnology Research and Applications for Sustainable Development. Chulalongkorn research Institute, Bangkok, Thailand.

Maïti, I.B., J.F. Murphy, J.G. Shaw, and G.A. Hunt. 1993. Plants that express a potyvirus VPg. proteinase gene are resistant to virus infection. Proc. Natl. Acad. Sci. 90 : 6110-6114.

Morel, G. and R. Whetmore. 1951. Fern callus culture. Am. J. Bot. 38 : 141-143.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15 : 437-497.

Pannetier, C., J. Tourneur, V. Le Tan, M. Mazier, and P. Couzi. 1993. Genetic engineering of cotton for insect pests management in an INRA-CIRAD research group, pp. 61-65. *In* M.C. Peeters (ed.). Cotton Biotechnolgy. Proceedings of a Meeting of the Working Group on Cotton Biotechnology. Leuven, Belgium.. REUR Technical Series 32. FAO-Rome.

Perlak, F., R.W. Deaton, T.A. Armstrong, R.L. Fuchs, S.R. Sims, J.T. Greenplate, and D.A. Fischhoff. 1990. Insect resistant cotton plants. BioTechnology. 8 : 939-943.

Perlak, F.J., R.L. Fuchs, D.A. Dean, S.L. McPherson, and D.A. Fischhoff. 1991. Modification of the coding sequence enhances

plant expression of insect control protein genes. Proc. Natl. Acad. Sci. 88 : 3324-3328.

Sardana, R., S. Dukiandjiev, M. Giband, X. Cheng, K. Cowan, C. Sauder, and I. Altossar. 1996. Construction and rapid testing of synthetic and modified toxin gene sequences *cryIA* (bande) by expression in maize endosperm culture. Plant Cell Rep. 15 : 677-681.

Trolinder, N.L. and J.R. Goodin. 1987. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep. 6 : 231-234.

Trolinder, N.L. and C. Xhixian. 1989. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep. 8 : 133-136.

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

Voo, K.S., C.L. Rugh, and J.C. Kamalay. 1991. Indirect somatic embryogenesis and plant recovery from cotton (*Gossypium hirsutum* L.). In Vitro Cell Dev. Biol. 27 : 117-124.