Obtaining Insect-resistant Cotton by Transformation with *Agrobacterium*

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ABSTRACT

A plant expression vector, pBinLK, carrying two insecticidal genes: pea lectin gene and soybean Kunitz trypsin inhibitor gene, were constructed and successfully transferred into 4 upland cotton (Gossypium hirsutum L.) cultivars, Xinluzao-1, Xinluzhong-2, Jihe-321 and Liao-9 via Agrobacterium-mediated transformation. Hypocotyl segments from aseptic seedlings were used as transgene recipients. After co-cultivation, kanamycin-resistant calli were screened, and somatic embryos and regenerated plants were obtained using various media. Transgenic cotton plants with two insecticidal genes were confirmed by ELISA, PCR and PCR Southern analysis. Bioassays demonstrated that the transgenic plants had significant resistance to larvae of cotton bollworm (Helicoverpa armigera Hübner).

Introduction

Crop plants are susceptible to attack by a wide range of herbivorous insects. It is estimated that around 13% of the total world crop production is lost to insect pests. Until recently, crop protection in an agricultural system relied exclusively on chemical pesticides that have undesirable side effects on both environmental pollution and human health (Zhou and Zhu, 1994). Transgenic technology is a useful tool that can be used in crop breeding to introduce resistance genes into host plant species.

Lectins are a group of proteins that occur widely in plants and can protect plants from insects in different ways (Etzler, 1985). Pea lectin (P-lec) comes from the seed of common bean and has very low toxicity to humans and animals, but it can remarkably inhibit growth and development of *Homopteran* insects, especially aphids. Lectins bind to the glycoproteins on the peritrophic membrane of an insect's digestive tract, affecting the insect's ability to assimilate nutrients. They can also damage the digestive tract (Chrispeels and Raikhel, 1991). Proteinase inhibitors are another group of natural insecticidal proteins from plants that inhibit the activity of proteinase in the pest mid-gut and block digestion of protein (Koide and Ikenaka, 1973; Gatehouse *et al.*, 1993; Song *et al.*, 1993). Soybean Kunitz trypsin inhibitor (SKTI) belongs to the serine proteinase inhibitor family, providing a wide spectrum of insect-resistance and can significantly inhibit *Lepidopteran* insects. Insect pests have difficulty developing tolerance to SKTI, but it is safe to humans and mammals (Pusztai *et al.*, 1992; Gatehouse, 1993).

The P-lec gene and SKTI gene that have been cloned in our laboratory (Liu *et al.*, 1995a,b; Gao *et al.*, 1997; 1998), are complementary in insecticidal mechanism and insecticidal spectra. In order to extend the insecticidal spectrum and increase pest-resistance of transgenic plants, a plant expression vector harbouring both of these genes and suitable to *Agrobacterium tumefaciens* - mediated transformation was constructed and used in cotton transformation.

Materials and Methods

Plant Recipients. Upland cotton cultivars Xinluzao-1, Xinluzhong-2, Jihe-321 and liao-9 were used.

Bacteria Strains and Plasmids. *E.coli* DH5 α was purchased from Stratagene Co., *A. tumefaciens* LBA4404 was a kind gift from Professor Bo Tian. The plasmid, pBin19 was purchased from Clonetech Laboratories Inc., while other plasmids pSKTI, pBS-LecI and pSPROK were constructed in previous research in our laboratory.

Challenging insect. The larvae of cotton bollworm used in the bioassays were bred on artificial diet at 25-28° C and 85% humidity.

Reagents. Restriction endonucleases were purchased from New England Biolabs Inc., Promega Co. and Gibco BRL Life technologies Inc. *Taq* DNA polymerase was prepared in our laboratory. *E. coli* polymerase Klenow fragment and T4 DNA ligase were purchased from Gibco BRL Life technologies Inc. DIG DNA Labelling and Detection Kit was purchased from Behringer Mannheim. ELISA (NPT-II) Kit was purchased from 5 Prime → Prime Co.

PCR Primer

P-Lec primer: 5'-ATAATGGCTTCTCTTCAAACCC-3'(5'end primer)

3'-CGTTCGACGTCTACGTATCA-5'(3'end primer)

SKTI primer: 5'-AAAATGAAGAGCACCATCTTC-3'(5'end primer)

3'-GAAAGAGCGTCACTCACTCTAGAC-5'(3'end primer)

Isolation and Clone of genes. The details of isolation and cloning of the P-lec and SKTI genes can be consulted in Lui *et al.* (1995a,b) and Gao *et al.* (1997, 1998).

Construction of anti-insect plant expression vector. A *HindIII/EcoRI* fragment of pBS-lecI including the 0.8kb P-Lec gene was inserted into pSPROK and further cloned into mini Ti plasmid pBin19 to form a P-lectin plant expression vector, pBRL3. A *BamHI/BglII* fragment from pSKTI carrying the 0.66 kb SKTI gene was inserted into the *BamHI* site of pSPROK to form pSRSK. Finally, a plant expression vector carrying both the P-lec and the SKTI gene, pBinLK (Fig.1), was obtained by inserting the *XhoI/BglII* fragment of pSRSK into the *SmaI* site of pBRL3. Finally pBinLK was transferred into *Agrobactium* LBA-4404 by electroporation.

Transformation and Regeneration. Hypocotyl segments from aseptic cotton seedlings were infected by *Agrobacterium*. After 2 days co-cultivation with *Agrobacterium* cultivation medium (MS salts + B_5 vitamin + 30g/L glucose + 0.1mg/L 2,4-D + 0.1mg/L kinetin (KT) + 0.2mM acetosyringone), callus induction and selection for transformed tissues were performed on selection medium (MS salts+B_5 vitamin + 30g/L glucose + 0.1mg/L 2,4-D + 0.1mg/L KT + 80mg/L kanamycin + 500mg/L cefotaxime). Somatic embryos and transgenic cotton plantlets were obtained on induction medium (MS salts + B_5 vitamin + 1.9g/L KNO_3 + 30g/L glucose) and differentiation medium (MS salts without NH_4NO_3 + B_5 vitamin + glucose 24g/L + 1.0g/L glutamine + 0.5g/L asparagine) using procedures described by Wang *et al.* (1998).

NPT-II ELISA. NPT-II detection followed directions of the manufacturer of NPT-II ELISA Kit.

PCR and PCR Southern. The genomic DNA was prepared from the regenerated plants by a modified CTAB method (Porebski *et al.*, 1997). PCR and PCR Southern hybridization were carried out according to conventional protocol (Southern, 1975).

Bioassay of leaf of transgenic cotton. For each bioassay fully expanded young leaves at the top of the transgenic cotton plants were harvested and placed in 20cm Petri dishes with 10 second instar larvae of bollworms. Insect survival was recorded on the 3rd, 5th, 7th, 9th, 11th days after infestation, and weights of the surviving larvae were taken on 11th day. The mortality, exuviation index, average weight and biomass of larvae were calculated, and the degree of leaf damage was measured using the method described by Wang *et al.* (1998).

Results and Discussion

The plant expression vector for insect-resistant genes. The plant expression vector pBinLK carried both the P-Lec and the SKTI gene under control of CaMV 35S promoters. These two genes complement each other in insecticidal function, and when both genes are present in a transgenic plant, stronger insecticidal

vulnerability and a broad insecticidal spectrum could be achieved than when a single gene is used. pBinLK also carried NPT-II gene as selective marker. It conferred resistance to aminoglycosides antibiotics to the transformed cells, so that kanamycin, neomycin, paromomycin, G418, etc. could be used to select for transformed cells.

Selection of transformed calli and plantlets regeneration. Resistant calli could be induced from the transformed hypocotyl segments after about 40-days cultivation on the selection medium containing kanamycin, while no any callus could be induced from the control. The resistant calli with grey and yellow colour were transferred to embryo inducing medium until embryos were formed. NPT-II detection was performed on km_r calli to further eliminate the non-transformed cells. The NPT-II positive clones were transferred to differentiation medium and plantlets were regenerated after 2-3 months. The regenerated plants were transplanted into a greenhouse and another NPT-II-ELISA was performed to confirm that they were transformed plants.

PCR and PCR Southern assays. Genomic DNA was isolated from leaves of NPT-II positive plants and amplified by PCR with P-Lec primers or SKTI primers, as appropriate. Bands of 0.8kb and 0.66kb could be amplified from DNA samples derived from NPT-II positive plants that represented the molecular weights of the P-lec and SKTI genes, respectively, while no corresponding band could be amplified from control samples. PCR Southern showed that those two bands hybridized with P-lec and SKTI gene probes, respectively. The above results confirmed that both the P-lec gene and the SKTI gene were present in the genome of the host plants.

Bioassay on transgenic plants. Bioassays were carried out on transgenic cotton plants using larvae of cotton bollworm. Eleven days after infestation, the transgenic plants showed notable resistance to larvae compared to non-transformed plants with transgenic leaves having less damage than the controls. The transgenic leaves were also significantly inhibited compared to those fed on control (Table 1).

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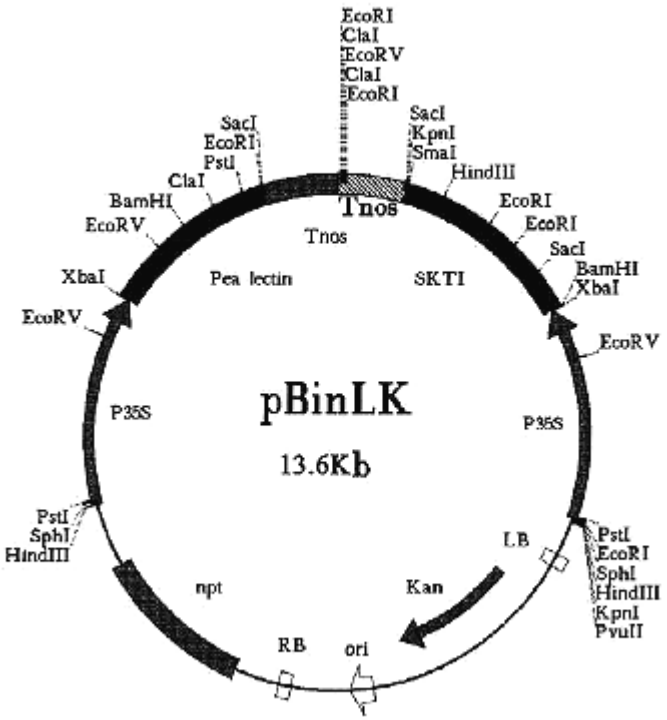
Table 1. Bioassay on transgenic cotton with two insecticidal genes*.

No. **	Mortality (%)	Exuviation index	Average weight(mg)	Biomass (mg)	Leaf Damage
CK-1	50	2.6	160.2	801	4
CK-2	40	3.2	169.0	1014	4
CK-3	40	3.1	150.6	904	4
CK-4	50	2.7	176.4	882	4
LK-1	100	1.8	0	0	0
LK-2	50	3.2	135.6	678	4
LK-3	70	1.9	42.7	128	4
LK-4	90	1.7	9	9	1
LK-5	70	2.4	51.0	153	3
LK-6	80	2.8	143.0	286	3
LK-7	90	1.3	27	27	1
LK-8	100	1.2	0	0	2
LK-9	100	1.2	0	0	1
LK-10	60	2.6	22.2	89	1
LK-11	90	1.5	20.0	20	2
LK-12	70	2.4	100.3	301	4

- Bioassay on transgenic cotton with both P-Lec gene and SKTI gene. 10 second instar larvae of *Helicoverpa armigera* Hubner were infested per leaf. ** CK-1~CK-4 represented non-transformed control plants, LK-1~LK-12 represented transgenic cotton plants. ***Leaf damage degree were demonstrated as levels 0, 1, 2, 3 and 4 which represented little damaged, mildly damaged, moderately damaged, severely damaged and completely destroyed, respectively.

Figure 1. Diagram of plant expression vector pBinLK.



- P35S: CaMV 35S gene promoter; Tnos: Poly(A) signal of nopaline synthetase gene; Pea lectin: Pea lectin gene; SKTI: Soybean Kunitz trypsin inhibitor gene; NPT: Neomycin phosphotransferase-II gene as plant selective marker; Kan: Kanamycin-resistant gene as bacterial selective marker; RB, LB: Right and left borders of T-DNA.