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Development of insect resistant transgenic cotton lines expressing *cry1EC* gene from an insect bite and wound inducible promoter

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ABSTRACT

Transgenic cotton lines were developed for high-level expression of a synthetic *cry1EC* gene from a wound inducible promoter. The tobacco pathogenesis related promoter PR-1a was modified by placing CaMV35S promoter on its upstream in reverse orientation. The resultant chimeric promoter CaMV35S(r)PR-1a expressed constitutively and was further up-regulated at the site of feeding by insects. It was induced more rapidly by treatment with salicylic acid (SA). The CaMV35S(r)PR-1a *cry1EC* expressing transgenic lines of cotton showed 100% mortality of *Spodoptera litura* larvae. The tightly regulated low-level expression of PR-1a was modified to a highly expressing constitutive expression by CaMV35S placed in reverse orientation. Salicylic acid treatment and wounding enhanced the expression further by the chimeric promoter. The leaves expressed more δ-endotoxin around the sites of insect bites. The levels of expression and induction varied among different transgenic lines, suggesting position effect. Some of the transgenic lines that expressed Cry1EC from the chimeric promoter at a low level also showed 100% mortality when induced with salicylic acid. A highly expressing insect bite and wound inducible promoter is desirable for developing insect resistant transgenic plants.

1. Introduction

Resistance to lepidopteran insect pests has been achieved in cotton by introducing genes encoding δ-endotoxin of *Bacillus thuringiensis*. Insect resistant transgenic cotton varieties expressing *cry1Ac* against the major lepidopteran pest, *Helicoverpa armigera* have been cultivated commercially in several countries. A novel gene *cry1EC* was reported by us earlier to cause 100% mortality of *Spodoptera litura* in transgenic cotton, tobacco (Singh et al., 2004), pigeon pea (Surekha et al., 2005) and ground nut (Tiwari et al., 2008). In all these cases, the insecticidal proteins were expressed constitutively by the CaMV35S promoter. However, regulated expression of δ-endotoxin gene(s) at the site of insect feeding is preferable for biological safety as a component of the insect management strategy. To achieve regulated high-level expression of *cry* gene(s), the salicylic acid inducible PR-1a promoter was modified in our study for expression of the δ-endotoxin coding gene *cry1EC*.

The PR-1a is a salicylic acid (SA) inducible promoter that is triggered in pathogenic response through salicylic acid pathway (Strompen et al., 1998). It is a tightly regulated promoter but is induced rather slowly. The expression becomes significant, 8 h after SA treatment and increases gradually to a high level till 96 h (Lodhi et al., 2008). This has been deployed earlier (Cao et al., 2001) to express δ-endotoxin gene in broccoli. However, the level of its expression was insufficient to give protection against the target pest. With an aim to enhance the level of expression of PR-1a, we provided an upstream enhancer to PR-1a, by making fusion with the CaMV35S promoter. Behaviour of the chimeric promoter construct was examined, using *gusA* reporter with and without induction by salicylic acid in tobacco plants. Finally, transgenic cotton plants expressing *cry1EC* from the chimeric PR-1a promoter were developed to evaluate their suitability in achieving high-level inducible expression of the δ-endotoxin gene.

2. Materials and methods

2.1. Binary vector

Plasmid constructs for plant transformation were prepared as described (Sambrook et al., 1989). The CaMV35S promoter with duplicate enhancer elements (Kay et al., 1987) was fused upstream of PR-1a in reverse orientation to give the chimeric promoter (CaMV35S(r)PR-1a) as shown in Fig. 1 (P_{1332}). A construct containing PR-1a promoter alone with *gusA* (P_{1329}) was used as control for comparing expression behaviour of the promoters in transgenic tobacco plants. The chimeric promoter CaMV35S(r)PR-1a was placed upstream of *cry1EC* (P_{1334}) and used to transform tobacco and cotton plants.

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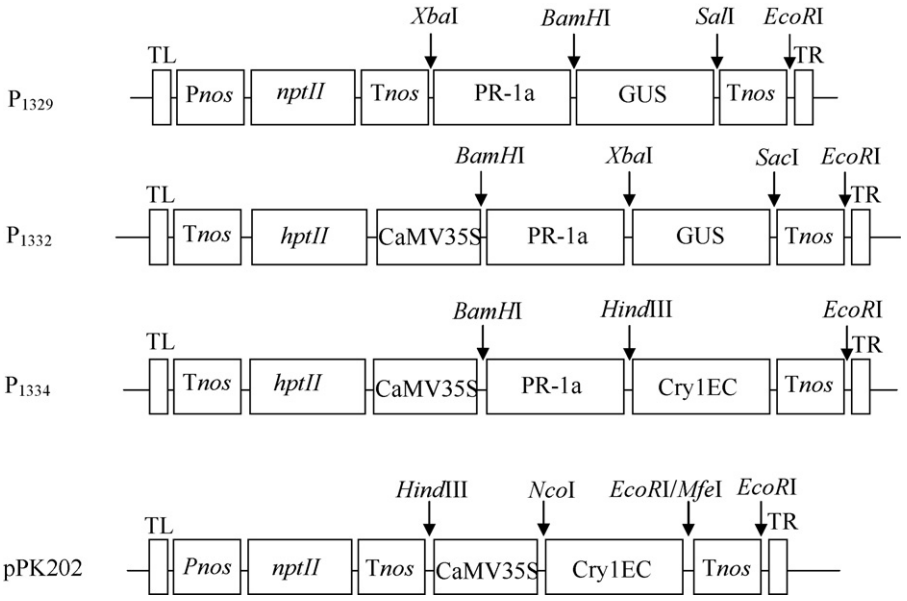

Fig. 1. Gene constructs used for transformation in tobacco and cotton. P_{1329} represents the PR-1a promoter with *gusA*; P_{1332} represents the CaMV35S(r)PR-1a promoter with *gusA*; P_{1334} represents CaMV35S(r)PR-1a promoter with *cry1EC*; pPK202 contains CaMV35S promoter with *cry1EC* gene flanked by the T-DNA borders, with nptII or hptII as selection markers, as shown.

2.2. Agrobacterium-mediated transformation of tobacco and cotton

Transgenic tobacco plants were raised after transforming *Nicotiana tabaccum* Petit Havana by *Agrobacterium*-mediated standard protocols (Horsch et al., 1985). Sterilization and germination of seeds of cotton variety Coker 310 was carried out as described earlier (Kumar and Tuli, 2004). *Agrobacterium tumefaciens* (LBA4404) harbouring binary vector (P_{1334}) was streaked on YEB medium, a single isolated colony was picked for primary culture in 5 mL YEP medium containing antibiotics rifampicin 50 mg L^{-1}, streptomycin 250 mg L^{-1} and kanamycin 50 mg L^{-1}. Secondary culture was inoculated in 50 mL of YEP medium and grown at 28 °C, 200 rpm in dark. It was harvested at an O.D. of 1.4 and centrifuged at 6000 rpm for 5 min. Pellet was resuspended in 100 mL IM (induction medium containing NH_4Cl_2 1 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.3 g L^{-1}, KCl 0.15 g L^{-1}, $CaCl_2$ 0.01 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 0.0025 g L^{-1}, KH_2PO_4 0.272 g L^{-1}, MES 0.39 g L^{-1} and glucose 5.0 g L^{-1} at pH 6.0) for 4 h at 175 rpm and 26 °C. *Agrobacterium* was pelleted, resuspended in 100 mL MSO medium (MS salts, B5 vitamins, MES 1.95 g L^{-1} and glucose 20 g L^{-1} at pH 5.65) and incubated for 2 h at 150 rpm and 25 °C.

The explants used for *Agrobacterium*-mediated transformation were 8 mm long hypocotyls and 1 cm^2 cotyledon pieces taken from 5- to 7-day-old healthy germinated seedlings. The explants were infected with *Agrobacterium* suspension for 15–20 min, blot dried with sterile Whatman filter paper (Cat. 3030917) and transferred to co-cultivation medium (MS salts, B5 vitamins, myoinositol 100 mg L^{-1}, glucose 30 g L^{-1} and agar 0.8%) for 3 days at 28 °C in diffused light. The explants were given cefotaxime (250 mg L^{-1}) wash for 5 min, followed by four rinses with autoclaved distilled water. The explants were blot dried and transferred to callus induction medium comprising MS salts, B5 vitamins, myoinositol 100 mg L^{-1}, 2,4D 0.5 mg L^{-1}, BAP 0.2 mg L^{-1} solidified with 2.2 g L^{-1} phytagel and 0.75 g L^{-1} $MgCl_2$ (Kumar and Tuli, 2004) containing hygromycin B 50 mg L^{-1} and augmentin 250 mg L^{-1}. All growth regulators were added before autoclaving at 121 °C for 15 min. The pH of the medium was adjusted to 5.8 prior to autoclaving.

In the callus induction medium, the explants were sub-cultured after intervals of 3 weeks. After 2–3 cycles on growth regulator medium, the actively growing calli were transferred for induction of embryogenic calli (loose, friable and cream-coloured) to the hormone free medium (MS salts, B5 vitamins, myoinositol 100 mg L^{-1}, glucose 30 g L^{-1} and agar 0.8%) containing hygromycin B 50 mg L^{-1} and augmentin 250 mg L^{-1} for callus proliferation.

The embryogenic calli were maintained by regular sub-culturing every 3 weeks, on hormone free medium containing hygromycin B 50 mg L^{-1} and augmentin 250 mg L^{-1}. Globular, heart-shaped and torpedo-shaped embryos were obtained on hormone free medium. Embryos longer than 5 mm were transferred to germination medium containing 1/2 strength MS salts, 1/2 strength B5 vitamins, myoinositol 100 mg L^{-1}, sucrose 20 g L^{-1}, $MgCl_2$ 750 mg L^{-1} and phytagel 2.2 g L^{-1} (MSG). At all stages, the cultures were incubated in 60 μmol m^{-2} s^{-1} light intensity, with 16 h photoperiod in petri-plates sealed with parafilm. Rooted embryos that produced shoot-apex were transferred to 7.5 cm × 7.5 cm × 10 cm Planton boxes from Tarsons Products Ltd. (Kolkata, India) containing 100 mL MSG medium. The plantlets were transferred to pots for hardening. Hardening of plants was carried out in plastic pots as described earlier (Kumar and Tuli, 2004).

2.3. Molecular analysis

2.3.1. PCR amplification

Genomic DNA was isolated from plants, 1 month after transfer to pots, by CTAB method (Murray and Thompson, 1980). The genomic DNA (100 ng) was used for PCR amplification of the insert with *cry1EC* specific forward (5′ CCA GAG AAC GAG ATC TTG GAC 3′) and reverse (5′ AGT ATT GTA CCA TCT AAC AGC GTA 3′) primers.

2.3.2. Southern hybridization and ELISA

About 20 μg of genomic DNA was digested with BamHI and separated by electrophoresis in 0.8% agarose gel. The gel was washed, depurinated and neutralized. The DNA was transferred to positively charged nylon membrane (Hybond N^+, Amersham Life Sciences). A fragment of 578 bp was amplified from 5′ site of *cry1EC* gene. This amplified product was eluted from the gel and radiolabelled with α^{32} P-dCTP by random priming. Since there is no BamHI cleavage site within *cry1EC*, the number of fragments hybridizing in Southern hybridization indicated the number of insertion