Engineering Turf Grass for Resistance against Certain Coleopteran Pests Using *Bacillus thuringiensis cry8Da* Gene

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A transgenic grass was engineered with the cry8Da gene from a newly discovered *B. thuringiensis* strain called SDS-502. The *cry8Da* gene was cloned in a vector, pBI221, by replacing the *gusA* gene. The plasmid vector containing cry8Da along with another plasmid harboring the GFP gene were introduced to calli derived from mature seed embryos using the particle bombardment method. GFP was used as an indicator of transformed grass cells. Grass cells showing green fluorescence were selected and grown on a plant tissue culture medium. The transgenic grass containing the *cry8Da* gene showed strong resistance against the feeding attack by the Japanese beetle.

The *cry8Da* gene was cloned from Bt SDS-502 following the method described in Asano *et al*. (1). A fragment of the *cry8Da* gene containing the active region was amplified by PCR using two primers having the sequences, 5'- GGATCCCATGAGTCCAAATAATCAAAATG, 5'-CCCGGGTCACACATCTAGGTCTTCTTCTGC, and the cloned *cry8Da* gene as a template. The PCR amplified gene fragment was then cloned in pGEM-T-Easy (Promega) following the instructions given by the plasmid manufacturer. The cloned gene was sequenced to confirm the sequence of the *cry8Da* gene. The PCR amplified *cry8Da* gene in pGEM-T-Easy was excised out with *Bam*HI and *Sac*I utilizing these sites provided in pGEM-T-Easy and cloned in pBI221 (Clonetech) which had been cut with *Bam*HI and *Sac*I to remove the *gusA* gene. The resulting plasmid derived from pBI221 in which the *cry8Da* gene cloned was called p35S-cry8DT and used in plant transformation. The other plasmid (p35S-GFP, Clonetech) used in the plant transformation. The concentration of p35S-cry8DT and p35S-GFP plasmids was adjusted to 2µg/ul before used in the turf grass transformation

Transformation was performed with particle gun transformation technology. The user manual provided by the particle gun (GIE-III IDER) manufacturer Frontier Science, was essentially followed. Calli grown on the callus induction medium was placed on a high osmotic pressure (HOP) medium consisting of the entire ingredients in the callus induction medium and 0.5 M mannitol overnight. The calli placed on the HOP medium were cut in small sizes of about 1 mm^3. About 40 callus pieces were placed on the callus-induction medium used in one shot. 10 µg of gold particles (1.5 micron) in 4 µl ethanol were coated with 1 µg of p35S-cry8DT and p35S-GFP and were shot once callus pieces from 12 cm above the sample stage.

The transformed callus was then transferred on the fresh medium. Each callus piece was placed on the medium in about 1 cm apart. Within a few days, transformed cells showed GFP fluorescence. After one week, cell masses showing strong GFP fluorescence were excised out from each lump of callus and transplanted on a regeneration medium. The regeneration medium is the same as for the callus induction media except that no hormones were added. The transformed cells were grown on the medium at 24 °C under 16 hr light per day. After 4 weeks, 3 GFP positive tall fescue callus pieces developed into whole plants with leaves and roots.

When transformed callus developed into whole plants, a portion of leaves was taken from each plant and DNA was extracted from the leaf samples using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The *cry8Da* gene in the samples were analyzed by PCR using two primers having the sequences, 5'-GGATCCCATGAGTCCAAATAATCAAAATG, 5'-CCCGGGTCACACATCTAGGTCTTCTTCTGC. If the *cry8Da* gene existed in a template DNA sample (plant leaf extracts), these primers should produce a 2 kb amplified fragment. All leaf samples derived from

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GFP positive callus pieces showed this 2-kb fragment by PCR analysis confirming the *cry8Da* gene inserted into the plant genome (Fig. 1). Three lines from tall fescue were selected based on this PCR analysis for insect resistance tests.

Regenerated whole plants from transformed calli were transferred to 15cm-diameter pots containing potting soil. About six plants were planted in each pot. In each pot, two third-instar Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabeidae), larvae which had been collected from a grass field were released and were allowed to feed on grass roots for one month. The insects consumed the roots of the plants that were not insect-resistant and killed the plants. On the other hand, plants that were positive for *cry8Da* by PCR analysis showed resistance to the Japanese beetle, which survived (Fig. 2).

Acknowledgments

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Reference

1. **Asano, S., C. Yamashita, T. Lizuka, K. Takeuchi, S. Yamanaka, D. Cerf, and T. Yamamoto.** 2003. A strain of *Bacillus thuringiensis* subsp. *galleriae* containing a novel *cry8* gene highly toxic to *Anomala cuprea* (Coleoptera: Scarabaeidae). Biological Control **28:** 191-196.

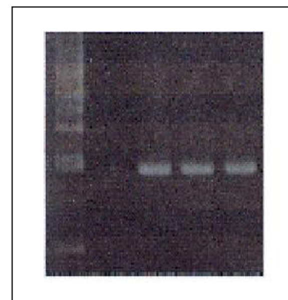
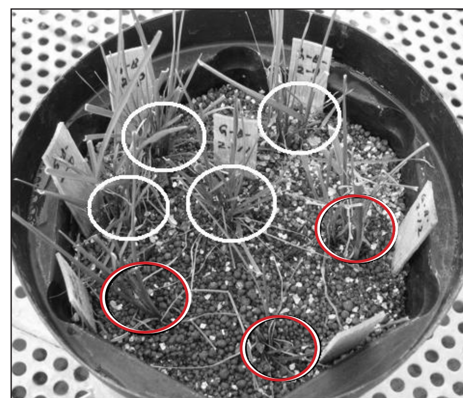


FIG. 1. PCR analysis of transformed turf grass showing the *cry8Da* gene in a form of a 2 kb band (right three lanes). The left lane is a size marker. The second lane from left is a negative control obtained from non-transgenic grass.

A



B

FIG. 2. Picture of a pot contains several lines of transgenic turf grass. The pot contains two third-instar Japanese beetle larvae **(A)** which were allowed to consume the grass roots for one month **(B)**. Some plants (top left four plants, white circled) showed resistance while the others (right and bottom plants, red circled) were killed by the insects.