J. Amer. Soc. Hort. Sci. 122(2):158–162. 1997.

# The Effect of Growth Regulators and Antibiotics on Eggplant Transformation

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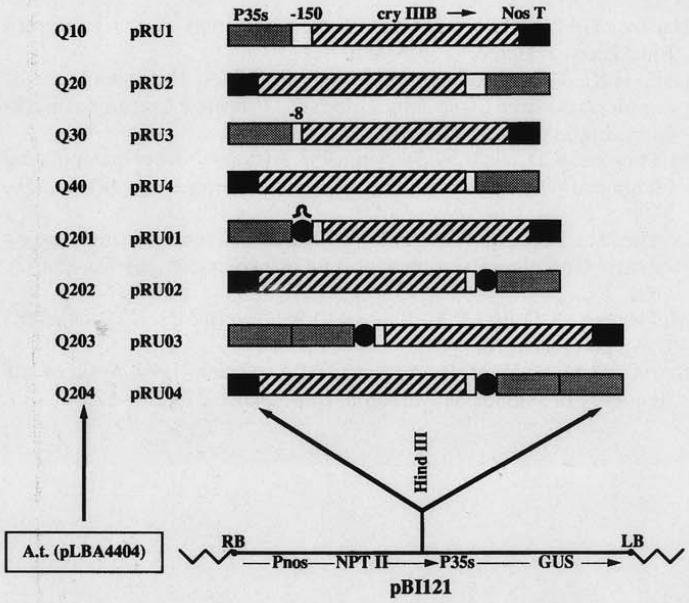
Additional index words. shoot regeneration, *Solanum melongena*

Abstract. **A protocol with a high rate of transformation and regeneration of 'Hibush' eggplant (*Solanum melongena* L.) has been developed. This protocol used leaves of in vitro-grown seedlings as a source of explants. The shoot regeneration culture medium contained 0.1 μM thidiazuron (TDZ) combined with 10 to 20 μM $N^6$-[isopentyl] adenine (2iP). Adding TDZ significantly improved regeneration efficiency and produced a mean of 15 buds and 3 to 4 shoots per explant. When explants were cocultivated with *Agrobacterium tumefaciens* strains Q10, Q20, Q30, Q40, Q201, Q202, Q203, or Q204 containing the native *cryIIIB Bacillus thuringiensis (Bt)*, neomycin phosphotransferase (*NPTII*), and β-glucuronidase (*uidA*) genes, a callus/bud regeneration frequency of 38.8% was observed on the selection medium. Kanamycin at 50 $\mu g \cdot mL^{-1}$ was most effective in selecting for transgenic buds and shoots. Augmentin at 300 $\mu g \cdot mL^{-1}$ was used to eliminate *A. tumefaciens*. Augmentin also enhanced shoot proliferation. A transformation/regeneration efficiency of 20.8% was observed for shoot production. More than 400 putative transgenic plants have been produced with this method. From 50 putative transgenic plants, gene integration has been confirmed with Southern blot analysis and progeny tests.**

Transferring genetic information into the genome of a plant species by recombinant DNA techniques has become an important strategy in basic plant biology studies as well as in improving cultivated plants. However, a severe impediment to the applicability of this approach with many plant species is the inefficacy of a reliable transformation and regeneration procedure in vitro. A review (Van Wordragen and Dons, 1992) describes *Agrobacterium tumefaciens*-mediated transformation of plant species, the most widely used transfer system in plants, and indicates that only a few species (model plant species) can be transformed and regenerated routinely with commonly used procedures. Most plants, the so-called recalcitrant species, require empirical development of a particular transformation protocol for a particular species. In developing such species-specific transformation protocols, efficiency should be an important attribute in evaluating various protocols. This is of particular importance in cases for which it is necessary to secure a large population of transformed genotypes to subsequently screen and select desired traits.

A previous eggplant transformation study (Rotino and Gleddie, 1990) reported a transformation efficiency of 7%. Repeated attempts to increase the number of eggplant transformants by their method and those of Guri and Sink (1988) were unsuccessful with our experimental material ('Hibush'). In the course of producing >400 primary transgenic eggplants carrying a native gene from *Bacillus thuringiensis* (*Bt* gene codes for an insecticidal protein), we investigated systematically the effect of some major factors involved in the outcome of a transformation procedure for 'Hibush' eggplant. As a result, we have developed a reliable protocol for transforming and regenerating eggplant explants in vitro. This paper presents the main operational steps in this protocol and the most critical factors responsible for making this procedure efficient.

Fig. 1. Q Series. Plasmid constructs pRU1 to pRU04 ligated into pBI121 at *Hind III* restriction site, producing new *Agrobacterium tumefaciens* strains Q10 to Q204.




## Materials and Methods

**Plant material and stock plants.** Seeds of 'Hibush' eggplant (*Solanum melongena*) were surface-sterilized in 1.050% sodium hypochlorite and a few drops of Tween-20 for 20 min followed by five rinses (5 min each) with sterilized-distilled water, incubated overnight in a small film of sterile distilled water (10 mL), and then surface-sterilized a second time with 0.525% sodium hypochlorite for 15 min followed by three final rinses (5 min/rinse) with sterilized-distilled water. Seeds were cultured on half-strength Murashige and Skoog (MS) (1962) basal salts and vitamins containing 2% sucrose and 0.6% agar (gum-agar, USB, Cleveland). Stock plants were maintained by excising 2 cm of the shoot tip and subculturing them into glass jars containing 30 mL MS medium. Once shoots developed six to eight vegetative leaves, leaves and internodal stem segments were excised and used for regeneration and transformation experiments.

**Regeneration from leaf and stem explants.** Leaf segments (3 × 8 mm) and stem segments (10 mm in length) were initially

Received for publication 8 Mar. 1996. Accepted for publication 14 Nov. 1996. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.
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cultured on media used by other researchers for eggplant transformation and regeneration. All these media used MS basal salts and vitamins, 2% sucrose, and 0.6% agar along with the following plant growth regulators: 10 μM zeatin (Guri and Sink, 1988), 5 μM zeatin (Rotino and Gleddie, 1990), and 5 and 10 μM BA along with either 0 or 1 μM 2,4-D (Gill, 1994). In addition, leaf discs were cultured on media containing 0, 0.01, 0.1, or 1.0 μM thidiazuron (TDZ) combined with 0, 5, 10, or 20 μM $N^6$-[isopentyl] adenine (2iP). After 4 weeks, bud primordia and shoots were counted. Shoot regenerants were then transferred to shoot elongation media consisting of MS salts and vitamins, 2% sucrose, and 0.6% agar without growth regulators or with either 0.5 or 1.0 μM zeatin.

**Bacteria and plasmids.** Some of the plasmid constructs with the native *cryIIIB* gene used in these transformation experiments have been described recently (Chen et al., 1995). For these experiments, eight constructs were used that contained the coding region of the *cryIIIB* gene driven by the CaMV 35S and NOS regulatory elements (Fig. 1). PRU1 and pRU2 contained a 150-bp leader sequence, while the remaining constructs contained an 8-bp leader sequence. PRU01, pRU02, pRU03, and pRU04 contained a translation enhancer (Gallie et al., 1987). PRU03 and pRU04 contained double-35S promoters. In all odd numbered constructs, the *Bt* gene was inserted in the same orientation of flanking selective marker and reporter genes; in even numbered constructs, the orientation was in the opposite direction. All constructs were inserted into the *Hind III* polylinker site of pBI121 (Clontech, Palo Alto, Calif.) and transferred into *A. tumefaciens* LBA4404 (Clontech). The new strains were consecutively identified as Q10, Q20, Q30, Q40, Q201, Q202, Q203, and Q204 (Q series). Bacterial cells were grown in YEB (yeast extract–beef extract) medium (1 g yeast, 5 g beef extract, 5 g peptone, 5 g sucrose, 0.5 g $MgSO_4$, pH 7.2 in 1 L) containing 25 μg·mL$^{-1}$ kanamycin, at 28 °C and at 250 rpm for 48 h. Bacterial cells were pelleted and resuspended in liquid MS medium at its original titer (between 1 to 1.5 $OD_{600}$).

**Plant transformation.** Following the protocol of McCormick et al. (1986), leaf segments (3 × 8 mm) and stem explants (10 mm in length) were excised and placed on a shoot regeneration (SR) medium consisting of MS basal salts and vitamins, 10 μM 2iP, 0.1 μM TDZ, 2% sucrose, and 0.6% agar, pH 5.8, for 48 h. Explants were inoculated for 1 min with one of the *A. tumefaciens* strains of the Q series (resuspended in liquid MS medium), blotted, and placed back onto the SR medium. After 48 h, the explants were blotted again and cultured on SR selection medium containing 300 μg·mL$^{-1}$ augmentin and 50 μg·mL$^{-1}$ kanamycin. Explants were cultured in petri plates (100 × 15 mm) at 24 °C, for a 16-h photoperiod under fluorescent lights with a photon flux of 50 μmoL·m$^{-2}$·s$^{-1}$, and subcultured every 2 to 3 weeks onto fresh selection medium. After 6 to 8 weeks, larger petri plates (100 × 25 mm) were used. Ten separate *A. tumefaciens* cocultivation experiments, with all eight strains, were conducted with 1250 leaf explants. Once small shoots (1 cm in length) were observed, these were subcultured onto an elongation medium consisting of MS basal salts and vitamins, 1 μM zeatin, 2% sucrose, 0.6% agar, and 300 μg·mL$^{-1}$ augmentin. After 2 weeks, nonrooted shoots (3 to 4 cm in length) were dipped in 0.1% IBA (hormodin #1, Merck Chemicals) and then transferred to a 2 sphagnum peat : 1 vermiculite (by volume) mix and placed under intermittent mist for 1 to 2 weeks to induce rooting.

**Effect of antibiotics on regeneration.** Leaf segments (3 × 8 mm) were cultured on SR medium containing the following antibiotics: 150 μg·mL$^{-1}$ augmentin (4 amoxicillin : 1 clavulanic acid, SmithKline Beecham, Philadelphia), 300 μg·mL$^{-1}$ augmentin, 250 μg·mL$^{-1}$ cefotaxime (Calbiochem, La Jolla, Calif.), 500 μg·mL$^{-1}$ cefotaxime, 250 μg·mL$^{-1}$ carbenicillin, or 500 μg·mL$^{-1}$ carbenicillin. Three plates of each treatment with 10 leaf discs per plate along with three plates with SR medium without any antibiotic were used. After 3 weeks, data on number of buds and shoots per explant were taken and observations on morphology and health of shoots were made. In a second experiment, leaf discs were cocultivated with *A. tumefaciens* LBA4404 containing pBI121 (Clontech) prepared as described above. After cocultivation for 48 h, explants were blotted and placed on plates containing the various antibiotics listed above, but also containing 50 μg·mL$^{-1}$ kanamycin. Two plates per treatment with 10 leaf discs per plate were used. For 3 months, explants were subcultured every 2 weeks, and the used plates were immediately washed with 3 mL of sterile distilled water. A

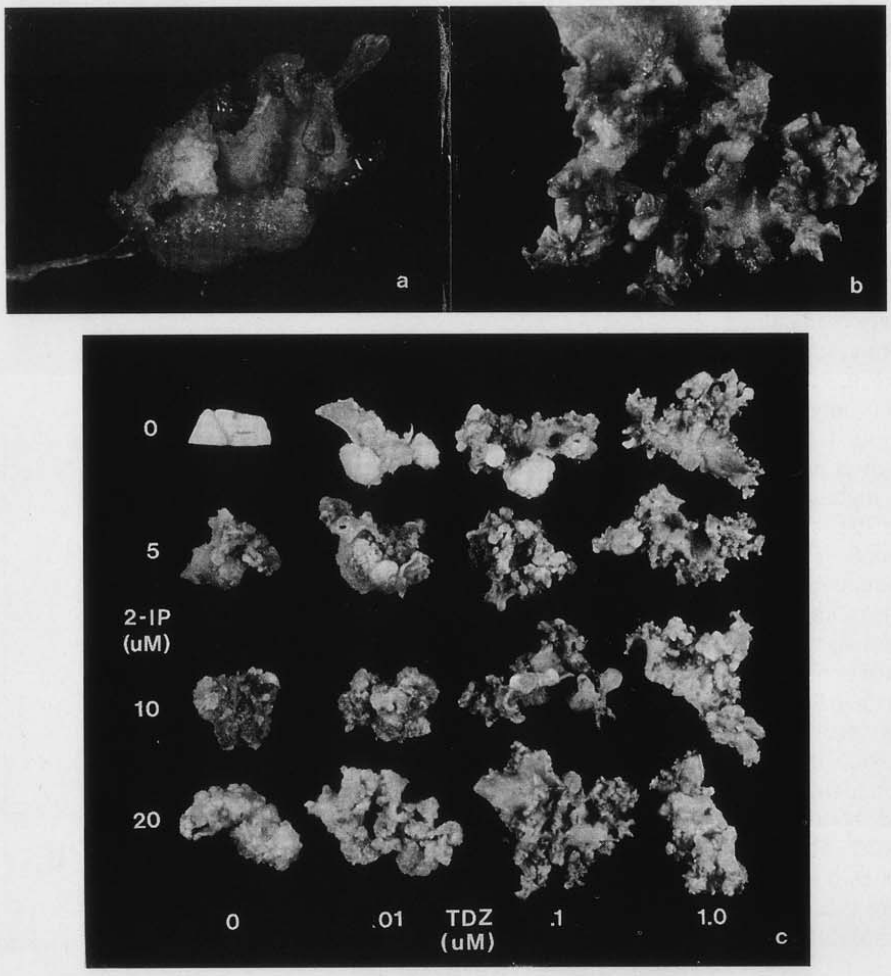



Fig. 2. (**a**) Leaf explant from media containing 2 μM zeatin. Note large amount of calli and few buds or shoots. (**b**) Leaf explant from media containing 0.1 μM thidiazuron (TDZ) and 10 μM $N^6$-[isopentyl] adenine (2iP). Note large number of bud primordia. (**c**) Leaf explants from factorial media with TDZ (X axis, left to right, 0, 0.01, 0.1, 1.0 μM) and 2iP (Y axis, top to bottom, 0, 5, 10, 20 μM) 4 four weeks culture.

mL of bacterial slurry was removed, and the bacterial titer was determined by dilution and plating onto YEB medium containing 25 $\mu g \cdot mL^{-1}$ kanamycin.

In a third experiment, the effect of kanamycin concentration on regeneration from cocultivated plant material was investigated. Leaf discs were cocultivated in *A. tumefaciens* containing pBI121 prepared as described above. After 48 h, the leaf discs were blotted and cultured on SR medium containing 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 $\mu g \cdot mL^{-1}$ kanamycin along with 300 $\mu g \cdot mL^{-1}$ augmentin. Control, noninoculated explants were cultured on SR medium containing the same concentrations of kanamycin. Tissue was subcultured every 2 weeks. After 8 weeks, putative transformed growing points (callus and/or buds) were counted. The number of GUS-positive plants was determined after 5 months.

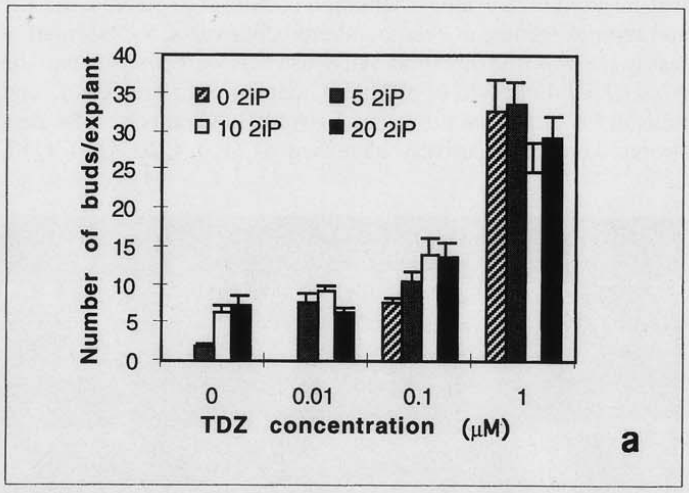
**Transgenic plant assays.** Leaf tissue from shoots was assayed by the fluorogenic GUS assay (Jefferson, 1987). Primary transgenic plants and $R_1$ progenies were subjected to the GUS assay. Genomic DNA of putative transformed plants was extracted according to Junghans and Metzlaff (1990). Five to ten micrograms DNA was restricted with *Eco RV* and electrophoresed on a 0.9% agarose gel. The DNA was blotted onto a nylon membrane according to Maniatis (1982). Prehybridization, hybridization, detection, and randomly primed DIG-labeled probe preparation were carried out according to the Boehringer Mannheim Genius System (Indianapolis). The DNA probe was a 1.2-kb segment of the native *cry IIIB* gene random primed-labeled with digoxigenin-11-dUTP.

## Results and Discussion

**The effect of growth regulators on regeneration from leaf tissue.** Leaf explants incubated on regeneration media containing 5 to 10 μM BA (Gill, 1994) developed mostly callus and a few vitrified shoots. Incorporating 5 μM zeatin (Rotino and Gleddie, 1990) or 10 μM zeatin (Guri and Sink, 1989) into the medium produced abundant callus on almost all the explants, but only a few normal shoots were observed (Fig. 2a). In contrast, a high frequency of regeneration was observed on the media containing TDZ and 2iP. After only 2 weeks in culture, many tiny green buds could be seen along the cut edge of the explant tissue (Fig. 2b). Many these buds developed into shoots after 4 weeks in culture. Differences in production of callus, bud primordia, and shoots were clearly seen between different treatments (Fig. 2c). Media containing 0 or 0.01 μM TDZ produced either callus only or callus with a few buds and shoots. All media containing 1 μM TDZ, regardless of 2iP concentration, produced the highest number of bud primordia (Fig. 3a). Media that produced the highest number of shoots contained 0.1 μM TDZ with either 10 or 20 μM 2iP (Fig. 3b). All bud primordia did not develop into shoots. Therefore, numbers of shoots per explant were lower than numbers of buds per explant. After 4 weeks, the former medium had produced 13.9 bud primordia and 3.2 shoots per disc, while the latter had produced 13.5 primordia and 4.1 shoots per disc. Twice as many buds formed on explants in media with 1.0 μM as those on 0.1 μM TDZ. However, more than twice as many normal shoots were recovered from media that contained 0.1 μM TDZ. These results unequivocally indicated that TDZ was the critical component of these media in bud production. The optimal media for bud and shoot development contained 0.1 μM TDZ and either 10 or 20 μM 2iP. The former medium was selected for transformation studies as the SR medium. These results support previous observations that TDZ enhances shoot regeneration of other plant species (Fiola et al., 1990; Szasz et al., 1995; Tsai et al., 1994).

Further development and elongation of 0.5- to 1.0-cm shoots was enhanced by adding 1.0 μM zeatin to MS medium (data not presented). In general, leaf explants produced more shoots than stem explants. Out of 96 stem explants, only 46% regenerated normal shoots; whereas, all leaf explants regenerated multiple shoots. It was observed that the apical ends of stem segments regenerated buds and shoots, while the basal ends produced only friable callus.

**Comparison of shoot development from controls and cocultivated tissues.** Differences in regeneration capacity were observed between control and inoculated explants. With control explants, shoots developed directly from explant tissue with little callus production (Fig. 2b). With cocultivated explants, callus developed first along the cut surface, primarily along the midvein, from which buds differentiated and developed into normal shoots. With cocultivated explants cultured on kanamycin, it took 4 to 6 weeks for callus development, 7 to 11 weeks for bud differentiation, and 12 to 15 weeks for shoot development and elongation (Fig. 4). Regeneration from control explants was observed 2 weeks following incubation, and unrooted plantlets were transferred to the greenhouse during the sixth week. Similar observations have



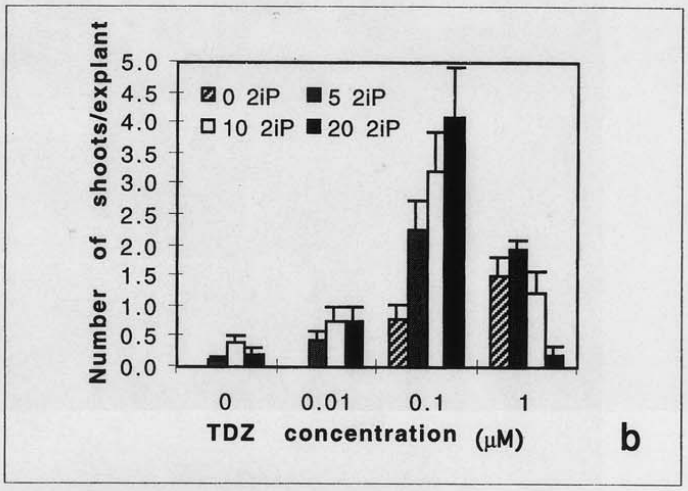




Fig. 3. Combined thidiazuron (TDZ) and 2iP effect on bud (**a**) and shoot (**b**) regeneration from eggplant leaf discs.

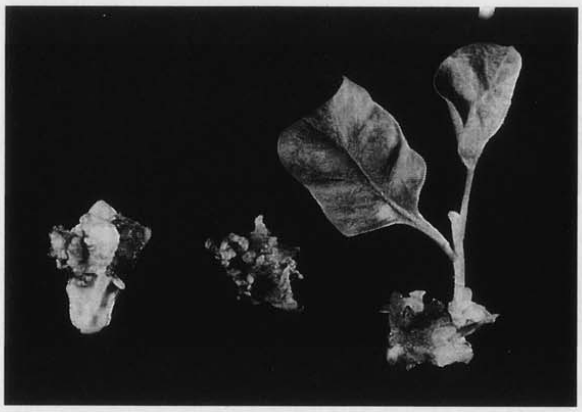


Fig. 4. Development of callus, buds, and shoots from cocultivated leaf discs on shoot regeneration (SR) selection medium. Left to right, after 4 to 6 weeks, 7 to 11 weeks, and 12 to 15 weeks respectively.

been reported by Rotino and Gleddie (1990).

**Effect of antibiotics on regeneration.** Augmentin was used to eliminate *Agrobacterium* after cocultivation. Since most recent plant transformation protocols have used either carbenicillin or cefotaxime for this purpose and because augmentin has not been extensively used, its effects on regeneration were examined. Leaf segments cultured on SR medium containing 300 $\mu g \cdot mL^{-1}$ augmentin induced high numbers of buds, usually clustered along the cut surface. Bud quality and the time it took to produce the buds were similar to those from control tissue cultured on SR medium without augmentin. However, there were significantly more buds when the medium contained 300 $\mu g \cdot mL^{-1}$ augmentin when compared to controls or media containing 500 $\mu g \cdot mL^{-1}$ cefotaxime (Table 1). Lower levels of augmentin (150 $\mu g \cdot mL^{-1}$) and cefotaxime (250 $\mu g \cdot mL^{-1}$) produced similar numbers of buds and shoots as their respective higher concentrations. Carbenicillin was almost as effective as augmentin in bud production, but it was only half as effective in shoot production (data not presented). Therefore, it is evident that augmentin produced no inhibitory effects on regeneration and possibly enhanced regeneration. Augmentin (300 $\mu g \cdot mL^{-1}$) was extremely effective in eliminating *Agrobacterium* from the explants after 3 months, and it was similar to 500 $\mu g \cdot mL^{-1}$ cefotaxime in this respect (data not presented). Carbenicillin at either concentration did not effectively eliminate *Agrobacterium,* since a new burst of bacterial growth was observed after 8 weeks in culture, presumably due to the development of resistance to the antibiotic. Neither 150 $\mu g \cdot mL^{-1}$ augmentin nor 250 $\mu g \cdot mL^{-1}$ cefotaxime completely eliminated *Agrobacterium*. In our protocol, once shoots were transferred to the elongation medium and after 3 months of culture, augmentin was removed from the SR medium and no bacterial growth was observed.

'Hibush' eggplant was sensitive to kanamycin. No growth of any kind (swelling, callus, or buds) was observed with control noninoculated leaf discs grown on 10 to 100 $\mu g \cdot mL^{-1}$ kanamycin. However, once leaf explants were cocultivated with *Agrobacterium,* their ability to produce callus and buds in the presence of kanamycin increased. Regression analysis showed that the number of growing points (callus or buds) from cocultivated leaf discs significantly decreased at 70 $\mu g \cdot mL^{-1}$ kanamycin after 2 months (Fig. 5). The number of transformed shoots was determined by GUS assay from the total population of regenerated and rooted shoots in the same experiment after 5 months. At relatively low concentrations of kanamycin, (30 or 40 $\mu g \cdot mL^{-1}$) 22% or 45%, respectively, of shoots tested positive for GUS activity. This was expected, since selection of transformants was presumably not adequate at low concentrations, which led to competition from escapees. The highest percent (68%) of GUS-positive shoots was obtained on medium containing 50 $\mu g \cdot mL^{-1}$ kanamycin, while regenerating explants on 60, 70, and 80 $\mu g \cdot mL^{-1}$ produced 43%, 0%, and 0% GUS-positive plants respectively. Thus, at higher kanamycin concentrations, even transformed tissue failed to regenerate shoots. In almost all previously reported eggplant transformation experiments, a high kanamycin concentration (100 to 200 $\mu g \cdot mL^{-1}$) was used to select for transformants (Fari et al., 1995; Filippone and Lurquin, 1989; Guri and Sink, 1988; Rotino and Gleddie, 1990). Our study showed that a lower kanamycin concentration effectively selected for transformants.

**Regeneration and transformation efficiency.** Based on the above results, transformation was routinely carried out as follows: 1) axenic leaf tissue was excised and precultured for 48 h on SR medium; 2) leaf explants were cocultivated with *A. tumefaciens* for 1 min; 3) after blotting, tissue was cultured on SR medium for another 48 h and then transferred to a selection medium containing 50 $\mu g \cdot mL^{-1}$ kanamycin and 300 $\mu g \cdot mL^{-1}$ augmentin. There was considerable variability in the percentage of explants producing callus and/or buds among different experiments. Regeneration efficiency, defined as percentage of explants producing growing

Table 1. The effect of different antibiotics on shoot regeneration from eggplant leaf explants.

| | Concn | Mean no.[z] ±SE | |
|---|---|---|---|
| Antibiotic | ($\mu g \cdot mL^{-1}$) | Buds/explant | Shoots/explant |
| Augmentin | 300 | 36.6 ±2.8 | 4.0 ± 0.3 |
| Control | 0 | 19.3 ± 1.7 | 3.9 ± 0.3 |
| Cefotaxime | 500 | 17.1 ± 1.3 | 2.5 ± 0.4 |

[z]Fisher's protected LSD at $P = 0.05$ (n = 30).

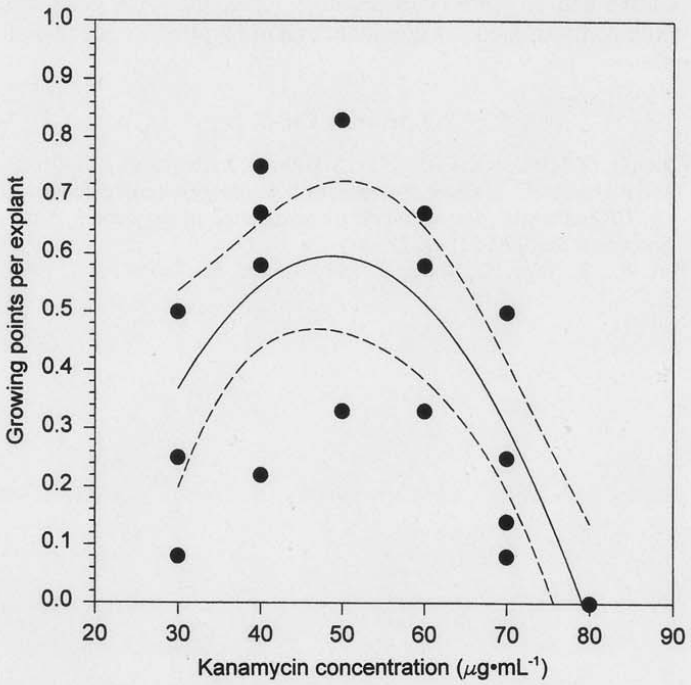



Fig. 5. Effect of kanamycin concentration on percent of cocultivated leaf discs to producing growing points after 2 months. Predicted values of Y are represented by solid line ($Y = -0.95 + 0.06 \times Rate_1 - 0.0006 \times Rate_2$, $R^2 = 0.60$). Dashed lines represent 95% confidence band.

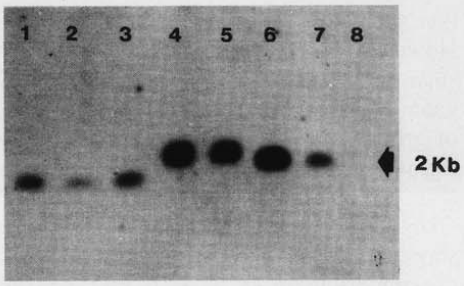



Fig. 6. Southern blot of eggplant DNA digested with *Eco RV*. The blots were probed with a 1.2-kb DIG-labeled *cryIIIB* gene. Washing and detection was according to the nonradioactive Genius system (Boehringer-Mannheim). DNA from putative transgenic and control plants as follows (T = transgenic plant): lane 1) T Q20; lane 2) T Q30; lane 3) T Q40; lane 4) T Q201; lane 5) T Q202; lane 6) T Q203; lane 7) T Q204; lane 8) control, not cocultivated plant.

points (callus or buds), varied between 20% to 63% with a mean of 38.8%. This may have been due to physiological and developmental conditions of leaflets at the time of explant excision, which is an important factor in transformation efficiency (Van Wordragen and Dons, 1992). In addition, the titer of the bacterial culture might have influenced transformation (Lin et al., 1994).

Shoots that emerged in the presence of kanamycin were screened for GUS. A total of 456 GUS-positive transformed plants from 10 different experiments was identified. Southern blot analysis conducted on 50 putative transgenic plants confirmed integration of the *cryIIIB* gene into the genomes (Fig. 6). Out of 305 GUS-positive plants from one of the experiments, 100 independent transformants have been identified from individual explants. In other words, some callus nodules produced multiple sister plants. In this protocol, transformation efficiency was defined as the number of independently transformed GUS-positive plants produced per number of cocultivated explants, and it was 20.8% for this particular experiment. Integration of the *cryIIIB* gene was further verified by GUS analysis of $S_1$ offspring from one of the primary transformants ($S_0$). Out of 67 seedlings, 51 were GUS-positive and 16 were GUS-negative. Thus, the GUS gene was sexually transmitted as a dominant trait in a typical 3:1 Mendelian ratio.

## Literature Cited

Chen, Q., G. Jelenkovic, C.-K. Chin, S. Billings, J. Eberhardt, J. Goffreda, and P. Day. 1995. Transfer and transcriptional expression of coleopteran *cryIIIB* endotoxin gene of *Bacillus thuringiensis* in eggplant. J. Amer. Soc. Hort. Sci. 120:921–927.

Fari, M., I. Nagy, C. Marta, J. Mityko, and A. Andrasfalvy. 1995. *Agrobacterium* mediated genetic transformation and plant regeneration via organogenesis and somatic embryogenesis from cotyledon leaves in eggplant (*Solanum melongena* L. cv. 'Kecskemeti lila'). Plant Cell Rpt. 15:82–86.

Filippone, E. and P.F. Lurquin. 1989. Stable transformation of eggplant (*Solanum melongena* L.) by cocultivation of tissues with *Agrobacterium tumefaciens* carrying a binary plasmid vector. Plant Cell Rpt. 8:370–373.

Fiola, J.A., M.A. Hassan, H.J. Swartz, R.H. Bors, and R. McNicols. 1990. Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell. Tiss. Org. Cult. 20:223–228.

Gallie, D.R., D.E. Sleat, J.W. Watts, P.C. Turner, and M.A. Wilson. 1987. The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucleic Acids Res. 15:3257–3273.

Gill, J.M. 1994. Transformation of eggplant (*Solanum melongena* L.) and chinese kale (*Brassica alboglabra* Bayley) using *Agrobacterium tumefaciens* as a vector. PhD thesis, Cook College, Rutgers Univ., New Brunswick, N.J.

Guri, A. and K.C. Sink. 1988. Agrobacterium transformation of eggplant. J. Plant Physiol. 133:52–55.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rpt. 5:242–255.

Junghans, H. and M. Metzlaff. 1990. A simple method for the preparation of total plant DNA. Biotechnology 8:176.

Lin, J-J., N. Assad-Garcia, and J. Kuo. 1994. Effects of *Agrobacterium* cell concentration on the transformation efficiency of tobacco and *Arabidopsis thaliana*. Focus: Life Technologies 16(3):72–22.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

McCormick, S., J. Niedermeyer, J. Fry, A. Barnason, R. Horsch, and R. Fraley. 1986. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rpt. 5:81–84.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497

Rotino, G.L. and S. Gleddie. 1990. Transformation of eggplant (*Solanum melongena* L.) using a binary *Agrobacterium tumefaciens* vector. Plant Cell Rpt. 9:26–29

Szasz, A., G. Nervo, and M. Fari. 1995. Screening for *in vitro* shoot-forming capacity of seedling explants in bell pepper (*Capsicum annuum* L.) genotypes and efficient plant regeneration using thidiazuron. Plant Cell Rpt. 14:666–669.

Tsai, C-J., G.K. Podila, and G.K. Podila. 1994. *Agrobacterium*-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants. Plant Cell Rpt. 14:94–97.

Van Wordragen, M.F. and H.J.M. Dons. 1992. *Agrobacterium tumefaciens*-mediated transformation of recalcitrant crops. Plant Mol. Biol. Rpt. 10:12–36.