Short Communication

A Highly Efficient Transformation Protocol for Micro-Tom, a Model Cultivar for Tomato Functional Genomics

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We report a highly efficient protocol for the *Agrobacterium*-mediated genetic transformation of a miniature dwarf tomato (*Lycopersicon esculentum*), Micro-Tom, a model cultivar for tomato functional genomics. Cotyledon explants of tomato inoculated with *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) C58C1Rif[R] harboring the binary vector pIG121Hm generated a mass of chimeric non-transgenic and transgenic adventitious buds. Repeated shoot elongation from the mass of adventitious buds on selection media resulted in the production of multiple transgenic plants that originated from independent transformation events. The transformation efficiency exceeded 40% of the explants. This protocol could become a powerful tool for functional genomics in tomato.

Keywords: *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) — Functional genomics — *Lycopersicon esculentum* L. — Micro-Tom — Transformation.

Abbreviations: AS, acetosyringone; EMS, ethyl methanesulfonate; EST, expressed sequence tag; GUS, β-glucuronidase; MS, Murashige and Skoog.

With the successful conclusion of the *Arabidopsis* and rice genome projects, researchers in plant science recognize that genomic information is a powerful tool for both basic studies and technological developments. A specific feature of tomato (*Lycopersicon esculentum* L.), i.e. fruit development, is impossible to study fully in *Arabidopsis* or rice. In addition, tomato is an economically important food worldwide. For these reasons, tomato has become the next target for plant genomics studies. However, compared with *Arabidopsis* and rice, tomato has a relatively large genome (approximately 950 Mb), which makes it difficult for a single institution to undertake the genome project. Therefore, tomato genome initiatives are currently in progress, involving the action of an international consortium called the 'International Solanaceae Genome Project' (SOL). Among other accomplishments, SOL has been generating information on genome resources related to tomato, such as the

results obtained by genome sequencing and expressed sequence tag (EST) sequencing (Fei et al. 2004), including sequencing of full-length cDNA and mutants induced by a variety of mutagenic treatments, such as the application of ethyl methanesulfonate (EMS; Emmanuel and Levy 2002, Menda et al. 2004). Using these resources, functional genomics have been used to study the function of genes related to tomato development (Gidoni et al. 2003, Shibata 2005, Takahashi et al. 2005).

Genetic transformation is a key technology for functional genomics. To confirm the functions of genes isolated using map-based cloning of mutant alleles and omics analysis, functional complementation using genetic transformation is required. Previous studies on the genetic transformation of tomato have reported transformation efficiencies ranging from 6 to 37% (Hamza and Chupeau 1993, Van Roekel et al. 1993, Frary and Earle 1996, Ling et al. 1998, Vidya et al. 2000, Hu and Phillips 2001, Park et al. 2003). Nevertheless, many research groups that are using tomato as an experimental plant are having difficulty in generating transgenic tomato plants (Dr. Y. Kubo, Okayama University; Dr. A. Itai, Tottori University; Dr. K. Matsui, Yamaguchi University; and Dr. K. Aoki, Kazusa DNA Institute; personal communications at SOL and JSOL workshop), indicating that an efficient reliable protocol is required.

Micro-Tom is a miniature dwarf tomato cultivar that was originally bred for home gardening (Scott and Harbaugh 1989). This cultivar has several unique features, such as a small size that enables it to grow at a high density (1,357 plants m^{-2}), seed setting under fluorescent light and a short life cycle that allows for mature fruit to be harvested within 70–90 d after sowing. These features are similar to those of *Arabidopsis*; consequently, this tomato is considered to be a model cultivar for tomato functional genomics.

Tomato researchers in Japan have established a consortium for tomato genomics called Japanese SOL (JSOL). JSOL is currently establishing shared genomic resources for tomato and is participating in the international SOL activity. The genomic resources include genome sequences, macroarray profiling data sets using Micro-Tom (Van der Hoeven et al. 2002,
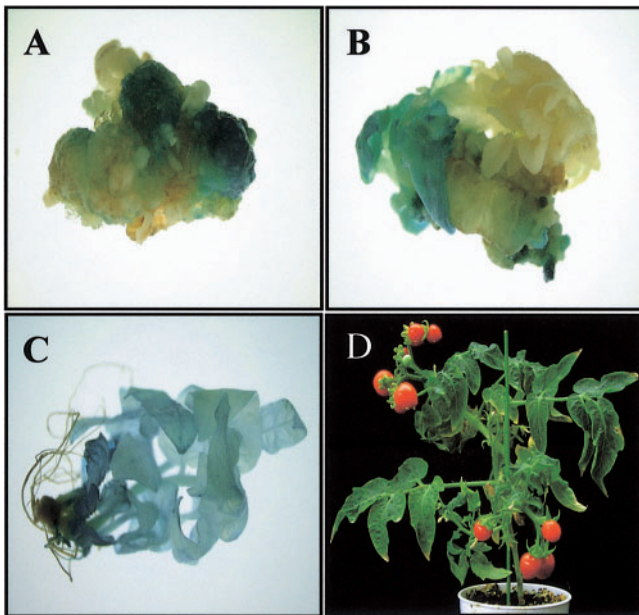
Fig. 1 Observation of GUS expression at several stages in the transformation of tomato and regenerated transgenic plant. (A) GUS expression in a callus on selection medium after about 4 weeks of culture. (B) GUS expression in shoots on selection medium after about 6 weeks of culture. (C) GUS expression in a regenerated tomato plant after about 8 weeks of culture. (D) A mature transgenic plant after 12 weeks of culture in soil.

Yamamoto et al. 2005), Micro-Tom-based mutants generated using EMS treatment and full-length cDNA sequences of Micro-Tom. Using these genetic resources, many candidate genes of interest have been identified (Giovannoni 2004). The final confirmation of such genes requires genetic transformation. In addition, directed mutagenesis involving the integration of T-DNA into a plant genome requires a high-throughput transformation method. *Agrobacterium*-mediated transformation of Micro-Tom has been reported as a part of studies on transposon or activation tagging and promoter-trapping studies (Meissner et al. 1997, Meissner et al. 2000, Mathews et al. 2003). However, detailed protocols were not provided in these studies, and other research groups have had difficulty in reproducing the methods (Dr. Y. Kubo, Okayama University; Dr. A. Itai, Tottori University; Dr. K. Matsui, Yamaguchi University; and Dr. K. Aoki, Kazusa DNA Institute; personal communications at SOL and JSOL workshop). Although many research groups have attempted to produce transgenic tomato plants, they have been unable to obtain sufficient numbers of transgenic tomato with the gene of interest for functional complementation studies. Functional genomics studies of the tomato therefore clearly require a high-throughput transformation protocol.

In this study, we established a high-throughput transformation protocol for Micro-Tom by modifying and refining a protocol reported for other tomato cultivars (Ohyama et al. 1995). The established protocol is reproducible and simple, and

enabled us to produce transgenic plants at a frequency exceeding 40% of the explants inoculated with *Agrobacterium*. This protocol could therefore become a powerful tool for functional genomics in tomato.

Cotyledon explants from 7- to 10-day-old seedlings were inoculated with *Agrobacterium tumefaciens* C58C1Rif^R harboring pIG121-Hm. The explants were placed on co-cultivation medium with the abaxial side down, and incubated in the dark for 3–4 d. The explants were then subcultured in callus induction medium containing 100 mg l^{-1} kanamycin, 1.5 mg l^{-1} zeatin and 375 mg l^{-1} Augmentin (GlaxoSmithKline, Uxbridge, UK). Augmentin, which is a mixture of amoxicillin and clavulanate potassium, was used to eliminate *Agrobacterium* cells from the inoculated explants as it is less expensive than the antibiotics commonly used for *Agrobacterium*-mediated transformation in tomato, such as carbenicillin (Meissner et al. 1997, Mathews et al. 2003), Clavamox (Park et al. 2003), cefotaxime and ticarcillin/potassium clavulanate (Ling et al. 1998). In addition, Augmentin has a minimal effect on plant regeneration compared with cefotaxime, which frequently induces hyperhydration on explants, reducing the plant regeneration frequency (Kikuchi et al. 2005). Therefore, Augmentin is suitable for producing a large number of transgenic lines.

Explants commenced callus development at the edges 2 weeks after *Agrobacterium* inoculation, which was followed by the regeneration of a mass of shoot buds.

Histochemical staining to detect β-glucuronidase (*GUS*) gene expression showed the development of transgenic and non-transgenic calli (Fig. 1A), indicating that the developed calli are chimeras of transgenic and non-transgenic cells. Consequently, the elimination of chimeric status is critical for the efficient generation of stable transgenic shoots. The mass of calli produced multiple shoots when transferred to shoot elongation medium containing 100 mg l^{-1} kanamycin, 1.0 mg l^{-1} zeatin and 375 mg l^{-1} Augmentin, although small buds still existed on the calli. To enhance shoot elongation, we tested the concentration of zeatin in the shoot induction medium. Shoot elongation from a mass of shoot buds was enhanced by reducing the zeatin concentration from 1.5 to 1.0 mg l^{-1}. This modification shortened the period of shoot elongation. The elongated multiple shoots consisted of transgenic and non-transgenic shoots based on a histochemical GUS assay (Fig. 1B). The elongated shoots were excised from the calli and transferred to rooting medium. After 10–14 d, rooted shoots were selected as putative transgenic plants. Most of the rooted plants showed the GUS staining reaction throughout (Fig. 1C), indicating that these plants were not chimeras. In addition, mature fruits were obtained from these transgenic plants after 12 weeks of culture in soil (Fig. 1D). The unrooted shoots on the first rooting medium were re-cut at the basal end and transferred to new rooting medium. Some of the unrooted shoots rooted on the second rooting medium. The mass of calli with small shoot buds remaining after excision of the elongating shoots was transferred to new shoot elongation medium, and

Table 1   Transformation frequency of tomato with three different transgenes

Transgenes		No. of explants	No. of explants forming calli	No. of independent calli producing rooted plants (total no. of rooted plants)	Transformation frequency [a] (%)
GUS	Exp.1	80	42	39 (154)	48.8
	Exp.2	80	41	30 (137)	37.5
SVP	Exp.1	80	40	25 (42)	31.3
	Exp.2	80	31	20 (41)	25.0
LHY	Exp.1	120	62	51 (104)	42.5
	Exp.2	80	28	23 (50)	28.8

GUS, β-glucuronidase gene; *SVP* and *LHY*, genes that play key roles in the control of flowering and the circadian clock, respectively, in *Arabidopsis* (provided by Dr. T. Mizoguchi, University of Tsukuba).
[a] Percentage of explants that formed rooted plants on rooting medium.

the remaining buds also elongated into shoots. Some of the shoots were rooted on rooting medium. Consequently, many rooted plants were obtained from the mass of calli using this repeated shoot selection. For example, 39 independent calli induced from 80 explants produced rooted shoots, giving a transformation efficiency of 48.8% for explants inoculated with *Agrobacterium* harboring the binary plasmid pIG121Hm (Table 1, Fig. 2A). Moreover, the 39 independent calli produced 154 rooted shoots. If each of these rooted plants originated from independent transformation events, the transformation efficiency was 192.5%. To test this possibility, we performed genomic Southern analysis on 12 randomly selected regenerated plants that originated from five independent calli (Fig. 2B). Southern analysis showed that eight of the 12 plants probably originated from independent transformation events. Therefore, the estimated transformation frequency was 128.3%. In a second experiment, a similar transformation frequency was obtained (Table 1).

The *Agrobacterium*-mediated transformation protocol for Micro-Tom developed in this study is summarized in Fig. 3B, and the repeated rooting selection of transgenic shoots is shown in Fig. 3A. Cotyledon explants prepared from in vitro germinated seedlings were inoculated with *Agrobacterium* cells in the presence of acetosyringone (AS) and co-cultivated on co-cultivation medium for 3–4 d. The abaxial surface of the explants was placed in contact with the medium and then incubated in the dark. The preparation and treatment of explants were performed carefully to avoid damage that reduces the subsequent callus induction frequency. After co-cultivation, the explants were subcultured in callus induction medium containing 375 mg l^{-1} Augmentin and 100 mg l^{-1} kanamycin to select transgenic cells. Transgenic callus initiation was observed 2–3 weeks after inoculation. After 3–4 weeks, calli with small shoot buds were induced from the cut end of the explants. To promote shoot elongation, calli with a mass of shoot buds were excised from the explants and transferred to shoot elongation medium. These calli were subcultured every 2 weeks for shoot regeneration. After 2 weeks, shoots elongated from the mass of

calli with shoot buds. Shoots 1–2 cm long were excised from the mass of shoot buds and transferred to rooting medium. In addition, calli with shoot buds that remained after excising the elongated shoots were subcultured in fresh shoot elongation
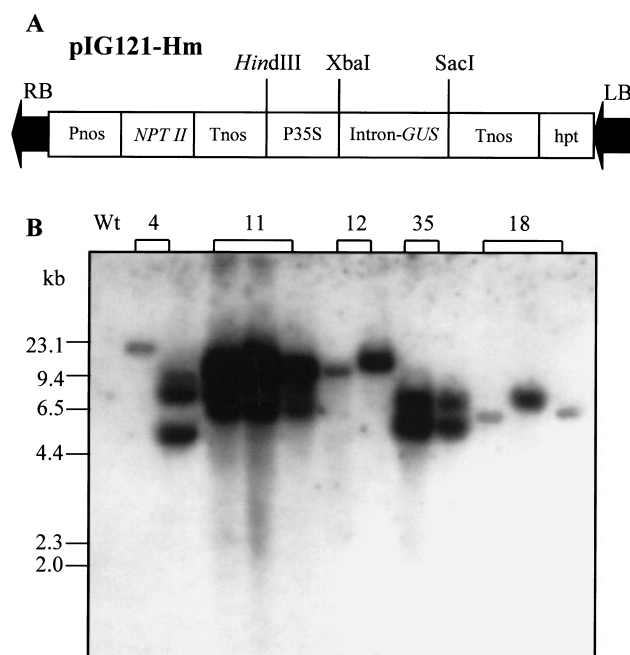

Fig. 2   Map of the binary vector pIG121Hm and Southern blot analysis of transgenic Micro-Tom plants. (A) Map of the T-DNA region of binary vector pIG121Hm. The *GUS* coding region was used as a probe. RB, right border of T-DNA; LB, left border of T-DNA; Pnos, nopaline synthase gene promoter; *NPTII*, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; P35S, CaMV 35S promoter; *GUS*, β-glucuronidase gene; hpt, hydromycin phosphotransferase gene. (B) Southern blot analysis of 12 randomly selected regenerated tomato plants that originated from five independent calli. Wt, untransformed tomato plant; 4, 11, 12, 35 and 18, transgenic tomato plant lines. Two or three regenerated plants derived from five independent calli were used in this analysis.
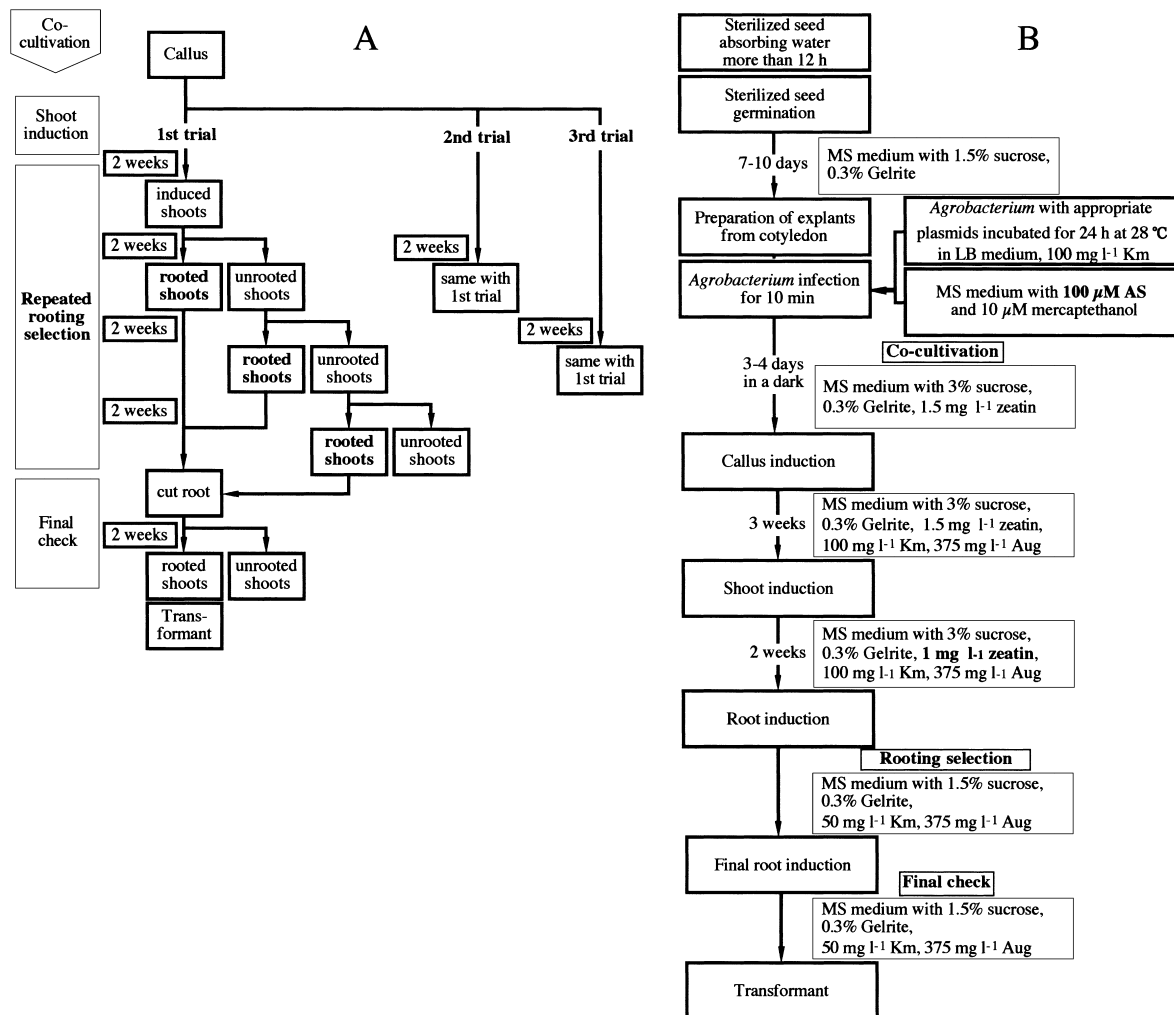
Fig. 3 Repeated rooting selection of transgenic shoots (A) and schematic representation of *Agrobacterium*-mediated transformation of tomato (B). The time line is indicated on the arrow.

medium, which promoted further shoot elongation. The additional elongated shoots were excised and transferred to rooting medium. The transgenic shoots rooted in 7–14 d. When the expansion of the roots in rooting medium was slow or stopped and the top of root turned brown without producing side roots, the possibility of escape was very high. Unrooted shoots were therefore re-cut at the basal end and transferred to new rooting medium; some of these shoots then rooted. The repeated rooting selection of transgenic shoots overcame the chimeric status of the mass of shoot buds, and transgenic Micro-Tom plants were obtained with an efficiency of approximately 40% of the explants about 3 months after commencing in vitro seed germination. We provided this protocol to other groups, who obtained transgenic tomato with similar frequencies (Dr. Y. Kubo, Okayama University; Dr. A. Itai, Tottori University; Dr. K. Matsui, Yamaguchi University; and Dr. K. Aoki, Kazusa DNA Institute; personal communications), indicating that this protocol is reproducible. Research on functional genomics in

tomato is currently in progress as a part of the SOL initiative; this activity will generate genomic resources that are useful for basic and applied studies. This protocol could become a powerful tool for functional genomics in Micro-Tom, a model cultivar for the tomato genomics initiative.

To test the adaptability of our protocol to introducing genes that affect plant development, we introduced two genes, *SVP* and *LHY*, which play key roles in the control of flowering and the circadian clock, respectively, in *Arabidopsis* (kindly provided by Dr. T. Mizoguchi, University of Tsukuba), and found that the transformation efficiency ranged from 25.0 to 42.5% (Table 1). The transgenic tomatoes had altered phenotypes (elongated calyx and elongated fruit) in terms of plant development, implying that these genes were functional. Detailed analyses of those transgenic tomato plants will be reported elsewhere. Although the transformation efficiency was reduced slightly compared with that obtained by introducing the *GUS* gene, the efficiency was still sufficiently high to

obtain enough transgenic plants in a small transformation experiment. This result suggests that the protocol presented here is useful for introducing functional genes into Micro-Tom tomato.

We have also tested the adaptability of the protocol developed for Micro-Tom tomato transformation in another tomato cultivar, Money Maker, which is frequently used as a model cultivar for developmental and physiological studies of tomato (Ali et al. 2002, Cristescu et al. 2002, Li and Steffens 2002, Carbone et al. 2005, Sobczak et al. 2005). We introduced the gene encoding the taste-modifying protein miraculin into this cultivar and found that the transformation frequency was 20–30% of explants (unpublished data), suggesting that the *Agrobacterium*-mediated transformation protocol developed for Micro-Tom can be adapted to other important cultivars for genomics and physiology studies in tomato.

Materials and Methods

Seeds of the *L. esculentum* Mill. cultivar Micro-Tom were surface sterilized in 70% ethanol for 2 min and in 10% commercial bleach with a detergent (Kitchen Haiter, Kao, Tokyo, Japan) for 45 min or in 1% (v/v) sodium hypochlorite solution with two drops of Tween-20 for 20 min and then rinsed with sterilized water three times for 5 min each. The seeds were germinated in a plant box with 40 ml of MS medium (Murashige and Skoog 1962) with 15 g l^{-1} sucrose and 3 g l^{-1} Gelrite. The pH was adjusted to 5.8 before autoclaving. All the cultures were maintained at 25°C under a 16 h light/8 h dark cycle with fluorescent light (irradiance of 60 μmol m^{-2} s^{-1}). For the genetic transformation, the seedlings were used when the cotyledons had expanded fully and the true leaves were several millimeters long.

Agrobacterium tumefaciens strain C58C1Rif^R with appropriate plasmids (Fig. 2A) was used for this study. This strain was grown for 24 h at 28°C in 2 ml of LB medium containing 100 mg l^{-1} kanamycin. The bacterial suspension (1 ml) was centrifuged and the bacteria were resuspended in the same volume of MS medium with 30 g l^{-1} sucrose. The bacterial cultures were diluted 20–40 times with MS culture medium containing 100 μM AS and 10 μM 2-mercaptoethanol, and used for bacterial inoculation.

Cotyledons were sectioned into two halves at the mid-vein region. The end of each cotyledon was cut off to allow it to adsorb the bacterial suspension. The explants were dipped in the bacterial suspension for 10 min and blotted dry on a sterilized paper towel. The explants were placed on co-cultivation medium with the abaxial surface of the leaf in contact with the medium. The co-cultivation medium contained MS salts, 30 g l^{-1} sucrose, 3 g l^{-1} Gelrite and 1.5 mg l^{-1} zeatin, at pH 5.8. The plate was wrapped with aluminum foil and incubated for 3–4 d at 25°C. The explants were then subcultured onto callus induction medium containing MS salts, 30 g l^{-1} sucrose, 3 g l^{-1} Gelrite, 1.5 mg l^{-1} zeatin, 100 mg l^{-1} kanamycin and 375 mg l^{-1} Augmentin at pH 5.8. Every 2 weeks, the explants were subcultured onto selection medium containing MS salts, 30 g l^{-1} sucrose, 3 g l^{-1} Gelrite, 1.5 or 1.0 mg l^{-1} zeatin, 100 mg l^{-1} kanamycin and 375 mg l^{-1} Augmentin at pH 5.8. When calli with shoot buds formed from the cotyledon fragment, the zeatin concentration was adjusted to 1 mg l^{-1} (shoot elongation medium). When calli with adventitious buds developed from the explant, the cotyledon was cut off and transferred to new plate (with a height of about 4 cm) containing shoot elongation medium for shoot elongation. When a stem developed in addition to a leaf, the stem was cut off at the possible maximum length and transferred to rooting medium. The rooting medium contained half-strength MS medium, 15 g l^{-1} sucrose, 3 g l^{-1} Gelrite, 50 mg l^{-1} kanamycin and 375 mg l^{-1} Augmentin at pH 5.8. Subsequently, the rooted plants were subjected to molecular analysis as putative transgenic plants, and unrooted shoots were cut off at the thin end and then transferred to new rooting medium. If there was a newly developed shoot on a callus, the shoot was cut and transferred to rooting medium. The callus was also transferred to new shoot elongation medium. If a shoot did not produce roots after two subcultures, it was discarded, while those with vigorous leaves could be transferred to new rooting medium up to three times. This treatment eliminated chimeras. If the rooted plant grew, it was transferred to a plant box containing rooting medium.

A histochemical assay to detect GUS activity was performed on kanamycin-resistant calli, shoots and regenerated tomato plants using the histochemical GUS staining procedure of Jefferson et al. (1987).

Total DNA was isolated from 0.5 g of fresh young leaves using the CTAB extraction method of Rogers and Bendich (1985). A 10 μg aliquot of genomic DNA digested with *Hin*dIII was fractionated on a 1% agarose gel and then transferred to a nylon membrane by capillary transfer. A digoxigenin-labeled *GUS* gene-specific probe was generated using a PCR DIG probe synthesis kit, according to the manufacturer's protocol (Roche, Diagnostics GmbH, Mannheim, Germany). The following primer sets were used to amplify the GUS gene-specific probe: forward primer, 5′-GGTGGGAAAGCGCGTTACAAG-3′; reverse primer, 5′-CGGTGATACATATCCAGCCAT-3′. The blots were hybridized at 45°C overnight in high SDS concentration hybridization buffer (DIG System, Roche, Diagnostics GmbH, Mannheim, Germany), and washed twice in 2× SSC for 10 min each at room temperature and in 0.1× SSC for 15 min at 60°C. Signals were detected by a chemiluminescent method using CDP^{star} (Roche, Diagnostics GmbH, Mannheim, Germany). The membrane was then exposed to X-ray film (Hyperfilm ECL™, Amersham Biosciences, Piscataway, NJ, USA).

Acknowledgments

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