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Improvement of Transformation Efficiency of Agrobacterium Mediated Gene Transfer in Tomato

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ABSTRACT

The transformation/regeneration system was developed for *Agrobacterium tumefaciens* mediated gene transfer in tomato. Transformation efficiency depended on variety and type of tissue of tomato. Cotyledon disc transformation was superior to leaf disc transformation. Transformants derived from cotyledon disc transformation yielded 1-10 plantlest per tissue within 2-3 weeks. Transgenic tomato plants expressed kanamycin resistant gene and GUS (β-glucuronidase) activity.

บทคัดย่อ

การพัฒนาระบบ transformation/regeneration เพื่อการถ่ายยีนให้กับมะเขือเทศโดย *Agrobacterium tumefaciens* พบว่าประสิทธิภาพของการถ่ายยีน ขึ้นอยู่กับพันธุ์และชนิดของเนื้อเยื่อ การถ่ายยีนให้กับส่วนใบเลี้ยงจะมีประสิทธิภาพดีกว่าการถ่ายยีนให้กับส่วนใบ โดยที่สามารถสร้างต้นพืชจำลองพันธุ์ได้ตั้งแต่ 1-10 ต้น จากเนื้อเยื่อ 1 ชิ้น ภายในระยะเวลา 2-3 สัปดาห์ มะเขือเทศจำลองพันธุ์ที่ผลิตได้มีการแสดงออกของยีนต้านทานต่อ kanamycin และยีน GUS (β-glucuronidase) ในทุกส่วนของต้นที่นำมาตรวจสอบ

INTRODUCTION

Recent advances in genetic engineering technology and plant tissue culture system promise to meet the way of incorporating foreign genes for desired agronomic traits while preserving the existing characteristic of improved genotypes. This plant is called "transgenic plant". Plant transformation procedure can be devided into two systems, direct gene transfer and gene transfer by vector. At present, *Agrobacterium tumefaciens* is the most common vector used for genetic transformation of higher plants (Uchimiya et al, 1989). the bacterium is capable of transfering a piece of tumour-inducing (Ti) plasmid (T-DNA) into the genome of host plants. The foreign genes inserted into T-DNA through Ti-plasmids are co-transferred and integrated into the host genome. The success of Agrobacterium-mediated gene transfer depends on the susceptibility of target crop to Agrobacterium, expression of *vir* genes on the Ti-plasmid that controls the transfer of T-DNA region which induced by substances secreated by susceptible plant cells, competent plant cells for transformation with a high rate of plant DNA synthesis and cell division , and availability of high frequency shoot regeneration from plant tissues. Various types of tissues are being tested for the most efficient mean of transformation such as leafdisc (Horsch *et al.*, 1985 Rotino and Gleddie, 1990), cotyledon (Michelmore *et al.*, 1987; Enamoto *et al.*, 1990), stem segment (Jordan and Mc Hughen, 1988), merstematic tissue (Schraameijer *et al.*, 1990) and tuber (Sheerman *et al.*, 1988). For tomato (*Lycopersicon esculentum* L.) transformation had been reported since 1986 (McCormick *et al.*, 1986). Transgenic tomato plants resistant to insect pests and tolerant to glyphosate herbicide have been developed (Fischhoff *et al.*, 1987, Fillatti *et al.*, 1987). At plant Genetic Engineering

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Unit (PGEU), we are working on the production of tomato plant resistant to tomato yellow leaf curl virus (TYLCV). Our approach is to transform tomato plant with TYLCV-coat protein gene to induce resistance. Therefore, the improvement of transformation efficiency of Agrobacterium mediated gene transfer is the important aspect of this study to assure the success of the production of transgenic tomato plant.

MATERIALS AND METHODS

Bacterial strains. - *Agrobacterium tumefaciens* with a binary vector carrying chimaeric genes including NPT-II, nopaline synthase, and GUS was used for transformation. Agrobacterium was grown on antibiotic containing LB-medium at 28°C. Overnight culture was diluted to the concentration of 1 x 10^9 cfu/ml for explant transformation.

Plant materials and transformation.- Leaflets of greenhouse grown tomato plants and cotyledons of 7-10 days old seedlings germinated on MS medium were used for transformation. Samples were surface sterilized for 15 min in 10% clorox and 0.1% Tween-20, and washed 3 times with sterile distilled water. They were then cut into square approximately 1 x 1 cm^2, placed on the surface of KDMS medium (MS medium with B5 vitamins, 3% sucrose, 0.8% agar, 0.2 mg/l 2,4-D and 0.1 mg/l kinetin) and kept in the dark for 2 days. Green and fresh tissues were removed from the KDMS plate and edges of tissues were gently cut. Selected tissues were mixed with the bacterial suspension for 5-10 min then transferred for blot dry on filter paper. Bacterial treated tissues were placed on the feeder plate prepared by spreading tomato cell suspension over the KDMS plate and covered with #1 Whatman filter paper. The culture plates were incubated for 48 hr at 25 ± 2 °C under low light conditions. After that tissues were transferred to the selection plate containing MS basal salts, B5 vitamins, 3% sucrose, 0.8% agar, 1 mg/l zeatin, 500 mg/l carbenicillin, 100 mg/l cefotaximer and 50 mg/l kanamycin for 2 weeks. Regenerated callus or shoots were transferred every 2-3 weeks to the selection plate with kanamycin concentration increased up to 100 mg/l. Rooted planlets were planted in a potting medium for further analysis.

Assay of gene expression :

GUS assay.- GUS (β-glucuronidase) gene activity was determined by histochemical localization method (Jefferson et al., 1987). Thin sections (1 mm thick) of tomato tissues were placed in the microwell culture plate, incubated in X-GLUC solution containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (Sigma), 50 mM phosphate buffer pH 7.0, 10 mM Na_2 EDTA, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide at 37°C over night. Samples were rinsed twice with sterile distilled water and observed blue color development with light microscope.

Kanamycin resistant assay.- Leaf and stem tissues of transgenic plants grown on the selection plate were collected and cultured onto the selection plate with 100 mg/l kanamycin. Tissues from non-transformed plants were used as control. Results were recorded 4 weeks after culturing.

RESULTS

Effects of plant variety and tissue on Agrobacterium mediated gene transfer.- Transformation efficiency of tomato plants by Agrobacterium depends on varieties and tissues being used. Results obtained from leafdisc transformation showed that Tn #3 and VF 134-1-2 varieties were transformed better than those of Cl 5915-206D4-2-5-0, Seeda and Rutgers. No transformation was observed in SVRDC-4 and 1131-0-0-483-1 varieties (Table. 1). It is noted that tissues showing small necrotic lesions after bacterial inoculation will generate transgenic plant more efficient than those with large necrotic lesions.

Types of tissue significantly affected transformation efficiency.- Cotyledon disc transformation was superior to leafdisc transformation (Table. 1). For Seeda variety, number of transformants obtained from cotyledon disc transformation was 6 times higher than leafdisc transformation. Regeneration of plantlets from cotyledon was faster (2-3 weeks) than from leafdisc (4-6 weeks). Moreover, number of plantlets derived from cotyledon was 1-10 plantlets/tissue while 1-5 plantlets were obtained from each leafdisc.

Development of transgenic plants.- When transformed tissues were cultured on kanamycin containing medium, plantlets may develop directly from tissues within 3 weeks (Fig. 1a) or from green compact callus within 8 weeks (Fig. 1b). Non transformed tissues became chlorosis and subsequently died (Fig. 1c). Only transgenic plantlets generated roots within 2 weeks in 50 mg/l kanamycin containing medium (Fig. 1d). Rooted transgenic plants can be transferred onto potting medium 2 weeks after root induction (Fig. 1 e,f).

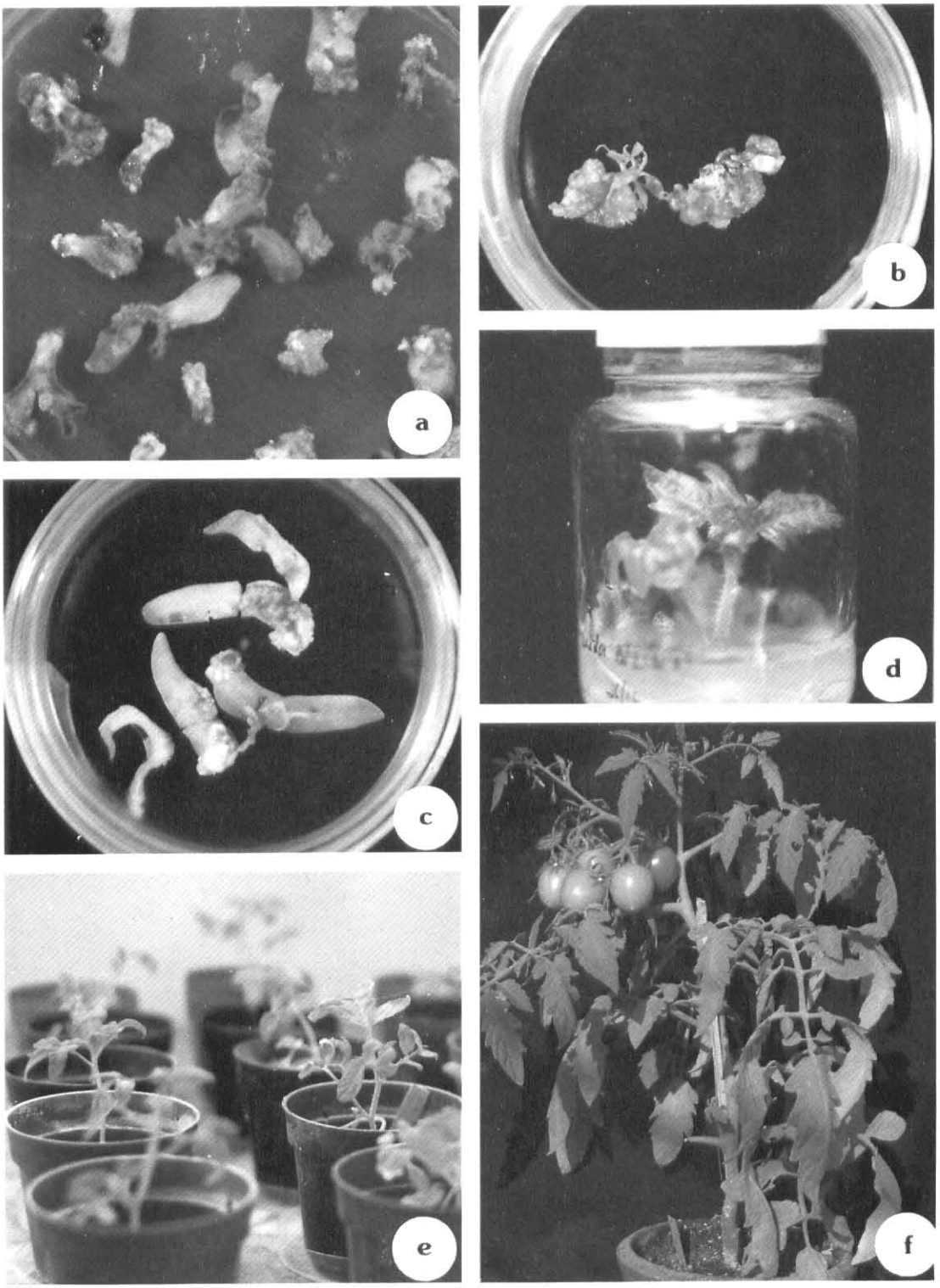

Figure 1 **Development of *A. tumefaciens* transformed tomato plants.**

a) plantlets regeneration from cotyledon after 3 weeks culture on 100 mg/l kanamycin containing medium
b) development of green conpact calli after 8 weeks culture on 100 mg/l kanamycin containing medium
c) non-transformed cotyledons showing tissue chlorosis on 100 mg/l kanamycin containing medium
d) root induction of transgenic plant on 50 mg/l kanamycin containing medium
e) 1-2 weeks old transgenic plants grown on vermiculite
f) 2 months old transgenic plant grown in glasshouse

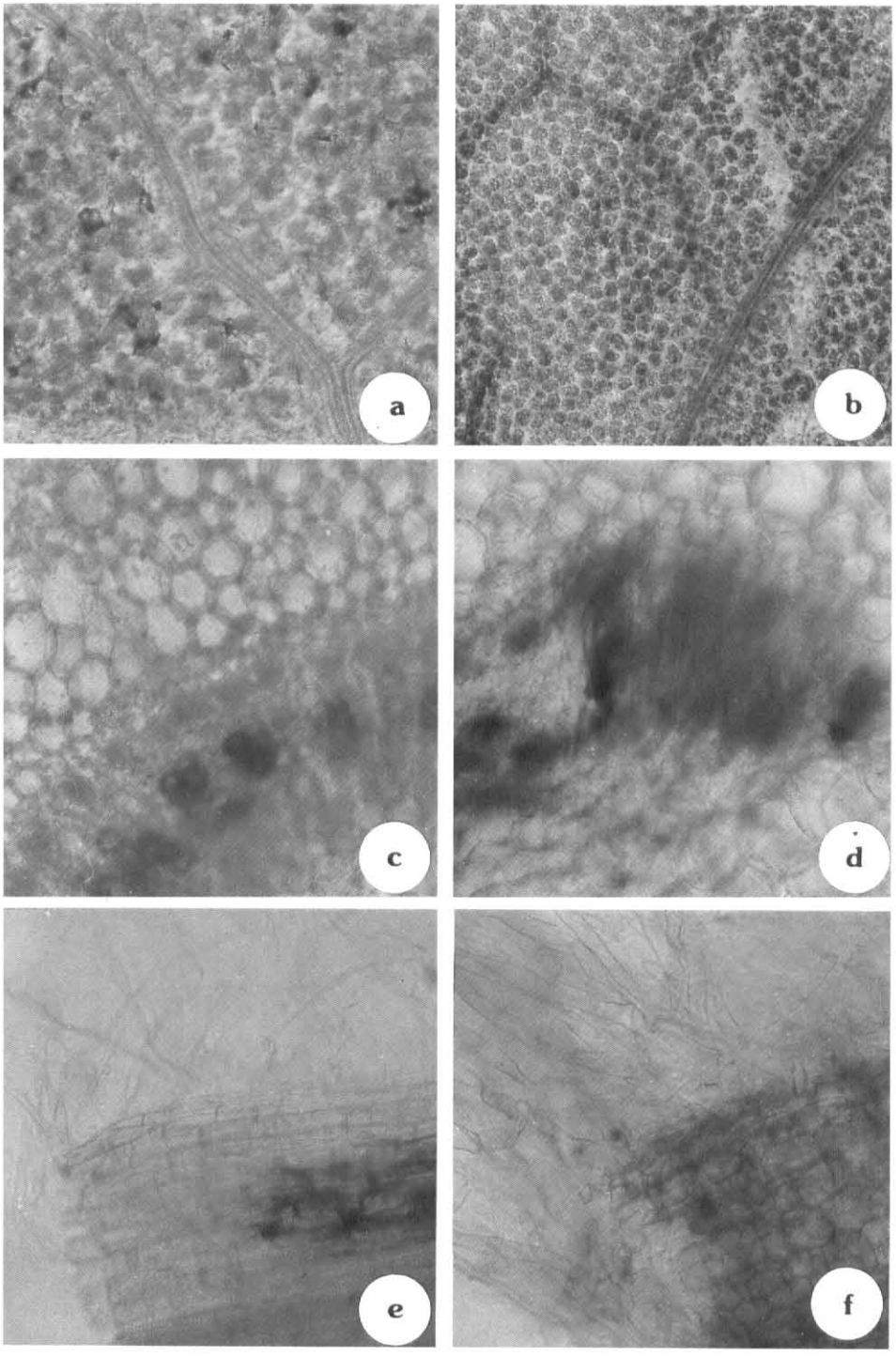

Figure 2 Expression of GUS (β-glucuronidase) gene in tomato tissue.
a) leaf tissue of non-transformed plant
b) leaf tissue of transgenic plant
c) stem tissue of non-transformed plant
d) stem tissue of transgenic plant
e) root tissue of non-transformed plant
f) root tissue of transgenic ransgenic plant

Table 1 Effects of variety and tissue of tomato on *A. tumefaciens* mediated gene transfer.

Variety	Leaf disc [1]		Cotyledon disc [2]	
	# transformants /total	(%)	# transformants /total	(%)
Tn #3	12/45	(26.67)	26/35	(74.28)
VF134-1-2	5/31	(16.13)	71/92	(77.17)
Seeda	2/52	(4.00)	24/92	(26.08)
SVRDC-4	0/56	(0.00)	---	---
1131-6-0-483-1	0/37	(0.00)	---	---
CI5915-206D4-2-5-0	4/63	(6.35)	---	---
Rutgers	1/37	(2.70)	---	---

1 12 weeks after cultured on 100 mg/l kanamycin containing medium
2 8 weeks after cultured on 100 mg/l kanamycin containing medium
--- no test

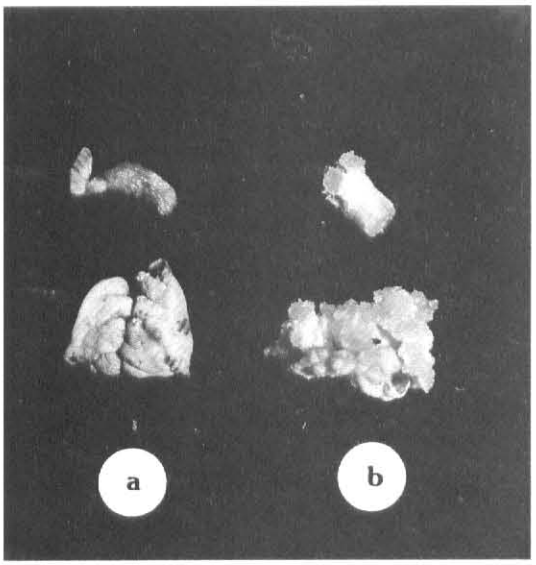

Figure 3 Assay of kanamycin resistant gene in transgenic tomato tissues on 100 mg/l kanamycin containing medium.
a) petiole and leaf tissues from non-transformed plant
b) petiole and leaf tissue from transgenic plant

GUS assay.- Histochemical staining of transformed tissues revealed the blue color in 90% of examined samples. GUS activity was observed in leaf tissue (Fig. 2b), stem (Fig. 2d) and root (Fig.2f). The expression of GUS gene seemed to be localized in vascular tissues of leaf, petiole, stem and root but dispersed in callus tissue. No GUS activity was observed in non-transformed tomato tissue (Fig. 2a, c, e).

Kanamycin resistant assay.- Tissues collected from transgenic plants developed white to light green, loosely bounded callus after 4 weeks culture on 100 mg/l kanamycin containing medium. Those which did not from callus were still fresh and green. Tissues obtained from non-transgenic plants became chlorosis rapidly without any callus development (Fig. 3).

DISCUSSION

We have shown that the efficiency of *A. tumefaciens* to transform tomato plants depends on tomato variety and type of tissue. Cotyledon disc transformation is more efficient method than leaf disc transformation. Cotyledon may have the advantage of planlet regeneration over leaf tissue in term of the ease of surface sterilization, higher numbers of plantlet produced and shorter regeneration time. Synthesis of *vir*-induction factors also requires young tissue and the active stage of cell growth and development (Stachel *et al.*, 1986). This may indicate that cotyledon is the better tissue for transformation. Cotyledon disc transformation was shown to be suitable for other crop plants like lettuce (Enamoto *et al.*, 1990) and muskmelon (Fang and Grumet, 1990).

We have noted that rapid elimination of bacteria from tissues right after inoculation was necessary for the high efficiency transformation. Since Agrobacterium multiplied so rapidly on tomato tissue resulting in cell necrosis, antibiotic treatment of Agrobacterium after inoculation became essential. Cotyledon proved to be ideal for tissue sterilization without causing much damage to the cells and the production of large amount of homogenous plant tissues. Cotyledon disc transformation is considered time saving and cost reducing as compared to leaf disc transformation. However, cotyledon has to be 7-14 days old after germination. If cotyledon became old the efficiency

of transformation was greatly reduced.

Seeda variety was susceptible to *A. tumefaciens* thus making leaf disc transformation unsuccessful. However, the transformation can be overcome by using cotyledon disc and lower bacterial concentration to approximately 1-5 x 10^8 cfu/ml.

Results showed that *A. tumefaciens* mediated cotyledon disc transformation is the efficient transformation regeneration system for gene transfer in our commercial tomatoes. Transfer of useful genes for the development of disease resistant tomato is being investigated.

ACKNOWLEDGEMENT

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