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Transgenic Plant Production from Embryogenic Callus of Sweet Potato (*Ipomoea batatas* (L.) Lam.) Using *Agrobacterium tumefaciens*

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Abstract

Transformed sweet potato plants were obtained from embryogenic calli following *Agrobacterium tumefaciens*-mediated transformation. *A. tumefaciens* strain EHA101/pIG121-Hm used in the present study contained a binary vector with genes for β-glucuronidase (*gus*A) and hygromycin resistance (*hpt*). Around ten hygromycin resistant cell clusters were produced from 1 g fresh weight of the infected embryogenic calli. The hygromycin resistant plantlets were regenerated from 53.1% of the hygromycin-resistant calli. Histochemical GUS assay and Southern hybridization analysis indicated that these plants were stably transformed with a copy number of introduced genes of one to three. Transgenic plants grew normally and formed storage roots after 3 months of cultivation in a green house.

1. Introduction

The sweet potato, *Ipomoea batatas* (L.) Lam., is an important crop [1], but, the conventional breeding program based on sexual hybridization of this plant species has not been developed well because of its sterility and cross incompatibility. To overcome the limitations, novel approaches such as somatic hybridization and genetic transformation must be incorporated into sweet potato breeding.

On the other hand, sweet potato is an attractive plant species as a target of 'molecular farming' because of its high production yield of biomass. Recent developments in genetic engineering enable the production of various biomolecules such as carbohydrates, fatty acid, high-value pharmaceutical polypeptides, industrial enzymes and biodegradable plastics from other organisms in transgenic plants [2]. Transgenic plants may become attractive and cost effective alternatives to microbial and animal systems for the production of biomolecules.

In sweet potato, an efficient and reproducible transformation system has not been established, although a few reports on the production of transgenic plants has been published [3-8].

We have established an efficient method for embryogenic callus production from meristem tissues of sweet potato using altered plant growth regulators, picloram, dicamba or 4FA in the medium [9]. Our preliminary experiments suggested that these embryogenic calli could be suitable target materials for *Agrobacterium*-mediated transformation. Recently, Gama *et al.* [8] reported the production of the transgenic plants of sweet potato cv. White Star using embryogenic callus and *Agrobacterium tumefaciens*. However the efficiency is still low and they did not confirm the stability of integrated genes.

The present study describes a simple, efficient and reproducible method for the production of transgenic sweet potato plants by *Agrobacterium tumefaciens*-mediated transformation using the embryogenic callus. We also describe the morphology of transgenic plants and stability of integrated genes.

2. Materials and Methods

2.1 Embryogenic callus induction and tissue culture media

Ipomoea batatas (L.) Lam. cultivar Kokei 14 was grown in media used tissue culture and transformation as shown in **Table 1**. Embryogenic calli were induced from shoot meristems on LS medium [10] containing 1 mg/*l* 4- fluorophenoxyacetic acid (4FA) according to the methods of Otani and Shimada [9]. These embryogenic calli were maintained at 26°C in the dark and proliferated by subculture on the same fresh medium.

2.2 Bacterial strain and plasmid

The sweet potato embryogenic calli was infected using *Agrobacterium tumefaciens* strain EHA101 harboring the binary vector plasmid pIG121-Hm, which carries the chimeric neomycin phosphotransferase

Table 1. Media used for tissue culture and transformation of sweet potato.

Stage	Medium composition
Embryogenic callus induction and proliferation	LS medium, 1 mg/*l* 4FA, 30 g/*l* sucrose, 3.2 g/*l* gellan gum, pH5.8
Bacterial infection	LS medium, 1 mg/*l* 4FA, 10 mg/*l* acetosyringone, 30 g/*l* sucrose, pH 5.8
Co-culture	LS medium, 1 mg/*l* 4FA, 10 mg/*l* acetosyringone, 30 g/*l* sucrose, 3.2 g/*l* gellan gum, pH 5.8
Selection	LS medium, 1 mg/*l* 4FA, 25 mg/*l* hygromycin, 500 mg/*l* carbenicillin, 30 g/*l* sucrose, 3.2 g/*l* gellan gum, pH 5.8
Somatic embryo formation	LS medium, 4 mg/*l* ABA, 1 mg/*l* GA_3, 25 mg/*l* hygromycin, 500 mg/*l* carbenicillin, 30 g/*l* sucrose, 3.2 g/*l* gellan gum, pH 5.8
Plant formation	LS medium, 0.05 mg/*l* ABA, 25 mg/*l* hygromycin, 500 mg/*l* carbenicillin, 30 g/*l* sucrose, 3.2 g/*l* gellan gum, pH 5.8

(*nptII*), β-glucuronidase (*gus*A) and hygromycin phosphotransferase (*hpt*) genes [11]. *A. tumefaciens* strain LBA4404/pTOK233 [12] and *A. tumefaciens* strain R1000/pBI121 [13] were also examined for transient GUS expression in embryogenic callus.

2.3 *Transformation*

The transformation procedure was performed according to the method of Rashid *et al.* [14] with some modification. The *Agrobacterium* was grown for two days at 27°C on LB medium supplemented with 50 mg/*l* kanamycin, 50 mg/*l* hygromycin and 1.5% (W/V) agar. The colony of bacteria was transferred to liquid LS medium supplemented with 10 mg/*l* acetosyringone and 1 mg/*l* 4FA, and shaken at 100 rev./minute for one hour in the dark at 27°C. The embryogenic calli were soaked in a bacterial suspension for two minutes, blotted dry with sterile filter paper to remove excess bacteria. Then the calli were transferred onto co-culture medium which was LS medium supplemented with 1 mg/*l* 4FA, 10 mg/*l* acetosyringone, 3% (W/V) sucrose and 0.32% (W/V) gellan gum. After three days of co-cultivation, the infected calli were washed three times with sterile distilled water supplemented with 500 mg/*l* carbenicillin and then transferred onto selection medium which was LS medium containing 1 mg/*l* 4FA, 25 mg/*l* hygromycin, 500 mg/*l* carbenicillin, 3% (W/V) sucrose and 0.32% (W/V) gellan gum. The cultures were carried out at 26°C in the dark.

2.4 *Selection and regeneration of transgenic plants*

After selection for two weeks, the calli were washed three times with sterile distilled water supplemented with 500 mg/*l* carbenicillin and then transferred to the fresh selection medium. The calli were subcultured onto the fresh medium every two weeks. After 60 days of culture on the selection medium, the calli were transferred onto the somatic embryo formation medium which was LS medium supplemented with 4 mg/*l* abscisic acid (ABA), 1 mg/*l* gibberellic acid (GA_3), 25 mg/*l* hygromycin, 500 mg/*l* carbenicillin, 3% (W/V) sucrose and 0.32% (W/V) gellan gum. Somatic embryos formed from hygromycin-resistant calli were transferred onto LS medium supplemented with 0.05 mg/*l* GA_3, 25 mg/*l* hygromycin, 500 mg/*l* carbenicillin, 3% (W/V) sucrose and 0.32% (W/V) gellan gum after 21 days of culture on the somatic embryo formation medium. Regenerated plants derived from somatic embryos were cultured on LS medium supplemented with 25 mg/*l* hygromycin, 500 mg/*l* carbenicillin, 3% (W/V) sucrose and 0.32% (W/V) gellan gum. The cultures were maintained at 26°C under a 16-hour photoperiod at 38 μmol/m^2/s by daylight fluorescent tubes.

2.5 *Histochemical β-Glucuronidase (GUS) assay*

Histochemical (GUS) assay of sweet potato cells was performed by the method of Jefferson *et al.* [15]. The embryogenic callus infected by *Agrobacterium*, the expanded leaves and the storage root disks of regenerated plants from hygromycin-resistant calli were incubated in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) solution at 37°C for five hours. Then they were soaked in 99.5% (V/V) ethanol for chlorophyll removal and maintained at 4°C before observation using an optical microscope.

2.6 *DNA isolation and Southern hybridization*

Total DNA was isolated from leaves of *in vitro* plants by the sodium dodecyl sulfate (SDS) extraction method according to Honda and Hirai [16]. After complete digestion with *Hind* III, DNA fragments were separated by 1% agarose gel electrophoresis, and transferred to Amersham's Hybond-N nylon membrane as described by Southern [17]. The filter

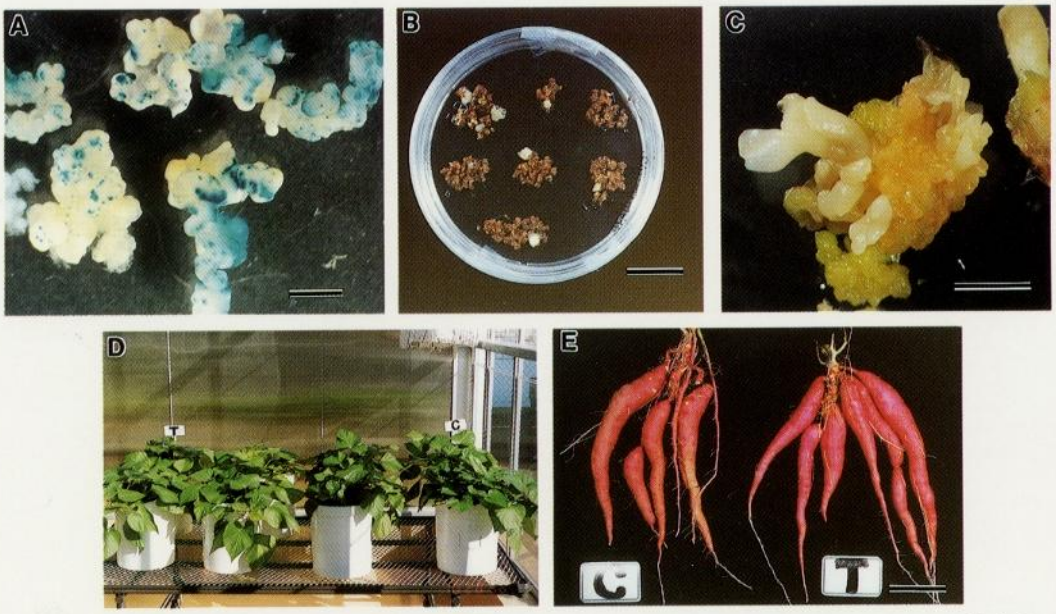

Fig. 1 Production and histochemical GUS assay of transgenic plants of sweet potato cv. Kokei 14 by *Agrobacterium tumefaciens* strain EHA101/pIG121-Hm.
(A) Transient GUS expression in embryogenic callus of sweet potato after three days of co-cultivation with *Agrobacterium*. Bar=15 mm.
(B) Hygromycin-resistant cell clusters developing on the selection medium containing 25 mg/*l* hygromycin. Bar=20 mm.
(C) Numerous somatic embryos produced from hygromycin-resistant calli. Bar=20 mm.
(D) Transgenic plants (left three pots) and an untransformed plant (right pot) established in soil and grown for 2 months in a green house.
(E) Storage roots formed on untransformed (left) and transgenic plants (right). Bar=50 mm.

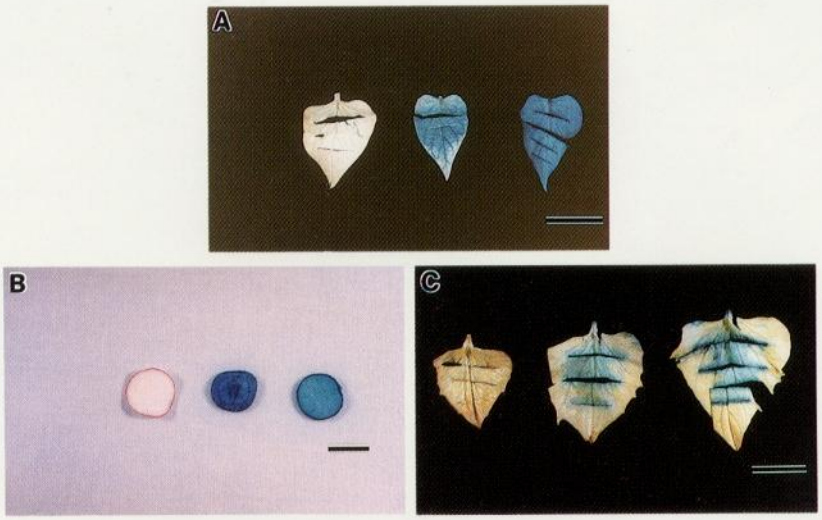

Fig. 2 Histochemical GUS assay of transgenic plants.
Leaves (A) and storage roots (B) of two independent transgenic plants (center and right) and untransformed plant (left).
(C) Leaves of freshly sprouting shoots from harvested storage roots of two independent transgenic plants (center and right) and untransformed plant (left). Bar=15 mm.

was hybridized with a GUS DNA fragment labeled with [α-^{32}P]dCTP by Megaprime™ DNA labeling system (Amersham International plc, England).

3. Results

3.1 Factors affecting transient expression of GUS

We examined three factors, strains of *Agrobacterium*, inclusion of acetosyringone and culture period of embryogenic calli before bacterial inoculation, for efficient transformation in sweet potato. Transient expression of GUS was examined for 1.5 g fresh weight of embryogenic calli per treatment soon after 3 days of co-cultivation. Experiments were repeated at least two times. The transient GUS expression varied among the bacterial strains. *A. tumefaciens* strain EHA101/pIG121-Hm gave the highest GUS spots, while another bacterial strains gave no (for LBA4404/pTOK233) or very few spots (average 6 spots per g fresh weight for R1000/pBI121).

The effect of acetosyringone on transient GUS expression was tested using 14-day-old calli of a cultivar Kokei 14 and *A. tumefaciens* strain EHA101/pIG121-Hm (**Fig. 1-A, Table 2**). The number of GUS expressing unit (GUS spots) per g fresh weight was 1526.6±266.7 (standard deviation) when acetosyringone was included during infection and co-cultivation, while that of treatment which acetosyringone was not added was 382.2±115.4 (standard deviation). Addition of acetosyringone to both infection and co-cultivation media clearly promoted the transient expression of GUS.

Embryogenic calli were infected by *A. tumefaciens* strain EHA101/pIG121-Hm at 3, 6, 10, 14 and 21 days after the beginning of sub-culture. The calli cultured for a short period (3 and 6 days) gave few GUS spots, while 14-day-old calli gave the most (1526.6±266.7 spots per g fresh weight (standard deviation), **Table 2**). These findings clearly showed a culture period before bacterial infection of 14 days was needed to obtain efficient expression of *gus*A gene in embryogenic callus of Kokei 14.

3.2 Plant regeneration from hygromycin-resistant calli

The embryogenic calli infected with *A. tumefaciens* strain EHA101/pIG121-Hm were cultured on hygromycin-containing media for 60 days, and these embryogenic calli could produce several hygromycin-resistant calli, while uninfected-embryogenic calli failed to form them on the same media. The average number of hygromycin-resistant calli were produced at 10.7 per g fresh weight of infected embryogenic calli (**Fig. 1-B**). These hygromycin-resistant calli produced numerous somatic embryos on the somatic embryo formation medium containing hygromycin (**Fig. 1-C**). The hygromycin-resistant plantlets were developed from these somatic embryos on the plant formation medium. An average of 53.1% of hygromycin-resistant calli regenerated plantlets (**Table 3**). All of the regenerated plants grow further and rooted on the LS plant growth regulator-free

Table 2. Effect of acetosyringone and pre-culture period on transient GUS expression in embryogenic callus of sweet potato.

	No. of GUS spots/g fresh weight of embryogenic calli	
Culture period (days)	Acetosyringone (+)	Acetosyringone (−)
3	514.7± 21.5	—
6	840.9±242.2	—
10	1054.4±255.6	—
14	1526.6±266.7	382.2±115.4
21	922.8±132.4	—

*− and − denote the presence and absence of acetosyringone in the medium, respectively.

Table 3. Transformation efficiency of sweet potato by using *Agrobacterium tumefaciens* strain EHA101/pIG121-Hm.

Experiements	No. of hyg^r calli obtained[a]	No. of hyg^r calli produced plants	No. of hyg^r plants regenerated
		(%)	
1	20	9 (45.0)	26
2	12	8 (66.7)	14

[a]Number of hygromycin-resistant cell clusters obtained from 1.5 g fresh weight of embryogenic calli.

medium supplemented with 25 mg/*l* hygromycin. Regenerated plants were transferred to pots containing a vermiculite and perlite mixture (3: 1) and maintained at 26°C under a 16h-photoperiod in a growth chamber for 14 days. Then these regenerated plants were grown in a greenhouse. These transgenic plants grew normally and formed storage roots after three months (**Fig.1-D, E**). Five transgenic plants regenerated from independent hygromycin-resistant calli were analyzed to determine various phenotypic characteristics, such as apical immature leaf color, mature leaf color, mature leaf shape, stem color, number of storage roots per plant, fresh weight of a storage, skin color of storage roots and flesh color of storage roots. No morphological differences were observed between untransformed plants and the transgenic plants.

3.3 Histochemical GUS assay

Histochemical analysis of GUS activity was carried out on fully expanded leaves and storage roots of the regenerated plant from hygomycin-resistant calli. The tissue of a regenerated plants were stained blue indicating the expression of *gus*A gene, but none of those of control plants (**Fig.2-A**). GUS expression was also observed in storage roots harvested after three months cultivation in pots, while those of untransformed control plants did not show any GUS activity (**Fig.2-B**).

Leaves of freshly sprouting shoots from harvested storage roots of transgenic plants also showed GUS activity (**Fig.2-C**), suggesting that the *gus*A gene was transmitted to their vegetatively propagated progenies. Since sweet potato is commonly propagated using storage roots, this suggests the usefulness of genetically engineered sweet potato for the practical breeding of this plant species.

3.4 Integration of foreign DNA in the genome of transgenic plants

To measure the copy number of the integrated T-DNA directly, genomic blot analysis was performed for transgenic plants regenerated from five independent hygromycin-resistant callus lines. A *Sac* I / *Xba* I fragment of the *gus*A gene was used as a hybridization probe (**Fig.3-A**). Since there is only one *Hind* III site in the T-DNA region, hybridizing fragment(s) of different lengths indicated that the T-DNA was integrated at different location(s) in the sweet potato plant genome. The copy number was determined by the number of hybridizing fragments. **Fig.3-B** shows the number and size of hybridizing fragments varied among the different transgenic plant lines. Transgenic plant lines possessed one to three copies of T-DNA.

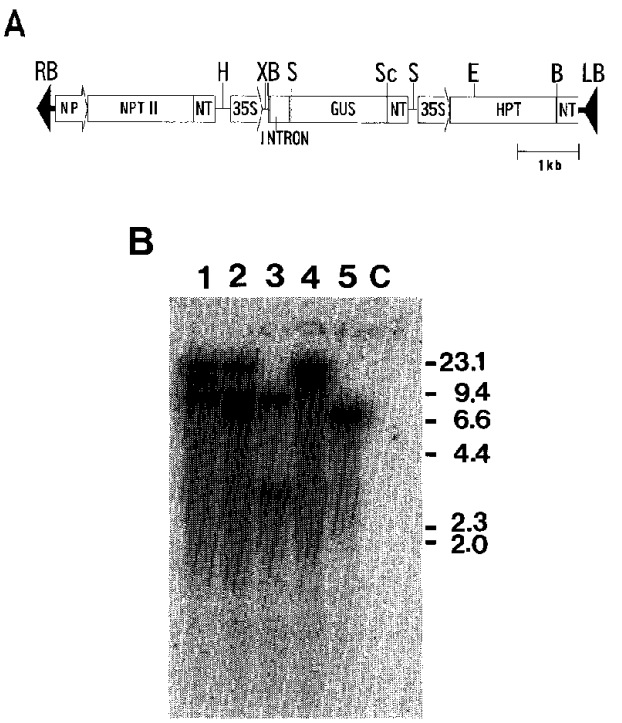

Fig. 3 Transformation vector and Southern blot analysis.
(A) Schematic diagram of a part of the T-DNA region of transformation vector pIG121-Hm. RB, right border; LB, left border; NP, nopaline synthase promoter; NT, nopaline synthase terminator; 35S, 35S promoter of cauliflower mosaic virus; INTRON, the first intron of catalase gene of castor bean; NPTII, gene for neomycin phosphotransferase; GUS, gene for β-glucuronidase; HPT, gene for hygromycin phosphotransferase. Cutting sites of restriction enzymes are indicated; *Bam*H I (B), *Eco*R I (E), *Hind* III (H), *Sal* I (S), *Sac* I (Sc), *Xba* I (X).
(B) Southern blot analysis of 5 independent transgenic plants. DNA was digested with *Hind* III and allowed to hybridize to the *gus* probe. Lanel-5, transgenic plants, which were regenerated from independent hygromycin-resistant calli; Lane C, untransformed control plant.

4. Discussion

In the present study we succeeded in the transformation of sweet potato mediated by *A. tumefaciens*. There are various factors which affect the transient GUS expression on embryogenic callus of sweet potato cv. Kokei 14. We examined three factors, inclusion of acetosyringone, culture period of embryogenic calli before bacterial inoculation and the differences of *Agrobacterium* strains, and confirmed

that all of these factors affected the transformation efficiency of sweet potato cells. The most interesting finding was the differences in transformation efficiency among the bacterial strains. The transient GUS expression varied with the bacterial strain. *A. tumefaciens* strain LBA4404 harboring a 'super-binary' vector pTOK233 has been reported to be a more efficient strain than EHA101/pIG121-Hm in rice transformation [12] and *A. tumefaciens* strain R1000 harboring pRiA4 and pBI121 an efficient strain for transformation of *Ipomoea trichocarpa* [13], a wild relative of sweet potato. However, *A. tumefaciens* 'super-virulent' strain EHA101 has the advantages in genetic transformation of sweet potato using embryogenic callus. Gama *et al.* [8] also succeeded in obtaining transgenic sweet potato plants by using same bacterial strain, EHA101.

Recently, Murata *et al.* [5] and Okada *et al.* [7] obtained transformed sweet potato plants from electroporated-protoplasts and biolistic transformed suspension cultures, respectively. However, some problems such as genotypic differences in transformation efficiency and inefficient selection of transformed cells still remain. Moreover, transformation by direct gene transfer methods such as electroporation and particle bombardment often leads to complex integration of multiple copies of the introduced genes [18, 19]. The production of the transgenic plants having a low copy number (one to three) of integrated genes by *A. tumefaciens*-mediated transformation method presented here was an advantage of this method.

In contrast to the direct gene transfer method, Newell *et al.* [6] obtained seven transgenic plants from 140 disks of storage roots of sweet potato cv. Jewel by *Agrobacterium*-mediated transformation. The storage root may not be a good material for obtaining regenerated plants in sweet potato, because the culture responses varied among the genotypes and moreover cultivars with the ability to regenerate plants from storage root disks were rare [20]. Using the present transformation method of sweet potato, based on the culture system of embryogenic callus induction from meristem tissue at high frequency using the medium containing 4FA, more than 50% of meristem tissues formed embryogenic callus in all eleven cultivars tested [9]. Therefore, the method of *A. tumefaciens*-mediated transformation using embryogenic callus might overcome the genotypic differences in genetic transformation of sweet potato.

Although, it is difficult to compare the results of Gama *et al.* [8] in which they selected transformed embryogenic cells by using kanamycin, in this study the transformation efficiency have been considerably improved by using hygromycin for the selection of transformed cells.

We have obtained transgenic Kokei 14 plants possessing the coat protein gene of sweet potato feathery mottle virus [21], a severe pathogen of Kokei 14 in Japan. The *A. tumefaciens*-mediated gene transfer system using embryogenic callus may be useful as a routine method for the genetic modification of sweet potato.

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References

[1] Henderson, J. H. M., Phills, B. R., Whatley, B. T., 1984. In "Handbook of plant cell culture vol. 2" (eds. by Sharp, W.R. *et al.*), p. 302-326, Macmillan, New York.

[2] Goddijin, O. J. M., Pen, J., 1995. Trend in Biotech., **13**: 379-387.

[3] Dodds, J. H., Merzdorf, C., Zambrano, V., Siguenas, C., Jaynes, J., 1991.In "Sweet potato pest management: a global perspective" (eds. by Jansson, R.K., Raman, K.V.), p. 203-219, Westview, Boulder.

[4] Otani, M., Mii, M., Handa, T., Kamada, H., Shimada, T., 1993. Plant Sci., **94**: 151-159.

[5] Murata, T., Okada, Y., Fukuoka, H., Saito, A., Kimura, T., Mori, M., Nishiguchi, M., 1995. In "Proceeding of the 1st Chinese-Japanese Symposium on sweetpotato and potato, Beijin, August 30- September 2, 1995" (eds. by Liu, Q.C., Kokubu, T.), p. 369-374, Beijin Agricultural University Press, Beijin.

[6] Newell, C.A., Lowe, J.M., Merryweather, A., Rooke, L.M., Hamilton, W.D.O., 1995. Plant Sci., **107**: 215-227.

[7] Okada, Y., Murata, T., Saito, A., Kimura, T., Mori, M., Nishiguchi,M., Fukuoka, H., 1995. Breed. Sci., **46** (Suppl.1): 256.

[8] Gama, M. I. C. S., Leite Jr., R. P., Cordeiro, A. R., Cantliffe, D. J., 1996. Plant Cell Tissue Organ Cult., **46**: 237-244.

[9] Otani, M., Shimada, T., 1996. Breeding Science, **46**: 257-260.

[10] Linsmaier, E. M., Skoog, F., 1965. Physiol Plant., **18**: 100-127.

[11] Akama, K., Shiraishi, H., Ohta, S., Nakamura, K., Okada, K., Shimura, Y., 1992. Plant Cell Rep., **12**: 7-11.

[12] Hiei, Y., Ohta, S., Komari, T., Kumashiro, T.,

1994. The Plant J., **6**: 271-282.

[13] Otani, M., Shimada, T., Kamada, H., Teruya, H., Mii, M., 1996. Plant Sci., **116**: 169-175.

[14] Rashid, H., Yokoi, S., Toriyama, K., Hinata, K., 1996. Plant Cell Rep., **15**: 727-730.

[15] Jefferson, R. A., Kavanagh, T. A., Bevan, M. W., 1987. EMBO J., **6**: 3901-3907.

[16] Honda, H., Hirai, A., 1990. Jpn. J. Breed., **40**: 339-348.

[17] Southern, E. M., 1975. J. Mol. Biol., **98**: 503-517.

[18] Klein, T. M., Kornstein, L., Sanford, J. C., Fromm, M. E., 1989. Plant Physiol., **91**: 440-444.

[19] Register, J. C., III, Peterson, D. J., Bell, P. J., Bullock, W. P., Evans,I. J., Frame, B., Greenland, A. J., Higgs, N. S., Jepson, I., Jiao, S., Lewnau, J. L., Sillick, J. M., Wilson, H. M., 1994. Plant Mol. Biol., **25**: 951-961.

[20] Yamaguchi, T., 1978. Bull. Univ. Osaka Pref., Ser. B, **30**: 54-88.

[21] Mori, M., Sakai, J., Kimura, T., Usugi, T., Hayashi, T., Hanada, K., Nishiguchi, M., 1995. Arch. Virol., **140**: 1473-1482.