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Expression Patterns of CaMV 35S Promoter-GUS in Transgenic Potatoes and Their Clonal Progenies

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Two potato (*Solanum tuberosum* L.) cultivars were transformed by *Agrobacterium tumefaciens* harboring cauliflower mosaic virus (CaMV) 35S promoter and β-glucuronidase (GUS) gene. Expression patterns of the CaMV 35S promoter according to tissue types and deveplopmental stages, and genetic stability of GUS gene were investigated in the clonal progenies of transgenic potatoes. Kanamycin-resistant shoot emerged from tuber disc after 4 weeks of culture, and root was induced 6 weeks after culture on the selection medium. Shooting frequency of cvs. Superior and Dejima were 43% and 27%, respectively. Mature transformants and their clonal progenies showed no phenotypical abnormality. GUS activity was expressed primarily at parenchymatous cells of phloem tissue around the vascular cambium in the stem and root, and higher activity was found at the apical meristem of shoot, root and adventious shoot bud. GUS activity was higher at tubers of young explants than at stored tubers. These facts indicate that expression level of the CaMV 35S promoter differred according to tissue types and developmental stages of the organs. The GUS gene was stably inherited to each clonal progeny and normally expressed.

Key words : Solanum tuberosum L., GUS, CaMV 35S, clonal progeny of transgenic potato

Potato (*Solanum tuberosum* L.) has been regenerated from the callus of various explants (Lam, 1975; Binding *et al.*, 1978) and mesophyll protoplasts (Shepard and Totten, 1977). However, the regeneration efficiency of potato was relatively low, and a wide range of phenotypic and chromosomal variation has been reported among regenerated potatoes (Sree-Ramulu *et al.*, 1985; Sree-Ramulu, 1986). In spite of its low regeneration capacity, production of transgenic potatoes by *Agrobacterium*-mediated transformation has been possible in European cultivars (Horsch *et al.*, 1985; Rogers *et al.*, 1986).

In gene transfer studies on potato as well as other plants, because the cauliflower mosaic virus 35S promoter is constitutively transcribed in transgenic plant cells (Odell *et al.*, 1985), it is often used to express selectable marker genes or other useful genes. Recent research reported that three functional upstream fragments located between −343 and −46 were responsible for the high transcriptional activity of CaMV 35S promoter (Fang *et al.*, 1989; Odell *et al.*, 1988). However, it was showed that the expression of fusion genes directed by CaMV 35S promoter was the highest in the actively dividing tissue such as vascular cambium (Jefferson *et al.*, 1987; Williamson *et al.*, 1989). It was also reported that the expression of the chloramphenicol acetyl transferase (CAT) gene directed by CaMV 35S promoter was strong at the S phase in the transformed tobacco protoplasts (Nagata *et al.*, 1987).

In this study, we produced morphologically normal transgenic potatoes, and investigated the expression patterns of CaMV 35S-GUS gene according to tissue types and developmental stages.

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MATERIALS AND METHODS

Plant material and reagents

Two potato (*Solanum tuberosum* L.) cultivars, Superior and Dejima, were obtained from Alpine Experiment Staion, Rural Development Administration, Hoengye, Korea. After these potatoes had been stored at 4℃ for about 1 month, they were used as a culture explant. Restriction enzymes and random primer DNA labelling kit were purchased from Promega and used according to manufacturer's specifications. α-^{32}P dCTP was obtained from New England Nuclear, and 5-bromo-chloro-indolyl-β-D-glucuronide (X-gluc) and *p*-nitrophenyl-β-D-glucuronic acid (*p*NPG) were purchased from Jersey Lab Supply and Sigma, respectively.

Transformation and growth of *Agrobacterium*

pLS201 described previously (Lee and Sung, 1992) was transformed to *Agrobacterium* by freeze-thaw method (Hofgen and Willmitzer, 1988), and used for potato transformation. *A. tumefaciens* PC2760 (Hoekema *et al.*, 1983) was cultured in a YEP medium (10 g/L bacto-tryptone, 10 g/L yeast extract, and 5 g/L NaCl, pH 7.2) at 28℃.

Potato transformation and regeneration

Tuber discs of potato were transformed and regenerated as described by Lee and Sung (1992) except for the change of the shoot induction medium and propagation of transgenic plants in a liquid medium. The co-culture medium for *Agrobacterium* infection contained the MS salts (Murashige and Skoog, 1962), 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 30 g/L sucrose, 8 g/L agar, 0.6 mg/L indole-3-acetic acid, 1.1 mg/L zeatin, pH 5.9, and was supplemented with tobocco cell suspension culture. One week after co-culture, explants were placed onto the shoot induction medium (the same composition of the co-culture medium containing 50 μg/mL kanamycin and 500 μg/mL carbenicillin). Induced shoots were cut and transferred on the root induction medium (MS basal medium, 50 μg/mL kanamycin and 500 μg/mL carbenicillin). Regenerated plantlets were transferred in the liquid medium containing MS salts, 30 g/L sucrose, 100 mg/L myo-inositol, 0.01 mg/L GA_3, 0.5 mg/L kinetin, 50 μg/mL kanamycin and 300 μg/mL carbenicillin, and shaked with 80 rpm for 2 weeks. The regenerated plants were hardened and grew to whole plants in the pots.

Investigation of GUS expression by histological analysis

To investigate the expression pattern of CaMV 35S promoter in the various tissues of transgenic potato, histological analysis was done according to Jefferson (1987) using X-gluc as a substrate. Leaf, petal, stamen and pistil were immersed into *in situ* assay solution [100 mM sodium phosphate (pH 7.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3 % X-gluc, and 0.5% Triton X-100] for 24 h at 26℃, and then washed with 70% ethanol, and observed under a stereoscopic microscope.

GUS spectrophotometric assay

Protein extraction and GUS spectrophotometric assay from the various organs of transgenic potatoes were performed as described by Lee and Sung (1992).

Extraction of genomic DNA and DNA gel blot

Genomic DNA was isolated from leaves and tubers using the method described by Murray and Thompson (1980). Ten microgram of genomic DNA was digested with *Pst*I or *Hin*dIII, seperated by electrophoresis on a 0.8% agarose gel, and transferred to a nylon membrane in 20X SSC. The membrane was prehybridized at 65℃ in hybridization solution [5X SSC, 10X Denhardt's solution, 0.5% SDS and 100 μg/mL denatured salmon sperm DNA] for 3 h, and hybridized in the same solution containing GUS probes for 16 h. Filters were washed two times at 65℃ for 30 min each with 2X SSC and 0.5% SDS, two times for 30 min each with 0.2X SSC and 0.5% SDS, and exposed at −70℃ for 5 d. For the probe, the 4.4 kb *Hin*dIII fragment containing the GUS coding region was seperated from pLS201 (Lee and Sung, 1992) by gel electrophoresis, eluted, and radioactively labeled by random priming method (Feinberg and Vogelstein, 1983).

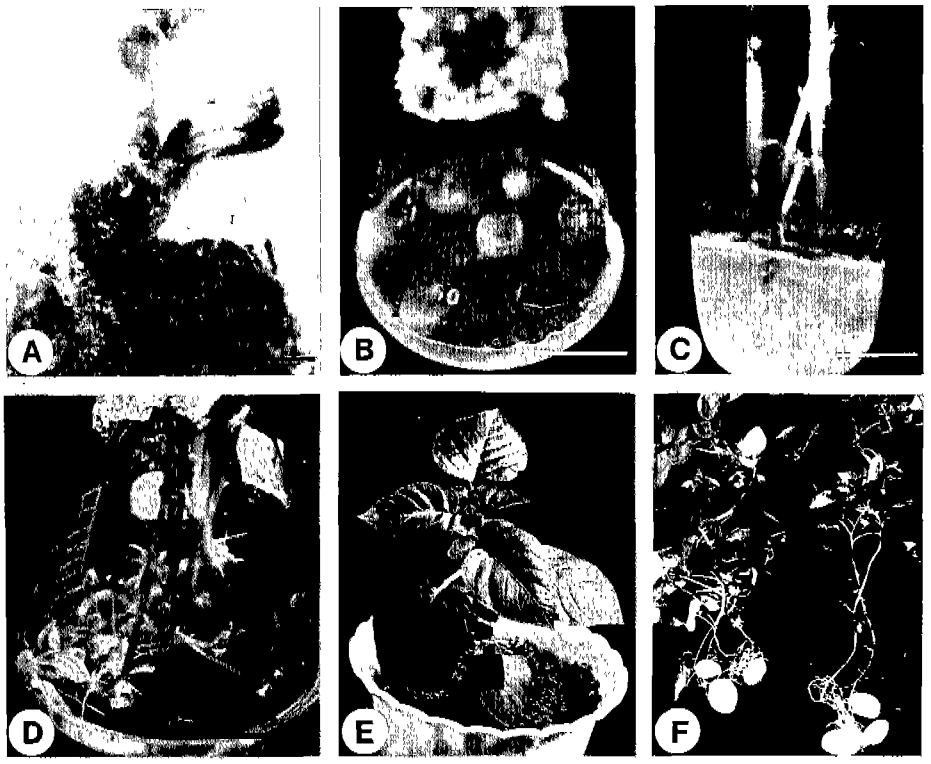

Fig. 1. Various stages during potato transformation on the selective medium and maturing process of transformed plant. A, tuber disc 3 weeks after incubation with *Agrobacterium*. Shoot primordia emerged from the green protuberance (bar=2 mm). B, shoots appeared from tuber discs after 4 to 5 week culture (bar=2 cm). C, shoot excised from disc as in (B) and subcultured on the root induction medium (bar=1 cm). D, rapid shooting and rooting formation occurred at the liquid culture (bar=2 cm). E, hardened transgenic potato obtained from the *in vitro* cultures. F, transgenic plants after 15 week culture at green house.

RESULTS AND DISCUSSION

Potato transformation and induction of clonal progenies

Green protuberances from tuber discs transformed with binary vector appeared after 10 to 14 d after culture on the selection medium (Fig. 1A), and shoot primordia were formed 3 weeks after culture (Fig. 1B). Procedure for the potato transformation used in this experiment shortened the period for shoot induction by 3 weeks when compared with that in the previous report (Lee and Sung, 1992). This result indicates that use of nurse culture and modification of the shoot induction medium improved the efficiency of the *Agrobacterium*-mediated transformation. Shoots were cut and placed onto the root induction medium. Roots were formed one week after culture in the root induction medium (Fig. 1C). Regenerated plants were propagated in a liquid medium for 10 to 14 d (Fig. 1D), and then hardened and grown to whole plants in pots (Fig. 1E). After 15 weeks, tubers were harvested from these plants (Fig. 1F). Tubers of transgenic potatoes were sowed and observed the morphological variances in their clonal progenis. Shoot from the second clonal progeny was not different from that of wild type plant (Fig. 2A), showing that morphological changes did not occur during regeneration (Fig. 2).

We also examined the differences between two cultivars during the induction of shoot and root. Shoots in cvs. Superior and Dejima were formed after 4 and 5 weeks, respectively. But rooting frequency of Superior was 3.6 times higher than that of Dejima (Table 1). These results demonstrated that Superior was more suitable for gene transfer than Dejima. The culture period for the production of shoot from tuber discs in the European cultivars, Desiree and

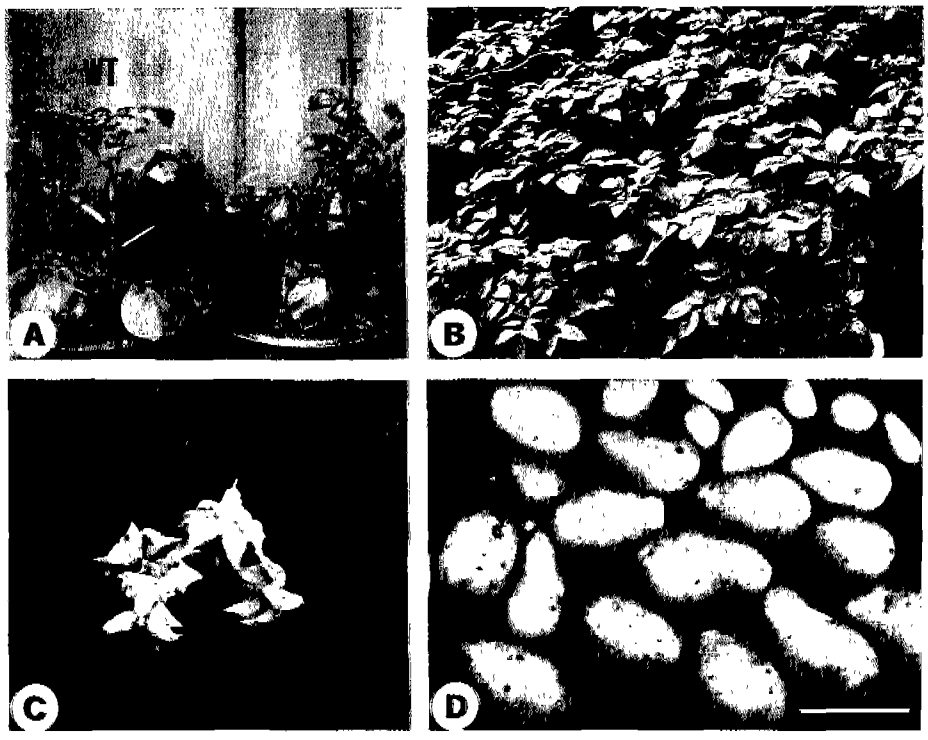

Fig. 2. Morphological view of second clonal progenies of the transformed potatoes. A, comparison of morphology of wild-type (WT) with transformed (TF) plants. B, C and D, the progenies of transgenic plants were vigorous, phenotypically normal and produced a good crop of tubers (bar=1 cm).

Table 1. Shooting and rooting efficiency of potato tuber discs in cv. Superior and cv. Dejima on the shoot and root induction medium containing 50 μg/mL kanamycin

Cultiver	Shooting frequency (%)[a] (No. of shoots/ No. of discs)	Rooting frequency (%)[b] (No. of roots/ No. of discs)
Superior	42.9 (66/154)	30.5 (47/154)
Dejima	27.2 (52/191)	8.4 (16/191)

[a]Shooting frequency was counted after 8 week culture. [b]Rooting frequency was counted after 10 week culture.

Bintje was 4 weeks, and shooting frequency was 20% in the kanamycin-containing medium (Sheerman and Bevan, 1988). In addition, polyploidy appeared in the transgenic potatoes (Sheerman and Bevan, 1988; Stiekema *et al.*, 1988). In our result, the period for shoot induction of Superior and Dejima was similar to the European cultivars (Sheerman and Bevan, 1988), while their shooting frequency was higher than that of European cultivars to 43% and 27%, respectively (Table 1). These results indicated that the transformation method used in this experiment was satisfactory and very useful.

Expression pattern of CaMV 35S promoter in different tissue type

We had previously performed the histological analysis in transformed calluses and stem, root and microtuber of transgenic potatoes (Lee and Sung, 1992), and examined here in detail the localization of GUS activity in various tissues of the second clonal progeny of the transformed potato. In stem, GUS was highly expressed in vascular cambium, especially in parenchyma cells of phloem and xylem, but weakly detected in cortex and epidermis (Fig. 3A, B and C). GUS activity was high in phloem parenchyma cells around the vascular cambium of petiole (Fig. 3D and E). Apical meristem of shoot showed extremely high activity (Fig. 4G), while leaf, sepal and petal contained high activity in veins (Fig. 4A, B and C). GUS activity was high in immature flower bud (Fig. 4D) and stigma of mature pistil (Fig. 4E), but weak in filament of mature stamen (Fig. 4F). In the underground part, actively growing regions such as root apical meristem (Fig. 5A), phloem parenchyma cells around cambium contained high GUS

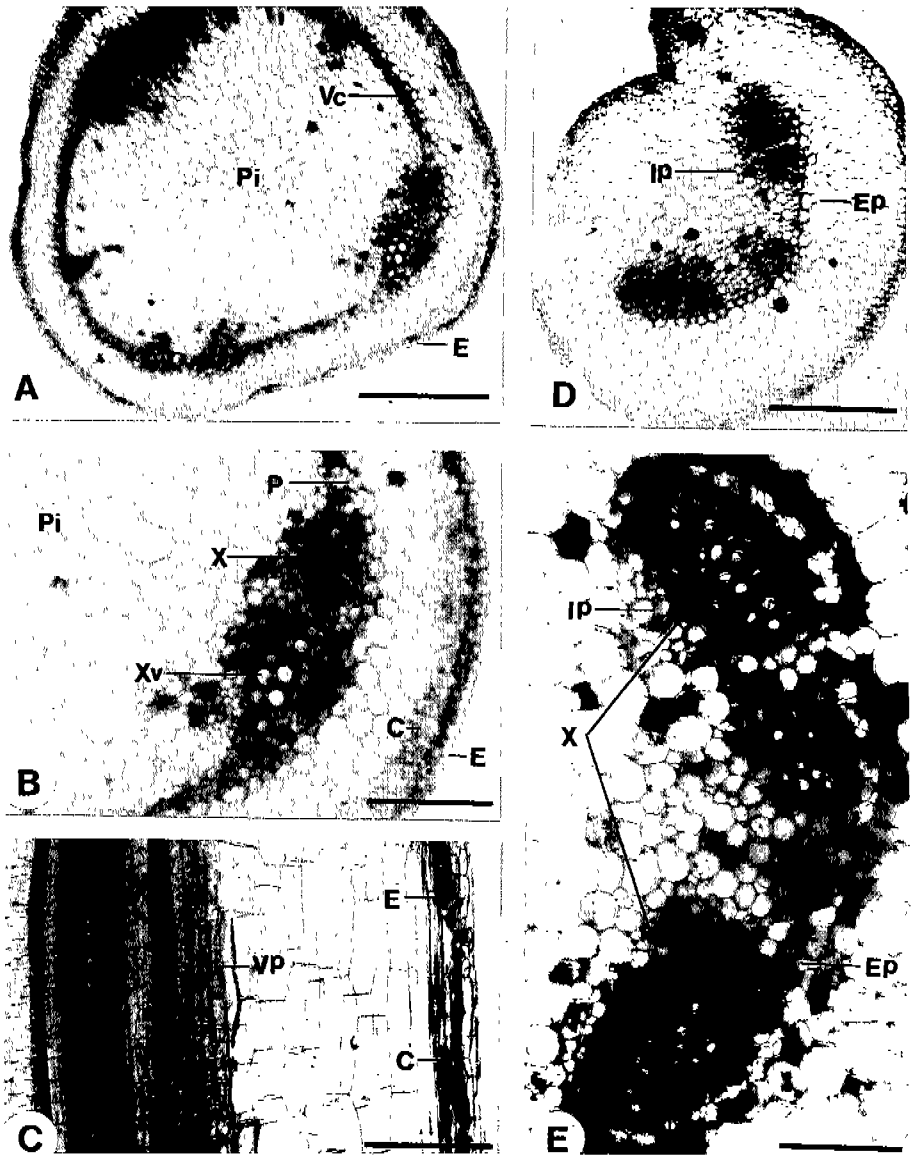

Fig. 3. Histochemical localization of GUS in the stem and petiole of second clonal progeny of the transgenic potato. A, transverse section of stem (bar=0.4 mm). B, magnification of the vascular bundle of the stem (bar=0.3 mm). C, longitudinal section of stem (bar=0.3 mm). D, transverse section of petiole (bar=0.4 mm). E, magnification of the vascular bundle of the petiole (bar=0.1 mm). Abbreviations: C, collenchyma; Co, cortex; E, epidermis; Ep, external phloem; Ip, internal phloem; P, phloem; Pi, pith; Vc, vascular cambium; Vp, vascular parenchyma; X, xylem; Xv, xylem vessel.

activity.

The pattern of GUS expression in rhizome was very similar to that in stem, but the expression level was lower than that in stem (Fig. 3A, B, C and Fig. 5B). In adventitious shoot or its primordium, GUS activity was the highest owing to their active cell division (Fig. 5C and D). Also, GUS activity in tubers on rest appeared around the vascular ring (Fig. 5E), but existed around all parts in the tubers of the seedling (Fig. 5F).

Several points could be summarized from the histological GUS assay as follows. 1) Actively dividing cells of shoot, rhizome and root, specifically phloem parenchyma cells, had high GUS activity. 2) Veins of leaf, sepal and carpel had also high GUS activity. 3) GUS expression in the apical meristem and adventitious shoot primordium was very high. 4) Tubers of young plants had higher GUS expression level

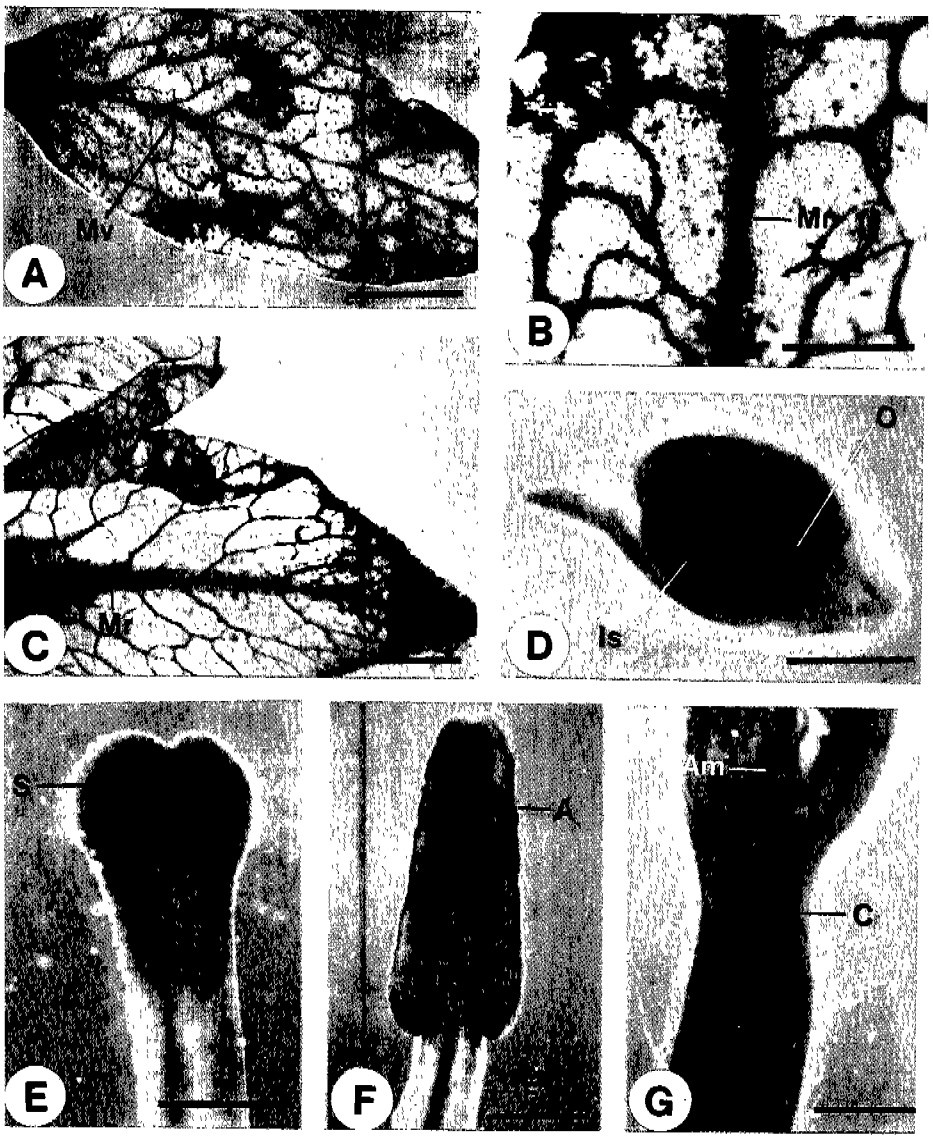

Fig. 4. Histochemical localization of GUS in the leaf and floral parts of second clonal progeny of the transgenic potato. A, leaf (bar=3 mm). B, sepal (bar=1.2 mm). C, mature flower (bar=2 mm). D, longitudinal section of immature flower bud (bar=3 mm). E, pistil (bar=0.8 mm). F, stamen (bar=2 mm). G, longitudinal section of apical region of stem (bar=2.5 mm). Abbreviations: A, anther; Am, apical meristem; C, cambium; F, filament; Is, immature stamen; Mr, midrib; Mv, midvein; O, ovule; S, stigma.

in comparison with those on rest.

It was reported that the expression pattern of CaMV 35S promoter-GUS or-CAT gene depended on the division capacity of cells (Jefferson *et al.*, 1987; Williamson *et al.*, 1989). Jefferson *et al.* (1987) suggested that the difference of expression according to tissue types was due to the difference in the metabolic activity of each cell. In all tissues that we examined, there was a good correlation between higher GUS activity driven by the CaMV 35S promoter and cell division capacity. Our data suggest that CaMV 35S promoter activity might be located to actively dividing tissues such as meristem and vascular cambium of shoot and root in the transgenic potatoes. Also the data that GUS activity was higher in tubers of young plant than the mature tubers on rest explained that CaMV 35S promoter depend on the developmental stage as well as the tissue type.

GUS activity at different organs

The protein extracts from the upper, middle and

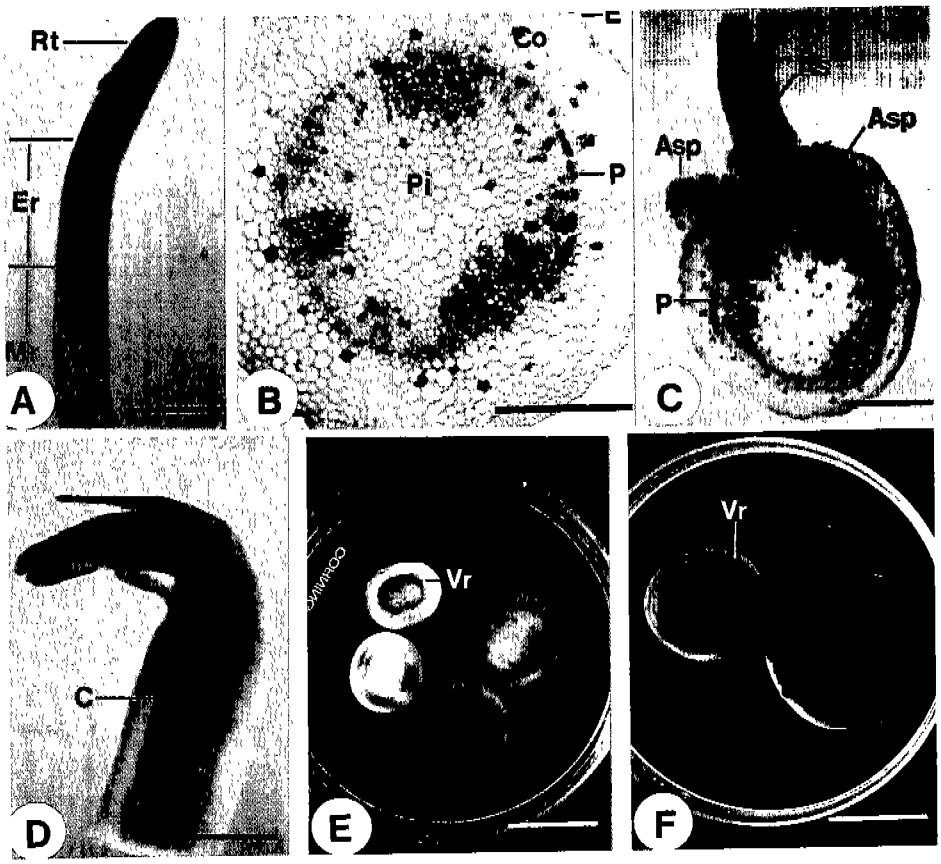

Fig. 5. Histochemical localization of GUS in the underground parts of second clonal progeny of the transgenic potato. A, root (bar=1.5 mm). B, transverse section of rhizome (bar=0.5 mm). C, transverse section of rhizome with adventitious shoot (bar=1.2 mm). D, longitudinal section of adventitious shoot bud (bar=1 mm). E, section of tubers stored at 4℃ for one month after harvest (bar=3 cm). F, section of tubers of young plants (bar=2 cm). Abbreviations: As, adventitious shoot; Asp, adventitious shoot primordium; C, cambium; Co, cortex; E, epidermis; Er, elongation region; P, phloem; Pi, pith; Mr, maturation region; Vr, vascular ring.

Table 2. GUS activity in different stages of leaves in transgenic potatoes (cv. Superior)

No.[a]	Young leaf (5-20×10-25)[b] mm	Expanded leaf (25-40×35-55)[b] mm	Mature leaf (45-50×60-70)[b] mm
1	13.91± 1.27	26.76± 1.07	25.49± 4.02
2	13.68± 1.23	25.64± 1.34	18.11± 2.68
3	39.24± 3.67	98.47± 8.25	54.01± 3.85
4	36.08± 3.20	35.98± 1.95	7.72± 0.08
Mean	25.73± 5.36	46.71± 15.0	26.33± 8.59

GUS activity was expressed as nmole *p*-nitrophenol·min^{-1}·mg protein^{-1}. [a]Each transgenic plant was grown in green house. [b]Size of the leaves.

lower leaves were used for spectrophotometric GUS assay (Table 2). From this assay, it was showed that the leaves of middle part had higher activity than those of upper and lower parts. This result concurred with the views that GUS protein has been persisted for a long time (Jefferson *et al.*,1986), and accumulated within tissue (Hensgens *et al.*, 1992). The GUS activity in leaf, stem, root and tuber of mature plants was assyed with *p*NPG as a substrate. When GUS activity per protein content was measured, it was identified that GUS was expressed highly in the order of leaf, stem, root and tuber (Table 3). Difference of GUS activity among these organs may be due to the distribution and proportions of tissue types.

GUS expression in clonal progenies

To find out if GUS gene of transgenic plants was transmitted to the next generations, genomic DNAs from the second and the third generations of T-7 and T-8 lines were southern hybridized. In the second generation of T-7 and T-8 line, 1.9 kb *Pst*I frag-

Table 3. GUS activity in different organs of transgenic potatoes (cv. Superior)

No.[a]	Leaf	Stem	Root	Tuber
Wild-type	0.78± 0.06	0.22± 0.03	0.00± 0.0	0.00± 0.0
T7	63.3± 1.2	68.3± 0.3	63.0± 0.6	27.2± 0.7
T8	39.0± 1.0	64.1± 3.9	46.7± 0.6	28.1± 1.2
T10	85.7± 1.2	116.3± 0.3	77.3± 1.5	35.9± 0.8
T11	84.1± 3.1	22.2± 0.4	24.2± 0.4	39.0± 1.7
Mean	68.0± 9.5	67.7± 16.7	52.8± 9.9	32.6± 2.5

GUS activity was expressed as nmole *p*-nitrophenol·min^{-1}·mg protein^{-1}. [a]Each transformed plant was grown in green house, and T7-T11 are the progeny lines of the transgenic potato plants.

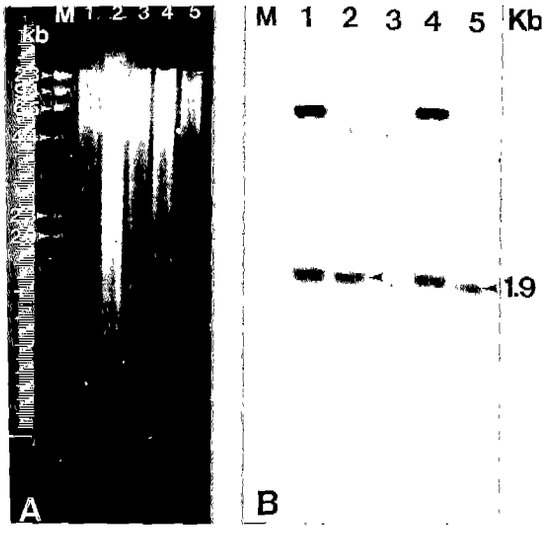

Fig. 6. Southern blot analysis of genomic DNA extracted from the second clonal progeny of the transgenic potato. Restriction enzyme (*Pst*I) digestion patterns (A) and corresponding blot hybridized to GUS-probe (B). Lane M, lambda DNA digested with *Hin*dIII. Lane 1, T-7 line tuber. Lane 2, T-7 line leaf. Lane 3, wild type leaf. Lane 4, T-8 line tuber. Lane 5, T-8 line leaf. Arrow head represents GUS gene.

ment of GUS coding region was positively detected (Fig. 6). In their third generation, 4.4 kb *Hin*dIII fragment of GUS coding region was dectectd (Fig. 7). From genomic DNA gel blot, it was demonstrated that GUS gene was inherited stably to their progenies.

In conclusion, we certified CaMV 35S promoter was expressed differently according to tissue types and developmental stages. And it was identified that GUS gene was stably inherited to the clonal progenies of potato.

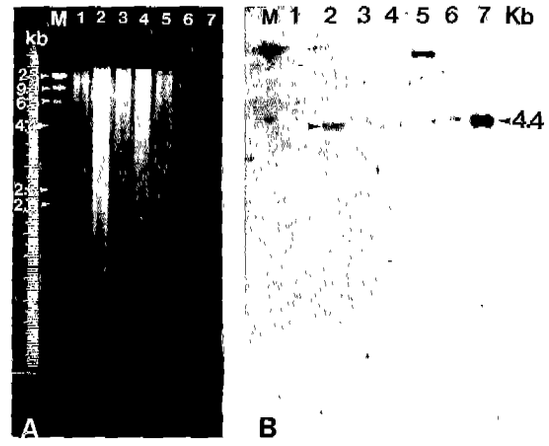

Fig. 7. Southern blot analysis of genomic DNA extracted from the third clonal progeny of the transgenic potato. Restriction enzyme (*Hin*dIII) digestion patterns (A) and corresponding blot hybridized to GUS-probe (B). Lane M, lambda DNA digested with *Hin*dIII. Lane 1, wild type leaf. Lane 2, T-7 line leaf. Lane 3, T-7 line tuber. Lane 4, T-8 line leaf. Lane 5, T-8 line tuber. Lane 6 and 7 are one and five copy reconstruction of 4.4 kb DNA containing GUS coding region, respectively. Arrow head represents GUS gene.

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形質轉換된 감자의 後代 植物體에서 CaMV 35S Promoter-GUS 遺傳子 發現

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적 요

감자 (*Solanum tuberosum* L.) 괴경을 재료로 하여 cauliflower mosaic virus (CaMV) 35S promoter와 β-glucuronidase (GUS)를 포함하는 *Agrobacterium tumefaciens*를 매개로 형질전환 개체를 유도하였던 바, 선별 배지에서 캘러스 과정을 거치지 않고 배양 4주 후 shoot가, 배양 6주 후 정상적인 재분화 개체가 유도되었다. Kanamycin 저항성 shoot의 형성률은 수미 (cv. Superior)의 경우 43%, 대지 (cv. Dejima)의 경우 27%로 나타났다. 조직 유형에 따른 CaMV 35S promoter의 발현 양상을 조사하기 위하여 GUS 분포를 살펴본 결과, 줄기와 뿌리의 형성층을 따라 체관부 유조직 세포에서 높은 발현을 보였으며, 세포 분열이 활발한 줄기 정단부 및 근단에서 매우 강한 발현을 나타내었다. 잎, 꽃잎, 꽃받침에서는 주맥을 따라 높은 발현을 보였고, 근경의 경우 새로 형성되는 부정아 시원 조직에서 매우 강하게 발현되었다. 또한 4℃에 보관 중인 휴지 상태의 괴경에서는 중심주 부위에서만 GUS가 발현된 반면, 유식물체의 괴경에서는 전체적으로 균등한 발현을 보임으로써 CaMV 35S promoter에 의한 GUS의 발현은 조직 유형에 따른 발현의 차이와 함께 기관 발달에 따라서도 발현의 차이를 나타냄을 알 수 있었다. 형질전환된 후대 식물체에서 도입된 GUS 유전자를 조사한 결과, 3세대까지의 식물체에도 GUS 유전자는 안정하게 전달되었다.

주요어 : 감자, GUS, CaMV 35S, 형질전환된 감자의 후대 식물체

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