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Transformation of Plant Cells by Gene Transfer : Construction of a Chimeric Gene Containing Deleted Maize Alcohol Dehydrogenase Intron and β-Glucuronidase Gene and Its Expression in Potato

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유전자 도입에 의한 식물세포의 형질전환 : 옥수수 알코올 탈수소효소 유전자의 절단된 인트론 및 β-Glucuronidase 유전자를 함유하는 키메라 유전자의 제조와 감자에서의 발현

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ABSTRACT

To understand the properties of the cauliflower mosaic virus (CaMV) 35S promoter and the effect of the deleted maize alcohol dehydrogenase 1-S (*Adh*1-S) intron 1 on the expression of the CaMV 35S-β-glucuronidase (GUS) gene in potato (*Solanum tuberosum* L. cv. Superior), we constructed a chimeric gene and transferred it into potato with *Agrobacterium tumefaciens* mediated method. The pLS201, a gene transfer vector of 17.7 kilobase pairs, was composed of the CaMV 35S promoter, the 249 base pairs of deleted maize *Adh*1-S intron 1, the GUS reporter gene, and the kanamycin resistance gene as a selectable marker for transformation. The GUS activity was examined by histochemical and spectrophotometric assay in transformed potato plants. The GUS activity was found primarily around the vascular tissue cells in stem and root. In the spectorophotometric assay, the level of GUS activity of transgenic potato transformed with CaMV 35S/249 bp of intron 1 fragment-GUS (pLS201) was compared with that of potato transformed with CaMV 35S-GUS (pBI121). The quantitative spectrophotometric assay showed that the level of GUS activity in potato transformed with pLS201 was higher in leaf, stem and root by 30-, 34- and 42-fold, respectively than those in potato transformed with pBI121. This results indicate that the inclusion of the deleted maize *Adh*1-S intron 1 resulted in increament of the GUS gene expression in transgenic potato.

INTRODUCTION

The production of transgenic plants by gene transfer has become an important tool for genetic engineering of agronomically important crop species and for elucidating mechanisms of gene expression. In gene transfer studies, the cauliflower mosaic virus (CaMV) 35S promoter exhibits high level of transcriptional activity in a variety of plant species, and is one of the most widely studied and utilized promoters. Since the 35S promoter activity was described as constitutive (Odell *et al.*, 1985), this promoter is used in the construction of plant expression vectors (Jefferson *et al.*, 1987) and expression of agronomically useful genes such as those for viral coat

protein (Abel *et al.*, 1986), insect resistance (Fischhoff *et al.*, 1987) and herbicide resistance (Shah *et al.*, 1986).

Although the CaMV 35S promoter activity has been described as constitutive, recent studies suggest that the expression of genes fused to the 35S promoter is tissue specific, cell type specific in particular organs and the specific expression depends on developmental stages (Jefferson *et al.*, 1987; Nagata *et al.*, 1987; Williamson *et al.*, 1989). Futhermore, several elements affecting the transcriptional level of this promoter were identified including putative upstream activating sequences (Kay *et al.*, 1987; Benfey *et al.*, 1989, 1990). In addition, introns were shown to stimulate expression of reporter gene constructs, even when the reporter genes were derived from bacteria and did not contain introns (Callis *et al.*, 1987; Luehrsen and Walbot, 1991).

To evalute the expression pattern driven by the 35S promoter and identify wheather or not the deleted maize alcohol dehydrogenase 1-S (*Adh*1-S; S=slow electromorph) intron 1 affects the GUS gene expression in plant, we constructed a chimeric gene comprising the CaMV 35S promoter, deleted *Adh*1-S intron, and GUS gene and expressed it in potato. At the various organs in transgenic potato plants transformed with the CaMV 35S promoter-deleted intron (*Adh*1-S) 1 indicated that GUS expression was detected in all organs and the level of GUS activity driven by 35S promoter/249 bp of the *Adh*1-S intron 1 fragment was 30-42 fold higher than that without intron fragment (pBI121) in transgenic potato plants. Also, the histochemical study showed that GUS activity was primarily localized around vascular tissue in the stem and root of the transgenic potato.

MATERIALS AND METHODS

Reagents. All restriction and DNA modifying enzymes were obtained from New England Biolabs (NEB) and Promega and they were used according to the manufacturers' specifications. 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt (X-gluc) and *p*-nitrophenyl β-D-glucuronide were purchased from Research Organics Inc.(Cleveland, OH, USA) and Sigma.

Bacterial strains and transformation. *Escherichia coli* strains K802, JM101 and HB101 were used throughut the experiment for the construction and maintenance of the plasmids. The *Agrobacterium tumefaciens* strain LBA 4404 (pAL4404) was used as a host for all Ti plasmid-derived vectors. Procedures for transformation of *E. coli* were those described by Sambrook *et al.* (1989). The constructed vectors were transferred to *A. tumefaciens* by the freeze-thaw method (Holsters *et al.*, 1978). *A. tumefaciens* carrying the plasmids was grown on the sterile medium which was composed of 5 g/*l* NaCl, 10 g/*l* tryptone, 10 g/*l* yeast extract (pH 7.2) and 50 μg/m*l* kanamycin.

Plasmid construction. All plasmids were constructed using routine DNA manipulation protocols (Sambrook *et al.*, 1989). Recombinant plasmids were multiplied in *E. coli*, isolated by alkali lysis and phenol extraction, and purified by cesium chloride/ethidium bromide density gradient centrifugation.

Potato transformation and regeneration. Tuber discs of potato (*Solanum tuberosum* L. cv. Superior) were transformed as described by Sheerman and Bevan (1988). Tubers were peeled and surface sterilized for 20 min in 0.5% sodium hypochlorite containing a few drops of Tween 20. They were then washed five times with sterile distilled water. Samples were taken using a sterile cork borer (1 cm diameter) and columnar sections were sliced into discs approximately 2-3 mm thick. The tuber discs were soaked in MS liquid medium (Murashige and Skoog, 1962) containing suspensions of the mid phase liquid culture of the agrobacteria (final density, OD at 550 nm=0.01). After 10 min excess liquid was removed by blotting on Whatman paper and the tuber discs were transferred onto modified MS medium (Jarret *et al.*, 1980) containing 0.03 μg/m*l* α-naphthaleneacetic acid, 0.5 μg/m*l* 6-benzylaminopurine, 1 μg/m*l* kinetin, 0.4 μg/m*l* zeatin, 1 μg/m*l* indoleacetic acid and 0.5 μg/m*l* gibberellic acid and then incubated at 20℃ under cool-white lamps (60 $\mu E/m^2/sec$) for 16 h/day photoperiod until the slight bacterial ring developed at the cut-edge surfaces of the tuber discs. The discs were then placed on the identical medium containing 500 μg/m*l* carbenicillin and 50 μg/m*l* kanamycin for selection of transformed cells. Tuber discs were subcultured onto fresh regeneration medium at 4 week intervals, lowering the carbencillin concentration from 500 μg/m*l* to 300 μg/m*l* after 4 weeks in culture. Developing shoots were removed and replated on the basal MS medium containing 300 μg/m*l* carbenicillin and 50 μg/m*l* kanamycin for root induction.

GUS histochemical assay. Sections were cut by hand from stems and roots of kanamycin resistant plants, and fixed with 0.3% formaldehyde in 10 mM MES (pH 5.6), 0.3 M mannitol for 45 min at room temperature, followed by several washes with 50 mM sodium phosphate buffer (pH 7.0). Histochemical reactions were performed with 1 mM X-gluc in 50 mM sodium phosphate buffer (pH 7.0) at 37℃ for 3 h. After staining, sections

were rinsed with 70% ethanol for 5 min and then mounted for microscopy.

Protein extraction. Total proteins were extracted from tissues for assays in 50 mM Na_2PO_4 (pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% triton X-100 (extraction buffer) by freezing with liquid nitrogen and grinding with a pinch of polyclar (insoluble polyvinyl pyrollidone). The extracts were centrifuged at 12,000 rpm for 30 min at 4℃. Supernatants were used for protein concentration and spectrophotometric assays. Protein concentrations of extracts were determined by the dye-binding method of Bradford (1976).

GUS spectrophotometric assay. The spectrophotometric reaction was carried out with a reaction volume of 5 m*l* containing 20 μg of protein and 1 mM *p*-nitrophenyl β-D-glucuronide. The reaction was carried out at 37 °C, and aliquots of 1 m*l* were removed at zero time and at subsequent times, and the reaction was terminated with the addition of 0.4 m*l* of 2.5 M 2-amino-2-methyl propanediol. Absorbance was measured at 415 nm against a substrate blank. Under these conditions the molar extinction coefficient of *p*-nitrophenol is assumed to be 14,000.

RESULTS AND DISCUSSION

Construction of the CaMV 35S promoter/deleted *Adh* 1-S intron 1-GUS gene. It had been reported that the inclusion of the 535 bp of maize *Adh*1-S intron 1 within the transcribed region of chimeric gene constructs stimulated the effect of the gene expression in maize cells (Callis *et al.*, 1987; Luehrsen and Walbot, 1991). Also, the CaMV 35S promoter is constitutively active in several different species and has been used to express a number of foreign genes in transgenic plants (Shah *et al.*, 1986). To test the effect of the deleted intron (*Adh* 1-S) 1, an expression vector containing a chimeric CaMV 35S-GUS gene with the 221 bp of *Adh*1-S intron 1 fragment in the 5′ untranslated region was constructed and tested for the degree of GUS expression. Plasmid pAI1 GusN (Klein *et al.*, 1988) which contains 535 base pairs (bp) of maize *Adh*1-S intron 1 (Dennis *et al.*, 1984) and 1.87 kilobase pairs (kb) of GUS coding gene (Jefferson *et al.*, 1986) is 6.5 kb long. Plasmid pKCH1 (Chung *et al.*, 1989) which contains CaMV 35S promoter and nopaline synthase (*nos*) termintor gene is 5 kb long. pAI1 GusN was digested with *Bgl*II and the fragment (2.4 kb) containing the 249 bp of the intron (*Adh*1-S intron 1)

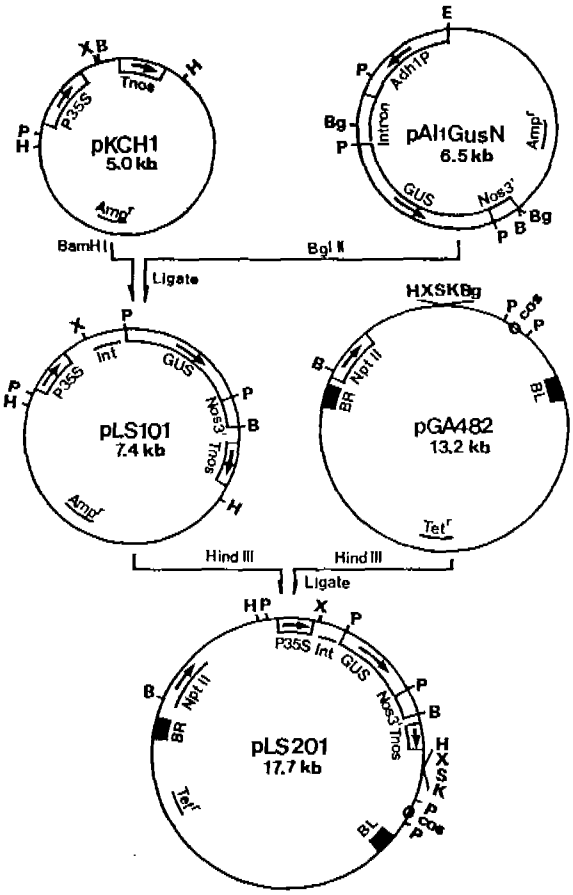

Fig. 1. The strategy for the construction of the CaMV 35S/deleted *Adh*1 intron-GUS chimeric gene in pGA482. The Symbols used are: P35S, CaMV 35S promoter; *Adh*1 P, alcohol dehydrogenase I promoter; Intron, 535 bp of *Adh*1-S intron 1; Int, 249 bp of intron (*Adh*1-S) 1 fragment; GUS, β-glucuronidase gene; *npt* II, kanamycin resistance gene; Amp′, ampicillin resistance gene; Tet′, tetracycline resistance gene; nos3′ and Tnos, nopaline synthase terminator; *cos*, λ *cos* site; BR and BL, the right and left border of T-DNA. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sac*I; X, *Xba*I.

fragment and GUS gene was ligated to the *Bam*HI site in front of the *nos* terminator gene of pKCH I to yield pLS101 (Fig. 1). Plasmid pLS101 had the 249 bp of intron fragment and GUS coding gene was adjoined to the 3′ of the CaMV 35S promoter. The junction sequence of the CaMV 35S promoter and remaining region deleted from the *Adh*1-S intron 1, and GUS was represented in the Fig. 2. In the sequence reading, a putative translation initiation codon existed in the intron fragment, and 221

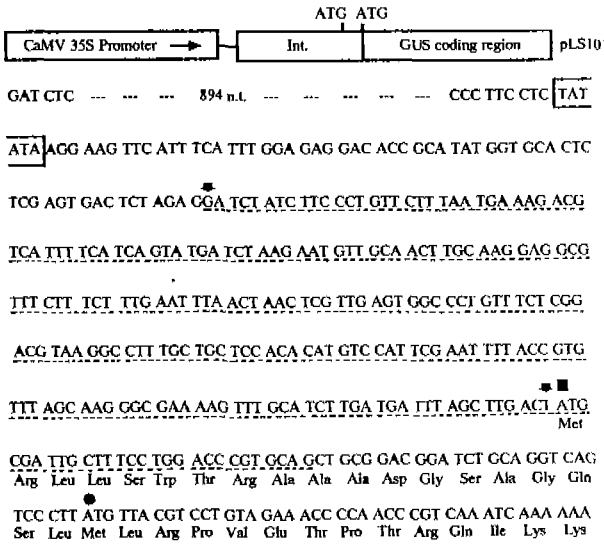

Fig. 2. Nucleotide sequence of the CaMV 35S promoter/deleted *Adh*1-S intron 1 and GUS gene insert of pLS 101. The 'TATA' box is indicated with bracket. The putative translation initiation site (ATG) is indicated with a closed squrare and the GUS coding region is indicated by a closed circle. The scquence with dotted underline is remaining region deleted from the *Adh*1-S intron 1 (Dennis *et al.*, 1984), and the 221 bp between vertical arrow is assumed to be untranslated region. The unrecorded 894 nucleotides are CaMV 35S promoter sequences from the published data (Robertson *et al.*, 1983).

bp of the upstream region was assumed to be untranslated (Fig. 2).

Construction of a gene transfer vector. Plasmid pLS 101 was cleaved with *Hin*dIII and the fragment (4.4 kb) containing the 35S promoter/249 bp of intron (*Adh*1-S) 1 fragment-*GUS* gene was cloned in the *HindIII site of* pGA482 (An, 1987) to yield pLS201 and the orientation of the insert fragment was confirmed by double digestion with *Eco*RI and *Bam*HI. In the resulting, the kanamycin resistance gene and CaMV 35S promoter/249 bp of deleted intron (*Adh*1-S) 1 fragment-GUS gene were located in between the right and left borders in pLS201 (Fig. 1). The plasmid pLS201 is 17.7 kb long and contains the kanamycin resistance gene as a selective marker gene, the CaMV 35S/249 bp of intron (*Adh*1-S) 1 fragment-GUS gene, multiple cloning sites, and the border sequences for transformation by *A. tumefaciens* as a mediator. This vector was able to maintain in *E. coli* and *A. tumefaciens* in the presence of 50 μg/ml kanamycin or 10 μg/ml tetracycline and 25 μg/ml kanamycin.

Table 1. Shooting and rooting frequencies from potato tuber discs between control (non-transformants) and transformants with *A. tumefaciens*

	Shooting frequency (%)[1] (No. of shoots/ No. of discs)	Rooting frequency (%)[2] (No. of roots/ No. of discs)
Control	86 (172/200)	63 (126/200)
Transformants	11.7 (14/120)	6.7 (8/120)

[1]Shoot induction frequency was counted at 6 weeks after culture. [2]Root induction frequency was counted at 10 weeks after culture.

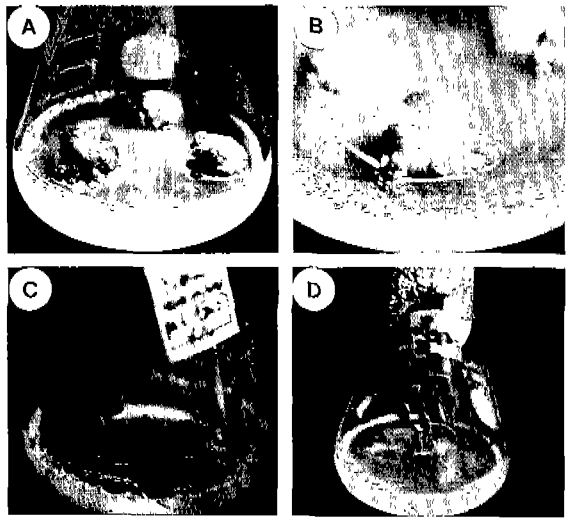

Fig. 3. Various developmental stages transformed potato. A, Tuber discs on the selection medium 4-5 weeks after incubation with *Agrobacterium* (Green protuberances were evident before shoot formation). B, Shoot induced from tuber discs 6-7 weeks after incubation with *Agrobacterium* (Indepandent shoot has grown from the protuberances). C, Shoot and green protuberances excised from B and subcultured (10 weeks after incubation with *Agrobacterium*). D, Shoot excised and cultured on root induction medium. Prolific root formation was seen 12 weeks after incubation with *Agrobacterium*.

Plant transformation and regeneration. In preliminary experiments, the formation of adventitious shoots and roots was studied from tuber discs which were not infected with *A. tumefaciens* on the kanamycin-free medium. The results showed that higher incidence of morphogenesis occurred (Table 1). Shoots were initiated in 6 weeks after culture without callus phase and roots were

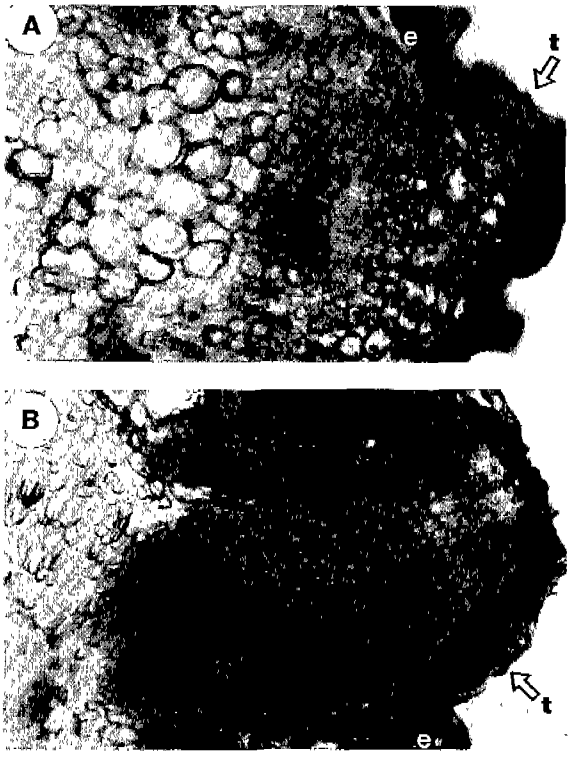

Fig. 4. Histochemical assay of GUS activity on stems of transformed potato plants. A, Transformed with A281 (pTiB$_6$542): pGA482; B, Transformed with A281 (pTiB$_6$ 542): pLS201. Symbols: e, epidermis; t, tumor cells.

initiated 10 weeks after culture, respectively (Data not shown). In contrast, the response from the cut edges of pototo tuber discs was obtained 4 weeks after infection with *Agrobacterium* (Fig. 3A). The tuber discs gave rise to compact green protuberances from which shoots were formed directly after 6-7 weeks in culture (Fig. 3B), and prolific root formation was seen 12 weeks after culture (Fig. 3D), but the transformation efficiency was low (Table 1). However, some of the discs formed calluses, and shoot regeneration was observed from them (Fig. 3C). Although whole plants can be regenerated from the callus, it tends to be genetically unstable and usually lose their morphogenetic potential during prolonged culture (Karp *et al.*, 1982; Thomas *et al.*, 1982). For these reasons, it was required to develop a more efficient and rapid method of gene transfer based on the direct selection and shoot induction method which avoids the callus stage.

Localization of GUS expression by the histochemical assay. Sections from callus, stem, root, and microtuber were stained with X-gluc. No blue-colored products were detected in the tissue sections of kanamycin resistance plants which were transformed with pGA482 (Fig. 4A). The blue-colored products of GUS activity were visible after incubation for 30 min (Fig. 4B).

The GUS activity was found in all organs of the transformed potato, and its activity was observed in and around the vascular bundle of the stem (Fig. 5A) and root (Fig 5B). In the stem, the GUS activity was primarily localized at phloem tissues along the inside and outside of the vascular bundles, and lighter staining was also observed in the cortex and epidermis (Fig. 5A). In root, the GUS activity was primarily localized at phloem tissues along the inside of vascular bundles (Fig. 5B), and root tip and hairy root were intensely stained (Fig. 5C). In microtubers, the GUS activity was localized at premedullary tissues along the inside of vascular ring (Figs. 5D and 6B) and calluses were stained throughout all cells (Fig. 5E). This phenomenon was similar to the stem sections of tobacco plants transformed with CaMV 35S-GUS (pBI121), in which the phloem tissues were highly stained (Jefferson *et al.*, 1987). Also, in the histochemical study in a monocotyledonous plant (rice) transformed with CaMV 35S-GUS (pBI121), the GUS activity was primarily localized at or around the vascular tissue in leaf, root and flower organs (Terada and Shimamoto, 1990). These results indicate that the level of GUS expression which was driven by the CaMV 35S promoter depended on cell types in transgenic monocotyledonous and dicotyledonous plants.

Analysis of GUS expression by spectrophotometric assay. The level of GUS activities of transgenic potato transformed with that CaMV 35S/249 bp of intron 1 fragment-GUS (pLS201) was compared with transformed with CaMV 35S-GUS (pBI121). The quantitative spectrophotometric assay showed that the level of GUS activities at leaves, stem and root of potato plants which were transformed with pLS201 was increased approximately 30-, 34- and 42-fold than pBI121, respectively (Tables 2 and 3). This result was similar with the histochemical assay, showing that the GUS staining of pLS201 was stronger than pBI121 at callus, stem and microtuber tissues (Figs. 5 and 6). Using a series of vector pairs, which were intron-minus and intron-containing constructs, it was demonstrated that the inclusion of alcohol dehydrogenase introns (*Adh*1-S introns 1 and 6) resulted in increment at the level of both RNA and protein in cultured transgenic maize and breadwheat cells (Callis *et al.*, 1987; Oard *et al.*, 1989; Luehrsen and Walbot, 1991). Also, the 5'

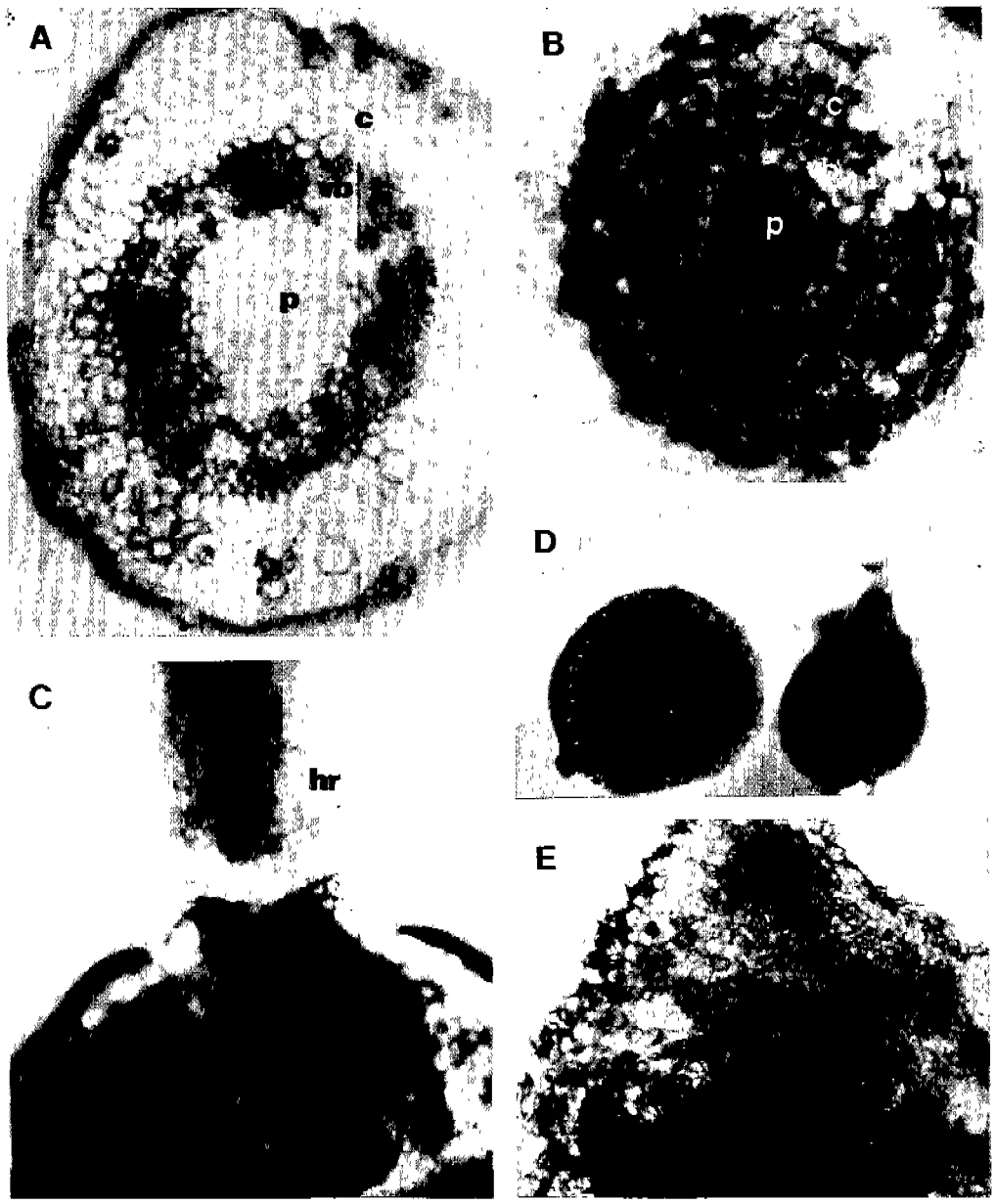

Fig. 5. Expression patterns of the CaMV 35S/249 bp of intron fragment-GUS (pLS201) at various organs of transgenic potato. Thin sections of each organ from kanamycin resistance plants were stained with X-gluc (see the Materials and Methods). A, Cross section of stem; B and C, Cross section of hairy root and root; D, Section of microtubers; E, Section of callus. Symbols: vb, vascular bundle; hr, hairy root; p, pith; c, cortex.

untranslated leader sequences of several plant RNA viruses enhanced expression of the contiguous open reading frames for chloramphenicol acetyltransferase (CAT) or GUS in transformed tobacco mesophyll protoplasts (Gallie *et al.*, 1987). The ability of a viral leader sequence to enhance translation was not strictly dependent on its capacity to bind more than one ribosome. And sequence comparisons between the leaders showed no significant homologies other than a high A,U-content, a common feature of viral leader sequences (Gallie *et al.*, 1987). Gallie

Table 2. GUS activity (unit) in different organs of transgenic potato (cv. Superior) plants which were transformed by pLS201

No.#	Leaf	Stem	Root
Untransformed	<0.2	<0.2	<0.2
1	28.9	33.3	82.2
2	112.8	250.6	199.2
3	42.0	101.3	212.1
4	113.6	184.3	444.6
5	65.5	55.1	102.9
6	68.7	39.8	189.5
Mean	71.9	110.7	205.1

The GUS enzymatic assay was performed according to Jefferson *et al.* (1987) using 0.2-1 g of tissue. Activity is expressed as nmole *p*-nitrophenol/min/mg protein. #=Transformed plant grown *in vitro*.

Table 3. GUS activity (unit) in different organs of transgenic potato (cv. Superior) plants which were transformed by pBI121

No.#	Leaf	Stem	Root
Untransformed	<0.2	<0.2	<0.2
1	3.3	5.1	–
2	1.5	1.3	2.4
3	4.6	5.7	9.3
4	1.1	1.9	1.8
5	1.7	2.4	6.2
Mean	2.4	3.3	4.9

The GUS enzymatic assay was performed according to Jefferson *et al.* (1987) using 0.2-1 g of tissue. Activity is expressed as nmole *p*-nitrophenol/min/mg protein. #=Transformed plant grown *in vitro*.

and co-workers suggested, the lack of homology between the various viral leader sequences may indicated that no one strategy is followed by all, but that there may be several ways to achieve the enhancement.

Interpretation of histochemical GUS staining is not simple as discussed by Jefferson *et al.* (1987). Closely packed small cell such as phloem cell tends to be stained more effectively because the number of cells per unit area is greater. Also, the position effect in the chromosome and the copy numbers of inserted DNA affect the foreign gene expression in transformed plants. Nevertheless, the quantitative assay and GUS staining at various organs suggest that the inclusion of the 249 bp of intron (*Adh*1-S) 1 fragment increased the levels of GUS gene

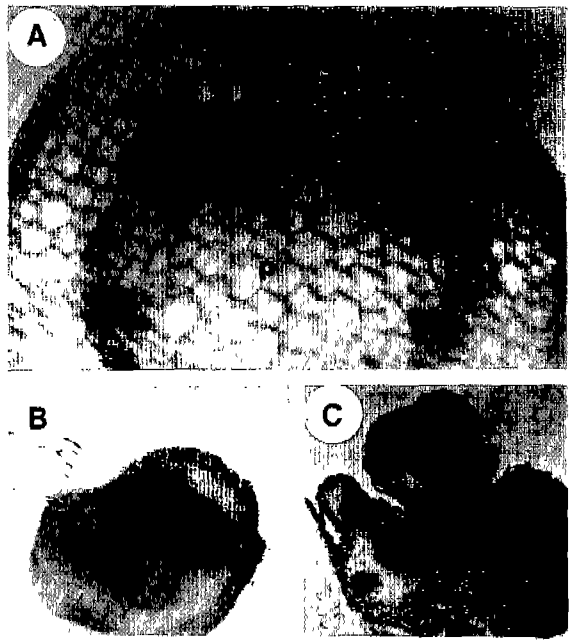

Fig. 6. Expression patterns of the CaMV 35S-GUS (pBI 121) at various organs of transgenic potato. Thin sections of each organ from kanamycin resistance plants were stained with X-gluc (see Materials and Methods). A, Cross section of stem; B, Section of microtuber; C, Section of callus. Symbols: p, pith; vb, vascular bundle; c, cortex.

expression in transgenic potato plants. It is tempting to speculate that the 5′ untranslated sequences may circumvent the need for some rate-limiting initiation factors, or that it acts as an enhancing element for the association of ribosomes or initiation factors. Secondary structures within the 5′ untranslated leaders of some eukaryotic mRNAs have been claimed to promote (Ahlquist *et al.*, 1979; Darlix *et al.*, 1982) or inhibit (Kozak, 1986) translation initiation. Clearly substantial additional work is required to elucidate the mechanisms whereby the 5′ untranslated sequence can enhance expression of contiguous coding regions in eukaryotic translation systems.

적 요

감자 (*Solanum tuberosum* L. cv. Superior)에서 cauliflower mosaic virus (CaMV) 35S promoter의 발현 양상 및 외래 유전자의 발현에 미치는 intron fragment의 효과를 조사하기 위하여 옥수수의 alcohol dehydrogenase 1-S (*Adh*1-S) intron 1의 249 base pairs 와 β-glucuronidase (GUS) 유전자를 결합한, CaMV 35S/deleted *Adh*1 intron-

GUS 구조의 유전자 전달벡터를 제조하고 이를 *Agrobacterium tumefaciens*를 매개로 형질전환을 유도하였다. 유전자 전달벡터인 pLS201는 17.7 kilobase pairs로서 형질전환의 초기 선별에 용이한 kanamycin 저항성 유전자와 GUS 유전자를 갖는 구조로 제조되었다. 형질전환된 개체의 조직화학적 분석 결과 CaMV 35S promoter에 의한 GUS 유전자는 모든 기관에서 발현되었고, 줄기 및 뿌리에서는 세포분열이 활발한 유관속 형성층을 중심으로 강한 발현을 나타내었다. GUS 유전자의 발현에 미치는 intron fragment의 효과를 조사하기 위하여 CaMV 35S/GUS 구조의 plasmid (pBI121)로 형질전환된 개체를 대조구하여 GUS 활성을 조사한 결과 pLS201이 잎, 줄기, 뿌리에서 각각 30, 34, 42배 높은 활성을 보여, 옥수수 탈수소 효소 유전자의 절단된 인트론이 GUS 유전자의 발현을 증가시킴을 알 수 있었다.

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