Korean Journal of Life Science
Vol. 12. No. 1. 96~105, 2002

Expression of an artificial gene encoding a repeated tripeptide lysyl-glutamyl-tryptophan in Tobacco plant

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Abstract

To investigate expression of the artificial gene encoding a repeated tripeptide lysyl-glutamyl-tryptophan in tobacco plant, the plant binary vector, pART404 has been constructed, which contains the duplicated CaMV 35S promoter, an artificial gene coding for repetitive polymer $(Lys\text{-}Glu\text{-}Trp)_{64}$, and nopaline synthase (*nos*) terminator. The recombinant expression vector was introduced in *Nicotiana tabacum* (var. Xanthi) via *Agrobacterium tumefaciens*-mediated transformation. The transgenic calli selected by kanamycin containing medium were then regenerated to whole plants. Southern blot analysis indicated that five transgenic plants (No. 1, 7, 9, 43, 45) showed the hybridizing signals at 1.1 kb of the expected size on *Eco*RI digestion and each of the transgenic plants contained 1 or 3 copies of the artificial gene inserted into its genome. By northern blot analysis, the size of the hybridized total RNA was estimated to be approximately 1.2 kb and the RNA appeared generally to have the integrity. Western blot indicated that the protein was detected at the position of 33 kDa and the expression level of the polypeptide in the transgenic plant (No. 45) was measured to approximately 0.1% of the total protein.

Key words – artificial gene, repetitive polymer $(Lys\text{-}Glu\text{-}Trp)_{64}$, *Agrobacterium tumefaciens*, transgenic plant

Introduction

Generally, the essential amino acids which are found to be most limiting in plants are lysine, methionine, threonine, and tryptophan [21]. Since a number of proteins for both human and animal nutrition are obtained from plant proteins, the composition of its essential amino acids is very important for optimal growth and development of humans or livestock [1]. And also, because protein malnutrition can usually be ascribed to a deficiency in the diet of one or more of the essential amino acids [2], polypeptide, with elevated levels of essential amino acids, could be useful as partial supplements to food and feeds. Accordingly, it would be highly profitable to engineer the plant producing proteins with high essential amino acid content.

There are some approaches to improve the quality of plant storage protein : (1) modifying the existing storage protein genes to code for essential amino acids [20,26]; (2) transferring genes for nutritionally rich storage protein from heterologous species into the desired crops [1]; (3) trans-

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ferring synthetic genes coding for polypeptides with the essential amino acid composition [27]; (4) expressing desensitized biosynthetic enzyme of an essential amino acid pathway to increase the limiting content of the amino acid [24].

Using chemically synthesized gene coding for simple polypeptide or synthetic genes for nutritionally artificial proteins, gene expressions have been attempted to improve protein quality [1,5,8,14]. We have also tried to express the artificial genes encoding polypeptide containing essential amino acids in *E. coli* [18]. The artificial genes coding for repetitive polypeptides $(Glu\text{-}Trp\text{-}Lys)_n$, $(Glu\text{-}Trp\text{-}Lys\text{-}Thr)_n$, and $(Met\text{-}Trp\text{-}Lys\text{-}Met\text{-}Trp\text{-}Asp)_n$ were fused to λ *cro* and *lac*I/*lac*Z sequence. When expressions of the *cro*-artificial gene-*lac*I/Z plasmids were initiated from the *lac* promoter in the presence of IPTG, the fusion proteins were produced upto 5 to 10% of total cell protein. Accordingly, cloning and expression of synthetic DNA, containing repeated codons for essential amino acids, could be used as a means for improvement of the amino acid profiles of single protein and supplementing cereal diets and feed grain rations.

In this study, in order to investigate expression of an artificial gene for the nutritional improvement of plant protein, the plant expression vector containing the artificial gene coding for repetitive polypeptide $(Glu\text{-}Trp\text{-}Lys)_n$ was constructed, introduced into tobacco cells, and expression characteristics were analyzed in transgenic tobacco.

Materials and Methods

Bacterial strains, media, and plasmids

Escherichia coli HB101 (*hsdR, hsdM, leu, pro, recA, supE*) strain was used as a primary host for transformation and propagation of plasmids. *E. coli* cells harboring plasmid were grown in Luria-Bertani (LB) medium supplemented with 50 μg of ampicillin per ml.

Plasmid pUT4-4 [18] which contains 0.8 kb insert DNA was a source of artificial gene coding for repetitive polypeptide $(Glu\text{-}Trp\text{-}Lys)_n$. The pAGUS1 vector which has the duplicated CaMV 35S promoter, β-glucuronidase (GUS), and *nos* terminator was used as a source of plant expression promoter. The pGA482 [3] which is a binary vector derived from Ti plasmid of *A. tumefaciens* and carries the neomycin phosphotransferase II (*npt*II) gene for kanamycin was used for the cloning of promoter and artificial gene, resulting in the plant expression vector, pART4-4. The constructed plasmid was mobilized to *Agrobacterium tumefaciens* LBA4404 [11] carrying the *vir* helper plasmid by freeze-thaw method [12] and selected in YEP medium (10 g of bactopeptone, 10 g of yeast extract, and 5 g of NaCl per 1 liter) containing 5 μg/ml of tetracycline and 10 μg/ml of kanamycin.

Materials

All restriction enzymes and DNA modifying enzymes were purchased from Promega (USA), NEB (USA), and Boeringer Mannheim (Germany). [α-^{35}S]dATP (1,000 Ci/mmol) and [α-^{32}P]dCTP (3,000 Ci/mmol) were obtained from Amersham (UK), prime-a-gene labelling kit from Promega (USA), and sequencing kit was from United States Biochemical (USA). Alkaline phosphatase labeled secondary antibody, BCIP(5-bromo-4-chloro-3-indolyl-phosphate), NBT (nitroblue tetrazolium) were purchased from Pierce (USA). Oligonucleotides were synthesized in Bioneer (Korea). Acrylamide, molecular weight markers, urea, lysozyme, agarose, and ampicillin were from Sigma (USA). All other materials were reagent grade and obtained from commercial source.

DNA sequencing

DNA sequencing was carried out by the dideoxy chain termination methods on double-stranded template [23] by using sequenase 2.0 as recommended by the manufacturer (United State Biochemical). For sequencing the internal regions, synthetic oligonucleotide primers were designed from the DNA sequence previously determined.

Plant transformation

Leaf discs of *Nicotiana tabacum* (var. Xanthi) were in-

fected with *A. tumefaciens* harboring co-integrated Ti vector by the method of Horsch *et al.* [13]. Transformants were selected on MS [19] agar medium supplemented with 500 mg/L carebnicillin, 100 mg/L kanamycin, 0.5 mg/L α-naphthalene acetic acid (NAA), and 2 mg/L 6-benzylaminopurine (BAP). Regenerated plantlets obtained from hormone-free MS medium with 100 mg/L of kanamycin were checked for correct integration of the construct in the tobacco genome by southern blot analysis.

Southern blot analysis

Total cellular DNA was extracted from young tobacco leaves by a modification of the method of Draper *et al.* [9]. Frozen leaves were ground in the presence of liquid nitrogen and the powder was mixed in CTAB (cetyltrimetylammonium bromide) extraction buffer containing 50 mM Tris-HCl, pH 8.0, 70 mM sodium chloride, 10 mM EDTA, 1% CTAB, 20 mM β-mercaptoethanol (6 ml/g tissue). The mixture was incubated at 56℃ for 20 min and then, equal volume of phenol/chloroform (1:1) was added and mixed. The two phases were separated by centrifugation at 6,000×g for 5 min and the aqueous phase collected to a new tube. One to tenth volume of 10% CTAB buffer was added and followed by extraction with chloroform/isoamylalcohol (24:1). The supernatant was ethanol precipitated. The purified genomic DNA (10 μg) was digested with *Eco*RI and subjected to electrophoresis on a 0.8% asgarose gel. The DNA was transferred to membrane filter (Genescreen plus, NEN) and hybridized with ^{32}p-end labeled synthetic oligonucleotide (36 mer) of the artificial gene (5'-CCATTCTTTCCACTCCTT-CCATTCTTTCCACTCCTT-3'). After hybridization at 42℃, the blot was washed twice with 2×SSC (0.3 M NaCl and 0.03 M sodium citrate) at room temperature and with 0.2 ×SSC and 0.2% SDS at 50℃ for 20 min. The filter was then autoradiographed at -70℃ using intensifying screen.

Northern blot analysis

Total RNA was prepared from 100 mg of tobacco tissue using a small scale procedure for the rapid isolation of plant RNA [25]. Isolated total RNA was denatured, fractionated in 1.2% agarose-formaldehyde gel and transferred to GeneScreen-plus membrane according to a laboratory manual of Molecular Cloning [22]. The amounts of total RNA loaded were normalized with those of ribosomal RNA. The membrane was hybridized at 42 ℃ in 2 x PIPES (0.8 M NaCl and 20 mM PIPES, pH 6.5), 50% deionized formamide, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA using ^{32}p-end labeled synthetic oligonucleotide of the artificial gene as a probe. The final washing was carried out in 0.1×SSC (15 mM NaCl and 1.5 mM sodium citrate, pH 7.0) and 0.1% SDS at 60℃ for 15 min. The filter was visualized by autoradiography.

SDS-PAGE and Western blot analysis

Total protein was extracted from tobacco leaves as described by Barton *et al.* [4] and the protein content of leaf tissue was measured as described by Bradford [6]. Prior to fractionation on SDS-PAGE, protein sample (60 μg) was denatured at 100℃ for 5 min in a solution containing 100 mM dithiothreitol, 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.1% bromphenol blue. Electrophoresis was performed at 20 mA in 12% polyacrylamide gel containing 0.1% SDS [17] and electroblotted to Immobilon-P membrane (Millipore, Bedford, MA). Blot was blocked for 1 hr in PBS buffer containing 5% skim milk and incubated with polyclonal anti-artificial polypeptide antibody (1:200 dilution) for 1 hr. After washing unbound primary antibodies with washing buffer (PBS+0.5% Triton X-100) three times for 10 min each, the blot was treated with alkaline phosphatase-conjugated goat anti rabbit antiserum and developed with BCIP/NBT substrate solution.

Results and Discussion

Construction of plant expressior vector containing the artificial gene

In order to achieve constitutive expression of the ar-

tificial gene in tobacco plants, the coding sequence for an artificial polypeptide was fused to a duplicated CaMV 35S promoter, which is known to raise transcriptional activity approximately 10 fold higher than that of the natural promoter. First of all, to facilitate the cloning of the artificial gene into the CaMV 35S promoter-*nos* terminator cassette, β-glucuronidase (GUS) gene was removed. As can be seen in Fig. 1, pAGUS1 vector was

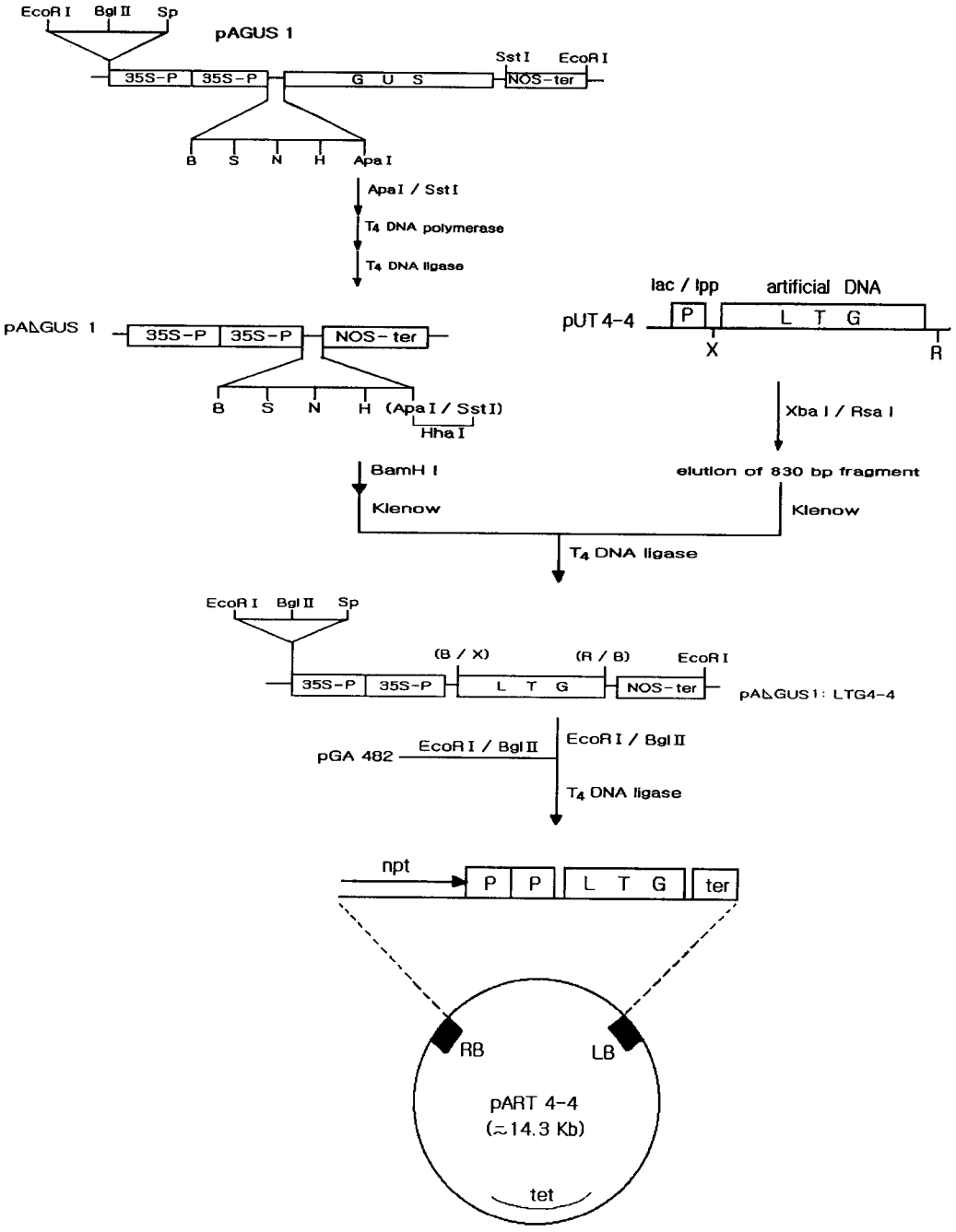

Fig. 1. Diagrammatic representation of the construction of plant expression vector, pART4-4. The plasmid contains the duplicated CaMV 35S promoter, artificial gene (LTG), and nopaline synthase (*nos*) terminator.

digested with *Apa*I and *Sst*I, treated with T4 DNA polymerase to remove overhanging ends and self-ligated to form pA△GUS1. Plasmid pUT4-4 containing the artificial gene was digested with *Xba*I and *Rsa*I, 0.8 kb fragment coding for artificial polypeptide was obtained, and the sticky ends were blunted with large fragment of DNA polymeraseI (Klenow). For cloning of the artificial gene, the pA△GUS1 vector was digested with *Bam*HI, followed by filling-in with Klenow enzyme, and ligated with the above 0.83 kb fragment to construct pA△GUS1: LTG4-4 intermediate plasmid. And then, a 2 kb *Eco*RI-*Bgl*II fragment containing the CaMV 35S promoter, artificial gene, and *nos*-terminator was obtained from the intermediate plasmid and ligated with pGA482 digested with same restriction enzymes, which is a binary vector derived from Ti plasmid of *A. tumefaciens* and carries the neomycin phosphotransferase II (*npt*II) gene for kanamycin. Finally, the obtained clone was designated as pART4-4. As shown in Fig. 2, complete nucleotide sequence was determined. Eight amino acid residues at *N*-terminus were derived from the lipoprotein (*lpp*) gene of pINIIIA3 vector, 2 amino acids from bacteriophage λ-*cro* gene, and 3'-noncoding region from *nos* terminator [18]. The nucleotide sequence of the artificial gene shows one open reading frame of 735 bp encoding a protein of 245 amino acids (calculated MW, 33 kDa).

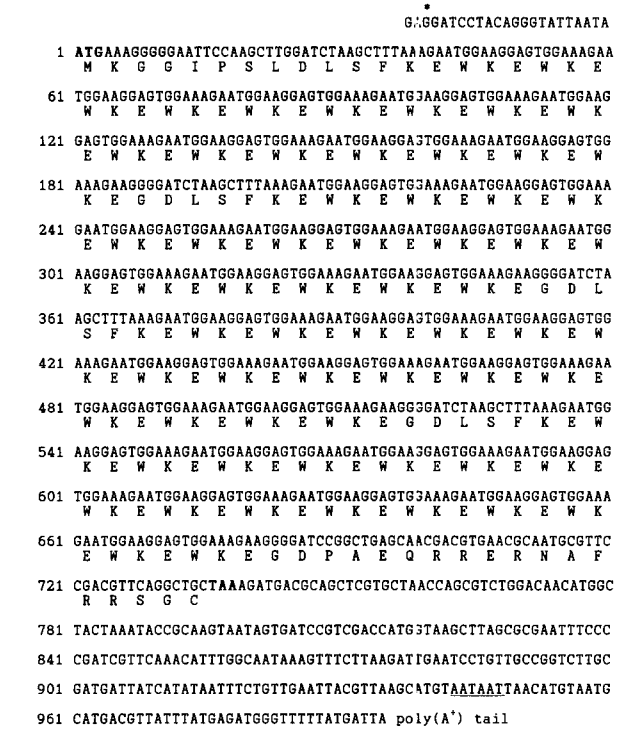

Fig. 2. Nucleotide and deduced amino acid sequence of the artificial gene encoding lysine, glutamic acid, and tryptophan-rich polypeptide. The initiation and termination codons are indicated in bold type and the asterisk means transcription initiation site. The polyadenylation recognition sequence in *nos* terminator is underlined.

Plant transformation and analysis of the genomic DNA

The constructed expression vector has the artificial gene, dual CaMV 35S promoter, the neomycin phosphotransferase II (*npt*II) gene conferring kanamycin resistance upon the transformed tobacco cells, nopaline synthase (*nos*) terminator, and the sequence of left and right border. The plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404 that contains the *vir*-helper plasmid by freeze-thaw method. Transformants harboring the plasmid were confirmed by the presence of 1.1 kb insert DNA when digested with *Eco*RI (data not shown). *Agrobacterium tumefaciens* harboring the expression plasmid was cocultivated with leaf discs of *Nicotiana tabacum* (var. Xanthi), maintained on MS selective medium containing kanamycin sulfate (100 μg/ml) to promote the growth of kanamycin resistant transgenic callus tissue, and grown to form shoot (Fig. 3). The transformed transgenic calli had a normal appearance and similar growth characteristics as the control calli, and kanamycin resistant plants were grown to maturity along with nontransformed plants obtained by leaf disc culture, No morphological differences were observed between the transformed and nontransformed plants.

In order to identify the integration of the artificial gene into tobacco genome, the fifty kanamycin resistant plants were analyzed by southern blot. Genomic DNAs

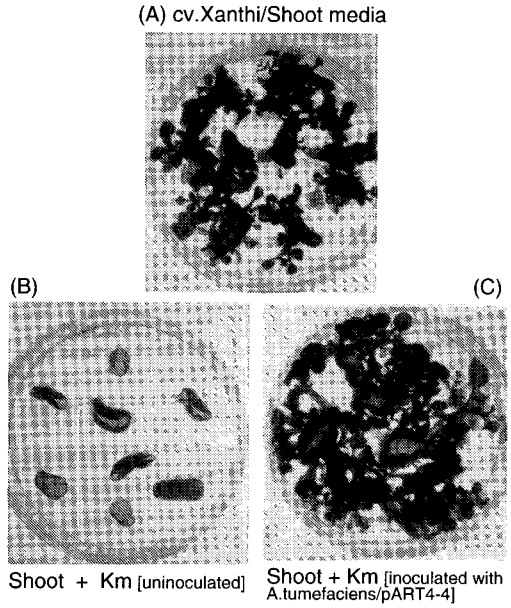

Fig. 3. Morphological characteristics of the transformed tobacco leaf disc on various media after 4 weeks culture: (A) Uninoculated leaf disc on the shoot regeneration medium; (B) Uninoculated leaf disc on the shoot regeneration medium containing kanamycin (100 μg/ml); (C) Discs inoculated with *Agrobacterium tumefaciens* LBA4404/pART4-4 on the shoot regeneration medium containing kanamycin (50 μg/ml)and carbenicillin (500 μg/ml).

extracted from the transformed plants were digested with *Eco*RI, fractionated in agarose gel, and blotted to membrane filter. The blot was probed with ^{32}p-end labeled synthetic oligonucleotide of the artificial gene. As can be seen in Fig. 4A, five transgenic plants (No. 1, 7, 9, 43, 45) showed the hybridizing signals at 1.1 kb of the expected size on *Eco*RI digestion although the intensities were different according to the copy number integrated into the genome of transgenic plants. To quantitate the copy number of artificial gene in genomes of transgenic plants, pART4-4 plasmid equivalent to 1 or 5 copies of the artificial gene per haploid genome was included in southern blot. As a result, it was estimated that each transgenic plant might contain one or three copies of the artificial gene into its genome.

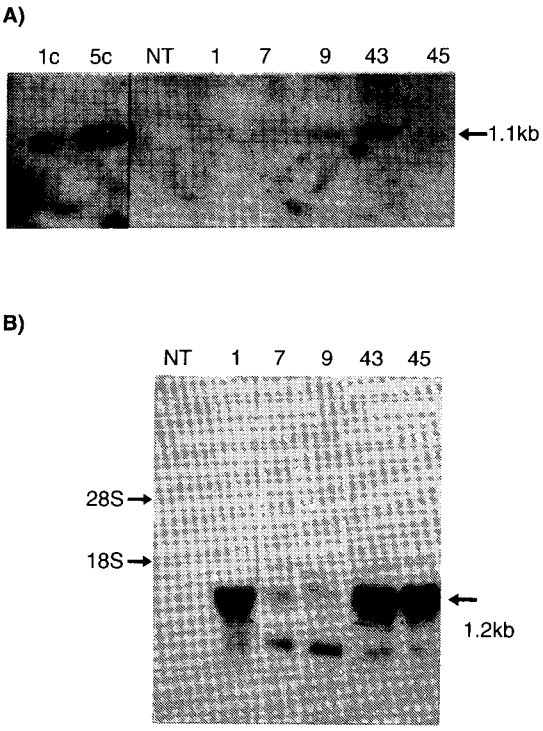

Fig. 4. Southern and Northern analysis of the transgenic tobacco plants: (A) Southern blot analysis of the transgenic tobacco plants. Genomic DNA (10 μg) was digested with *Eco*RI and size-fractionated in 0.8% agarose gel, transferred onto Genescreen plus memebrane (NEN) and probed with ^{32}p-end labeled synthetic oligonucleotide (36 mer) of the artificial gene (5'-CCAT TCTTTCCACTCCTTCCATTCTTT-CCACTCCTT-3'). Plasmid pART4-4 was loaded at the calculated concentration equivalent to 1 or 5 copies per cell after mixing with 10 μg of tobacco genomic DNA. NT indicates the genomic DNA isolated from non-transformed control plant. The arrow shows the position of expected size by *Eco*RI digestion; (B) Northern blot analysis. Isolated total RNAs (30 μg) were denatured, fractionated in 1.2% agarose formaldehyde gel and transferred to Gene-Screen-plus membrane. The membrane was hybridized with ^{32}p-end labeled synthetic oligonucleotide. The amounts of total RNA loaded were normalized with those of ribosomal RNA. The arrow indicates the position of the transcripts when the artificial gene was expressed in tobacco plant.

Northern blot analysis

In order to primarily screen the transgenic plants

which express the artificial gene-specific transcript, the fifty kanamycin resistant plants were analyzed for the expression of artificial gene by dot blot hybridization using total RNA with a specific probe DNA. Total RNAs were isolated from leaves of transgenic plants. Most transformants showed signals of artificial gene-specific mRNA with different levels of hybridization intensity whereas hybridization signal could not be detected in the RNA isolated from the non-transformed tobacco plant (data not shown). The five individual transformants which showed the hybridizing signals in southern blot and the different levels of transcripts in dot blot hybridization were subsequently analyzed by northern blot. Total RNA was isolated from leaves, thirty micrograms of total RNA was fractionated by formaldehyde-agarose gel electrophoresis, and probed with synthetic oligonucleotide (36 mer) labeled with γ-P^{32}-ATP. As shown in Fig. 4B, the radiolabeled probes were hybridized strongly with total RNAs from the transgenic plants, No. 1, 43, and 45, whereas No. 7 and 9 transformants showed weak signals. However, no signal could be seen in total RNA from non-transformed plant. It was previously reported that the magnitude of inter-transformant variability is somewhat dependent upon the introduced gene [7,15]. The reason of this variability is not understood, although it is generally considered to result from the different sites of integration of the introduced gene in the recipient genome. Compared with rRNA marker, the size of the hybridized total RNA from transgenic plants was approximately estimated to be 1.2 kb. While the mRNA of artificial gene appeared generally to have integrity and the predicted size in most plants, additional signals of small size were also examined in the RNA of most plants.

The duplicated CaMV 35S promoter contains two types of promoter different in the length. One is ranged from -390 to -90 region of the promoter conferring the specificity of expression in leaves and stem. The other is composed of -390 to +1 region giving the specificity in leaves, stem and root [16]. Therefore, the expression may be higher levels in leaves and stem and relatively lower in root tissue. To investigate this possibility, the total RNAs from various organs of tobacco plant (No. 45) were analyzed by northern blot. As can be seen in Fig. 5, the artificial gene was highly expressed in the leaves as expected and lower level in stem. On the other hand, the expression was almost extinguished in roots caused by transcripts instability, indicating that the duplicated CaMV 35S promoter has tissue specificity in the promoter strength.

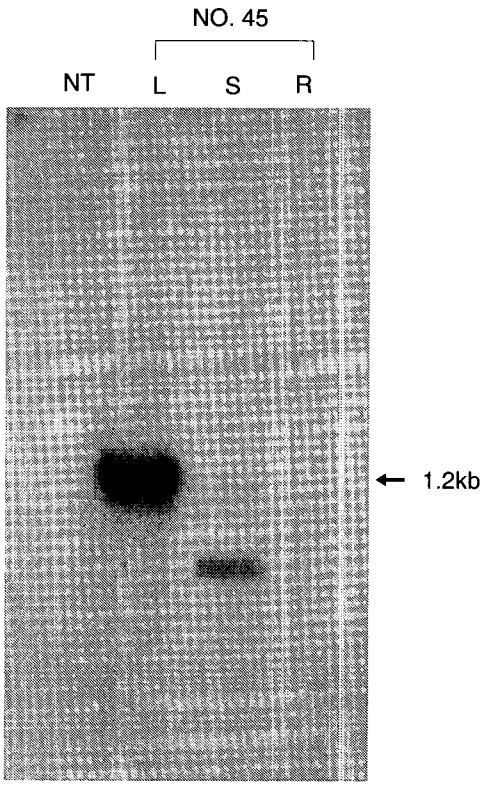

Fig. 5. Northern blot analysis of total RNAs isolated from various tissues of the transgenic tobacco plant (No. 45): NT, total RNA from non-transformed plant ; L, total RNA from leaf of the transgenic plant ; S, total RNA from stem of the transgenic plant ; R, total RNA from root of the transgenic plant.

Expression of artificial polypeptide in transgenic plants

Crude extracts from transgenic plants were analyzed to confirm whether the transgenic plant produces a specific immunoreactive polypeptide by western blot or not. Protein extracts (60 μg/lane) were loaded onto 12% SDS-PAGE, transferred onto Immobilon-P membrane, and reacted with polyclonal artificial polypeptide (LTG) antibodies. As shown in Fig. 6, a specific band of 33 kDa was detected in transgenic plant (No. 45) and co-migrated with the artificial polypeptide produced in *E. coli*, whereas the non-transformed plant did not produce the polypeptide. Based on the intensities of coomassie blue staining and by comparison with the amount of the loaded artificial polypeptide produce in *E. coli*, the expression level of the polypeptide in the transgenic plant No. 45 was estimated to approximately 0.1% of the total protein by densitometer scanning (data not shown). Northern and western analysis showed that the amount of mRNA was not proportional to the expression level of artificial gene and the differences of expression between transgenic plants might be due to the efficiency of translability or other unknown factors [1]. And also, low level expression of the artificial gene in transgenic tobacco plants might result from inefficient processing or transport of the artificial protein [10]. Indeed, Guerche *et al.* [10] noted that the level of methionine-rich protein accumulated in the canola seeds (0.02-0.06% of total seed protein) was much lower than would have been expected from the mRNA levels (0.2% of total developing seed mRNA) and suggested that the heterologous protein might be incorrectly processed or targeted.

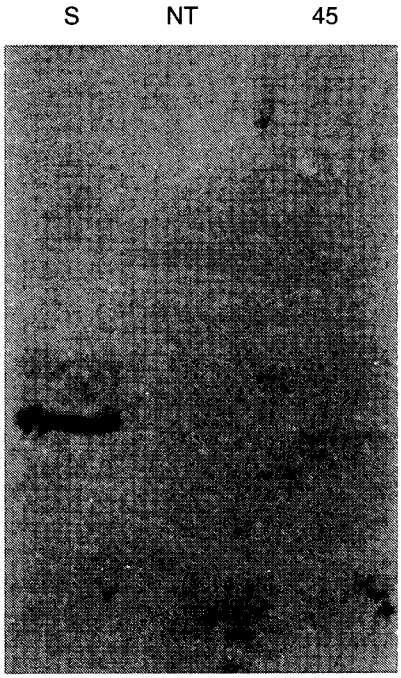

Fig. 6. Western blot analysis of the artificial polypeptide expressed in transgenic plant (No. 45). Crude protein extracts (60 μg per lane) were loaded on 12% polyacrylamide gel containing 0.1% SDS, electroblotted to Immobilon-P membrane, and reacted with polyclonal artificial polypeptide antibody. S indicates the artificial polypeptide purified from *E. coli*. NT and 45 mean proteins from non-transformed plant and transgenic plant No. 45, respectively.

The ultimate goal of this study is to investigate the expression of an artificial gene encoding repetitive lysine, glutamic acid, and tryptophan residues in plant and to improve lysine and tryptophan deficiencies found in the seeds of many crop plants. Since it was not possible to apply directly the expression of artificial gene in transgenic cereal plants such as rice, maize, and wheat, tobacco plant was chosen to examine factors affected on the expression of the artificial gene as a model system. Although the expression level of the artificial gene is not satisfying at present in tobacco plant, the feasibility of this molecular approach to enhance the limiting amino acid composition has been demonstrated. Accordingly, in order to increase the expression level, further study remains to elucidate factors affected on translational efficiency of the artificial gene.

Acknowledgement

This research was supported by the Taegu University

research grant, 2000.

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(Received January 25, 2002; Accepted February 18, 2002)

초록 : 담배식물체에서 필수아미노산인 lysyl-glutamyl-tryptophan을 암호화하는 인공유전자의 발현

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식물 단백질의 영양가 향상을 위한 일환으로 필수아미노산의 조성이 풍부한 인공단백질을 암호화하는 인공유전자를 담배 식물체에서 발현을 시도하기 위하여, 식물에서 외래유전자의 발현에 널리 사용되는 Cauliflower mosaic virus (CaMV)의 35S promoter를 이중으로 중첩되도록 하고, (Lys-Glu-Trp)이 64번 반복되는 인공유전자 및 nopaline synthase (*nos*) terminator를 갖고있는 binary vector pART4-4를 구성하였다. 이 재조합 플라스미드는 *Agrobacterium tumefaciens*를 이용한 형질전환에 의해 *Nicotiana tabacum* (Var. Xanthi)으로 도입되었다. Kanamycin이 포함된 신초 유도배지 및 뿌리 유도배지를 이용하여 정상적으로 재생된 담배 식물체로부터 도입된 인공유전자의 발현을 분석하였다. 추출한 genomic DNA를 *Eco*RI으로 자른 다음 Southern blot 분석에 의하면, 효소 절단 시 예상되는 1.1 kb에서 band를 형성하였으며 각각의 형질전환 식물체에 인공유전자가 1 또는 3 개씩 도입되어 있음을 확인하였다. Northern blot 분석에 의하면 약 1.2 kb 전사체가 비교적 안정하게 발현되었으며, 잎, 줄기, 뿌리로부터 RNA를 분리하여 promoter의 조직 특이성 발현을 분석한 결과, 잎에서 생성되는 RNA가 줄기나 뿌리 조직보다 안정하게 발현되었다. 형질전환 식물체에서 Western blot에 의한 단백질 분석 결과, 잎에서 추출한 단백질로부터 원하는 크기인 33 kDa의 인공단백질이 생성됨을 확인하였으며 발현 수준은 전체 세포 단백질의 0.1%로서 낮은 수준이었다.