Plant Biotechnology 32, 47–53 (2015)
DOI: 10.5511/plantbiotechnology.14.1217a

Original Paper

High-level expression of sucrose inducible sweet potato sporamin gene promoter: β-glucuronidase fusion gene in transgenic *Nicotiana plumbaginifolia*

Youhei Honma, Takashi Yamakawa*

Department of Global Agricultural Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
*E-mail: ayama@mail.ecc.u-tokyo.ac.jp Tel: +81-3-5841-7515 Fax: +81-3-5841-5304

Received August 7, 2014; accepted December 17, 2014 (Edited by T. Aoki)

Abstract We produced transgenic *Nicotiana plumbaginifolia* plants which contained Spo^{min} (sporamin minimal promoter)-GUS fused chimeric gene constructs with 5 types of signal sequences, such as cytosol, apoplast, ER, vacuole and plastid, and analyzed the GUS expression patterns after sucrose treatment. Spo^{min} induced extremely high GUS activities after 6% and 10% sucrose treatment, especially in leaves. The high GUS activities were observed in leaves of the Spo^{min}-ER-GUS construct treated with 6% or 10% sucrose. These were over 200 times higher than those in leaves with the 35S promoter-ER-GUS construct. The 10% sucrose treatment significantly altered GUS activities in all Spo^{min} and 35S promoter constructs compared with those in the 6% sucrose treatment; some increased and some decreased. GUS activities in 2 months old plants were almost the same as 8 months old plants, indicating that GUS expression driven by Spo^{min} was stably maintained. Also, even when sucrose treatment was stopped, GUS gene expression by Spo^{min} continued for 10 days.

Key words: GUS, sporamin promoter, sucrose inducible promoter, *Nicotiana plumbaginifolia*.

Following early studies on transgenic plant experiments in the 1983 (Fraley et al. 1983), many plant species have been genetically modified for production of useful proteins (Guan et al. 2013). The production of useful proteins in plants has many benefits (Fisher and Schillberg 2004), such as; 1) posttranslational modification of proteins is carried out, 2) conventional agricultural technology can be used, thereby enabling easy (low cost) scaling up of production, 3) products can be stably stored in the plant storage organs (seeds or tuberous roots) for long periods at room temperature, refrigeration is not required during storage or transportation, 4) plants have a much lower risk of contamination by human or animal pathogens. However, the current accumulation levels of exogenous proteins in transgenic plants are not high (Guan et al. 2013).

At present, constitutive promoters are usually used in plant genetic engineering; for example, cauliflower mosaic virus 35S RNA promoter (35S) is mainly used for dicotyledonous plants, and actin promoter of rice and ubiquitin promoter of maize are mainly used for monocotyledonous plants (Guan et al. 2013). These constitutive promoters induce constitutive expressions of genes in all plant tissues, and they are useful tools for developing protein production systems using plants. However, constitutive expressions of exogenous genes can be detrimental to plant growth. For example, constitutive expression may result in an inability to regulate the expression of exogenous genes. Thus, to improve the temporal and spatial expression patterns of exogenous genes and to maximize production of exogenous proteins, it is essential to choose suitable promoters. In previous studies, many researchers reported that tissue-specific or stress inducible promoters, which were able to induce expressions of genes in specific organs or specific conditions, effectively improved the expression levels of exogenous genes and plant growth. For example, the cholera toxin B (CTB) subunit was expressed in rice (*Oryza sativa* L. cv. Nipponbare), under the control of the rice endosperm specific storage protein glutelin *GluB-1* promoter, that enabled high accumulation of up to 3.37 mg/g in rice seeds (Kajiura et al. 2013). However, CTB expression in tobacco (*Nicotiana tabacum* L. cv TI560) under the control of ubiquitin promoter was much lower, resulting in an accumulation of only 1.8% of total soluble protein

Abbreviations: GUS, β-glucuroidase; Spo^{min}, sugar inducible minimal promoter; ER, endoplasmic reticulum; TP1, transit peptide of sweet potato ADP-glucose pyrophosphorylase; UTR, untranslated region; Semi qRT-PCR, semi quantitative reverse transcription polymerase chain reaction; HPT, hygromycin phosphotransferase; TSP, total soluble protein; 35S, cauliflower mosaic virus 35S RNA promoter.

This article can be found at http://www.jspcmb.jp/
Published online February 20, 2015

(TSP) in tobacco leaves (Kang et al. 2006). Similarly, in tobacco (*N. tabacum* cv K326) the 35S promoter only produced an accumulation level of 0.0095% of TSP in tobacco leaves (Wang et al. 2001). Furthermore, 35S was unsuitable for inducing high expression of genes in cassava (*Manihot esculenta* Crantz) storage roots (Zhang et al. 2003). In contrast, an alternative promoter, the cassava granule-bound starch synthase (GBSSI) promoter, which has light and sugar responsive cis elements, has been isolated; even though the GBSSI promoter induced very low expression in leaves, stems and roots, it did produce very high expression in storage roots (Koehorst-van Putten et al. 2012). It has also been shown that when a dexamethazone inducible promoter was used instead of 35S to drive waterlogging resistance genes, arabidopsis waterlogging tolerance to hypoxia was enhanced (Dennis et al. 2000). Furthermore, studies have shown that replacing 35S with the chloroplast *psbA* promoter increased the accumulation of human serum albumin in tobacco leaves (*N. tabacum* cv. Petit Havana) from 0.02 to 11.1% of TSP (Fernández-San Millán et al. 2003; Sijmons et al. 1990).

It has been reported that sporamin is the most abundant storage protein in the tuberous storage roots of sweet potato (*Ipomoea batatas* L. Lam.); it comprised about 80% of TSP in sweet potato tuberous roots (Maeshima et al. 1985). Although sporamin gene expression is normally specific to tuberous roots (Maeshima et al. 1985), expression has also been induced in sweet potato leaves and petioles by sucrose, glucose and fructose treatment (Hattori et al. 1990, 1991). Morikami et al (2005) determined the 204 bp sucrose inducible minimal region (Spo^{min}) in the sporamin gene promoter region. These studies showed that Spo^{min} might be a useful tool to develop plant bioreactors.

This paper assesses the potential of applying the sugar inducible sporamin promoter to produce exogenous proteins in transgenic *Nicotiana plumbaginifolia*. In the study, we analyze GUS gene expression by sucrose treatment in transgenic *N. plumbaginifolia* containing Spo^{min}-GUS constructs (Spo^{min} was located upstream of the GUS gene).

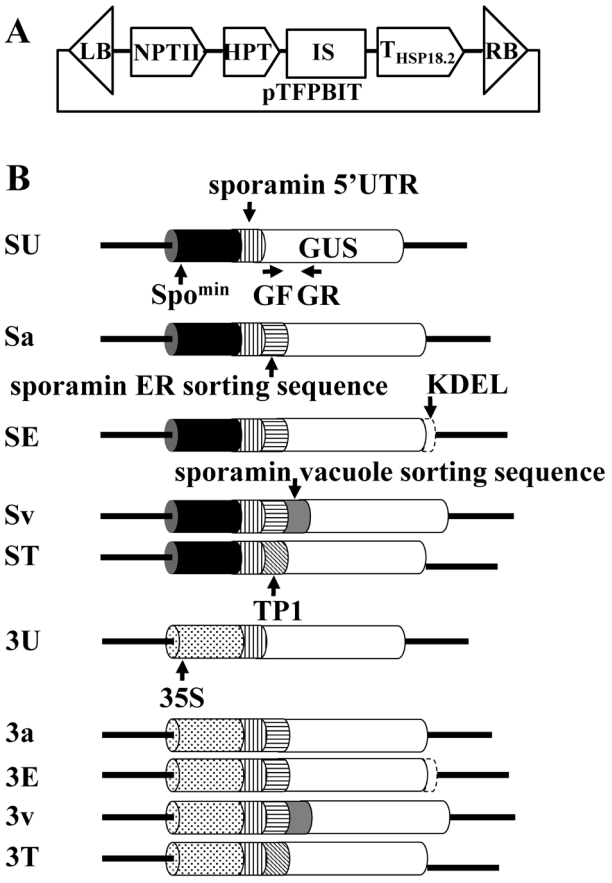

Figure 1. Constructs of pTFPBIT vector for GUS introduction with Spo^{min} or 35S and signal sequences. (A) The pTFPBIT vector was constructed from a pBI121 vector by adding HPT selection marker, and replacing $T_{HSP18.2}$ from the NOS terminator following GUS gene. $T_{HSP18.2}$ was the terminator of *A. thaliana* heat shock protein 18.2. IS was insertion site of Spo^{min} or 35S+signal sequences+GUS. (B) 10 GUS constructs were shown. GF: 5′-AAATCAAAAAACTCGACGGCCTGTG-3′ and GR: 5′-TATAAAGACTTCGCGCTGATA-3′ were GUS gene specific primers. GUS gene (uidA): white boxes, Spo^{min}: black boxes (Morikami et al. 2005), 35S (dot boxes), sporamin 5′UTR: vertical stripe boxes (accession number, X13509), sporamin ER sorting sequences: horizontal stripe boxes (accession number, X13509), ER retention signal KDEL: broken line boxes, sporamin vacuole sorting sequences: gray box (accession number, X13509) and transit peptide of sweet potato ADP-glucose pyrophosphorylase (TP1) (Kwak et al. 2008): slash box (accession number. AY544766). Signal sequences were collected from National Center for Biotechnology Information.

Materials and methods

GUS expression constructs

The pTFPBIT vector was constructed from the pBI121 vector by adding a HPT selection marker, and replacing terminator of *Arabidopsis thaliana* heat shock protein 18.2 ($T_{HSP18.2}$) from the NOS terminator following the GUS gene (Figure 1A). We made 5 constructs to sort the GUS, which was expressed by Spo^{min}, to cytosol (SU), apoplast (Sa), ER (SE), vacuole (Sv) and plastid (ST) (Figure 1B). We also made 5 constructs to sort the GUS, which was expressed by 35S, to cytosol (3U), apoplast (3a), ER (3E), vacuole (3v) and plastid (3T) (Figure 1B).

Plant transformation

N. plumbaginifolia plants grown in pot (25°C in a 16 h light/8 h dark cycle) were used to produce transformants. The binary vector pTFPBIT that contained each expression construct was introduced into *Agrobacterium tumefaciens* EHA105 by the freeze-thaw method (Jyothishwaran et al. 2007). *N. plumbaginifolia* was transformed with the *A. tumefaciens* using the leaf-disk method (Lloyd et al. 1986). Hygromycin B-resistant (Hyg^r) shoots were selected on a Linsmaier and Skoog (LS) regeneration solid medium (Linsmaier and Skoog 1965) containing 1% agar, 0.2 mg/l naphthaleneacetic

acid, 2 mg/l kinetin, 25 mg/l hygromycin B, and 500 mg/l carbenicillin sodium. Selected transgenic plants were grown on LS solid medium containing 1% agar, 25 mg/l hygromycin B, and 500 mg/l carbenicillin sodium at 25°C in a 16 h light/8 h dark cycle. Every 3 months, approximately 2 cm long tip sections of the transgenic plant stems, were cut out from upper parts of stems and transplanted to new LS solid medium containing 1% agar, 25 mg/l hygromycin B, and 500 mg/l carbenicillin sodium.

Expression analysis of introduced genes in transgenic plants

Total RNA was extracted from leaves with Sepasol RNA I Super (Nacalai Tesque, Japan) according to the manufacturer's protocol. After treatment with Recombinant DNase I (RNase-free) (Takara, Japan), first-strand cDNA was synthesized with the PrimeScript RTreagent kit (Perfect Real Time) (TaKaRa, Japan), using oligo dT primer and random hexamer primer. Semi qRT-PCRs were performed with *hpt* specific primers (HPTF: 5′-GAA AGT TCG ACA GCG TCT CC-3′ and HPTR: 5′-GTC CAT CAC AGT TTG CCA GTG-3′) (Tsugawa et al. 2004), and *actin* (FM244697) was amplified with specific primers (actinF: 5′-CTG TTC TCT TGA CTG AAG CAC-3′ and actinR 5′-TAA CCT TCA TAG ATA GGG ACG GT-3′), to be used as an internal standard. The amplification was performed using a Thermal Cycler (LifePro, BIOER, USA), with an initial denature at 94°C for 4 min, followed by a cycling stage (30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s), and then a following stage of 72°C for 7 min, after which the samples were stored at 4°C. Signal intensities of the electrophoresed bands were determined using Image J software (Figure 2A).

Treatment with sucrose solution

Excised leaves, stems, and roots were incubated in deionized water (5 ml) containing 0, 6, or 10% sucrose under static and sterilized conditions for 7 days at 25°C in a 16 h light/8 h dark cycle. The treated tissues were used for the GUS assay.

Cessation of sucrose treatment in leaves which were treated with 6% sucrose solution

After 6% sucrose treatment for 7 days, leaves were rinsed by deionized water and transferred to 15 ml tube containing 5 ml deionized water. The GUS assay was also conducted on these leaves.

Protein extraction and fluorimetric assay of GUS activity

Extraction of proteins and fluorimetric assays of GUS activity were carried out on all excised leaves, stems, and roots using the method described by Jefferson et al. (1987). Extraction solution contains 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% TritonX-100 ($v\,v^{-1}$), 0.1% sodium *N*-laurylsarcosinate ($mg\,ml^{-1}$), and 10 mM 2-mercaptoethanol. The protein concentration in the extracts was determined according to the method of Bradford (1976), and the GUS activity was expressed as pmol of 4-methylumbelliferone (MU) produced per minute per milligram of proteins.

Expression analysis of GUS gene in transgenic plants after sucrose treatment

After 0 or 6% sucrose treatment for 7 days, total RNA was extracted from some leaves. Semi qRT-PCRs were performed with GUS gene specific primers (GF: 5′-AAA TCA AAA AAC TCG ACG GCC TGT G-3′ and GR: 5′-TAT AAA GAC TTC GCG CTG ATA-3′) to confirm GUS gene expressions. And *actin* primers (actinF and actinR) were used as an internal standard.

Statistical analysis

Mann–Whitney *U* test was performed to determine differences.

Results

Introduction of GUS gene constructs and selection of transgenic lines

In each construct, over 7 transgenic lines were obtained and confirmed by genomic PCR (data not shown). One transgenic plant of each line was selected and their transcript levels of *hpt* were examined by semi qRT-PCR (Figure 2A). The analysis of these transgenic lines showed similar transcript levels. Moreover growth of each transgenic line was indistinguishable from the wild type plants (Figure 2B).

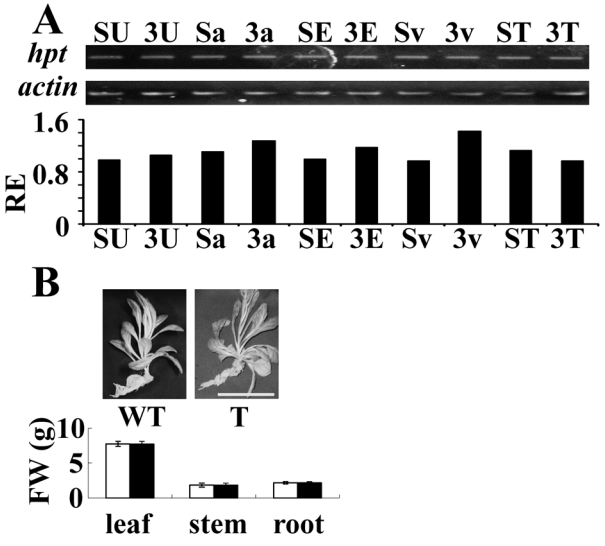

Figure 2. Relative expression levels of constructs in transgenic *N. plumbaginifolia*. (A) Relative expression levels (RE) of *hpt* in transgenic *N. plumbaginifolia* by semi qRT-PCR. Upper bands show *hpt* transcripts and lower bands show *actin* transcripts. The graph shows RE of *hpt*/*actin*. (B) Wild type *N. plumbaginifolia* (WT) or transgenic *N. plumbaginifolia* with SE construct (T) at 3 months after cultivation. Scale bar=10 cm. The graph shows fresh weight (FW) of WT (white bars) or T (black bars) at 3 months after initial cultivation. Error bars show standard error, $n=4$.

Sucrose induced GUS activities in excised organs

As shown in Figure 3, GUS activities in the transformants of Spo^{min} constructs such as SU, Sa, SE, Sv and ST plant segments were induced by sucrose treatment. Significantly high level GUS activities were found in these leaf segments, and were higher than those in the transformants of 35S constructs such as 3U, 3a, 3E, 3v and 3T leaves. The high GUS activities were observed in SE leaves treated with 6% or 10% sucrose, and were over 200 times higher than those in 3E. Small increases in GUS activities were also found in the stems and roots. In the Spo^{min} construct plants such as SU, Sa, SE, Sv and ST plants, even though the total GUS activities were much higher in the leaves than in stems and roots, all tissues showed similar patterns under different sucrose treatments. GUS activities in all tissues (leaves, stems and roots) were higher after 6% or 10% sucrose treatment than after 0% sucrose treatment.

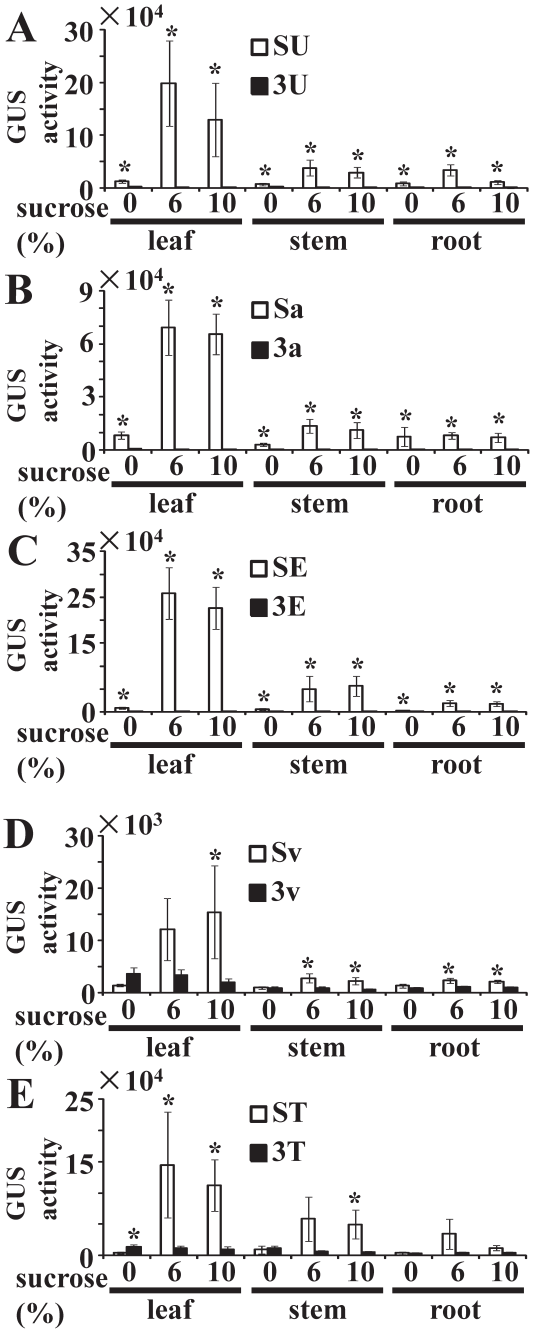

Figure 3. Expression of GUS gene in leaves, stems and roots of transgenic *N. plumbaginifolia* after sucrose treatment. (A) SU and 3U, (B) Sa and 3a, (C) SE and 3E, (D) Sv and 3v and (E) ST and 3T. White bars show SU, Sa, SE, Sv and ST. Black bars show 3U, 3a, 3E, 3v and 3T. Plants were cultured on LS medium for 2–4 months. Vertical axes show GUS activities (pmol MU min^{-1} mg protein^{-1}). Error bars show standard error, n=7. Astarisks (*) indicate significant differences between Spo^{min} and 35S (*p<0.05).

GUS activities in leaves over long periods of cultivation

It has been reported that GUS expression induced by the promoter *A. thaliana* heat shock protein 18.2 (HSP18.2) decreased with aging in transgenic *N. plumbaginifolia* (Moriwaki et al. 1999). Therefore, we investigated whether Spo^{min} could maintain consistent gene expression during extended subculture. Our analyses showed no differences in GUS activities between the plants after 2 or 8 months from the plant induction (Figure 4). Furthermore, the GUS activities of SE leaves which were treated either with 6% and 10% sucrose were significantly higher than those with 0% sucrose treatment. There were no differences in growth and developmental characteristics between the plants (data not shown).

GUS activities and GUS expression after cessation of sucrose treatment

GUS expression and activity have been detected up to 40 h after heat shock treatment in transgenic *N. plumbaginifolia* leaves that had received the *A. thaliana* HSP18.2 promoter-GUS (Moriwaki et al. 1999). Therefore we analyzed GUS activities and GUS gene expression levels in SE leaves after cessation of the sucrose treatment (Figure 5). After cessation of sucrose treatment, GUS activities increased until day 10 (Figure 5A). Semi qRT-PCR analysis of GUS gene expression

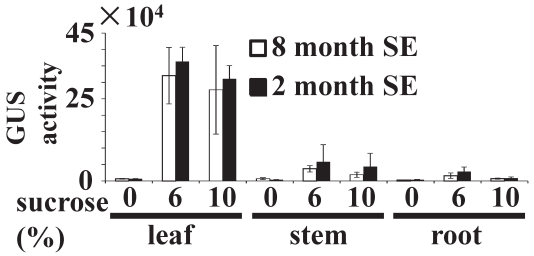

Figure 4. GUS activities of transgenic *N. plumbaginifolia* in 2 and 8 months from the plant induction. 2 month SE (black bars) and 8 month SE (white bars) were treated with 0, 6 or 10% sucrose and then examined for GUS activities. Vertical axis shows GUS activity (pmol MU min^{-1} mg protein^{-1}). Error bars show standard error, n=4.

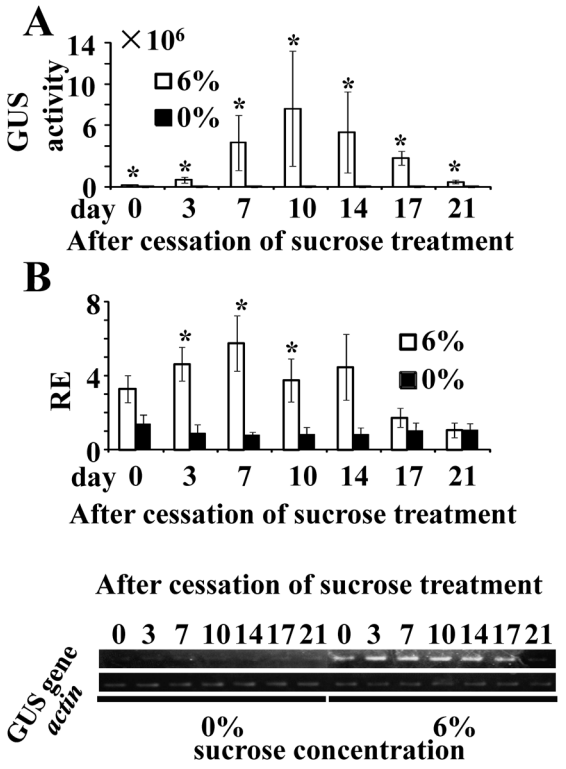

Figure 5. GUS activity and GUS expression after cessation of sucrose treatment. (A) GUS activities after SE leaves were treated with 0% or 6% sucrose for 7 days. Leaves were analyzed for GUS activities at 0, 3, 7, 10, 14, 17 and 21 days after cessation of sucrose treatment. Vertical axis shows GUS activities (pmol MU min^{-1} mg $protein^{-1}$). (B) Relative expression levels (RE) of GUS gene after 7 days sucrose treatment by semi qRT-PCR. The graph shows RE of GUS gene/*actin* graph. The gel bands show GUS gene transcripts and *actin* transcripts. Error bars show standard error, $n=4$. Astarisks (*) indicate significant differences between 0% sucrose treatment and 6% sucrose treatment (* $p<0.05$)

showed higher expression levels in 6% sucrose treated leaves than those in 0% sucrose treated at day 17 (Figure 5B). Obvious GUS gene transcripts were detected until day 17 in 6% sucrose treated leaves, with some faint GUS gene transcripts at day 21 (Figure 5B). Similarly faint GUS gene transcripts were detected over the whole 21-day period in 0% sucrose treated leaves (Figure 5B).

Discussion

Generally, heterologous proteins retained in the cytosol are usually degraded (Benchabane et al. 2008). In order to overcome degradation of heterologous proteins, previous studies presented some potential solutions, such as to transport heterologous proteins to organelles. For example, the taste-modifying protein, miraculin was able to be accumulated in tomato fruit apoplast at levels over 150 μg/g fresh weight (Hiwasa-Tanase et al. 2011; Kim et al. 2010). Also, in the case of chloroplast, cry2Aa2 accumulated in tobacco leaf chloroplasts at a level over 70% of TSP (Oey et al. 2009). Furthermore, Hamorsky et al. (2013) showed that CTB subunit was accumulated in ER of *Nicotiana benthamiana* leaves, at levels of up to 1 g/kg of fresh leaf. In our studies, we employed 2 kinds of promoters (Spo^{min} and 35S) and 5 kinds of signal sequences (cytosol, apoplast, ER, vacuole and plastid) to accumulate GUS abundantly in *N. plumbaginifolia* (Figure 3). We showed that GUS activities of SE leaves were higher than GUS activities of Sa, Sv and ST leaves in 6% and 10% sucrose treatments (Figure 3). The GUS activities increased to about 80 times and GUS protein (μg) increased from about 0.26 to 20.0 (calculated by data according to Kim et al. 1995) by 6% sucrose treatment in SE leaves in comparison with 0% sucrose treatment. The TSP (μg) in SE leaves by 6% sucrose treatment was 792 and it in SE leaves was 841 by 0% sucrose treatment. There was not so much difference between both of them while the GUS activities increased to about 80 times. It would be caused by the increase of GUS protein amount in the tissue and/or increase of relative enzyme activity of GUS protein. As there were reports on sucrose treatment which did not promote GUS activities in bacteria (Ku et al. 2011; Mariscal et al. 1998), we presumed that the GUS protein amount increased and it would be from 0.31 to 214 μg by 6% sucrose treatment in SE leaves by calculation from the data according to Kim et al. (1995). These results are supported by previous reports. For example, Nausch et al. (2012) showed that accumulation of human interleukin 6 was over 10 times greater in ER than in apoplasts or vacuoles, comprising up to 0.005% of TSP in *N. benthamiana* leaves. Also, in maize seeds, the accumulation of heat-labile toxin was about 5 times higher in ER than in plastids, reaching 0.0028% of TSP (Streatfield et al. 2003).

In SU leaves, the high GUS activities that were observed (Figure 3A) may have been caused by lack of *N*-glycosylation of GUS protein. GUS protein (accession number, S69414) has one site which is glycosylated with *N*-glycosylation, and GUS activity has been decreased by *N*-glycosylation (Iturriaga et al. 1989). Sporamin protein was transported to vacuoles by way of Golgi apparatus (Yang et al. 2005) and was glycosylated during transportation (Shimizu et al. 2005). Therefore, in our study it is possible that decrease of GUS activities were induced in the Sa, 3a, SE, 3E, Sv and 3v transformants because the GUS proteins had sorting sequences of sporamin and were glycosylated by sorting. The relatively higher GUS activities in the SU transformants may have occurred because the GUS proteins of SU do not have sorting sequences of sporamin. And Lee et al. (2007) reported that rice ADP-glucose pyrophosphorylase was located in plastid. However, it has not been uncovered whether ADP-glucose pyrophosphorylase was glycosylated.

A previous study showed that GUS activities induced by the sporamin promoter (0.95 kb 5′-upstream region

of gSpo-A1, one of the genes for A-type sporamins) were predominantly found in stems of transgenic *N. tabacum* which were treated with 2% sucrose (Ohta et al. 1991). The same study also showed that GUS staining was restricted to the inside of the vascular system. In contrast, our results showed that Spo^{min} induced low GUS activities of stems treated with 6% or 10% sucrose (Figure 3). However, we did find strong GUS stainings in leaf veins (data not shown). Therefore, this may still be consistent the results from Ohta et al. (1991) because the leaf veins are part of the plant vascular system.

Sucrose inducible promoters, for example, patatin promoter, are used for expression of foreign proteins in transgenic potatoes, but expression levels are much lower, below 1% of TSP, than by using 35S promoter (Deryabin et al. 2003; Shulga et al. 2004). On the other hand, sporamin promoter induced high levels of phytase expression in potato, up to 7.4% of TSP (Hong et al. 2008). Moreover, phytase activities in transgenic potato tubers were shown to be stable over 3 growing seasons (Hong et al. 2008). In our study, Spo^{min} also showed stable GUS activities (Figure 4) and continued GUS expression after cessation of sucrose treatment (Figure 5), whereas these results were not found with *A. thaliana* HSP18.2 promoter. GUS activity was observed up to 21 days after the 6% sucrose treatment, but hardly any GUS transcript level was detected (Figure 5). This is consistent with a previous report which showed that GUS protein is very stable (Martin et al. 1992). Therefore, we have confirmed that Spo^{min} is an excellent promoter for making plant bioreactors. Previous reports have shown that ER is a suitable location to accumulate heterologous protein in transgenic Nicotiana plants leaves (Benchabane et al. 2008; Hamorsky et al. 2013; Nausch et al. 2012; Yang et al. 2005). Our study also showed that ER was suitable for accumulating heterologous GUS protein in an expression system using Spo^{min} and *N. plumbaginifolia* and also that leaves might be a more suitable organ than stems and roots because of high GUS activities of SE, especially in SE leaves (Figure 3).

In conclusion, we have developed transgenic *N. plumbaginifolia* which can accumulate GUS by sucrose treatment using a Spo^{min} expression system. Further experiment will be needed for this high-performance gene expression system, which permits accumulation of high-value protein in plant tissue.

Acknowledgement

We would like to express our thanks to Dr. Elizabeth Hood of Prodigene Inc. (Present address: Arkansas state university) for providing *Agrobacterium tumefaciens* strain EHA105.

References

Benchabane M, Goulet C, Rivard D, Faye L, Gomord V, Michaud D (2008) Preventing unintended proteolysis in plant protein biofactories. *Plant Biotechnol J* 6: 633–648

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254

Dennis ES, Dolferus R, Ellis M, Rahman M, Wu Y, Hoeren FU, Grover A, Ismond KP, Good AG, Peacock WJ (2000) Molecular strategies for improving waterlogging tolerance in plants. *J Exp Bot* 51: 89–97

Deryabin AN, Trunova TI, Dubinina IM, Burakhanova EA, Sabel'nikova EP, Krylova EM, Romanov GA (2003) Chilling tolerance of potato plants transformed with a yeast-derived invertase gene under the control of the B33 patatin promoter. *Russ J Plant Physiol* 50: 449–454

Fernández-San Millán A, Mingo-Castel A, Miller M, Daniell H (2003) A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. *Plant Biotechnol J* 1: 71–79

Fischer R, Schillberg S (Eds.) (2004) *Molecular Farming: Plant-made Pharmaceuticals and Technical Proteins*. WILEY-VCH Verlag GmbH & Co., KGaA, Weinheim

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, et al. (1983) Expression of bacterial genes in plant cells. *Proc Natl Acad Sci USA* 80: 4803–4807

Guan ZJ, Guo B, Huo YL, Guan ZP, Dai JK, Wei YH (2013) Recent advances and safety issues of transgenic plant-derived vaccines. *Appl Microbiol Biotechnol* 97: 2817–2840

Hamorsky KT, Kouokam JC, Bennett LJ, Baldauf KJ, Kajiura H, Fujiyama K, Matoba N (2013) Rapid and scalable plant-based production of a cholera toxin B subunit variant to aid in mass vaccination against cholera outbreaks. *PLoS Negl Trop Dis* 7: e2046

Hattori T, Fukumoto H, Nakagawa S, Nakamura K (1991) Sucrose-induced expression of genes coding for tuberous root storage protein, sporamin, of sweet potato in leaves and petioles. *Plant Cell Physiol* 32: 79–86

Hattori T, Nakagawa S, Nakamura K (1990) High-level expression of tuberous root storage protein genes of sweet potato in stems of plantlets grown in vitro on sucrose medium. *Plant Mol Biol* 14: 595–604

Hiwasa-Tanase K, Nyarubona M, Hirai T, Kato K, Ichikawa T, Ezura H (2011) High-level accumulation of recombinant miraculin protein in transgenic tomatoes expressing a expressing a synthetic miraculin gene with optimized codon usage terminated by the native miraculin terminator. *Plant Cell Rep* 30: 113–124

Hong YF, Liu CY, Cheng KJ, Hour AL, Chan MT, Tseng TH, Chen KY, Shaw JF, Yu SM (2008) The sweet potato sporamin promoter confers high-level phytase expression and improves organic phosphorus acquisition and tuber yield of transgenic potato. *Plant Mol Biol* 67: 347–361

Iturriaga G, Jefferson RA, Bevan MW (1989) Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. *Plant Cell* 1: 381–390

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901–3907

Jyothishwaran G, Kotresha D, Selvaraj T, Srideshikan SM, Rajvanshi PK, Jayabaskaran C (2007) A modified freeze-thaw method for efficient transformation of *Agrobacterium tumefaciens*. *Curr Sci* 93: 770–772

Kajiura H, Wasai M, Kasahara S, Takaiwa F, Fujiyama K (2013) N-glycosylation and N-glycan moieties of CTB expressed in rice seeds. *Mol Biotechnol* 54: 784–794

Kang TJ, Kim BG, Yang JY, Yang MS (2006) Expression of a synthetic cholera toxin B subunit in tobacco using ubiquitin promoter and bar gene as a selectable marker. *Mol Biotechnol* 32: 93–100

Kim DH, Jin YH, Jung EA, Han MJ, Kobashi K (1995) Purification and characterization of beta-glucuronidase from *Escherichia coli* HGU-3, a human intestinal bacterium. *Biol Pharm Bull* 18: 1184–1188

Kim YW, Hirai T, Kato K, Hiwasa-Tanase K, Ezura H (2010) Gene dosage and genetic background affect miraculin accumulation in transgenic tomato fruits. *Plant Biotechnol* 27: 333–338

Koehorst-van Putten HJ, Wolters AM, Pereira-Bertram IM, van den Berg HH, van der Krol AR, Visser RG (2012) Cloning and characterization of a tuberous root-specific promoter from cassava (*Manihot esculenta* Crantz). *Planta* 236: 1955–1965

Ku S, Zheng H, Park MS, Ji GE (2011) Optimization of *β*-Glucuronidase Activity from *Lactobacillus delbrueckii* Rh2 and Its Use for Biotransformation of Baicalin and Wogonoside. *J Korean Soc Appl Biol Chem* 54: 275–280

Kwak MS, Oh MJ, Paek KH, Shin JS, Bae JM (2008) Dissected effect of a transit peptide of the ADP-glucose pyrophosphorylase gene from sweetpotato (ibAGP2) in increasing foreign protein accumulation. *Plant Cell Rep* 27: 1359–1367

Lee SK, Hwang SK, Han M, Eom JS, Kang HG, Han Y, Choi SB, Cho MH, Bhoo SH, An G, et al. (2007) Identification of the ADP-glucose pyrophosphorylase isoforms essential for starch synthesis in the leaf and seed endosperm of rice (*Oryza sativa* L.). *Plant Mol Biol* 65: 531–546

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. *Physiol Plant* 18: 100–127

Lloyd A, Barnason A, Rogers SG, Byrne MC, Fraley RT, Horsch RB (1986) Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. *Science* 234: 464–466

Maeshima M, Sasaki T, Asahi T (1985) Characterization of major proteins in sweet potato tuberous roots. *Phytochemistry* 24: 1899–1902

Mariscal A, Gómez-Aracena J, Varo MC, Fernández-Crehuet J (1998) Interference by carbohydrate substrates, flavonoids, and monosaccharide derivatives on bacterial beta-D-glucuronidase assays. *Arch Environ Contam Toxicol* 35: 588–593

Martin T, Woehner RV, Hummel S, Willmitzer L, Frommer WB (1992) The GUS reporter system as a tool to study plant gene expression. In: Gallagher SR (eds) *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression*. Academic Press, London, pp 23–43

Morikami A, Matsunaga R, Tanaka Y, Suzuki S, Mano S, Nakamura K (2005) Two *cis*-regulatory elements are involved in the sucrose-inducible expression of the sporamin gene promoter from sweet potato in transgenic tobacco. *Mol Genet Genomics* 272: 690–699

Moriwaki M, Yamakawa T, Washino T, Kodama T, Igarashi Y (1999) Delayed recovery of *β*-glucuronidase activity driven by an *Arabidopsis* heat shock promoter in heat-stressed transgenic *Nicotiana plumbaginifolia*. *Plant Cell Rep* 19: 96–100

Nausch H, Mikschofsky H, Koslowski R, Meyer U, Broer I, Huckauf J (2012) High-level transient expression of ER-targeted human interleukin 6 in *Nicotiana benthamiana*. *PLoS ONE* 7: e48938

Oey M, Lohse M, Kreikemeyer B, Bock R (2009) Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant J* 57: 436–445

Ohta S, Hattori T, Morikami A, Nakamura K (1991) High-level expression of a sweet potato sporamin gene promoter: *β*-glucuronidase (GUS) fusion gene in the stems of transgenic tobacco plants is conferred by multiple cell type-specific regulatory elements. *Mol Gen Genet* 225: 369–378

Shimizu M, Igasaki T, Yamada M, Yuasa K, Hasegawa J, Kato T, Tsukagoshi H, Nakamura K, Fukuda H, Matsuoka K (2005) Experimental determination of proline hydroxylation and hydroxyproline arabinogalactosylation motifs in secretory proteins. *Plant J* 42: 877–889

Shulga NY, Rukavtsova EB, Krymsky MA, Borisova VN, Melnikov VA, Bykov VA, Buryanov YI (2004) Expression and characterization of hepatitis B surface antigen in transgenic potato plants. *Biochemistry (Mosc)* 69: 1158–1164

Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ, Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. *Biotechnology* 8: 217–221

Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, Lamphear BJ, Drees CF, Jilka JM, Hood EE, et al. (2003) Corn as a production system for human and animal vaccines. *Vaccine* 21: 812–815

Tsugawa H, Kagami T, Suzuki M (2004) High-frequency transformation of *Lobelia erinus* L. by *Agrobacterium*-mediated gene transfer. *Plant Cell Rep* 10: 759–764

Wang XG, Zhang GH, Liu CX, Zhang YH, Xiao CZ, Fang RX (2001) Purified cholera toxin B subunit from transgenic tobacco plants possesses authentic antigenicity. *Biotechnol Bioeng* 72: 490–494

Yang J, Barr LA, Fahnestock SR, Liu ZB (2005) High yield recombinant silk-like protein production in transgenic plants through protein targeting. *Transgenic Res* 14: 313–324

Zhang P, Bohl-Zenger S, Puonti-Kaerlas J, Potrykus I, Gruissem W (2003) Two cassava promoters related to vascular expression and storage root formation. *Planta* 218: 192–203