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

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Abstract We produced transgenic Nicotiana plumbaginifolia plants which contained Spo\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{min} was stably maintained. Also, even when sucrose treatment was stopped, GUS gene expression by Spo\textsuperscript{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 T1560) under the control of ubiquitin promoter was much lower, resulting in an accumulation of only 1.8% of total soluble protein.
GUS high-expression tobacco by sporamin promoter

(TSP) in tobacco leaves (Kang et al. 2006). Similarly, in tobacco \textit{(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 \textit{(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 \textit{(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 \textit{(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 \textit{(Spo\textsuperscript{5\textprime}UTR)} in the sporamin gene promoter region. These studies showed that \textit{Spo\textsuperscript{5\textprime}UTR} 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 \textit{Nicotiana plumbaginifolia}. In the study, we analyze GUS gene expression by sucrose treatments in transgenic \textit{N. plumbaginifolia} containing \textit{Spo\textsuperscript{5\textprime}UTR}-GUS constructs (\textit{Spo\textsuperscript{5\textprime}UTR} was located upstream of the GUS gene).

Materials and methods

\textbf{GUS expression constructs}

The \textit{pTFPBIT} vector was constructed from the \textit{pBI121} vector by adding a HPT selection marker, and replacing terminator of \textit{Arabidopsis thaliana} heat shock protein 18.2 (\textit{T\textsubscript{HSP18.2}}) from the NOS terminator following the GUS gene (Figure 1A). We made 5 constructs to sort the GUS, which was expressed by \textit{Spo\textsubscript{5\textprime}UTR}, 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).

\textbf{Plant transformation}

\textit{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 \textit{pTFPBIT} that contained each expression construct was introduced into \textit{Agrobacterium tumefaciens} EHA105 by the freeze-thaw method (Jyothishwaran et al. 2007). \textit{N. plumbaginifolia} was transformed with the \textit{A. tumefaciens} using the leaf-disk method (Lloyd et al. 1986). Hygromycin B-resistant (Hyg\textsuperscript{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’-CTGTTTCTCTGAATGACAC-3’ and actinR 5’-TAA CCT TCA TAG ATG GGG ACC 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 AACTGACGCCTGTG-3’ and GR: 5’-TAT AAA GAC TCTGCGTTG-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 Spomin 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 Spomin 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.

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 Spomin 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 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. Asterisks (*) indicate significant differences between Spomin and 35S (*p<0.05).

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.
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). Spoamin 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 Spomnin 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, Spomnin 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 Spomnin 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 Spomnin 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 Spomnin expression system. Further experiment will be needed for this high-performance gene expression system, which permits accumulation of high-value protein in plant tissue.

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**References**


