Synthesis and Assembly of *Escherichia coli* Heat-labile Enterotoxin B Subunit in Transgenic Rice (*Oryza sativa* L.)

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Abstract *Escherichia coli* heat-labile enterotoxin B subunit (LTB) can be used as a potent mucosal immunogen and immunoadjuvant for co-administered antigens. The synthetic LTB (sLTB) was modified based on plant optimized codon usage, and fused to a translation signal (the Kozak sequence) in the front of start codon and the ER retention signal, SEKDEL, in the c-terminus of sLTB gene. The sLTB and the wild-type LTB gene (wLTB) were located into plant expression vectors under the control of the wheat Bx17 HMW (High Molecular Weight) glutenin endosperm-specific promoter containing the first intron of the rice actin1 gene. Both genes were introduced into rice cells (*Oryza sativa* L.) via particle bombardment mediated transformation. The integration of LTB gene into the chromosome of transgenic plants was confirmed by genomic DNA PCR amplification methods. The transcription and translation of the LTB genes were demonstrated by reverse-transcription PCR (RT-PCR) and Western blot analyses, respectively. The LTB proteins produced in the seed tissues of transgenic rice showed binding affinity for G_{M1} ganglioside, a receptor for biologically active LTB, suggesting the plant-produced LTB are capable of forming active pentamers. The expression level of sLTB was higher than wLTB in transgenic rice plants and was up to 2.7% of the total soluble proteins of the seed tissues.

Keywords: Escherichia coli *heat-labile enterotoxin B subunit*, *rice*, *endosperm specific promoter*

INTRODUCTION

The *Escherichia coli* heat-labile enterotoxin (LT) is composed of one copy of the A subunit, which exhibits ADP-ribosylation activity, and a homopentamer of B subunits (LTB), which binds to the G_{M1} gangliosides located on the surfaces of enterocytes. LTB is comprised of five identical, 103-amino acid (11.6 kDa) peptides [1], which form a donut-shaped pentamer via non-covalent association [2]. LTB is strongly immunogenic and stimulates mucosal and systemic immune responses and already has been useful as a vaccine against enterotoxigenic *E. coli*-induced diarrhea [3]. In addition, LTB has shown a higher capacity to function as a potent mucosal adjuvant than cholera toxin B subunit when co-administered to mice intranasally with hen-egg lysozyme [4].

The structure and characteristics of genes expressed in the endosperm of wheat and other cereals have been studied extensively [5,6]. Most of these proteins are storage proteins or prolamins. Storage proteins are expressed only in the starchy endosperm during the middle and late developmental stages. Promoters of the so-called HMW (High Molecular Weight) prolamin genes are endosperm-specific, and the sequences and strength of the promoters have been evaluated and characterized [7,8]. These promoters appear to be ideal candidates for the enhancement of tissue-specific expression level of transgenes in the endosperm of wheat and other cereals, regardless of the origin of promoter and target plant [9,10]. It has been reported in transient expression assays that the addition of the first intron of rice actin1 gene exerted

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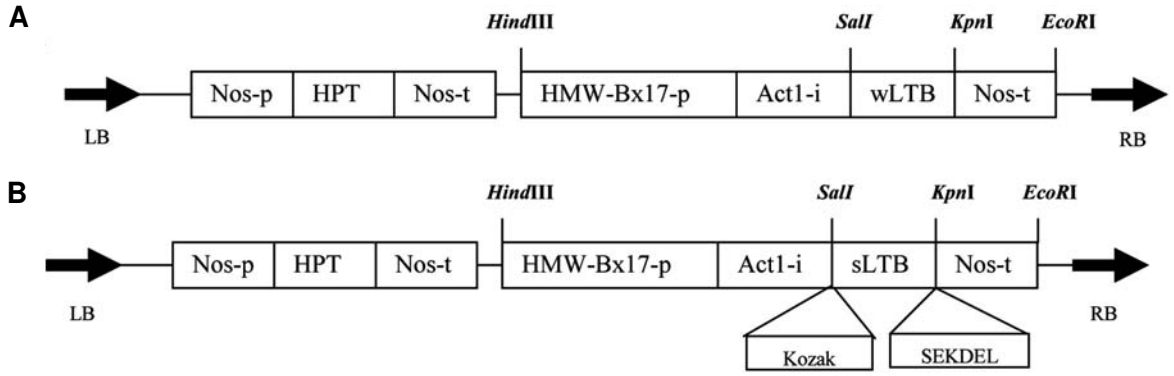

Fig. 1. Schematic diagram of the pMYN-sLTB (A) and pMYN-wLTB (B) containing genes encoding for sLTB or wLTB, respectively, under the control of the Bx17 HMW endosperm specific promoter. LB and RB are left and right border of T-DNA. NOS-p is promoter of nopaline synthase gene. *HPT* is hygromycin phosphotransferase gene. NOS-t is terminator of nopaline synthase gene. HMW-Bx17-p is the Bx17 HMW endosperm specific promoter. Kozak is translation signal sequence. SEKDEL is ER-retention sequence motif. Act1-i is the first intron of rice actin1 gene.

a positive effect on the strength of the Bx17 HMW glutenin promoter, increasing the expression level by a factor of four in the immature wheat endosperm [11].

Recently, plants have been used to produce heterologous proteins and secondary metabolites [12-16]. Plant-based vaccines provide promising examples of a new strategy which combines innovations in medical science and plant biology to generate pharma-ceutical products. Plant-based vaccines are particularly attractive because plants are not susceptible to human or ani-mal diseases, which reduce the costs normally associated with screening for viruses and bacterial toxins [17,18]. LTB has been produced in tobacco, potato, maize, lettuce, and soybean [19-26]. Rice is one of the most important foodstuffs worldwide and is consumed by 60% of the world's population. It represents the principal staple food in Asia, Africa, and South America. The development of rice-based vaccine is important because vaccination programs are not financially viable in these regions.

In this study, we tested the feasibility of expression and assembly of LTB in the seed tissues of rice plants under the control of wheat endosperm-specific promoter in the first step of rice-based edible vaccine development. The G_{M1}-ganglioside binding assay showed that LTB proteins were capable of successfully assembling into their active form within the rice seeds and were able to function as an antigen.

MATERIALS AND METHODS

Construction of Plant Expression Vectors

The synthetic LTB (sLTB) was amplified by PCR from pMYO51 [27] containing sLTB gene modified with plant optimized codon usage, and fused to the Kozak sequence [28] and the ER retention signal as a template. The wLTB gene was amplified by PCR from pMYO47 [17] which contains the LT operon (GenBank Locus ABO11677) as a template. PCR products were introduced into plant expression vector using *Sal*I and *Kpn*I restriction enzymes included in the PCR primers (underlined). The primers for the sLTB gene were msLTB-F (5′-GGGGTCGACGCCACCATGGTGAAGGTGAAGTGC-3′) and msLTB-R (5′-GGGGGTACCTCATAGCTCATCTTTCTCAGAGTA-3′), and the primers for the wLTB gene were mwLTB-F (5′-GGGGTCGACATGAATAAAGTAAAATGT-3′) and mwLTB-R (5′-GGGGGTACCTAGTTTTCCATACTGAT-3′). These plant expression vectors contain the marker gene (*hpt*) resistant for hygromycin B and an endosperm-specific Bx17 HMW glutenin promoter which is fused to the first intron of the rice actin1 gene [10]. The plant expression vector harbouring the sLTB gene was designated as pMYN-sLTB, whereas the other harbouring the wLTB gene was designated as pMYN-wLTB (Fig. 1).

Plant Transformation

Mature seeds of rice (*Oryza sativa* L.) were de-hulled and then surface-sterilized for 20 min in 20% (v/v) commercial bleach (5.25% sodium hypochlorite). After washing with sterile water to remove any residual bleach, the seeds were positioned on N6-based callus induction media [29] containing 2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid). The scutellum was separated from the shoots and roots after 9 days of incubation under dim lighting at 25°C and transferred to fresh N6CO medium [30]. Rice calli were transformed with pMYN-sLTB and pMYN-wLTB plasmids by the particle bombardment mediated transformation method [31]. The plants were recovered via *in vitro* tissue culture via hygromycin-B selection, in accordance with the protocols established by Cho *et al.* [32]. Juvenile plants that selected positively on the medium containing hygromycin B were transferred to soil and grown to maturity.

Detection of the LTB Gene in Transformed Plants

The total genomic DNA was isolated from the leaf tissues

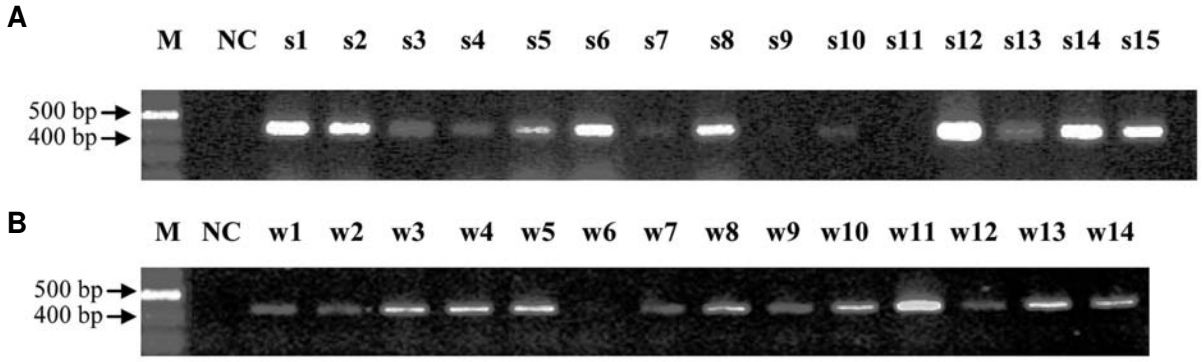

Fig. 2. Detection of sLTB and wLTB gene in transformed rice plants. Genomic DNA was isolated from the leaf tissues of non-transformed and transformed rice and used as templates for genomic DNA PCR amplification. (A) s1-15, PCR products with the genomic DNA from putatively transformed rice plants with sLTB gene as template. (B) w1-14, the PCR products from putative transformed rice plants with wLTB gene. M, 1 kb plus 100 bp ladder marker (ELPIS BIOTECH, Korea). NC, PCR products with genomic DNA from non-transformed rice plant as template.

of putative transformed plants using the methods developed by Kang and Fawley [33]. Genomic DNA (200 ng) was used as template in PCR to detect the LTB gene and the conditions used were as follows: 1 cycle of denaturation at 94°C for 5 min, followed by 36 cycles of 95°C for 1 min, annealing at 62°C for 30 sec and extension at 72°C for 1 min, and a final 10 min extension cycle at 72°C. The primers for the sLTB gene were msLTB-F and msLTB-R, and the primers for the wLTB gene were mwLTB-F and mwLTB-R. The PCR products were analyzed on 1% agarose gel.

Reverse Transcription PCR

Total RNA was extracted from transformed rice seeds showing positive signal in genomic DNA PCR amplification via the methods established by Li and Trick [34]. The first strand of complementary DNA was synthesized using the SuperScript Choice System for cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The forward primer specific for 5′ UTR of LTB gene and the reverse primer used in genomic DNA PCR amplification were utilized to amplify LTB transcripts from cDNA. The amplified PCR products were analysed on 1% agarose gel.

Western Blot Analysis

Total soluble proteins (TSP) were extracted from mature transformed rice seeds, roots and leaves. Approximately 100 mg of pulverized material was mixed with 500 μL of extraction buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol). The samples were incubated at 50°C for 5 min and then centrifuged for 10 min at 10,000 × g. The protein extracts were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Promega, Madison, WI, USA) in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol) using a mini-transblot apparatus (Bio-Rad Inc., Hercules, CA, USA) at 130 mA for 2 h. The membranes were incubated with anti-LTB antiserum developed in rabbits in accordance with the method described in [25]. The membrane was incubated with anti-rabbit IgG conjugated with alkaline phosphatase as secondary antibody (Promega S3731). The membrane was developed using BCIP/NBT (USB, Cleveland, OH, USA) in TMN buffer.

Quantification of LTB Protein Level in Transformed Rice Seeds

The expression level of LTB protein in transformed rice seeds were determined via indirect ELISA in accordance with the methods established previously by Kang *et al.* [20]. The wells of plates were coated with protein extracts from the seeds of the non-transformed and transformed plants, and were then incubated overnight at 4°C. The plates were washed three times with PBS buffer containing 0.1% Tween 20 (PBST), followed by blocking with 3% (w/v) bovine serum albumin (BSA) for 2 h and washing with PBST. The wells were incubated in PBS buffer containing rabbit anti-LTB serum (Immunology Consultant Lab, OR, USA) diluted to 1:5,000 for 2 h at 37°C. After washing, the plates were added with goat anti-rabbit IgG-HRP conjugate (Promega G-7641) diluted to 1:7,000 in PBST at 37°C for 2 h. After a final washing with PBST, the plates were developed with TMB substrate (PharMingen 2606 and 2607KC, USA) in a buffer (pH 9.8) containing 10% (v/v) diethanolamine, 10% (w/v) $MgCl_2$, and 0.02% (w/v) sodium azide for 30 min at room temperature in darkness. The expression levels of LTB protein were quantified via comparisons with known amounts of bacterially-expressed LTB protein.

G_{M1}-Ganglioside Binding Assay

G_{M1}-ELISA was conducted to determine the affinity of the plant-produced LTB proteins for G_{M1}-ganglioside receptor [35]. The wells of plates were coated with 100 μL of mo-

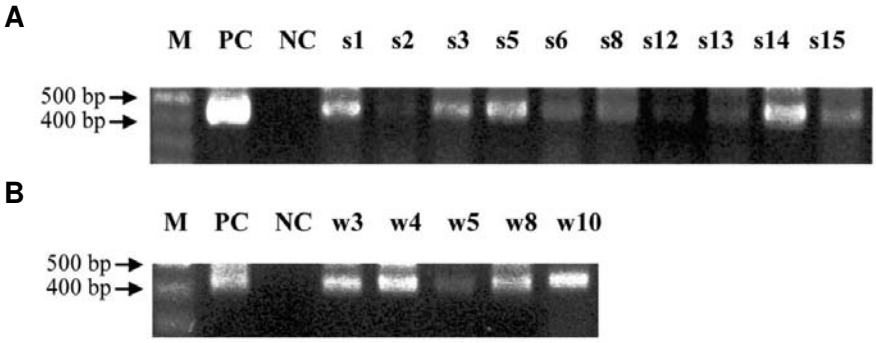

Fig. 3. The detection of sLTB and wLTB mRNA expression using the RT-PCR. Total RNA was isolated from the seed tissues of transformed and non-transformed rice and used as templates for RT-PCR. (A) s1-3, 5, 6, 8, and 12~15 showed RT-PCR products with total RNA from the seed tissues of transformed plants with sLTB. (B) w3-5, 8, and 10 showed RT-PCR products with total RNA from the seed tissues of transformed plants with wLTB gene. M, 1 kb plus 100 bp ladder marker (ELPIS BIOTECH). NC, RT-PCR product with total RNA from non-transformed plant seed. PC, PCR product from plasmid containing target gene (without intron) as template.

nosialganglioside-GM1 (0.3 μg/mL) (Sigma G-7641, St. Louis, MO, USA). After washing, the wells were blocked with 300 μL of blocking buffer containing BSA and then incubated for 2 h at 37°C. The remaining procedures were conducted in accordance with the previously described methods in indirect ELISA.

RESULTS

Analysis of Transformed Rice Lines

Twenty-nine plants out of about 1,000 explants were obtained on the medium containing hygromycin-B. Fifteen out of 29 putative transformed plants were derived from bombardment with pMYN-sLTB and 14 were the result of bombardment with pMYN-wLTB. The results of genomic DNA PCR amplifications indicated that PCR products of the expected size (approximately 400 bp) were detected on agarose gel in 13 out of 15 transformed plants with sLTB gene and 13 out of 14 transformed with wLTB gene (Fig. 2). No bands were amplified in non-transformed plants as negative control. The 12 sLTB- and 8 wLTB-harboring transformed plants were fertile and generated seeds.

RT-PCR of Transformed Rice

Total RNA from different tissues of the non-transformed and transformed rice plants were isolated to demonstrate the transcription of the LTB gene. The samples were subjected to RT-PCR with primers specific for 5′ UTR and LTB gene. No bands were detected in the RNA samples extracted from the leaves or roots (data not shown) indicating that the endosperm-specific wheat HMW glutenin promoter was not functional in leaf or root. However, PCR products were observed in the RNA samples purified from the seed tissues of transformed rice plants (Fig. 3). The amplified DNA fragment was approximately 450 bp in length confirming that splicing had been correctly executed during the post-transcriptional modification process. The expression of LTB gene was detected in 10 independent transformed plants with pMYN-sLTB and in 5 transformed plants with pMYN-wLTB (Fig. 3).

Western Blot Analysis and ELISA

Based on the results of RT-PCR, 5 plants (lines s1, s3, s5, s14, s15) from the transformed plants with pMYN-sLTB and 4 plants (lines w3, w4, w8, w10) from the transformed plants with pMYN-wLTB were selected to determine the LTB protein production. The productions of recombinant LTB in protein extracts from leaf, root, and seed tissues of each transformed rice plants were confirmed by Western blot analysis with anti-LTB antiserum after electrophoresis in 12% PAGE. As expected from the results of RT-PCR, no LTB proteins were detected in the leaf and root protein extracts (data not shown). The LTB band with molecular weight of approximately 55 kDa was detected in protein extracts from the seed tissues (Figs. 4A and 4B), which is slightly less than the expected that of the LTB pentameric protein form [35].

Two independent lines from both the sLTB and wLTB gene-transformed rice plants (sample s5, s14 and w8, w10, respectively) were selected to determine the expression level of LTB protein in the transformed seed tissues. The relative quantity of expressed LTB protein was compared with the total soluble proteins of the seed tissue in three replicates. The expression level of LTB protein equalled 2.7% (s5) and 2.4% (s14) of TSP in the seed tissues of transformed rice plants with sLTB gene, and equalled 0.3% (w8) and 0.5% (w10) of total soluble protein (TSP) in the seed tissues of transformed rice plants with wLTB gene (Fig. 5A).

Binding Assay of the Plant-produced LTB Protein to G_{M1} Ganglioside

G_{M1}-ELISA was conducted to determine the biological

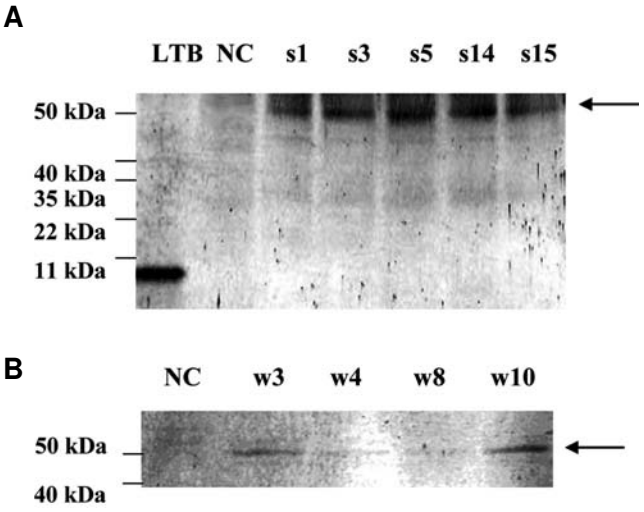

Fig. 4. Western blot analysis of LTB protein expressed in the seed tissues of transformed plants. Fifteen μg of total soluble proteins extracted from transformed seed tissues was separated on SDS-PAGE. (A) s1, 3, 5, 14, and 15 are protein extracts from transformed seed tissues with sLTB. (B) w3, 4, 8, and 10 are protein extracts from transformed seed tissues with wLTB without boiling prior to electrophoresis. LTB, purified bacterially-expressed LTB protein as a positive control. NC, protein extracts from non-transformed seeds as a negative control.

function of the LTB protein produced in transformed rice, such as its binding to the G_{M1}-ganglioside receptor. G_{M1} binding capacity of LTB protein was detected both in the transformed plant with sLTB and wLTB. Unboiled protein extracts showed the binding activity but boiled samples did not show the binding activity indicating that the plant-produced LTB were properly assembled into pentameric structures (Fig. 5B). The binding activity in the transformed plants with sLTB was higher than that of transformed with wLTB due to the high expression level of LTB.

DISCUSSION

The expression of recombinant LTB has been reported in various plants with different expression levels [19-26]. In this study, the highest level of LTB protein accumulation was 2.7% of total soluble proteins (TSP) in the T1 seed tissues of transformed plants with plant-optimized codon synthetic LTB fused with Kozak sequence and ER retention signal compared to that in transformed plants with bacterial LTB (0.3~0.5% of TSP). This result is consistent with previously published results that the plant codon-optimized LTB and the ER retention signal, SEKDEL accumulated at a higher level in transformed tobacco compared to unmodified gene [27]. The Kozak sequence (GCCACC) should also have contributed to these elevated expression levels, as was demonstrated earlier [28]. Although it is difficult to compare

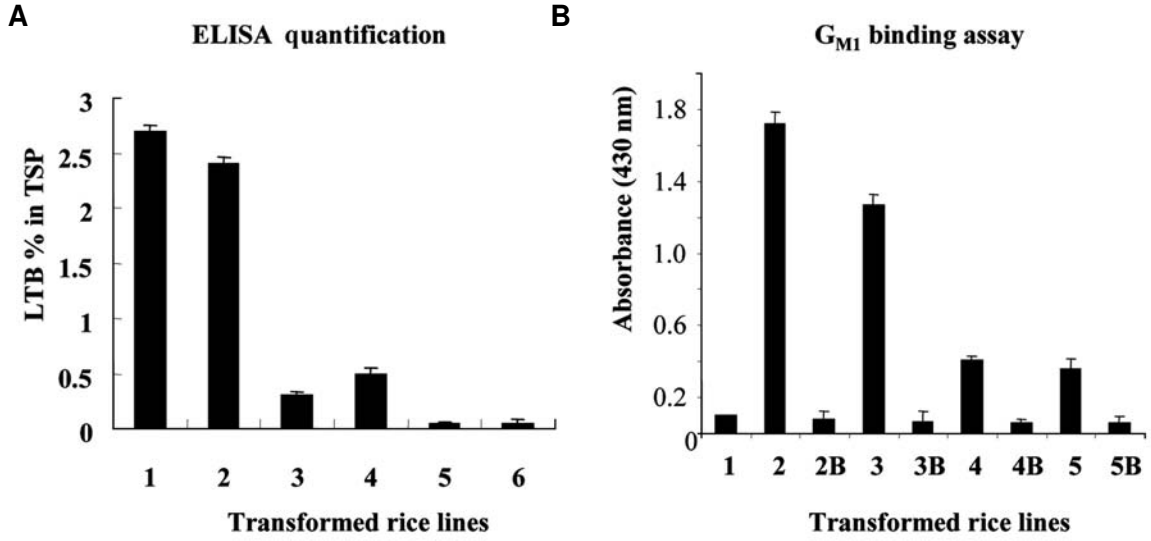

Fig. 5. The quantification (A) and biological activity (B) of LTB expressed in transformed plants. (A) The LTB expression level in the mature transformed rice seeds (T1) (% of total soluble protein, TSP) was determined via ELISA in triplicate. 1 and 2, s5 and s14 of transformed rice seeds with sLTB gene; 3 and 4, w8 and w10 of transformed rice seeds with wLTB gene. 5, leaf tissue of transformed plant s5; 7, root tissue of transformed plant s5. Error bars represent standard deviation of the mean. (B) G_{M1} binding assay of sLTB and wLTB proteins. The G_{M1}-ELISA was conducted with coating the plates with G_{M1} ganglioside as receptor molecules. 1, non transformed rice seeds; 2 and 3, s5 and s14 of transformed rice seeds with sLTB gene without boiling; 2B and 3B, s5 and s14 of transformed rice seeds with sLTB gene after boiling; 4 and 5, w8 and w10 of transformed rice seeds with wLTB gene without boiling; 4B and 5B, w8 and w10 of transformed rice seeds with wLTB gene after boiling. Error bars represent standard deviation of the mean.

the exact amount of target proteins observed in different laboratories, the production of LTB in the transgenic potato tubers at the level of 0.01% of TSP was sufficient to elicit both systemic and mucosal immune responses in mice [18]. Recently, it was reported that LTB derived from transgenic maize was immunogenic at nanogram levels when orally administered to mice [36]. Thus, it is expected that the expression level of LTB in the seed tissues of transformed rice plant at 2.7% of TSP (in a range of 1.2~1.35 mg/g) is enough to stimulate significant immune responses in mice and other animals.

Cholera toxin B subunit expressed in transgenic rice elicited CTB-specific serum IgG and mucosal IgA antibodies with neutralizing activity when mucosally fed [37]. The LTB binds to a broader receptor population on mammalian cells than CTB, which binds only to ceramide-galactose sugar receptor molecules such as G_{M1} ganglioside [38]. It is expected that LTB expressed in transgenic rice is able to show a higher capacity to function as a potent mucosal adjuvant than CTB when mucosally administered.

The expression of the target gene was under the control of the endosperm-specific promoter of the wheat Bx17 HMW glutenin gene and this promoter was fused to the first intron of rice actin1 gene [10]. The promoters for the Bx type HMW glutenin subunit, which is identified in the wheat genome, showed the strongest endosperm specific promoter activity and the addition of the first intron of the rice actin1 gene exerted a positive effect on protein expression levels [11]. The root, leaf, and seed tissues of transgenic rice were analyzed for the expression of LTB on the RNA and protein level. The LTB transcripts and protein were observed only in the seed tissues (data not shown). In addition, the ELISA confirmed the expression of LTB only in seed tissues of transgenic rice plants (Fig. 5A). The primers for RT-PCR were designed to determine whether splicing had been correctly executed during post-transcriptional modification; one is specific for the 5′ untranslated region (UTR) and another is specific for the LTB gene. The PCR products with appropriate length (450 bp) instead of 920 bp which is result of failure for splicing of LTB primary transcripts was detected on the agarose gel confirming that mRNA of the target gene had, in fact, gone through the proper maturation procedure.

Twenty-nine regenerated independent putative transformed lines showed the positive signal in PCR amplification and only 20 transgenic plants proved to be fertile and produced seeds. Fifteen out of 20 transgenic plants with PCR-positive signal showed the transcripts of LTB gene in their seed tissues with different levels (Fig. 3). This different LTB gene expression levels depended on different incorporation sites of target gene in the chromosome of different transgenic plants, a phenomenon referred to as the 'position effect' [39,40].

The LTB protein was detected neither in the seed tissues of non-transgenic rice, nor in the leaf and root tissues of transformed rice plants. However, the LTB protein was produced in the seed tissues of transgenic rice. The results of Western blot analysis showed the pentmeric LTB protein without LTB monomer indicating that the LTB proteins were capable of successful assembly in the seed tissues. Although the size of the LTB pentamer expressed in transformed rice was slightly smaller than calculated (55 rather than 58 kDa), the G_{M1}-ganglioside binding assay confirmed that the LTB pentamer was capable of binding to G_{M1} ganglioside indicating that transgenic rice-expressed LTB can function as an antigen.

Plants have been generally recognized as a safe and inexpensive system for the production of pharmaceutical proteins, including vaccines. One of the primary advantages of the production of functional proteins in cereal grains is the stability of protein under long-term storage conditions. Rice is the principal source of energy and protein for developing countries, and transformed rice expressing antigens may play a key role in the future in the production of edible vaccines.

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