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ORIGINAL PAPER

Production of double repeated B subunit of Shiga toxin 2e at high levels in transgenic lettuce plants as vaccine material for porcine edema disease

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Abstract Pig edema disease is a bacterial disease caused by enterohemorrhagic *Escherichia coli*. *E. coli* produces Shiga toxin 2e (Stx2e), which is composed of one A subunit (Stx2eA) and five B subunits (Stx2eB). We previously reported production of Stx2eB in lettuce plants as a potential edible vaccine (Matsui et al. in Biosci Biotechnol Biochem 73:1628–1634, 2009). However, the accumulation level was very low, and it was necessary to improve expression of Stx2eB for potential use of this plant-based vaccine. Therefore, in this study, we optimized the *Stx2eB* expression cassette and found that a double repeated Stx2eB (2× Stx2eB) accumulates to higher levels than a single Stx2eB in cultured tobacco cells. Furthermore, a linker peptide between the two Stx2eB moieties played an important role in maximizing the effects of the double repeat. Finally, we generated transgenic lettuce plants expressing 2× Stx2eB with a suitable linker peptide that accumulate as much as 80 mg per 100 g fresh weight, a level that will allow us to use these transgenic lettuce plants practically to generate vaccine material.

Keywords Pig edema disease · Edible vaccine · Lettuce · Shiga toxin 2e B

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Introduction

Pig edema disease is caused by enterohemorrhagic *Escherichia coli* (STEC), mainly serotypes O138, O139, and O141, which produce Shiga toxin 2e (Stx2e) (Makino et al. 2001). The predominant symptoms include edema, diarrhea, hemorrhagic colitis, and neurological symptoms. Edema disease occurs mainly in baby pigs, 1–2 weeks after weaning,

and lethality is as high as 50–90%. Stx2e is composed of one A subunit (Stx2eA), which has *N*-glycosidase activity, and five B subunits (Stx2eB), which play an important role in internalization of the toxin into gut cells (Fraser et al. 2006). Stx2e binds to glycosphingolipid receptor on gut cells via Stx2eB and is subsequently endocytosed and transported in a retrograde manner through the Golgi apparatus to the endoplasmic reticulum (ER), where toxic Stx2eA is released into the cytosol. Stx2eA removes a specific adenine base from the 28S ribosomal RNA (rRNA) of the 60S ribosomal subunit and causes toxicity.

Today, we treat edema disease mainly by administering antibiotics such as ampicillin. However, this treatment is associated with higher risk of drug-resistant *E. coli* strains, and antibiotics can stimulate toxin release from *E. coli* cells. Because of these complications with antibiotic therapy, a vaccine against edema disease is considered the most effective means to control this disease. Among various vaccine strategies, a mucosal vaccine that elicits immune responses in the small intestine, which is the primary site of STEC infection, would be particularly efficacious. The nontoxic Stx2eB is a candidate mucosal vaccine protein because the B subunits of cholera toxin (CTB) and heat-labile toxin (LTB), which belong to the AB5 toxin family along with Stx2e, are highly immunogenic and effective vaccine proteins (Sanchez and Holmgren 1989; Williams et al. 1997). Furthermore, we found that clinical symptoms in baby pigs that were challenged with STEC cells improved upon oral administration of a purified Stx2eB-GST fusion protein (Kawamoto et al. 2009). Administering at least 4 mg Stx2eB-GST three times per head was sufficient to prevent edema disease.

Currently, rapid progress is being made in the field of plant-made pharmaceuticals (PMPs). The advantages of plants include lower production costs compared with microorganisms or animals and reduced risk of contamination with animal viruses (Daniell et al. 2001; Yoshida et al. 2004). When a recombinant vaccine protein is produced in *E. coli*, the antigen must be purified to prevent induction of unintended immune responses. However, in principle, mucosal vaccine antigens can be produced in dietary plants without cumbersome and expensive purification steps. Lettuce is an edible and suitable host plant for an edible vaccine. In addition, lettuce can be cultivated in a plant factory setting that uses a closed system to grow plants under tightly controlled conditions. We previously reported production of Stx2eB for an edible vaccine using lettuce (*Lactuca sativa*) as host plant (Matsui et al. 2009). In this system, the *Stx2eB* expression cassette contained the 5′-untranslated region (UTR) of *Nicotiana tabacum alcohol dehydrogenase* gene (*NtADH* 5′-UTR), which functions as a translational enhancer (Satoh et al. 2004), and a sequence encoding an ER localization signal. However, this transgenic lettuce system yielded suboptimal expression, and Stx2eB accumulated to less than 100 ng per 100 g fresh weight (FW), which was too low to administer to pigs as a vaccine. Therefore, it is necessary to further optimize the quantity and/or quality of the vaccine protein to use transgenic lettuce for practical applications.

In general, the immunogenicity of an antigen is increased by conjugating the antigen to a carrier protein. For example, the nontoxic CTB and the closely related LTB are often used as mucosal carriers/adjuvant molecules, and antigens are conjugated chemically or genetically to CTB and LTB to induce oral tolerance (Bublin et al. 2007; O'Dowd et al. 1999; Yu and Langridge 2001). Arranging the antigen in a tandem repeat is another strategy that can increase immunogenicity (Jin et al. 2008; Oscherwitz et al. 1999; Rubio et al. 2009; Yankai et al. 2006). In the case of a candidate vaccine peptide that was designed to induce antitumor antibodies against the β-subunit of human chorionic gonadotropin (β-hCG), one copy of the peptide fused to HSP65 induced only very low-titer anti-β-hCG antibodies. However, administering ten copies of this peptide fused to HSP65 induced much higher levels of anti-β-hCG antibodies that had significant antitumor effects in mice (Yankai et al. 2006).

In this study, we further optimized the *Stx2eB* expression cassette to obtain transgenic lettuce plants that accumulated greater amounts of Stx2eB with higher immunogenicity. To evaluate different expression cassettes rapidly, we used two experimental systems: a transient gene expression system in lettuce protoplasts and a stable gene expression system in cultured tobacco cells. We constructed genes that encoded tandem repeated Stx2eB (single, double, triple, and quadruple *Stx2eB*) to improve the immunogenicity of Stx2eB and then expressed these various genes in plant cells. Although we have not

yet completed the immunogenicity studies, we found that the double Stx2eB accumulated to higher levels than the single Stx2eB. Placing a spacer peptide between the Stx2eB proteins was important to maximize the effects of the tandem arrangement. We also found that the Stx2eB expression level slightly increased with GC-rich codons. With these modifications, we generated stable transgenic lettuce plants using a binary vector containing a codon-modified gene that encoded a double Stx2eB with a suitable linker peptide. These transgenic lettuce plants accumulated as much as 2 mg Stx2eB per 100 g FW of plants. Furthermore, we optimized the expression cassette of *Stx2eB*. Transcriptional terminator derived from *Arabidopsis thaliana heat shock protein 18.2* (HSP-T) was incorporated into the cassette, and nucleotides immediately upstream of the initiating AUG (positions −3 to −1) were changed for efficient translation initiation. Finally, transgenic lettuce plants generated using this optimized cassette accumulated as much as 80 mg Stx2eB per 100 g FW of plants.

Materials and methods

Expression plasmids for the ER-targeted tandem repeat Stx2eB

Sequences of primers used for construction of plasmids are listed in supplementary Table S1, and a diagram of plasmids construction is shown in supplementary Fig. S1. A hemagglutinin (HA) tag (YPYDYPDYA) was fused at the C-terminus of Stx2eB to detect the protein. To add the HA tag to the C-terminus of Stx2eB, oligonucleotides HA-F and HA-R were annealed and phosphorylated with T4 polynucleotide kinase (PNK) (New England Biolabs, Hitchin, UK). The resulting phosphorylated HA fragments were inserted into the *Bgl*II site of plasmid 14 (Matsui et al. 2009) to generate the ER-targeted Stx2eB expression vector. Hereinafter, we refer to this ER-targeted Stx2eB plasmid as plasmid 20 to facilitate our descriptions of the constructs described below.

To add a PG12 spacer (Arg–Ser–Pro–Gly–Ser–Gly–Pro–Gly–Ser–Pro–Arg–Ser) between Stx2eB and the HA tag, oligonucleotides PG12-F and PG12-R were annealed and phosphorylated with T4 PNK. The resulting phosphorylated PG12 fragments were inserted into the *Bgl*II site of plasmid 20 to generate ER 1× Stx2eB (Fig. 1a).

The *Bam*HI–*Eco*RI fragments for Stx2eB–PG12–HA–HDEL–NOST were obtained from ER 1× Stx2eB and inserted into the *Bgl*II–*Eco*RI sites of ER 1× Stx2eB to generate ER 2× Stx2eB (Fig. 1a). The *Bam*HI–*Eco*RI fragments for Stx2eB–PG12–Stx2eB–PG12–HA–HDEL–NOST were obtained from ER 2× Stx2eB and inserted into the *Bgl*II–*Eco*RI sites of ER 1× Stx2eB to generate ER 3× Stx2eB (Fig. 1a). The *Bam*HI–*Eco*RI fragments for Stx2eB–PG12–Stx2eB–PG12–HA–HDEL–NOST were obtained from ER 2× Stx2eB and inserted into the *Bgl*II–*Eco*RI sites of ER 2× Stx2eB to generate ER 4× Stx2eB (Fig. 1a).

To express Stx2eB stably in transformed plant cells, each gene was subcloned into pBI121 (Clontech, Palo Alto, CA) using *Xba*I and *Sac*I under a cauliflower mosaic virus 35S RNA promoter (p35S) and a nopaline synthase gene transcription terminator (NOS-T).

Expression plasmids for ER-targeted 2× Stx2eB linked with various spacers

Several different spacers were inserted using the following procedures. ER 2× Stx2eB (PG12) is the same as ER 2× Stx2eB mentioned above (Figs. 1a, 2a). The *Bam*HI–*Eco*RI fragments of Stx2eB–PG12–HA–HDEL–NOST were obtained from ER 1× Stx2eB and inserted into the *Bgl*II–*Eco*RI sites of plasmid 14 (Matsui et al. 2009) to generate ER 2× Stx2eB (RS) (Fig. 2a).

To generate DNA fragments for the PG7 spacer (Arg–Ser–Pro–Gly–Ser–Arg–Ser), oligonucleotides PG7-F and PG7-R were annealed and phosphorylated with T4 PNK. The resulting phosphorylated PG7 fragments were inserted into the *Bgl*II site of plasmid 20 and ER 1× Stx2eB to generate ER 1× Stx2eB (PG7) and ER 1× Stx2eB (PG17), respectively (Fig. 2a). The phosphorylated PG12 fragments were inserted into the *Bgl*II site of ER 1× Stx2eB to generate ER 1× Stx2eB (PG22) (Fig. 2a). To generate DNA fragments for the SG12 spacer (Arg–Ser–Gly–Ser–Gly–Ser–Gly–Ser–Gly–Ser–Arg–Ser), oligonucleotides SG12-F and SG12-R were annealed and phosphorylated with T4 PNK. The resulting phosphorylated SG12 fragments were inserted into

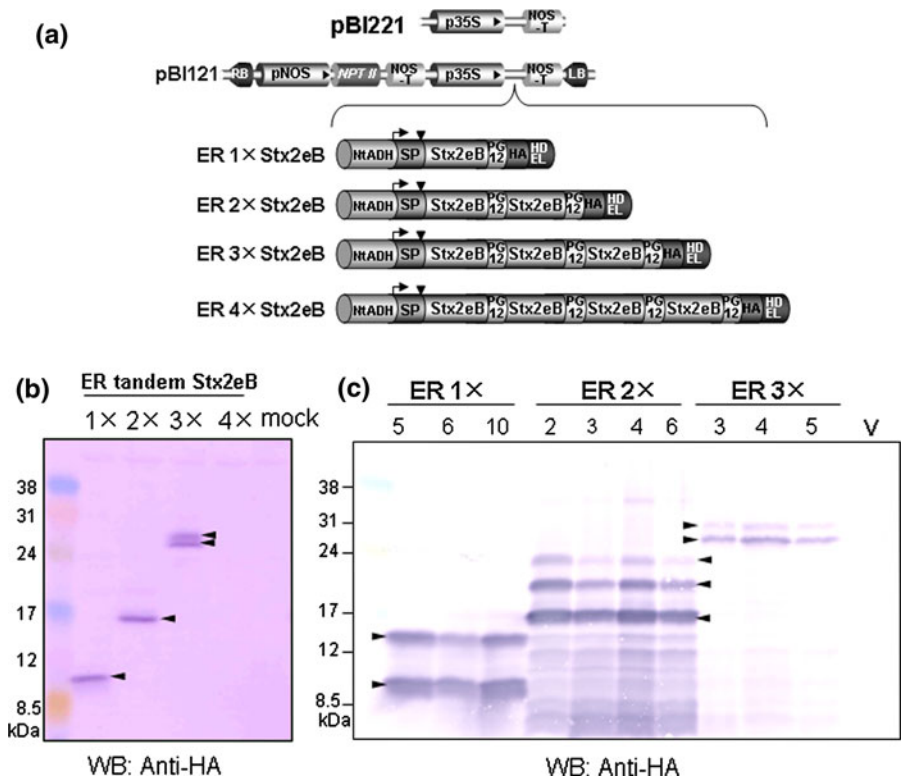

Fig. 1 Expression of tandem-repeated Stx2eB. **a** Schematic representation of the tandem-repeated Stx2eB expression constructs. pBI221 and pBI121 were used for transient expression and to generate stable transformants, respectively. Single (1×), double (2×), triple (3×), and quadruple (4×) Stx2eB were constructed. PG12, linker peptide (Arg–Ser–Pro–Gly–Ser–Gly-Pro–Gly–Ser–Pro–Arg–Ser). p35S, Cauliflower mosaic virus 35S RNA promoter; NOS-T, transcription terminator from *A. tumefaciens nopaline synthase* gene; ADH, 5′-untranslated region (UTR) of tobacco (*Nicotiana tabacum*) *alcohol dehydrogenase* gene; SP, signal peptide for ER translocation derived from tobacco *β-D-glucan exohydrolase*; HA, HA peptide tag to detect the Stx2eB protein (Tyr–Pro–Tyr–Asp–Tyr–Pro–Asp–Tyr–Ala); HDEL, ER retention signal (Ser–Glu–His–Asp–Glu–Leu). An *arrow indicates* the translation start site, and a *triangle* indicates the putative cleavage site after translation. **b** Transient expression in lettuce protoplasts. Mock, protoplasts transfected with unrelated *GUS* plasmids were analyzed in a similar manner. **c** Western blot analysis of transgenic BY2 cells. V, as a negative control, a protein sample of transgenic BY2 cells generated using *GUS* vector was incorporated. For Western blotting (**b**, **c**), an anti-HA antibody was used as the primary antibody. The *number* adjacent to each *lane* represents the clone number. *Arrowheads* in **b** and **c** indicate signals

the *Bgl*II site of plasmid 20 to generate ER 1× Stx2eB (SG12) (Fig. 2a).

The *Bam*HI–*Eco*RI fragments of Stx2eB–PG12–HA–HDEL–NOST were obtained from ER 1× Stx2eB, and inserted into the *Bgl*II–*Eco*RI sites of ER 1× Stx2eB (PG7), ER 1× Stx2eB (PG17), ER 1× Stx2eB (PG22), and ER 1× Stx2eB (SG12) to generate ER 2× Stx2eB (PG7), ER 2× Stx2eB (PG17), ER 2× Stx2eB (PG22), and ER 2× Stx2eB (SG12), respectively. Each gene was subcloned into pBI121 using *Xba*I and *Sac*I under a p35S with a NOS-T.

Expression plasmids for the reporter-fused Stx2eB, organelle markers, and effectors

The DNA fragment encoding yellow fluorescent protein (YFP) was polymerase chain reaction (PCR)-amplified using pEYFP (Clontech) as a template and primers YFP-F and YFP-R. The resulting DNA fragment was digested with *Bam*HI and *Bgl*II, and inserted into the *Bam*HI–*Bgl*II sites of plasmid 20 (ER YFP, Fig. 3a). The DNA fragment encoding monomeric red fluorescent protein (mRFP) (Campbell et al. 2002) was PCR-amplified using A60-mRFP (Ono et al.

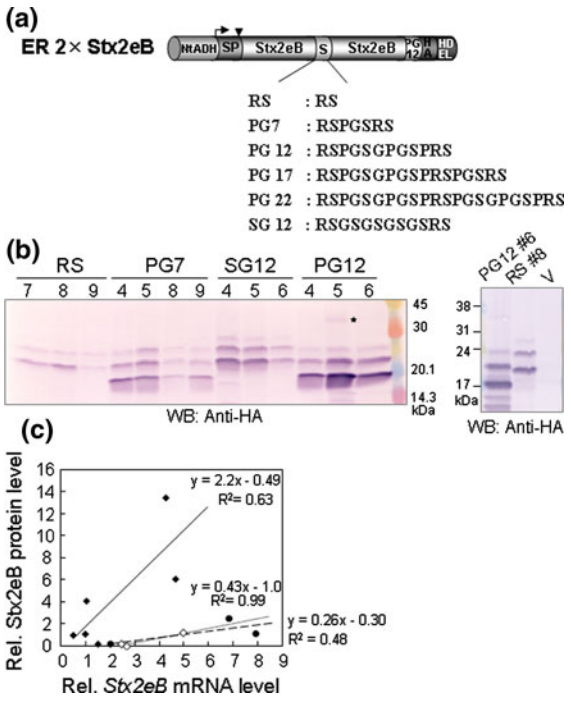

Fig. 2 Effects of a spacer peptide on accumulation of ER 2× Stx2eB. **a** Schematic representation of the constructs. The listed spacers were inserted between two Stx2eB moieties. Three or four transgenic BY2 clones for each construct were randomly picked and analyzed by Western blotting using an anti-HA antibody (**b**). Cells grown on solidified Murashige and Skoog (MS) medium were mixed with sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) sample buffer at a ratio of 100 μL sample buffer to 100 mg fresh weight of cells, followed by thermal denaturation at 95°C for 15 min. After centrifugation, 15 μL supernatant was loaded in PAGE gel. *Asterisks* indicate signals corresponding to higher-molecular-weight Stx2eB signals. The *number* adjacent to each *lane* represents the clone number. V, as a negative control, a protein sample of transgenic BY2 cells generated using *GUS* vector was incorporated. **c** Quantification of messenger RNA (mRNA) and protein accumulation levels. We serially diluted extracts of ER 2× Stx2eB (PG12) #4, analyzed them by Western blotting, and compared them with dilutions of other samples. The relative accumulation levels were calculated from the signal intensities on the Western blots as indicated in supplementary Fig. S2. Values represent mean values of three replicate measurements of Stx2eB proteins and those of *Stx2eB* mRNA in an extract. *Solid circles*, *solid diamonds*, and *open diamonds* represent ER 1× Stx2eB, ER 2× Stx2eB (PG12), and ER 2× Stx2eB (RS), respectively. Best fit lines for ER 1× Stx2eB, ER 2× Stx2eB (PG12), and ER 2× Stx2eB (RS) are drawn as a *long-dashed line*, *continuous line*, and *dotted line*, respectively

2006) as a template and primers mRFP-F and mRFP-R. The resulting DNA fragment was digested with *Bam*HI and *Bgl*II, and inserted into the *Bam*HI–*Bgl*II sites of plasmid 20 (ER mRFP, supplementary Fig. S9).

Markers for the *medial*-Golgi were constructed as follows. Oligonucleotides HA-F and HA-R were annealed and phosphorylated with T4 PNK. The resulting phosphorylated HA fragments were inserted into the *Bgl*II site of plasmid 13 (Matsui et al. 2009) to generate a vector for Apo-type Stx2eB containing an HA tag at the C-terminus (plasmid 22). The *Bam*HI–*Bgl*II fragments for YFP and mRFP were inserted into the *Bam*HI–*Bgl*II sites of plasmid 22 to generate Apo-YFP and Apo-mRFP, respectively. The DNA fragments encoding the cytosolic and transmembrane domains of *Arabidopsis thaliana* β1, 2-xylosyltransferase, which are *medial*-Golgi localization signals (Saint-Jore-Dupas et al. 2006), were PCR-amplified using XylT-F and XylT-R. These XylT fragments were digested with *Xba*I and *Bam*HI and inserted into the *Xba*I–*Bam*HI sites of Apo-YFP and Apo-mRFP to generate XylT-YFP and XylT-mRFP, respectively (supplementary Fig. S9).

The *Bam*HI–*Eco*RI fragments for YFP–HA–HDEL–NOST were obtained from ER YFP and inserted into the *Bgl*II–*Eco*RI sites of ER 1× Stx2eB, ER 2× Stx2eB(PG12), and ER 2× Stx2eB (RS) to generate ER 1× Stx2eB-YFP, ER 2× Stx2eB (PG12)-YFP, and ER 2× Stx2eB (RS)YFP, respectively (Fig. 3a).

The DNA fragments encoding *A. thaliana* ARF1, which plays important roles in ER-to-Golgi protein trafficking (Takeuchi et al. 2002), were PCR-amplified using primers ARF1-F and ARF1-R. The resulting DNA fragment was subcloned into the *Eco*RV site in pBluescript (Stratagene, Cedar Creek, TX). Another PCR was performed using an ARFQL-F primer and an ARFQL-R primer to substitute a Glu residue at position 71 with a Leu residue. Each resulting ARF1 fragment was subcloned into the transient expression vector pBI221 (supplementary Fig. S9).

Expression plasmids for codon-modified double Stx2eB

The codon-modified Stx2eB gene (mStx2eB5) (supplementary Fig. S10) was constructed as follows. Two oligo nucleotides were annealed at their 3′ complementary termini and then subjected to a polymerase reaction with T4 DNA polymerase

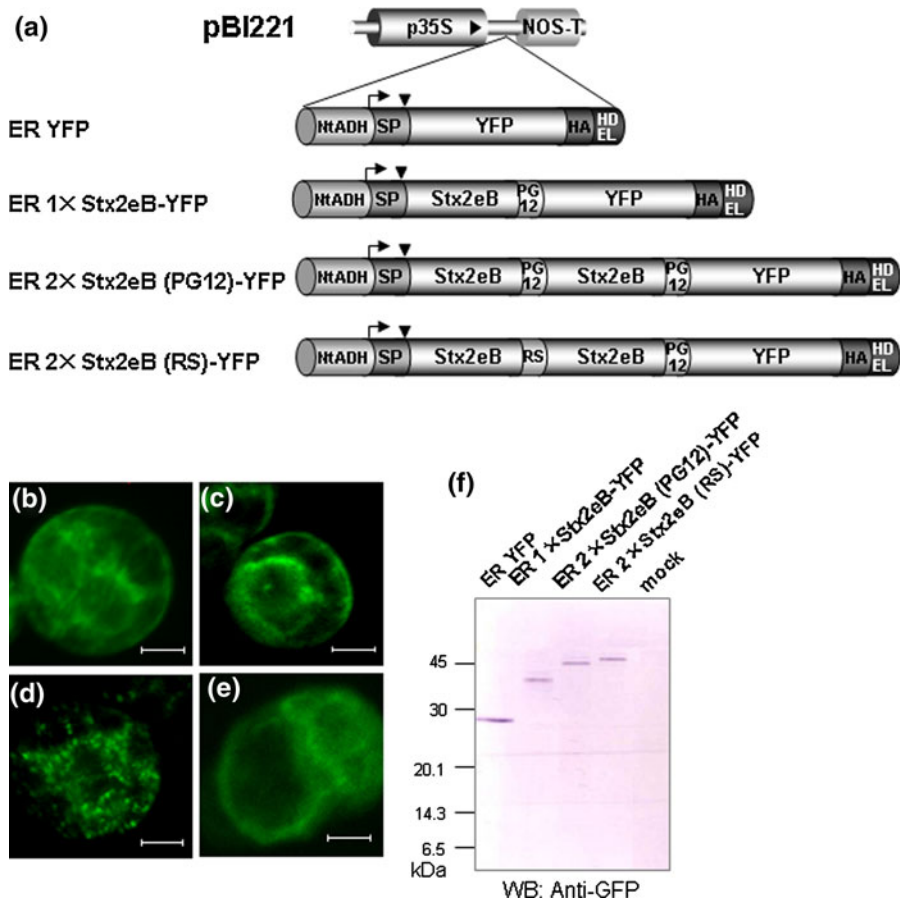

Fig. 3 Expression of YFP-fused Stx2eB in BY2 protoplasts. **a** Schematic representations of expression plasmids for the YFP and mRFP reporters. p35S, Cauliflower mosaic virus 35S RNA promoter; NOS-T, transcription terminator from *A. tumefaciens nopaline synthase* gene; ADH, 5′-untranslated region (UTR) of tobacco (*Nicotiana tabacum*) *alcohol dehydrogenase* gene; SP, signal peptide for ER translocation derived from tobacco *β-D-glucan exohydrolase*; HA, HA peptide tag to detect the Stx2eB protein (Tyr–Pro–Tyr–Asp–Tyr–Pro–Asp–Tyr–Ala); HDEL, ER retention signal (Ser–Glu–His–Asp–Glu–Leu). *Arrows* indicate the translation start sites, and *triangles* indicate the putative cleavage sites after translation. Typical images of BY2 protoplasts with yellow fluorescent protein (YFP) fluorescence are shown. Fluorescence expression patterns corresponding to ER YFP (**b**), ER 1× Stx2eB-YFP (**c**), ER 2× Stx2eB (PG12)-YFP (**d**), and ER 2× Stx2eB (RS)-YFP (**e**). Each image represents one protoplast expressing the GFP protein. Scale bars represent 10 μm. Western blot analysis of protoplasts expressing YFP were performed using an anti-GFP antibody (**f**). Mock, protoplasts transfected with unrelated *GUS* plasmids were analyzed in a similar manner

(Toyobo, Fukui, Japan). The first round of reactions was performed using a combination of oligonucleotides mStx2eB5-A and mStx2eB5-B, oligonucleotides mStx2eB5-C and mStx2eB5-D, and oligonucleotides mStx2eB5-E and mStx2eB5-F to synthesize the A + B, C + D, and E + F gene fragments, respectively. Subsequently, the second reaction was performed using A + B and C + D in combination to synthesize the A + B + C + D gene fragment. Finally, the third round was performed using A + B + C + D and E + F in combination to synthesize the full-length gene of A + B + C + D + E + F. The *Bam*HI–*Bgl*II fragment of mStx2eB5 was inserted into the *Bam*HI–*Bgl*II sites of ER 1× Stx2eB (plasmid 21). The phosphorylated PG12 fragment was inserted into the *Bgl*II site of plasmid 21 (ER 1× mStx2eB5). The *Bam*HI–*Eco*RI fragments for mStx2eB5–PG12–HA–HDEL–NOST were obtained from ER 1× mStx2eB5 and inserted into the *Bgl*II–*Eco*RI sites of ER 1× mStx2eB5 to generate ER 2× mStx2eB5. To express Stx2eB stably in transformed plant cells, DNA fragment for ER 2×

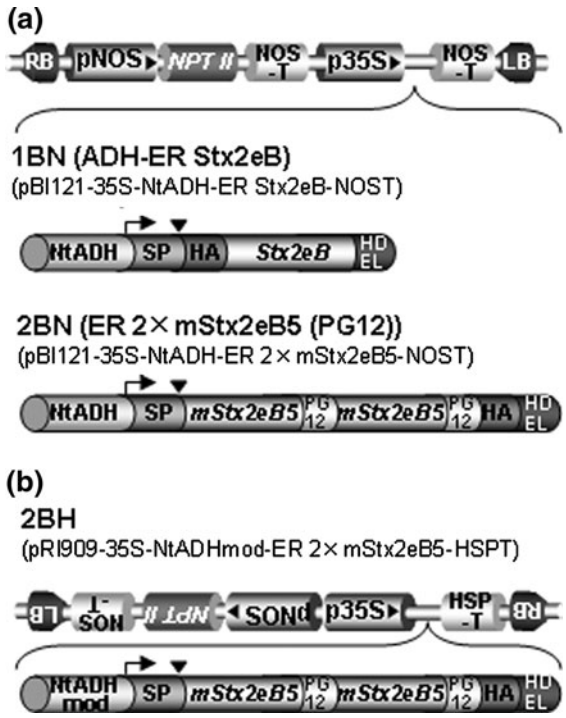

Fig. 4 Schematic representations of plasmids used for the transformation of lettuce plants. Genes for Stx2eB were subcloned into pBI121 (**a**) or pRI909 (**b**). HSP-T, transcription terminator from *Arabidopsis thaliana heat shock protein 18.2* gene; *mStx2eB5*, codon-modified *Stx2eB* gene; NtADHmod, modified *NtADH* 5′-UTR in which three nucleotides immediately upstream of the initiating AUG were changed from UAA to AAG

mStx2eB5 was subcloned into pBI121 using *Xba*I and *Sac*I, and placed between a p35S and a NOS-T (2BN in Fig. 4a).

Plasmid for 2BH (Fig. 4b) was constructed as follows. The DNA fragments for p35S were PCR-amplified using primers 35S-F and 35S-R, and subcloned into pRI909 (TaKaRa Bio Inc., Shiga, Japan) using *Xba*I and *Kpn*I (plasmid 23). The DNA fragments for HSPT (Nagaya et al. 2010) were PCR-amplified using primers HSPT-F and HSPT-R, and inserted into plasmid 23 using *Sac*I and *Eco*RI (plasmid 24). The DNA fragments for modified *NtADH* 5′-UTR (NtADHmod), in which three nucleotides immediately upstream of the initiating AUG were changed from UAA to AAG, were PCR-amplified using primers NtADH-XbaIKpnI-F and NtADHmod-NsiI-R, and inserted into plasmid 1 (Matsui et al. 2009) using *Xba*I and *Nsi*I (plasmid 25). Plasmid 25 was treated with *Nsi*I, and termini were blunted with T4 DNA polymerase, followed by self-ligation so that the initiation codon of *NtADH* corresponded to the initiation codon of the signal peptide (plasmid 26). *Kpn*I–*Bam*HI fragment of NtADHmod obtained from plasmid 26 and *Bam*HI–*Sac*I fragment of 2× mStx2eB5 were together inserted into *Kpn*I–*Sac*I sites of plasmid 24 to generate 2BH (Fig. 4b).

Transformations of lettuce plants and cultured tobacco cells

Stable transformations of lettuce plants (*L. sativa* L. cv. green wave) and cultured tobacco cells (*Nicotiana tabacum* L. cv. BY2) were performed as previously described (Nakayama et al. 2000; Matsui et al. 2009).

Transient expression in lettuce and BY2 protoplasts

Transient expression in lettuce protoplasts and BY2 protoplasts was performed as previously described (Matsui et al. 2009; Satoh et al. 2004).

Western blot analysis

For the analysis of protoplasts, all protoplasts precipitated by centrifugation (9,000×*g*, 1 min at 4°C) were mixed with 30 μL sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer [4% (w/v) SDS, 20% (w/v) glycerol, 0.05% (w/v) bromophenol blue, 300 mM β-mercaptoethanol, 125 mM Tris–HCl, pH 6.8], followed by thermal denaturation at 95°C for 2 min. For analysis of transgenic BY2 cells, cells grown on solidified MS medium were mixed with SDS–PAGE sample buffer at a ratio of 100 μL sample buffer to 100 mg fresh weight of cells, followed by thermal denaturation at 95°C for 15 min. After centrifugation (20,000×*g*, 20 min at 4°C), supernatant was collected and analyzed by Western blotting. In this case, loads were not normalized by measuring soluble proteins. For analysis of transgenic lettuce plants, leaves were ground in liquid nitrogen and added with denaturation extraction buffer (DEB; 6 M urea, 5 mM imidazole, 500 mM sodium chloride, 20 mM Tris–HCl, pH 7.8). After centrifugation (20,000×*g*, 20 min at 4°C), supernatant was collected. Extracted proteins were

quantified by Bradford (1976) analysis, and 0.3 μg total soluble protein (TSP) was loaded onto an SDS–PAGE gel for Western blotting.

Protein samples were applied to 15% acrylamide gel for reducing SDS–PAGE, and proteins were separated. After electrophoresis, gel was incubated in transfer buffer [48 mM Tris–HCl (pH 8.3), 39 mM Glycine, 20% methanol], followed by blotting onto a polyvinylidene fluoride (PVDF) membrane (Hybond-P; Amersham, Piscataway, NJ) using an electrotransfer system (NA-1512, Nihon EIDO, Japan). As primary antibodies, anti-HA antibody (final concentration 50 ng/mL, clone 3F10; Roche, Mannheim, Germany) or anti-green fluorescent protein (GFP) antibody (diluted 1:5,000, code no. 1 814 460; Boehringer Mannheim, Mannheim, Germany) were used. Membranes were incubated with primary antibodies in TBS-T (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature for 2 h, followed by three 10-min washes with TBS-T. Alkaline phosphatase-conjugated secondary antibodies, anti-rat IgG (diluted 1:5,000, code no. S3831; Promega, Madison, WI) or anti-mouse IgG (diluted 1:5,000, code no. S3721; Promega), were treated at room temperature for 2 h, followed by three 10-min washes with TBS-T. For detection of signals, membranes were incubated at 37°C in detection solution, which was prepared by mixing 15 mL AP buffer [100 mM Tris–HCL (pH 9.5), 100 mM KCl, 0.05% Tween20], 100 μL NBT solution [5% (w/v) 4-nitro blur tetrazolium chloride, 70% (v/v) dimethylformamide], and 50 μL BCIP solution [5% (w/v) 5-bromo-4-chloro-3-indolyl-phosphate in dimethylformamide].

Details of quantification of Stx2eB proteins in transgenic BY2 cells by SDS–PAGE and Western blotting are shown in supplementary Fig. S2.

Preparation of standard Stx2eB proteins for quantification by dot-blot

The 6His-BB protein was purified as follows. The DNA fragment encoding 6His-BB was PCR-amplified using ER 2× Stx2eB as a template, a Stx2eB-F primer containing no Stx2eB signal sequence, and a Stx2eB-R primer containing the Stx2eB termination codon. The PCR amplicon was purified from an agarose gel and then cloned using the Champion™ pET151 Directional TOPO® Expression Kit (Invitrogen, Carlsbad, CA). The resulting plasmid was transformed into *E. coli* BL21 (DE) cells following the manufacturer's protocol. 6His-BB expression was induced by incubating the cells in 1 liter PlusGrow medium (Nacalai Tesque, Kyoto, Japan) containing 1 mM ampicillin for 24 h at 25°C with shaking. The *E. coli* cells expressing 6His-BB were harvested by centrifugation (7,000×*g* for 20 min at room temperature) and resuspended in DEB. The cell extracts were subjected to three intermittent sonication cycles (3-min sonication followed by 2-min rest) on ice with the output set to 3 and the duty time set to 60 using a Sonifire 250 (Branson Ultrasonics Corporation, Danbury, CT) and then centrifuged (20,000×*g* for 20 min at 4°C). The extract was subjected to nickel column chromatography using the Novagen® His-bind purification kit (Novagen, Darmstadt, Germany) to obtain pure 6His-BB. The purity of 6His-BB was examined by SDS–PAGE, and the concentration was determined by Bradford assay.

The 6His-BB-HA-HDEL protein was prepared as follows. The DNA fragment containing a ribosome binding site and the original signal peptide of *Stx2eB* was PCR-amplified using primers Stx2eB-SDSP-F and Stx2eB-SP-R. This fragment was digested with *Xba*I and *Bam*HI, and inserted into the *Xba*I–*Bam*HI sites of ER 2× mStx2eB5 (plasmid 27). To generate the DNA fragment encoding the hexa His tag, oligonucleotides His-F and His-R were annealed and phosphorylated with T4 PNK. The resulting phosphorylated His fragment was inserted into the *Bam*HI site of plasmid 27 (plasmid 28). The *Xba*I–*Xho*I fragment was obtained from plasmid 28, and inserted into the *Xba*I–*Xho*I sites of pET-15b (Novagen). Expression of 6His-BB-HA-HDEL was induced using the same method as for 6His-BB. After induction, the cells were collected, and 6His-BB-HA-HDEL was extracted in DEB.

Quantification of Stx2eB protein expression

To quantify 2× Stx2eB protein levels, dot-blot analysis was performed. First, the 6His-BB-HA-HDEL protein was expressed in *E. coli* and quantified using a known amount of purified 6His-BB protein. Second, the 2× Stx2eB protein in lettuce extracts was quantified using the 6His-BB-HA-HDEL protein as standard. Lettuce leaves were obtained from plants sterile-cultured in plant box, immediately frozen in liquid nitrogen, and stored at −80°C until protein extraction. Total protein was extracted from frozen lettuce leaves as previously described (Shultz

et al. 2005). The dried protein precipitate was suspended in DEB, and the supernatant was collected as the total protein fraction after centrifugation. The total protein concentration was adjusted to 0.5 mg/mL. The total protein was serially diluted twofold in DEB, and 1 μL was applied to a nitrocellulose membrane (Trans-Blot transfer medium 0.45 μm; Bio-Rad, Hercules, CA). In addition, serial twofold dilutions of a standard protein purified from *E. coli* expressing tandem Stx2eB (4.50, 2.25, 1.13, 0.563, 0.281, 0.141, 0.0703 ng/μL) were also applied to the membrane to construct a standard curve. The membrane was dried, and incubated in Blocking Solution in TBS (Nacalai Tesque) at room temperature for 1 h. After blocking, the membrane was washed three times with TBS-T (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 5 min at room temperature. The membrane was incubated with an anti-HA High Affinity Rat monoclonal antibody (clone 3F10, Roche) diluted 1:1,000 in TBS-T at room temperature for 2 h and then washed as described above. Next, the membrane was incubated with an anti-Rat IgG (H + L) Alkaline Phosphatase Conjugate (Promega) diluted 1:2,500 in TBS-T at room temperature for 1 h, and then washed. Colorimetric detection was performed by incubating the membrane with the substrate solution (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.33 mg/mL nitro blue tetrazolium, 0.33 mg/mL 5-bromo-4-chloro-3-indolyl phosphate) at room temperature for 7 min. The membrane was scanned with PM-A900 (Seiko Epson, Nagano, Japan) at 600 dpi, and the signal intensities were quantified using the CS Analyzer version 3.0 (ATTO, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was extracted from BY2 cells using the RNeasy® Mini Kit (Qiagen, Huntsville, AL). After treating with DNase, complementary DNA (cDNA) was synthesized using oligo-dT primers with the Transcriptor Reverse Transcriptase Kit (Roche). Quantitative real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). To quantify the *Stx2eB* mRNA levels, primers ADHrealtime-F (5′-gtgatcagtgaaggaaatcaagaaa-3′) and SPrealtime-R (5′-cataacaccacaaaacccatcat-3′) were used. To normalize the *Stx2eB* expression levels in BY2 cells, the mRNA of the tobacco *actin* gene was quantified using primers Actrealtime-F (5′-tggcatcatacattttacaacga-3′) and Actrealtime-R (5′-aggtgcttcagtgagtagtacagg-3′), synthesized based on the aforementioned nucleotide sequences. The relative mRNA level for 1× Stx2eB was further divided by two.

Results and discussion

Expression of tandem-repeated Stx2eB

In general, increasing the molecular weight of an antigen by adding a carrier protein or using a tandem-repeat arrangement increases the immunogenicity of the antigen. In an attempt to improve the immunogenicity of Stx2eB, we constructed genes that encode tandem-repeated Stx2eB proteins as indicated in Fig. 1a. Single (1×), double (2×), triple (3×), and quadruple (4×) Stx2eB were designed so that they were targeted to the endoplasmic reticulum (ER). A linker peptide containing Pro residues, which destabilizes the secondary structure of the peptide and provides flexibility (von Heijne 1991), was inserted between the Stx2eB moieties.

These constructs were transiently expressed in lettuce protoplasts, and the accumulated Stx2eB protein levels were analyzed by Western blotting using an anti-HA antibody to detect the HA tag that was fused to the C-terminus of Stx2eB. Similar signals were detected with 1×, 2×, and 3× Stx2eB, but the signal for quadruple Stx2eB was below the detection limit (Fig. 1b). The molecular weights of the detected bands were approximately 9, 17, and 25 kDa for 1×, 2×, and 3× Stx2eB, respectively.

Genes for tandem-repeated Stx2eB were also expressed in stable cultured tobacco cells (*N. tabacum* L. cv BY2). We found that ER 2× Stx2eB tended to accumulate to higher levels than ER 1× Stx2eB and ER 3× Stx2eB (Fig. 1c). When ER 1× Stx2eB was expressed, two bands were detected. The lower band corresponded to a nonmodified Stx2eB, while the upper band corresponded to Stx2eB containing an *N*-glycan, because the upper band disappeared on treating cellular extracts with glycopeptidase F (GPF), which removes *N*-glycans from Asn residues, before Western blotting (data not shown). Three bands were detected when ER 2× Stx2eB was expressed. The molecular weight of the

lowest band (17 kDa) corresponds to the predicted molecular weight of mature 2× Stx2eB, and Stx2eB in this band migrated slightly faster than 6His-tagged 2× Stx2eB produced in *E. coli* (supplementary Fig. S3). The upper two bands (approximately 20 and 23 kDa) could be interpreted as Stx2eB containing one or two *N*-glycans. These three bands for ER 2× Stx2eB were recognized by anti-Stx2e antiserum, too (supplementary Fig. S4).

We also constructed genes for a tandem-repeated Stx2eB that was designed to accumulate either in the cytoplasm or plastids. 2× Stx2eB also accumulated to a higher level than 1× Stx2eB in both the cytoplasm and plastids of tobacco cells (supplementary Fig. S5), indicating that the enhanced accumulation by the double repeat is not specific to the ER. Accumulation level of 3× Stx2eB was comparable in plastids and even superior in cytoplasm to that of 2× Stx2eB (supplementary Fig. S5). We currently have no idea why ER 3× Stx2eB accumulates less efficiently than ER 2× Stx2eB, but it is considered that ER-specific factors contribute to this situation. Although we do not have data on the immunogenicity of 2× Stx2eB, we can conclude that the double repeat arrangement is a good strategy to obtain a higher level of Stx2eB in plant cells.

Spacers between the two Stx2eB moieties affect the protein accumulation level

Next, we analyzed the effects of a spacer peptide between two Stx2eB moieties on the accumulation of ER 2× Stx2eB. The spacers listed in Fig. 2a were evaluated. RS, PG7, PG17, and PG22 were examined to determine the effects of linker length. In addition, a 12-amino-acid linker peptide that did not contain Pro residues (SG12) was also used to analyze the flexibility of the linker. Cultured tobacco cells were used as a host for the *Agrobacterium*-mediated transformation. The resulting kanamycin-resistant clones were randomly picked for analysis. Clones expressing ER 2× Stx2eB (PG12) tended to accumulate higher levels of the Stx2eB protein than clones expressing ER 2× Stx2eB (RS), ER 2× Stx2eB (PG7) or ER 2× Stx2eB (SG12) (Fig. 2b). These results suggest that the length and amino acid composition of the spacer between the two Stx2eB moieties are important factors that can maximize the effects of the double repeat. The PG17 and PG22 spacers seemed to be comparably effective as the PG12 spacer (supplementary Fig. S6). For all constructs, three main bands for Stx2eB were detected, presumably due to the *N*-glycosylations as mentioned above. For ER 2× Stx2eB (PG12), ER 2× Stx2eB (PG17), and ER 2× Stx2eB (PG22), additional bands with molecular weight of approximately 35 kDa were also detected (asterisks in Fig. 2b; supplementary Fig. S6). These bands were more prominent for clones with higher expression levels. We speculate that the 35-kDa band corresponded to an intermolecular homodimer of 2× Stx2eB. When the blots were subjected to longer detection reaction, additional bands that were equivalent in size to trimers and tetramers of 2× Stx2eB were also detected (supplementary Fig. S7). When the samples were loaded onto PAGE gels without boiling, the signal intensities of these additional bands increased (supplementary Fig. S7).

We studied the mechanism by which the double repeat enhanced protein accumulation. The level of Stx2eB accumulation was semiquantified for each ER 1× Stx2eB, ER 2× Stx2eB (RS), and ER 2× Stx2eB (PG12) clone. Protein samples were relatively quantified by SDS–PAGE/Western blotting (WB) analysis using PG12#9 as a standard (Supplementary Fig. S2). The *Stx2eB* mRNA levels were also quantified by real-time PCR and plotted against the Stx2eB protein levels (Fig. 2c). We found that ratio of the Stx2eB protein to *Stx2eB* mRNA was higher for ER 2× Stx2eB (PG12) than for ER 2× Stx2eB (RS) and ER 1× Stx2eB. Based on these results, we conjectured that the 2× Stx2eB (PG12) protein was more stable than 1× Stx2eB or 2× Stx2eB (RS). This hypothesis also explains why there were more marked differences in the accumulation levels of 2× Stx2eB and 1× Stx2eB in stable transformants than in protoplasts transiently expressing the *Stx2eB* genes (Fig. 1b, c). The stability of a translated protein is thought to have a greater impact on its accumulation level in stable transformants than in protoplasts. In addition, the higher-molecular-weight bands in the Western blot analysis mentioned above suggest that ER 2× Stx2eB (PG12) proteins more easily oligomerize and therefore are more stable in the ER.

The double Stx2eB joined by an optimized peptide linker facilitates aggregation

Previous reports have shown that, when a large amount of secretory proteins aggregate in the ER,

these proteins are deposited into specialized membrane-bound organelles called protein bodies (PBs) (Muntz 1998; Saito et al. 2009). We tried to determine whether 2× Stx2eB could form PBs using a visible reporter protein. The C-terminus of each modified Stx2eB protein was fused to yellow fluorescent protein (YFP) (Fig. 3a), and transiently expressed in protoplasts derived from BY2 cells. When ER YFP, ER 1× Stx2eB-YFP, and ER 2× Stx2eB (RS)-YFP were expressed, the ER network exhibited a normal pattern (Fig. 3b, c, e). On the other hand, when ER 2× Stx2eB (PG12)-YFP was expressed, there were many punctate structures scattered throughout the cell (Fig. 3d). In a Western blot analysis, the Stx2eB signals had the expected molecular weight, and no degradation products were detected (Fig. 3f). The band intensities for the YFP-fused Stx2eB proteins were highly similar, which indicates that the formation of the punctate structures was not simply due to higher levels of protein accumulation. The punctate pattern was also observed when ER 2× Stx2eB (PG17)-YFP and ER 2× Stx2eB (PG22)-YFP were expressed, but not when ER 2× Stx2eB (PG7)-YFP and ER 2× Stx2eB (SG12)-YFP were expressed (supplementary Fig. S8). There was a positive correlation between the ability of the 2× Stx2eB proteins to form punctate structures and their accumulation level in stable BY2 transformants.

We further characterized these punctate structures. By collocalization analyses, we found that the YFP spots were located on the ER (supplementary Fig. S9) and were distinct from the Golgi apparatus (supplementary Fig. S9). To investigate whether the formation of these structures required vesicular transport processes, a dominant negative form (DN) or native form (WT) of ARF1, which plays an important role in vesicular transport between the ER and the Golgi (Takeuchi et al. 2002), was coexpressed with the Stx2eB reporters. In cases of vacuolar-type GFP (Di Sansebastiano et al. 1998) and *medial*-Golgi-type YFP, ER-to-Golgi transport of the reporter proteins was inhibited when they were coexpressed with ARF1 DN, and an ER-like pattern was observed (supplementary Fig. S9). On the other hand, the formation of punctate structures containing ER 2× Stx2eB (PG12)-YFP was not affected by ARF1 DN (supplementary Fig. S9). It was further determined that the formation of the punctate structures was independent of ordinary vesicular transport processes, because these structures still formed upon expression of ER 2× Stx2eB (PG12)-YFP, which does not have a C-terminal ER retention signal (supplementary Fig. S8). These characteristics of the YFP punctate structures are consistent with PBs, which originate from the ER by a mechanism that is distinct from the normal vesicular transport process that occurs when cargo proteins are highly aggregated (Muntz 1998; Saito et al. 2009). Furthermore, we speculate that 2× Stx2eB containing a preferable linker peptide (PG12, PG17, and PG22) forms aggregates more easily than 1× Stx2eB and 2× Stx2eB containing a linker peptide such as RS, PG7, and SG12, and this aggregation could be the reason for the higher accumulation levels.

Expression of codon-modified double Stx2eB in transgenic lettuce

We found that the Stx2eB expression level slightly increased with GC-rich codons (supplementary Fig. S10). Using a combination of tandem repeats and codon modifications, we constructed a gene encoding ER 2× mStx2eB5 (PG12) (2BN in Fig. 4a), and produced transgenic lettuce plants by the *Agrobacterium* method. Among a total of 104 kanamycin-resistant clones, 45 clones were selected based on their *Stx2eB* mRNA levels (data not shown) and then examined for Stx2eB protein expression (Fig. 5a). Stx2eB proteins in these lettuce plants were quantified by dot-blot analysis. We did not perform conventional enzyme-linked immunosorbent assay (ELISA) at this time, because both ER 2× Stx2eB (PG12) in transgenic lettuce and a standard 2× Stx2eB protein produced in *E. coli* were not solubilized under nondenaturing conditions. Among these clones analyzed, we obtained a T0 plant that accumulated up to 2 mg Stx2eB per 100 g fresh weight (FW) of plant. The accumulation levels of Stx2eB in a transgenic lettuce that were transformed using *Agrobacterium* harboring the 1BN construct (same as ADH-ER Stx2eB #7 in Matsui et al. 2009) were below the detection limit (10 μg/100 g FW) of the quantification method used in this analysis (1BN-7 in Fig. 5a).

To obtain transgenic lettuce plants with higher accumulation levels of Stx2eB, we further optimized the expression cassette of *Stx2eB* (2BH in Fig. 4b).

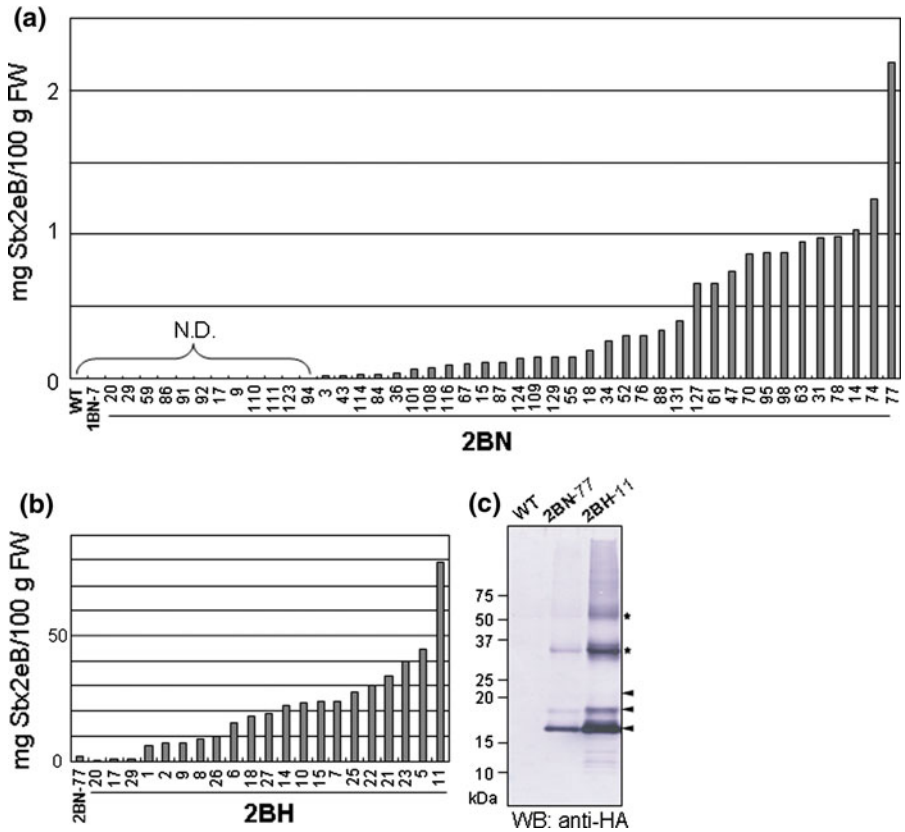

Fig. 5 Accumulation of Stx2eB protein in lettuce plants. **a**, **b** Quantification of Stx2eB was performed by dot-blot analysis using anti-HA antibody. Stx2eB protein in a young T0 plant sterile-cultured in a plant box was quantified for each clone. Serial twofold dilutions of a standard protein from *E. coli* expressing tandem Stx2eB were applied to the membrane to construct a standard curve. A mean value of three replicate measurements of Stx2eB in an extract is shown for each clone. Data of wild-type lettuce (WT), 1BN-clone 7, and 2BN plants are shown in **a**, and those of 2BN-clone 77 and 2BH plants are shown in **b**. The number adjacent to each bar represents the clone number. N.D., not detected by this method. **c** Western blot analysis of transgenic lettuce plants. Protein samples were prepared from three representative plants, separated by SDS–PAGE, and then blotted onto polyvinylidene fluoride membranes and probed with an anti-HA antibody. Amount of total soluble protein (TSP) loaded in each lane was 0.3 μg. *Arrowheads* indicate signals for Stx2eB. The *asterisk* denotes higher-molecular-weight Stx2eB signals

Firstly, transcriptional terminator derived from *Arabidopsis thaliana heat shock protein 18.2* gene (HSP-T) was incorporated in the cassette. The HSP-T increases mRNA levels of transgenes approximately twofold more than transcriptional terminator derived from *A. tumefaciens nopaline synthase* gene (NOS-T) in *A. thaliana* and in rice (Nagaya et al. 2010). We also evaluated the HSP-T in lettuce cells by transient gene expression system, and found that HSP-T increases the expression of p35S-driven *Renilla luciferase* (*Rluc*) about threefold more than NOS-T (supplementary Fig. S11). Secondly, nucleotides immediately upstream of the initiating AUG (positions −3 to −1) were changed for efficient translation initiation. In *A. thaliana*, AAGAUG was a good sequence context for expression of *E. coli β-glucuronidase* (Sugio et al. 2010). We constructed *Rluc* gene fused with original *NtADH* 5′-UTR (UAAAUG) and modified *NtADH* 5′-UTR (NtADHmod, AAG AUG), and each *Rluc* gene was transiently expressed in lettuce protoplasts. Rluc activity was increased by 1.4-fold by this modification (supplementary Fig. S11). Finally, we chose to use pRI909 as a binary vector for the transformation. Its multicloning site is located at the position closer to right border (RB) of T-DNA than the *NPTII* cassette, so that target gene is not deleted in transformation events. In this plasmid, an *NPTII* cassette and an *Stx2eB* cassette

were arranged in head-to-head orientation, intending to avoid transcriptional interference (Fig. 4b).

Transgenic lettuce plants were generated using *Agrobacterium* harboring 2BH construct. The majority of the transgenic plants accumulated higher amount of Stx2eB than clone #77 of 2BN, and clone #11 of 2BH accumulated as much as 80 mg Stx2eB per 100 g FW of a plant (Fig. 5b). In Western blotting, more than one band was detected as in BY2 cells (Fig. 5c). The lowest band (17 kDa) is presumably a nonmodified Stx2eB, and the upper two bands (approximately 20 and 23 kDa) probably correspond to *N*-glycosylated Stx2eB as mentioned above. Bands that corresponded to dimer (35 kDa) and trimer (52 kDa) of 2× Stx2eB were also detected, and these bands were more prominent in clone #11 of 2BH than in clone #77 of 2BN (Fig. 5c).

Conclusions and perspectives

In our previous study, transgenic lettuce plants accumulating Stx2eB were generated using a binary vector containing an *Stx2eB* gene with the ER-targeting signal and a translational enhancer *NtADH* 5′-UTR (Matsui et al. 2009), but the accumulation level was too low to administer to pigs as a vaccine. In this study, the expression cassette was upgraded as follows: (1) two Stx2eB moieties were translationally joined by an optimized peptide linker, (2) GC-rich codons were used, (3) HSP-T was incorporated, (4) nucleotides immediately upstream of the initiating AUG (positions −3 to −1) were optimized, and (5) pRI909 was used as a binary vector, with a *NPTII* cassette and an *Stx2eB* cassette arranged in head-to-head orientation. By combining these modifications, we obtained a transgenic lettuce plant that accumulated up to 80 mg Stx2eB per 100 g FW (#11 of 2BH). This lettuce was micropropagated and cultivated in a plant factory setting. Fully grown T0 lettuce (#11 of 2BH) accumulated more than 35 mg Stx2eB per 100 g FW (equivalent to one fully grown plant) (data not shown). Administering at least 4 mg Stx2eB-GST three times per head was sufficient to prevent edema disease (Kawamoto et al. 2009), and therefore one lettuce plant (100 g FW) is sufficient to treat three pigs by simple arithmetic.

In a preliminary analysis of subcutaneously treated rabbits, we found that 2× Stx2eB, which was expressed using *E. coli* and purified, induced higher anti-Stx2eB antibody titers than 1× Stx2eB. It is important to see whether the structure of Stx2eB moiety in ER 2× Stx2eB protein is the same as native Stx2eB. We are currently determining the efficacy and safety of these lettuce materials as a potential vaccine by orally administering a freeze-dried powder of these plants to pigs. For practical purposes, it is important to obtain homogeneous vaccine materials in addition to accumulating large amounts of protein. For this reason, we are analyzing the *N*-glycosylation sites of ER 2× Stx2eB, and trying to create nonglycosylated form of 2× Stx2eB. We are also analyzing the stability of *Stx2eB* gene expression in progeny plants.

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