Lettuce-based production of an oral vaccine against porcine edema disease for the seed lot system

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Abstract Plant-made oral vaccines can be a cost-effective method to control infectious diseases of humans and farm animals. Pig edema is a bacterial disease caused by enterohemorrhagic Escherichia coli producing the toxin Shiga toxin 2e (Stx2e). In our previous report, we chose the non-toxic B subunit of Stx2e (Stx2eB) as a vaccine antigen, and Stx2eB was expressed in lettuce (Lactuca sativa L., cv. Green wave). We found that a double repeated Stx2eB (2×Stx2eB) accumulates to higher levels than a single Stx2eB. In this study, we analyzed progeny plants introduced with 2×Stx2eB in which the gene was expressed under the control of conventional cauliflower mosaic virus 35S RNA (CaMV 35S) promoter, and found that the lettuce underwent transgene silencing and bore few seeds. We resolved these problems by using a transgene cassette which harbored a transcriptional promoter derived from the lettuce ubiquitin gene and a longer version of HSPT. The lettuce harboring this expression construct will be valuable in establishing the seed lot system on the basis that thousands of seeds can be obtained from one plant body and the resulting progeny plants accumulate 2×Stx2eB at high levels without the transgene silencing.

Key words: edema disease, Lactuca sativa, oral vaccine, seed, silencing.

Introduction

Nowadays, many recombinant proteins are commercialized for use as pharmaceuticals, industrial enzymes, and research tools. Plants are among the available host organisms for recombinant expression. The maize-derived bovine trypsin (Woodard et al. 2003), TrypZean®, has been commercialized since 2002 as an animal-free alternative to bovine or porcine trypsin. As a drug for human use, the United States Food and Drug Administration (FDA) approved a carrot cell culture-based recombinant taliglucerase alfa, ELELYSO®/ UPLYSO™, for the treatment of type 1 Gaucher’s disease in May 2012. This is the first example of a practical application of plant-made pharmaceuticals (PMPs) for human use (Maxmen 2012). During the Ebola virus outbreak in West Africa in 2014, an anti-Ebola antibody produced in tobacco, ZMapp, was applied to humans (Arntzen 2015). In addition, some human vaccines and pharmaceuticals are undergoing clinical trials (Murad et al. 2020; Shim et al. 2019).

For commercialization of PMPs, the accumulation level of a target protein is critical, because it directly affects the production cost. To increase the expression level using a molecular biological approach, transcriptional, translational, and post-translational regulation of the target gene is very important. We found that the transcriptional terminator derived from the Arabidopsis thaliana heat shock protein 18.2 gene (HSPT250) increases the mRNA levels of transgenes by approximately twofold compared to the frequently used transcriptional terminator derived from the Agrobacterium tumefaciens nopaline synthase gene (NOS-T) in A. thaliana, lettuce, and rice (Matsui et al. 2011; Nagaya et al. 2010). In addition, longer version of HSPT (HSP878), which possibly contains a matrix attachment region (MAR), contributes to higher expression of stably integrated transgenes in cultured tobacco cells, torenia (Torenia fournieri), petunia (Petunia hybrida) and chrysanthemum (Chrysanthemum morifolium) (Chin et al. 2018; Kishikaboshi et al. 2017; Matsui et al. 2014; Sasaki et al. 2014).

At the translational step, the 5′-untranslated region (5′-UTR), untranslated region; Stx2eB, B subunit of Shiga toxin Stx2e.
UTR) plays an important role because the nucleotides immediately upstream of the initiating AUG (−3 to −1) exert a considerable influence on translation initiation (Sugio et al. 2010). We also found that some 5′-UTRs function as translational enhancers in plant cells (Matsui et al. 2012, 2015; Satoh et al. 2004; Sugio et al. 2008). At the post-translational level, sending the protein into a vesicular transport pathway often leads to increased accumulation of the protein (Matsui et al. 2003, 2006; Yoshida et al. 2004).

Pig edema disease (ED) is a bacterial disease caused by enterohemorrhagic Escherichia coli (STEC) producing the toxin Shiga toxin 2e (Stx2e). The symptoms of ED include eyelid edema, emaciation, neurological disorders such as ataxia or paralysis, and sudden death in severe cases (Imberechts et al. 1992). Prophylactic administration of antibiotics to healthy piglets is associated with an increase of drug-resistant bacteria, and developing an effective vaccine to prevent ED is desired. We chose the non-toxic B subunit of Stx2e (Stx2eB) as a vaccine antigen, and Stx2eB was expressed in lettuce (Lactuca sativa L., cv. Green wave) (Matsui et al. 2009). We found that a double repeated Stx2eB (2×Stx2eB) accumulates to higher levels than a single Stx2eB (Matsui et al. 2011). The expression cassette of 2×Stx2eB was fine-tuned to increase the accumulation of the Stx2eB protein, and the HSPT and 5′-UTR derived from Nicotiana tabacum alcohol dehydrogenase gene were used in combination with the cauliflower mosaic virus 35S RNA promoter (CaMV 35S pro.). We later confirmed that recombinant Stx2eB is functional as a vaccine (Sato et al. 2013), and furthermore, three times of oral administration of approximately 1 g dry weight of freeze dried lettuce powder containing 2.3 mg Stx2eB protein relieves the pathogenic symptoms of edema disease in piglets challenged with a virulent STEC strain (Hamabata et al. 2019).

A constant production system, in addition to the expression levels, is crucial for a reliable commercial supply of recombinant proteins, especially pharmaceuticals. One important reason why we chose lettuce as a host was that we can cultivate the vaccine lettuce in plant factory (PF). In a PF, environmental parameters such as temperature, humidity, and light intensity can be tightly controlled. The production system is isolated from the external environment, and we can cultivate the lettuce throughout the year. We have found that the system is isolated from the external environment, and the productivity of the vaccine using a chilled transport was modified for plant production and outfitted with environmental control systems (Okamura et al. 2014; Takahashi et al. 2012).

For vaccine production, consistency in production can be ensured by establishing a "seed lot system", where successive batches of a product are derived from the same “master seed lot” at a given passage level, and a “working seed lot” is prepared from the “master seed lot” for routine production. The final product is derived from the “working seed lot” and has not undergone more passages from the “master seed lot” than the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy. Some animal vaccines made from a virus or bacterium using the seed lot system are already approved, and we therefore hypothesized that the system is also applicable to transgenic plant-based vaccines and the "seed lot" can literally be seeds that have the same productivity of a vaccine. Lettuce plants usually bear seeds on the order of thousands, so we intended to scale up the production system through seeds; however, we faced two technical problems: the transgene was silenced in the progeny plants, and the transgenic lettuce plants bore few seeds. In this study, we resolved these problems by using a transgene cassette contained a transcriptional promoter derived from the lettuce ubiquitin gene, which was reported to be successfully used for stable expression of miraculin in lettuce (Hirai et al. 2011), and a longer version of HSPT. Our results provide strong evidence for seed-mediated constant production of the vaccine material using this expression system in lettuce.

Materials and methods

Gene construction

2BN and 2BH have been reported previously (Matsui et al. 2011). 2BU was constructed as follows: the DNA fragment for the transcriptional promoter of the lettuce ubiquitin gene (NCBI accession No. AB500086) was amplified by PCR using a UBQ-F primer (5′-TCT AGA GGC GCG CCA AGC TTG CTC AGG AAA CAA GTG-3′, XhoI site underlined) and UBQ-R (5′-GTT ACC AAC CAT ATATA AAC ATATA TTA A-3′, KpnI site underlined). The fragment was subcloned into pCR2.1-TOPO (Thermo Fisher Scientific Inc.), and the nucleotide sequences were confirmed. The UBQ promoter was digested with XhoI and KpnI, and was replaced with a CaMV 35S promoter in the expression cassette of Stx2eB–Stx2eB where the longer version of HSPT (HSPT878) was incorporated (Matsui et al. 2014).

For luciferase analysis, Expression cassettes, in which Renilla reniformis luciferase (Rluc) and firefly (Photinus pyralis) luciferase (Fluc) were placed in a head-to-tail orientation in a binary vector pRl909 (TaKaRa Bio Inc., Shiga, Japan). As a reference construct, Fluc without Rluc was also used. For Fluc expression, the CaMV 35S promoter and HSPT250, a 250-bp DNA element that includes the entire 3′-UTR and an additional downstream sequence of the A. thaliana heat shock protein 18.2 gene (Nagaya et al. 2010), and a translational enhancer derived from A. thaliana alcohol dehydrogenase (AtADH) were fused. Expression cassettes for Rluc were associated with various terminator sequences as follows: 81-4T is a 250-bp DNA element that includes the entire 3′-UTR and an additional
downstream sequence of the *A. thaliana heat shock protein 81-4* gene (Tachiki et al. 2009); 81-4TM, which contains 81-4T and a downstream DNase I hypersensitive site and a matrix attachment region (MAR) corresponding to regions D to J of a previous report (Tachiki et al. 2009); and HSPT250 and HSPT878, which are described previously (Matsui et al. 2014).

**Genetic transformation of lettuce plants and analysis of the Stx2eB-Stx2eB protein**

Lettuce (*Lactuca sativa*, cv. Green wave) was transformed using *Agrobacterium tumefaciens* EHA 105 harboring a binary vector according to the method we have previously described (Matsui et al. 2009). Accumulation of Stx2eB−Stx2eB protein was analyzed by semi-quantitative western blotting as described previously (Matsui et al. 2011).

**Collection of lettuce seeds**

Lettuce plants were grown by hydroponic culture (Otsuka A formula; Otsuka Chemical Co., Osaka, Japan) under a 16-h light with a metal halide lamp/8-h dark photoperiod at 22.5°C. The CO₂ concentration was kept at 1,000 ppm. Mature seeds were collected in plastic tubes and were stored in a refrigerator at 4°C with silica gels.

**Results**

**Transgene silencing in progeny plants in which Stx2eB expression is driven by the CaMV 35S promoter**

We previously reported that Stx2eB accumulates at high levels in transgenic lettuce plants when the expression cassette for translationally double-repeated Stx2eB was used. The transcriptional terminators incorporated in the expression cassette was either NOST (2BN) or HSPT250 (2BH) (Figure 1; Matsui et al. 2011). We chose one line from each construct (2BN-clone 77 and 2BH-clone 21), with criteria that Stx2eB accumulation is high and a single copy of the transgene is integrated. Next, the accumulation level of Stx2eB was analyzed in the progeny plants of these lines. For both lines, a decrease in Stx2eB accumulation was observed in the T₃ and T₄ lines compared to the T₁ generation, and the expression level in the T₄ plants 2BN-clone 77 or 2BH-clone 21 were approximately a tenth or a third of the expression level in T₁ plants, respectively (Figure 2).

**Evaluation of longer version of the Arabidopsis thaliana heat shock protein 18.2 gene terminator**

There are some reports that 35S promoter-driven transgenes are silenced in *Asteridae* plants such as Gentian (Mishiba et al. 2010) and lettuce (Dubois et al. 2005; Hirai et al. 2011; McCabe et al. 1999; Okumura et al. 2016; Pniewski et al. 2011). The NOST is reported to be an inefficient terminator and transcriptional readthrough is observed in various plant species including tomato, tobacco *Nicotiana tabacum* cv. Xanthi, and *A. thaliana* (Bhullar et al. 2009; Hedtke and Grimm 2009; Rang et al. 2005; Thompson and Myatt 1997). When the NOST was incorporated in an expression cassette, improperly terminated, unpolyadenylated readthrough transcripts were recognized by RNA-dependent RNA polymerase 6 (RDR6), and the transgene was targeted for degradation by the RDR6 system.

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**Figure 1.** Schematic representation of the expression plasmid used in this study. 2BN and 2BH are described in our previous report (Matsui et al. 2011). In 2BN, expression cassettes for NPTII and Stx2eB were arranged in a head-to-tail orientation in a pBI211 plasmid. In both 2BH and 2BU, expression cassettes for NPTII and Stx2eB were arranged in a head-to-head orientation in a pH1909 plasmid. NOST, transcription terminator from *A. tumefaciens nopaline synthase* gene; NOS pro., *A. tumefaciens nopaline synthase* gene promoter; NPTII, neomycin phosphotransferase gene for kanamycin resistance, derived from *E. coli*; RB, right border; LB, left border; MCS, multiple cloning site; CaMV 35S pro., cauliflower mosaic virus 35S RNA promoter; LsUBQ pro., lettuce (*Lactuca sativa*) ubiquitin promoter; NADH 5′-UTR, 5′-untranslated region of tobacco (*Nicotiana tabacum*) alcohol dehydrogenase gene; SP, signal peptide for ER-translocation derived from tobacco β-D-glucan exohydrolase; Stx2eB, B subunit of Siga toxin Stx2e; HA, HA epitope tag for the detection of Stx2eB protein; HDEL, ER-retention signal (Ser-Glu-His-Asp-Glu-Leu); HSPT, transcription terminator from *A. thaliana heat shock protein 18.2* gene. Arrows indicate translation initiation sites. Triangles indicate signal peptide cleavage sites.
Figure 2. Gene silencing in progeny transgenic lettuce plants in which a CaMV 35S promoter was used. Stx2eB protein accumulation was analyzed in progeny lettuce plants of 2BN-clone 77 (A) and 2BH-clone 21 (B). The number of lettuce plant bodies analyzed is indicated on each bar. For T1, plants with homo- and hemi-insertions are included. Student’s t-test was performed and significant differences were indicated by asterisks. *p<0.01; **p<0.005.

Figure 3. Evaluation of transcriptional terminators. Luciferase constructs used in this analysis (A). Cultured tobacco cells (*Nicotiana tabacum* L. cv. BY2) were transformed using *Agrobacterium tumefaciens* EHA105 harboring each construct. Five and four independent infections were performed for each construct. From each infection, at least 11 kanamycin-resistant clones were randomly picked for the measurement of Rluc and Fluc activities. For each construct, average Rluc and Fluc activities were calculated for the clones from each infection, and then the average Rluc and Fluc activities were calculated for all infection events (B). Different letters indicate significant differences between treatments at p<0.05 as determined with the Tukey–Kramer’s test.
silencing in *A. thaliana* (Luo and Chen 2007). Therefore efficient terminator is thought to boost the expression of the gene through proper polyadenylation and simultaneously the avoidance of transgene silencing.

We previously reported that the longer version of the *Arabidopsis thaliana* heat shock protein 18.2 gene with a length of 878 bp (HSPT878) contributes to a higher accumulation of Stx2eB than a shorter version of HSPT with a length of 250 bp, that is HSPT250, in transgenic cultured tobacco cells (*Nicotiana tabacum* L., cv. BY2) (Matsui et al. 2014). The HSPT878 also successfully used for expression of a fluorescent protein in torenia and petunia (Chin et al. 2018; Sasaki et al. 2014). In order to re-evaluate transcriptional terminators, we constructed *Renilla reniformis* luciferase (*Rluc*) expression cassette associated either with NOST, HSPT250, HSPT878, or *A. thaliana* heat shock protein 81-4 gene terminator with a length of 250 bp (Tachiki et al. 2009) (Figure 3A). The Rluc activity was analyzed in stable transgenic tobacco cells. Among terminators tested, HSPT878 exhibited the highest average Rluc activity (Figure 3B; R::HSPT878).

For the evaluation of readthrough, we also constructed tandem expression cassettes in which *Rluc* with each terminator and downstream firefly (*Photinus pyralis*) luciferase (*Fluc*) were placed in a head-to-tail orientation. The Rluc and Fluc activities were analyzed. If the terminator used in upstream *Rluc* cassette was inefficient, readthrough occurred and expression of downstream *Fluc* was thought to be decreased by so-called transcriptional interference. For tandemly arranged expression cassettes, we observed decreasing Fluc activity in the following order: HSPT878 > HSPT250 > NOST > 81-4T. From these results, the HSPT878 is thought to be most promising terminator and we used it as the terminator for the expression of Stx2eB in lettuce in following development.

### Avoidance of gene silencing in transgenic plants with the Stx2eB expression construct with LsUBQ promoter and HSPT878

For avoidance of transgene silencing, promoter is of course also an important element to be modified. Hirai et al. (2011) reported that for miraculin expression in transgenic lettuce, transgene silencing was observed when a CaMV 35S promoter-driven cassette was used, but stable expression in progeny plants was achieved.
when the promoter and terminator were replaced with those derived from the lettuce (*Lactuca sativa*) *ubiquitin* (*LsUBQ*) gene. Therefore, we constructed a double-repeated Stx2eB expression cassette in which the *LsUBQ* promoter was incorporated in combination with HSPT878 (2BU; Figure 1).

We generated approximately 50 lines of 2BU-transgenic lettuce. The maximum accumulation of Stx2eB was approximately half of 2BH. For further analyses, we selected two independent lines (2BU-clone 18 and 2BU-clone 5), which are single-copy integrated lines (Supplementary Figure S1). As shown in Figure 4, silencing of Stx2eB was not observed in either line, in the T₆ generation in 2BU-clone 18 and in the T₃ generation in 2BU-clone 5.

**Stable seed collection from 2BU transgenic lettuce plants**

Another prerequisite for scaling up seed-mediated lettuce vaccine production is that the lettuce bears many seeds. The cultivar we use, *Lactuca sativa* L., cv. Green wave, bears as many as 8,000 mature seeds per plant body in hydroponic culture under a metal halide lamp. In a normal seed maturation process, seeds become black (Figure 5A, left), but defective seeds remain white (Figure 5A, right) and never germinate when they are sown. As for mature seed collection from transgenic plants, thousands of mature seeds were obtained from a plant body of 2BU clones 5 and 18, while 2BH clones 2 and 21 bore few seeds (Figure 5B). The total number of seeds closely correlates to the number of mature seeds per flower head (Figure 5C). The number of flower heads per plant body was not significantly different between 2BU, 2BH and wild type plants, and vegetative growth of 2BU and 2BH plants were comparable to wild type plants (data not shown). These results indicate that oral vaccine materials can be produced reliably using 2BU transgenic lettuce because it bears many seeds and transgene silencing does not occur in 2BU.

**Discussion**

In this report, we obtained Stx2eB-lettuce that thousands of seeds can be obtained from one plant body and the resulting progeny plants accumulate 2×Stx2eB at high levels without the transgene silencing. A possible explanation for the high efficiency of HSPT878 is that it contains a nuclear matrix attachment region (MAR) (Heessen and Fornerod 2007). There are some reports that MAR boosts transcription in plant cells (Abranches et al. 2005; Butaye et al. 2004; Fukuda and Nishikawa 2003; Halweg et al. 2005; Mankin et al. 2003; Nowak et al. 2001; Xue et al. 2005). We found that experimentally identified HSP81-4 MAR (81-4TM; Tachiki et al. 2009) contributed to the alleviation of transcriptional readthrough even though its polyadenylation activity
seemed to be very low (Figure 3). The HSPT878 is AT-rich sequences like other known MARs, and therefore HSPT878 possibly alleviated transgene silencing through its activity as the MAR.

In 2BH plants, the number of flower heads per plant body was comparative to wild type plants, but the number of mature seeds per flower head was significantly reduced as compared to wild type, which means seed maturation process is disturbed in 2BH plants. There is no experimental evidence, but we speculate that 2BH plants might have defects in pollination process. In pollination, polarized growth of pollen tube is necessary and endomembrane trafficking plays important roles (Zhang et al. 2010). Our vaccine antigen, 2×Stx2eB, is designed to accumulate in the ER which is entrance of endomembrane system, and the antigen might disturb the pollination process when it was expressed under strong CaMV 35S promoter. On the other hand, in 2BU, the negative effect of the antigen accumulation might be slighter than 2BH, possibly owing to lower expression of UBQ promoter in pollen.

Regarding the industrial supply of the Stx2eB-lettuce vaccine, we should produce more than 10,000 heads of lettuce per year according to our calculations as follows: There are more than 9,000,000 heads of pigs produced in Japan in 2018. If we apply the vaccine to 10% of piglets, 900,000 doses are needed. We need 6.9 mg of Stx2eB protein to protect one piglet (Hamabata et al. 2019), and the accumulation level of Stx2eB in the lettuce is 6 mg per 1 g dry weight (Figure 4). We can obtain about 10 g dry weight of lettuce powder from one lettuce head with approximately 200 g fresh weight, and therefore more than 100,000 heads of lettuce is needed for 900,000 doses. Nowadays, huge lettuce plant factory is in commercial operation for supply of non-transgenic lettuce as human foods. For example, one operated by MIRAI in Japan has a capacity of 10,000 heads of lettuce a day (https://www. igrow.news/igrownews/largest-indoor-farm-shigeharu- shimamuras-indoor-farm-sets-world-record). So, the plant factory setting can be applicable to the industrial mass production of the Stx2eB lettuce vaccine, and rate limiting step will be preparation of seedlings to be planted. Pniewski et al. (2017) reported mass-scale production of lettuce-based Hapatitis B Virus vaccine by micropropagation of the lettuce, and they implied potential production of up to 6,600 plant clones within a maximum 5 months. But the micropropagation procedure is cumbersome, and we dare say that the process is far from industrially applicable for animal farming. On the other hand, we can get 4 to 6 thousands of seeds from one plant for about half year, and seed-mediated enlargement of production scale will be possible.

These results indicate that seed-mediated, reliable, and highly efficient production is possible using transgenic lettuce plants in which an LsUBQ promoter and HSPT878 are incorporated in the expression cassette.

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