The Optimal Harvesting Time of Vaccine-Producing Transgenic Lettuce Cultivated in a Closed Plant Factory

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The amount of growth and the vaccine productivity of vaccine-producing lettuce with different cultivation periods were examined in order to determine the optimal harvesting time of transgenic lettuce cultivated in a closed plant factory. Lettuce was planted in a hydroponic system and harvested at 20, 30, 40, and 50 d, and the concentrations of the total soluble protein (TSP) and the double repeated B subunit of Shiga toxin 2e (2 × Stx2eB) were measured. The dry-matter weight of leaves per plant increased in a linear fashion until 50 d. Although the TSP concentration decreased continually from day 20 to 50 and 2 × Stx2eB concentration decreased from day 40 to 50, the yield per plant of both TSP and 2 × Stx2eB increased exponentially until day 50. According to the calculation based on these results, the optimal harvesting time to maximize the annual production of 2 × Stx2eB was revealed to be 30 d. Since the optimal 30-d harvesting time is the same to the general harvesting time for commercial lettuce production in a closed plant factory, the capability to utilize the existing closed plant factory lettuce production system could be a big advantage for vaccine-producing lettuce in terms of the cost performance.

Keywords: annual production, biopharmaceutical, harvesting cycle, Shiga toxin 2e B, veterinary vaccine

INTRODUCTION

Researches on the production of pharmaceutical ingredients using transgenic plants have been conducted (Fischer and Emans, 2000; Ma et al., 2003). The advantages of the utilization of plant systems include a vast reduction in production costs, and a lower risk of contamination with infectious microbes or toxins (Giddings et al., 2000; Daniell et al., 2001). In 2012, the FDA approved Elelyso (taliglucerase alfa), a recombinant enzymatic medicine for type 1 Gaucher’s disease, produced in transgenic carrot cells (Maxmen, 2012). However, this type of biopharmaceutical is produced in plant cell culture systems, and so the production cost remains high. In order to reduce the cost, it is necessary to utilize whole plant cultivation systems. However, there are numerous restrictions on outdoor production of transgenic plants especially for pharmaceuticals, such as measures to prevent dissemination. In order to resolve these issues, carrying out all stages from plant production to processing within closed factories is currently under examination (Yasuno and Matsumura, 2007; Goto and Matsumura, 2009). In 2013, canine interferon-α-producing transgenic strawberry plants grown in a closed plant factory were approved as an animal medicine (National Institute of Advanced Industrial Science and Technology, 2013).

Well-studied transgenic host plants, such as cereal crops and fruiting vegetables, require high light intensity. Moreover, in the case of expression of recombinant proteins in seeds or fruits, flowering is necessary to produce seeds or fruits; therefore, a means of preventing the release of pollens and eliminating gene flow by pollen transfer are needed in the cultivation system. In contrast, leafy crops including lettuce require relatively low light levels, and are harvested before flowering. In addition, lettuce has a short cultivation cycle, resulting in several harvests a year in a plant factory. Since the stable cultivation conditions, such as temperature, humidity, carbon dioxide concentration and light, are maintained year-around, highly uniform lettuce can be produced not affected by seasonal variations in a closed plant factory. Considering all these factors, we have been developing a production system for transgenic lettuce in a plant factory as part of a project to produce a veterinary vaccine against swine edema disease (ED) in transgenic lettuce. ED is caused by enterohemorrhagic Escherichia coli (EHEC) producing Shiga toxin 2e (Stx2e), and is a cause of economic loss in the pig industry worldwide (MacLeod et al., 1991; Mosley, 2000). ED often occurs mostly in piglets within the first 2 weeks post-weaning. Administration of antibiotics such as ampicillin is commonly used to prevent ED. However, use of antibiotics may lead to the development of antibiotic-resistant bacteria. Thus, alternative methods of disease control such as vaccines are needed. Non-toxic Stx2e recombinant protein expressed in E. coli is one of the candidates for practical use in such vaccines (Makino et al., 2001). In addition, Matsui et al. (2009; 2011) produced a transgenic lettuce expressing a potent ED vaccine, using only the subunit B of non-toxic Stx2e, Stx2eB. Oral administration of a lyophilized powder of

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this transgenic lettuce to pigs was sufficient to protect from challenge by EHEC (Sawada et al., 2012). We have used this transgenic lettuce line to investigate ways to increase vaccine productivity in the cultivation environment (Takahashi et al., 2012; Okamura et al., 2013).

Edible lettuce cultivated in plant factories is commercially produced with a target weight for shipping of 80–100 g per plant and the general harvesting time is about 30 d. However, the optimal harvesting time suitable for the Stx2eB is not known. In the present study, we examined the amount of growth and vaccine productivity (concentration and yield) between vaccine-producing lettuce plants with different cultivation periods in order to determine the optimal harvesting time to maximize the Stx2eB yield per area in a closed plant factory.

**MATERIALS AND METHODS**

*Plant materials*

Matsui et al. (2011) established a transgenic lettuce line (2BN-77) using leaf lettuce (*Lactuca sativa* L. cv. Greenwave) as a host. The transgenic lettuce expresses the double repeated B subunit of Shiga toxin 2e (2 × Stx2eB), a candidate vaccine against ED, fused with a hemagglutinin tag to detect 2 × Stx2eB. The Stx2eB gene is driven by a cauliflower mosaic virus 35S promoter, so the recombinant protein accumulates in all plant tissues. The 2BN-77 plantlets (T0) were self-fertilized and their T1 progeny were also self-fertilized to obtain homozygous T2 seeds. Homozygous T2 plants were used in this study.

*Growth conditions*

Transgenic lettuce was cultivated in a closed-type plant factory, termed a plant factory in this study, by remodeling a transportation container ordinarily used for chilled product. It has a two-layer hydroponic system for growth of lettuce. Lighting was provided by fluorescent lamps (FHF32EX-D-HX-S; NEC Lighting, Ltd., Tokyo, Japan). The light intensity at the top of the plant was measured using a quantum sensor (LI-190; LI-COR Inc., Lincoln, NE).

T2 seeds were germinated on polyurethane cubes at 23/18°C (light/dark), with 14 h of light at 100 ± 50 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) and 10 h of dark in the plant factory. Five d after sowing, seedlings were transplanted to floating panels in the hydroponic system. Otsuka A solution (Otsuka Chemical Co., Ltd., Osaka, Japan) was used as a hydroponic nutrient solution. The pH and electrical conductivity of the nutrient solution were maintained at 6.0 and 2.0 dS m⁻¹, respectively. Lighting was 200 ± 50 μmol m⁻² s⁻¹ PPFD provided by fluorescent lamps (FHF32EX-D-HX-S; NEC Lighting, Ltd., Tokyo, Japan). During the cultivation period, day length (light 14 h/dark 10 h), air temperature (23°C for light/18°C for dark), relative humidity (70 ± 10% for the light/90 ± 10% for the dark), and the CO₂ level (about 1,000 μmol mol⁻¹ of air) were maintained. The plant cultivation density was adjusted to ensure that the lettuce plants did not touch each other during the cultivation period.

**RESULTS AND DISCUSSION**

The relationship between plant growth or level of maturity and expression of target proteins has been...
investigated in other transgenic plants (Okayama et al., 2009; Colgan et al., 2010; Hikosaka et al., 2013). In particular, it has been suggested that when using tomato plants as hosts for recombinant protein production, the optimum harvest time differs according to the combination of target protein and host (Matsuda et al., 2010; Kato et al., 2011). In the present study, the relationship between cultivation period and vaccine productivity in vaccine-producing transgenic lettuce was investigated.

The amount of growth in vaccine-producing lettuce plants cultivated for 20–50 d is shown in Table 1. Plant height and maximum leaf length increased respectively with increased cultivation period. The leaf number of vaccine-producing lettuce plants increased in a linear fashion during the cultivation period, and there were approximately 50 leaves on day 50. From 40 to 50 d, new leaves no longer emerged horizontally and instead developed into a head, while the lower leaves began to turn yellow. The fresh weight and dry-matter weight of leaves per plant increased exponentially during the cultivation period, such that the weight on day 50 was approximately 20 times the weight on day 20. During this period, there was a slight reduction in dry-matter percentage.

The concentration of TSP (mg gDW⁻¹) in the leaves was highest on day 20, and decreased significantly during the cultivation period (Fig. 2A). On day 50, the concentration of TSP was approximately half that of day 20. The concentration of 2 × Stx2eB (mg gDW⁻¹) in the leaves began to decrease from day 40, and was reduced significantly by day 50 (Fig. 2C). The concentration of TSP and 2 × Stx2eB declined as leaves became older (Fig. 3A, 3B). While, on day 40 and day 50, an overall reduction including the lower and higher leaves was observed. Our previous study showed that the concentration of vaccine per plant decreases when plants are cultivated at higher density (Okamura et al., 2013). When plants are cultivated over an extended period of time, the newly emerging upper leaves developing within the interior of the plant would form a head. It may be conjectured that insufficient supply of light, nutrients and CO₂ to the head de-

### Table 1

<table>
<thead>
<tr>
<th>Cultivation period (day)</th>
<th>Plant height (cm)</th>
<th>Number of leaves</th>
<th>Maximum leaf length (cm)</th>
<th>Leaf weight (g/plant</th>
<th>Dry-matter percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fresh</td>
<td>Dry</td>
</tr>
<tr>
<td>20</td>
<td>9.9a</td>
<td>13.3a</td>
<td>13.3a</td>
<td>19.8a</td>
<td>1.1b</td>
</tr>
<tr>
<td>30</td>
<td>16.0b</td>
<td>21.3b</td>
<td>18.4b</td>
<td>82.4b</td>
<td>4.2b</td>
</tr>
<tr>
<td>40</td>
<td>25.7c</td>
<td>37.7c</td>
<td>25.0c</td>
<td>199.9c</td>
<td>10.0b</td>
</tr>
<tr>
<td>50</td>
<td>26.7d</td>
<td>50.3d</td>
<td>26.5d</td>
<td>497.6d</td>
<td>23.3d</td>
</tr>
</tbody>
</table>

Plants were grown hydroponically for 20–50 days after transplanting. Letters denote data that differ with statistical significance (P < 0.05) by the Tukey-Kramer multiple test (n = 10).
increased productivity of TSP or $2 \times \text{Stx2eB}$ in the upper leaves. Despite the decrease in TSP (mg gDW$^{-1}$, Fig. 2A) and $2 \times \text{Stx2eB}$ (mg gDW$^{-1}$, Fig. 2C) concentrations, the yield of TSP (mg/plant, Fig. 2B) and $2 \times \text{Stx2eB}$ (mg/plant, Fig. 2D) in lettuce plants increased exponentially and these yields on day 50 was approximately 10 times the level on day 20.

For commercial production of the recombinant protein in the closed plant factory, the maximizing annual vaccine production (vaccine yield per area per year) is required to minimize the cultivation space efficiently. In this study, we focused on the annual production calculated based on the parameters such as annual cultivation cycle and number of plants per unit area in Table 2. Despite the leaf dry-matter yield (kgDW m$^{-2}$ year$^{-1}$) was at the maximum when the production was carried out in a 50-d cycle, the annual production of TSP (g m$^{-2}$ year$^{-1}$) and $2 \times \text{Stx2eB}$ (mg m$^{-2}$ year$^{-1}$) in a 30-d cycle were both highest. These data indicate that the 30-d harvesting time is the optimal to maximize the productivity of the vaccine in a plant factory. Also, the annual production of vaccine was calculated based on the cultivation plant density (16 plants m$^{-2}$) in which the transgenic lettuces do not touch each other. Our previous study showed that the combination of cultivation condition, the plant cultivation density (44.4 plants m$^{-2}$) and PPFD 200±50 μmol m$^{-2}$ s$^{-1}$, was optimal in terms of maximizing the $2 \times \text{Stx2eB}$ yield and minimizing the electrical consumption of lighting in a 30-d cycle (Okamura et al., 2013). The annual production was 2.7 times higher than that of this study.

In conclusion, we revealed that conducting the 30-d cycle for the vaccine production would result in obtaining the most abundant vaccine protein. Since the concentration of vaccine protein in transgenic lettuce on day 30 was also greatest, it would help to reduce the doses of the lyophilized transgenic lettuce powder orally administered to piglets directly after weaning. Minimizing the applying dosage would decrease the stress to the piglets.

Although the 30-d cycle was set as the general harvesting time in the previous study (Okamura et al., 2013), our findings in this study support that altering the harvesting time is not necessary in order to maximize the annual production of vaccine. Thus, the capability to utilize the existing closed plant factory lettuce production techniques could be a big advantage for vaccine-producing lettuce plants in terms of the cost performance.

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REFERENCES
