Effects of Plant Cultivation Density and Light Intensity on the Production of a Vaccine Against Swine Edema Disease in Transgenic Lettuce

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(Received November 14, 2012; Accepted November 21, 2013)

Production technologies using closed-type plant production systems have been studied to assess their suitability for stable and uniform expression of biopharmaceutical materials in transgenic plants. We have developed a production system for a veterinary vaccine candidate against swine edema disease, using transgenic plants. In this paper, we report the combined effects of plant cultivation density and light intensity on the production levels of a vaccine candidate, the double repeated B subunit of Shiga toxin 2e (2×Stx2eB), in transgenic lettuce cultivated in a closed-type plant factory. Leaf dry-matter yield and total soluble protein (TSP) yield increased at higher plant cultivation densities, but in contrast, the 2×Stx2eB concentration in the plants tended to decrease with an increase in plant cultivation density, so that the 2×Stx2eB yield per unit area at lower plant cultivation density (44.4 plants m⁻²) was similar to or even higher than that obtained at the highest plant density (222.2 plants m⁻²). In addition, at the cultivation density (44.4 plants m⁻²), a photosynthesis photon flux density (PPFD) 200 (200±50 μmol m⁻² s⁻¹) was optimal in terms of maximizing the 2×Stx2eB yield and minimizing the electrical consumption of lighting. These results show that an optimal combination of plant cultivation density and light intensity is important in improving the productivity of recombinant protein expression systems in transgenic lettuce leaves when grown in a plant factory.

Keywords: biopharmaceutical, oral vaccine, plant factory, Shiga toxin 2e B, veterinary vaccine

INTRODUCTION

Recombinant proteins as biopharmaceuticals have been commercially produced in *Escherichia coli*, yeasts, insect cells and mammalian cells. Although the production systems are well established, there are some issues concerning production costs and safety. The high production costs are due to the expensive facilities required, such as tanks and purification systems to remove impurities or toxins, and maintaining cell lines is costly. There are also some issues regarding infection risks by viral contamination.

One possible solution for these issues is to produce recombinant proteins in transgenic plants. 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). Tomatoes, tobacco, rice and corn have been used as hosts when developing production technologies for biopharmaceuticals (Giddings et al., 2000; Daniell et al., 2001; Sala et al., 2003). In 2006, the USDA approved a new oral vaccine against Newcastle disease in chickens, produced in transgenic tobacco cells. 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, these two biopharmaceuticals are produced in plant cell culture systems, and so the production costs remain high. In order to reduce these costs it is necessary to utilize whole plant cultivation systems. When using whole transgenic plants, it is also necessary to prevent the potential-flow of transgenes from the cultivation room to the surrounding environment. In addition, high levels of uniformity of harvested materials, and stable plant production, not affected by seasonal variations, are required. This can be achieved by using a closed-type plant production system, termed a plant factory in this study. The plant factory can maintain stable cultivation conditions, such as temperature, humidity, and light, all year round. In addition, the plant factory can create a specialized cultivation environment not occurring in nature, such as a high CO₂ concentration. Optimizing the cultiva-

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tion conditions in the plant factory can maximize the production of recombinant proteins in transgenic plants. Several studies for the production of biopharmaceutical products in transgenic plants in plant factories have been conducted (Yasuno and Matsumura, 2007; Okayama et al., 2010; Hirai et al., 2010; Kato et al., 2011). Furthermore, a fully automated factory for plant-based recombinant protein production has been developed that meets current Good Manufacturing Practice (cGMP) guidelines (Wirz et al., 2012).

Plants requiring high light intensities, such as tobacco, corn, and rice are commercially used as transgenic host plants (Giddings et al., 2000; Daniell et al., 2001). However, in a plant factory, these plants need a cultivation system with high-intensity light sources, entailing both large initial investments, and high operating costs. On the other hand, lettuce is already commercially produced in plant factories because it requires relatively low light levels, and has short cultivation cycles resulting in several harvests a year. Since transgenic lettuce is harvested before flowering, negative pressure in the cultivation room to prevent pollen dispersal is not needed. In addition, using an edible plant for production of useful materials can be relatively inexpensive because processing costs for extraction and purification are not required (Walmsley and Arntzen, 2000; Daniell et al., 2001; Sala et al., 2003; Ma et al., 2005). Therefore, extensive studies on the expression and accumulation of recombinant proteins using transgenic lettuce have been conducted (Kapusta et al., 2001; Webster et al., 2006; Kim et al., 2007; Rosales-Mendoza et al., 2010; Piiewski et al., 2011; Huy et al., 2011; Lim et al., 2011).

We have been developing a production system for transgenic lettuce, to produce a veterinary vaccine against swine edema disease (ED). ED occurs mostly in piglets, and 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; Moxley, 2000). Administration of antibiotics such as ampicillin is commonly used to prevent ED. Since antibiotic use is associated with an increased risk of developing resistant bacteria, alternative methods of disease control such as vaccines are needed. Makino et al. (2001) reported that vaccination of pigs with non-toxic Stx2e recombinant protein expressed in E. coli was effective in protecting against ED. Matsu et al. (2009; 2011a) 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 the transgenic lettuce to pigs was sufficient to protect from challenge by EHEC (Matsui et al., 2011b; Sawada et al., 2012).

Environmental factors affecting the growth of plants can also alter the gene expression pattern and protein levels (Jamal et al., 2009; Colgan et al., 2010; Okayama et al., 2010). The production of Stx2eB in transgenic lettuce may vary with environmental conditions that strongly affect plant growth (Takahashi et al., 2012), although as yet this has not been investigated experimentally.

For cost-efficient production of recombinant proteins by transgenic plants in a plant factory, improving the usability of cultivation space and reducing both the initial and operating cost are important. In this study, we investigated the combined effects of plant cultivation density and light intensity on the yield and concentration of Stx2eB in transgenic lettuce, using a plant factory.

MATERIALS AND METHODS

Controlled environmental conditions in the plant factory

We constructed a plant growth facility for transgenic lettuce by remodeling a transportation container for chilled products, to meet the requirements as stipulated by the Cartagena Protocol for the cultivation of transgenic plants in a plant factory. The facility was divided into two rooms, a front room that was the control room for the environmental conditions in the cultivation room and the cultivation room with hydroponic systems for growth of lettuce. Lighting was provided by fluorescent lamps (FFH32EX-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). 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 light / 90±10 % for dark), and the CO2 level (about 1,000 µmol mol⁻¹ of air) were maintained.

Plant materials

We used leaf lettuce (Lactuca sativa L. cv. Greenwave) as the host plant. The transgenic lettuce expressing the double repeated B subunit of Shiga toxin 2e (2×Stx2eB), a candidate vaccine against ED, fused with a hemagglutinin (HA) tag to detect 2×Stx2eB was made by stable nuclear transformation as shown in Fig. 1a (Matsui et al., 2011a). These lettuce lines possess the stx2eB gene driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter, and non-toxic Stx2eB protein accumulates in all plant tissues (Fig. 1b). The transgenic line ‘2BN-77’ was self-fertilized and their T1 progenies were also self-fertilized to obtain homozygous T2 seeds. The homozygous T2 plants were used in this study.

T2 seeds were germinated in the plant factory on polyurethane cubes thoroughly saturated with water, with 14 h of light at 100±50 µmol m⁻² s⁻¹ PPFD and 10 h of dark. Five days 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 was maintained at 6.0 and 2.0 dS m⁻¹, respectively. Cultivation test was repeated twice for each set of cultivation conditions.

Experiment 1: Effect of plant cultivation density on the production of recombinant protein

Transgenic lettuce seedlings were transplanted to the floating panels (0.6 m×0.9 m) at four cultivation densities: 12, 24, 48, and 120 plants panel⁻¹ (Fig. 2). These densities were equivalent to 22.2, 44.4, 88.9, and 222.2 plants m⁻². Light intensity was 200±50 µmol m⁻² s⁻¹. All plants were harvested 30 days after transplanting. Plant height, number
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of leaves and maximum leaf length were measured at the time of the harvesting.

Experiment 2: Effect of light intensity on the production of recombinant protein

The lettuce seedlings were cultivated in different light conditions (100 ± 50 (PPFD 100), 200 ± 50 (PPFD 200), 300 ± 50 (PPFD 300) and 400 ± 50 µmol m⁻² s⁻¹ (PPFD 400)). For this experiment, a plant cultivation density of 24 per panel (44.4 plant m⁻²) was used. All plants were harvested 30 days after transplanting. Plant height, number of leaves and maximum leaf length were measured at the time of harvesting.

Quantification of the 2×Stx2eB protein expression

In both experiments, after measurements of the weight of fresh leaves were taken, all the lettuce leaves from plants grown under each set of experimental conditions were freeze-dried under vacuum using a freeze dryer (Labconco FZ-6; Labconco Corp., Kansas City, MO) and dry leaf weights were measured. Freeze-dried leaves were ground into a fine powder using a mill (WB-1; Osaka Chemical Co., Ltd., Osaka, Japan). Total soluble protein (TSP) was extracted from the dried leaf powder by the TCA-Acetone method (Shultz et al., 2005). In brief, the dried protein precipitate was suspended in denaturation extraction buffer (6 M urea, 5 mM imidazole, 500 mM sodium chloride, 20 mM Tris-HCl, pH 7.8), and the supernatant liquid was collected after centrifugation (20,000 g for 20 min at 4°C). The TSP concentrations in clarified plant extracts were determined using a Bradford assay kit (Bio-Rad., Hercules, CA).

To determine 2×Stx2eB protein levels in the transgenic lettuces, dot-blot analysis using an anti-HA antibody was performed as previously described (Matsui et al., 2011a). The HA tag as a general epitope tag was facilitated the detection with high sensitivity of the target protein using anti-HA antibody. A standard protein purified from E. coli expressing 2×Stx2eB, was used to construct a calibration curve.

Leaf dry-matter yield per unit area (g DW m⁻²) was calculated based on both leaf dry-matter (g DW plant⁻¹) and
plant cultivation density (plants m$^{-2}$). TSP or 2×Stx2eB yield per unit area (mg m$^{-2}$) was calculated based on TSP or Stx2eB concentration (mg gDW$^{-1}$) and leaf dry-matter yield (gDW m$^{-2}$). Comparisons of data were made using the Tukey-Kramer multiple test ($P<0.05$).

RESULTS AND DISCUSSION

Effect of plant cultivation density on the production of recombinant protein

Plant shapes of transgenic lettuces cultivated at different plant cultivation densities are shown in Fig. 3. Plant height and maximum leaf length were respectively higher and longer at high cultivation density (88.9, 222.2 plants m$^{-2}$) than at low density (22.2, 44.4 plants m$^{-2}$, Table 1). It is a well-known fact that spindly growth and decreasing dry mass per plant with increasing plant cultivation density are due to the lowered light intensity caused by mutual shading (Sasaki, 1984) with increasing plant cultivation density. In this study, at cultivation densities of 222.2, 88.9, 44.4 and 22.2 plants m$^{-2}$, the mutual shading occurred at 7, 10, 16 and 17 days after transplanting, respectively (data not shown). Therefore, the ventilation rate in the lettuce communities may also decrease with increasing plant cultivation density. The number of leaves and leaf dry weight per plant decreased with the increase in plant cultivation density in our study. It is considered that these results were caused by limited light and gas exchange. When the gas exchange in plant communities is limited by an increase in plant cultivation density, the CO$_2$ fertilization effect, known to enhance growth, is decreased. Our previous study (Takahashi et al., 2012) showed that controlling air flow from the bottom to leaves at 1.0 m s$^{-1}$ to the individual plants enhanced the growth of transgenic lettuces in the plant factory. If this air flow system could improve the gas exchange in plant communities, the number of leaves and leaf dry weight per plant may increase. The effect of plant cultivation density with air flow should be investigated in a future study.

The plant cultivation density did not significantly affect TSP concentration per leaf dry-matter (Fig. 4A), and the leaf dry-matter yields (g m$^{-2}$) increased with increasing plant cultivation density, with the result that the overall TSP yields (g m$^{-2}$, Fig. 4B) were greater with increasing plant cultivation density. In contrast, the 2×Stx2eB concentration in dry-matter (Fig. 4C) decreased when plants were cultivated at higher density. The 2×Stx2eB concentrations in dry-matter at the lower plant cultivation densities (22.2 or 44.4 plants m$^{-2}$) were approximately 2.4 times higher than that at the highest plant cultivation density (222.2 plants m$^{-2}$). In addition, the 2×Stx2eB yields (g m$^{-2}$, Fig. 4D) at 44.4 plants m$^{-2}$ were as high as that at 222.2 plants m$^{-2}$. These results showed that if the cultivation density is decreased from 222.2 to 44.4 plants m$^{-2}$, almost the same amount of 2×Stx2eB will be produced in the same area, and this would lead to a reduction in costs by decreasing the numbers of transplants (a cut of 80%) and harvesting processes. Since preparing seeds in closed conditions needs both time and space, a requirement for fewer seeds is also an important material cost saving. Therefore, at a light intensity of 200±50 μmol m$^{-2}$s$^{-1}$, we decided on 44.4 plants m$^{-2}$ as the suitable plant cultivation density to produce 2×Stx2eB in our plant factory system.

Effect of light intensity on the production of recombinant protein

The effects of light intensity on the production of recombinant protein were examined at the suitable plant cultivation density (44.4 plants m$^{-2}$) as determined above, and the results are shown in Table 2. Plant height at PPFD 400 was shorter than at other PPFDs. Leaf length was shorter in plants grown with increased light intensity. In contrast, the number of leaves and the weight of leaf dry-matter

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**Fig. 3** Lettuces producing 2×Stx2eB, grown at various cultivation densities in the plant factory, 30 days after transplanting. Cultivation densities were 22.2 (A), 44.4 (B), 88.9 (C), and 222.2 plants m$^{-2}$ (D). The scale bar indicates 5 cm in length.

**Table 1** Effects of plant cultivation density on plant growth, 30 days after transplanting into the plant factory.

<table>
<thead>
<tr>
<th>Plant cultivation density (plants m$^{-2}$)</th>
<th>Plant height (cm)</th>
<th>Maximum leaf length (cm)</th>
<th>Number of leaves</th>
<th>Leaf dry-matter (g plant$^{-1}$)</th>
<th>Leaf dry-matter (g m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.2</td>
<td>12</td>
<td>15.4$^a$</td>
<td>18.3$^a$</td>
<td>24.8$^a$</td>
<td>5.25$^a$</td>
</tr>
<tr>
<td>44.4</td>
<td>24</td>
<td>16.6$^a$</td>
<td>18.7$^a$</td>
<td>21.7$^a$</td>
<td>4.03$^a$</td>
</tr>
<tr>
<td>88.9</td>
<td>48</td>
<td>20.9$^a$</td>
<td>23.8$^a$</td>
<td>19.4$^a$</td>
<td>2.63$^a$</td>
</tr>
<tr>
<td>222.2</td>
<td>120</td>
<td>22.3$^a$</td>
<td>26.4$^a$</td>
<td>17.3$^a$</td>
<td>1.94$^a$</td>
</tr>
</tbody>
</table>

Plants were grown hydroponically at four cultivation densities. Light intensity was 200±50 μmol m$^{-2}$s$^{-1}$ PPFD (photosynthesis photon flux density). Letters denote data that differ with statistical significance ($P<0.05$) by the Tukey-Kramer multiple test (n=10).
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Tended to increase with increasing light intensities, showing that overall growth of transgenic plants was enhanced by increased light intensity. The TSP concentration (Fig. 5A) and the $2\times$ Stx2eB concentration at PPFD 200 and 300 (Fig. 5C) were slightly higher than those at PPFD 100 and 400. Stevens et al. (2000) reported that both TSP and recombinant IgG protein concentration in transgenic tobacco were increased in conditions of high light intensity (PPFD 400) compared with the concentrations seen at low light intensity (PPFD 100). On the other hand, the concentration of recombinant miraculin protein in the tomato pericarp decreases with increasing light intensities (Kato et al., 2011). However, in our study, high light intensity (PPFD 400) did not increase the concentration of both TSP and the $2\times$ Stx2eB when compared with other PPFDs. Hence, it is necessary to determine the optimum cultivation environments for each host-recombinant protein combination, because each combination may require different optimal cultivation conditions.

As shown in Table 2, the yield of the leaf dry-matter increased with increasing light intensity, while the TSP and $2\times$ Stx2eB yield at PPFD 200-400 were similar, and decreased at PPFD 100 (Fig. 5B and 5D). These data show that higher light intensity does not always enhance the yield of a protein of interest. For the production of $2\times$ Stx2eB, PPFD 200 or 300 at a cultivation density of 44.4 plants m$^{-2}$ was a suitable light intensity in our plant factory system. However both the initial cost and ongoing electricity costs for $2\times$ Stx2eB production at PPFD 300 were higher than at PPFD 200 because many light fixtures were needed. Cost performance is very important for commercial production. Thus, PPFD 200 in a cultivation density of 44.4 plants m$^{-2}$ was the most suitable light intensity in our factory.

Vol. 51, No. 4 (2013)
Matsui et al. (2011b) reported that for each animal, three doses of at least 2.3 mg of Stx2eB were needed to protect pigs from challenge by EHEC. From this, it is estimated that approximately 15 g of the dried transgenic lettuce is required for each animal. Fifteen g of the dried lettuce corresponds to 3.4 plants. Since the vaccine against ED is administered to piglets directly after weaning, a reduction in the doses of the lettuce powder by increasing Stx2eB concentration would help to decrease stress on the piglets. In addition, the number of piglets receiving Stx2eB could be increased by increasing Stx2eB concentration and yield in same cultivation space.

In conclusion, we investigated suitable cultivation environments such as plant cultivation density and light intensity in a plant factory, for the production of double repeated Stx2eB. Our results indicated that a suitable cultivation environment increase both Stx2eB concentration and yield simultaneously. Our findings would contribute to a reduction in production costs in a plant factory both by improving the usability of cultivation space and reducing the energy cost of light.

This work was supported by the development of Fundamental Technologies for the Production of High-Value Materials Using Transgenic Plants project (2006–2010) managed by the Ministry of Economy, Trade and Industry of Japan.

Fig. 5 Effects of light intensity on the production of TSP and 2×Stx2eB, 30 days after transplanting into the plant factory. Plants were grown hydroponically at four light intensities. Plant cultivation density was 44.4 plants m⁻². Letters denote data that differ with statistical significance (P<0.05) by the Tukey-Kramer multiple test (n=8). Vertical bars represent SD.

### REFERENCES


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