Effects of environmental conditions before gene transfer on the amount of influenza hemagglutinin transiently expressed in *Nicotiana benthamiana* leaves

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**Abstract**

We investigated the effects of photosynthetic photon flux density (PPFD), air temperature, and CO₂ concentration before gene transfer on the accumulation level of hemagglutinin (HA), an influenza vaccine antigen, in *Nicotiana benthamiana* leaves in a transient gene expression system. Plants were treated for two weeks before gene transfer with different levels of PPFD, air temperature or CO₂ concentration in separate experiments. Leaf fresh mass (FM), leaf HA content per unit FM, and leaf HA content per plant at 6 d post infiltration (6 DPI) for gene transfer all tended to increase with increasing average PPFD during the treatment period. Leaf FM and leaf HA content per plant at 6 DPI were significantly greater at an average day air temperature of 25°C–30°C during the treatment period than at 20°C–25°C. No clear effects of CO₂ concentration were found on either growth or leaf HA content under the conditions tested in the present study. There was a positive correlation between leaf HA content per unit FM at 6 DPI and leaf HA content at 0 or 6 DPI in plants grown under different PPFDs and air temperatures. Preparing plants with increased leaf FM by applying high PPFD and/or high air temperature before gene transfer should increase not only total HA yield per plant but also HA content per unit leaf biomass at harvest.

**Key words:** Air temperature, Carbon dioxide concentration, Influenza subunit vaccine antigen, Photosynthetic photon flux density, Plant-made pharmaceutical

1. Introduction

Plants have become a viable platform to produce recombinant proteins such as biopharmaceuticals. Compared with conventional transgenic animal-based or mammalian cell culture-based systems, whole plant-based systems offer advantages for biopharmaceutical protein production, including low production cost, high production scalability, and low risk of contamination with human pathogens, whereas animal and plant cells possess a similar eukaryotic protein synthesis pathway (Fischer and Emans, 2000; Ma et al., 2003; Twyman et al., 2003; Desai et al., 2010). There are two plant-made biopharmaceuticals currently available on the market (Drake et al., 2017), taliglucerase alfa (ELELYSO®) and interferon-α (Interberry α°), approved for human and animal use, respectively. In addition, it has been announced by Medicago Inc. that a plant-made quadrivalent seasonal influenza vaccine is being subjected to a Phase 3 efficacy study in multiple countries. Several other candidates have initially shown safety and efficacy in early-phase clinical trials (Fischer et al., 2012). The two main biotechnologies for transgene expression in plants are stable transformation of the nuclear or chloroplast genome and transient gene expression using plant viral or non-viral vectors. Among these technologies, transient gene expression with viral vectors in *Nicotiana* species such as *N. benthamiana* enables rapid mass production of recombinant proteins (Pogue et al., 2002, 2010; Gleba et al., 2004, 2007; Lico et al., 2008; Matoba et al., 2011; Chen et al., 2013; Peyret and Lomonossoff, 2015).

It has been reported that the accumulation of recombinant proteins in plants in transient gene expression systems is influenced by plant growth conditions (for a review, see Fujiuchi et al., 2016a). Several studies have reported that air temperature (Buyel and Fischer, 2012; Matsuda et al., 2012, 2017a, 2017b) and humidity (Plesha et al., 2007; Fujiuchi et al., 2016b) after gene transfer significantly influence recombinant protein accumulation. Compared with the growth conditions after gene transfer, a limited number of studies have examined the effects of those before gene transfer. Those studies reported that light source (Lai and Chen, 2012), nitrate concentration of a nutrient solution (Fujiuchi et al., 2014), and plant density (Fujiuchi et al., 2017) influenced recombinant protein content per unit leaf biomass. Optimizing plant growth conditions, not only after but before gene transfer, is thus crucial to efficient recombinant protein production in transient gene expression systems. Taking into consideration that plants play a role in supplying the energy and resources required for protein synthesis, it is important to prepare plants for gene transfer treatment such that they possess the appropriate characteristics for subsequent transgene expression, yet what specific characteristics are appropriate is unknown.

In practical plant-made biopharmaceutical protein production, plants are generally grown before gene transfer in greenhouses (e.g., Medicago Inc.) or indoor plant production facilities with artificial lighting (e.g., Kentucky BioProcessing, Inc., iBio, Inc.; see also Holtz et al., 2015) to ensure uniform batch-to-batch productivity as far as possible. Environmental factors such as photosynthetic photon flux density (PPFD), air temperature, and CO₂ concentration are controllable in such facilities, though to a lesser extent in greenhouses. Very recently, Shang et al. (2018) examined the effects of CO₂ enrichment, supplemental
lighting, and a high plant density before gene transfer on growth and productivity of hemagglutinin (HA), an influenza vaccine antigen, of \textit{N. benthamiana} plants. They reported positive effects of supplemental lighting and dense planting on HA productivity per unit area. In their research, only greenhouses were targeted, and plants were actually grown and treated in greenhouses before gene transfer. To our knowledge, no information is available on the effects of basic environmental factors prior to gene transfer in indoor facilities with artificial lighting on recombinant protein production in plants after gene transfer.

The aim of the present study was therefore to investigate the effects of PPFD, air temperature, and CO$_2$ concentration before gene transfer on the accumulation of a recombinant protein in leaves of \textit{N. benthamiana} plants in a transient gene expression system. Assuming environmental conditions in indoor plant production facilities, experiments were carried out using growth chambers equipped with artificial light sources. Plants were treated for two weeks before gene transfer. The two-week treatment period was divided into the first and second weeks, and treatments consisted of combinations of different levels of PPFD, air temperature, or CO$_2$ concentration in each week. HA was used as a model recombinant protein. As indicators of HA accumulation, we evaluated both HA content per total leaves of a plant and HA content per unit fresh mass (FM) of leaves. The former should be maximized as it directly relates to the harvestable HA yield. The latter itself is also important because high HA content per unit FM not only contributes to high HA productivity of a plant and HA content per unit fresh mass but it can also reduce the cost of the downstream protein purification process by reducing the use of consumables (Buyel and Fischer, 2012; Tüse et al., 2014). We also measured leaf TSP content, because a positive correlation between TSP and HA contents was sometimes observed (Matsuda et al., 2012; Fujiuchi et al., 2014), suggesting a possible contribution of a high amount of TSP to increasing HA.

2. Materials and methods

2.1 Plant material

Seeds of \textit{N. benthamiana} were sown into rockwool cubes (AO36/40, ROCKWOOL B.V., Roermond, the Netherlands). For each experiment (see below), two seeds were sown into each of 96 cubes moistened with tap water (192 seeds in total). The cubes were placed in a temperature-controlled room. Light was provided by natural white fluorescent lamps (FPL55EX-N, Iwasaki Electric Co., Ltd., Tokyo, Japan) for 16 h d$^{-1}$ at a PPFD of 200 µmol m$^{-2}$ s$^{-1}$ measured at the surface of the cubes. Air temperatures were 25 ± 2°C/20 ± 2°C (day/night). The rockwool cubes were subirrigated with tap water for the first week and subsequently with a nutrient solution (prescription A, OAT Agrio Co., Ltd., Tokyo, Japan) with an electrical conductivity of 0.18 S m$^{-1}$ and pH of 6.0. At 7 d post sowing (DPS), cubes with two seedlings were thinned to one per cube. At 14 DPS, 30 seedlings at a similar growth stage were selected, transplanted onto rockwool blocks (Delta 6.5G, ROCKWOOL B.V.), and grown further in the same room until 22 DPS. The rockwool blocks were subirrigated with the above nutrient solution for 20 min once a day. Lateral shoots and flower buds were removed once a week.

2.2 Treatments

Plants were subjected to treatments of different PPFDs (Exp. 1), air temperatures (Exp. 2) or CO$_2$ concentrations (Exp. 3) from 23 to 36 DPS (hereafter, treatment period). At 22 DPS, plants were transferred to incubators (MIR-553, SANYO Electric Co., Ltd., Osaka, Japan) equipped with LED panels (580 × 440 mm, HMWD10DC6 (1N-40Y), Kyoritsu Densho Co., Ltd., Osaka, Japan) composed of 120 phosphor-based natural white LEDs (GSPW1651NSE-40Y-TR, Stanley Electric Co., Ltd., Tokyo, Japan). Internally the incubators were separated into upper and lower compartments, and the LED panels were fixed to the roof surface of each compartment. The LEDs were driven by direct-current power supplies (PR70-1A, Texitio Technology Corp., Kanagawa, Japan). Each incubator was ventilated with an air pump to produce an air exchange rate of 0.5 h$^{-1}$ except for Exp. 3. In all experiments, photoperiod was 16 h d$^{-1}$ and relative humidity was 75 ± 15% throughout the treatment period. There were five treatments in all experiments, and six plants were subjected to each treatment. The plant density was 71 plants m$^{-2}$.

In Exp. 1, for the first (from 23 to 29 DPS) and second (from 30 to 36 DPS) weeks in the treatment period, respectively, plants were grown at PPFDs of 100 and 100 µmol m$^{-2}$ s$^{-1}$ (P100-100), 200 and 100 µmol m$^{-2}$ s$^{-1}$ (P200-100), 200 and 200 µmol m$^{-2}$ s$^{-1}$ (P200-200), 200 and 400 µmol m$^{-2}$ s$^{-1}$ (P200-400), or 400 and 400 µmol m$^{-2}$ s$^{-1}$ (P400-400) (Table 1). PPFD at the tops of the plants was measured using a quantum sensor (LI-190SA, LI-COR Inc., NE, USA). Air temperature and CO$_2$ concentration were 25 ± 1°C/20 ± 1°C (day/night) and 500 ± 100 µmol mol$^{-1}$, respectively, throughout the treatment period.

In Exp. 2, for the first and second weeks in the treatment period, respectively, plants were grown at day air temperatures of 20 ± 1°C and 20 ± 1°C (T20-20), 25 ± 1°C and 20 ± 1°C (T25-20), 25 ± 1°C and 25 ± 1°C (T25-25), 25 ± 1°C and 30 ± 1°C (T25-30), or 30 ± 1°C and 30 ± 1°C (T30-30) (Table 1). Night air temperature was set 5°C below respective day air temperature.

| Experiment number | Environmental factor tested | Level of environmental factor | Treatment code |
|-------------------|-----------------------------|-----------------------------|----------------|
| 1                 | PPFD [µmol m$^{-2}$ s$^{-1}$] | 23–29 DPS | 30–36 DPS |
|                   | 100                          | 100                         | P100-100       |
|                   | 200                          | 100                         | P200-100       |
|                   | 200                          | 200                         | P200-200       |
|                   | 200                          | 400                         | P200-400       |
|                   | 400                          | 400                         | P400-400       |
| 2                 | Day/night air temperature [°C] | 23–29 DPS | 30–36 DPS |
|                   | 20/15                        | 20/15                       | T20-20         |
|                   | 25/20                        | 20/15                       | T25-20         |
|                   | 25/20                        | 25/20                       | T25-25         |
|                   | 25/20                        | 30/25                       | T25-30         |
|                   | 30/25                        | 30/25                       | T30-30         |
| 3                 | CO$_2$ concentration [µmol mol$^{-1}$] | 23–29 DPS | 30–36 DPS |
|                   | 400                          | 400                         | C400-400       |
|                   | 600                          | 400                         | C400-600       |
|                   | 800                          | 800                         | C400-800       |
|                   | 800                          | 800                         | C600-800       |
|                   | 800                          | 800                         | C800-800       |
for all treatments (Table 1). Air temperature was measured using calibrated type-T thermocouples. PPFD measured at the tops of the plants and CO₂ concentrations were 200 µmol m⁻² s⁻¹ and 500 ± 100 µmol mol⁻¹, respectively, throughout the treatment period.

In Exp. 3, for the first and second weeks in the treatment period, respectively, plants were grown at CO₂ concentrations of 400 ± 100 and 400 ± 100 µmol mol⁻¹ (C400-400), 400 ± 100 and 600 ± 100 µmol mol⁻¹ (C400-600), 400 ± 100 and 800 ± 100 µmol mol⁻¹ (C400-800), 600 ± 100 and 600 ± 100 µmol mol⁻¹ (C600-600), or 800 ± 100 and 800 ± 100 µmol mol⁻¹ (C800-800) (Table 1). CO₂-free air was made by passing air through soda lime canisters and introduced continuously into the incubators at an air exchange rate of 1 h⁻¹. CO₂ from a cylinder was injected based on the CO₂ concentration inside the incubators measured by nondispersive infrared CO₂ analyzers (ZFP, Fuji Electric Co., Ltd., Kanagawa, Japan) so that the CO₂ concentrations were maintained within the target ranges. PPFD measured at the tops of the plants and air temperature were 400 µmol m⁻² s⁻¹ and 25 ± 1°C/20 ± 1°C (day/night), respectively, throughout the treatment period.

2.3 Vector construction and vacuum infiltration for gene transfer

A “deconstructed” tobamoviral replicon system (Marillonnet et al., 2004, 2005) (magnICON, ICON Genetics GmbH, Halle (Saale), Germany) was used to express HA transiently in N. benthamiana. Vector construction and Agrobacterium tumefaciens (GV3101::pMP90, Koncz and Schell, 1986) transformation have been described previously (Matsuda et al., 2012, 2017a). Briefly, the HA construct was designed to target the ectodomain of HA derived from influenza A virus (subtype H1N1, strain A/California/07/2009) to the endoplasmic reticulum, using an N-terminal secretory signal peptide and a C-terminal HDEL ER-retention signal peptide. The construct was subcloned into the plasmid vector (pICH26212), which contained genes encoding tobamovirus-derived RNA-dependent RNA polymerase and movement protein, to form the HA expression vector (pNM216). Suspension of the transformed A. tumefaciens carrying the HA expression vector was vacuum-infiltrated into all leaves of 36-d-old plants for gene transfer, as described previously (Matsuda et al., 2012, 2018a). The optical density of the bacterial suspension at 600 nm was adjusted to 0.03. Three plants per treatment were subjected to vacuum infiltration in each experiment.

2.4 Growth conditions after gene transfer

Plants subjected to vacuum infiltration were grown in incubators (MIR-554-PJ, PHC Holdings Corp., Tokyo, Japan) for 6 d until harvest. Light was provided by the LED panels for 16 h d⁻¹ at 200 µmol m⁻² s⁻¹ PPFD measured at the tops of the plants. Air temperature, relative humidity, and CO₂ concentration were 21 ± 1°C, 75 ± 15%, and 400 ± 100 µmol mol⁻¹, respectively. According to our previous studies (Matsuda et al., 2017a, 2018b), leaf HA content was expected to reach almost its maximum level at 6 DPI under these conditions. The rockwool blocks were subirrigated with 0.5 L tap water per block every two days.

2.5 Growth analysis

Growth analysis was performed immediately before infiltration and at 6 d post infiltration (DPI). Total leaf area was measured using an automatic area meter (AAM-9, Hayashi Denko Co., Ltd., Tokyo, Japan). Leaves were cut in half along the primary vein. One half was first weighed to determine FM and then oven-dried at 100°C for 1 h followed by 80°C for 3 d to determine dry mass (DM). The other half was first weighed to determine FM and then subjected to quantification of total soluble protein (TSP) (0 and 6 DPI) and HA (6 DPI) as described below.

2.6 Biochemical assays

In a blender the leaf portions were homogenized in 5 mL g⁻¹ (FM)⁻¹ of 50 mmol L⁻¹ sodium phosphate buffer at pH 7.0 containing 0.8% (v/v) 3-mercapto-1,2-propanediol, 5% (v/v) glycero, and 2 mmol L⁻¹ sodium iodoacetate. TSP and HA contents were quantified by Bradford assay and sandwich enzyme-linked immunosorbent assay, respectively, as previously described by Fujiuchi et al. (2016b).

2.7 Statistical analysis

In each experiment, individual plants were considered independent biological replicates. Significant differences in mean among the treatments were tested by Tukey-Kramer’s HSD test at P < 0.05 using statistical software (R 3.4.3, R Core Team, 2017).

3. Results and discussion

3.1 Effects of PPFD

At 0 DPI, leaf DM for the two highest PPFD treatments (P200-400 and P400-400) was significantly greater than that for the other three treatments (Table 2), whereas no significant differences were found among the treatments for leaf FM (Fig. 1a). At 6 DPI, leaf FM and DM in P200-400 and P400-400 were significantly greater than those in P100-100 and P200-100 (Fig. 1b, Table 2). There was no significant difference in total leaf area among the treatments either at 0 or 6 DPI (Table 2). Leaf HA content per unit FM was significantly greater in P400-400 than in P100-100 and P200-100 (Fig. 1c). Leaf HA content per unit FM tended to increase as the average PPFD, rather than the PPFD during a particular period (e.g., for a few days before gene transfer), affected the accumulation level of HA in leaves. These results for leaf HA content per unit FM are consistent with results from our recent study in which leaf HA content per unit FM was significantly lowered when plant density was high (400 plants m⁻², Fujiuchi et al., 2017), as dense planting decreases the amount of light received by a plant. In contrast, Shang et al. (2018) reported that supplemental lighting with LEDs and dense planting in a greenhouse before gene transfer had little or no effect on leaf HA content per unit of biomass. Effects of light intensity may be different between indoor and greenhouse conditions and/or between transgene expression systems used. Leaf HA content per plant, which is the product of leaf FM and leaf HA content per unit FM at 6 DPI, was significantly greater in P200-400 and P400-400 than in P100-100 and P200-100 (Fig. 1d). It appears that a high PPFD contributed not only to an increase in leaf biomass but also to an
increase in leaf HA content per unit FM, resulting in a substantial increase in total HA yield per plant. Leaf TSP content per unit FM in P200-400 was significantly greater than that in P100-100 at both 0 and 6 DPI (Table 2), but no clear trend was observed between the PPFD level and leaf TSP content. Although a high leaf HA content per unit FM can reduce the cost of the downstream protein purification process, a high PPFD would increase the cost of lighting in the upstream plant production process. An appropriate PPFD level should be determined based on the calculation regarding this trade-off.

3.2 Effects of air temperature

Leaf FM at 0 DPI in T25-30 and T30-30 was significantly greater than that in T20-20 (Fig. 2a). Similarly, leaf FM at 6 DPI in T25-30 and T30-30 was significantly greater than that in T20-20 and T25-20 (Fig. 2b). Leaf FM both at 0 and 6 DPI tended to increase as average air temperature during the treatment period increased. Leaf DM at 6 DPI and total leaf area at 0 and 6 DPI also tended to increase with increasing air temperature, whereas leaf DM at 0 DPI showed no significant differences among treatments (Table 3).

Leaf HA content per unit FM was significantly greater in T25-25 and T25-30 than in T20-20 (Fig. 2c). Leaf HA content per plant was significantly greater in T25-30 and T30-30 than in T25-20 and T20-20 (Fig. 2d). This implies that, to achieve high total HA yield per plant, average day air temperature before gene transfer should be regulated in the range 25°C to 30°C; air temperatures between 20°C and 25°C are much less productive. This is in contrast to our previous reports on the effects of air temperature after gene transfer: an air temperature of 20°C–22°C was suitable for accumulation of HA in leaves (Matsuda et al., 2012, 2017a, b) and an air temperature higher than those levels significantly decreased leaf HA content at 6 DPI (Matsuda et al., 2017b). Clearly, the appropriate air temperature settings differ

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**Table 2.** Leaf dry mass (DM), total leaf area, and total soluble protein (TSP) content per unit leaf fresh mass (FM) of *Nicotiana benthamiana* plants at 0 and 6 d post infiltration (DPI) grown at different photosynthetic photon flux densities before infiltration in Exp. 1.

| Treatment code | Leaf DM per plant [g plant⁻¹] | Total leaf area [cm² plant⁻¹] | Leaf TSP content per FM [mg g⁻¹] |
|---------------|-------------------------------|------------------------------|----------------------------------|
|               | 0 DPI                         | 6 DPI                        |                                  |
| P100-100      | 0.55 ± 0.067 b                | 1.44 ± 0.088 d               | 434 ± 43.4 a                     |
| P200-100      | 0.76 ± 0.067 b                | 1.69 ± 0.056 cd              | 525 ± 57.7 a                     |
| P200-200      | 1.02 ± 0.007 b                | 1.97 ± 0.046 bc              | 383 ± 31.8 a                     |
| P200-400      | 1.86 ± 0.187 a                | 2.53 ± 0.044 a               | 382 ± 65.8 a                     |
| P400-400      | 1.88 ± 0.289 a                | 2.28 ± 0.191 ab              | 410 ± 73.1 a                     |
| P200-100      | 0.76 ± 0.067 b                | 1.69 ± 0.056 cd              | 525 ± 57.7 a                     |
| P200-200      | 1.02 ± 0.007 b                | 1.97 ± 0.046 bc              | 383 ± 31.8 a                     |
| P200-400      | 1.86 ± 0.187 a                | 2.53 ± 0.044 a               | 382 ± 65.8 a                     |
| P400-400      | 1.88 ± 0.289 a                | 2.28 ± 0.191 ab              | 410 ± 73.1 a                     |

*See Table 1 for treatment codes

Means ± standard errors of biological triplicates. Means with different small letters in each panel are significantly different by Tukey–Kramer’s HSD test at P < 0.05.

**Fig. 1.** Effects of photosynthetic photon flux density (PPFD) before gene transfer by vacuum infiltration on growth and hemagglutinin (HA) content in leaves of *Nicotiana benthamiana* plants in Exp. 1. a Leaf fresh mass (FM) per plant at 0 d post infiltration (DPI). b Leaf FM per plant at 6 DPI. c Leaf HA content per unit FM at 6 DPI. d Leaf HA content per plant at 6 DPI. See Table 1 for treatment codes. Data represent means with standard errors of biological triplicates. Means with different small letters in each panel are significant different by Tukey–Kramer’s HSD test at P < 0.05.
between plant growth stages before and after gene transfer, at least for the expression system we employed. Leaf TSP content per unit FM at 0 DPI was not significantly different among treatments, and that at 6 DPI in T25-30 was significantly greater than that in T20-20 (Table 3).

### 3.3 Effects of CO$_2$ concentration

We did not observe any significant differences in growth parameters (leaf FM and DM and total leaf area) among different CO$_2$ concentrations either at 0 or 6 DPI (Fig. 3a, b, Table 4). It is likely that dry matter productivity of *Nicotiana benthamiana* plants was nearly at maximum at 400 µmol m$^{-2}$ s$^{-1}$ PPFD and 400 µmol mol$^{-1}$ CO$_2$ concentration therefore CO$_2$ enrichment caused no further growth promotion. In fact, we recently found that net photosynthetic rates of *N. benthamiana* leaves at CO$_2$ concentrations between 400 and 800 µmol mol$^{-1}$ are not very different, irrespective of PPFD levels (250 and 1,200 µmol m$^{-2}$ s$^{-1}$) (Matsuda et al., 2018b), supporting the null growth promotion under high CO$_2$ concentrations seen in the present study. There was no significant difference in leaf HA content per unit FM, leaf HA content per plant, or leaf TSP content per unit FM among treatments (Fig. 3c, d, Table 4). Similar results of negligible effects of CO$_2$ enrichment were reported by Shang et al. (2018). Thus, no clear effects dependent upon CO$_2$ enrichment before gene transfer were found under the conditions tested in the present experiment.

### 3.4 Factors potentially involved in leaf HA content differences

We performed regression analysis to ascertain the parameter(s) potentially involved in the difference in leaf HA content per unit FM (or per unit DM) observed among the different environmental conditions tested. Among those analyzed, relatively high coefficients of determination ($r^2$) were found between leaf HA content per unit FM and leaf FM at 6 DPI in...
Exp. 1 ($r^2 = 0.728$, Fig. 4b) and Exp. 2 ($r^2 = 0.769$, Fig. 4d), and weak positive correlations were observed between leaf HA content per unit FM and leaf FM at 0 DPI ($r^2 = 0.501$ and 0.715 for Exps. 1 and 2, respectively, Fig. 4a, c). We also found relatively high $r^2$ between leaf HA content per unit DM and leaf FM at 0 or 6 DPI in Exps. 1 and 2 (Table 5). In Exp. 3, no clear correlation was found for either relationship (data not shown) because there were few variations in leaf HA content per unit FM among CO2 treatments (Fig. 3c). In Exps. 1 and 2, increased leaf FM at 0 DPI arising from higher PPFD or air temperature should contribute to increased leaf FM at 6 DPI even though growth conditions after gene transfer for 6 d were the same, and the increased leaf FM at 6 DPI could be related to the high-level accumulation of HA in leaves. The reason for the positive correlation between leaf FM and leaf HA content per unit FM is unclear. Resources that can be utilized for protein synthesis, such as nonstructural carbohydrates, could be enriched in leaves with increased FM. This possibility should be verified in future research. From a practical point of view, preparing plants with increased leaf FM by applying high PPFD and high air temperature before gene transfer is beneficial. This is because it would increase not only total HA yield per plant but also HA content per unit leaf biomass at harvest, contributing to downstream process cost reduction (Buyel and Fischer, 2012; Tusé et al., 2014).

We had thought that leaf TSP content per unit FM at 0 DPI should affect leaf HA content per unit FM at 6 DPI, because it could be expected that a higher TSP content at the time of gene transfer might have a positive effect on subsequent recombinant protein production. However, we did not find any significant correlations between leaf HA content per unit FM at 6 DPI and leaf TSP content per unit FM at 0 or 6 DPI in all experiments (data not shown).

In this study, we did not examine a possibility that
environmental conditions before gene transfer influenced DPI at which time leaf HA content reached the maximum level. This possible effect should be investigated in future research.

4. Conclusions

PPFD and air temperature before gene transfer affected both HA content per unit FM of leaves and HA content per total leaves of a plant. HA content per total leaves tended to increase with increasing average PPFD or air temperature over the two weeks before gene transfer. Such increases in HA content per total leaves were due not only to increased leaf biomass but also to increased HA accumulation level per unit FM. No effects of CO$_2$ enrichment were found on either growth or HA content under the conditions tested in the present study. Increasing leaf biomass per plant at the timing of gene transfer by appropriate environmental control before gene transfer should be effective in obtaining high HA production per plant while reducing the cost of the downstream protein purification process.

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