Inter-batch variability of hemagglutinin content transiently expressed in *Nicotiana benthamiana* plants grown under sole-source lighting and sunlight conditions before gene transfer

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**Abstract**

I investigated the effects of seasonal fluctuations in light availability for plants before gene transfer on the inter-batch variation of target recombinant protein productivity in a viral vector-based transient gene expression system. *Nicotiana benthamiana* plants were grown five times in different seasons, either in a growth chamber (GC) under sole-source (solely electric) lighting or in a temperature-controlled greenhouse (GH) under sunlight until agroinfiltration for gene transfer. The plants were then further grown in a shared growth chamber to allow accumulation of hemagglutinin (HA) until harvest. The coefficient of variation of leaf HA content per plant among GC batches was more than twice that among GH batches. The greater variation of leaf HA content per plant in GH was due to the higher coefficient of variation (CV) of leaf biomass and the slightly higher CV of leaf HA content per biomass, the former being primarily due to the variation in cumulative photosynthetic photon flux density before gene transfer. There was a significant linear regression between leaf HA content per biomass at harvest and leaf biomass at the timing of gene transfer across growth conditions (GC or GH) and seasons. This regression indicates that the variation of leaf biomass or any related variables immediately before gene transfer can account for a significant part of the observed variation of leaf HA accumulation per plant at harvest. Thus, strictly regulated plant growth conditions before gene transfer are crucial to reducing the inter-batch variation of HA productivity. I conclude that indoor facilities with sole-source lighting are more appropriate than greenhouses, not only for plant cultivation after gene transfer but also before gene transfer.

**Key words:** Greenhouse, Growth analysis, Influenza hemagglutinin, Photosynthetic photon flux density (PPFD), Plant-made pharmaceuticals (PMPs)

1. Introduction

Recombinant proteins used as biopharmaceuticals, including vaccine antigens, therapeutic proteins, and diagnostic agents, are highly valuable products that living organisms can produce. They have conventionally been produced in microbial and mammalian cell culture-based systems, but plants have attracted increasing attention as an alternative platform for producing recombinant proteins. The advantages of the plant-based production systems over conventional systems include low production costs, high production scalability, and low risk of contamination with human pathogens during the production process, while both animal and plant cells have a similar eukaryotic protein synthesis pathway (Fischer and Emans, 2000; Ma et al., 2003; Twyman et al., 2003; Desai et al., 2010; Fauster-Bovendo and Kobinger, 2021). Two fundamental biotechnologies exist to introduce transgenes into plant cells and express them: stable transformation and transient gene expression. In the former, transgenes are stably introduced into the plant genome, while in the latter, transgenes are transferred into growing plants with viral or non-viral vectors and expressed transiently. Transient gene expression in *Nicotiana benthamiana*, a wild relative of tobacco (*N. tabacum*), with *Agrobacterium*-assisted viral vector systems 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; Tusé et al., 2020). Plant-derived vaccines for influenza and COVID-19 produced with the transient expression system have reportedly shown positive results in human phase 2/3 clinical trials (Ward et al., 2020; Gobeil et al., 2021), and notably the latter (COVIFENZ®) has recently been granted approval by Health Canada. These results demonstrate the feasibility and usefulness of this technology.

It is well recognized that plant growth conditions before and after vacuum infiltration of an *Agrobacterium* suspension for gene transfer significantly affect the target protein accumulation level at harvest (for a review, see Fujuchi et al., 2016). The primary objectives in the upstream production process of plant-based recombinant protein production include stabilizing the recombinant protein yield from batch to batch and maximizing the level of recombinant protein accumulating in harvestable plant parts. It is therefore essential to elucidate the effects of plant growth conditions on target protein productivity. Among various environmental and cultural conditions before and after agroinfiltration, the light environment before agroinfiltration is crucial. For example, the light source (Lai and Chen, 2012), plant
density (Fujuchi et al., 2017), supplemental lighting (Shang et al., 2018), photosynthetic photon flux density (PPFD) (Matsuda et al., 2019), and photoperiod (Matsuda, 2021) before agroinfiltration reportedly influence recombinant protein productivity.

In plant-derived pharmaceutical protein production using transient gene expression technology, plants before gene transfer are generally grown in greenhouses (e.g., Shang et al., 2018; Goulet et al., 2019) or in indoor plant production facilities with artificial lighting (vertical farms) (e.g., Wirz et al., 2012; Holtz et al., 2015), while plants after gene transfer are incubated in contained facilities to prevent transgene flow to the outside. In greenhouses, environmental factors including PPFD have considerable inter- and intra-daily fluctuations, probably leading to a substantial batch-to-batch variability of target protein production. Using transgenic plants, Knödler et al. (2019) examined the effects of seasonal weather changes on target protein yields under greenhouse conditions. They grew transgenic tobacco plants expressing DFE, a fusion protein of the HIV-neutralizing antibody 2FS and the fluorescent protein DsRed, in different seasons in two years in a greenhouse under sunlight. The results showed that the yield was highly variable depending on the season, and that there was a negative correlation between the yield and the integrated light level. They suggested that controlled cultivation environments such as vertical farms may help to reduce such seasonal effects and ensure consistent protein yields across batches. Another study also reported inter-batch variability in the production of a human monoclonal antibody with transgenic tobacco grown in a greenhouse (Sack et al., 2015). For transient gene expression, however, there is no available data on whether and how much seasonal changes in meteorology, particularly the light environment, before agroinfiltration influence target protein productivity. Given that there is a subsequent plant cultivation process after agroinfiltration in transient gene expression systems, which in general takes place in vertical farms under a stable environment, the extent of the batch-to-batch variability due to sunlight before agroinfiltration might be different from that for transgenic plant systems. The plant cultivation process in a vertical farm after agroinfiltration may mitigate the extent of the batch-to-batch variability of target protein production at harvest, compared with the variability of biomass production at agroinfiltration. However, the extent has been neither quantified nor compared to pre-infiltration sole-source lighting conditions, i.e., the conditions where electric lighting provides the sole source of light for plant growth and development.

In this study, N. benthamiana plants were grown in a greenhouse or a “simulated” vertical farm, i.e., a growth chamber equipped with light-emitting diodes (LEDs), before agroinfiltration. After infiltration, plants from both conditions were grown in a common growth chamber with sole-source lighting to allow for the accumulation of influenza hemagglutinin (HA). The HA is surface protein of influenza viruses and used as a vaccine antigen of the viruses, and was used as a model protein in this study. Cultivation was repeated five times across different seasons to observe the inter-batch variability of plant growth and HA productivity under the two pre-infiltration growth conditions. The greenhouse was temperature controlled, so the primary difference in the inter-batch variability between the two pre-infiltration growth conditions was whether irradiation was from sunlight or sole-source artificial light. I also performed growth analysis (Peterson and Neofotis, 2004; Radford, 1967), which decomposes the difference in the growth rates of the plants into “physiological” (net assimilation per unit leaf area) and “morphological” (light availability of leaves) components, to analyze the factors responsible for the differences in plant growth.

2. Materials and Methods

2.1 Experimental design

The whole experiment described below was repeated five times between July 2018 and March 2019. Experiment numbers and cultivation schedules are shown in Table 1.

2.2 Plant material

N. benthamiana seeds were sown into moistened rockwool cubes (AO36/40, ROCKWOOL B.V., Roermond, the Netherlands) in a plug tray placed in a plastic container. The bottom of the rockwool cubes was soaked in deionized water, and the top of the container was covered with transparent plastic wrap to keep the cubes moistened. They were placed in a temperature-controlled growth chamber (GC) (MIR-553, SANYO Electric Co., Ltd., Osaka, Japan). The light was provided by an LED panel (HM120DC6(1N-40Y), Kyoritsu Densho Co., Ltd., Osaka, Japan) composed of phosphor-converted white LEDs (GSPW1651NSE-40Y-TR, Stanley Electric Co., Ltd., Tokyo, Japan). The PPFD was 200 µmol m⁻² s⁻¹ for a photoperiod of 16 h d⁻¹, which was measured at the tops of plants using a quantum sensor (LI-190SA, LI-COR Inc., NE, USA) and adjusted using a direct-current power supply (PMC-A, Kikusui Electronics Corp., Kanagawa, Japan). The air temperature was set at 25 °C/20 °C (day/night). At 7 d post-seeding (DPS), the plastic wrap was removed. The rockwool cubes were thereafter subirrigated with a nutrient solution (prescription A, OAT Agrio Co., Ltd., Tokyo, Japan) with an electrical conductivity of 0.18 S m⁻² and pH of 6.0.

2.3 Growth conditions before gene transfer

At 14 d post-seeding (DPS), each of 36 seedlings was transplanted onto a rockwool block (Delta 6.5G, ROCKWOOL B.V.). Half of the seedlings were further grown in the same GC, and the other half were moved to a greenhouse (GH). The seedlings were grown under these respective conditions until subjected to vacuum infiltration (see the following subsection) at 35 DPS.

Table 1. Experiment numbers and cultivation schedules.

| Experiment number | Seeding (0 DPS) | Transplanting (14 DPS) | Vacuum infiltration (35 DPS) | Harvest (41 DPS) |
|-------------------|----------------|------------------------|-----------------------------|-----------------|
| 1                 | 2018/7/25      | 2018/8/8               | 2018/8/29                   | 2018/9/4        |
| 2                 | 2018/7/25      | 2018/8/8               | 2018/10/3                   | 2018/10/9       |
| 3                 | 2018/8/29      | 2018/9/12              | 2018/11/7                   | 2018/11/13      |
| 4                 | 2019/1/4       | 2019/1/18              | 2019/2/8                    | 2019/2/14       |
| 5                 | 2019/2/13      | 2019/2/27              | 2019/3/20                   | 2019/3/26       |

*DPS: days post-seeding.
Environmental conditions in the GC were set as follows: PPFD at the tops of plants of 200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for a photoperiod of 16 h d\(^{-1}\) and an air temperature of 25 °C/20 °C (day/night). The GC was ventilated with external air using an air pump (APN-110R, IWAKI Co., Ltd., Tokyo, Japan) to maintain an inside CO\(_2\) concentration close to the atmospheric level.

The GH was located at the top of a four-storied building in Bunkyo, Tokyo, Japan (35°43' N). It was a half-span lean-to GH, and the north side was attached to walls. The covering material was glass panes. The floor area was 25 m\(^2\). The GH was equipped with an air conditioning system. The air temperature was controlled at 25 °C/20 °C (day/night). The inside air was humidified when the relative humidity was lower than 50%. The GH was also equipped with four 400-W high-intensity discharge lamps, and they were turned on between 6 a.m. and 8 a.m. and 4 p.m. and 8 p.m. in Exps. 4 and 5. The PPFD of the supplemental light around the plants was 80 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

In both the GC and GH, the plant density was 65 plants/m\(^2\). The rockwool blocks were subirrigated with the above nutrient solution. Lateral shoots and flower buds were removed weekly.

The PPFD was measured using the above quantum sensors. In the GC, the PPFD was measured and adjusted twice in each experiment: before seeding and before vacuum infiltration. In the GH, the PPFD of sunlight and supplemental light was recorded every 1 min using a data logger (GL220, Graphitec Corp., Yokohama, Japan). Daily PPFD integrals in the GC and GH during the experiments are shown in Table 2. The air temperature, relative humidity, and CO\(_2\) concentration were measured and recorded every 1 min using measurement devices (MCH-383SD, FUSO Co., Ltd., Tokyo, Japan). Daily mean air temperature, calculated water vapor pressure deficit (VPD), and CO\(_2\) concentration during the experiments are shown in Tables S2–4. In each growth condition (GC or GH), eight plants were subjected to growth analysis as described below.

2.4 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 in _N. benthamiana_ transiently. Construction of the plasmid vector pNM216 and _Agrobacterium tumefaciens_ (GV3101::pMP90, Koncz and Schell, 1986) transformation have been described previously (Matsuda et al., 2012, 2017). The ectodomain of HA derived from influenza A virus (subtype H1N1, strain A/California/07/2009) was targeted to the endoplasmic reticulum (ER) in plant cells using an N-terminal secretory signal peptide and a C-terminal HDEL (His-Asp-Glu-Leu) ER-retention signal peptide. Suspension of the transformed _A. tumefaciens_ carrying pNM216 was vacuum-infiltrated into all leaves of 35-d-old plants for gene transfer, as described previously (Matsuda et al., 2012, 2018a). In each pre-infiltration growth condition, nine plants were subjected to vacuum infiltration.

2.5 Growth conditions after gene transfer

After vacuum infiltration, plants were grown in a GC equipped with the LED panel for 6 d, irrespective of the pre-infiltration growth condition. Environmental conditions were set as follows: PPFD at the tops of plants of 200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for a photoperiod of 16 h d\(^{-1}\) and an air temperature of 21 °C throughout the day. The rockwool cubes were subirrigated with the above nutrient solution.

### Table 2. Mean daily integral of photosynthetic photon flux density (PPFD) at the tops of plants.

| Experiment number | Treatment | 0–13 DPS \(^{\text{a}}\) | 14–34 DPS | 35–41 DPS |
|-------------------|-----------|-----------------|------------|------------|
| 1                 | GC \(^{1}\) | 12.0            | 12.0       | 12.0       |
|                   | GH \(^{1}\) | 15.1 ± 6.14    | 11.5       | 11.5       |
| 2                 | GC        | 12.1            | 12.1       | 12.1       |
|                   | GH        | 7.5 ± 4.81     | 11.6       | 11.6       |
| 3                 | GC        | 12.3            | 12.3       | 12.3       |
|                   | GH        | 6.9 ± 2.99     | 11.7       | 11.7       |
| 4                 | GC        | 11.7            | 11.7       | 11.7       |
|                   | GH        | 6.3 ± 1.32     | 11.6       | 11.6       |
| 5                 | GC        | 11.7            | 11.7       | 11.7       |
|                   | GH        | 11.1 ± 5.27    | 11.6       | 11.6       |
| CV \(^{\text{w}}\) [%] | GC | 2.3             | 2.3        | 0.7        |
|                   | GH        | 39.3           | 0.7        |

\(^{1}\)DPS: days post-seeding.  
\(^{2}\)GC: growth chamber; GH: greenhouse.  
\(^{3}\)Mean ± standard deviation.  
\(^{w}\)CV: coefficient of variation among five experiments.

### 2.6 Growth and developmental measurements

At 34 DPS, eight plants per treatment were harvested. Leaf number, stem length, leaf area, and fresh mass (FM) of leaf lamina and stem plus petiole (hereafter referred to as leaf and stem, respectively) were measured. Leaves and stem were oven-dried at 100 °C for 1 h followed by 80 °C for 3 d to determine the dry mass (DM).

At 41 DPS, eight plants per treatment were harvested. Leaf number, stem length, leaf area, and stem FM and DM were measured as described above. Each leaf lamina was divided into two portions along the primary vein. Halves per plant were weighed to determine FM and subsequently oven-dried to determine DM, and the leaf dry matter ratio (DMR, DM/FM) was calculated. The other halves per plant were weighed to assess FM and subsequently subjected to HA quantification as described below. Whole-leaf lamina DM per plant was estimated using the calculated leaf DMR of the plant.

So-called “growth analysis” (Peterson and Neofotis, 2004; Radford, 1967) of shoots (leaves and stem) for pre- and post-infiltration was carried out using the DM of each organ and leaf area at 14, 34, and 41 DPS. Relative growth rate (RGR, \( g \ g^{-1} d^{-1} \)) is calculated on the assumption of exponential growth using the following formula:

\[
RGR = \frac{1}{W} \frac{dW}{dt} = \frac{\ln W_t - \ln W_i}{t_f - t_i}
\]

where \( W \) and \( t \) are shoot DM (g) and time (d), respectively, and \( W_i \) and \( W_t \) denote \( W \) at \( t_i \) and \( t_f \) (\( t_i < t_f \)), respectively. The RGR
is expressed as the product of the net assimilation rate (NAR, g m\(^{-2}\) d\(^{-1}\)) and leaf area ratio (LAR, m\(^{2}\) g\(^{-1}\)):

\[
\text{RGR} = \text{NAR} \times \text{LAR} \tag{2}
\]

\[
\text{NAR} = \frac{1}{\Delta} \frac{dW}{dt} = \frac{\ln W_2 - \ln W_1}{\Delta t} - \frac{\ln W_1 - \ln W_0}{
\Delta t - \Delta t_1}
\tag{3}
\]

\[
\text{LAR} = \frac{A}{W} = \frac{A_2 - A_1}{\ln A_2 - \ln A_1} \frac{W_2 - \ln W_1}{W_2 - W_1} \tag{4}
\]

where \(A\) is leaf area (m\(^2\)), and \(A_1\) and \(A_2\) denote \(A\) at \(t_1\) and \(t_2\), respectively. The NAR represents the rate of dry matter production per unit leaf area, while the LAR represents the morphological aspect, leaf area per unit of shoot DM. The LAR is further divided into leaf mass ratio (LMR, g g\(^{-1}\)) and specific leaf area (SLA, m\(^2\) g\(^{-1}\)):

\[
\text{LAR} = \text{LMR} \times \text{SLA}
\tag{5}
\]

\[
\text{LMR} = \frac{L}{W} = \frac{L_2 - L_1}{\ln L_2 - \ln L_1} \frac{W_2 - \ln W_1}{W_2 - W_1}
\tag{6}
\]

\[
\text{SLA} = \frac{A}{L} = \frac{A_2 - A_1}{\ln A_2 - \ln A_1} \frac{\ln W_2 - \ln W_1}{W_2 - W_1}
\tag{7}
\]

where \(L\) is leaf DM (g), and \(L_1\) and \(L_2\) denote \(L\) at \(t_1\) and \(t_2\), respectively. The inverse of SLA, called leaf mass per area (LMA, g m\(^2\)), is often used as an indicator of leaf thickness (e.g., de la Riva et al., 2016).

### 2.7 Hemagglutinin quantification

Halves of all leaf laminae per plant were homogenized in a buffer mixture (Matsuda et al., 2019) with a blender, and the supernatant of the surfactant-added homogenate (Matsuda et al., 2012) was subjected to sandwich enzyme-linked immunosorbent assay (ELISA) using a kit (SEK001, Sino Biological Inc., Beijing, China) to quantify HA. The capture and detection antibodies were mouse anti-HA (influenza A H1N1) monoclonal antibody and rabbit HA (influenza A H1N1) polyclonal antibody conjugated to horseradish-peroxidase (HRP), respectively. Calibration curves were drawn with standard recombinant HA (influenza A H1N1). Clear flat-bottom 96-well plates (Life Technologies Japan Ltd., Tokyo, Japan) were used for assays. After color development using a TMB peroxidase EIA substrate kit (Bio-Rad Laboratories, Inc., CA, USA), the absorbance at 450 nm was measured using a microplate reader (MTP-310, Hitachi High-Tech Science Corporation, Tokyo, Japan). As HA contents in petioles and stems are much lower than that in leaves and negligible (Fujuchi et al., 2017), HA content per whole leaf laminae was considered as HA content per plant.

### 2.8 Statistical analysis

The coefficient of variation (CV) among five experiments (Exps. 1–5) was calculated for respective pre-infiltration growth conditions of GC and GH (\(CV_{GC}\) and \(CV_{GH}\), respectively). The significance of the difference between GC and GH in each experiment was tested by Welch’s \(t\) test at a significance level of 0.05. Individual plants were dealt with as experimental units (\(n = 8\)).

### 3. Results and Discussion

#### 3.1 Environmental conditions

Table 2 shows the daily PPFD integral for each growth stage from seeding to harvest. The \(CV_{pre}\) of the cumulative PPFD for Exps. 1–5 was 39.3\% due to fluctuations in PPFD of sunlight during treatment (14–34 DPS), while \(CV_{pp}\) was only 2\%. The difference in the cumulative PPFD between GC and GH depended on the experiment; it was higher in GH than in GC in Exp. 1, lower in Exps. 2–4, and comparable in Exp. 5. The daily PPFD integral was similar before (14–34 DPS) and after (35–41 DPS) infiltration in GC but not in GH in most experiments. Time course...
of daily integral of PPFD is shown in Fig. S1. Daily mean air temperature during treatment was slightly (0.4–1.1 °C) lower in GH than in GC, irrespective of the experiment (Table S1). Daily mean VPD during that period was substantially higher in GH than in GC in all experiments (Table S2). Daily mean CO₂ concentration during treatment tended to be slightly higher in GH than in GC (Table S3). The $CV_{GH}$ values of air temperature and CO₂ concentration during treatment were not much different from $CV_{GC}$ and less than 3%, which was substantially smaller than that for PPFD of 39.3% (Table 2). On the other hand, the $CV_{GH}$ of VPD was unignorable (13.5%) yet almost half of $CV_{GC}$ (26.1%).

**Fig. 2.** Leaf fresh mass (FM) (A, B), dry mass (DM) (C, D), dry matter ratio (DMR, E, F) and leaf area (G, H) one day before vacuum infiltration (34 d post-seeding (DPS)) (A, C, E, G) and at harvest (41 DPS) (B, D, F, H) for the pre-infiltration growth-chamber (GC) and greenhouse (GH) treatments. Vertical bars represent standard errors of the means ($n = 8$). Asterisks (*) represent significant differences between GC and GH in each experiment. $CV_{GC}$ and $CV_{GH}$ represent the coefficients of variation among five experiments for GC and GH, respectively.
3.2 Leaf Hemagglutinin content

The highest HA production per plant was found under the GC condition in Exp. 5 (Fig. 1A). The $CV_{GH}$ of leaf HA content per plant (57.9%) was more than twice that of $CV_{GC}$ (20.6%) (Fig. 1A), clearly indicating that the sole-source lighting conditions of GC before infiltration enabled more stable HA production than the sunlight conditions of GH. The higher CV of mean daily PPFD integral in GH should be the primary cause of the greater inter-batch variation of HA productivity, as the CVs of temperature and CO₂ concentration were similar to those in GC and that of VPD was even lower than GC. This result of the significant inter-batch target protein productivity found in GH is consistent with a study of transgenic plants (Knödler et al., 2019), suggesting the importance of controlled-environment plant cultivation to reduce seasonal effects and ensure consistent yields across batches.

Leaf HA content per plant is the product of leaf HA content per biomass and leaf biomass at harvest. The $CV_{GH}$ of leaf HA content per DM was more significant than that of leaf HA content per plant.

Despite the same number of batches, may be partly accounted for by the inter-assay CV of per plant. The HA content per biomass was one of the sources, but not the only one, of the large inter-batch variation of leaf HA content per plant. The $CV_{GC}$ of leaf HA content per biomass (20.6%) was substantially smaller than that of leaf HA content per plant (57.9%). This indicates that, for the pre-infiltration GH treatment, the inter-batch variation of leaf HA content per biomass was one of the sources, but not the sole source, of the large inter-batch variation of leaf HA content per plant. The $CV_{GC}$ of leaf HA content per biomass of 20%, even though growth conditions were controlled to be similar among batches, may be partly accounted for by the inter-assay CV of ELISA, of which one acceptable range is reported to be 15% (Thomsson et al., 2014).

3.3 Biomass production and plant development

The CV of leaf FM measured at 1 d before infiltration (34 DPS) was more remarkable for GH (37.8%) than for GC (22.5%) (Fig. 2A). At harvest after infiltration (41 DPS), the $CV_{GH}$ (33.7%) was still greater than $CV_{GC}$ (13.2%). The CVs decreased from 34 to 41 DPS for GC and GH, associated with the one-week plant growth in the growth chambers (Fig. 2B). Similar trends were found in leaf DM (Fig. 2C, D) and leaf area (Fig. 2G, H). Leaf DMR in GH at 34 DPS was similar to or lower than that in GC (Fig. 2E). However, at 41 DPS, leaf DMR became significantly higher in GH than in GC (Fig. 2F). The one-week post-infiltration growth under sole-source lighting tended to increase leaf DMR in plants grown in GH before infiltration but not in GC. In GH, CVs of leaf biomass (33.7% and 23.7% for FM and DM, respectively) were comparable to those of leaf HA content per biomass (26.4 and 34.9%). The inter-batch variation of leaf biomass at harvest in GH, most of which was due to variation in the amount of light during the pre-infiltration process (Table 2), was thus another significant source of variation in leaf HA content per plant.

Surprisingly, in Exp. 1, leaf FM and DM in GH at 34 DPS was significantly greater than that in GC, although the cumulative PPFD was lower in GC than in GH (Table 2). Although the reason remains undetermined, the inter- and/or intra-daily fluctuations in PPFD in GH might have a negative influence on dry matter production (Morales and Kaiser, 2020). For example, daily integral of net photosynthesis of a leaf under conditions where PPFD considerably fluctuates can be lower than that estimated based on a steady-state PPFD-response curve of net photosynthesis of the leaf, due to the delayed response of photosynthesis to a sudden increase in PPFD (photosynthetic induction) (Taylor and Long, 2017; Viallet-Chabrand et al., 2017; Tanaka et al., 2019), although the significance of the effect may be fluctuating pattern-dependent (Matsuda et al., 2021). Also, under a given cumulative PPFD condition, PPFD fluctuations will generally lead to a lower temporal integral of net photosynthesis than a constant PPFD, given a nature that a PPFD-response curve of net photosynthesis is convex upward (Murakami and Jishi, 2022).

Table 3. Leaf number and stem length one day before vacuum infiltration (34 DPS) and at harvest (41 DPS).

| Experiment number | Treatment | Leaf number | Stem length [cm] |
|-------------------|-----------|-------------|------------------|
|                   |           | 34 DPS *    | 41 DPS          |
|                   |           | 34 DPS *    | 41 DPS          |
| 1                 | GC †      | 11 ± 0.2 *  | 14 ± 0.2        |
|                   | GH †      | 11 ± 0.2    | 14 ± 0.3        |
| 2                 | GC        | 11 ± 0.3    | 14 ± 0.3 *      |
|                   | GH        | 10 ± 0.2    | 13 ± 0.3        |
| 3                 | GC        | 11 ± 0.2 *  | 14 ± 0.3 *      |
|                   | GH        | 10 ± 0.1    | 12 ± 0.2        |
| 4                 | GC        | 12 ± 0.2    | 15 ± 0.4 *      |
|                   | GH        | 12 ± 0.2    | 14 ± 0.3        |
| 5                 | GC        | 12 ± 0.2    | 15 ± 0.3        |
|                   | GH        | 12 ± 0.2    | 15 ± 0.2        |
| CV (%)            | GC        | 7.5         | 3.7             |
|                   | GH        | 8.2         | 7.0             |

*DPS: days post-seeding.
†GC: growth chamber; GH: greenhouse.
*Mean ± standard error of the mean. Asterisks (*) represent significant differences between GC and GH in each experiment.
*CV: coefficient of variation among five experiments.

Fig. 1A, 2A, 3A, 4A, 5A
Table 3 shows the leaf number and stem length measured at 34 and 41 DPS. The greater $CV_{GH}$ than $CV_{GC}$ was pronounced for stem length, while the difference between $CV_{GC}$ and $CV_{GH}$ was unclear for the leaf number. Leaf number was significantly smaller in GH than in GC in a few experiments. Stem length was significantly greater in GH than in GC at both 34 and 41 DPS in all experiments.

3.4 Growth before vacuum infiltration

Before infiltration, RGR, NAR, and LAR in GC were relatively consistent among experiments: $CV_{GC}$ was less than 5% (Fig. 3A, C, E). In GH, although $CV_{GH}$ of RGR was only 7.2%, $CV_{GH}$ of NAR and LAR was greater, 27.0 and 19.8%, respectively. The trend of NAR among experiments in GH was similar to that of the cumulative PPFD (Table 2). This is because NAR reflects net photosynthesis per unit leaf area of a plant, which is mainly dependent on the absorbed light energy of the plant. The trend of LAR among experiments in GH appears negatively correlated with that of NAR. This indicates that the lower NAR in Exps. 2–4 in GH was partly compensated for by the higher LAR, contributing to the relatively stable RGR among experiments in GH. Figures 4A and C show the inter-batch variation of LMR and SLA at infiltration. The inter-batch variation of LAR in GH was mostly due to variations in SLA, not LMR.

3.5 Growth after vacuum infiltration

After infiltration, RGR for both GC and GH treatments drastically declined from those before infiltration (Fig. 3B). The $CV_{GC}$ increased while the $CV_{GH}$ decreased compared with those before infiltration, although $CV_{GH}$ was still higher than $CV_{GC}$. The NAR and LAR were also reduced in GC and GH (Fig. 3D, F). The daily PPFD integral after infiltration was almost unchanged in GC and even increased in Exps. 2–4 for GH from before infiltration (Table 2). Although the light availability was...
not reduced, NAR decreased after the infiltration (Fig. 3A, B). This was probably because leaf photosynthetic activity was significantly downregulated by viral vector inoculation and foreign protein overexpression, as we reported previously (Matsuda et al., 2018b).

In Exps. 2 and 3, RGR after infiltration in GH was significantly higher than that in GC (Fig. 3B), although RGR before infiltration was lower in GH than in GC (Fig. 3A). This reversal was likely brought about by the higher LAR in GH at the timing of infiltration (Fig. 3E); the higher LAR contributed to the increased light absorption per plant, although the daily PPFD integral after infiltration was the same for both pre-infiltration GC and GH conditions (Table 2).

The trends of inter-batch variation of LMR and SLA after infiltration (Fig. 4B, D) were similar to those before infiltration (Fig. 4A, C). However, the difference in SLA after infiltration between GC and GH became smaller than that before infiltration. The one week of plant growth under a controlled environment in the growth chambers after infiltration might have alleviated the effects of climate conditions before infiltration on the leaf morphology. Nevertheless, the effects appeared too small to eliminate the batch-to-batch variation of leaf biomass at harvest (Fig. 2B, D).

3.6 Relationship between growth and leaf hemagglutinin content

I verified the relationships between leaf HA content and various growth parameters. I found a significant ($p = 0.04$) linear regression between leaf HA content per DM and leaf DM at vacuum infiltration across growth conditions (GC or GH) and seasons (Exps. 1–5) (Fig. 5). The $r^2$ of 0.49 implies that the variation of leaf DM or any related variable at infiltration may account for approximately 50% of the observed variation of leaf HA accumulation per DM after infiltration. We previously found a similar trend in HA-expressing $N. benthamiana$ plants grown under different PPFDs and air temperatures before infiltration under sole-source lighting (Matsuda et al., 2019). The positive correlation suggests that leaf biomass at the timing of agroinfiltration, which varies depending on environmental and cultural conditions before agroinfiltration, influenced not only leaf biomass at harvest directly but also leaf HA accumulation per biomass after infiltration indirectly. This demonstrates the importance of stable biomass production for minimizing the inter-batch variability of HA production per plant. The detailed mechanism underlying the role of environmental conditions including PPFD before infiltration in HA accumulation after infiltration is unclear. Resources that can be utilized for protein synthesis, such as nonstructural carbohydrates, could be enriched in leaves with increased biomass at infiltration due to a high cumulative PPFD condition (Matsuda et al., 2019).

4. Conclusions

Seasonal changes in cumulative PPFD of sunlight in a greenhouse for pre-infiltration plant growth led to a substantial inter-batch variability of leaf HA content per plant, which corresponded to 58% of CV and was significantly larger than under sole-source lighting condition in a controlled environment. A considerable variation of...
leaf biomass among batches and also a relatively greater variation of leaf HA content per biomass under sunlight contributed to the inter-batch variability of plant-level HA productivity. I also found that leaf HA content per biomass at harvest was positively correlated with leaf biomass at agroinfiltration. This correlation strongly suggests that strictly regulated plant growth conditions before agroinfiltration are crucial for obtaining minimal batch-to-batch variability of target protein production from transient gene expression. Although vertical farms require higher investments than greenhouses, the increased productivity and reproducibility that vertical farms can offer outweigh this disadvantage, as Huebbers and Buyel (2021) suggested. I conclude that in this transient gene expression system for HA production, light environment control before gene transfer to enable consistent PPFD conditions among batches is important for mitigating the inter-batch variability. Indoor facilities with sole-source lighting are therefore more appropriate than greenhouses for plant cultivation both before and after gene transfer.

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