Alternating Red/Blue Light Increases Leaf Thickness and Mesophyll Cell Density in the Early Growth Stage, Improving Photosynthesis and Plant Growth in Lettuce

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INTRODUCTION

Cultivation in a closed-type plant factory with artificial lighting enables year-round production of crops with a stable yield and uniform quality. This is in contrast to cultivation in fields and in sunlight-type plant factories where environmental fluctuations can reduce crop yield and quality. Therefore, closed-type plant factories are expected to be applicable to harsh environments inadequate for crop production and to lead to improved global food security (Kozai, 2013; Anpo et al., 2019). However, the high electricity cost due to the artificial lighting diminishes the benefit of improved sales, which hinders new entry into the business (Kozai and Niu, 2020). Identifying optimal irradiation methods to maximize crop yield without increasing electricity costs could enhance the benefits of closed-type plant factories and lead to an expansion in operations.

In most cases, artificial red (R) and blue (B) light from light-emitting diodes (LEDs) is used for cultivation in plant factories since these wavelengths are specifically absorbed by chlorophyll to drive photosynthetic processes (Plünderl and Baake, 1990; Massa et al., 2008). In addition, monochromatic R or B light alone is unsuitable for crop production, because compared to simultaneous R+B (RB) light or white (W) light, R light alone decreases photosynthetic rate and biomass, and leads to abnormal shape (Goins et al., 1998; Wang et al., 2015), and B light alone decreases stem length, leaf area, and photosynthetic rate due to chloroplast avoidance response (Wada et al., 2003; Kim et al., 2004). It is widely recognized that simultaneous RB light is a promising irradiation procedure for vegetable plants including pepper (Piper nigrum), lettuce (Lactuca sativa L.), spinach (Spinacia oleracea), radish (Raphanus sativus var. sativus), tomato (Solanum lycopersicum), rapeseed (Brassica napus), and cucumber (Cucumis sativus L.) (Brown et al., 1995; Yorio et al., 2001; Nanya et al., 2012; Li et al., 2013; Miao et al., 2019). Previous studies have attempted to find optimal controls of R and B light, including intensity of photosynthetic photon flux density (PPFD) (Yanagi et al., 1996; Zha and Liu, 2018), length of photo-period (Jao and Fang, 2004; Jishi et al., 2016), and ratio of R light to B light (Okamoto et al., 1997; Hogewoning et al., 2010; Borowski et al., 2015, Wang et al., 2016).

Recent studies also report that the patterns of R and B light irradiation affect plant growth. For instance, Shimo-kawa et al. (2014) found that alternating irradiation with R and B LEDs (12 hours R : 12 hours B) enhanced growth in leafy lettuce (Lactuca sativa L. cv. ‘Summer Surge’), compared with lettuce grown under W light or simultaneous RB light (12 hours R : 12 hours dark) with the same daily light integrals. This phenomenon cannot be explained by a difference in day length, because alternating irradiation of red and blue (R/B) light also promoted lettuce...
growth relative to RB under similar day length and daily light integral (Chen et al., 2017; Kuno et al., 2017; Ohtake et al., 2018; Takasu et al., 2019). More recently, Chen et al. (2019) reported that an alternating interval of 1 hour promoted growth and sugar accumulation in lettuce more than that under an alternating interval of 4 hours. These results suggest that the alternating R/B light procedure enables further enhancement of crop productivity and in other words, could shorten the cultivation period to decrease production costs in closed-type plant factories. However, the physiological mechanisms underlying the phenomenon remain unclear.

In our previous study, we compared the growth properties of lettuce ‘Summer Surge’ grown under W fluorescent light, RB light, and alternating R/B light with same daily light integrals (Ohtake et al., 2018). The results showed that the fresh weight, dry weight, and leaf area were enhanced in the later growing stage (22 to 31 days after sowing) under R/B conditions relative to that under W or RB conditions. Additionally, the plants under R/B had higher net assimilation rate, which might be partly explained by less self-shading and higher leaf photosynthetic rate. However, the photosynthetic measurements in that study were conducted at a single point in time in the later growth stage and no further assessments of leaf physiological or anatomical changes were conducted. In the current study, we examined the effects of alternating R/B irradiation on the anatomical properties at a cellular level, photosynthetic activity, and traits relating to photosynthesis at two growing stages (i.e., early and late growth stages) in comparison with W light and simultaneous RB irradiation.

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MATERIALS AND METHODS

Plant materials and growth conditions

Leaf lettuce seeds (‘Summer Surge’; Takii Seed Co., Kyoto, Japan) were sown in sponge blocks, and the seedlings were grown at 23°C under cool white fluorescent lamps with a PPFD of 140 μmol m⁻² s⁻¹ (FHF32-EX-N-H; Panasonic Corp., Osaka, Japan) and a 14-hour photoperiod per day. At 10 days after sowing (DAS), the seedlings were transplanted into growth chambers equipped with B and R LEDs (Showa Denko K.K., Tokyo, Japan) or white LEDs (LDL40S.N/24/39.N4; Mitsubishi Electric Corp., Tokyo, Japan) with an electrical conductivity of 2.1 dS m⁻¹ and a pH of 6.0–6.5. The air temperature, relative humidity, and CO₂ concentration were set to 23°C, 70%, and 1,000 μmol mol⁻¹, respectively. The planting density was 37.6 plants m⁻² from 10 to 16 days, and then decreased to 16.1 plants m⁻² from 16 to 35 days to minimize mutual shading between plants.

Plant growth analysis

 Marketable fresh weight, total dry weight, projected leaf area (PLA), maximum leaf length, and maximum leaf...
width were measured in plants harvested at 16 and 35 DAS. PLA was measured in image analysis software (LI-3100; Li-Cor). Leaf anatomy was described previously (Maeshiro et al., 2013). Leaf dry matter ratio (DMR) was calculated in the youngest expanded leaves as dry weight per fresh weight without petiole. DMR was used to derive the dry weight of leaf discs of approximately 1.0 cm² immersed in liquid nitrogen, and stored at −80°C until quantification. The leaf thickness and mesophyll cell density, which were measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Leaf anatomy

Samples were taken from the central portion of the youngest expanded leaves at 16 and 35 DAS and fixed in 50 mM phosphate buffer (pH 7.2) containing 3% paraformaldehyde and 2.5% glutaraldehyde for prefixation. The leaf samples were then transferred to 1% osmium tetroxide in the same buffer for postfixation. The samples were dehydrated in a graded ethanol series, and embedded in Araldite resin (Nissin EM, Tokyo, Japan). Semi-thin sections (3 μm thickness) were prepared with a diamond knife on a Leica EM UC7 ultramicrotome (Leica Microsystems, Wien, Austria), mounted on glass slides, and stained with 1% toluidine blue O. The sections were observed under a light microscope (Olympus EX43; Olympus, Tokyo, Japan), and photographed with a digital camera (Olympus DP26) connected to the light microscope. Eight leaves from each growth condition were sectioned (n = 8), and a single slice from each leaf was selected for image analysis. The leaf thickness and mesophyll cell density in the image of each slice were measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Determination of chlorophyll and Rubisco contents

Immediately after the measurements of gas exchange, leaf discs of approximately 1.0 cm² were sampled, immersed in liquid nitrogen, and stored at −80°C until quantification. The chlorophyll was extracted with 80% (v/v) acetone and quantified by the procedure of Porra et al. (1989), and the Rubisco content was quantified by SDS-PAGE according to the method described in Yamori et al. (2005).

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured in lettuce leaves at 16 DAS under three different light conditions by using imaging pulse amplitude modulated (PAM) fluorometry (Imaging-PAM; Hennig Walz, Effeltrich, Germany), which applied the same array of blue LED (peak wavelength, 470 nm) for fluorescence excitation, actinic illumination, and saturating light pulses. The light response curves of chlorophyll fluorescence parameters were obtained through the application of a series of light exposures with increasing of PPFD (37, 98, 189, 311, 467, and 655 μmol m⁻² s⁻¹). The leaves were acclimated for 5 minutes at each light level, and, during the time, they were exposed to saturating pulse every 30 seconds (in total 10 flashes). The quantum yield of photosystem II (ΦPSII = (Fm’−F)/Fm’) was calculated (Baker, 2008). The electron transport rate (ETR) was calculated as ETR = 0.5 × abSI × ΦPSII, where 0.5 is the fraction of absorbed light reaching PS II and abSI is absorbed irradiance taken as 0.85 of incident irradiance (Genty et al., 1989).

Measurement of gas exchange

Gas exchange was measured with a portable gas exchange system (LI-6400; Li-Cor Inc., Lincoln, NE, USA) as described previously (Yamori et al., 2012; Zhang et al., 2015). A clear-top leaf chamber was used to analyze leaf gas exchange under each of the growth light conditions. For the youngest expanded leaves of the plants from 28 to 35 DAS, CO₂ assimilation rate (A), stomatal conductance (gₛ), and intercellular CO₂ concentration (Cᵢ) at CO₂ concentration of 1,000 μmol mol⁻¹ in growth chambers were continuously measured for 6 hours after 30 minutes adaptation (measurements under R12/B12 were started at 3 hours before shifting R to B or B to R to obtain gas exchange data across both irradiation patterns). Mean daily carbon gains were estimated from the CO₂ assimilation rate under the three light conditions (the mean daily carbon gain under R12/B12 was estimated from the mean net photosynthetic rate of the R light period and the B light period as described previously) (Chazdon, 1986; Ellsworth and Reich, 1992).

Morphologies of stomata and pavement cells

At 16 and 30 DAS, the youngest fully expanded leaves under each light condition were sampled. The epidermal cell density was measured following the method of Ceulemans et al. (1995). Briefly, epidermal tissue near the central vein of the leaf was peeled off using colorless nail polish and adhesive cellophane tape, and observed under a light microscope (DMBA310; Shimadzu Co., Kyoto, Japan). The number and size of the epidermal cells and stomata were determined with Image J software. Surface cell density was calculated as the number of epidermal cells and stomata (e+s) per unit leaf area, and stomatal index (SI) was calculated as (s/[e+s])×100.

Statistical analysis

The obtained values among irradiation treatments were compared by Tukey’s post-hoc test or Dunnett’s test in Excel software (BellCurve for Excel, v. 2.15; Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Plant growth

The plant growth parameters for the leafy lettuce grown under each irradiation condition are shown in Table 1. Marketable fresh weight and dry weight at 35 DAS were the highest under R12/B12 treatment, followed by RB24 and then W24, while less difference was found among the treatments at 16 DAS. Consistent with these results, PLA at 35 DAS was the greatest under R12/B12 and was similar under RB24 and W24. Maximum leaf length under R12/B12 was already enhanced relative to RB24 and W24 at 16 DAS, and the difference was kept until 35 DAS (Table 1).

Leaf anatomy

The leaf thickness and mesophyll cell density, which were observed from light microscope images of leaf cross-sections (Fig. 2A), were highest under R12/B12, followed by RB24 and then W24 at 16 DAS (Fig. 2B, 2C).
Das, these values under R12/B12 were higher than under W24, and were similar to those under RB24 (Fig. 2B, 2C). LMA under R12/B12 was highest at 16 DAS among all treatments and was significantly higher than W24 at 35 Das (Fig. 2D).

The morphological changes in stomatal and epidermal cells of the abaxial leaf surface were also analyzed (Fig. S1). There was little variation in these traits among the treatments at 16 Das, whereas stomatal density and surface cell density significantly increased, and epidermal cell area significantly decreased under R12/B12 at 30 Das.

**Photosynthetic activity**

No significant difference in total Chlorophyll a+b content was observed at either 16 or 35 Das, while Rubisco content under R12/B12 was highest among the treatments at 16 Das, and it was higher than W24 and similar to RB24 at 35 Das (Fig. 3).

In the early stage at 16 Das, the response of ETR to PPFD was significantly higher under R12/B12 and RB24 than under W24, especially at PPFD of ≈98 µmol m⁻² s⁻¹ (Fig. 4, Supplemental Table S1).

We also analyzed the CO₂ assimilation rate of plants at 28–35 Das with gas exchange analysis (Fig. S). A₁₆₀₀ and gₛ were always higher under RB24 than under W24 (Fig. 5A, 5B). A₁₆₀₀ under R12/B12 showed a clear shift following the R to B light phase change: A₁₆₀₀ was highest in the R-light phase and lowest in the B-light phase among all treatments (Fig. 5A). The value of gₛ in the R-light phase decreased to a level similar to that of W24, and it increased in the B-light phase to a level similar to that of RB24 (Fig. 5B). Cᵢ values also showed a pattern similar to that of gₛ, under R12/B12 conditions (Fig. 5C). The daily carbon gain, which was estimated by the integration of A₁₆₀₀, was significantly greater under R12/B12 and RB24 than under W24 (Fig. 5D).

**DISCUSSION**

In closed-type plant factories with artificial light, improving production efficiency without increasing electricity costs is a challenging issue. Several recent studies have showed alternating R/B could be a promising approach to enhancing lettuce productivity and income in closed-type plant factories (Shimokawa et al., 2014; Chen et al., 2017, 2019; Kuno et al., 2017; Takasu et al., 2019). However, the physiological reason why alternating R/B can enhance the productivity is largely unknown. This study analyzed the effects of alternating R12/B12 irradiation on the anatomical properties at a cellular level, photosynthetic activity, and traits relating to photosynthesis.

In general, larger biomass production is caused by rapid expansion of leaf area or by enhanced photosynthetic rate per unit leaf area, or by both (Gifford and Evans, 1981). In our previous study, growth analysis showed that the net assimilation rate, which is the primary indicator of the rate of photosynthesis, was enhanced under R12/B12 relative to that under other treatments, especially during 16 to 31 Das, resulting in increased relative growth rate (Ohtake et al., 2018). In this study, we found that the ETR at 16 Das and daily carbon gain per unit leaf area at 28–35 Das were higher under R12/B12 and RB24 than under W24, indicating that the use of R and B lights promotes leaf photosynthetic rate (Figs. 4, 5). This is mainly due to the difference in the wavelength absorption efficiency of chlorophyll between R, B and W lights under the same DLI as mentioned in introduction. However, we observed less significant difference in ETR and daily carbon gain per unit leaf area between R12/B12 and RB24 both in the Imaging-PAM and in the gas exchange measurements (Figs. 4, 5). On the other hand, Rubisco content was significantly higher under R12/B12 at 16 Das, while less significant difference in Rubisco content was observed between R12/B12 and RB24 at 35 Das (Fig. 3A). Rubisco, the initial enzyme of carboxylation in C₃ plants, has long been considered a limiting factor of photosynthesis in several crop species (Makino et al., 1988; Evans, 1989). Furthermore, ETR does not correlate with CO₂ assimilation rate in some environments such as high light intensity and low CO₂ concentration, despite we used ETR as a proxy of CO₂ assimilation rate at 16 Das in this study (Beer and Axelson, 2004; Miyazawa and Yabuta, 2006). This suggests that the higher Rubisco content under R12/B12 reflects the higher photosynthetic carbon gain than under RB24, at least in the younger growth stage. We also observed that alternating R/B irradiation increased maximum leaf length and PLA across the growth stages relative to the other light conditions, which could be due to the delayed senescence of leaves under R12/B12.
conditions (Table 1), despite total leaf areas being similar among the growth light conditions (Ohtake et al., 2018). The longer leaf and greater PLA would facilitate light acquisition by minimizing self-shading, and would enhance photosynthetic carbon gain per plant (Takenaka, 1994; Enríquez and Pantoja-Reyes, 2004). Therefore, we conclude that the alternating R/B light improved plant biomass by the enhancement of net assimilation rate via the higher efficient light capture and leaf photosynthetic rate.

We found a significant change in leaf anatomy with changes in light conditions. Alternating R/B enhanced leaf thickness and mesophyll cell density across the growth period (Fig. 2). Simultaneous RB also improved these traits, especially in the later growth stage (Fig. 2). It is well known that an enhanced number of mesophyll cells increases leaf thickness and photosynthetic apparatus, thereby increasing photosynthetic rate (Reich et al., 1991; Poorter et al., 2014). In our results, the leaf thickness,
to CO2 assimilation rate is smaller than Rubisco content. Our result suggests that the influence of chlorophyll content is important proteins consisting photosynthetic machineries, DAS (Fig. 3B). Although chlorophyll is one of the most abundant pigments, the correlation between the irradiation treatments at both 16 and 35 days after sowing (DAS) was not significant (Fig. 2, 3). However, we found no significant difference in leaf chlorophyll content among the three light treatments by Dunnett’s test at \( P < 0.05 \). Columns and bars represent mean±SE (n = 8).

**Fig. 3** (A) Rubisco contents and (B) Chlorophyll (Chl) \( a+b \) under W24, RB24, and R12/B12 at 16 and 35 days after sowing (DAS). Columns labeled with the same letter are not significantly different among the three light treatments by Dunnett’s test at \( P < 0.05 \).

LMA, mesophyll cell density, and Rubisco content were well correlated in each growth stage, suggesting that the enhanced mesophyll cell density increases leaf photosynthetic rate under R12/B12 and RB24 (Fig. 2, 3). However, we found no significant difference in leaf chlorophyll content between the irradiation treatments at both 16 and 35 DAS (Fig. 3B). Although chlorophyll is one of the most important proteins consisting photosynthetic machineries, our result suggests that the influence of chlorophyll content to \( \text{CO}_2 \) assimilation rate is smaller than Rubisco content. This is consistent to Evans (1989) who reported that leaf chlorophyll content largely varied depending on the light intensity, and had minor effects on photosynthetic capacity relative to leaf nitrogen content and Rubisco content. R and B lights are received by different photoreceptors including phytochromes (for R), and crytrophoromes and phototropins (for B) (Liscum, 2016; Sánchez-Lamas et al., 2016; Gärtnner, 2017). Activation of these photoreceptors and the signal transduction pathways are expected to differ depending on the received light quality (Kami et al., 2010; Bantis et al., 2018). Growth under monochromatic R light is suppressed relative to that under W light, but supplementing R light with B light enhances plant growth (Kim et al., 2004; Hirai et al., 2006). In addition, LMA and photosynthetic rate are enhanced as the ratio of B to R light is increased in cucumber (Hogewoning et al., 2010; Hernández and Kubota, 2016) and in lettuce (Dougher and Bugbee, 2001, 2004), whereas leaf area is not promoted by B light in lettuce (Oshima et al., 2015). Thus, it seems that blue light contributes to increasing leaf thickness rather than increasing leaf area in lettuce cultivation. Considering the high correlation between LMA, leaf thickness and mesophyll cell density, it could be explained that relative to W light, besides the advantage of high absorption efficiency of chlorophyll to R and B lights, activated signaling pathways of B light should also promote the mesophyll cell division under monochromatic R light conditions, leading to the increase in the photosynthetic rate. Further, the alternating R12/B12 light enhanced leaf thickness and mesophyll cell number from the early growth stage relative to RB24 (Fig. 2). The reason for this is still unclear, but we hypothesize that there would be conflicts between R and B signal transduction, such as the antagonistic relationship between phytochromes and cryptochromes in the induction of flowering (Mockler et al., 1999). Shimokawa et al. (2014) and Chen et al. (2017) speculated that if there is any conflict between the photosresponse to R and B light, then alternating irradiation may resolve the conflict, leading to more efficient growth. This speculation can be applied to the enhanced leaf thickness and mesophyll density, as well as the increased leaf length under R12/B12 light. This hypothesis can be tested via omics and transgenic approaches in terms of light signaling cascades. Importantly, this is the first study to verify that alternating R/B light affects leaf anatomical traits.

We also found unique changes in epidermal morphologies. RB24 had less effect on the epidermal cells, while R12/B12 decreased epidermal cell area and increased epidermal and stomatal cell density, especially in
the later growth stage (Table S1). The higher stomatal density is probably linked to the increased number of mesophyll cells and the increased photosynthetic rate under R12/B12 (Kundu and Tigerstedt, 1999; Bergmann, 2004; Kondo et al., 2010; Pillitteri and Torii, 2012; Tanaka et al., 2013). Although it is reported that simultaneous RB light increases stomatal density relative to monochromatic R or B light (Wang et al., 2016), no report of effects of alternating irradiance of R/B light on stomatal and epidermal cell densities is available. The mechanisms and physiological implications of these changes need to be clarified.

**CONCLUSION**

In conclusion, the higher lettuce growth under alternating R/B lights is attributable to the higher leaf photosynthetic rate and more efficient light capture due to the minimal self-shading. The leaf morphological changes in mesophyll cells and epidermal cells induced by the alternating light could be associated with the photosynthetic difference. Further research using transgenics and omics approaches, including metabolome, hormonome, and transcriptome, will be needed to clarify the mechanisms underlying the photoresponses at the molecular level.

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