Study of the beneficial effects of green light on lettuce grown under short-term continuous red and blue light-emitting diodes

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Red and blue light are the most important light spectra for driving photosynthesis to produce adequate crop yield. It is also believed that green light may contribute to adaptations to growth. However, the effects of green light, which can trigger specific and necessary responses of plant growth, have been underestimated in the past. In this study, lettuce (*Lactuca sativa* L.) was exposed to different continuous light (CL) conditions for 48 h by a combination of red and blue light-emitting diodes (LEDs) supplemented with or without green LEDs, in an environmental-controlled growth chamber. Green light supplementation enhanced photosynthetic capacity by increasing net photosynthetic rates (P_n), maximal photochemical efficiency (F_v/F_m), electron transport for carbon fixation (J_{PSII}) and chlorophyll content in plants under the CL treatment. Green light decreased malondialdehyde and H_2O_2 accumulation by increasing the activities of superoxide dismutase (SOD; EC 1.15.1.1) and ascorbate peroxidase (APX; EC 1.11.1.11) after 24 h of CL. Supplemental green light significantly increased the expression of photosynthetic genes *LHCb* and *Psb*A from 6 to 12 h, and these gene expression were maintained at higher levels than those under other light conditions between 12 and 24 h. However, a notable down-regulation of both *LHCb* and *Psb*A was observed during 24 to 48 h. These results indicate that the effects of green light on lettuce plant growth, via enhancing activity of

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particular components of antioxidantive enzyme system and promoting of *LHCb* and *Psb*A expression to maintain higher photosynthetic capacity, alleviated a number of the negative effects caused by CL.

Abbreviations – APX, ascorbate peroxidase enzyme; CAT, catalase enzyme; Chl a, chlorophyll a; Chl b, chlorophyll b; CL, continuous light; F_v/F_m, maximal photochemical efficiency of PSII; F_v'/F_m', the efficiency of excitation capture by open PSII centres; J_{fD}, light-independent thermal dissipation and fluorescence rate; J_{NPQ}, rate of energy dissipated by light-dependent process; J_{PSII}, total electron transport rate; P_n, net photosynthetic rate; LEDs, light emitting diodes; *LHCb*, light-harvesting chlorophyll binding a/b-protein related gene; LMA, leaf mass per area; MDA, malondialdehyde; POD, peroxidase; *Psb*A, D1protein related gene; PSII, photosystem II; qP, photochemical quenching of PSII; R/B, red to blue light ratio.

Introduction

Light is the driving force of plant growth and development. The quantity and quality of light for photosynthesis is a combination of its duration, spectrum, and photosynthetically active radiation (Bian et al. 2015, Li and Kubota 2009). Continuous light (CL) is a potential method to increase crop production in a protected environment and is also a useful tool for speeding crop selection (Sysoeva et al. 2010, Velez-Ramirez et al. 2011). Therefore, the use of CL has been widely studied in many species, including tomato (Ohyama et al. 2005, Velez-Ramirez et al. 2014), eggplant (Murage et al. 1996), lettuce (Sysoeva et al. 2010) and pepper (Demers et al. 2002). However, CL has been shown to induce negative effects in several plant species, and the most visible negative effect induced by CL is chlorosis (Pettersen et al. 2010, Tibbitts et al. 1990). Long-term CL induces decreases in photochemical quenching (Van Gestel et al. 2005) and in the quantum yield of linear electron flux in plant leaves (Pettersen et al. 2010, Velez-Ramirez et al. 2011). Reductions in photochemical quenching and electron transport capacity not only lead to unfavourable dissipation of excess light energy but also lead to a greater propensity for light energy to generate reactive oxygen species (ROS) (Huner et al. 1998). In plants, photosystem II (PSII) is vulnerable to ROS at all light intensities, especially under excessive light stress (Murata et al. 2007), as an integral part of the reaction core of PSII, specifically the D1 protein (also known as PsbA), is sensitive to ROS generated by various abiotic stresses, such as excessive light stress, high light stress (Herbstová et al. 2012) and heat and cold stress (Sen et al.

2014). The accumulation of ROS not only inhibits the de novo synthesis of D1 protein (Qian et al. 2009) but also can stimulate the degradation of D1 protein (Nishiyama et al. 2004). Under abiotic stress, the imbalance between the synthesis and degradation of D1 protein is the main reason leading to photodamage and decreased photosynthetic capacity in plant leaves (Sen et al. 2014). The *Psb*A is the key gene that encodes the D1 protein, and *Psb*A expression under stress is critical in the de novo synthesis of the D1 protein and the repair of photodamage of PSII (Andersson and Aro 2001). In plants, *Psb*A transcription is light stimulated (Klein and Mullet 1990). However, previous studies reported that short-term CL leads to decreases in *Psb*A transcription (DuBell and Mullet 1995, He and Vermaas 1998). Besides being modulated by light intensity and light duration, *Psb*A expression in plants is affected by light spectra (Bissati and Kirilovsky 2001). However, the effects of light spectra on *Psb*A expression in lettuce under short-term CL are still unclear.

In addition to D1 protein, the light-harvesting chlorophyll a/b-binding protein of photosystem II (LHCb) is another important protein for maintaining high photosynthetic efficiency in plant leaves. The LHCb protein, which is encoded by the *LHCb* gene family, is the apoprotein of the light-harvesting complex of PSII (LHCII). LHCb collects and transfers light energy to photosynthetic reaction centres (Jansson 1999). Under abiotic stress, excessive photosynthetically active radiation can decrease *LHCb* expression to alleviate further stress-generated damage to LHCII (Karpinski et al. 1997). Thus, the modulation of *LHCb* expression is regarded as one of the most important mechanisms for plants to tolerate environmental stress (Ganeteg et al. 2004). However, there is limited knowledge reported in the literature on the effects of light spectra on *LHCb* expression under CL by light-emitting diodes (LEDs).

CL-induced injury can be attributed to several environmental factors (Velez-Ramirez et al. 2011). In addition to light intensity, light spectral distribution influences the degree of injury caused by CL, but CL-induced injury is more complex than light intensity-induced injury (Demers and Gosselin 2000). Continuous red light alone or a high percentage of blue light within CL can induce severe leaf injury and reduce photosynthesis in leaves (Murage et al. 1997). However, Globig et al. (1994) reported that far-red light supplementation reduced CL-induced injury generated by red light. Regarding light spectra, red and blue light are more efficient at regulating plant physiological processes, especially photosynthetic functions (Whitelam and Hallidy 2007). However, green light has been proven to drive

leaf CO_2 fixation more efficiently than red light when combined with other light qualities, especially in strong white light (Sun et al. 1998, Terashima et al. 2009). In addition, the effect of green light on plant growth depends on the intensity of the green light (Johkan et al. 2012). Kim et al. (2004) reported that red and blue LED supplied with 24% green light (approximately 36 μmol m^{-2} s^{-1}) showed higher efficiency at facilitating lettuce growth than did other treatments that consisted of different amounts of green light. Furthermore, green light can counteract stomatal opening, stem growth modulation and chloroplast gene expression directed by red and blue light (Folta and Maruhnich 2007). Thus, we hypothesize that green light supplementation might also show positive effects on alleviating the degree of photosynthetic capacity reduction and/or injury induced by CL under red and blue light.

We previously reported that green light supplementation had beneficial effects on the growth of lettuce under short-term continuous red and blue LED light treatment (Bian et al. 2016). However, little is known about the molecular mechanisms of green light regulating photosynthetic capacity, the transcriptional control of *D1*- and *LHCb*-related genes, and physiological processes during short-term CL. The aims of this study were to investigate whether green light can protect lettuce from photodamage caused by CL and to determine whether this protection involves the gene expression of *Psb*A and *LHCb*. The results should provide an insight into plant responses to differing light spectra and into the types of light sources to optimise plant viability, growth and yield when plants are grown in unfavourable conditions.

Materials and methods

Plant materials and growth conditions

Lettuce seeds (*Lactuca sativa* L. cv. Butterhead) were sown in plastic trays filled with a seed-peat mixture (1:1, v/v) substrate and then grown in an environmental-controlled growth chamber. Fluorescent lamps (TL-D 36 W, Philips) were used as light sources for seedling growth. The day/night temperature, photosynthetic photon flux density (PPFD), photoperiod, CO_2 level, and relative humidity in the growth chamber were 25/20°C, 200 μmol m^{-2} s^{-1}, 12 h, 400 μmol mol^{-1} and 75%, respectively. Water was added daily to maintain the moistness of the substrate and replenish evapotranspiration losses. At 14 d after sowing, similarly sized lettuce seedlings that had two true leaves were grown in

40-l containers of Hoagland solution (pH 6.5 ± 0.2, EC 1.9 ± 0.1 dS m^{-1}). Then the plants were randomly grown under mixtures of red (R; peak wavelength: 660 nm, peak broadness at half peak height: 15 nm) and blue (B; peak wavelength: 460 nm, peak broadness at half peak height: 15 nm) LEDs (R:B = 4:1) as well as mixtures that included green (G; peak wavelength: 530 nm, peak broadness at half peak height: 21 nm) LEDs (R:B:G = 1:1:1). No-reflect black separators were placed between the different light treatments to eliminate light contamination. The light spectra and PPFD were monitored daily by a spectroradiometer (Avaspec-2048CL, Avantes, Apeldoorn, The Netherlands). The PPFD was maintained at 200 μmol m^{-2} s^{-1} by adjusting the distance between the light sources and the plant canopies. To minimize the angle impact, the containers of plants under the same light sources were systematically moved every other day. The day/night temperature, PPFD, photoperiod, CO_2 level and relative humidity were maintained at similar levels as those at the seedling stage. The solutions were replaced with freshly prepared solutions every 7 d.

Light treatment

At the end of the dark period, at 20 d after being transplanted, the plants were transferred to the experimental conditions that consisted of a PPFD of 200 μmol m^{-2} s^{-1} and temperature of 25°C. The plants grown under red and blue LEDs were treated with CL by red and blue LEDs as before (RB-CL, R:B = 4:1) or were treated with supplemental green LEDs (RBG-CL, R:B:G = 4:1:1). The plants grown under red, blue and green LEDs received CL treatment using LED light sources as before (rbg-CL, r:b:g = 1:1:1) or were treated with the same LED light sources as before but without the green light LEDs (rb-CL, r:b = 1:1). The light duration of CL and light intensity for all treatments were 48 h and 200 μmol m^{-2} s^{-1}, respectively. The details of these treatments are summarized in Table 1. RBG-CL was used to investigate whether green light induced positive effects on the photosynthetic capacity and on *Psb*A and *LHCb* expression under short-term CL versus RB-CL. rb-CL was used to investigate the effects of the red to blue light ratio (R/B) on plant photosynthetic capacity versus RB-CL and further to assay the effects of green light under CL versus rbg-CL. There were four replicates per treatment and a total of 48 plants per treatment. During the experiment, other environmental conditions were maintained at similar levels as those during the seedling stage.

Leaf area and plant growth determination

Shoot and root fresh weight, total fresh weight and leaf number of the lettuce plants were measured before (0 h) and after (48 h) treatment. The leaf area of the lettuce plants before (0 h) and after treatment (48 h) was determined by a portable leaf area meter (LI-3100C, LI-COR, Lincoln, NE). Leaf mass per area (LMA) was determined as the method of Fan et al. (2013). Eight plants (two plants per replicate, four replicates per light treatment) were randomly selected for each determination.

Chlorophyll content measurements

Leaf samples were collected from the second youngest and fully expanded leaves before treatment (0 h) and after treatment (12, 24, 36 and 48 h). The sample leaf tissue (100 mg) was subjected to extraction in 5 ml of 80% (v/v) acetone buffer at 4°C for 72 h. Four replicates were performed for chlorophyll (Chl) measurements. The absorbance of the extraction solution was determined at 645 and 663 nm by a UV-VIS spectrophotometer (UV-180, Shimadzu, Japan). The equations described by Torrecillas et al. (1984) were used to estimate the contents of Chl a, Chl b and Chl a + b.

Antioxidantive enzyme and H_2O_2 level determination

Fresh leaf samples collected before (0 h) and after (24 and 48 h) treatment were immediately frozen in liquid nitrogen and stored at –80°C, after which they were used to determine antioxidantive enzyme and H_2O_2 levels. Leaf tissue (0.1 g) was ground in 1% (w/v) polyvinylpolypyrrolidone using a chilled mortar and pestle, after which the tissue was then homogenized in 1.2 ml of ice-cold 50 m*M* phosphate buffer (pH 7.8) containing 1 m*M* EDTA-Na_2 and 0.3% Triton X-100. For ascorbate peroxidase (APX; EC 1.11.1.11) determination, 1 ml of ascorbate was added to the mixture. The extract was centrifuged at 20 000 *g* for 30 min at 4°C. The supernatant, referred to as the 'crude extract', was used to determine superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and APX enzyme activities. Four replicates were performed per measurement.

To determine the SOD activity, 3 ml of reaction mixture containing 100 µl of enzyme extract, 0.1 µ*M* EDTA, 13 m*M* methionine, 75 µ*M* nitro blue tetrazolium, and 2 µ*M* riboflavin, 50 m*M* phosphate buffer (pH 7.8) was shaken before being illuminated by 15-W fluorescent lamps. The absorbance monitored at 560 nm was used to calculate the SOD activity. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the rate of nitroblue tetrazolium chloride reduction (Wu et al. 2007). The CAT activity was determined in accordance with the methods of Bisht et al.

(1989). The APX activity was assayed using the methods of Nakano and Asada (1981). The level of H_2O_2 was spectrophotometrically determined as described by Sergiev et al. (1997).

Measurement of malondialdehyde (MDA) content

The MDA content, as a marker of lipid peroxidation, in plant leaves was determined using the method described by Yang et al. (2010). Four replicates were measured. Leaf samples were extracted using thiobarbituric acid (TBA) and were boiled at 100°C for 20 min. The supernatants were cooled to room temperature and then centrifuged at 15 000 *g* for 10 min. The absorbance monitored at 450, 532 and 600 nm was used to calculate the MDA content.

RNA isolation and gene expression assays

The total RNA was isolated from each sample using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The extracted total RNA was treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) to prevent any genomic DNA contamination before reverse transcription, in accordance with the manufacturer's instructions. The total RNA was quantified using a NanoDrop™ 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA) before and after DNase I treatment. The quality and integrity of the total RNA were checked using electrophoresis via a 1% agarose gel stained with SYBR green dye. The total RNA was reverse-transcribed using a RevertAid First Stand cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) and a 20-μl reaction mixture containing 1 μl of total RNA from each individual sample. A further check for genomic DNA with cross-intron primers of PBGD was performed to ensure the cDNA in the samples did not contain genomic DNA. The cDNA fragments were then used as templates to test their transcripts. An initial denaturing temperature at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 56°C for 30 s and a melt curve 65–95°C using a QuantiTect SYBR Green PCR Kit (Qiagen). Primers of actin were used as an internal conference.

qRT-PCR was performed independently four times, and each sample was analysed in triplicate by PCR using a 7500 Real-Time PCR System (Applied Biosystems). The quantification of gene expression was analysed by the ABI PRISM 7500 Software Tool (Applied Biosystems). Threshold values (CT) were used to quantify relative gene expression by the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Plants were randomly collected before treatment (0 h) and after treatment (6, 12, 24, 36 and 48 h). The second youngest and fully expanded leaves were used to investigate the expression of the *Psb*A and *LHCb*. Target gene sequences corresponding to the top BLAST hits were identified within the Compositae Genome Project EST database via sequence homology to known light absorption and transfer genes from existing *Lactuca sativa* L. sequence data in GenBank. Primers of the *Lactuca sativa* L. *ACTIN* gene (Accession number: AB359898.1) reported by Ebisawa et al. (2008) were used. The primers for *Psb*A and *LHCb* were designed by Primer-Premier 6.0 (Biosoft International, Palo, CA). The primers used for the qRT-PCR assays are shown in Appendix S1.

Gas exchange and chlorophyll fluorescence determination

Leaf gas exchange and Chl fluorescence of the second youngest and fully expanded leaves were determined simultaneously using an integrated fluorescence fluorometer (LI-6400XT, Li-Cor, NE) before treatment (0 h) and after treatment (12, 24, 36 and 48 h) as described by Weng et al. (2008). The minimal (F_o'), maximal (F_m'), and steady (F_s) fluorescence parameters and the net photosynthetic rate (P_n) were simultaneously monitored. Furthermore, the minimal (F_o) and maximal (F_m) fluorescence of dark-adapted leaves were measured when the leaves were dark-adapted for 30 min. During these measurements, the temperature, light intensity and CO_2 concentration in the leaf chamber of the LI-6400XT were controlled at 25°C, 200 μmol m^{-2} s^{-1} and 400 μmol mol^{-1}, respectively. The actinic light in the leaf chamber was supplied by a red/blue light source. Each measurement comprised four to six replicates.

The response of electron transport and the utilization absorbed by photosystem II (PSII) were calculated in accordance with the methods of Hendrickson et al. (2004) and Maxwell and Johnson (2000). The equations for each process are described as follows: the maximal photochemical efficiency of PSII in dark-adapted leaves (F_v/F_m) = ($F_m - F_o$)/F_m; the efficiency of excitation capture by open PSII centres (F_v'/F_m') = ($F_m' - F_o'$)/F_m'; the photochemical quenching of PSII (qP) = ($F_m' - F_s$)/($F_m' - F_o'$); the quantum efficiency of PSII (Φ_{PSII}) = ($F_m' - F_s$)/F_m'; the fraction of PSII-absorbed light energy dissipated either by light-independent thermal dissipation or by fluorescence (Φ_{fD}) = F_s/F_m; and the fraction of PSII-absorbed light energy is dissipated by light-dependent processes (Φ_{NPQ}) = $F_s/F_m' - F_s/F_m$.

The rate of energy dissipated by light-dependent process (J_{NPQ}) was calculated as $J_{NPQ} = \Phi_{NPQ} \times PPFD \times I_A \times 0.5$; the rate of light-independent thermal dissipation and fluorescence (J_{fD}) was determined as $J_{fD} = \Phi_{fD} \times PPFD \times I_A \times 0.5$; the rate of total electron transport via photochemistry (J_{PSII}) was calculated as $J_{PSII} = \Phi_{PSII} \times PPFD \times I_A \times 0.5$, where PPFD is the photosynthetic photon flux density, I_A is the absorbed irradiance assuming an average leaf absorptance of 0.85 (Zhou et al. 2007), and 0.5 is the assumed proportion of absorbed quanta used by PSII reaction centres (Melis et al. 1987). The excess energy was estimated according to Demmig-Adams et al. (1996) using the equation (Excessive energy) $= (1 - qP) \times F_v'/F_m'$.

Lincomycin treatment

To further verify the effect of green light on lettuce growth under CL, lincomycin treatment was performed. Plants (34 days after germination) were either concomitantly exposed to CL and lincomycin (0.2 g l^{-1}) or without lincomycin. Lincomycin solution was prepared by dissolving lincomycin hydrochloride (Sigma Aldrich, Munich, Germany) into water. The expression of *Psb*A and *LHCb* under the combined CL with lincomycin treatment was investigated. F_v/F_m and the rapid light response curve (corresponding to the following light intensities: 0, 30, 50, 100, 200, 500, 800 and 1200 μmol mol^{-1}) were monitored with an interval of 12 h using an integrated fluorescence fluorometer (LI-6800F, Li-Cor, Lincoln, NE).

Statistical analysis

All of the data were evaluated by analysis of variance (ANOVA) using SAS software (Version 8.1, SAS Institute, Cary, NC), and significant differences between means were assessed by Duncan's multiple range test at $P < 0.05$.

Results

Continuous light increases shoot biomass especially when red, blue and green light are combined

Before CL treatment (0 h), there were no significant differences in leaf area, leaf number, LMA, shoot fresh weight or total fresh weight of lettuce plants, but the root fresh weight under rb-CL (red:blue 1:1) and rbg-CL (red:blue:green 1:1:1) was lower than that under the other light treatments (Table 2). Total fresh weight, LMA, shoot and root fresh weight increased after the CL treatment for 48 h. The

RBG-CL (red:blue:green 4:1:1) treatment at 48 h showed higher fresh weight and LMA than did the rbg-CL and rb-CL treatments. The values of total fresh weight, LMA and leaf area under RBG-CL were higher than these under RB-CL (red:blue 4:1) , although there were no significant differences in these parameters between RBG-CL and RB-CL. Compared with the RB-CL treatment, the RBG-CL treatment especially caused a significant increase in shoot fresh weight. In addition, leaf area under rbg-CL was higher than that under rb-CL. However, green light had little effect on leaf number and root fresh weight during 48 h of CL treatment (Table 2).

Addition of green light alleviates the negative effect of red and blue light on chlorophyll content

Before CL treatment (0 h), no significant difference was observed in Chl content among the different treatments (Fig. 1). The amounts of Chl a, Chl b and Chl a + b under RB-CL and rb-CL showed constant decreases after CL treatment for 24 h, but these decreases were markedly alleviated by adding green light to the red and blue light. Much higher amounts of chl a, Chl b and Chl a + b were observed in RBG-CL and rbg-CL than in RB-CL and rb-CL between 24 and 48 h (Fig. 1A-C). The Chl a to Chl b ratio gradually increased after 24 h of CL treatment, but the ratios under RBG-CL and rbg-CL were lower than under RB-CL and rb-CL (Fig. 1D). Furthermore, the ratio of Chl a to Chl b and contents of Chl a and Chl a + b were higher under RBG-CL than these under rbg-CL between 24 and 48 h (Fig. 1A, C, D).

Photosynthetic performance is improved by addition of green light

There was no significant difference in P_n under different light conditions before CL treatment (0 h). However, the values of P_n were sharply reduced after CL for 24 h. RBG-CL caused a marked increase in P_n during the first 24 h of CL treatment and then a decrease from 24 to 48 h (Fig. 2). However, the value of P_n for RBG-CL was higher than that for RB-CL. Interestingly, the treatment without green light resulted in a severe reduction in P_n, as shown by the lower P_n value under the rb-CL treatment. Throughout this study, the value of P_n under RBG-CL was higher than that under rbg-CL, but no significant difference was observed between RB-CL and rbg-CL (Fig. 2A).

There were decreasing trends in F_v/F_m and F_v'/F_m' after 24 h of CL treatment. Treatment with a higher percentage of blue light intensified the decreases in F_v/F_m and F_v'/F_m' induced by the CL treatment, as

shown by lower F_v/F_m and F_v'/F_m' values under rb-CL than under RB-CL. However, green light supplementation eliminated the reduction in the F_v/F_m and F_v'/F_m' values of plants exposed to CL treatment (Fig. 2B, C). The qP under RBG-CL was higher than that under other CL treatments between 12 and 48 h. Compared with the other treatments, the rb-CL treatment led to lower qP values at 36 and 48 h (Fig. 2D).

Addition of green light promotes the electron transport and utilization

Before the CL treatment (0 h), the level of J_{PSII} under rb-CL and rbg-CL was lower than that under RB-CL and RBG-CL, but there were no significant differences in J_{PSII} among these treatments. Between 24 and 48 h, the J_{PSII} for RBG-CL and rbg-CL was higher than that for RB-CL and rb-CL, respectively (Fig. 3A). These results suggest that green light showed positive effects on maintaining a high J_{PSII} under CL. The value of J_{fD} for RBG-CL was higher than that for RB-CL, and the treatment without green light from rbg-CL caused significant decreases in J_{fD}. There was no significant difference in J_{fD} between RB-CL and rb-CL except at 0 h, suggesting that a change in R/B had little effect on J_{fD} under the CL treatment (Fig. 3B).

Unlike J_{fD}, RBG-CL had a lower J_{NPQ} than did RB-CL at 12 and 36 h, but this parameter for rb-CL was higher than that for rbg-CL between 12 and 48 h. The highest value of J_{NPQ} was observed under rb-CL, followed by RB-CL, RBG-CL and then rbg-CL during the period from 24 to 48 h (Fig. 3C). Except RBG-CL, the CL treatments caused excessive energy accumulation in the leaves. The level of excessive energy for RBG-CL showed a constant deceasing trend between 0 and 36 h before reaching the same level as that at 0 h again at 48 h. Interestingly, the levels of excessive energy in plants under RBG-CL and rbg-CL were lower than those under RB-CL and rb-CL, respectively (Fig. 3D).

Green light supplementation enhances antioxidantive enzyme activity

The activities of the antioxidantive enzymes and H_2O_2 levels in lettuce leaves are presented in Fig. 4. The SOD activity for the RBG-CL treatment was higher than that for the other treatments at 24 and 48 h. However, no significant differences were observed in SOD activity between rb-CL and rbg-CL (Fig. 4A). The CAT activity for RBG-CL remained steady during the 48 h of CL. However during the same period, this parameter for other treatments showed an increasing trend, and the values were higher

than those for RBG-CL at 48 h (Fig. 4B). Unlike the CAT activity, the highest APX activity was obtained under RBG-CL, followed by rbg-CL, RB-CL and then rb-CL at 24 and 48 h. (Fig. 4C). There was an increasing trend in H_2O_2 levels during the 48 h of CL treatment. However, green light supplementation showed positive effects on alleviating H_2O_2 accumulation in lettuce leaves under the CL treatment, as shown by the lower H_2O_2 levels for RBG-CL and rbg-CL at 24 and 48 h. (Fig. 4D).

Addition of green light alleviates the negative effect of red and blue light on lipid peroxidation

Lipid peroxidation was revealed by the malondialdehyde (MDA) content in the lettuce leaves. CL for 48 h resulted in a constantly increasing MDA content. Green light supplementation slowed the increase in MDA content, but the lack of green light from the light source led to a significant accumulation in MDA content, as shown by higher MDA contents in the leaves under RB-CL and rb-CL than under RBG-CL and rbg-CL, respectively. Furthermore, the MDA content under rb-CL was higher than that under RB-CL after CL for 24 h. This finding indicates that high percentage of blue light in the CL treatment led to severe lipid peroxidation (Fig. 5).

Green light supplementation up-regulates *Psb*A and *LHCb* expression

Both the transcripts of *Psb*A and *LHCb* were down-regulated after 24 h of CL treatment compared with transcript levels at 0 h of CL treatment, but green light supplementation alleviated this down-regulation. A decrease of R/B in CL treatment intensified the down-regulation of the *Psb*A, as shown by the higher *Psb*A expression under RB-CL than under rb-CL (Fig. 6A). However, there was no significant difference in the expression of the *LHCb* between rb-CL and RB-CL (Fig. 6B). When the plants under CL were supplied with green light, the expression of the *Psb*A was up-regulated between 12 and 24 h, and the transcript of the *LHCb* was up-regulated between 6 and 24 h. The expressions of the *Psb*A and *LHCb* both peaked at 12 h under RBG-CL (Fig. 6A, B).

Green light can partially reverse the inhibitory effect of lincomycin on the photosystem II efficiency

Lincomycin-treated plants showed a significant decrease in maximal photochemical efficiency (F_v/F_m) under RB-CL and RBG-CL after 6 h, but interestingly, the value of F_v/F_m with RBG-CL treated in the

presence of lincomycin was 10-45% higher than that of RB-CL (Fig. S1). This suggested that green light plays an important role in maintaining a higher PSII efficiency and protecting against photoinhibition, even when the D1 synthesis is blocked by lincomycin. The light response curve experiments indicated light-induced damage of photosystem II. However, the effect of the inhibition was partly relieved by green light during 6-24 h (Fig. S2). For *Psb*A gene expression, no significant difference was found between lincomycin treated and non-lincomycin treated plants under same light condition (RB-CL or RBG-CL), but RBG-CL combined with lincomycin treatment (RBG-CL-linc) resulted in higher gene expression than under RB-CL-linc, and the highest expression strength was observed during 12-24 h. (Fig. S3A). In addition, lincomycin-treated plants exhibited a significant decrease of *LHCb* gene expression compared to plants without lincomycin treatment. RBG-CL-linc showed a higher level expression of *LHCb* than RB-CL-linc during 6-24 h light (Fig. S3B).

Discussion

Green light increases plant growth by maintaining a higher photosynthetic capacity

Light is one of the most important factors affecting plant growth and development. The contribution of green light to plant growth and development has been proven in many species, especially in *Arabidopsis thaliana* (Kudo et al. 2009, Wang and Folta 2013, Zhang et al. 2011). In the present study, we found that green light played a positive role in lettuce growth under CL conditions. CL can lead to reductions in photosynthetic capacity and maximum electron transport rates (Pettersen et al. 2010, Van Gestel et al. 2005). In general, F_v/F_m, F_v'/F_m' and qP are parameters that reflect photochemical quenching in PSII (Baker 2008). The long-term decrease of F_v/F_m reflected the rate of PSII-damage and is an indicator for photo-inhibition. In this study, the levels of F_v/F_m, F_v'/F_m' and qP were all reduced by CL, but these decreases were alleviated or even absent after adding green light to red and blue LED light (Fig. 2B-D). Treating plants with specific inhibitors of the chloroplastic translation, such as lincomycin, also can block the PSII repair process, especially D1 protein synthesis in the chloroplast (Ding et al. 2012, Mulo et al. 2003, Kato et al. 2015, Tian et al. 2017). In this study, it was shown that the positive effect of green light on alleviating F_v/F_m was still observed after treatment with lincomycin under CL (Fig. S1). This finding suggested that green light supplementation can reduce the photo-inhibition by alleviating the PSII damage and improving the photochemical efficiency in lettuce exposed to CL from red and blue LEDs. A previous study also proved that green light supplementation

was more efficient at promoting J_{PSII} to drive photosynthesis in sunflower leaves (*Helianthus annuus*) (Terashima et al. 2009).

Green light alleviates injury caused by continuous light

CL has positive effects on increasing the productivity of plant species, including *Arabidopsis* (Lepistö et al. 2009), lettuce (Gaudreau et al. 1994) and some potato cultivars (Wheeler et al. 1986). However, CL also induces injury and damages plants (Velez-Ramirez et al. 2011). Under stress conditions, the accumulated excess energy in plant leaves leads to the generation of ROS (Cakmak and Kirkby 2008), and photo-oxidative damage caused by ROS is responsible for chlorosis and membrane lipid peroxidation (Sevengor et al. 2011). Globig et al. (1994) reported that adding far-red light to red light can reduce the CL injury caused by red light alone. In nature, green light accounts for a large percentage of solar light. If CL is partially or completely provided by solar light, the injury is less than that caused by CL from artificial light sources or is even absent (Demers and Gosselin 2000). This study confirms that in addition to far-red light, green light also has a positive effect on reducing the injury caused by CL via red and blue LEDs. The data produced by this study demonstrate that green light supplementation can alleviate Chl decrease by increasing activities of SOD and APX to scavenge ROS generated by CL, as shown by the higher Chl content, SOD and APX activities and lower H_2O_2 under RBG-CL and rbg-CL. However, Haque et al. (2105) reported that CL for 12 d did not affect APX activity in tomato plant leaves. Furthermore, the significant differences in above-mentioned parameters between RBG-CL and rbg-CL indicates that under CL treatment, the effect of green light on regulating lettuce growth and development is subjected to its percentage in the total light spectra. A similar result in lettuce was reported previously by Kim et al. (2004).

Green light supplementation increases photosynthetic capacity by promoting the expression of *Psb*A and *LHCb*

In plants, repairing photodamage of PSII is important for alleviating photo-inhibition and for maintaining high photosynthetic capacity in plants under abiotic stress (Zavafer et al. 2015). The transcript of *Psb*A is critical for both the de novo synthesis of the D1 protein and the repair of photodamage of PSII (Andersson and Aro 2001). Down-regulation of the *Psb*A transcript can lead to photo-inhibition (Murata et al. 2007, Sen et al. 2014) and cause P_n reduction (Powles 1984). The

expression of the *Psb*A was down-regulated after CL for more than 24 h (Fig. 6A). The decrease in P_n under RB-CL and rb-CL might be caused by the down-regulation of the *Psb*A. However, green light supplementation up-regulated *Psb*A expression at 12 and 24 h and alleviated the down-regulation of the *Psb*A caused by long durations of CL (Fig. 6A). In this study, lincomycin treatment flattened the slope of the linear portion of the photosynthesis response curve to light, even under green light supplement treatment (Fig. S2), reflecting the inactivation and/or disassembly of PSII cores, especially the D1 protein (Adams et al. 2008). Since green light supplement showed strong effect on *Psb*A expression under CL in lincomycin-treated plants (Fig. S3A), it was suggested that green light could promote the de novo synthesis of D1 protein by stimulating the expression of the *Psb*A to repair the photodamage of PSII caused by CL, thereby maintaining a higher photosynthetic capacity. This phenomenon could partly explain why the addition of green light led to an increase in P_n during the first 24 h and alleviated the reduction in P_n caused by CL (Fig. 2A). The positive effect of green light on promoting *Psb*A expression was also reported by Efimova et al. (2013). Furthermore, compared with rbg-CL, the significant higher *psb*A transcript for RBG-CL suggests that the positive effect of green light on *Psb*A expression depends on the percentage of green light in the total light spectra of CL.

LHCb, encoded by the *LHCb* gene, is essential for the regulation and distribution of excitation energy within the photosynthetic apparatus (Melis 1996). In the present study, plants under green light supplementation produced significantly more *LHCb* transcripts (Fig. 6B), which perform two important functions: a higher efficient collection of light energy for photosynthesis and a higher capacity of dissipating excessive excitation energy from PSII (Fan et al. 2011, Kong et al. 2016). This view was further supported by the evidence of down-regulation of *LHCb* transcripts under lincomycin treatment (Fig. S3B) and the concomitant decrease in maximum of J_{PSII} (Fig. S2) under green light supplementation. Similar results were also reported in *Arabidopsis* by Dhingra et al. (2006). Furthermore, the changes in J_{PSII}, J_{fD} and J_{NPQ} among CL treatments led to different accumulations of excess energy in the lettuce leaves (Fig. 3D). Excessive energy in leaves causes reduced *LHCb* expression under abiotic stress (Ganeteg et al. 2004, Karpinski et al. 1997). In this sense, the higher *LHCb* expression under RBG-CL might be a consequence of lower excess energy in lettuce leaves compared with rbg-CL (Fig. 6B).

With increased light duration, CL led to the accumulation of excessive energy in plant leaves (Fig. 3D). Under excessive light conditions, more absorbed light energy is used for generating ROS (Huner et al. 1998). After CL for 36 h, the down-regulation of *Psb*A and *LHCb* expression and the rapid accumulation of MDA and H_2O_2 in plants indicate an excessive accumulation of ROS induced by CL led to membrane lipid peroxidation and the down-regulation of *Psb*A (He and Vermaas 1998, Qian et al. 2009) and *LHCb* (Mackerness et al. 1999). CL is a type of environmental stress that disturbs the natural photoperiod of plants and interferes with their inherent circadian rhythm and gene expression (Velez-Ramirez et al. 2011). Green light supplementation has been proven to promote shade avoidance-related gene expression, and cryptochrome receptors participate in the acclimation to green light-enriched environments (Zhang et al. 2011). Under CL, green light can also affect the gene expression of blue and red light photoreceptor genes (Folta and Maruhnich 2007). Therefore, further studies and genetic analyses on the expression of major circadian clock genes (e.g. *CCA*1, *LHY* and *TOC*1) and photoreceptor genes (e.g. *PHYB* and *CRY*1) together with enzyme activity analyses during either natural stress (e.g. high light and high temperature) or CL will help us understand more about the regulation of green light on plant growth and development. Given the available plant genomes and recent advances in RNA-Seq as a method of transcriptome profiling, it is possible to expand our understanding of the regulatory mechanisms controlling plant growth by LED light.

Conclusion

CL for more than 24 h in the presence of red and blue light led to membrane lipid peroxidation of mesophyll cells and reduced the photosynthetic capacity in lettuce leaves, but green light supplementation enhanced antioxidantive enzyme activities to alleviate these negative effects. Green light supplementation enhanced electron transport for carbon fixation (J_{PSII}) and promoted lettuce growth under CL treatment. CL with red and blue LEDs led to the down-regulation of the *Psb*A and *LHCb* transcripts, but green light supplementation facilitated the expressions of the *Psb*A and *LHCb* to maintain a higher photosynthetic capacity in lettuce. Furthermore, green light can still induce *Psb*A gene expression, when lincomycin blocks the PSII repair process by inhibiting D1 protein synthesis. Therefore, our study confirms that green light plays a positive role in plant processes and the regulation of photosynthetic genes. As such, green light could be used to stimulate photosynthetic capacity and other critical features to enhance photosynthesis during key stages under light stress

conditions and/or other photosynthetically unfavourable conditions.

Author contributions

Z. B. conducted the measurements and performed the data analysis. Z. B., T. L. and R. C. prepared the manuscript. C. L., Y. B. and Q. Y. made substantial contributions to the conception and experimental design and critically revised the manuscript.

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Supporting Information

Appendix S1. Primers used during this study.

Fig. S1. The maximum photosynthetic efficiency in dark-adapted leaves (F_v/F_m) of leaves in response to continuous light (CL) treatment with/without lincomycin (linc).

Fig. S2. The J_{PSII} of lettuce plants under continuous light (CL) treated with/without lincomycin (linc).

Fig. S3. Expression levels of the *Psb*A (A) and *LHCb* (B) in response to continuous light (CL) treated with/without lincomycin (linc).

Figure legends

Fig. 1. Changes in the contents of chlorophyll (Chl) a (A), Chl b (B) and Chl a + b (C) and in the ratio of Chl a to Chl b (D) under continuous light (CL). Data points indicate the mean± SE (n = 4)

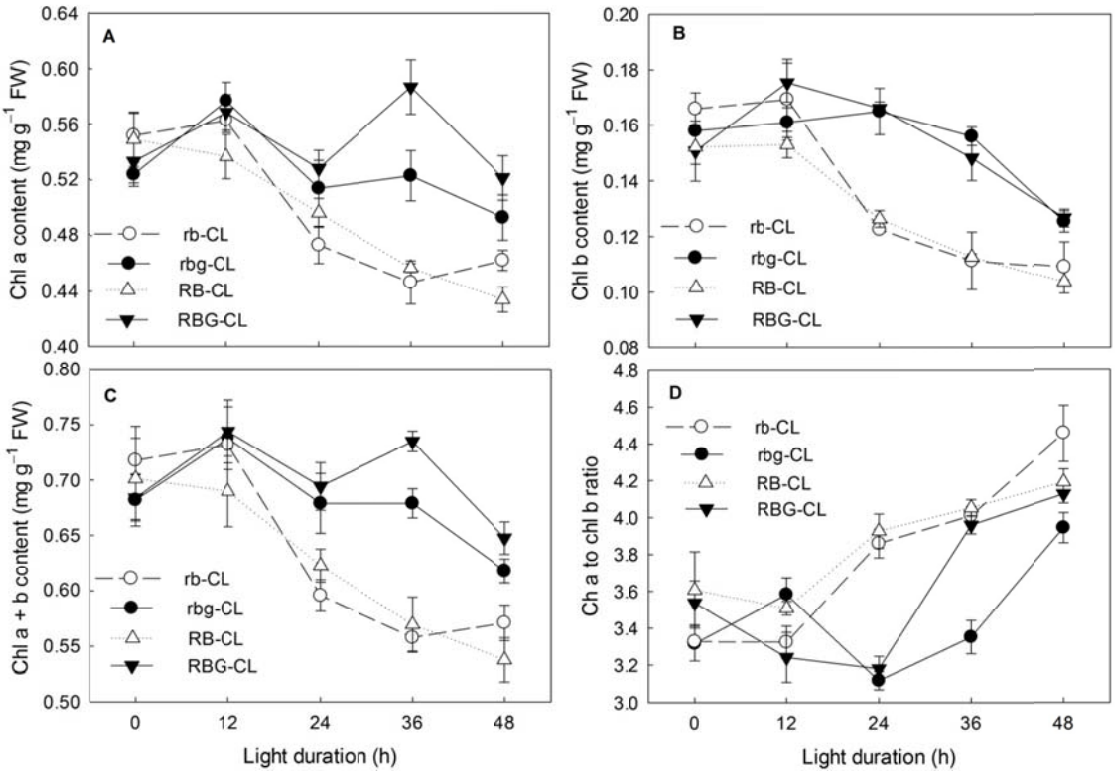

Fig. 2. Net photosynthetic rate (P_n, A), maximum photosynthetic efficiency in dark-adapted leaves (F_v/F_m, B) and in light-adapted leaves (F_v'/F_m', C) and photochemical quenching (qP, D) of leaves in response to continuous light (CL). Data points indicate the mean± SE (n = 4 or 6).

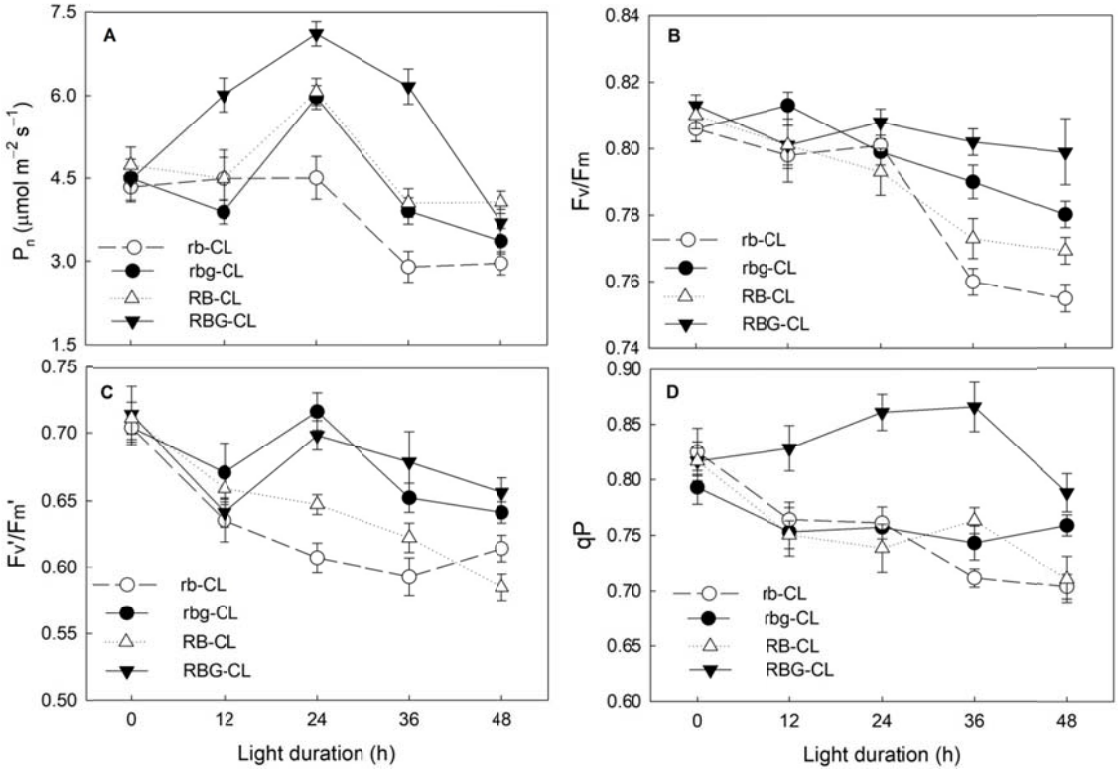

Fig. 3. Rate of total electron transport (J_{PSII}, A), light-dependent thermal dissipation (J_{NPQ}, B), light-independent thermal dissipation (J_{fD}, C) and excess energy (D) in leaves in response to continuous light (CL). Data points indicate the mean± SE (n = 4 or 6).

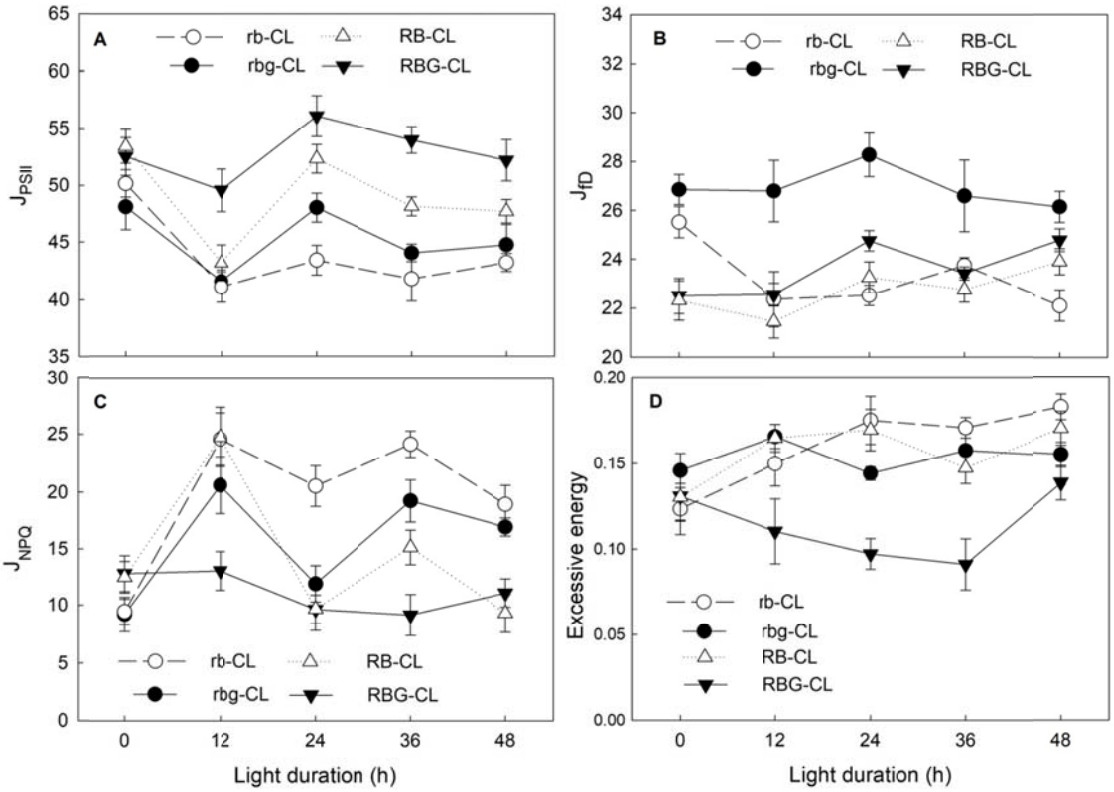

Fig. 4. Superoxide dismutase (SOD, A), catalase (CAT, B) and ascorbate peroxidase (APX, C) enzyme activities and hydrogen peroxide levels (H_2O_2, D) in leaves before (0 h) and after (24 and 48 h) continuous light (CL). Histogramm show the means ± SE (n = 4). Different letters indicate significant differences between treatments ($P < 0.05$).

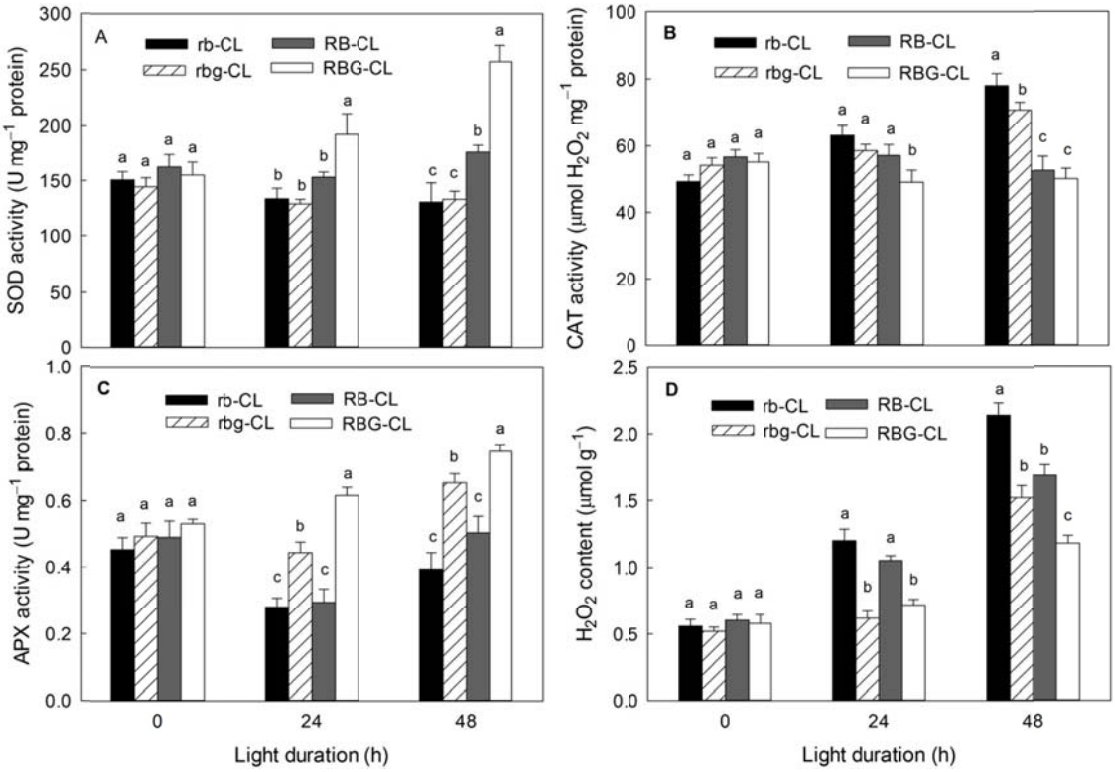

Fig. 5. Changes in MDA content in lettuce leaves under continuous light (CL). Data points indicate the mean± SE (n = 4).

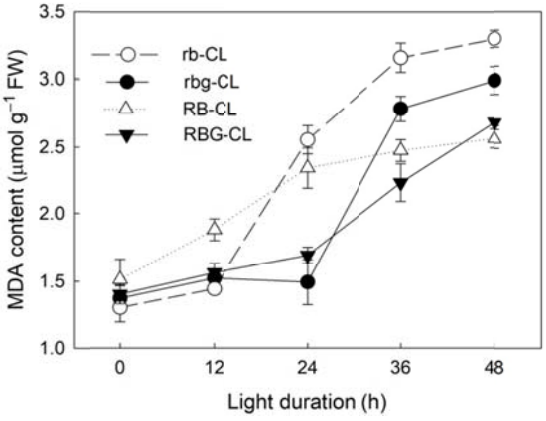

Fig. 6. Expression levels of the *Psb*A (A) and *LHCb* (B) in response to continuous light (CL). The expression of the lettuce *ACTIN* gene at each time point was used as a reference. Data points indicate the mean± SE (n = 4).

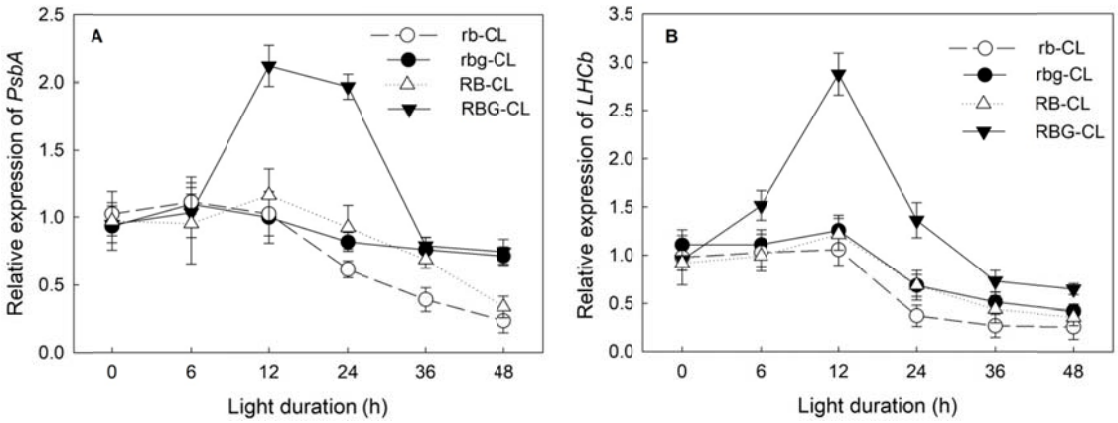

Table 1. List of light spectral data and light duration applied in the continuous light treatment. The light intensity of all treatments during the experiment was 200 μmol m^{-2} s^{-1}. Before light treatment, the photoperiod in all the treatments was 12 h. R, red light; B, blue light; G, green light; CL, continuous light.

Treatments	Before light treatment (from transplanting until the light treatment)		Light treatment (from the end of the dark period until 20 d after transplanting)	
	Light source	Light quality ratio	Light source	Light quality ratio
RB-CL	RB LEDs	R:B= 4:1	RB LEDs	R:B = 4:1
RBG-CL			RBG LEDs	R:B:G = 4:1:1
rbg-CL	RBG LEDs	R:B:G =1:1:1	RBG LEDs	R:B:G = 1:1:1
rb-CL			RB LEDs	R:B = 1:1

Table 2. Leaf area, leaf number, leaf mass per area (LMA), shoot and root fresh weight and total fresh weight of lettuce before (0 h) and after (48 h) continuous light treatment. Data represent mean± SE (n = 8). Different letters indicate significant differences between treatments ($P < 0.05$).

Time (h)	Treatment	Leaf area (cm^2)	Leaf number	LMA ($g\ m^{-2}$)	Total fresh weight (g)	Fresh weight (g)	
						Shoot	Root
0	rb-CL	337.3 ± 40.7^{a}	13.0 ± 0.40^{a}	22.5 ± 2.4^{a}	16.3 ± 0.95^{a}	14.1 ± 0.95^{a}	2.2 ± 0.25^{b}
	rbg-CL	325.3 ± 36.6^{a}	13.3 ± 0.43^{a}	19.7 ± 3.3^{a}	16.1 ± 0.82^{a}	14.3 ± 0.64^{a}	1.8 ± 0.25^{b}
	RB-CL	332.8 ± 39.9^{a}	15.0 ± 0.47^{a}	21.1 ± 2.7^{a}	16.9 ± 0.79^{a}	14.5 ± 0.40^{a}	2.4 ± 0.51^{a}
	RBG-CL	321.5 ± 36.0^{a}	14.5 ± 0.47^{a}	22.4 ± 3.1^{a}	17.3 ± 0.81^{a}	14.8 ± 1.1^{a}	2.5 ± 0.34^{ab}
48	rb-CL	363.3 ± 30.4^{b}	13.8 ± 0.70^{a}	29.7 ± 1.6^{b}	22.3 ± 0.68^{c}	20.0 ± 1.3^{b}	2.4 ± 0.54^{b}
	rbg-CL	394.8 ± 17.2^{a}	14.5 ± 0.49^{a}	29.1 ± 2.3^{b}	25.1 ± 0.65^{b}	22.4 ± 1.0^{b}	2.7 ± 0.26^{b}
	RB-CL	406.5 ± 19.2^{a}	15.3 ± 0.52^{a}	31.6 ± 2.8^{ab}	25.4 ± 0.90^{ab}	21.9 ± 1.2^{b}	3.5 ± 0.63^{a}
	RBG-CL	427.3 ± 19.7^{a}	15.8 ± 0.43^{a}	35.3 ± 1.5^{a}	27.5 ± 0.50^{a}	24.3 ± 0.80^{a}	3.2 ± 0.19^{a}