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Stomatal Opening and Growth in Tomato Seedlings Treated with Different Proportions of Red and Blue Light.

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Running title: Blue light induces stomatal opening

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ABSTRACT

Stomatal opening/closure plays a key role in balancing a plant's need to conserve water, while still allowing for exchange of photosynthetic and respiratory gasses with the atmosphere. Stomatal opening/closure can be induced by differences in light quality but detailed knowledge of the role of light in stomatal regulation in tomato is limited. In this study, we evaluated red and blue light-dependent stomatal opening processes in tomato seedlings and explored the mechanisms involved using different light-quality treatments. After 10 h of darkness, tomato seedlings were subjected to the following five treatments: monochromatic red light (R), 33% blue (2R1B), 50% blue (1R1B), 67% blue (1R2B) and monochromatic blue light (B) at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. The highest stomatal conductance recorded were for 1R1B under $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Stomatal aperture under 1R1B showed a 92.8% increase after 15 min and a 28.6% increase after 30 min, compared with under R. Meanwhile, the study shows that expressions of the plasma membrane H^{+} -ATPase in the leaf were regulated by different proportions of blue light. The results show the expressions of HA1 and HA4 increased under 1R1B and 1R2B after 15 min exposure, compared with under R. Under 1R1B of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ intensity, our results also show net photosynthesis increased compared with R and B after longer treatments, which may be related to chloroplast ultrastructure, and leaf dry weight increased compared with under 1R2B or B but there were no differences under R and 2R1B light treatments.

Keywords: photosynthesis, stomata, *Solanum lycopersicum*, H^{+} -ATPase, light-emitting

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diodes (LEDs)

ABBREVIATIONS

HA: H⁺-ATPase genes; *g_s*: stomatal conductance; *P_n*: net photosynthetic rate; *C_i*: leaf intercellular CO₂ concentrations; *PHs*: plant height; *SD*: stem diameter

INTRODUCTION

Stomata are bounded by a pair of guard cells. These cells control the exchange of water vapor and of respiratory gasses between the atmosphere and the intercellular spaces of the leaf in such a way as to optimize the often conflicting needs to conserve water and to maximize photosynthesis. Stomata are able to adjust their apertures rapidly in response to the ever-changing environment (Streck, 2005; Merilo et al., 2014). Light serves both as a signal and as an energy source for plants (Shimazaki et al., 2007; Roelfsema et al., 2002). Light is not only an essential factor for the growth and leaf development of plants (Fan et al., 2013) but is also a very variable factor governing stomatal movement (Assmann, 1988; Talbott et al., 2002; Wang et al., 2010; O'Carrigan et al., 2014). In comparison with light intensity, light quality has very complex effects on stomatal opening (Aasamaa and Aphalo, 2016; O'Carrigan et al., 2014). Phototrophins (phot1 and phot2) are plant-specific blue light receptors for phototropism, chloroplast movement, leaf expansion, and stomatal opening (Takemiya et al., 2005). Another plant-specific blue light receptor, Cryptochromes, which are flavoprotein photoreceptors, play key roles in growth and development (Chaves et al.,

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2011). Light energy transformation, light signal transduction, membrane ion transport and an abundance of plasma membrane H^+ -ATPase (*HA*) in leaves work together to regulate the process of stomatal opening (Shimazaki et al., 2007). Overexpression of plasma membrane H^+ -ATPase in leaves can promote stomatal opening induced by light (Wang et al., 2014) and enhance plant growth.

Specific light spectra stimulate different stomatal responses (Sharkey and Raschke, 1981; Kim et al., 2004; Busch, 2014; Frechilla et al., 2000). Red light and blue light and a mixture of red and blue light can induce stomatal opening. Distinct mechanisms underlie stomatal opening responses to these different wavelengths (Eisinger et al., 2000; Horrer et al., 2016; Wang et al., 2010). However, there are differences among plants in their responses to variation in the percentage of blue light. Hogewoning et al. (2010) found the stomatal conductance (g_s) of cucumber leaves was highest under 50% blue light and 50% red light. Similarly, the highest g_s of lettuce was found under blue light treatments (Wang et al., 2016). It is well known that red and blue light can promote plant net photosynthetic rate (P_n) acting as an energy source. Blue light plays a crucial role in the membrane potential hyperpolarization with simultaneous apoplast acidification and plasma membrane H^+ -ATPase activation, while red light may result in guard cell responses to the reduction in the intercellular partial pressure of CO_2 (Assmann et al., 1988) and a direct response of the guard cell chloroplasts to red light driving photosynthesis (Roelfsema et al., 2002), which induces stomatal opening. The specific mechanisms of stomatal responses to red light are still a controversial issue

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(Baroli et al., 2008; Wang et al., 2011).

Previous studies have concluded that plasma membrane H⁺-ATPase plays a critical role in regulating stomatal opening (Ueno et al., 2005; Yamauchi et al., 2016). Different isozymes of HA encoded by multiple paralogous genes have been identified and characterized in certain model plants. For example, 11 H⁺-ATPase genes have been described in *Arabidopsis thaliana* (Ueno et al., 2005), eight in tomato (Liu et al., 2016) and seven in tobacco (Oufattole et al., 2000). These isoforms of H⁺-ATPase characterize tissue-specific expression (Liu et al., 2016) and can respond to different stimuli (Portillo, 2000; Kinoshita and Shimazaki, 2002). Ueno et al. (2005) confirms that in *Arabidopsis*, blue light activates HA through C-terminus phosphorylation and the penultimate phosphorylated Thr subsequently bound 14-3-3 protein.

Tomato is a major fresh and processed vegetable worldwide because of its unique flavor and nutrition value. As a crop it is of very high commercial value, particularly in the off season (Liu et al., 2004). Increasing the efficiency of stomatal opening in tomato has the potential to promote CO₂ uptake and, ultimately, to increase net photosynthesis and thus may increase yield. Duan et al. (2014) found close associations between damage of chloroplast ultrastructure in the leaves and decrease in photosynthesis. It is well known that stomatal opening and chloroplast ultrastructure change in leaves have been shown to be sensitive to light quality (Giliberto et al., 2005; Li et al., 2017), and Liu et al. (2011) suggested that light quality seemed to be an essential factor on regulation of chloroplast ultrastructure of tomato leaves. The purpose of our study was to determine

the influences of different proportions of red and blue light on g_s , stomatal aperture, net photosynthesis, chloroplast ultrastructure and various morphological indices of plant growth (plant height (PHs), stem diameter (SD) and leaf and shoot fresh and dry weights). The results of this study are likely to be useful for research in relation to the modulation of light quality for tomato plants cultivated in greenhouse or plant factory.

MATERIALS AND METHODS

Plant material and treatments

We used the tomato (*Solanum lycopersicum* L.) cultivar ‘Jinpeng chaoguan’ as the experimental material. Seeds were surface-sterilized by soaking in 2% NaClO for 15 min and then rinsed thoroughly in clean water. They were then placed in a Petri dish on moistened filter paper and incubated at 25°C until germination. After this, seeds were sown in seedling trays (72 wells) filled with a culture medium (peat:vermiculite:perlite; 3:1:1; v:v:v) and placed in a controlled environment at a constant temperature of 23°C, and the relative humidity ranged from 65% to 75% with a 14/10 h photoperiod (white light, $200 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$). After the first leaf was fully expanded, plants of similar size were selected and transferred to black plastic pots (5×5×8 cm, one seedling per pot) filled with the same culture medium. Finally, plants were placed in a climate chamber (Container Plant Growth Cabinet, Xutian CO., Xi an, China) at Northwest A&F University, Yangling, Shaanxi, China (North latitude: 34.2624 ° ; East longitude: 108.0673 °). The chamber was completely enclosed and equipped with air-conditioning. The climate chamber temperature was set at 23°C, $200 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic

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photon flux density (PPFD) and 65%-75% relative humidity. Plants were irrigated daily with $\frac{1}{4}$ strength Yamasaki nutrient solution (pH $\approx$ 6.5, electrical conductivity $\approx$ 1.6 dS $\cdot$ m $^{-1}$) (Yamazaki, 1982).

Each climate chamber was divided into five sections randomly with different ratios of red and blue light and covered with an opaque black curtain to avoid light pollution (one climate chamber as a replicate). Plants ($n = 30$) were selected randomly and placed in each section. There were three plants in each replication. Illumination panels (970 $\times$ 570 mm) were constructed containing light-emitting diodes (LEDs) whose intensities can be adjusted through dimming. Panels were placed horizontally 20 cm above the plants and covered with the opaque black curtains. The light photosynthetic photon flux density and spectra distributions were measured using a spectro-radiometer (PAR-NIR, Apogee Instruments Inc, Logan, UT) and shown (**Fig. 1**). Tomato seedlings that had just grown to the first two true leaves are just visible stage were subjected (thirty seedlings per treatment) to one of five different light treatments: 0% blue(R), 33% blue(2R1B), 50% blue(1R1B), 67% blue(1R2B) and 100% blue(B) (peak illumination at 455 nm) and red light (peak illumination at 660 nm). The light treatments were at 200 ± 10 μ mol m $^{-2}$ s $^{-1}$ light intensity for 14 h from 08:00 to 22:00 inside opaque black curtains which prevented light contamination among the different treatments. The seedlings were harvested after 15 days. Three independent biological replicates from different seedlings were used for measurements and every treatment was repeated three times. Data were analyzed using one-way ANOVA and compared using Duncan's multiple range test

($p < 0.05$) with SPSS 20 (IBM, USA).

Measurement of growth and development

Plants were treated with different blue-light proportions for 15 d, plant height (PHs) was measured using a ruler with 0.1 cm accuracy and SD was measured using a digital caliper (Digimatic Caliper, The great wall Co., Ltd., Zejiang, China) with 0.01 mm accuracy at the first internode. After 15 d treatment, leaf and stem fresh weights (FW) and dry weights (DW) was measured individually in 10 plants per treatment using a 0.01 g accuracy balance (JY10002, Shanghai Liangping Instrument Co., Ltd., Shanghai, China). Tissues were dried at 70°C for 2 d before DW were recorded.

Leaf gas exchange, transpiration rate and net photosynthetic rate measurements

The net photosynthetic rate (P_n), gas exchange (g_s), transpiration rate (T_r) and leaf intercellular CO_2 concentrations (C_i) were measured simultaneously on the second fully-developed leaf using an infrared gas analyzer-based portable photosynthesis system (LI-6400, Li-Cor; Lincoln, NE, USA) equipped with a 6400-40 Leaf Chamber Fluorometer. Microclimatic conditions in the leaf chamber were adjusted to be consistent with the conditions of the growth environment. The air temperature, relative humidity and PPFD were set at 23 °C, 65%-75% and 200 $\mu mol\ m^{-2}\ s^{-1}$ through block temperature, the desiccant (manual bypass control), light control option which Values between 0 and 2000 $\mu mol\ m^{-2}\ s^{-1}$ can be specified, and are maintained by the control

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system, respectively. Meanwhile, the spectral quality of LI-6400 was set according to the growth unit (treatment) through the light control option which can set red, green blue light fraction, and CO₂ reference concentrations of LI-6400 were set through CO₂ control system, which include CO₂ Scrubber and steel cylinder filled with high pressure liquid CO₂. Values of P_n, Tr, Ci and g_s were measured inside the growth sections. In total, six plants were measured for P_n, Tr and Ci under each light-quality treatment after a short time (60 min) and a long time (15 d), and the g_s was measured during a dark period (10 min) and different light treatments (90 min).

Field-emission scanning electron microscopy (FESEM)

The specimens for FESEM were prepared following standard procedures (Meng et al., 2014) with some modifications. Three replicates of healthy tissue from fresh young leaves (from the second node) were cut from the same area of different leaves in the control and treatment groups to ensure specimen uniformity. Small pieces of leaf (approximately 5×5 mm) were washed in distilled water and prepared for FESEM. Segments of leaves were fixed overnight with 4% glutaraldehyde in 0.2 M phosphate buffer solution (pH 6.8) for more than 6 h at 4°C. They were then washed four times with 0.1 M phosphate buffer solution (pH 6.8), for 10 min each. Following dehydration in a graded ethanol series (30%, 50%, 70%, 80%, 90% and three times in 100%), they were displaced with isopentyl acetate for 15 min, then dried in a critical-point dryer (Emitech K850; Quorum, Kent County, UK). A S4800 field-emission scanning electron

microscope (Hitachi, Ltd., Tokyo, Japan) was used to investigate and analyze sections of the stomata from leaves of cuttings after metal coating.

Transmission electron microscope (TEM) analysis

The specimens for TEM were prepared following standard procedures (Meng et al., 2014) with some modifications. Healthy tissue samples of fresh young leaves (about 1-3 mm³) were fixed at 4% glutaraldehyde in phosphate buffered saline (PBS, 0.2 M, pH 6.8) for 8 h at 4°C. The specimens rinsed with PBS (0.1 M, pH 6.8) for 5, 10, 15, 20, and 30 min, respectively, before being post-fixed using 1% osmium tetroxide (OsO₄) in PBS (0.2 M, pH 6.8) for 2.5 h at 4°C. After rinsing again with PBS (0.1 M, pH 6.8) for 5, 10, 15, 20, and 30 min, samples were dehydrated for 15 min in a series of ethanol solutions (30%, 50%, 70%, 80%, 90%, and 100% (V/V)), and infiltrated overnight in a mixture of LR-White resin (London Resin Company, Reading, U.K.) and ethanol (1:1, V/V), followed by infiltration with pure LR-White resin twice (for 2 h and 1 h, respectively) at room temperature. Next pure LR-White resin was used for embedding and the samples were incubated for 48 h at 60°C. Ultrathin sections of 50 - 100 nm were obtained using a diamond knife on the Leica EM UC7 ultramicrotome (Leica, Nussloch, Germany), and picked up on 200-mesh formvar-carbon coated grids.

cDNA preparation and quantitative real-time RT-PCR analysis

To obtain cDNA, total RNA was extracted from leaves using the Plant RNA kit

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(OMEGA), then reverse transcribed applying the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa). A Step one Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used to quantify the relative transcription abundance of target genes. qRT-PCR was conducted using a SYBR® Premix EX Taq™ II kit, 20 µL reaction system (TaKaRa) contain 10 µL of SYBR Green premix (2×), 2 µL of cDNA template (50 ng µL⁻¹), 0.8 µL of each gene-specific primer, 0.4 µL ROX Reference Dye and 6 µL dH₂O. The PCR procedure consisted of 40 cycles of 95°C for 5 s, 60°C for 30 s after an initial incubation at 95°C for 30 s, and a dissociation stage was added to ensure specific amplification. The ($2^{-\Delta \Delta C_t}$) method was used to calculate the relative transcription level of each target gene. **Supplementary table S1** shows the specificity of primer sets for the qRT-PCR (Liu et al., 2016).

Statistical analyses

The data are reported as means ± standard errors (SE), analyzed using SPSS statistical software (version 20), One way ANOVA and compared using Duncan's multiple range test ($p < 0.05$). Graphing was performed in OriginPro (version 8.0, Origin Lab, MA, USA).

RESULTS

Effects of different proportions of red and blue light on tomato leaf stomatal conductance

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Stomatal conductance increased rapidly after light treatment and then remained stable after about 60 min (**Fig. 2A-D**). Also, the additional light intensity increased the stomatal responses to different proportions of red and blue light. Firstly, plants failed to increase their stomatal conductance efficiently when subjected to $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity with different proportions of red and blue light (**Fig. 2A**). However, they increased their stomatal conductance effectively under 200, 400 and $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity (**Fig. 2B-D**). Moreover, under $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ intensity, they increased their stomatal conductance more under 1R1B and 1R2B than that under 2R1B. Meanwhile, the g_s value under 2R1B was higher than that under R and B alone (**Fig. 2B**). The values of g_s under 1R1B and 1R2B treatments were 62.0% and 54.0% higher, respectively, than those under R for $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity after 60 min lighting. The same trends were observed in the results for the 400 and $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity treatments.

Under the same light intensity, 1R1B and 1R2B both increased stomatal conductance efficiently (**Fig. 3**). The g_s values under 1R1B increased compared with R and B other than under $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ (**Fig. 3A**). The same trends were observed for 1R2B (**Fig. 3B**). Meanwhile, there were no differences between 200, 400 and $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ under 1R1B. In contrast, differences were observed between $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 200, 400 and $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ (**Fig. 3A**). The values of g_s increased from $0.089 \text{ mol m}^{-2} \text{s}^{-1}$ for $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ under 1R1B to $0.195 \text{ mol m}^{-2} \text{s}^{-1}$ under $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (**Fig. 3A**). The values of g_s under 1R2B showed a similar trend under 1R1B. However, it increased

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from $0.095 \text{ mol m}^{-2} \text{ s}^{-1}$ under $50 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ to $0.185 \text{ mol m}^{-2} \text{ s}^{-1}$ under $200 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ (**Fig. 3B**).

Effects of different proportions of red and blue light on stomatal aperture

Stomata opened quickly after exposure to different proportions of red and blue light under $200 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ (**Fig. 4**). **Fig. 5A** shows stomatal aperture increased with increasing proportions of blue light with the highest aperture value for 1R1B. After 15 min exposure to 1R1B, stomatal aperture was 93.38% higher than that under R and after 30 min exposure it was 28.9% higher. Meanwhile, after receiving 30 min pretreatments with different proportions of red and blue light, stomatal aperture reached a plateau with slight fluctuations (**Fig. 5A**). Under 1R2B of $200 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ intensity, no differences in stomatal aperture were detected between 30 and 60 min. However, stomatal aperture increased sharply from 0.807 to $3.193 \text{ } \mu\text{m}$ and there were differences between 30 min and in darkness (**Fig. 5B**). Stomatal aperture under 1R1B showed a similar trend with only a slight decline after 60 min of treatment, but the differences were not statistically significant (**Fig. 5B**).

In contrast, there were no differences in stomatal density among different treatments (**Fig. 5C** and **Supplementary Fig. S1**).

Expression pattern of H^+ -ATPase genes in tomato leaves under 1R1B and 1R2B

Transcript analyses revealed that HA1 and HA4 play major roles in promoting stomatal

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opening when plants were exposed to blue light (**Fig. 6**). Compared to R, exposure to 1R1B and 1R2B upregulated the transcripts of HA4 after 15 and 30 min. The transcripts of HA1 showed similar trends but showed no increase under 1R1B compared to under R. Meanwhile, the relative transcript abundance of HA2 showed no change with time under 1R1B and 1R2B compared with in the dark. However, the transcripts of HA2 under R showed decreases compared with in the dark.

Compared with R, **Fig. 6** shows little change in the transcripts of HA1 after 15 min under 1R1B but these increased from 1.47 to 2.38 under 1R2B. After 15 min, the HA4 transcripts increased from 0.88 under R, to 1.62 under 1R1B and to 1.78 under 1R2B. The relative transcript abundances of HA4 after 30 min showed similar trends to those after 15min.

Effects of different proportions of red and blue light proportions on Pn, Ci and Tr

Different proportions of red and blue light affected Pn after long (15 d) treatment periods (**Fig. 7B**). Exposure to 1R1B had highest effect, increasing Pn from 6.07 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for R, to 7.33 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for 1R1B. The values for 1R1B and 1R2B were 20.96% and 15.02% higher than for R, respectively. Plants grown under R and B had lower values for Pn compared with the other treatments but the Pn value was not different among the various red and blue light proportion treatments after short-term (60 min) exposures (**Fig. 7A**).

Referring to **Fig.8**, no differences of Ci were detected among 2R1B, 1R1B, 1R2B and B

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after short time treatment (60 min), and there were no differences between R and 2R1B.

However, being treated after long time (15 d), Ci under 2R1B and 1R1B decreased compared with that under B and R treatments.

There were no differences of Tr among R, 2R1B, 1R1B, 1R2B and B after short time treatment (60 min) (**Fig.9A**). After the long time treatment (15 d), Tr under R had highest value, which was higher than other treatments (**Fig. 9B**).

Effects of different proportions of red and blue light on Chloroplast ultrastructure

Chloroplasts play an essential role in plant photosynthesis. Ultrastructure of chloroplasts was different under various treatments (**Fig. 10**). More starch grains in chloroplasts were detected under R and 1R2B conditions, whereas there were less starch grains under B treatment. Moreover, the boundary between stromathylakoid and granathylakoid was clearly distinguished in the chloroplasts and granathylakoid forms several layers stacked densely in an orderly pattern. Futhermore, thicker granathylakoids were observed under 2R1B and 1R1B than those under B, and they were obscure under B. Compared with chloroplasts under B, their widths increased under other treatments, and were round in shape, with numerous plastoglobuli were formed in the Chloroplasts.

Effects of different proportions of red and blue light on vegetative growth

We found the red/blue combination of treatments were most effective in inhibiting

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spindly stems. Exposing the first expanded leaf to the various blue light proportion treatments, we found that plants exposed to 2R1B, 1R1B and 1R2B were more robust than those under monochromatic red and blue light (**Fig. 11**). After 15 days exposure to the R, 2R1B, 1R1B and 1R2B treatments, we found the PHs increased by 147.3, 27.5, 27.1 and 10.4% respectively, compared with the value under B (**Table 1**).

Leaf dry weights (DW_L) and shoot dry weights (DW_s) were affected by the blue light proportion treatments (**Table 1**). After 15 days exposure to 1R1B, R, 2R1B and 1R2B, DW_L increased by 106.45, 90.32, 80.65 and 29.03%, respectively, compared with under blue light. There were no differences among R, 2R1B and 1R1B treatments. Also, when exposed to R, 2R1B, 1R1B and 1R2B, DW_s increased by 466.67, 166.67, 133.33 and 66.67%, respectively, compared with under B, but the dry weight of stems between 1R2B and B treatments were the same.

DISCUSSION

Plant growth and quality are much affected by light quality (Massa et al., 2008; Olle and Virsile, 2013; Arena et al., 2016; Jason et al., 2018), and the growth of seedlings is pivotal for vegetative and reproductive developmental in later stage (Wang et al., 2016). Stomata play an important role in the processes through which light quality regulates plant growth (Wang et al., 2014). Shimazaki et al. (2007) have shown that values of g_s in leaves illuminated with a combination of red and blue light are larger than under red or blue light alone. It can be seen from **Fig. 2A-D** and **5A**, that increasing the

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percentage of blue light up to 1R2B, increases both g_s and stomatal aperture. Similar effects have been reported by others (Hernández and Kubota, 2016; Hogewoning et al., 2010; Doi et al., 2015). For instance, Hogewoning et al. (2010) demonstrated that in all treatments, values of g_s under red light were low and did not respond efficiently to increase in light intensity. Similarly, cucumbers grown under monochromatic red light had very low values of g_s (Hogewoning et al., 2010; Savvides et al., 2012). Our study agrees with that of Wang et al. (2016) that the highest values of g_s are observed under high proportions of blue light.

Stomatal responses are among the most rapid as plants adapt to environmental stimuli, such as to air humidity (Aasamaa and Söber, 2011; Bauer et al., 2013), ambient CO₂ concentration (Hashimoto et al., 2006; Messinger et al., 2006), vapor pressure deficit (Creese et al., 2014) and light intensity (Doi et al., 2015; Aasamaa and Aphalo, 2016). Here, we examined the role of stomatal aperture in tomato plants in relation to the different proportions of red and blue light. Under 1R1B and 1R2B, stomatal aperture is higher than that under R, 2R1B and B (**Fig. 4** and **5A-B**). Similar results have been reported in studies with lettuce (*Lactuca sativa* L.) (Wang et al., 2016). Wang et al. (2016) found that adding blue light to red background light, increased stomatal opening compared with pure red light. Changes in stomatal density, in stomatal size and in stomatal location on the leaf surface can respond to variations in the environment and all these can affect stomatal conductance (Maherali et al., 2002). Irrespective of the size, shape, and number of epidermal cells filling the spaces between stomata on a leaf

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surface, stomatal aperture is virtually the only epidermal characteristic regulating CO₂ and water transfer between leaf and atmosphere. Stomatal opening is known to be regulated by a combination of light intensity and light quality (Frechilla et al., 2000; Muneer et al., 2014). Moreover, stomatal opening responses are regulated by a complex combination of light energy conversion, light signaling and ion transport across guard cell membranes (Takemiya et al., 2005; Wang et al., 2016). In this study, light intensity levels under 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ led to rapid stomatal opening after 30 min under 1R1B and 1R2B.

Previous studies have found that plasma membrane HAs play a crucial and primary role in regulating plant growth (Zhao et al., 2000) and responses to environmental stimuli (Portillo, 2000; Janicka-Russak et al., 2008). Also, AHA1 plays a major role in the opening of stomata induced by blue light in *Arabidopsis* (Yamauchi et al., 2016; Shimazaki et al., 2007). However, there has been no evidence yet of HA change among the HA isogenes. In our study, the relative expressions of HA1, HA2 and HA4 in tomato leaves are revealed (**Fig. 6**). Compared to R, the expression of HA1 is upregulated under 1R2B after 15 min. However, there were no differences between 1R1B and 1R2B after 30 min. Similar trends were found for HA4 under 1R1B and 1R2B. Tissue-specific expressions of eight encoding H⁺-ATPase genes (*SLHA1-8*) have also been observed in tomato (Liu et al., 2016). In all tissues, SLHA1, 2 and 4 were widely expressed, while SLHA5, 6 and 7 were expressed only in flowers. Under normal growth conditions, transcripts of SLHA8 are barely detectable, but activated only in

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arbuscular mycorrhizal fungal-colonized roots. There are 11 functional H⁺-ATPases (AHA1-11) in *Arabidopsis* guard cell protoplasts, there are a further eight AHA genes in the green leaves (AHA1, 2, 3, 5, 7, 8, 10 and 11), eight in the roots (AHA1, 2, 3, 4, 7, 8, 10 and 11) and four in the mesophyll cells protoplasts (AHA1, 2, 10 and 11) (Ueno et al., 2005). These isoform genes of HA may all have different functions and respond to different external stimuli. Ueno et al. (2005) reported that *Arabidopsis* AHA5 was expressed predominantly in guard cells in response to blue light. Such discrepancies in HA expression obtained in different studies may explain which HA is responsive to blue light and so induces stomatal opening.

Our study shows quantitative increases in Pn with increasing proportions of blue light up to 1R1B when plants are exposed to different proportions of red and blue light over long periods. Similar results have been found for cucumber (*Cucumis sativus* L.) (Hogewoning et al., 2010). We suppose that the effects of different proportions red and blue light on Pn are not only affected by stomatal movement, but also chloroplasts ultrastructure change (**Fig. 10**). Wang et al. (2016) reported that increasing blue light ratio could increase Ci, which was inconsistent with our results in that the leaves have the low Ci under the combination red and blue light. We found that in tomato seedlings treated with different proportions of blue light over long periods of time, the internal structure of the leaves is altered in such a way as to increase the utilization of intercellular CO₂. It had been found that cherry tomato leaves under 75% blue light and 25% red light contained more chloroplasts. Moreover, ultrastructure of chloroplasts

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changed into a form, which may increase the sensitivity of leaf net photosynthesis to light quality, as to increase the ability of leaf to capture and utilize light energy (Liu et al., 2011). Meanwhile, Deram et al. (2014) found that higher fruit production was obtained using the 5:1 ratio (red to blue), which suggests that there are connections between fruit production and tomato seedlings exposed to different ratios of red and blue light.

Stomata can be effectively stimulated by combinations of red and blue light. Meanwhile, the process of stomatal opening involves crucial roles played by HA1 and HA4. In addition, monochromatic red light may lead tomato plants to become spindly and monochromatic blue light may result in plants that grow slowly, while appropriate combinations of red and blue light allow them to grow more healthily. Compared to other light treatments, the stomata of tomato leaves can be induced to open maximally under 1R1B to 1R2B within 30 min. Thus, the light environment between 1R1B to 1R2B appeared more beneficial for stomatal opening and for promoting better leaf growth than red or blue light.

In this study, stomata can be significantly opened under 1R1B and 1R2B, and tomato seedlings under 2R1B, 1R1B and 1R2B treatments exhibited the higher Pn, compared with monochromatic red and blue light. In conclusion, 1R1B and 1R2B treatments can be beneficial for stomatal opening and seedlings growth, which will provide theoretical basis for industrialized seedling cultivation and efficient use of LED light source for tomatoes in greenhouse.

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Figure legends

Figure 1. Spectral distribution characteristics of different ratios of blue and red LEDs used for treatment.

Figure 2. Effects of different ratios of blue and red LEDs on stomatal conductance (g_s) of tomato leaves under 50, 200, 400 and 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity ($n=6$). A, B, C and D represent the above four light intensities, respectively. Gray bands represent dark period.

Figure 3. Effects of 1R1B and 1R2B on stomatal conductance in tomato leaves. (A) 1R1B (B) 1R2B. Data are means $\pm$ standard error (SE). Different letters indicate significant differences according to Duncan's multiple range test ($p<0.05$; $n=6$).

Figure 4. Effects of different ratios of blue and red LEDs on stomatal aperture of tomato leaves. Light intensity 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. $\times 45000$ magnification, scale bars= 10.0 μm .

Figure 5. (A) Effects of different ratios of blue and red LEDs on stomatal aperture of tomato leaves; (B) Effects of 1R1B and 1R2B on stomatal aperture after different time compared to dark. (C) Effects of different ratios of blue and red LEDs on stomatal density of tomato leaves. Data are means of 30 fields of view for six samples from each treatment $\pm$ standard error (SE). Different letters indicate significant differences according to Duncan's multiple range test ($p<0.05$, $n=6$).

Figure 6. Expression analysis of tomato HA genes. The RNA samples were prepared

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from tomato leaves. Values are the means of three biological replicates with SEs. In each individual graph, different letters indicate significant difference ($p < 0.05$, $n = 3$).

Figure 7. Leaf net photosynthetic rate (Pn) under different ratios of blue and red LEDs after short (60min) (A) and long periods of treatment (15d) (B). Data are means $\pm$ standard error (SE). Different letters indicate significant differences according to Duncan's multiple range test ($p < 0.05$; $n = 6$).

Figure 8. Leaf intercellular CO₂ concentration (Ci) under different ratios of blue and red LEDs after short and long periods of treatment. Data are means $\pm$ standard error (SE). ($p < 0.05$; $n = 6$).

Figure 9. Leaf Transpiration rate (Tr) under different ratios of blue and red LEDs after short and long periods of treatment. Data are means $\pm$ standard error (SE). ($p < 0.05$; $n = 6$).

Figure 10. Effects of the different ratios of blue and red LEDs on structure of chloroplasts in tomato leaves. R = red LEDs; B = blue LEDs; RB = red and blue LEDs. MC = Mesophyll cells; CW = cell wall; P = Plastoglobulus; SG = starch grain; GT = granathylakoid; ST = stromathylakoid

Figure 11. Effects of different ratios of blue and red LEDs on tomato seedlings.

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Table 1. Effects of different ratios of blue and red LEDs on plant height (PHs), stem diameter (SD), leaf fresh weight (FW_L), leaf dry weight (DW_L), shoot fresh weight (FW_S) and shoot dry weight (DW_S).

Blue light proportion	R	2R1B	1R1B	1R2B	B
PHs (cm)	20.3 ± 0.49 _a	10.4 ± 0.24 _b	10.4 ± 0.25 _b	9.0 ± 0.22 _c	8.2 ± 0.26 _c
SD (mm)	5.40 ± 0.09 _a	4.43 ± 0.08 _c	4.86 ± 0.10 _b	3.81 ± 0.02 _d	3.92 ± 0.06 _d
FW _L (g)	5.05 ± 0.37 _a	4.04 ± 0.16 _b	5.45 ± 0.29 _a	2.78 ± 0.12 _c	2.80 ± 0.14 _c
DW _L (g)	0.59 ± 0.05 _a	0.56 ± 0.02 _a	0.64 ± 0.04 _a	0.40 ± 0.02 _b	0.31 ± 0.02 _b
FW _S (g)	3.41 ± 0.18 _a	1.17 ± 0.05 _b	1.32 ± 0.08 _b	0.74 ± 0.03 _c	0.60 ± 0.04 _c
DW _S (g)	0.17 ± 0.01 _a	0.08 ± 0.01 _b	0.07 ± 0.01 _{bc}	0.05 ± 0.01 _{cd}	0.03 ± 0.01 _d

Note: Data are means ± standard error (SE), different letters indicate significant difference using the Duncan’s Multiple Range Test ($p<0.05$; $n=10$)

Fig. 1

289x209mm (300 x 300 DPI)

Fig. 2

294x209mm (300 x 300 DPI)

Fig. 3

207x294mm (300 x 300 DPI)

Fig. 4

294x209mm (300 x 300 DPI)

Fig. 5

293x209mm (300 x 300 DPI)

Fig. 6

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Fig. 7

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Fig. 8

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Fig. 9

294x205mm (300 x 300 DPI)

Fig. 10

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