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RESEARCH PAPER

Smart glass impacts stomatal sensitivity of greenhouse *Capsicum* through altered light

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Abstract

Optical films that alter light transmittance may reduce energy consumption in high-tech greenhouses, but their impact on crop physiology remains unclear. We compared the stomatal responses of *Capsicum* plants grown hydroponically under control glass (70% diffuse light) or the smart glass (SG) film ULR-80, which blocked >50% of short-wave radiation and ~9% of photosynthetically active radiation (PAR). SG had no significant effects on steady-state (g_s) or maximal (g_{max}) stomatal conductance. In contrast, SG reduced stomatal pore size and sensitivity to exogenous abscisic acid (ABA), thereby increasing rates of leaf water loss, guard cell K^+ and Cl^- efflux, and Ca^{2+} influx. SG induced faster stomatal closing and opening rates on transition between low (100 µmol m^{-2} s^{-1}) and high PAR (1500 µmol m^{-2} s^{-1}), which compromised water use efficiency relative to control plants. The fraction of blue light (0% or 10%) did not affect g_s in either treatment. Increased expression of stomatal closure and photoreceptor genes in epidermal peels of SG plants is consistent with fast stomatal responses to light changes. In conclusion, stomatal responses of *Capsicum* to SG were more affected by changes in light intensity than spectral quality, and re-engineering of the SG should maximize PAR transmission, and hence CO_2 assimilation.

Keywords: Abscisic acid, *Capsicum annuum*, greenhouse horticulture, light spectrum, smart glass, stomatal conductance.

Introduction

Efficient climatic control in protected cropping can be achieved by alterations in greenhouse structures. These include the even-span greenhouse designed for crop cultivation at high latitude (Sethi, 2009), optimal orientation allowing plants to receive more radiation (Xu *et al.*, 2015), different greenhouse shapes to improve the ventilation (Katsoulas *et al.*, 2006), and building materials utilizing a special plastic film to block UV radiation and enhance light diffusion (Hemming *et al.*, 2004).

Other techniques such as vent, fog, and fan cooling systems, dehumidification, and the regeneration process of the liquid desiccant also improve glasshouse climatic control (White, 2014; Lefers *et al.*, 2016; Rabbi *et al.*, 2019; Samaranayake *et al.*, 2020). However, the high cost of these solutions indicates that an innovative alternative technique of using a low emissivity 'smart glass' (SG) film should significantly reduce the costs while maintaining adequate climate control in glasshouses (Lin *et al.*, 2020).

The special glass film materials are optically engineered in a nanometre-scale, adjusting light transmittance to allow for high potential of reducing energy cost in high-technology greenhouses (Lin *et al.*, 2020). We recently showed that the SG film ULR-80 blocks the majority of UV light and a proportion of far-red and red light, which can reduce energy load required for heating and cooling in a protected cropping situation (Chavan *et al.*, 2020). However, reducing the photosynthetically active radiation (PAR) potentially decreases the growth and productivity of horticultural crops. In our recent study using eggplant (aubergine) grown in a high-tech glasshouse, the application of the SG film led to a net reduction in heat load, water and nutrient consumption, and therefore improved energy and resource use efficiency. However, the 19% decrease in PAR reduced fruit yield of eggplants under SG glass by 25% compared with normal control glass (Chavan *et al.*, 2020). Whilst SG consistently reduced photosynthetic rates, the response of stomatal conductance was less consistent, decreasing in one season and remaining unaffected in another (Chavan *et al.*, 2020). Guard cells are highly specialized cells that form the stomatal pore and determine stomatal conductance. By integrating environmental cues with endogenous signals, guard cells ensure optimal stomatal function by mediating physiological trade-offs to minimize water loss while maximizing carbon gain in the light. Optimal stomatal function is crucial for plant photosynthesis (Farquhar and Sharkey, 1982) and water use efficiency (Lawson and Vialet-Chabrand, 2019), but can be compromised under adverse light conditions (O'Carrigan *et al.*, 2014). Another important limitation in this process is the rate at which stomata open in the light or close in darkness (Drake *et al.*, 2013). To what extent the altered light conditions generated by an SG film affect the stomatal morphology and physiology remains unclear.

PAR, including wavelengths between 400 nm and 700 nm, supplies the essential photons utilized by plants during photosynthesis, which is highly dependent on its intensity (McCree, 1981). Light directly and indirectly (via photosynthesis) regulates stomatal function (Assmann and Jegla, 2016). Plants have developed sensing mechanisms for both light quantity and quality (Aasamaa and Sõber, 2011; Düring and Harst, 2015; Ballard *et al.*, 2019) to adjust stomatal aperture, allowing CO_2 absorption for carbon fixation. Light also plays important roles in stomatal formation, as well as closing and opening of the guard cells (Roelfsema and Hedrich, 2005). Rapid stomatal responses to light help to optimize plant photosynthesis (Lawson and Vialet-Chabrand, 2019). Photosynthetic capacity is well linked with the theoretically maximum stomatal conductance (g_{max}), which is estimated from the anatomical features of stomatal size and density, and reflects the maximum leaf diffusive (stomatal) conductance of CO_2 to the site of assimilation (Franks and Beerling, 2009), along with the operational stomatal conductance (g_{op}) which reflects the measured values of stomatal conductance for the given environmental conditions (McElwain *et al.*, 2016).

Long-term effects of light quantity and quality on stomatal density and conductance have been well studied (Savvides *et al.*, 2012). Stomatal density increases under high light (Gay and Hurd, 1975), leading to increased stomatal conductance and CO_2 assimilation (Baroli *et al.*, 2008). Excessive light stimulates a rapid stomatal closure along with a rapid production of reactive oxygen species (ROS) (Devireddy *et al.*, 2018). ROS accumulation in guard cells activates key ion channels such as the slow anion channel (SLAC1) and the outward rectifying K^+ channel (GORK) for stomatal closure (Brandt *et al.*, 2012; Deger *et al.*, 2015; Lind *et al.*, 2015; Zhao *et al.*, 2018). Moreover, photoreceptors are key players in the response of plant growth and yield to changes of the light environment (Casal, 2013; Babla *et al.*, 2019). Blue light-induced stomatal opening is mediated by the light receptor phototropins and cryptochromes (Wang *et al.*, 2010), while red light-induced stomatal opening is mediated by increased expression of phytochromes (Wang *et al.*, 2010). Other light-related genes such as *UV-B Photoreceptor 8* (*UVR8*), *Light-Harvesting Component B* (*LHCB*), and *Ribulose Bisphosphate Carboxylase Small Chain 1* (*RBCS1*) can regulate plant photosynthetic rates (Borkowska, 2005; Baroli *et al.*, 2008; Wang *et al.*, 2010; Davey *et al.*, 2012; Xu *et al.*, 2012; Tossi *et al.*, 2014). Their responses to SG may elucidate the potential mechanisms that control the stomatal regulation in *Capsicum*.

In particular, abscisic acid- (ABA) induced stomatal signalling has been widely reported to play roles in stomatal response to environmental stimulus and in light-induced stomatal regulations (Garcia-Mata and Lamattina, 2007). ABA stomatal signalling involves regulations of upstream elements, such as ABA receptors, and downstream elements, such as ROS metabolites and ion channels (Hosy *et al.*, 2003; An *et al.*, 2008; Vahisalu *et al.*, 2008; Gonzalez-Guzman *et al.*, 2012; Merilo *et al.*, 2013; Deger *et al.*, 2015). Besides, given the accumulated evidence about the effects of light on stomatal development and response, our overarching hypothesis was that altered light conditions under SG reduce stomatal density and aperture, and affect stomatal sensitivity and guard cell ion fluxes due to regulation of ABA and photoreceptor signalling networks. To address this hypothesis, we used *Capsicum annuum* L. for studying stomatal morphology and physiology. *Capsicum*, also known as sweet pepper, is the second most cultivated crop after tomatoes in protected cropping in many countries including Australia. Studies on *Capsicum* have mainly focused on developmental responses to temperature, humidity, and water stress (Bakker,

1989*a*, *b*; Hawa, 2003). In this study, we cultivated *Capsicum* plants for 8 months with and without an SG film and measured stomatal density, size, guard cell ion fluxes, rate of stomatal response to exogenously applied ABA, and the expression of genes involved in ABA and light signalling networks. We next tested whether signalling pathways triggered by light transitions were altered in a manner that would affect stomatal regulation. We demonstrate that the SG-induced reduction in PAR altered stomatal morphonology, behaviour, and downstream signalling cascades.

Materials and methods

Plant culture and experimental design

The *Capsicum* crop was grown from April (autumn in Australia) to December 2019 (summer in Australia) in the state-of-the-art glasshouse facility at Western Sydney University (33°S, 150°E, Hawkesbury Campus, Richmond, NSW, Australia). A detailed description of the facility, including software and climate control, was presented by Chavan *et al.*, (2020) and Samaranayake *et al.* (2020). We used four research bays (105 m^2 each) with precise environmental control of atmospheric CO_2, air temperature, relative humidity, and hydroponic nutrient and water delivery. The hydroponic nutrient recipe was based on the European nutrient guideline booklet for glasshouse crops, (https://www.yara.nl/siteassets/toolbox/nutrient-solutions/nutrient-solutions-for-greenhouse-crops-dec2017.pdf/). *Capsicum annuum* L. seeds (variety Ghia, Syngenta, Australia) were grown in a nursery centre (Withcott Seedlings, Withcott, QLD, Australia) for 6 weeks. The seedlings were transplanted in Rockwool slabs and transferred into two control 'hazed' glass (control) and two SG (treatment) bays (Supplementary Fig. S1A).

The control bays were fitted with HD1AR diffuse 'haze' glass (roof 70% diffuse light; side walls 5% diffuse light). The two treatment bays had HD1AR diffuse glass, but were also coated with ULR-80 film, known as 'smart glass' (Solar Gard, Saint-Gobain Performance Plastics, Sydney, Australia). According to the manufacturer's specifications, the SG film ULR-80 has a low thermal emissivity (0.87) which blocks the light that mainly contributes to heat, but transmits most of the wavelengths of light used by plants for growth in the PAR region. In a previous study, we comprehensively measured light quality and quantity and generally validated the manufacturer's SG specifications (Chavan *et al.*, 2020). In the current experiment, SG reduced net short-wave radiation (measured by an SN 500 net radiometer), roof-level PAR, and canopy-level PAR (measured by PAR sensor LI190) by an average of 56, 29, and 9%, respectively, relative to the control during the 3 months of measurements (Supplementary Fig. S2).

Each bay had six gutters with length at 10.8 m and width at 25 cm (AIS Greenworks, Castle Hill, NSW, Australia), which were fitted with 10 Rockwool slabs (90×15×10 cm, Grodan, The Netherlands) per gutter. Three plants per slab were planted in the four middle gutters, and two plants per slab were planted in the two side gutters which served as buffer plants. Plants were grown in glasshouse light and photoperiod conditions, 25/20 °C (day/night) air temperature, 70/80% (day/night) relative humidity, and non-limiting nutrient and water (fertigation) supplied at industry standards. For sample collection and stomatal morphological measurement, unless otherwise specified, top canopy leaves fully exposed to light from each of the two bays were investigated. Sample collections were completed during sunny conditions on the same day or continuous days to minimize weather effects. The measurements described in this study were conducted between June and September 2019 (Supplementary Fig. S2).

Relative water loss measurement

Top canopy *Capsicum* leaves which were fully exposed to the glasshouse light were investigated for relative water loss rate (RWL). RWL was measured using the following equation with modifications (Weatherley, 1950), $RWL=(FM–FM_t)/FM×100\%$. Fresh mass (FM) was determined immediately after samples were collected and sealed on excision using a small piece of foil paper which minimizes the water loss through the petiole, and the samples were weighed and recorded as FM_t on a scale every 10 min for 90 min. Overall, 10 time points (including 0 min as control) were recorded and the ratio was used to determine differences in the rate of water loss from plants in SG and control plants. RWL investigations were conducted in a PC2 laboratory where temperature, humidity, and PAR are consistent. Five independent leaves from the top canopy of five independent *Capsicum* plants from two bays were collected at around 09.00 h on the same day for RWL investigations.

Stomatal assay

Stomatal aperture was measured using *Capsicum* epidermal peels from fully expanded top canopy leaves, according to O'Carrigan *et al.* (2014). Epidermal peels were collected under the respective glasshouse light and attached to 35 mm glass bottom Petri dishes (MatTek Corporation, MA, USA) using silicone adhesive (B-521, Factor II, InC Lakeside, AZ, USA) and bathed in maintaining solution, referred to as 'MS' (50 mM KCl, 5 mM MES at pH 6.1 with KOH) for ~20 min. Afterward, epidermal peels were imaged in MS under a Nikon microscope attached with a camera and a DS-U3 controller (Nikon, Tokyo, Japan). Images of stomatal apertures and sizes were measured and processed with ImageJ software (National Institute of Health, USA). Stomatal density investigations followed a simplified method (Schlüter *et al.*, 2003). Nail polish imprints were taken from the abaxial surface of mature leaves from plants grown under control and SG treatments. Stomatal densities were determined by light microscopy from leaf imprints of at least five individual plants from both SG and control growth rooms. Three independent counts were carried out on each leaf.

Given that stomata mediate the majority of plant water loss, more water loss from leaves of SG plants indicates a change in stomatal responses. Thus, the stomatal closure rate in response to ABA was investigated using epidermal peels. The method for measuring ABA-induced stomatal aperture changes followed Cai *et al.* (2017). Manually collected epidermal peels (the same as above) were incubated in MS for 1 h then rinsed with measuring buffer, referred to as 'MB' [10 mM KCl, 5 mM MES at pH 6.1 with $Ca(OH)_2$], three times within 10 min. Epidermal peels were imaged in MB for 10 min under light microscopy as a control. Then, 100 μM ABA treatment was applied and the peels were imaged for another 50 min. Images were taken every 5 min and stomatal apertures were measured and analysed with ImageJ. Thirteen time points (including 0 min as control) were recorded, and the ratio was used to reflect stomatal aperture changes; 30–80 stomata from at least three independent epidermal peels were analysed. Epidermal peels were collected at 09.00 h on sunny days.

Gas exchange measurement

Leaf gas exchange measurements utilized top canopy leaves which were fully exposed to their respective glasshouse light. Net assimilation rate (A_{net}) and stomatal conductance (g_s) were measured using a Li-6400XT (Li-Cor, Lincoln, NE, USA) infrared gas analyser at 25 °C and a vapour pressure deficit of ~1 kPa. Intrinsic water use efficiency (WUE_i) was calculated as the instantaneous ratio between net CO_2 assimilation rate and stomatal conductance. We measured the response of photosynthetic parameters to changes in light intensity over three stages that lasted ~140 min recorded at 60 s intervals. Initially, steady-state measurements were made under 1500 μmol m^{-2} s^{-1} PAR during 20 min of stabilization (control stage).

Subsequently, the light intensity was reduced to 100 μmol m^{-2} s^{-1} PAR and maintained for 1 h. Finally, the light intensity was returned to 1500 μmol m^{-2} s^{-1} PAR and measurements were continuously recorded for another hour. At the control stage, after 20 min of recording, gas exchange parameters stabilized and the average values were used for calculating stomatal conductance, photosynthesis rate, and WUE_i at the control stage.

The PAR decrease under SG was mostly in the blue spectrum (Chavan *et al.*, 2020). Therefore, we investigated the effect of blue light on stomatal conductance by removing blue light from the measurement light. Initially, 1500 μmol m^{-2} s^{-1} PAR [1350 μmol m^{-2} s^{-1} PAR using a red LED light and 150 μmol m^{-2} s^{-1} PAR using a blue LED light (10%)], was employed. After 20 min of measurement, 10% blue light was switched off and recording continued for 1 h before the 10% blue light ratio was returned for another hour. Average g_s was calculated and used for normalizing the relative g_s changes to the initial stage. Gas exchange measurements were conducted between 09.00 h and 15.00 h on sunny days and four individual *Capsicum* plants were measured from both SG and control treatments.

Stomatal morphological trait measurement and calculation of g_{max}

Operating stomatal conductance (g_{op}) was measured according to Drake *et al.* (2013) and McElwain *et al.* (2016) with modifications using top canopy *Capsicum* leaves. The g_{op} measurements were taken in the morning on sunny days with a Li-6400XT at 1500 μmol m^{-2} s^{-1} PAR, 25 °C, 70% ambient humidity, 300 μmol m^{-2} s^{-1} air flow, and a VPD of ~1 kPa. It is noted that the *Capsicum* stomatal opening phase took ~100 min to reach a steady-state g_{op}, and values are means of g_{op} measurements from four independent *Capsicum* plants using top canopy leaves from both SG and control treatments. Maximum theoretical stomatal conductance (g_{max}) calculation followed Drake *et al.* (2013) and McElwain *et al.* (2016), and utilized stomatal morphological parameters collected based on the stomatal assay:

$$g_{max} = \frac{dw * SD * pa_{max}}{v * \left(pd + \frac{\pi}{2} * \sqrt{\left(\frac{pa_{max}}{\pi}\right)}\right)}$$

where dw=diffusivity of water vapour at 25 °C (0.000025 m^2 s^{-1}) and v=molar volume of air (0.022 m^3 mol^{-1}) are both constants (McElwain *et al.*, 2016); SD is stomatal density (m^{-2}) observed from our stomatal assay; stomatal pore sizes (m^2) were calculated as an elipse using stomatal pore length (m) as the long axis and 1/2 stomatal pore width (m) as the short axis; pa_{max} (maximum stomatal pore size) was recorded from each replicate among four independent plants; and pd is stomatal pore depth (m) considered to be equivalent to the stomatal width of a fully turgid guard cell (McElwain *et al.*, 2016). Similarly, stomatal sizes (μm^2) were calculated following an ellipse using stomatal length (μm) as the long axis and 1/2 stomatal width (μm) as the short axis. Maximum stomatal sizes (SS_{max}, μm^2) were recorded accordingly from each replicate among four independent *Capsicum* plants.

Stomatal opening half-time ($t_{1/2}{}^{O}$, min) and closing half-time ($t_{1/2}{}^{C}$, min) were calculated as the time taken to achieve half the minimum (or maximum) of stomatal conductance following light transitions from four independent *Capsicum* plants of both SG and control treatments. Total stomatal opening time was calculated as the time *Capsicum* stomata took to reach the maximum stomatal conductance in response to the light transition from 100 μmol m^{-2} s^{-1} to 1500 μmol m^{-2} s^{-1} PAR. Total stomatal closing time was calculated as the time it took to reach the minimum stomatal conductance in response to the light transition from 1500 μmol m^{-2} s^{-1} to 100 μmol m^{-2} s^{-1} PAR.

Measurement of guard cell ion fluxes

For guard cell ion flux measurements, the preparation of epidermal peels was identical to that for the stomatal bioassay. Net fluxes of K^+, Cl^-, Ca^{2+}, and H^+ were measured using non-invasive, ion-selective microelectrodes (MIFEs) on guard cells of *Capsicum* according to Pornsiriwong *et al.* (2017) and Zhao *et al.* (2019). Specific details related to the MIFE theory, electrode fabrication, and calibration are described in Shabala *et al.* (2013). Epidermal peels were pre-treated with MS for 20 min before blue light treatment. The peels were fixed on a coverslip coated with silicone adhesive and then placed in a long, flat 5 ml measuring chamber containing MB. Electrodes with fine tips (resistance=4–6 GΩ) were filled with ion-selective ionophore cocktails (Sigma, Buchs, Switzerland) and their tips were moved towards and away from the sample in a slow (5 s cycle, 80 μm amplitude) square-wave by a computer-driven micromanipulator. Net fluxes of ions from guard cells were calculated from the measured differences in electrochemical potential for these ions between two positions. Net K^+, Ca^{2+}, H^+, and Cl^- fluxes from guard cells were measured for 10 min as a control to ensure initial, steady values before implementing the blue light treatment, and then measurements were conducted for another 30–40 min. At least five individual stomatal guard cells from independent plants were investigated for ion flux measurements.

Quantitative real-time PCR

Quantitative real-time PCR was performed as previously described (Chen *et al.*, 2016) but with modifications based on two reference genes optimized for *Capsicum* (Wan *et al.*, 2011). We measured the transcripts of key genes using abaxial epidermal peels of *Capsicum* leaves. The details of tested genes can be found in Supplementaty Table S1. Epidermal peels were collected for gene expression investigations to minimize the effect of mesophyll cell mRNA and to enrich the guard cell mRNA (Cai *et al.*, 2017). Fully expanded leaves from the top canopy of 4-month-old *Capsicum* plants were selected for sample collection. Under normal light inside growth rooms, the epidermal peel was immediately collected and stored in liquid nitrogen. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Australia) following the manufacturer's procedure, and the residual genomic DNA was removed with amplification grade DNase I (Ambion). First-strand cDNA was synthesized with the SensiFAST Kit (Bioline, Alexandria, Australia). Fluorescence reflecting expression of target genes was determined by the SensiFAST SYBR No-ROX Kit (Bioline, Australia) using gene-specific primers (Supplementary Table S1) by employing a Rotor-Gene Q6000 (Qiagen). qPCR conditions were composed of three steps of cycling: polymerase activation at 95 °C for 15 min; 40 cycles were set up for denaturation at 94°C for 15 s, annealing for 15 s at 55 °C, extension at 72 °C for 15 s; SYBR green signal data were acquired at the end. The ubiquitin-conjugating gene (*UBI-3*) and β-tubulin (β-*TUB*) were used as suitable references for normalization of relative gene expression in *Capsicum* (Wan *et al.*, 2011). Data were expressed as the average of four independent plants from two research bays with two technical replicates.

Statistical analysis

Statistical significance between SG and control plants, before and after treatment, was analysed using Student's *t*-test, and SPSS one-way ANOVA test using Turkey's method was applied for statistical analysis of ion flux measurement. All data were presented as means with standard errors.

Results

Smart glass reduced stomatal pore size but not stomatal conductance or density

Light conditions are vital for stomatal formation and development. To investigate stomatal morphological changes induced

by SG, we measured stomatal parameters from both control and SG-grown plants. During the measurement period of the current study, the SG treatment reduced net short-wave radiation, roof-level PAR, and canopy-level PAR by an average of 56, 29, and 9%, respectively, relative to the control (Supplementary Fig. S2). Accordingly, the SG chambers appeared light blue and the control chambers appeared white from the aerial view (Supplementary Fig. S1A). The *Capsicum* crop appeared visually similar in the control and SG bays (Supplementary Fig. S1B–D). The canopy-level PAR reductions reported here (~9%) with *Capsicum* are lower than what we previously reported (~19%) for the eggplant crop (Chavan *et al.*, 2020) because our measurements were conducted during the winter growing season (Supplementary Fig. S2).

Compared with control plants grown under normal 'hazed' glass, plants grown under SG had similar steady-state stomatal conductance measured at high light (Fig. 1B). However, SG significantly decreased stomatal pore size (P=0.036) by 13% relative to the control (Fig. 1C), due to reduced stomatal pore length rather than width (Fig. 1A; Supplementary Table S2). Stomatal size and density were not statistically different between the control and SG treatments (Fig. 1D, E). These results partially support our hypothesis that altered light conditions under SG will reduce stomatal aperture, indicated by decreased stomatal pore size (length) but not stomatal size or density.

Smart glass led to greater leaf water loss, slower ABA-induced stomatal closure, and up-regulation of ABA signalling genes

Did changes in stomatal morphology observed under SG induce physiological or molecular changes in the stomatal response? To answer this question, we compared water loss rate between SG and control plants and subsequently investigated

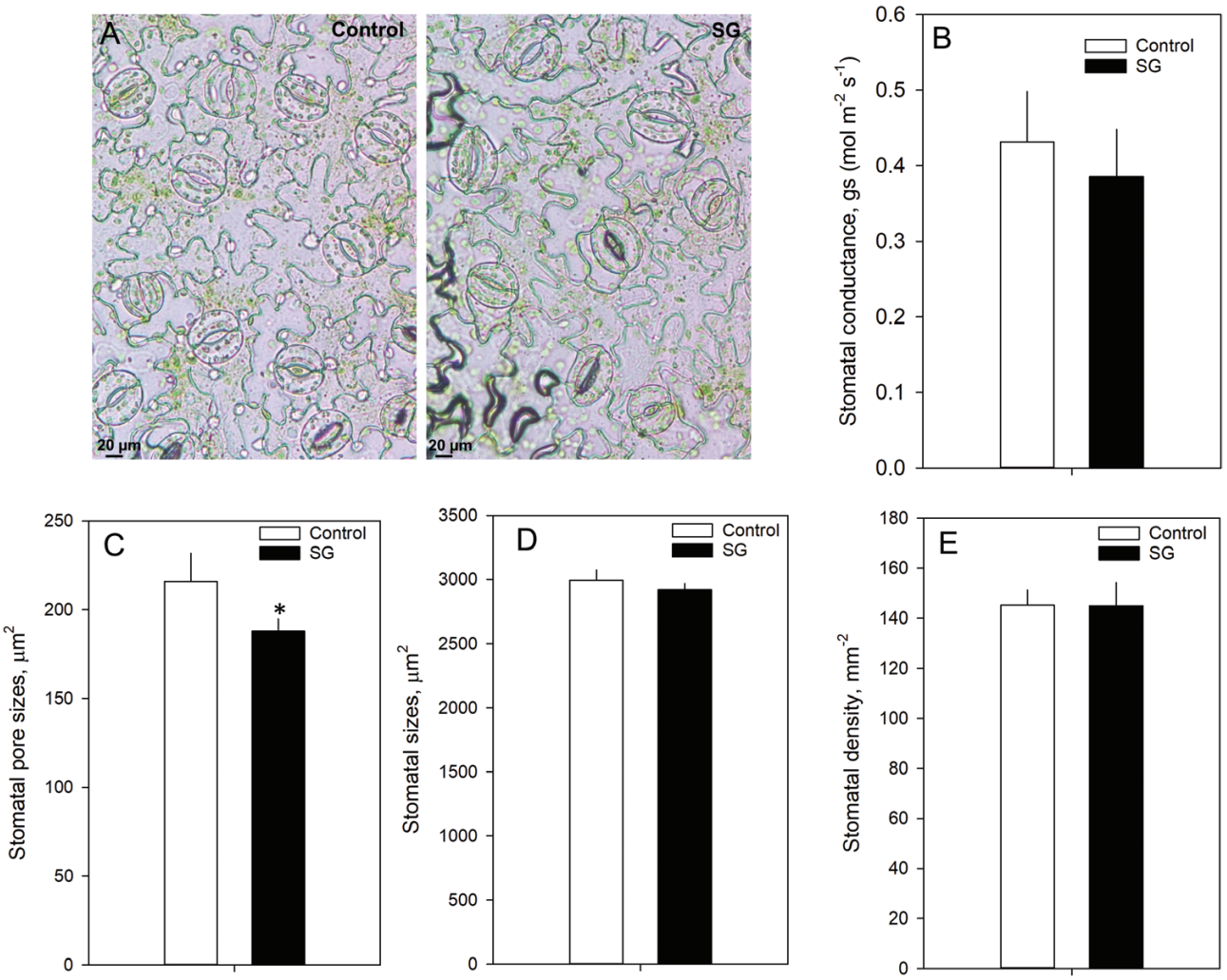

Fig. 1. Effect of smart glass (SG) on stomatal traits of *Capsicum*. (A) Photographs of stomata collected from epidermal peels of both control and SG plants. Scale bar=20 µm. (B) Stomatal conductance in the control and SG treatments measured under high light (1500 PAR). (C) Stomatal pore size, (D) stomatal size, and (E) stomatal density in control and SG plants (n=5 biological replicate with 50–80 stomata). Values are means ±SE. *P<0.05.

genetic transcripts relating to ABA signalling networks. During the initial 40 min following leaf detachment, SG and control leaves had similar rates of relative water loss. After 60 min, SG leaves transpired water faster than control leaves (P=0.012 at 90 min) (Fig. 2A). The initial stomatal aperture before ABA application was not significantly different between the control and SG treatments (Fig. 2B). Exogenously applied ABA caused a slower stomatal closure in SG plants, especially after 40 min of incubation with ABA (Fig. 2B).

We then quantified the expression of genes involved in ABA signalling in epidermal peels. *PYL8* and *CHLH* are vital ABA receptors whose mutations lead both to severe stomatal opening and an ABA-insensitive phenotype, whilst overexpression of *PYL8* or *CHLH* leads to high degrees of stomatal closure (Shen *et al.*, 2006; Gonzalez-Guzman *et al.*, 2012; Lim *et al.*, 2013). Relative to the control, there were significant increases (~4-fold) in *PYL8* and *CHLH* transcripts (Fig. 2C). Since ROS accumulation has been identified as a central network component for stomatal closure (Sierla *et al.*, 2016), core genes encoding ROS metabolism were also investigated [e.g. superoxide dismutase (SOD) which catalyses the decomposition of hydrogen peroxide (H_2O_2), and the GTP-binding protein ADP-ribosylation factor 1 (ARF1) (Dana *et al.*, 2000)]. SG generated a 3-fold up-regulation of *SOD1*, and *ARF1* expression was enhanced by 4-fold in SG compared with the control (Fig. 2C; Supplementary Fig. S3). However, the SG treatment showed no significant effect on the expression of *Catalase 3* (*CAT3*), which catalyses the breakdown of H_2O_2 into water and oxygen (Supplementary Fig. S3). Finally, the expression of *SLAC1*, whose protein contributes to stomatal closure (Deger *et al.*, 2015), was 4-fold higher in SG compared with control epidermal peels (Fig. 2C). Taken together, these results support our hypothesis that SG will affect stomatal sensitivity to water stress and ABA-mediated signalling processes.

Smart glass-grown stomata responded faster to light transitions without changes in SS_{max}, g_{max}, *or* g_{op}

To investigate if the SG treatment has altered the stomatal sensitivity, we measured changes in leaf gas exchange in response to light transitions between 1500 µmol m^{-2} s^{-1} and 100 µmol m^{-2} s^{-1} PAR (Fig. 3). On average, stomata of SG plants showed lower opening and closing half-times relative to the control (Fig. 3D). In particular, SG stomata closed faster in response to the transition to low (100 µmol m^{-2} s^{-1}) PAR, and opened faster in response to the subsequent transition to high PAR (1500 µmol m^{-2} s^{-1}) (Fig. 3A). After 140 min of both light transitions, stomatal conductance was significantly higher in SG relative to control plants (Fig. 3A; Supplementary Fig. S4A; Supplementary Table S2, P<0.01). Photosynthetic rates did not differ significantly between the control and SG treatments during the light transitions (Fig. 3B; Supplementary Fig. S4B; Supplementary Table S2). Consequently, SG plants had lower WUE_i relative to the control during the stomatal opening phase on transition to high light (Fig. 3C; Supplementary Fig. S4C; Supplementary Table S2).

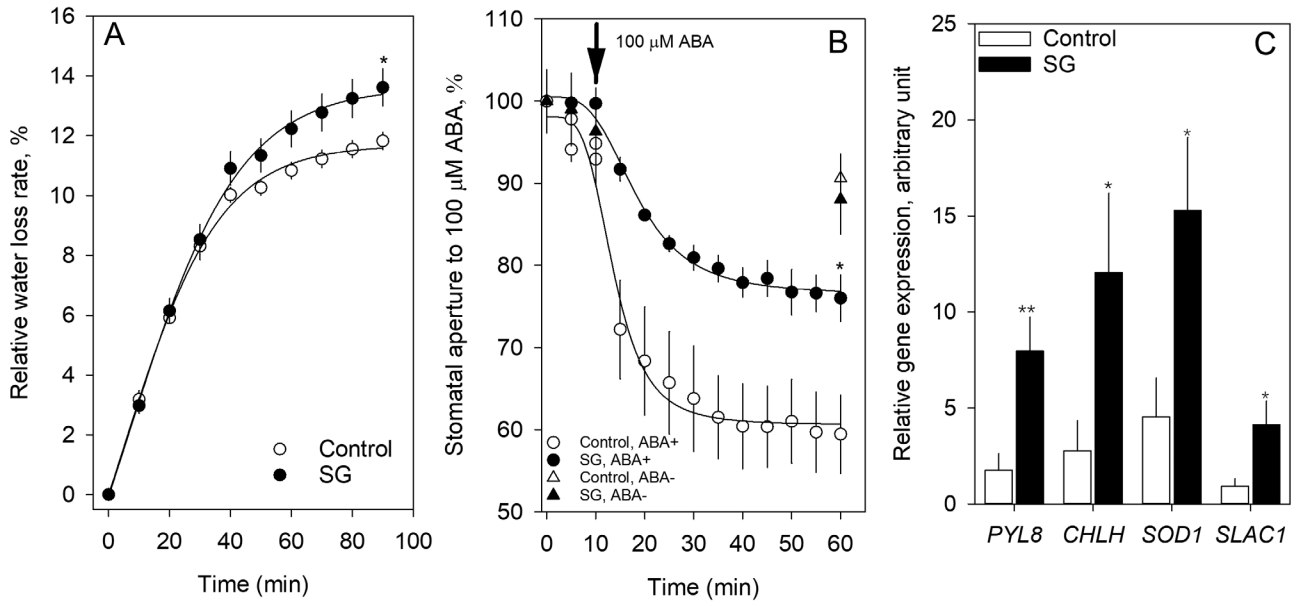

Fig. 2. Smart glass (SG) alters relative water loss, ABA-induced stomatal closure, and expression of genes of ABA signalling in *Capsicum*. (A) Relative water loss was calculated based on the mass loss every 10 min. Values are means ±SE (n=5). (B) Stomatal response to exogenous ABA (100 µM) application. Circles represent measurement with ABA while triangles represent control measurements without ABA treatment. Data are means ±SE (n=4 biological replicates from 30–80 stomata). (C) Relative expression of genes of ABA signalling from the epidermis. *Pyrabactin resistance8* (*PYL8*), *Mg-chelatase H subunit* (*CHLH*), *superoxide dismutase* (*SOD*), and *slow anion channel-associated 1* (*SLAC1*). Data are means ±SD (n=4 biological replicates with two technical replicates). *P<0.05; **P<0.01.

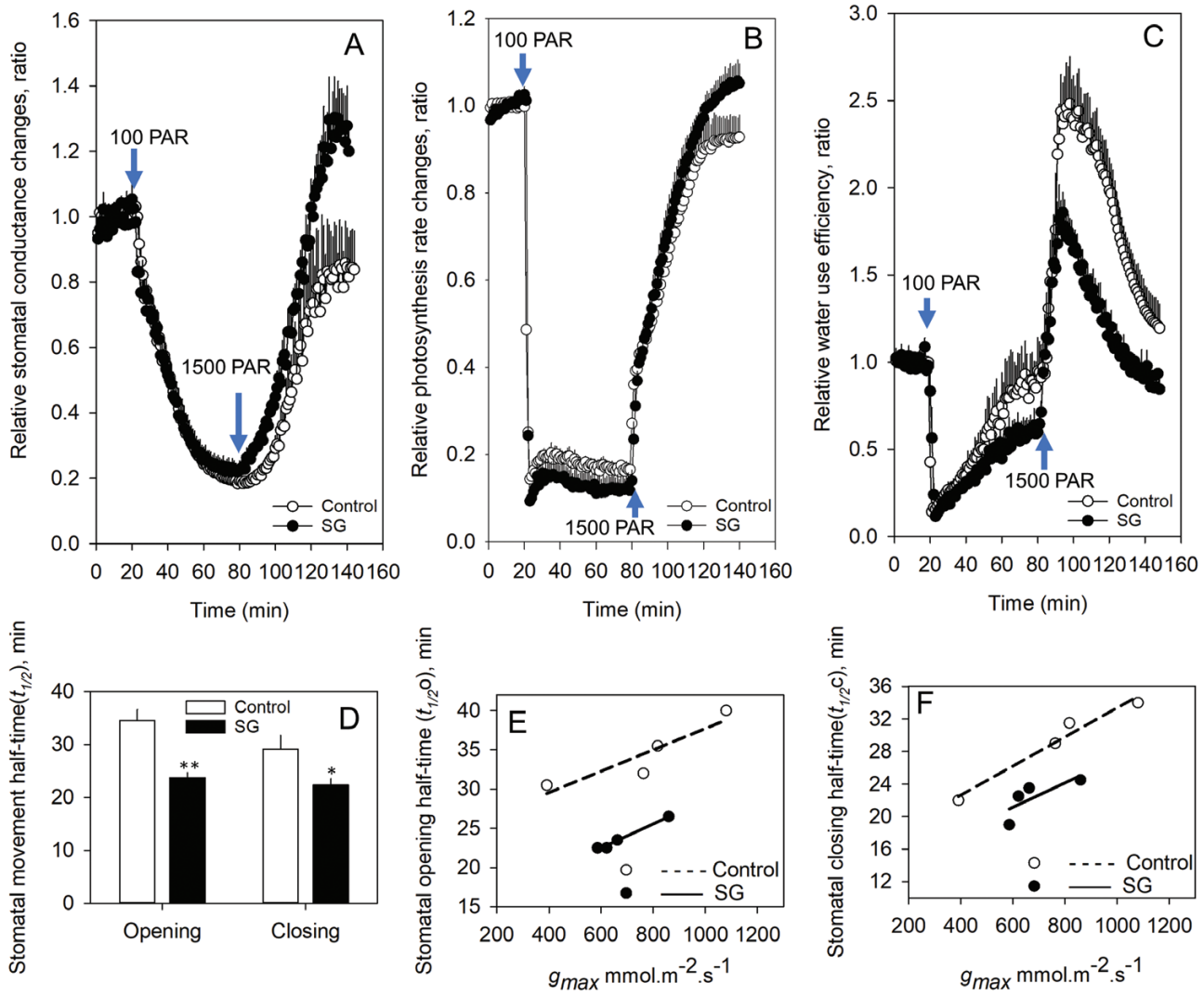

Fig. 3. Effect of smart glass (SG) on the responses of leaf gas exchange to short-term light transitions in *Capsicum*. Measurements were initially stabilized and recorded under 1500 PAR then light was reduced to 100 PAR for 1 h and increased to 1500 PAR for another 1 h. Averaged (A) stomatal conductance, (B) photosynthesis rates, and (C) WUE_i measured during the initial 20 min were used for normalizing ratios during the transition stages. (D) Stomatal opening half-time ($t_{1/2}^{O}$) and stomatal closing half-time ($t_{1/2}^{C}$). (E and F) Relationships between maximum theoretical stomatal conductance (g_{max}) and stomatal opening and closing half-times ($t_{1/2}$). Data are means ±SE (n=4). *P<0.05; **P<0.01.

Given the clear link between light conditions and stomatal development (Fu *et al.*, 2010; O'Carrigan *et al.*, 2014), we correlated stomatal parameters with maximum theoretical stomatal conductance (g_{max}) and operational stomatal conductance (g_{op}). SG and control plants maintained similar g_{op} and g_{max} (Supplementary Table S2). The relationship between g_{op} and g_{max} was steeper in SG than in control plants (Supplementary Fig. S5C). Both treatments showed parallel relationships between opening and closing half-times with g_{max} (Fig. 3E, F) and g_{op} (Supplementary Fig. S5D, E). The maximal stomatal size (SS_{max}) also showed similar relationships with g_{op} in both treatments (Supplementary Fig. S5B). Overall, SG produced more active and less water use-efficient stomata in response to light intensity changes (Fig. 3), with smaller aperture, but not size (Fig. 1), supporting our hypothesis that stomatal sensitivity to light intensity will increase due to the altered light condition under SG.

Smart glass enhanced expression of photoreceptor and photosynthesis genes in epidermal peels

SG reduced blue light transmission (Chavan *et al.*, 2020). Accordingly, stomatal conductance was investigated in response to changes in blue light fraction, which is required for inducing stomatal opening (Inoue and Kinoshita, 2017). SG and control plants responded similarly to blue light (Fig. 4A). During the measurement, there were no differences in stomatal conductance under 1500 µmol m^{-2} s^{-1} PAR (Fig. 4B). Removing 10% blue light (1350 µmol m^{-2} s^{-1} PAR) generally induced stomatal closure in both SG and control leaves, while the retrieval

of 10% blue-light increased stomatal conductance similarly in both treatments (Fig. 4B). Accordingly, our hypothesis about increased stomatal sensitivity to blue light under SG is rejected.

Capsicum leaf epidermal peels were used to assess the expression of photoreceptor- and photosynthesis-associated genes such as *PHOT1*, *PHYA*, and *RBCS1*. Compared with the control, SG-grown plants had enhanced gene expression by 27% in *PHOT1*, 148% in *PHYA*, and 265% in *RBCS1* (Fig. 4C; Supplementary Fig. S3). Further, SG plants exhibited increased gene expression of *UV light response element* (*UVRB*) and *UVR8* relative to control plants (Fig. 4C). These are crucial genes regulating photosynthesis and differential gene expression patterns between SG and control plants, indicating that a different factor was deployed by SG plants to adapt to changed light conditions. Overall, SG stomata maintained a higher sensitivity to light at physiological and molecular levels under the current glasshouse conditions.

Higher guard cell flux of K^+, Ca^{2+}, and Cl^- is induced by smart glass but suppressed by blue light

Stomatal opening and closing are regulated by ion fluxes across membranes of guard cells. We investigated ion fluxes from guard cells of both treatments. In normal light, guard cells from SG plants showed approximately three times greater efflux of K^+ and Cl^- compared with control plants (Fig. 5A, D), which reduced stomatal aperture and contributed to closure. The Ca^{2+} influx of guard cells from SG plants was about twice as high as that from control plants (Fig. 5B). The H^+ efflux of guard cells was similar between SG and control plants under normal light (Fig. 5C).

Blue light significantly suppressed K^+ efflux of guard cells by 35% in control and 53% in SG plants (Fig. 5A). Moreover, blue light also suppressed Cl^- efflux by 72% in SG and 28% in control plants (Fig. 5D). Meanwhile, Ca^{2+} influxes were suppressed by 41% and 60% in control and SG, respectively (Fig. 5B). In contrast, blue light slightly induced H^+ efflux in the control treatment, but slightly suppressed H^+ efflux in SG, indicating similar effects on guard cells of SG and control plants (Fig. 5C). Under SG, epidermal peels indicated enhanced solute loss under normal light conditions but maintain the ability to response to blue light. This agrees with our hypothesis that long-term altered light conditions under SG will affect guard cell ion fluxes determining stomatal status.

Discussion

In this study, we compared stomatal functions in upper canopy leaves from *Capsicum* plants grown under SG and control lighting environment. Our results can be categorized into four main findings. Firstly, SG reduced stomatal pore size and increased guard cell fluxes (K^+ and Cl^- efflux, and Ca^{2+} influx) and the expression levels of *SLAC1* involved in cellular ion homeostasis without appreciably affecting g_s, g_{op}, g_{max}, stomatal size, or stomatal density. Secondly, SG reduced stomatal sensitivity to ABA, leading to relatively more water loss in detached leaves, and this response was underpinned by up-regulation of ABA- (*PYL8* and *CHLH*) and ROS- (*SOD1* and *ARF1*) related genes. Thirdly, SG stomata responded faster to PAR transitions and became less water use efficient, such that stomatal opening

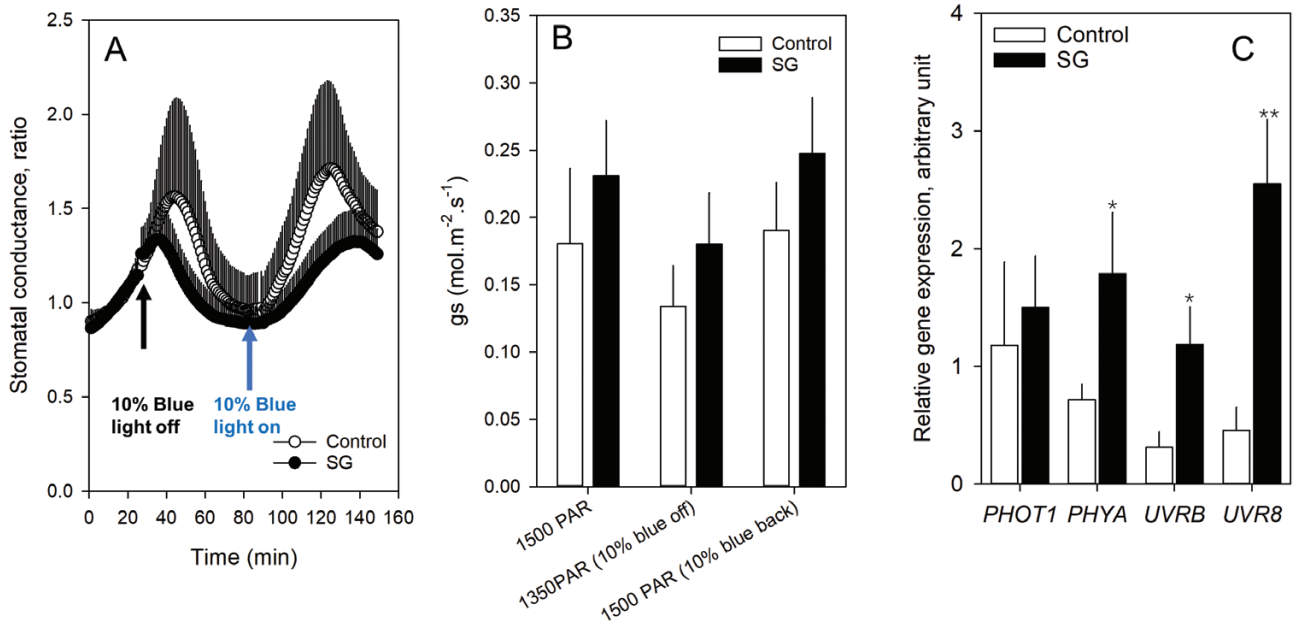

Fig. 4. Smart glass (SG) induces different stomatal and gene expression responses to blue light in *Capsicum*. (A) Stomatal conductance was monitored under three light regimes: normal light (1500 PAR: 1350 red+150 blue) for 20 min, 1350 red PAR for 1 h, and normal light for another 1 h. The averaged stomatal conductance value from the initial 20 min measurement was used for normalizing ratios during transitions. (B) Values at the end of each light condition. Data are means ±SE (n=4). (C) Gene expression of *Phototropin 1* (*PHOT1*), *Phytochrome A* (*PHYA*), *UV response element* (*UVRB*), and *UV-B Receptor 8* (*UVR8*). Data are means ±SE (n=4 biological replicates with two technical replicates). *P<0.05; **P<0.01.

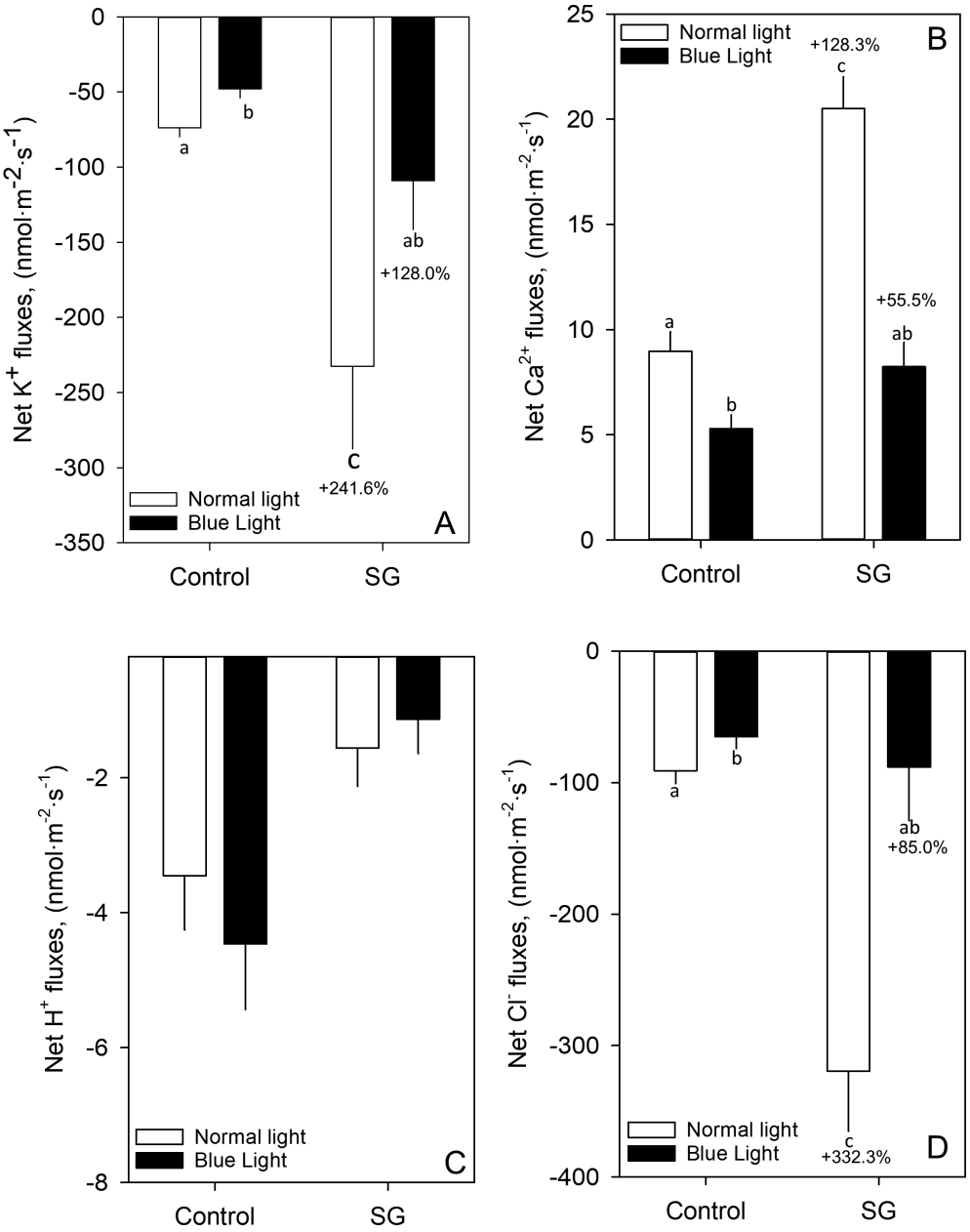

Fig. 5. Smart glass (SG) affects ion fluxes and their regulation by blue light in guard cells of *Capsicum*. Net fluxes of K^+ (A), Ca^{2+} (B), H^+ (C), and Cl^- (D) were recorded from guard cells in leaf epidermal peels. Data are means ±SE (n=5–7 plants). Different lower case letters represent a statistical difference.

and closing speed was proportional to g_{max}, whilst the relationship between g_{op} and g_{max} was steeper in SG plants. Fourthly, even though SG filtered out light most efficiently in the blue spectrum, dependence of stomatal conductance on blue light was similar between SG and control treatments. Yet, guard cell fluxes showed selectively greater (K^+ and Ca^{2+}) or different (H^+) blue light sensitivities in the SG plants, and this was associated with increased expression of photoreceptor genes (*PHOT1* and *PHYA*) and UV-B light response genes (*UVRB* and *UVR8*). Combining all these findings, the SG light condition did not impair stomatal ability to respond to light changes in *Capsicum* leaves; instead, the adaptation of SG *Capsicum* stomata to the altered light condition involved a more active response to PAR changes, ABA signalling, and solute loss to maintain a decreased stomatal pore size under SG light condition.

Decreased stomatal pore area in smart glass is underpinned by enhanced guard cell solute loss and anion channel activity rather than changes in stomatal morphology

Stomata regulate plant water use efficiency by affecting CO_2 uptake and photosynthesis as well as transpiration (Brodribb *et al.*, 2009). Under low light conditions, where

light reception is limited, full stomatal opening may not be necessary for photosynthesis (Pasternak and Wilson, 1973). A study in sweet pepper suggests that partial shade induced lower stomatal aperture (Jaimez and Rada, 2011). Under SG, where the light intensity was lower than in the control, stomatal pore sizes were significantly smaller in SG than in control leaves, due to decreased stomatal pore length (Fig. 1C). We found that there was no difference in stomatal density between SG and control plants (Fig. 1E). Moreover, no significant difference was observed in most of the stomatal morphological parameters (Supplementary Table S1), suggesting that ion flux changes may affect the stomatal aperture.

Stomatal opening requires activation of potassium inward channels, such as KAT1, KAT2 (Ronzier *et al.*, 2014), and AKT1 (Nieves-Cordones *et al.*, 2012), as well as decreased activity of the potassium outward channel GORK (Hosy *et al.*, 2003). SLAC1 plays a vital role in regulating stomatal response to light (Hiyama *et al.*, 2017), CO_2 (Lind *et al.*, 2015), and humidity (Vahisalu *et al.*, 2008), and stomatal closure in response to drought (Geiger *et al.*, 2009), salinity (Qiu *et al.*, 2016), and darkness (Merilo *et al.*, 2013). Here, SG stomata exhibited significantly higher guard cell efflux of K^+ and Cl^-, which suggests that SG plants close stomata more rapidly than control plants (Fig. 5A, D). Compared with control, SG plants take up about twice more Ca^{2+} into guard cells (Fig. 5B), which is in agreement with other studies showing that increased cytosolic Ca^{2+} activates anion channels (Asano *et al.*, 2012) and deactivates potassium inward channels (Ronzier *et al.*, 2014) for stomatal closure (Asano *et al.*, 2012; Zhao *et al.*, 2018). In our study, the higher guard cell efflux of K^+ and Cl^- and influx of Ca^{2+} under SG reduced cell turgor, thereby decreasing stomatal pore area. SG plants also showed significantly higher expression of *SLAC1* responsible for Cl^- efflux (Brandt *et al.*, 2012), and higher expression of ABA receptor genes (Fig. 2C). Hence, we propose that SG-induced prolonged low light conditions increased solute loss, leading to a decrease in stomatal pore area and reduced stomatal conductance (Supplementaty Fig. S6).

Smart glass deceased stomatal sensitivity to exogenously applied ABA due to up-regulated ABA signalling leading to higher water loss from Capsicum *leaves*

Sensing adverse environments and producing ABA for closing stomata has been well established during plant evolution (Lind *et al.*, 2015), and the speed for closing stomata reflects the plant's ability to adapt to a new environment (Pantin *et al.*, 2013; Wang and Chen, 2020). A higher relative water loss rate in detached SG leaves, together with stomata that do not open as wide and close more slowly following ABA application, indicates that a modified acclimation mechanism for closing stomata developed in plants grown in SG.

We investigated transcripts of the critical components of ABA signalling networks (Supplementary Fig. S6). The ABA-induced signalling network consists of critical components, including ABA receptors (Gonzalez-Guzman *et al.*, 2012; Merilo *et al.*, 2013), ROS production (An *et al.*, 2008), Ca^{2+} signalling (Asano *et al.*, 2012; Ronzier *et al.*, 2014), and regulation of ion channels (Hosy *et al.*, 2003; Vahisalu *et al.*, 2008; Deger *et al.*, 2015). In our study, SG increased expression of the ABA receptor gene *PYL8* and the ABA signalling genes *CHLH* and *ARF1* (Fig. 2C; Supplementary Fig. S3), indicating that their roles are affected by SG (Mishra *et al.*, 2006; Liu *et al.*, 2013). SOD1 functions as a strong ROS remover in plants, which also affects stomatal activity through ROS accumulation (An *et al.*, 2008; Jiang and Yang, 2009; Jannat *et al.*, 2011). Therefore, the up-regulated gene expression of *SOD1* may suggest ROS accumulation in SG guard cells as part of the SG-affected ABA signalling (Fig. 2C; Supplementary Fig. S3) to regulate anion channels for stomatal closure (Sierla *et al.*, 2016; Zhao *et al.*, 2018). This was confirmed by increased expression of *SLAC1* in SG plants, enhanced guard cell Cl^- efflux, and the slow stomatal response to exogenous ABA treatment. As the ABA-induced stomatal signalling elements were already enhanced, exogenously applied ABA failed to induce further significant stomatal closure in SG plants, leading to a higher water loss rate in SG detached leaves.

Faster smart glass-induced stomatal response to light transitions correlates with g_{max} *without affecting photosynthesis rate*

Stomatal morphology (e.g. stomatal size), stomatal conductance, and photosynthesis rate are linked to g_{max} and g_{op} (Drake *et al.*, 2013), such that faster stomatal reaction speed to light increases g_{op}, thereby improving photosynthesis and possibly water use efficiency (Lawson and Matthews, 2020). We found that SG stomata exhibited a faster response rate to light transitions and these rates were well correlated with g_{max}. However, averages of g_{max} and g_{op} were not affected by SG. Low PAR generally reduces stomatal conductance and net photosynthetic rate (Pasternak and Wilson, 1973; Farquhar and Sharkey, 1982; Roelfsema and Hedrich, 2005). This is also supported by a previous study, where *Capsicum* plants grown in low light conditions (20% of control) had a reduced stomatal index and CO_2-saturated photosynthesis rate (Fu *et al.*, 2010). Here, we found that SG plants produced slightly smaller stomata, which partly agrees with the above findings.

The 'smaller but faster stomata' theory was supported by a multispecies study which found that smaller stomata were usually associated with faster stomatal dynamics (Franks and Beerling, 2009; Drake *et al.*, 2013), and has been summarized in a recent review (Lawson and Vialet-Chabrand, 2019). Evolution of a faster stomatal response promoted expansion of grasses (Chen *et al.*, 2017), leading to higher plant productivity, efficiency, and fitness (Lawson and Vialet-Chabrand, 2019). In

our study, SG plants exhibited a smaller stomatal pore size as well as faster opening and closing speed in response to light transitions between 1500 μmol m^{-2} s^{-1} PAR and 100 μmol m^{-2} s^{-1} PAR, which validate the 'smaller but faster stomata' theory (Drake *et al.*, 2013) in a greenhouse horticultural crop.

However, SG did not significantly affect the net photosynthetic rate (Supplementary Fig. S3B), whilst increasing the speed of stomatal closing and opening in response to light transitions (Fig. 3). Ultimately, this led to reduced WUE_i on switching to high PAR (Fig. 3C), in contrast to what has been previously stipulated (Lawson and Matthews, 2020). Faster stomatal opening, in particular, may allow more CO_2 diffusion inside the leaf, but may also lead to excessive water loss, especially if plants, such as *Capsicum*, already enjoy high stomatal conductance and are grown under non-limiting conditions. In accordance with this, detached SG leaves lost more water than control leaves (Fig. 2).

Enhanced expression of light-responsive genes underpins the effects of smart glass on Capsicum

Photoreceptors are closely linked with plasma membrane transport, determining plant ionic balance, affecting plant growth, development, and yield (Babla *et al.*, 2019). *PHOT1* and *PHOT2* were reported to regulate membrane transport via regulating cytosolic Ca^{2+} in plants (Briggs and Christie, 2002). Further evidence suggests that *PHOT1* joins blue light-induced Ca^{2+} influx to the cytoplasm and therefore affects significant changes of Ca^{2+} and H^+ fluxes (Babourina *et al.*, 2002). In our study, SG plants showed up-regulation of *PHOT1* along with increased Ca^{2+} influx. SG reduces 99% of UV light into the greenhouse bays, which also induced a significantly higher expression of UVR8 (Fig. 4C). In Arabidopsis, UVR8 plays important roles in UV light-induced stomatal closure by a mechanism involving both H_2O_2 and nitric oxide generation in guard cells (Tossi *et al.*, 2014). This further supports our observations of a higher basal level of expression of light-responsive genes in SG so that stomata maintain full capacity to respond to light alternations.

To confirm previously reported blue light-induced stomatal opening case studies, where crucial ion channels, such as K^+ (Takahashi *et al.*, 2013), Ca^{2+} (Ronzier *et al.*, 2014), H^+ (Inoue and Kinoshita, 2017), and Cl^- (Hiyama *et al.*, 2017), were regulated by blue light, we measured dynamic stomatal conductance during blue light transitions and ion flux changes in response to blue light with no red light background. SG plants exhibited a suppression of K^+ and Cl^- effluxes and Ca^{2+} influx (Fig. 5). Absence of blue light slightly decreased stomatal conductance in both SG and control plants, whilst blue light retrieval mildly increased stomatal conductance (Fig. 4B), indicating the key role of blue light in stomatal opening in *Capsicum* (Inoue and Kinoshita, 2017). However, compared with the light retrieval-induced stomatal dynamic changes (Fig. 3A), stomata of SG plants obviously responded more actively to light intensity than the blue light spectrum (Figs 3A, 4A). Overall, SG plants maintain a similar capacity of responding to the blue light spectrum but more actively respond to light intensity relative to the control, and this is highly linked with the increased expression of light-responsive genes (Fig. 4C).

Conclusions

Altered light condition under SG did not lead to strong stomatal morphological changes but decreased stomatal aperture, which is the consequence of vigorously activated ABA and light signalling networks as well as Ca^{2+} influx and K^+ and Cl^- effluxes from SG guard cells (Supplementary Fig. S6). Interestingly, SG-grown plants presented faster stomatal responses to changes in illumination, and this was associated with smaller stomatal pore sizes. However, the faster stomatal responses did not improve *Capsicum* photosynthesis whilst compromising WUE_i of attached leaves during light transitions and accentuating water loss of detached leaves under SG light conditions. The current study provides valuable physiological insight into the effects of SG material on *Capsicum* production in controlled-environment horticulture. Together with our previously published work (Chavan *et al.*, 2020), we conclude that SG films could potentially be suitable materials for growing *Capsicum* particularly in high-light countries, such as Australia.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Photographs of the high-tech glasshouse and *Capsicum* crop.

Fig. S2. Daily averages of the light conditions over the experimental period in the two control and two smart glass bays.

Fig. S3. Relative expression of genes relevant to ABA signalling, ROS metabolism, and photosynthesis in *Capsicum* epidermal peels under control and smart glass.

Fig. S4. Stomatal conductance, photosynthesis rate, and intrinsic water use efficiency changes during the transitions of light intensities.

Fig. S5. Correlation analysis of the effect of smart glass on stomatal traits in *Capsicum*.

Fig. S6. Schematic summary of smart glass-induced low-light condition on the guard cell signalling network in *Capsicum*.

Table S1. Primers and gene information in the quantitative RT-PCR experiment.

Table S2. Summary of stomatal traits, gas exchange parameters, and ion fluxes of *Capsicum* between control and smart glass conditions.

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Author contributions

DT, ZHC, CIC, and OG designed the smart glass experiment that supported this project. The project was conceived by CZ, ZHC, and OG. CZ and SC performed experimental research and data analyses. CZ, ZHC, OG, DT, and CIC wrote the manuscript with contributions from all co-authors.

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Conflict of interest

No conflict of interest is declared.

Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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