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Slow photosynthetic induction and low photosynthesis in *Paphiopedilum armeniacum* are related to its lack of guard cell chloroplast and peculiar stomatal anatomy

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Paphiopedilum and *Cypripedium* are close relatives in the subfamily Cypripedioideae. *Cypripedium* leaves contain guard cell chloroplasts, whereas *Paphiopedilum* do not. It is unclear whether the lack of guard cell chloroplasts affects photosynthetic induction, which is important for understory plants to utilize sunflecks. To understand the role of guard cell chloroplasts in photosynthetic induction of *Paphiopedilum* and *Cypripedium*, the stomatal anatomy and photosynthetic induction of *Paphiopedilum armeniacum* and *Cypripedium flavum* were investigated at different ratios of red to blue light. The highest stomatal opening and photosynthesis of intact leaves in *P. armeniacum* were induced by irradiance enriched with blue light. Its stomatal opening could be induced by red light 250 μmol m^{-2} s^{-1}, but the magnitude of stomatal opening was lower than those at the other light qualities. However, the stomatal opening and photosynthesis of *C. flavum* were highly induced by mixed blue and red light rather than pure blue or red light. The two orchid species did not differ in stomatal density, but *P. armeniacum* had smaller stomatal size than *C. flavum*. The stomata of *P. armeniacum* were slightly sunken into the leaf epidermis, while *C. flavum* protruded above the leaf surface. The slower photosynthetic induction and lower photosynthetic rate of *P. armeniacum* than *C. flavum* were linked to the lack of guard cell chloroplasts and specific stomatal structure, which reflected an adaptation of *Paphiopedilum* to periodic water deficiency in limestone habitats. These results provide evidence for the morphological and physiological evolution of stomata relation for water conservation under natural selection.

Introduction

The stoma is central to plant physiology as it plays a critical role in the regulation of gas exchange and hence evaporation from leaf mesophyll to atmosphere (Webb and Baker 2002). A stoma is formed by a pair of specialized parenchyma cells known as guard cells which are responsible for regulating stomatal opening. As plants require sufficient CO_2 to enter the leaf for photosynthesis, stomatal size and density are positively linked to leaf conductance to vapor and CO_2 and hence photosynthetic carbon gain (Büssis et al. 2006). Maximum leaf conductance can be determined by the stomatal size and density which are linked

Abbreviations – AQE, apparent quantum efficiency; ETR, electron transport rate; Lcp, light compensation point; LMA, leaf mass per unit area; Lsp, light saturation point; PPFD, photosynthetic photon flux density; PSII, Photosystem II; qP, photochemical quenching.

to the variation in atmospheric CO_2 concentration over geologic time (Franks and Beerling 2009). For this reason, stomata have significant implications for global hydrological and carbon cycle (Fraser et al. 2009). Knowledge of stomatal function is critical to determine the plant responses to environmental stresses, particularly reduced water availability and is necessary for variety selection and plant breeding to enhance water use and yields in dry environments (Lawson 2009).

Plants inevitably lose water from their leaves through transpiration during the simultaneous uptake of CO_2 for photosynthesis from the atmosphere. Accordingly, the efficient adjustment of stomatal movement is essential for the balance between maintaining water and CO_2 uptake. The stomatal movement is regulated by guard cells and influenced by many environmental and endogenous signals, such as light, temperature, CO_2, water status and plant hormones (Assmann and Shimazaki 1999, Gehring et al. 1997, Lawson et al. 2003). The responses of guard cell chloroplasts to environment factors are similar to those in mesophyll cells (Lawson et al. 2003), but guard cell chloroplasts accumulate starch in the dark, and hydrolyze it in the light. This opposite time course to that of mesophyll chloroplasts suggests different carbon assimilation mechanisms (Talbott and Zeiger 1998, Zeiger et al. 2002). Guard cell chloroplasts contribute to stomatal opening by providing reductants, osmotic modulators or a blue-light photoreceptor (Lawson 2009). Although significant advances in the understanding of guard cell function and stomatal responses have been made over the past century, many gaps in the knowledge regarding guard cell metabolism and its role in stomatal behavior remain (Lawson 2009).

In the majority of plant species, guard cells contain well-developed chloroplasts, but the species of *Paphiopedilum* do not have guard cell chloroplasts (Nelson and Mayo 1975, Zeiger et al. 2002). The ecophysiological implications of the lack of guard cell chloroplasts in *Paphiopedilum* are intriguing (Chang et al. 2011, Zeiger et al. 1985). Previous studies suggested that guard cell chloroplasts are needed to sustain high conductance rates at moderate to high irradiances (Donovan and Arditti 1984, Williams et al. 1983).

The stomatal response to light in the isolated leaf peels is regulated by the photosynthesis in guard cell chloroplasts, specific blue light response and phytochrome (Schwartz and Zeiger 1984, Talbott et al. 2002). As *Paphiopedilum* do not have guard cell chloroplasts in stomata, they lack a photosynthesis-dependent opening response but have a blue light-specific opening (Zeiger et al. 1983). The stomatal response to blue light in *Paphiopedilum harrisianum* can be enhanced by red light, reduced intercellular CO_2 concentrations (C_i) and low vapor pressure differences (Assmann 1988). Zeiger et al. (1983) showed that high fluence rate of red light does not stimulate stomatal opening in *P. harrisianum*, while Talbott et al. (2002) found that stomatal opening of two *Paphiopedilum* species is greatest in red light at 10 μmol m^{-2} s^{-1} and declines at higher fluence rate. Blue light-stimulated opening can be completely reversed by green light in the presence of far red light. Previous study showed that the guard cell plastids operate a xanthophyll cycle, and zeaxanthin concentration increases upon blue light irradiation, indicating that green light activates both a phytochrome response and the blue-green reversibility response in *Paphiopedilum* (Zeiger et al. 2002). The existing findings in stomatal movement induced by light quality remain ambiguous, especially in terms of fluence rate of red light. Previous studies suggested that a fluence rate of red light 10 μmol m^{-2} s^{-1} is advantageous for stomatal opening in *Paphiopedilum* (Talbott et al. 2002), but would not be favorable for photosynthesis and growth in nature. In fact, several *Paphiopedilum* species, such as *P. armeniacum*, can grow well under higher light condition in forest margins or on rocks. Clearly, some of the previous results do not agree well with the observations of *Paphiopedilum* species in their natural habitats, as most studies are conducted in isolated leaf peels. As mesophyll cells play an important role in stomatal opening, the sensitivities of stomata in the intact leaf to environmental factors such as light and CO_2 are different from those in the detached epidermis (Lee and Bowling 1995, Zeiger et al. 2002). On the basis of the analysis above, we speculated that the lack of guard cell chloroplasts would influence photosynthetic induction, as it can affect the stomatal response to light. However, no study has assessed the effect of lack of guard cell chloroplasts on photosynthetic induction, which is important for understory plants to utilize sunflecks and increase carbon gain.

Paphiopedilum has a close phylogenetic relationship with the genus *Cypripedium* (Cox et al. 1997) that contains well-developed guard cell chloroplasts. A comparison of *Paphiopedilum* and related orchid genera that have guard cell chloroplasts should be helpful for the understanding of the role of guard cell chloroplasts in stomatal function (Assmann and Zeiger 1985, Williams et al. 1983). In this study, we investigated the stomatal anatomy, photosynthetic induction and photosynthetic performance of *Paphiopedilum armeniacum* and *Cypripedium flavum* at different ratios of red and blue light. The aims were to understand the effects of light quality on the photosynthetic induction and photosynthetic performance of *P. armeniacum* and

C. flavum. We hypothesized that the photosynthetic induction of *P. armeniacum* due to the lack of guard cell chloroplasts was slower than that of *C. flavum*.

Materials and methods

Plant materials

Both *C. flavum* and *P. armeniacum*, known as lady's slipper orchids, are endemic to China and belong to the subfamily Cypripedioideae (Cox et al. 1997). *Cypripedium flavum* is a deciduous herb and can be found in the understory of sparse woods and forest margins at an altitude between 1800 and 3700 m in the west of China, and on brown forest soils with abundant humic matter, and pH 6.1–6.8. *Paphiopedilum armeniacum*, an evergreen herb, is found in western Yunnan of China, at elevations from 1350 to 2050 m. It grows on limestone cliffs and slopes in semi-shady forests. The habitat is characterized with a constant light fog in the winter and heavy rains in the summer. Stable carbon isotope ratio ($\delta^{13}C$) analysis is widely employed as a rapid screening method to discriminate photosynthetic type (Ehleringer and Osmond 1989). The values of $\delta^{13}C$ in *P. armeniacum* and *C. flavum* were $-26.52 \pm 0.08‰$ and $-27.86 \pm 0.64‰$, respectively, indicating C_3 photosynthesis for both species.

About 50 mature plants of *P. armeniacum* and *C. flavum* were collected from Baoshan (altitude 1650 m, 99°10′E, 25°07′N) and Shangri-La (altitude 3200 m, 99°33′E, 27°55′N) in Yunnan province, respectively. Then, these plants were cultivated in plastic pots with bark mixture in the greenhouse at Kunming (altitude 1900 m, 102°41′E, 25°01′N), Yunnan province, and were shaded with nylon netting to give about 40% full sunlight. In the greenhouse, the air temperature was 20–25°C in the day and 10–15°C at night, and a misting system was used to maintain 70–80% relative humidity. The plants were watered as needed and fertilized once a month. After cultivation for 1–2 years, 30 plants per species were used for measurements of this study.

Stomatal observation

The adaxial and abaxial epidermis of the middle part of mature leaves were peeled from fresh leaves, and images were taken using an Olympus U-CMAD3 light microscope (Olympus Optical Co. Ltd, Tokyo, Japan). Stomatal number was counted in 30 randomly selected fields per species and stomatal density (d) was calculated. The length and width of guard cells, referred to as stomatal length (l) and width (w), were measured for 30 stomata selected randomly. Stomatal aperture area (A_s) was calculated as $1/4 \times \pi \times l \times w$ (Shelley and David 2001).

As chloroplasts can emit fluorescence, the epidermal peels were observed under green light of 500–530 nm using a Zeiss Axioplan2 fluorescence microscope (Zeiss, Oberkochen, Germany) to detect the presence of chloroplasts in guard cells of stomata following the method of Nelson and Mayo (1975).

Tissues from mature leaves were fixed in a solution consisting of 70% formaldehyde, acetic acid and alcohol (5:5:90, v/v/v) for 1 day. They were then dehydrated in an alcohol series and embedded in paraffin wax for sectioning. Transverse sections were examined using a light microscope (Olympus Optical Co. Ltd).

Leaf fragments were mounted on a stub in a low temperature tissue Tek and transverse sections were taken using a cryo-microtome. Then, the sections were transferred to the cold stage of a KYKY Amray 1000B scanning electron microscope (KYKY Inc., Beijing, China), etched for several minutes at −90°C, cooled to −170°C and coated with gold for observation at 30 kV.

Photosynthetic measurement

Photosynthetic measurements were conducted on fully developed, healthy leaves using a Li 6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE) that is attached with a 6400-40 fluorescence chamber. Before taking measurements, each leaf was adapted in the dark using a dark-adapted clip for 1 h to ensure complete stomata closure. Afterwards, the minimal fluorescence (F_o) was determined by a weak modulated light, then a 0.8-s saturating light of 8000 μmol m^{-2} s^{-1} was used on a dark-adapted leaf to determine the maximal fluorescence (F_m). Then photosynthetic gas exchanges were induced by illuminating the leaves with actinic light of 250 μmol m^{-2} s^{-1}. The actinic lights were configured to different red-blue light ratios (B0, full red light; B30, 30% blue + 70% red; B70, 70% blue + 30% red; B100, full blue light) by using the red-blue light source built into the Li-6400. During the photosynthetic induction measurements, relative air humidity and temperature of the leaf in the leaf chamber were maintained at 70% and 20°C, respectively. Photosynthetic rate (A) and stomatal conductance (g_s) were recorded every 3 min from five leaves of different plants of each species using an automated protocol built into the Li-6400.

After completion of photosynthetic induction, response curves of photosynthesis and chlorophyll fluorescence to photosynthetic photon flux density (PPFD) were made simultaneously from five leaves of different plants of each species using an automated protocol built

into the Li-6400. These measurements were performed under controlled levels of CO_2 (370 μmol CO_2 mol^{-1}), flow rate (200 μ mol s^{-1}), leaf temperature (20°C) and vapor pressure deficit (1.0–1.5 kPa). Effective quantum yield of Photosystem II (PSII) (φ PSII) was calculated as $(F_m' - F_s)/F_m'$, where F_s is steady-state fluorescence and F_m' is maximum fluorescence in the light. The rate of electron transport of PSII (ETR) was calculated as $0.5\varphi PSII \times Q_{abs}$, where Q_{abs} was the absorbed light energy that was calculated as PPFD × leaf absorbance, and leaf absorbance was taken as 0.85. Photochemical quenching (qP) was calculated as $(F_m' - F_s)/(F_m' - F_o')$, where F_o' is minimum fluorescence in the light. A–PPFD curves were fit by a non-rectangular hyperbola (Prioul and Chartier 1977), and apparent quantum efficiency (AQE) was estimated by PHOTOSYN ASSISTANT software (Dundee Scientific, Scotland, UK).

Stable carbon isotope

To determine the photosynthetic pathway and estimate the mesophyll conductance (g_m) of the two species, the stable carbon isotope ($\delta^{13}C$) ratio of leaf tissues was determined using a mass spectrometer (Finnigan MAT 253, Gainesville, FL). Mesophyll conductance (g_m) was estimated by using the approach of Evans et al. (1986) as modified by Lloyd et al. (1992).

$$g_m = \frac{(b - b_s - a_1)A/P_a}{(\Delta_{exp} - \Delta_{obs}) - f\Gamma^*/P_a} \quad (1)$$

where Δ_{obs} is sample $\delta^{13}C$, Δ_{exp} is $\delta^{13}C$ of air CO_2 (−4.4‰), b the discrimination associated to carboxylation reactions (27.5‰), b_s the fractionation occurring when CO_2 enters solutions (1.1‰), A the photosynthetic rate, a_1 the fractionation during diffusion in water (0.7‰), P_a the CO_2 partial pressure in the ambient air, f the fractionation associated with photorespiration (7‰) and Γ^* the CO_2 compensation saturation point in the absence of dark respiration (33.06 μmol mol^{-1} at 20°C).

Statistical analysis

Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL). The differences in photosynthetic parameters among treatments were tested using one-way ANOVA and least significant difference multiple comparisons tests, and the differences between species were tested by independent sample *t*-test.

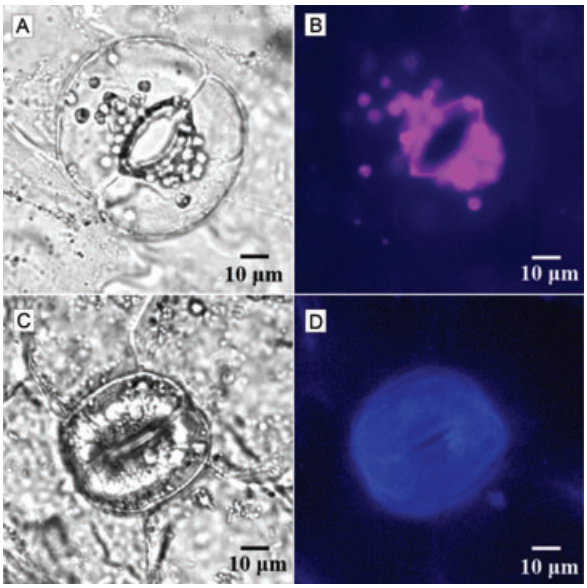

Fig. 1. Stomata and guard cell chloroplasts of *Paphiopedilum armeniacum* and *Cypripedium flavum*. (A) Kidney-shaped stomata in *C. flavum*; (B) red fluorescence indicating the presence of guard cell chloroplasts in *C. flavum*; (C) kidney-shaped stomata in *P. armeniacum* and (D) no detectable fluorescence indicating the absence of guard cell chloroplasts in *P. armeniacum*.

Results

Fluorescence microscopy studies indicated that chlorophyll fluorescence was absent from the guard cells of stomata in *P. armeniacum*, but red fluorescence was present in guard cells of *C. flavum* (Fig. 1), suggesting that the guard cells of *P. armeniacum* did not contain chlorophyll, while those of *C. flavum* did.

The stomatal density of *P. armeniacum* (29.1 ± 1.9 mm^{-2}) was not significantly different from that of *C. flavum* (34.1 ± 1.8 mm^{-2}) ($P > 0.05$), but *P. armeniacum* had smaller stomatal area ($t < 0.001$). The stomatal area per pore in *P. armeniacum* and *C. flavum* were 2687 ± 43 and 3783 ± 91 μm^2, respectively. The stomatal length and width were 63.6 ± 0.6 and 53.6 ± 0.5 μm in *P. armeniacum*, and 71.2 ± 1.2 and 67.3 ± 0.8 μm in *C. flavum*, respectively. Both *P. armeniacum* and *C. flavum* had elliptic stomata, but the stomata of *P. armeniacum* sank slightly into the leaf epidermis, while *C. flavum* protruded slightly above the leaf surface (Fig. 2). Furthermore, *P. armeniacum* had thicker walls of guard cells than *C. flavum* (Fig. 2). The upper cuticular lips of guard cells were very pronounced, resulting in a very large antechamber above the stoma. In *C. flavum*, however, the cuticular lips were not so prominent.

There was a significant difference in photosynthetic induction under different light qualities between

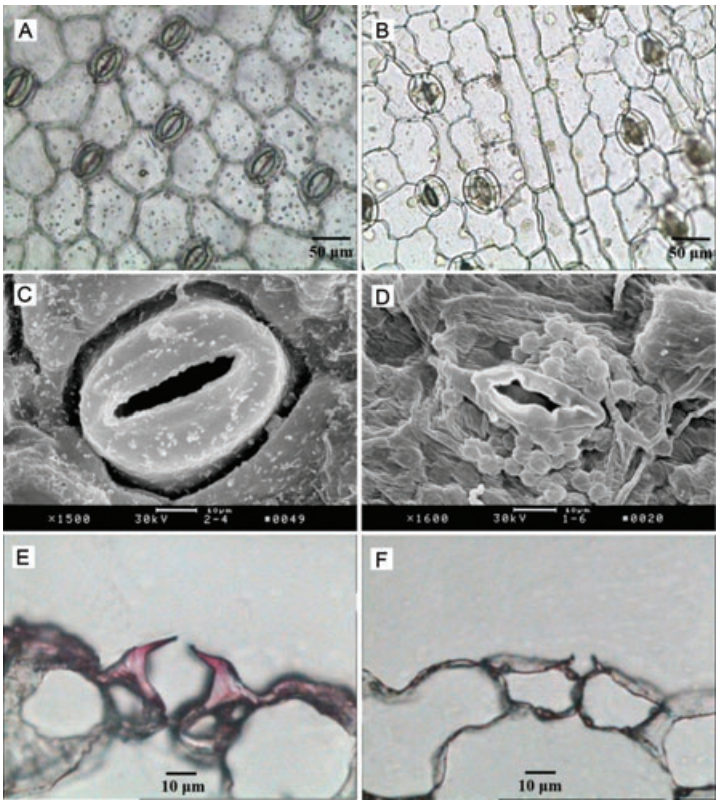

Fig. 2. Stomatal anatomy of *Paphiopedilum armeniacum* and *Cypripedium flavum*. (A) Stomata on the lower leaf surface of *P. armeniacum*; (B) stomata on the lower leaf surface of *C. flavum*, (C) sunken stomata in *P. armeniacum*, (D) raised stomata in *C. flavum*, (E) thick wall of guard cell in *P. armeniacum* and (F) thin wall of guard cell in *C. flavum*.

P. armeniacum and *C. flavum* (Fig. 3). In *P. armeniacum*, maximal stomatal conductance (g_s) was observed under full blue light and B70 (70% blue + 30% red) conditions, while maximal photosynthetic rate (A) was obtained under full blue light condition (B100). Under full red light (B0), *P. armeniacum* had the smallest g_s and A among light qualities. The value of maximal g_s in *P. armeniacum* under full red light was less than 50% of those under full blue light. Compared with that under B0 (full red light) condition, the increments of g_s at full induction for *P. armeniacum* under B30 (30% blue + 70% red), B70 (70% blue + 30% red) and B100 conditions were significantly larger (Fig. 4). The initial values of g_s in *C. flavum* were larger than in *P. armeniacum*, and the increments of g_s at full induction in *C. flavum* were smaller than those in *P. armeniacum*, and not significantly different among light qualities ($P > 0.05$). In *C. flavum*, the maximal g_s and A were found under B30 condition at full induction, and the minimal g_s and A were under B0 and B100 conditions (Figs 4 and 5). In general, *P. armeniacum* had lower g_s and A than *C. flavum* at any light treatments under full photosynthetic inductions. Besides, the mesophyll conductance (g_m) of *P. armeniacum* was also lower than that of *C. flavum* (0.091 ± 0.006 vs 0.159 ± 0.009 mol m^{-2} s^{-1}).

On the whole, the induction times to reach maximum g_s and A in *P. armeniacum* were significantly longer than those of *C. flavum* (Fig. 3). Average induction times of *P. armeniacum* to reach maximal g_s ranged from 32 to 40 min depending on light quality, while from 12 to 18 min in *C. flavum*. The times to reach maximal A were shorter than those of g_s for both species. The g_s of *P. armeniacum* showed the fastest induction under full blue light, and the slowest under full red light. In *C. flavum*, the completions of stomatal induction under mixed lights were earlier than under single light conditions.

The photosynthetic light response measurements showed that both photosynthetic rate and ETR in *P. armeniacum* and *C. flavum* increased with increasing PPFD at any light treatment (Fig. 5). Maximum A was obtained at average PPFD of 550 ± 22 μmol m^{-2} s^{-1} for *C. flavum* and 240 ± 9 μmol m^{-2} s^{-1} for *P. armeniacum*. The photosynthetic light saturation point (Lsp) of *P. armeniacum* was lower than that of *C. flavum*, but photosynthetic light

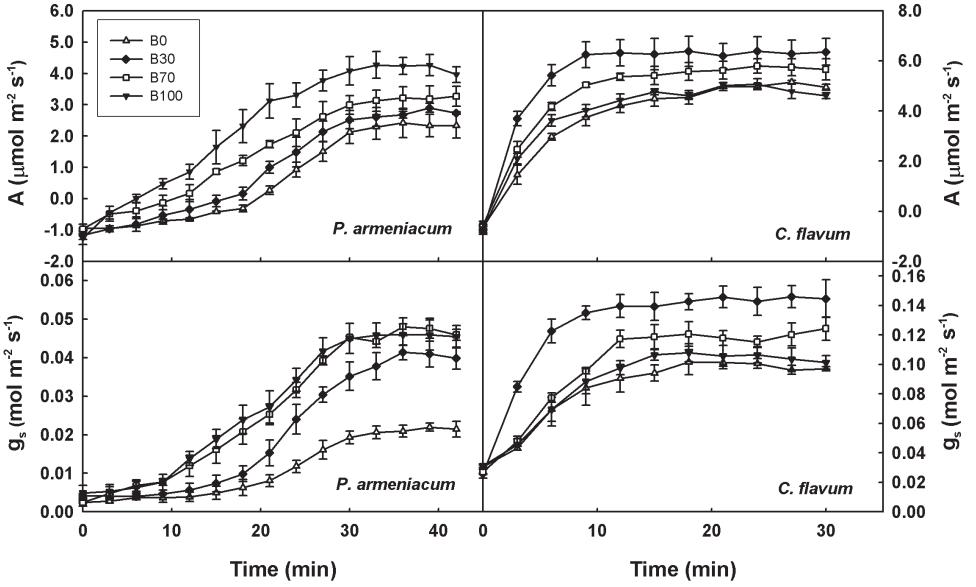

Fig. 3. Photosynthetic rate (A) and stomatal conductance (g_s) during induction by different light qualities at light intensity of 250 μmol m^{-2} s^{-1} for *Paphiopedilum armeniacum* and *Cypripedium flavum*. B0, full red light; B30, 30% blue + 70% red; B70, 70% blue + 30% red; B100, full blue light.

compensation point (Lcp) showed a converse trend. At low irradiance, there was little difference in qP between the two species, but above 400 μmol m^{-2} s^{-1} the values for *C. flavum* were higher than those of *P. armeniacum*. The ETR of *P. armeniacum* reached its maximum at 300 μmol m^{-2} s^{-1} light intensity, *C. flavum* above 800 μmol m^{-2} s^{-1}. *Cypripedium flavum* obtained a higher photosynthetic rate than *P. armeniacum* under any light treatment. In *P. armeniacum*, there was no significant difference in Lcp among light treatments, but AQE under full red light was lower than under the other light qualities. For *C. flavum*, AQE under full blue light was the lowest among light treatments.

Discussion

This study reports the significant difference in photosynthetic induction and stomatal anatomy between *P. armeniacum* and *C. flavum*. The slower photosynthetic induction of *P. armeniacum* than *C. flavum* is linked to the lack of guard cell chloroplasts and to the special stomatal anatomy of *P. armeniacum*.

Our study confirmed the previous observation that the stomata of *P. armeniacum* did not contain guard cell chloroplasts, while its close relative, *C. flavum*, possessed guard cell chloroplasts in stomata (Nelson and Mayo 1975, Rutter and Willmer 1979). This indicates that guard cell photosynthesis is not essential for normal stomatal functioning in *P. armeniacum* (Zeiger et al. 2002).

The lack of guard cell chloroplasts, however, can affect the response of stomatal movement to light quality and photosynthetic induction. The stomata of *P. armeniacum* were more responsive to blue light than red light but still partially responsive to full red light, whereas the stomata of *C. flavum* were more responsive to mixed blue and red light than either pure red or blue light. Our results obtained from intact leaves contradicted the results from isolated leaf peels of Talbott et al. (2002), who found that stomatal opening in *Paphiopedilum* is largest in red light at 10 μmol m^{-2} s^{-1} and is indistinguishable from baseline levels at 100 μmol m^{-2} s^{-1}. In our study, the stomata of *P. armeniacum* still opened considerably at 250 μmol m^{-2} s^{-1} red light, but the magnitude and speed of stomatal opening were lower than those at the other light qualities. Blue light activates the plasma membrane H^+-ATPase, hyperpolarizing the membrane potential with simultaneous apoplast acidification, and drives K^+ uptake through voltage-gated K^+ channels. Red light likely mediates stomatal opening via reduction in the intercellular CO_2 concentration (C_i) by mesophyll photosynthesis, but the role of guard cell chloroplasts in the response could not be excluded (Shimazaki et al. 2007, Zeiger et al. 2002). It is likely that both C_i and guard cell chloroplasts play roles in the synergistic effect of blue and red light on stomatal opening. Reducing C_i increases the magnitude of blue light response (Assmann 1988, Lascève et al. 1993). Consequently, the lack of guard cell chloroplasts decreased the sensitivity of

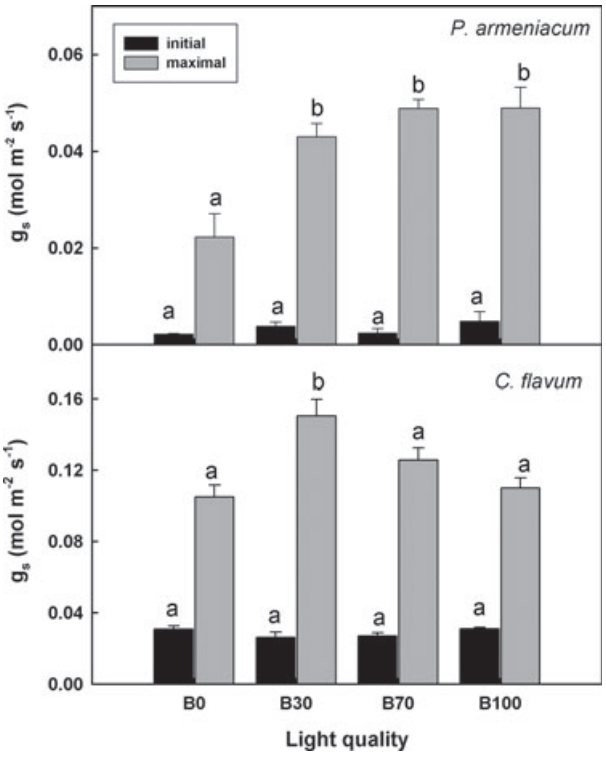

Fig. 4. Initial stomatal conductance (g_s) and maximal g_s at full induction at light intensity of 250 μmol m^{-2} s^{-1} in *Paphiopedilum armeniacum* and *Cypripedium flavum*. A different letter in the same parameter indicates mean statistically different $P < 0.05$ as determined by one-way ANOVA. B0, full red light; B30, 30% blue + 70% red; B70, 70% blue + 30% red; B100, full blue light.

stomatal opening to red light. The stomatal opening in *P. harrisianum* is more dependent on a specific blue light response, phytochrome and mesophyll photosynthesis (Talbott et al. 2002), but red light responses in stomata from intact leaves of *P. harrisianum* result from indirect effects, such as depletion of intercellular CO_2 concentration by mesophyll photosynthesis (Zeiger et al. 1983).

Photosynthetic induction of *P. armeniacum* was significantly slower than *C. flavum* at any light quality. However, the slow photosynthetic induction of *P. armeniacum* could not be completely explained by the lack of guard cell chloroplasts. Apart from light environment and successional status of plants affecting photosynthetic induction, photosynthetic induction is related to leaf structure and stomatal density (Cao and Booth 2001, Kursar and Coley 1993, Zipperlen and Press 1997). *Paphiopedilum armeniacum* had higher leaf mass per unit area (LMA) and lower mesophyll conductance (g_m) than *C. flavum* (Chang et al. 2011, Guan et al. 2011). Mesophyll conductance is negatively correlated with LMA that is related to leaf thickness, thickness of mesophyll cell walls and mesophyll cell density. Mesophyll CO_2 diffusion limitation increases the CO_2 drawdown from substomatal cavities to chloroplasts in the sclerophyllous leaves with higher LMA (Niinemets et al. 2009). Consequently, slow drawdown of C_i resulting from low g_m in *P. armeniacum* would slow down the induction of stomatal conductance. However, the thick walls of the guard cells and the stomata sunken beneath the leaf surface would hinder stomatal opening. Rutter and Willmer (1979) noted the striking thickening of guard cell walls of several *Paphiopedilum* species and supposed that the extensive wall thickening and sculpturing of the guard cells limited the extent of stomatal opening. Photosynthetic gas exchange can be affected by the mechanical properties of stomata (Franks and Farquhar 2007). The walls of guard cells of plants on dry land are exceptionally strong and must undergo large and reversible deformation during stomatal opening and closing (Jones et al. 2003). In *Bulbophyllum sessiliforum*, some outer cuticular ledges fuse to form a ring. Two extensions bulge out from the polar ends of the cavity, segregating upper and lower portions of the cavity, which are connected by a narrow canal (Solereder and Meyer 1930). Similarly, the prominent cuticular lips in *P. armeniacum* would result in a very large antechamber above the stomata (Fig. 2). When the guard cells close, the cuticular lips touch, thus providing an outer barrier, apart from the actual closed guard cells (Linder and Volk 1990).

The photosynthetic rate (A) and stomatal conductance (g_s) of *P. armeniacum* were lower than *C. flavum*. The mesophyll conductance (g_m) of *P. armeniacum* was also lower than that in *C. flavum*. Obviously, both low g_s and g_m contributed to low A in *P. armeniacum*. Williams et al. (1983) suggested that guard cell chloroplasts are needed to sustain high g_s at moderate to high irradiances. Flexas et al. (2008) suggested that g_m is correlated negatively with LMA. Meanwhile, g_m and stomata respond to all same environmental variables, and in a similar manner. It is likely that the co-regulation of g_s and g_m results in adjustment in CO_2 availability in the chloroplasts in response to environments, which contributes to ecological adaptation of the plant. In addition, the slow drawdown of C_i resulting from low g_m would increase the magnitude of blue light response (Assmann 1988, Lascève et al. 1993). Blue light enrichment resulted in significantly higher growth rates of *Paphiopedilum* over a 3–4-week growing period due to the blue light response of stomata (Zeiger et al. 1985). Consequently, the low photosynthetic rate in *Paphiopedilum insigne* is postulated as an adaptation to low-light habitat (Williams et al. 1983). The lack of guard cell chloroplasts in *P. harrisianum*

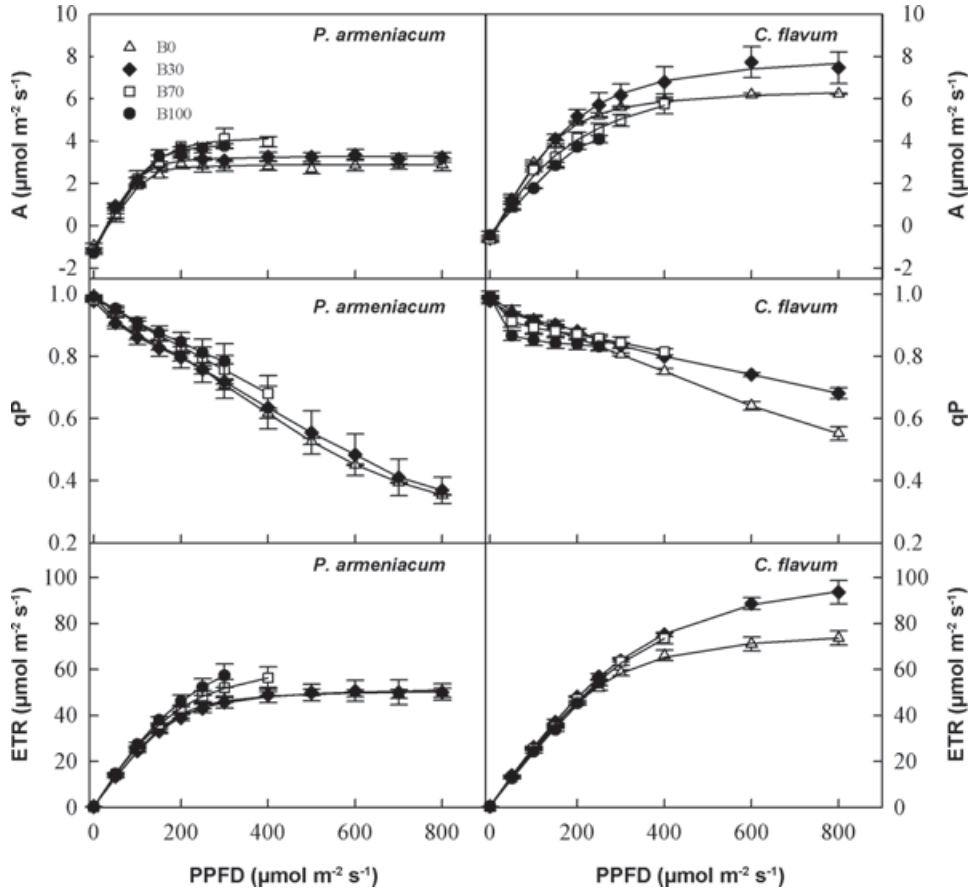

Fig. 5. Photosynthetic and chlorophyll fluorescence light response curves for *Paphiopedilum armeniacum* and *Cypripedium flavum* at 370 μmol mol^{-1} CO_2 concentration and 20°C leaf temperature under different light qualities. The ranges of irradiance for the response curves under B70 and B100 conditions were up to 400 μmol m^{-2} s^{-1} and 250 μmol m^{-2} s^{-1}, respectively. Each point represents the mean ± 1 SE of five measurements. B0, full blue light; B30, 30% blue + 70% red; B70, 70% blue + 30% red; B100, full blue light.

would play an important role in increasing stomatal conductance in the morning, and preventing high stomatal conductance under high irradiance to reduce water loss (Assmann 1988, Williams et al. 1983). *Paphiopedilum armeniacum* often grows on limestone cliffs and slopes in semi-shady forests, which is characterized by periodic water deficit. The ability to minimize water loss from leaf surfaces during drought represents an important survival feature for the plants in low-water availability habitats (Holbrook and Putz 1996). In *P. harrisianum*, the blue light response is decreased by an increased vapor pressure difference (Assmann 1988). Several studies suggested that the magnitude of the blue light response decreases from morning to afternoon, consistent with early morning stomatal opening, when ambient radiation is enriched with the blue wavelengths, and also consistent with the theory of varying osmoregulatory pathways, and changes in guard cell fluorescence transients through the day (Assmann and Shimazaki 1999, Doi et al. 2004, Talbott and Zeiger 1998). Zeiger et al. (1985) confirmed that growth and carbon gain in *P. harrisianum* are enhanced in the blue light-enriched environment. The blue light response of *Paphiopedilum* is considered as a modulator for adjusting the balance between carbon gain and water conservation (Assmann 1988). Our study supported the notion that lack of guard cell chloroplasts in *Paphiopedilum* is an ecophysiological adaptation to water deficit (Assmann and Zeiger 1985).

In conclusion, the photosynthetic induction and performance in *P. armeniacum* were significantly different from those of *C. flavum*. The slow photosynthetic induction and low photosynthetic rate in *P. armeniacum* were linked to the lack of guard cell chloroplasts and specific stomatal structure, which reflected an adaption to low soil water availability. Our results provided evidence of the morphological and physiological evolution of

stomata relating to water-conserving traits under natural selection.

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