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Front cover: Flowers of columbine (*Aquilegia coerulea*). Anthers of columbine produce abundant phased secondary small interfering RNAs (phasiRNAs) during the meiotic stage of development. Columbine is a strong candidate as a model system for the study of reproductive phasiRNAs in eudicots because of its extensive number of *PHAS* loci and its diversity of triggering microRNAs (pp. 1332-1345). Photo by Suresh Pokhrel.

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Mesophyll conductance variability of rice aquaporin knockout lines at different growth stages and growing environments

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SUMMARY

The plasma membrane subfamily of aquaporins [plasma membrane intrinsic proteins (PIPs)], which facilitates the CO_2 diffusion across membranes, is proposed to play an important role in mesophyll conductance to CO_2 (g_m), a major limiting factor of photosynthesis. However, recent studies implied no causal relationship between g_m and PIPs because they failed to repeat the previous observed differences in g_m between PIP knockout lines and their wild-type. The contrasting results on the role of PIPs in g_m were interpreted as the different growth conditions among studies, which has never been tested. Here, we assessed whether the differences in g_m between wild-type and PIP knockout lines, *Ospip1;1*, *Ospip1;2* and *Ospip2;1*, varied with growth condition (field versus pot condition) and growth stages in rice. Under field conditions, no differences were observed in plant performance, photosynthetic rate (A) and g_m between PIP knockout lines and the wild-type. However, in agreement with previous studies, two out of three knockout lines showed significant declines in tiller number, plant height, A and g_m under pot conditions. Moreover, we found that the differences in A and g_m between PIP knockout lines and the wild-type varied with the growth stage of the plants. Our results showed that the differences in g_m between PIP knockout lines and wild-type were depending on the growth environments and stage of the plants, and further efforts are required to reveal the underlying mechanisms.

Keywords: mesophyll conductance, photosynthesis, aquaporins, *Ospip1;1*, *Ospip1;2*, *Ospip2;1*, growth environment.

INTRODUCTION

Mesophyll conductance to CO_2 (g_m), referring to the movement of CO_2 from the intercellular air spaces to the site of carboxylation inside the chloroplast, has been recognized as an important limiting factor of photosynthetic assimilation rate in C_3 plants (Flexas et al., 2012). Over the past decade, g_m has been estimated in hundreds of species and, according to quantitative photosynthetic limitation analysis, it can limit photosynthesis across a range of 25–80% (Gago et al., 2019). The g_m variation across species is linked to the mesophyll structures (for review, see Evans, 2021), and two of the most important structural traits are mesophyll cell wall thickness and the total chloroplast surface area exposed to mesophyll intercellular air spaces per leaf area. Beyond structural traits, the roles of membrane permeability in regulating g_m, especially under dynamic environmental conditions, have been highlighted recently (Flexas et al., 2006; Xu et al., 2019). Behind each mesophyll cell wall lies the plasma membrane, and chloroplasts in mesophyll cells are enclosed by a double membrane. As these membranes typically contain a diversity of proteins, very limited lipid surface area exists for CO_2 free diffusion, and CO_2 across membranes is then suggested to be regulated by protein pores that are known as aquaporins (AQPs). The plasma membrane intrinsic proteins (PIPs), a subfamily of AQPs, have been demonstrated playing the key role in CO_2 diffusion across membranes (for review, see Grondin et al., 2016).

The regulation of PIPs on CO_2 across membranes in plants has been widely investigated by previous studies (Grondin et al., 2016). Some of the studies suggested that single AQP-mediated membrane permeability to CO_2 represents a significant proportion of the mesophyll resistance (the reciprocal of g_m) by comparing the g_m values of AQP lacking or overexpressed mutants to their wild-types. For instance, Arabidopsis mutants lacking *AtPIP1;2* had a 10-fold decline in CO_2 diffusion efficiency across the mesophyll plasma membrane compared with the wild-type

(Heckwolf et al., 2011; Uehlein et al., 2012), and g_m increased nearly 200% by overexpressing the *OsPIP1;2* in rice (Xu et al., 2019). However, a recent study implied no causal relationship between AQPs and g_m (Kromdijk et al., 2020). In contrast to the observation in Heckwolf et al. (2011) and Uehlein et al. (2012), Kromdijk et al. (2020) reported no difference of g_m values between AtPIP1:2 knockout line and its wild-type by estimating g_m using multiple methods. The reason underlying the different observation in these studies on the role of AtPIP1;2 in g_m is unclear. As proposed in Kromdijk et al. (2020) and in Evans (2021), one possibility is that the growth conditions (light irradiances, photoperiod, temperature) were different in the two studies, and the plasticity response of other traits may offset the effect of lacking *AtPIP1;2* in mutants. Indeed, many PIP isoforms exist in Arabidopsis and other species, and the expression patterns of PIP isoforms have been shown to be shaped by growth conditions (Wu et al., 2015). If the target PIP gene does not express under a given growth condition, the PIP knockout mutants should show no functional difference from the wild-type. Another possibility might be that the plants were measured in different ontogenetic stages (no information about the estimated leaf and plant age was provided in those studies), as the expression patterns of AQPs vary with growth stages (Xu et al., 2019).

In addition, although the g_m has been suggested to be important for plant growth and development (Evans, 2021), the growth performance of the PIP knockout lines was typically evaluated by leaf level photosynthetic measurements, which were usually conducted on newly expanded leaves under controlled environment conditions. However, plant growth performance is more closely related to canopy photosynthesis expressing as the sum of the photosynthetic rates of all leaves in the canopy (Terashima and Hikosaka, 1995). The complexity of canopy photosynthesis was frequently described, as the leaves inside the canopy are exposed to different environmental conditions, and have different functional traits and ontogeny (Slattery and Ort, 2021; Wu et al., 2019). Therefore, the impacts of PIP genes knockout on plant performance may better be evaluated by measuring the growth rather than the photosynthetic rate on newly expanded leaves.

Here, we conducted the study to assess whether the g_m and growth performance of PIP knockout lines are impacted by growth environments and/or growth stage. To do so, we used three rice (*Oryza sativa*) PIP knockout lines that have been reported to be involved in g_m regulation (Ding et al., 2016, 2019; Xu et al., 2019) and the wild-type by measuring gas exchange and growth traits under both field and pot conditions, and at three growth stages under pot conditions. Rice rather than Arabidopsis was selected in the present study because: (i) rice is a major staple crop for almost half the global population, and improving g_m and then enhancing photosynthesis is proposed to be an important strategy for yield improvement (Long, 2014); (ii) compared with Arabidopsis, rice typically has larger leaves, which are favored more for gas exchange measurement using commercially available infrared gas analysis systems (Flexas et al., 2007); and (iii) rice, as a crop, has a higher photosynthetic rate and stomatal conductance than Arabidopsis, which is important for a precise mesophyll conductance estimation (Gu and Sun, 2014).

RESULTS

Growth performance

As expected, the plant growth performance differed significantly in the two environments. Overall, the plants grown under field conditions have less tillers and lower plant height than those grown under pot conditions. Importantly, no difference in tiller number between wild-type and knockout lines was observed under field conditions at Sanya (Figure 1). The average plant heights of the knockout lines were lower than wild-type, but only the *Ospip1;1* line was statistically significant. In contrast, both tiller number and plant height of knockout lines were lower than the wild-type in pot conditions at Wuhan, expect for the plant height of *Ospip1;2* (Figure 2). Moreover, the tiller number and plant height differed significantly among knockout lines in pot conditions. The tiller number of *OsPIP1;2* knockout line was almost twice that of *OsPIP1;1* knockout line, and the plant height of *OsPIP1;2* knockout line was also higher than that of *OsPIP1;1* knockout line. The difference in tiller number between wild-type and PIP knockout lines disappeared at 56 days after sowing, except for the *Ospip1;1* line, which had the lowest tiller number and plant height over the growth cycle in pot conditions. When comparing the plant performance growing in two conditions, tiller number and plant height of wild-type showed larger plasticity than knockout lines, and no plasticity was observed for *Ospip1;1* line (Figure 3).

Photosynthetic traits

Overall, the plants grown under field conditions had a higher photosynthetic rate (A) than those in pots (Figure S4). No differences were observed among plant lines in A and mesophyll conductance (g_m) under field conditions (Figure 4). However, the stomatal conductance under field conditions differed significantly, where *Ospip1;2* line had the highest g_s and the *Ospip2;1* line had the lowest g_s. The parameters fitted from the light response curves further confirmed that no significant difference in photosynthetic capacity existed among the lines under field conditions (Figure S5). However, under pot conditions, the photosynthetic parameters differed significantly among the lines (Figure 4). Similar to the

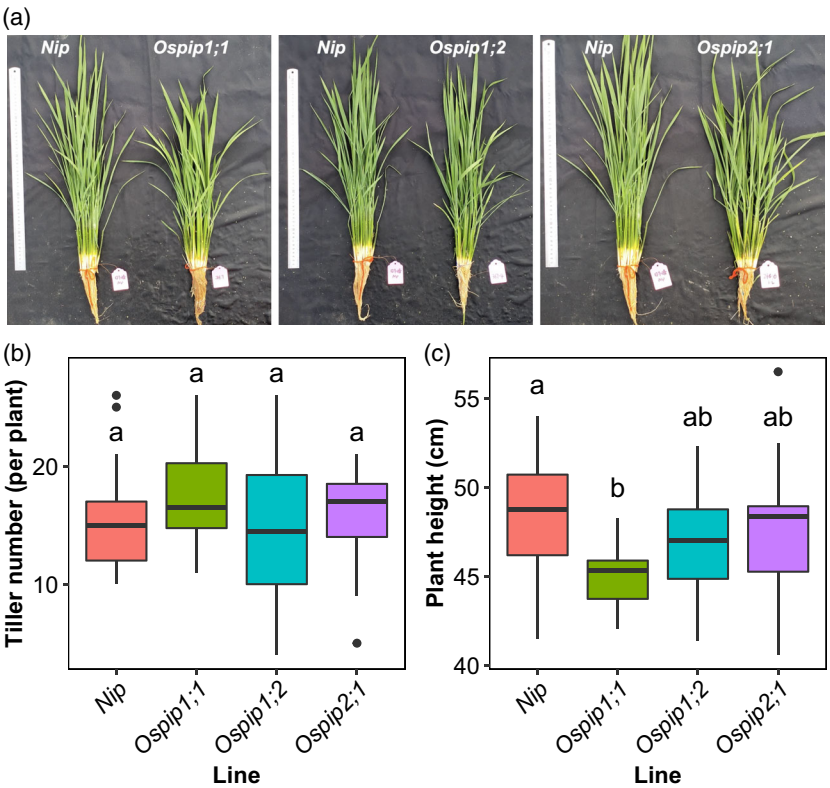

Figure 1. (a) Photograph of the representative plants, (b) tiller number and (c) plant height of wild-type and *OsPIP* knockout lines. Plants were growing in a paddy field in Sanya, Hainan. Photos and measurements were taken at 43 days after sowing. Different letters represent statistical significance ($P < 0.05$, $n = 16–24$). ANOVA, analysis of variance.

growth performance traits, the *Ospip1;1* had the lowest *A* over the estimated growth cycle when plants were grown in pots. The differences between knockout lines and the wild-type in photosynthetic parameters at ambient CO_2 conditions varied with plant growth stages, and the net photosynthetic rate tended to decline over the growth cycle. However, according to the CO_2 response curves (Figure 5), we found that the photosynthetic capacities (both V_{cmax} and J_{max}) of the mutants were similar to the wild-type for all three estimated growth stages (Figures S6–S8). The *A*, V_{cmax} and J_{max} of the wild-type and mutants declined over the growth stages (Figures S6–S8), but the patterns of g_{sw} and g_m were genotype dependent (Figure 4).

Leaf functional traits

Regarding leaf morphological traits, *Ospip1;1* and *Ospip2;1* had smaller leaves at the tiller stage, but no difference in flag leaves comparing with the wild-type (Table S3). In this study, we also investigated the chemical components, Rubisco content and stable isotopes of carbon for pot-growing plants at three growth stages. The results, however, showed that PIP knockout lines had no significant difference from the wild-type, except for N content and Rubisco content of *Ospip1;1* at the tiller stage (Table S3). Similar to *A*, the C content, N content and Rubisco content tended to decease over growth stages in all the genotypes; however, the $\delta^{13}C$ increased over the growth stages.

DISCUSSION

Photosynthetic assimilation in C_3 plants under atmospheric conditions is typically limited by the CO_2 concentration in chloroplasts, which is determined by the diffusion conductance of CO_2 through boundary layer, stomata and mesophyll tissues (Flexas et al., 2012). As AQPs are functioning to enhance the permeability of membranes to substances such as water and CO_2, the contribution of AQPs to CO_2 diffusion conductance, especially to the mesophyll conductance (g_m), have been assessed using mutants in several species (for review, see Groszmann *et al.*, 2017). Several AQP genes were suggested to have a role in CO_2 diffusion across membranes in those studies; however, the results were rarely replicated by other researchers. Most recently, Kromdijk et al. (2020) reinvestigated the role of *AtPIP1;2* on g_m in Arabidopsis, and their result, surprisingly, contrasted with previous studies (Heckwolf et al., 2011; Uehlein et al., 2012), which showed strong decline of g_m in *Atpip1;2* mutant comparing with the wild-type. Although no empirical data are available, the contrasting results of the role of the AQP genes on g_m were proposed to be arisen by the different growth environments and/or the growth stage of the plants (Evans, 2021; Kromdijk et al., 2020). Here, three

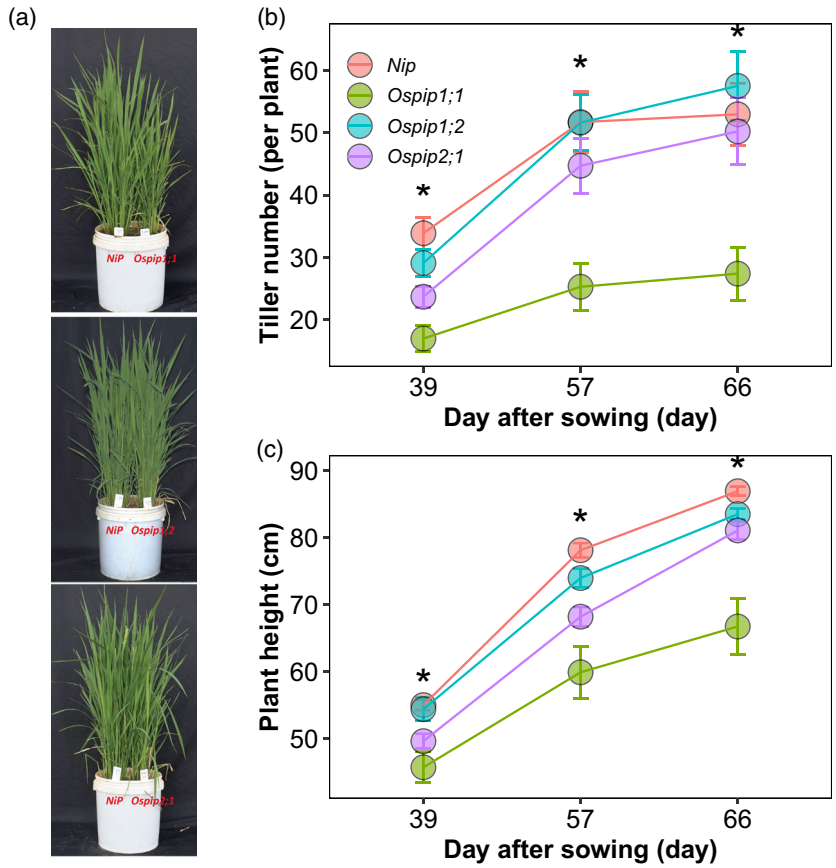

Figure 2. (a) Photograph of the representative plants, (b) tiller number and (c) plant height of wild-type and *OsPIP* knockout lines. Photos were taken 56 days after sowing. Measurements were taken on plants growing in pot conditions at 39, 57, 66 days after sowing in Wuhan. Means ± SE (n = 10–16). SE, standard error. * represents $P < 0.05$ (ANOVA)

previous reported g_m-related PIP genes were knocked out to investigate the effects of AQP on the plant performances and the g_m under different environments and at different growth stages in rice. We found that the differences in plant growth traits and photosynthetic traits between PIP knockout lines and the wild-type were influenced by the growth stage of the plants and the planting conditions.

In rice, 11 PIP genes have been identified, and only the roles of *OsPIP1;1*, *OsPIP1:2* and *OsPIP2;1* in photosynthetic CO_2 diffusion efficiency have been investigated (Ding et al., 2016, 2019; Xu et al., 2019). In this study, the knockout lines of three photosynthesis-associated PIP genes *OsPIP1;1*, *OsPIP1:2* and *OsPIP2;1* were generated using the CRISPR/Cas9 system (Figure S1). Field and pot experiments showed that the plant performance of lines was significantly affected by growth conditions. The tiller number and plant height of plants grown in field conditions were much lower than those grown in pot conditions, except for *Ospip1;1*, which had no difference under two conditions. The result agreed with a previous meta-analysis showing that pot-grown plants generally had faster growth rates and different morphology (Poorter et al., 2016). Interestingly, we found that trait plasticity in response to growth environment was strongly affected by PIP genes, as the wild-type showed the highest plasticity and the *Ospip1;1* showed no plasticity at all (Figure 3). Although the mechanisms involved in how PIP genes modulate rice phenotypic plasticity remain to be further revealed, our result suggests that the PIP family may play a role in regulating rice phenotypic plasticity induced by environmental changes. While no difference in A was observed, the tiller number and plant height were obviously higher in pot growth conditions than in field growth conditions, indicating the light-saturated leaf level photosynthesis does not predict the whole-plant growth performance of the rice plants.

The *OsPIP1;1* and *OsPIP1;2* were suggested to play an important role in g_m and photosynthesis of rice (Ding et al., 2016; Xu et al., 2019), but the plants lacking in *OsPIP2;1* had no change in g_m in previous studies (Ding et al., 2019). Consistent with the previous study, we found no difference in g_m between the wild-type and the *Ospip2;1* line across the investigated growth environment and growth stages. Moreover, we found that the stomatal conductance (g_s) of the *OsPIP2;1* knockout line was lower than the wild-type under field conditions and pot conditions in contrast to the previous study (Ding et al., 2019). The PIP2 type of AQP have been suggested to have greater water permeability than the PIP1 type (Groszmann *et al.*, 2017). In rice, the *PIP2;1* is mainly expressed in the endodermis of roots, where aplastic water flow is blocked by

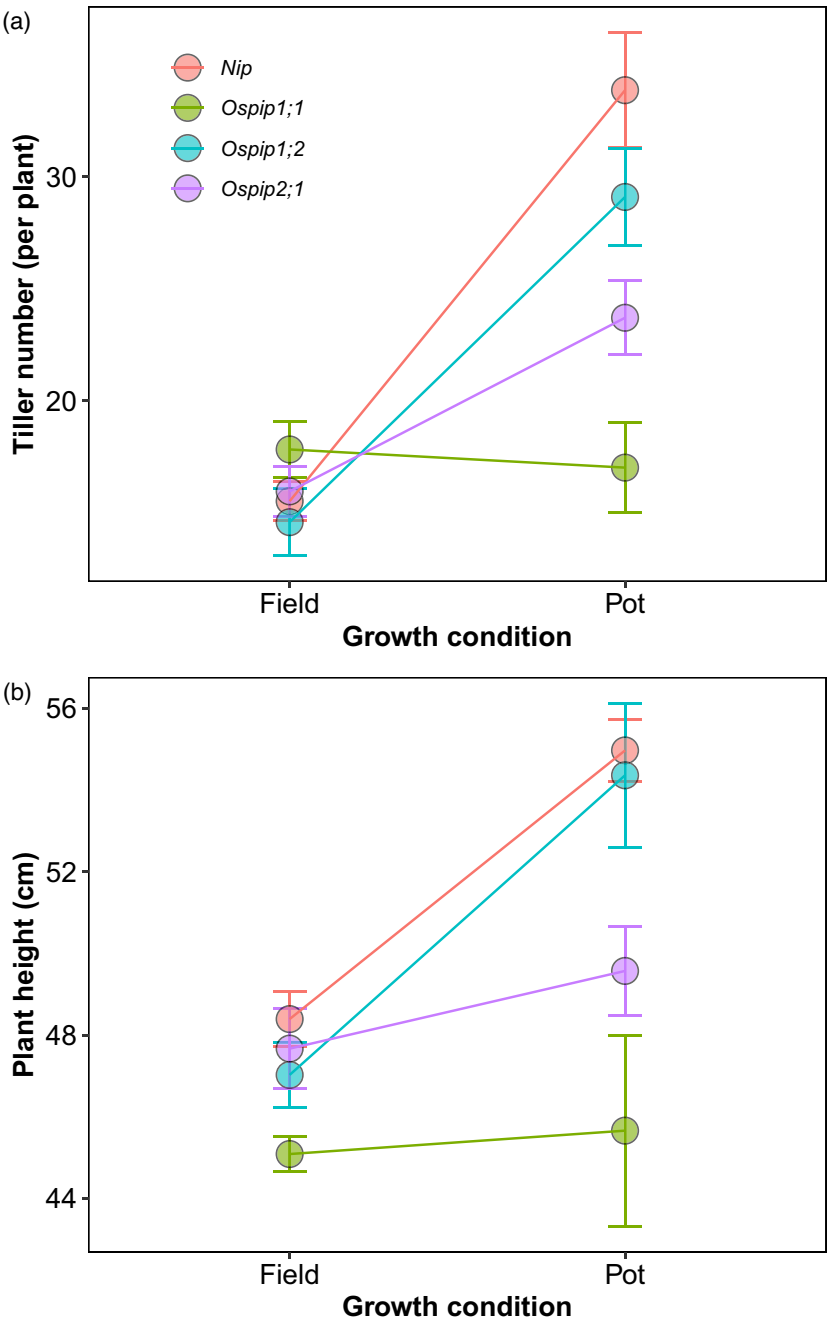

Figure 3. (a) Tiller number and (b) plant height of wild-type and *OsPIP* knockout lines under filed and pot conditions. Measurements were taken on plants at about 40 days after sowing. Means ± SE. SE, standard error.

hydrophobic substances, and several previous studies have shown that the root hydraulic conductance declined by 80% in the *Ospip2;1* line (Ding et al., 2019; Ishikawa-Sakurai et al., 2017; Sakurai-Ishikawa et al., 2011). Therefore, the declined g_s in *Ospip2;1* might be related to the decreased water transport capacity (plant hydraulics) from root surface to transpiration site inside the leaves. Interestingly, the decline in g_s had limited impact on *A*, resulting in a higher water-use efficiency (WUE). The result indicated that the stomatal pores were over-open at normal conditions, as observed in the previous study (Caine et al., 2019). Therefore, decreasing the g_s by mutating *OsPIP2;1* might be a potential way to enhance WUE of paddy field rice, which consume up to 90% of the total water used for irrigation in Asia (Khepar et al., 2000).

Unlike the *OsPIP2;1*, here we observed that the influences of *OsPIP1;1* and *OsPIP1;2* on *A* and g_m were depending on the growth conditions and the growth stages of the plants. Given the high homology of AQP isoforms in rice, the compensation between different AQP isoforms to mask effects of single gene loss would be expected. If this is the case, the question becomes why the compensation effect differs with

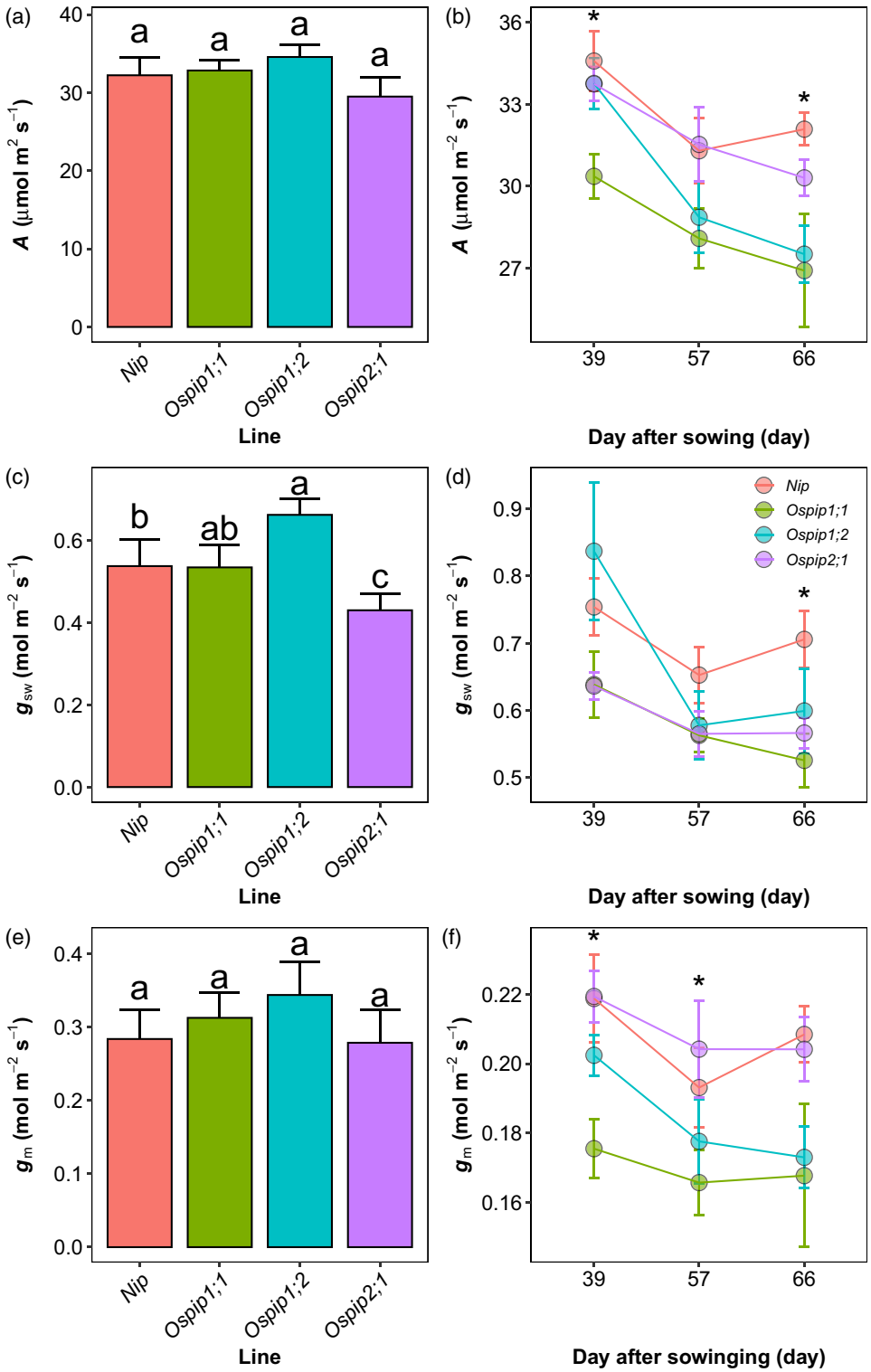

Figure 4. The photosynthetic rate (*A*), stomatal conductance (g_{sw}) and mesophyll conductance (g_m) of wild-type and *OsPIP* knockout mutants at the photosynthetic photon flux density (PPFD) of 1500 mol m^{-2} sec^{-1} in Sanya (a,c,e) and Wuhan (b,d,f). Means ± SE. Different letters near the bars and asterisks near the points represent statistical significance (ANOVA, $P < 0.05$, $n = 4$–8).

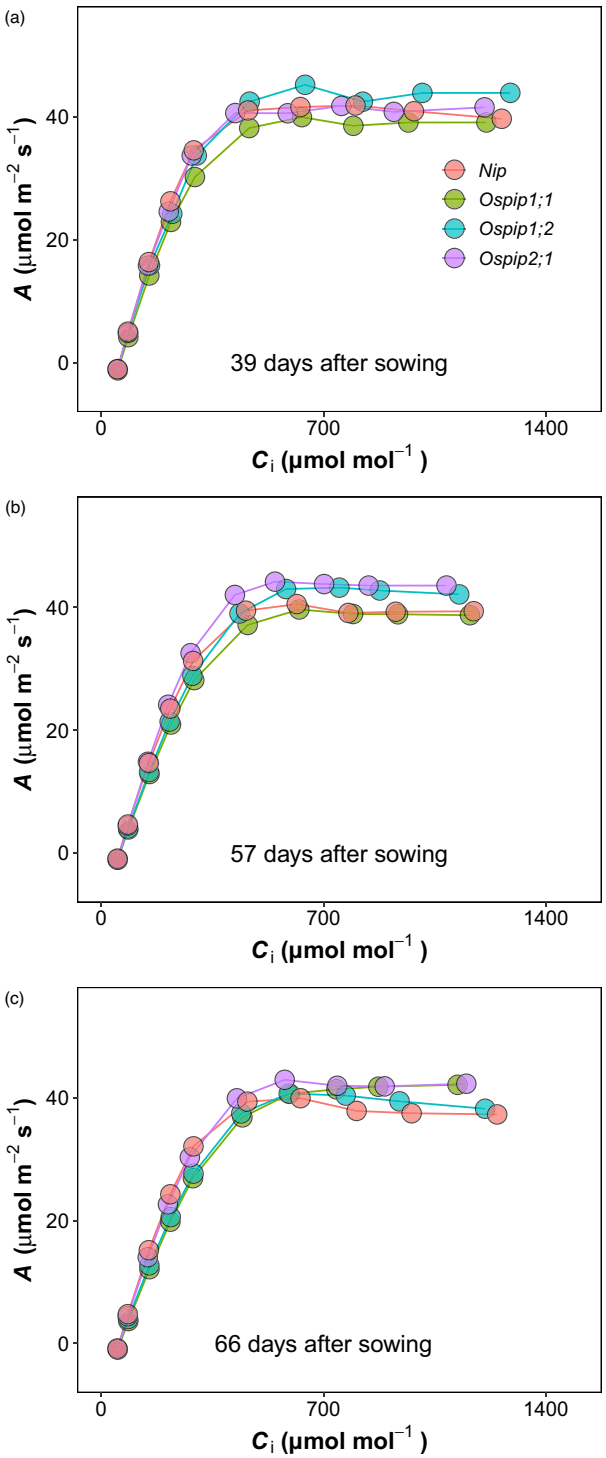

Figure 5. CO_2 response curves of wild-type and *OsPIP* knockout lines. *A* and C_i are net photosynthetic rate and intercellular CO_2 concentration, respectively. Measurements were conducted on plants grown under pot conditions. The mean values were shown (n = 4–8).

growth conditions. By combination of biochemical and cellular biology techniques, the AQP activities have been confirmed to be regulated by post-translational modifications and protein interactions (for review, see Chaumont and Tyerman, 2014). Plants perceive and process environmental simulation signals by plasma membrane receptor-like protein kinases (RLKs), which comprise a major gene family in plants with over 1131 members in rice (Shiu et al., 2004). Previous studies have demonstrated that the activities of AQPs are regulated by RLKs, and a given RLK may regulate some specific AQPs (Grison et al., 2019; Rodrigues et al., 2017; Wu et al., 2015). For instance, Wu et al. (2015) reported that the activities of *OsPIP1;1, OsPIP1;3* and *OsPIP2;3* are regulated by *LP2*, a leucine-rich repeat RLK member, in responding to soil water change. Indeed, the climate of the rice-growing season in Sanya was warming (over the growth season, the maximum air temperature, the minimum air temperature and the average air temperature were 28.5˚C, 12˚C, 21.5˚C, respectively), cloudy and moist; but the climate in Wuhan was hot (over the growth season, the maximum air temperature, the minimum air temperature and the average air temperature were 37.5˚C, 15.8˚C, 26.5˚C, respectively), sunny and dry. Therefore, different RLKs may express in two growth conditions, and the RLK-dependent modulation of AQP activities might correspond to the variable functional performances in different growth conditions as well as different growth stages. Although future investigations are required, the lower g_m values of wild-type as well as the knockout lines growing in pots compared with those growing in field conditions might relate to the interactions between PIPs and RLKs. It is worthy of note that leaf morphological and biochemical traits differed between plants grown in field and pot conditions, which could also contribute to the difference in physiological traits. Moreover, the highly dynamic expression pattern of AQP genes may also play a role in the variable functional performances at different growth stages (Sakurai et al., 2005; Xu et al., 2019).

Photosynthetic traits are complex because they are affected by many structural, biochemical and physiological traits (Flexas et al., 2012; Xiong and Flexas, 2018; Xiong and Nadal, 2020). In fact, the leaf area and Rubisco content varied significantly over the growth stages as well as among the lines, and those modifications could have an impact on photosynthetic performance. Similar to OsPIP2;1, both OsPIP1;1 and OsPIP1;2 have been reported to act as water channels (Liu et al., 2007, 2013; Yu et al., 2006) and, therefore, they may play a role in plant hydraulic conductance. Plant hydraulic conductance is an important determinant of photosynthesis because it sets up the maximum g_s (Brodribb et al., 2007). In fact, the g_s values of *Ospip1;1* were significantly lower than the wild-type over the growing season in pot conditions. In addition, g_s values for plant growing in field conditions were much lower than those in pot conditions. As the coordination of plant hydraulic conductance, g_s and g_m (Flexas et al., 2013; Xiong et al., 2017), the differences between knockout lines and the wild-type in photosynthetic parameters over the growth conditions and

growth stages might be related to the AQPs-mediated plant hydraulic conductance adjustment.

EXPERIMENTAL PROCEDURES

OsPIP1;1, *OsPIP1;2* and *OsPIP2;1* knockout line creation

Sequences of three rice PIP genes, *OsPIP1;1*, *OsPIP1;2* and *OsPIP2;1*, were obtained from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) for CRISPR/Cas9 targets design. Two targets for each gene were designed using the tool CRISPR-GE (https://skl.scau.edu.cn). All the target sequences were listed in Table S1. For each construct, the synthesized oligonucleotide fragments were introduced into tRNA intron and sgRNA expression cassette, driven by OsU3 promoter, in the Y1 vector (from Biorun Bio-technology, Wuhan, China), respectively. Then the two targets expression cassettes were then ligated to one vector based on Golden Gate cloning. The CRISPR/Cas9 constructs were then introduced into *Agrobacterium tumefaciens* strain EHA105 after being verified by sequencing, and then separately transferred into the rice cultivar *Nipponbare* background by Agrobacterium-mediated transformation. The transgenic lines (T_0) were transplanted into the 10-L pots and grown in a growth chamber (Conviron, Controlled Environments, Manitoba, Canada) with a 12 h light/12 h dark cycle at 25–28°C Genomic DNA was extracted from transformant seedlings for polymerase chain reaction (PCR) using specific primers (Table S2). Mutations in the PCR products were detected through the direct Sanger sequencing method. Next, the PCR products were identified by comparing sequences with the Nipponbare reference using the online tool–DSDecode (http://skl.scau.edu.cn/dsdecode/). The progenies of the plants, which are biallelic mutations (Figure S1) of each gene, were screened out, and the seeds from the T_1 generation plants were used in the current research after being verified with genotyping.

Growth conditions

A field and a pot experiment were conducted to investigate the influences of growth environments on photosynthetic and growth performance. The field experiment was carried out at Sanya, Hainan between November 2019 and February 2020. The average temperature and relative humidity in Sanya was 21.5°C and 79.9%, respectively, during the experiment. Seeds were germinated and grown in seedling trays for 10 days, and then the seedlings were transplanted to the paddy field in a randomized block design. The field management, nutrient and irrigation followed local practices. The pot experiment was conducted at Huazhong Agricultural University (HZAU, Wuhan, China) between August and November 2020. The average temperature in Wuhan was 26.5°C, while the relative humidity (80.46%) was similar to that in Sanya during the experiment. Seeds were also germinated and grown in seedling trays for 10 days prior to transferring into 13-L plastic pots containing 10 kg of soil which was applied with 10.0 g of compound fertilizer ($N:P_2O_5:K_2O$ = 1:1:1). In each pot, a mutant seedling was planted in a pair with a wild-type seedling. Plants were grown outdoors, and the pots were rearranged weekly to avoid edge effects. The photosynthetic performances at the different growth stages were only investigated in the pot experiment, as to have the gas exchange measured precisely in paddy field conditions is quite difficult (Du et al., 2020).

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and chlorophyll fluorescence parameters were measured on newly and fully expanded leaves using a LI-6800 portable photosynthesis system (LI-COR, Lincoln, NE, USA). Forty-three days after sowing, the light response curves were measured on newly and fully expanded leaves of plants growing in the paddy field. The leaf temperature in the gas exchange chamber was set to 30°C, the reference CO_2 concentration was set to 400 μmol mol^{-1}, and the leaf-to-air vapor pressure deficit was set to 1.5 kPa. Leaves were firstly acclimated at the photosynthetic photon flux density (PPFD) of 1500 μmol m^{-2} sec^{-1}, and then the auto-progress of the light response curve was adopted. The PPFD were set to 1500, 800, 600, 200 and 0 μmol m^{-2} sec^{-1} in a series with an interval of 60–90 sec. Measurements were conducted between 09:00 and 15:00 h. For each measurement, the gas exchange parameters, steady-state fluorescence (F_s') and maximum fluorescence (F_m') were recorded, simultaneously.

The gas exchange and chlorophyll fluorescence in the pot experiment were measured in a growth chamber (Conviron, Controlled Environments), where the air temperature was 28 ± 5°C, the PPFD was set to 1500 μmol m^{-2} sec^{-1} using a lab-made LED light source, and relative humidity was about 60%. One day before the measurements were performed, pots were moved into the growth chamber. Gas exchange and chlorophyll fluorescence parameters were measured at 39, 57 and 66 days after sowing, respectively. The environmental conditions inside the gas exchange system were set as described above. After the leaf reached a steady state (the fluctuation of stomatal conductance – g_s – being less than 0.05 mol m^{-2} sec^{-1} during a 10-min period), the auto-progress of the CO_2 response curve was adopted. The reference CO_2 concentrations were subsequently set at 400, 300, 200, 100, 50, 400, 600, 800, 1000, 1200, 1500, 2000, 400 μmol CO_2 mol^{-1} air. The CO_2 response curve measurements were performed between 08:30 and 17:00 h each day.

The actual photochemical efficiency of photosystem II (Φ_{PSII}) was calculated as:

$$\Phi_{PSII} = \frac{(F'_m - F_s)}{F'_m}.$$

The electron transport rates (J) were computed as follows:

$$J = \Phi_{PSII} \cdot \mathrm{PPFD} \cdot \alpha\beta,$$

where α and β are the leaf absorption and the distribution of electrons between photosystem I and photosystem II, respectively. The αβ was determined from the slope of the relationship between Φ_{PSII} and the quantum efficiency of CO_2 uptake (Figure S2), which was obtained by varying light intensity under non-photorespiratory conditions at less than 2% O_2 (Valentini et al., 1995; Yin et al., 2009).

The mesophyll conductance of CO_2 (g_m) was calculated based on the variable J method described by Harley et al. (1992), as follows:

$$g_m = \frac{A}{C_i - \frac{\Gamma^*(J+8(A+R_d))}{J-4(A+R_d)}},$$

where A is the net rate of CO_2 assimilation, C_i is the intercellular CO_2 concentration, Γ^* is the CO_2 compensation point in the absence of respiration, and R_d is the day respiration. In the present study, a Γ^* value of 40 μmol mol^{-1} and Rd value of 1 μmol m^{-2} sec^{-1}, which were typical for rice plants, were adopted (Xiong and Flexas, 2018; Xiong et al., 2017). The maximum carboxylation rate (V_{cmax}) and maximum electron transport rate (J_{max}) were estimated from A–C_i curves using *plantecophys* package (Duursma, 2015).

In the current study, the g_m of pot-growing plants was also estimated by using the CO_2 response curve-fitting method, and the

relationship between two estimates of g_m was shown in Figure S3. As the g_m values from both methods were very similar, we used the values obtained by the variable *J* method to compare with other parameters.

Plant growth and leaf morphology

Plant height (cm) was measured from ground level to the tip of the longest leaf, and tiller numbers were counted for PIP knockout lines and the wild-type. For the field experiment, plant height and tiller number were measured at the 43rd day after sowing. Plant height and the tiller number were investigated at the 39th, 57th and 66th days after sowing under pot conditions. To have leaf morphological traits measured, leaves were scanned, and then the leaf width, leaf length and area were manually done in an open-source Java image processing program, ImageJ (https://imagej.net).

Leaf N, C, $\Delta^{13}C$ and Rubisco content

Leaf disks of known area were collected after the gas exchange measurement and then oven dried at 80°C for 72 h. Dry samples were ground before leaf chemical component and the carbon isotope measured using an isotope ratio mass spectrometry (IRMS; IsoPrime 100 IRMS; Isoprime, Stockport, UK). The Rubisco content of newly expanded leaves was measured using the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) method. Leaf tissues for Rubisco concentration measurement were immersed in liquid nitrogen and then stored at −80°C before measuring. The frozen leaf sample was ground with liquid nitrogen on ice and homogenized in an extraction buffer [50 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol and 12.5% (v/v) glycerol]. After centrifugation (rcf: 21130 ***g***, 15 min, 4°C), 0.5 ml supernatant solution was separated and then 0.5 ml dissolving buffer containing 2% (w/v) SDS, 4% (v/v) β-mercaptoethanol and 10% (v/v) glycerol was added. The Rubisco samples were loaded onto SDS–PAGE containing a 12.5% (w/v) polyacrylamide gel. After electrophoresis, the gels were washed with deionized water several times and then dyed in 0.25% (w/v) Coomassie blue staining solution (Coomassie dissolved in the buffer with water:ethanol:acetic acid = 5:4:1) for 12 h and decolorized (in the buffer with water:ethanol:acetic acid = 5:4:1) until the background was colorless. The washed solutions were measured at 595 nm (Infinite M200; Tecan U.S., Männedorf, Switzerland) using the background glue as a blank.

Data analysis

Light response curve parameters, including the maximum net photosynthetic rate (A_{sat}), light compensation point and PPFD at the 75% saturation photosynthetic rate were fitted using the non-rectangular hyperbola-based model.

$$A=\frac{\Phi\times \mathrm{PPFD}+A_{gmax}-\sqrt{(\Phi\times \mathrm{PPFD}+A_{gmax})^2-4\theta\times\Phi\times \mathrm{PPFD}\times A_{gmax}}}{2\theta}-R_n$$

where Φ is the quantum yield at PPFD = 0 μmol (photon) m^{-2} sec^{-1}, A_{gmax} is the maximum gross photosynthetic rate, θ is the convexity factor, and R_n is dark respiration. The model was fitted to the data using the Orthogonal Non-linear Least-Squares Regression (*onls*) function. Statistical analysis was performed using packages of *agricolae*. Other analyses and plots were conducted using the *tidyverse* package. All analyses were performed in R 3.6.3 platform.

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AUTHOR CONTRIBUTIONS

DX designed the research; XH and ZW performed the experiments; XH and DX analysed data; and DX wrote the paper with inputs from all the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

DATA AVAILABILITY STATEMENT

All relevant data supporting the results presented in this work are available within the article and the supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1 Designed targets for the three *OsPIP* genes.

Table S2 Primers used for the CRISPR/Cas9 mutant identification.

Table S3 Leaf morphological and biochemical features at different growth stages.

Figure S1. Identification of the O*spip* mutants in T_0 CRISPR/Cas9 edited plants.

Figure S2. Calibration relationship *A* versus PPFD $\Phi_{PSII}/4$ measured under non-photorespiratory conditions.

Figure S3. Correlation between mesophyll conductance (g_m) fitted by the *Either method* and mesophyll conductance (g_m) calculated by variable *J* methods.

Figure S4. Gas exchange traits varied between plants grown under field and pot conditions.

Figure S5. Light response curves of wild-type and *OsPIP* knockout lines.

Figure S6. CO_2 response curves of wild type and *OsPIP* knockout lines.

Figure S7. CO_2 response curves of wild-type and *OsPIP* knockout lines at the 39th day after sowing.

Figure S8. CO_2 response curves of wild-type and *OsPIP* knockout lines at the 57th day after sowing.

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