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Overexpression of rice aquaporin OsPIP1;2 improves yield by enhancing mesophyll CO₂ conductance and phloem sucrose transport

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Abstract

Aquaporins are involved in CO₂ transport from the leaf intercellular air space to the chloroplast, which contributes to CO₂ assimilation. However, the mechanism of CO₂ transport by rice (Oryza sativa L.) aquaporins is unknown. Here, we investigated the function of the aquaporin OsPIP1;2 in CO₂ diffusion-associated photosynthesis and phloem sucrose transport. Moreover, the grain yield of rice lines overexpressing OsPIP1;2 was determined. OsPIP1;2 was localized to the plasma membrane and the relative expression of OsPIP1;2 was approximately 5-fold higher in leaves in the presence of an elevated CO₂ concentration. Overexpression of OsPIP1;2 increased mesophyll conductance by approximately 150% compared with wild-type (WT) rice. The OsPIP1;2-overexpressing lines had higher biomass than the WT, possibly due to increased phloem sucrose transport. In addition, the grain yield of OsPIP1;2-overexpressing lines was approximately 25% higher than that of the WT in three-season field experiments, due to the increased numbers of effective tillers and spikelets per panicle. Our results suggest that OsPIP1;2 modulates rice growth and grain yield by facilitating leaf CO₂ diffusion, which increases both the net CO₂ assimilation rate and sucrose transport.

Keywords: Aquaporin, elevated CO₂, grain yield, mesophyll conductance, rice, sucrose.

Introduction

The atmospheric CO₂ concentration ([CO₂]) has increased significantly from 318 to >400 ppm since 1959 (Meinshausen et al., 2011). The rate of increase in atmospheric [CO₂] may accelerate (Tripathi et al., 2009), and is predicted to reach 550–700 ppm by 2050 (Meinshausen et al., 2011). Therefore, the effect of elevated [CO₂] on crop production has been intensively
investigated in recent decades (Kimball, 2016). Rice (Oryza sativa L.) is a major staple food crop for almost half the global population (Kurai et al., 2011). The yield of various rice cultivars is reportedly improved by an elevated [CO₂], as indicated by increased growth, tiller number, and leaf area (Kimball, 2016; Hasegawa et al., 2013). An elevated [CO₂] had a positive effect on leaf gas exchange and net photosynthetic rate (Norby et al., 2016), and thus is important for plant growth and development.

In general, CO₂ entering chloroplasts must pass through leaf stomata, plasma membranes, cytoplasm, and chloroplast membranes; these steps are collectively reflected by stomatal conductance (gₛ) and leaf mesophyll conductance to CO₂ (gₘ) (Evans and Loreto, 2000; Evans et al., 2009). In rice, gₛ is a limiting factor in photosynthesis (Kusumi et al., 2012), and enhanced gₛ increased biomass in Arabidopsis (Wang et al., 2014). Although gₘ was long considered to be constant, it is now known to vary according to environmental conditions (Montpied et al., 2009; Singh et al., 2014). Flexas et al. (2008) reported that gₘ is an important determinant of the photosynthetic rate, indicating that CO₂ diffusion from the leaf intercellular air space to the chloroplast is a limiting factor in photosynthesis. Aquaporin NtAQP1 from tobacco leaf facilitates CO₂ transport across the plasma membrane in vivo, which in turn modulates membrane permeability to CO₂ and mesophyll conductance (Uehlein et al., 2003). In addition, NtAQP1 is located in the inner chloroplast membrane, and reduced expression of NtAQP1 resulted in a 20% decrease in CO₂ conductance (Uehlein et al., 2008). By contrast, overexpression of NtAQP1 in tobacco significantly increased gₘ. In Arabidopsis, T-DNA insertion of atpip1;2 reduced leaf CO₂ conductivity, indicating that AtPIP1;2 facilitates CO₂ transport (Heckwolf et al., 2011). Moreover, overexpression of the barley aquaporin HvPIP2;1 in rice plants resulted in an increased gₘ value (Hanba et al., 2004).

OsPIP1;2 is a plasma membrane intrinsic protein (PIP) localized to cellular plasma membranes (Lian et al., 2006). Sakurai et al. (2005) reported that rice plants possess 33 aquaporin-encoding genes and stop-flow spectrophotometry analysis revealed that OsPIP2;4 and OsPIP2;5, but not OsPIP1;2, have high water-channel activity. In addition, OsPIP1 members are localized mainly to mesophyll cells (Sakurai et al., 2005). According to the sequence homology of PIP genes in various plant species, the role of OsPIP1;2 may be related to CO₂ diffusion in rice plants; however, the evidence is sparse. In this study, we evaluated the function of OsPIP1;2 in CO₂ permeability using OsPIP1;2-overexpressing (OE) rice lines under ambient and elevated [CO₂] by determining the biomass, photosynthesis-related physiological parameters, and phloem sucrose-transport rate. Moreover, the contribution of OsPIP1;2 to rice yield was analysed in a field experiment. We aimed to determine the function of OsPIP1;2 in rice plants and its potential for agriculture.

Materials and methods

Plant growth conditions

Rice seeds were sterilized as described by Zhu et al. (2009) for hydroponic experiments. After 10 d, seedlings of OE lines and the wild type (WT) were transplanted into 7-litre plastic containers. Rice plants were grown in a growth chamber (Saifu DRX-680E-DG-CO₂, Ningbo, China) under a light intensity of 300 μmol m⁻² s⁻¹ at shoot height, a relative humidity of α 70%, and a 14 h light (26 °C)/10 h dark (22 °C) photoperiod. Each experiment was randomized and involved three replicates of five plants each at ambient [CO₂] (400 μmol mol⁻¹) and elevated [CO₂] (800 μmol mol⁻¹). The nutrient solution contained: 1.25 mM NH₄NO₃, 0.3 mM K₂SO₄, 0.3 mM NaH₂PO₄, 1 mM CaCl₂, 1 mM MgSO₄, 9 μM MnCl₂, 0.39 μM Na₃MoO₄, 20 μM H₃BO₄, 0.77 μM ZnSO₄, 0.32 μM CuSO₄, and 20 μM EDTA-Fe. Nutrient solution was exchanged every 3 d and its pH was maintained at 5.5. The expression level of OsPIP1;2 was determined in cv. Nipponbare at the tillering, booting, flowering, and grain-filling stages grown in soil pots from June to October 2016; three replications were used.

Homology modeling and sequence alignment

We performed homology modeling using the workspace at the SwissModel website (http://swissmodel.expasy.org/). The X-ray crystal structures of SoPIP2;1 (Protein Data Bank [PDB] codes: 2DSF and 1Z98) served as templates for homology modeling (Törnroth-Horsefield et al., 2006). The amino acid sequence of aquaporin OsPIP1;2 was aligned using Jalview software version 1.6 (http://www.jalview.org/).

Construction of OsPIP1;2-transgenic rice plants

For β-glucuronidase (GUS) expression analysis, the OsPIP1;2 (Osa4g72220) promoter (1977 bp) was amplified from rice (Oryza sativa L. cv Nipponbare) genomic DNA using the primers listed in Supplementary Table S1 at JXB online. The fragment was ligated into the SalI/KpnI sites of the vector pS1aG3 to replace the cauliflower mosaic virus 35S promoter (Tang et al., 2012). The open reading frame (ORF) sequence of OsPIP1;2 was amplified using the primers listed in Supplementary Table S1. Generation of the OsPIP1;2-OE vector was described by Patron et al. (2015).

Histochemical localization of GUS expression

Histochemical analysis was performed as described previously (Ai et al., 2009). Leaves and roots of rice plants were collected in triplicate after 3 weeks. Inflorescences were selected prior to flowering, and seeds were selected 30 d after pollination. Tissues were immersed in GUS reaction mix for 30 min and subsequently incubated at 37 °C for 2 h. GUS-stained tissues were visualized using an Olympus BX51T stereomicroscope equipped with a color charge-coupled device camera.

Transient expression of OsPIP1;2 and fluorescence microscopy

The ORF of OsPIP1;2 without the stop codon was cloned into the C-terminus of the pCAMBIA (GFP) vector at HindIII/PstI sites. Next, the 35S::OsPIP1;2::GFP expression vector was transferred into rice protoplasts using polyethylene glycol-mediated transformation. Rice protoplasts were obtained from etiolated seedlings and transfected as described previously (Jia et al., 2011). OsMCA1 was used as a plasma membrane localization marker (Takamitsu et al., 2012). A confocal laser scanning microscope (LSM410; Carl Zeiss, Oberkochen, Germany) was used to obtain fluorescence images.

Reverse transcription-polymerase chain reaction and real-time quantitative PCR

To investigate the expression pattern of OsPIP1;2, samples were taken from rice plants at different growth stages (Yamaji et al., 2013). To determine the expression level of OsPIP1;2 in leaf and the effect of CO₂ on its expression, rice seedlings (2 weeks old) were exposed to ambient [CO₂] (400 μmol mol⁻¹) or elevated [CO₂] (800 μmol mol⁻¹) for 1 week in a growth chamber (Saifu DRX-680E-DG-CO₂). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). OsPIP1;2 and OsActin were subjected to reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR using the primers in Supplementary Tables S2 and S3 and the protocol of Zeng et al. (2012).
Field experiments

Transgenic T4- and T6-generation rice plants were cultivated in plots at the Nanjing Agricultural University experimental site from June to October in 2016 and 2017. T5-generation rice plants were grown in plots of the experimental site of Sanya Nanjing Agricultural University (tropical climate) from December 2016 to April 2017. The soil at the experimental site contained 29.42 g kg⁻¹ organic carbon, 25.78 mg kg⁻¹ Olsen-P, and 140 mg kg⁻¹ exchangeable potassium. The pH of the soil was 6.4. Nitrogen (urea, 200 kg ha⁻¹), phosphorus (P₂O₅, 90 kg ha⁻¹), and potassium (K₂O, 150 kg ha⁻¹) were applied for the present experiment. Nitrogen fertilizer was split into basal dressing, panicle initiation, and inflorescence formation. The ambient [CO₂] and potassium (K₂O, 150 kg ha⁻¹) were applied for the present experiment.

Gas exchange and chlorophyll fluorescence measurements

The LI-6400 system (LI-COR, Lincoln, NE, USA) was used for measuring gas exchange and chlorophyll fluorescence in plants grown in the growth chamber and the field. The temperature of the leaf chamber was maintained at 25 °C with a photosynthetically active radiation (PPFD) of 1500 μmol m⁻² s⁻¹. The ambient CO₂ concentration was adjusted to 400 μmol mol⁻¹ under saturating light (1500 μmol m⁻² s⁻¹). Relative humidity in the leaf chamber was maintained at 50–60% under non-photorespiratory conditions in the presence of less than 2% CO₂ (Valentini et al., 1992). Daytime respiration rate (Rd) was calculated as the intercept of the linear regression of the photosynthetic rate against PPFD × ΦPSII/4 using light-response curve data (Yin et al., 2009). The Rd values of transgenic plants are listed in Supplementary Fig. S1.

Measurement of leaf chlorophyll content, stomatal density, stomatal size, relative water content, dry mass per unit area and water use efficiency

Chlorophyll was extracted with ethanol and quantified using a SpectraMax M5 spectrometer (Molecular Devices, Sunnyvale, CA, USA) (Sartory and Grobbelaar, 1984; Xiong et al., 2015). The stomatal density (n=15) and size (n=30) of leaf adaxial and abaxial surfaces were measured as described by Wáng et al. (2011, 2014). Briefly, three fully expanded leaves of rice plants were selected, and the stomatal density and size were determined in five randomly selected microphotographs of the adaxial or abaxial surface of the lamina. Leaf number of three plants was measured. In the field experiment, after the leaf net rate of CO₂ assimilation was measured for plants, the leaf chlorophyll content was measured using a chlorophyll meter (SPAD 502 Plus; Spectrum Technologies, Japan). The leaf relative water content and dry mass per unit area were measured according to Heckwolf et al. (2011). Water use efficiency (WUE) was calculated as the ratio between net rate of CO₂ assimilation and transpiration rate.

Measurement of leaf N content

Leaf samples were harvested, heated at 105 °C for 30 min, and dried at 80 °C for 3 d. The leaf samples were digested with 18.4 M H₂SO₄ at 260–270 °C and their N contents were determined using an Auto Analyzer 3 digital colorimeter (Bran + Luebbe GmbH, Germany).

Measurement of phloem sucrose transport and sucrose content in tissues

Phloem exudates were collected as described previously (King and Zeexvaart, 1974). Petioles were cut in 10 M EDTA (pH 6.0), transferred to a cup containing 1.0 ml EDTA, and incubated in the dark for 1 h for exudation. The sucrose content of rice-plant tissues were measured in five biological replicates according to Stitt et al. (1989). Samples (0.1 g) were extracted three times with 4 ml of 80% v/v ethanol for 20 min at 80 °C. Next, the samples were incubated for 10 min in boiling water. The sucrose content was measured using a SpectraMax M5 spectrometer.

Statistical analysis

The data were subjected to analysis of variance and Duncan’s multiple-range test using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). A value of P<0.05 was considered indicative of statistical significance.

Results

Localization and expression pattern of OsPIP1;2 in rice plants

The amino acid sequences of OsPIP1;2 and other aquaporins (NtAQP1, AtAQP1;2, HvPIP2;1, and HvPIP2;3) are...
highly conserved. A homology model of OsPIP1;2 was generated using the crystal structure of SoPIP2;1 (PDB codes: 2D5F and 1Z98) as a template (Supplementary Fig. S2). To analyse the expression of OsPIP1;2 in rice tissue, a 1977 bp fragment immediately upstream of the translation start site of OsPIP1;2 was used for GUS reporter (Fig. 1A–G). OsPIP1;2 was expressed in the roots of rice plants (Fig. 1A–C). GUS activity was high in the leaf, panicle, and embryonic primary tissue (Fig. 1D, E, G). In the cross-section of the leaf blade, GUS activity was detected in mesophyll cells (Fig. 1E).

Fig. 1. OsPIP1;2 expression pattern and subcellular localization. (A–G) Rice transformed with OsPIP1;2 promoter::GUS. GUS activity in the root (A), lateral root elongation zone (B), cross-section of the root tip (C), leaf blade (D), cross-section of the leaf blade (E), floret (F), and germinating seeds (G). Scale bars: 100 μm (C, E) and 1 mm (A, B, D, F, G). (H) Response of OsPIP1;2 expression to CO2 in leaf blade by quantitative real-time PCR. The internal reference gene was OsActin. Values are means ±SD (n=3); different letters indicate significant differences (P<0.05). (I–L) Subcellular localization of OsPIP1;2 in rice protoplasts. Scale bars: 10 μm. (I) Fluorescence signal of 35S::OsPIP1;2-GFP. (J) fluorescence signal of OsMCA1 (Takamitsu et al., 2012). (K) bright-field image. (L) merged image. (M) Expression levels of OsPIP1;2 in the indicated tissues and at the indicated growth stages of the wild type (WT) (cv. Nipponbare) grown in soil, as determined by quantitative real-time PCR with OsActin as an internal reference. Values are means ±SD (n=3). (This figure is available in color at JXB online.)
By real-time quantitative RT-PCR, the relative expression level of *OsPIP1;2* in the rice leaf blade was 5-fold higher in the presence of an elevated [CO₂] (Fig. 1H). The expression of *OsPIP1;2* was next investigated in different organs and growth stages (Fig. 1M). *OsPIP1;2* showed the highest expression in the leaf blade at all growth stages. In addition, the expression of *OsPIP1;2* in the leaf blade was highest at the flowering stage. *OsPIP1;2* was also expressed in other organs at various levels (Fig. 1M).

To determine the subcellular localization of *OsPIP1;2*, an *OsPIP1;2–GFP* fusion was expressed in mature rice protoplasts isolated from culms of rice seedlings grown in the dark. The GFP fluorescence signal was detected at the plasma membrane (Fig. 1I–L), indicating co-localization with the plasma membrane marker Ca²⁺-permeable mechanosensitive channel OsMAC1 (Takamitsu et al., 2012). Therefore, *OsPIP1;2* is localized to the plasma membrane.

Gas-exchange parameters of rice plants

To characterize the physiological function of *OsPIP1;2* in rice plants, OE lines were constructed. The relative expression level of *OsPIP1;2* was significantly 6.3–7.1-fold higher in the OE lines than in the WT (Fig. 2).

We next examined the net rate of CO₂ assimilation (*Aₙₚ*) at different substomatal CO₂ concentrations (*Cᵢ*) and PPFDs using a gas-exchange system (Fig. 3; Supplementary Fig. S3). The *Aₙₚ* values revealed that the response of the OE lines to a low PPFD (<200 μmol m⁻² s⁻¹) was similar to that of the WT (Supplementary Fig. S3). However, at a PPFD of 1500 μmol m⁻² s⁻¹, the *Aₙₚ* of the OE lines was 17–19% higher than that of the WT (Supplementary Fig. S3). Under these conditions, *Aₙₚ* was light-saturated and limited by the carboxylation rate. The light-use efficiency of the OE lines was higher than that of the WT under light-saturated conditions. The *Aₙₚ* of the OE lines was also higher than that of the WT at a *Cᵢ* of >150 μmol CO₂ mol⁻¹ (Fig. 3A). The maximum *Aₙₚ* of the OE lines was ~37 μmol CO₂ m⁻² s⁻¹, compared with ~31 μmol m⁻² s⁻¹ in the WT. Therefore, the OE lines had greater CO₂ for photosynthesis. Based on the chlorophyll fluorescence and gas exchange data, the leaf *gₛ* of the OE lines increased by approximately 150% that of the WT (Fig. 3D). The *gₛ* values of the OE lines were 26–38% higher than that of the WT (Supplementary Fig. S4). By contrast, water-use efficiency did not differ significantly between the OE lines and the WT (Supplementary Fig. S5). Further, there was no significant difference between the OE lines and the WT in the electron transport rate (*fₗ*) and maximum electron transport rate (*Jₘₐₓ*) (Fig. 3B; Table 1). The *Cᵢ* and maximum Rubisco activity (*Vₐₘₐₓ*) of the OE lines were 25–30% and 28–33%, respectively, higher than those of the WT (Table 1).

In the field experiment, the *Aₙₚ* values of the OE lines were 9–15%, 13–42%, and 19–34% higher than those of the WT at the flowering, middle-filling, and end-filling stages, respectively. In addition, the *Aₙₚ* values of the OE lines and the WT decreased from the flowering stage to the end of the grain-filling stage. There was no significant difference in chlorophyll content at any growth stage between the OE lines and the WT (Supplementary Fig. S6).

The OE lines and the WT did not display significant differences in chlorophyll content, number of leaves per plant, relative water content, leaf dry mass per unit area (Supplementary Table S4), or stomatal density and size on the adaxial or abaxial surface (Fig. 3C; Supplementary Table S5). In addition, the leaf N content was similar between the OE lines and the WT (Supplementary Fig. S7).

![Fig. 2. Expression level of OsPIP1;2 in selected rice transgenic lines. (A) Phenotype of 7-day-old transgenic lines (overexpressing (OE) 1, OE2, and OE3). Scale bar: 10 cm. (B, C) Expression level of OsPIP1;2 in the OE lines by RT-PCR (B) and real-time quantitative PCR (C) with OsActin as an internal reference. Values are means ±SD (n=3); different letters indicate significant differences (P<0.05). (This figure is available in color at JXB online.)](https://academic.oup.com/jxb/article-abstract/70/2/671/5238162)
Response of OsPIP1;2 to ambient and elevated [CO₂]

We determined the growth of the OE lines and the WT under ambient and elevated [CO₂] over 4 weeks (Fig. 4). Compared with ambient [CO₂], the total dry weight of the OE lines and the WT were significantly higher in the presence of an elevated [CO₂]. Moreover, the total dry weight of the OE lines was 13–18% and 15–20% higher than that of the WT under ambient and elevated [CO₂], respectively (Fig. 4C, D).

Carbohydrate content and sucrose transport under ambient and elevated [CO₂]

Phloem sucrose transport and sucrose content were measured in the WT and OE lines under ambient and elevated [CO₂] (Fig. 5). The shoot sucrose content, root sucrose content, ratio of root/shoot sucrose, and phloem sucrose transport activity in the OE lines were 10–18%, 17–20%, 10–15%, and 18–21% higher, respectively, than those of the WT under ambient [CO₂]. Under elevated [CO₂], the shoot sucrose content, root sucrose content, and ratio of root/shoot sucrose of the OE lines were 19–28%, 24–34%, 11–20%, and 13–16%, respectively, higher than those of the WT. Phloem sucrose transport activity in the OE lines was 13–16% higher than that in the WT (Fig. 5), indicating that the OE lines allocate more carbon from source to sink. Therefore, we investigated phloem sucrose transport from shoot (source) to panicle (sink). The panicle sucrose content of the T4-, T5-, and T6-generation OE lines was 41–49%, 51–66%, and 37–50% higher than that of the WT, respectively (Table 2); this may influence the rice yield.

Table 1. Net rate of CO₂ assimilation parameters of rice plants under ambient [CO₂]

|       | \(A_{\text{net}}\) (μmol CO₂ m⁻² s⁻¹) | \(C_i\) (μmol CO₂ mol⁻¹) | \(C_c\) (μmol CO₂ mol⁻¹) | \(V_{\text{cmax}}\) (μmol CO₂ m⁻² s⁻¹) | \(J_{\text{max}}\) (μmol photons m⁻² s⁻¹) |
|-------|--------------------------------------|--------------------------|--------------------------|--------------------------------------|--------------------------------------|
| WT    | 28.05 ± 1.3b                         | 305 ± 9a                 | 158 ± 16b                | 55.9 ± 4.8b                         | 242 ± 11a                           |
| OE1   | 33.43 ± 1.6a                         | 267 ± 18b                | 198 ± 15a                | 71.8 ± 4.6a                         | 251 ± 9a                            |
| OE2   | 32.95 ± 1.5a                         | 272 ± 16b                | 204 ± 8a                 | 74.1 ± 5.8a                         | 246 ± 17a                           |
| OE3   | 33.19 ± 1.1a                         | 265 ± 14b                | 205 ± 10a                | 72.4 ± 6.5a                         | 248 ± 26a                           |

Rice plants were grown under ambient [CO₂] in the chamber for 4 weeks. Values are means ±SD (n=5). Different letters indicate significant differences at the P<0.05 level in rice plants. \(A_{\text{net}}\), net rate of CO₂ assimilation; \(C_i\), substomata CO₂ concentration; \(C_c\), chloroplastic CO₂ concentration; \(V_{\text{cmax}}\), maximum RuBisco activity; \(J_{\text{max}}\), maximum electron transport rate; \(\epsilon_{\text{m}}\), maximum Rubisco activity.
Alteration of OsPIP1;2 expression affects rice grain yield

To examine the influence of OsPIP1;2 on rice grain yield, OsPIP1;2 WT and OE lines were cultivated in a field (Fig. 6; Table 2). The agricultural traits of the T4–T6 generations of the OE lines and the WT were investigated at Nanjing City, Jiangsu Province and Sanya City, Hainan Province from 2016 to 2017 (Table 2). The effective tiller number and spikelets per panicle of the OE lines were 17–40% and 12–23% higher than those of the WT. The grain yield of the OE lines was enhanced by 13–25% (T4 generation) at Nanjing, by 18–23% (T5 generation) at Sanya, and by 13–36% (T6 generation) at Nanjing, relative to the WT (Fig. 6). Therefore, overexpression of OsPIP1;2 enhances rice yield in the field.

Discussion

We report here that overexpression of OsPIP1;2 enhances the net CO₂ assimilation rate by improving CO₂ diffusion in the leaf, which increases the growth and yield of rice plants. Rice OsPIP1;2 belongs to the PIP1 family, among which PIP1;2 had a function in CO₂ diffusion in tobacco and Arabidopsis (Uehlein et al., 2003; Heckwolf et al., 2011; Sade et al., 2014). We reported previously that oocytes transfected with OsPIP1;2 did not show water-transport activity (Ding et al., 2016), suggesting that water transport activity was not enhanced in the OE lines. Interestingly, the amino acid sequences of OsPIP1;2 and other aquaporins related to CO₂ permeability are highly conserved (Supplementary Fig. S2). According to Mori et al. (2014), OsPIP1;2 had the same amino acid residue at the C-terminal end of the E-loop as barley aquaporin, which was permeable to CO₂ in the Xenopus laevis oocyte expression system. In addition, the relative expression of OsPIP1;2 was significantly increased under elevated [CO₂] (Fig. 1). Therefore, OsPIP1;2 may be associated with CO₂ permeability. In addition, gₛ, related closely to the modification of aquaporins in tobacco and Arabidopsis (Uehlein et al., 2003; Heckwolf et al., 2011; Sade et al., 2014). In our study, OsPIP1;2 expression was significantly up-regulated in transgenic plants relative to the WT (Fig. 2). The OsPIP1;2-OE transgenic rice lines had higher gₛ and Aₕₑₙ, and the gₛ of OE lines was 1.5-fold higher than that of the WT (Fig. 3D; Table 1).
Our results are consistent with the observation in NtAQP1 (NtAQP1:2) OE tobacco plants (Uehlein et al., 2003). Although, \( g_m \) and \( g_s \) are in general correlated (Lauteri et al., 1997; Loreto et al., 2003), the \( g_s \) of the OE lines was observed to be only 0.3-fold higher than that of the WT (Supplementary Fig. S4), indicating that a high \( A_{net} \) is mainly contributed by increased \( g_m \). The chloroplastic CO2 concentration was also higher in the OE lines than in the WT (Table 1), indicating that OsPIP1;2 influences mesophyll CO2 conductance. In general, high \( g_s \) usually causes lower WUE (Lawson and Blatt, 2014). However, in our study, the OsPIP1;2 OE lines did not show decreased WUE even though the \( g_s \) increased. The reason is the enhanced net rate of CO2 assimilation in overexpression of OsPIP1;2, which showed high \( g_m \) (Supplementary Figs S4 and S5). Therefore, a potential approach for crop plants is to increase \( g_s \), maintaining WUE without substantial cost in overexpression of OsPIP1;2.

Statistical analysis of data is from T4–T6 generations. Values are means ±SD (n=12). Different letters indicate significant differences at the \( P<0.05 \) level in rice plants.
increasing $C_i$ was enhanced during the first phase in the OE lines (Fig. 3A), consistent with the reduced $C_i$ in atpip1;2 lines reported by Heckwolf et al. (2011). According to the $A_{net}$–$C_i$ curves, $V_{cmax}$ and CO$_2$ assimilation rate of the OE lines were increased (Table 1), but this is not due to the difference in leaf N content (Supplementary Fig. S7). In addition, under the FACE system, $V_{cmax}$ decreases as an acclimatory response to long-term elevated [CO$_2$] (Ainsworth and Long, 2005). In the present study, $g_{max}$ and $V_{cmax}$ were increased in OE lines, which suggested that $g_{max}$ enhancement may increase $V_{cmax}$ as the early response to CO$_2$ in rice plants.

Sucrose is the major translocated photosynthetic product and the main form of carbon on which plant sinks grow (Sung et al., 1989). In this study, the shoot sucrose content of the OE lines was higher than that of the WT (Fig. 5A). Sucrose acts as a signaling molecule and provides energy for root growth and development (Chiou and Bush, 1998). Efficient sucrose transport from shoot to root via phloem plays a key role in root growth (Salerno and Curatti, 2003). Plant acclimation to elevated [CO$_2$] has been associated with an increase in carbohydrate content (Weigel and Manderscheid, 2012). In the present study, the OE lines had higher biomass than the WT under both ambient and elevated [CO$_2$] (Fig. 4), consistent with the results of Kim et al. (2001). This is because the OE lines showed higher carbohydrate content than the WT (Fig. 5). Our results suggest that OsPIP1;2 is involved in the response to elevated [CO$_2$] and increases the root/shoot sucrose concentration ratio (Figs 4 and 5). Although the root/ shoot sucrose concentration ratio is not a good proxy for phloem export, it is also affected by the sucrose consumption rates in organs. Thus, we examined phloem transport in rice plants. Under ambient [CO$_2$], the OE lines showed a higher root sucrose content than the WT (Fig. 5D). Therefore, the OE lines exhibited higher phloem sucrose transport activity compared with the WT, suggesting greater carbon allocation from source to sink (Fig. 5D).

Sucrose is an important determinant of the number of grains in rice spikelets, and an increased number of spikelets per panicle is key for enhancing grain yield (Kato et al., 2007). In the field experiment, the panicle sucrose content of the OE lines was markedly higher (Table 2), suggesting greater sucrose export from leaves to seeds, relative to the WT. This suggests that the OE lines were source limited, but not sink limited, in grain yield increase. Further, the OE lines had a larger number of spikelets per panicle than the WT (Table 2), which may be due to their greater sink capacity. This finding is consistent with prior reports that the yield potential of rice is enhanced by its large sink capacity, itself related to the large number of spikelets per panicle (Peng et al., 2008). Therefore, overexpression of OsPIP1;2 increased the number of spikelets per panicle in rice plants by enhancing sucrose transport from leaf to panicle by increasing the net CO$_2$ assimilation rate. This likely...
contributed to the increased yield of the OE lines in the field experiment (Fig. 6).

In addition, the leaf relative water content and dry mass per unit area reflect the water status of the plant (Jones, 2007). The leaf relative water content and dry mass per unit area did not differ between the OE lines and the WT (Supplementary Table S4), indicating that water transport in leaves was unaffected by overexpression of OsPIP1;2. The number of leaves per plant also did not differ between the OE lines and the WT (Supplementary Table S4), suggesting that the OE lines have the same developmental rates. Our results indicate that the effect of OsPIP1;2 on rice growth and yield is largely due to the facilitation of CO₂ transport rather than the modulation of water transport and developmental rate. In conclusion, our results indicate that overexpression of OsPIP1;2 modulates the number of spikelets per panicle by increasing leaf CO₂ diffusion, photosynthetic performance, and phloem sucrose transport; together, these effects have a positive effect on rice yield.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Daytime respiration rate ($R_d$) of the transgenic rice plants.

Fig. S2. Homology modeling of OsPIP1;2.

Fig. S3. Net rate of CO₂ assimilation ($A_{net}$) to PPFD of rice plants under 400 ppm CO₂.

Fig. S4. Stomatal conductance ($g_s$) of rice plants under 400 ppm CO₂.

Fig. S5. Water-use efficiency of rice plants under 400 ppm CO₂.

Fig. S6. Chlorophyll content and net rate of CO₂ assimilation ($A_{net}$, μmol m⁻² s⁻¹) of newly and fully expanded leaves of rice plants at the flowering stage (Flowering), middle grain-filling stage (Mid-Fill), and end grain-filling stage (End-Fill).

Fig. S7. Leaf N content of rice plants under 400 ppm CO₂.

Table S1. Primers used for construction of vectors.

Table S2. Primers used for RT-PCR analysis.

Table S3. Primers used for real-time quantitative PCR analysis.

Table S4. Morphological and physiological parameters of the rice plants.

Table S5. Stomatal size in leaves of the transgenic rice plants.

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