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ORIGINAL ARTICLE

Contrasting root traits and native regulation of aquaporin differentially determine the outcome of overexpressing a single aquaporin (*OsPIP2;4*) in two rice cultivars

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Abstract

Overexpressing *OsPIP2;4* in the two rice cultivars Giza178 and IR64 resulted in contrasting cultivar-dependent physiological attributes under control and drought conditions in the field. In the T3 plants, *PIP2;4* expression was significantly higher in the leaves and roots of Giza178 under control but only in the roots under drought condition and higher in the leaves and roots of IR64 under control but not under drought condition compared with that in the corresponding wild types. The transgene improved the plant growth in Giza178 under both growth conditions but had no significant effect in IR64 under either condition. The transgenic lines of Giza178 recovered their leaf relative water content faster than those of the wild type in the afternoon and showed improved gas exchange parameters, water use efficiency, and grain yield, as a result of improved root hydraulic conductivity (Lp_r) and xylem sap flow. No comparable responses were found in IR64 although Lp_r and xylem sap flow were enhanced in the transgenic lines under the control condition only, suggesting that the positive effect of *PIP2;4* on the well-watered leaves of IR64 was offset by the low root/shoot ratio and the inherent expression of other aquaporins. In the transgenic plants of IR64 under drought, *PIP2;4* expression was not induced in the roots presumably due to an overriding post-transcription regulatory mechanisms, leading to the lack of changes in the Lp_r and xylem sap flow and consequently, the plant growth, water relations, gas exchange, and grain yield were similar to the wild type. The data suggest that the outcome of overexpressing a single aquaporin gene depends on the plant architecture, internal responses to drought, and native expression of other aquaporins.

Keywords Aquaporins; *PIP2;4* · Rice · Drought · Water relations · Gas exchange, yield

Introduction

Aquaporins are a family of membrane proteins in plants. Plant aquaporins have been classified on the bases of sequence homology and localization in the cell into the plasma membrane intrinsic proteins (PIPs), which were further subdivided into two subfamilies (PIP1s and PIP2s), the tonoplast intrinsic proteins (TIPs), the peribacteroid membrane proteins (Nod26-like intrinsic proteins abundant in N-fixing root nodules, NIPs), the small and basic intrinsic proteins in the membranes of endoplasmic reticulum (SIPs) (Ishikawa et al. 2005; Johanson et al. 2001; Quigley et al. 2002), the X-intrinsic proteins (XIPs), the hybrid-intrinsic proteins (HIPs), and the GIpF-like intrinsic proteins (GIps) (Danielson and Johanson 2008). Aquaporins have been well characterized in many plants leading to the identification of 35 members in Arabidopsis (Johanson et al. 2001), 36 in maize (Chaumont et al. 2001), and 33 in rice (Sakurai et al. 2005).

The extensively studied PIPs and TIPs have been proved to increase the water permeability of cell membranes by acting as selective transmembrane water channels (Niemietz and Tyerman 1997; Maurel et al. 1997; Ohshima et al. 2001). PIP2s have been shown to have higher water transport activity than most PIP1s (reviewed in Yaneff et al. 2015), except for a limited number of PIP1 isoforms (Suga and Maeshima 2004; Zhang et al. 2007). Some PIP1s are functional CO_2 channels, which are important in CO_2 stomatal and mesophyll conductance and thereby sustaining high photosynthetic rate (Heinen

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et al. 2014; Sade et al. 2014). However, PIP1s have regulatory role upon PIP2s where complexes between PIP1s and PIP2s have higher water transport activity than pure PIP2s (Fetter et al. 2004). Evidence also exists that aquaporins can transport small molecules such as CO_2 (Ding et al. 2016; Hanba et al. 2004; Flexas et al. 2006), glycerol and ammonia particularly in NIPs (Wallace et al. 2006; Niemietz and Tyerman 2000), boron (Pang et al. 2010), and H_2O_2 (Henzler and Steudle 2000) across cell membranes.

Because of their water transport activity, aquaporins have been assigned a central role in plant water relations under normal and drought conditions. In the light of the composite model put forward by Steudle and Peterson (1998) for water movement through plant roots, the radial path (i.e., the path of water through the different cell layers and types in the root starting from the epidermis and ending into the stele) is more resistant to water flow than the axial path (xylem vessels). Moreover, the most resistant part of the radial path is the endodermis due to the presence of Casparian strip, which blocks the apoplastic path, thereby restricting the flow of water to be through cell membranes. The abundance of PIP2 proteins (*PIP2;1*, *PIP2;2*, *PIP2;3*, *PIP2;5*) in the endodermis more than in other root tissues of rice unambiguously highlights a crucial role in water transport to the stele and consequently water uptake by root (Sakurai et al. 2008; Sakurai-Ishikawa et al. 2011). In the developing leaves of maize, the abundance of aquaporins was found to be higher in the elongating zones than in the mature ones, also suggesting a role in regulating water movement among the different leaf zones over a path of development (Hachez et al. 2008). Although aquaporins are not expected to contribute to water flow through xylem vessels, their distribution in the xylem parenchyma suggests a role in regulating water movement in and out of xylem vessels to the surrounding tissues in the shoot system (Sack and Holbrook 2006).

Under drought stress, the contribution of aquaporins to water uptake by roots has been reported to be even greater than under well-watered conditions. Root-to-leaf hydraulic conductance was more sensitive to mercury (which blocks some aquaporin channels; Frick et al. 2013) in the stressed than in the well-watered plants, indicating more dependence on aquaporins for water transport under stress conditions (Lu and Neumann 1999). Given that abscisic acid (ABA) is a major signal under drought stress, the induction of aquaporin expression in response to drought and ABA provides a strong evidence of essential contribution of aquaporins to plant water relations under drought (Parent et al. 2009). Overall, these findings prove that aquaporins contribute positively to plant water uptake and transport at the cell and tissue level.

Nonetheless, manipulation of the expression of single aquaporin genes in different plants has resulted in contrasting effects on whole plant growth and water relations. In many plants, overexpressing an aquaporin improved salt and/or drought tolerance as indicated by enhancing plant growth, water uptake, and transpiration: in Arabidopsis overexpressing the barley *HvPIP2;5* (Alavilli et al. 2016), tomato overexpressing the tomato *SlTIP2;2* (Sade et al. 2009), and tobacco overexpressing the wheat *TaAQP8* (Hu et al. 2012) or *TaAQP7* (Zhou et al. 2012). In other cases, overexpressing the *PIP1* or *PIP2* from Jatropha did not alter salt or drought tolerance in Arabidopsis (Jang and Ahn 2015). However, altering the expression of a single aquaporin (*PIP1b*) caused an increased sensitivity to drought in tobacco where increasing the water flow through cell membranes decreased drought tolerance (Aharon et al. 2003). These findings suggest that at the whole plant level, the function(s) of aquaporins is still poorly understood and that the outcome of altering the expression of a single aquaporin gene depends on internal factors besides the activity of the aquaporin of interest.

Lowland rice has unique features compared with other cereal crops in that it has excessive transpiration from leaves (Tanguilig et al. 1987) and lower hydraulic conductance of the root as a result of excessive suberization of root cells (in addition to the shallow root system) (Miyamoto et al. 2001; Schreiber et al. 2005). These features render rice plant so sensitive to water deficit that it experiences symptoms of drought stress (such as midday stomatal depression on sunny days) even in submerged soil. In a comparative study on the expression of aquaporins in low land and upland rice (which is more resistant to drought), Lian et al. (2006) reported that the expression of aquaporins with high water transport activity (PIP2s), along with the regulatory PIP1s, was more abundant in roots of upland rice, suggesting that they improve the capacity of root water uptake and tolerance of whole plant to water deficit. Aquaporins were also found to be induced by transpiration demand in rice (Sakurai-Ishikawa et al. 2011). Moreover, we reported that aquaporins constitute a determining factor of water use efficiency (WUE) in rice under well-watered and drought conditions where the unbalanced expression of aquaporins in Indica rice cultivars resulted in increased drought sensitivity and reduced WUE compared with japonica cultivars (Nada and Abogadallah 2014). However, overexpression of a single aquaporin in rice resulted in either enhancing drought tolerance by improving root hydraulic conductivity and presumably water uptake by roots (Lian et al. 2004) or raising salt sensitivity by decreasing root/shoot ratio in spite of increasing the root hydraulic conductivity (Katsuhara et al. 2003).

These findings highlight the need of further studies to understand the function(s) of aquaporin genes at whole plant level as affected by internal factors. We hypothesized that the native expression of aquaporin complement along with root traits (most importantly root/shoot ratio) strongly influences the outcome of overexpressing a single aquaporin gene using rice as a model plant. We therefore overexpressed the rice aquaporin *OsPIP2;4* in a japonica (Giza178) and an Indica (IR64) rice cultivars for which we have previously quantified the expression of eight PIP and four TIP aquaporins

under normal and drought conditions (Nada and Abogadallah 2014). The transformed plants responded differently to drought stress. We explained the contrasting effects of the transgene on the whole plant in the light of those previous findings.

Materials and methods

Plant material

Two rice cultivars were used in this study, namely Giza178 and IR64, which were kindly supplied by the Rice Research Institute at Sakha, Egypt, and the International Rice Research Institute (IRRI, Philippines), respectively.

Preparation of *PIP2;4* construct and plant transformation

RNA extraction and cDNA synthesis was done as previously mentioned in Nada and Abogadallah (2014) from leaves of IR64 rice cultivar. *PIP2;4* from IR64 rice cultivar was amplified using forward primer: CATGCCATGGATGG GCAAAGAGGTGGACGTG and reverse primer: CGCG GATCCAGACGAGCTCAACTACGCGTTG. The amplified fragment was purified using PCR column (Thermo-Scientific, USA) and then digested by NcoI and BamHI (the underlined sequence in forward and reverse primers) to be NcoI: *PIP2;4*: BamHI. The same two enzymes were used to digest pPZP3425 vector (Szakasits et al. 2007). After digestion, the purified fragment was ligated to pPZP3425 vector replacing *GUS* gene under CaMV 35S promoter (Fig. 1). Chemically competent *Escherichia coli* bacteria (TOP10 strain, Life Technologies, USA) were transformed by adding 10 μL of the ligated mixture to the bacterial vial (50 μL) using heat shock method. The extracted construct from positive colonies (for full methods see Nada 2016) was then mobilized to electroporant *Agrobacterium tumefaciens* (LBA4404, Takara, Japan).

Preliminary experiments showed that kanamycin resistance was not reliable for the selection of transformants where there was no correlation between kanamycin resistance and transformation and also frequent regeneration of albino plantlets was observed in the presence of kanamycin. For this reason, we relied on amplification of CaMV::*PIP2;4* for screening and confirmation of transformants. Plants of Giza178 were transformed and regenerated essentially as described by Hiei et al. (1994). Briefly, callus was induced from seeds on MS medium (Muraahige and Skoog 1962) containing 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and solidified with 6 g L^{-1} agarose for 3 weeks in the dark at 25 °C. The fast-growing calli were incubated for 10 min with a suspension of *A. tumefaciens* prepared by harvesting the bacteria followed by washing with MS medium containing 20 g L^{-1} sucrose; 5 g L^{-1} glucose; 2 mg L^{-1} 2,4-D; 0.25 mg L^{-1} benzyladenine; 0.3 g L^{-1} glutamine; and 0.3 g L^{-1} casein hydrolysate and by re-suspending in a medium with the same composition supplemented with 15 mg L^{-1} acetosyringone (co-cultivation medium) at 25 °C. The calli were then transferred to Petri dishes containing sterile filter papers moistened with the co-cultivation medium for 2 days at 23 °C in the dark. The calli were then washed with sterile water and subcultured onto a medium composed of MS basal medium supplemented with 20 g L^{-1} sucrose; 2 mg L^{-1} 2,4-D; 0.25 mg L^{-1} BA; 0.5 g L^{-1} casein hydrolysate; and 250 mg L^{-1} cefotaxime and solidified with 6 g L^{-1} agarose in the dark at 25 °C. After 3 weeks, the calli were subcultured onto embryo a maturation medium composed of MS basal medium plus 20 g L^{-1} sucrose; 25 g L^{-1} sorbitol; 1 mg L^{-1} 2,4-D; 0.5 mg L^{-1} BA; 0.5 g L^{-1} glutamine; 0.3 g L^{-1} casein hydrolysate; and 50 mg L^{-1} kanamycin and solidified with 8 g/l agarose. The calli were subcultured every 3 weeks onto the same medium. Calli which showed developing embryos were subcultured onto a regeneration medium containing MS basal medium 20 g l^{-1} sucrose, 1 g L^{-1} casamino acid, and 1 mg L^{-1} kinetin and was solidified with 6 g L^{-1} agarose under continuous light (200 μmol m^{-2} s^{-1}). After 3–4 weeks, plantlets with distinct leaves and roots were transferred to pots containing compost and grown under controlled conditions (350 μmol m^{-2} s^{-1} light intensity, 60–65% relative humidity, 14-h photoperiod) until used for subsequent analysis. This procedure was repeated five times and a total of about 120 plantlets were regenerated.

Because we were unable to regenerate IR64 plantlets from callus cultures, we opted to use the *in planta* method described in by Supartana et al. (2005) for the transformation of IR64.

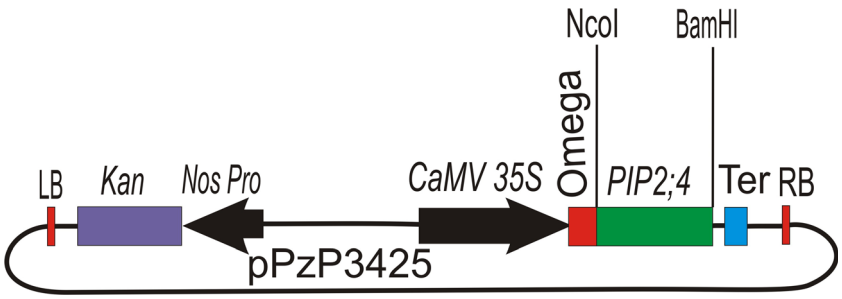

Fig. 1 Construction of *PIP2;4* in pPZP3425 vector. *PIP2;4* was ligated between NcoI and BamHI restriction sites under *CaMV 35S* (Cauliflower mosaic virus 35S) promoter. The kanamycin (*Kan*) is plant selectable marker under *Nos* (nopaline synthase) promoter

The seeds of IR64 were de-husked, sterilized with 0.1% mercuric chloride for 20 min, and germinated in distilled water for 2 days. The *Agrobacterium* was prepared as described above and then used for *in planta* transformation. Each seed was pierced with a needle of a syringe filled with the *Agrobacterium* suspension at the point of shoot tip emergence. The seeds were then plated onto MS medium supplemented with 10 g L^{-1} sucrose and 15 mg L^{-1} acetosyringone and incubated in the dark at 23 °C for 9 days. The seedlings were then transferred to pots containing perlite and watered with Ruakura nutrient solution (Smith et al. 1983) for 5 days. Plants with green leaves were transferred to pots containing compost and incubated as above until used for subsequent analysis. This procedure was repeated 8 times (200–300 seeds each) and about 140 plants were obtained.

Confirmation of transformation

The incorporation of the construct into the genome of the putative transformants of both cultivars was confirmed by polymerase chain reaction (PCR) of *CaMV 35S*::*PIP2;4*. Briefly, DNA was extracted from the leaf tissues as described by Sika et al. (2015) and *CaMV 35S*::*PIP2;4* was amplified by using the forward primer: CAGATTAGCCTTTTCAATTT CAG and the reverse primer: AGACGAGCTCAACT ACGCGTTG. The expected fragment size was about 1500 bp. At least 25 plants from each cultivar with positive PCR test of *CaMV 35S*::*PIP2;4* were isolated.

For more confirmation, the mRNA transcript level of *PIP2;4* was also quantified as described by Nada and Abogadallah (2014) for the positive lines obtained from the previous test. To check the linearity of PCR reaction, we calibrated the band volumes (normalized based on *18S rRNA* as an internal control) of PCR products against the number of cycles and then performed the quantification of *PIP2;4* expression at a number of cycles within the linear phase of amplification. To make sure the selection procedure was rigorous, we collected leaf samples form 15 wild-type plants from each cultivar, extracted mRNA, and then quantified *PIP2;4* by RT-PCR as mentioned above. We then compared the expression of *PIP2;4* in leaf samples from the putative transformants of Giza178 and IR64 with the corresponding wild types. A plant was considered transgenic if it showed at least 50% higher expression of *PIP2;4* than all of the corresponding 15 wild-type plants. At least 15 transgenic plants were obtained for each cultivar.

The confirmed transgenic plants were transplanted into a wire-mesh greenhouse under field conditions with a soil mixture of 50% clay and 50% peat moss. The soil was 35-cm deep on a concrete basement. The average climatic conditions over the experiment period were 25–29/22–24 °C day/night temperature, 58–65% relative humidity, and 2150 μmol m^{-2} s^{-1} maximum light intensity. To acclimate, the plants were covered with transparent plastic cones with top ventilation holes for 3 days, where new leaves appeared expanding. The cones were then removed. The plants were watered every day until they produced seeds. For each plant, only the main tiller was kept and other tillers were excised regularly. During anthesis, each panicle was wrapped with a paper bag until all flowers were self-pollinated. The seeds were then collected (T1) and grown next year as described above to produce T2 seeds. The T2 seeds were also grown next year under the same conditions to produce T3 seeds which were used for the subsequent work. The presence of the transgene in T3 plants was confirmed as previously mentioned.

Growth of T3 plants and drought treatment

The wild-type and T3 seeds of Giza178 and IR64 were germinated and grown as described above. After 15 days of sowing, the seedlings were transplanted into 3 independent plots; each plot included two blocks. Each block included 40 seedlings of each cultivar that were 25-cm apart. The plants were watered every day for 10 days until they have established. For drought treatment, each block was divided into two sections (20 plants each). One section was watered every day and used as a control. The other section was not watered until the field capacity (FC, measured by dividing the fresh weight of a soil sample by its water saturated weight%) decreased down to 72%. This FC value was selected because preliminary experiments showed that the wild-type plants showed permanent leaf rolling when the soil FC was 69%. The soil FC was monitored once a day and water was added through 20-cm-deep holes between rows to maintain the soil FC at 72%. This treatment lasted until the plants produced seeds.

Sampling

All analyses described below were carried out on intact plants or samples collected 20 or 21 days after the onset of drought treatment, i.e., when the plants were 45–46 days old, except for samples used for analysis of grain yield that were collected from fully mature plants (105–110 days old). Measurements of gas exchange, xylem sap flow, and root Lp_r were performed on the same day and samples for the measurement of biomass, leaf RWC, and *PIP2;4* expression were collected on the next day.

Quantification of *PIP2;4* expression

Leaf and root samples for the quantification of *PIP2;4* expression were collected at 9:00 to 10:30 am. The samples were frozen immediately in liquid nitrogen and then stored at − 80 °C until used. RNA extraction, reverse transcription, and amplification of *PIP2;4* transcripts were carried out as

described previously (Nada and Abogadallah 2014). Three independent RNA extracts were used for this analysis.

Measurement of biomass attributes and leaf RWC

Intact plants were removed from soil (by using a shovel), washed briefly with cold water for drought-treated plants, or normal water for control plants to remove soil remains and then sealed into plastic bags. The roots were then separated from shoots and each was weighed. The total biomass (fresh weight, FW) and root/shoot ratio were then calculated for each plant. Five plants for each treatment were used. Samples for measuring leaf RWC were collected at predawn (about 03:00), 09:00, 13:00, 15:00, 17:00, and 19:00. The leaves were collected and sealed immediately into pre-weighed plastic bags. The bags were weighed and the fresh weights (FW) were calculated. The leaves were submerged in distilled water at 4 °C and stored in a fridge overnight and then weighed (saturated weight, SW). After drying at 60 °C for constant weights after 3 days, the leaves were weighed (dry weights, DW). The leaf RWC was calculated from the formula RWC% = [(FW-DW)/(SW−DW)] × 100. Five samples for each treatment were used.

Measurement of root hydraulic conductivity (Lp_r) and xylem sap flow

For measuring the hydraulic conductivity, samples of intact 4-cm terminal part of roots (i.e., including the root tip) were collected at 09:30 to 10:30. The root hydraulic conductivity was measured as described by Katsuhara et al. (2003). Xylem sap flow was measured as described by Soejima et al. (1992) with some modifications. Briefly, for each plant, the shoot was clipped evenly at 4 cm above the soil surface (and weighed) and the undisturbed shoot bases were capped with pre-weighed absorbent cotton. The cotton was wrapped in tin foil and sealed with plastic tape and each shoot base along with the cotton was covered with an inverted black pot so that no light penetrated in. After 6 h, the cotton was weighed and the xylem sap flow was calculated as the increase in the weight of cotton (milligrams of water) per gram of shoot. The measurement of xylem sap flow was performed at 08:00 to 14:00. The measurements were made for five plants from each treatment.

Measurement of gas exchange

Gas exchange parameters namely rates of photosynthesis (A), transpiration (E), stomatal conductance (g_s), and leaf internal CO_2 concentration (C_i) were measured by using the LCi-SD gas exchange system (Analytical Development Company Ltd., England). Measurements were made at 09:00 to 10:30 from the first fully expanded leaves in each plant. The water use efficiency (WUE) was calculated as A/E. Measurements were made from five randomly chosen plants for each treatment.

Measurement of grain yield

Panicles from mature plants were collected separately from each plant and dried in air for 8 days. The grains were extracted from each plant and weighed. Measurements were made for five plants from each treatment.

Statistical analysis

Measurements were replicated as mentioned in each section. To compare between samples, one-way ANOVA was run by using SPSS v 18 at a significance level of $P < 0.05$.

Results

Overexpression of *PIP2;4*

Ten to fifteen transgenic plants from each cultivar were selected and grown over the next 2 years to set seeds (T2 and T3 seeds). The total time required to obtain a putative transgenic plantlet was about 59 days for Giza178 and 16 days for IR64. However, the transformation frequency was higher in Giza178. In both cultivars, preliminary experiments showed that not all kanamycin-resistant plantlets were transgenic. Therefore, we did not rely on this type of selection in proving the transformation, but rather, we performed PCR of the *CaMV 35S*::*PIP2;4* (Fig. 1 and 2) and also quantified the expression of the transgene in the transgenic plants (T1–T3). The T3 seeds from three transgenic lines (the presence of transgene in these lines was confirmed) from each cultivar were used for the present study. Data from three lines from each cultivar is presented in this report. Figure 2 shows that the *CaMV 35S::PIP2;4* was present in the genome of the transgenic lines of both Giza178 and IR64. Figure 3 shows that the expression of *PIP2;4* was significantly higher (at least 50% higher) in the leaves of transgenic plants of Giza178 than in the wild-type ones under well-watered condition but not under drought. In the roots, the expression of *PIP2;4* was significantly (several folds) higher in the transgenic than in the wild-type plants under both conditions. In the transgenic plants of IR64 (Fig. 4), the expression of *PIP2;4* in the leaves was significantly higher (several folds) than in the wild-type ones under well-watered condition, but was similar in both types of plants under drought because of the strong induction of *PIP2;4* by drought in the wild type. In the roots of IR64, the expression of *PIP2;4* was significantly higher (several folds) in the transgenic plants than in the wild-type ones. In the roots of IR64 under drought, the expression of *PIP2;4* was not detectable in the wild type, L1, and L2 but low expression was detected in L3.

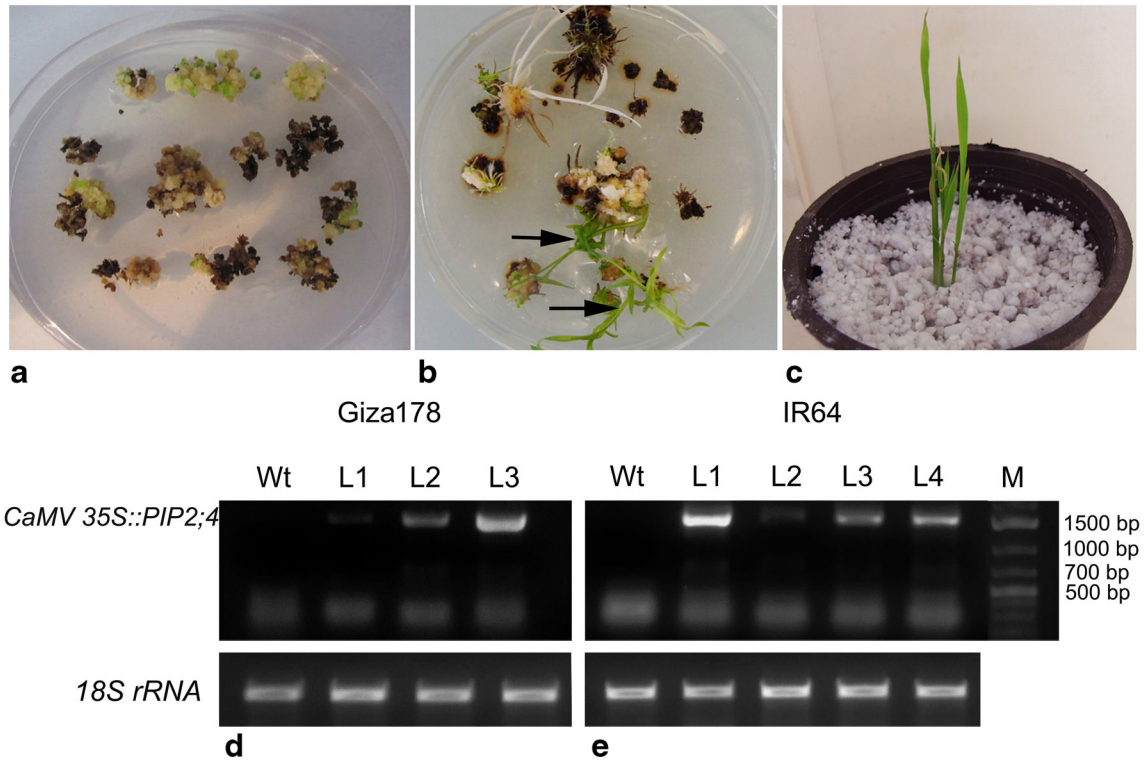

Fig. 2 a: *Agrobacterium*-treated calli of Giza178 after 3 weeks of incubation on selection medium. b: developing plantlets of Giza178 after 4 weeks of growth on embryo development medium. c: fully developed plantlet (arrows in b) transferred to soil. d and e: confirmation of transformation (in T3 lines used in the subsequent analyses) by PCR of the *CaMV 35S*::*PIP2;4* fragment in Giza178 and IR64 using *18S rRNA* gene as an internal control. The expected fragment size was 1500 bp. M is DNA ladder. Subsequent data on line 4 in IR64 was not presented in this report and we did not remove it in order to minimize image manipulation

Biomass attributes and RWC

The growth of the transgenic Giza178 plants in terms of plant FW was significantly higher than that of the wild type under the well-watered (by 16–26%) as well as drought (by 24–31%) conditions (Fig. 5). No significant difference was found between the transgenic and the wild-type plants of IR64 under control or drought condition.

Overexpression of *PIP2;4* did not result in changing the root/shoot ratio in Giza178 or IR64 in the control or in plants under drought (Fig. 6). However, the root/shoot ratio was significantly and consistently higher in Giza178 than in IR64.

The leaf RWC decreased steeply from predawn to mid-day (13:00) in both Giza178 and IR64 (Fig. 7) and then started to recover from 15:00 up to 19:00 in Giza178, but remained at a minimum at 15:00 and 17:00, where it recovered only at 19:00 in IR64. The recovery of leaf RWC in Giza178 was significantly greater in L3 and L1, L2, and L3 than in the wild type at 15:00 and 17:00, respectively. No significant difference in the recovery of leaf RWC was found between the wild-type and transgenic plants of IR64 (Fig. 7). A closely similar trend was also observed under drought but data from plants under drought are not presented for simplicity.

Root hydraulic conductivity and xylem sap flow

In Giza178, overexpression of *PIP2;4* significantly increased the Lp_r in the control (by 21, 25, and 19% in L1, L2, and L3, respectively) and in plants under drought (by 28, 32, and 22% in L1, L2, and L3, respectively) compared with the wild type (Fig. 8). In IR64, the transgenic plants showed significantly higher Lp_r under well-watered condition (by 21, 16, and 19% in L1, L2, and L3, respectively) compared with the wild type but not under drought (Fig. 8).

The transgenic plants of Giza178 showed significantly higher xylem sap flow per unit shoot biomass compared with the wild type under control (by 28, 27, and 26% in L1, L2, and L3, respectively) and drought (by 38, 34, and 44% in L1, L2, and L3, respectively) conditions (Fig. 9). In IR64, xylem sap flow of the transgenic lines increase compared with that in the wild type in the well-watered plants (by 14, 15, and 12% in L1, L2, and L3, respectively). None of the transgenic lines of IR64 had significantly higher xylem sap flow than the wild type under drought (Fig. 9).

Gas exchange

Overexpression of *PIP2;4* led to a significant increase in the rate of photosynthesis (*A*) in L1, L2, and L3 of Giza178 in the

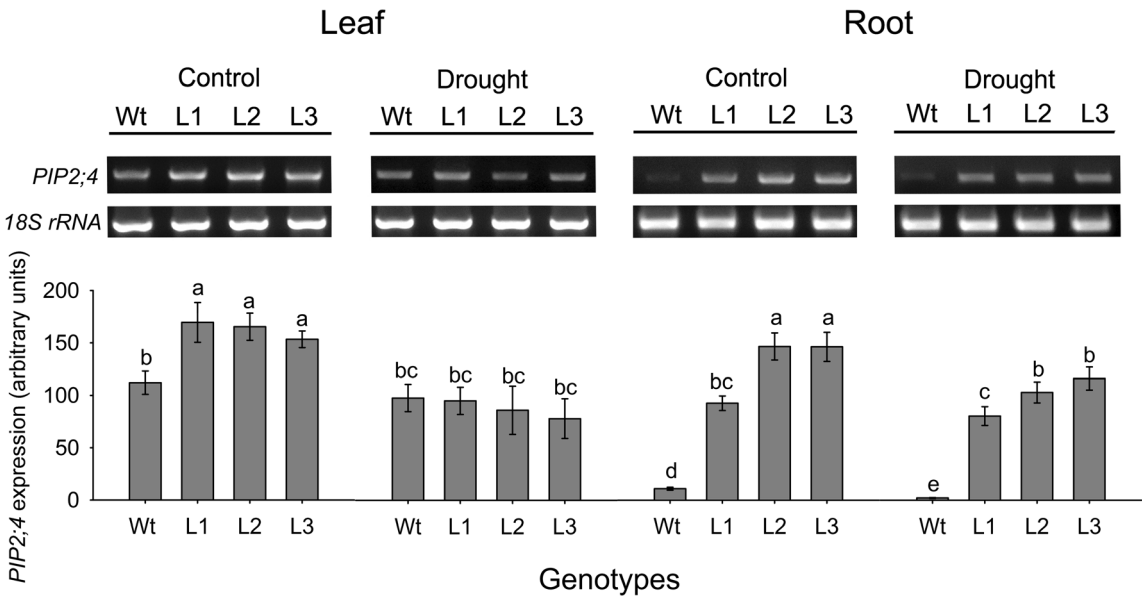

Fig. 3 Quantification of *PIP2;4* expression in leaves and roots of Giza178 wild-type and transgenic lines under control and drought conditions. *PIP2;4* expression was quantified in the linear phase of PCR by measuring band volumes normalized on the basis of *18S rRNA* as an internal control. Bars are means of three independent measurements ± SE. Bars ± SE not sharing similar small letters are significantly different at $P < 0.05$

well-watered (by 31, 26, and 31% of the wild type, respectively) and plants under drought (by 31, 34, and 33% of the wild type, respectively). However, no significant differences were found between the wild-type and transgenic lines of IR64 under control or drought condition (Fig. 10A, B). The same trend was found for the rate of transpiration (E) (Fig. 10C, D) and stomatal conductance (g_s) (Fig. 10E, F) in both cultivars. The transgenic lines of both cultivars had similar values of leaf internal carbon dioxide concentration (C_i) to those of the corresponding wild types under control condition (Fig. 10G, H). Drought stress led to a significant reduction of C_i in the wild-type and transgenic lines of both cultivars compared with those of the corresponding controls (except in L2 of Giza178), but no significant differences were found between the wild-type and transgenic lines in either cultivar. The water use efficiency (WUE, A/E) was significantly higher in the well-watered plants than that of the wild type for the three transgenic lines of Giza178 but not those of IR64 (Fig. 10I, J). Drought stress caused significant increase in the WUE of the wild type and L2 but not L1 and L3 of Giza178, but led to significant decrease in WUE of the wild type, L1, L2, and L3 in IR64 compared with that of the corresponding controls.

Grain yield

Overexpression of *PIP2;4* led to a significant increase in grain yield of L1, L2, and L3 of Giza178 under control (by 21, 13, and 18% of the wild type) and drought (by 17, 20, and 21% of the wild type) conditions (Fig. 11). No significant differences in the grain yield were found between the wild-type and transgenic lines of IR64 either in the control or plants under drought (Fig. 11). The wild type of IR64 significantly out yielded (18% higher) that of Giza178 under well-watered condition but not under drought.

Discussion

In a comprehensive study on the expression of aquaporin genes (involving PIP1s, PIP2s, and TIPs) in rice, we have shown that the expression of *PIP2;4* in leaves was higher than that in roots of Giza178 and IR64. We also found that *PIP2;4* expression was induced in the leaves but greatly depressed in the roots by drought in both cultivars. This was also true for *PIP2;1*, *PIP2;2*, *PIP2;4*; *PIP2;6*, and *PIP2;7* (Nada and Abogadallah 2014). Because PIP2s have been shown to have high water transport activity than other aquaporins (Fetter et al. 2004), we suggested that this pattern of expression is the main reason of the exceptional sensitivity of rice to water deficit and consequently its low WUE. We thought that overexpressing a PIP2 aquaporin would increase the root water permeability under normal and drought conditions and enhance the whole plant performance in the field. However, due to the quantitative cultivar-dependent differences in aquaporin expression and in plant architecture (Nada and Abogadallah 2014), we expected varying effects of the transgene in Giza178 and IR64.

In this study, overexpression of *PIP2;4* in Giza178 and IR64 was screened and confirmed by detection of the *CaMV 35::PIP2;4* in the genomes of the transformants (Fig. 2) and

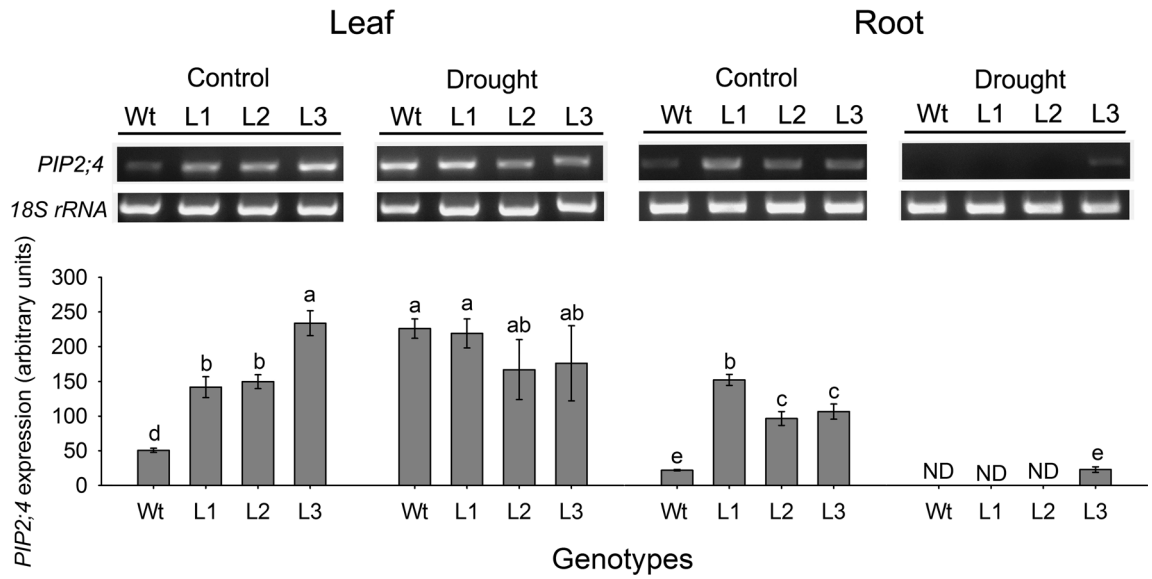

Fig. 4 Quantification of *PIP2;4* expression in leaves and roots of IR64 wild-type and transgenic lines under control and drought conditions. *PIP2;4* expression was quantified in the linear phase of PCR by measuring band volumes normalized on the basis of *18S rRNA* as an internal control. Bars are means of three independent measurements ± SE. Bars ± SE not sharing similar small letters are significantly different at $P < 0.05$. ND, not detectable

then quantification of *PIP2;4* expression in the leaves and roots of the transgenic lines (Figs. 3 and 4). It is noteworthy that hygromycin or herbicides (Hiei et al. 1994; Ozawa 2009; Lin et al. 2009) are commonly used as selective agents in rice transformation. However, when we initiated this work, a vector with hygromycin or herbicide resistance gene was not available to us. We thus constructed a kanamycin tolerance curve for the wild types of Giza178 and IR64 and then used 50 m L^{-1} of kanamycin for screening because it brought about 50% reduction in the growth of the wild types. Remarkably, there was no correlation between kanamycin resistance and transformation; i.e., not all kanamycin-resistant plantlets were transgenic. Alternatively, we relied on the amplification of *CaMV 35S*::*PIP2;4* by PCR, coupled with quantification of the *PIP2;4* transcripts by RT-PCR, in the putative transformants in order to verify transformation. These findings suggest that kanamycin is not appropriated for the selection of transgenic plants in the rice cultivars used in this study which agrees with earlier reports on rice (Dekeyser et al. 1989).

The data in Figs. 3 and 4 suggest that the transcript abundance of *PIP2;4* in the leaves of well-watered Giza178 and IR64 is not under tight control, where the transgenic lines showed increased expression compared with the wild types. This contrasts to the leaves under drought in which the transcript abundance was similar in the wild type and transgenic lines of both cultivars. On the other hand, overexpressing *PIP2;4* consistently increased the transcript abundance in the well-watered roots of Giza178 and IR64 and in the roots under

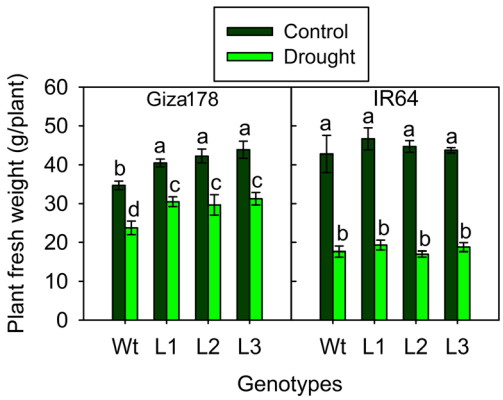

Fig. 5 Changes in growth in terms of plant fresh weight of the wild-type and transgenic lines of Giza178 and IR64 in response to drought. Bars are means of plant fresh weight ± SE. Bars ± SE labeled with different small letters are significantly different at $P < 0.05$. Statistical analysis was calculated for each cultivar separately

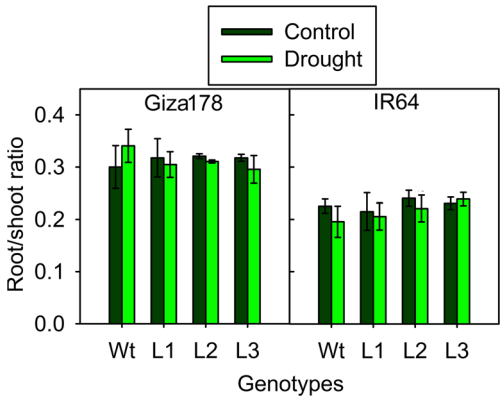

Fig. 6 The root/shoot ratios of the wild-type and transgenic lines of Giza178 and IR64 grown under control or drought condition. Bars are means of root/shoot ratios ± SE. Genotypes in each cultivar were significantly similar at $P < 0.05$. Statistical analysis was calculated for each cultivar separately

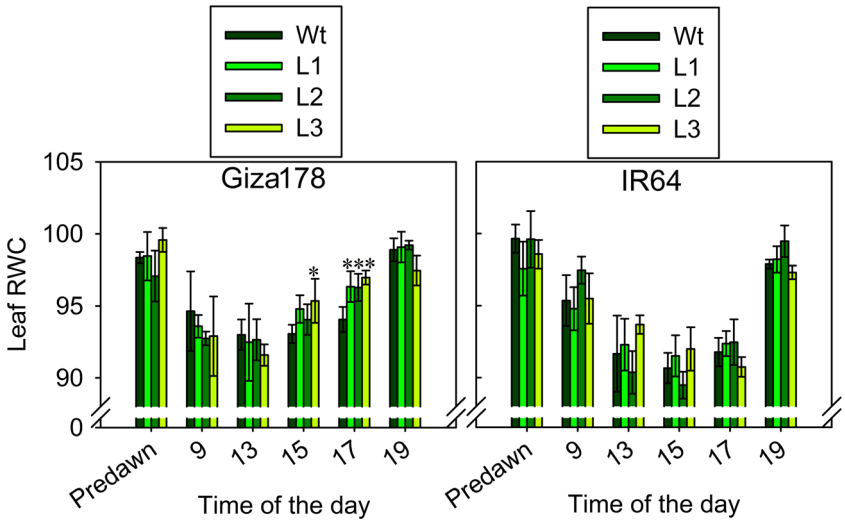

Fig. 7 The leaf RWC of the well-watered plants of the wild-type and transgenic lines of Giza178 and IR64 measured at predawn, 9:00, 13:00, 15:00, 17:00, and 19:00. Bars are means of leaf RWC ± SE. Bars ± SE labeled with an asterisk are significantly different from the corresponding wild types at $P < 0.05$. Statistical analysis was calculated for each cultivar separately

drought of Giza178 but not those of IR64 (except for only one line). These data suggest that regardless of the increased transcription rate (by the constitutive promoter), the steady-state transcript abundance is dictated possibly by a post-transcription mechanism at the level of mRNA longevity that is species- and organ-dependent. Aquaporin gene expression and activity have been reported to be controlled at post-transcription, translation, and post-translation (Zardoya 2005). Drought has been also reported to induce the expression of aquaporin genes via ABA-dependent and independent mechanisms (Aroca et al. 2006; Olaetxea et al. 2015). Our data imply that expression control mechanisms at post-transcription are overriding to those at transcription under drought but not under well-watered condition.

At the leaf level, the effect of overexpressing *PIP2;4* (and the expected increase in tissue water transport activity) would be minor if any, given the high transcript abundance of several PIPs and TIPs in the leaves reported previously (Nada and Abogadallah 2014). Contrarily in the roots, the increased expression of *PIP2;4* improved root water uptake under both growth conditions in Giza178 (as indicated by the enhanced root hydraulic conductivity and xylem sap flow, see below) but only under control condition in IR64. The lack of *PIP2;4* induction in roots of IR64 under drought seems to be related to the whole plant response to drought compared with Giza178. Because IR64 is a drought-sensitive cultivar (Liu et al. 2013; Henry et al. 2012), suppression of root hydraulic conductivity by downregulation of aquaporins (Lu and Neumann 1999) may lead to water retention in root tissues rather than its loss to the drying soil (Rodríguez-Gamir et al. 2011). However, this response seems to be within the framework of plant survival rather than sustained growth, in contrast to Giza178 (a moderately drought-tolerant cultivar, unpublished data) which responded to drought by increasing the root hydraulic conductivity (by accumulation of the *PIP2;4* transcripts in the transgenic lines) in order to keep growing. Overall, the data in Figs. 3 and 4 suggest that the *PIP2;4* transgene increases the transcript abundance regardless of the genetic background under

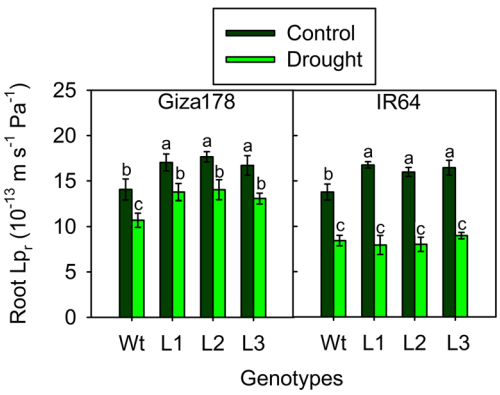

Fig. 8 The root Lp_r of the wild type and transgenic lines of Giza178 and IR64 grown under normal or drought condition. Bars are means of Lp_r ± SE. Bars ± SE labeled with different small letters are significantly different at $P < 0.05$. Statistical analysis was calculated for each cultivar separately

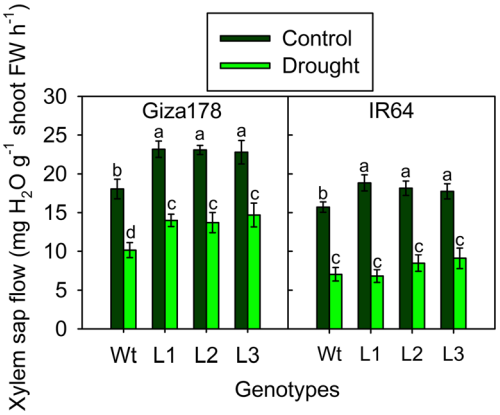

Fig. 9 Changes in xylem sap flow of the wild-type and transgenic lines of Giza178 and IR64 grown under normal or drought condition. Bars are means of xylem sap flow per unit shoot FW ± SE. Bars ± SE not sharing small letters are significantly different at $P < 0.05$. Statistical analysis was calculated for each cultivar separately

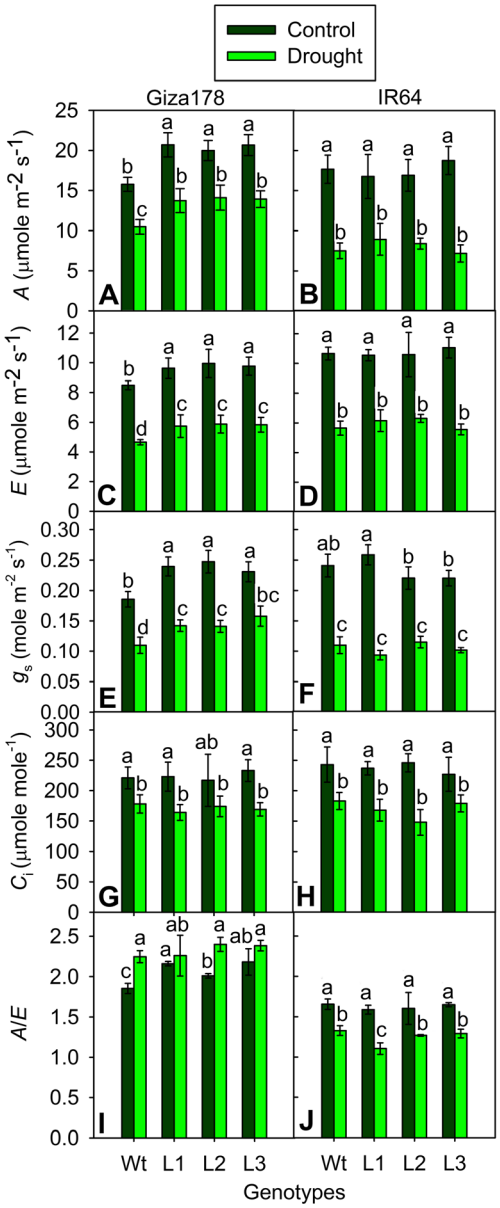

Fig. 10 Changes in rate of photosynthesis (A, B), transpiration (C, D), stomatal conductance (E, F), C_i (G, H), and WUE (I, J) of the wild-type and transgenic lines of Giza178 and IR64 grown under normal or drought condition. Bars are means ± SE. Bars ± SE not sharing small letters are significantly different at $P < 0.05$. Statistical analysis was calculated for each cultivar separately

control condition, but its effect under drought appears to be under native post-transcription control that complies with the whole plant response to stress.

Overexpression of *PIP2;4* consistently improved plant growth under normal and drought conditions in Giza178 but not in IR64 (Fig. 5). The enhanced growth in the transgenic lines of Giza178 apparently has resulted from improved leaf water status (leaf RWC) and rate of photosynthesis (Fig. 10A) compared with the wild type (Fig. 7). This in turn correlated with higher Lp_r (Fig. 8) and xylem sap flow (Fig. 9) which together indicates improved water uptake by roots. Remarkably, the transgene did not change the plant growth (Fig. 5), leaf water status (Fig. 7), or photosynthesis (Fig. 10B) in IR64 even under control condition where the expression of *PIP2;4* was significantly higher and the Lp_r (Fig. 8) and xylem sap (Fig. 9) flow were significantly enhanced in the transgenic lines compared with the wild type. A possible explanation for this is that the increase in xylem sap flow in the control IR64 did not translate into enhanced biomass because it was offset by the low root/shoot ratio of IR64 compared with Giza178 (Fig. 6). It is also possible that the xylem sap flow that was measured in the absence of the transpiration demand from shoot (because the shoot was clipped) which mainly depends on aquaporin activity was too little to improve the whole plant water status during periods of high transpiration. It has been reported that water flow through plant tissues during periods of high transpiration is mainly through the apoplast. During the night (or when the hydraulic forces are removed as in the present case), the water flow is mainly driven by osmotic forces through cell-to-cell path, i.e., through aquaporins (Steudle and Peterson 1998; Sakurai-Ishikawa et al. 2011). A third possible explanation is that the enhanced water uptake by the roots in IR64 may have been offset by the extremely induced aquaporins (PIP1s, PIP2s, and TIPs) in the leaves with the resulting excessive transpiration from leaves of IR64 (Fig. 10) compared with those of Giza178 (Nada and Abogadallah 2014). If this was true, then the contrasting effects of the transgene in Giza178 and IR64 can be attributed to the increased expression of *PIP2;4* per se, the root/shoot ratio, and the native expression of aquaporin complement in each cultivar. Jang and Ahn (2015) reported that Arabidopsis plants overexpressing a *PIP1* or a *PIP2* gene showed no change in tolerance to salt or drought obviously, because these genes alone were not sufficient to affect the whole plant response to stress since the expression of other stress-responsive genes was not altered in these transgenic

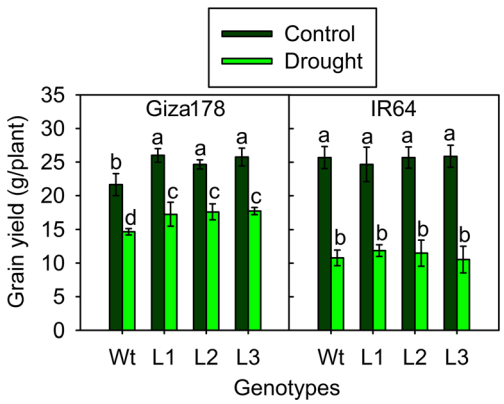

Fig. 11 Grain yield of the wild-type and transgenic lines of Giza178 and IR64 grown under normal or drought condition. Bars are means of grain yield per plant ± SE. Bars ± SE labeled with different small letters are significantly different at $P < 0.05$. Statistical analysis was calculated for each cultivar separately

plants. It has also been reported that overexpression of the barely *PIP2;1* in rice resulted in an increased salt sensitivity because the transgene resulted in excessive water loss from leaves while not enhancing water uptake by roots (Katsuhara et al. 2003). This contrasts to the results of Lian et al. (2004) and Liu et al. (2013) who reported that overexpressing a single aquaporin improved salt or drought tolerance, respectively in rice. This suggests that the effect of an aquaporin transgene depends on factors other than the gene of interest. Up to the best of our knowledge, this is the first comparative report of contrasting effects of one aquaporin in two different rice cultivars.

The enhanced *A* in the transgenic lines of Giza178 compared with the wild type (Fig. 10A) apparently has resulted from increased g_s, and thereby more CO_2 availability for carboxylation, at least under drought stress. We have shown previously (Nada and Abogadallah 2014) that C_i is limiting the photosynthesis in rice under drought. Although data in Fig. 10G do not show significant differences in C_i between the wild-type and transgenic lines, the increase in *A* in the transgenic lines but not in the wild type suggests more CO_2 availability for fixation. It is well-established that g_s and *E* are indicators of the rate of water uptake and plant water status in rice (Taylaran et al. 2011) and in other plants (Arbona et al. 2005; García-Sánchez et al. 2007). This also supports a positive role for *PIP2;4* in improving the rate of water uptake by roots and hence the plant water status as shown by the faster recovery of the leaf RWC in the afternoon (Fig. 7) in the transgenic lines of Giza178 compared with the wild type. Nonetheless, no comparable effects of the transgene on gas exchange parameters were found in the transgenic lines of IR64 obviously because the effect of *PIP2;4* was offset by several factors as mentioned above.

The WUE of Giza178 was generally higher than in IR64 (Fig. 10I, J). Overexpressing *PIP2;4* improved the WUE in the transgenic lines only under well-watered condition, suggesting that some factors other than water availability per se regulate the WUE under drought. We suggest that under drought, the decrease in *A* was greater relative to that in *E* in the transgenic lines, presumably because of the limited sink strength resulting from growth retardation (Paul and Foyer 2001), a response that is not related to aquaporin expression but rather is related to expression of other-stress related genes. In contrast to Giza178 in which the WUE increased under drought, the WUE of IR64 was depressed by drought in the wild-type and the transgenic lines. We suggested above that IR64 responded to drought by restricting water movement through tissues and halting growth, a response that presumably resulted in severe inhibition of photosynthesis in all genotypes and hence reduced WUE. The improved plant growth, water status, and gas exchange in the transgenic lines of Giza178 but not in those of IR64 have translated into increased grain yield per plant under normal and drought conditions supporting the positive effect of the transgene in Giza178.

We conclude that the contrasting effect of overexpressing *PIP2;4* in Giza 178 and IR64 resulted from (i) the failure of IR64 to induce the expression of *PIP2;4* under drought in the transgenic lines, (ii) the low root/shoot ratio in IR64 that diluted the positive effect of the transgene on water uptake by roots, and (iii) the unbalanced expression of aquaporin complement in the leaves of IR64.

Author contribution R. Nada: study plan, sampling, *PIP2;4* cloning, overexpression and other molecular analyses, preparation of the figures with statistics, and helped in writing of the mauscript. G. Abogadallah: tissue culture, physiological analyses, preparation of the figures with statistics, and writing of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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