The Role of Aquaporin RWC3 in Drought Avoidance in Rice

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Although the discovery of aquaporins in plants has resulted in a paradigm shift in the understanding of plant water relations, the relationship between aquaporins and drought resistance still remains elusive. From an agronomic viewpoint, upland rice is traditionally considered as showing drought avoidance. In the investigation of different morphological and physiological responses of upland rice (*Oryza sativa* L. spp *indica* cv. Zhonghan 3) and lowland rice (*O. sativa* L. spp *japonica* cv. Xiushui 63) to water deficit, we observed young leaf rolling and the remarkable decline of cumulative transpiration in the upland rice. The expression of water channel protein *RWC3* mRNA was increased in upland rice at the early response (up to 4 h) to the 20% polyethylene glycol (PEG) 6000 treatment, whereas there was no significant expression changes in lowland rice. Protein levels were increased in upland rice and decreased in lowland rice at 10 h after the water deficit. The up-regulation of *RWC3* in upland rice fits well with the knowledge that upland rice adopts the mechanism of drought avoidance. The physiological significance of this *RWC3* up-regulation was then explored with the overexpression of *RWC3* in transgenic lowland rice (*O. sativa* L. spp *japonica* cv. Zhonghua 11) controlled by a stress-inducible *SWPA2* promoter. Compared to the wild-type plant, the transgenic lowland rice exhibited higher root osmotic hydraulic conductivity (Lp), leaf water potential and relative cumulative transpiration at the end of 10 h PEG treatment. These results indicated that RWC3 probably played a role in drought avoidance in rice.

Keywords: Aquaporin — Drought avoidance — Hydraulic conductivity — Rice (*Oryza sativa*).

Abbreviations: A_r, surface area of root; J_v, water flow; Lp, hydraulic conductivity; MIP, major intrinsic protein; Osm_{in}, osmotic pressure in oocyte; Osm_{out}, osmotic pressure out of oocyte; PEG, polyethylene glycol; P_f, osmotic permeability coefficient; PIP, plasma membrane intrinsic protein; S_0, initial oocyte surface area; UBI, ubiquitin; V_0, initial oocyte volume; V_w, molar volume of water; II_b, osmotic pressure of bath medium; Π_s, osmotic pressure of sap.

Introduction

Aquaporins, which show the ability to facilitate and regulate passive exchange of water across membranes, belong to a highly conserved membrane protein family MIP (major intrinsic protein) (Maurel 1997, Tyerman et al. 1999, Tyerman et al. 2002). In plants, aquaporins are present in the plasma membrane and in the vacuolar membrane where they are abundant proteins. The structural analysis of aquaporins has revealed general mechanism of protein-mediated membrane water transport (De Groot and Grubmuller 2001, Sui et al. 2001). Studies on plant aquaporins and plant water relations have been carried out for years. Hg^{2+} is one of inhibitors for aquaporins, and there is a large body of reports on Hg^{2+} decreasing plant root hydraulic conductivity (Lp), suggesting that aquaporins might play a major role in overall root water uptake (Javot and Maurel 2002). However, we should bear in mind that mercurial compounds have inevitable metabolic toxicity and not all aquaporins have been shown to be sensitive to mercury, e.g. *Arabidopsis* RD28 (Daniels et al. 1994). Reverse genetics provides an elegant approach to address aquaporin roles in plant water relations. In the aquaporin *PIP1b* antisense *Arabidopsis*, the reduced expression of the specific aquaporins decreases the cellular osmotic water permeability coefficient (P_f) by approximately three times, and the root system is five times as abundant as that of control plants. It has been proposed that the phenotypic traits of antisense *Arabidopsis* plants reflect a compensation of reduced root P_f by an increase in root surface area (Kaldenhoff et al. 1998). *Arabidopsis* aquaporin *PIP1b* is also constitutively over-expressed in tobacco. The over-expression of the plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions, but the *PIP1b* over-expression has no beneficial effect under salt stress, and even negative effect, causing fast wilting during water stress (Aharon et al. 2003). The aquaporin *NtAQP1* antisense tobacco shows decreased cellular water permeability, reduced root hydraulic conductivity and lower water stress resistance (Siefritz et al. 2002). Some genes for aquaporins or putative aquaporins, for instance, rice *rTip1* (Liu et al. 1994), tomato *TRAMP* (Fray et al. 1994), tobacco *NeMip2* and


NeMip3 (Yamada et al. 1997) and radish *RsPIP2* (Suga et al. 2002), are up-regulated under drought stress. However, the simple accumulation of aquaporin does not equate with adaptive function for aquaporins. It has also been revealed that phosphorylation (Johansson et al. 1998), Ca^{2+} (Gerbeau et al. 2002) and pH (Gerbeau et al. 2002, Tournaire-Roux et al. 2003) can influence aquaporin activity. Although the discovery of aquaporins in plants has resulted in a paradigm shift in the understanding of plant water relations (Maurel and Chrispeels 2001), the relation between aquaporins and plant drought resistance still remains elusive.

Drought avoidance is one of mechanisms of drought resistance. From an agronomic viewpoint, upland rice is traditionally regarded as showing drought avoidance (Price et al. 1997, Price et al. 2002). At the cellular level, the osmotic adjustment, where solutes accumulate in cells and decrease osmotic potential to favor water uptake and maintain cell turgor as water potential falls (Turner et al. 1986), has been well known. A large number of studies have centered on cell solute accumulation. These osmolytes include amino acid (e.g. proline), sugar alcohols (e.g. pinitol), other sugars (e.g. fructans) and quaternary ammonium compounds (e.g. glycine betaine) (Bray 1997). Recently, interest has turned to the water transport across membranes facilitated by aquaporins. To obtain an insight into the relationship between aquaporins and plant drought resistance, this study tried to elucidate the role of RWC3 (NCBI accession number: AB029325), one of the rice PIP1 subfamily, in rice drought resistance. The deduced amino acids of RWC3 had 87% homology to RWC1, which has been demonstrated to be a rice water channel protein (Li et al. 2000). The results of GUS staining of *RWC3* promoter-GUS fusion transgenic plants indicated that distribution of RWC3 was wide in rice including both vegetative organs (mainly root, stem and also leaf) and reproductive ones (flower and seed) (Sun et al., unpublished data).

In this study, we observed that upland rice and lowland rice presented different physiological responses to water deficit induced by 20% polyethylene glycol (PEG) 6000. These different physiological responses confirmed that upland rice and lowland rice adopt different mechanisms for drought resistance. We also observed the upland rice and the lowland rice had different *RWC3* expressions pattern during water deficit at both mRNA and protein levels. To better understand the role of RWC3 in drought resistance in rice, we introduced *SWPA2* promotor-*RWC3* into lowland rice and investigated the physiological characteristics of transgenic plants. *SWPA2* is a gene encoding peroxidase in sweet potato, and *SWPA2* promoter contains several *cis*-element sequences implicated in oxidative stress and thus is stress-inducible (Kim et al. 2003). The overexpressed *RWC3* transgenic lowland rice had better water status under water deficit. Thus, it was presumed that RWC3 probably adjusted the water movement across plasma membrane during water deficit for drought avoidance in rice.
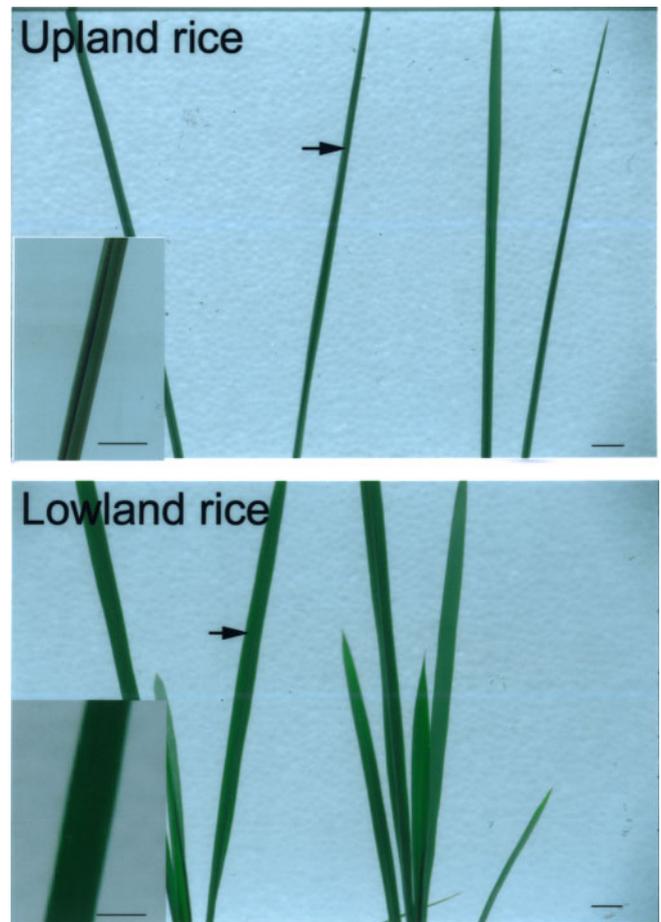
Fig. 1 Different response to water deficit between upland rice and lowland rice. The young leaves of upland rice were rolling, while those of lowland rice still maintained stretch during the 20% PEG 6000 treatment. Insert: the leaf parts indicated by solid arrows. Bars = 1 cm.

Results

Functional analysis of RWC3 in Xenopus oocytes

The water transport activities of RWC3 were assayed in *Xenopus* oocytes. Three d after cRNA or water injection, the osmotic P_f values were calculated from the rate of cell volume change upon osmotic gradient. The P_f of RWC3-expressing, AQP2-expressing and water-injected oocytes was $0.67\pm0.14\times10^{-2}$ cm s^{-1}, $2.77\pm0.43\times10^{-2}$ cm s^{-1}, and $0.28\pm0.07\times10^{-2}$ cm s^{-1}, respectively. Oocytes expressing RWC3 yielded P_f approximately 2- to 3-fold higher than water-injected oocytes, suggesting that RWC3 was a water channel protein that facilitated water flux across membranes.

Different physiological responses to water deficit between upland rice and lowland rice

Young leaf rolling was observed in the upland rice 15–30 min after the PEG treatment, but was not observed in the leaf of lowland rice (Fig. 1). Three other pairs of upland rice
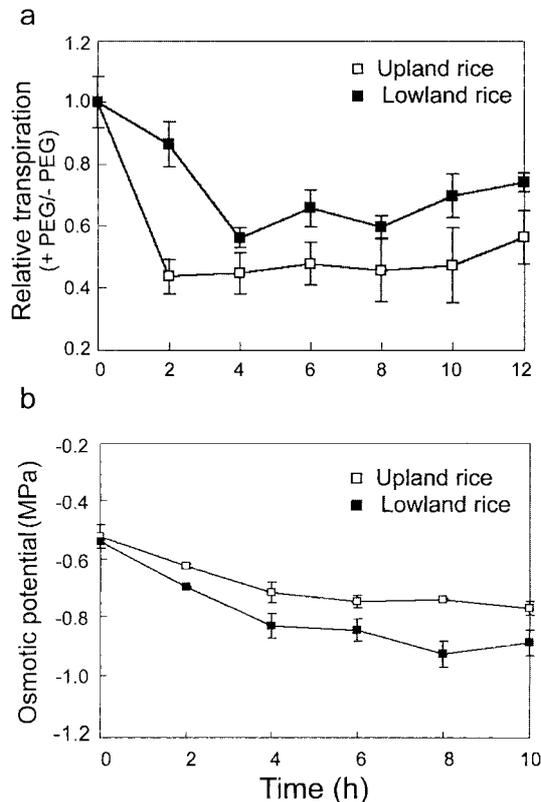
Fig. 2 The effect of water deficit induced by 20% PEG 6000 on the relative cumulative transpiration rate (a) and osmotic potential (b) of upland rice and lowland rice. Each point is the mean of measurements ($n = 5$), and the bars represent the standard error of the mean.

and lowland rice cultivars showed a similar tendency. In view of the great difference in transpiration between individual leaves, measurement of the whole plant would give more significant information on how the plant responds to water stress. The cumulative transpiration of lowland rice decreased less than that of upland rice during the water deficit (Fig. 2a).

During water deficit, the osmotic potential of root reduced from –0.54 MPa to –0.92 MPa and from –0.52 MPa to –0.77 MPa in the lowland rice and in the upland rice, respectively (Fig. 2b). After 4 h of PEG treatment, the osmotic potential of roots decreased to –0.83 MPa and this value was lower than the osmotic potential of PEG solution (–0.82 MPa) in the lowland rice; by contrast, this did not occur in the upland rice even after 10 h of the treatment (Fig. 2b). From these results, we predicted that the lowland rice might mobilize the remarkable osmotic adjustment to maintain the root cell turgor compared to the upland rice, although the root cell turgor and the osmotic potential of living cells were not determined in this study. According to the composite transport model (Steudle and Peterson 1998, Steudle 2000), the plant root was not a perfect osmometer, and unlike individual cells, roots had a low reflection coefficient. The transpiration pull resulted in remarkably

high tensions in the xylem and provided a pivotal driving force to pull water into plants. In addition, the PEG concentration in the unstirred layer surrounding the surface of roots was probably higher than that of the block PEG solution. Hence, roots could still take up water driven by hydrostatic pressure through apoplastic bypasses even when the osmotic potential of the roots was lower than that of the PEG solution.

The different expression pattern of RWC3 in upland rice and lowland rice during water deficit

Using reverse transcription PCR, the mRNA accumulation of *RWC3* was investigated during water deficit at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h. *RWC3* mRNA abundance increased and reached the highest level both in leaves and roots of upland rice treated with PEG for 4 h, whereas in lowland rice, *RWC3* mRNA level remained unchanged in leaves and reduced after slightly increasing in roots during the 10 h PEG treatment (Fig. 3a). As the polyclone antibody could specifically react with RWC3 protein (31 kDa), we immunochemically quantified the amounts of RWC3 protein during water deficit. As shown in Fig. 3b, the amount of RWC3 protein was much higher in roots than leaves of the non-stress plants. Both in leaves and roots, the amount of RWC3 protein increased in upland rice, while it decreased significantly in lowland rice 10 h after 20% PEG treatment.

### Molecular identification of transgenic plants

RWC3 was introduced into lowland rice under the control of the stress-induced promoter *SWPA2* (Fig. 4a) mediated by *Agrobacterium* and some physiological parameters of the resulting transgenic plants could be detected. PCR strategy was used to identify the transgenic plant. In order to avoid the detection interference resulting from endogenous *RWC3*, we designed the forward primer and the reverse primer based on the sequence of the *SWPA2* promoter and *RWC3*, respectively (Fig. 4a). In all, we obtained 14 transgenic lines by PCR identification. As representative examples, Fig. 4b shows that 730 bp PCR productions were amplified specifically from genomic DNA in four lines of the transgenic plant.

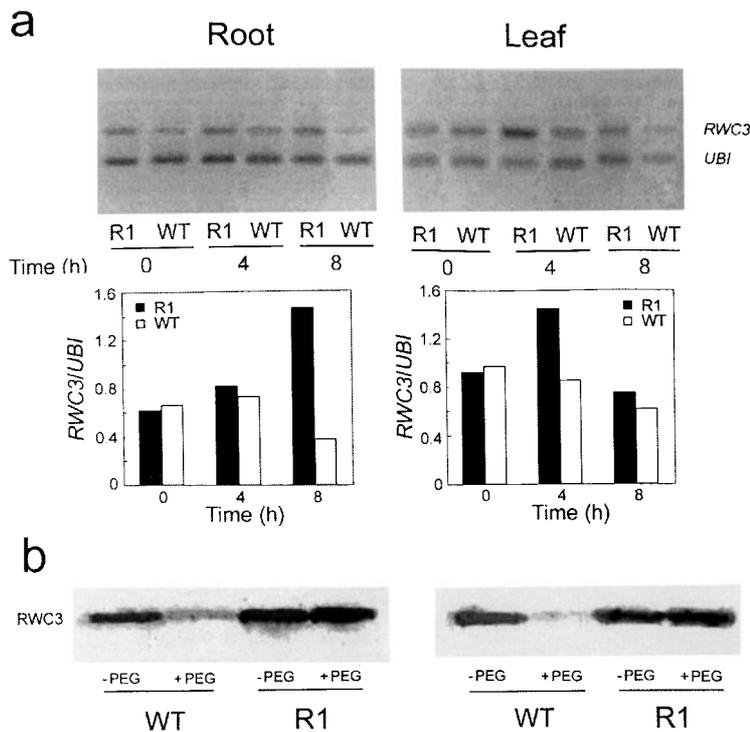### SWPA2 promotor-RWC3 expression induced by PEG treatment in transgenic plants

To verify the stress-inducible *SWPA2* promoter driving the expression of *RWC3* gene in transgenic plants under drought stress, we traced the expression pattern of RWC3 at both mRNA and protein levels after the plants were subjected to the water deficit mediated by PEG 6000 for 8 h or 10 h. The transgenic plants had approximately the equivalent amount of RWC3 mRNA or protein under non-stress conditions, while they had a clearly increased RWC3 amount compared with the wild-type plants under the water deficit (Fig. 5). In other words, the up-regulation of RWC3 in these transgenic plants was dependent on water deficit condition.
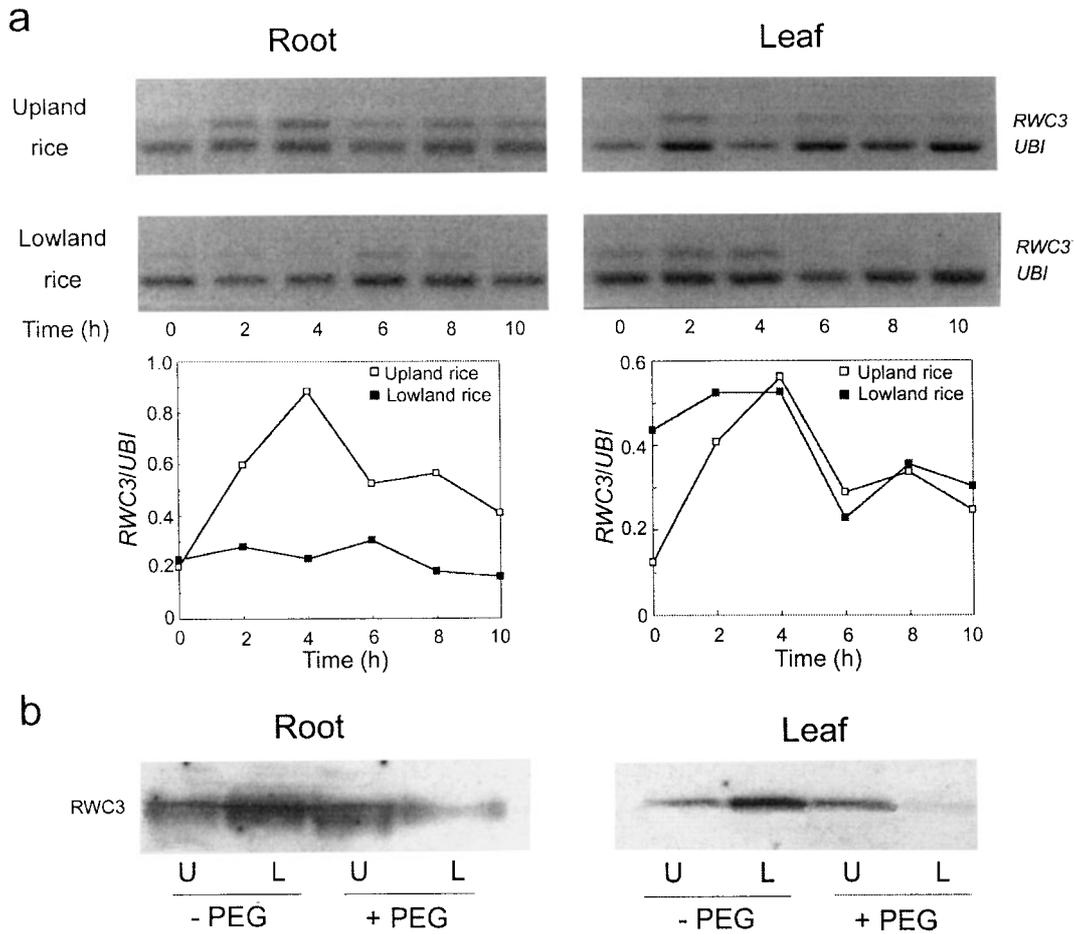
Fig. 3 RWC3 expression pattern in upland rice and lowland rice under water deficit induced by 20% PEG 6000. RT-PCR analysis of RWC3 mRNA expression (a) in leaf and root of upland rice and lowland rice during water deficit. RNA was extracted from leaves or roots collected in upland rice and lowland rice 0 h, 2 h, 4 h, 6 h, 8 h or10 h after water deficit treatment. Agarose gel separation of PCR production was amplified using specific primers P3 and P4. RT-PCR production of ubiquitin (*UBI*) is shown as standard primers. RWC3 immunoblot analysis of plasma membranes extracts (b) for roots and leaves of upland rice (U) and lowland rice (L) under the water deficit treatment (+ PEG) for 10 h or non-stress (– PEG). Plasma membrane protein (10 μg) was loaded in each lane.
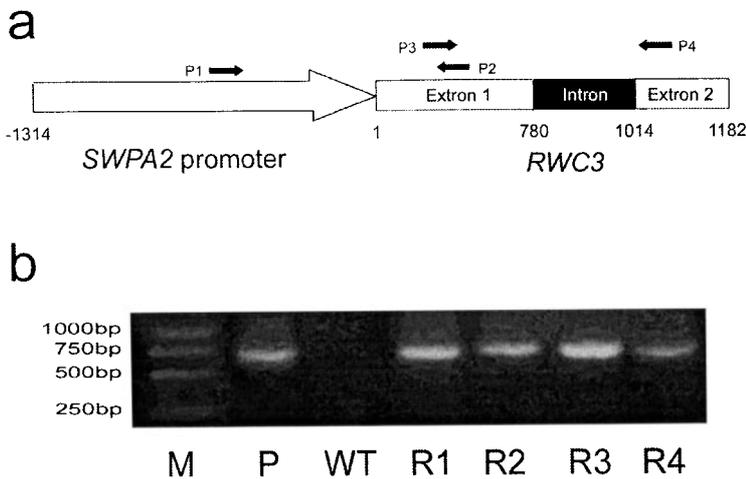
Fig. 4 Introduction of *SWPA2* promoter-*RWC3* into lowland rice. (a) The construction of the SWPA2::RWC3. The size of the SWPA2::RWC3 is not presented to scale. Solid horizontal arrows indicate the positions of primer sequences for PCR (P1 and P2) or RT-PCR (P3 and P4) analyses. (b) PCR analysis of the genomic DNA in transgenic plants using P1 and P2 as primers. Agarose gel separation of PCR products amplified from the genomic DNA of the wild type (WT), four lines of transgenic plants (R1 to R4) and plasmid PCAMBIA 1301-*SWPA2*::*RWC3* (P).

Fig. 5 RWC3 expression in *SWPA2* promoter-*RWC3* transgenic lowland rice under water deficit induced by 20% PEG-6000. RT-PCR analysis of the RWC3 mRNA expression (a). PCR analysis was performed using P3 and P4 as primers. Agarose gel separation of PCR products amplified from RNA that was extracted from roots and leaves in wild type (WT) and transgenic plants (R1) during the 20% PEG treatment for 4 h or 8 h. RWC3 immunoblot analysis of plasma membranes extracts under 20% PEG treatments for 10 h (b). Plasma membrane protein (10 μg) was loaded in each lane.

No phenotype differences between wild-type and transgenic lowland rice

However, no observable phenotypic difference was found between the transgenic plants and the wild-type plants, either under non-stress or during water deficit.

*Overexpression of RWC3 enhanced the root osmotic Lp and improved water status during water deficit*

Osmotic Lp is an index of cell-to-cell water flow driven by the osmotic force, and is strongly dependent on the density or activity of water channels in the plasma membranes of root cells (Tyerman et al. 1999, Kjellbom et al. 1999). Root osmotic Lp rather than hydrostatic Lp were measured to check whether RWC3 was involved in the water uptake under drought. After being subjected to water deficit for 10 h to drive *RWC3* expression by *SWPA2* promoter, the plants were transferred to normal nutrient solution, and then decapitated for root Lp measurement. No remarkable difference was found between the Lp of transgenic plants (4.14×10^{-8} m s^{-1} MPa^{-1}) and that of wild-type plants (4.49×10^{-8} m s^{-1} MPa^{-1}) under non-stress conditions. After being subjected to water deficit, transgenic plants showed higher Lp (6.70×10^{-8} m s^{-1} MPa^{-1}) than that (5.11×10^{-8} m s^{-1} MPa^{-1}) of wild-type plants (Fig. 6b). These results indicated that the transgenic lowland rice had the increased capacity for root water uptake under water deficit.

Transgenic lowland rice had higher leaf water potential than wild-type plants at 10 h after initiation of the PEG treatment, although there was no difference between those of transgenic plants and wild-type plants before the treatment (Fig. 6a).

Maintenance of transpiration was important in plants, especially during water stress, because it facilitated the dissipation of excess heat. At 10 h after the initiation of the PEG treatment, we observed that the relative cumulative transpiration rate of wild-type plants was 42% of that before the treatment, while the relative cumulative transpiration of the transgenic plants was 55% of that before the treatment. These data indicated the transgenic plants showed the improved water status during water deficit.

Discussion

Using the *Xenopus* oocyte expression system, the data demonstrated that RWC3 could facilitate water movement across membrane. By the morphological and physiological comparisons between upland rice and lowland rice under water deficit conditions, we confirmed that upland rice showed drought avoidance. Meanwhile, at both mRNA and protein levels, *RWC3* expression increased in upland rice, while it decreased in lowland rice under water deficit. Water deficit induced *RWC3* expression in the *SWPA2* promoter-*RWC3* transgenic lowland rice, and the increased root osmotic Lp (Fig. 6b) indicated a higher capacity of water uptake in the transgenic plants. The fact that the *SWPA2* promoter-*RWC3* transgenic lowland rice had an improved water status (e.g. higher water potential) (Fig. 6a) under water deficit gives us an insight into aquaporin roles in plant drought avoidance. We hypothesize that plasma membrane aquaporin RWC3 endowed,
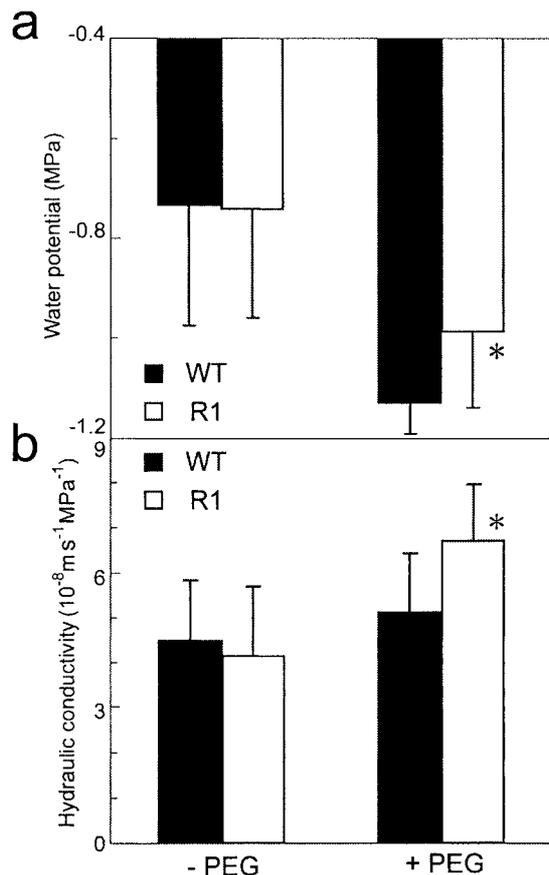
Fig. 6 *SWPA2* promoter-*RWC3* transgenic lowland rice showed higher water potential (a) and root hydraulic conductivity (b) under water deficit induced by 20% PEG 6000 for 10 h. Data are means ± SE for *n* = 20. Asterisk indicates values significantly different in the same group according to *t*-test (P ≥0.05).

at least partially, the cell with water uptake to maintain cell turgor when cells are subjected to water deficit.

RWC3 exhibited an osmotic water permeability of $0.67\pm0.14\times10^{-2}$ cm s^{-1} in oocytes compared with the water-injection oocytes ($0.28\pm0.07\times10^{-2}$ cm s^{-1}). The P_f value of RWC3 was relatively low compared with that of AQP2 from human, and this value was consistent with those of the plant PIP1 group (Kaldenhoff et al. 1998, Li et al. 2000, Yamada et al. 1997, Dixit et al. 2001). The P_f value ($2.77\pm0.43\times10^{-2}$ cm s^{-1}) of AQP2-expressing oocytes was about 10 times of that of water-injected oocytes ($0.28\pm0.07\times10^{-2}$ cm s^{-1}), and these data also accorded with those of Yang and Verkman (1997).

Before the PEG treatment, protein level of root RWC3 was only slightly higher in the transgenic lowland rice than that of wild type, whereas after the PEG treatment, RWC3 protein expression in transgenic plant was very much higher than that of wild type (Fig 5b). On the other hand, the root hydraulic conductivity of transgenic plants was similar to that of wild-type plants before water deficit while higher than that of wild-type plants after water deficit (Fig. 6b). These results were

understandable, when considering that the aquaporin super-family has a large number of members. The water permeability ($P_f = 0.67\pm0.07\times10^{-2}$ cm s^{-1}) of RWC3, a member of PIP1, showed a quite lower value than that of PIP2, for instance, spinach PM28A ($P_f = 1.1\times10^{-2}$ cm s^{-1}) (Johansson et al. 1998). Some reports (Kammerloher et al. 1994, Chaumont et al. 2000, Moshelion et al. 2002) had supported that PIP1 showed less efficient functional expression in *Xenopus* oocytes than PIP2. As one member in the PIP1 subfamily, rice aquaporin RWC3 involvement in root water uptake to a certain extent was established in this study although the contribution of RWC3 to root water uptake was uncertain.

Many morphological and physiological traits for drought resistance in rice were used as selective traits for breeding (Fukai and Cooper 1995), although the exact mechanisms of drought resistance of those traits still remain elusive. International Rice Research Institute (IRRI) was investigating strategies for selection of lines with deep, thick roots, leaf rolling and stomatal closure as a drought avoidance characteristic for upland conditions (Fukai and Cooper 1995, Price et al. 1997, Price et al. 2002). A deeper understanding of plant adaptation mechanisms to drought could be obtained from the comparison between upland rice and lowland rice. Here the fact that aquaporin *RWC3* was up-regulated during water deficit in upland rice provided us with a clue to explore drought resistance from the point of view of water uptake mediated by aquaporins at the cellular level, although it was also believed that there must be many genes involved in plant drought resistance. The cell-to-cell path dominated the water transport from the soil to the xylem under drought stress (Steudle and Peterson 1998, Steudle 2000). At the cellular level, many studies on plant responses to water deficit have been undertaken from the osmotic adjustment (solute) viewpoint (Bray 1997), while the water flux across membrane has drawn less attention. People took it for granted that the water physically passes through the membrane bilayers and that this passage was hardly regulated biologically until the plant aquaporins were characterized. To date, the knowledge that plant aquaporins functioned in the water uptake and transport was broadly accepted. By observing the physiological change in aquaporin *NtAQP1* antisense tobacco plant under drought, it was concluded that NtAQP1 contributed to water stress avoidance in tobacco (Siefritz et al. 2002). Here, the *SWPA2* promoter-*RWC3* transgene manipulation in lowland rice improved the water status under water deficit, suggesting that RWC3 played a role in drought avoidance in rice, probably thanks to a high level of RWC3 enhancing water uptake under water deficit and therefore maintaining the plant water balance.

In this study, upland rice showed sensitive physiological responses (e.g. leaf rolling) to the sudden and serious drought stress (20% PEG treatment for 10 h) after they had grown under abundant water supply for over 1 month. Under non-stress conditions, the upland rice plant had higher cumulative transpiration, higher leaf water potential and higher plasma

membrane total aquaporin expression than lowland rice (data not shown). To some extent, these results were similar to Aharon et al. (2003). Aharon et al. (2003) constitutively over-expressed the *Arabidopsis* plasma membrane aquaporin PIP1b in tobacco, and these transgenic plants had higher vigor and PIP1b expression when water was sufficiently supplied, but no beneficial effect under salt stress, and even negative effect, causing fast wilting during water stress. However, it must kept in mind that stress responses were dependent on the tissue, cell type and developmental stage of the plant (Bray 1997). No visible difference of responses to long-term (10 d) and mild (10% PEG 6000) stress was observed in the young upland rice seedlings (3 d after germination). However, the RWC3 expression pattern of the young upland rice seedlings was similar to those in this study, except that the enhancement of RWC3 expression was delayed to 1 d (data not shown). It was proposed that under both circumstances RWC3 was involved in the response to water deficit in upland rice, although other physiological processes for acclimation in the long-term adverse environment could not be excluded.

Aquaporin *RWC3* was introduced into the lowland rice under the control of the stress-inducible *SWPA2* promoter. As we expected, the RWC3 specifically increased during water deficit. It greatly benefited our detection of the physiological role of RWC3 during the stress with the similar background of inherent *RWC3* expression. Aharon et al. (2003) once constitutively over-expressed the *Arabidopsis* plasma membrane aquaporin PIP1b in tobacco. Nevertheless this PIP1b over-expression had no beneficial effect under salt stress, and even a negative effect, causing fast wilting during water stress. These results suggest that the all-round and constitutive increase in water transport (as expected from the expression of the 35S promoter) was "harmful" under drought stress. While in our experiment, the RWC3 was induced by stress-induced promoter *SWPA2*, and the RWC3 over-expression showed the positive effect during water deficit. These might partly contribute to the distinct effect of aquaporin over-expression on the plant during stress. An alternative explanation to the different effect of aquaporin over-expression might be the different drought treatment. We simulated drought by PEG treatment (a drought shock), while Aharon et al. (2003) exposed the plants to drought by stopping irrigation (a gradual stress).

Compared to high GUS expression in the *SWPA2* promoter-*GUS* transgenic tobacco induced by hydrogen peroxide, wounding and ultraviolet stress (Kim et al. 2003), *SWPA2* promoter only moderately enhanced *RWC3* expression in our experiments (Fig. 5). The optimum stress conditions in terms of *SWPA2* promoter should be explored in rice plants. Furthermore, we could not exclude the possibility that the *SWPA2* promoter showed moderate induction capability in the monocotyledon because *SWPA2* promoter was isolated from a dicotyledon sweet potato.

Materials and Methods

Plant materials and growth conditions

Physiological experiments were performed using lowland rice (*Oryza sativa* L. spp *japonica* cv. Xiushui 63) and upland rice (*O. sativa* L. spp *indica* cv. Zhonghan 3), and gene transformation experiments were performed using lowland rice (*O. sativa* L. spp *japonica* cv. Zhonghua 11). After germinating in the dark for 24 h at 28°C, all seedlings were grown in nutrient solution (Ni 1985) at photon flux density of 350–400 µmol m^{-2} s^{-1}, 60–80% relative humidity, 12 h/12 h day–night cycle at 28°C in a phytotron. Four-week-old plants were used for water deficit treatment, the nutrient solution contained 20% PEG 6000 giving about –0.82 MPa of stress determined by a Vapor Pressure Osmometer (Wescor, U.S.A.).

Microinjection of cRNA into oocytes and permeability analyses

Mature oocytes (stage V to VI) were isolated from adult *Xenopus laevis* and stored in Barth's buffer containing Na-penicillin (10 µg ml^{-1}) and streptomycin (10 µg ml^{-1}). The large and full-grown defolliculated oocytes were selected for injection with 50 nl of complementary RNA (1 µg µl^{-1}) or water using an automatic injector (Nanoject, Drummond, U.S.A.). The oocytes were incubated for 48–72 h at 18°C in fresh Barth's buffer before water permeability measurement.

Individual oocytes were transferred from Barth's buffer (200 mosmol) to threefold dilution of Barth's buffer with distilled water (70 mosmol) at 20°C. Oocytes swelling was determined with a stereomicroscope (Shimadzu, Japan). The successive pictures at 5-s intervals were automatically captured by video camera. The osmotic permeability coefficient (P_f) was calculated according to Yang and Verkman (1997) using the following formula:

$$P_f = V_0[d(V / V_0) / dt] / [S_0 \times V_w (Osm_{in} - Osm_{out})]$$

where Osm_{in} was 200 mosmol and Osm_{out} was 70 mosmol. V_0 was the initial oocyte volume. S_o was the initial oocyte surface area, and V_w was molar volume of water (18 cm^3 mol^{-1}).

Relative cumulative transpiration

The weight of tubes in which rice seedlings grew was measured and the moisture lost was replenished every 2 or 4 h. Moisture lost was taken as the amount of water transpired. The ratio of the water loss of seedlings in nutrient solution containing 20% PEG 6000 to that of seedlings in non-stress solution was calculated as the relative cumulative transpiration.

Water potential and osmotic potential

Leaf water potential was measured by a pressure chamber (Soilmoisture Equipment Corp. U.S.A.). Briefly, a mature leaf was sampled, and the leaf was quickly placed in the chamber with the cut end protruding through a rubber stopper sealing the chamber. At the bottom of the chamber, a little water was applied to moisten the chamber air. The atmospheric pressure in the chamber was then increased at the rate of 0.025–0.035 MPa s^{-1} by means of an external source of the compressed air until sap just appeared at the cut ends of the xylem elements. At that instant the hydrostatic pressure inside the xylem elements was exactly balanced by the applied external pressure, the chamber pressure was recorded.

Adventitious roots were harvested and put in a syringe and frozen in a freezer overnight. The root juice was squeezed and then the osmotic potential was measured with the Freezing-Point Osmometer (Instrumental Factory of Shanghai Medical University, China).

Reverse transcription PCR (RT-PCR)

For the *RWC3* mRNA analysis, cDNA was synthesized using AMV reverse transcriptase (TaKaRa, Japan) with oligo (dT)15 as a primer. The reactions were incubated at 25°C for 10 min, 42°C for 1 h and 4°C for 5 min. The cDNA was amplified by 20–24 cycles at 94°C for 40 s, 60°C for 50 s, and 72°C for 45 s. The primers were as follows (Fig. 4a): P3, 5′-acaagtcggcgtccaagtgc-3′; P4, 5′-gatgaacggaccaaccca-gaa-3′. The fragment of ubiquitin (*UBI*) (Nishi et al. 1993, NCBI accession number: D12776) was used as internal standard. The design of forward and reverse primers for *UBI* PCR amplification was based on the sequences 5′-gacggacgcaccctggctgactac-3′ and 5′-tgctgccaattac-catataccacgac-3′, respectively. According to these performances, the expected specific products are 396 bp for *UBI* and 563 bp for *RWC3*. The optical density ratio of *RWC3* and *UBI* amplification products was used for the relative quantitative value of *RWC3* mRNA expression. The software of LABWORKS digitized the results.

Preparation of plasma membranes

Plasma membrane fraction was prepared by the aqueous two-phase partitioning as described by Ohshima et al. (2001). Protein concentration was measured according to Bradford (1976).

Antibody preparation and immunoblot analysis

One peptide was synthesized corresponding to the N-terminal parts of RWC3. To obtain an antibody that reacted with RWC3, a specific sequence in RWC3 in the N-terminus, KDYREPRRRRCS-TAEEL, was used as an antigen. The synthesized oligopeptide was conjugated with keyhole limpet hemocyanin, and then injected into rabbits. The resulting antiserum was used as antibody against RWC3. Western blot was carried out essentially by the standard method according to Sambrook et al. (1989). Using standard procedures for SDS-PAGE in 12% polyacrylamide gels, proteins were electrophoretically transferred to nitrocellulose, bound antibodies were detected with goat anti rabbit IgG-HRP (1 : 1,000), which were used for chemiluminescence detection (West Pico West Blotting, Pierce, U.S.A.). The primary antibody was diluted to 1 : 500 for immunoblotting.

Plasmid construction and plant transformation

The *RWC3* genomic DNA (1.0 kb) was cloned into the binary vector pCAMBIA 1301 3′ to *SWPA2* promotor and 5′ to a nopaline synthetase terminus. The *SWPA2* gene had been found to encode an anionic POD which was highly expressed in response to oxidative stress in sweet potato, and the *SWPA2* promoter contained several *cis*-element sequences implicated in oxidative stress such as GCN-4, AP-1, HSTF, SP-1 reported in animal cells and a plant specific G-box. –1,314 bp *SWPA2* promoter had the strong induction ability in response to environmental stresses (Kim et al. 2003), and thus here we used –1,314 bp *SWPA2* promoter. *Agrobacterium tumefaciens* strain EHA105 was transformed this resulting plant expression vector. Transformation and regeneration of transgenic rice was performed according to the protocol provided by Liu et al. (1998).

PCR

PCR strategy was used to identify the transgenic plant. To avoid the interference of endogenous RWC3, the forward primer was based on the sequence of promoter *SWPA2*. The primers were as follows (Fig. 4a): P1, 5′-ttgccaaatcctatcatacac-3′; P2, 5′-acgccgacttgttcaccc-3′. The following PCR program was used: 94°C for 3 min; 30 cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 45 s, and 72°C for 10 min.

Root hydraulic conductivity

The measurement of root hydraulic conductivity was essentially based on the Miyamoto et al. (2001). In brief, after being subjected to

deficit treatment for 10 h, plants were decapitated, and the root systems were transferred to normal nutrient solution. Exuded sap was collected into a micropipette, and the flow rate (J_v) was measured over the next 120 min. The osmotic pressure of the bath medium (Π_b) and sap (Π_s) exuding from individual plants were measured by a Freezing-Point Osmometer (Instrumental Factory of Shanghai Medical University, China). The osmotic root hydraulic conductivity (Lp) was calculated from the following equation:

In this equation, σ was the root reflection coefficient for nutrient salts in the xylem and was estimated to be σ = 0.4 (Miyamoto et al. 2001). A_r was the overall root surface area, which was measured from projected areas of roots that were assumed to be cylindrical in shape. For precise measurement, root systems were scanned, and then the pixels of root projection were digitalized with software Adobe Photoshop 6.0. With the calibration of a metal wire of known length and diameter, the surface areas of roots were calculated.

Acknowledgment

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