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Overexpression of the potassium channel TPKb in small vacuoles confers osmotic and drought tolerance to rice

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Summary

- Potassium (K^+) is the most important cationic nutrient for all living organisms. Vacuolar two-pore K^+ (TPK) channels are important players in the regulation of cellular levels of K^+ but have not been characterised in rice.
- In order to assess the role of OsTPKb, a K^+ selective ion channel predominantly expressed in the tonoplast of small vacuoles, we generated overexpressing (OX) lines using a constitutive promoter and compared their phenotypes with control plants.
- Relative to control plants, OX lines showed better growth when exposed to low-K^+ or water stress conditions. K^+ uptake was greater in OX lines which may be driven by increased AKT1 and HAK1 activity. The enhanced K^+ uptake led to tissue K^+ levels that were raised in roots and shoots. Furthermore, energy dispersive X-ray (EDX) analyses showed a higher cytoplasm: vacuole K^+ ratio which is likely to contribute to the increased stress tolerance.
- In all, the data suggest that TPKb can alter the K^+ status of small vacuoles, which is important for general cellular K^+ homeostasis which, in turn, affects stress tolerance.

Introduction

OsTPKb is a K^+ selective vacuolar channel (Isayenkov *et al.*, 2011). Its activity is controlled by cytoplasmic factors such as Ca^{2+} and 14-3-3 proteins (Latz *et al.*, 2007; Isayenkov *et al.*, 2011) but also by mechano-stimuli (Maathuis, 2011). TPKb can catalyse both inward (into the cytoplasm) and outward current but, due to prevalent electrochemical gradients, is likely to mediate vacuolar K^+ efflux in most cells. In rice protoplasts, transient expression of TPKb is found predominantly in the tonoplast of small vacuoles (SVs; Isayenkov *et al.*, 2011) and relatively little expression is observed on the lytic vacuole (LV) tonoplast (Fig. 1).

Small vacuoles can be present in the same cell as the large lytic vacuole (LV). They have been shown to occur in a number of species (Okita & Rogers, 1996; Müntz, 1998; Herman & Larkins, 1999) and have been detected in leaves, seed pods, stems, cotyledons, bark and storage tubers. Plant species vary greatly with respect to the number of SVs found in vegetative cells: in Arabidopsis mesophyll there are often only one or two, whereas in tobacco and rice there are often > 4 (Di Sansebastiano *et al.*, 1998; Park *et al.*, 2004; Sohn *et al.*, 2007; Isayenkov *et al.*, 2011).

Lytic vacuoles have an acidic pH and are responsible for well-characterised functions such as turgor provision, protein degradation, storage of metabolites, minerals and xenobiotics and cell signalling (Marty, 1999). By contrast, SVs typically have a neutral pH (Swanson & Jones, 1996; Isayenkov *et al.*, 2011) and their physiological relevance is less clear. It has been suggested that SVs are a storage compartment for proteins such as lectins, sporamin and patatins that have been found in the leaves and bark of tree legumes (Staswick, 1994) and as such provide a nitrogen reserve in perennials. Alternatively, SVs may serve as deposits for defence proteins such as chitinases (Di Sansebastiano *et al.*, 2001). A further hypothesis states that SVs are semi-permanent structures that depend on vacuolar dynamics. For example, the occurrence of vacuolar 'bulbs' which resemble SVs are formed in osmotically stressed suspension culture cells suggesting that these structures play a role drought tolerance (Reisen *et al.*, 2005).

As yet, there is little hard evidence to support any of the above ideas. Because OsTPKb is found in all rice tissues (Genevestigator, https://genevestigator.com/gv/) and its predominant expression is in SVs, it provides a convenient marker for rice SVs (Isayenkov *et al.*, 2011). We used a transgenic approach to create a rice TPKb gain of function mutant and characterised this line to reveal more about the potential function of TPKb and consequently putative roles of rice LVs. Our data show improved growth of TPKb overexpressing rice during K^+ starvation and water stress, and suggest that SVs may function as 'cellular K^+ stores' which become important in water-deficient conditions.

Materials and Methods

Plant materials and growth

Rice (*Oryza sativa* L.) sub group *Japonica* cv. Nipponbare seeds were geminated on terra green and kept for 5 d in the dark at 28°C and 90% relative humidity. The germinated seedlings were

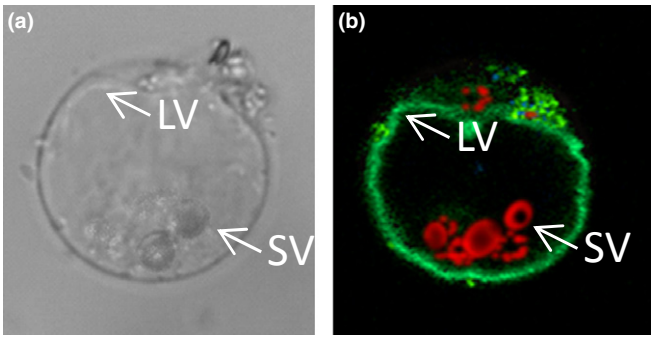

Fig. 1 Cellular expression of OsTPKb. Root rice protoplast cotransformed with OsTPKa:YFP and OsTPKb:RFP. TPKa expression in the large central vacuole (LV) and that of TPKb in multiple small vacuoles (SVs). (a) Bright field image and (b) superimposed fluorescence images from confocal microscope.

transferred to plastic boxes containing 2 l growth medium each. The standard growth medium consists of macronutrients (2.9 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1 mM $CaCl_2$, 1.6 mM $MgSO_4{\cdot}7H_2O$) and micronutrients (Yoshida *et al.*, 1976) and Na_2SiO_3 (0.18 g l^{-1}). K_2SO_4 in the standard medium was replaced with an equimolar quantity of Na_2SO_4 for the '0-K' condition. The standard medium was supplemented with additional KCl and NaCl to increase the concentration of Na^+ and K^+ up to 60 mM in the medium for the salt stress treatments, whereas for osmotic stress treatments it was supplemented with 5%, 10% or 15% PEG (Polyethylene glycol-4000). Solutions were prepared with deionised water and pH was adjusted to 5.6–5.7.

Glasshouse conditions were: 16 h : 8 h, light : dark; 28 : 24°C, day : night; 60% relative humidity with light radiation of *c.* 160 W m^{-2}. The growth medium was changed every 3 d. Seedlings were grown in standard medium for 3 wk before treatments which lasted for 2 wk unless otherwise indicated.

For 'low-K' growth assays in soil, 4-wk-old plants were weighed and transferred to pots containing silica sand (WBB Minerals Ltd, Lancaster, UK) in pots. Nontreated plants were fertilised with standard nutrient solution (2 mM K^+). Low-K^+ conditions were created by fertilisation with growth medium without K^+. After 4 wk exposure, fresh weights were recorded and relative growth rates (RGRs) were calculated.

Drought stress was measured by transferring plants to pots containing soil (John Innes No. 2 Compost + perlite 2–5 mm). Non treated plants were watered twice per week to 100% field capacity, whereas drought stress was applied by watering plants to *c.* 50% field capacity. After 6 wk exposure, plant FW was recorded and relative growth rate (RGR) determined. In all cases, RGR was calculated according to Poorter & Garnier (1996).

Rice transformation

The full-length OsTPKb open reading frame (ORF) was amplified with Phusion Hot Start DNA polymerase (New England Biolabs, Hitchin, UK) using TPKb cDNA (accession AK109604) as template and primers corresponding to the 5′ and 3′ ends of OsTPKb with added *Hin*dIII and *Eco*RI restriction sites (5′-GCGCAAGCTTATGGCGGCCCTCGACCAACA-3′ and 5′-GCGCGAATTCCTAACGCAGGGAAGGCGGCG-3′ respectively). The OsTPKb ORF was then ligated into the corresponding sites of the pART27 plasmid downstream of the CaMv-35S promoter. The promoter-TPKb cassette was restricted with *Eco*RV and inserted into the binary vector pGreen (Vain *et al.*, 2004). Subsequently, pGreen and pSoup were introduced into the Agrobacterium strain AGL1 to transform rice calli as described by Vain *et al.* (2004).

PCR analyses for validation of transgenic rice

Putative TPKb transgenic lines were tested by PCR using different sets of primers. To test the presence of the transgene, three sets of primers were used: hygromycin specific forward (5′-GGATATGTCCTGCGGGTAAA-3′) and reverse (5′-ATTTG TGTACGCCCGACAG-3′) primers, 35S promoter forward (5′-AAACCTCCTCGGATTCCATT-3′) and terminator reverse (5′-GCTCAACACATGAGCGAAAC-3′) primers and 35S promoter forward and TPKb gene specific reverse (5′-CTCCAGG TCCATGTTGGTG-3′) primers.

Analysis of the expression level of TPKb by RT-qPCR in transgenic rice

Hygromycin resistant primary transformants were selfed and homozygous lines of TPKb were identified in the T3 generation. The total RNA from leaf tissues of three plants of each genotype were extracted and cDNA was formed. Equal amounts of cDNA from the wild-type (WT) and transgenic lines were used as a template for the qRT-PCR to analyse TPKb transcript level using TPKb forward 5′-GCTGCACTCGCACACGAT-3′ and reverse 5′-CCCCGCCGTGTAGAGCTT-3′ primers. The quantitative analyses were carried out in triplicate using the SYBR Green master mix in an ABI 7300 sequence detection system. Rice histone3 (Os06g0130900) and actin1 (Os05g0438800) were used to normalise the data.

The progenies of the self-crossed heterozygous transgenic plants which lacked the transgene in the T3 generation were identified and used as control lines. These are denoted as 'control' or WT plants.

K^+ content analyses in root and shoot tissues

K^+ contents of roots and shoots were measured using flame photometry. Plants were separated into roots and shoots, and roots were washed with 20 mM $LaCl_3$ solution for 10 min. Samples were dried at 80°C for 3 d and extracted for 24 h with 10 ml of 20 mM $LaCl_3$. K^+ levels were determined with a flame photometer (Sherwood flame photometer-410, Cambridge, UK).

Net K^+ uptake and efflux assays

In order to determine net K^+ uptake, 4-wk-old plants ($n = 3$) grown in 0-K medium for 2 d were exposed to 200 ml medium

containing 50 μM K^+. Samples were taken regularly to determine changes in medium K^+. Loss of volume was corrected by addition of K^+ free medium. Where necessary, K^+ concentration measurements were corrected for differences in root FW. For K^+ efflux measurements, the same procedure was followed but plants were grown in full K^+ medium before being exposed to 200 ml of 0-K medium. Sample K^+ concentration was determined by flame photometry.

Expression analysis of K^+ transporters in roots

In order to determine expression levels of OsAKT1 (Os01g 45990), HAK1 (Os04g32920) and GORK (Os04g36740), root RNA was collected from control and transgenic plants (TPKb OX1 and TPKb OX2) as described above. RT-PCR was carried out using forward and reverse primers for AKT1 (5′-ACCA CATGGCTTGTTCTTGAC-3′ and 5′-ACGTAGCGAATCC ATAAGCTCC-3′), GORK (5′-TGCAGGAGCAGATCCGA GTA-3′ and 5′-GGCGTGTTTCCCCACCTATC-3′), HAK1 (5′-ACTGCATCCTGTTCCCATCG-3′ and 5′-GTCGTCAC CACACAGATCCC-3′) and signal was normalised using Actin1 (5′-TATCCTCCGGTTGGATCTTG-3′ and 5′-CCATGTTT CTGGAATTGCT-3′) and Histon H3 (5′-CGAGAAGCGA AGAGGAGATG-3′ and 5′-TCAACAAGTTGACCACGTC AC-3′) as constitutive controls.

Energy-dispersive X-ray spectroscopy (EDX) analysis

Three-week-old rice plants were exposed to standard or 0-K medium for 1 wk. Tissue was cut from the 4th leaf of each plant 4 cm from the leaf tip and sections were mounted in slotted cryo pins. Samples were cryo-planed using a Leica UC6 cryo-ultramicrotome. Samples were examined for suitable sites of interest from which spectra were acquired inside and outside the cell vacuole. About 7–10 spectra of each (vacuole and cytoplasm) were collected for all samples from several sites along the planed surfaces. The EDX analysis was performed using an INCA Energy 350 (Oxford Instruments, Abingdon, UK) system.

Water loss and tissue water determination

Four-week-old plants were weighed (W_p) and transferred to boxes containing 200 ml standard medium or 10% PEG medium. The weight of the box plus plants was recorded as initial weight (W_i). The final weight (W_f) was recorded after 5 d (T). Boxes without plants were kept in parallel to measure water loss (C) from evaporation. The total water loss per gram plant tissue was calculated as

$$\frac{(W_f - W_i) - C}{T \times W_p}$$

The relative % water content in the tissues was determined by the difference between FW and DW using the formula (FW – DW)/FW × 100.

Whole-leaf conductance measurements

Intact leaves from 4-wk-old plants were used to measure leaf conductance and rate of photosynthesis by using an Infrared Gas Analyser, Li-Cor 6400 (Li-Cor, Cambridge, UK). For each genotype, three leaves (second, third and fourth) per plant (at six-leaf stage) were used and these were derived from three separate plants ($n = 9$). The experiments were repeated three times.

Root hydraulic conductance

Root conductance (Lp) was determined using a pressure chamber as described by Javot *et al.* (2003) using $Lp = Jv/\Delta P$ and $Jv = A \times t$ (Jv, volume flow; P, pressure; A, root area; t, time). Root area was calculated from weight with a conversion factor of 5.18×10^{-4} m^2 g^{-1} FW based on root length and diameter measurements and assuming an average diameter of 300 μm.

Decapitated rice (30 mm above the root–shoot junction) was placed in the pressure chamber and pressure (P) was increased slowly until exudate appeared. Exudate was collected for 5 min and weighed. Pressure was then increased to 1 or 2 higher values for exudate collection and Lp was calculated from the slope of the regression line of Jv vs ΔP plots.

Statistical treatment

In all cases, data are from at least three independent experiments and the error bars in Figs 2–9 represent the standard errors. * Denotes a significant difference by Student's t-test at a probability level of $P < 0.05$ between the WT and overexpressor lines.

Results

TPKb overexpressing rice is less sensitive to K^+ deficiency

Transformation of Nipponbare with OsTPKb (Os07g01810) rice resulted in 12 independent TPKb overexpression lines, three of which were characterised in detail by comparing them to an azygous control line. The three lines (OX1, OX2 and OX3) showed a 18-, 14- and 9-fold increase in levels of transcript for OX1, OX2 and OX3, respectively. To assess growth performance, transgenic and azygous plants (denoted as WT henceforth) were grown in a number of hydroponic and soil conditions including high and low K^+, Na^+, osmotic and drought stress.

In standard medium, azygous and transgenic plants showed some variability in growth which may be due to nonspecific effects of the transformation (Kurusu *et al.*, 2004). When no K^+ was added to the growth medium ('0-K') *c.* 2 μM K^+ is present (as determined by flame photometry). In these conditions, both genotypes show drastically reduced growth rates but the reduction is less severe in the OX plants. With *c.* 57%, 38% and 35% RGR reduction for control, OX2 and OX3, respectively, this leads to a significantly higher RGR for OX2 and OX3 relative to the control plants. In case of OX1, growth is lower in standard conditions but, as observed for OX2 and OX3, RGR reduction (*c.* 40%) is far smaller than that in control plants. No significant

differences in growth were obtained when plants were grown in the presence of 60 mM NaCl or KCl (Fig. 2a).

Thus, it appears that TPKb overexpression limits low-K^+ nutritional stress. The provision of adequate tissue K^+ is crucial for both biochemical and biophysical processes that occur in plant cells and thus K^+ starvation has a negative impact on plant growth. To assess the extent of change in tissue K^+ after exposure to the 0-K medium, we measured root and shoot [K^+] using flame photometry. Figure 2(b) shows that OX lines accumulated more K^+ in their roots than control plants when grown in standard medium but also after growth in K^+-deficient medium. Leaf K^+ showed similar trends (Fig. 2c) with higher values for all three OX genotypes in 0-K-grown plants.

In order to determine whether the hydroponics-obtained phenotype also pertained to soil-grown plants, we grew control plants and the two highest expressing overexpressors (OX1 and OX2) in standard and low-K^+ soil for 6 wk. Figure 3 shows that in normal soil little difference in plant weight was obtained. When grown in low-K^+ soil, all plants developed considerably less well with yield being 20–30% lower. However, both OX1 and OX2 plants grew significantly better than their control counterparts. As was observed for hydroponically grown plants, K^+ content in roots and shoots was higher in the transgenic plants compared to the control lines (Fig. 3).

The greater accumulation of K^+ in root and shoot tissues of OX lines suggests that roots of OX plants have either a larger K^+ uptake capacity or a reduced K^+ leak. We did not obtain significant differences in K^+ efflux between the genotypes (Supporting Information Fig. S1). By contrast, short term K^+ depletion experiments from a medium containing 0.05 mM K^+ indicates that net K^+ uptake in the OX lines is consistently higher than that of control plants (Fig. 4). Increased K^+ uptake could be fuelled by a more negative membrane potential (Em). Root epidermal cells were therefore impaled with glass electrodes but no statistical difference in Em values was observed between genotypes (Table S1). We also tested if transcriptional regulation of the major K^+ transporters in roots was important. Fig. 5 shows that roots of TPKb-OX lines show 3–4 fold higher transcript levels of AKT1 than the control plants but no significant difference in GORK (the main route for K^+ efflux, Ache *et al.*, 2000) transcript level is apparent. The high affinity K^+ uptake system HAK1 (Chen *et al.*, 2015) shows a modest (1.4–1.6 fold) increase in transcript level. Increased AKT1 activity could contribute to augmented K^+ uptake during drought but not in the 0-K medium where energised K^+ uptake is necessary. In the latter condition, the slight increase in HAK1 transcript level could translate into greater uptake but other mechanisms may be at play such as post-transcriptional regulation.

The increased K^+ uptake capacity of TPKb OX plants grown in 0-K medium is likely to be an important factor in limiting the negative effect of K^+ starvation on RGR. However, the intracellular K^+ distribution is also likely to affect tolerance to low-K^+ conditions. The vacuolar location of TPKb means that it could influence intracellular K^+ distribution and we used energy dispersive X-ray (EDX) to test this notion. EDX only provides a relative element distribution profile and is therefore not suited to determine absolute K^+ concentrations, but it can be applied to reveal K^+ ratios between cytoplasm and the large central vacuole. For control plants grown in standard medium this ratio was *c.* 0.9 whereas it was *c.* 1.1 for TPKb-OX plants (Fig. 6). For control plants only a small increase in ratio was observed but a much larger shift in this ratio was observed for OX1 plants after growth

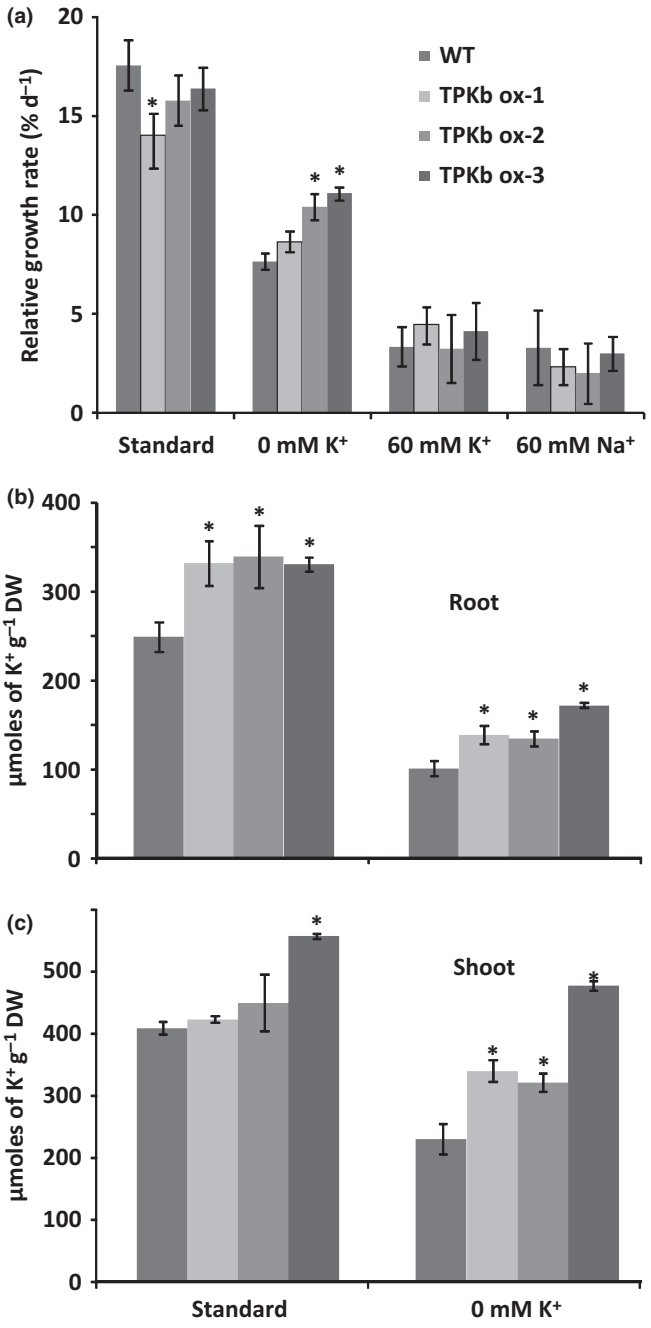

Fig. 2 Relative growth rate and tissue K^+ for hydroponically grown rice. (a) Relative growth rate (RGR) of 5-wk-old rice plants exposed to standard medium, and media containing 0 mM K^+, 60 mM K^+ and 60 mM Na^+. (b) Root K^+ content of plants grown in standard and 0-K^+ conditions. (c) Shoot K^+ content of plants grown in standard and 0-K^+ conditions. Data are from at least three independent experiments and the error bars represent ± SE. Significant difference between the wild-type (WT) and overexpressor lines (Student's *t*-test): *, $P < 0.05$.

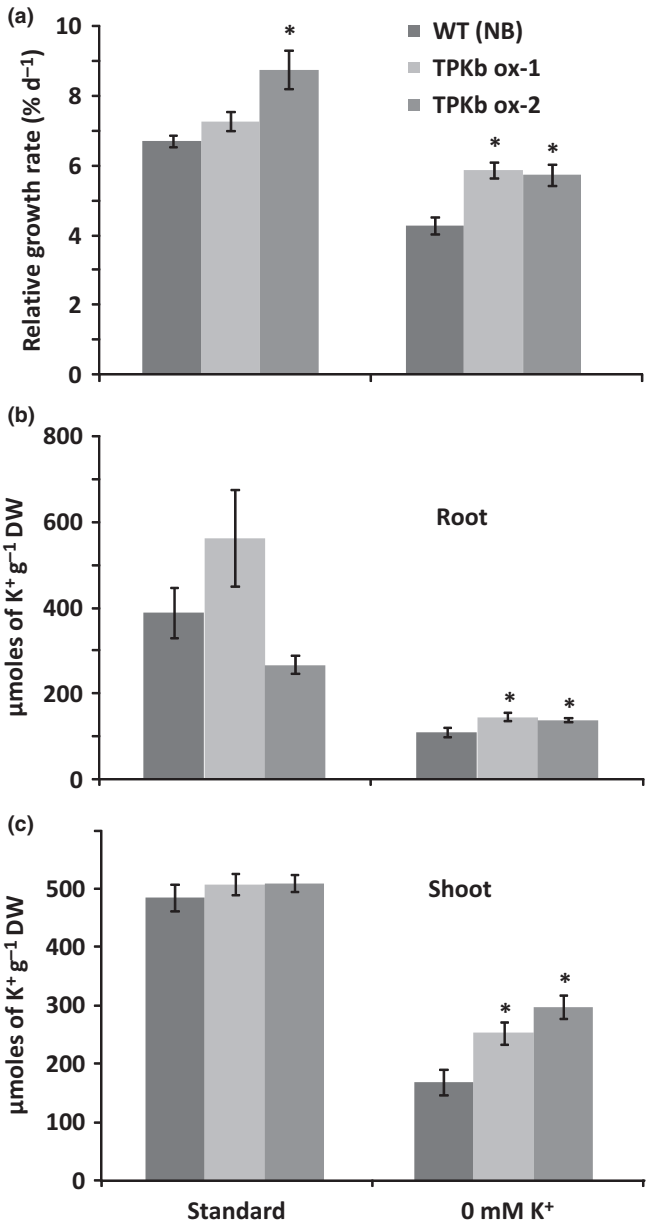

Fig. 3 Relative growth rate (RGR) and tissue K^+ for pot grown plants. (a) RGR of 4-wk-old rice plants that were transferred to pots and exposed for a further 4 wk to standard or low-K^+ conditions. (b) Root K^+ content of plants grown in the conditions mentioned under (a). (c) Shoot K^+ content of plants grown in the conditions mentioned under (a). Data are from at least three independent experiments and the error bars in the figure represent ± SE. Significant difference between the wild-type (WT) and overexpressor lines (Student's *t*-test): *, $P < 0.05$.

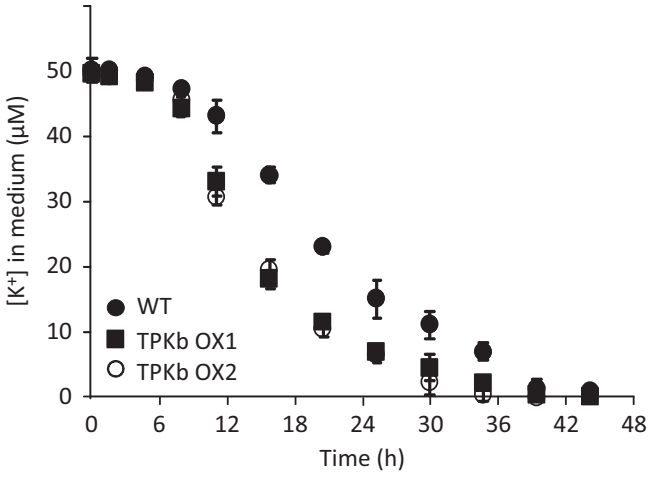

Fig. 4 K^+ depletion assay in rice. Net K^+ uptake was recorded for a period of 48 h in medium containing 50 µM K^+. Samples were collected at the indicated time points and were normalised to root FW. Data are from at least three independent experiments and the error bars represent ± SE.

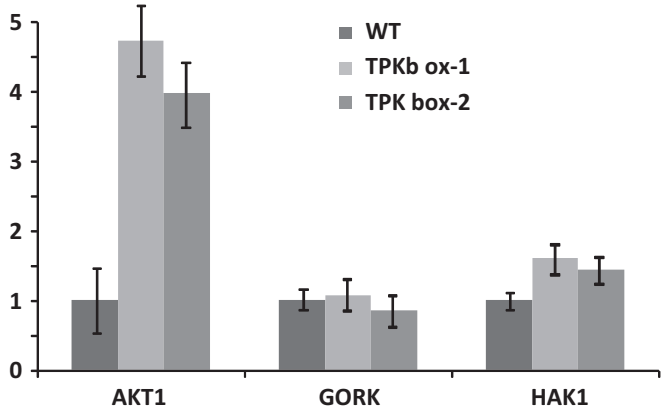

Fig. 5 Reverse transcription polymerase chain reaction (RT-PCR) analysis of rice roots. RT-PCR to determine relative expression levels was carried out on root RNA for the K^+ uptake channel AKT1 and high affinity K^+ uptake carrier HAK1 and the K^+ efflux channel GORK. Transcript levels were normalised to expression of Actin1 and HistonH3. Data are from at least three independent experiments and the error bars represent ± SE.

for 7 d in 0-K medium. These numbers show that (1) K^+ starvation leads to a relative reduction in vacuolar K^+, presumably at the expense of maintaining cytoplasmic K^+ (Walker *et al.*, 1996), and (2) K^+ distribution in the transgenic line favours cytoplasmic K^+ when compared to the control plants. Because the negative effect of K^+ starvation is most likely manifested via biochemical disruption in the cytoplasm, this relatively higher cytoplasmic K^+ level observed in the OX line would help alleviate this stress.

Transgenic rice shows greater tolerance to water stress

After treatments that impose osmotic stress (5%, 10% and 15% PEG) the OX plants showed a growth advantage relative to the control line for the 5% and 10% PEG levels (Fig. 7a). Higher concentrations of PEG (15%) led to severe and comparable growth restriction in all genotypes. One of the first responses to water deficit is the uptake of inorganic salts from the environment, particularly in the form of K^+ (Andersen *et al.*, 1992; Wang *et al.*, 2004). This will help increasing the cellular osmotic potential and therefore aids water retention. When grown in the presence of 5 or 10% PEG, OX lines accumulated more K^+ in both root and shoot tissue compared to their control counterparts (Fig. 7b,c). The pattern for drought-treated plants raised in soil was similar with superior growth for the OX lines and *c.* 10–20% more K^+ in both root and leaf tissue of the OX lines than control line (Fig. 8).

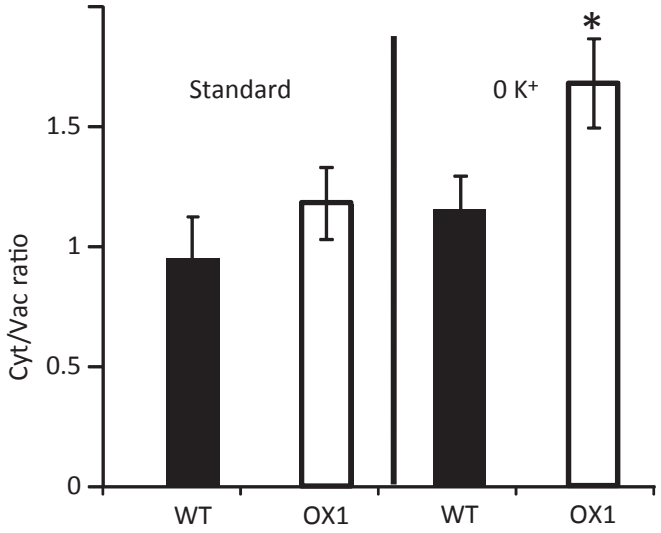

Fig. 6 Intracellular distribution of K^+. Energy dispersive X-ray (EDX) analyses were carried out on rice leaves from control and OX1 plants grown in standard and in 0-K medium. Ratios of the cytoplasmic and vacuolar K^+ are given for plants pre-grown in standard medium or in 0-K medium. Data are from at least three independent experiments and the error bars represent ± SE. Significant difference between the wild-type (WT) and overexpressor lines (Student's *t*-test): *, $P < 0.05$.

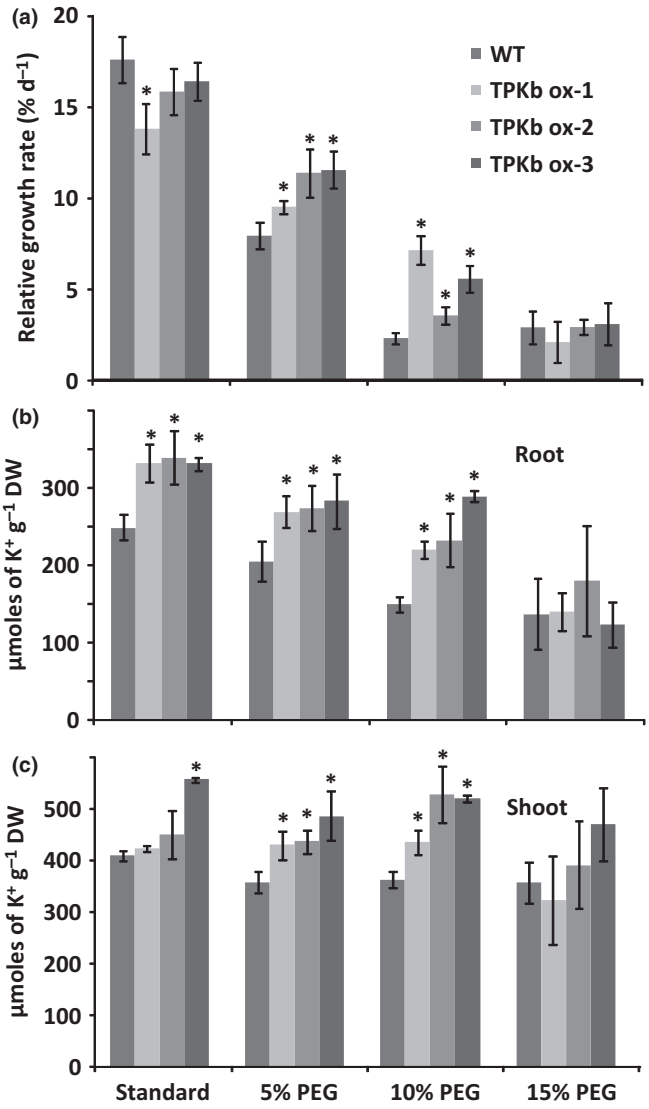

Fig. 7 Relative growth rate and K^+ content of osmotically stressed rice. (a) Relative growth rate (RGR) of 5-wk-old rice plants exposed to standard medium, and media containing 5%, 10% or 15% polyethylene glycol (PEG). (b) Root K^+ content of plants grown in the conditions mentioned under (a). (c) Shoot K^+ content of plants grown in the conditions mentioned under (a). Data are from at least three independent experiments and the error bars represent ± SE. Significant difference between the wild-type (WT) and overexpressor lines (Student's *t*-test): *, $P < 0.05$.

Because the relatively higher level of K^+ in root and shoot tissue of OX lines could contribute to the cell osmotic potential and therefore reduce water loss, we measured evaporation rates. In standard medium the TPKb-OX lines had similar (OX1) or slightly lower (OX2) rates of evaporation compared to control plants (Fig. 9a). In the medium containing 10% PEG, evaporation dropped for all genotypes but much more so for the transgenic rice. Not surprisingly, this resulted in a higher tissue water content for the transgenic plants (Fig. 9b,c). A difference in specific leaf area (SLA) could lead to changes in evaporation rates but SLA values were comparable between WT, OX1 and OX2 plants, amounting to *c.* 130 $cm^2\ g^{-1}$. To assess whether the differences in water loss correlated with stomatal conductance, an infrared gas analyser was used to record steady-state leaf conductance. Figure 9(d) shows that in standard medium TPKb-OX plants had significantly lower leaf conductance than control plants. However, after exposure to 10% PEG, the transgenic plants actually showed a greater leaf conductance than control plants. Thus, in water stress conditions, evaporation is relatively low in the OX lines, in spite of a relatively high leaf conductance.

The latter finding suggests that root conductance may vary between control and TPKb-OX lines. We therefore determined root hydraulic conductance (Lp) using a pressure chamber. Figure 8(e) shows that for all genotypes there is a large drop in conductance (4–5-fold) after osmotic stress (10% PEG). However, in both standard medium and 10% PEG grown plants, the OX lines exhibited significantly lower root conductance than the control line.

Discussion

We showed previously that the vacuolar channel OsTPKb is mostly expressed in small vacuoles (SVs) (Isayenkov *et al.*, 2011), whereas the very similar protein TPKa (63% identity) is primarily detected in lytic vacuoles (LVs). Because little is known about the physiological role of SVs it is difficult to speculate on the putative function of TPKb. We pursued a gain of function strategy by overexpressing TPKb under control of a constitutive promoter (35S) to study the role of TPKb and by extension, of SVs.

TPKb overexpression reduces the impact of low-K^+ conditions

Our growth analyses show that TPKb-OX plants grew slightly less vigorously in standard hydroponic medium but this was only

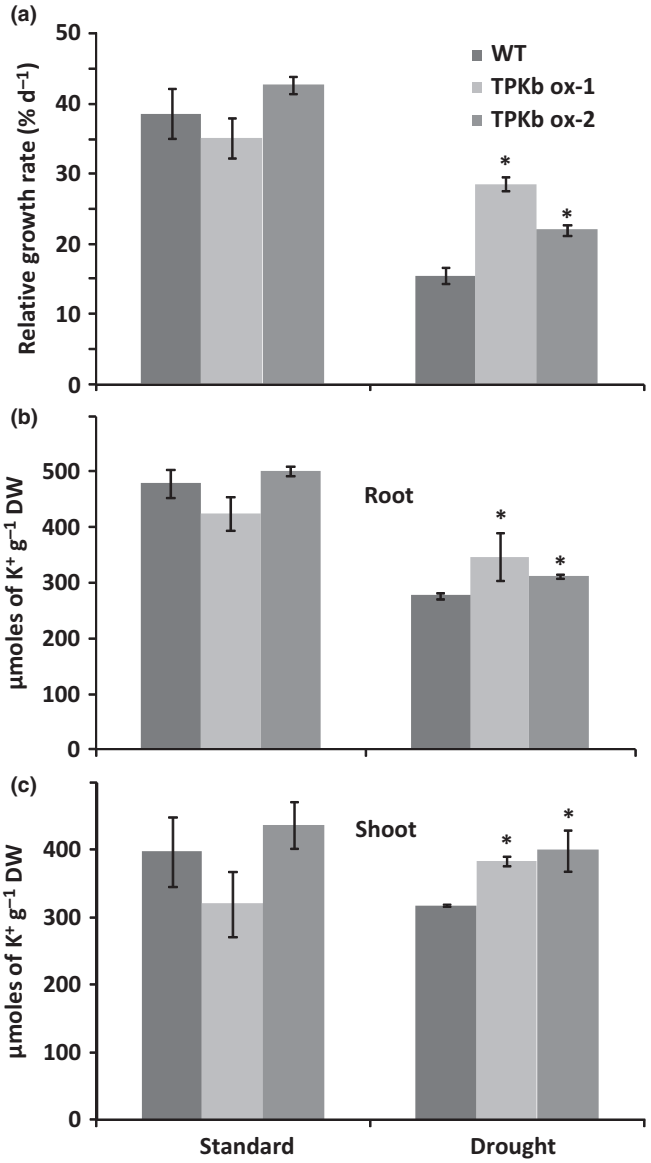

Fig. 8 Growth and K^+ content of drought treated rice. (a) Relative growth rate (RGR) of 4-wk-old plants that were transferred to pots and exposed for a further 6 wk to full watering (100% field capacity) or limited water supply (*c.* 40% field capacity). (b) Root K^+ content of plants grown in the conditions mentioned under (a). (c) Shoot K^+ content of plants grown in the conditions mentioned under (a). Data are from at least three independent experiments and the error bars represent ± SE. Significant difference between the wild-type (WT) and overexpressor lines (Student's *t*-test): *, $P < 0.05$.

significantly so for OX1 (Fig. 2a). This is likely to be a nonspecific effect of rice transformation and has been observed in other transgenic rice (Kurusu *et al.*, 2004). Furthermore, the growth data show large toxicity effects when rice was exposed to either 60 mM KCl or NaCl but no difference was detected between the control and three independent OX lines. This suggests that extra TPKb capacity does neither benefit nor harm plants in salt stress conditions.

When corrected for unequal growth in standard conditions, all TPKb-OX plants grow significantly better in low-K^+ conditions than control plants. A similar phenotype was found when plants

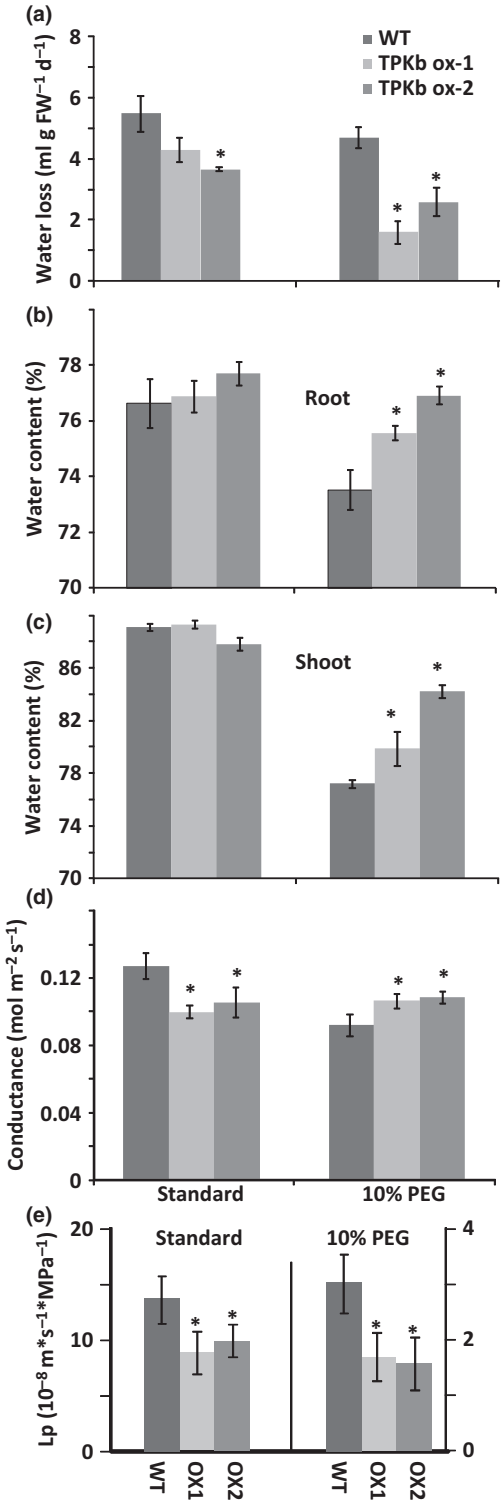

Fig. 9 Water relations and whole leaf conductance analyses. (a) Control and TPKb OX plants were incubated in either standard medium or 10% PEG medium. Total water loss through transpiration was recorded during a 5-d period. (b, c) Root and shoot water content of control and TPKb OX plants. (d) Steady-state leaf conductance was measured with an IRGA on leaf segments derived from 4-wk-old rice plants. (e) Root hydraulic conductance in plants exposed to standard and 10% PEG media. Data are from at least three independent experiments and the error bars represent ± SE. Significant difference between the wild-type (WT) and overexpressor lines (Student's *t*-test): *, $P < 0.05$.

were grown in pots containing low-K^+ soil which is highly relevant because agricultural land around the globe is, or is becoming, progressively more deficient in potassium (Mengel & Kirkby, 2001; Römheld & Kirkby, 2010). To counter this threat either requires application of expensive fertiliser or the development of crops which can thrive on low-K^+ soils.

Ion analyses indicate that higher tissue K^+ may be the reason for rendering TPKb-OX plants more resilient to K^+ stress than control plants. The depletion experiments show that OX plants have a greater capacity to take up K^+ at the root–soil boundary. Although the exact mechanism of this upregulated net K^+ uptake remains to be elucidated, it may very well be fuelled by a 2–3 fold increase in expression of AKT1, one of the major components of K^+ uptake.

The increased K^+ uptake could explain the relatively high tissue levels of K^+ in the OX lines. A second issue that could affect growth in K^+-deficient conditions is the intracellular distribution of K^+; EDX measurements point to a relatively higher cytoplasmic K^+ content in the transgenic plants, especially during K^+ deficiency. This could ameliorate the K^+ deficiency symptoms because the detrimental impact of low tissue K^+ is primarily manifested in the cytoplasm where K^+ fulfils specific biochemical roles (Maathuis, 2009; Ahmad & Maathuis, 2014). The mostly biophysical functions of vacuolar K^+ can be achieved by other cations (Isayenkov *et al.*, 2010). With multiple SVs potentially making up a significant fraction of the cellular volume (Fig. 1), the increase in cytoplasmic K^+ could depend on TPKb mediated K^+ release from SVs, a function that may be strengthened in the OX line.

TPKb affects tolerance to water deficit

Potassium nutrition is intimately connected to plant water homeostasis and water use efficiency (Kuchenbuch *et al.*, 1986; Tanguilig *et al.*, 1987). Enhanced K^+ uptake is one of the main responses of plants suffering from drought (Andersen *et al.*, 1992; Wang *et al.*, 2004) and limiting K^+ loss will improve water retention, ensure appropriate stomatal regulation and help maintain photosynthetic activity via the function of K^+ in photoassimilate translocation (Römheld & Kirkby, 2010; Zörb *et al.*, 2014). TPKb expression is greatly increased in response to drought, with *c.* 17-fold and 40-fold rises in roots and leaves, respectively, whereas there are very few other conditions that impact on TPKb transcript level (Genevestigator). Thus, the transcript data strongly suggest a role played by TPKb in water homeostasis. In agreement with these transcriptomics data we found that TPKb overexpression generated rice plants which are more resilient in the face of either osmotic stress or drought stress. Growth rates were higher and transpiration in polyethylene glycol (PEG)-grown plants was much lower in OX lines than in control plants.

A causal link between TPKb overexpression and improved water stress tolerance may stem from the higher K^+ accumulation that was observed in both root and shoot tissue of the transgenic rice compared to control plants. Increased K^+ retention lowers the cellular water potential and, as such, prevents excessive water loss, for example from roots to soil.

Altered gas exchange, and therefore stomatal functioning, may also contribute to water loss rates. However, although 10% PEG led to a *c.* 25% reduction of leaf conductance in control plants, virtually no reduction was observed in OX plants. This relatively greater leaf conductance was present in the transgenic lines in spite of a lower overall water loss and could sustain higher photosynthetic activity, and consequently, better growth. Thus, TPKb-OX plants are capable of limiting their water loss in spite of a relatively high leaf conductance. This finding suggests that components other than stomata dominate the hydraulic resistance of the root-to-atmosphere continuum. Indeed, root conductance in the OX lines is considerably lower. The exact mechanism responsible for this change is largely unknown but is possibly related to the relatively high root K^+ concentration observed in the overexpression lines. For several species, including rice, it has been reported that high tissue K^+ can lead to reduced hydraulic conductance in the root (Liu *et al.*, 2006).

In addition to a role of TPKb in K^+ tissue level and cellular K^+ distribution, the osmosensing capability of TPK channels may be important. Previous work showed that TPKb activity responds to changes in trans-tonoplast osmogradients (Maathuis, 2011). Although perturbations in trans-tonoplast osmogradients are likely to be brief they may form an important part of adaptation to turgor changes (MacRobbie, 2006) and or water homeostasis-related signalling events. Soil water potentials vary widely and can do so relatively quickly which may require rapid osmotic adjustment in root cells. The latter may involve TPKb-mediated K^+ flux in and out of the SVs, for example to charge balance osmotic movement of negative ions.

Conclusion

In summary, our data show that constitutive overexpression of the vacuolar K^+ channel TPKb affects rice growth in K^+-deficient conditions and alters tolerance to osmotic and drought stress. In turn this points to a function of SVs in K^+ homeostasis and rice water relations. It is not *a priori* evident how the activity of a tonoplast K^+ channel would lead to increased K^+ uptake and accumulation. Impalements of root cells did not reveal any significant difference in membrane potential between control and OX plants, ruling this out as a mechanism to drive increased K^+ influx. However, reverse transcription polymerase chain reaction experiments showed a clear increase in expression of AKT1, a main component of root K^+ uptake, in the transgenic lines. The latter points to a mechanism where TPKb activity affects the expression of other K^+ transporters, as was previously shown for K^+ uptake mechanisms (Nieves-Cordones *et al.*, 2010), possibly via its impact on cellular K^+ homeostasis. For example, the shift in cellular K^+ distribution or a relatively low vacuolar K^+ status might (transcriptionally) activate root uptake systems. Alternatively, the K^+ status of SVs themselves may impact on overall K^+ homeostasis and stress tolerance. For instance, in tomato the overexpression of the K^+:H^+ antiporter NHX2 in prevacuolar compartments affected both K^+ homeostasis and increased salt tolerance (Rodríguez-Rosales *et al.*, 2008). Thus, it appears that

the role of small vacuolar bodies may be farther reaching than hitherto recognised.

Author contributions

F.J.M.M. and I.A. planned and designed the research. F.J.M.M., I.A., R.M., J.D. and M.S. performed experiments and analysed data. F.J.M.M. and I.A. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 K^+ efflux assays for different rice genotypes.

Table S1 Membrane potential measurements in rice roots

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