Plant Cell Tiss Organ Cult (2013) 112:19–31
DOI 10.1007/s11240-012-0207-9

ORIGINAL PAPER

Overexpression of a poplar two-pore K^+ channel enhances salinity tolerance in tobacco cells

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Received: 31 January 2012 / Accepted: 9 July 2012 / Published online: 22 July 2012

Abstract *Populus euphratica* is a plant model intensively studied for elucidating physiological and molecular mechanisms of salt tolerance in woody species. Several studies have shown that vacuolar potassium (K^+) ion channels of the two-pore K^+ (TPK) family play an important role in maintaining K^+ homeostasis. Here, we cloned a putative TPK channel gene from *P. euphratica,* termed *PeTPK*. Sequence analysis of PeTPK1 identified the universal K-channel-specific pore signature, TXGYGD. Over-expression of *PeTPK1* in tobacco BY-2 cells improved salt tolerance, but did not enhance tolerance to hyperosmotic stress caused by mannitol (200–600 mM). After 3 weeks of NaCl stress (100 and 150 mM), *PeTPK1*-transgenic cells had higher fresh and dry weights than wild-type cells. Salt treatment caused significantly higher Na^+ accumulation and K^+ loss in wild-type cells compared to transgenic cells. During short-term salt stress (100 mM NaCl, 24-h), *PeTPK1*-transgenic cells showed higher cell viability and reduced membrane permeabilization compared to wild-type cells. Scanning ion-selective electrode data revealed that salt-shock elicited a significantly higher transient K^+ efflux from *PeTPK1*-transgenic callus cells and protoplasts compared to that observed in wild-type cells and protoplasts. We concluded that salt tolerance in *P. euphratica* is most likely mediated through PeTPK1. We propose that, under salt stress, PeTPK1 functions as an outward-rectifying, K^+ efflux channel in the vacuole that transfers K^+ to the cytosol to maintain K^+ homeostasis.

Keywords K^+ flux · NaCl · PeTPK1 · *Populus euphratica* · Protoplast · Tobacco BY-2 cells

Introduction

Potassium (K^+) represents 3–5 % of the total dry weight of plants (Marschner 1995), and it is essential for plant growth and survival (Kochian and Lucas 1988; Cha-um et al. 2012). K^+ fluxes support enzyme function, turgor pressure, stomatal movement, and cell elongation in cells and tissues (Schroeder et al. 1994; Maathuis and Sanders 1996; Very and Sentenac 2003). Under salt stress, Na^+ competes with K^+ for uptake into roots. A high cytosolic K^+/Na^+ ratio is important for maintaining cellular metabolism (Zhu 2003; Munns and Tester 2008). Thus, the ability to control intracellular K^+ homeostasis is arguably one of the most important features of salt-tolerant plant species (Shabala and Pottosin 2010). Volkov and colleagues found that K^+ content in mesophyll cells of *Thellungiella salsuginea* (a salt-tolerant species) increased under salt stress. In contrast, *Arabidopsis* (a salt-sensitive species) K^+ content dropped under the same saline

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conditions (Volkov et al. 2003). *Populus euphratica* has been widely considered a convenient plant model for elucidating physiological and molecular mechanisms of salt tolerance in woody species (Chen et al. 2001, 2002, 2003; Gu et al. 2004; Ottow et al. 2005a, b; Junghans et al. 2006; Wang et al. 2007, 2008; Sun et al. 2010a; Chen and Polle 2010). Similar to *T. salsuginea*, *P. euphratica* maintains high K^+ concentrations at both the tissue and cellular levels, compared to salt-sensitive poplars (Chen et al. 2001, 2002, 2003; Sun et al. 2010a, b). The relatively high K^+ levels in root and shoot tissues of *P. euphratica* are attributed to its high rates of net uptake and transport of K^+ (Chen et al. 2003). The high capacity for maintaining K^+ balance in the presence of high external Na^+ is suggested to result from preferential uptake of K^+ over Na^+ (Flowers et al. 1977; Mills et al. 1985). Moreover, flux studies have shown that the high capacity of *P. euphratica* for maintaining K^+ homeostasis is based on the ability to retain K^+ under NaCl stress (Sun et al. 2009a). For example, high saline conditions caused a significant net K^+ efflux in the root apex of *Populus popularis* (a salt-sensitive species); however, salt stress did not significantly alter the K^+ flux in the root apex of *P. euphratica* (Sun et al. 2009a). Expression analysis performed in a salt-sensitive poplar, *P.* × *canescens*, revealed that, when K^+ channels released K^+, they exhibited a remarkable transcriptional up-regulation of plant outward rectifying K^+ channels, PTORK (KORC), PTORK2 (KORC2), and the plant K^+ channel, PTK2, during the period of salt stress (Escalante-Pérez et al. 2009).

Several types of K^+ channels contribute to ion uptake and transport in plants (Voelker et al. 2010). The unique common feature of all K^+ channel subunits identified to date is the presence of a conserved pore region (P-domain) motif, TXGYGD, which contributes to K^+ conductivity. To date, two classes of K^+ selective channels have been reported in plants. The first class comprises channels formed from four, pore-forming α-subunits, where each subunit contains a hydrophobic core, six transmembrane domains (TMs), and a single P-domain (Very and Sentenac 2003). The second class, initially described in yeast, human, and *Drosophila*, comprises channels formed from α-subunits, where each subunit contains two P-domains (a tandem-pore; Ketchum et al. 1995; Goldstein et al. 1996; Lesage et al. 1996a). This configuration suggested that a K^+ selective channel could be formed by the association of only two α-subunits (Lesage et al. 1996b). Several tandem-pore K^+ channels have been cloned, and they comprise the TPK/KCO gene family. Arabidopsis family members include five TPK channel genes, with four TMs and two P-domains, and one inward rectifying K^+ channel gene (Kir-type) with two TMs and one P-domain (Goldstein et al. 2001; Patel and Honore 2001; Becker et al. 2004). Each P-domain contains a GYGD sequence (Schönknecht et al. 2002; Dunkel et al. 2008). The *Arabidopsis AtTPK1* channel was the first cloned plant K^+ channel gene that belonged to the TPK family; it has four putative TMs and a tandem, calcium-binding, EF-hand motif (Czempinski et al. 1997).

Several studies have shown that the TPK1 channel mediates vacuolar K^+ release and plays important roles in plant physiology (Gobert et al. 2007; Hamamoto et al. 2008; Isayenkov et al. 2011; Maîtrejean et al. 2011). TPK1 was found to participate in vacuolar K^+ release during stomatal closure, and it may be directly or indirectly activated by abscisic acid (ABA) (Gobert et al. 2007). TPK1 was found to be involved in radicle growth stages through vacuolar K^+ loading, which provided turgor-driven cell expansion during seed germination (Gobert et al. 2007). Given the electrochemical gradient for K^+ across the vacuolar membrane, compensatory K^+ efflux through channels could represent a rapid response to the onset of salt stress (Cuin et al. 2003). Coincidently, previous microarray data obtained by Ding et al. (2010) indicated that the ability of *P. euphratica* to retain K^+ was correlated with the transcript abundance of K^+ transporters and channels. A variety of K^+ transporters, particularly high-affinity K^+ transporters and stelar K^+ outward rectifiers, were more strongly expressed in salt-tolerant *P. euphratica* than in salt-sensitive *P. popularis* (Ding et al. 2010). Although multiple functions of TPK1 have been clarified, its contribution to salt resistance is only rudimentarily understood.

X-ray microanalysis of *Populus tomentosa* (a salt-sensitive poplar) showed that salt stress lowered K^+ in the leaf apoplast, but increased K^+ in the vacuole (Dai et al. 2006). In contrast, *P. euphratica* redistributed intracellular K^+ in the opposite direction (Dai et al. 2006). This suggested that *P. euphratica* could compensate for K^+ loss caused by Na^+ competition by shifting K^+ from the vacuole to the cytoplasm (Ottow et al. 2005b). The TPK-type K^+ channel has been widely recognized as a novel tonoplast K^+ channel in rice and tobacco (Hamamoto et al. 2008; Isayenkov et al. 2011). In the present study, we aimed to clone a TPK channel expressed in the salt-tolerant poplar model, *P. euphratica* and analyze its role in ion homeostasis in tobacco cells. Based on *Populus trichocarpa* sequence data (Tuskan et al. 2006), we cloned the TPK channel gene from *P. euphratica* (*PeTPK1*). This sequence was highly similar to that of *AtTPK1* (81 % identity). We studied PeTPK1 function in tobacco BY-2 cells, a model system for investigating ion homeostasis in plants, due to its conserved basic transport mechanism (Serrano and Rodriguez-Navarro 2001). We found that expression of the *PeTPK1* transgene in tobacco BY-2 cells resulted in a salt tolerant increment in contrast to wild-type cells. To gain insight into the biophysical properties and physiological functions

of PeTPK1, K^+ flux of *PeTPK1* transgenic cells were examined with the scanning ion-selective electrode technique (SIET).

Materials and methods

Plant material and treatments

Seedlings of *P. euphratica* Oliver were obtained from the XinJiang Uygur Autonomous Region of China. They were planted in individual pots (10 L) that contained loamy soil (1:1 sand:soil) and placed in a greenhouse at Beijing Forestry University. Potted plants were well irrigated and fertilized with 1.0 L, full-strength, Hoagland's nutrient solution every 2 weeks. The temperature in the greenhouse was maintained at 20–25 °C with a 16-h photoperiod (7:00 AM–11:00 PM). Photosynthetically active radiation was applied at 150 μmol m^{-2} s^{-1} during the plant culture period.

Tobacco BY-2 (*Nicotiana tabacum* cv. Bright Yellow 2) cells were grown in BY-2 growth medium, a modified Linsmaier and Skoog medium, supplemented with 200 μg L^{-1} 2,4-dichlorophenoxyacetic acid and 3 % sucrose. Cultures were maintained at 26 °C in the dark with constant shaking, and they were propagated by subculturing 1 mL of BY-2 cells into 100 mL of fresh medium every 14 day. BY-2 cells were also grown in solid medium (with 0.3 % phytagel added to the suspension culture) at 26 °C in the dark, and these were maintained by subculturing a portion of the BY-2 cells into fresh, solid medium every 3 weeks.

Cloning the full length *PeTPK1* gene

Total RNA was extracted from *P. euphratica* leaves with TRIzol reagent according to the manufacturer's protocol (QBio Technologies Inc., Beijing, China). The first-strand cDNA was synthesized by reverse transcription in a reaction with an oligo (dT) primer, M-MLV reverse transcriptase (Promega, Madison, WI, USA), and 1 μg total RNA template. The reverse transcription product was diluted with an equal volume of sterilized water. PCR was used to obtain the full length *P. euphratica TPK* cDNA with primers designed to anneal to sequences homologous to the *P. trichocarpa TPK* sequence: 5′-CACCAAAA CTGCAGATGGCTTCCAATGGTG-3′ and 5′-CTAGTC TAGATCACTTCTTCATTGGAGT-3′. With PCR amplification, we identified a 1060 bp cDNA that represented the full-length *PeTPK1* transcript.

Sequence alignment was conducted with the CLUSTALW program and the TMs were predicted with the TMHMM program (version 2.0), available at (http://www.cbs.dtu.dk/services/TMHMM/), and the SMART program, available at (http://smart.embl-heidelberg.de). The phylogenic tree was created with the MEGA program (version 4.0).

PeTPK1 overexpression in tobacco BY-2 cell lines

PeTPK1 was subcloned into the PENTR/D TOPO vector (Invitrogen, Carlsbad, CA, USA) and then transferred into the GATEWAY destination pk7WG2D.1 vector (Karimi et al. 2002). The latter contained two CaMV 35S promoters for respectively driving the target gene and a green fluorescent protein (GFP) reporter gene in an LR Clonase reaction (Invitrogen, Carlsbad, CA, USA). Subsequently, the *PeTPK1* construct was introduced into the *Agrobacterium tumefaciens* strain GV3101, and then transformed into wild-type BY-2 cells according to the Nocarova protocol (Nocarova and Fischer 2009). After incubation in the dark for 2 days, the transformed BY-2 cells were grown on selection medium with kanamycin (100 mM) for three weeks. After 3–4 subculture selections, total DNA was extracted from the transgenic cell lines with the hexadecyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987).

RT-PCR

To check for transgene expression, total RNA was extracted from the transgenic BY-2 lines with the TRIzol method (QBio Technologies Inc., Beijing, China). cDNA synthesis was performed via reverse transcription in a reaction with M-MLV Reverse Transcriptase and an oligo (dT) primer according to the manufacturer's instructions (Promega, Madison, WI, USA). The reverse transcription products were diluted (1:2) and 1 μL was used as template in subsequent PCR amplifications. The gene-specific forward and reverse primers were 5′-CCGTATGTTCGCT GTAGTTTGG-3′ and 5′-TCGTCTATGTTTGCTGCTT CG-3′ designed with Primer Premier 5.0. The PCR program ran for a total of 30 cycles and aliquots were removed after 27 cycles. PCR products were evaluated by normalization to an internal control cDNA that encoded the housekeeping gene, *elongation factor 1α* (*EF1α*), amplified under identical conditions. The forward and reverse primers for *EF1α* were 5′-TGCGAAAGAGCCCAGGTT-3′ and 5′-CAATGGTGGGTACGCAGAGAG-3′. The PCR products were separated in 2 % agarose gels, stained with ethidium bromide, and then photographed under UV illumination.

Salt and osmotic tolerance of BY-2 cells

Long term salinity and hyperosmotic experiments were conducted with wild-type and transgenic cells grown on

solid medium supplemented with NaCl (50, 100, and 150 mM) or mannitol (200, 400, and 600 mM). Control cells were grown without added NaCl or mannitol. All cells were transferred to identical culture dishes and grown for 3 weeks in the dark. Then cells were harvested, weighed, and dried at 65 °C for 2 days. Three replicate samples of BY-2 cells were harvested per treatment at each sampling time.

Cell viability

Salt stress was induced in BY-2 cell suspensions by inoculating with liquid BY-2 cell medium that contained 100 mM NaCl. Control cells were treated without additional NaCl. After 6, 12, and 24 h of treatment, the supernatant was discarded, and cells were stained with fluorescein diacetate (FDA) for 7 min at room temperature. The FDA working solution was freshly prepared (5 mg mL^{-1}, Sigma Chemical Co., St. Louis, MO, USA) in dimethyl sulfoxide (DMSO) at a concentration of 1 % (w/v) (Sun et al. 2010a). DMSO was selected as the permeabilizing agent for FDA staining (Brodelius and Nilsson 1983). Then, FDA-stained cells were examined with a Leica inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 50-W mercury lamp and appropriate filter settings. Cell viability, expressed as the percentage of living cells, was calculated in triplicate, based on analyses of about 200 cells on each slide.

Membrane permeability

The membrane permeability was examined in terms of conductivity. Wild-type and transgenic cells (0.2 g) were incubated in redistilled water at 25 °C for 2 h. Then, the conductivity (C1) in the bathing solution was measured. Next, callus samples were heated at 95 °C for 1 h to measure the total conductivity (C2). The relative membrane permeability (MP) was expressed as a percentage of the total conductivity; i.e., MP (%) = C1/C2 × 100.

Na^+ and K^+ analysis

Wild-type and transgenic cells were oven-dried, ground, and passed through a 1.0 mm sieve for mineral analysis. After digestion with H_2SO_4-H_2O_2, Na^+ and K^+ contents were determined with an atomic absorption spectrophotometer (Perkin-Elmer 2280) (Chen et al. 2001).

Protoplast isolation

Protoplasts were isolated from transgenic BY-2 lines and wild-type cells, as previously described by Sun et al. (2009b) and Dreyer et al. (2004). A brief description is given below. Cell suspensions were sedimented at 200×*g* for 3 min and subsequently resuspended in 4 mL of a basic solution (BS: 10 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM MES, 0.2 % bovine serum albumin (w/v), and 400 mM mannitol, pH 5.7 was adjusted with Tris) supplemented with the following enzymes (w/v): 1.0 % cellulase Onozuka R-10 (Yakult Honsha Co., Ltd., Tokyo, Japan), 0.2 % macerozyme R-10 (Yakult Honsha Co., Ltd., Tokyo, Japan), and 0.1 % pectolyase Y-23 (Yakult Honsha Co., Ltd., Tokyo, Japan). Then cells were gently shaken at 60 rpm/min in the enzyme solution for 3 h at 26 °C. After the enzyme digestion, BY-2 protoplasts were filtered through a nylon mesh with 50-mm-diameter pores, and were collected by centrifugation at 200×*g* for 2 min. Then protoplasts were resuspended and washed with 2–3 mL of a holding solution (×2). Finally, protoplast suspensions (1 mL) were diluted with a 2 mL holding solution and used for steady-state and transient kinetics flux measurements (Shabala 2000; Chen et al. 2005).

Prior to cellular ion flux recordings, protoplasts were fixed on the bottom of the measuring chamber to reduce mobility caused by movements of the electrode during measurements. Glass coverslips were treated with a poly-L-Lysine (Sigma, USA) solution (Mazea et al. 1975) at a concentration of 0.002 % (w/v) for 10 min. Coverslips were stored in a sealed container for air drying at room temperature. A 200 µL aliquot of protoplast suspension was dropped onto the middle of the poly-L-Lysine-pretreated coverslip in the measuring chamber. When protoplasts had settled on the surface (requiring several minutes), 3 mL of a K^+ measuring solution was slowly added to the measuring chamber.

Scanning ion-selective electrode technique

Transient K^+ fluxes were measured in tobacco cells and protoplasts with the scanning ion-selective electrode technique (the SIET system BIO-001A; Younger USA Sci. & Tech. Corp.; Applicable Electronics Inc.; and Science Wares Inc.) as described previously (Kühtreiber and Jaffe 1990; Kochian et al. 1992; Zonia et al. 2002; Vincent et al. 2005; Xu et al. 2006). The concentration gradients of the target ions were measured by moving the ion selective microelectrode between two positions close to the cell in a preset excursion (10 µm for protoplasts and callus cells in our experiment) at a programmable frequency in the range of 0.3–0.5 Hz. Pre-pulled and silanised glass micropipettes (2–4 µm aperture, Xuyue (Beijing) Sci. and Tech. Co., Ltd., Beijing, China) were treated with a backfilling solution (K^+: 100 mM KCl) to a length of 1.0 cm from the tip. Then the micropipettes were front-filled with 15 µm columns of selective liquid ion exchange cocktails (LIXs)

(K: Fluka 60398, Fluka Chemie GmbH, Buchs, Switzerland). An Ag/AgCl wire electrode holder (XYEH01-1; Xuyue (Beijing) Sci. and Tech. Co., Ltd., Beijing, China) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. The reference electrode was an Ag/AgCl half-cell (DRIREF-2; World Precision Instruments, Inc., Sarasota, FL) connected to the experimental solution by a 0.5 % agarose bridge containing 3.0 M KCl. Ion selective electrodes of K^+ were calibrated prior to flux measurements: 0.1 mM, 0.5 mM and 1.0 mM (K^+ concentration was 0.5 mM in the measuring solution). Only electrodes with Nernstian slopes >50 mV/decade were used in our experiments. Flux rate was calculated from Fick's law of diffusion:

$$J = -D(dc/dx)$$

where J represents the ion flux in the x direction, dc/dx is the ion concentration gradient and D is the ion diffusion constant in a particular medium (Kühtreiber and Jaffe 1990). Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software [Science Wares (East Falmouth, MA) and Applicable Electronics].

For transient measurements, steady fluxes of K^+ from transgenic and wild-type cells were recorded for about 10 min in control conditions. Then, salt shock (final concentration, 100 mM NaCl) was elicited by adding an appropriate amount of stock NaCl solution (0.2 M, pH 6.0 adjusted with NaOH and HCl). The transient ion fluxes were monitored for 15–20 min. The data measured during the first 2–3 min were discarded, due to diffusion of the NaCl stock solution (Shabala 2000).

Data analysis

Three-dimensional ionic fluxes were calculated with the MageFlux acquisition program, developed by Yue Xu (http://xuyue.net/mageflux). Positive values in the figures represent cation efflux. All mean data were subjected to analysis of variance. Significant differences between means were determined with Duncan's multiple range test. Unless otherwise stated, differences were considered statistically significant when $P < 0.05$.

Results

PeTPK1 cloning and sequence analysis

With primer sequences homologous to the TPK family of putative K^+ transporters from *P. trichocarpa*, the full-length *TPK* cDNA was cloned from *P. euphratica* via reverse transcription and a standard PCR-based cloning procedure. The cloned cDNA was designated *PeTPK1*. It contained a 990 bp open reading frame encoding a 330-amino acid protein with an ATG start codon at position 41 and a TGA termination codon at position 1,031. A BLAST procedure (NCBI) based on the predicted amino acid sequence for PeTPK1 showed that it was 97 and 81 % identical to the *Eucalyptus camaldulensis* KCO1 and *Arabidopsis thaliana* TPK1 channel sequences, respectively (Fig. 1). A multiple alignment showed a high degree of amino acid homology between the PeTPK1 and putative out-ward rectifying K^+ channel sequences of other higher-order plants (Fig. 1). A phylogenetic analysis of polypeptide TPK K^+ channel sequences produced an unrooted consensus phylogenetic tree. This tree was constructed with a set of 23 protein sequences shared among the whole family (five members) of TPK channels in *Arabidopsis*. Phylogenetic analysis indicated that PeTPK1 and AtTPK1 were in the same branch of the tree (Fig. 2). The hydropathic profile of PeTPK1, generated with the Kyte and Doolittle (1982) algorithm, predicted five potential TMs (Fig. 1). We speculated that TM2 and TM3 comprised a single, intact TM (TM23), because there were only four amino acids between these two putative TMs (Fig. 1). The predicted protein sequence contained two P-domains, P1 located between TM1 and TM23, and P2 located between TM4 and TM5. Within the two P-domains, we identified the K^+ channel selective motif, TXGYGD (Heginbotham et al. 1992, 1994).

Overexpression of *PeTPK1* improved the salt tolerance of BY-2 cells

To discern the role of the K^+ channel in the salt tolerance of *P. euphratica*, the *PeTPK1* gene was transformed into BY-2 cells. Semi-quantitative RT-PCR analysis indicated that *PeTPK1* was over-expressed in transgenic cells and not present in wild-type cells (Fig. 3). All cells showed reduced growth with increasing NaCl concentrations in the medium (Fig. 4a). However, at high salt concentrations, there were significant differences between *PeTPK1*-transgenic and wild-type cells. After 3 weeks of exposure to 100 and 150 mM NaCl, the fresh weight of transgenic cells was 2–2.5 fold greater than that of wild-type cells (Fig. 4b). Dry weight values showed the same trend (Fig. 4b, c). However, the cellular response to hyperosmotic stress differed from the response to NaCl stress. Hyperosmotic stress caused by mannitol significantly changed the fresh and dry weights of tobacco cells, but the effects were equivalent in transgenic and wild-type cells (Fig. 5a–c).

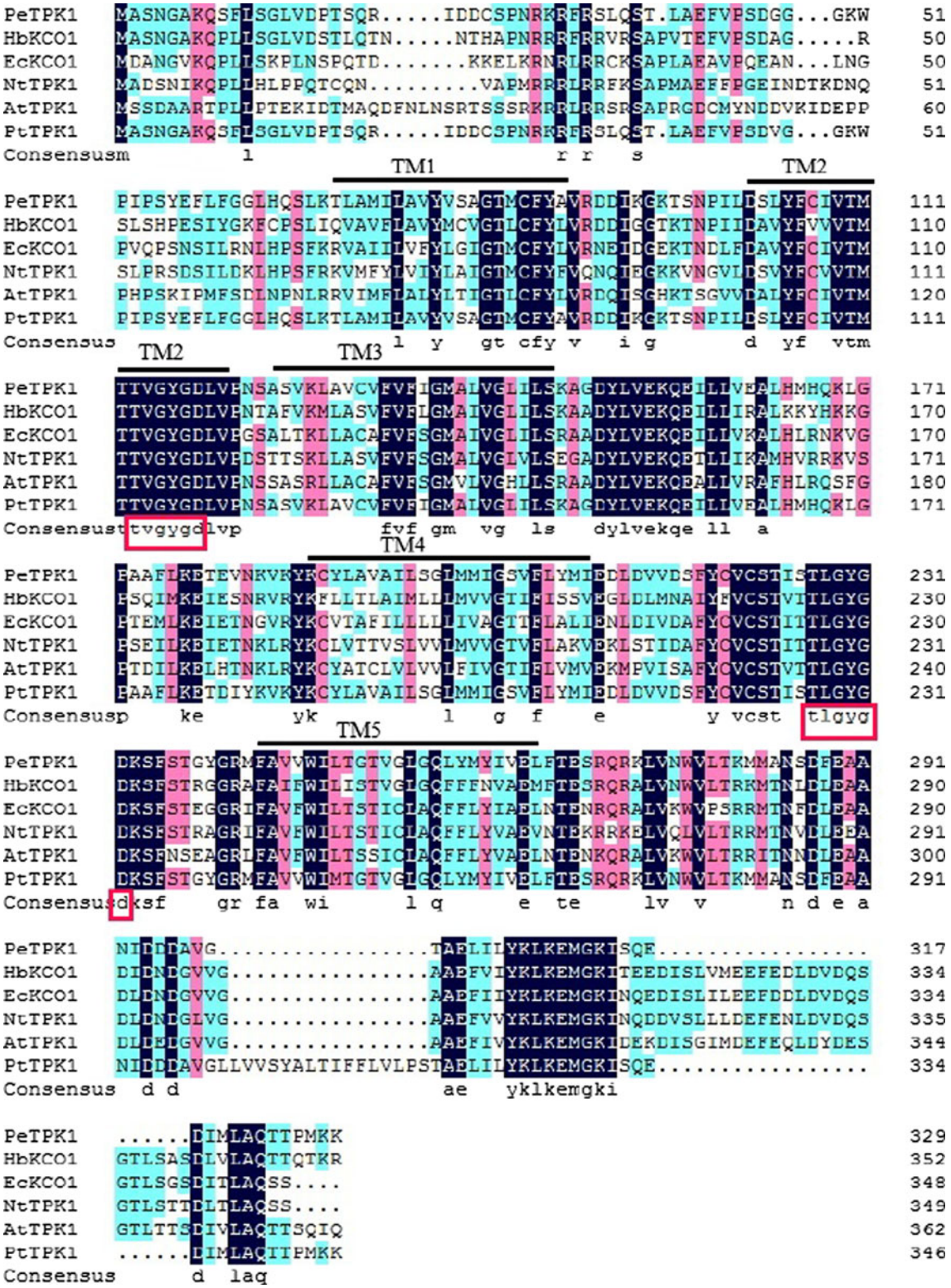

Fig. 1 Identification of the *P. euphratica* outward rectifying K^+ channel, PeTPK1. Multiple sequence alignment shows the similarities in deduced amino acid sequences of PeTPK1, HbKCO1 (*Hevea brasiliensis* KCO1), EcKCO1 (*Eucalyptus camaldulensis* KCO1), NtTPK1 (*Nicotiana tabacum* TPK1), AtTPK1 (*Arabidopsis thaliana* TPK1), and PtTPK1 (*Populus trichocarpa* TPK1). Among the five predicted transmembrane domains (TM1-TM5), the two pore domains are indicated by the K^+ channel-selectivity motif, TXGYGD, enclosed in *red boxes*. *Black* and *colored shading* indicate identical and conserved amino acid residues, respectively. (Color figure online)

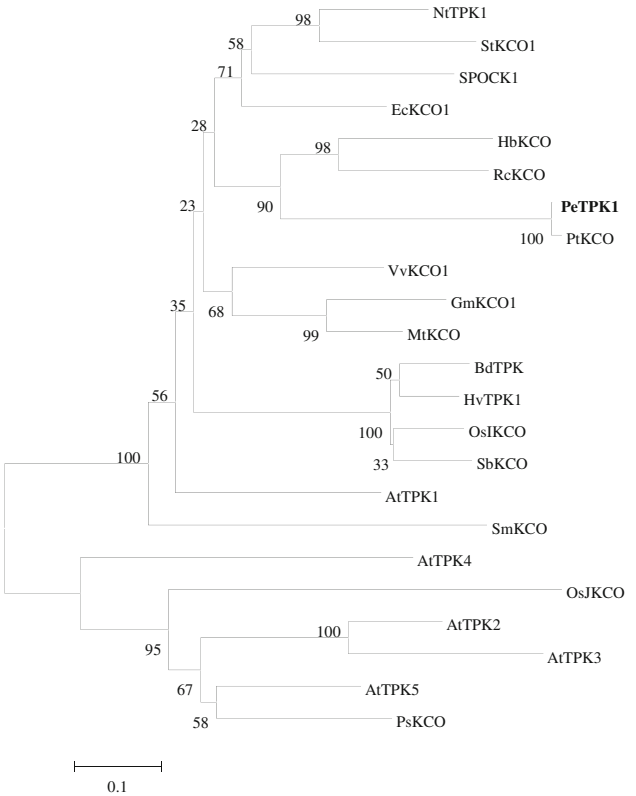

Fig. 2 Phylogenetic relationships between PeTPK1 and other representative TPK proteins from different plant species. The MEGA program (version 4.0) was used with the Neighborhood Joining Bootstrap method (Bootstrap analysis with 1,000 replicates) to construct the phylogenetic tree, based on the multiple alignments of the full-length amino acid sequences of TPKs from different plant species. The different species are indicated as follows: Nt, *Nicotiana tabacum*; St, *Solanum tuberosum*; Ss, *Samanea saman* (SPOCK1); Ec, *Eucalyptus camaldulensis*; Hb, *Hevea brasiliensis*; Rc, *Ricinus communis*; Pe, *Populus euphratica*; Pt, *Populus trichocarpa*; Vv, *Vitis vinifera*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Bd, *Brachypodium distachyon*; Hv, *Hordeum vulgare*; OsI, *Oryza sativa* Indica Group; Sb, *Sorghum bicolor*; At, *Arabidopsis thaliana*; Sm, *Selaginella moellendorffii*; OsJ, *Oryza sativa* Japonica Group; Ps, *Picea sitchensis*

Cell viability, membrane permeability, and K^+/Na^+ ratios under NaCl stress

We examined cell viability and MP (membrane permeability) after short-term exposure to 100 mM NaCl (6–24 h). Cell viability of wild-type BY-2 cells markedly decreased by 50 % after 24 h of salt treatment; in contrast, transgenic BY-2 cells maintained 70 % viability compared to controls grown without added salt (Fig. 6a). NaCl-induced MP increased sharply in both wild-type and transgenic cells after 6 h of salt stress, but wild type cells showed a more pronounced effect (Fig. 6b). At the end of salt treatment, MP increased by sixfold in wild-type cells, but by fourfold in transgenic cells (Fig. 6b). Ion analyses showed that NaCl stress caused significantly higher Na^+ accumulation and K^+ loss in wild-type cells compared to transgenic cells (Fig. 7a, b). As a result, the reduction in the K^+/Na^+ ratio was more pronounced in wild-type than in transgenic cells (Fig. 7c).

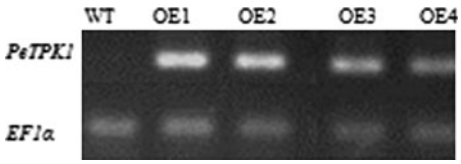

Fig. 3 Semi quantitative RT-PCR analysis of transgenic *PeTPK1* expression. The *PeTPK1* gene was over-expressed in tobacco BY-2 cells (OE1-4) compared to no expression in wild-type (WT) tobacco BY-2 cells under normal growth conditions. *PeTPK1* expression was evaluated by normalization to expression of the housekeeping gene, *elongation factor 1α* (*EF1α*) (see "Materials and methods" section)

Cellular K^+ flux upon salt shock

SIET was used to measure salt-induced alterations in K^+ fluxes in transgenic and wild-type tobacco cells. No-salt control cells exhibited a steady influx of K^+ before the salt shock with 100 mM NaCl (Fig. 8a). The salt shock induced K^+ efflux in transgenic and wild-type cells; this efflux exhibited a biphasic pattern over the observation period (Fig. 8a). The efflux of K^+ abruptly increased after the onset of salt treatment, then decreased, but remained constant during the recording period (Fig. 8a). However, the *PeTPK1*-transgenic cells showed a marked difference from wild-type cells in the flux rate change elicited by the salt shock. The transgenic cells exhibited a mean K^+ efflux of 80 pmol cm^{-2} s^{-1} with a peak of 500 pmol cm^{-2} s^{-1}, significantly higher than the mean ($\sim$20 pmol cm^{-2} s^{-1}) and peak (100 pmol cm^{-2} s^{-1}) observed for wild-type cells (Fig. 8a,b).

Salt-shocked protoplasts showed a trend similar to that of callus cells. After exposure to 100 mM NaCl, protoplasts from transgenic and wild-type tobacco cells showed a rapid increase in K^+ efflux, followed by a gradual decrease that finally reached a stable level (Fig. 8c). The K^+ efflux elicited by the shock ranged from 200 to 440 pmol cm^{-2} s^{-1} in protoplasts from transgenic cells and from 50 to 150 pmol cm^{-2} s^{-1} in protoplasts from wild-type cells during the recording period (Fig. 8c, d).

Discussion

PeTPK1 expression enhances salt tolerance of tobacco cells

In this study, we determined the characteristics of PeTPK1. We used tobacco BY-2 cells, because they are an ideal

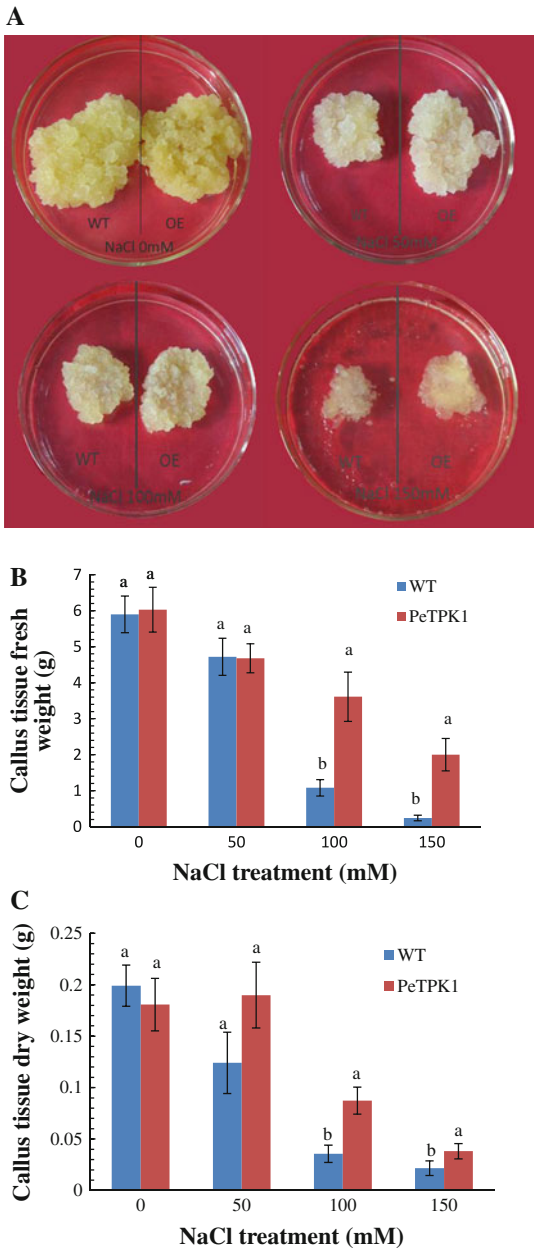

Fig. 4 Effects of NaCl on growth of *PeTPK1*-over-expressing (OE) and wild-type (WT) tobacco BY-2 cells. (**a**) Photographs of representative cultures show cell growth on culture medium supplemented without (0 mM) or with NaCl (50, 100, 150 mM, 3 weeks). Comparisons of (**b**) fresh weights and (**c**) dry weights show significant differences between *PeTPK1* transgenic and WT cells (*different letters*, *a*, *b*, indicate significant differences at $P < 0.05$). The means of three independent experiments are shown with the standard error of the mean (In each experiment 4–6 measurements were carried out for each treatment)

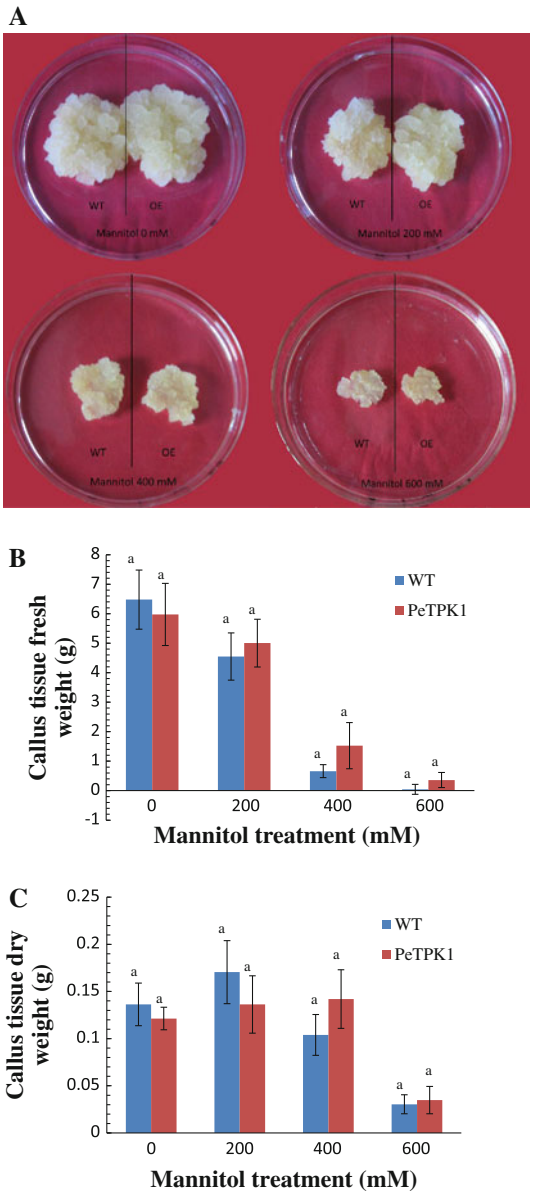

Fig. 5 Effects of mannitol on growth of *PeTPK1*-over-expressing (OE) and wild-type (WT) tobacco BY-2 cells. (**a**) Photographs of representative cultures show cell growth on culture medium supplemented without (0 mM) or with mannitol (200, 400 and 600 mM, 3 weeks). Comparisons of (**b**) fresh weights and (**c**) dry weights show significant differences between *PeTPK1* transgenic and WT cells (*different letters*, *a*, *b*, indicate significant differences at $P < 0.05$). The means of three independent experiments are shown with the standard error of the mean (In each experiment 4–6 measurements were carried out for each treatment)

system for studying the electrophysiological properties of subcellular, membrane-associated channels in plant cells (Serrano and Rodriguez-Navarro 2001). A BLAST procedure showed that the predicted amino acid sequence for PeTPK1 was highly homologous to the *Eucalyptus* EcKCO1 and *Arabidopsis* AtTPK1 (Fig. 1). TPK1 channels from *Arabidopsis*, rice and tobacco are characterized by a structure with four transmembrane domains and two pore loops (Hamamoto et al. 2008; Isayenkov et al. 2011; Czempinski et al. 1997). We noticed that the hydropathic profile of PeTPK1 with predicted five potential TMs (Fig. 1). We speculated that TM2 and TM3 comprised a

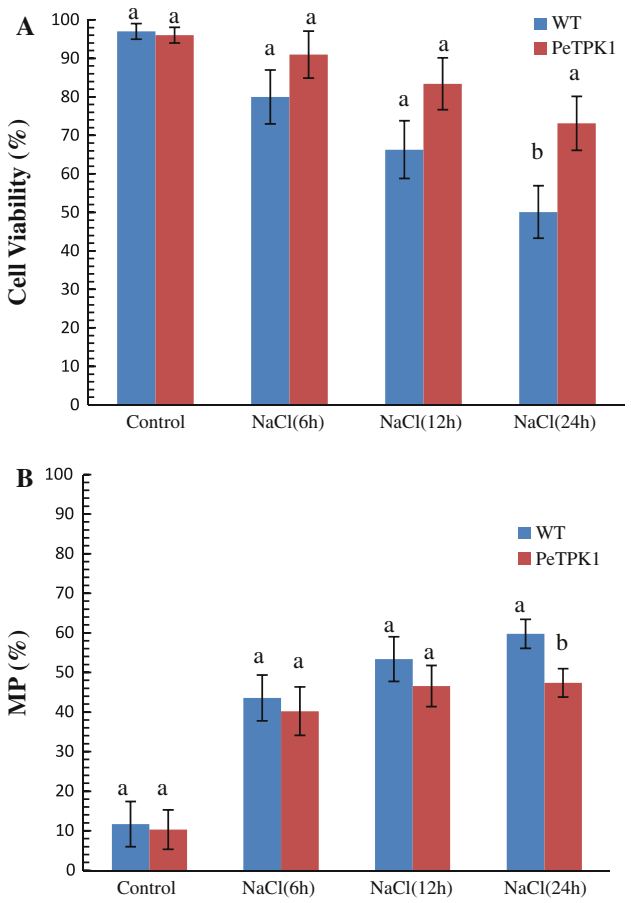

Fig. 6 Effects of salt stress on cell viability and membrane permeability (MP) in *PeTPK1*-transgenic (PeTPK1) and wild-type (WT) tobacco BY-2 cells. High NaCl (100 mM) conditions (**a**) reduced cell viability and (**b**) increased MP. The means of three independent experiments are shown with the standard error of the mean (In each experiment 4–6 measurements were carried out for each treatment). *Different letters*, *a*, *b*, indicate significant differences ($P < 0.05$) between transgenic and WT cells

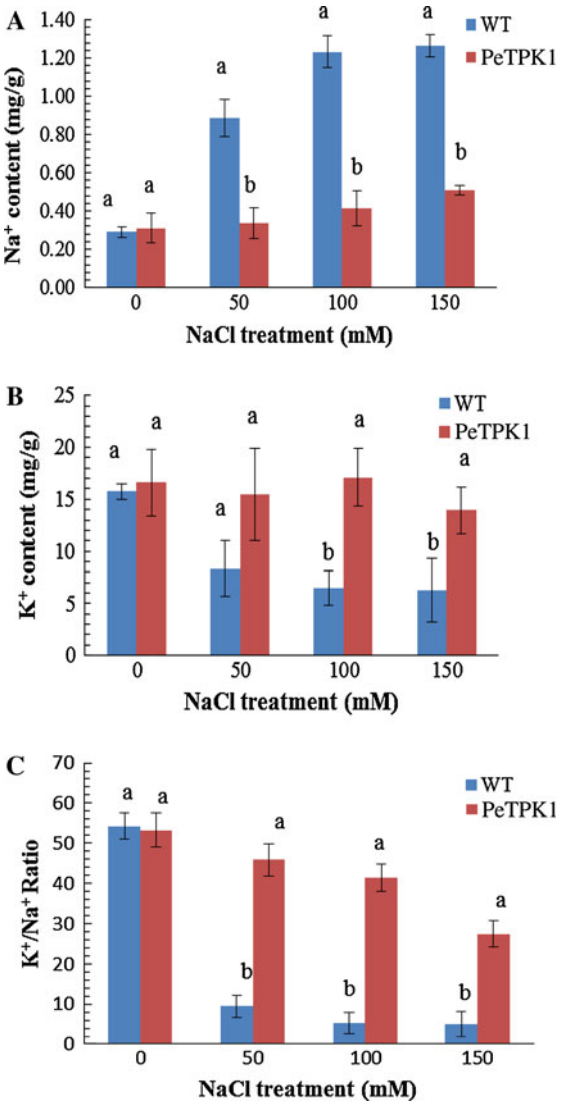

Fig. 7 Effects of salt stress on K^+ and Na^+ contents in *PeTPK1*-transgenic (PeTPK1) and wild-type (WT) tobacco BY-2 cells. High NaCl (100 mM) conditions (**a**) increased the Na^+ content, (**b**) reduced the K^+ content, and (**c**) altered the K^+/Na^+ ratios. Columns labeled with *different letters* (*a*, *b*) indicate significant differences ($P < 0.05$) between transgenic and WT cells. The means of three independent experiments are shown with the standard error of the mean (in each experiment three measurements were carried out for each treatment)

single TM as there were only four amino acids between these two putative TMs (Fig. 1). A phylogeny tree indicated that PeTPK1 shared high sequence similarity to PtKCO (*P. trichocarpa* outward rectifying potassium channel), HvTPK1 (*Hordeum vulgare* two-pore potassium channel 1), NtTPK1 (*N. tabacum* two-pore potassium channel 1) and AtTPK1 (*A. thaliana* two-pore potassium channel 1) (Fig. 2). When *PeTPK1* was over-expressed in tobacco cells, transgenic cells had higher fresh and dry weights than wild-type cells under high salt conditions (Fig. 4). Additionally, under high salt conditions, the *PeTPK1*-transgenic cells showed greater viability, higher resistance to membrane permeabilization, and greater maintenance of the K^+/Na^+ ratio than wild-type cells (Figs. 6, 7). These data demonstrated that the PeTPK1 channel contributed to salt-tolerance in tobacco cells. Our results were consistent with other studies that showed that over-expression of the K^+ uptake channel, *OsKAT1* conferred salt tolerance in rice cell lines (Obata et al. 2007). Interestingly, our results showed that *PeTPK1* expression under the CaMV 35S promoter did not increase the capacity of tobacco cells to tolerate osmotic stress in the presence of high mannitol (Fig. 5). These results were inconsistent with the notion that BY-2 cells might harbor the NtTPK1 promoter-A or promoter-B. Those promoters control a tonoplast-located NtTPK1 channel located in flowers, leaves, and roots, where channel expression was increased by 2-threefold under salt stress or strong osmotic

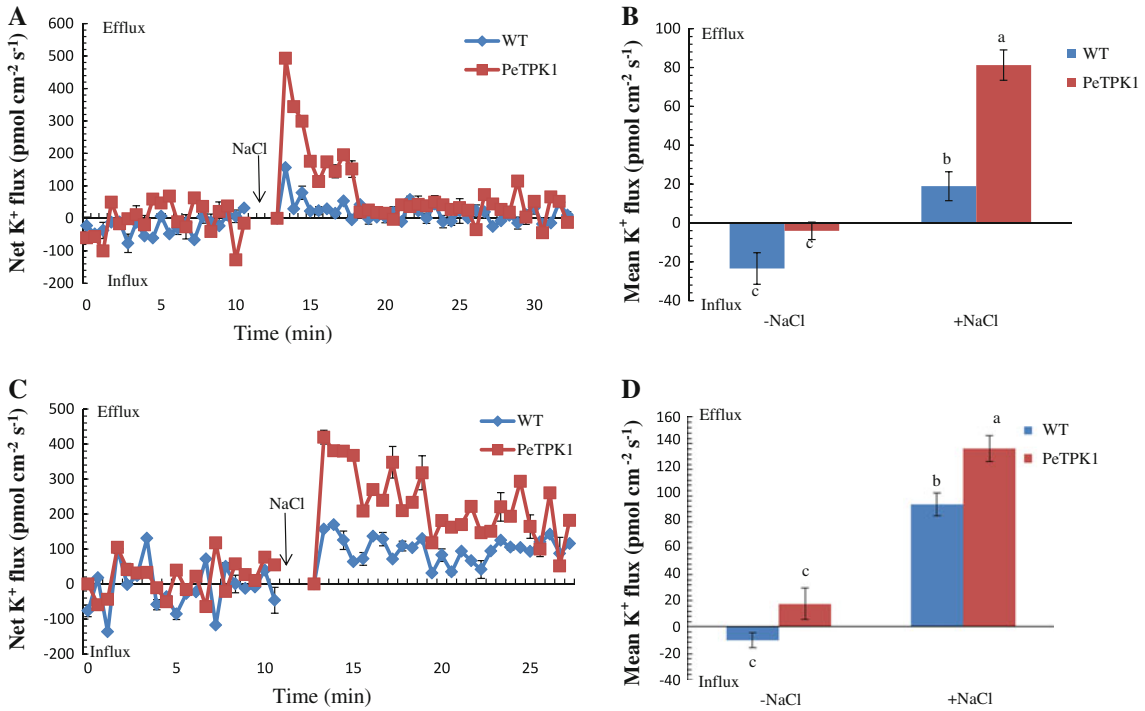

Fig. 8 Transient K^+ kinetics elicited by salt shock. *Left panels* After addition of a NaCl stock solution (0.2 M; final concentration: 100 mM NaCl), K^+ fluxes were recorded from *PeTPK1*-transgenic (PeTPK1) and wild-type (WT) tobacco BY-2 (**a**) callus cells and (**c**) isolated protoplasts for 15–20 min. For approximately 10 min prior to the salt shock (*arrow*), steady K^+ fluxes of callus cells and protoplasts were recorded. Each point represents the mean of five to six individual cells or protoplasts, and *bars* represent standard error of the mean. *Right panels* The mean K^+ fluxes before and after the salt shock are shown for (**b**) callus cells and (**d**) protoplasts. Each column is the mean of five to six individual cells or protoplasts and bars represent standard error of the mean. Columns labeled with *different letters*, *a*, *b*, *c*, indicate significant differences ($P < 0.05$)

shock (Hamamoto et al. 2008). Thus, the NtTPK1 promoters conferred tolerance to both salt and osmotic stress. Overexpression of *PeTPK1* increased salt tolerance of tobacco cells but did not enhance their capacity to tolerate hyperosmotic treatment, implying that *P. euphratica* employs different strategies to tolerate salinity and osmotic stress. In accordance, salt-induced alterations of ion flux in *P. euphratica* usually differ from the response to hyperosmotic stress (Sun et al. 2009b).

PeTPK1 expression contributes to K^+ homeostasis in salinized tobacco cells

Under high saline conditions, the plasma membrane is highly depolarized, and this causes high K^+ efflux from both root (Chen et al. 2005; Cuin and Shabala 2005) and mesophyll (Shabala 2000; Shabala et al. 2006) cells; this substantially reduces the cytosolic K^+ pools (Carden et al. 2003; Cuin et al. 2003; Shabala et al. 2006). Most authors agree that K^+/Na^+ homeostasis is a key feature of plant salinity tolerance (Rubio et al. 1995; Dubcovsky et al. 1996; Maathuis and Amtmann 1999; Volkov and Amtmann 2006; Chen et al. 2012; Wei et al. 2012). Plants with efficient cytosolic K^+ homeostasis maintain a high cytosolic K^+/Na^+ ratio. Our previous studies showed that high salinity caused a significant increase in K^+ efflux in the root apex of *P. popularis*, but not in the apical regions of the salt-tolerant *P. euphratica* (Sun et al. 2009a). Moreover, microanalysis of leaf cells showed that, under salt stress, *P. euphratica* maintained K^+ concentrations in the cytoplasm, but not in the cell wall and vacuole. This suggested that *P. euphratica* compensated for K^+ loss caused by Na^+ competition by shifting K^+ from the vacuole to the cytoplasm (Ottow et al. 2005b). In the present study, SIET data revealed that, under salt shock, *PeTPK1*-transgenic cells exhibited a transient K^+ efflux that was significantly higher than that observed in wild-type cells (Fig. 8a, b). We also noticed that shocked protoplasts of transgenic cells maintained a higher mean K^+ efflux than wild-type protoplast cells (Fig. 8c, d). The high rate of cellular K^+ efflux detected in the transgenic cells suggested that the cytoplasmic K^+ concentration was supplemented with K^+ from other sources. Accordingly, it is reasonable to assume that the cytoplasmic K^+ in *PeTPK1* transgenic cells was enriched by the over-expression of *PeTPK1*, which enhanced the transfer of K^+ from internal stores to the cytoplasm during the period of salt treatment. A previous study in yeast (*Saccharomyces cerevisiae*) showed that expression of *AtTPK1* conferred

strong selectivity for K^+ over Na^+, independent of membrane voltage over the range of ±80 mV (Bihler et al. 2005). We found that the PeTPK1 amino acid sequence shared high similarity with the TPK-type K^+ channels, AtTPK1 and NtTPK1, which function as tonoplast K^+ channels (Hamamoto et al. 2008; Isayenkov et al. 2011; Maîtrejean et al. 2011). Therefore, we speculated that, under high salt conditions, K^+ translocation is likely mediated through the PeTPK1 channel, which functions as an outward rectifying channel that transfers K^+ from the vacuole to the cytoplasm, thus enabling tobacco BY-2 cells to maintain cytosolic K^+ homeostasis under salt stress.

Acknowledgments The research was supported jointly by the Fundamental Research Funds for the Central Universities (JC2011-2), the National Natural Science Foundation of China (31170570, 30872005), the Foundation for the Supervisors of Beijing Excellent Doctoral Dissertations (YB20081002201), the Beijing Natural Science Foundation (6112017), and the Key Projects of the Ministry of Education, PR China (209084). We thank Ms. Junqi Zhang and Meiqin Liu for their assistance in confocal analysis.

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