The Plant Journal (2004) **37**, 828–838 doi: 10.1111/j.1365-313X.2003.02008.x

The poplar K^+ channel KPT1 is associated with K^+ uptake during stomatal opening and bud development

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Received 30 September 2003; revised 26 November 2003; accepted 9 December 2003.

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Summary

To gain insights into the performance of poplar guard cells, we have measured stomatal conductance and aperture, guard cell K^+ content and K^+-channel activity of the guard cell plasma membrane in intact poplar leaves. In contrast to *Arabidopsis*, broad bean and tobacco grown under same conditions, poplar stomata operated just in the dynamic range – any change in conductance altered the rate of photosynthesis. In response to light, CO_2 and abscisic acid (ABA), the stomatal opening velocity was two to five times faster than that measured for *Arabidopsis thaliana, Nicotiana tabacum* and *Vicia faba*. When stomata opened, the K^+ content of guard cells increased almost twofold, indicating that the very fast stomatal opening in this species is mediated via potassium uptake. Following impalement of single guard cells embedded in their natural environment of intact leaves with triple-barrelled microelectrodes, time-dependent inward and outward-rectifying K^+-channel-mediated currents of large amplitude were recorded. To analyse the molecular nature of genes encoding guard cell K^+-uptake channels, we cloned K^+-transporter *Populus tremula* (KPT)1 and functionally expressed this potassium channel in a K^+-uptake-deficient *Escherichia coli* mutant. In addition to guard cells, this K^+-transporter gene was expressed in buds, where the *KPT1* gene activity strongly correlated with bud break. Thus, *KPT1* represents one of only few poplar genes associated with bud flush.

Keywords: K^+ channel, poplar, guard cell, bud, quantitative RT-PCR, EDXA.

Introduction

Populus advanced to the model angiosperm tree in view of the possibility to transform poplars, increasing expressed sequence tag (EST) collections, quantitative trait loci (QTL) analysis and proceeding genome project. Because some of the approximately 30 species within the genus *Populus* exhibit the fastest growth rates among the temperate trees, this woody species is well suited to answer tree-specific questions (for review, see Taylor, 2002). In order to select QTL linked to drought resistance and response to elevated CO_2, hybrid aspen have been screened for stomatal density and action (Chen *et al.*, 1997; Ferris *et al.*, 2002; Noormets *et al.*, 2001). A strong correlation between rate of photosynthesis and quantity/quality of stomata on one side and biomass production on the other indicates the possibility of employment of screenable parameters in early selection for high-vigour wood production.

Because of the long generation time of trees, their domestication has only just begun. To accelerate the knowledge about the basic principles of domestication of trees, molecular genetic approaches have focused on *Populus* species and hybrids. In addition and contrast to other plant models, such as the dicot weed *Arabidopis* and the monocot crop rice, *Populus* has genuine value as a tree for timber, plywood, pulp and paper.

Trees fundamentally differ from other plant species in that they are adapted to survive on a long timescale (up to 5000 years for some species). The most obvious difference to herbal species is the development of wood, a secondary xylem originating from the vascular cambium. In addition, trees undergo consecutive phases of growth and dormancy in vascular cambium and buds. A bud is a short axis generating densely packed series of leaf primordia

produced by the shoot apical meristem (Rohde *et al.*, 2000). While in dormant buds, which are surrounded by protective bud scales, the axis does not elongate and primordia do not develop, active buds elongate and embryonal leaves develop. Bud set and bud burst are the result of a complex integration between environmental (temperature and day length) and endogenous (hormone and metabolite status) signals (Rohde *et al.*, 2000). In spring, when buds break, the arranged embryonic leaves need to rapidly expand to enable stomata-regulated gas exchange. Up to now, little is known about the molecular basis of bud flush, while several molecular processes have been shown to be involved in bud set (cf. Rohde *et al.*, 2000, 2002). Dormancy is initiated by decreasing day length, resulting in peak levels of abscisic acid (ABA; Olsen *et al.*, 1997). Concomitantly, the expression of *PtABI3* is induced in young embryonic leaves, where it was shown to be required for the relative growth rate and differentiation, and in the subapical meristem and procambial strands (Rohde *et al.*, 2002). Other insights into these tree-specific questions have been gained by molecular breeding of trees through marker-assisted selection. A number of QTL's for yield-related traits have already been co-located on molecular genetic maps (Frewen *et al.*, 2000), e.g. the genes *ABI3* and *PhyB* were mapped and co-located with a QTL for bud burst and bud set (Frewen *et al.*, 2000). To study the pivotal role of potassium in cell elongation (cf. Peuke *et al.*, 2002), we here analysed annual changes in the K^+-transporter expression profiles of poplar buds and associated the K^+ channel KPT1 (K^+ transporter *Populus tremula* 1), to bud flush.

Besides QTL approaches, complementary models based on photosynthesis and gas exchange (stomatal conductance and activity) have been developed (Rauscher *et al.*, 1990). Screens with poplar ecotypes and hybrids on biomass production include, among other parameters, stomatal responsiveness to prevailing environmental conditions (Braatne *et al.*, 1992). Thereby, the effects to water stress, increased atmospheric CO_2, UV irradiation and ozone pollution were studied. Whereas the existence of a CO_2 sensor in the two guard cells surrounding the stomatal pore is accepted among plant physiologists (Roelfsema *et al.*, 2002 and papers cited therein), the nature of the cell type targeted by O_3 is discussed controversially (Noormets *et al.*, 2001; Torsethaugen *et al.*, 1999). The discrepancy may have arisen from data recorded on the herbal species *Vicia faba* on one side and *Populus* tree on the other. Thus, the advancement in stomatal physiology of trees requires cell biological, molecular and electrophysiological analyses of poplar guard cells.

To bridge this gap, we have adapted the double-barrelled voltage-clamp technique to single poplar guard cells in the natural surrounding of the intact poplar leaf, characterised the plasma membrane K^+ channels, identified the K^+ channel gene *KPT1*, and expressed it in an *Escherichia coli* K^+-transport-deficient mutant.

Results

Dynamics and velocity of stomatal movement

To determine the stomatal opening and closing velocity, we excised young, fully expanded leaves from 1- to 2-year-old poplar plants and mounted them into a gas exchange chamber. Using infrared gas analysers, we followed the light-, CO_2- and ABA-dependent changes in photosynthesis (CO_2 uptake) and transpiration (cf. Hedrich *et al.*, 2001; Szyroki *et al.*, 2001). Upon illumination of dark-adapted leaves, stomata opened, and CO_2 uptake and transpiration rapidly increased (Figure 1a, left and right). In the absence

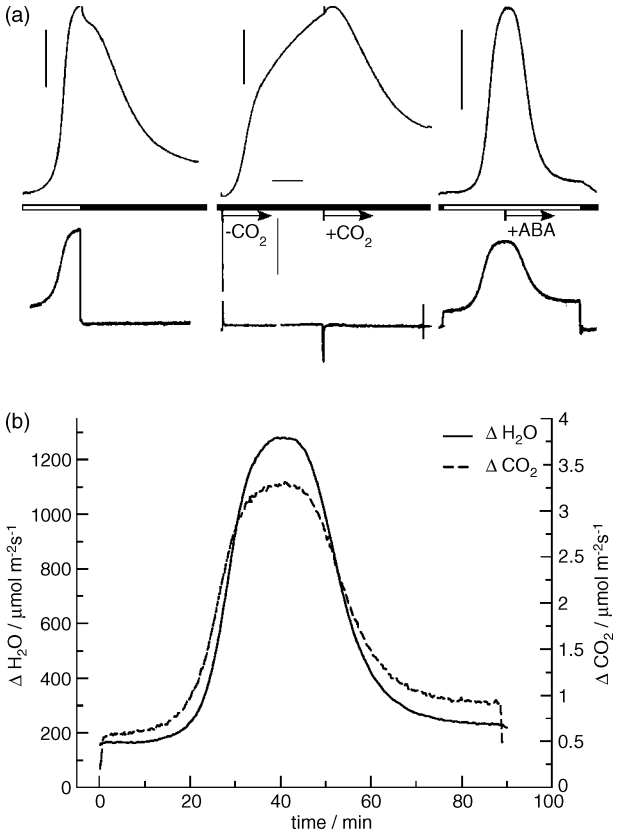

Figure 1. Transpiration and photosynthetic activity of poplar leaves.
(a) Horizontal bar denotes 20 min; vertical bars in upper figures reflect an evaporation of 0.4 mmol m^{-2} sec^{-1} and in lower figures a change in CO_2 uptake of 3 µmol m^{-2} sec^{-1} (left) at 300 p.p.m. CO_2. Stomata open upon light exposure and close again in darkness (as indicated by the bar). In CO_2-free air (middle), stomata open in darkness and close when an atmosphere of 300 p.p.m. of CO_2 is re-established. In the light and 300 p.p.m. CO_2 (right), stomata open and close after addition of 10 µM ABA.
(b) Dynamics of evaporation (continuous line) and CO_2 uptake (dashed line) upon exposure to white light at $t = 0$ min and after addition of 10 µM ABA to the leaf at $t = 40$ min.

Table 1 Physical parameters of guard cells

	A. thaliana	*V. faba*	*N. tabacum*	*P. tremula* × *P. tremuloides*
Stomata per mm^2	188 ± 44	72 ± 21	90 ± 55	191 ± 28
Guard cell volume (pl)	0.5 ± 0.0	3.4 ± 0.1	3.4 ± 0.1	0.3 ± 0.0
Guard cell surface (μm^2)	390 ± 9	1380 ± 20	1300 ± 30	267 ± 6
Guard cell surface/volume (μm^{-1})	0.75	0.39	0.38	0.94
Opening surface per cell pair, open (μm^2)	39 ± 2	93 ± 4	92 ± 7	21 ± 1
Opening surface per cell pair, closed (μm^2)	4 ± 0.3	26 ± 1.6	17 ± 2.0	9 ± 0.5
Water conductance, open (mm sec^{-1})	1.0 ± 0.2	2.7 ± 0.6	2.4 ± 0.2	5.3 ± 0.2
Water conductance, closed (mm sec^{-1})	0.2	0.5	0.5	1.1
Opening rate (μmol m^{-2} sec^{-2})	1.0 ± 0.3	0.7 ± 0.1	0.5 ± 0.1	2.5 ± 1.4
Closure rate (μmol m^{-2} sec^{-2})	4.4 ± 0.3	1.9 ± 0.1	1.4 ± 0.2	2.3 ± 1.3
K^+ channels per μm^2	1.4–2.3	1.2 ± 0.7	2.5 ± 1.5	2.8–9.1
Single K^+ channel conductance (pS)	6–10	4–8	3–5	4–13
I/*A* (pA μm^{-2})	1.7	1.2	1.4	4.4
I/*V* (pA/pl)	1210	490	540	4034
I_{ges} (pA)	640	1660	1820	1170

[a]Closure rate was measured in response to ABA application (cf. Figure 1a, upper panel, right trace).
The long axis and perpendicular to this, the widest distance between the cuticular lips of guard cells from *A. thaliana*, *V. faba*, *N. tabacum* and *P. tremula* × *P. tremuloides* were measured and physical properties calculated. Electrophysiological data were taken from Dietrich *et al.* (1998); Kwak *et al.* (2001) and in the case of *Populus* calculated from total current of single guard cells of intact leaves. Values are mean ± SEs, $n \geq 10$. Note that the data (guard cell volume) on *V. faba* are in agreement with Humble and Raschke (1971).

of CO_2, stomatal opening rates in the dark were as fast as with ambient CO_2 in the light (Figure 1a, middle). When compared to *Nicotiana tabacum* grown under similar conditions, poplar stomatal opening velocity triggered by light- and CO_2-free air was up to five times higher than that measured for this herb species (Table 1). In all other species, tested stomatal opening was slower. When superimposing the light- and ABA-induced changes in water vapour and CO_2 exchange, it is apparent that poplar stomata operate just in the dynamic range – any change in conductance altered the rate of photosynthesis (Figure 1b). In response to a sudden illumination of pre-shaded leaves, the increase in photosynthesis preceded stomatal opening. This is most likely because of a drop in leaf CO_2 concentration and thus increase in the inward-directed gradient for CO_2 uptake (Hanstein and Felle, 2002). In this context, it should be mentioned that in a previous study on *V. faba* leaves, we could demonstrate that photosynthetically active red light affects stomatal opening via a change in the substomatal CO_2 concentration (Roelfsema *et al.*, 2002).

The extremely fast gas exchange of poplar guard cells could result from a high stomatal density and optimal guard cell surface to volume ratio compared to the other species tested. In poplar, the number of guard cells per square millimetre lower epidermis was about 190 and thus similar to *Arabidopsis* (Table 1). On the basis of stomata size, poplar is comparable to the tiny *Arabidopsis* guard cells (surface of poplar guard cell 267 μm^2 compared to 390 μm^2 in *Arabidopsis*; Table 1). In contrast, the surfaces of broad bean and tobacco guard cells were up to five times larger. In the model weed *Arabidopsis thaliana*, however, the opening velocity of guard cells was 2.5-fold lower than in the poplar tree (Table 1). The closing rate of *Populus* guard cells was 2.3 μmol m^{-2} sec^{-2}, a velocity similar to that of *V. faba* (1.9 μmol m^{-2} sec^{-2}), about two times lower than in *Arabidopsis* and almost twofold higher than in *Nicotiana* (Table 1). The calculated change in surface area for an open–close transition is about twofold, a number in the range of values measured for *Vicia* (fourfold), but much lower than those obtained for *Arabidopsis* and *Nicotiana* (10- and 5-fold).

Potassium and chloride content of guard cells in open and closed stomata

To address the question about difference in stomatal opening velocity between poplar and other species, the potassium and chloride content of poplar guard cells was determined by X-ray microanalysis. Open stomata were analysed in illuminated leaves exposed to CO_2-free air. Leaves with closed stomata were harvested in the dark 2 h after the leaves were exposed to ABA. For scanning electron microscopy and energy dispersive X-ray (EDX) analysis, mature leaves were harvested. Small sections of leaf tissues were excised and immediately shock-frozen in liquid isopentane. After freeze-drying, the samples were coated with chromium and examined in a scanning electron microscope (Figure 2b, upper) attached to an EDX microanalysis system. Element-specific X-ray spectra for K, Cl, Ca and P (Figure 2a) were determined with guard cells of open and closed stomata (Figure 2b). Relative potassium and chloride concentrations were expressed as peak values from five recorded spectra (Figure 2b, lower). In open

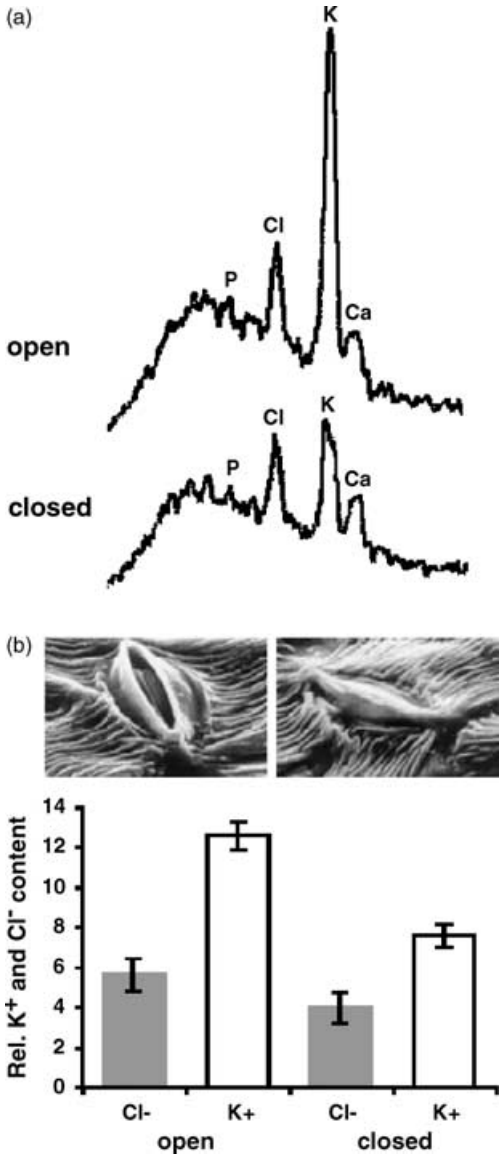

Figure 2. EDX analysis of poplar guard cells.
(a) Element-specific EDX spectra of poplar guard cells in open (upper) and closed (lower) state. Compared to closed stomata potassium levels reach threefold higher contents when stomata open.
(b) Relative chloride and potassium concentrations of stomata in open and closed states expressed as peak values from five recorded spectra. After stomatal closing, guard cell chloride concentration was reduced to 70% and potassium to 60%. Upper: scanning electron micrograph of primary open and closed poplar stoma.

stomata, the potassium content was 1.6 and the Cl content was 1.4 times higher than in the dark.

Dietrich *et al.* (1998) have shown that the properties of potassium uptake channels in guard cells differ among species. As poplar guard cells drive their osmotic motor on the basis of potassium salts, just like the few herb species investigated before, unknown tree-specific transporter properties or densities might explain the difference in stomatal opening velocity between poplar and *Arabidopsis*.

Electrical properties of the guard cell K^+-uptake channel

Studies on the stomatal physiology and membrane biophysics of woody species require proper access to the individual guard cells. Best suited for minimal invasive studies on the properties of ion channels and pumps in single guard cells of intact plants are multibarrelled microelectrodes (Roelfsema *et al.*, 2001, 2002). Therefore, we mounted young 2–4-cm long leaves from 8- to 10-week-old poplar plants onto an upright microscope. Under microscopic inspection, microelectrodes were inserted into selected lower epidermis guard cells (Figure 3a). After gaining access to the cytoplasm (indicated by the ionophoretically injected fluorescent dye Lucifer yellow through one of the barrels; Figure 3b), time-dependent inward and outward K^+ currents could be evoked by membrane potential changes (Figure 3c,d). With the membrane potential clamped at a holding potential of −80 to −100 mV, hyperpolarising voltage pulses (2 sec) activated inward-rectifying currents. Current activation was visible at membrane potentials negative of −140 mV (Figure 3e). Membrane depolarisation positive of −80 mV elicited outward-rectifying currents with a slow activation kinetic (Figure 3c,e). Both steady-state K^+ currents did not inactivate during prolonged depolarisation (data not shown but cf. Ache *et al.*, 2000). Tail current analysis revealed reversal potentials of −70 mV for outward ($n = 7$) and −100 mV ($n = 6$) for inward-rectifying currents in 10 mM K^+ bath solution. Increasing the external K^+ concentration shifted the reversal potential (V_{rev}) in a K^+-dependent manner in line with the K^+-selectivity of these channels (data not shown). Thus, the inward rectifier represents an ion channel, capable to mediate K^+ uptake into poplar guard cells, driven by a hyperpoplarised membrane potential. From the steady-state inward K^+ currents, a current density of 4.4 pA μm^{-2} of poplar guard cell surface was calculated (Table 1). This current density and thus the number of active K^+ channels are higher to those found with the other species.

Molecular analysis of Shaker-type K^+ channels in poplar guard cells

Previous studies on guard cells of the model plant *Arabidopsis* have shown that the guard cell inward rectifier is composed of different K^+-channel α-subunits belonging to the plant *Shaker* channel family with *A. thaliana* inward rectifying potassium channel 1 (KAT1) representing the dominant transcript (Szyroki *et al.*, 2001). In contrast, the outward rectifier is assembled from just one channel subunit *A. thaliana* guard cell outward rectifying K^+ channel (GORK) (Ache *et al.*, 2000; Hosy *et al.*, 2003). To examine the molecular basis of channel-mediated K^+ transport in poplar, we searched the EST database from *P. tremula* × *P. tremuloides* (Sterky *et al.*, 1998), for

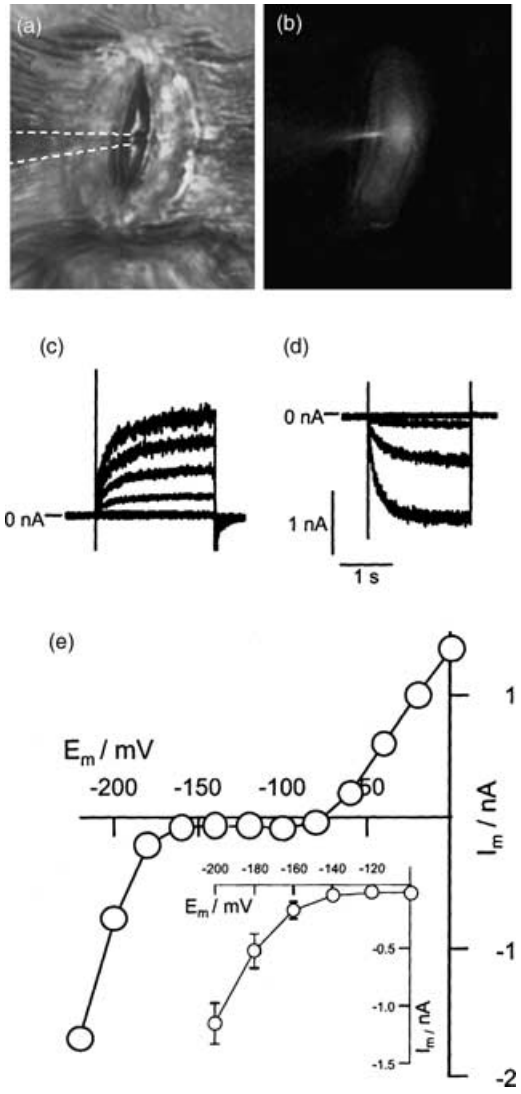

Figure 3. K^+-channel currents in poplar guard cells.
Populus guard cell, impaled with triple-barrelled microelectrode under (a) transmission and (b) fluorescent light. The cell was electrophoretically loaded with Lucifer yellow. Dye concentration in loading barrel was 0.5 mM. (c) K^+ currents carried by outward rectifier upon depolarisation. (d) Inward-rectifying K^+ currents elicited by hyperpolarisation. (e) *I–V* relation corresponding to (c) and (d). Traces on (c–e) are from the same cell. (e, inset) Average *I–V* curve for inward rectifier. Error bars represent ±SE ($n = 7$).

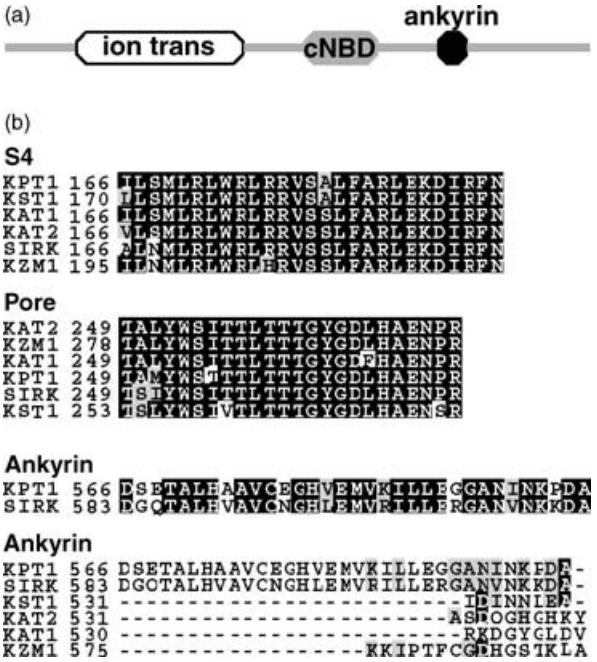

Figure 4. The *KPT1* gene encodes a *Shaker*-like K^+ channel.
(a) Schematic presentation of the predicted domains of KPT1. The ion channel consists of a transport domain (ion *trans*) with six linked transmembrane domains and a conserved pore region, a cyclic nucleotide-binding site (cNBD) and additionally an ankyrin domain.
(b) Alignment of KPT1 (AJ344623), SIRK (AF359521), KAT1 (NM_123993), KAT2 (NM_117939), KZM1 (AJ421640) and KST1 (X79779) S4, pore and ankyrin. Identical amino acids are labelled in white with black background; similar amino acids are shown in black on a grey background. Alignments were generated with CLUSTALW on EBI server (http://www.ebi.ac.uk/clustalw/) and represented on Boxshade Server (http://www.ch.embnet.org/software/BOX_form.html).

sequence homologies, to K^+ transporters known. Thereby, we isolated DNA fragments with relevant homologies to *Arabidopsis* guard cell potassium channels (Szyroki *et al.*, 2001). Following complete sequencing of these fragments, we identified distinct orthologues to guard-cell-expressed K^+ channel of the KAT1-type, *A. thaliana* potassium channel (K^+ transporter AKT)2/3 and GORK (for review, see Very and Sentenac, 2002). Based on ESTs and degenerated primers against conserved K^+-channel domains (Ache *et al.*, 2001), we cloned the corresponding full-length cDNAs and named the KAT1 orthologue KPT1 (GenBank Accession number AJ344623; Langer *et al.*, 2002). KPT1 exhibited all structural features of members of the 'green' *Shaker* channel family: six transmembrane domains and an amphiphilic linker – containing the K^+ selectivity filter – between transmembrane segments 5 and 6 (Hedrich and Becker, 1994), and an ankyrin-like domain at the C-terminus (cf. Pratelli *et al.*, 2002). Based on amino acid alignments with known plant K^+ channels, KPT1 showed highest homologies to guard cell K^+-uptake channels of the KAT1-type from *Arabidopsis* (Figure 4), potato, maize and grapevine (Mueller-Roeber *et al.*, 1995; Philippar *et al.*, 2003; Pratelli *et al.*, 2002). So far, KAT1-type channel with ankyrin-like domains have been identified in *Populus* and *Vitis vinifera* only. In a previous study on the molecular mechanism of potassium-dependent wood growth, we identified *P. tremula* potassium channel (PTK)2 as an AKT2/3 orthologue and *P. tremula* outward rectifying potassium channel (PTORK) as a member of the *A. thaliana* stelar K^+ outward rectifier (SKOR)/GORK family (Langer *et al.*, 2002). KPT1, sharing closest homologies to the KAT1-like guard cell K^+ channels, was found in guard-cell-enriched epidermis peels (Figure 5a). In roots and shoots, almost no *KPT1*-specific

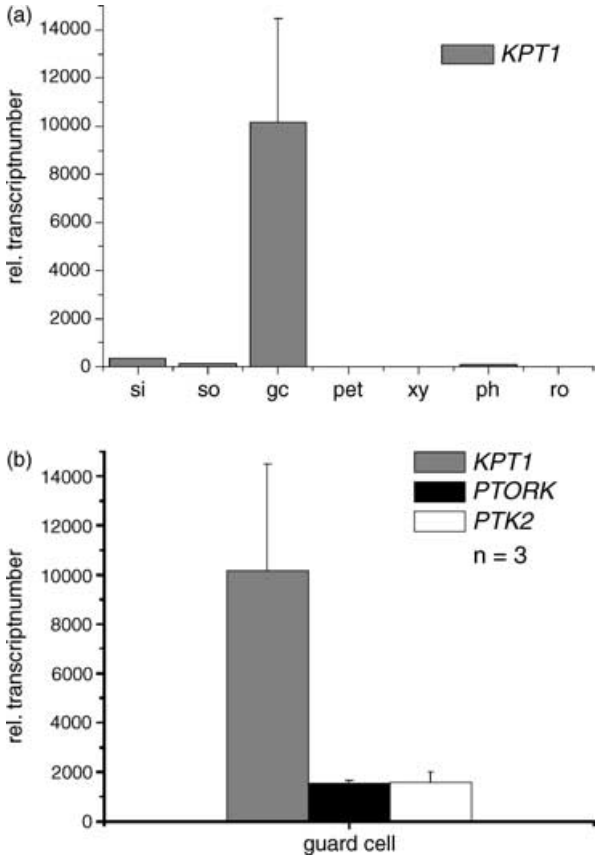

Figure 5. Expression pattern of *KPT1* analysed by quantitative RT-PCR. Quantification of *KPT1* transcripts calculated by external standards relative to actin.
(a) High amounts of *KPT1* transcripts were detected in guard-cell-enriched fractions only. Total RNAs isolated from sink (si) and source leaves (so), guard cells (gc), petioles (pet), xylem (xy), phloem (ph) and roots (ro) were analysed with primers specific for poplar potassium transporters.
(b) *KPT1*, *PTORK* and *PTK2* expression in guard-cell-enriched epidermal fractions.

mRNA could be detected by quantitative real-time PCR. In the 'guard cell' fractions, *PTK2* and *PTORK* co-localised with *KPT1* (Figure 5b). Close inspection of the *KPT1*, *PTK2*, as well as the *PTORK* promoter, identified guard cell localisation elements (cf. Langer, 2003; Mueller-Roeber *et al.*, 1995; Plesch *et al.*, 2001). This indicates that PTK2 and PTORK, which have been functionally expressed in *Xenopus* oocytes and characterised as K^+-selective channels before (Langer *et al.*, 2002), mediate K^+ transport in poplar guard cells just as their counterparts in *Arabidopsis*.

KPT1 functionally complements K^+-uptake-deficient E. coli *mutant*

Unfortunately, *KPT1*, like *SPICK1*, *SPICK2* and *SPORK1* from the rain tree *Samanea saman* (Moshelion *et al.*, 2002a), when injected into *Xenopus* oocytes, did not mediate K^+ currents. To test whether KPT1 is capable to serve as a K^+-uptake channel, we expressed KPT1 in the *E. coli* strain LB2003, lacking the K^+-uptake systems, Trk, Kup and Kdp. This mutant does not grow on K^+-limited media (Uozumi *et al.*, 1998), but grows on media containing high K^+ contents (Figure 6a). Cells transformed with the empty vector pCRII TOPO did not grow in media supplemented with 3 mM K^+, while *E. coli*, expressing the KAT1 orthologue KPT1, formed colonies (Figure 6b). These results demonstrate that KPT1 subunits from poplar guard cells form functional K^+-uptake channels in *E. coli*.

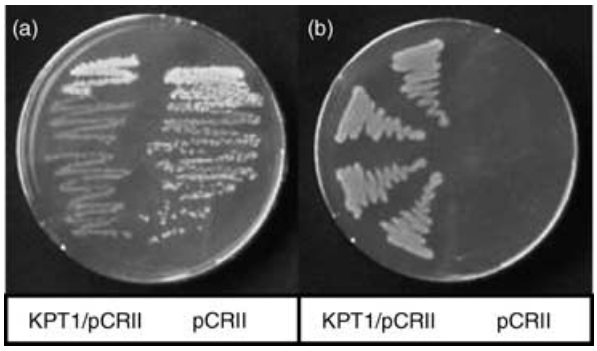

Figure 6. Functional expression of KPT1.
(a) Growth of LB2003 (lacking the bacterial K^+-uptake systems Kdp, TrkA and Kup) transformed with KPT1 (left) and the empty plasmid pCRII TOPO (right) on KML plates with high potassium content (about 134 mM).
(b) On low potassium medium (3 mM K^+), only KPT1 complements growth of *E. coli* strain LB2003. Growth of LB2003 transformed with KPT1 (left) or the empty plasmid pCRII TOPO as control (right).

Co-expression of KPT1 with other α-subunits

The injection of *KPT1* cRNA into *Xenopus* oocytes did not result in any detectable K^+ whole cell currents compared to water-injected oocytes (data not shown). This behaviour is in agreement with studies on AKT1 and *V. faba* potassium channel (VFK1), which do not exhibit K^+-channel activity in oocytes either (Ache *et al.*, 2001 and references cited therein). Co-expressing VFK1 with the KAT1 mutant T256G (Uozumi *et al.*, 1995), however, fundamentally changed the electrical properties of the KAT1 mutant, indicating the formation of heteromeric channels between the subunits of VFK1 and KAT1 T256G (Ache *et al.*, 2001). To test if KPT1 could also form heteromeric channels and thereby alter the biophysical properties of K^+-conducting subunits, we studied KPT1 in the presence of KAT1 T256G, PTK2 and PTORK (Langer *et al.*, 2002; Figure 7a–c). The KAT1 mutant T256G exhibits a higher permeability for Rb^+ compared with K^+ (Uozumi *et al.*, 1995). This mutant characteristic (data not shown), as well as the protein expression level of KAT1 T256G, was not changed in the presence of KPT1 (Figure 7a). Co-expressing KPT1 together with the outward-rectifying K^+ channel PTORK, however, decreased the whole cell K^+ current by 73 ± 9% at a membrane potential

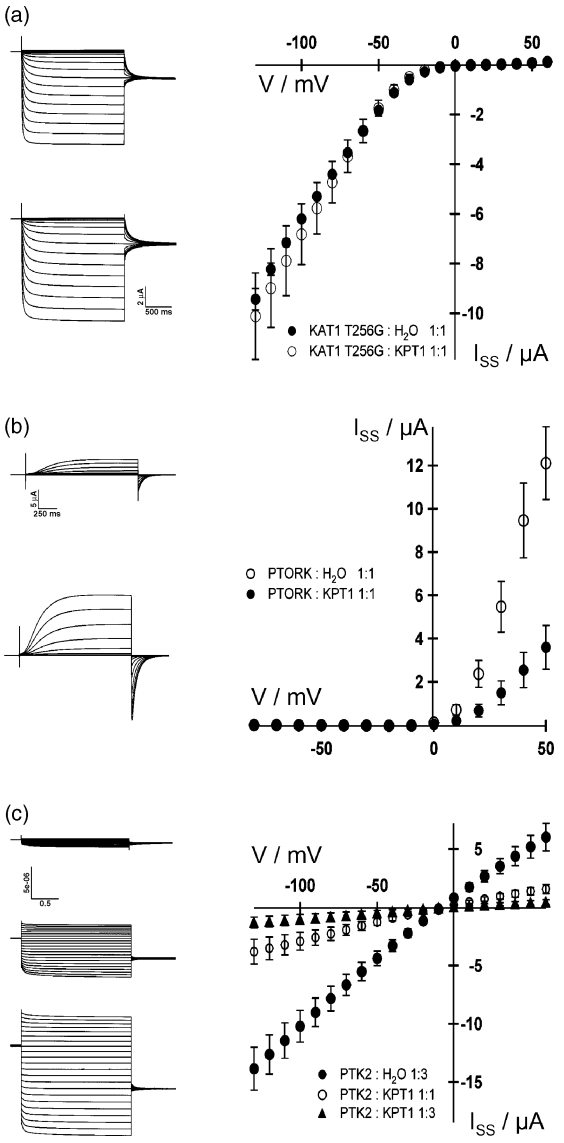

Figure 7. KPT1 co-expressed in *Xenopus* oocytes.
(a) Current responses to voltage pulses from 60 to −130 mV (10 mV increments), followed by a voltage pulse to −60 mV, are shown for representative oocytes injected with KAT1 T256G (lower traces) or co-injected with KAT1 T256G and KPT1 (upper traces). The corresponding steady-state current amplitudes and voltage dependence of the KAT1 mutant remain unaffected by KPT1.
(b) Whole cell currents of *PTORK* (lower traces) injected and *KPT1–PTORK* (upper traces) co-injected oocytes. From a holding potential of −100 mV, the membrane voltage was successively stepped from −110 to 50 mV in 10 mV decrements. The PTORK steady-state current amplitude of the co-injected oocytes is reduced by 73 ± 9% at a membrane potential of 40 mV compared to oocytes injected with *PTORK* cRNA alone (cf. *I–V* plot; mean ± SE, $n = 6$).
(c) Whole cell currents of *PTK2* (lower traces) injected and *KPT1–PTK2* (1 : 1, middle traces; 3 : 1, upper traces) co-injected oocytes. Alike PTORK, PTK2 currents in the presence of KPT1are reduced up to 90 ± 3% at −120 mV. 2.5 sec voltage pulses were applied in the range of 60 to −130 mV in steps of 10 mV. The *I–V* plot depicts the reduction of PTK2 currents with increasing amounts of injected *KPT1* cRNA. The ratio between the injected amount of *PTK2* cRNA and *KPT1* cRNA is indicated. Data in (a) and (b) are presented as the mean ± SE ($n = 3$).

of 40 mV, compared to oocytes injected with the same amount of *PTORK* cRNA (Figure 7b). Biophysical properties like voltage dependence and activation kinetics of PTORK retained unchanged. This indicates that KPT1 reduces the number of active channel complexes. Alike PTORK, PTK2 steady-state currents (I_{SS}) decreased in the presence of KPT1 subunits by 90 ± 3% at −120 mV (Figure 7c). The co-injection of *KPT1* with *PTK2* did not result in a change of the voltage dependence of PTK2. In order to exclude changes in the protein expression level of PTK2, we subjected oocyte membranes to SDS page and Western blot analysis. Membranes of *PTK2-/KPT1*-injected oocytes exhibit signals comparable to *PTK2*-injected ones (data not shown). These results point to the formation of heteromeric channels between KPT1 subunits and PTORK, as well as PTK2 subunits. Thus, KPT1 seems to exert a dominant negative effect on ankyrin domain containing K^+-channel α-subunits, but not the ankyrin-free KAT1.

KPT1 *is expressed during bud burst*

To localise additional sites of *KPT1* expression other than guard cells, we isolated RNA from leaves, roots, epidermal fragments, petioles, xylem, phloem and buds from young branches (Figures 5 and 8) for quantitative RT-PCR analyses. Thereby, we found *KPT1* expressed in buds too. Young branches were cut in early spring before bud burst and incubated in a climate chamber under long-day conditions (22°C/day and 17°C/night). When comparing dormant buds with early active or opened buds, the highest amounts of *KPT1* transcripts were measured in opened buds (Figure 8a,b). *KPT1* transcription associated with 'climate chamber-controlled' bud burst shows that this K^+-uptake channel is associated with K^+-dependent expansion during early leaf development (Krabel, 2000). To test this hypothesis, we compared the expression level of *KPT1* in buds from the same poplar hybrid growing in the botanical garden throughout the year (Figure 8b). From bud set in early June until April, the *KPT1* mRNA level remained at a low background level. Within the few days in April, the temperature rose from about 10 to 20°C, inducing bud burst. Bud flush was accompanied by a sudden increase in *KPT1* message, which rapidly decayed when bud burst was completed. In contrast, the *PTK2* and *PTORK* messages in buds did not change throughout the year (data not shown).

Discussion

KPT1 represents the major K^+-uptake channel in guard cells of poplar (Figure 5; Langer *et al.*, 2002). The plasma membrane potassium conductance recorded in guard cells of intact poplar leaves resulted in inward- and outward-rectifying K^+ currents of large amplitude (Figure 3). But alike AKT1, VFK1 and the K^+ channel isolated from *S. saman*

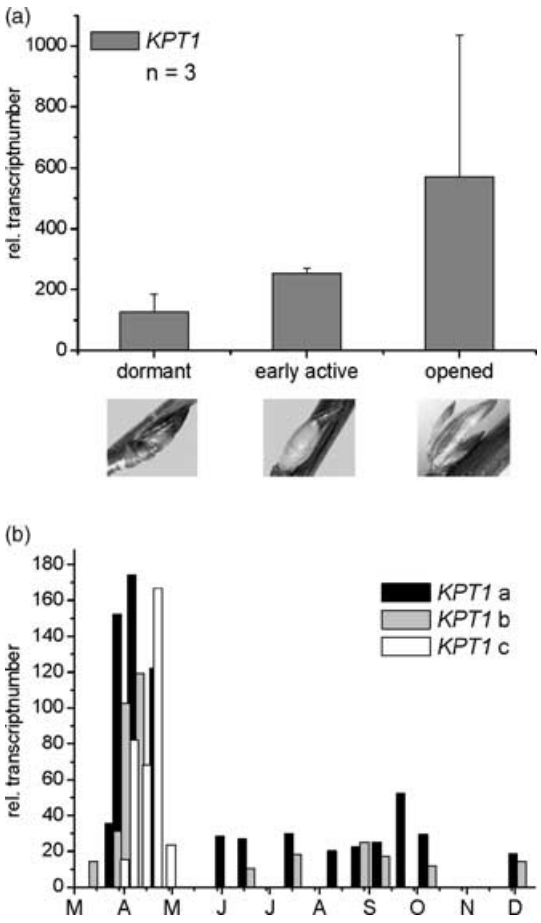

Figure 8. Changes in *KPT1* transcripts of poplar bud RNA.
RNA samples were analysed by quantitative RT-PCR and calculated relative to actin using external standards.
(a) Transcript level of *KPT1* upon artificial maturation increases before and during bud burst. Young branches with buds were cut at the end of February and incubated in a climate chamber under long-day conditions until they opened. Total RNA from non-green buds, harvested in the dormant stage, in an early active, green stage and when buds burst, was analysed by quantitative RT-PCR. Error bars indicate SEs.
(b) Seasonal changes in *KPT1* transcripts of poplar buds. All transcript levels are low during summer, autumn and winter, while *KPT1* mRNA rises in spring before buds open in April (11th and 25th). New buds did not appear before the beginning of June. Total RNA was isolated from buds of 1- and 3-year-old trees in 2002 (a,b) and of a 4-year-old tree in 2003 (c).

(Moshelion *et al.*, 2002a,b), KPT1 did not express K^+-channel activity in *Xenopus* oocytes (Ache *et al.*, 2001 and references cited therein). As already shown for KAT1/AKT3 heteromers (Baizabal-Aguirre *et al.*, 1999; Dreyer *et al.*, 1997), however, KPT1, PTORK and PTK2 subunits interact with each other, resulting in decreased PTORK and PTK2 currents in *Xenopus* oocytes compared to oocytes injected with only *PTORK* and *PTK2* (Figure 7c; Langer *et al.*, 2002). Oocytes co-injected with KPT1 and KAT1 T256G, however, resembled just the electrical properties of the KAT1 mutant rather than a dominant negative reduction of the K^+-current amplitude (Figure 7a). These results point to a species-specific interaction of KPT1 and PTK2 contributing to active guard cell K^+-uptake channels.

In contrast to the inward rectifier, the outward-rectifying potassium channel of *Arabidopsis* guard cells is formed by just one subunit, GORK. Studies by Ivashikina *et al.* (2001) and Hosy *et al.* (2003) provided clear evidence that the guard cell and root hair K^+-release channel represents the gene product of GORK only. As PTORK shares properties with GORK expressed in *Xenopus* oocytes, as well as the guard cell outward rectifier in its natural environment of the poplar leaf, PTORK very likely represents the guard cell K^+-release channel. In contrast to its *Arabidopsis* orthologue, however, PTORK currents are reduced in *Xenopus* oocytes if PTORK is co-expressed together with the KAT1-like KPT1 inward rectifier (Figure 7b), indicating an interaction of these two K^+-channel subunits *in vivo*, probably via their ankyrin-like domains.

Besides the reduced current amplitude, the co-expression of neither PTK2 nor PTORK together with KPT1 led to changed biophysical properties of PTK2 or PTORK currents in oocytes. Thus, in contrast to *E. coli*, in oocytes, the channel-forming domains seem not to have been functionally inserted into the membrane. However, the reason why some K^+ channels do not functionally express or insert in the membrane remains unclear.

KPT1 shares sequence homology with the KAT1-type *Shaker* channel subfamily. Among them, the K^+ channels from the woody species, KPT1 from poplar and SIRK from grapevine (stomatal inward-rectifying K^+ channel) represent the only members which contain an ankyrin domain (Pratelli *et al.*, 2002). Besides guard cells, *SIRK* is expressed in the berries during the first stages (before veraison) of berry growth. Similar to *KPT1* – one of only a few genes so far shown to be associated with bud break – *SIRK* is expressed in a very narrow window of berry development characterised by K^+-dependent cell expansion. Thus, the transient induction of *KPT1* before bud burst (Figure 8b) seems to provide for uptake of osmotically active potassium ions, turgor-driven cell expansion and stomatal opening. In this context, it should be mentioned that KAT1-related channels in *Arabidopsis* have been shown to be regulated by the growth hormone auxin (Philippar *et al.*, 2004). This may indicate that during bud flush, changes in auxin concentration affect the transcript regulation.

Transpiration is governed by a number of biophysical and environmental factors, including size and density of stomata, degree of stomatal opening, solar radiation, temperature, humidity and boundary-layer conditions. In contrast to densely packed societies of herbal species or crop stands, solitaire trees are often exposed to dry air and wind. Under well-watered conditions, 5-year-old poplar trees transpire about 100–200 l per day or 600 mmol m^{-2} sec^{-1} (Chappell, 1997). In response to atmospheric and soil–water

deficits, however, poplar trees in general and some hybrids in particular rapidly close their stomata. Increased drought avoidance enable hybrids to maintain higher leaf areas for longer periods during a drought cycle than native species (Braatne *et al.*, 1992). The optimisation of carbon gain (photosynthesis) relative to water loss requires fast responding guard cells. This capability of poplar guard cells is reflected by the very rapid stomatal opening and closure in response to light (Figure 1). Furthermore, trees exhibit a dynamic response to sun flecks. Depending on the local light environment, sun and shade leaves differ in the rate at which they open their stomata (Naumburg *et al.*, 2001 and references therein). As the increase of guard cell potassium during stomatal opening and K^+-channel-mediated current densities were comparable to the herb species tested, poplar guard cells seemed to operate extremely fast light-signalling pathways. Thus, *Populus* guard cells represent a good model system for stomatal action of trees. Stomata are autonomous cell pairs that integrate signals received from the plant on one side and the atmosphere on the other. Future single guard cell studies on poplar hybrids differing in the stomatal density, water conductance and velocity of guard cell response to light and drought/ABA will provide new insides into the interaction of tree stomata with their biotic and abiotic environments.

Experimental procedures

Plant growth conditions

Populus tremula × *P. tremuloides* plants (clone T89) were grown in soil under natural conditions or in hydroponic culture under long-day conditions (16 h light (22°C) : 8 h darkness (17°C); TLD 58 W/840 Super 80; Philips, the Netherlands and 58 W L58/77, Osram, Germany). Buds (apical and axillary) were collected from two different trees of the same hybrid (1 and 3 years old at the beginning of analysis) over a period of 2 years in 2002 on 28 February; 14 and 28 March; 2, 11 and 25 April; 5 and 19 June; 18 July; 14 and 29 August; 11 and 26 September; 10 October and 5 December. On 11 (1-year-old tree) and 25 (3-year-old tree) April, buds opened. In June, new buds developed. In 2003, buds were collected from a meanwhile 4-year-old tree on 13 and 27 March, and 10, 17 and 25 (bud burst) April. Twigs with buds for 'climate-chamber-controlled' bud development were cultured, after natural chilling, in a climate chamber under long-day conditions in hydroponic culture.

V. faba, *N. tabacum* and *A. thaliana* plants were grown in a green house for 4–6 weeks.

Gas exchange measurements

Gas exchange measurements were performed as described before by Hedrich *et al.* (2001).

Physical parameters of guard cells

For measurement of stomatal aperture and guard cell size, whole leaves or epidermal peels were kept in darkness and incubated with 5 μM ABA for 30 min (closed stomata) or illuminated with 300 μmol m^{-2} sec^{-1} white light (open stomata). After the respective treatments, samples were immediately transferred to a video microscope to collect images for off-line analysis of stomatal aperture and guard cell shape. From these images, the long axis, and perpendicular to this, the widest distance between the cuticular lips were measured, and two circle segments were calculated that led through the end points of the long axis and the point of widest opening on each side. The area between these two segments was taken as the opening area of one stoma. By measuring the width of a guard cell and its bending radius, a torus with two half spheres at the ends was used to approximate the guard cell's volume and surface area ($n \geq 10$).

Determination of guard cells' ionic content

To analyse open stomata, potted *P. tremula* × *P. tremuloides* plants were kept under CO_2-free air in a controlled environment at 20°C and a photon flux density of 300 μE m^{-2} sec^{-1}. Mature leaves were harvested at noon for light-electron microscopy and X-ray microanalysis. Leaves with closed stomata were gained at night 2 h after the leaf was exposed to 100 μM ABA.

Specimen preparation for scanning electron microscopy. For scanning electron microscopic observations, small pieces of leaves were freeze-dried and coated with chromium. A scanning electron microscope (AMR 1200, Leitz, Wetzlar, Germany) was utilised at 15 keV.

X-ray microanalysis. Small leaf sections were immediately shock-frozen in liquid isopentane. After freeze-drying, the samples were coated with chromium and examined with a scanning electron microscope (AMR 1200, Leitz) equipped with an EDX microanalysis system (KEVEX 4000). Element-specific X-ray spectra were obtained from open and closed guard cells using a reduced scan raster area at 1.000× magnification. Relative potassium and chloride concentrations were expressed as peak values from five recorded spectra.

Impalement measurements of poplar guard cells

Electrical measurements on intact leaves were carried out as described by Roelfsema *et al.* (2001), with minor modifications. Triple-barrelled microelectrodes were pulled from borosilicate glass capillaries (GC100F-10, Clark Electromedical Instruments, Pangbourne, Reading, UK). Resistance of each single barrel filled with 300 mM KCl was around 100 MΩ. Electrophoretic dye injection was achieved by application of constant or pulsing negative current of up to 1 nA, depending on initial velocity of fluorescence intensity rise. Time- and voltage-dependence of inward- and outward-rectifying currents were studied under voltage-clamp condition. Starting from holding potential of −100 mV, a series of 2 sec voltage steps to −220 mV, respectively, 0 mV were applied in 20 mV increments. To identify the ionic nature of the currents observed, the reversal potentials were determined. After a preactivation pulse to −240 mV (for inward-rectifying current) or +40 mV (for outward-rectifying current), the membrane potential was stepped to depolarising, respectively, hyperpolarising potentials in 10 mV increments. All measurements were corrected for surface potential changes to analyse the reversal of the tail currents (cf. Roelfsema *et al.*, 2001), using blunt microelectrodes in contact with the guard cell wall. The mean surface potential of leaves of *Populus* guard cell was around −35 mV.

Expression analysis by quantitative real-time RT-PCR

RNA of sink and source leaves, guard-cell-enriched fractions (for details, see Becker *et al.*, 1993), petioles, xylem, phloem (for details, see Tuominen *et al.*, 2000), roots and buds was isolated using the Plant RNeasy Extraction Kit (Qiagen, Germany). DNA was digested on-column during RNA purification (RNase- Free DNase Kit; Qiagen, Germany). cDNA was prepared using the M-MLV Reverse Transcriptase (Promega, USA) and an Oligo $(dT)_{25}$ primer, and diluted 20-fold in water for quantitative RT-PCR. PCR was performed in a LightCycler™ (Roche Molecular Biochemicals, Switzerland) with the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals, Switzerland). Primers used were (TIB MOLBIOL, Germany): PtACT2fwd 5′-CCCAGAAGTCCTCTT-3′, PtACT2rev 5′-ACTGAGCACAATGTTAC-3′; KPT1LCfw 5′-GATGTCCCCATGATAGG-3′, KPT1LCrev 5′-CATGATGTATTGCGCT-3′; PTORKLCfw 5′-CAGGGGCATCACTGGCA-3′, PTORKLCrev 5′-GGTAACCACCTGAAGAT-3′ and PTK2LCfw 5′-ATGCGATATACACCTG-3′, PTK2LCrev 5′-TGCTCACCCTAATACA-3′.

All quantifications were normalised to actin cDNA fragments amplified by PtACT2fwd and PtACT2rev. These fragments are homologous to the constitutively expressed *Arabidopsis* actins 2 and 8 (for details, see Szyroki *et al.*, 2001 and references therein). Each transcript was quantified using individual standards. To enable detection of contaminating genomic DNA, PCR was performed with the same RNA as template, which was used for cDNA synthesis. All kits were used according to the manufacturers' protocols.

Two-electrode voltage clamp

For heterologous expression in *Xenopus laevis* oocytes, the *KPT1*-cRNA was prepared using the mMESSAGE mMACHINE™ RNA Transcription Kit (Ambion, USA). Oocyte preparation and cRNA injection have been described elsewhere (Becker *et al.*, 1996). The ratio of the co-injected K^+-channel cRNAs is indicated in the figures. In two-electrode voltage-clamp studies, oocytes were perfused with 100 mM KCl-, 1 mM $CaCl_2$- and 1.5 mM $MgCl_2$-containing solutions, based on Tris/Mes buffers (pH 7.5). The osmolarity of the solution was adjusted to 220 mosmol kg^{-1} using D-sorbitol.

Complementation tests of KPT1 in potassium-uptake deficient E. coli *strain LB2003*

After insertion of *KPT1* full-length cDNA into the expression vector pCRII TOPO (Invitrogen, USA), the resultant plasmid KPT1/pCRII TOPO was expressed in *E. coli* LB2003 lacking all K^+-uptake systems, Trk (TrkG and TrkH), Kup (TrkDa) and Kdp. As a control, the LB2003 strain was transformed with the empty pCRII TOPO vector.

Transformed *E. coli* LB2003 strains were grown at 28°C on solid KML medium containing 10 g of tryptone, 5 g of yeast extract and 10 g KCl l^{-1} (Epstein and Kim, 1971). Transformants were grown on medium containing low (3 mM) potassium (10 g of tryptone, 2 g of yeast extract and 100 mmol mannitol l^{-1}; pH 7.0) for 2 days. K^+ concentrations were determined by ICP-OES-elementaranalysis.

Acknowledgements

We are very grateful to M. Rob G. Roelfsema for help with stomatal density analysis in intact poplar leaves and critical reading of the manuscript. We thank A. Latz for Western blot analysis of membranes gained from K^+-channel cRNA-expressing oocytes. This work was supported by grants of the Deutsche Forschungsgemeinschaft to the German Poplar Research Group and Körber Award to R.H.

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