Poplar potassium transporters capable of controlling K\(^+\) homeostasis and K\(^+\)-dependent xylogenesis

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Summary

The cambial K\(^+\) content of poplar increases during the growth period in a K\(^+\) supply dependent manner. Upon K\(^+\) starvation or application of tetraethylammoniumchloride (TEA\(^+\)), a K\(^+\) channel blocker, the average vessel lumen and expansion zone area were significantly reduced. In search for the molecular basis of potassium-dependent xylogenesis in poplar, K\(^+\) transporters homologous to those of known function in Arabidopsis phloem- and xylem-physiology were isolated from a poplar wood EST library. The expression profile of three distinct K\(^+\) channel types and one K\(^+\) transporter, *Populus tremula* K\(^+\) uptake transporter 1 (PtKUP1), was analysed by quantitative RT-PCR. Thereby, we found *P. tremula* outward rectifying K\(^+\) channel (PTORK) and *P. tremula* K\(^+\) channel 2 (PTK2) correlated with the seasonal wood production. K\(^+\) transporter *P. tremula* 1 (KPT1) was predominantly found in guard cells. Following the heterologous expression in *Xenopus* oocytes the biophysical properties of the different channels were determined. PTORK, upon membrane de-polarization mediates potassium release. PTK2 is almost voltage independent, carrying inward K\(^+\) flux at hyperpolarized potential and K\(^+\) release upon de-polarization. PtKUP1 was expressed in a K\(^+\) uptake-deficient *Escherichia coli* strain, where this K\(^+\) transporter rescued K\(^+\)-dependent growth. In order to link the different K\(^+\) transporters to the cambial activity and wood production, we compared the expression profiles to seasonal changes in the K\(^+\) content of the bark as well as xylem vessel diameter. Thereby, we found PTORK and PTK2 transcripts to follow the annual K\(^+\) variations in poplar branches. PtKUP1 was expressed at a low level throughout the year, suggesting a housekeeping function. From these data, we conclude that K\(^+\) channels are involved in the regulation of K\(^+\)-dependent wood production.

Keywords: K\(^+\) channel, poplar, wood formation, quantitative RT-PCR, DEVC, patch-clamp technique.

Introduction

The most abundant cation in plants is potassium, playing a central role in many aspects of plant physiology. Potassium is transported within cells, tissues and organs, and its uptake and transport mechanisms have been studied extensively in different cell types of the root, shoot and leaf (Hedrich and Roelfsema, 1999). Potassium taken up by the root is transported to the shoot via the xylem. A high percentage of the K\(^+\) content received by mature leaves is further transported to young leaves, flowers, seeds, fruits or growing roots via the phloem (Ache et al., 2001; Deeken et al., 2000; Fromm and Bauer, 1994).

Potassium is taken up from the soil via potassium uptake channels of the AKT1- and AtKCl-type (*Arabidopsis thaliana* K\(^+\) channel) and carriers of the high-affinity K\(^+\) transporter (HKT1) and K\(^+\) uptake (KUP) family (Brüggemann et al., 1999; Gassman et al., 1996; Hirsch et al., 1998; Ivashikina et al., 2001; Kim et al., 1998; Reintanz et al., 2002; Rodriguez-Navarro, 2000; Schroeder and Fang, 1991; Spalding et al., 1999). The requirement of three-root Shaker-like K\(^+\) channels for K\(^+\) uptake into and transport within roots was shown by the use of loss-of-channel function mutants (Gaymard et al., 1998; Hirsch et al.,...
activity of the trees. Their width can be increased by potassium and re-pressed by the dormancy hormone ABA and conditions of K⁺ depletion (Gaymard et al., 1998). In contrast to SKOR, the high-affinity potassium carrier A. thaliana K⁺ uptake transporter (AtKUP1) is enhanced by K⁺ depletion (Fu and Luan, 1998; Kim et al., 1998). The membrane potential of the phloem, as measured with the aphid technique, has been shown to be dominated by K⁺ conductance (Ache et al., 2001). This led to the identification of corresponding specifically light-regulated AKT2/3-like K⁺ channels (K⁺ transporter A. thaliana), involved in phloem transport. These channels have been identified in several species such as Arabidopsis, maize and broad bean (Ache et al., 2001; Bauer et al., 2000; Deeken et al., 2000, 2002; Marten et al., 1999).

Annual rings in wood are caused by periodic growth activity of the trees. Their width can fluctuate strongly from year to year depending on various environmental factors. The amount of wood produced by a tree depends on its cambial activity. Fusiform initial cells differentiate to form axial elements such as tracheides, vessels, fibres, parenchyma cells and sieve elements, whereas ray initials produce radial transporting rays (e.g. Aloni, 1987; Hampp et al., 1990; Krabel et al., 1994; Larsson, 1994; Roberts et al., 1988; Savidge, 1996, 2000). Potassium homeostasis in higher plants in general, and trees in particular, depends on nutrient availability, degree of mycorrhizal association, and physiological state of the plant. Potassium ions are involved in various aspects of tree growth and wood formation. In the cambial region and xylem differentiation zone, a strong potassium demand has been shown (Dünisch and Bauch, 1994a,b; Kuhn et al., 1997), especially during cell enlargement when the symplastic potassium content increases (Dünisch et al., 1998). This behaviour suggests that differentiating xylem cells involved in early-wood formation represent a strong sink for potassium and that the accumulation of this osmolyte provides the driving force for cell expansion during primary wall formation. Similarly, fertilized spruce stands, develop 30% more biomass and enlarged annual rings compared to unfertilized controls (Dünisch and Bauch, 1994a,b). The cambium showed prolonged cambial activity characterized by an elevated periclinal division rate and radially enlarged early tracheids. Kuhn et al. (1995, 1997) analysed the distribution of potassium in xylem, cambium and phloem of spruce wood and found the highest levels in the cambium. Moreover, a detailed X-ray microprobe analysis revealed a radial interchange of mineral nutrients between xylem, cambium and phloem (Kuhn et al., 1997). The high levels of potassium in the rays were suggested to indicate that rays are the major re-loading point in wood tissue of trees. During leaf senescence in the fall, evidence for a rapid potassium export out of the leaf blade into the sieve tubes of the subtending stem was shown in beech (Eschrich et al., 1988). Upon onset of vegetative dormancy K⁺ accumulates in the rays from where it can be re-mobilized in the spring and used for wood production.

Energy dispersive X-ray analysis (EDXA) of seasonal changes in the cambial potassium and calcium content of the balsam poplar (Populus trichocarpa) revealed that the re-activation of the cambium in spring is accompanied by high concentrations of potassium and calcium (Arend and Fromm, 2000). In summer, when the cambium starts to develop latewood, the K⁺ concentration remained high, whereas calcium decreased after cambial re-activation.

While some information on seasonal changes in the K⁺ content within trees is available, the molecular mechanism of K⁺ transport in these perennial plants remains unknown. To characterize the fundamental processes involved in potassium supply during xylogenesis, molecular and biophysical techniques have been used to analyse K⁺ transporters of poplar. We have isolated one K⁺ uptake channel (KPT1), one K⁺ release channel (PTORK) and one weak voltage-independent channel (PTK2) as well as a broadly expressed KUP-type of K⁺ transporter from a poplar wood EST library (Sterky et al., 1998). Transporter functions were verified by heterologous expression in Xenopus oocytes or Escherichia coli and their properties and expression patterns are discussed in the context of potassium-dependent wood formation.

Results

Distribution and seasonal changes in potassium content

The K⁺ content of different poplar tissues was analysed by EDXA (Eschrich et al., 1988; Fromm et al., 1987). In spring, the increase in metabolic activity is accompanied by the initiation of K⁺ uptake (Fromm and Eschrich, 1986) and by changes in the membrane potential of cortex and phloem cells (Fromm and Spanswick, 1993). Similarly, we found a seasonal variation in potassium levels in the poplar cambium, where a strong reduction of K⁺ content was found in winter and high levels were found in summer (Figure 1a). Experimental changes in potassium nutrition showed that the K⁺ content of cambial and differentiating xylem cells depends on the K⁺ supply (Figure 1b). Plants grown under limiting K⁺ concentrations (0.05 mM) showed low and equally distributed K⁺ contents in different cell types. Elevating the K⁺ supply to 5 or 10 mM led to higher potassium contents in general (Figure 1b). In 10 mM K⁺, however, the distribution was altered towards maximal K⁺ content
Figure 1. Effect of K⁺ supply on wood production of P. tremula × P. tremuloides.
(a) Seasonal changes in relative cambium K⁺ content, EDXA peakbackground ratios (n = 10, mean ± SD).
(b) Relative K⁺ content of cambial cells, differentiating fibres and vessels grown in different potassium concentrations, EDXA peakbackground ratios (n = 10, mean ± SD).
(c) EDXA-linescan of relative K⁺ distribution in active twig tissue (sequential EDXA from 100 overlaid single scans). A = 10 mM K⁺ supply, B = 0.05 mM K⁺ supply.
(d and e) The extension of the vessel cell-expansion zone is affected by the potassium supply (arrows). Transverse sections of poplar stems of the same age grown with 0.05 mM K⁺ (d) or 10 mM K⁺ (e). Note: under K⁺ limiting conditions cambial and cell-expansion zones lack 2–3 cell layers each. Secondary cell wall formation under potassium depletion starts early.

Figure 2. Relative cambium K⁺ content and potassium-dependent vessel lumen.
(a) EDXA: Relative potassium concentrations of the cambium. Peakbackground ratios increase with root potassium supply from 1 to 11 mM.
(b) Effect of 2 weeks root K⁺ supply and TEA⁺ (5 mM) on vessel size. Vessel lumen area (black bars) depends on K⁺ supply. TEA⁺ reduces vessel size. Note: the fibre lumen (white bars) does not significantly alter with different K⁺ levels or TEA⁺ treatment.
(c and d) K⁺ channel blocker TEA⁺ reduces vessel size. Cross-section of the active cambial zone after treatment of twigs with 5 mM TEA⁺ (c). The vessel size is significantly reduced when compared with the untreated control (d), taken from the same twig below the TEA⁺-treated zone. Bar, 20 μm.
in differentiating vessels. When we analysed the distribution of potassium in actively growing twigs, we found the highest K\(^+\) concentrations in the cambium and the xylem differentiation zone (Figure 1c). In line with the potassium distribution shown in Figure 1(b), this pattern was most pronounced in plants grown in nutrient solution with 10 mM potassium (scan A) rather than with those supplied with 0.05 mM potassium (scan B). In addition, the zone of expanding xylem cells was three-fold larger when trees were grown in 10 mM K\(^+\) versus 0.05 mM K\(^+\) (Figure 1d,e). Coinciding with the narrow expanding xylem cell zone in the K\(^+\) starved trees was an earlier initiation of secondary cell walls (Figure 1d).

**Influence of potassium supply on cell enlargement of cambial cell derivatives**

Rooted cuttings of *P. trichocarpa* were cultivated either on 1 or 11 mM K\(^+\) during the time of active cambial growth. The amount of potassium supplied via the nutrient solution determined the potassium levels in the cambial region as measured by X-ray analysis (Figure 2a). The effect of potassium on the enlargement of xylem cells derived from the cambium was investigated by measurements of the lumen area of newly formed vessel and fibre cells. Vessel cells showed a distinct tendency to have an increased lumen area with increasing potassium levels in the nutrient solution (Figure 2b, black bars). After treatment of the stem with 5 mM tetraethylammoniumchloride (TEA\(^+\)), a K\(^+\) channel blocker, in the presence of 6 mM K\(^+\), the vessel lumen area did not expand as much as untreated stems (Figure 2b–d). In contrast to vessel cells, the lumen area of newly formed fibre cells was not affected by potassium nutrition or TEA\(^+\) treatments (Figure 2b, white bars).

**Molecular analysis of poplar K\(^+\) transporters**

The content of free and bound potassium and the respective buffering/exchange capacity of the cell walls were analysed throughout the year using percolation analysis (Sauter, personal communication). With the onset of...
growth in late winter/early spring, the potassium concentration rose dramatically, whereas it dropped in the fall and winter. Potassium channel blockers, such as Cs\(^+\) and TEA\(^+\), led to a 20% reduced potassium uptake (Sauter, personal communication), pointing to the involvement of K\(^+\) channels in these processes.

To examine the molecular basis of K\(^+\) transport in poplar, we searched the EST database from the cambial region of *P. tremula × P. tremuloides* (Sterky *et al*., 1998) for sequence homologies to known K\(^+\) transporters (Figure 3). We identified DNA fragments with relevant homologies to *Arabidopsis* potassium channels and carriers. Following complete sequencing of these fragments we identified distinct homologues to SKOR, the gene for the outward rectifier expressed in endodermis and xylem parenchyma cells (Gaymard *et al*., 1998), the plasmolymph channels of the AKT2/3 type (Aceh *et al*., 2001; Deeken *et al*., 2000; Lacombe *et al*., 2000; Marten *et al*., 1999), the guard cell channel of the KAT1 type (K\(^+\) channel A. thaliana, Anderson *et al*., 1992), and to AtTrh1 which mediates K\(^+\) transport (Rigas *et al*., 2001). We cloned the corresponding full-length cDNAs and named the SKOR homologue PTORK (*P. tremula* outward rectifying K\(^+\) channel), the AKT2/3 homologue PTK2 (*P. tremula* K\(^+\) channel 2), the KAT1 homologue KPT1 (Figure 3a), and the Trh1 homologue PtKUP1 (*P. tremula* K\(^+\) uptake transporter) (Figure 3b). The deduced proteins PTORK, PTK2 and KPT1 (GenBank accession numbers AJ271446, AJ271447 and AJ344623) exhibited all structural features of members of the ‘green’ Shaker channel family (Hedrich and Becker, 1994).

**Functional expression of PTORK and PTK2 in oocytes**

KPT1 is most similar to the KAT1-like guard cell K\(^+\) channel and was only found in the leaf epidermis (data not shown). The *Arabidopsis* homologues of PTORK and PTK2 are involved in xylem and phloem K\(^+\) transport. To better understand the functional properties of the putative K\(^+\) channels in xylogenesis, we thus focused on PTORK and PTK2 rather than KPT1. PTORK and PTK2 cRNA’s were injected into *Xenopus* oocytes and gene products were analysed 3–5 days post-injection using the double-electrode voltage-clamp technique (cf. Aceh *et al*., 2000; Geiger *et al*., 2002).

![Figure 4. PTORK mediates outward currents in PTORK cRNA-injected oocytes.](image)

(a) Using the double-electrode voltage-clamp technique, time-dependent, outwardly rectifying currents were elicited upon de-polarizing voltage steps between −120 and 60 mV in 10 mV increments, starting from a holding potential of −100 mV. The bath solution contained 30 mM K gluconate, 1.5 mM MgCl\(_2\), 1 mM CaCl\(_2\) and 10 mM Tris–MES pH 7.4.

(b) Steady-state current–voltage curve of PTORK-mediated currents shown in (a).

(c) K\(^+\)-dependent activation curves. Relative open probabilities *P*\(_o\) in 10, 30 and 100 mM K\(^+\), pH 7.4 plotted against the applied membrane voltage. *P*\(_o\) was measured at the onset (t = 0) of the tail pulse at −100 mV and normalized to the maximal open probability (*P*\(_o\) = 1). Solid lines represent the best Boltzmann fits (gating parameters: 10 mM K\(^+\): *V*\(_{1/2}\) = −11.44 ± 2.12, apparent gating charge *z*\(_g\) = 1.46 ± 0.15; 30 mM K\(^+\): *V*\(_{1/2}\) = 12.33 ± 5.69, *z*\(_g\) = 1.56 ± 0.08; 100 mM K\(^+\): *V*\(_{1/2}\) = 31.10 ± 2.58, *z*\(_g\) = 1.73 ± 0.19). Error bars indicate standard deviation (n = 3). LiCl was added to the external solutions to adjust the ionic strength to 100 mM in each solution.

(d) Shift in reversal potentials (*V*\(_{rev}\)) and half-activation potential (*V*\(_{1/2}\)) in response to changes in extracellular K\(^+\) concentrations. Upon increase in K\(^+\) concentration the activation potential (*V*\(_{1/2}\)) shifts positive. A 10-fold increase in K\(^+\) concentration resulted in a 40-mV shift of the half-maximal activation potential (*V*\(_{1/2}\)) (c). The reversal potential (*V*\(_{rev}\)) shifted 45 mV upon a 10-fold increase in K\(^+\) concentration.
Membrane de-polarization elicited an outward rectifying current with a slow sigmoidal activation kinetic in PTORK-expressing oocytes (Figure 4a,b). The steady-state value was reached within 1.5 sec and K⁺ channels did not inactivate during prolonged de-polarization (10 sec at +50 mV, data not shown). Increasing the external K⁺ concentration from 10 to 100 mM resulted in a positive shift of the activation threshold (V₁/₂) following the K⁺ equilibrium potential (E钾) (Figure 4c,d). The reversal potential (Vrev) shifted in a K⁺ dependent manner too (Figure 4d), which, together with the susceptibility of PTORK towards K⁺ channel blockers (data not shown but cf. Ache et al., 2000; Gaymard et al., 1998), classifies PTORK as a K⁺ selective channel. Both external and internal acidification led to a decrease in the steady-state currents as observed with SKOR and GORK (data not shown; but cf. Ache et al., 2000; Lacombe et al., 2000). Thus, PTORK represents an outward rectifying K⁺ channel the activity of which is under control of the membrane potential and external K⁺ concentration.

In contrast to PTORK but similar to its Arabidopsis homologue AKT2/3, PTK2 was active at positive and negative membrane potentials. Figure 5(a) depicts the typical instantaneous and time-dependent current components mediated by PTK2 in response to stepwise changes in membrane potential. At voltages more positive than the K⁺ equilibrium potential, outward K⁺ currents were elicited. The steady-state currents (Iₚ) plotted against the membrane voltage revealed the weak voltage dependence and rectification of PTK2 (Figure 5b). In contrast to AKT2/3 but similar to ZMK2, PTK2 exhibits almost no rectification (Figure 5a,b). The Nernstian behaviour of the reversal potential to K⁺ concentration changes, lack of inward current in Na⁺- and Li⁺-based external media and susceptibility to K⁺ channel blockers Cs⁺ and TEA⁺, classifies PTK2 as a K⁺-selective channel (data not shown; but cf. Geiger et al., 2002; Lacombe et al., 2000; Marten et al., 1999).

Another characteristic of the AKT2/3 family is their susceptibility towards voltage-dependent blocking by Ca²⁺ (Marten et al., 1999). In order to prove whether PTK2 is blocked by extracellular Ca²⁺ tail current recordings were performed. After pre-activating the channels at a membrane voltage of −150 mV followed by tail pulses (t = 0) in the range of 20 to −170 mV revealed a voltage-dependent Ca²⁺ block (Figure 5c). Similar results were obtained with 10-fold lower K⁺ and Ca²⁺ concentrations (not shown). When the extracellular proton concentration was increased from pH 7.4 to 5.6, currents through PTK2 were reduced (Figure 5d) a behaviour characteristic for members of the AKT2/3 family.

**Figure 5.** Calcium and protons block K⁺ currents mediated by PTK2.

(a) Representative macroscopic recordings of inward and outward currents obtained from PTK2-RNA-injected Xenopus oocytes. Typical instantaneous and time-dependent current components were mediated by PTK2. From a holding potential of Vh = −30 mV, the membrane voltage was successively changed during 2.5 sec pulses from +30 mV to −160 mV in 10 mV decrements. The bath solution was composed of 30 mM K gluconate, 1 mM CaCl₂, 1.5 mM MgCl₂ and 10 mM Tris-MES (pH 7.4).

(b) Corresponding current-voltage curve: Steady-state currents Iₚs determined in (a) plotted against the membrane voltage. Note: the weak voltage dependence and rectification of PTK2.

(c) Relative (rel.) instantaneous tail-current amplitudes Iₚ plotted against the membrane voltage in the presence of 30 mM CaCl₂ (●) or 30 mM MgCl₂ (○). Iₚ currents were normalized to the currents at −110 mV in the control solution. The Ca²⁺ solution contained 20 mM KCl, 10 mM Tris-MES, pH 7.2 and 30 mM CaCl₂.

(d) K⁺ currents through PTK2 in responses to single voltage pulses of −150 mV (Vh = −30 mV) with the bath solution buffered to pH 5.6 and 7.4. Note: protons block both, instantaneous and time-dependent PTK2 currents. The pH solutions were composed of 30 mM K gluconate, 1 mM CaCl₂, 1.5 mM MgCl₂ buffered with 10 mM MES-TRIS to pH 5.6 or pH 7.4, respectively.
PtKUP1 functionally complements a K⁺ uptake-deficient E. coli mutant

E. coli LB2003, lacking the K⁺ uptake systems, Trk, Kup, and Kdp, does not grow on K⁺-limited media (Uozumi et al., 1998). This triple K⁺ transport deficient strain requires 25 mM K⁺ for half-maximal cell growth (Epstein and Kim, 1971). Therefore, cells transformed with the empty vector pCRII TOPO did not grow in media supplemented with 3 mM K⁺, while E. coli expressing the Trh1 homologue PtKUP1 formed colonies (Figure 6a). To further characterize the transport properties of PtKUP1, the effects of calcium and K⁺ channel blockers on growth were tested by placing an impregnated paper disk on a nascent lawn of E. coli cells suspended in growth agar. After incubation for 36–48 h, growth inhibition was reflected by a decreased cell density (halo) around the disk (Figure 6b). Ca²⁺ and Cs⁺, but not TEA⁺ and Ba²⁺, strongly inhibited growth of the PtKUP1 strain. These results demonstrate that PtKUP1 represents a functional K⁺ uptake transporter sensitive to Ca²⁺ and Cs⁺ ions.

Localization and seasonal changes in K⁺ transporters expression

To localize the site of PTORK, PTK2 and PtKUP1 expression, we isolated mRNA from leaves, epidermal fragments, petioles, xylem and phloem of young branches, and roots.

for quantitative RT-PCR analyses (Figure 7a). The highest levels of all three transcripts were detected in the vascular-rich petioles and in the phloem. Following the separation of the bark from annual branches into cambium/phloem/bast and cambium/xylem/wood, PTORK and PTK2 were found in both fractions, but PTK2, was predominantly found in the bast and only at background levels in the root. PtKUP1 in contrast seems to be expressed ubiquitous but at low levels.

In order to determine whether the seasonal changes in cambial activity, xylegenesis and thus wood formation are accompanied with changes in the K⁺ transporter expression, branch segments were collected throughout the year and mRNA was isolated from each sample. The expression profile depicted in Figure 7b shows quantitative RT-PCR of poplar stem RNA collected in February and June. PTORK and PTK2 were highly expressed when temperatures increased above 10–15 °C and wood production was initiated, while PtKUP1 expression remained at low levels throughout the seasons.
From this behaviour, we may thus conclude that PTORK and PTK2 initiate and/or accompany xylogenesis. The fact that PtKUP1 transcript levels were low but constant throughout the year might point to a housekeeping function of this transporter in \(K^+\) homeostasis.

**\(K^+\) currents in callus cells**

Studies on the physiology, molecular biology and biophysics of poplar ion channels *in vivo* require proper access to the individual cell types. Best suited for this purpose are patch-clamp studies on isolated protoplasts. Therefore, we isolated protoplasts from suspension cultures expressing PTORK and PTK2. Poplar branches were induced to build callus and the resulting meristematic tissues to generate suspension cultures. When analysing mRNA isolated from this cell culture, the \(K^+\) channel PTORK and the \(K^+\) transporter PtKUP1 were highly expressed, the PTK2 gene was weakly expressed and KPT1, the KAT/guard cell homologue was not detected (data not shown). Since this culture represents a model system for poplar cells expressing PTORK and PTK2, protoplasts were isolated and the plasma membrane potassium conductance’s were compared to the electrical properties of *Xenopus* oocytes expressing PTORK and PTK2 individually. The whole cell configuration of the patch-clamp technique was established with 150 mM \(K^+\) in the cytoplasm (pipette solution) and 30 mM in the extracellular medium, and both inward and outward \(K^+\) currents were observed (Figures 8 and 9). With the membrane potential clamped to \(-45\) mV, hyperpolarizing 1.5 sec voltage pulses activated inward rectifying currents (Figure 8a). To further characterize this channel type, we challenged the suspension cells with 5 mM Cs\(^+\) in the bathing solution. Under these conditions inward currents were completely blocked (Figure 8b), while a reduction from 30 mM potassium to 3 mM in the bathing solution of protoplasts resulted in a decrease of the inward current (Figure 8c). The Nernstian behaviour of the reversal potential to \(K^+\) changes (not shown) and \(K^+\)-dependent current amplitude together with the susceptibility to \(K^+\) channel blocker Cs\(^+\) classified the inward rectifier as a \(K^+\)-selective channel.

**Figure 8.** Patch-clamp analyses on cultured poplar cells show PTK2-like features.

(a) Whole cell recordings of representative \(K^+\) currents on protoplasts isolated from suspension culture in standard bathing medium containing 30 mM K gluconate. Voltage- and time-dependent inward \(K^+\) currents could be observed at voltages less than \(-100\) mV using the standard pulse protocol.

(b) After the addition of 5 mM CaCl\(_2\) to the standard solution inward currents were completely abolished. The corresponding inward currents without Cs\(^+\) are shown in (a).

(c) Upon lowering the external \(K^+\) concentration from 30 to 3 mM \(K^+\) inward currents decreased at hyperpolarized voltages. Currents were recorded in response to a pulse of \(-185\) mV.

(d and e) A rise in external calcium from 1 mM (d) to 20 mM (e) caused a voltage-dependent current inhibition. Compared to the time-dependent activation in the presence of 1 mM Ca\(^{2+}\), a pulse to \(-185\) mV in the presence of 20 mM Ca\(^{2+}\) induced current activation followed by voltage-dependent block.

(f) Quantification of the Ca\(^{2+}\) block shown in (d and e). Steady-state currents \(I_{ss}\) of three independent experiments were plotted against the membrane potential. In the presence of 20 mM Ca\(^{2+}\) (●) a voltage-dependent block of the inward currents could be observed, whereas in 1 mM Ca\(^{2+}\) (○) no reduction was visible. The steady-state currents were normalized to the currents at \(-145\) mV in 1 mM CaCl\(_2\). Error bars indicate standard errors.

Following the rise in external calcium from 1 to 20 mM, inward currents progressively declined with more negative membrane potentials (Figure 8d–f). This type of voltage-dependent calcium block has so far only been observed with AKT2/3-type channels and not with other members of the plant Shaker family (Hoth et al., 2001; Marten et al., 1999). In contrast to AKT2/3 channels, the K^+ currents in cultured cells were time-dependent and strongly inward rectifying (Figure 8).

When the membrane potential was clamped at −85 mV and 1.5 sec pulses stepped to de-polarizing potentials were applied, outward K^+ currents could be observed (Figure 9a). These currents were strongly outward rectifying and characterized by a sigmoidal activation kinetic, a feature reminiscent of PTORK and other members of the SKOR family when expressed in oocytes (Figure 4a; Ache et al., 2000; Gaymard et al., 1998). A reduction from 30 mM potassium to 3 mM in the bathing solution of protoplasts resulted in a negative shift of the voltage dependence of the outward rectifier (Figure 9b) which was also found in the PTORK-expressing oocytes (Figure 4c,d). The positive shift of the reversal potential was induced by an elevation of external potassium from 3 to 30 and 90 mM (Figure 9d). Both the shift of the half maximal activation potential and the Nernstian behaviour of the reversal potential classified this outward rectifier as K^+ permeable channel.

**Discussion**

Cell division and expansion are potassium dependent (Philippar et al., 1999) and the K^+ nutrition status was shown to strongly affect the development of wood producing cells. The expansion of vessel initials under potassium depletion ceased early followed by untimely secondary cell wall formation. The lack of 1–2 cell divisions in the vessel development region and limited cell expansion resulted in reduced wood formation. The application of the potassium channel blocker TEA^+ led to the same effect and, therefore, pointed to a link between potassium channels and wood production.

The data presented here and in previous experiments by others reveal a strong K^+ dependency of wood formation. To determine which K^+ transporters might control potassium-dependent xylogenesis, we screened a poplar cambium EST database and identified two K^+ channel-like sequences that we called PTORK, PTK2 and one potential K^+ carrier, PtKUP1. PTORK shared closest structural and functional similarities with its *Arabidopsis* counterpart.
SKOR, a stelar K⁺ outward rectifier, expressed in the root xylem parenchyma. Thus, PTORK enables potassium release in a voltage- and potassium-dependent manner. PTK2 was classified as a member of the AKT2/3 phloem K⁺ channel family with similar structures and functions. Like AKT2/3, PTK2 is able to mediate both uptake and release of potassium in response to changes in membrane potential in a calcium- and pH-dependent fashion.

When we compared the properties of outward rectifying K⁺ channels in PTORK-expressing poplar suspension cells and PTORK-injected Xenopus oocytes they were shown to share basic features and furthermore were similar to other plant de-polarization-activated K⁺ release channels (Ache et al., 2000; Gaymard et al., 1998). The inward K⁺ channel from poplar suspension culture showed a voltage-dependent calcium block and was highly sensitive to Cs⁺. Although these features were characteristic for PTK2 and AKT2/3, its voltage dependency differed from that recorded in PTK2 expressing oocytes. Inward rectification was weak in PTK2-injected oocytes but strong in PTK2-expressing poplar cells. This feature could reflect the finding that functional Shaker K⁺ channels are formed by four alpha subunits (MacKinnon, 1991), and that members of different subfamilies are able to form hetero-tetramers (Daram et al., 1997; Dreyer et al., 1997; Ehrhardt et al., 1997). In this context, it should be mentioned that when CAT1 (KPT1 homologue expressed in guard cells) was co-expressed with AKT3 (PTK2 homologue expressed in the phloem and guard cells) in Xenopus oocytes, the voltage dependence was dominated by the strong inward rectifier CAT1 (Baizabal-Aguirre et al., 1999 and own unpublished data). In guard cells that express several different Shaker channel types including AKT2/3, a calcium-sensitive and highly caesium-sensitive inward rectifier represents the dominant inward K⁺ conductance (Szyroki et al., 2001). Arabidopsis plants, however, lacking the AKT2/3 subunit were no longer blocked by external calcium ions (Ivashikina et al., 2001). Furthermore, a protein phosphatase interacting with the AKT3 has been identified (Vranova et al., 2001). When co-expressed with AKT2/3 this phosphatase turns the weak voltage-dependence of this channel type into an inward rectifier (Chérel et al., 2002). We, thus, propose that poplar suspension cells express an additional K⁺ channel alpha subunit or a channel modulator which transforms PTK2 into an inward rectifier.

Both K⁺ channel genes are expressed in young poplar twigs, while PTK2 was predominantly found in the phloem fraction PTORK was detected in both phloem and xylem fractions. The seasonal changes in expression levels of both channels coincided with cambial activity and xylogenesis and the functions of their Arabidopsis homologues in xylem and phloem transport, point to a role of PTORK in K⁺ release from xylem parenchyma and of PTK2 in K⁺ uptake of cambium and phloem cells.

### Experimental procedures

#### Plant growth conditions

*Populus tremula* × *P. tremuloides* plants were grown in soil under natural conditions. Suspension cell cultures from shoots were grown in liquid MS medium (≈20 mM K⁺), containing 5 μM 2,4-dichlorophenoxyacetic acid (2,4 D) or in modified Hoagland nutritional solution (1 mM K⁺) with 5 μM 2,4-D. Cell cultures were shaken at 133 g in darkness at 26°C.

Rooted cuttings from mature poplar trees (*P. trichocarpa*) were cultivated hydroponically at different K⁺ concentrations (modified Hoagland nutritional solution) in a controlled environment chamber at 20°C with a photon flux density of 300 μE m⁻² s⁻¹ and were used for anatomical analysis of fibre and vessel lumen areas. Additionally, a few cuttings grown in Hoagland nutritional solution with 6 mM K⁺ were treated with 5 mM TEA⁺ for 2 weeks. To avoid systemic responses TEA⁺ was applied on the scraped twig surface and not within the nutrition solution. Controls were treated with water (not shown).

#### Light microscopy and image analysis

Twig tissue was sampled and fixed with 3% formaldehyde in phosphate-buffered solution (PBS) for 2 h, washed in buffer and dehydrated in a graded series of ethanol. After embedding in LR White acryl resin, semithin sections were cut with a diamond knife and stained with Toluidine Blue for light microscopy. The lumen areas of fibres and vessels of newly formed wood tissue were measured by digital image analysis using a Zeiss Axio Vision system.

#### X-ray microanalysis

Small sections of twig tissue were cut with a razor blade and immediately shock frozen in liquid isopentane at its melting point. After freeze drying, the samples were coated with chromium and examined in a Leitz AMR 1200 scanning electron microscope fitted with a Kevex 4000 X-ray analyser. Element specific X-ray spectra were obtained from a reduced scan raster area at 1000× magnification. Relative potassium concentrations were expressed as peak to background ratio from 10 recorded spectra. For visualizing the distribution of potassium in the twig tissue, potassium specific X-ray signals were recorded using the element specific scan modus of the microscope.

#### Cloning

*Populus tremula* × *P. tremuloides* cDNA fragments homologous to potassium channels and transporters were identified from the expressed sequence tag (EST) database using BLAST (Altschul et al., 1990). The poplar data base entries of the selected *Populus* ESTs are as follows: A020P20, B007P19, A043P54. Amplification of 5’cDNA ends were performed by RACE technique (Marathon™, cDNA Amplification Kit, SMART™RACE cDNA Amplification Kit, Clontech) using the following gene-specific primers: PTORK Mrev position 195 (5’T-AAG AAA CTT CCC CAA ATG -AAG AAA CTT CCC CAA ATG-3’), PTK2 Mrev position 172 (5’-GTC CAT TCT TGT TCC CCT TCA C-3’) and PKUP1 Mrev position 39 (5’-CAC GGG AAT CCT TGT ATG TTG-3’).

Full-length cDNAs were amplified from reverse transcribed RNA derived from xylem, cambium and phloem tissues of *P. tremula* × *P. tremuloides*.
Cloning of KPT1

Within the increasing number of plant K⁺ uptake channels cloned so far, amino acid sequences within the second (S2) and sixth (S6) putative transmembrane domain and the amphipathic linker between SS and S6 (H5) are highly conserved (Hedrich and Roelfsema, 1999). Using degenerative oligonucleotides (Ache et al., 2001), we cloned a 200-bp (H5 ± S6) fragment of the KPT1 cDNA from a leaf cDNA library. Amplification of 5′- and 3′-cDNA ends were performed by RACE technique (SMART™ RACE cDNA Amplification Kit, Clontech) using the following generic-special primers:

3′-KPT1GSP1 (5′-ATA CCT TGA TCC GAA GAG AAC C-3′), Nested 3′-KPT1 GSP2 (5′-TAC AAC ATC ATT AAC AAC AGG G-3′), 5′-KPT1 GSP1 (5′-CCC ATA CCC TGT TGT GGT T-3′), Nested 5′-KPT1 GSP2 (5′-TGC AGT CAC GTA TCT ATT CCA TAG T-3′)

The full length cDNA was amplified with KPT VL fw (5′-TGA GAA TTC AAG CAA CCA GTG-3′) and KPT VL rev (5′-CAC TTG GCC ATG ATG TAG TAC TGC-3′) Primers.

Heterologous expression in Xenopus oocytes

The cRNAs of PTORK and PTK2 were generated by in vitro transcription (T7-Megascript kit; Ambion Inc., Austin, TX) and injected into oocytes of *Xenopus laevis* (Nasco, Fort Atkinson, WI) using a Picospritzer II microinjector (General Valve, Fairfield, NJ). Oocyte preparation and cRNA injection have been described elsewhere (Becker et al., 1996). In two-electrode voltage-clamp studies oocytes were perfused with potassium gluconate containing Tris–MES buffers. Further used solutions are described in the figure legends. All media were adjusted to a final osmolality of 215–235 mosmol kg⁻¹ with D-sorbitol. Analyses of voltage dependence, pH dependence, selectivity and Ca²⁺ block were performed as described previously (Hoth et al., 1997; Marten et al., 1999).

Expression analysis by quantitative RT-PCR

RNA of stem fragments (see below) was isolated using the Plant RNAeasy Extraction kit (Qiagen, Hilden, Germany) DNA was digested on-column during RNA purification (RNase-Free DNase kit, Qiagen, Hilden, Germany).

First-strand cDNA was prepared using the Superscript RT kit (Gibco_BRL) and diluted for RT-PCR 20-fold in water. PCR was performed in a LightCycler (Roche Molecular Biochemicals) with the LightCyclerFastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals). Primers used: PACT2fwd (5′-GCC AGT CCT CCT-3′) and PACT2rev (5′-ACT GAG CAC AAT GTT AC-3′), PTORKLfw (5′-CAG GGG CAT CAC TGG CA-3′) and PTORKLrev (5′-GGT AAC CAC CTC AAG AT-3′), PTK2 Lfw (5′-ATG CTA TAT ACA CCT G-3′) and PTK2 Lrev (5′-TGC TCA CCC TAA TAC A-3′) and KPTLCfw (5′-GAT GCC CCC ATG ATA GG-3′), KPTLCrev (5′-CAT GAT GTA TTG CGC T-3′)

All quantifications were normalized to actin cDNA fragments amplified by PtACT2fwd and PACT2rev. 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 manufacturer’s protocols.

Complementation tests of PtKUP1 in *E. coli*

The PtKUP1 cDNA was inserted into the expression vector pCRII TOPO (Invitrogen), the resultant plasmid containing the entire PtKUP1 coding region was designated PtKUP1.

The plasmid was expressed in *E. coli* LB2003, which lacks the three K⁺ uptake systems, Trk (TrkG and TrkH), Kup (TrkDa), and Kdp (a kind gift from K. Altendorf, University of Osnabruck, Germany). The *E. coli* strain was grown at 28 °C on solid KML-medium (10 g tryptone, 5 g yeast extract, and 10 g KCl l⁻¹) (Epstein and Kim, 1971). As a control *E. coli* LB2003 strain was transformed with the empty pCRII TOPO vector. Transformants were tested for their ability to grow in medium containing low (3 mM) potassium (10 g KCl l⁻¹) and 100 mmol of mannitol per liter, pH 7.0, for 2 days. K⁺ concentrations were determined by ICP–OES–Eletromass analysis.

The effect of monovalent and divalent cations on growth was detected by the halo assay (Becker et al., 1996). A lawn of 10⁵ cells of LB2003 expressing PtKUP1 was plated in 0.7% agarose on KML plates with 3 mM K⁺. A paper disk containing 10 μl of the test cation solution (TEA⁺ (1 M), upper disk; Cs⁺ (1 M), left disk; Ba²⁺ (1 M), right disk; Ca²⁺ (1 M), lower disk) was placed on the agar surface and the plates were incubated at 28 °C for 36–48 h. In the growth zone around the disk, inhibition creates a dark halo.

Cell culture and protoplasts isolation

Cell culture protoplasts were enzymatically isolated from young and white tissue at the border of callus pieces. Two to five days after exchange of the nutrient solution, the young tissue was separated from the old, brown coloured tissue using a razorblade.

The enzyme solution contained 0.8% (w/v) Cellulase (Onozuka R10), 0.1% (w/v) Pectolyase (Sigma), 0.5% (w/v) BSA (Serva), 0.5% (w/v) PVP (Sigma), 1 mM CaCl₂, 8 mM MES/KOH ph 5.5, 100 mosmol kg⁻¹ (D-sorbitol). The protoplasts were filtered through a 50-μm nylon-net, washed 2 times with a solution containing 1 mM CaCl₂± 280 mosmol kg⁻¹ (D-sorbitol) and finally they were centrifuged at 600 g for 10 min at 4 °C.

Patch-clamp

Ion fluxes were studied in the whole-cell configuration of the patch-clamp technique. Current measurements were performed using an EPC-7 patch-clamp amplifier (HEKA, Lambrecht, Germany). The patch pipettes were prepared from Kimax-51 glass (Kimble products, Vineland, NY, USA) and coated with silicone (Sylgard 184 silicone elastomer kit; Dow Corning, USA).

Whole cell measurements were performed by stepwise voltage pulses starting from 75 to 185 mV in 20 mV decrements. After a preactivating voltage pulse of 75 mV, tail currents were observed by changing the membrane voltage successively from 75 to 185 mV in 20 mV steps. Voltage values were corrected for liquid junction potential 5 mV (Neher, 1992). The difference between the liquid junction potential correction of voltage values and Rg compensation corrected voltage values was smaller than 5 mV, so this difference was not taken into consideration.
Solutions: The standard pipette solution (cytoplasm) contained 150 mM K gluconate, 2 mM MgCl₂, 2 mM MgATP, 10 mM HEPES/Tris pH 7.4. Cytosolic Ca²⁺ was buffered with 10 mM EGTA. The standard bathing medium contained 30 mM K gluconate, 1 mM CaCl₂ and 10 mM MES–Tris pH 5.8. In order to test the calcium dependence of the inward rectifiers we increased the CaCl₂ concentration from 1 to 20 mM. By changing the external potassium concentration from 3 to 30 and 90 mM the K⁺ selectivity of inward and outward rectifiers was studied. 5 mM of the specific K⁺ channel blocker CsCl₂ was added to the standard bathing medium to test the susceptibility towards Cs⁺. All solutions were adjusted to a final osmolality of 280 mosmol kg⁻¹ with d-sorbitol.

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References


