The K\(^+\) Channel KZM1 Mediates Potassium Uptake into the Phloem and Guard Cells of the C\(_4\) Grass Zea mays*

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In search of K\(^+\) channel genes expressed in the leaf of the C\(_4\) plant Zea mays, we isolated the cDNA of KZM1 (for K\(^+\) channel Zea mays 1). KZM1 showed highest similarity to the Arabidopsis K\(^+\) channels KAT1 and KAT2, which are localized in guard cells and phloem. When expressed in Xenopus oocytes, KZM1 exhibited the characteristic features of an inward-rectifying, potassium-selective channel. In contrast to KAT1- and KAT2-type K\(^+\) channels, however, KZM1 currents were insensitive to external pH changes. Northern blot analyses identified the leaf, nodes, and silks as sites of KZM1 expression. Following the separation of maize leaves into epidermal, mesophyll, and vascular fractions, quantitative real-time reverse transcriptase-PCR allowed us to localize KZM1 transcripts predominantly in vascular strands and the epidermis. Cell tissue separation and KZM1 localization were followed with marker genes such as the bundle sheath-specific ribulose-1,5-bisphosphate carboxylase, the phloem K\(^+\) channel ZMK2, and the putative sucrose transporter ZmSUT1. When expressed in Xenopus oocytes, ZmSUT1 mediated proton-coupled sucrose symport. Coexpression of ZmSUT1 with the phloem K\(^+\) channels KZM1 and ZMK2 revealed that ZMK2 is able to stabilize the membrane potential during phloem loading/unloading processes and KZM1 to mediate K\(^+\) uptake. During leaf development, sink-source transitions, and diurnal changes, KZM1 is constitutively expressed, pointing to a housekeeping function of this channel in K\(^+\) homeostasis of the maize leaf. Therefore, the voltage-dependent K\(^+\) uptake channel KZM1 seems to mediate K\(^+\) retrieval and K\(^+\) loading into the phloem as well as K\(^+\)-dependent stomatal opening.

Since the first isolation of a plant K\(^+\) channel gene 10 years ago, plant science has focused on their cell-specific localization and structure-function relationship. Therefore, new insights into the physiological role of the different K\(^+\) channel genes have been gained. The Arabidopsis thaliana genome contains at least 15 K\(^+\) channel genes (1). Among them, the Shaker family of Arabidopsis K\(^+\) channels consists of nine members (for review see Ref. 2). According to their localization, structure, and function, these genes can be assigned to different subfamilies. In 1992, the first plant K\(^+\) channel genes isolated were AKT1 and KAT1 (3, 4). Both proteins represent K\(^+\)-uptake channels (5, 6). AKT1-like channels are involved in K\(^+\) uptake into growing roots (AKT1) (7, 8) and pollen tubes (SPIK) (9), and KAT1 plays a role in Arabidopsis guard cells (10–12). Recently, the inward rectifier KAT2 could be characterized as the closest relative to KAT1 (13). KAT2 is expressed in guard cells, too, but in contrast to KAT1, KAT2 transcripts were identified in the phloem parenchyma of the leaf. A coding sequence of the channel gene AKT5 (AKT subfamily) could be isolated from hypocotyl tissue, but its function still remains unknown. The AtKCI gene splits into another subfamily and together with AKT1 subunits seems to generate the functional properties of the root hair K\(^+\)-influx channel (14, 15). In contrast to the mentioned inward rectifiers within the AKT1 and KAT1 family, members of the AKT2/3 subfamily are characterized by weak voltage dependence, a Ca\(^2+\) and H\(^+\) block, and seem to control phloem function (16–19). The K\(^+\) channel genes SKOR, localized in xylem vessels of the root (20), and GORK, in guard cells, vasculature, and roots (14, 21), build the subfamily of outward-rectifying K\(^+\) channels in Arabidopsis.

Because the Arabidopsis genome reveals the complete set of Shaker-like K\(^+\) channel genes in plants, we can assign the orthologs from different plant species to the respective subfamilies. So far K\(^+\) channel genes have been isolated from 12 different plants (compare with Ref. 22) including the C\(_3\) plant Zea mays. In maize, the two K\(^+\) channel genes ZMK1 and ZMK2, isolated from the coleoptile, belong to the AKT1- and AKT2/3-type subfamilies, respectively (23). ZMK1 is involved in auxin-induced K\(^+\) uptake, coleoptile growth, and tropisms. ZMK2 displays the voltage-independent features of the AKT2/3-type K\(^+\) channels and thus seems to serve phloem-associated functions (24). Besides rice, the maize plant is not only a model system for monocotyledonous crops but C\(_4\) photosynthesis as well. This involves a special anatomic feature called Kranz anatomy: mesophyll cells, involved in the pre-fixation of CO\(_2\), transport C\(_4\) compounds to the bundle sheath cells, which surround the vascular strands and finally fix CO\(_2\) in the Calvin cycle (reviewed in Ref. 25). Due to this cell and chloroplast dimorphism, C\(_4\) plants are characterized by a better water-use efficiency than C\(_3\) plants. The carbohydrate transport between mesophyll, bundle sheath, and vascular parenchyma cells of the maize leaf is accomplished by numerous

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1 S. Scheuermann, unpublished results.
plasmodesmata (26–28). The loading of sucrose from the vascu-
lar parenchyma to the thin-walled sieve tubes, representing the site of assimilate export, however, is thought to involve an apoplastic step (reviewed in Refs. 29 and 30). To characterize the sugar import machinery, Aoki et al. (31) isolated ZmSUT1, a putative sucrose transporter from source leaves of maize. By heterologous expression in Xenopus oocytes, we showed that ZmSUT1 indeed represents a sucrose/H⁺ symporter under the voltage control of the AKT2/3 ortholog ZMK2.

Because the AKT2/3-type channels such as AKT23 from Arabidopsis, VFK1 from Vicia faba, and ZMK2 from Z. mays seem to play an important role in the control of phloem sucrose loading and unloading (16, 17, 24, 32), we here studied K⁺ channels expressed in the dimorphic structure of the maize leaf. In addition to ZMK2 (23), we isolated the cDNA of KZM1. KZM1 represents the maize ortholog to KAT23 from Arabidopsis. Like KAT2 we found this new maize K⁺ channel gene expressed in vascular/bundle sheath strands as well as guard cell- and subsidiary cell-enriched epidermal fractions of the maize leaf. However, KZM1 is characterized by unique functional properties that enabled us to discriminate between the function of KAT2 in the dicotyledonous plant Arabidopsis and KZM1 in the monocotyledonous C₃ plant maize. The K⁺-uptake channel KZM1 is able to mediate phloem K⁺ loading and retrieval as well as K⁺-dependent stomatal movement. The function of the inward-rectifier KZM1 in combination with ZMK2 and the sucrose/H⁺ symporter ZmSUT1 as well as its expression pattern point to a housekeeping function of KZM1 for K⁺ homeostasis in the phloem of the maize leaf.

MATERIALS AND METHODS

Plant Material—Cloning, Northern blot procedures, and quantita-
tive real-time RT-PCR analysis were performed on tissues isolated from maize plants (Z. mays L., hybrid corn cv. “Oural FA2030,” Deutsche Saatveredelung, Lippstadt, Germany). Seeds were sown in soil and grown in a greenhouse with a 16-h light (25 °C) and 8-h dark (18 °C) cycle. The white light used had a photon-flux density of 210 μmol·m⁻²·s⁻¹ (LiCOR Quantum Sensor LI-250, Walz GmbH, Effeltrich, Germany). After harvesting, all maize tissues were stored in liquid nitrogen prior to RNA extraction. The age of plants or organs is denoted in days after sowing.

Cloning of KZM1 cDNA—Degenerated oligonucleotide primers, di-
rected toward homologous regions of known plant inward-rectifying K⁺ channels, were used to amplify a corresponding region of potassium channel-encoding cDNA from reverse-transcribed maize leaf RNA (RT-PCR). By using the SMART RACE cDNA Amplification kit (Clontech, Heidelberg, Germany) in combination with gene-specific primers, we amplified overlapping N- and C-terminal K⁺ channel fragments according to the RACE technique. The corresponding full-length cDNA was generated in a single PCR step using primers flanking the 5′- and 3′-ends of the coding sequence of KZM1 and ligated into pcRII-TOPO TA vector (Invitrogen). Besides the N- and C-terminal clones of KZM1, 15 ng of doped poly(A)⁺ was hybridized against a (γ³²P)dATP end-labeled oligo(dT) probe as described (23).

Separation of Leaf Tissues—Tissue from the last fully developed leaf of 5-week-old maize plants was separated into epidermis, mesophyll cells, and vascular strands by a procedure modified according to Keunecke and Hansen (34). The central vascular strand was excised, and the lower epidermis was collected in 1 ml CaCl₂, 5 ml Mes/KOH, pH 6.5, adjusted with mannitol to 530 mmol·kg⁻¹ and frozen in liquid nitrogen for mRNA extraction. To test the vitality of epidermal cells before freezing, an aliquot of the epidermal fraction was stained with neutral red (see Fig. 4). To isolate mesophyll protoplasts, the remaining leaf sections were incubated for 90 min at 30 °C in enzyme solution containing 1.5% cellulase (Cellulase R-10, Yakult Honsha, Tokyo, Japan), 2% pectolytic enzyme (Yakult Honsha), 0.1% LiCl, 5 ml Mes/KOH, pH 6.2, adjusted with n-sorbitol to 480 mmol·kg⁻¹. The digestion was stopped before the bundle sheath cells were released from the vascular strands. Isolated vascular/bundle sheath strands were pooled and frozen in liquid nitrogen. Mesophyll protoplasts were sedimented at 60 × g for 5 min at 4 °C and frozen in liquid nitrogen.

Quantitative Real-time RT-PCR—For real-time RT-PCR experi-
ments, total RNA from the fractionated maize leaves was isolated using the Plant RNeasy Extraction kit (Qiagen, Hilden, Germany). To mini-
mize DNA contaminations, mRNA was purified twice with the Dyna-
beads mRNA Direct kit (Dynal). By using an RNase-free digestion with the RNase-free DNase kit (Qiagen), mRNA was purified with the Dynabeads mRNA Direct kit (Dynal). First-strand cDNA synthesis and quantitative real-time RT-PCR were performed as described before (12) using a LightCycler (Roche Molecular Biochemi-
cal). The following K⁺ channel-specific primers were used: KZM1 LCfw (5′-aagagacagtgtgrtaa-3′), KZM1 LCrev (5′-tgaaaccaagaggctc-3′), ZM2 LCfw (5′-gagagttatcattc-3′), and ZM2 LCrev (5′-aggagattcattc-3′). For detection of the coding sequence of the small subunit of the ribulose-1,5-bisphosphate carboxylase (ZmRuBPCssu, GenBank accession number X06535) and the 3′-translated region of the C₃ form phosphoenolpyruvate carboxylase (ZmPepC, GenBank accession number X15228), we used the primers RuBCPssu LCfw (5′-acaacaaaggtgagacg-3′), RuBPcssu LCrev (5′-ggtggagatttgagct-3′), and C₃-PepC LCfw (5′-ggtttccttacctc-3′), C₃-PepC LCrev (5′-tcatcttacctct-3′), respectively. All quantifications were normalized to the signal of actin cDNA fragments generated by the primers ZmAkt 81/83fw (5′-accaagcagcttcacat-3′) and ZmAkt 81/83rev (5′-acttgagacagcttc-3′), which amplified cDNA from the maize actin ZmAkt 81 (GenBank accession number A41003) and ZmAkt 83 (GenBank accession number A240105) respectively. The relative amount of channel cDNA was calculated from the correlation 2ⁿ (actin) = n (channel) with n = threshold cycle of the respective PCR product. To identify contaminating genomic DNA, the primers for ZM2 were selected to flank an intron.

Two-electrode Voltage Clamp Experiments—For heterologous expression in Xenopus laevis oocytes, the cDNAs of KZM1 in pcRII and ZmSUT1 in pBS SK(–) were subcloned as XhoI/BgiIII and BamHI/Xhol fragments, respectively, into the pGEMHE vector (35). Expression of ZM2K in oocytes was performed as described (23). The respec-
tive cDNA was generated by in vitro transcription (TT7-Megascript kit, Ambion Inc., Austin, TX) and injected into Xenopus oocytes (CRBM, CNRS, Montpellier, France) using a PicospritzerII microinjector (General Valve, Fairfield, NJ). Two to 6 days following injection, double-electrode voltage clamp recordings were performed with a Turbo-21C amplifier (NPI Instruments, Tann, Germany). The electrodes were filled with 3 M KCl and had typical input resistance on the order of 2–4 megohm. Source DNA (Agilent Technologies, Germany), Poly(A)⁺ RNA was purified from total RNA using Dyna-
beads (Dynal, Hamburg, Germany) and subjected to Northern blot analysis as described (33). The blotted poly(A)⁺ RNA was hybridized against ³²P-radiolabeled full-length cDNA probes of the K⁺ channel genes KZM1 and ZMK2 as described in Philipp et al. (23). For the sucrose transporters ZmSUT1, a 539-bp-long 3′-terminal cDNA frag-
ment, amplified between the primers ZmSUT LCfw (5′-cccaagag-
gcaaa-3′) and ZmSUT LCrev (5′-ttggggtgaagc-3′), served as a probe. Each probe exhibited specific signals at 2.5 kb for KZM1, 2.8 kb for ZMK2, and 2.0 kb for ZmSUT1. To standardize transcript abundance,

² The abbreviations used are: RT, reverse transcriptase; RACE, rapid amplification of cDNA ends; Mes, 2-morpholinoethanesulfonic acid; NaAc, sodium acetate; RuBPcssu, ribulose-1,5-bisphosphate carboxylase small subunit; PEPc, phosphoenolpyruvate carboxylase.
FIG. 1. KZM1 belongs to the KAT1 subfamily of plant Shaker potassium channels. A, sequence comparison of Shaker K⁺ channels from maize and Arabidopsis. Alignment of the deduced amino acid sequences of KZM1 cDNA (GenBank™ accession number AJ421640) with the K⁺ channels KAT2 from Arabidopsis and ZMK2 from maize is shown. The start and stop codons of KZM1 were used as end positions of the alignment. Amino acids identical in all 3 channel proteins are shown as black-boxed letters, and residues conserved in 2 sequences are shown as gray-boxed letters. The predicted transmembrane regions (S1 to S6) and the pore region (P) are marked with solid lines. The C-terminal region of all 3 channels contains a conserved cyclic nucleotide binding motive (cNMP, dashed line), whereas only ZMK2 exhibits a putative ankyrin binding domain (ANK, asterisks). Blocks denote hydrophobic (KH) and acidic (KA) core sequences according to Ref. 40. The alignment was generated using ClustalX (41) and GeneDoc 2.0 (42), and protein domains were identified with InterPro (43).

B, phylogenetic tree, demonstrating that KZM1 is a member of the KAT1-type plant K⁺ channel subfamily. The 9 Shaker-type K⁺ channels from A. thaliana KAT1 (M86890), KAT2 (AJ288900), AKT1 (X62907), SPIK (AJ309323), AKT5 (AJ249479), AtKC1 (Z83202), AKT2/3 (AJ243703, U44745), SKOR (AJ223357), and GORK (AJ279009) group into 5 subfamilies (highlighted by gray backgrounds). A. thaliana members, which named the subfamilies, are underlined. Whereas ZMK1 (Y07632) from maize is similar to AKT1 and ZMK2 (AJ132686) is the ortholog to AKT2/3, KZM1 and KZM2 belong to the KAT1-type subfamily. GenBank™ accession numbers of the respective channels are shown in parentheses. The alignment was generated using ClustalX (41); the tree was drawn with TreeView (44).
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mm MgCl\(_2\), 1 mm CaCl\(_2\), and 10 mm Tris/Mes, pH 7.5. Pipettes were filled with solution containing 100 mm KCl, 2 mm MgCl\(_2\), 1 mm CaCl\(_2\), and 10 mm Tris/Mes, pH 7.5. Currents were recorded in the cell-attached configuration using an EPC-9 amplifier (HEKA, Lambrecht, Germany) as described previously (37).

RESULTS

KZM1 Represents a KAT1-type Shaker K~+~ Channel Gene—To study the role of K~+~ channels in C\(_4~\) leaves, characterized by Kranz anatomy, we isolated KZM1 from maize leaf cDNA via RT-PCR and RACE techniques. The cloning strategy took advantage of highly conserved regions in the Shaker gene family of plant K~+~ channels (for review see Ref. 2). Sequence analysis of the open reading frame of the KZM1 cDNA (2274 bp) revealed the basic features of the KAT1 subfamily (Fig. 1A) as follows: six putative transmembrane domains (S1–S6) with a proposed voltage sensor in segment 4 and a K~+~-selective pore (P), formed by the amphiphilic linker between S5 and S6 (for structure-function analysis of plant K~+~ channels see Refs. 38 and 39). The deduced KZM1 protein spans 758 amino acids with a predicted molecular mass of 86.7 kDa. When compared on the amino acid level to the Arabidopsis K~+~-selective channel, KZM1 showed highest similarity to KAT2 (48% identity, Ref. 13) and KAT1 (47% identity, Ref. 3), whereas the identity to the previously identified maize K~+~ channels ZMK1 and ZMK2 (23) was only 36 and 34%, respectively. Thus, KZM1 represents a member of the KAT1 subfamily of plant K~+~ channels (Fig. 1B). The proposed cytoplasmic C terminus of KZM1 contains a region, which shares structural homologies to cyclic nucleotide binding domains (Fig. 1A). In contrast to ZMK2 (five ankyrin repeats), the sequence of KZM1 did not contain an ankyrin binding domain (Fig. 1A), a feature that is conserved among K~+~-selective channels of the KAT1 subfamily (1), beside the SIRK protein from Vitis vinifera (22) and KPT1 from Populus tremula (GenBank\(^\text{TM}\) accession number AJ344483, for details see “Discussion”). In the 5' region of the open reading frame of KZM1, we could identify two possible translational start positions (“ATG”), a structural element also found with the Arabidopsis ortholog KAT2 (Fig. 1A). In addition, plant-specific hydrophobic and acidic C-terminal domains, involved in plant K~+~ channel clustering (40, 45), could be identified. Based on Southern blot analysis with maize DNA, we characterized KZM1 as a single copy gene within the maize genome (not shown). Besides KZM1 we could also identify KZM2, the second maize member of the KAT1 subfamily (Fig. 1B), most likely representing the ortholog to KAT1 from Arabidopsis.

KZM1 Is a Voltage-dependent K~+~-uptake Channel—When expressed in Xenopus oocytes, the gene product of KZM1 showed the characteristic properties of a voltage-dependent, inward-rectifying plant K~+~ channel (Fig. 2). In two-electrode voltage clamp experiments, KZM1 activated upon hyperpolarization to membrane potentials negative to −60 mV (Fig. 2A). The steady-state current-voltage curve of the data shown in Fig. 2A underlines the strong inward rectification of KZM1 (Fig. 2B). From activation curve analyses, a half-maximal activation voltage \(U_{1/2} = -105.4 \pm 6.9\) mV \((n = 5)\) was calculated. Recordings in the cell-attached patch clamp configuration allowed us to resolve single KZM1 channel-fluctuations (Fig. 2C). The channel amplitude and time-dependent activity increased with increasing negative voltages. From the current-voltage relationship of the single channels (Fig. 2D), a unitary conductance of \(20 \pm 0.7\) pS \((n = 3, \text{mean } \pm S.E. \text{ with } 100 \text{ mK}^{+} \text{ in the pipette})\) was deduced. Thus, KZM1 exhibits a 2–4-fold higher conductance than the previously characterized K~+~-channels of the KAT1 subfamily (6.7 pS for KAT2 (13), 5 pS for KAT1 (37), 7 pS for KST1 (46), and 13 pS for SIRK (22)).

In agreement with a K~+~-selective channel, K~+~ currents through KZM1 increased as a function of the external K~+~ concentration (not shown) with the current reversal potential following the Nernst potential for potassium (60.8 ± 4.7 mV per 10-fold change in external K~+~ concentration, Fig. 3A). Replacing K~+~ by Rb~+~ (100 mM) caused a drop in the inward current (at −150 mV, \(I_{\text{Rb}}/I_{\text{K}} = 0.190 \pm 0.041, n = 4\)). Comparison of the reversal potential in either K~+~ or Rb~+~-solutions allowed us to determine the permeability ratio \(P_{\text{Rb}}/P_{\text{K}} = 0.437 \pm 0.085, n = 4\). In contrast to other KAT1-like channels, the permeability ratios for Na~+~ and Li~+~ ions could not be determined, because KZM1 did not even conduct outward currents in Na~+- or Li~+-based media (Fig. 3, B and C). Similar results were obtained with N-methyl-D-glucamine solution, pointing to gating properties shared with the AKT3 channel (compare with Ref. 39).

KAT1-like channels are stimulated by external acidification (13, 22, 47). When we analyzed the sensitivity of KZM1 to extracellular pH changes in Xenopus oocytes, however, the relative open probabilities \((P_{o})\) obtained at different external proton concentrations, rendered KZM1 pH-insensitive (Fig. 3D). Thus, KZM1 seems to represent the first KAT1-type K~+~ channel not affected by external pH changes (for details see “Discussion”). In contrast, K~+~ currents through KZM1 increased upon cytoplasmic acidification of the oocyte in response to 10 mM sodium acetate, pH 5.6 in the bath solution (Fig. 3E, compare with Refs. 18 and 38). As shown in Fig. 3F, the cytosolic acid activation of KZM1 results from a shift of the half-maximal activation voltage \(U_{1/2} \Delta U_{1/2} = 36.9 \pm 10.3\) mV, \(n = 3\) toward more positive values.

KZM1 Is Expressed in Vascular Strands and Epidermis of the Maize Leaf—To identify KZM1-expressing tissues, mRNA from different organs of the maize plant was isolated. In North-
Fig. 3. KZM1 is K⁺-selective and independent on external pH. A, shift in reversal potential (U_{rev}) in response to changes in extracellular K⁺ concentration. In tail-current experiments, KZM1-expressing oocytes were challenged with an activating prepulse to −150 mV. In subsequent voltage jumps to potentials ranging from +40 to −180 mV, tail currents were elicited that reversed direction (U_{rev}) around the predicted Nernst potential for K⁺. Changing the K⁺ concentration 10-fold caused a shift in U_{rev} of 60.8 ± 4.7 mV. Error bars indicate S.D. (n = 8). B, tail-current recordings of KZM1-injected oocytes at 20 mV after a 1-s preactivating pulse to −130 mV in 100 mM K⁺ or Li⁺, respectively. The bath solution was composed of 100 mM KCl/LiCl, 1 mM CaCl₂, 2 mM MgCl₂, and 10 mM Tris/Mes, pH 7.5. Note that the outward currents in Li⁺ decay, although the driving force for K⁺ release increases. C, relative instantaneous tail-current amplitudes (rel. I_T) plotted against the membrane voltage (U) revealed outward currents positive from the reversal potential in 100 mM K⁺ (closed circles), but not in 100 mM Li⁺ or Na⁺ (closed diamonds and open circles, respectively). KZM1-expressing oocytes were challenged with an activating prepulse to −130 mV. In subsequent voltage jumps to potentials from +30 to −150 mV in 10-mV decrements, relative instantaneous tail-current amplitudes were measured at t = 0.1 s, normalized to the values in 100 mM K⁺ at −140 mV. Results represent mean ± S.D., n = 6. D, Boltzmann analysis of voltage-dependent gating at various external pH values (pH 7.5, 5.6, and 4.5, open and closed circles and squares, respectively) normalized to the maximal conductance of 1.0 obtained by Boltzmann fittings. The relative open probabilities (rel. P_o) plotted against the membrane voltage (U). Solid lines represent best Boltzmann fits to the data (gating parameters: half-maximal activation voltage U_{0.5}, gating charge Z: pH 7.5: U_{0.5} − 105.4 ± 6.9 mV, Z = 1.17 ± 0.12; pH 5.6: U_{0.5} − 108.8 ± 11.4 mV, Z = 1.05 ± 0.13; pH 4.5: U_{0.5} − 110.1 ± 10.1 mV, Z = 0.97 ± 0.07). Error bars indicate S.D. (n = 5), pH changes had no effect on the gating of KZM1 channels. E, lowering the internal proton concentration by perfusion with 10 mM NaAc at pH 5.6 (+NaAc) the inward currents, elicited by 2-s voltage pulses to −160 mV, increased with respect to those in the absence of acetate (−NaAc). F, the voltage-dependent gating in response to internal acidification was analyzed with a Boltzmann function as described in B (gating parameters: half-maximal activation voltage U_{0.5}, gating charge Z: +NaAc (open circles): U_{0.5} = −84.8 ± 3.5 mV, Z = 1.08 ± 0.17; −NaAc (closed circles): U_{0.5} = −121.6 ± 7.1 mV, Z = 1.22 ± 0.22). Error bars indicate S.D. (n = 3). KZM1 is activated by internal acidification due to a positive-going shift of the half-maximal activation voltage (36.9 ± 10.3 mV).

er analyses KZM1 transcripts were found in developing (1-week-old) and mature (5-week-old) leaves (not shown). High transcript levels were also detected in nodes, husks, and silks, whereas KZM1 mRNA was rare in internodes and not detectable in young cobs and developing tassels (not shown). To study KZM1 expression within the C₁ leaf in more detail, we fractionated the last fully developed leaf of 5-week-old maize plants into epidermal tissue, mesophyll protoplasts, and vascular strands (Fig. 4A). During enzymatic digestion, the bundle sheath cells remained attached to the vascular strands (compare with Ref. 34). In the following, the latter fraction will be addressed as “vascular/bundle sheath strands.” To estimate contaminations of this fraction, the small subunit of the ribulose-1,5-bisphosphate carboxylase (ZmRuBPCssu), specifically expressed in maize bundle sheath cells, was used as marker for vascular/bundle sheath strands. In addition the C₂ form of the phosphoenolpyruvate carboxylase (ZmC₂-PEPC) served as marker for mesophyll cells (compare with Refs. 25 and 48). By using quantitative real-time RT-PCR to determine the transcript density of those two marker genes, we could show that the epidermis and mesophyll protoplast fractions were contaminated by less than 1% of the vascular/bundle sheath RuBPCssu transcripts (Fig. 4B). In contrast, 35 and 40% of the mesophyll-specific C₂-PEPC were detected in epidermis and vascular/ bundle sheath strands, showing that these tissue preparations contained residual mesophyll fractions, probably from protoplasts still attached to the epidermal strips or vascular strands (for separation of mesophyll and bundle sheath cells compare Refs. 49 and 50). We cannot, however, exclude that the C₄ form of PEPC is expressed in guard cells and/or subsidiary cells as well (for discussion see Refs. 51 and 52).

Quantitative real-time RT-PCR on mRNA from the three different samples demonstrated that KZM1 expression in the maize leaf is restricted to vascular/bundle sheath strands and the epidermis (Fig. 4B). The transcripts were about 13 times more abundant in the vascular/bundle sheath strands than in the epidermis. In mesophyll protoplasts the KZM1 mRNA level was at the detection limit of the real-time RT-PCR method. Upon peeling of the epidermis, common epidermal cells ruptured, whereas viable guard and subsidiary cells, visualized by neutral red staining (Fig. 4A, inset, compare with Ref. 53), survive this mechanical treatment. Thus, KZM1 expression in the maize leaf is restricted to guard/subsidiary cells and the phloem-enriched vascular/bundle sheath strands. As a phloem marker we used the K⁺ channel gene ZMK2 (23, 24), which was detected in vascular bundles only (not shown, compare for phloem localization of AKT2/3 (16, 18, 19)). These results suggest that KZM1 displays an expression pattern similar to its Arabidopsis ortholog KAT2 (phloem tissue and guard cells (13), for differences see “Discussion”).

Expression of KZM1 during Development, Sink-Source Transitions, and Diurnal Changes—To explore the role of KZM1 in phloem physiology, we followed the expression of this K⁺ channel gene along different developmental stages of the primary
leaf and the 4th leaf of the maize plant (Fig. 5). Here the juvenile, just emerging organs of the leaves represent carbohydrate sinks (54), whereas the mature leaves serve as source tissue. In contrast to the broad pattern of KZM1 expression, transcripts of the phloem K⁺ channel gene ZMK2 were restricted to sink tissues such as young leaves (Fig. 5) and coleoptiles (23, 24). The sucrose transporter gene ZmsUT1 (31), however, was prominent in the RNA fraction isolated from mature source leaves (Fig. 5). Because ZmsUT1 expressed in oocytes mediates proton-coupled sucrose uptake (compare Fig. 8), this may indicate a source-specific function of ZmsUT1 for sucrose loading into the phloem.

The tip region of the maize leaf contains the oldest cells and mostly minor veins, in which thin-walled sieve tubes in combination with companion cells serve as the source site of apoplastic phloem loading (25, 29). In contrast, the leaf base is dominated by young cells and thick vascular bundles. In young leaves the latter are involved in phloem unloading (sink) to support growth in this expanding leaf zone, and in mature leaves they mediate long distance transport of carbohydrates in the thin-walled sieve tubes (compare with Refs. 28 and 54). In contrast to the leaf blade, characterized by sucrose production and phloem loading in a C₄-specific manner, the sheath of the maize leaf does not show the C₄ intrinsic Kranz anatomy (55).

Here the sheath contains mostly large vascular bundles with thin-walled sieve tubes, considered to mediate transport of photosynthates out of the leaf. Moreover, veins of the leaf blade consist of thick-walled sieve tubes, not present in the leaf sheath (55). Thick-walled sieve tubes in combination with vascular parenchyma cells are thought to mediate retrieval of solutes leaking out of the xylem vessels (56, 57). Northern blot analyses with mRNA extracted from 6 different zones of a 20-day-old maize leaf blade showed ZM1 to be evenly expressed from tip to base. With leaf sheath mRNA, however, no signal was obtained (Fig. 6), pointing to a role for KZM1 in K⁺ retrieval from xylem vessels of the leaf blade via phloem parenchyma and thick-walled sieve tubes. As noticed for leaf development before (Fig. 5), ZMK2 was predominantly expressed in the sink and transport regions of the leaf, represented by the leaf base and sheath (Fig. 6). In addition ZMK2 mRNA was seen in the very tip. As was shown by Anki et al. (31), transcripts of the source-specific sucrose transporter ZmsUT1 in expanding leaf blades increase from the unexpanded base (sink) toward the expanded tip region (source).

During the day, ZmsUT1 transcript levels in the leaf increase, reach a maximum at the end of the light period, and decrease during the night (31). Thus, the expression pattern of this sugar transporter correlates with carbohydrate synthesis and phloem loading. In line with a constitutively active gene, KZM1 expression in the leaf blade was not affected by diurnal changes (Fig. 7). In contrast, ZMK2 transcripts accumulated during the dark period, again pointing to an important function of this phloem K⁺ channel in sink control.

These studies show that KZM1 expression in contrast to the source- (ZmsUT1) and sink-specific (ZMK2) genes is not affected by leaf development, sink-source transitions, or diurnal changes. However, expression of KZM1 is clearly associated with K⁺ retrieval by phloem parenchyma cells of the leaf blade and not with long distance transport of photosynthates in the leaf sheath or internodes. Based on this expression pattern, one would conclude that KZM1 has a housekeeping function in the vascular bundles of the maize leaf, controlling K⁺ retrieval into the phloem and K⁺ homeostasis.

Cooperation of Phloem K⁺ Channels and Sucrose Transporters—To gain insight into the feedback control between K⁺ channels and sucrose transporters in the phloem of the maize

Fig. 5. KZM1 is constitutively expressed during leaf development. Northern blot with 0.7 µg mRNA, isolated from young (7 days (7d)) and mature (11 days (11d)) maize primary leaves (left) and from the 4th leaf of the maize plant (right) at juvenile (15 days (15d)), intermediate (19 days (19d)), and mature stage (28 days (28d)). Young leaves were just emerging from the plant (sink tissue), and the mature leaves were characterized by a visible leaf collar (source tissue). The RNA was blotted against radiolabeled cDNA probes of KZM1, ZMK2, and ZmsUT1 (GenBank™ accession number AB008464). To standardize mRNA levels, 15 ng of dotted mRNA were hybridized against a radiolabeled oligo(dT) probe (lower panel).
leaf, we studied the functional properties of the sucrose transporter ZmSUT1 alone and in the presence of either KZM1 or ZMK2 in *Xenopus* oocytes (Fig. 8). When the phloem-specific sucrose/H\(^+\) co-transporter ZmSUT1 (31) was expressed in *Xenopus* oocytes, a sucrose-induced depolarization of the membrane was monitored. Properties like the sucrose specificity of ZmSUT1, as well as its concentration and pH dependence (not shown), were well in agreement with studies on the phloem sucrose/H\(^+\) symporter ortholog AtSUC2 from *Arabidopsis* (17). However, when oocytes expressing both the voltage-independent phloem K\(^+\) channel ZMK2 (23) and ZmSUT1 were challenged with sucrose, the drop in membrane potential was prevented (Fig. 8). This indicates that ZMK2, because of its peculiar kinetics and voltage dependence (compare Refs. 23 and 24), stabilizes the membrane potential in the presence of the sucrose-fueled sugar/H\(^+\) symporter. In vivo this phloem K\(^+\) channel thus very likely repolarizes the membrane rather than catalyzing the bulk flow of potassium, a function of which KZM1 is capable (see below). In contrast to ZMK2, KZM1 is activated at hyperpolarizing potentials only (compare Fig. 2). Coexpression of the sucrose/H\(^+\) symporter with the inward rectifier KZM1 therefore did not prevent the sucrose-dependent depolarization of the membrane potential. Thus, voltage-independent, proton-blocked K\(^+\) channels like ZMK2, which clamp the membrane to the Nernst potential for K\(^+\), during phloem loading and unloading processes interact with the sucrose transporter ZmSUT1 via the membrane potential and extracellular pH. The voltage-dependent but pH-insensitive inward rectifier KZM1 is able to maintain the K\(^+\) homeostasis of the leaf phloem. Under physiological conditions the phloem H\(^+\)-ATPase at the source site often hyperpolarizes the membrane negative to \(-80\) mV. Membrane polarization in the long run is accompanied by a drop in external pH. The latter effect will inhibit ZMR2, but not KZM1 which is activated negative to \(-60\) mV (compare Fig. 2A). Thus, the inward rectifier provides for K\(^+\) uptake as well as for a membrane control unit at more hyperpolarized potentials. With the voltage-independent, H\(^+\)-blocked ZMK2 and the voltage-dependent, H\(^+\)-insensitive KZM1 working hand in hand, membrane potential and K\(^+\) homeostasis can be controlled over a broad voltage and pH range.

### DISCUSSION

In this study we have focused on KZM1, a new K\(^+\) channel gene from *Z. mays*, belonging to the *Shaker* family of plant K\(^+\) channels. KZM1 displays structural, functional, and expression patterns reminiscent of the KAT2 K\(^+\) channel from *Arabidopsis*. Thus, KZM1 represents the first KAT-type K\(^+\) channel isolated from a *C\(_4\)* species. In *Arabidopsis* the KAT1 subfamily consists of two members, KAT1 and KAT2 (compare with Fig. 1). With the isolation of KZM2, another KAT-type K\(^+\) channel gene from maize, most likely representing the orthologous gene to KAT1, the genome of *Z. mays* also seems to harbor two KAT1 subfamily members.

The KZM1 protein is characterized by the absence of an ankyrin repeat domain, a feature that was assigned to be specific for the KAT1-type *Shaker* K\(^+\) channels (1). Only very recently it was reported that SIRK, a KAT-like K\(^+\) channel from grapevine (*V. vinifera*), contains an ankyrin repeat (22). Because this ankyrin repeat is truncated (one repeat and two half-motifs) compared with complete ankyrin binding domains of the AKT1-, ART2-3-, and SKOR-like channels (five to six repeats), it was concluded that this atypical feature of SIRK gives insight into the evolution of the plant *Shaker* K\(^+\) channel family. The KAT-type channel KPT1 from poplar trees (*P. tremula*) contains such a truncated ankyrin domain as well, indicating that the KAT1 subfamily of woody species may be identified by this motif. KZM1 from the monocot *Z.

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4 K. Langer, unpublished results.
mays, however, is more closely related to KAT1, KAT2 from Arabidopsis, and KST1 from Solanum tuberosum, all characterized by the absence of ankyrin repeat motifs. Like KAT2 (13) and AKT2 (18), KZM1 contains two possible translational start positions within the 5'-region of the open reading frame. The cRNA including both methionines was functional in Xenopus oocytes. Future experiments similar to those performed with AKT2 will address the role of the second possible translational start position (compare with Ref. 18).

When expressed in Xenopus oocytes, KZM1 showed the characteristic properties of a voltage-dependent, inward-rectifying plant K⁺ channel. Differences between the monocotyledonous KZM1 and the dicotyledonous KAT-type K⁺ channels KAT1, KAT2, KST1, and SIK could be assigned to the higher single channel conductance of KZM1 (20 pS) and the fact that the gating of KZM1 required potassium. The latter property could recently be recognized for AKT3, another phloem K⁺ channel (for discussion see Ref. 39). These channels, sensing the K⁺ concentration in the sink and source and along the transport phloem, are able to control K⁺ release into growing tissue and resorption from mature or even senescing leaves.

Another unique feature of KZM1 is displayed by its insensitivity to external pH changes. All previously characterized KAT1-like K⁺ channels are activated by an increase in the extracellular proton concentration (13, 22, 38), whereas channels from the AKT2/3 and SKOR subfamilies are proton blocked (19, 21, 58). Thus, structural elements of KZM1 might provide a new molecular tool for future structure-function experiments to access the external pH sensor of plant K⁺ channels. This proton sensor of the potato guard cell K⁺ channel KST1 could be assigned to two extracellular histidine residues in the outer pore (His-271) and between the linker of the transmembrane helices S3 and S4 (His-160) of the protein (36). The histidine of the outer pore is unique to all plant K⁺ channels. However, KZM1 lacks the histidine residue in the linker between S3 and S4 but possesses a more positive charged arginine at this site. When the adequate mutation H160R was introduced into KST1, the protein lost its pH sensitivity almost completely (36). Therefore, it is tempting to speculate that the absence of histidine residue 160 might contribute to the insensitivity of KZM1 to external pH changes. In contrast, we found the histidine of the internal pH sensor, originally identified for the Arabidopsis KAT1 (59), located in the intracellular loop between the transmembrane helices S2 and S3 of KZM1, very likely responsible for the sensitivity of KZM1 to internal protons. The insensitivity of KZM1 toward changes in the extracellular proton concentration clearly distinguishes this channel from its Arabidopsis ortholog KAT2 and therefore might reflect a feature required to operate a monocotyledonous plant like maize, characterized by Kranz anatomy and C₄-acid metabolism (see below).

By quantitative real-time RT-PCR analysis, we could identify guard cells, subsidiary cells, and vascular/bundle sheath strands as sites of KZM1 expression. By using the patch clamp technique, KZM1-like, inward-rectifying K⁺ channels have been identified in maize guard cells (60, 61) and subsidiary cells (53). Thus KZM1, together with KZM2, seems to carry a major part of the inward K⁺ current in Z. mays guard cells and subsidiary cells during stomatal movement (compare Refs. 12 and 13). Likewise in bundle sheath cells, KZM1 might contribute to the inward K⁺ currents recorded by Keunecke and Hansen (34) and Keunecke et al. (62).

Because KZM1 expression was most pronounced in vascular/bundle sheath strands, we investigated the role of this channel protein for carbohydrate export and import during leaf development, sink-source transitions, and diurnal changes. Sugar transport from the bundle sheath cells of the C₄ plant Z. mays to the phloem parenchyma cells occurs symplastically (26–28), whereas the subsequent loading to the thin-walled sieve element-companion cell complex is believed to involve an apoplastic step (29, 30, 56, 57). Aoki et al. (31) provided evidence for a role of the sucrose transporter ZmSUT1 during phloem loading of carbohydrates exported from source leaf blades. This hypothesis is supported by our finding that ZmSUT1 encodes a H⁺/sucrose transporter, and its expression is restricted to source tissues (e.g., mature leaves). In contrast, the K⁺ channel gene ZMK2 displayed an inverse expression pattern pointing to a more sink-specific function. The phloem K⁺ channel ZMK2 belongs to the AKT2/3-type subfamily of Shaker K⁺ channels (compare Fig. 1B). In Arabidopsis, AKT2/3 is expressed predominantly in source organs and plays a role in sugar loading of the phloem (16, 17). In contrast to AKT2/3 but in line with ZMK2, their ortholog VKF1 from V. faba is found in sink tissues and during transition from source to sink and therefore related to phloem unloading (32). However, we localized ZMK2 expression also in the tip of the leaf (source), indicating that ZMK2 can be involved in phloem loading as well, as discussed for AKT2/3-like channels (18, 32).

The expression of KZM1 in the maize leaf was highest in the vascular/bundle sheath. In contrast to ZmSUT1 or ZMK2 this gene was constitutively expressed during leaf development, sink-source transitions, and diurnal changes. The tip of the maize leaf, responsible for loading of sucrose to the phloem, alkalinizes the apoplast, whereas the expansion growth of young cells in the leaf base results in an acidification of the extracellular medium (63). Moreover during phloem unloading in sink tissues, the apoplastic pH increases, activating K⁺ channels like ZMK2 to control the membrane potential (for discussion see Ref. 32). In such an environment, the K⁺-uptake channel KZM1 is insensitive to external pH changes, providing a mechanism that is robust to sink-source changes, day-night cycles, and even development and thus maintains phloem K⁺ uptake and homeostasis. Testing the potential feedback loops between the maize phloem K⁺ channels and the sucrose/H⁺-symporter ZmSUT1 in Xenopus oocytes, we could show that the voltage-independent ZMK2 prevents a collapse of membrane potential during H⁺/sucrose transport via ZmSUT1. In contrast, KZM1 in the presence of ZmSUT1 is able to mediate the bulk flow of potassium into the maize phloem. At hyperpolarized potentials and acidic apoplast following enhanced H⁺-ATPase activity, KZM1 is able to substitute ZMK2 in control of phloem potential. In addition, KZM1 gene expression is linked to K⁺ retrieval from xylem vessels in the leaf blade and not to long distance transport in the leaf sheath or stem internodes. The orthologous K⁺ channel gene KAT2 in Arabidopsis shows a similar expression pattern as KZM1 but is characterized by distinct functional properties such as activation by external protons, K⁺-independent gating, and a smaller single channel conductance (13). Up to now data on the regulation and phloem function of the KAT2 gene are lacking. Here we could show that KZM1 in contrast is equipped with unique functional characteristics (K⁺-dependent gating, insensitivity to external pH, and high single channel conductance) and a specific expression pattern in the leaf phloem, all pointing to a housekeeping function of KZM1 for K⁺ homeostasis in the phloem of a C₄ leaf and K⁺ transport required for the related organic acid-based metabolism.

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The $K^+$ Channel KZM1 Mediates Potassium Uptake into the Phloem and Guard Cells of the C$_4$ Grass Zea mays
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