Functions of AKT1 and AKT2 Potassium Channels Determined by Studies of Single and Double Mutants of Arabidopsis

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A reverse genetic strategy was used to isolate Arabidopsis plants containing “knockout” mutations in AKT1 and AKT2, two members of a K+ channel gene family. Comparative studies of growth and membrane properties in wild-type and mutant seedlings were performed to investigate the physiological functions of these two related channels. The growth rates of plants supplied with rate-limiting concentrations of K+ depended on the presence of AKT1 but not AKT2 channels. This result indicates that AKT1 but not AKT2 mediates growth-sustaining uptake of K+ into roots, consistent with the expression patterns of these two genes. K+-induced membrane depolarizations were measured with microelectrodes to assess the contribution each channel makes to the K+ permeability of the plasma membrane in three different organs. In apical root cells, AKT1 but not AKT2 contributed to the K+ permeability of the plasma membrane. In cotyledons, AKT1 was also the principal contributor to the K+ permeability. However, in the mesophyll cells of leaves, AKT2 accounted for approximately 50% of the K+ permeability, whereas AKT1 unexpectedly accounted for the remainder. The approximately equal contributions of AKT1 and AKT2 in leaves detected by the in vivo functional assay employed here are not in agreement with previous RNA blots and promoter activity studies, which showed AKT2 expression to be much higher than AKT1 expression in leaves. This work demonstrates that comparative functional studies of specific mutants can quantify the relative contributions of particular members of a gene family, and that expression studies alone may not reliably map out distribution of gene functions.

The most abundant inorganic solute in plant cells is K+. The transport in and out of cells of this essential element is a highly regulated process mediated by specific transporters within the plasma membrane (Maathuis et al., 1997; Chrispeels et al., 1999). Present at concentrations on the order of 100 mM, K+ serves as an osmoticum important to turgor pressure, and may act as an essential cofactor for certain enzymes. Its abundance contributes to the electrolyte character of cytoplasm and affects electrostatic interactions between charged entities such as proteins and other biopolymers. The transport of K+ helps set the electric potential difference across the plasma membrane, which powers the transport of other substances. Because K+ serves such fundamental functions throughout the plant, understanding the molecular mechanisms of its uptake and redistribution is an important goal. Progress in this regard may also spawn novel strategies for improving plant mineral nutrition and fertilizer application in the field.

The first isolation of Arabidopsis genes encoding plasma membrane K+ channels by complementation of yeast K+ uptake mutants marked a major step toward this goal (Anderson et al., 1992; Sentenac et al., 1992). The transport properties displayed by AKT1 and KAT1 channels expressed in heterologous systems (Schachtman et al., 1992; Bertl et al., 1995, 1997; Gaymard et al., 1998a) indicated that they probably function in plants as K+ uptake pathways in the tissues that express them; primarily guard cells in the case of KAT1 and root cells for AKT1 (Nakamura et al., 1995; Lagarde et al., 1996). Comparisons of genome sequences revealed that AKTI and KATT are members of a family of plant channels similar in structure and sequence to the shaker superfamily of...
animal voltage-dependent K\(^+\) channels (Anderson et al., 1992; Sentenac et al., 1992).

The field now faces the challenge of ascribing physiological functions to the various other family members. One standard approach to this problem has been to determine the tissue expression pattern for particular family members, at the protein level with isoform-specific antibodies, or at the mRNA level with gene-specific hybridization probes or promoter-reporter gene constructs. Hypotheses about function then are based on the observed expression patterns. For example, KAT1 is primarily expressed in guard cells so it is logical to propose that it encodes the well-studied inward-rectifying K\(^+\) channels that mediate stomatal opening. Although this strategy has proven useful, it does not directly indicate the distribution of activity for each family member’s gene product.

A more direct means of determining the function of specific gene family members is to isolate null mutants for each of the genes and then to assess the phenotypes of these homozygous “knockout” plants by performing in planta assays of the encoded protein’s catalytic function. This “reverse genetic” strategy relies on a PCR-based method of screening DNA pools from large numbers of T-DNA-mutagenized plants to isolate individuals containing a mutation in the gene of interest (Krysan et al., 1999). A plant with an insertion in the AKT1 gene was isolated previously and used to study the function of the channel it encodes (Hirsch et al., 1998). Consistent with the AKT1 expression pattern, electrophysiological experiments revealed that root cells of the knockout mutant, \( akt1-1 \), lacked inward-rectifying K\(^+\) channel activity, displayed significantly reduced plasma membrane K\(^+\) permeability, and grew more slowly than wild type on media containing rate-limiting concentrations of K\(^+\). These results indicated that AKT1 mediates K\(^+\) uptake into roots in parallel with one or more as-yet-unidentified, NH\(_4\)\(^+\)-sensitive transporters (Hirsch et al., 1998; Spalding et al., 1999).

Among the several shaker-like channel genes identified in plants is AKT2 (Cao et al., 1995; Ketchum and Slayman, 1996), a K\(^+\) channel related to AKT1 but whose mRNA is predominantly located in the phloem of stems and leaves (Marten et al., 1999; Lacombe et al., 2000). Here, we report the isolation of an \( akt2 \) mutant by reverse genetics and the generation of an \( akt1\Delta\Delta \) double mutant. Using this set of channel mutants and a microelectrode-based technique for assessing K\(^+\) permeability of the plasma membrane, we were able to quantitatively map the distribution of AKT1 and AKT2 activities in roots, cotyledons, and leaves of Arabidopsis seedlings. The degree to which growth rate depends on each of these channels was also assessed. Our results demonstrate that insight into the “division of labor” between members of a gene family can be obtained by combining reverse genetics and assays of in vivo function.

**RESULTS**

**Mutant Isolation and Identification**

To study the function of the \( AKT1 \) and \( AKT2 \) genes, plants containing T-DNA insertion alleles of each were isolated. Sequence analysis revealed that in both cases the T-DNA disrupted the gene within the coding region, as shown in Figure 1A. The \( akt1-1 \) allele contains a T-DNA insert in the 3’ end of the gene, in a region predicted to encode a regulatory domain in the C terminus. Plants homozygous for this mutation appear to lack AKT1 activity completely (Hirsch et al., 1998). The \( akt2-1 \) allele contains...
a T-DNA insert in the third exon of the coding sequence, a region predicted to encode the third transmembrane domain of the protein (Fig. 1A). Because the T-DNA disrupted the gene before the pore-forming region would be transcribed, the akt2-1 allele is not expected to make functional protein. A double mutant homozygous for both the akt1-1 and akt2-1 mutations was constructed as described in “Materials and Methods.” Figure 1B shows that the akt1-1 and akt2-1 parental lines did not contain genomic DNA capable of producing AKT1- and AKT2-specific PCR products, respectively. Plants of the F₁ generation were heterozygous at each locus, as expected (Fig. 1B). An akt1ΔΔ akt2ΔΔ double-mutant line was identified in an F₂ population as an individual lacking wild-type copies of AKT1 and AKT2 (Fig. 1B).

Contributions of AKT1 and AKT2 to Growth Rate

When grown on soil replete with nutrients, neither of the single mutants nor the double mutant displayed an overt phenotype. Previous work had demonstrated that under specific conditions, growth of akt1-1 seedlings was strongly impaired relative to wild type (Hirsch et al., 1998; Spalding et al., 1999). Experiments were performed to determine if a similar phenotype would become apparent in akt2-1 seedlings when they were grown in conditions that made K⁺ uptake the rate-limiting step in the growth process, i.e. at concentrations less than 1,000 μM and in the presence of NH₄⁺. Examinations of akt1ΔΔ plants under these same conditions would test for genetic interactions between the two channel genes. For example, evidence of one channel compensating for the lack of another could be obtained if the double mutant was affected in ways not explicable by the sum of the single-mutant phenotypes.

Figure 2A shows that in the absence of NH₄⁺ the growth rate of wild-type seedlings increased as [K⁺]ext increased between the concentrations of 10 and 1,000 μM. This established the important point that under these conditions, the growth rate of seedlings was limited by the availability of K⁺. Loss of either AKT1 or AKT2 had little effect on growth rate at any of the K⁺ concentrations when NH₄⁺ was absent. Figure 2B shows that addition of NH₄⁺ greatly inhibited the growth rate of akt1-1 plants without affecting growth of akt2-1 seedlings. As found previously, increasing [K⁺]ext ameliorated the inhibitory effect of NH₄⁺, consistent with the notion that NH₄⁺ inhibits a non-AKT1 K⁺ uptake pathway by competing for a K⁺ binding site (Spalding et al., 1999). It is presumable that because akt2-1 seedlings possess a functional AKT1 channel, their growth is not sensitive to NH₄⁺ and is similar to or greater than wild type under all combinations of K⁺ and NH₄⁺ (Fig. 2, A–C). Taken together, these results indicate that AKT1, but not AKT2, contributes to the ability of seedlings to take up K⁺ when its availability limits growth of seedlings.

The growth rate of the akt1ΔΔ/2Δ double mutant was examined under the same conditions to determine if the absence of AKT2 was of more consequence in a genetic background lacking AKT1 than it was in a wild-type background. A consistent exacerbation of the akt1-1 phenotype by the akt2-1 mutation was observed but the small effect was not statistically significant. A reasonable conclusion to be drawn from these data is that AKT2 does not contribute importantly to seedling growth rate on K⁺-limiting media even in the absence of AKT1, and even when growth is made more dependent on AKT1 by the presence of NH₄⁺. For the most part, the mutations in these two related channels act independently of each other.
Contributions of AKT1 and AKT2 to Cotyledon K⁺ Permeability

Cotyledons were also examined to determine which of the two AKT channels contributed more to the K⁺ permeability of the plasma membrane in these photosynthetic organs. Figure 4 demonstrates that the small ΔVₑ𝐦 induced by shifting [K⁺]ₑₓ from 10 to 100 μM in wild-type cotyledons was essentially eliminated in akt1-1 cotyledons. The akt1-1 mutation had no significant effect on the membrane potential response to this same K⁺ shift. The higher K⁺ shift (100–1,000 μM) induced a small depolarization in akt1-1 cotyledons and a response in akt2-1 cotyledons that was 65% of the wild-type value. Thus, the K⁺ permeability of cotyledon cells behaves as the sum of a large AKT1 component and a small AKT2 component.

Contributions of AKT1 and AKT2 to Leaf K⁺ Permeability and Growth

The K⁺ permeability of mesophyll cells was assessed in leaves by the same method of measuring K⁺-induced changes in Vₑ. The ΔVₑᵐ induced by 10-fold shifts in [K⁺]ₑₓ in wild-type leaves, shown in Figure 5, were significantly smaller than the responses measured in roots or cotyledons, consistent with previous demonstrations that the K⁺ permeability of mesophyll cells is relatively low (Bei and Luan, 1998). Although smaller in magnitude than the AKT1-dependent response of root cells, the ΔVₑᵐ measured in leaf mesophyll probably also reflects the activity of plasma membrane K⁺ channels, which were characterized by patch-clamp studies (Spalding et al., 1992; Spalding and Goldsmith, 1993). Shifts of [K⁺]ₑₓ from 10 to 100 μM induced a small response of 4 mV in wild-type leaves. The responses of akt1-1 and akt2-1 mutants to the same treatment were almost undetectable. Mesophyll cell membranes of the double mutant responded with a slight hyperpolarization, indicating that deletion of both AKT channels and the scarcity of K⁺ created a situation in which the permeability of one or more other ions exceeded that of K⁺. Chloride ions are likely candidates and the theory supporting this interpretation was recently explained in Spalding et al. (1999).

Shifts from 100 to 1,000 μM K⁺ induced a 20-mV response in wild-type leaves. The responses mea-
Figure 5. K⁺ permeability of leaf mesophyll cells in wild-type and mutant seedlings. Values are mean depolarization in response to shifts in [K⁺]ext from 10 to 100 μM, and from 100 to 1,000 μM for between six and eight independent trials. Error bars represent s.e. of the mean.

Figure 6. Phenotypes of wild-type and mutant plants at the leaf development stage. Photographs of representative individual plants grown for 22 d on agarose medium containing 100 μM K⁺ and 2 mM NH₄⁺. Leaf growth and development is not affected by the akt2-1 mutation under the same K⁺-limiting condition that strongly inhibits akt1-1 growth.
transcriptional regulation of AKT1 or AKT2 equalizes their contributions. This insight into the operation of proteins encoded by paralogous genes is a unique result of combining reverse genetics and quantitative assays of function.

Posttranslational regulation of activity is very common in channels. Some types rarely adopt their open-state conformation until an event such as ligand binding, phosphorylation, or a change in membrane potential increases many fold their probability of opening. An abundant channel may not contribute to the permeability of the membrane to the extent indicated by its mRNA or protein levels if it is rarely open. Likewise, a rare channel could contribute more than expected if its open probability were high. It is possible that AKT1, expected to be much the less abundant of the two channels, has a relatively high open probability, which would increase its contribution to membrane permeability. Patch-clamp experiments performed with mesophyll protoplasts isolated from wild-type and mutant plants could test this possibility. An independent explanation is that protein levels of AKT1 and AKT2 are not proportional to their mRNA concentrations. This could be tested with immunohistochemical methods.

It is prudent to consider how the functional assays used here are affected by the leaf’s anatomy. To make measurements in leaves, the tip of the electrode was advanced through the organ until a highly negative and stable $V_m$ was obtained. Although the electrode tip could not be visualized during this process, it was probably located inside a mesophyll cell because the first cell encountered (epidermis) rarely produced a stable recording and cells associated with the vascular bundles are far fewer and smaller than mesophyll cells. Any path for current to flow between the electrode tip and the grounded bathing solution must cross a plasma membrane. Shifts in $[K^+]_{ext}$ produce a $\Delta V_m$ that is proportional to the $K^+$ permeability of the plasma membrane(s) in the least resistive of all such paths. That membrane is probably the plasma membrane of the impaled mesophyll cell because it separates the cytoplasm from a low-resistance, apoplastic path to the bathing solution. (Although the electrode tip was undoubtedly located in the vacuole in the majority of recordings, this does not affect the interpretation of $\Delta V_m$.)$[K^+]_{ext}$ shifts in $[K^+]_{ext}$ could not alter the tonoplast voltage and therefore its effect on the measured $\Delta V_m$ can be considered negligible [Bates et al., 1982].) In this simplest case, the measured $\Delta V_m$ reflects the $K^+$ permeability of the impaled cell. However, the existence of plasmodesmatal connections between mesophyll cells has the effect of producing a weighted average of the $\Delta V_m$ responses of adjacent cells, the contribution of each cell to the measured value being weighted by the extent to which it is electrically coupled to the impaled cell (Spanswick, 1972). In the extreme case of perfect intercellular coupling, the measured $\Delta V_m$ would be the average response of all connected mesophyll cells. The real situation undoubtedly lies somewhere between the extremes of nonexistent and perfect coupling so the recorded $\Delta V_m$ may be viewed as the average response of a few interconnected mesophyll cells.

The AKT2 gene, previously also referred to as AKT3 (the nomenclature is explained by Lacombe et al. [2000]) is not expressed uniformly throughout the leaf. Reporter gene constructs and mRNA measurements indicate that AKT2 expression is considerably higher in phloem than mesophyll of Arabidopsis leaves (Marten et al., 1999; Lacombe et al., 2000). Did an electrode located in the mesophyll detect membrane responses in cells of other leaf tissues such as the phloem and therefore give a distorted view of the degree to which the two channels contribute to mesophyll membrane properties? If there were significant intercellular coupling between phloem and mesophyll cells, the AKT2 contribution in mesophyll would be overestimated and the AKT1 contribution detected here would be even more out of line with its expression level. Also, if the mesophyll and phloem had extensive symplastic connections, the active concentration of sugars into the latter tissue could not occur, at least by the currently accepted mechanisms. The most straightforward interpretation of the data in Figure 5 is that the $K^+$ permeability of the mesophyll of Arabidopsis leaves, on average, is approximately equally determined by AKT1 and AKT2, despite their very different mRNA expression levels.

A scenario that could complicate the present assessment of channel contributions is that expression of AKT family members change when one is mutated. There is no evidence that such compensation occurs in roots or cotyledons because removing AKT1 channels has a large effect, whereas loss of AKT2 has little or no effect. AKT1 appears to be important in these organs, and AKT2 does not appear to compensate for the loss of AKT1. The results obtained with leaves (Fig. 5) also hold no evidence that any consistent compensatory changes in expression of these two genes occurred. That is not to say no changes in gene expression occur as a result of a particular channel mutation. A very appropriate use of current DNA microarrays would be to determine the impact of the mutations presented here on genome-wide expression profiles. Suites of genes related to $K^+$ nutrition and the control of membrane permeability may be revealed.

An electrophysiological phenotype of the akt2-1 mutant was uncovered here, although a corresponding impairment of growth was not observed. When grown on nutrient-replete soil, all of the mutants studied here resembled the wild type. This indicates that neither channel is required for leaf growth under those conditions. When grown in $K^+$-limiting conditions to the leaf-producing stage, akt2-1 and wild-type plants were similar (Fig. 6). The 50% reduction
in K⁺ permeability caused by the akt2-1 mutation apparently is not sufficient to impair growth. In fact, in the presence of NH₄⁺, the lack of AKT2 activity appears to be somewhat beneficial. The possibility that AKT1 provides an activity required for leaf expansion is not readily tested with these mutants because akt1-1 seedlings do not reach the leaf expansion stage in K⁺-limiting conditions, presumably because the uptake ability of their roots has been greatly impaired.

Perhaps loss of AKT2 does not affect leaf expansion because AKT1 can provide the necessary uptake function, or perhaps AKT2 does not function primarily as a K⁺ uptake mechanism in leaves analogous to the AKT1 function in roots. A link between K⁺ permeability and photosynthesis in leaf mesophyll cells has long been established (Jeschke, 1976) and previous patch-clamp studies of Arabidopsis mesophyll cells indicated that AKT2-like K⁺ channels at the plasma membrane are activated by photosynthesis, possibly via ATP (Spalding and Goldsmith, 1993). It has not yet been determined whether photosynthesis benefits from the increase in K⁺ permeability it causes. The new mutants described here may prove to be useful in studies of the importance of K⁺ permeability to photosynthesis. They may also be used to genetically test the suggestion that AKT2 activity is important to sugar translocation in phloem (La-combe et al., 2000). There are undoubtedly other K⁺-dependent physiological processes that will be better understood by studying channel “knockout” mutants with appropriate functional assays.

**MATERIALS AND METHODS**

**Identification of T-DNA Mutant Lines**

The akt1-1 and akt2-1 plants were isolated from a T-DNA mutagenized population of Arabidopsis (a combination of the Arabidopsis Biological Resource Center and DuPont collections) using a PCR-based, reverse genetic strategy (Krystan et al., 1996). DNA sequencing of PCR-amplified fragments that spanned the insertion site determined the position of the T-DNA insert in each gene.

Plants homozygous for the akt1-1 allele were crossed to plants homozygous for the akt2-1 allele. Individuals of the F₁ generation were grown and allowed to self-fertilize to produce a population of F₂ plants in which the two mutant alleles were independently segregating. The genotypes of individual F₂ plants were determined by examining the edge of the leaf. Experiments proceeded with shifts in external potassium concentration ([K⁺]ₑ) only if the membrane potential (Vₑ) stabilized at a value more negative than −175 mV. (The resting potentials of the mutants and wild-type plants were not significantly different.) If a few basic assumptions are adopted, changes in membrane potential (ΔVₑ) due to shifts in [K⁺]ₑ are indicative of the relative permeability of the plasma membrane to K⁺ (Spalding et al., 1999).

**Growth Rate Measurements**

To measure growth rate, 25 surface-sterilized seeds each of akt1-1, akt2-1, akt1ΔΔ, and the Arabidopsis Wasilewskija ecotype were sown on a complete nutrient medium (Spalding et al., 1999) containing 0.8% (w/v) agarose in square petri plates such that all four genotypes were represented on each of two plates. After 4 d of growth, one plate was harvested and the fresh weights of each genotype group were measured to the nearest 0.1 mg. The second plate was harvested after 8 d of growth, the seedlings were weighed, and the difference between the 4- and 8-d masses was divided by 4 to obtain a growth rate value in units of milligrams per day for 25 seedlings. This experiment was repeated at least three times to obtain the averages presented. Potassium was varied as additions of 10-, 100-, and 1,000-μM concentrations of KCl to the nominally K⁺-free nutrient medium. The concentration of NH₄⁺ was varied from 0 to 2 mM by adding NH₄H₂PO₄, and to 4 mM by adding additional NH₄Cl.

To measure the growth rates of older plants, nine seeds were sown on 50 mL of the complete medium (100 μM KCl and 2 mM NH₄Cl) in transparent plastic “magenta” boxes (100 × 65 × 65 mm). Duplicate boxes with seeds were placed in a growth chamber set to deliver 16-h-light/8-h-dark cycles. The culture boxes were sealed to prevent evaporation, which could alter critical ion concentrations of the medium during the course of the experiment. The mass of the nine seedlings was determined after 18 or 22 d of growth and growth rates calculated from the differences as stated above. The average rate of growth during these 4 d was primarily due to expansion of the five or six leaves wild-type seedlings had produced at this stage in development. The experiment was repeated three times per genotype.

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