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# Increased Functional Diversity of Plant K<sup>+</sup> Channels by Preferential Heteromerization of the Shaker-like Subunits AKT2 and KAT2\*

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Assembly of plant Shaker subunits as heterotetramers, increasing channel functional diversity, has been reported. Here we focus on a new interaction, between AKT2 and KAT2 subunits. The assembly as AKT2/KAT2 heterotetramers is demonstrated by (i) a strong signal in two-hybrid tests with intracytoplasmic C-terminal regions, (ii) the effect of KAT2 on AKT2 subunit targeting in tobacco cells, (iii) the complete inhibition of AKT2 currents by co-expression with a dominant-negative KAT2 subunit in *Xenopus* oocytes, and reciprocally, and (iv) the appearance, upon co-expression of wild-type AKT2 and KAT2 subunits, of new channel functional properties that cannot be explained by the co-existence of two kinds of homotetrameric channels. In particular, the instantaneous current, characteristic of AKT2, displayed new functional features when compared with those of AKT2 homotetramers: activation by external acidification (instead of inhibition) and weak inhibition by calcium. Single channel current measurements in oocytes co-expressing AKT2 and KAT2 revealed a strong preference for incorporation of subunits into heteromultimers and a diversity of individual channels. *In planta*, these new channels, which may undergo specific regulations, are likely to be formed in guard cells and in the phloem, where they could participate in the control of membrane potential and potassium fluxes.

Animal voltage-gated K<sup>+</sup> channels of the Shaker superfamily (1) and their plant homologs (2) result from the assembly of four so-called  $\alpha$ -subunits. Plant and animal Shaker  $\alpha$ -subunits display a hydrophobic core surrounded by hydrophilic N- and C-terminal regions, both located in the cytoplasm. The hydrophobic core of Shaker  $\alpha$ -subunits, which contributes to the transmembrane moiety of the channel, includes six transmembrane segments, named S1 to S6, with a so-called P loop

between S5 and S6. P lines the outer part of the channel pore and determines its ion selectivity (3, 4). Positively charged residues are found in S4, allowing this part of the polypeptide to move within the membrane upon changes of the electrical field therein. Movements of this so-called voltage sensor lead to the channel conformational changes that result in channel opening or closing (5).

Data obtained with animal channels (K<sup>+</sup> Shakers, cyclic nucleotide-gated channels, and so forth) have demonstrated the existence of heterotetramerization between different types of subunits (6–8). In the Shaker (Kv) family, this generally occurs between channels of the same subfamily (9) but not systematically (10, 11). Animal cyclic nucleotide-gated channels, more closely related to plant Shakers, are tetramers of two kinds of subunits (A and B). Their subunit assembly process was studied using tandem dimers, pharmacological and cross-linking agents, and fluorescence resonance energy transfer. Heterotetramers were previously thought to have a 2:2 stoichiometry (12, 13) and to assemble as dimers of dimers (14), but more recent studies rather support a 3:1 stoichiometry of A and B subunits (15–17). The A subunits first assemble as a trimer through the binding of their leucine zipper domains (17).

Animal Shaker channels (tetramers of  $\alpha$ -subunits) bind to regulatory  $\beta$ -subunits. These  $\beta$ -subunits also assemble as tetramers (18) and bind to either the N-terminal region (19, 20) or the C-terminal region (21) of  $\alpha$ -subunits. Some  $\beta$ -subunits have been shown to change the inactivation rate of their target channel (22, 23), whereas others behave as chaperones promoting channel maturation and surface expression on the plasma membrane (24). In plants, homologs of  $\beta$ -subunits have been found and characterized (25–27).

In *Arabidopsis*, a family comprising nine genes encodes Shaker  $\alpha$ -subunits (28). All these polypeptides share the common structure described above. The GORK and SKOR  $\alpha$ -subunits form depolarization-activated Shaker channels displaying a strong outward rectification. The KAT1, KAT2, AKT1, AKT5, SPIK, AKT2, and AtKC1 have been shown (or are believed) to form hyperpolarization-activated Shaker channels, all displaying a strong inward rectification except AKT2 channels, which display a weak inward rectification (29). Plant  $\alpha$ -subunits have a long C-terminal region constituting more than half of the protein (28). This C-terminal region includes three remarkable domains believed to be involved in molecular

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interactions: (i) a putative cyclic nucleotide-binding domain, which participates in interactions between subunits within the channel tetramer (2, 30); (ii) an ankyrin domain (found in six out of the nine *Arabidopsis* Shaker subunits), which may bind to other proteins (31); and (iii) a distal region, involved in tetramerization (2, 30, 32) and in clustering of channels via the conserved  $K_{HA}$  domain (33). These  $\alpha$ -subunits can generally assemble as homotetramers, but evidence has also been provided, by functional analyses in *Xenopus* oocytes or two-hybrid tests in yeast, for heterotetramerization. Interactions have been found between KAT1 and AKT1, KAT1 and AtKC1 (34), AKT2 and KAT1 (35), KAT1 and KAT2 (36), AKT1 and AKT2, AKT1 and AtKC1, and AKT2 and AtKC1 (37). Heterotetramerization has been reported only between subunits of the same functional type (inward or outward). It has been shown that the C-terminal region is involved in discrimination between outward and inward channel-forming subunits (30), preventing formation of heteromeric structures between the two subunit types. In the case of AKT1 and KAT1, heteromer formation has been reported in oocytes (34) and in yeast using the split ubiquitin system (38), but this phenomenon is not observed in two-hybrid tests and *in vitro* under conditions where homotetrameric associations are detected, suggesting that some discrimination can exist for the formation of stable associations (39).

*In planta*, Shaker  $\alpha$ -subunits seem to be expressed in all organs and tissues (29). The different genes display specific expression patterns. The expression of a given gene is restricted to a limited number of cell types but overlaps between the different patterns occur so that each tissue expresses a specific set of  $\alpha$ -subunits (29). Confrontation of expression pattern analyses and of interaction tests suggests numerous possibilities of interactions in plant cells, especially between inward channel-forming subunits, which display overlapping patterns in a number of cell types or tissues. In *Arabidopsis* root hairs, where the two Shaker genes *AKT1* and *AtKC1* are co-expressed at high levels, formation of AKT1/AtKC1 heteromers has been shown to occur and to result in channels with new functional properties (40). The situation is, however, less clear for other sets of inward subunits and cell types. For instance, five Shaker genes encoding inward channel-forming subunits, *KAT1*, *KAT2*, *AKT1*, *AtKC1*, and *AKT2*, are expressed in guard cells (41), and only little information is available on the actual homomeric and/or heteromeric structure of the channels operating in these cells. Indeed, little is known on the actual interactions and assembly patterns of Shaker subunits underlying the voltage-dependent currents recorded in plant cells and even less is known on the gating properties of the resulting heterotetrameric channels.

The attention is focused here on interactions involving AKT2, especially AKT2/KAT2 interaction. AKT2 (42–44) and KAT2 (36, 45) cDNAs were cloned by cDNA library screenings with nucleotide channel probes and 5'-rapid amplification of cDNA ends-PCR (5'-RACE-PCR). The two genes are co-expressed in the phloem vasculature. *AKT2* is expressed in phloem throughout the whole plant (44, 46, 47), and *KAT2* is expressed in the leaf minor vein phloem (36). *AKT2* and *KAT2* transcripts are also co-localized in guard cells (41). In this study, we show that heteromeric AKT2/KAT2 channels are actually

formed, giving rise to a combination of functional features inherited from the two individual  $\alpha$ -subunits. Moreover, evidence is provided that AKT2 and KAT2 subunits are preferentially incorporated into heterotetramers when *AKT2* and *KAT2* are co-expressed.

## EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid cDNA Library Screening**—Plasmids pGBT9-KAT1 and pGBT9-KAT2 allowing, respectively, the synthesis of KAT1 and KAT2 channel C-terminal parts fused to the DNA-binding domain of GAL4 were described previously (36). These constructs were used to screen an *Arabidopsis thaliana* cDNA library prepared with the GAL4 activation domain plasmid pGAD10 (MATCHMAKER, Clontech), using for transformation the lithium acetate method, as described in Chérel *et al.* (48). Transformed cells were spread directly on minimal medium lacking histidine, leucine, and tryptophane (plating medium described in Ref. 2, without histidine) supplemented with 50 mM 3-aminotriazole. Positive colonies were screened for  $\beta$ -galactosidase activity using a filter-lift assay (49). pGAD10 plasmids were isolated from yeast colonies displaying a positive phenotype and amplified in *Escherichia coli*. Plasmid inserts were sequenced and compared with the GenBank<sup>TM</sup> data base by BLAST search analysis (50).

**Yeast Two-hybrid Assays**—The C-terminal region of AKT2 was previously cloned in pGBT9 (48), yielding the (bait) construct named pGBT9-AKT2. The plasmids used for the expression of AKT2 and KAT2 C-terminal regions fused to the GAL4 activation domain (prey constructs) are the previously described plasmids pACT2-AKT2 and pACT2-KAT2 (36, 37). Binary combinations of bait/prey plasmids were incorporated into yeast by the lithium acetate procedure (51). Standard methodology was used to isolate transformants and to perform quantitative assays of  $\beta$ -galactosidase (49). This enzymatic activity was measured by following the hydrolysis of *o*-nitrophenyl- $\beta$ -D-galactopyranoside in liquid medium.

**GFP Imaging**—*AKT2* and *KAT2* cDNAs were amplified by PCR and cloned into pLoc and pFunc (52) for expression of GFP<sup>3</sup>-tagged and untagged channel subunits, respectively. The resulting plasmids were introduced by co-transfection into tobacco mesophyll protoplasts according to Hosy *et al.* (52). Protoplasts were visualized for GFP fluorescence under a Zeiss confocal microscope (LSM510 AX70 Zeiss, Göttingen, Germany). The excitation was obtained with a beam splitter HFT 488 nm, and the emitted radiations were selected with two filters: BP 505–530 for GFP; LP 585 for chlorophyll.

**Plasmids Used for Electrophysiological Measurements**—All constructs were made according to standard methods (53) and verified by sequencing. The *AKT2* and *KAT2* cDNAs were cloned in pGEMHE (54). The *AKT2* and *KAT2* cDNA dominant-negative mutant constructs (pGEMHE-AKT2-DNM and pGEMHE-KAT2-DNM) were obtained as described previously (30).

<sup>3</sup> The abbreviations used are: GFP, green fluorescent protein; DNEM, dominant-negative mutant; MES, 4-morpholineethanesulfonic acid; WT, wild type.

## AKT2/KAT2 Heteromerization in Arabidopsis

**Expression in *Xenopus* Oocytes and Electrophysiology**—*In vitro* transcriptions were performed using the mMESSAGE mMACHINE kit (Ambion) following the manufacturer's instructions. *Xenopus* oocytes were purchased from the Centre de Recherche en Biochimie Macromoléculaire (CNRS, Montpellier, France). Stage V-VI oocytes were injected with a final volume of 50 nl (50 ng) of various cRNA *AKT2-KAT2* combinations using a 10–15- $\mu$ m tip diameter micropipette and a pneumatic injector. Co-injections were systematically performed at an equimolar concentration ratio. Injected oocytes were then maintained at 18 °C for 3–5 days in modified ND-96 solution (2 mM KCl, 96 mM NaCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, 2.5 mM sodium pyruvate, pH 7.5) supplemented with gentamycin sulfate (50  $\mu$ g/ml).

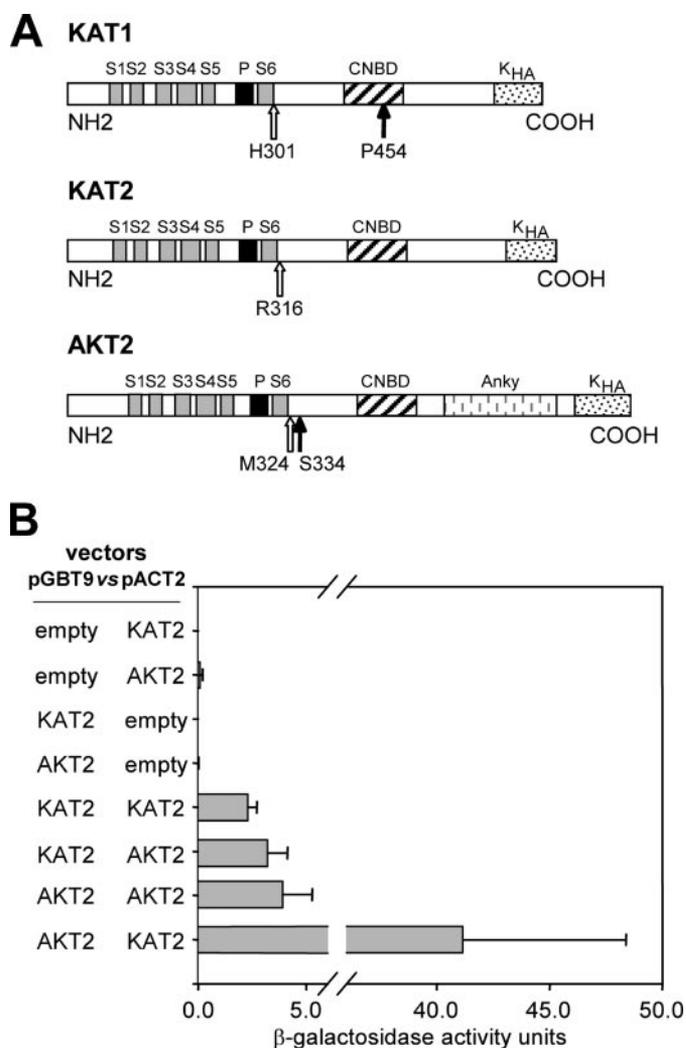
Whole-cell currents from oocytes were recorded 3–5 days after injection as described previously (55) using the two-microelectrode voltage clamp technique. The bath solution, containing (unless otherwise stated) 10 mM KCl, 90 mM NaCl, 1 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, and 10 mM HEPES-NaOH (pH 7.5), except in the pH 6.0 experiments of Fig. 5, in which the medium was buffered with MES-NaOH (pH 6.0) was continuously perfused through the chamber. For patch clamp experiments, cell-attached patch recordings were performed on devittellinized oocytes based on methods previously described (56). Voltage-pulse protocol application, data acquisition, and data analysis were performed using pClamp software version 9.0 (Axon Instruments) and Sigmaplot software (Jandel Scientific).

Control electrophysiological recordings in media supplemented with 10 mM CsCl were performed systematically, as described previously (44), to ensure that currents recorded were generated essentially by potassium-selective channels. All experiments were performed at room temperature (20–22 °C).

## RESULTS

**Detection of Interactions by Two-hybrid Tests in Yeast**—Large C-terminal intracytoplasmic regions of KAT1 and KAT2 (Fig. 1A) were used as baits to screen an *Arabidopsis* cDNA library using the two-hybrid system in yeast. Expected outcomes of such a screening procedure were partner proteins interacting with these two subunits of *Arabidopsis* Shaker-like K<sup>+</sup> channels. With both baits, two different cDNAs were isolated: *KAT1* and *AKT2* (not shown). Interactions between KAT1 and KAT2 (36) and between KAT1 and AKT2 (35) had already been reported but not between KAT2 and AKT2. This last interaction appeared interesting for two reasons: the very high level of reporter gene activation in the two-hybrid test and the known co-localization of *AKT2* and *KAT2* gene expression in phloem and guard cells.

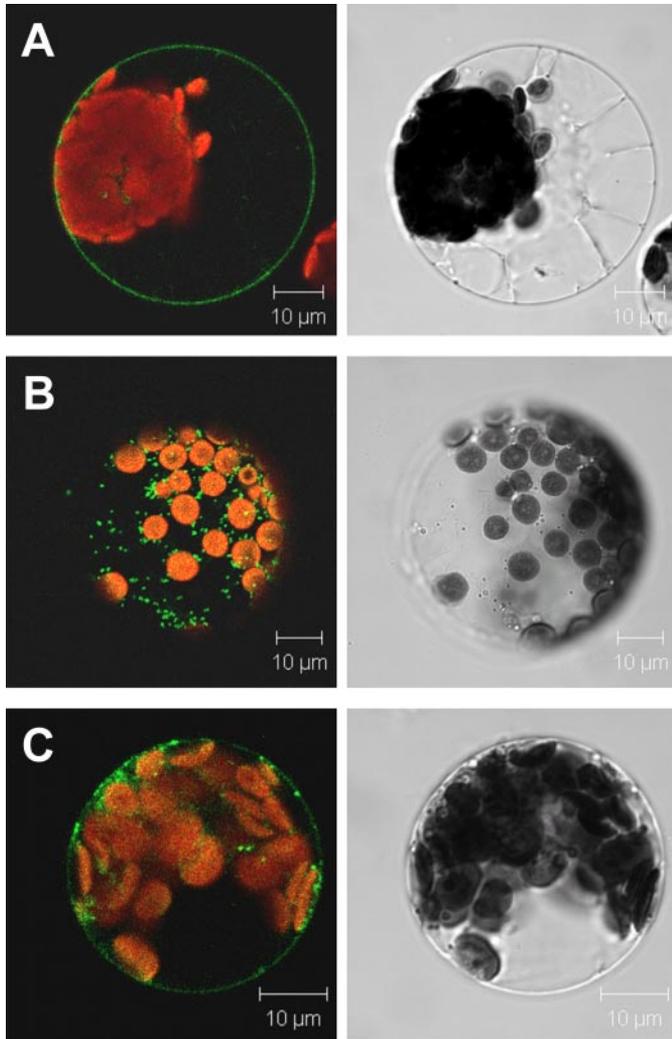
The interaction between AKT2 and KAT2 was further studied in a series of two-hybrid tests in which the C-terminal region of each of these two putative partners was fused either to the DNA-binding domain (*i.e.* as the bait, pGBT9 vector) or to the activation domain (*i.e.* as the prey, pACT2 vector) of the GAL4 transcription factor (Fig. 1B). In negative controls (when one of the pGBT9 or pACT2 vectors was left empty), there was no activity of the reporter gene (Fig. 1B, four upper lanes). When KAT2 was used as the bait, an interaction was observed with both KAT2 and AKT2 (*fifth* and *sixth* lanes, respectively).



**FIGURE 1. Yeast two-hybrid experiments reveal interactions between KAT1, KAT2 and AKT2.** A, screenings of cDNA libraries. The positions on the channel structural schemes of the first amino acid of KAT1 and KAT2 C-terminal regions used as baits (His-301 and Arg-316, respectively) are indicated by open arrows. Both screenings resulted in the cloning of cDNAs encoding the same KAT1 and AKT2 C-terminal polypeptides, beginning at the amino acid indicated by a black arrow (Pro-454 for KAT1 and Ser-334 for AKT2). An open arrow also indicates the first amino acid (Met-324) of the AKT2 polypeptide used in B. S1–S6 indicate the six transmembrane segments of the channel hydrophobic core, with a pore-forming domain (P) present between S5 and S6. CNBD, putative cyclic nucleotide-binding domain; K<sub>HA</sub>, domain rich in hydrophobic and acidic amino acids; anky, ankyrin domain (in AKT2 only). B, interactions between the C-terminal domains of KAT2 and AKT2 evidenced by reciprocal two-hybrid tests with complete C-terminal intracytoplasmic regions (downstream from S6). The pGBT9 and pACT2 plasmids bearing the AKT2 or KAT2 C-terminal insert (from Met-324 and Arg-316, respectively) or no insert (empty) were tested in the different combinations indicated.  $\beta$ -Galactosidase activities were measured following the hydrolysis of o-nitrophenyl- $\beta$ -D-galactopyranoside in liquid medium. The data (mean  $\pm$  S.E., n = 3) are given in the following units: 1000  $\times$  (A<sub>420 nm</sub>/reaction time in min  $\times$  A<sub>600 nm</sub> of the yeast culture).

AKT2 as the bait also interacted with AKT2 and KAT2 (*seventh* and *eighth* lanes, respectively). The highest expression level of the reporter gene was obtained in the latter of these combinations.

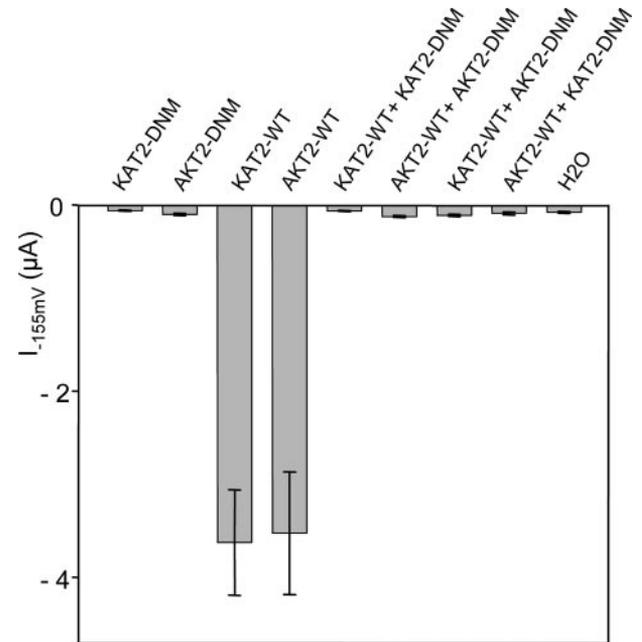
**Driving of AKT2 to the Plasma Membrane by KAT2 Subunits in Plant Cells**—When KAT2-GFP was expressed alone in tobacco protoplasts, it was targeted to the plasma membrane (Fig. 2A). In the same conditions, when expressed alone or co-



**FIGURE 2. Photographs of tobacco cells expressing GFP fused to channel subunits.** *Left panels*, detection of GFP fluorescence by confocal imaging; *right panels*, corresponding pictures obtained with transmitted light. *A*, protoplasts expressing KAT2-GFP alone. *B*, protoplasts co-expressing AKT2-GFP and AKT2. *C*, protoplasts co-expressing AKT2-GFP and KAT2.

expressed with untagged AKT2, AKT2-GFP remained in intracellular structures that might be identified as Golgi vesicles or aggregates (Fig. 2*B*). A plasma membrane localization was never observed, either in the 41 protoplasts we have scanned or in the much larger population of protoplasts we have observed under the microscope. On the contrary, co-transfection of untagged KAT2 with AKT2-GFP often resulted in a shift of part of the fluorescence to the plasma membrane (Fig. 2*C*), the absence of plasma membrane localization in some cells probably being due to absence of KAT2 expression. The day following transfection (24–30 h), 40–60% of the protoplasts displayed (partial) plasma membrane localization of the fluorescence.

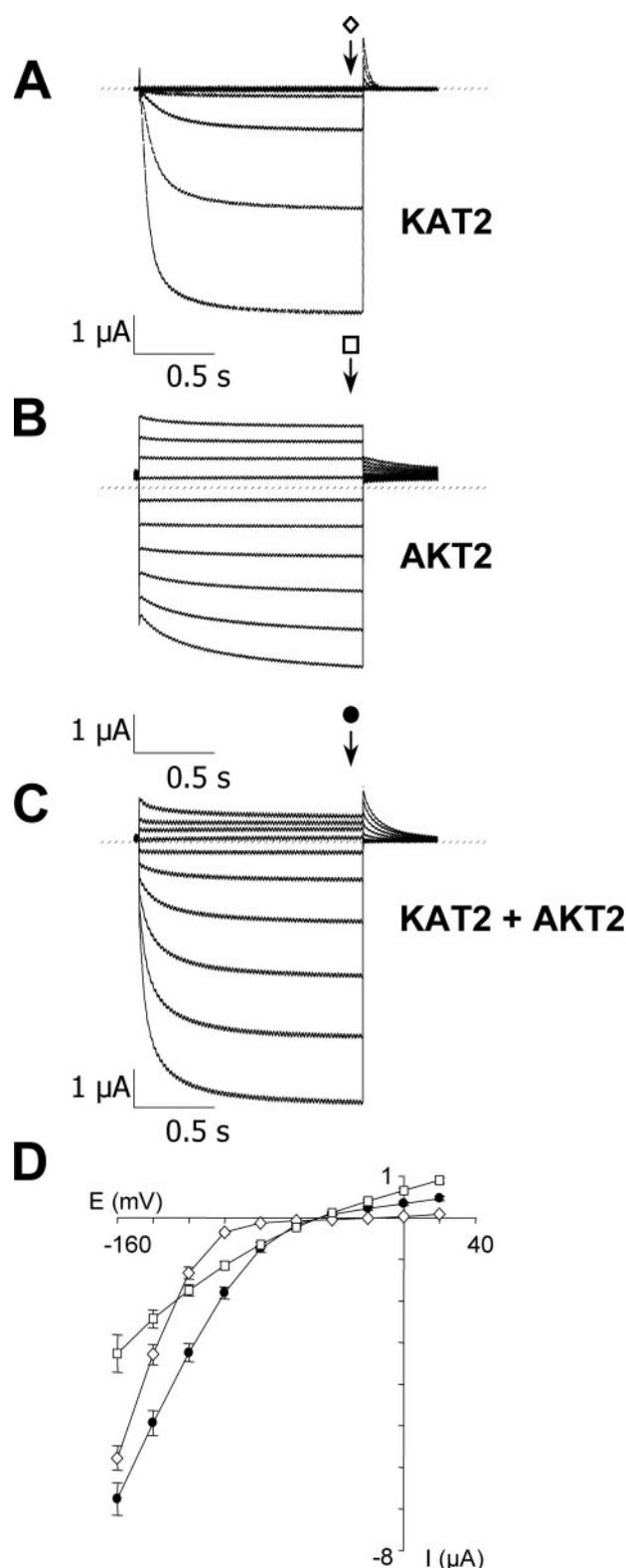
**Use of Dominant-negative Mutants to Reveal Co-assembly of AKT2 and KAT2 in *Xenopus* Oocytes**—Point mutations in the pore (P-domain) of plant Shaker subunits have been reported to yield dominant-negative mutant (DNM) subunits (35, 57), able to inactivate the channels in which they are incorporated. In other words, co-expression of such DNM subunits with wild-type subunits results in the absence of channel activity (*i.e.* of exogenous current). We introduced mutations similar to those



**FIGURE 3. KAT2 and AKT2 subunit interactions revealed by a dominant-negative approach in *Xenopus* oocytes.** Shown are two-electrode voltage clamp recordings, in a 10 mM K<sup>+</sup> external solution, of currents from oocytes injected with either a single mutant (KAT2-DNM, AKT2-DNM) or a single wild-type (KAT2-WT, AKT2-WT) cRNA or co-injected with equimolar amounts of a wild-type and a mutant cRNA (see the labels above the figure). Control oocytes were injected with H<sub>2</sub>O. All current values were sampled at the end of an hyperpolarizing pulse of 2 s at  $-155$  mV. Results are displayed as mean  $\pm$  S.E. ( $n = 6-9$ ).

carried by the previously studied GORK-DNM subunit (35, 57) in the P-domains of KAT2 and AKT2; the GYGD motif, hallmark of K<sup>+</sup> selectivity, was mutated to RRGD. It was first checked that expression of the resulting mutant subunits produced electrically silent (or not properly targeted or unstable) channels in *Xenopus* oocytes (Fig. 3, KAT2-DNM and AKT2-DNM). Although control oocytes expressing only wild-type subunits displayed a large inward conductance (Fig. 3, KAT2-WT and AKT2-WT), co-expression of mutant subunits with the corresponding wild-type subunits resulted in a virtually null inward current (Fig. 3, KAT2-WT + KAT2-DNM and AKT2-WT + AKT2-DNM). Thus, the whole set of data indicated that the KAT2-DNM and AKT2-DNM subunits had indeed a dominant-negative capability, *i.e.* that they interacted with the wild-type polypeptide, leading to formation of channels that were not functional. Co-expressing AKT2-DNM with KAT2 or KAT2-DNM with AKT2 also resulted in the absence of any significant inward currents (Fig. 3). Along with the results of the two-hybrid tests, this provided further evidence that AKT2 and KAT2 polypeptides can actually interact and form heteromeric channels when they are co-expressed in *Xenopus* oocytes.

**Functional Properties of AKT2/KAT2 Heterotetramers**—It was then investigated whether operation of AKT2/KAT2 heteromeric channels could be assessed from currents recorded in *Xenopus* oocytes after injection with a mix of wild-type KAT2 and AKT2 cRNA solutions. In parallel, control currents were recorded in oocytes from the same batch, injected with pure water, KAT2 cRNA, or AKT2 cRNA. Typical recordings are shown in Fig. 4, A–C.

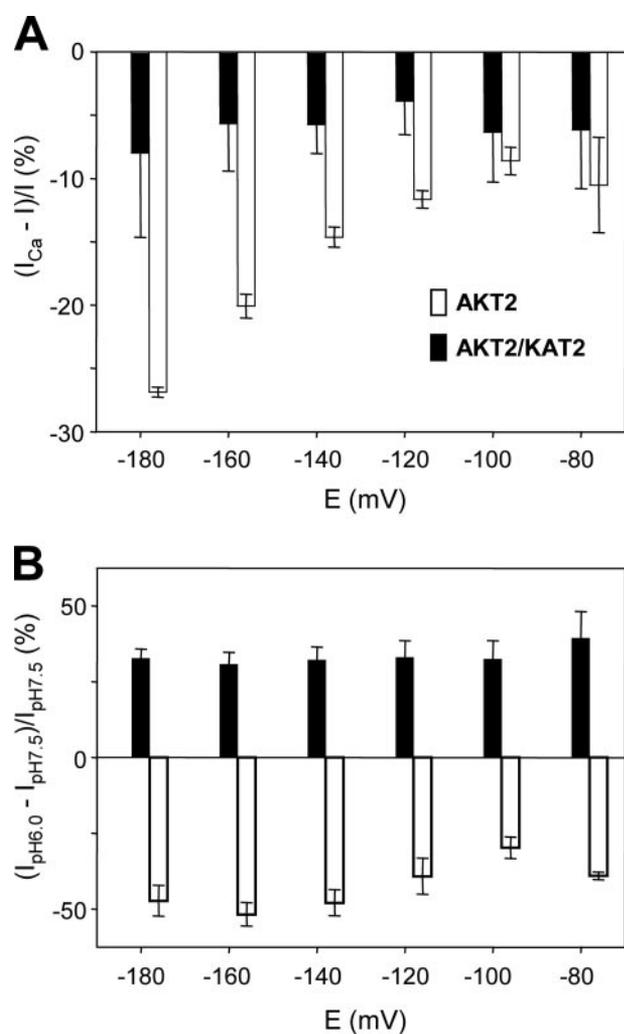


**FIGURE 4. Co-expression of KAT2 and AKT2 cRNAs in *Xenopus* oocytes.** A–C, representative currents elicited in a 10 mM K<sup>+</sup> external solution by 1.4-s voltage pulses from +20 to −160 mV (−20 mV steps, −40 mV holding potential) on oocytes injected with KAT2 (A), AKT2 (B), or co-injected with equimolar ratio of KAT2 and AKT2 (C). Note the same scale for all recordings. D, steady-state I/V plots from recordings obtained in a 10 mM K<sup>+</sup> external solution. Steady-state currents were sampled at the end of the 1.4-s pulses for KAT2- (open diamonds), AKT2- (open squares) or (AKT2 + KAT2)- (closed circles) injected oocytes. All data are means ± S.E. ( $n = 18$  for KAT2,  $n = 16$  for AKT2, and  $n = 15$  oocytes for AKT2 + KAT2).

As reminded by data displayed in Fig. 4, A and B, KAT2 and AKT2 belong to different functional subgroups of the Shaker family of *Arabidopsis* voltage-gated K<sup>+</sup> channel subunits (29). KAT2 forms channels mediating a time-dependent current below a negative membrane potential threshold (36, 58, 59), whereas AKT2 forms channels mediating both a time-dependent current (as do KAT2 channels) and an instantaneous current present at any physiological membrane potential (44, 46, 60–62), these two types of current being generated by two AKT2 populations differing by their phosphorylation state (61). Concerning the kinetics and voltage dependence of the macroscopic currents recorded in the AKT2/KAT2 co-injected oocytes (Fig. 4C), a simple hypothesis was that the channel activity corresponded to the summed contributions of KAT2 and AKT2, both present as homotetramers. The averaged I–V curves (steady-state current *versus* membrane potential, Fig. 4D) led, however, to rule out this hypothesis. Indeed, no given linear combination of the two AKT2 and KAT2 I–V curves ( $x \cdot \text{AKT2} + y \cdot \text{KAT2}$ ) could account for the shape of the AKT2/KAT2 I–V average curve in the whole voltage range. Similarly, although the deactivation kinetics of the tail current recorded at the holding potential was mono-exponential in A and B, it seemed to be bi-exponential in panel C, but this could not be ascribed to linearly combined contributions of AKT2 and KAT2 homomers (not shown). These results suggested that heteromeric channels were functional in AKT2/KAT2 co-injected oocytes and contributed to the recorded currents.

In AKT2/KAT2 co-expressing oocytes, six kinds of tetramers could be theoretically formed: KAT2 and AKT2 homotetramers, which can respectively be named KKKK and AAAA, and four different heterotetramers, which can be labeled AAKK, AKAK, AAKK, KKKA (where K and A indicate the presence of a KAT2 or an AKT2 subunit in the tetrameric structure, respectively). Since KAT2 mediates time-dependent current only, instantaneous currents recorded in oocytes co-expressing KAT2 and AKT2 can be ascribed to the activity of AKT2 homomers or, possibly, of AKT2/KAT2 heteromers (if the presence of AKT2 subunits in heteromeric channels confers the ability to mediate instantaneous current). Thus, it was investigated whether the instantaneous fraction of the currents recorded either in AKT2-expressing or in AKT2/KAT2 co-expressing oocytes showed differences that could reveal the existence of at least one kind of functional heteromer in the latter oocytes.

The AKT2 instantaneous current has been shown to decrease when the external concentration of Ca<sup>2+</sup> or H<sup>+</sup> is increased (44, 46, 63), whereas the KAT2 current has been reported to be essentially insensitive to external Ca<sup>2+</sup> (59) and stimulated by lowering the external pH (36). We have therefore compared the effect of extracellular Ca<sup>2+</sup> and pH on the instantaneous fraction of the currents recorded on AKT2-expressing or AKT2/KAT2 co-expressing oocytes (Fig. 5). In our experimental conditions, 10 mM Ca<sup>2+</sup> produced a voltage-dependent block of the instantaneous inward current recorded on AKT2-expressing oocytes (Fig. 5A, *white bars*), in line with previously published data (44, 46). In AKT2/KAT2 co-expressing oocytes, the instantaneous inward current was poorly affected (less than 10% inhibition) in an essentially voltage-independent way (Fig.

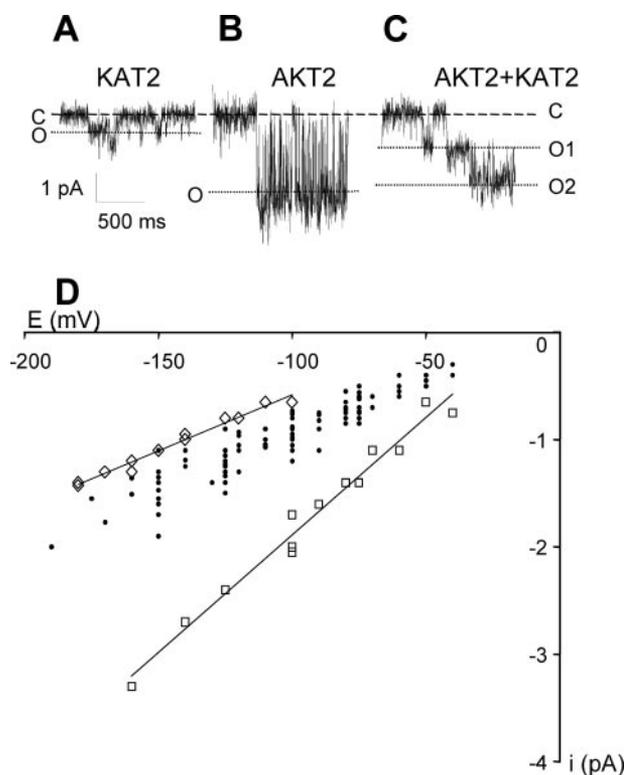


**FIGURE 5. pH and  $\text{Ca}^{2+}$  sensitivity of the AKT2/KAT2 instantaneous currents.** *A*, inhibition by 10 mM extracellular  $\text{Ca}^{2+}$  of the instantaneous fraction of the  $\text{K}^+$  current recorded on AKT2- (white bars) or (AKT2 + KAT2)- (black bars) injected oocytes. All current values were obtained at the beginning of pulses at a voltage ranging from  $-80$  to  $-180$  mV. Results are displayed as mean  $\pm$  S.E. ( $n = 3$  for AKT2 and  $n = 8$  for (AKT2/KAT2)). *B*, effect of lowering the external pH from 7.4 to 6.0 on the instantaneous fraction of the  $\text{K}^+$  current recorded at the beginning of pulses at a voltage ranging from  $-80$  to  $-180$  mV, on AKT2- (white bars) or (AKT2 + KAT2)- (black bars) injected oocytes. Results are displayed as mean  $\pm$  S.E. ( $n = 3$  for AKT2 and  $n = 7$  for (AKT2 + KAT2)). Holding potential:  $-40$  mV.

5A, black bars). Since as stated above, KAT2 homomers are not contributing to the instantaneous current, it can be concluded that heteromers associating AKT2 and KAT2 subunits are functional in AKT2/KAT2 co-expressing oocytes and have functional properties different from those of AKT2 homomers.

Similar experiments were performed, based on the same kind of rationale, regarding the effect of the extracellular pH on the instantaneous current. Although the instantaneous fraction of the current was inhibited by acidification of the bath solution (from pH 7.5 to 6.0) in oocytes expressing AKT2 only, as reported previously (44, 46, 63), it was stimulated in AKT2/KAT2 co-expressing oocytes (Fig. 5B). This provides further support to the hypothesis that AKT2/KAT2 heteromers contribute to the current when both subunits are co-expressed.

Single channel patch clamp experiments were then performed with the aim of getting access to the properties of



**FIGURE 6. AKT2/KAT2 single channel recordings.** *A–C*, typical cell-attached recordings at  $-125$  mV from *Xenopus* oocytes expressing KAT2 (*A*), AKT2 (*B*), or (AKT2 + KAT2) (*C*). *D*, single channel current-voltage relationship for AKT2- (open squares,  $n = 3$ ), KAT2- (open diamonds,  $n = 3$ ), or (AKT2 + KAT2)- (black dots,  $n = 44$ ) injected oocytes. The single channel current values displayed were obtained by analyzing histograms of current amplitude for each recorded patch. The pipette and bath solutions contained 100 mM  $\text{K}^+$ .

AKT2/KAT2 heteromers at the molecular level. The cell-attached configuration was used because AKT2 and KAT2 currents display rundown after patch clamp excision (44, 46, 60). The single channel conductance of AKT2 and KAT2 homomers obtained in AKT2- or KAT2-expressing oocytes ( $22 \pm 2$  picosiemens,  $n = 3$ , and  $10.6 \pm 0.2$  picosiemens,  $n = 3$ , respectively) were similar to those already published (36, 44, 46, 60). Single channel recordings obtained on AKT2/KAT2 co-injected oocytes yielded complex data; plotting the single channel currents against membrane potential demonstrated a strong variation of the current level at any given potential (Fig. 6). This variability was likely to be due to the activity of different channel types, probably resulting from different heteromeric combinations of AKT2 and KAT2 subunits (64). Currents typical of AKT2 or KAT2 homotetrameric channels were not recorded in AKT2/KAT2 co-expressing oocytes. Out of 44 recorded single channel currents (in 19 independent patches), none corresponded to the conductance of 22 picosiemens, which would have been generated by AKT2 homotetramers. This indicates that AKT2 homotetramers were rare or absent in our AKT2/KAT2 co-injected oocytes. Similarly, in most of the recorded patches, analysis of the voltage dependence of the current fluctuations led to a slope-conductance value reminiscent of that of KAT2, around 10 picosiemens, but the underlying channels remained active at membrane potential above (more positive than)  $-80$  mV, *i.e.* in a voltage range where KAT2 homomers are normally closed, indicating that these unitary

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currents were not mediated by KAT2 homotetramers but probably by AKT2/KAT2 heterotetramers.

### DISCUSSION

**Predominance of Heterotetrameric Forms**—In two-hybrid tests, the intensity of the signal depends on the binding of the bait to the target DNA sequence and also on the amount and affinity of the prey. When AKT2 is used as the bait, a much stronger signal is observed with KAT2 in the prey construct than with AKT2 (Fig. 1B). This could merely result from a much higher amount/stability of KAT2 when it is expressed as the prey. However, in reciprocal tests performed with KAT2 as the bait, KAT2 as prey does not lead to higher signals than AKT2 (the signal being even slightly lower). The simplest explanation to these results is that the AKT2 C-terminal region has a higher affinity for that of KAT2 than for itself.

In oocytes, the ability of KAT2-DNM or AKT2-DNM to induce formation of non-functional channels when they were co-expressed with the wild-type form of the other subunit (Fig. 3C) was as high as when they were co-expressed with their own wild-type subunit (Fig. 3B). In both cases, the current was almost totally inhibited. This indicates that the mutation results in complete inhibition of channels comprising at least one mutant subunit. This experiment also allows us to conclude that there is no preference for associations between identical subunits.

Our results, however, hardly enable us to predict what kind of heterotetrameric channels (ratio and disposition of subunits) can be formed from a mixture of two different subunits. Single channel recordings on oocytes co-injected with the same amount of *AKT2* and *KAT2* cRNAs reveal a strong heterogeneity of current levels at each given potential. This high diversity of currents provides further evidence that formation of homotetrameric channels is not the predominant process but also suggests that several types of heteromeric channels are formed (64). In other words, there seems to be no strict preference for a single type of heteromeric association. Thus, along with the heteromerization process, the absence of preference for a single type of assembly would further increase the diversity of channel activity.

**Heterotetramerization in Plant Cells**—In tobacco protoplasts, AKT2-GFP (alone or in combination with untagged AKT2) was not found at the plasma membrane, thus indicating that the targeting machinery of tobacco is not effective on AKT2. The mechanism of plant channel targeting is still unknown. In *Arabidopsis*, a chaperone-like role has been suggested for the KAB1 protein, homologous to animal  $\beta$ -subunits (27). Targeting of AKT2-GFP to the plasma membrane was only detected in the presence of KAT2. This indicates that AKT2/KAT2 heteromers can be formed in a plant cell type (here, tobacco mesophyll protoplasts). This, however, does not bring the definitive proof that AKT2/KAT2 heteromers are actually formed *in planta*, in their native context, in the absence of any overexpression. Previous observations in *Arabidopsis* guard cells of the ecotype Wassilevskija (59), in which KAT2 transcripts dominated the  $K^+$  channel mRNA pool, indicated that the *akt2* mutation induced qualitative changes of inward currents: shift of the activation potential to more negative volt-

ages and loss of voltage-dependent calcium block. This was interpreted as a possible heterotetramerization of AKT2 with other  $K^+$  channel subunits. Along with the shift of AKT2-GFP fluorescence to the plasma membrane of the tobacco protoplast in the presence of KAT2, these observations support the hypothesis that AKT2/KAT2 heterotetrameric channels are indeed formed in plant cells. Definitive demonstration of the heteromeric assembly in the native context would, however, involve purification of native channel proteins from plant cells, a result that has never been reported so far, probably because channels are very low abundant proteins in plants.

**Physiological Impact of Heterotetramer Formation**—Single channel recordings performed on oocytes co-expressing KAT2 and AKT2 indicated that AKT2/KAT2 heteromers have actually inherited some gating properties of their AKT2 subunits as they were found active at voltage values less negative than the activation threshold of pure KAT2 channels (Fig. 6). On the other hand, the AKT2/KAT2 heteromers underlying the currents shown in Fig. 5 clearly displayed sensitivities to  $Ca^{2+}$  and  $H^+$  both reminiscent of KAT2 rather than AKT2 ones. In addition, the AKT2/KAT2 heteromers studied here displayed properties inherited from both of their constitutive subunits. It can then be concluded that such channels are of a novel functional type.

Such formation of heteromeric channels can increase channel diversity *in planta*. Little is known on  $K^+$  currents in phloem cells, which are the main site of *AKT2* expression. In the *akt2-1* mutant, the  $K^+$  dependence of the membrane potential of the companion cell/sieve tube complex, involved in control of sucrose uptake, is altered (58). In protoplasts of companion cells from main leaf veins, *AKT2* is expressed essentially along with *KAT1*, the twin of *KAT2*. Instantaneous current of AKT2-type was not detected in these cells, but the time-dependent inward current (KAT1 type) recorded displayed a voltage-dependent  $Ca^{2+}$  block attributable to AKT2 subunits (47). The absence of instantaneous current might be explained by the weakness of the leak current in AKT2/KAT1 heteromers, as shown in Baizabal-Aguirre *et al.* (35). In guard cells, dominant  $K^+$  currents are generated by the activity of voltage-gated  $K^+$  channels (65–68). There is co-existence of *KAT1*, *GORK*, *KAT2*, *AKT1*, *AKT2*, and *AtKCI* transcripts (41, 69). KAT1 and KAT2 are thought to be the main contributors to  $K^+$  uptake in guard cells (36, 70). The role of KAT1 in stomatal opening has been demonstrated by the expression in *Arabidopsis* of  $Cs^+$ -resistant or dominant-negative KAT1 mutant subunits (70, 71). Heteromerization between KAT1 and KAT2 has been shown in *Xenopus* oocytes and can be predicted to occur in guard cells, provided that the *KAT1* and *KAT2* expression patterns overlap in time (36). Our data allow us to complete the interaction diagram; AKT2, KAT1, and KAT2 can all interact with each other. The relatively low level of *AKT2* transcripts when compared with those of *KAT1* and *KAT2* and the easy heteromerization of AKT2 subunits would explain why typical AKT2 currents are not recorded in guard cells of wild-type plants.

Currents without rectification mediated by AKT2 subunit-containing channels might play a role in the control of cell membrane resting potential since they are recorded at potentials where channels mediating rectifying currents are closed.

Activation by acidic external pH and relative insensitivity to external calcium might confer to AKT2 subunit-containing channels a specific role in membrane potential regulation, different from that expected for homotetrameric AKT2 channels.

Interestingly, participation of AKT2 subunits to heterotetramers should confer to the corresponding channel population susceptibility to regulation by the protein phosphatase AtPP2CA (48), a feature that is not shown by homotetramers of KAT2 subunits. It will be worth studying whether AKT2/KAT2 heteromers show such regulation by AtPP2CA and, beyond this, phosphorylation-dependent regulation of their gating as described for AKT2 homomeric channels (62).

In conclusion, our results reveal a diversity of current types indicative of the activity of heterotetrameric AKT2/KAT2 channels. Depending on the expression level of the two genes, different homo- and heterotetrameric channel populations could be produced, exhibiting different gating properties and sensitivity to channel regulators. Heteromeric AKT2/KAT2 (and probably AKT2/KAT1) channels are thus likely to play specific roles by conferring increased diversity in channel activity and regulation in guard cells, phloem cells, and other cell types co-expressing these subunits.

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**Increased Functional Diversity of Plant K<sup>+</sup> Channels by Preferential Heteromerization of the Shaker-like Subunits AKT2 and KAT2**

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