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FOCUS PAPER

Regulation of K^+ channel activities in plants: from physiological to molecular aspects

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Abstract

Plant voltage-gated channels belonging to the Shaker family participate in sustained K^+ transport processes at the cell and whole plant levels, such as K^+ uptake from the soil solution, long-distance K^+ transport in the xylem and phloem, and K^+ fluxes in guard cells during stomatal movements. The attention here is focused on the regulation of these transport systems by protein–protein interactions. Clues to the identity of the regulatory mechanisms have been provided by electrophysiological approaches *in planta* or in heterologous systems, and through analogies with their animal counterparts. It has been shown that, like their animal homologues, plant voltage-gated channels can assemble as homo- or heterotetramers associating polypeptides encoded by different *Shaker* genes, and that they can bind auxiliary subunits homologous to those identified in mammals. Furthermore, several regulatory processes (involving, for example, protein kinases and phosphatases, G proteins, 14-3-3s, or syntaxins) might be common to plant and animal Shakers. However, the molecular identification of plant channel partners is still at its beginning. This paper reviews current knowledge on plant K^+ channel regulation at the physiological and molecular levels, in the light of the corresponding knowledge in animal cells, and discusses perspectives for the deciphering of regulatory networks in the future.

Key words: K^+ channel, potassium, protein–protein interaction, regulation.

Introduction

Potassium is the most abundant cation in the cytosol of plants and animals. The cell requires this inorganic cation, compatible with protein structure at high concentration, to neutralize dissociated organic acids and anionic groups of macromolecules (nucleic acids and phospholipids) and to lower water potential. To fulfil these requirements, K^+ must be readily transported and, therefore, this cation plays a role in the control of membrane potential. Consistent with these basic functions (Clarkson and Hanson, 1980; Maathuis and Sanders, 1996), K^+ plays important roles at the cell and whole organism levels. In plants, direct evidence has been obtained that K^+ is involved in processes such as cell elongation, stomatal movements and regulation of gas exchanges, and the transduction of various signals (Clarkson and Hanson, 1980; Zimmermann and Sentenac, 1999; Véry and Sentenac, 2003). Several families of K^+ transport systems were identified during the 1990s, and the integration of various molecular, electrophysiological and reverse genetics approaches has already revealed the functions of some of these systems, including those of K^+ channels belonging to the so-called Shaker family (Véry and Sentenac, 2003). This has opened the way to the molecular analysis of the regulation of membrane K^+ transporters. Most of the present knowledge in this field concerns Shaker channels.

Shaker channels in plants

The *Arabidopsis* K^+ channels AKT1 and KAT1, cloned in 1992 by functional complementation of yeast mutants defective for K^+ uptake, were the first nutrient ion transport systems identified in plants (Sentenac *et al*., 1992; Anderson *et al*., 1992). They are related to animal K^+ channels initially cloned from *Drosophila* and named

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Shakers. The Shaker family comprises nine members in *Arabidopsis*, and members of this family have also been identified in a number of other plant species (Véry and Sentenac, 2003).

Plant Shaker polypeptides typically display a rather short (*c.* 60 amino acid) intracytoplasmic N-terminal domain, followed by a hydrophobic core composed of six transmembrane segments (S1 to S6, the pore domain being inserted between S5 and S6), and a long intracytoplasmic region representing more than half of the sequence. The fourth transmembrane segment harbours positively charged amino acids (R and K) and is expected to act as a voltage sensor. A highly conserved pore domain, carrying the hallmark GYGD/E motif of highly K^+ selective channels, is present between S5 and S6. The long C-terminal region harbours a putative cyclic nucleotide-binding domain and, in most Shaker channels, an ankyrin domain potentially involved in protein-protein interactions.

Shaker channels are voltage-gated. They are either hyperpolarization-activated and thus inwardly rectifying (i.e. mediating an inward K^+ current) or depolarization-activated and thus outwardly rectifying (allowing K^+ efflux from the cell). Based on their voltage-dependence, selectivity and sensitivity to pharmacological agents, they are believed to mediate most of the major K^+ currents described in the plasma membrane of plant cells (Véry and Sentenac, 2002). They also represent the best characterized family of plant channels and transporters at the molecular level.

Studies of channel expression patterns, the generation and characterization of mutant and transgenic plants, together with electrophysiological analyses of channels in heterologous systems have been undertaken to address the role of each of the nine Shaker channel genes. *AKT1* encodes an inwardly rectifying channel (Gaymard *et al*., 1996). A knock-out mutant in which the *AKT1* gene is disrupted displays impaired growth on a low-K^+ medium (in the presence of NH_4^+) and reduced K^+ (Rb^+) uptake from the medium (Hirsch *et al*., 1998). Expression studies indicate that the promoter is active in root cortex and epidermal cells (Lagarde *et al*., 1996). Thus, the whole set of data indicates that AKT1 plays a role in K^+ uptake from the soil solution. The *AtKC1* gene, the expression of which co-localizes with that of *AKT1* in roots, might also take part in K^+ influx from the soil solution (Reintanz *et al*., 2002). Once K^+ has been taken up, its secretion into the root xylem for delivery to the shoot involves the outwardly rectifying SKOR channel, which could mediate the delivery of up to 50% of the K^+ in the xylem sap (Gaymard *et al*., 1998). At least two inwardly rectifying Shakers, KAT1 and KAT2, and one outwardly rectifying Shaker, GORK, are expressed in guard cells (Nakamura *et al*., 1995; Ache *et al*., 2000; Pilot *et al*., 2001; Szyroki *et al*., 2001). Expression of mutated KAT1 subunits displaying decreased sensitivity to the channel blocker Cs^+ in transgenic plants resulted in a decreased sensitivity to Cs^+ of both the inward K^+ current in guard cells and light-induced stomatal opening (Ichida *et al*., 1997). Consistently, the expression of a dominant negative KAT1 subunit resulted in a significant reduction of the inward K^+ current and light-induced stomatal movement (Kwak *et al*., 2001). However, stomatal functioning does not seem to be affected in a knock-out mutant disrupted in the *KAT1* gene (Szyroki *et al*., 2001). This might be explained by the fact that other inwardly rectifying Shakers (at least KAT2: Pilot *et al*., 2001) are expressed in guard cells (Szyroki *et al*., 2001). Conversely, GORK has been shown to be the only outwardly rectifying Shaker channel present in guard cells. In a knock-out mutant defective for the *GORK* gene, guard cell outward K^+ currents are abolished, stomatal closure is impaired, and plants have higher transpiration rates than in the wild type (Hosy *et al*., 2003). In phloem tissues, the expression of *KAT2* can be detected (Pilot *et al*., 2001), along with that of *AKT2*, which encodes a weakly inwardly rectifying channel (Marten *et al*., 1999; Lacombe *et al*., 2000*b*). In an *akt2* knock-out mutant, the K^+ content of the phloem is not modified compared with that of the wild type, but the sucrose transport and K^+-dependence of membrane potential in phloem tissues are reduced, suggesting a role for AKT2 in the control of the phloem cell membrane potential, and, thereby, in the regulation of sucrose loading/unloading into/from the phloem sap (Deeken *et al*., 2002). The AKT2 channel also accounts for about 50% of K^+ permeability of mesophyll cells, AKT1 being responsible for the remaining 50% (Dennison *et al*., 2001). The inwardly rectifying Shaker SPIK is specifically expressed in pollen where it mediates K^+ uptake. Pollen tube growth and, therefore, pollen competitive ability, are dependent on the activity of this channel (Mouline *et al*., 2002). A single *Arabidopsis* Shaker gene (At4g32500) has not yet been characterized. The only available data concern its expression pattern: the transcript was mainly detected in flowers (Lacombe *et al*., 2000*b*).

Evidence has been obtained that Shaker K^+ channel activity is regulated both at the transcriptional and post-translational levels, the sensitivity to stimuli as well as the kind of response differing from one channel to another. The transcript levels can fluctuate in response to different environmental and hormonal factors such as light, abscisic acid (ABA), auxin and salt stress (Véry and Sentenac, 2003). At the post-translational level, channel activity is controlled by membrane polarization and intracellular factors such as H^+ (Hoshi, 1995; Marten *et al*., 1999; Lacombe *et al*., 2000*a*), calcium (Marten *et al*., 1999), and cyclic nucleotides (Hoshi, 1995; Gaymard *et al*., 1996; reviewed in Véry and Sentenac, 2003). An increasing amount of data indicates that channel activity is also modulated *in planta* by protein–protein interactions.

Operationally, these interactions can be divided into two categories: formation of heteromeric channels, due to the assembly of polypeptides encoded by different *Shaker* genes, and interaction with *bona fide* regulatory proteins. This review will focus on these aspects of Shaker K^+ channel regulation in plants. Before this, the corresponding information in animal cells will be surveyed since it has provided clues for investigating similar interactions in plants.

Shaker channel regulation in *Drosophila* and mammals

The animal Shaker superfamily comprises the so-called Kv, KvLQT, SK, Slo, HCN, EAG, and CNGC members. The best characterized subfamily with respect to structure and regulation is that of the Kv channels (voltage-gated Shakers), which are involved in, for example, the propagation of action potentials in the heart and brain. The homology of Kv channels with plant Shaker channels is restricted to the transmembrane core, and mainly to the pore domain (Fig. 1). The closest relatives of plant Shakers (Pilot *et al*., 2003*b*) are members of the EAG (Ether-a-gogo) family, which comprises EAG, ELK (EAG-like) and ERG (EAG-related) channels (Ganetzky *et al*., 1999), and of the HCN (hyperpolarization-activated cation) and CNG (cyclic nucleotide-gated) channel families (Biel *et al*., 2002; Kaupp and Seifert, 2002). Like their plant counterparts, all these channels bear a putative cyclic nucleotide-binding domain in their C-terminal region. It is worth noting that the region of sequence homology of the *Arabidopsis* KAT1 with animal ERG channels is clearly larger than with animal Kv channels since it encompasses part of the cytoplasmic N-terminus, the whole transmembrane domain and the cyclic nucleotide domain (Fig. 1). Although present knowledge of animal cells mainly concerns members of the Kv family, particular attention will be drawn here to animal channels displaying some

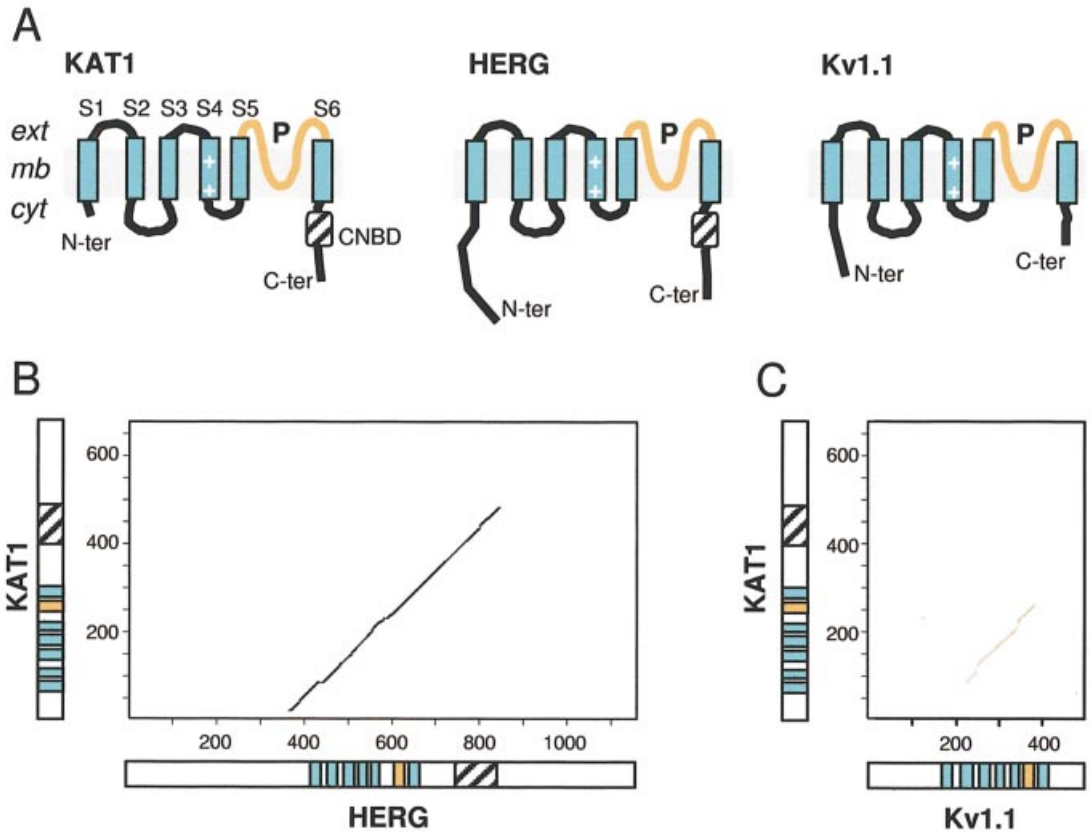

Fig. 1. Structure and sequence conservation between a plant channel (KAT1) and animal channels from the Shaker superfamily. (A) Structure of the *Arabidopsis* KAT1, human HERG and mouse Kv1.1. The six transmembrane segments (S1 to S6) are represented in green. The (+) on the S4 segments indicate positively charged residues involved in voltage sensing. P, pore domain; CNBD, cyclic nucleotide-binding domain; Mb, membrane; ext and cyt, external and cytoplasmic sides. (B) and (C) Comparisons of sequences for local similarity and plot of local sequence alignment (Matrix Blosum 50, PLALIGN program http://fasta.bioch.virginia.edu/fasta/plalign2.htm with default parameters) between KAT1 and HERG, and KAT1 and Kv1.1, respectively. Blue boxes represent the transmembrane segments and the orange one the pore domain. The cyclic nucleotide-binding domain is hatched. The thickness and darkness of the lines in the matrix are indicative of the strength of the homology. For the KAT1/HERG alignment, in the overlapping region: score 490, E(10,000): 2.2 e-32; for the KAT1/Kv1.1 alignment: score 86, E(10,000): 8.2. The E(10,000) values are the pairwise-alignment probabilities multiplied by 10 000.

homologies with plant Shakers in the N- and C-terminal cytoplasmic regions, which are the most likely to interact with regulatory proteins.

Associations between animal Shaker subunits

Channels from the animal Shaker superfamily are multimeric proteins. The *sensu stricto* channel hydrophobic core, that is, the minimal quaternary structure containing the functional aqueous pore, is formed by the assembly of polypeptides called α subunits. These α subunits are encoded by genes from the *Shaker* superfamily themselves. The mode of assembly of channel α subunits constitutes the first level of the regulation of K^+ currents. Direct evidence has been obtained that Kv polypeptides (MacKinnon, 1991; Liman *et al*., 1992; Li M *et al*., 1994), human ERG (HERG) polypeptides (Li *et al*., 1997) and CNG polypeptides (Liu *et al*., 1996) all assemble as tetramers. It has been shown that heterotetrameric channels can be formed, comprising α subunits encoded by different genes and exhibiting functional features different from those of the homotetrameric channels formed by identical α subunits (Isacoff *et al*., 1990; Ruppersberg *et al*., 1990). This process generates diversity in K^+ channel activity (Jan and Jan, 1990). It can also allow the properties of some channel subunits to be modulated by bringing them into close proximity with regulatory proteins that bind to neighbouring subunits in the heterotetrameric structure (Nitabach *et al*., 2001). Heteromeric associations generally occur solely between members of the same subfamily of animal Shaker polypeptides (Xu *et al*., 1995) but some Kv subunits make exceptions to this behaviour (Hugnot *et al*., 1996; Salinas *et al*., 1997*b*).

Modulation of channel activities by regulatory proteins

Once assembled, tetrameric channels are the targets of signalling cascades leading to their activation or inactivation in response to different stimuli. Initially, the search for regulatory proteins that affected K^+ channel activity was undertaken by pharmacological and biochemical approaches. Pharmacological agents specific for a given family of regulatory proteins, such as G proteins, protein kinase A (PKA), or a class of protein phosphatases, were tested for their effects on the channel current. Examples of this kind of approach are provided, for instance, by reports on the regulation of the HERG channel by protein kinase A (Cui *et al*., 2000) and protein kinase C (Barros *et al*., 1998) activities. However, these data do not necessarily infer a direct effect of the regulatory proteins. Identification of protein partners that interact directly with animal K^+ channels to regulate their activities was achieved initially by classical biochemical approaches, including affinity purification and co-immunoprecipitation, and later by screening cDNA libraries using the two-hybrid system. Some proteins observed to interact directly with members of the Shaker superfamily in animals are listed below.

β subunits: A polypeptide of low molecular weight (39 kDa), called a β subunit, was found to be associated with the bovine brain Kv1.2 channel during affinity purification (Parcej *et al*., 1992; Scott *et al*., 1994). Later on, other related polypeptides were identified (Xu and Li, 1998). These β subunits (Kvβ) are not required for the channel to be active. However, they can confer different properties to the channel, such as modification of current amplitudes or inactivation kinetics, and some of them have been reported to promote channel assembly and targeting to the cell surface (Rettig *et al*., 1994; Xu and Li, 1998; Martens *et al*., 1999). Sequence analysis indicates that they are related to NAD(P)H oxidoreductases (McCormack and McCormack, 1994). Mutations in the oxidoreductase active site of Kvβ subunits modify their effects on channel inactivation (Bähring *et al*., 2001). Generally, the β subunit association is selective (Xu and Li, 1998). For example, Kvβ1 and Kvβ2 bind to all members of the Kv1 subfamily tested but not to Kv2.1 and Kv3.1 (Nakahira *et al*., 1996). Their interaction with Kv4.2, which is biochemically distinct from that observed with Kv1 family members, might not be physiologically relevant (Nakahira *et al*., 1996). Kvβ4 interacts with Kv2.2 but not with Kv2.1 (Fink *et al*., 1996). A site essential for the binding of the Kvβ1 subunit has been localized within the T1 tetramerization domain of Kv1 channels, in the N-terminal cytoplasmic region (Sewing *et al*., 1996; Yu *et al*., 1996). By contrast, the interaction between Kv2.2 and Kvβ4 involves the COOH-terminal end of the Kv subunit (Fink *et al*., 1996). Although EAG channels do not harbour Kv-like interaction domains, a β subunit from *Drosophila* physically interacts with members of this family. This interaction leads to increased current amplitudes (Wilson *et al*., 1998).

The minK subunit and related proteins: MinK is a small protein with a single transmembrane segment that regulates cardiac K^+ currents by binding to the Shaker KvLQT1 channel (Barhanin *et al*., 1996; Sanguinetti *et al*., 1996). When co-expressed with KvLQT1, MinK modifies the channel current in such a way that it becomes identical to the slow activating (I_{Ks}) current measured in mammalian cardiac cells. MinK also associates with the HERG channel which generates the rapidly activating current I_{Kr} in the same cardiac cells (McDonald *et al*., 1997). However, I_{Kr} currents cannot be produced by co-expression of HERG with minK in *Xenopus* oocytes. In fact I_{Kr}-like currents are obtained by the association of HERG with a minK-related subunit, MirP1 (Abbott *et al*., 1999), which, it has been suggested, binds to the cyclic nucleotide-binding domain of the channel (Cui *et al*., 2001).

Cytoskeleton-associated proteins: Membrane-associated guanylate kinases such as PSD-95 are synaptic proteins

associated indirectly with microtubules and probably also with microfilaments (Hata and Takai, 1999). They have been identified as physically interacting with Kv1 Shaker polypeptides in two-hybrid screens and shown to be responsible for the formation of channel clusters at the plasma membrane (Kim *et al*., 1995). Other cytoskeleton-associated proteins, such as α-actinin-2 (Maruoka *et al*., 2000), filamin (Petrecca *et al*., 2000) and cortactin (Hattan *et al*., 2002), have been shown to interact directly with Kv channels, and they are thought to regulate channel localization and current density at the cell surface. Kvβ subunits can also bind to the actin skeleton (Nakahira *et al*., 1998). Physiological studies indicate that the attachment to the cytoskeleton of the Kv1.1-Kvβ1 channel controls current inactivation by the Kvβ1 subunit (Levin *et al*., 1996; Martens *et al*., 1999).

Kinases, phosphatases and 14-3-3 proteins: Evidence has been obtained, since the early 1990s, that voltage-gated Shaker channels are regulated by phosphorylation, resulting in modifications of inactivation kinetics and current amplitude (Jonas and Kaczmarek, 1996). Most data concerning the effects of kinases and phosphatases on K^+ channels have been provided by pharmacological studies. However, the formation of stable complexes between cytoplasmic Src tyrosine kinases and Kv1.5 (Holmes *et al*., 1996; Nitabach *et al*., 2001), as well as channels of the EAG family (Santoro *et al*., 1997; Cayabyab and Schlichter, 2002), has been observed. A direct phosphorylation of the Kv1.2 channel by the PYK2 tyrosine kinase has been revealed by the use of anti-phosphotyrosine antibodies (Lev *et al*., 1995). Mutations in phosphorylation sites combined with physiological studies also provided evidence that Shaker channels are substrates for PKA (Drain *et al*., 1994), PKC (Covarrubias *et al*., 1994) and CaMKII kinase (Roeper *et al*., 1997), and that HERG is a substrate for PKA (Cui *et al*., 2000).

Phosphorylation of α subunits can modulate the effects of other regulatory proteins such as β subunits (by impairing the association of channels with microfilaments; Levin *et al*., 1996; Jing *et al*., 1997; Martens *et al*., 1999) and 14-3-3 proteins (Kagan *et al*., 2002). The HERG channel is activated by 14-3-3 proteins that bind to N- and C-terminal phosphorylated PKA sites (Kagan *et al*., 2002).

G proteins: Signalling pathways often involve receptor-mediated G protein regulations. A Gβγ complex has been shown to bind to both Kv1.1 and Kvβ1.1 subunits and to promote their assembly. This would favour detachment of the Kv channels from actin microfilaments and promote channel inactivation (Jing *et al*., 1999). A monomeric G protein binding to Kv2.1 has also been identified. It modulates the channel phosphorylation by a tyrosine kinase (Cachero *et al*., 1998).

Proteins of the secretory pathway: Another mode of regulation involves direct interaction with the exocytotic machinery. Kv1.1 and Kvβ1.1 subunits have been shown to bind to syntaxin 1A (Fili *et al*., 2001). The effect of syntaxin is modulated by direct binding of Kv1.1 to the Gβγ complex (Michaelevski *et al*., 2002). A model in which concomitant binding of syntaxin and Gβγ to the channel αβ complex leads to channel inactivation (by a mechanism involving the release of the inactivating ball of β subunits initially bound to actin microfilament) has been proposed by Michaelevski *et al*. (2002).

Other regulatory proteins identified by systematic screening: Screening cDNA libraries using the two-hybrid system in yeast has identified, besides guanylate kinases, members of the KChIP family of calcium sensors (An *et al*., 2000) and the KCHAP chaperone (Wible *et al*., 1998) as partners of some Kv channels. The Slob protein, identified as interacting with the *Drosophila* Slowpoke channel, also recognizes the EAG channel (Schopperle *et al*., 1998). KCR1, which belongs to the α-glucosyltransferase family, was isolated by functional cloning in *Xenopus* oocytes. It binds the rat EAG channel by its C-terminal region (Hoshi *et al*., 1998).

Summary

These studies suggest approaches to decipher the regulatory networks impacting on plant Shakers. From the above survey, the regulatory network acting on the Kv1.1 channel involves β subunits and the actin skeleton, their effect on the α subunits being modulated by kinases, G proteins, and syntaxins. Other regulatory pathways also begin to emerge, especially for the cardiac HERG channel, which is under the control of protein kinases and 14-3-3s, and is associated to the mirP1 subunits.

Functional and pharmacological analyses identify regulatory processes in plants

Electrophysiological analyses, in combination with pharmacological characterizations, performed *in planta* or in heterologous systems have allowed the regulatory processes involved in the tuning of membrane K^+ channel activity in plants to be identified. These studies cannot, however, establish whether the effects of the pharmacological agents on channel activity are direct (the channel protein being the actual target of the drugs) or indirect (the actual target being a protein that interacts with the channel through more or less complex networks). Furthermore,

when the studies are carried out *in planta*, the molecular identity of the channels affected by the regulatory processes cannot be determined definitively unless knock-out mutant plants are used. It is, however, worth noting that the effects of pharmaceuticals on K^+ conductances *in planta* are likely to impact mainly on the regulation of Shaker channels since most studies are carried out on cells (e.g. guard cells) in which Shaker channels account for most of the membrane K^+ conductance (Véry and Sentenac, 2002).

Phosphorylation/dephosphorylation

Whole cell current recordings in the presence of kinase and phosphatase effectors have revealed the importance of phosphatases in the regulation of plant Shakers. The inactivation of guard cell inward K^+ currents in response to elevation of cytoplasmic Ca^{2+} is blocked by specific inhibitors of (Ca^{2+}-dependent) protein phosphatase 2B (Luan *et al*., 1993). Effects of calyculin A and okadaic acid, two inhibitors of phosphatase 1 and 2A activities, have been analysed in guard cells and mesophyll cells of *Vicia faba*. In guard cells, both okadaic acid (Thiel and Blatt, 1994; Li *et al*., 1994*b*) and calyculin A (Li *et al*., 1994*b*) inhibited inward K^+ currents, the outward currents being either unaffected (Li *et al*., 1994*b*) or inhibited (Thiel and Blatt, 1994). In mesophyll cells, in which inward K^+ currents were almost undetectable, these phosphatase inhibitors activated outward currents (Li *et al*., 1994*b*). Thus, K^+ channel activity appears to be regulated by members of the phosphatase 1 and 2A families in different ways depending on the cell type. Protein phosphatases from the 2C family (especially ABI1 and ABI2) have also been shown to play a crucial role in stomatal closure in response to ABA. Unfortunately, no specific inhibitor is available for these phosphatases. In *Nicotiana benthamiana* transgenic plants over-expressing the *abi1.1* dominant mutant gene from *Arabidopsis*, it was observed by Armstrong *et al*. (1995) that guard cell K^+ currents had become insensitive to ABA, thus pointing to involvement of the ABI1 phosphatase in signalling events leading to channel regulation.

The involvement of (de)phosphorylation processes in K^+ channel regulation is also suggested by experiments showing that ATP and non-hydrolysable ATP analogues can affect K^+ channel activities. Measurements performed on cell patches revealed 'membrane-delimited' regulatory mechanisms that occur in the vicinity of the plasma membrane. In patches from *Arabidopsis* mesophyll cells, ATP activated a weakly inwardly rectifying K^+ conductance (Spalding and Goldsmith, 1993) which might be ascribed to AKT2 (Dennison *et al*., 2001; Véry and Sentenac, 2002). In *Vicia faba* guard cells, although ATP had no effect on K^+ currents measured in the whole-cell configuration, single-channel recordings revealed an activation of inward K^+ currents triggered by this nucleotide (Wu and Assmann, 1995). This discrepancy could be due to the presence in whole cell patches of low ATP levels that would be sufficient for channel activation (Wu and Assmann, 1995). In whole-cell protoplasts or single channel patches isolated from *Samanea saman* pulvini, the rundown of outward K^+ current could be reversed by application of MgATP at the cytoplasmic side (Moran, 1996). A similar requirement for ATP to counteract rundown process has also been observed for cloned K^+ channels expressed in heterologous systems. Using KAT1 as a model, Hoshi (1995) found that the rundown observed after patch excision (macro-patch recording) was reversed by ATP but not by a non-hydrolysable analogue, suggesting that ATP hydrolysis was necessary for KAT1 activation. Prevention of rundown by addition of ATP to the pipette solution was also observed for AKT1 expressed in *Sf*9 cells (Gaymard *et al*., 1996).

The hypothesis that protein kinases are actually involved in K^+ channel regulation has been checked using commercially available bovine protein kinase A (PKA) and effectors of this enzyme. Cyclic AMP, which binds to the regulatory subunit of PKA, or the catalytic subunit of PKA alone, had a positive effect on outward K^+ currents in mesophyll protoplasts from *Vicia faba*. Conversely, PKA inhibitors routinely used in the animal field inhibited these currents (Li *et al*., 1994*a*). Evidence that KAT1 is stimulated by ATP through a phosphorylation mechanism when expressed in *Xenopus* oocytes was provided by the fact that addition of the catalytic subunit of PKA prolonged the ATP effect (rundown inhibition), and the replacement of PKA by alkaline phosphatase led to an acceleration of the rundown (Tang and Hoshi, 1999).

Other regulation mechanisms revealed by functional analyses in planta

Proteins involved in signalling processes have been shown to regulate K^+ channel activities. 14-3-3 proteins from *Vicia faba*, when over-expressed in tobacco, increased the outward K^+ conductance of mesophyll protoplasts (Saalbach *et al*., 1997). During whole-cell path-clamp experiments on protoplasts prepared from tomato suspension cells, the introduction of a purified 14-3-3 into the patch pipette resulted in a 2-fold increase in outward K^+ currents, which was interpreted as resulting from the recruitment of 'sleepy' channels (Booij *et al*., 1999). The role of G proteins in K^+ channel regulation was first investigated in patch-clamped protoplasts using GTP and GDP analogues which lock G proteins either in their active form (GTPγS) or in their inactive form (GDPβS). In *Vicia faba*, GTPγS reduced inward guard cell K^+ currents (Fairley-Grenot and Assmann, 1991) and outward K^+ currents from mesophyll cells (Li and Assmann, 1993), whereas the opposite was observed with GDPβS.

Conversely, in xylem parenchyma cells, inward K^+ conductances have been shown to be increased by a G protein activator (Wegner and de Boer, 1997). Similar results using isolated guard cell membrane patches suggested that the effect of G proteins was membrane-delimited (Wu and Assmann, 1994; Wegner and de Boer, 1997). Consistent with the inhibition of inward K^+ channels by G proteins in *Vicia* guard cells, *Arabidopsis* guard cells from a knock-out mutant for the *GPA1* gene, which encodes a putative Gα subunit, do not exhibit an ABA-induced reduction of inward K^+ currents and the stomata of this mutant open in the presence of ABA (Wang *et al*., 2001). Calcium might modulate the effects of G proteins on plant K^+ channels (Li and Assmann, 1993; Kelly *et al*., 1995).

The actin skeleton is thought to regulate guard cell K^+ channel activity since changes in cell turgor affect both the organization of actin filaments and K^+ currents (Liu and Luan, 1998). In *Vicia* guard cells, inward K^+ channels are activated by cytochalasin D, which induces actin depolymerization, whereas they are inhibited by phalloidin which stabilizes actin polymers (Hwang *et al*., 1997). Actin microfilament-depolymerizing drugs also affect K^+ currents in BY2 cells (Stoeckel and Takeda, 2002). Syntaxins, possibly in connection with the cytoskeleton and G proteins, might also be involved in K^+ channel regulation. The Nt-SYR1 syntaxin has been isolated by functional screening in *Xenopus* oocytes of a tobacco cDNA library on the basis of Ca^{2+}-induced activation of an endogenous Cl^- conductance in response to ABA. When added as a truncated form in *Nicotiana* guard cells, it suppresses the effect of ABA on K^+ currents, thus revealing a new role for this vesicle-trafficking protein in ABA signalling (Leyman *et al*., 1999).

The demonstration in animal cells of an association between sulfonylurea receptors (belonging to the family of ABC transporters) and inward K^+ channels of the Kir family (Inagaki *et al*., 1995) led Leonhardt and colleagues to examine the impact of effectors of sulfonylurea receptors on K^+ currents in plant cells. These pharmacological agents were found to affect guard cell outward currents and stomatal closure in *Vicia* (Leonhardt *et al*., 1997). The mechanism underlying this phenomenon might involve an interaction of outward K^+ channels with the slow anion channel that would be tightly linked to (or equivalent to) an ABC transporter (Leonhardt *et al*., 1999).

In summary, functional approaches *in planta* or in heterologous systems, coupled to pharmacological analyses inspired by the corresponding state of the art in the animal field, have provided information that is likely to be valuable in attempts to understand the regulation of plant Shakers at the molecular level, by allowing plant scientists to select, sort and test candidates among proteins identified by *in silico* searches or by biochemical or DNA-based strategies.

Towards molecular analysis of regulatory networks

Biochemical evidence for KAT1 phosphorylation in guard cells

KAT1 was one of the first two plant K^+ channels cloned. This guard cell channel has often been used as a model, for example, in elucidating the relationships between protein structure and channel activity, because it functions and is easy to characterize when expressed in heterologous systems, including *Xenopus* oocytes.

Direct phosphorylations can be revealed by incorporation of ^{32}P from labelled ATP into proteins. Surprisingly, this kind of approach applied to the KAT1 and AKT1 channels expressed in *Sf*9 insect cells revealed that only AKT1 was phosphorylated, while electrophysiological analyses indicated that both channels were active in the membrane of the transfected insect cells (Urbach *et al*., 2000). On the other hand, KAT1 channel activity was shown to be enhanced by phosphorylation events in *Xenopus* oocytes (see above). The KAT1 protein has been reported to be a substrate for at least two kinds of plant kinases. A 57 kDa kinase isolated from *Vicia* guard cell protoplasts was shown to phosphorylate KAT1 in a calcium-dependent manner. It was identified as a calcium-dependent protein kinase (CDPK) on the basis of its physical properties and recognition by antibodies against the calmodulin domain of soybean CDPK (Li *et al*., 1998). When expressed in *Xenopus* oocytes together with KAT1, this soybean CDPK inhibited KAT1 currents (Berkowitz *et al*., 2000). Another kinase from *Vicia* guard cells, of 48 kDa, induced by abscisic acid in guard cell protoplasts, was reported to phosphorylate a carboxy-terminal fragment of KAT1 immobilized in polyacrylamide gels (Mori *et al*., 2000). Unfortunately, neither the CDPK nor the 48 kDa kinase induced by abscisic acid have yet revealed their molecular identity. The exact role of KAT1 phosphorylation in channel activation or inhibition and guard cell functioning has also to be determined.

Shaker subunits and heterotetrameric channels

The first indication of heterotetramer formation between plant Shaker polypeptides came from co-expression studies in *Xenopus* oocytes showing that the recorded currents could not be fitted simply by summing homotetrameric channel currents (Dreyer *et al*., 1997). Interactions between the C-terminal regions of Shaker channels from potato and *Arabidopsis* were also revealed by two hybrid experiments in yeast (Ehrhardt *et al*., 1997; Pilot *et al*., 2001, 2003*a*) and by biochemical tests (Ehrhardt *et al*., 1997). As in animal cells, the occurrence of stable associations between two members of the family, it has been suggested, depend on their polypeptide sequence and the presence of domains acting as molecular barriers,

allowing or preventing the assembly. AKT1 and KAT1 subunits do not physically bind to each other in biochemical tests, which allowed the observation of homologous AKT1-AKT1 and KAT1-KAT1 interactions (Urbach *et al*., 2000). Phylogenetic proximity of subunits does not seem to be a prerequisite for the ability to form heteromeric associations. Five Shaker subgroups can be defined in plants (Pilot *et al*., 2003*b*). Group I, II, III, IV, and V correspond to relatives of the *Arabidopsis* AKT1, KAT1, AKT2, AtKC1 and SKOR, respectively. Channels of subgroups I and II, I and III, II and III and I and IV have been shown to interact, at least in heterologous systems (Dreyer *et al*., 1997; Baizabal-Aguirre *et al*., 1999; Pilot *et al*., 2001, 2003*a*; Zimmermann *et al*., 2001). Deletion experiments have led to the identification of a short sequence at the C-terminus of the potato SKT1 (Group I type) polypeptide, named the KT domain, as a necessary element for the assembly with KST1 (Group II type) subunits (Zimmermann *et al*., 2001).

Analyses of the expression patterns of the different *Arabidopsis* Shaker genes combined with interaction tests (e.g. two hybrid experiments or co-expression of channel subunits in *Xenopus* oocytes) allow the prediction of which channel polypeptides might interact and form heterotetramers *in planta*. The KAT1 and KAT2 channels are both expressed in guard cells and they have been shown to interact in two-hybrid tests in yeast and co-expression experiments in *Xenopus* oocytes (Pilot *et al*., 2001). The formation of hybrid KAT1/KAT2 channels might explain why the over-expression of KAT1 mutant subunits results in detectable effects on inward guard cell currents and stomatal functioning (Ichida *et al*., 1997; Kwak *et al*., 2001) while disruption of the KAT1 gene does not affect guard cell movements (Szyroki *et al*., 2001). On the basis of the expression patterns and two hybrid experiments, AKT1 might interact with AKT2 in mesophyll cells, and with AtKC1 in root epidermal and cortical cells (Pilot *et al*., 2003*a*). Disruption of the *AKT1* gene results in complete loss of the inward K^+ currents in root hairs (thus indicating that functional expression of *AKT1* is required for the formation of the major inward K^+ conductance in these cells), while disruption of the *AtKC1* gene results in qualitative changes of the current, such as changes in the current sensitivity to external Ca^{2+} or pH (Reintanz *et al*., 2002). Since *AtKC1* has never been found to form functional channels per se in heterologous systems, the AtKC1 polypeptide was suggested to act only as an integral component of heteromultimeric K^+ channels (Reintanz *et al*., 2002). It would thus play a role reminiscent of that of the silent modulatory α subunits of mammalian Kv channels (Hugnot *et al*., 1996; Salinas *et al*., 1997*a*, *b*) and cyclic nucleotide-gated channels (Kaupp and Seifert, 2002). However, one of its putative orthologues, KDC1 (from *Daucus carota*), seems to form functional, inwardly rectifying channels when expressed alone in mammalian cells (Downey *et al*., 2000). In potato, KST1 and SKT1, which are reported to interact in co-expression tests in *Xenopus* oocytes, are both expressed (and thus are likely to form heterotetramers) in guard cells (Zimmermann *et al*., 2001).

Another aspect of the interaction between channel subunits is the formation of clusters of tetrameric channels. The membrane of insect cells expressing GFP tagged KST1 channels displayed fluorescence spots taken as an indication of the formation of such clusters (Ehrhardt *et al*., 1997). Deletion of the conserved C-terminal end of KST1, harbouring the so-called K_{HA} domain (rich in hydrophobic and acidic residues), did not abolish channel activity, but resulted in the disappearance of the fluorescence spots (the GFP signal being distributed evenly along the membrane in these cells). The deletion also resulted in a lack of interaction between KST1 C-terminal fragments. Thus, the channel C-terminal end seems to play a role in clustering active channels at discrete loci in the membrane, and it has been suggested that this would affect the functional features of these channels (Ehrhardt *et al*., 1997). In the case of KAT1, this kind of phenomenon could explain the dependence of current properties with respect to the level of channel expression in *Xenopus* oocytes (Véry *et al*., 1994).

Protein partners interacting directly with plant Shakers and regulating channel activity

Significant advances towards the identification of regulatory partners of Shaker channels have been achieved in *Arabidopsis* with the identification of homologues of animal β subunits and of a phosphatase, AtPP2CA, which physically and functionally interacts with the AKT2 channel.*?*

In silico analyses of the *Arabidopsis* genome revealed the existence of a gene, *KAB1*, encoding a putative homologue of animal Kvβ subunits (Tang *et al*., 1995). The KAB1 protein binds to KAT1 (Tang *et al*., 1996) and AKT1 (Tang *et al*., 1998) *in vitro*, but not to the human channel HERG (Tang *et al*., 1998). When co-expressed with KAT1 in *Xenopus* oocytes, KAB1 moderately enhanced KAT1 currents, suggesting that it might stabilize the channel complex in the membrane as do some animal Kvβ subunits (Zhang *et al*., 1999). Expression data suggest that KAB1 is present in different plant aerial organs, and especially well represented in guard cells where the *KAT1* gene is expressed (Tang *et al*., 1996). With antibodies raised against KAB1, immunostaining was found to be associated with several membranes including the chloroplast and mitochondrial inner membrane, tonoplast and plasma membrane (Tang *et al*., 1998). This could result from association of KAB1 with multiple targets present in different compartments, or from cross-reaction of the antibodies with KAB1 homologues. A rice counterpart of

KAB1, KOB1, is present mainly in young leaves and its abundance is correlated with the K^+ status of the leaf (Fang *et al*., 1998).

Two hybrid screens allowed the identification of the AKT2 channel as a putative partner of the AtPP2CA protein phosphatase (Vranova *et al*., 2001), and, conversely, AtPP2CA as a putative partner of AKT2 (Chérel *et al*., 2002). AtPP2CA is a close relative of the ABI1 phosphatase known to affect ABA-induced responses of guard cell K^+ currents (Armstrong *et al*., 1995). The channel binds the phosphatase at its intracytoplasmic C-terminal sequence, and the AtPP2CA/AKT2 interaction results in decreased AKT2 currents and alteration of the channel gating properties (Chérel *et al*., 2002). Since AtPP2CA, like ABI1 and ABI2, has also been reported to be involved in ABA signalling (Sheen, 1998; Tahtiharju and Palva, 2001), this effect is likely to take part in the regulation of K^+ fluxes in response to the hormone.

Conclusion and perspectives

Little is currently known about the regulation of K^+ channel activity at the molecular level in plants. The roles of the two channel-interacting proteins identified so far in *Arabidopsis*, KAB1 and AtPP2CA, need to be clarified. Protein kinases directly modulating channel activities, physical links with the cytoskeleton and pieces of regulatory networks involving G proteins, 14-3-3s or syntaxins have still to be identified.

A strategy for identifying such channel regulatory partners in plants consists of testing proteins that have been found to regulate animal channel activities since the basic types of regulation seem to be conserved between plants and animals. However, the extrapolation of data obtained with animal K^+ channels is not straightforward. First, as discussed above, plant channels display unique features such as the ankyrin domain which should take part in specific regulatory mechanisms. Second, plant homologues of animal regulatory proteins often differ from the classical form observed in animals (Table 1). For instance, serine/threonine kinases homologous to PKA exist in the *Arabidopsis* genome, but a canonical protein kinase A (with its cAMP-binding regulatory subunit) cannot be found. The family of α subunits of heterotrimeric G proteins seems to be restricted to a single member, GPA1 (Assmann, 2002), as well as that of G protein-coupled receptors (Colucci *et al*., 2002). Instead, the numerous small GTPases (93 in *Arabidopsis*) appear to play major roles in plant signalling (Yang, 2002), but their contribution to plant channel regulation is still poorly documented. Also, no *Arabidopsis* counterpart could be identified for membrane-associated guanylate kinases, KChips, Slob, and KChap (Table 1).

The yeast two-hybrid system has proven to support powerful systematic strategies for identifying proteins that interact with a given channel in plants as in animal systems. Its main limitations are the possible activation of the reporter gene by the hybrid bait protein in the absence of activator domain, the difficulty for eliminating false positives, and the requirement for hybrid protein expression in the nucleus. In the case of membrane proteins such as channels, transmembrane segments have to be removed in order to allow the targeting of the bait protein to nucleus. To overcome this problem, methods allowing the detection of interactions between constructs in the yeast cytoplasm have been developed, such as Sos/Ras systems (Aronheim *et al*., 1997; Broder *et al*., 1998). However, suitable *Arabidopsis* cDNA libraries are not yet available, and these new techniques apparently need to be improved before being used to screen cDNA libraries routinely (Causier and Davies, 2002). The split-ubiquitin system (Stagljar *et al*., 1998) has proven to be efficient in detecting interactions between sucrose transporters (Reinders *et al*., 2002; Schulze *et al*., 2003). It has been used as a screening method for yeast genomic fragments (Laser *et al*., 2000), but awaits confirmation that it can be used for plant cDNA libraries. The development of mass spectrometry for the analysis of proteins and protein complexes has boosted biochemical approaches for the study of protein–protein interactions (Pandey and Mann, 2000). The coupling of classical methods for the isolation of protein complexes (using tagged proteins, fusions with GST or antibody-based strategies) to analyses by mass spectrometry seems to be promising. Last, in the case of channels, cloning by functional co-expression in *Xenopus* oocytes (Vallet *et al*., 1998) or yeast cells (Ueda *et al*., 1996) can provide an alternative to methods based on physical associations between proteins, by directly identifying regulatory proteins controlling the channel activity.

Detection of interactions *in vitro* or in heterologous systems does not constitute by itself a demonstration that the proteins interact *in planta*. Other arguments such as co-localization of the candidate protein with the channel have to be provided, together with phenotypic analyses of mutants defective for the candidate gene. Fluorescence imaging techniques suitable for monitoring protein–protein interactions in plant cells are just beginning to emerge (Subramaniam *et al*., 2001; Hink *et al*., 2002). Their application to plant cells is still limited, and seems to be restricted to non-physiological conditions (over-expression in transfected protoplasts). However the use of the FRET (Gadella *et al*., 1999) could increase with progress and accessibility of the technology. The combination of data from systematic screens, physiological studies and reverse genetics should allow the identification of new channel-binding proteins and the physiological roles of their interactions with plant Shakers to be addressed. Delineation of the relationships between the different

Table 1. *K^+ channel-interacting proteins in animals and plants, and their homologues in* Arabidopsis

Regulatory proteins	Regulated channels	Presence of regulatory protein homologues in the *Arabidopsis* genome	Information source
In animal cells			
β subunits (NAD(P)H oxidoreductases)	Kv1 subunits[a] (Sewing *et al.*, 1996; Xu and Li, 1998) EAG[a] (Wilson *et al.*, 1998)	KAB1 (+17 more distantly related aldo-keto reductases)	Blast at TAIR with mouse Kvβ1
minK (IsK) and relatives	KvLQT1[a] (Barhanin *et al.*, 1996) HERG[a] (McDonald *et al.*, 1997)	None	Blast at TAIR with mouse IsK
Serine/threonine protein kinases, PKA- and PKC-like	Kv channels[b] (Covarrubias *et al.*, 1994; Drain *et al.*, 1994) HERG[b c] (Cui *et al.*, 2000)	IRE/NPH/PI dependent/S6 kinase family (41 members in *Arabidopsis*)	Blast at TAIR with bovine PKA-α and PKC-α + PlantsP
PKA regulatory subunits		None	Blast at TAIR with P00514 (bovine, type I-α)
CamKII	Kv1.4[b c] (Roeper *et al.*, 1997) HERG[b c] (Wang *et al.*, 2002)	34 calcium-dependent protein kinases	Cheng *et al.*, 2002
Tyrosine kinases	Kv1.2[c] (Lev *et al.*, 1995) Kv1.5[a] (Holmes *et al.*, 1996) rat erg1[a] (Cayabyab and Schlichter, 2002)	No typical tyrosine kinase identified; existence of putative serine/threonine/ tyrosine kinases	Luan, 2002; PlantsP
Tyrosine phosphatases	Kv1.2[a] (Tsai *et al.*, 1999)	~ 20 genes	Kerk *et al.*, 2002; Luan, 2002
Heterotrimeric G proteins	Kv1.1[a] (Jing *et al.*, 1999; Michaelevski *et al.*, 2002)		
G protein α subunits		1 member, GPA1	Assmann, 2002
G protein β subunits		1 member, AGB1	Assmann, 2002
G protein γ subunits		At least two members, AGG1 and AGG2	Assmann, 2002
Small GTP-ases	Kv1.2[a] (Cachero *et al.*, 1998)	93 members	Yang, 2002
14-3-3 proteins	HERG[a] (Kagan *et al.*, 2002)	15 members	Sehnke *et al.*, 2002
Syntaxins	Kv1.1[a] (Fili *et al.*, 2001; Michaelevski *et al.*, 2002)	25 members	TAIR
Membrane-associated guanylate kinases	Kv1 subunits[a] (Kim *et al.*, 1995)	3 proteins with guanylate kinase signature, no PDZ channel-interacting domain	Blast at TAIR with P78352 (human PSD-95)
α-actinin-2	Kv1.5[a] (Maruoka *et al.*, 2000)	None (but similar to calmodulins)	Blast at TAIR with human α-actinin-2
Filamin	Kv4.2[a] (Petrecca *et al.*, 2000)	None (similar to fimbrins)	Blast at TAIR with AAA92644
Cortactin	Kv1.2[a] (Hattan *et al.*, 2002)	None	Blast at TAIR with AAA19689
KCHIPs	Kv4.2 and Kv4.3[a] (An *et al.*, 2000)	None (homologies with calcineurins in Ca^{2+}-binding domains)	Blast at TAIR with AAL92564
Slob	dSlo and eag[a] (Schopperle *et al.*, 1998)	None	Blast at TAIR with Slob
KChAP	Kv1 and Kv2[a] channels (Wible *et al.*, 1998)	None	Blast at TAIR with rat KChAP (NM_031784)
KCR1	Rat EAG (Hoshi *et al.*, 1998)	At5g02410	Blast at TAIR with KCR1
In plant cells			
β subunits	KAT1[a] (Tang *et al.*, 1996) AKT1[a] (Tang *et al.*, 1998)	See above	
Calcium-dependent protein kinases	KAT1[c] (Li *et al.*, 1998)	34 members	Cheng *et al.*, 2002
Protein phosphatases 2C	AKT2[a] (Chérel *et al.*, 2002)	Group 6.3, 69 members	PlantsP

[a] Biochemical evidence for direct physical interaction.
[b] Evidence for the channel being a substrate for the kinase obtained by mutations in phosphorylation sites.
[c] Evidence for the channel being a substrate for the kinase obtained by *in vitro* phosphorylation tests.
TAIR: http://www.arabidopsis.org
PlantsP: http://plantsp.sdsc.edu/

regulatory proteins (such as those detected in animal cells between the actin skeleton and kinases, G proteins and syntaxins) is a prerequisite for a global understanding of the mechanisms controlling K^+ transport in plant cells.

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