FEBS Letters 409 (1997) 166–170 FEBS 18650

Association of plant K^+_{in} channels is mediated by conserved C-termini and does not affect subunit assembly

Thomas Ehrhardt, Sabine Zimmermann, Bernd Müller-Röber*

Max-Planck-Institut für Molekulare Pflanzenphysiologie (MPI-MOPP), Karl-Liebknecht-Straße 25, Haus 20, D-14476 Golm, Potsdam, Germany

Received 3 April 1997; revised version received 14 April 1997

Abstract Inward rectifying potassium (K^+_{in}) channels play an important role in turgor regulation and ion uptake in higher plants. Here, we report a previously unrecognized feature of these proteins: K^+_{in} channel C-terminal polypeptides mediate channel protein interactions. Using a C-terminal fragment of potato guard cell K^+_{in} channel KST1 in a yeast two-hybrid screen two novel putative K^+_{in} channel proteins (SKT2 and SKT3) were identified by interaction of their C-termini which contained a conserved domain (K_{HA}). Interactions were confirmed by Western blot-related assays utilizing K^+_{in} channel C-termini fused to green fluorescence protein. Although deletion of the K_{HA}-domain abolished these interactions, K^+_{in} currents were still detectable by patch-clamp measurements of insect cells expressing these KST1 mutants, indicating that formation of a functional channel does not depend on this C-terminal domain.

Key words: Guard cell; Insect cell; Potassium channel; Two-hybrid system; Potato

1. Introduction

Potassium channels take part in important processes of higher plants, including opening and closing of stomatal pores and leaf movement [1–3]. The molecular cloning of several plant voltage-gated inward rectifying K^+ (K^+_{in}) channels has been reported during the recent years [4–8]. So far, investigations focused mainly on studies of their expression patterns throughout the plant [6,7,9–11] and their electrophysiological characterization after heterologous expression in e.g. *Xenopus* oocytes [2,6–8,12] and Baculovirus-infected insect cells [13,14]. Besides K^+_{in} channel regulation by pH and nucleotides [12], K^+ fluxes across the plasma membrane of stomatal guard cells are regulated by several proteins of signal transduction cascades. The involvement of protein phosphatases of the families 1 and 2A [15], 2B and C and of GTP-binding proteins (cf. [16]) as well as cytoskeletal proteins [17] has been reported, which all imply protein–protein interactions, but it remains unknown whether these proteins act directly on the channels. The ankyrin-like domains of some plant K^+_{in} channels have been proposed to be targets for interactions with structural proteins [4,7]. Further, association with a β-subunit has been described for *Arabidopsis* KAT1 but no function has been assigned to this interaction [18].

On the basis of its guard cell-specific expression pattern and its electrophysiological characteristics, KST1 has been considered as a key element of the signalling leading to stomatal opening [6]. To study KST1 in terms of protein interactions which may be involved in channel regulation or localization, we utilized the yeast two-hybrid system [19].

2. Materials and methods

2.1. Two-hybrid analysis

Yeast two-hybrid screening was performed as described using the GAL4-based system [19,20] and yeast strain YRG2 (Stratagene, Heidelberg, Germany). An oriented cDNA library in vector pAD-GAL4 was established from epidermal fragments of *Solanum tuberosum* L. cv. Désirée leaves [21] using the HybriZAP Kit (Stratagene). LacZ assays were performed as described (Stratagene). Plasmids KSTA, KSTB and KSTC were constructed via PCR in binding-domain vector pGBT9 (Clontech, Palo Alto, USA) and KSTD in pBD-GAL4 (Stratagene). KSTC, SKT2C-e and SKT3C fragments were transferred as *Eco*RI/*Pst*I fragments to pBD-GAL4 and a 0.8-kb *Sma*I-fragment of pSKT1 was ligated to pAD-GAL4 and pBD-GAL4 to obtain pADSKT1C and pBDSKT1C. Cloning procedures were performed as described [22].

2.2. Isolation of skt2 full-length and skt3 partial-size cDNAs

$\approx 5\times10^5$ plaque-forming units of a λ ZAP II cDNA library established from potato epidermal fragments [6] were screened with *skt2C-a* and *skt3C* cDNA fragments, respectively. Rescued phagemids were sequenced on both strands [23].

2.3. Protein expression in insect cells

Spodoptera frugiperda Sf21 cells were cultured as described [24]. Protein expression was performed with help of the Bac-to-Bac expression system (Gibco-BRL, Eggenstein, Germany). Transfer plasmids were constructed as follows: pGFP: the *Nhe*I/*Hin*dIII fragment of pGFP-C2 (Clontech) was subcloned into pFastBac1 (Gibco-BRL) cut with *Spe*I and *Hin*dIII. pKST1: the *Not*I/*Pst*I fragment of pKST8-1 [6] was subcloned to pFastBac1 cut with the same enzymes. pGFP-KST1: the first two codons of the *kst1* coding region were modified by PCR, generating a *Bam*HI restriction site. A *Bam*HI/*Pst*I fragment of this plasmid was ligated into vector pBlueBacHisB (Invitrogen, Leek, The Netherlands). From this construct a *Bam*HI/*Hin*dIII fragment was transferred to pGFP-C3 (Clontech) cut with *Bgl*II/*Hin*dIII. Finally, the GFP-KST1 coding region was cloned as a *Nhe*I/*Pst*I fragment into pFastBac1 cut *Xba*I/*Pst*I. pGFP-KST1ΔK$_{HA}$: pGFP-KST1 was cut *Bbr*PI/*Hin*dIII, ends were filled with T4 DNA polymerase and religated. pGFP-KSTC, pGFP-SKT2C-e and pGFP-SKT3C: *Eco*RI/*Pst*I fragments of the respective pAD-GAL4 constructs were ligated to pGFP-C2 and subsequently transferred as *Xba*I/*Pst*I fragments to pFastBac1. pGFP-SKT1C: an *Eco*RI fragment was transferred from pADSKT1C into pGFP-C2 and subsequently subcloned as a *Nhe*I/*Pst*I fragment into pFastBac1 cut with *Xba*I/*Pst*I.

Fluorescence imaging of insect cells was performed using an Olympus Provis AX70 microscope equipped with Olympus filters BP420-480 and BA515.

2.4. 'Green Western' blot analysis

Insect cells expressing GFP, GFP-KSTC, GFP-SKT1C, GFP-SKT2C-e or GFP-SKT3C, respectively, were harvested 2–3 days post-infection, solubilized in 2×SDS-loading buffer [25] by sonifica-

*Corresponding author. Fax: (49) (331) 977 2301.
E-mail: bernd@mpimp-golm.mpg.de

Abbreviations: K^+, potassium; GFP, green fluorescence protein; Bait, protein fusion to Gal4-binding domain; Prey, protein fusion to Gal4 activation domain

PII S0014-5793(97)00502-4

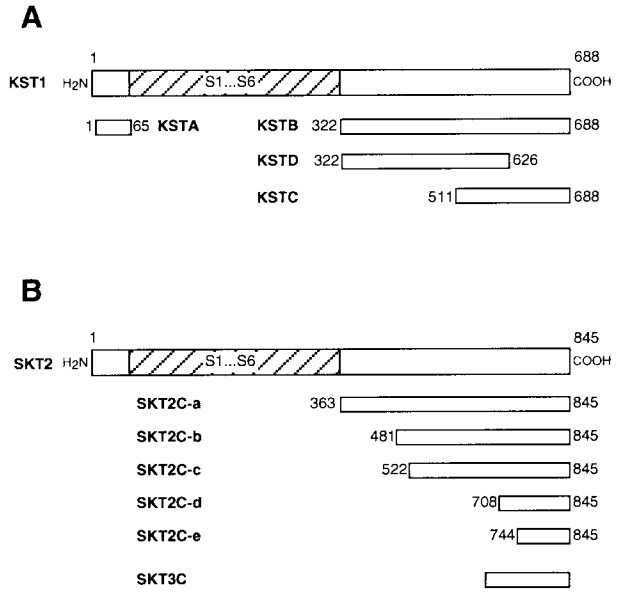

Fig. 1. Yeast two-hybrid analysis with guard cell K^+ channel KST1. (A) KST1 subfragments KSTA, -C, -D were used to screen an activation domain cDNA library. (B) cDNA clones identified with bait KSTC in the two-hybrid screen. In A and B, the hatched boxes indicate the transmembrane segments S1-S6. Numbers indicate amino-acid positions in KST1 and SKT2, respectively. SKT3C represents the C-terminal 154 amino acids of SKT3.

tion and heat denatured at 80°C for 20 min to irreversibly destroy the GFP fluorescence. Equal amounts of GFP fusion proteins were separated on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and renatured as described [26]. After incubation in 5% skimmed milk in buffer A (10 mM HEPES, 60 mM KCl, 1 mM EDTA pH 7.2) for 1 h, filters were incubated with native protein extracts obtained from Sf21 cells, expressing the same proteins (see above) for 2 h at 4°C. Native extracts were prepared by sonicating cells in buffer A supplemented with 1 mM 2-mercaptoethanol and 0.1% Triton X-100. Filter images were recorded with a CCD camera under illumination with 2 hand-lamps (365 nm, 6 W each).

2.5. Electrophysiology

Insect cells expressing KST1, KST1ΔK_{HA}, GFP, GFP-KST1, GFP-KST1ΔK_{HA} or uninfected Sf21 cells were monitored in the whole-cell configuration 2 days post-infection as described [27]. The bath solution contained 30 mM K^+-gluconate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 225 mM sorbitol, 10 mM Mes-Tris pH 6.2 and the pipette solution contained 150 mM K^+-gluconate, 2 mM $MgCl_2$, 10 mM MgATP, 10 mM EGTA, 10 mM HEPES-Tris pH 7.4.

3. Results

3.1. C-termini of plant K^+_{in} channels mediate protein interactions

Because transmembrane domains can not be used efficiently in the two-hybrid system [28] four different *kst1* cDNA fragments encoding KST1 polypeptides with cytosolic localization were fused to the coding region of the Gal4-binding domain (Fig. 1A). Except for KSTB, which turned out to self-activate the reporter genes, each 'bait' construct was used to screen $1–5\times10^6$ clones of a potato epidermal fragment activation domain cDNA library. Only with bait plasmid KSTC several partial cDNAs were isolated, all of which encoded C-terminal fragments of proteins (designated as SKT2 and SKT3) with strong homology to previously cloned plant K^+_{in} channels (Fig. 1B). Subsequently, a full-length 2.8-kb *skt2* cDNA and a 1.4-kb partial-size *skt3* cDNA were isolated from a potato epidermal fragment cDNA library [6]. The encoded protein SKT2 (845 residues) showed highest homology (65% identity, 81% similarity) to *Arabidopsis thaliana* AKT2 [7] while the 480 amino acids encoded by partial *skt3* cDNA shared highest homology with SKT2 (75% identity, 88% similarity) and to a lesser extent with AKT2 (61% identity, 77% similarity). Southern blot experiments indicated the presence of *skt2* and *skt3* genes as single copies in the potato genome (data not shown).

A comparison of KST1, SKT2 and SKT3 with the *Arabidopsis* K^+_{in} channels and with SKT1, a further potato K^+_{in} channel cloned by our laboratory (unpublished results) revealed conserved protein sequences in their C-termini close to the stop codons (Fig. 2). Two high-homology blocks in this region (K_{HA}) are enriched for hydrophobic and acidic amino-acid residues, respectively. Homologous regions were also observed in K^+_{in} channels from tomato [29] and maize (D. Becker and R. Hedrich, personal communication). In contrast, the structurally related *Shaker*-type K^+ channels from animals and the outward rectifying K^+ channel KCO1 from *A. thaliana* [27] lacked this motif. No additional homologous sequences were found in databases, indicating that the K_{HA}-domain displays a conserved and unique motif in K^+_{in} channels from mono- and dicotyledonous plants.

To extend the correlation between sequence similarity and protein interaction of plant K^+_{in} channels, we used KSTC, SKT1C, SKT2C-e and SKT3C fused to the Gal4-binding and activation domains to check all cross-wise interactions in the two-hybrid system. The resulting interaction matrix displayed several cross- and self-interactions in addition to those identified in the initial interaction screen (Fig. 3A). Signals were independent of the bait/prey distribution indicating true positive interactions [30]. We confirmed protein interactions by a novel in vitro system ('Green Western' blotting) using GFP as a reporter. Fusion proteins of GFP located N-terminally to KSTC, SKT1C, SKT2C-e and SKT3C expressed in insect cells were utilized in filter overlay assay-related experiments (cf. [26]. Unfused GFP did not yield any interaction signals (not shown). The established interaction

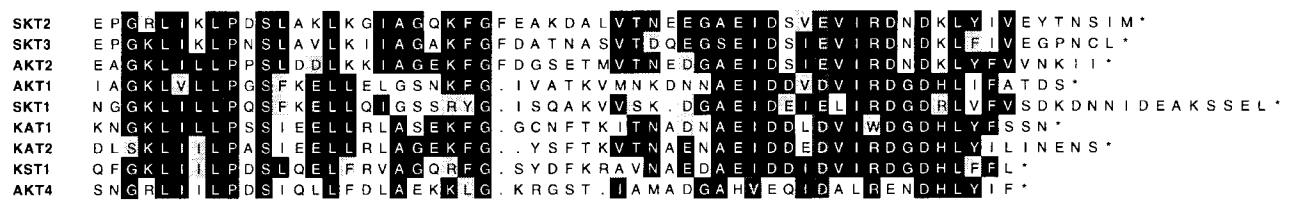

Fig. 2. C-terminal amino-acid sequence homology of plant K^+_{in} channel proteins (K_{HA}-domain). Identical or similar residues are indicated by black or grey boxes, respectively. Stop codons are indicated by *. GenBank accession numbers: AKT1, U06745; AKT2, U40145; AKT4, Z83202; KAT1, U25008; KAT2, U25694; KST1, X79779; SKT1, X86021; SKT2, Y09699; SKT3, Y09818.

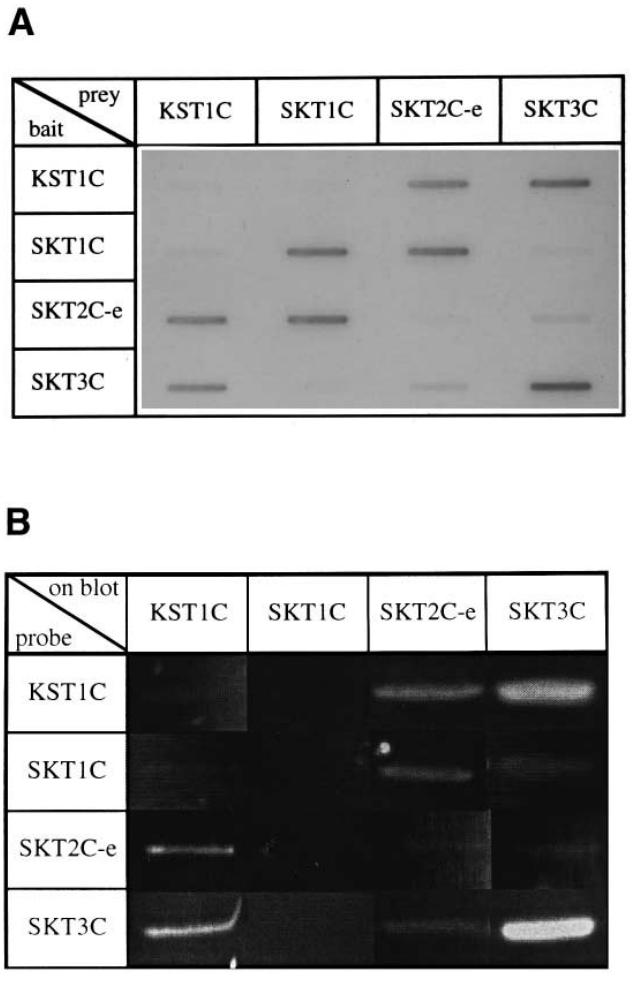

Fig. 3. Protein interaction matrices of potato K^+_{in} channel C-terminal polypeptides: (A) Interactions determined with the yeast two-hybrid system: LacZ assay performed on 0.5 ml of co-transformed yeast clones, grown to $OD_{600} = 1$ and blotted to a nylon membrane. (B) Interactions detected with 'Green Western' blotting (black and white representation of green flourescence signals on nitrocellulose membranes reflecting protein–protein interactions).

matrix (Fig. 3B) confirmed and supplemented the interactions detected with the two-hybrid system (Fig. 3A). Fluorescence signal intensities approximated the signal strength of the LacZ assay indicating different binding affinities for different combinations of K^+_{in} channel C-termini. Signal intensities were similar when the respective binding partners on the blot and in the probe where switched. Further, the 'Green Western' assay appeared to be more sensitive than the two-hybrid system, since the weakest *in vitro* signals were not detected in vivo. Binding to GFP-SKT1C on the *blot* was not detectable, which might be due to renaturation problems. However, when GFP-SKT1C was used as the *probe*, interactions with GFP-KSTC, GFP-SKT2C-e and GFP-SKT3C were detectable. Taken together, these data suggested that the plant K^+_{in} channel C-termini investigated here were capable to interact universally.

3.2. *Conserved domain K_{HA} confers clustering to KST1 expressed in insect cells*

The comparison of fluorescence microscopy images of insect cells expressing GFP (Fig. 4A) or a fusion of GFP located N-terminally to the complete KST1 protein (GFP-KST1, Fig. 4B) visualized the formation of K^+_{in} channel clusters within the plasma membrane. Contrary, a fusion protein deleted for the K_{HA}-domain (GFP-KST1ΔK_{HA}) clearly displayed an even distribution of fluorescence along the plasma membrane (Fig. 4C). Similarly, in 'Green Western' blot experiments self-interaction of GFP-KSTC was abolished when the K_{HA}-domain was deleted (data not shown). In conclusion, self-interaction of KST1, visible as cluster formation in the insect cell plasma membrane, depended on the K_{HA}-domain. Similar experiments with SKT1 and SKT2 were impaired by the minute amount of these channels located in the insect cell plasma membrane (data not shown).

3.3. *KST1 lacking the K_{HA}-domain is electrophysiologically active in insect cells*

Patch-clamp experiments on insect cells expressing the complete KST1 or a truncated version of the protein (lacking the K_{HA}-domain) were performed. Expressed KST1 (or a GFP-KST1; not shown) elicited large slowly activating inward K^+ currents upon membrane hyperpolarization ($n = 13$, Fig. 5B) similar to currents previously shown in *Xenopus* oocytes [6] and comparable with currents described for KAT1 or AKT1 expressed in insect cells [13,14]. Tail current analysis displayed the K^+ selectivity of KST1 (not shown but cf. Fig. 5A). The calculated reversal potential of -41 ± 4 mV ($n = 8$) was in good accordance with the Nernst potential for K^+ (−42 mV). K^+-selective inward currents with almost identical amplitudes and current–voltage relationships (Fig. 5A,B) were also observed when KST1ΔK_{HA} ($n = 10$) (or GFP-KST1ΔK_{HA}, not shown) were expressed. Only little background currents

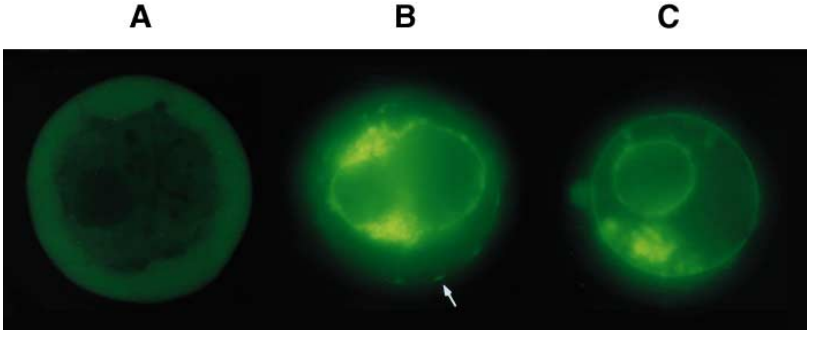

Fig. 4. Microscopic analysis of fluorescence localization in insect cells expressing the following proteins: (A) GFP, (B) GFP-KST1, (C) GFP-KST1ΔK_{HA}. Fusion proteins localized to endogenous membranes and the plasma membrane. Deletion of the K_{HA} domain abolishes cluster formation (indicated by an arrow) of KST1 in the plasma membrane.

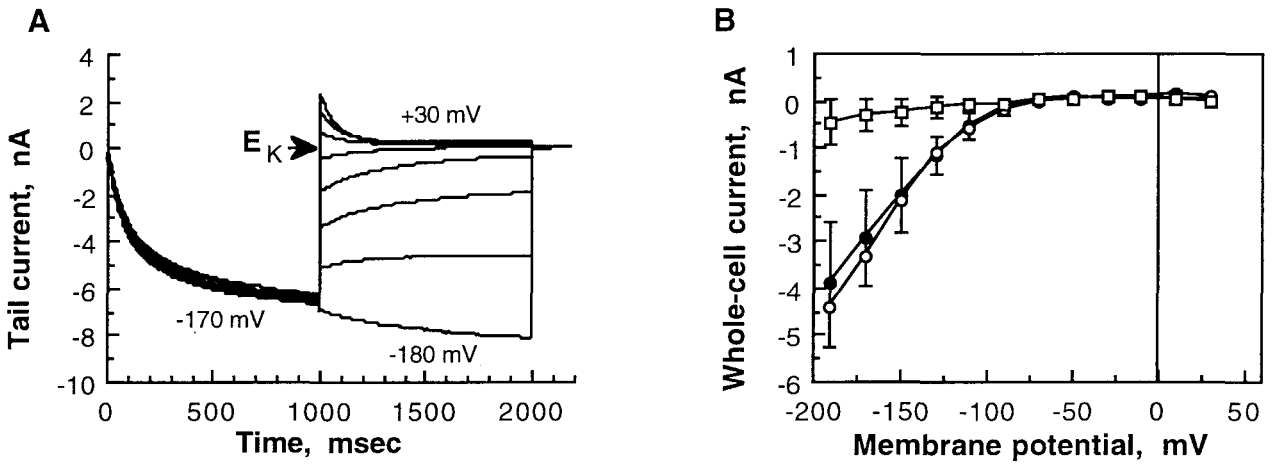

Fig. 5. Electrophysiological properties of KST1 and KST1ΔK$_{HA}$ expressed in insect cells. (A) Tail currents elicited by double-voltage pulses in insect cells expressing KST1ΔK$_{HA}$. The current direction reversed, as indicated, at E_K. (B) Current–voltage relations of the mean steady-state currents at the end of 1.5-s voltage pulses derived from control insect cells (open squares), and cells expressing KST1 (open circles) or KST1ΔK$_{HA}$ (filled circles), respectively.

were detected in uninfected or GFP-expressing insect cells ($n = 10$, Fig. 5B). The voltage-dependent activation kinetics for a −170 mV pulse were fitted by two exponentials for KST1 ($\tau_1 = 54 \pm 23$ ms, $\tau_2 = 510 \pm 130$ ms, $n = 7$) and similarly for KST1ΔK$_{HA}$ ($\tau_1 = 55 \pm 24$ ms, $\tau_2 = 402 \pm 78$ ms, $n = 7$). Also, the deactivation kinetics of KST1 ($\tau = 129 \pm 34$ ms, $n = 5$) and KST1ΔK$_{HA}$ ($\tau = 128 \pm 40$ ms, $n = 6$) showed no significant difference (calculated for a pulse to −110 mV after pre-activation for 400 ms at −190 mV).

These data indicated that the K$_{HA}$-domain did not contribute to the basic function of KST1. Hence, formation of a functionally active K^+_{in} channel is not a function of the K$_{HA}$-domain.

4. Discussion

In this work, we identified two new putative K^+_{in} channel proteins (SKT2 and SKT3) in the yeast two-hybrid system by interaction with a C-terminal fragment of KST1. In contrast to AKT2 and AKT3, which turned out to be identical [7,8], SKT2 and SKT3 represent different proteins of high similarity. Further, our data indicate indiscriminate self- and cross-interactions between the C-termini of the four potato K^+_{in} channels KST1, SKT1, SKT2 and SKT3. To what extend these interactions can occur *in planta* clearly depends on the distribution of the respective channel proteins in different cell types and membranes. Several studies on the described K^+_{in} channels have indicated differential but overlapping expression patterns within the plant (cf. [31]) but their subcellular targeting is largely unaddressed. Evidence for location in plasma membranes is present only for AKT1 [11] and circumstancially (by direct in vitro/in vivo comparison of K^+ currents) for KST1 [6]. Thus, at this point only self-interaction of proteins from a single K^+_{in} channel species in the respective membrane appears relevant, which we showed here for KST1 in insect cells. This function correlates with the presence of a homology domain in the interacting C-termini which is unique to plant K^+_{in} channels and has been noticed before as a sequence homology region between KAT1 and AKT1 [10]. We presented first evidence that the K$_{HA}$-domain is essential for interaction of plant K^+_{in} channels by monitoring KST1 self-interaction in insect cells (Fig. 4). However, at this point, the involvement of other factors acting in *cis* or in *trans* to the K$_{HA}$-domain can not be excluded.

While tetramerization has been related to N-terminal parts of the related *Shaker*-type K^+ channels in animals [32], channel formation has not been studied for plant K^+_{in} channels. Here, we showed that self-interactions, which depended on the C-terminal K$_{HA}$-domain, did not participate in subunit assembly to functional K^+_{in} channels of potato KST1 expressed in insect cells.

The clustering of KST1 expressed in insect cells indicates a contribution of K^+_{in} channel interaction on localization or concentration of functional channels in the membranes. The necessity for localization which also involves clustering is generally accepted for neuronal ion channels (cf. [33]). *Shaker*-type K^+ channels of animals show co-clustering with the PDZ-domains of linker proteins, which is also mediated by C-terminal amino acids [34]. Our data for the first time suggest similar processes for plant ion channels, albeit mechanisms additional to self-interactions have to be postulated. A proper channel localization might for example be of importance for the low-affinity K^+ uptake to which AKT1 has been related [11]. Since channel-mediated K^+ uptake becomes thermodynamically unfavoured when the exterior K^+ concentration is < 0.3–1 mM [2,35,36] the number of properly localized K^+ channels (i.e. channels facing the soil) may have a substantial influence on K^+ uptake efficiency. Interestingly, the *Arabidopsis thaliana akt1-1* mutant has been presented [37], in which a T-DNA insertion deletes the entire K$_{HA}$-domain of AKT1. Growth of these mutants was reduced in comparison to the wild type on low concentrations of K^+. If (in analogy to our data with KST1) the inward rectification properties of AKT1-1 are not influenced, deletion of the K$_{HA}$-domain in the *akt1-1* mutant might cause a channel delocalization in the plasma membrane, thereby reducing the K^+ uptake ability. This mutant might become a valuable tool to study K^+ channel association.

Acknowledgements: We thank J. Ellenberg, E. Reichel and M. Matcan for technical assistance and K. Czempinski, M. Klein, U. Menke, G. Plesch and N. Weigmann for helpful comments on the manuscript.

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