Distinct fluorescent pattern of KAT1::GFP in the plasma membrane of Vicia faba guard cells

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Abstract

The organisation of membrane proteins into certain domains of the plasma membrane (PM) has been proposed to be important for signalling in yeast and animal cells. Here we describe the formation of a very distinct pattern of the K⁺ channel KAT1 fused to the green fluorescent protein (KAT1::GFP) when transiently expressed in guard cells of Vicia faba. Using confocal laser scanning microscopy we observed a radially striped pattern of KAT1::GFP fluorescence in the PM in about 70% of all transfected guard cells. This characteristic pattern was found to be cell type and protein specific and independent of the stomatal aperture and the cytoskeleton. Staining of the cell wall of guard cells with Calcofluor White revealed a great similarity between the arrangement of cellulose microfibrils and the KAT1::GFP pattern. Furthermore, the radial pattern of KAT1::GFP immediately disappeared when turgor pressure was strongly decreased by changing from hypotonic to hypertonic conditions. The pattern reappeared within 15 min upon reestablishment of high turgor pressure in hypotonic solution. Evaluation of the staining pattern by a mathematical algorithm further confirmed this reversible abolishment of the radial pattern during hypertonic treatment. We therefore conclude that the radial organisation of KAT1::GFP depends on the close contact between the PM and cell wall in turgid guard cells. These results offer the first indication for a role of the cell wall in the localisation of ion channels. We propose a model in which KAT1 is located in the cellulose fibrils intermediate areas of the PM and discuss the physiological role of this phenomenon.

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Introduction

Structural conditions which cause a heterogeneous distribution of membrane proteins are believed to be important factors for the regulation of numerous signalling and transport events, especially at the plasma membrane (PM). So far a number of different mechanisms that induce a heterogeneous distribution of
membrane proteins have been described mainly in mammalian cell lines and yeast.

These mechanisms include the lateral separation of specific membrane lipid species (i.e., mainly cholesterol and glycosphingolipids) which leads to the formation of specialised microdomains, so-called lipid rafts. Certain proteins accumulate in these microdomains (e.g., glycosyl phosphatidyl inositol (GPI)-anchored proteins) while others are not affected. This accumulation can be explained by a slowdown of protein mobility by a factor of ~2 (Dietrich et al., 2002) which results in a heterogeneous distribution of proteins in the PM. Many proteins involved in signalling cascades are found in lipid rafts (Brown and Rose, 1992). This provided the first hint on the physiological significance of microdomain formation. The slowdown and accumulation of proteins in lipid rafts is believed to increase the probability of dimer, multimer and cluster formation which is important for many signalling events at the PM. Nevertheless, the exact role of lipid rafts in cellular signalling, trafficking, and structure has yet to be determined.

Lipid rafts and raft-associated proteins have also been identified in plants (Borner et al., 2003; Mongrand et al., 2004). However, like for lipid raft formations in mammalian and yeast cells, the physiological meaning of these microdomains remains to be confirmed.

A second factor that was found to limit free diffusion of membrane proteins is the cytoskeleton. It can determine the localisation of PM proteins via direct attachment to the protein or indirectly. A direct connection to the cytoskeleton is for example of particular importance for the localisation and functioning of the cellulose synthase complex in the PM of plant cells (Gardiner et al., 2003). In the case of certain mechanosensitive ion channels the attachment to the cytoskeleton is also proposed to function as a signalling component in mammalian cells (Barratt and Rychkov, 2005; Ghazi et al., 1998). Apart from the direct connection to PM proteins it is believed that the actin cytoskeleton can confine the movement of proteins with enlarged cytosolic domains by generating “fenced” microenvironments without the direct attachment to the diffusing components (Ritchie and Kusumi, 2004). While such a mechanism has so far not been described in plant cells, a cortical cytoskeleton—the only determinant of this effect—is also present in plant cells (Staiger and Lloyd, 1991).

The third factor that can contribute to heterogeneous distribution of PM proteins is the extracellular matrix (ECM). It is able to directly influence the distribution of proteins in the PM of eukaryotic cells (Arnold et al., 2004). For plant cell walls, which can be viewed as the plant ECM, a role in distribution of PM proteins has not yet been described.

PM ion channels which play an important role in signal transduction have been implicated to be distributed non-homogenously in the PM of plant and animal cells (Tester, 1990; Deutsch, 2002). In animal cells lipid rafts have been described as an important factor for the localisation of ion channels in certain domains in the PM (Martens et al., 2004). For plant ion channel mechanisms which determine their localisation in microdomains have not been identified.

Recently Sutter et al. (2006) demonstrated that the K⁺ inward rectifier KAT1 from Arabidopsis thaliana is localised in clusters in the PM when transiently expressed in tobacco epidermal cells. The authors detected KAT1 protein in a ‘moderately’ detergent-resistant fraction, indicating its association with lipid rafts. The KAT1 cluster showed nearly no lateral mobility. Investigations of the role of SNAREs (soluble NSF [N-ethylmaleimide-sensitive factor] attachment protein receptors) on trafficking of KAT1 indicated that SNAREs are involved in cluster formation and mobility of KAT1 (Sutter et al., 2006). However, the mechanism which anchors KAT1 in the PM remains to be determined.

KAT1 plays an important role in guard cell functioning. We therefore analysed turgid guard cells transiently expressing KAT1 fused to green fluorescent protein (GFP). KAT1::GFP was organised in clusters in the PM similar to what we previously described for guard cell protoplasts (Hurst et al., 2004). In addition we found a radial distribution of KAT1::GFP clusters which was dependent on a close contact between the PM and the cell wall. In animal cells contacts of the PM with the ECM are mediated by substrate adhesion molecules such as fibronectin, vitronectin, collagen, and others, via the short amino acid sequence Arg-Gly-Asp (RGD) (D’Souza et al., 1991) that interacts with integrins. The integrins in turn link the ECM to the cytoskeleton (Ruoslahti, 1996). For plant cell walls plant biologists are just beginning to understand how cell wall-to-membrane interactions are established to acquire cell- and tissue-specific characters and how this affects cell function and polarity and cell-to-cell interactions. So far, in plants only few homologues of classical adhesion molecules, e.g. β-integrin or fibronectin that revealed a RGD-mediated membrane matrix adhesion have been identified (Canut et al., 1998; Faik et al., 1998; Gens et al., 1996; Pellenc et al., 2004). This points to a similar interaction between the PM and the ECM or cell wall of animal and plant cells, respectively. In addition a number of plant PM proteins have been proposed to directly bind to both the PM and extracellular carbohydrates and may thus anchor the cell to the cell wall (for review see Kohorn, 2000)). Among these is the cellulose synthase complex (Kohorn, 2000), which also requires cortical microtubule arrays for normal localisation in the PM (Gardiner et al., 2003). This indicates that a continuous cytoplasm–cell wall scaffold is essential to control key events in plant development and growth.
Our data clearly demonstrate the crucial role of the guard cell wall in the pattern formation of KAT1. We propose a model in which KAT1 clusters are located in the intermediate areas of the cellulose microfibrils and discuss the physiological role of such a localisation.

Materials and methods

Construction/design of the KAT1::GFP fusion protein

The \textit{kat1} cDNA was amplified by PCR for directed cloning into \textit{pAVA393} (NcoI restriction site) in frame with the mGFP\textsubscript{5} (Haseloff et al., 1997) for expression of the fusion protein KAT1::GFP under the control of two \textit{P\textsubscript{35S}} promoters, as described in detail by Hurst et al. (2004). The plasmid was then cloned in \textit{Escherichia coli}/DH5\textsubscript{a}, followed by preparation of plasmid DNA (Qiagen high speed Midi-Kit, Qiagen, Germany). The purified vector was used for ballistic bombardment of guard cells and epidermal cells as described by Hurst et al. (2004).

The construct GFP::TM23 was kindly provided by Nadine Paris (University of Rouen, France). It represents the transmembrane domain of human LAMP1 (lysosome-associated membrane protein-1) fused to the green fluorescent protein in the vector pSGFP6K (Brandizzi et al., 2002).

The \textit{P-ATPase} PMA\textsubscript{4} from \textit{Nicotiana plumbaginifolia} fused to GFP in the vector pTZ19U-gfp (Lefebvre et al., 2004) was a kind gift from M. Boutry, Université Catholique de Louvain, Belgium. The talin::YFP construct (Kost et al., 1998) was kindly provided by B. Kost (University of Heidelberg, Germany), and the plasmid with TOR1::GFP (Buschmann et al., 2004) by A. R. Schäffner (GSF Research Center, Germany), respectively. All fusion constructs were expressed under \textit{P\textsubscript{35S}}.

Transfection of guard cells

Guard cells were transfected via ballistic bombardment as described by Hurst et al. (2004). Briefly, whole leaves of \textit{Vicia faba} L. cv. Bunyan were placed upside down on solid Murashige Skoog Medium and bombarded with 2 mg gold (1 \,\mu m particle diameter) coated with 10 \,\mu g DNA according to the manufacturer’s instructions (BioRad, Munich, Germany), at a pressure of 650 Psi, a distance of 6 cm, and a vacuum of 25 in Hg.

Confocal microscopy

Confocal microscopic analysis of transfected guard cells was carried out 16 to 24 h after ballistic bombardment as described by Meckel et al. (2004). Briefly, abaxial epidermal peels from bombarded leaves were placed in small dishes in a standard buffer solution consisting of 10 mM MES-KOH (pH 6.1), 45 mM KCl, and 100 \mu M CaCl\textsubscript{2}. Analysis was carried out using a confocal laser scanning microscope (Leica TCS SP, Leica Microsystems GmbH, Heidelberg, Germany), equipped with a 63x water immersion objective (plan apo, N.A. 1.2). For excitation of mGFP\textsubscript{5}, the 488-nm line of a 25-mW Ar/Kr-Ion-Laser was used; emission was detected at 505–535 nm. The confocal aperture was adjusted to give optical sections with a full-width at half-maximum of around 0.68 \,\mu m. Images were processed using the Leica Confocal Software 2.00 (LCS, Leica Microsystems GmbH, Heidelberg, Germany).

Analysis of guard cells stained with Calcofluor White

For staining of cellulose fibrils Calcofluor White (Sigma, Munich, Germany) was added to epidermal peels bathed in standard buffer to a final concentration of 0.1% (w/v). Epidermal peels were incubated in Calcofluor White for 5 min and washed 5 times with standard buffer. Epidermal peels were analysed using the confocal laser scanning microscope Leica TCS SP2 AOBS (Leica Microsystems GmbH, Heidelberg, Germany) with a 63x water immersion objective. For excitation the 405-nm line of a 50-mW Ar-UV-Laser was used, emission was detected at 415–440 nm.

Cytoskeleton inhibitors

Latrunculin B (Calbiochem, Darmstadt, Germany) and propyzamide (Sigma, Munich, Germany) were prepared in dimethyl sulphoxide (DMSO) as 25 and 50 mM stocks, respectively. The toxins were diluted with standard buffer solution to a final concentration of 10 \mu M latrunculin B and 50 \mu M propyzamide. The final concentration of DMSO was 0.04% (v/v) and 0.05% (v/v), respectively.

Hyper- and hypotonic treatment of guard cells

Epidermal peels were placed in standard buffer (see above) which was hypotonic (100 mosmol/kg), ensuring that cells were fully turgescent. For hypertonic treatment of cells the standard buffer plus sorbitol (1000 mosmol/kg) was added until plasmolysis was visible just at the tips of guard cells. The final osmolarity of the bath solution was between 500 and 800 mosmol/kg. To restore turgor pressure hypotonic conditions were induced by replacing the bath solution with standard buffer.
Determination of radial distribution versus random distribution via mathematical methods

The algorithmic challenge in quantifying the degree of radial organisation in these fluorescence distribution patterns is due to three features of the data: (i) inspection by eye shows that the degree of radial organisation varies gradually between the image sets, (ii) noise content (i.e. randomly excited pixels in the fluorescence images) varies strongly within the data and (iii) the image sizes are too small for an informative spectral analysis. These circumstances are calling for a quantification of the pattern to attach a reproducible value to each image which is independent of the observer. Our method exploits the fact that the radially organised patterns and the random spots transform differently under an Ising algorithm (i.e. a well-defined domain-forming dynamical process). For all analysis steps we use a binarised fluorescence image, obtained by substituting the value of each image point by 1, if it exceeds a certain threshold and 0 otherwise. In all cases we have chosen the average fluorescence (without background) as binarisation threshold. The Ising algorithm is an early thermodynamic model of ferromagnetism. In the original model of a ferromagnet the implementation of this algorithm leads to domain formation. Here it emphasises existing radial patterns. The resulting transformed image is then evaluated using a standard method from information theory, namely the mutual information. This process assigns a number (the information content of the image, as given by the mutual information) to this transformed image. The value is related to the radial organisation of the pattern, due to the different transformation properties of radial stripes and random dots. While this procedure is sufficient to assess the organisational features of a pattern at fixed noise intensity of the image, the varying noise content of the data requires an additional analysis step. We estimate the noise content of each (binarised) image with the help of a melting algorithm. The application of this algorithm gives the percentage of image points which are more likely to be noise rather than signal. Consequently, application of our algorithms to a given fluorescence image yields two numbers, namely the noise content of the original (binarised) image and the information content of the Ising-transformed image as a measure of stripe formation.

We calibrated the full algorithm on simulated data where the stripe-to-dot ratio and the noise content have been varied systematically. This led to the reference curves shown in Fig. 7G. A more detailed description and application of the Ising-algorithm in this context is given by Hütt (2001).

Results

Radial distribution of KAT1::GFP in the PM of guard cells

Figs. 1A–C show intact turgid guard cells of V. faba transfected with the K⁺ channel KAT1 fused to GFP (KAT1::GFP). The expression of KAT1::GFP resulted in a distinct staining pattern of the PM which in many cells appeared as stripes originating from the dorsal side of the guard cells and radially centred towards their ventral side (Fig. 1A). For further analysis we categorised the stripe formation as follows: guard cells showing a clear radial pattern over the whole PM were classified as type 3 (Fig. 1A); guard cells with less prominent stripe formation only visible in part of the PM or displaying shorter stripes not forming a continuous line from the dorsal to ventral side were classified as type 2 (Fig. 1B); guard cells without any clear stripe formation with KAT1::GFP appearing in large randomly distributed dots in the PM were classified as type 1 (Fig. 1C). According to this classification 36% out of the 157 guard cells analysed were classified as type 3 and 32% each as type 2 and type 1. In addition to staining of the PM, guard cells often exhibited labelling of intracellular compartments (mainly ER and nuclear envelope). This most likely resulted from protein expression under the strong 35S promoter which can lead to accumulation of the fusion protein in the ER (Hawes et al., 2001).

![Fig. 1. Distribution of KAT1::GFP in guard cells and epidermal cells. Maximum projection of cells expressing KAT1::GFP (green). (A) Guard cell of stomata displaying a clear radial staining pattern (type 3). (B) Guard cell of stomata displaying radial staining pattern only in part of the cell (type 2). (C) Guard cell displaying random staining pattern (type 1). (D) Epidermal cell displaying random staining pattern. Scale bars: 10 μm.](image-url)
Distribution pattern of KAT1::GFP is cell type and protein specific

In addition to guard cells we also analysed the distribution of KAT1::GFP fluorescence in transfected epidermal cells. Epidermal cells expressing the KAT1::GFP fusion protein displayed a strikingly different staining pattern compared to guard cells. The stripe formation typical of guard cells was never observed in epidermal cells ($n > 50$). All epidermal cells displayed random distribution of KAT1::GFP fluorescence in large dots in the PM (Fig. 1D). This implicates that the radial distribution of KAT1::GFP is cell type specific. To examine whether PM proteins in guard cells are generally distributed in a radial pattern we analysed the expression of two different PM proteins fused to GFP. These proteins included the 23 amino acid long single-pass membrane domain from human LAMP1 (lysosome-associated membrane protein-1; GFP::TM23; Brandizzi et al., 2002) and the PM ATPase from Nicotiana plumbaginifolia (PMA4::GFP; Lefebvre et al., 2004). Fig. 2 shows typical expression patterns from guard cells transfected with these fusion constructs. Both fusion proteins tested displayed an even distribution of the fluorescence in the PM without any formation of radial stripes.

Together these results implicate that the radial distribution of KAT1::GFP in the PM of transfected guard cells is cell type and protein specific.

Distribution pattern of KAT1::GFP in guard cells is independent of stomatal aperture and cytoskeleton inhibitors

Investigations of the cytoskeleton in guard cells of open stomata revealed that both actin filaments and microtubules are radially distributed (Fukuda et al., 1998; Kim et al., 1995). In closed stomata the radial distribution pattern disappears because actin filaments and microtubules depolymerise. The distribution pattern of KAT1::GFP in transfected guard cells is at first glance similar to the distribution of actin filaments and microtubules in open guard cells indicating that the cytoskeleton may be involved in the distribution of KAT1::GFP. This would implicate that the radial staining pattern can only be found in guard cells of open stomata.

However, analysis of the relationship between stomatal aperture and stripe formation revealed no correlation between the type of KAT1::GFP pattern and opening of the stomatal pore (Fig. 3). Fig. 3 shows the distribution of the stomatal aperture from type 1, type 2 and type 3 cells. Stomatal aperture was determined by measuring the inner width of the stomatal pore from maximum projections of 157 guard cells. All distributions were fitted with a Gaussian function displaying a peak value at 9.7, 9.8 and 10.4 µm for type 1, type 2 and type 3, respectively. Kolmogorov–Smirnov two-sample tests revealed no significant difference between the three distributions ($z = 0.05$). This suggests that the distribution pattern is unrelated to the stomatal aperture and thus independent of the cytoskeleton.

To further investigate the participation of the cytoskeleton in the formation of the radial distribution of KAT1::GFP, we applied latrunculin B.
(final concentration: 10 μM), an actin-depolymerising agent and propyzamide (final concentration: 50 μM) as a microtubule-destabilising drug. The effect of these inhibitors on the cytoskeleton was tested in guard cells transfected with talin::YFP (Kost et al., 1998) or TOR1::GFP (Buschmann et al., 2004) to visualise the distribution of the actin or microtubules, respectively. Incubation of guard cells in 10 μM latrunculin B for 20 min was sufficient to completely destroy the actin meshwork (Figs. 4A and B). The radial distribution of the microtubule marker significantly changed already after 10 min in 50 μM propyzamide with complete disappearance of smaller filamentous structures (Figs. 4C and D). This demonstrates that the arrangement of the cytoskeleton in guard cells is indeed destroyed by the inhibitors applied.

However, neither the application of latrunculin B nor the incubation in propyzamide had an appreciable effect on the radial distribution of KAT1::GFP. The striped pattern remained unchanged even after prolonged treatment for 70 and 140 min in propyzamide or latrunculin B, respectively (Figs. 5B and D). Together these results demonstrate that the cytoskeleton is not involved in maintenance of the radial distribution of KAT1::GFP.

Possible involvement of the cell wall in the radial distribution of KAT1::GFP

In guard cells not only actin filaments and microtubules but also cellulose fibrils are radially distributed (Raschke, 1979). Fig. 5 shows Calcofluor White staining of cellulose fibrils in the cell wall of guard cells and epidermal cells. Guard cells displayed a parallel and radial organisation of cellulose fibrils (Fig. 6A). In epidermal cells such a pattern could not be observed, and cellulose fibrils were found to be arranged in a disordered manner (Fig. 6B). The arrangement of the cellulose fibrils in guard cells shows the same radial orientation as KAT1::GFP stripes in the PM, indicating a causal relation between the two staining patterns.

Further indications for the participation of the cell wall in the radial distribution of KAT1::GFP can be derived from images of transfected guard cell protoplast. In contrast to turgid guard cells protoplasts lacked the radial fluorescence pattern in the PM (Hurst et al., 2004).

To further investigate the participation of the cell wall in the distribution of KAT1::GFP, we incubated epidermal peels in strong hypertonic solution to decrease turgor pressure of guard cells. Under this condition the PM is no longer tightly pressed against the cell wall. In order to avoid infolding of the PM we increased the osmolarity of the bath solution until the PM started to retract from the cell wall only at the cell tips. At this stage there was no visible detachment of the PM from the cell wall in the rest of the cell. The changes in staining pattern from three guard cells before and after hypertonic treatment are displayed in Figs. 7A–F.
Hypertonic treatment resulted in an immediate loss of
the radial pattern in the PM (Figs. 7B, D and F).

To quantitatively analyse the systematic features of
the distribution patterns in guard cells we have
developed an image analysis algorithm (see Materials
and methods). The radial organisation seems to exist in
gradually varying degrees, ranging from cases, where it
is a very prominent feature of the image, to cases, where
it is almost completely masked by other features and
hard to distinguish from randomly distributed fluores-
cent spots. This fact particularly emphasises the need for
a quantitative algorithmic look at these patterns. This
algorithm allows distinguishing cells with a radial
staining from cells with a more random distribution of
KAT1::GFP. Results from analysis of the cells shown in
Figs. 7A–F are represented in Fig. 7G (circles marked
with characters). The reference grid in Fig. 7G has been
obtained by analysing simulated data, where noise
content and the stripe-to-dot ratio have been varied
(see Materials and methods). Note that the length scales
(i.e. the “thickness” of typical stripes) determines the
absolute position of this grid. This additional depend-
once on other (but constant) features than stripe-to-
dot-ratio and noise has two important consequences for
our interpretation of the data: (1) for a given experi-
mental data set, individual reference grids have to be
simulated and (2) only the relative position in the plane
for images within a single experiment can be interpreted.
Thus, for the further examples given in Fig. 8 only the
relevant region of the plane is shown without an
additional reference grid.

The guard cells shown in Figs. 7A, C and E are well
above the reference line in this quantitative analysis and,
therefore, represent stripe formation whereas Figs. 7B,
D and F are clearly below this reference line and,
therefore, lack this feature of radial organisation. This
quantitative analysis confirms that loss of turgor is
associated with a decrease in the degree of stripe-like
distribution of KAT1::GFP.

**Fig. 6.** Radial distribution of cellulose fibrils in guard cells.
Confocal images of cells stained with Calcofluor White.
(A) Partial view from a maximum projection of four
consecutive stacks of a guard cell showing radial distribution
of cellulose fibrils. (B) Partial view from a guard cell (left) and
dermal cell (right) showing radial and random distribution
of cellulose fibrils, respectively. (C) Overview of the epidermis
utilizing Normarski optics. The selected area corresponds to
image (B). Scale bars: 10 µm.

**Fig. 7.** Loss of radial pattern of KAT1::GFP after hypertonic
treatment. (A), (C) and (E) Partial view from maximum
projections of four consecutive stacks of guard cells expressing
KAT1::GFP before hypertonic treatment. (B), (D) and (F)
Partial view from guard cells shown in (A), (C) and (E),
respectively, 3 to 8 min after hypertonic treatment. (G) Result
from mathematical analysis of images shown in (A)–(F);
reference grid has been obtained by analysing simulated data;
values above and below dashed reference line correspond to
stripe and random formation, respectively, of KAT1::GFP;
(1) and (2) indicate increasing noise and dottiness, respectively,
in reference data. Scale bar: 10 µm.
To test for the reversibility of the loss in stripe formation we replaced the hypertonic solution by the hypotonic standard bath solution. Figs. 8A–C show part of the cortical section of a KAT1::GFP-transfected guard cell before and after hypertonic treatment, and 15 min after replacing the hypertonic solution by the standard solution. The radial fluorescent pattern which vanished upon loss of turgor (Fig. 8B) started to recur when high turgor pressure was restored in standard solution (Fig. 8C). Quantitative analysis of the images confirmed that the decrease in radial organisation observed during hypertonic treatment is reversible (Fig. 8D). Together the results demonstrate that the formation of the radial fluorescence distribution in guard cells is associated with a close contact of the PM to the surrounding cell wall.

**Discussion**

In this study we heterologously expressed the K\(^+\) inward rectifier KAT1 from *Arabidopsis thaliana* fused to the green fluorescent protein (GFP) in *V. faba* guard cells, and observed a cell type- and protein-specific fluorescent pattern. In most guard cells KAT1::GFP was organised in distinct radial stripes in the PM. Our results lead to the hypothesis that this staining pattern is due to interaction of KAT1 with the cell wall of guard cells.

**Distinct KAT1::GFP pattern in the PM of guard cells does not refer to overexpression artefact**

Ever since GFP in fusion with functional proteins was applied in plant cell biology, artefacts resulting from overexpression have been a critical point to take into account while interpreting such data. This is especially true for the expression of constructs under a strong promoter like the 35S promoter used in our studies. At first sight the observed KAT1::GFP distribution may thus be explained by a non-physiological association of fusion proteins into clusters mediated by GFP. However, our results from two other GFP fusion proteins expressed in guard cells under the same strong promoter contradict this hypothesis. When fused to GFP neither the single-pass transmembrane domain TM23 from LAMP1 nor the functional protein PM-ATPase showed this striped fluorescent pattern in transfected guard cells. In addition KAT1::GFP was always found in an evenly dotted distribution in transfected epidermal cells. GFP fluorescence intensity per PM area did not differ with cell type or expressed fusion protein. Hence, varying protein amounts cannot account for the different expression patterns observed (for the quantification procedure see Meckel et al., 2007). We therefore conclude that the expression pattern of KAT1::GFP is cell type and protein specific. In addition, guard cell protoplasts transfected with KAT1::GFP never showed a striped pattern of KAT1::GFP. Taken together the results demonstrate that the striped orientation of KAT1::GFP is clearly not the result of an overexpression artefact, but rather points to a distinct mechanism that determines the distribution of KAT1 in the PM of turgid guard cells.

**Stomatal aperture and the cytoskeleton are not involved in pattern formation**

From animal cells, a PM-cytoskeleton-ECM continuum is proposed in which various membrane proteins are functionally included (Gumbiner, 1996). The role of the cytoskeleton in guard cell function is somewhat controversial. Marcus et al. (2001) demonstrated a diurnal cycle in microtubule formation in guard cells that was dependent on stomata aperture. Furthermore this cycle could be blocked by application of propyzamide, an inhibitor of microtubule formation (Marcus et al., 2001). In contrast, Assmann and Baskin (1998)
found no role for microtubules in guard cell functioning. Also actin filaments seem to have a role in guard cell function. Their distribution changes from a more radial pattern in open stomata to a less organised distribution in closed stomata (Hwang et al., 1997). Hwang et al. (1997) also propose that actin is involved in the regulation of K⁺ channels in guard cells. Despite the striking similarity of the KAT1::GFP distribution to the arrangement of the cytoskeleton (microfilaments and microtubules) observed in this study, we found no evidence for the involvement of the cytoskeleton in the distribution of KAT1. The staining pattern was not dependent on the stomatal aperture. Additionally neither the application of propyzamide as an inhibitor of microtubule formation nor latrunculin B as an inhibitor of microfilament formation could abolish the radial pattern of KAT1::GFP. The concentrations of latrunculin B and propyzamide used in our study were in the same range of what has been shown to destroy microfilaments in inner cortex cells of maize root apices (Baluska et al., 2004) and cortical microtubules in A. thaliana (Naoi and Hashimoto, 2004), respectively. Furthermore, sequence predictions from KAT1 do not reveal an ankyrin motif which would allow direct binding of actin to KAT1 (Nakamura et al., 1995). We therefore conclude that neither microtubules nor actin microfilaments are direct participants in the formation of the striped pattern of KAT1::GFP in the PM of guard cells.

**Pattern formation is related to a KAT1–cell wall interaction**

Our analysis of guard cells suggests that a tight contact between the PM and the cell wall is essential for the striped distribution of KAT1. When turgid guard cells were incubated in strong hypertonic solution resulting in a loss of turgor and consequently the PM being no longer pressed against the cell wall the radial fluorescent pattern disappeared immediately and dissolved into randomly distributed dots. Restoration of the turgor pressure by exchange of the hypertonic bath solution led to the reappearance of the radial pattern. Using a mathematical algorithm the pattern of fluorescence before and after loss of turgor could clearly be separated by “grade of their homogeneity” (with the fluorescence being more ordered before loss of turgor independent of noise and background). Together with the observation that KAT1::GFP-expressing guard cell protoplasts displayed no radial staining pattern (Fig. 1B; Hurst et al., 2004) this demonstrates that the formation of stripes depends on a close contact of KAT1 with the cell wall. The radial arrangement of KAT1 is very similar to the organisation of cellulose microfibrils in the guard cell wall. In epidermal cells cellulose fibrils displayed no parallel pattern but showed a rather disordered arrangement. The observed cell type-specific difference in the fluorescent pattern of KAT1::GFP in epidermal and guard cells (random versus radial distribution) may thus be the result from the disordered or radial arrangement of cellulose fibrils in the respective cell type. We therefore suggest that KAT1 is directly or indirectly associated with the cellulose fibrils in the cell wall. The stripes formed by KAT1::GFP show the same orientation as the cellulose fibrils but are much broader than single cellulose fibrils which have a diameter of only about 10 nm. This can be explained by an interaction of several parallel arranged cellulose fibrils with KAT1 or by the restriction of KAT1 to the wider gaps found between bundles of cellulose fibrils.

The observation that about 30% of transfected guard cells showed no stripe formation supports the hypothesis that KAT1 is not linked directly to the cellulose fibrils and that this link is not obligatory. A direct binding of KAT1 to the cellulose should have led to a radial distribution in all transfected guard cells because all fully developed guard cells show a radial distribution of the cellulose microfibrils. The KAT1–cell wall association is thus most likely mediated by additional protein(s) which are apparently not active in all cells.

In animal cell PM proteins, such as integrins, link intracellular proteins to the ECM. Recently, it has been shown that integrins are also physically and functionally connected to some classes of ion channels and that this association is of general importance for cell physiology (for review see (Arcangeli and Becchetti, 2006)). For the GIRK K⁺ channel the link between the channel and integrins most likely occurs via an RGD motif in the extracellular loop (McPhee et al., 1998). Other K⁺ channels do not contain an RGD motif. Some of these channels may interact with integrins via their N-terminal domain (Cherubini et al., 2005).

Even though only few homologues of classical adhesion molecules like integrins have been identified in plants, for a number of plant PM proteins links to the cell wall have been suggested on the basis of their molecular interaction with cell wall carbohydrates. Among these are the membrane intrinsic cellulose synthase complex and cell wall-associated kinases (WAK), and further arabino-galactan proteins (AGP), which are bound to the outer leaflet of the PM via a GPI anchor (Kohorn, 2000). Also, the formation of Hechtian strands during plasmolysis shows that attachment sites between the PM and the cell wall exist. These attachment sites cannot be restricted to plasmodesmata since guard cells that in general lack plasmodesmata also develop Hechtian strands (Oparka et al., 1996). However, the nature of these attachment sites is not clear. Results from Lang et al. (2004) demonstrated that attachment sites of Hechtian strands are lost during
digestion of cellulose which implicates that they are formed by a tight connection between PM proteins and cellulose. Likewise, digestion of cellulose diminished the spatial pattern of KAT1::GFP (Hurst et al., 2004). We suggest that some of the KAT1 channels may be contributing to these attachment sites. This is also supported by the observation that the proposed KAT1–cell wall association and the formation of Hechtian strands are both independent of the cytoskeleton (Lang-Pauluzzi, 2000; Lang-Pauluzzi and Gunning, 2000).

In animal cells there is evidence that the interaction between ion channels and integrins is accompanied by the formation of macromolecular complexes that are located in microdomains in the PM (Cherubini et al., 2005). Recently, Sutter et al. (2006) analysed the distribution of KAT1 transiently expressed in tobacco leaf tissue. In tobacco epidermal cells KAT1 was localised in small microdomains in the PM similar to what we observed in epidermal cells of Vicia faba. Using photoactivatable GFP Sutter et al. (2006) were able to show that KAT1 was largely immobile in the PM. They suggest that SNAREs which are known to be involved in vesicle fusion may also participate in microdomain formation and mobility of KAT1 in the PM of epidermal cells. Our results show that the radial distribution of KAT1 but not the microdomain formation was dependent on a close contact between the PM and the cell wall. This implies that microdomain formation and radial distribution of KAT1 are two separate processes. The protein(s) that mediate the association of KAT1 with cell wall components yet remain to be determined.

Possible physiological relevance of the distinct localisation of KAT1 in the PM

So far we can only speculate on the physiological importance of the protein- and cell type-specific distribution of KAT1 in the PM of guard cells. Rapid freezing methods on plant cells showed that the distance between the cell membrane and the cell wall is smaller than previously observed. More recent electron micrographs revealed that the PM is appressed against the cell wall, thus they are apparently in tight contact (Roberts, 1990). This is particularly true for guard cells as in this specialised cell type the turgor is extremely high (4–5 MPa; Franks et al., 2001; Raschke, 1979). Since the cell wall serves as an external K⁺ store the tight contact between the cell wall and the PM may affect the accessibility of K⁺ for ion channels in the PM. We therefore propose the following model for the physiological role of the observed K⁺ channel distribution in the PM (Fig. 9). In membrane areas where the PM is pressed tightly to the cellulose fibrils, the high resistance for K⁺ fluxes, whereas in PM areas that reach into the intermediate space of the cellulose fibrils, K⁺ can move freely and therefore resistance for ion flow is low. Hence in these areas the ion accessibility for K⁺ channels is sufficient for guard cell function. The K⁺ inward rectifier in guard cells KAT1 plays a key role in K⁺ uptake during stomatal movement. The radial distribution may reflect the arrangement of KAT1 in the intermediate areas of cellulose fibrils and the attachment to the fibrils would assure the stable localisation of the protein in the PM.

However, electrophysiological measurements on protoplasts show that the cell wall link is not essential for KAT1 function. Gutknecht et al. (1978) proposed a turgor sensor in the PM of guard cells. Considering the tight association of KAT1 with the cell wall found in our study, KAT1 may participate in turgor sensing. This underlines the physiological importance of an intimate interaction between PM and cell wall. The identification of the molecular nature of the link between KAT1 and the cell wall is therefore of great importance to further dissect stomatal functioning.

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