ER export of the plant K$^+$ channel KAT1:

Identification and functional analysis of acidic signal motifs

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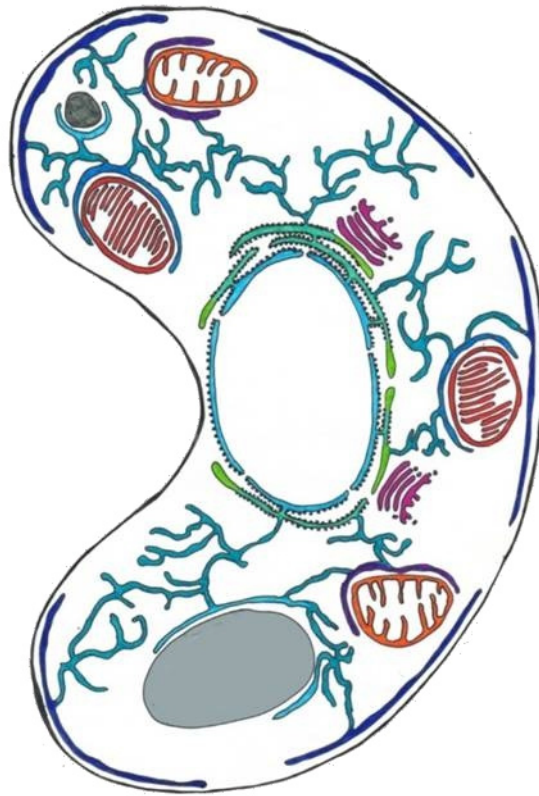
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Chapter 1   General Introduction

Drawing of a plant guard cell with the endoplasmic reticulum (ER) (blue), several Golgi stacks (magenta), nucleus (surrounded by the ER), chloroplasts (red) mitochondria (orange) and vacuole (grey). Different blue colours correspond to different parts of the ER to underline the compartmentalization of this organelle.

1.1 The secretory pathway

Every single cell is strongly compartmentalised. These compartments differ immensely in structure and in protein as well as lipid composition. To maintain the character of each organelle the cell has to regulate the transport of lipids and proteins.

A substantial proportion of all eukaryotic proteins and most integral membrane proteins travel through the secretory pathway to their intra- and extracellular destination.
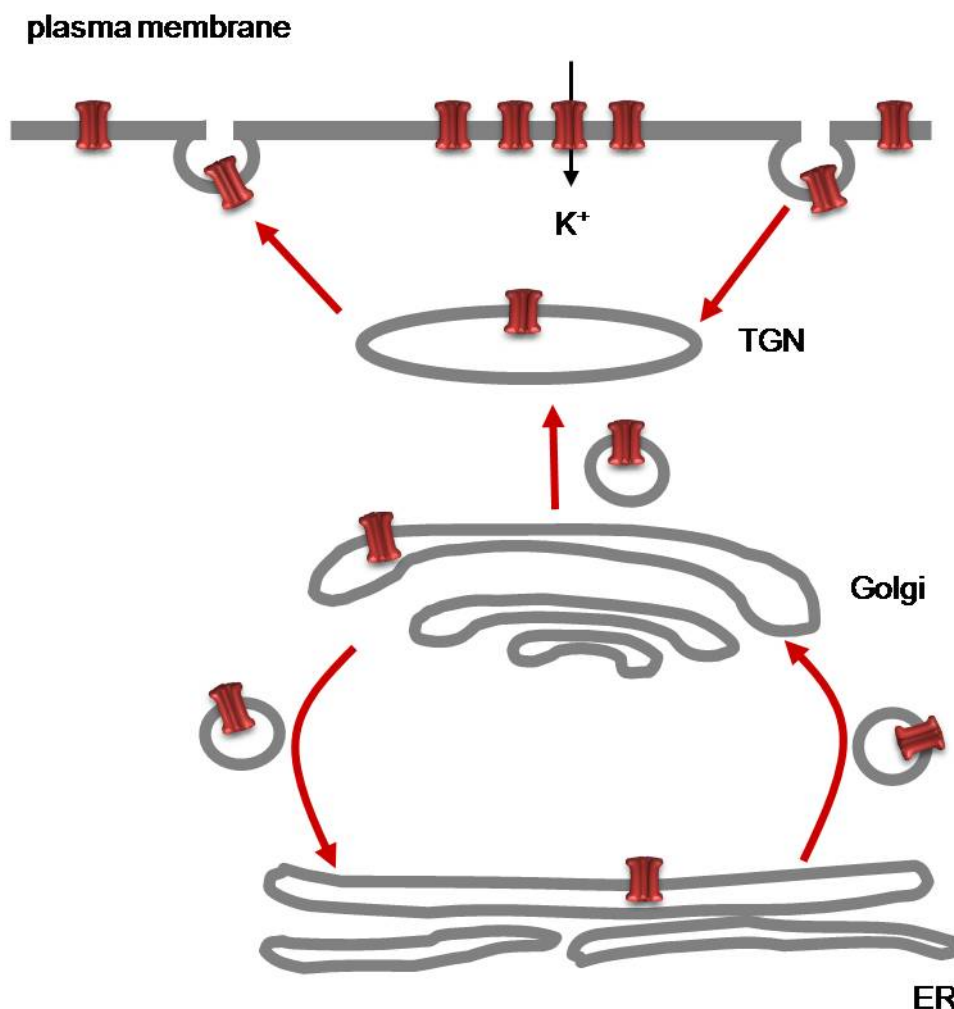
Figure 1.1: General model of the secretory pathway. Membrane proteins like the K$^+$ channel KAT1 are translated into the membrane of the endoplasmic reticulum (ER) and enriched in specialised ER export sites (ERES). Here the cargo proteins are sorted in COPII vesicles that are destined to fuse with the Golgi apparatus. From the trans-Golgi network (TGN) clathrin coated vesicles bud and fuse again with the plasma membrane and release their cargo.

The secretory pathway represents the main route for proteins to the plasma membrane (Figure 1.1). Membrane proteins are synthesised at ribosomes and cotranslationally inserted into the membrane of the endoplasmic reticulum (ER). There they are packed into coat protein II (COPII) coated vesicles that fuse with the cis-Golgi cisternae (Barlowe *et al*., 1994). After translocation through the Golgi apparatus and further transport to the trans-Golgi network the proteins are transported via clathrin coated vesicles to their final destination.

1.1.1 ER

The ER is the first compartment of the secretory pathway. It forms a large membrane network which comprises almost half of the total membrane of a cell. The ER can be divided into several interconnected subcompartments which differ substantially in their lipid and protein composition. These subcompartments have different function and morphology. The ER was subdivided by electron microscopy into the rough ER where ribosomes are attached to the ER membrane and the smooth ER which is ribosome free. Part of the smooth ER accounts for so called ER export sites (ERES) where vesicles bud off for further transport to the Golgi apparatus. These ERES are not found in *Saccharomyces cerevisiae*, suggesting that ER export can occur over the whole ER surface. In contrast *Pichia pastoris* has clearly defined subdomains of the ER responsible for ER export like plant and mammalian cells. A structure between the ER and the Golgi apparatus the ER Golgi intermediate compartment (ERGIC) is unique to higher eukaryotes and so far has not been found in plant cells (Kirk and Ward, 2007).

1.1.2 Golgi

Nearly all proteins which have been synthesised at the ER are transported to the Golgi apparatus. The primary function of the Golgi apparatus is to sort and process proteins and lipids that are synthesised at the ER. The Golgi apparatus is composed of different cisternae which have individual functions and a corresponding set of proteins and enzymes. The Golgi apparatus can be divided into cis-, medial- and trans-Golgi cisternae and the adjacent trans-Golgi network.

Proteins are transported from ERES towards the cis-Golgi cisternae and then they are subsequently transported through the Golgi apparatus to the trans-Golgi network from where proteins are again sorted into vesicles to their final destination.

There is a huge difference between the plant and human Golgi apparatus. Plant cells contain many individual Golgi stacks that are mobile organelles which travel along actin filaments. It is not well understood how COPII coated vesicles which form at the ERES reach the mobile Golgi stacks but it has been shown that the Golgi stacks in plants move along together with an individual ERES (daSilva *et al.*, 2004). In mammalian cells the ERES are distributed all over the ER but the single Golgi apparatus is found at the microtubule organising centre (MTOC) in the middle of the cell and distinct from the ERES.

1.2 ER export

Regulation of membrane protein transport at the level of the ER is under intense investigation but major aspects are still unknown. Mechanisms like concentration of cargo molecules at ERES and recruiting of participating factors are largely unidentified. Until now several specific amino acid sequences like ER export motifs have been shown to be involved in cargo concentration and recruiting of the COPII coat. However, for most of these motifs the mechanism of action is still unclear. As expected, all motifs are located in cytoplasmic domains of the proteins because they are supposed to interact with cytoplasmic receptor proteins. In contrast to membrane proteins which are supposed to interact directly with COPII components, soluble cargo proteins rely on transmembrane adapter proteins for regulated ER to Golgi apparatus transport. Therefore soluble cargo proteins contain specific cargo encoded sorting signals to interact with transmembrane receptors which in turn interact with the cytosolic components of the COPII coat.

1.2.1 ER export motifs

Only for few highly abundant soluble proteins has the transport out of the ER been shown to be non selective and to occur via bulk flow (Wieland *et al*., 1987; Martinez-Menarguez *et al*., 1999; Oprins *et al*., 2001). However, transport via bulk flow is very inefficient. For most proteins the transport is therefore regulated. Cargo proteins are actively sorted and enriched into COPII coated vesicles by means of specific export signals. Up to now, many ER export signals have been identified. They are frequently found in membrane proteins like ion channels, receptors and transporters. Among the ER export motifs are four major groups: diacidic, dihydrophobic, dibasic ER export motifs and a terminal valine (V) (Table 1.1).

Dihydrophobic signals have been identified in the ERGIC53 receptor that is necessary for secretion of the coagulation factors V and VII. But these signals are not sufficient to promote ER export of the protein. For efficient ER export, oligomerisation of the protein is needed as well (Baines and Zhang, 2007). A terminal V is very common in mammalian membrane proteins like the stem cell factor Kit1 (Pauhle *et al*., 2004).

The diacidic ER export motifs have been identified in several membrane proteins like ion channel proteins and transporters, most of them finally localised in the plasma membrane. Nishimura and Balch showed for the first time in 1997 that diacidic ER export motifs are required for efficient ER export of the vesicular stomatitis virus glycoprotein (VSVG). Ion channels like $K_{ir}2.1$ and TASK3 (Ma *et al*., 2001; Zuzarte *et al*., 2007) have been found to contain ER export motifs for regulated ER export. The yeast SNAREs Sys1 and Gap1 also use diacidic ER export motifs in their cytoplasmic domains for efficient trafficking out of the ER (Aridor *et al*., 2001; Votsmeier and Gallwitz, 2001). The function of diacidic ER export motifs in plant membrane proteins was demonstrated for the Golgi apparatus localised proteins CASP and GONST1, the aquaporins ZmPIP2,4 and ZmPIP2,5 and for the K^+ channel KAT1 (Hanton *et al*., 2005; Zelazny *et al*., 2008; Mikosch *et al*., 2006). For some of these motifs a direct interaction with the COPII coat has been shown.

Table 1.1: Proteins with different identified ER export motifs

Protein	Origin	Motif	
Diacidic (D/ExD/E)			
CFTR	Human	DxD	Wang *et al*. (2004)
K_{ir} 1.1	Human	ExD	Ma *et al*. (2001)
K_{ir} 2.1	Human	ExE	Ma *et al*. (2001)
K_{ir} 2.4	Human	ExE	Hofherr *et al*. (2005)
K_{ir} 3.2a	Human	DxE	Ma *et al*. (2002)
K_{ir} 3.4	Human	DxE	Ma *et al*. (2002)
TASK 3	Human	ExE	Zuzarte *et al*. (2007)
KAT1	Plant	DxE	Mikosch *et al*. (2006)
GAP1	Yeast	DxD	Malkus *et al*. (2002)
Sys1	Yeast	DxE	Votsmeier and Gallwitz (2001)
Can1	Yeast	DxD	Malkus *et al*. (2002)
Hip1	Yeast	DxD	Miller *et al*. (2003)
Yor1	Yeast	DxE	Pagant *et al* (2007)
GONST1	Plant	DxE	Hanton *et al*. (2005)
VSVG	Virus	DxE	Nishimura and Balch (1997)
CASP	Plant	DxE	Hanton *et al*. (2005)
ZmPIP2;4	Plant	DxE	Zelazny *et al*. (2008)
ZmPIP2;5	Plant	DxE	Zelazny *et al*. (2008)
Dihydrophobic			
ERGIC53	Human	FF	Nufer *et al*. (2003)
P24 family	Human	FF	Dominguez *et al*. (1998)
Erv41p	Human	FF	Otte and Barlowe (2002)
Erv46p	Human	FF	Otte and Barlowe (2002)
Dibasic (R/KxR/K)			
GAT1	Human	RL	Farhan *et al*. (2007)
SERT	Human	RI	Farhan *et al*. (2007)
DAT	Human	KL	Farhan *et al*. (2007)
GnTI	Plant	R	Schoberer *et al*. (2008)
GMII	Plant	K	Schoberer *et al*. (2008)
Terminal V			
Kit1	Human	V	Pauhle *et al*. (2004)
HLA-F	Human	V	Boyle *et al*. (2006)

1.2.2 Interaction with COPII

The COPII coat is responsible for the first step of the anterograde transport pathway, the transport of proteins from the ER to the Golgi apparatus. The coat is composed of five proteins that comprise three subunits: Sar1, Sec23/Sec24 and Sec13/Sec31. Assembly of the COPII coat is initiated by the small GTPase Sar1. The activation of the Sar1 protein occurs by a GDP to GTP exchange which is mediated by the integral ER membrane protein Sec12, a Sar1-GEF. Sec12 is ER membrane bound and restricts the Sar1 activation and consequential COPII assembly to the ER. Activated Sar1-GTP becomes membrane bound and recruits the first heterodimer of the COPII coat Sec23/Sec24. The Sar1-Sec23/Sec24 complex is called pre-budding complex and is sufficient to sort several cargo proteins into nascent COPII vesicles. The pre-budding complex recruits the Sec13/Sec31 heterodimer which provides the outer layer of the coat (Figure 1.2). The complete COPII coat forces the membrane curvature which leads to vesicle budding from the ERES. Final budding of the vesicle requires additional energy in the form of ATP and several additional proteins.
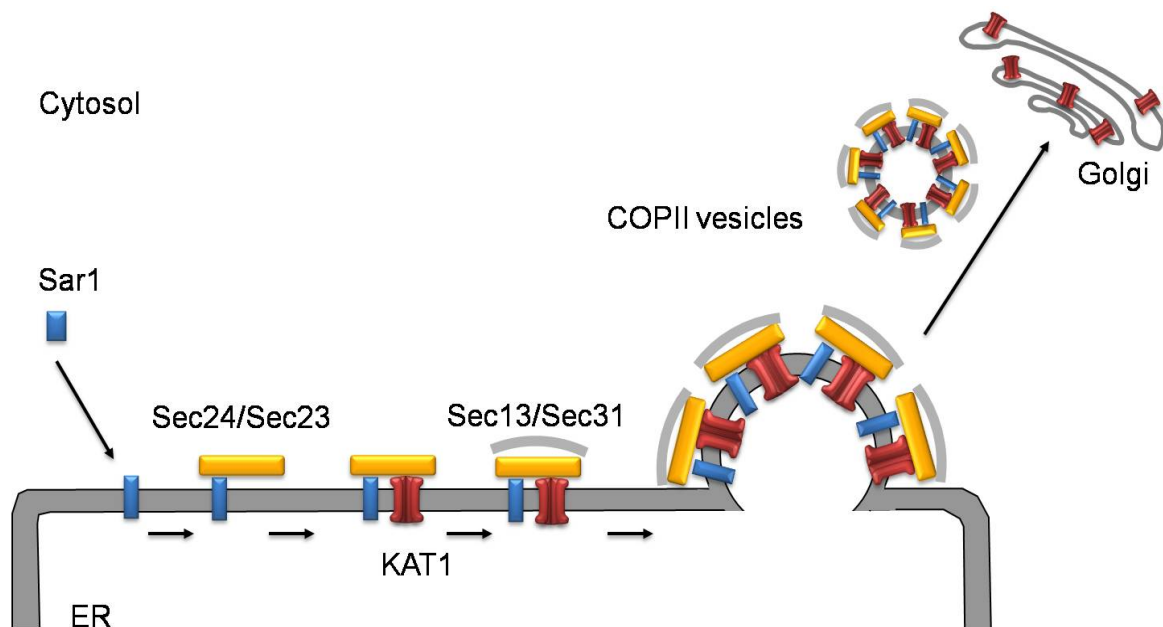
Figure 1.2: Formation of the COPII coat at ER export sites (ERES). Upon activation the small GTP binding protein Sar1 is inserted into the membrane of the ER and recruits the Sec23/Sec24 heterodimer. Sec24 selectively binds cargo proteins like the K$^+$ channel KAT1 and concentrates them into COPII vesicles. After binding of the heterodimer Sec13/Sec31 the COPII coated vesicle can bud of from the ER towards the Golgi apparatus.

COPII vesicle formation occurs in specialised regions –the ERES- that are enriched in COPII components and cargo molecules. These structures can be marked with the Sec16 protein (Watson *et al.*, 2006; Bhattacharyya and Glick, 2007). It has been shown that ERES are very dynamic cargo sensitive structures whose formation is induced by cargo molecules.

ERES have been shown to exist in several eukaryotic organisms like mammalia, yeast and plant and certain proteins have been identified as markers for ERES. Cargo selection at ERES occurs through COPII coated vesicles. Several cargo proteins are concentrated in COPII coated vesicles with the aid of ER export motifs. For many ER export motifs a direct interaction with the COPII coat has been shown. Most of them interact with the heterodimer Sec23/Sec24 and in many cases Sec24 was identified as the receptor. A possible role for the small GTPase Sar1 in cargo selection has also been suggested (Aridor *et al.*, 2001; Giraudo and Maccioni, 2003).

Table 1.2: Isoforms of Sec24 in different species (modified after Baines and Zhang, 2007).

Saccharomyces cerevisiae	Sec24, LST1 and ISS1
Caenorhabditis elegans	Sec24.1 and Sec24.2
Drosophila melanogaster	Sec24 and CG10882
Homo sapiens	Sec24a, Sec24b, Sec24c, Sec24d
Arabidopsis thaliana	At3g07100, At3g44340 and At4g32640

Investigations into the mechanism of recognition of the ER export motif by Sec24 demonstrated that Sec24 harbours at least three cargo binding sites (Miller *et al.*, 2003; Mossessova *et al.*, 2003) and interacts with several cargo proteins (Barlowe, 2003).

The B-Site has been shown to interact with diacidic motifs of yeast SNARE Sys1 and mammalian CFTR channel (Mossessova *et al.*, 2003; Wang *et al.*, 2004). Furthermore, mutation of the B-Site disrupts the binding to the ER export motif of the yeast SNARE Bet1 (Mossessova *et al.*, 2003). Wang *et al.* (2004) proved the binding of CFTR to Sec24 *in vitro* by homology modelling.

Another element of specificity in cargo recognition is provided by the existence of homologues of Sec24. In the genome of *Homo sapiens* four homologous proteins have been identified, three homologous proteins in *Saccharomyces cerevisiae* and

three in *Arabidopsis thaliana* (Table 1.2). Diversity of the COPII coat could be further increased by alternative splicing. These set of COPII coat components ensures a wide range of cargo recognition specific for different tissues and cell types.

1.3 KAT1

K^+ channels are very important in cell biology. They belong to a huge mulitigene family. One subfamily is formed by the inward rectifying K_{ir} K^+ channels which form a tetramer and were originally identified in animal cells. Each subunit of the K_{ir} channel tetramer has two transmembrane domains with the pore domain in between the two transmembrane domains. The pore domains of four subunits in one tetramer provide together the selective ion pore of the channel (Laine *et al*., 2004). Another subfamily consists of voltage gated K^+ channels (K_v channels). These channel are made up of four subunits each consisting of six transmembrane domains. Transmembrane domain four comprises many positively charged amino acids and provides the voltage sensor. The pore loop is situated between transmembrane domains five and six and contains the characteristic K^+ channel signature sequence TXXTXGYG (Heginbotham *et al*., 1994).

K^+ channels are particularly important in plant cell biology, since K^+ is essential for plant development, cell growth, photosynthesis and gas exchange. K^+ channels allow rapid flux of K^+ ions across the plasma membrane of guard cells and mediate stomata movement. These ion fluctuations enable guard cells to regulate the stomata aperture osmotically by water influx and efflux. The K^+ channels mediating K^+ flux in guard cells all belong to the K_v type K^+ channels. In *Arabidopsis thaliana* just a single gene, GORK, encodes for a K^+ outward conducting channel (Hosy *et al*., 2003) whereas for inward rectifying channels at least five subunits have been identified (KAT1, KAT2, AKT1, AKT2/3 and AtKC1). One prominent member is KAT1 which was first cloned by Schachtman *et al*. in 1992 and became an accepted model channel in plant cell biology. As a member of the K_v channel family KAT1 function as a tetramer with each subunit consisting of six transmembrane domains (Figure 1.3). KAT1 has a 297 amino acids long cytosolic C-terminus with a putative cyclic nucleotide binding domain (cNBD) at

amino acid position 377 to 494. This cNBD domain is a common feature of the cNBD channels like the hyperpolarisation activated, cyclic nucleotide modulated channel (HCN) and ether a go-go (EAG) channels. Members of this channels family show a very high similarity in the cytosolic C-terminus (Latorre *et al*., 2003; Cherel, 2004).

Several studies illustrated that the cNBD is important for the trafficking of the membrane protein to the plasma membrane. For HCN channels, Nazzari *et al*. (2008) demonstrated that a EEYP motif in the B-helix of the cNBD motif is responsible for the ER export of HCN2. The CFTR channel contains a diacidic ER export motif within the nucleotide binding domain (NBD) (Wang *et al*., 2004). The H$^+$ ATPase from *Nicotiana plumbaginifolia* shows blocked ER export after deletion of the complete cNBD (Lefebvre *et al*., 2004). Deletion studies from Marten and Hoshi (1997) indicated that the cNBD region is also critical for the plasma membrane expression of KAT1.
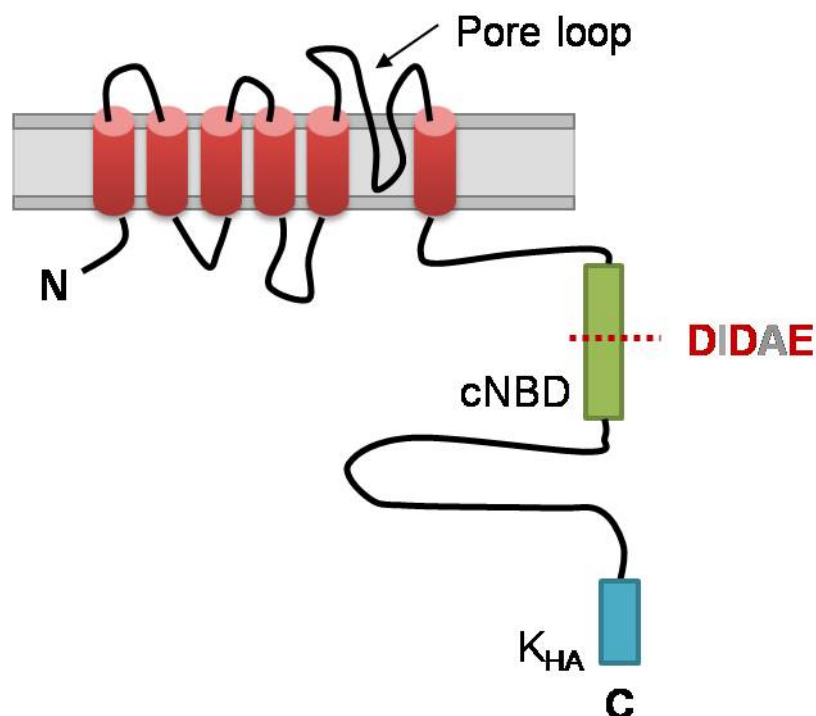
Figure 1.3: Topology of one monomer of the K$^+$ channel KAT1. Each subunit of the tetramer contains six helical transmembrane domains and a cytosolic N- and C-terminus. The pore loop is located between the transmembrane domains five and six. Transmembrane domain four contains several positively charged amino acids and provides the voltage sensor of the channel. The long C-terminus contains a putative cyclic nucleotide binding site (cNBD). The acidic ER export motif DIDAE is marked.

An interaction domain (K_{HA}) which is possibly involved in channel clustering in the plasma membrane (Ehrhardt *et al*., 1997), resides in the final part of the C-terminus.

The exact region important for the tetramerization of the KAT1 K^+ channel is still unknown. The shaker channel has a tetramerization domain (T1) in the N-terminus (Kreusch *et al*., 1998; Varshney *et al*., 2004; Robinson and Deutsch, 2005). But many other channels including the cNBD family of ion channels do not possess this domain and the tetramerization ability is reflected in parts of the C-terminus (Tsuruda *et al*., 2006). Although the transmembrane regions of shaker-type channels have a high similarity to KAT1, their N- and C-terminal parts are quite different. This region of KAT1 is much more related to the cNBD channels. The C-terminus of the cNBD channel HCN2 reveals a high sequence similarity to the KAT1 C-terminus and crystallisation studies by Zagotta *et al*. (2003) showed a tetramerization domain in this part of the protein. Another indication for a C-terminal tetramerization domain of KAT1 is the ability of a very closely related plant channel, AKT1, to build tetramers with the C-terminus of the protein only (Daram *et al*., 1997).

KAT1 also contains several putative phosphorylation residues. Yang *et al*. (2008) showed that phosphorylation of the $K_v1.2$ channel regulates the transport of the channels together with other channels in the heterotetramer. This may be due to the interaction of phosphorylated residues with 14-3-3 proteins. Recently, Sottocornola *et al*. (2008) discovered that the KAT1 also interacts with 14-3-3 and that this interaction influences ion channel trafficking. Although it is not known at which step of the secretory pathway this interaction occurs.

Altogether KAT1 is one of the best characterised plant ion channels. Therefore, it provides an excellent model to study ion channel trafficking in plants.

1.4 Aim of the work

The aim of this study was to identify factors regulating the trafficking of membrane proteins to the plasma membrane. It is widely accepted that the number of membrane proteins in the plasma membrane - like ion channels, transporters and receptors - is highly regulated by the rate of endocytosis, recycling and degradation. Surprisingly, up to now, the role of the secretory pathway in this process is not fully understood. In order to analyse regulatory steps along the secretory pathway, the K^+ inward rectifying channel KAT1 from *Arabidopsis thaliana* was chosen as a model membrane protein. Ion channels are particularly suitable to study regulation of trafficking because their number in the plasma membrane has to be tightly controlled.

The first trafficking step after cotranslational insertion of membrane proteins into the ER membrane is the transport of proteins from the ER to the Golgi apparatus. This step was chosen as the main focus of the current work.

For the efficient ER export of proteins, their recruitment into COPII vesicles is essential. This process involves specific amino acid motifs - the so called ER export signals. For ion channels in yeast and mammalian cells it was shown that so called diacidic ER export motifs regulate their trafficking at the level of ER export. As ER export motifs had so far not been identified for plant proteins the sequence of KAT1 was analysed for specific trafficking determinants. Their role in ER export was analysed via patch clamp measurements and confocal fluorescent microscopy.

Yeast membrane proteins have been shown to be incorporated into COPII vesicles by specific binding to the COPII component Sec24. In order to investigate the mechanism of ER export of KAT1, the interaction of KAT1 with Sec24 and the role of the ER export motif in this process were studied. Further analysis concentrated on the sequence specificity of the acidic motif.

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Chapter 2 DIACIDIC MOTIF IS REQUIRED FOR EFFICIENT TRANSPORT OF THE K$^+$ CHANNEL KAT1 TO THE PLASMA MEMBRANE
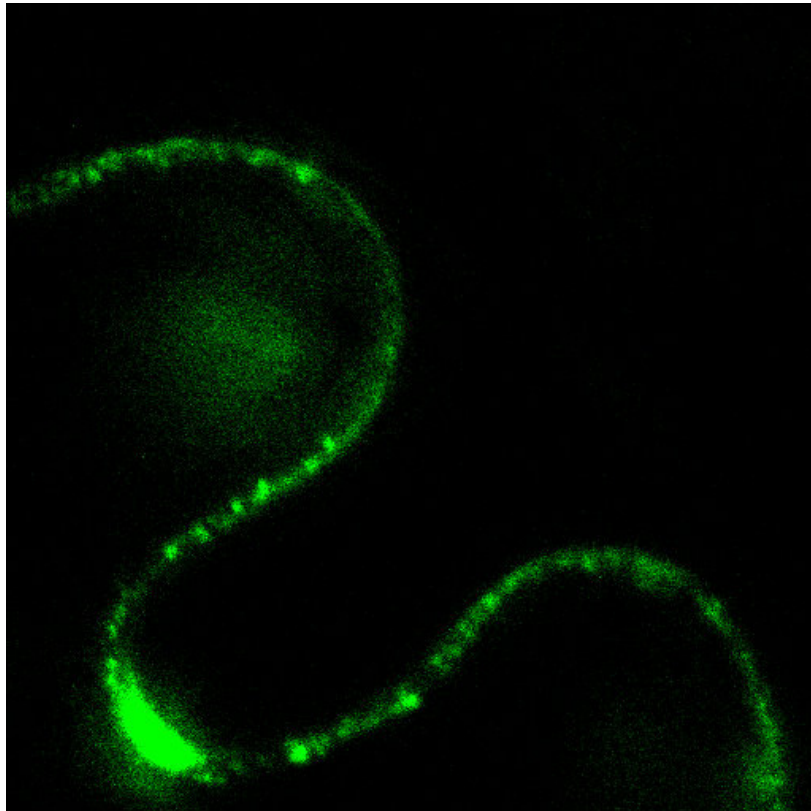
Localisation of the KAT1 K$^+$ channel fused to the green fluorescent protein (GFP) in the plasma membrane of *Vicia faba* epidermal cells after transfection by particle bombardment. The channel is located in distinct punctuate structures which could be clusters of the K$^+$ channel in the plasma membrane or vesicles containing the K$^+$ channel close to the plasma membrane.

2.1 Abstract

For a number of mammalian ion channels, trafficking to the plasma membrane was found to be controlled by intrinsic sequence motifs. Among these sequences are diacidic motifs that function as endoplasmic reticulum (ER) export signals. So far it is unclear if similar motifs also exist in plant ion channels. In this study the function of four diacidic DxE/DxD motifs of the plant potassium (K^+) channel KAT1 were analysed. Mutation of the first diacidic DxE motif resulted in a strong reduction of the KAT1 conductance in both, guard cell protoplasts and HEK293 cells (human embryonic kidney cells). Confocal fluorescence microscopy of guard cells expressing the mutated KAT1 fused to green fluorescent protein (GFP) revealed localization of the mutated channel only in intracellular structures around the nucleus. These structures could be identified as part of the ER via coexpression of KAT1 fused to yellow fluorescent protein (YFP) with an ER retained protein (HDEL) fused to cyan fluorescent protein (CFP). Blocking the vesicle formation from the ER by overexpression of the small GTP-binding protein Sar1 fixed in its GDP bound form led to retention of wildtype KAT1 in similar parts of the ER. Mutation of the three other diacidic motifs had no effect.

Together the results demonstrate that one diacidic motif of KAT1 is essential for ER export of the functional K^+ channel in both, guard cell protoplasts and HEK293 cells. This suggests that trafficking of plant plasma membrane ion channels is controlled via a conserved mechanism.

2.2 Introduction

Plasma membrane K^+ channels are crucial for cellular ion homeostasis, osmotic regulation and excitability of cells. Their correct functioning depends not only on the control of their activity in the plasma membrane but also on the regulation of their number in the plasma membrane. Ion channels are transported to the plasma membrane along the secretory pathway via the endoplasmic reticulum (ER) and the Golgi apparatus. Until recently, plasma membrane proteins including ion channels were considered to leave the ER by default (Wieland *et al.*, 1987). However, studies on trafficking of plasma membrane ion channels in mammalian

cells revealed that transport from the ER to the Golgi apparatus is highly regulated and plasma membrane channel density may be adjusted by controlling their export from the ER (Ma *et al*., 2001; Wang *et al*., 2004). Among the few motifs identified as ER export signals in ion channels are the diacidic D/ExD/E motifs which have also been shown to function as ER export signals in other plasma membrane proteins in yeast and animal cells (Nishimura and Balch, 1997; Votsmeier and Gallwitz, 2001). Mutation of these diacidic motifs resulted in a strong reduction of proteins in the plasma membrane and an accumulation in the ER.

ER export motifs are probably critical for enrichment of cargo proteins into COPII (coat protein complex II) vesicles which are responsible for the transport of proteins to the Golgi apparatus. A good candidate for the interaction of cargo proteins with the COPII coat complex is the coat protein Sec24, which can bind to a variety of ER export motifs (Bickford *et al*., 2004).

Homologues of the COPII coat proteins have also been identified in plants (Bar-Peled and Raikhel, 1997; Movafeghi *et al*., 1999; Contreras *et al*., 2004; Yang *et al*., 2005). However, knowledge about the molecular mechanism of COPII mediated transport from the ER in plants is still limited (for review sees Aniento *et al*., 2006). The small GTP-binding protein Sar1 which has been shown to be crucial for formation of COPII in yeast and animal cells has also been found to play an important role in ER to Golgi apparatus trafficking in plants (Takeuchi *et al*., 2000; Phillipson *et al*., 2001; daSilva *et al*., 2004). Investigation on the trafficking of the plasma membrane H^+ ATPase from *Arabidopsis thaliana* has demonstrated that transport to the plasma membrane is not achieved by default but requires cytosolic domains (Lefebvre *et al*., 2004). However, the motif responsible for targeting the H^+ ATPase to the plasma membrane was not identified. ER export signals in plants have so far only been identified for Golgi apparatus proteins (Contreras *et al*., 2004; Yuasa *et al*., 2005; Hanton *et al*., 2005). Recently, Hanton *et al*. (2005) demonstrated that transport of Golgi apparatus localised membrane proteins out of the ER was reduced to about 60% by mutation of a diacidic DxE motif.

Previous studies on trafficking of the plant K^+ channel KAT1 from *Arabidopsis thaliana* revealed that the K^+ channel is subject to a constitutive and pressure-driven turnover (Hurst *et al*., 2004; Meckel *et al*., 2004). Clusters of KAT1 were found to be inserted into and retrieved from the plasma membrane during

constitutive and pressure-driven exo- and endocytosis of small vesicles. Here the role of diacidic motifs in trafficking of KAT1 was investigated using patch clamp measurements and confocal fluorescence microscopy. The former technique allowed the analysis of the functioning of the channel in its target compartment plasma membrane while the latter provided information on the subcellular localization of the channel. Amino acid sequence analysis predicts four diacidic motifs in the cytosolic C-terminus of KAT1: I. DAE (394-396) located inside the putative cyclic nucleotide binding domain (cNBD), II. DTE (555-557) and two more inside the so called K_{HA} domain, III. DLD (662-664) and IV. DGD (668-670), which is possibly involved in channel tetramerization (Daram *et al*., 1997) or channel clustering (Ehrhardt *et al*., 1997). Here it is shown that the ER export was strongly dependent on the first diacidic motif. Mutation of this motif almost completely abolished the transport of functional KAT1 to the plasma membrane and resulted in the accumulation of the channel in the ER. These results demonstrate that ER export can act as a site of regulation of ion channel trafficking in plants.

2.3 Results

2.3.1 Mutation of a diacidic motif reduces the number of active KAT1 K^+ channels in the plasma membrane of guard cell protoplasts

Sequence analysis of the plant K^+ inward rectifier from *Arabidopsis thaliana* revealed two diacidic DxE motifs and two diacidic DxD motifs in the cytosolic carboxyl-terminal tail of the channel. To determine the role of these diacidic motifs four fusion constructs between green fluorescent protein (GFP) and KAT1 mutants were constructed. Each of these mutants contained the acidic amino acids aspartate (D) and glutamate (E) of one DxE or DxD motif substituted by alanine (A). The resulting mutants KAT1[(I)]::GFP (DAE[394-396] mutated to AAA), KAT1[(II)]::GFP (DTE[555-557] mutated to ATA), KAT1[(III)]::GFP (DLD[662-664] mutated to ALA), KAT1[(IV)]::GFP (DGD[668-670] mutated to AGA) and wildtype KAT1 fused to GFP (KAT1::GFP) were transiently expressed in guard cell protoplasts. Transfected cells were analysed by whole cell patch clamp measurements.
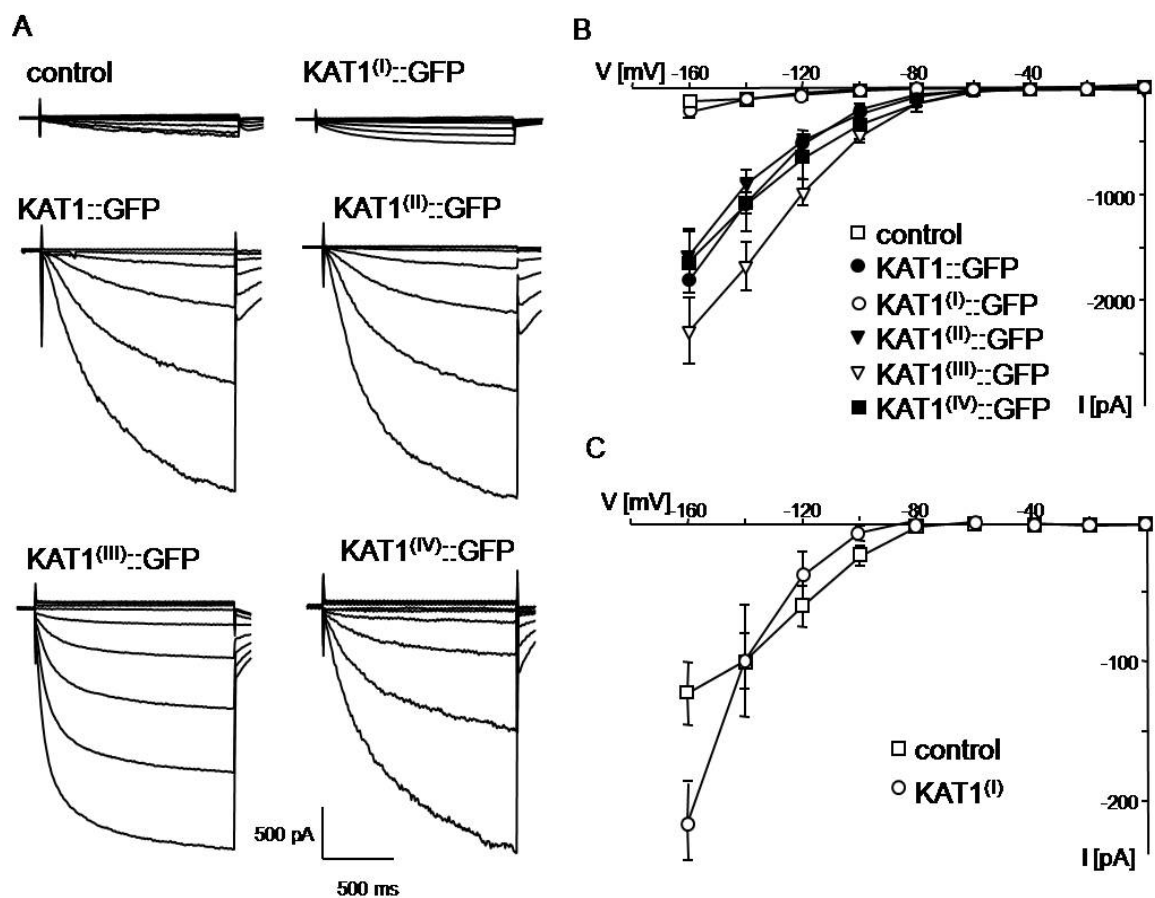
Figure 2.1: Mutation of the diacidic motif (I) of KAT1 reduces inward conductance of transfected guard cell protoplasts. A: Current response to voltage steps from a holding voltage of -55 mV to test voltages of 0 to -160 mV in 20 mV increments of untransfected guard cell protoplasts (control) and protoplasts transfected with GFP fusion constructs of wildtype KAT1 or either of the four mutants of KAT1 as indicated above traces. **B:** Current-voltage relation of mean steady state current minus instantaneous current recorded from untransfected guard cell protoplasts (control, n=15) and protoplasts transfected with KAT1::GFP (n=5), KAT1(I)::GFP (n=5), KAT1(II)::GFP (n=5), KAT1(III)::GFP (n=8) or KAT1(IV)::GFP (n=7). **C:** Current-voltage relation of mean steady state current minus instantaneous current recorded from untransfected guard cell protoplasts (control, n=15) and protoplasts transfected with KAT1(I)::GFP (n=5). Error bars correspond to ± s.e.m..

At voltages more negative than -80 mV, control and transfected guard cell protoplasts exhibit a time- and voltage-dependent inward conductance (Figure 2.1). In KAT1::GFP expressing protoplasts, the average time-dependent inward current at -160 mV exceeded those of untransfected protoplasts by a factor of about 10 (Figure 2.2) as demonstrated before (Hurst *et al.*, 2004). This large inward conductance corresponds to K$^+$ influx through KAT1. A similar increase in inward conductance was recorded from guard cell protoplasts expressing the mutated channels KAT1(II)::GFP, KAT1(III)::GFP and KAT1(IV)::GFP (Figure 2.1A, B

and 2.2). The time-dependent activation of K^+ inward currents and the current-voltage relation of KAT1$^{(II)}$::GFP and KAT1$^{(IV)}$::GFP expressing protoplasts resembled that of KAT1::GFP (Figure 2.1A, B). In protoplasts expressing KAT1$^{(III)}$::GFP activation of the K^+ inward current already occurred at -80 mV and was faster than the time-dependent activation of wildtype KAT1 and of the other two mutants described above (Figure 2.1A, B). This suggests that the voltage-dependent activation of KAT1 is affected by mutation of the diacidic motif (III). However, the focus of the present investigation is on trafficking of KAT1 which seems not to be affected by the mutation.
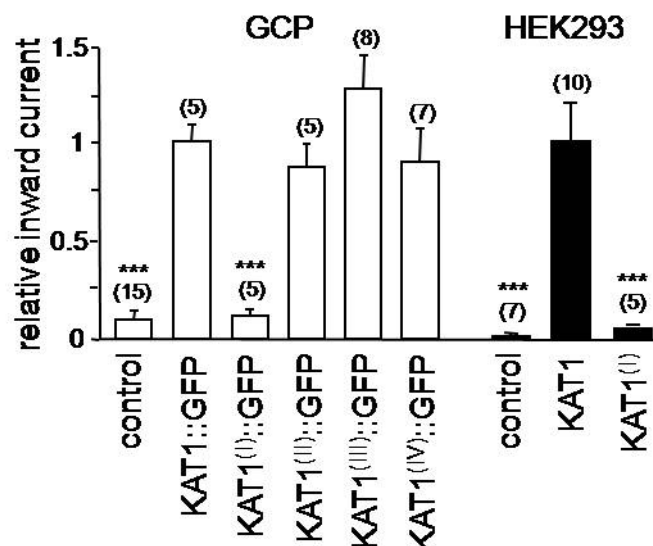
Figure 2.2: Comparison of average steady state inward currents. Currents were measured at -160 mV (guard cell protoplasts [GCP]) or -140 mV (HEK293 cells) in untransfected cells (control) and cells expressing KAT1 or KAT1 mutants (as indicated below bars). Average steady state currents were normalised to the current recorded in cells expressing wildtype KAT1. Numbers of independent experiments are given in brackets. Error bars correspond to ± s.e.m. Asterisks indicate significant differences compared to cells transfected with wildtype KAT1 (***P<0.005, Students t-test).

Measurements of protoplasts expressing KAT1$^{(I)}$::GFP revealed a completely different picture. The average time-dependent inward current at -160 mV was much lower than the one recorded from KAT1::GFP transfected protoplasts and was similar to untransfected control protoplasts (Figure 2.2). This suggests that KAT1$^{(I)}$::GFP is inactive or not incorporated into the plasma membrane. However, in contrast to the endogenous K^+ inward rectifier which exhibits saturation of the

conductance at voltages more negative than -140 mV the inward conductance in protoplasts transfected with KAT1[(I)]::GFP revealed no saturation in the voltage range analysed (Figure 2.1C). The current-voltage relation of KAT1[(I)]::GFP expressing protoplasts was very similar to the current-voltage relation of wildtype KAT1 and the other KAT1 mutants (Figure 2.1B, C). This implies that KAT1[(I)]::GFP is indeed incorporated and active in the plasma membrane of guard cells albeit to a very low extent. Due to the large variability in endogenous K^+ inward conductance the additional KAT1[(I)]::GFP conductance is not seen as a significant increase in the average inward current.

Together the results demonstrate that mutation of the diacidic motif (I) largely reduced the number of active KAT1 channels in the plasma membrane of guard cells while mutation of the three other diacidic motifs had no effect on the KAT1 conductance in transfected guard cell protoplasts.

2.3.2 Mutation of a diacidic motif results in ER retention of KAT1

The results described above imply that mutation of the diacidic motif (I) of KAT1 strongly affects the number of active channels in the plasma membrane. This can in principle result from an inhibition of channels in the plasma membrane or from a reduced incorporation of channels into the plasma membrane. The latter explanation seems more likely as diacidic motifs have been shown to act as ER export signals (Barlowe, 2003). To investigate whether the reduction in the number of channels in the plasma membrane of KAT1[(I)]::GFP expressing guard cell protoplasts results indeed from retention of the channel in the ER, the subcellular distribution of wildtype KAT1 and KAT1 mutants fused to GFP or yellow fluorescent protein (YFP) were compared in transfected guard cells using confocal laser scanning microscopy.
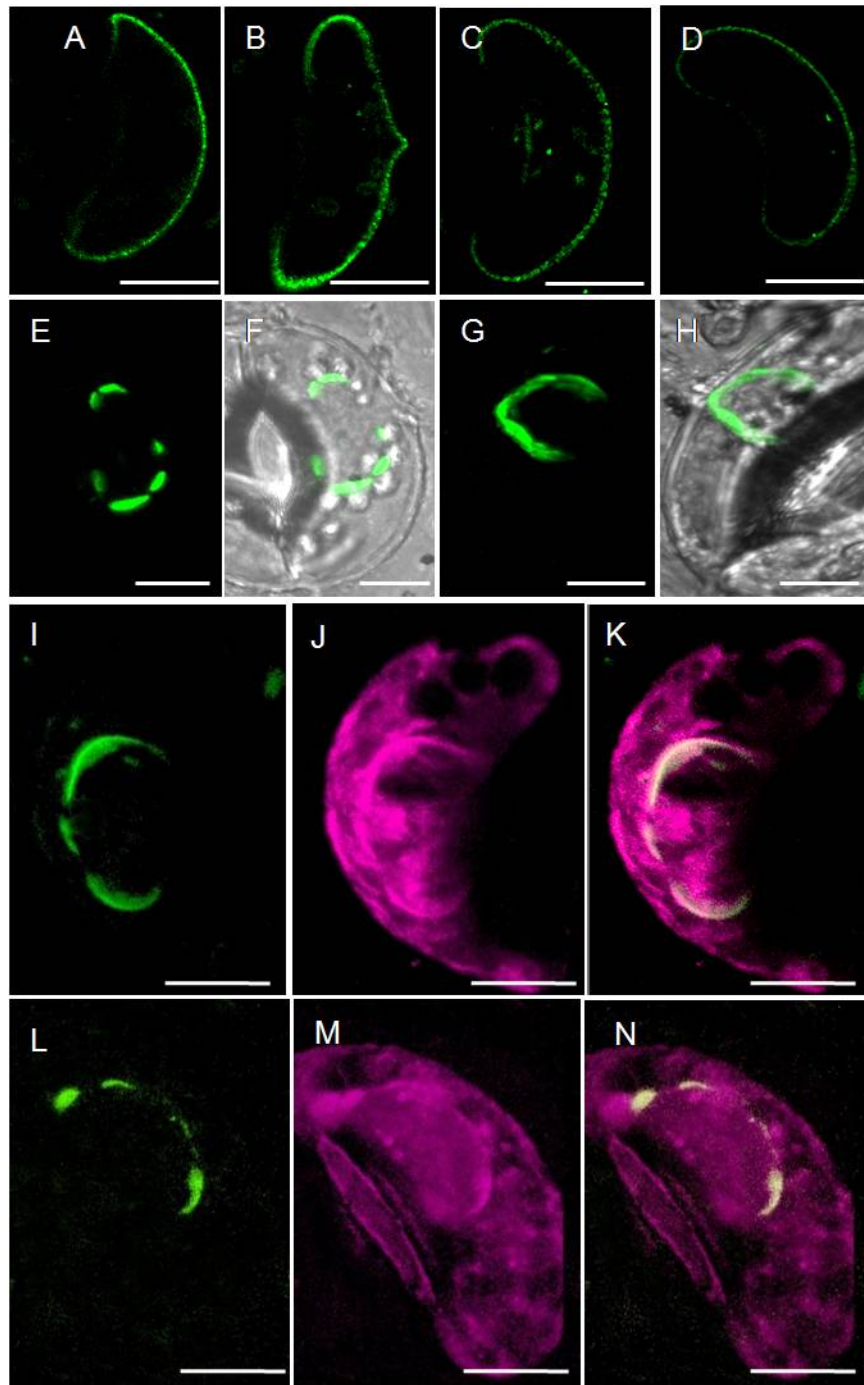
Figure 2.3: Mutation of the diacidic motif (I) of KAT1 results in ER retention A-D: Projection of two optical sections through the equatorial region of guard cells expressing KAT1::GFP (A), KAT1[(II)]::GFP (B), KAT1[(III)]::GFP (C) or KAT1[(IV)]::GFP (D). **E-H:** Overlay of transparency and fluorescent projection of four optical sections through the equatorial region of a guard cell expressing KAT1[(I)]::GFP (E,F) or wildtype KAT1::GFP and GDP-fixed Sar1 (G,H). **I-K:** Projection of four optical sections through the equatorial region of a guard cell cotransfected with KAT1[(I)]::YFP and CFP::HDEL; KAT1[(I)]::YFP fluorescence is shown in I, CFP::HDEL fluorescence is shown in J and K represents overlay of both images. **L-N:** Projection of four optical sections through the equatorial region of a guard cell cotransfected with GDP fixed Sar1, KAT1::YFP and CFP::HDEL; KAT1::YFP fluorescence is shown in L, CFP::HDEL fluorescence is shown in M and N represents overlay of both images. Scale bar corresponds to 10 μm.

In guard cells expressing KAT1::GFP or KAT1[(II)]::GFP, KAT1[(III)]::GFP and KAT1[(IV)]::GFP fluorescence was mainly found in the plasma membrane (Figure 2.3A to D). In addition to the bright labelling of the plasma membrane some guard cells also exhibit staining of intracellular compartments mainly around the nucleus (data not shown). Expression of KAT1[(I)]::GFP led to a completely different staining pattern. None of the guard cells transfected with this mutant displayed labelling of the plasma membrane. Instead only intracellular compartments, mainly around the nucleus were brightly labelled by GFP (Figure 2.3E, F). A comparable staining pattern was observed in guard cells cotransfected with wildtype KAT1::GFP and Sar1^{T39N}, a mutant of the small GTP-binding protein Sar1 fixed in the GDP bound form (Figure 2.3G, H). Sar1 has been shown to be essential for the formation of COPII vesicles and thus for export of proteins from the ER. Blocking Sar1 in its GDP bound form led to the inhibition of ER export in tobacco and *Arabidopsis thaliana* cultured cells (Takeuchi *et al*., 2000). The fluorescent intracellular structures shown in Figures 2.3E to H therefore most likely correspond to part of the ER. This was confirmed by colocalization studies of KAT1::YFP or KAT1[(I)]::YFP and the ER retention signal HDEL fused to secretory cyan fluorescent protein (CFP) as an ER marker. Figures 2.3I to N show that all structures labelled by KAT1[(I)]::YFP (Figure 2.3I to K) or KAT1::YFP expressed in the presence of the GDP fixed Sar1 mutant (Figure 2.3L to N) were also stained by CFP::HDEL. The largest part of the CFP::HDEL labelled ER did not show any KAT1[(I)]::YFP or KAT1::YFP staining. In particular KAT1[(I)]::YFP or KAT1::YFP was never localised to the cortical ER. Together the results demonstrate that inhibition of ER export of KAT1 is associated with the accumulation of the channel in restricted areas of the ER mainly around the nucleus. The localization of KAT1[(I)]::YFP was not time-dependent. Even 48 h after transfection neither the current of KAT1[(I)]::GFP transfected guard cell protoplasts nor the staining pattern were any different compared to measurements carried out 15 h after transfection. This implies that the distribution of channels reached a steady state soon after start of expression and did not change over time. In conclusion, the localization studies imply that the reduced K$^+$ inward conductance observed in KAT1[(I)]::GFP transfected protoplasts results from the inhibition of ER export and consequently a decrease in the number of KAT1[(I)]::GFP in the plasma membrane. The diacidic motif (I) thus most likely functions as an ER export signal.

2.3.3 Mutation of a diacidic motif also affects KAT1 conductance in HEK293 cells

To investigate whether the function of the first diacidic motif of KAT1 as an ER export signal is conserved among the plant and animal kingdom HEK293 cells (human embryonic kidney cells) transfected with wildtype or mutant KAT1 were analysed. HEK293 cells exhibit only a low plasma membrane conductance at negative voltages and no endogenous time-activated K^+ inward current (Figure 2.4). They therefore provide an excellent system to study the K^+ inward rectifier KAT1. HEK293 cells expressing wildtype KAT1 showed large time-dependent inward currents at voltages more negative than -80 mV (Figure 2.4A, B) as demonstrated before (Hertel *et al.*, 2005). The inward currents displayed the typical activation kinetic and voltage-dependence recorded from wildtype KAT1 expressing guard cell protoplasts (Hurst *et al.*, 2004). Measurements of HEK293 cells transfected with KAT1[I] showed a completely different current response which was at first glance similar to the one recorded from untransfected cells (Figure 2.4A). However, a blow up of the current traces clearly revealed a time and voltage depended activation of an inward current which was never observed in untransfected HEK293 cells (Figure 2.4A). The activation kinetic of this current qualitatively matches the kinetics of the current recorded from wildtype KAT1 transfected cells. All transfected cells exhibit similar voltage-dependence with an increase in current at voltages negative of -80 mV (Figure 2.4B, C). However, in KAT1[I] expressing cells the current at -140 mV was reduced to only about 5% of the current recorded in wildtype KAT1 expressing cells (Figure 2.2). This demonstrates that KAT1[I] is inserted into the plasma membrane of HEK293 cells albeit to a lower extent.
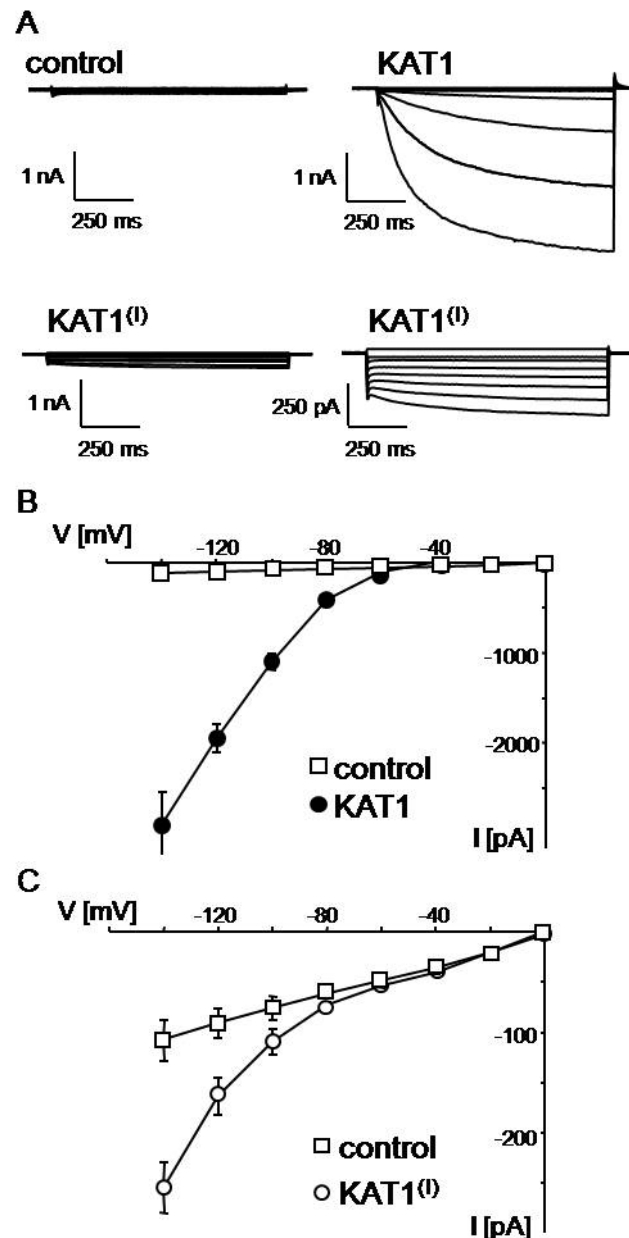
Figure 2.4: Mutation of the diacidic motif (I) of KAT1 reduces inward conductance of transfected HEK293 cells A: Current response to voltage steps from a holding voltage of -10 mV to test voltages of 0 to -140 mV in 20 mV increments of untransfected HEK293 cells (control) and of HEK293 cells transfected with wildtype KAT1 or KAT1[(I)] as indicated above traces. B: Current-voltage relation of mean steady state current minus instantaneous current recorded from untransfected HEK293 cells (control, n=7) and HEK293 cells transfected with wildtype KAT1 (n=10). C: Current-voltage relation of mean steady state current including instantaneous current recorded from untransfected HEK293 cells (control, n=7) and HEK293 cells transfected with KAT1[(I)] (n=5). Error bars correspond to ± s.e.m..

Together these results imply that mutation of the diacidic motif (I) of KAT1 affects ER export in HEK293 and in guard cells, suggesting that the mechanism of ER export is conserved among plant and animal cells.

2.4 Discussion

2.4.1 The diacidic motif of KAT1 is essential for efficient ER export

In a number of proteins diacidic motifs have been shown to be crucial for efficient transport of these proteins from the ER to their target compartments (Nishimura and Balch, 1997; Votsmeier and Gallwitz, 2001; Ma *et al*., 2001; Wang *et al*., 2004; Hanton *et al*., 2005). In the cytosolic C-terminus of the plant K^+ channel KAT1 four diacidic motifs have been identified. Patch clamp analysis of guard cell protoplasts and HEK293 cells revealed that only mutation of one (diacidic motif (I)) out of the four diacidic motifs of KAT1 dramatically reduced the inward conductance of transfected cells. The reduction in inward conductance could be quantified in HEK293 cells which do not exhibit any endogenous inward conductance. In HEK293 cells, expressing the mutant channel KAT1[(I)], the inward conductance was reduced to only about 5% compared to wildtype KAT1 transfected cells. The reduced current recorded from cells expressing KAT1[(I)] showed a qualitatively similar kinetic and voltage-dependence as wildtype KAT1. This implies that the lower inward conductance of KAT1[(I)] transfected cells results from a reduced number of functional channels in the plasma membrane suggesting that transport but not function of the channel to the plasma membrane is inhibited.

This was confirmed by confocal images of KAT1[(I)]::GFP expressing guard cells which showed a bright staining of intracellular compartments mainly around the nucleus without any detectable staining of the plasma membrane. Coexpression studies with the ER marker CFP::HDEL confirmed that these intracellular structures correspond to the ER, demonstrating that the largest amount of the mutated channel is indeed retained in the ER. The fluorescence of the remaining channels that still reached the plasma membrane was too low to be detected by confocal laser scanning microscopy.

In principle the retention of KAT1[(I)]::GFP in the ER could result from misfolding of the mutated protein which is kept in the ER for subsequent degradation.

However, the fact that in guard cell protoplasts as well as in HEK293 cells functional KAT1[(I)] channels with similar voltage-dependence and time-dependent activation kinetics as wildtype KAT1 can be detected in the plasma membrane argues against this hypothesis. Our results rather implicate that the observed retention of KAT1[(I)]::GFP in the ER is due to a reduction of ER export of fully functional KAT1 channels. This is consistent with our analysis of wildtype KAT1 expressing guard cells where ER export has been blocked by coexpression of the GDP-fixed Sar1 mutant. These cells showed the same staining pattern as KAT1[(I)]::GFP expressing cells. Therefore, it can be concluded that the diacidic motif (I) of KAT1 acts as an ER export signal in both, HEK293 and guard cells. This also suggests that the mechanism of ER export is conserved among plant and animal cells.

2.4.2 ER retention of KAT1 is very efficient and may be restricted to certain areas

Recently, a diacidic motif has been shown to affect ER export of two plant Golgi-localised membrane proteins (Hanton *et al*., 2005). Using imaging of transfected tobacco leaves Hanton *et al*. (2005) demonstrated that mutation of a diacidic motif led to a reduction of the Golgi-localization of these proteins by about 40%. The authors therefore suggest that factors other than diacidic motifs also influence the ER export of these proteins. In our studies mutation of the diacidic motif (I) nearly completely blocked transport of the channel to the plasma membrane without affecting its functioning. This implicates that efficient transport of KAT1 from the ER to the Golgi apparatus is highly dependent on the first diacidic motif. Similar results have been described for the function of diacidic motifs in trafficking of plasma membrane transporters in mammalian cells (Ma *et al*., 2001; Wang *et al*., 2004). Investigation of trafficking of mammalian plasma membrane transporters suggest that binding of scaffold proteins, such as PDZ domain-containing proteins can change the relative effectiveness of ER export signals and thus allow regulation of the number of transporters in the plasma membrane (Ma and Jan, 2002). A tight regulation of the protein density is of particular importance for plasma membrane ion channels as small changes in the number of channels can have a pronounced effect on the conductance and thus function of the cell. Most

likely the diacidic motif (I) of KAT1 is part of such a regulatory mechanism that controls the density of this channel in the plasma membrane of plant cells.

Analysis of the localization of KAT1[(I)]::GFP reveals that the channel is mainly retained in the ER around the nucleus. Similar results were found for guard cells transfected with KAT1::GFP when ER export was blocked by coexpression of GDP fixed Sar1. This distinct localization of ER retained KAT1 was also found in epidermal cells (data not shown). As the outer nuclear membrane is continuous with the ER it is not unexpected that proteins that are retained in the ER can also be found in the nuclear envelope. However, previous investigations on proteins retained in the ER revealed localisation of these proteins in the nuclear envelope only in addition to the distribution throughout the rest of the ER (Batoko *et al.*, 2000, Hermann *et al.*, 1990). The reasons for retention of KAT1 in restricted areas around the nucleus remain to be identified.

2.4.3 Function of the diacidic motif is position-dependent

From the four diacidic motifs found in KAT1 only mutation of the motif (I) had an effect on both, the KAT1 conductance and the cellular localization of the mutated channel. This implies that the position of the diacidic motif in the protein is important for its functioning. Position-dependence of the function of diacidic motifs has also been described for the yeast plasma membrane protein Sys1 (Votsmeier and Gallwitz, 2001) and for the plant Golgi localised membrane proteins GONST1 and CASP (Hanton *et al.*, 2005).

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KAT1    385 FLFQLVSDIDAEYFPPKEDIIL 486 ALMDAIHKEDTEMV  658 AEIDDLDVIWDGDHLYFS
KAT2    396 FLFQLVSDIDAEYFPPREDVIL 560 IDMGSTEWRDSRRS  686 AEIDDEDVIRDGDHLYIL
KST1    398 LLFQLVPEMEAEYFPPKQEVIL 353 KTMNDLSLNLENKS  671 AEIDDIDVIRDGDHLFFL
KPT1    385 FLFQLVSEMEAEYFPPKEDVIL 613 KSIHDLLLNYENRN  731 AEIDGISVIRDGDHLFLL
SIRK    385 FLFQLVSEVEAEYFPPREDVIL 630 KSIYDLLLSYENRR  759 AEIDDISVIRDGDHLFLL
KMT1p   127 SLFQLVSEMEAEYFPPREDVVL 296 WNPYDDLRSYQNGD  427 AELDDISVIRDGD
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Figure 2.5: Alignment of amino acid sequences of plant K^{+} inward rectifier. **Comparison of the amino acid sequences of the potential diacidic ER export signals found in the C-terminal region of KAT1 (bold letters) with the corresponding region of other closely related plant K^{+} inward rectifying channels. The GeneBank accession numbers of the aligned channels sequences are as followed: KAT1, M86990; KAT2, AJ288900; KST1, X79779; KPT1, AJ244623; SIRK, AF359522; KMT1p AAF81250.**

The diacidic motif (I) of KAT1 is located in the putative cyclic nucleotide binding domain (cNBD). Recent investigations on disruption of the cNBD in the animal K^+ channels HCN and HERG implied that highly conserved regions in the cNBD are generally critical for ion channel trafficking (Akhavan *et al.*, 2005) This is supported by the fact that the diacidic ER export motif of the CFTR channel is also located within the NBD (Wang *et al.*, 2004). Using structural models of the NBD of the CFTR channel Wang *et al.* (2004) demonstrated the ability of the loop containing the diacidic motif to insert directly into the diacidic code binding pocket of the COPII coat complex Sec23/Sec24. In plant cells the cNBD was found to be required for efficient transport of a H^+ ATPase from *Nicotiana plumbaginifolia* to the plasma membrane even though ER export motifs have so far not been identified (Lefebvre *et al.*, 2004). An alignment of KAT1 with other related plant K^+ inward rectifiers revealed that the diacidic motif (I) and the diacidic motif (IV) are highly conserved among these channels (Figure 2.5). However, only the diacidic motif (I) which is located in the cNBD was found to affect ER export suggesting that the cNBD is per se important for ER export of plant ion channels. Together the results demonstrate that a diacidic motif of KAT1 acts as an ER export signal in plant and animal cells probably via a conserved mechanism.

2.5 Methods

2.5.1 Vectors for KAT1 expression

For the expression of a KAT1::GFP fusion protein in guard cells the cDNA of KAT1 was cloned into the pAVA393 expression vector in frame with $mGFP_5$ or YFP under the control of two strong 35S promoters as previously described by Hurst *et al.* (2004).

Expression of KAT1 in the mammalian cell line HEK293 (human embryonic kidney cells) was obtained with KAT1 cDNA cloned into the pCB6 (Acc.No.: ATCC37274) eukaryotic expression vector at the *NcoI* restriction site under control of a CMV promotor. HEK293 cells were cotransfected with the $pEGFPN_2$ vector (Clontech, Pharmingen, Germany) to express cytosolic GFP as a transfection control.

2.5.2 Mutagenesis of putative ER export motifs in KAT1

Mutations in the DxE and DxD motif of channel protein were created by PCR based site directed mutagenesis (QuikChange site directed mutagenesis kit; Stratagene, LaJolla, USA) and confirmed by sequencing. The expression vectors pAVA393KAT1 and pCB6KAT1, both containing the KAT1 cDNA as described above served as templates. The plasmids were cloned into *E. coli*/DH5α and isolated with Qiagen high speed Midi-Kit (Qiagen, Germany) for cell transfection.

2.5.3 Transfection of intact guard cells via particle delivery and isolation of protoplasts

Vicia faba L. cv. Bunyan were grown under controlled climate conditions with 18 °C, 70 % relative humidity and a 14/10 h photoperiod at 350-400 µmol photons/m^2/sec. Transfection of intact guard cells via particle delivery was performed as described earlier (Hurst *et al*., 2004).
Cotransfection was performed via coating of gold with equal molar amounts of each plasmid DNA to give a total amount of 15 µg DNA. Guard cell protoplasts were prepared from transfected leaves after overnight incubation at room temperature as described previously (Homann, 1998).

2.5.4 Cultivation and transfection of mammalian cell line HEK293

HEK293 cells were grown at 37 °C and 5 % CO_2. For transient expression of KAT1 and KAT1 mutants HEK293 cells were transfected with each 0.75 µg of pCB6KAT1 and pEGFPN$_2$ vector using the liposomal transfection reagent Metafectene (Biontex, Munich, Germany) according to manufacturer's instructions.

2.5.5 Patch clamp measurements

HEK293 cells:

Experiments were performed on cells incubated at 37°C in 5% CO_2 for 2-3 days after transfection (for details see Hertel *et al.*, 2005). Cells were bathed in a solution containing: 20 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES at pH 7.4. Choline-Cl was used to adjust the osmolarity to 300 mOsmol/kg. Patch pipettes contained: 130 mM K^+-gluconate, 10 mM NaCl, 5 mM Hepes, 0.1 mM Na_2GTP, 0.1 µM $CaCl_2$, 2 mM $MgCl_2$, 5 mM $Na_2Phosphocreatin$, 2 mM K_2ATP at pH 7.4 with an osmolarity of approximately 330 mOsmol/kg.

Guard cell protoplasts:

Guard cell protoplasts were bathed in 10 mM KCl, 10 mM $CaCl_2$ and 5 mM MES, pH 6.0/KOH. The osmolarity was adjusted to 520 mOsmol/kg with Sorbitol. Patch pipettes were filled with 150 mM K^+-gluconate, 10 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES, 2 mM K_2ATP, pH 7.8/KOH. Osmolarity was adjusted to 560 mOsmol/kg with Sorbitol.

In both cell systems measurements were performed in a standard whole-cell patch clamp experiment as described in detail previously (Homann and Thiel, 2002; Hurst *et al.*, 2004; Hertel *et al.*, 2005).

2.5.6 CLSM

Confocal microscopic analysis of transfected turgid guard cells was performed after overnight incubation as described earlier using a confocal laser scanning microscope (Leica TCS SP, Leica Microsystems GmbH, Heidelberg, Germany) (for details see Meckel *et al.*, 2004). For excitation of fluorescent proteins the following lines of a 25 mW argon laser were used: 488 nm for GFP, 458 nm for CFP and 514 nm for YFP. Fluorescence was detected at 505-535 nm for GFP, 465-490 nm for CFP and 600-650 nm for YFP. Images were processed using the Leica Confocal Software 2.00 (LCS, Leica Microsystems GmbH, Heidelberg, Germany). For the microscopic analysis guard cells were bathed in 0.1 mM $CaCl_2$, 10 mM MES, 45 mM KCl, pH 6.1/KOH.

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Chapter 3 EFFICIENCY OF ER EXPORT OF THE K$^+$ CHANNEL KAT1 DEPENDS ON THE NUMBER OF ACIDIC AMINO ACIDS WITHIN A TRIACIDIC MOTIF
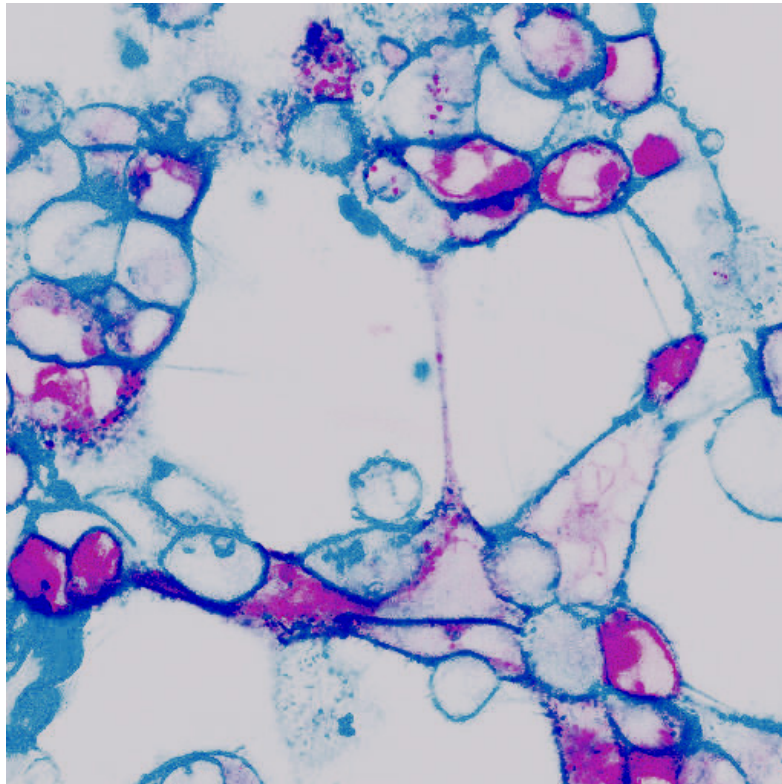
HEK293 cells transiently expressing KAT1 fused to the green fluorescent protein (purple) stained with the plasma membrane marker FM4-64 (light blue). Colocalisation of both fluorescent proteins in the plasma membrane is indicated in dark blue.

3.1 Abstract

For a number of ion channels, including the potassium (K^+) inward rectifying channel KAT1 from *Arabidopsis thaliana,* diacidic ER export motifs have been identified. The aim of this study was to specify the role of single acidic amino acids for efficient ER export of KAT1. Therefore, a sequence of KAT1, containing two aspartate (D) and one glutamate (E), was analysed. This sequence included the originally identified diacidic ER export motif (DxE) of KAT1 and an additional D just downstream of the diacidic motif. Analysis of single, double and triple mutations of the acidic amino acids of the DxDxE motif revealed a gradual reduction of ER export depending on the number of mutated acidic residues. The amount of reduction in ER export was not related to the position but only to the number of mutated acidic amino acids. Furthermore, it could be demonstrated that plasma membrane expression of ER export mutants can be rescued by heterotetrameric assembly with wildtype KAT1. This implies that not all subunits of the KAT1 tetramer need to carry a functional ER export motif for trafficking to the plasma membrane. Together this study shows that a triacidic motif functions as an ER export signal of KAT1. In addition, it suggests that ER export may not only function in regulation of channel density but also in controlling channel composition in the plasma membrane.

3.2 Introduction

Ion channels are integral membrane proteins which facilitate the diffusion of ions across biological membranes. They play a key role in many cellular processes including signal transduction. Therefore, the control of ion channel activity but also regulation of the number and composition of channels in the membrane is of particular importance for cell functioning. Recent investigations have highlighted the transport of ion channel from the endoplasmic reticulum (ER) to the Golgi apparatus as an important step for regulation of surface expression of ion channels (Schwappach, 2008). For the efficient ER export proteins are recruited into coat protein complex II (COPII) vesicles which bud off the ER membrane. This process involves recognition of specific amino acid motifs, so-called ER export

signals, by components of the COPII coat (Otte and Barlowe, 2004). Until now several ER export motifs have been identified in membrane proteins including ion channels, receptors and transporters. Most of them fall into four groups: diacidic, dihydrophobic and dibasic signal sequences as well as a terminal valine (V) at the C-terminus. The diacidic ER export motif consist of the two acidic amino acids aspartate (D) or glutamate (E) separated by any amino acid (x) (D/ExD/E). Ion channels and transporters are accounting for the largest part of proteins for which diacidic ER export motifs have been identified. For several members of the K^+ channel family (Ma *et al.*, 2001 and 2002; Hofherr *et al.*, 2005; Zuzarte *et al.*, 2007) and also for the chloride channel CFTR (Wang *et al.*, 2004) diacidic ER export motifs where found to be essential for surface expression of the channel.

Recently, it was demonstrated that ER export of the plant K^+ channel KAT1 also strongly depended on a diacidic motif. Mutation of the DxE (394-396) motif in the C-terminus of the K^+ channel reduced the transport of functional KAT1 to the plasma membrane by about 90 % and resulted in the accumulation of the channel in the ER (Mikosch *et al.*, 2006).

Further investigations regarding the function of the ER export signal revealed a direct interaction of the diacidic motif of KAT1 with the COPII component Sec24 (Sieben *et al.*, 2008). Sec24 contains at least three binding sites for ER export motifs. The B-site was found to be the binding site for the diacidic ER export motif of a number of proteins like Sys1 and Yor1 (Mossessova *et al.*, 2003; Miller *et al.*, 2003; Pagant *et al.,* 2007). However, mutation of the B-site of Sec24 from yeast suggests that other proteins like the amino acid permease Gap1 which also contains a diacidic motif, do not bind to the B-site (Miller *et al.,* 2003). These studies suggest that recognition of the diacidic ER export motif by Sec24 may not be consistent for all diacidic motifs. This may also explain the sometimes contradictory results on sequence specificity of the diacidic ER export motif (Mikosch and Homann, 2009).

Therefore, the role of single acidic amino acids for ER export of KAT1 was analysed. Sequence comparison of several plant ion channels from different organisms showed that the diacidic ER export motif is located in a highly conserved part of the protein. Noticeably a third acid residue ahead of the diacidic motif is found in nearly all of these channels (Figure 3.1). Hence, this residue was also included in the site directed mutagenesis studies. To complete the

investigation double and triple mutation of the acidic amino acids were performed. All mutations resulted in a reduction in ER export of KAT1, where the degree of reduction was dependent on the number of mutated acidic residues.

```
KAT1        392DIDAE
KAT2        403DIDAE
AKT5        411EMKAE
SPIK        413EMKAE
AKT1        387EMKAE
AKT2/3      409KMKAE
AtKC1       420QIQAE
KST1        405EMEAE
KPT1        392EMEAE
SIRK        392EVEAE
KMT1        334EMEAE
```

Figure 3.1: Alignment of inward rectifying potassium channels from *Arabidopsis thaliana*, *Vitis vinifera*, *Solanum tuberosum*, *Populus tremula* and *Mesembryanthemum crystallinum* with the acidic ER export sequence of the KAT1 channel. The acid amino acids are highly conserved among the potassium channels of different plant species.

For mammalian K^+ channels of the K_{ir} family it has been demonstrated that ER export motifs can not only affect the number but also the subunit composition of ion channels in the plasma membrane. Studies by Ma *et al*. (2002) revealed that formation of heterotetramers containing both, K_{ir} subunits with and without ER export signals, leads to expression of the heterotetramer in the plasma membrane while homotetramers of subunits that do not contain an ER export signal were accumulating in the ER. A similar mechanism may be involved in trafficking of the plant K^+ channel AtKC1. When expressed on its own, this channel is retained in the ER. Only upon formation of heterotetramers with other subunits like KAT1, KAT2 and AKT1 it reaches the plasma membrane (Reintanz *et al*., 2002; Pilot *et al*., 2003; Duby *et al*., 2008). To analyse the importance of heterotetramerisation for channel targeting in plants wildtype KAT1 together with an ER export mutant were expressed. The results demonstrate that the heterotetramerisation of subunits with and without ER export signals can rescue the targeting of ER export mutants to the plasma membrane. Together our data imply that not all subunits of channel heterotetramers need to contain ER export signals in order to ensure transport of the channel to the plasma membrane. For KAT1 efficient ER export most likely depends on the interaction of a triacidic motif with components of COPII vesicles.

3.3 Results and Discussion

3.3.1 Number of mutated acidic amino acids affects KAT1 conductance

Previously it was demonstrated that mutation of the two acidic amino acids of the diacidic motif DAE (394-396) of the K^+ channel KAT1 strongly reduces export of the channel from the ER (Mikosch *et al*., 2006). In order to investigate the role of each acidic amino acid within this motif mutants where glutamate (E) or aspartate (D) were substituted by an alanine (A) (KAT1^{AlaAE} and KAT1^{AlDAa}) were constructed. Also an additional aspartate (D392) downstream of the originally identified DAE (394-396) motif was included into the analysis (KAT1^{alDAE}). In addition to these three mutants all combinations of mutation of two acid amino acids to A (KAT1^{alaAE}, KAT1^{alDAa}; KAT1^{DlaAa}) and a triple mutant lacking all three acidic amino acids (KAT1^{alaAa}) were made. The resulting mutants were transiently expressed in HEK293 cells and transfected cells were analysed by whole-cell patch clamp measurements. Untransfected HEK293 cells (control) exhibit only a low plasma membrane conductance at negative voltages and no endogenous time activated K^+ inward current (Figure 3.2A). They provide an excellent system to study the K^+ inward rectifier KAT1. As demonstrated before, HEK293 cells expressing wildtype KAT1 showed large time-dependent inward conductance at voltages more negative than about -80 mV (Figure 3.2A) (see also Hertel *et al*., 2005 and Mikosch *et al*., 2006). All measurements of HEK293 cells transfected with one of the mutants revealed reduced conductance at negative voltages (Figure 3.2A). Only recordings of the triple mutant lacked a time-activated inward conductance and resembled measurements from untransfected cells (Figure 3.2A). The activation kinetics of all other KAT1 mutants qualitatively matches the kinetics of the current recorded from wildtype KAT1 transfected cells. All transfected cells exhibit similar voltage-dependence with an increase in current at voltages negative of -80 mV (Figure 3.3A). This suggest that the lower conductance recorded from cells transfected with KAT1 mutants does not result from an altered function of KAT1 K^+ channels in the plasma membrane but from a reduced number of functional channels in the plasma membrane and that the transport of the KAT1 K^+ channel is inhibited.
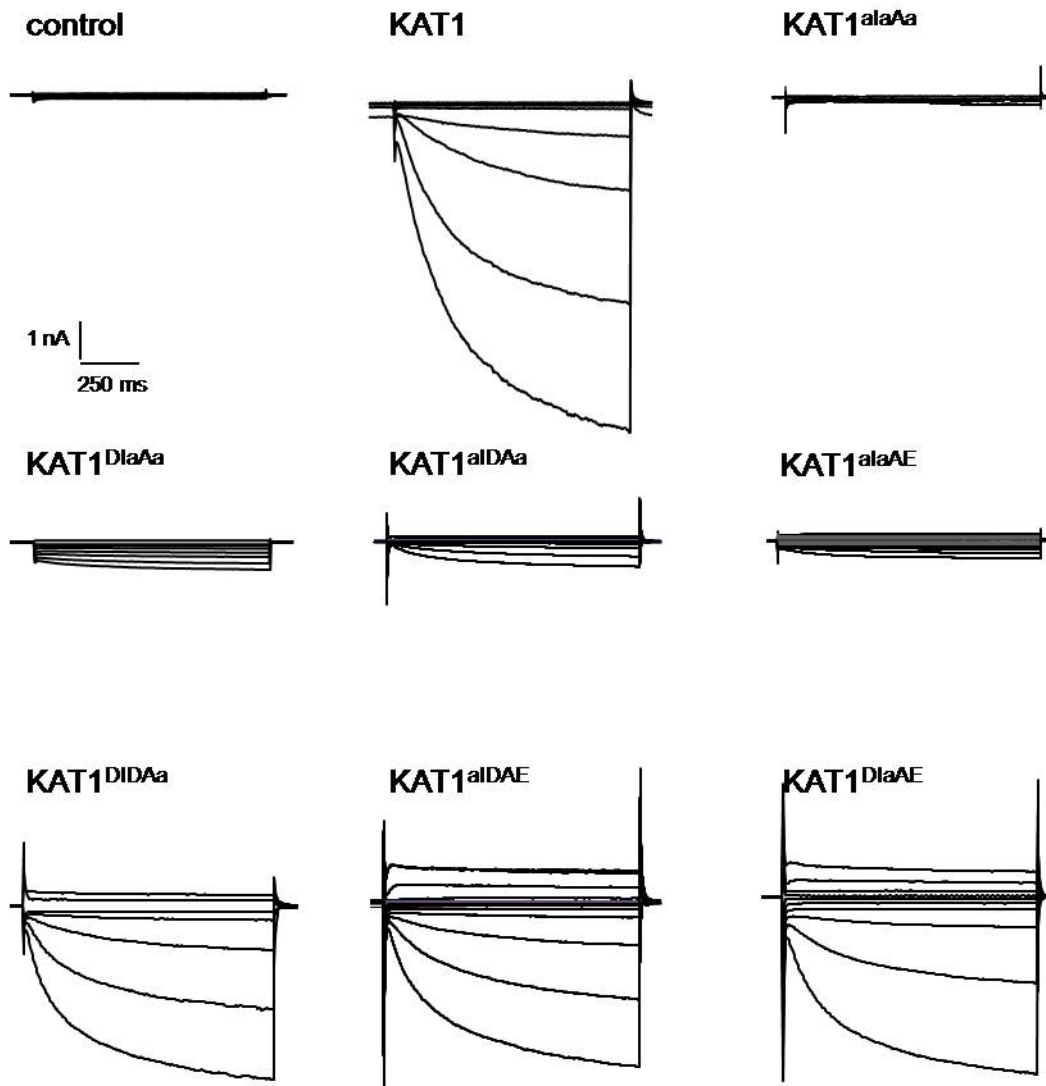
Figure 3.2: Mutation of the DIDAE motif of KAT1 reduces inward conductance of transfected HEK293 cells. Current responses to voltage steps from a holding voltage of -10 mV to test voltages of +40 mV to -160 mV in 20 mV increments of untransfected HEK293 cells (control) and HEK293 cells transfected with GFP fusion constructs of wildtype KAT1 or either of the seven mutants of KAT1 as indicated above traces.

A comparison of the average steady state current recorded at -160 mV demonstrates that all mutants exhibited a significantly lower current compared to wildtype KAT1 (P<0.005, Students t-test) and that the extent of reduction in the average inward current was dependent on the number of mutated acidic amino acids. HEK293 cells transfected with KAT1 mutants where only one of the three acid amino acids was mutated to A (KAT1^{aIDAE}, KAT1^{AlaAE} and KAT1^{AIDAa}) showed a decrease in the average inward current by about 30 to 50 % compared to wildtype KAT1 (Figure 3.3B). The decrease in the average current was much more

pronounced in cells expressing KAT1 mutants processing only one instead of three acidic amino acids within the examined array (KAT1^{alaAE}, KAT1^{alDAa}; KAT1^{DlaAa}) (Figure 3.3B). In these cells the current was reduced by 65 to 85 %. Expression of the triple mutant KAT1^{alaAa} lead to the strongest reduction in conductance with an average inward current reduced to 2-3 % of wildtype KAT1 (Figure 3.3B). The dependence of the inward conductance on the number of amino acids becomes even more obvious when the measured average current is plotted against the number of acidic amino acids (Figure 3.3C). The wildtype and mutant K$^+$ channels clearly fall into four groups represented by K$^+$ channels with zero, one, two or three acidic amino acids located within the examined array. Together these results demonstrate that all three acidic amino acids analysed affect KAT1 conductance and that the extent of reduction in conductance is determined by the number of mutated acidic amino acids with all three acidic amino acids analysed being equally important.
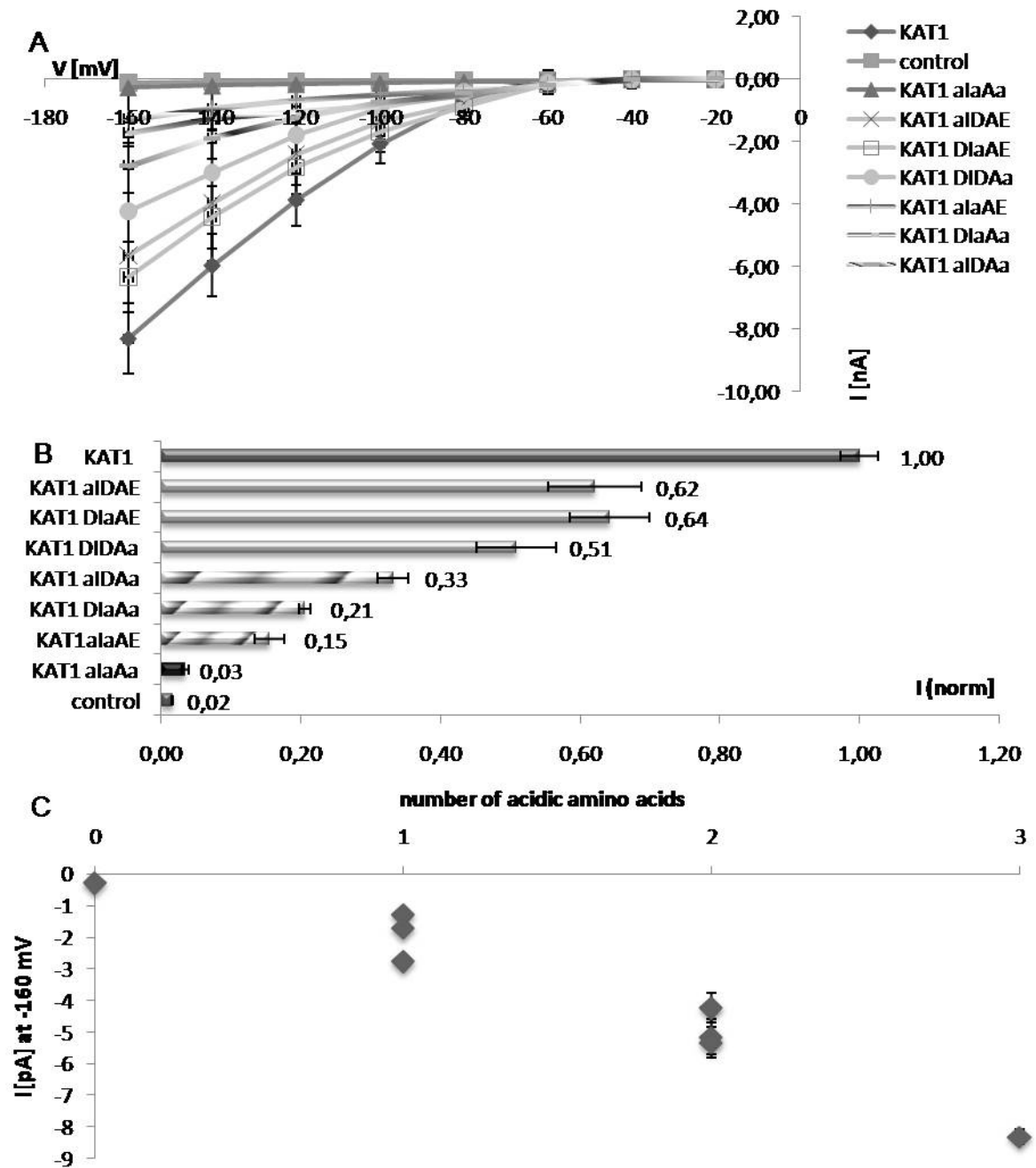
Figure 3.3: Reduction in inward current depends on the number of mutated acidic amino acids. A: Current-voltage relation of mean steady state current recorded from untransfected HEK293 cells (control, n=5) and HEK293 cells transfected with KAT1::GFP (n=10), KAT1^{alDAE}::GFP (n=7), KAT1^{DlaAE}::GFP (n=9), KAT1^{DlDAa}::GFP (n=8), KAT1^{AlDAa}::GFP (n=11), KAT1^{DlaAa}::GFP (n=8), KAT^{alaAE}::GFP (n=10) and KAT1^{alaAa}::GFP (n=6). Error bars correspond to ± s.e.m.. B: Comparison of the average steady state inward current recorded at -160 mV from untransfected HEK293 cells (control) and HEK293 cells expressing wildtype KAT1 and KAT1 mutants (as indicated beside bars). Average steady state currents were normalised to the current recorded in cells expressing wildtype KAT1. Relative size of the average current is given next to the bars. Error bars correspond to ± s.e.m.. C: Correlation between number of acidic amino acids contained in the analysed motif and average current recorded at -160 mV from HEK293 cells expressing wildtype KAT1 or one of the KAT1 mutants. Error bars correspond to ± s.e.m..

3.3.2 Mutation of acidic amino acids alters localisation of KAT1 in HEK293 cells

Judging from previous studies on diacidic motifs of KAT1 (Mikosch *et al*. 2006) the observed reduction of the inward conductance of the KAT1 mutants is most likely due to a reduced number of active channels in the plasma membrane. Therefore, the subcellular distribution of wildtype KAT1 and KAT1 mutants fused to GFP in HEK293 cells was compared using confocal laser scanning microscopy. In HEK293 cells expressing KAT1::GFP the channel was located in the plasma membrane but also to a great extent in intracellular compartments (Figure 3.4A). A similar distribution has previously been described for expression of KAT1::GFP in Chinese hamster ovary (CHO) cells (Szabo *et al.,* 2000). Colocalisation of KAT1 with the plasma membrane marker FM4-64 confirmed its plasma membrane localisation (Figure 3.4A and B). Figure 3.4B also reveals some intracellular colabelling of KAT1::GFP and FM4-64 located close to the plasma membrane. These internal structures most likely correspond to some endosomal compartments because KAT1 as well as FM4-64 are known to be internalised via endocytosis (Meckel *et al.,* 2004). To identify the largest part of the intracellular compartments labelled by KAT1::GFP but not by FM4-64, also colocalisation studies with the ER tracker red were performed. The partial colocalisation of KAT1::GFP with the ER tracker demonstrates that some of the intracellular retained channel is located in the ER but it can also be found in other intracellular compartments which may correspond to the Golgi apparatus and/or endosomes (Figure 3.5A).

Localisation analysis was also performed for the KAT1 mutants. HEK293 cells expressing KAT1 mutants with only one acidic amino acid mutated to alanine showed a very similar distribution of the K^+ channel compared to cells expressing wildtype KAT1 (Figure 3.4C and Figure 3.6C, E and G). Again the K^+ channel is found in intracellular compartments as well as in the plasma membrane. As a representative example for these mutants a more detailed analysis of the distribution of KAT1^{aIDAE}::GFP is displayed in Figure 3.5C. Colocalisation analysis with FM4-64 clearly confirmed the plasma membrane localisation (Figure 3.4C and D).
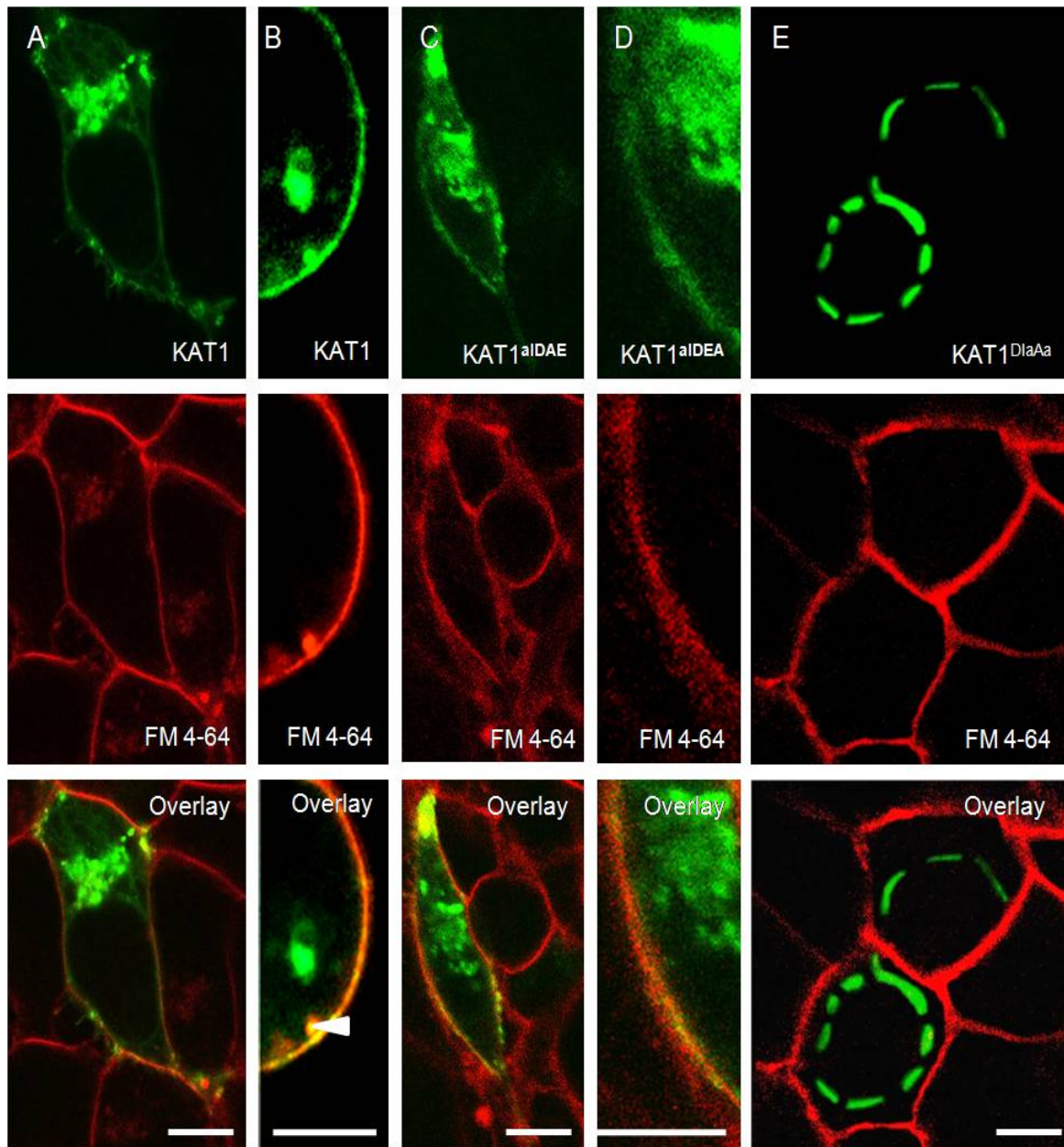
Figure 3.4: Localisation of KAT1 in the plasma membrane depends on acidic amino acids. A: HEK293 cell expressing wildtype KAT1, staining of cells with the plasma membrane marker FM4-64 and overlay of both images. KAT1 colocalises with FM4-64 in the plasma membrane **B:** Magnification of the plasma membrane region of a HEK293 cell transfected with wildtype KAT1 supporting the localization of the channel in the plasma membrane. Some wildtype KAT1 can also be found in endosomal compartments (arrow) **C:** HEK293 cell expressing KAT1^{aIDAE} as a representative example of KAT1 mutants where only one of the acidic of the DIDAE motif is mutated to alanine, staining of cells with the plasma membrane marker FM4-64 and overlay of both images. **D:** Magnification of part of the cell shown in C. This construct also clearly colocalises with the plasma membrane marker FM4-64. **E:** HEK293 cell expressing the mutant KAT1^{DIaAa} as a representative example of KAT1 mutants where two of the acidic amino acids of the DIDAE motif are mutated to alanine, staining of cells with the plasma membrane marker FM4-64 and overlay of both images. KAT1^{DIaAa} mutant does not localise to the plasma membrane. Scale bars correspond to 10 µm.

Expression of the mutants of KAT1 with two of the three acidic amino acids being mutated to A led to a completely different staining pattern (Figure 3.4E, Figure 3.5B and Figure 3.6D, F and H). As a representative example for this group of mutants the distribution of KAT1^{DIaAa} was analysed in more detail. KAT1^{DIaAa}::GFP was restricted to a small perinuclear ring with no detectable fluorescence in the plasma membrane as revealed by staining with the plasma membrane marker FM4-64 (Figure 3.4E). Almost no additional fluorescent intracellular structures were observed. Using the ER tracker red the perinuclear ring could be identified as part of the ER (Figure 3.5B). This mutant has previously been identified as an ER export mutant (Mikosch *et al.,* 2006). The distribution of KAT1^{DIaAa}::GFP in HEK293 cells is very similar to the localisation of the same mutant in *Vicia faba* guard cells (Mikosch *et al.,* 2006). A retention of the channel exclusively in the perinuclear region of the ER was also found for the mutant of KAT1 where all three acidic amino acids in the analysed motif have been replaced by A (Figure 3.6B). Thus, the localisation studies support the hypothesis that the reduced K$^+$ inward conductance observed in HEK293 cells expressing one of the KAT1 mutants results from the inhibition of transport to the plasma membrane and consequently a decrease in the number of K$^+$ channels in the plasma membrane. The inhibition of transport most likely occurs at the side of ER export. Previously, diacidic motifs have been identified as ER export signals in a number of ion channels including KAT1 (Mikosch and Homann, 2009). The consensus motif of these diacidic ER export motifs is generally believed to be D/ExD/E. The results presented above demonstrate that in addition to the two acidic amino acids identified before as part of the ER export motif of KAT1 (Mikosch *et al.,* 2006) a third acidic amino acid is equally important. The complete ER export motif is therefore most likely represented by the triacidic motif DIDAE (392-396). This is supported by the observation that the extent of ER retention was strictly dependent on the number of mutated amino acids and only mutation of all three acidic amino acids completely abolished the KAT1 conductance. This leads to the question how the ER export motif may function. In order to affect ER export the motif has to be recognised by Sec24, a component of the COPII coat which selects proteins for the anterograde transport from the ER to the Golgi apparatus. Using FRET measurement it was recently demonstrated that KAT1 indeed interacts with Sec24 at the ER and that this interaction was dependent on the

diacidic DAE (394-392) motif (Sieben *et al.,* 2008). The fact, that KAT1 mutants with two mutated acidic amino acids still elicited a small inward conductance may suggest that some mutated channels are exported out of the ER via bulk flow. Alternatively, the mutated KAT1 may still exhibit a weak binding to Sec24 which cannot be detected via FRET measurements but still allows some channels to be incorporated into COPII vesicles for further transport to the plasma membrane. The latter hypothesis is more likely since bulk flow should also enable the triple mutant to reach the plasma membrane with an equal efficiency. However, this mutant remained nearly completely in the ER. This also implies that binding of KAT1 to Sec24 involves all three acidic amino acids and that bulk flow does not play a significant role in transport of the channel out of the ER.
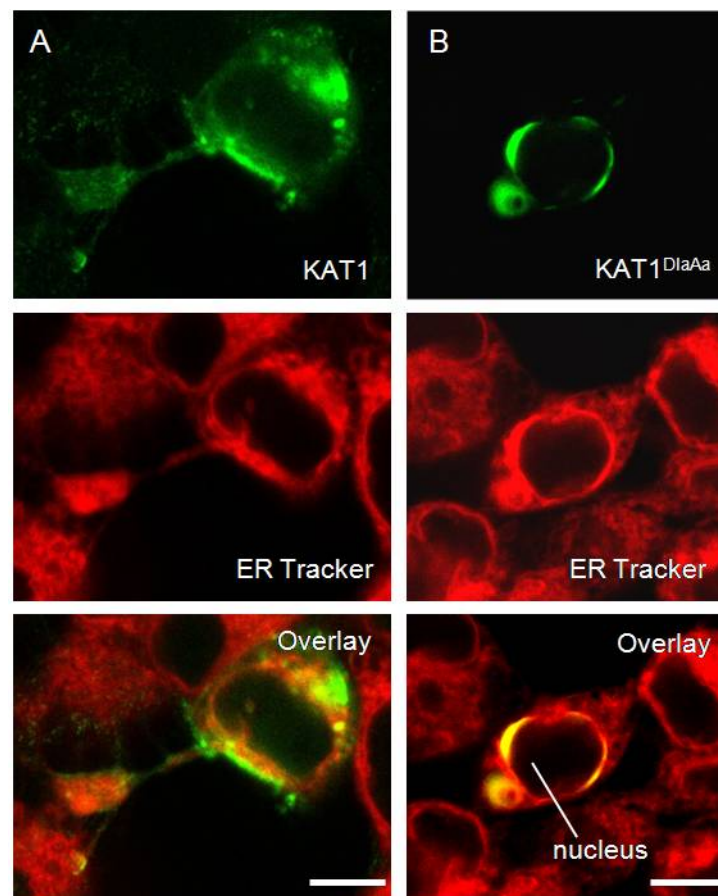
Figure 3.5: Mutation of two acidic amino acids of KAT1 results in ER retention. A: HEK293 cell expressing wildtype KAT1, staining of cells with ER tracker red and overlay of both images. The overlay shows a partial colocalisation. B: HEK293 cells expressing the KAT1[DIaAa] **mutant, staining of cells with ER tracker red and overlay of both images. The overlay shows complete colocalisation of the mutated channel with the part of the ER around the nucleus. Scale bars correspond to 10 μm.**

So far, only few studies have been carried out on the sequence specificity of the diacidic ER export motif. The results of these studies did not reveal any consistent pattern for the role of the respective amino acids. Studies on the ER export of the K$_{ir}$3.4 channel clearly demonstrate that mutation of the first as well as mutation of the last acidic amino acid greatly reduced ER export (Ma *et al.,* 2002). On the other hand mutation of both, the DxE motif of VSVG and the DxD motif of Gap1 to ExE let to retention of the proteins in the ER despite the E at the final position (Nishimura *et al.*, 1997 and Malkus *et al.*, 2002).

In addition, an effect of amino acids in the neighbourhood of the diacidic motif on ER export has clearly been demonstrated for the VSVG protein (Nishimura *et al.,* 1999). Together, this implies that the diacidic motif itself may be much more complex than just two acidic amino acids. This is in line with the results of this study which demonstrate for the first time the importance of an additional third acidic amino acid for efficient ER export.
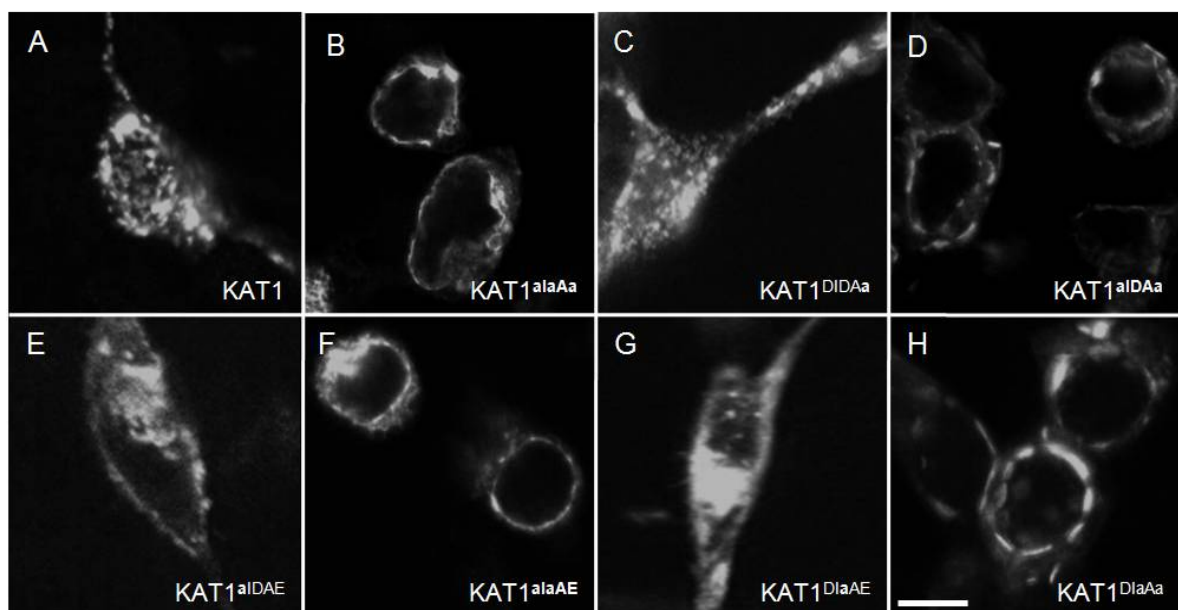
Figure 3.6: Localization of KAT1 depends on the number of acidic amino acids in the analysed motif. A-H: HEK293 cells expressing fusion products of wildtype KAT1 or one of the KAT1 mutants as indicated in the picture. KAT1 wildtype (A) is localised in the plasma membrane and in intracellular compartment of the cell. A very similar expression pattern is found in the mutants where only one of the acidic amino acids is mutated to alanine (C, E and G). Mutants with two or all three acidic amino acids mutated to alanine are restricted to the perinuclear region of the ER (B, D, F and H). Scale bars correspond to 10 µm.

3.3.3 Rescue of ER export mutant by heterotetramerisation with wildtype KAT1

KAT1 belongs to the shaker-type K^+ channel family which functions as homo- or heterotetramers. Tetramerization is supposed to already occur in the ER. Previous investigations on trafficking of mammalian K_{ir} channels which also function as tetramers have demonstrated that not all subunits of heterotetramers need to contain ER export signals in order to ensure efficient transport to the plasma membrane (Ma *et al.,* 2001). To analyse the effect of heterotetramerisation on targeting of ER export mutants of KAT1, fusions of KAT1 and KAT1^{DlaAa} with GFP and RFP were constructed.

Coexpression of both wildtype KAT1 fusion constructs or of both ER export mutants fused to RFP and GFP, respectively, resulted in a distribution of the channels which resembled those found upon expression of each construct on its own (Figure 3.7A and B). KAT1::RFP and KAT1::GFP were both located in the plasma membrane and in some intracellular compartments while KAT1^{DlaAa}::RFP and KAT1^{DlaAa}::GFP were retained in a part of the ER (Figure 3.7A and B).

In contrast, coexpression of the ER export mutant KAT1^{DlaAa} fused to either GFP or RFP together with the RFP or GFP fusion construct of wildtype KAT1 led to a completely different distribution of KAT1^{DlaAa}. As shown in a representative example in Figure 3.7C and D the ER export mutant was no longer retained in part of the ER but showed the same distribution as wildtype KAT1 including localisation at the plasma membrane. This implies that plasma membrane localisation of the ER export mutant could be rescued via heterotetramerisation with subunits that do contain an ER export signal. More generally, the results suggest that subunits, which are retained in the ER as homotetramers, can be targeted to the plasma membrane via heterotetramerisation with subunits that do contain an ER export signal. The physiological importance of such a mechanism is revealed by investigations of AtKC1, which is a member of the K^+ inward rectifying family from *Arabidopsis thaliana*. AtKC1 is only targeted to the plasma membrane when coexpressed with other members of the K^+ channel family (Reintanz *et al.*, 2002, Pilot *et al.*, 2003, Duby *et al.,* 2008). When expressed on its own, AtKC1 localised to the ER (Duby *et al.*, 2008). This conditional targeting of AtKC1 most likely plays an important role in the regulation of the physiological activity of other K^+ channel subunits in *Arabidopsis thaliana* (Duby *et al.,* 2008).

In conclusion, the results implicate that ER export signals may not only play a role in regulating the number of channels in the plasma membrane but also affect the subunit composition of ion channels in the plasma membrane.
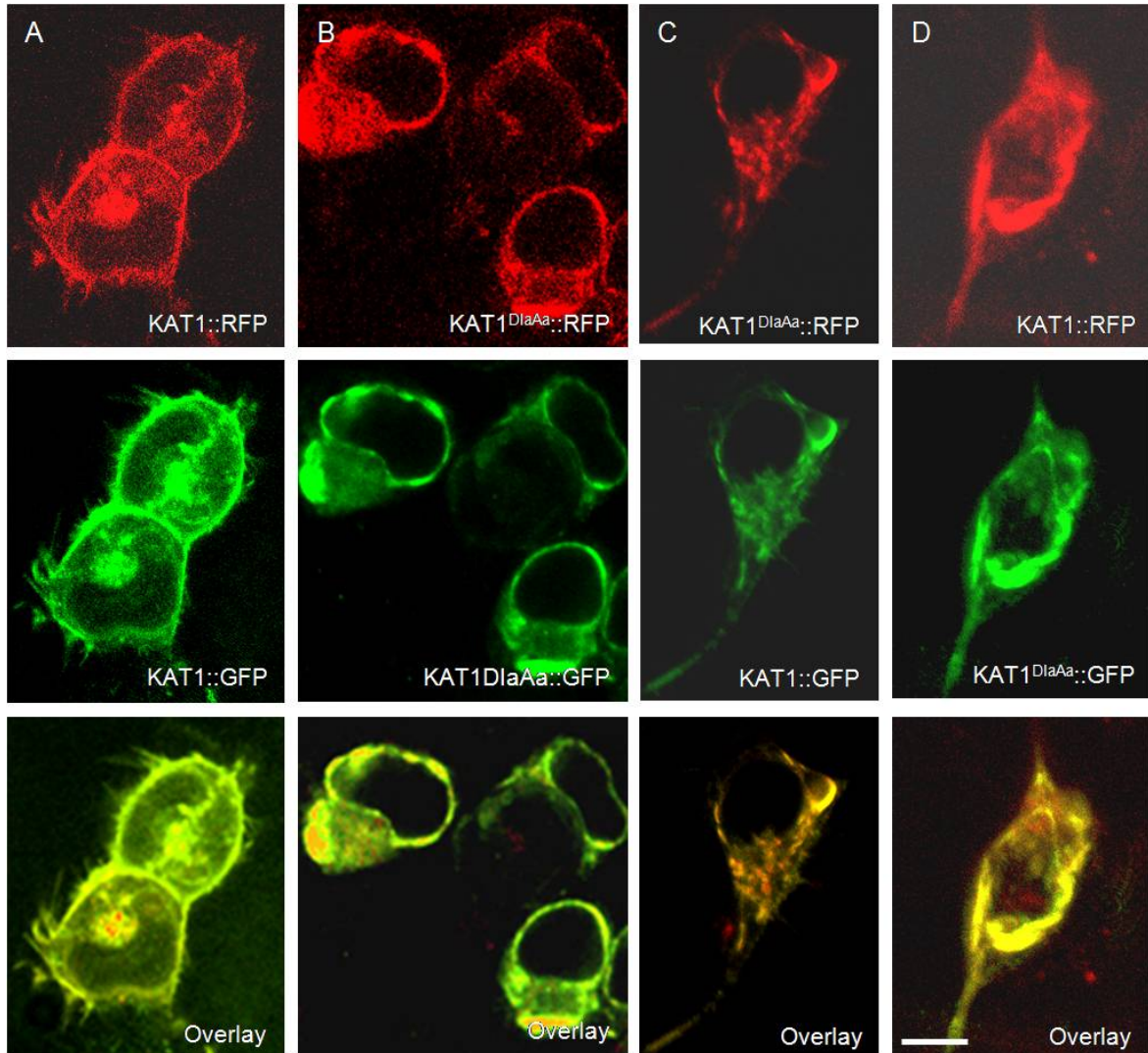
Figure 3.7: Retention of the mutant KAT1^{DIaAa} in the ER can be rescued by coexpression with wildtype KAT1. A: Coexpression of KAT1 fused to either RFP or GFP in HEK293 cells. Both construct colocalise in the plasma membrane and internal compartments. B: Coexpression of the ER export mutant KAT1^{DIaAa} fused to either RFP or GFP. Both constructs localised in the ER. C and D: Wildtype KAT1 and KAT1^{DIaAa} fused to GFP or RFP coexpressed with the respective other fluorophore in HEK293 cells. All constructs localised in the plasma membrane and in internal compartments. Scale bar corresponds to 10 µm.

3.4 Conclusion

Our results show that the previously identified diacidic ER export motif (DxE) of KAT1 more likely corresponds to a triacidic ER export motif (DxDxE). It was demonstrated that all three acidic residues have a similar impact on the function of the motif and that their effect on ER retention is almost additive. This implies that all acidic amino acids interact with a binding site of Sec24. However it is still unknown, which site of Sec24 this interaction occurs at and which role each acidic amino acid plays in this process. Coexpression analysis of wildtype and ER export mutant of KAT1 suggests that heterotetramers of wildtype and mutant KAT1 are still recognised by Sec24 for sorting into COPII vesicles. How many functional motifs have to be present in the heterotetramer for effective ER export remains to be shown. Overall, the results underline the importance of a triacidic ER export motif for density and subunit composition of channels in the plasma membrane.

3.5 Methods

3.5.1 Construction of pEGFPN$_2$KAT1

For expression as C-terminal GFP or RFP fusion in HEK293 cells the KAT1 gene was cloned into the *XhoI* and *BamHI* sites of the pEGFPN$_2$ or pmRFPN$_1$ vector (Clontech) in frame with the downstream GFP or RFP gene. Mutations in the DIDAE motif of KAT1 were achieved by PCR based site directed mutagenesis (QuikChange site directed mutagenesis kit; Stratagene) and confirmed by sequencing. The expression vectors pEGFPN$_2$KAT1 and pCB6KAT1 served as templates. The plasmids were cloned into *E*. coli/DH5α and isolated with Qiagen high speed Midi-Kit (Qiagen) for cell transfection.

3.5.2 Cell culture and transfection of HEK293 cells

HEK293 cells were grown at 37°C and 5% CO_2. For transient expression of KAT1 and KAT1 mutants HEK293 cells were transfected with 1 µg of $pEGFPN_2KAT1$ or $pmRFPN_1KAT1$ using the liposomal transfection reagent Transfektin (Biorad) according to manufacturer's instructions.

3.5.3 Patch clamp measurements

Recordings of membrane conductance in HEK293 cells were performed in a standard whole cell patch clamp experiment as described as described previously (Hertel *et al.,* 2005).

3.5.4 CLSM

HEK293 cells were investigated approximately 24 h after transfection with a Leica TCS SP spectral confocal microscope (Leica Microsystems). Images were acquired with an HCX PL APO 63×/1.2w objective. GFP was excited with the 488 nm line of a 25 mW argon laser and fluorescence was detected at 505-530 nm. RFP was excited with a 543 nm line of a helium neon laser and emission collected at 600–630 nm. For plasma membrane staining, cells were incubated in 10 µM FM4-64 (Invitrogen) before imaging. For ER staining cell were incubated in 10 µM ER Tracker red (Invitrogen). Images and colocalisation were analysed with ImageJ software (National Institutes of Health) and the Leica Confocal Software 2.00 (LCS, Leica Microsystems)

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Chapter 4 Interaction of the K⁺ channel KAT1 with the COPII coat component Sec24 depends on a diacidic ER export motif
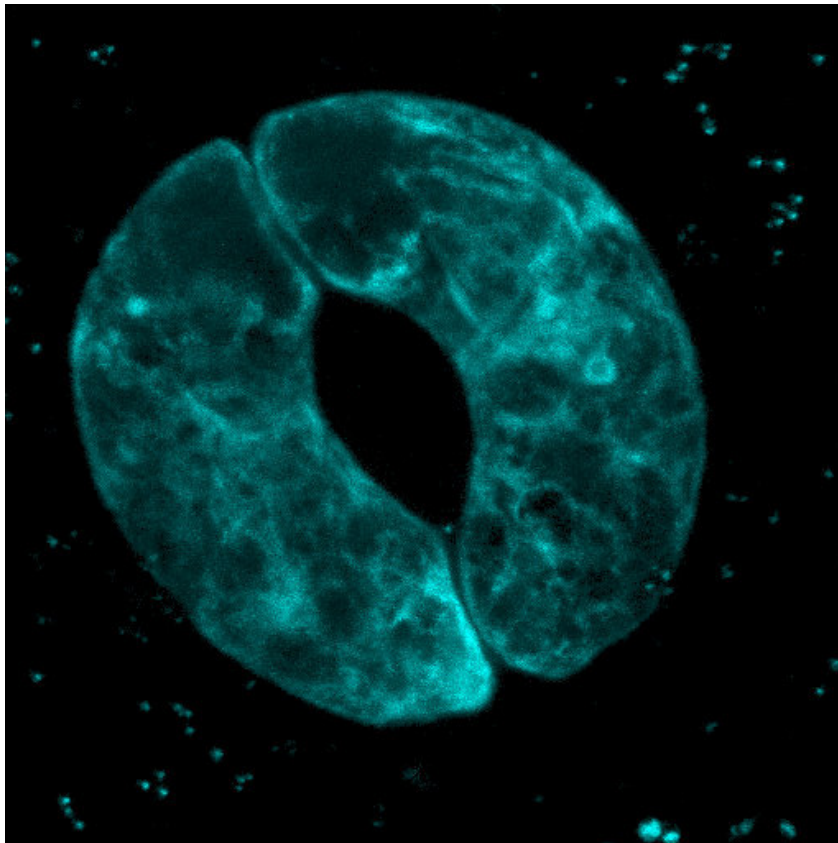
Maximum projection of a guard cell of *Vicia faba* transfected with the ER marker HDEL::CFP via particle bombardment. The network like structure is well visible in addition to the enlarged perinuclear ER with a ring like extension in the right cell due to overexpression of a KAT1 ER export mutant.

4.1 Abstract

The correct functioning of ion channels depends not only on the control of their activity but also on the regulation of their number in the membrane. For example, it has been proposed that the density of the plant potassium (K^+) channel KAT1 may be adjusted by controlling the export from its site of synthesis, the endoplasmic reticulum (ER). Efficient transport of the K^+ channel to the plasma membrane was found to depend on a diacidic ER export signals in the C-terminus of the protein. Studies in yeast and mammals indicate that diacidic ER export motifs are essential for enrichment of proteins into ER derived coat protein complex II (COPII) vesicles and are recognised by Sec24, a component of the COPII coat. To investigate whether similar mechanisms also exist in plants the interaction of KAT1 with Sec24 *in vivo* was analysed using FRET (fluorescence resonance energy transfer) measurements in *Vicia faba* guard cells. These measurements revealed a FRET signal between KAT1 and Sec24 fused to the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP), respectively, indicating an interaction between KAT1 and Sec24. The FRET signal did only occur in the perinuclear region of the ER and was dependent on the diacidic ER export motif of KAT1. Together the results point to a highly conserved mechanism for ER export of KAT1 whereby the channel is recruited into COPII vesicles via binding of the diacidic motif to Sec24.

4.2 Introduction

Ion channels are integral membrane proteins which allow the movement of ions across cell membranes. They play an important role in physiological functioning of cells and are key components of multiple signal transduction pathways. Previous investigations on the regulation of ion channels have mainly focused on the control of channel activity. However, recent work on membrane transport has highlighted the importance of the control of ion channel trafficking in adjusting channel density and thus membrane conductance (Heusser and Schwappach, 2005; Sivaprasadarao *et al*., 2007). In order to understand how channel density is adjusted it is important to identify the molecular mechanisms involved in every

step along the secretory and endocytotic pathway of ion channels. Recently, particular attention has been paid to the first station of the secretory pathway where ion channels are transported from the endoplasmic reticulum (ER) to the Golgi apparatus (Ma and Jan, 2002). Cargo transport from the ER to the Golgi apparatus is mediated by coat protein complex II (COPII) vesicles that bud from the ER. For the efficient ER export of proteins their recruitment into COPII vesicles is essential. This process involves specific amino acid motifs so called ER export signals (Otte and Barlowe, 2004). For a number of plasma membrane ion channels including the plant K^+ channel KAT1 diacidic D/ExD/E motifs have been shown to function as ER export signals (Ma *et al*., 2001; Stockklausner *et al*., 2001; Wang *et al*., 2004; Mikosch *et al*., 2006; Zuzarte *et al*., 2007). Mutation of these diacidic motifs resulted in a strong reduction of the channel in the plasma membrane and an accumulation in the ER. Even though regulation of ER export of ion channels is now widely recognised as a mechanism for adjusting the composition and density of channels in the plasma membrane detailed information about the underlying mechanism are still very limited. In general, ER export signals are thought to be recognised by distinct cargo binding sites of Sec24, a component of the COPII coat. In yeast the B-site of Sec24 has been identified as a binding site of the diacidic motif of the vesicular stomatitis virus glycoprotein (VSVG) and the yeast membrane protein Sys1 (Mossessova *et al.,* 2003; Miller *et al.,* 2003). So far, information about the recognition of diacidic ER export motifs of ion channels relies on *in silico* analysis. Using homology modelling of the CFTR channel Wang *et al*. (2004) have illustrated the ability of diacidic motif of CFTR to insert into the binding pocket at the B-site of yeast Sec24.

To further investigate the mechanism of COPII-dependent ER export of ion channels the interaction of a plant Sec24 and the plant ion channel KAT1 *in vivo* was analysed using FRET (fluorescence resonance energy transfer) measurements in *Vicia faba* guard cells. The interaction between KAT1 and Sec24 fused to the fluorophores CFP and YFP, respectively, could be demonstrated. This interaction was restricted to subdomains of the ER and was dependent on the diacidic ER export motif of KAT1. These results provide new insights into the mechanisms that regulate trafficking of ion channels from the plant ER and consequently affect the density of K^+ channels in the plasma membrane.

4.3 Results

4.3.1 FRET by acceptor bleaching provides a suitable tool for analysis of protein-protein interaction in plant cells in vivo

To study the interaction between KAT1 and Sec24 in guard cells, it was decided to coexpress KAT1::CFP and the known COPII marker, YFP::Sec24 (Stefano *et al*., 2006) to perform FRET (fluorescence resonance energy transfer) measurements. FRET is a suitable tool for establishing *in vivo* the interaction of two proteins tagged with a donor and an acceptor fluorophore (Karpova *et al*., 2003, Kluge *et al*., 2004). The two fluorophores CFP and YFP are the most widely used FRET pairs. In case of an interaction between KAT1 and Sec24 excitation of CFP should lead to an energy transfer between the donor fluorophore CFP and the acceptor fluorophore YFP resulting in an increase of YFP emission (Periasamy *et al*., 1999).

Figure 4.1: FRET between KAT1::CFP and KAT1::YFP in *Vicia faba* guard cells. *Vicia faba* guard cells coexpressing KAT1::CFP (A) and KAT1::YFP (B). Both signals are localised in the plasma membrane. The CFP emission (C) was measured over 5 scan cycles before and after bleaching (red arrowhead). CFP was excited at 458 nm and detected between 470 and 500 nm. Acceptor bleaching was performed over 15 scan cycles with 514 nm laser line at maximum intensity. YFP emission was recorded between 530 and 550 nm (D). Scale bar 10 µm.
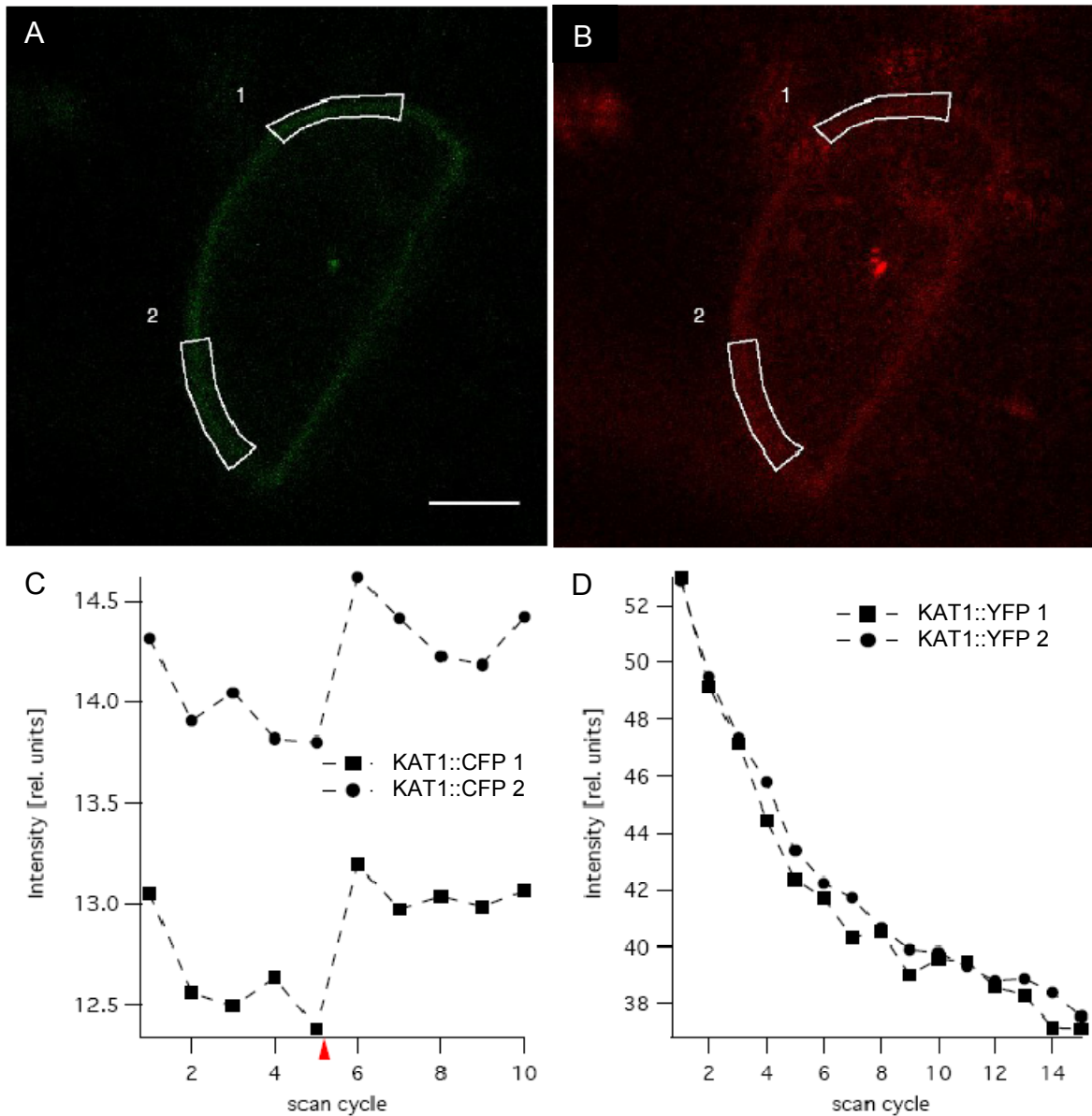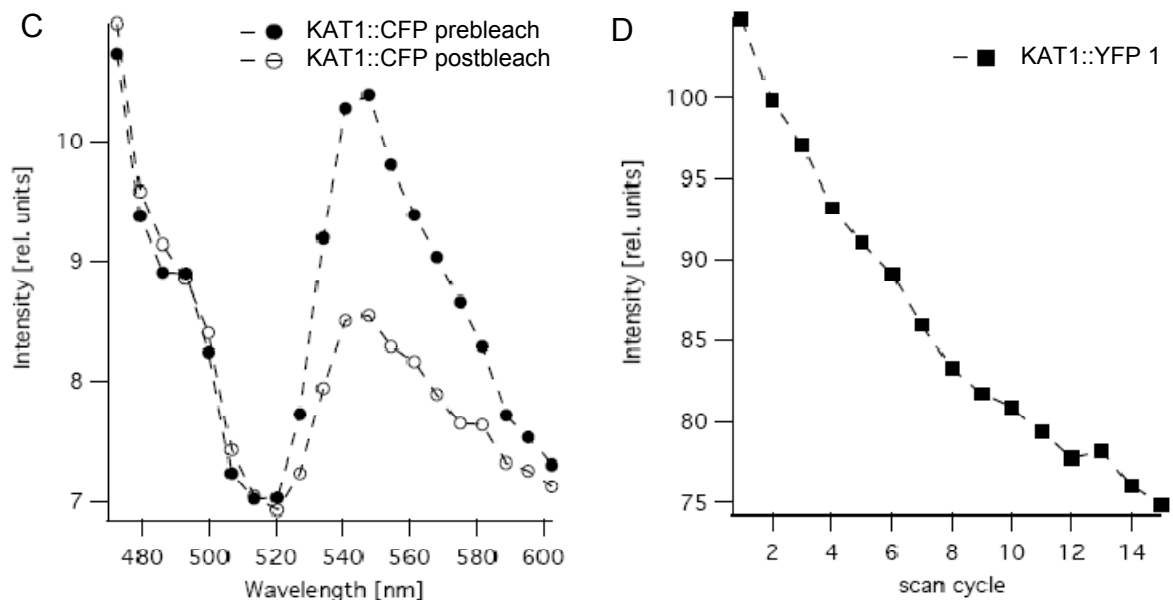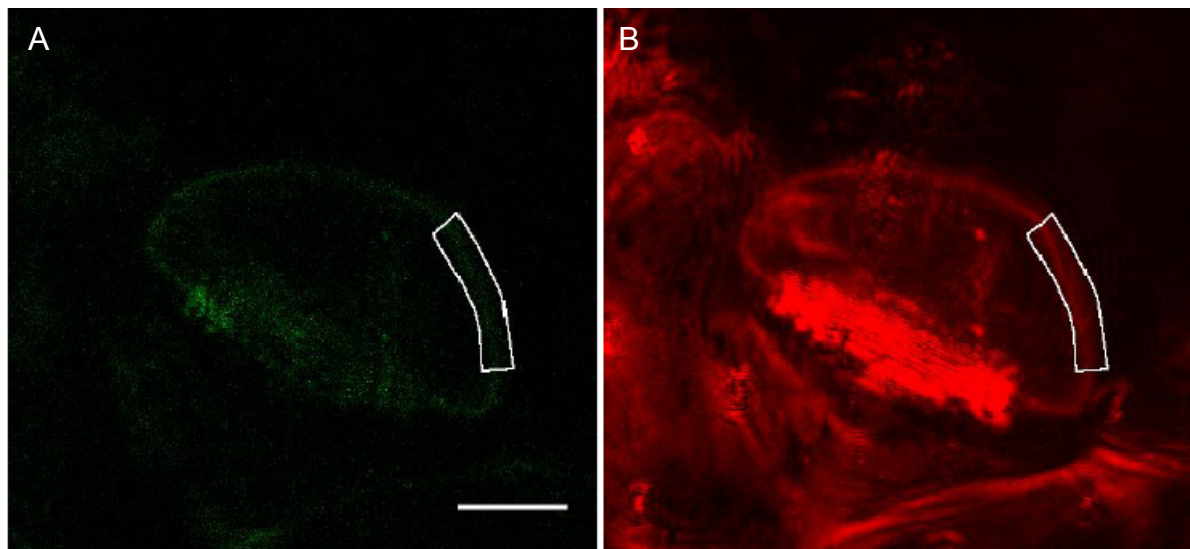
Figure 4.2: Spectral-FRET between KAT1::CFP and KAT1::YFP in *Vicia faba* guard cells. Guard cells coexpressing KAT1::CFP (A) and KAT1::YFP (B). CFP was excited at 458 nm and detected between 470 and 600 nm. The spectrum was measured before and after acceptor bleaching (C). Between the spectra the acceptor was bleached over 15 scan cycles with 514 nm excitation at maximum intensity and its emission was recorded between 530 and 600 nm (D). Scale bar 10 μm.

To verify the reliability of FRET measurements the extent of cross-talk caused by excitation of YFP using settings for CFP excitation, and the amount of bleed-through of CFP fluorescence into the YFP channel were estimated. For cross-talk analysis *Vicia faba* guard cells expressing YFP::Sec24 were excited with 458 nm argon laser line for CFP excitation and the emitted YFP fluorescence was filtered at 530-550 nm. These measurements revealed a high amount of YFP emission after excitation at 458 nm (Figure 4.8). In addition, bleed-through led to the detection of a considerable amount of CFP emission (about 30 %) in the YFP

channel (Figure 4.9). These results demonstrate that it is not possible to sufficiently separate the CFP and YFP signal under the given experimental conditions. Reliable FRET measurements could therefore not be carried out by simply exciting CFP and detecting the CFP and YFP emission. Therefore, it was decided to use FRET by acceptor photobleaching. To demonstrate that protein-protein interaction could indeed be detected via this method *Vicia faba* guard cells cotransfected with KAT1::CFP and KAT1::YFP were analysed. KAT1 belongs to the shaker-like K^+ channel family. The members of this family function as tetramers. Due to the stochastic nature of the transfection process, guard cells expressing both KAT1::CFP and KAT1::YFP could be found. In these conditions, KAT1 tetramers containing both fluorophores should form and allow an energy transfer from CFP to YFP. Bleaching of YFP should consequently lead to an increase of CFP fluorescence due to a reduced energy transfer between CFP and YFP.

In transfected guard cells KAT1 was located predominantly in the plasma membrane, as expected. (Figure 4.1A, B). For FRET analysis emission of the donor KAT1::CFP was measured in selected ROIs (regions of interest) before and after photobleaching of the acceptor KAT1::YFP (Figure 4.1C). Cells were bleached with 514 nm laser line at maximum intensity for 15 scan cycles (Figure 4.1d). After bleaching a steep rise of donor emission by about 7 % could be detected (Figure 4.1C) providing direct evidence for an energy transfer between CFP and YFP.

The energy transfer between CFP and YFP was also analysed by spectral-FRET measurements. Spectral-FRET provides a more accurate mean to determine FRET because it is not affected by certain background components (Goedhart *et al.*, 2000). In addition, the spectrum ensures that indeed the fluorescence ratios determined before and after bleaching reflect YFP to CFP fluorescence emission ratios and not other autofluorescent components.

The emission spectrum of guard cells coexpressing KAT1::CFP (Figure 4.2A) and KAT1::YFP (Figure 4.2B) showed two peaks (Figure 4.2C). The first peak corresponds to the emission maximum of CFP (475 nm) and the second peak to YFP emission (540 nm) resulting from an excitation of YFP via energy transfer from CFP to YFP. Upon bleaching of YFP the ratio of the fluorescence emitted at 475 nm (CFP) to that emitted at 540 nm (YFP) (475/540 ratio) changed from 0.92

to 1.13. This change can best be explained by an increase in CFP fluorescence due to a reduced energy transfer from CFP to YFP as a consequence of YFP bleaching. As a control, it was found that guard cells expressing KAT1::CFP alone did not display any unspecific FRET signals (Figure 4.10). Together these measurements demonstrate that the experimental set-up allows the recording of FRET signals that signify protein-protein interaction events such as the interaction of KAT1 in a tetramer assembly.

4.3.2 FRET measurements indicate interaction between KAT1 and Sec24 at ER export sites of *Vicia faba* guard cells

To determine whether KAT1 interacts with Sec24 FRET analysis of *Vicia faba* guard cells cotransfected with KAT1::CFP and YFP::Sec24 were performed via particle bombardment. Figure 4.3 shows a guard cell expressing KAT1::CFP (Figure 4.3A) and YFP::Sec24 (Figure 4.3B). Again, KAT1 was located predominantly in the plasma membrane. In addition the K^+ channel accumulated in restricted areas inside the cell mainly around the nucleus. These areas have previously been shown to correspond to the ER (Mikosch *et al*., 2006). The amount of intracellular localised KAT1 is generally very variable. For analysis of the interaction between KAT1 and Sec24 guard cells which show a clear staining of the ER were chosen as an interaction with Sec24 should occur on the surface of this organelle. YFP::Sec24 was visible throughout the cytosol and in structures (Figure 4.3B) which correspond to ER export sites (ERES) (Stefano *et al*., 2006; Hanton *et al*., 2007). ERES are subdomains of the ER where cargo is thought to be packed into COPII vesicle for further transport to the Golgi apparatus. For FRET analysis emission of the donor KAT1::CFP was measured before and after bleaching of YFP::Sec24. At sites of interaction between KAT1::CFP and YFP::Sec24 bleaching should result in an increase of CFP fluorescence due to a reduced energy transfer between the donor CFP and the acceptor YFP, as shown in Figure 4.1. Figure 4.3 shows a representative example of the change in CFP fluorescence after bleaching of YFP. A clear rise in the CFP emission could be detected in the ER around the nucleus (Figure 4.3A, ROI 1 and Figure 4.3C). Similar results were found in all other cells analysed (n=7) suggesting that an interaction between KAT1 and Sec24 occurs at the ERES located in the

perinuclear ER. This supports a previous hypothesis that this region of the ER may be implicated in the export of KAT1 (Mikosch *et al*., 2006). As a control, the emission of a FRET signal from the plasma membrane (Figure 4.3A, ROI 3 and 4) was tested as well as from an area just below the plasma membrane (Figure 4.3A, ROI 2). It was expected that a FRET signal would not be generated as Sec24 should not interact with protein cargo in distal compartments of the secretory pathway. Consistent with this hypothesis, emission of CFP recorded from these areas did not change after photobleaching (Figure 4.3C and E) suggesting that KAT1 does not interact with Sec24 at or near the plasma membrane.

Figure 4.3: FRET between KAT1::CFP and YFP::Sec24 in *Vicia faba* guard cells. Guard cell coexpressing KAT1::CFP (A) and YFP::Sec24 (B). The CFP emission (C, E) was measured over 5 scan cycles before and after YFP bleaching (red arrowhead). CFP was excited at 458 nm and detected between 470 and 500 nm. The intensities were taken from four different ROIs represented by the highlighted areas in (A) and (B). The ROIs correspond to the perinuclear region of the ER (ROI 1) a compartment close to the plasma membrane (ROI 2) and the plasma membrane (ROI 3 and ROI 4). Acceptor bleaching was performed over 15 scan cycles with 514 nm laser line at maximum intensity. YFP emission was recorded between 530 and 550 nm (D, F). Arrowheads in (B) represent ERES. Scale bar 10 µm
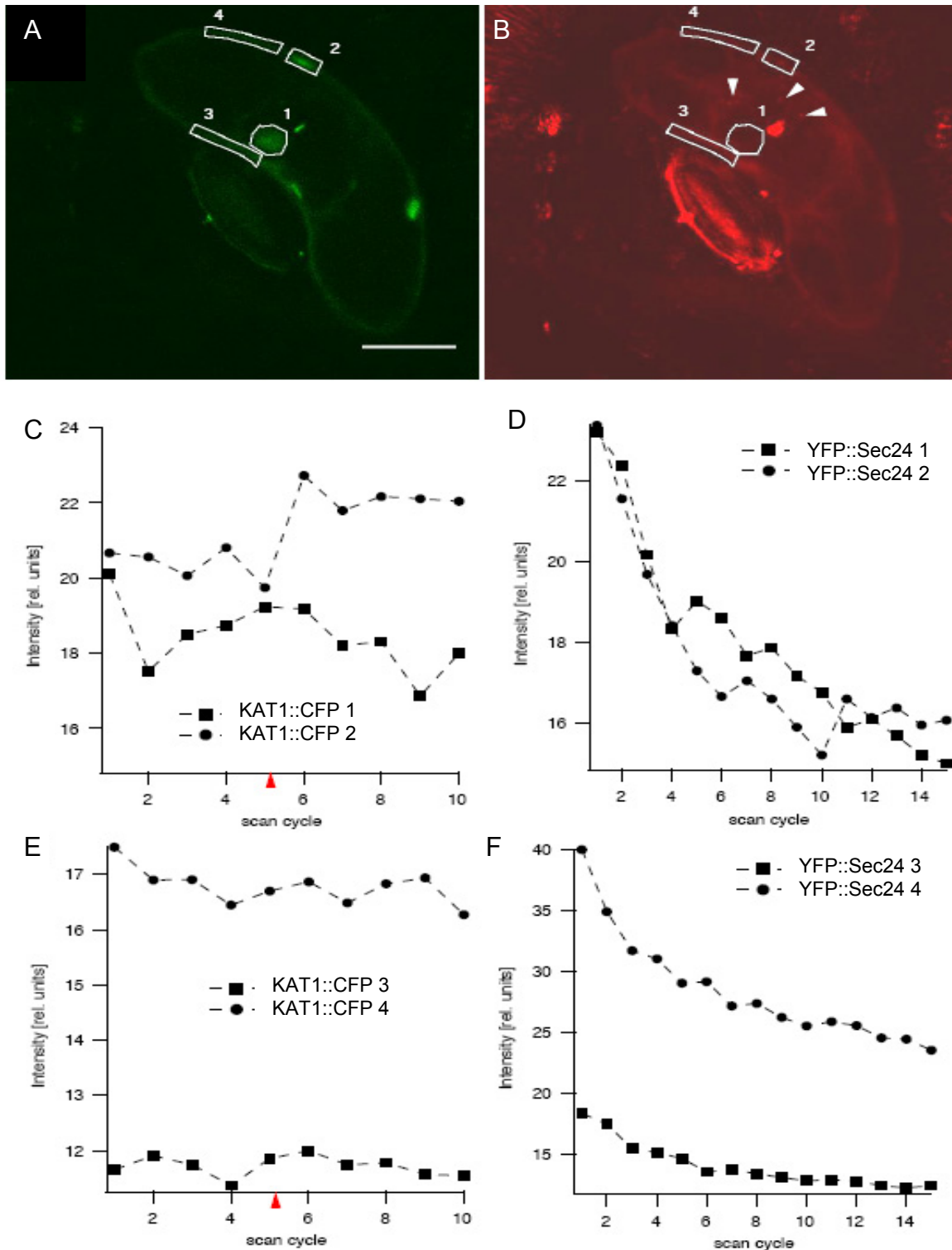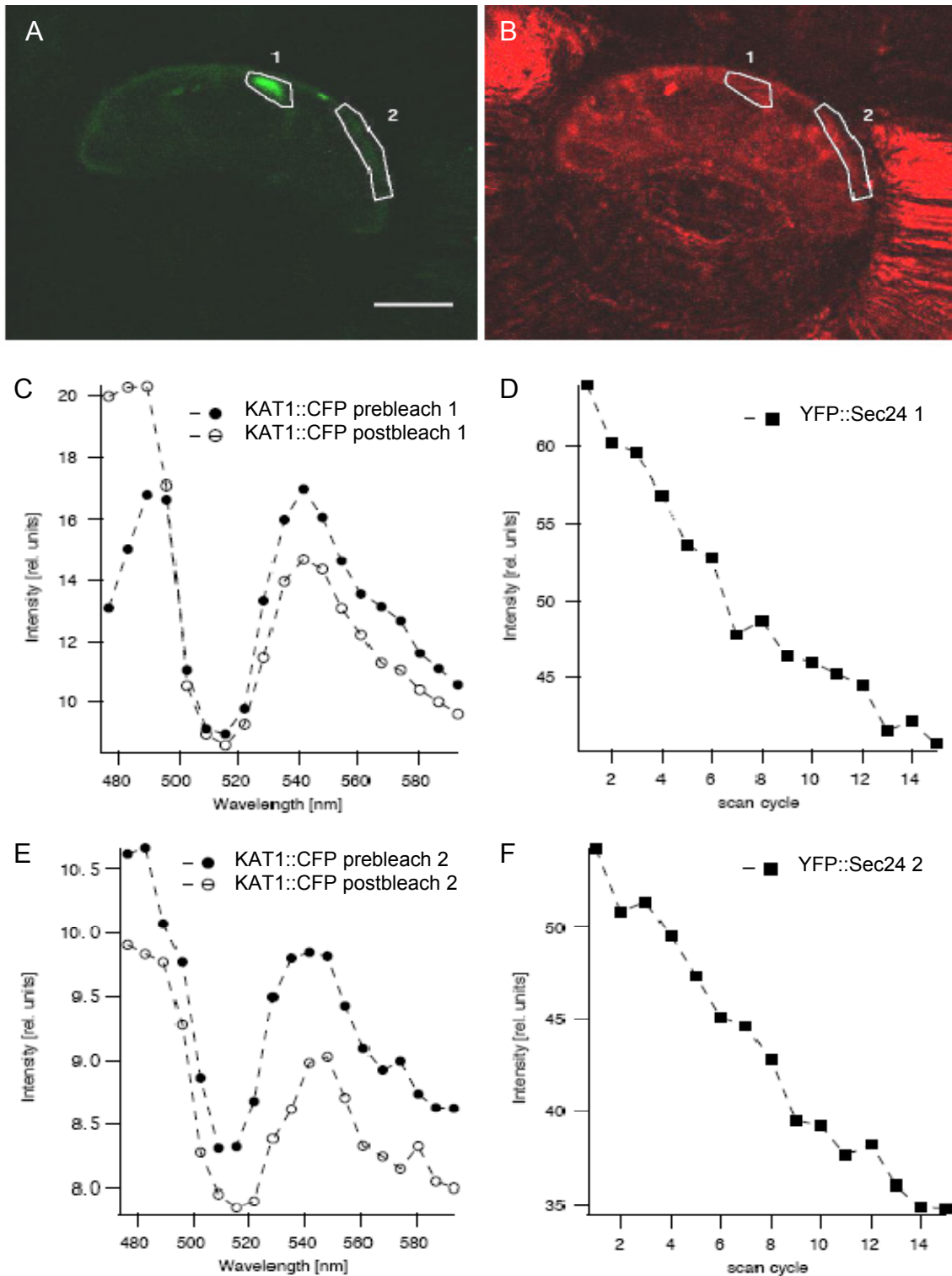
Figure 4.4: Spectral-FRET between KAT1::CFP and YFP::Sec24 in *Vicia faba* guard cells. Guard cell coexpressing KAT1::CFP (A) and YFP::Sec24 (B). CFP was excited at 458 nm and detected between 470 and 600 nm. The spectrum was measured before and after acceptor bleaching (C, E). The intensities were taken from ROI 1 (perinuclear region of the ER) and ROI 2 (plasma membrane) represented by the highlighted areas in (A) and (B). Between the spectra the acceptor was bleached over 15 scan cycles with 514 nm excitation at maximum intensity and its emission was recorded between 530 and 600 nm (D, F). Scale bar 10 µm.

The results were confirmed by spectral-FRET measurements. The CFP emission spectrum of a guard cell coexpressing KAT1::CFP (Figure 4.4A) and YFP::Sec24 (Figure 4.4B) is shown in Figure 4.4C. The ratio of the two peaks recorded at the perinuclear region (Figure 4.4A, ROI 1) was 1.1 before bleaching (Figure 4.4C, closed symbols). After acceptor bleaching (Figure 4.4D) the CFP maximum increased and the FRET peak decreased. The ratio of the two maxima changed to 1.43. This change can be explained by a reduced energy transfer from CFP to YFP and further indicates a close contact between KAT1::CFP and YFP::Sec24. It therefore supports the first FRET results (Figure 4.3) and argues for an interaction between KAT1 and Sec24 at ERES. As a control, the analysis was also performed at the plasma membrane (Figure 4.4A, ROI 2). In this region the emission spectrum before bleaching was similar to the one measured at the perinuclear region (Figure 4.4E, closed symbols). However, YFP bleaching (Figure 4.5F) did not result in any significant change in the ration between the CFP and the FRET peak (1.12 before bleaching, 1.04 after bleaching). After YFP bleaching the overall emission was reduced by 9.3% (Figure 4.4E, open symbols) as a consequence of photobleaching by the 458 nm laser line during scanning. A reduction of the emission without any significant change of the ratio between the two maxima clearly shows that there is no interaction between KAT1 and Sec24 at the plasma membrane. The spectral-FRET results therefore confirm the FRET measurements described above. Together they demonstrate that KAT1 interacts with Sec24 *in vivo* and that this interaction is restricted to perinuclear subdomains of the ER which most likely correspond to ERES. This implies that the function of diacidic motifs in ER export of ion channels is accomplished by direct interaction with Sec24.

4.3.3 FRET between KAT1 and Sec24 depends on the diacidic motif

It has been shown earlier that mutation of the diacidic motif (I) in the C-terminus of KAT1 leads to retention of the mutant channel KAT1[(I)] in the ER (Mikosch *et al*., 2006). As diacidic motifs are believe to interact directly with Sec24 in yeast (Votsmeier and Gallwitz, 2001) this retention may be caused by an insufficient interaction of KAT1 with Sec24. To test this hypothesis FRET analysis was performed in *Vicia faba* guard cells coexpressing the ER export mutant KAT1[(I)]::CFP and YFP::Sec24. In the ER around the nucleus the labelling pattern of wildtype KAT1 and KAT1[(I)] was very similar. However, differently from wildtype KAT1 the ER export mutant exhibits no staining of the plasma membrane (Figure 4.5a) which is consistent with previous observations (Mikosch *et al*., 2006). FRET measurements showed that in contrast to cells expressing wildtype KAT1, cells expressing the ER export mutant revealed no increase in CFP emission after acceptor bleaching (Figure 4.5c). This evidence suggests that even though KAT1[(I)] is localised to the same regions of the ER as KAT1 wildtype, it does not interact with Sec24.

Figure 4.5: FRET between the ER export mutant KAT1(I)::CFP and YFP::Sec24 in *Vicia faba* guard cells. Guard cell co-expressing KAT1(I)::CFP (A) and YFP::Sec24 (B). The CFP emission (C) was measured over 5 scan cycles before and after YFP bleaching (red arrowhead). CFP was excited at 458 nm and detected between 470 and 500 nm. The intensities were taken from ROI 1 and ROI 2 represented by the highlighted areas in (A) and (B). Acceptor bleaching was performed over 15 scan cycles with 514 nm laser line at maximum intensity. YFP emission was recorded between 530 and 550 nm (D). Scale bar 10 μm
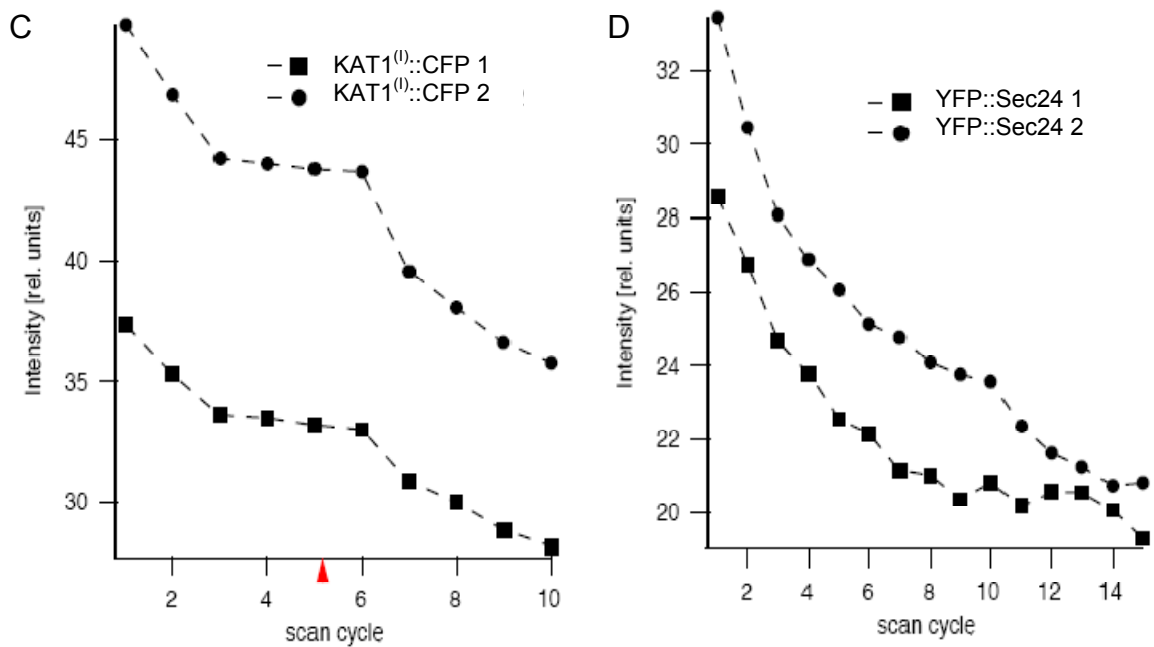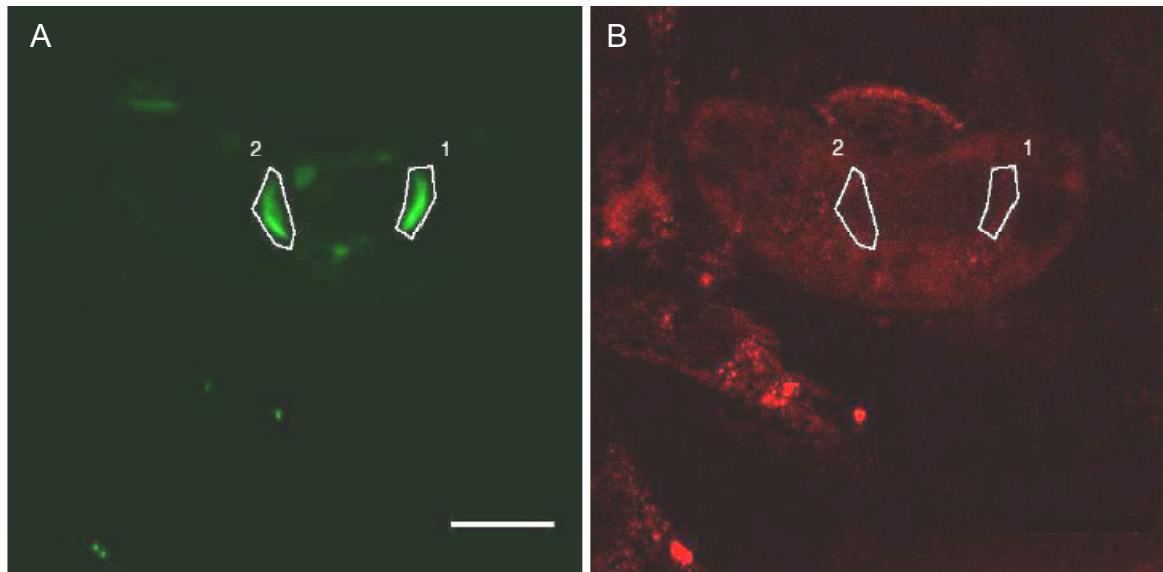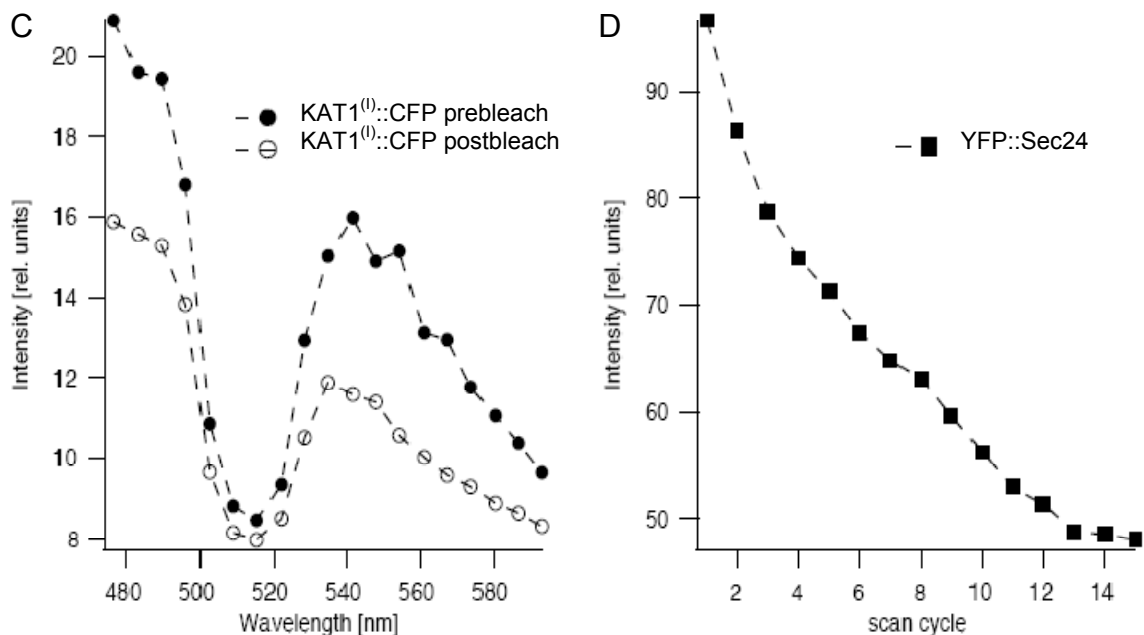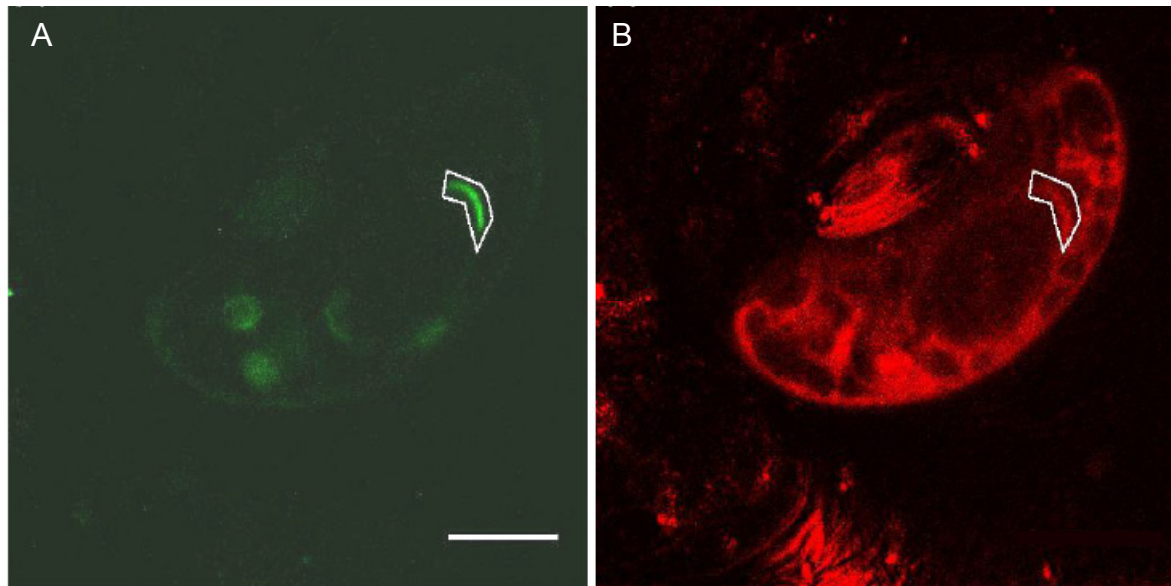
Figure 4.6: Spectral-FRET between the ER export mutant KAT1[(I)] and Sec24 in *Vicia faba* guard cells. Guard cell coexpressing KAT1::CFP (A) and YFP::Sec24 (B). CFP was excited at 458 nm and detected between 470 and 600 nm. The spectrum was measured before and after acceptor bleaching (C). The intensities were taken from the ROI represented by the highlighted area in (A) and (B). Between the spectra the acceptor was bleached over 15 scan cycles with 514 nm excitation at maximum intensity and its emission was recorded between 530 and 600 nm (D). Scale bar 10 µm.

The interaction of Sec24 with the mutant channel was also analysed by spectral-FRET as described above for the KAT1 wildtype. Figure 4.6 shows a guard cell coexpressing KAT1[(I)]::CFP (Figure 4.6A) and YFP::Sec24 (Figure 4.6B). Again KAT1[(I)] was mainly located in internal compartments. The emission spectrum recorded from the perinuclear region before photobleaching of YFP (Figure 4.6C,

closed symbols) was similar to the one measured in guard cell expressing KAT1 wildtype. After bleaching of YFP the overall emission was reduced by 18.9 % (Figure 4.6C, open symbols). The ratio between the two maxima did not change significantly after bleaching (1.34 before bleaching, 1.31 after bleaching). This implies that KAT1[(l)] did not interact with Sec24 and that the interaction between KAT1 and Sec24 depends on the first diacidic motif in the C-terminus of KAT1. The lack of interaction of KAT1[(l)] and Sec24 most likely leads to the retention of the ER export mutant in the ER. Together the results support the hypothesis that the interaction between the channel protein and Sec24 depends on the diacidic ER export motif and that this interaction is essential for ER export.

4.4 Discussion

4.4.1 The mechanism of cargo recruitment into ER derived COPII carriers is highly conserved among eukaryotes

For the efficient ER export of proteins their recruitment into ER derived COPII carriers is essential. This process involves specific amino acid motifs so called ER export signals (Otte and Barlowe, 2004), which are recognised by components of the COPII coat. The first signal found to be involved in selection of cargo into COPII vesicles was the diacidic ER export motif of the VSVG (Nishimura and Balch, 1997). Since then diacidic signals have been proven to be necessary for export of a number of proteins in yeast and mammals (see for example Votsmeier and Gallwitz, 2001; Miller *et al*., 2003; Ma *et al*., 2001; Wang *et al*., 2004). Among these proteins are several ion channels from different families. Current knowledge of protein export in plant cells indicates that for type I, II and multispanning membrane proteins such as the K$^+$ channel KAT1 the presence of intact diacidic motifs is also crucial for ER export (Hanton *et al*., 2005; Mikosch *et al*., 2006). Together this underlines the importance of the diacidic signal sequence for trafficking of proteins in general and for efficient targeting of ion channels to the plasma membrane in particular. However, how the function of these signals is accomplished in plants has yet to be demonstrated. Moreover, information about the mechanism of ER export of ion channels is limited to results from *in silico* analysis (Wang *et al*., 2004). In this study it was aimed to advance the

understanding of the mechanisms that lead to ER export of ion channels in order to improve our knowledge of the regulation of channel density and trafficking of plant membrane proteins in general. Using an *in vivo* approach based on FRET analyses it could be shown that KAT1 interacts with the COPII coat component Sec24. Mutants of KAT1 bearing a disrupted ER export signal failed to interact with Sec24 and to be exported from the ER. Taken together these results suggest that KAT1 is exported via a COPII-mediated mechanism and that the interaction of KAT1 with COPII proteins is mediated by the diacidic motif DxE. Therefore, these results provide evidence for a direct role of a DxE signal of ion channels in COPII coat recruitment *in vivo*. They furthermore advance our understanding of COPII regulation of ER export in plants. To date, evidence of COPII mediated ER export in plants has been based on the interference of the pathway using dominant negative mutants of the small regulatory GTPase Sar1 or overexpression of the Sar1-GAP, Sec12 (Takeuchi *et al.*, 2000; Phillipson *et al.*, 2001; daSilva *et al.*, 2004; Yang *et al.*, 2005). This approach has provided additional insights in the mechanisms of COPII mediated transport in plants by showing that a productive Sec24 cargo interaction via diacidic motifs leads to COPII mediated export from the ER. In particular, these data argue that the diacidic motif in the C-terminus of KAT1 provides the binding site for Sec24. It is known that the B-site of yeast Sec24 binds to the Lxx-L/M-E motifs in yeast SNAREs Bet1 and Sed5 as well as to the diacidic motif in Sys1 (Mossessova *et al.*, 2003). Furthermore, homology modelling of the mammalian ion channel CFTR suggests that the B-site is also important for binding of ion channels to Sec24 (Wang *et al.*, 2004). The mechanism of cargo binding by Sec24 in plants is still unknown. There are three homologous of Sec24 in plants (Robinson *et al.*, 2007) compared to four in humans and two in yeast (Pagano *et al.*, 1999). Sequence comparison of the B-site of Sec24 isoforms from plants, yeast and humans revealed a high homology (Figure 4.7). In particular, the amino acids which have been demonstrated to be crucial for binding of cargo to the B-site in yeast (Miller *et al.*, 2003) are highly conserved. Whether these domains are important for establishment of an interaction of Sec24 with the DxE motif of KAT1 has yet to be shown. It seems however highly plausible considering the likelihood of conservation of ER export mechanisms shown in our study and in previous investigations of ER export of KAT1 (Mikosch *et al.*, 2006).

```
Sec24C    427 ESGPLRCNRCKAYMCPFM 444   758 FDAVMRVRTSTGIRA 772   816 QCALLYTSCAG 826
Sec24D    418 ESGPVRCNRCKAYMCPFM 435   749 FDAIMRVRTSTGFRA 763   807 QCAVLYTTISG 817
Sec24A    425 SSTIVRCRSCRTYINPFV 442   746 FEAVMRIRCTKGLSI 760   805 QSALLYTSSKG 815
Sec24B    599 SNTIVRCRSCRTYINPFV 616   921 FEAVMRIRCTKGLSM 935   980 QTALLYTSSKG 990
Sec24     225 DGLIVRCRRCRSYMNPFV 242   554 METVMRARGSTGLRM 568   612 QVAVLLSLNNS 622
Iss1      158 DGVIVRCRRCRSYMNPFV 175   510 MEAVMRVRCSTGLRA 524   568 QVSTLLTLNTG 578
At3g07100 365 STGIIRCRRCRTYVNPFV 382   687 WEAVMRIRCGKGIRF 701   745 QVALLYTASCG 755
At3g44340 427 ESGPVRCSRCKGYVNPFM 444   752 FEAVMRVRCSQGIQV 766   810 QCALLYTTISG 820
At4g32640 414 EGGPVRCSRCKGYINPFM 431   749 FEAVMRVRCSQGIQV 763   795 QCALLYTTIYG 805
Lst1      214 DGTPLRCRRCRAYANPKF 231   538 YQALLKVRCSTGLSV 552   597 QTALLYTDIDG 607
```

Figure 4.7: Sequence comparison of the B-site of Sec24 shows high homology among different species. Amino acids which have been shown to be critical for cargo binding in yeast are highlighted by boxes. Sec24A, Sec24B, Sec24C and Sec24D are from *Homo sapiens*, Sec24, Iss1 and Lst1 are from *Saccharomyces cerevisiae*, At3g07100, At3g44340 and At4g32640 are from *Arabidopsis thaliana*.

4.4.2 Export of KAT1 occurs mainly at the perinuclear region of the ER

These results show that KAT1 accumulates preferentially at the perinuclear region in addition to its localisation at the plasma membrane which is in agreement with previous findings (Mikosch *et al.*, 2006). The fact that FRET signals between YFP::Sec24 and KAT1::CFP are only generated in this region rather than in compartments located close to the plasma membrane argues that this part of the ER is export competent. This is supported by previous observations showing that inhibition of ER export of KAT1 either by inhibition of the COPII-dependent pathway or by mutation of the diacidic ER export signal led to accumulation of the channel exclusively in the perinuclear region (Mikosch *et al.*, 2006). Together the results reinforce the hypothesis that the perinuclear region of the ER is the site where KAT1 is packaged in COPII carriers to facilitate its export from the ER. Whether the entire perinuclear region is ER export competent cannot be inferred by the data. In order to resolve a FRET signal the fluorescence from a large part of this region had to be analysed. Because of working with live cells, a diffuse FRET signal from the perinuclear area may be due to protein mobility in and over the perinuclear ER membranes.

The observation that wildtype and ER export mutant of KAT1 accumulate in the same subdomain of the ER suggests that the export of the channel occurs as a two step process. During the first step, the channel protein accumulates at ERES. This step is not affected by the ER export motif. Sorting and concentration of KAT1 into COPII vesicles for further transport along the secretory pathway may be the subsequent step. This process would involve interaction of the channel with Sec24

mediated by diacidic ER export motif. The evidence that both, FRET between KAT1 and Sec24 and ER export of the channels occurred only for wildtype KAT1, supports this hypothesis.

Together the results clearly show that mutation of the diacidic ER export motif does not prevent accumulation of KAT1 at ERES but impedes concentration of the channel into COPII vesicles. While the factors responsible for the first process are unknown our present results imply that the COPII mediated ER export occurs via a highly conserved mechanism involving interaction of the channel with the COPII component Sec24.

4.5 Methods

4.5.1 Expression vectors

For expression of KAT1::CFP and KAT1::YFP in *Vicia faba* guard cells, the cDNA of KAT1 was cloned into the pAVA393 expression vector in frame with CFP or YFP under the control of a 35S promoter as described previously by Hurst *et al*. (2004). The binary vector pVKH18En6 was used for expression of Sec24 in guard cells (Stefano *et al*., 2004).

4.5.2 Transfection of intact guard cells by particle delivery

Vicia faba L. cv. Bunyan were grown under controlled climate conditions with 18 °C, 70 % relative humidity, and a 14/10 day/night cycle at 350 to 400 µmol photons $m^{-2}s^{-1}$. Transfection of intact guard cells via particle delivery was performed as described previously (Hurst *et al*., 2004). Cotransfection was performed via coating of gold with equal molar amounts of each plasmid DNA to give a total amount of 15 µg DNA.

4.5.3 FRET and CLSM

Confocal microscopy analysis of transfected turgid guard cells was performed after overnight incubation as described earlier (Meckel *et al.*, 2004) using a confocal laser scanning microscope (Leica TCS SP). The following 25 mW argon laser lines were used for excitation of fluorescent proteins: 458 nm for CFP and 514 nm for YFP. The fluorescence of CFP was detected at 470 to 500 nm and of YFP at 530 to 550 nm. FRET was performed by acceptor photo-bleaching. CFP fluorescence was monitored for 5 scan cycles before and after YFP bleaching. Acceptor bleaching was performed for 15 scan cycles at 514 nm excitation with maximum laser intensity. For spectral-FRET the CFP fluorescence was detected before and after bleaching between 470 and 600 nm. Image acquisition was carried out by Leica LSM 5 image browser. The images were analysed and processed using ImageJ.

4.6 Supplemental Figures
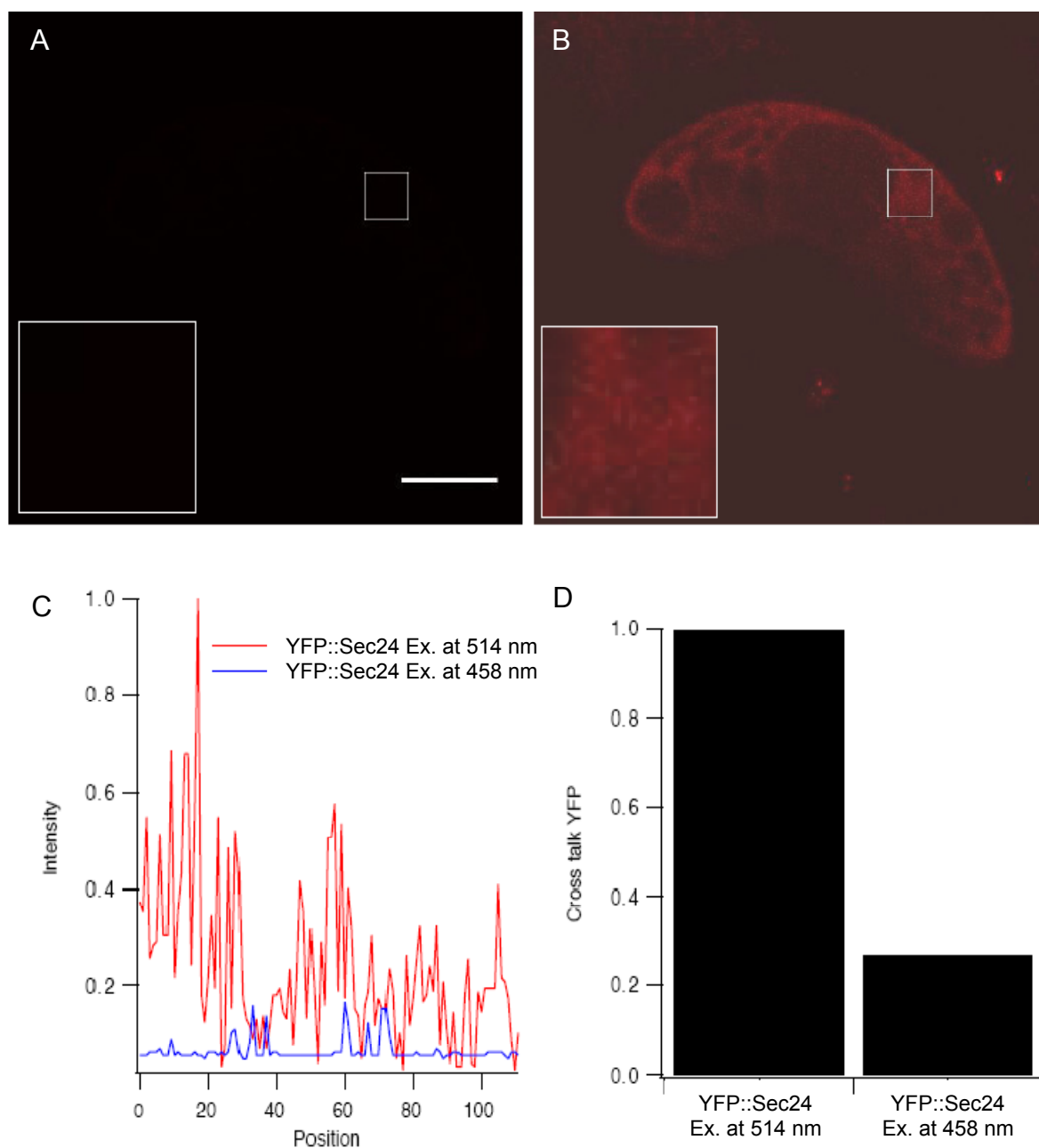
Figure 4.8: Cross-talk of YFP in *Vicia faba* guard cell expressing YFP::Sec24. YFP::Sec24 is clearly visible after excitation at 514nm (B) but to a much lesser extent after 458 nm excitation (A). Boxed areas refer to image inserts. A line plot along the line shown in A and B (inserts) was performed to quantify the extent of cross talk (C). D represents the sum of all intensity values along the line plot. Scale bar 10 μm.
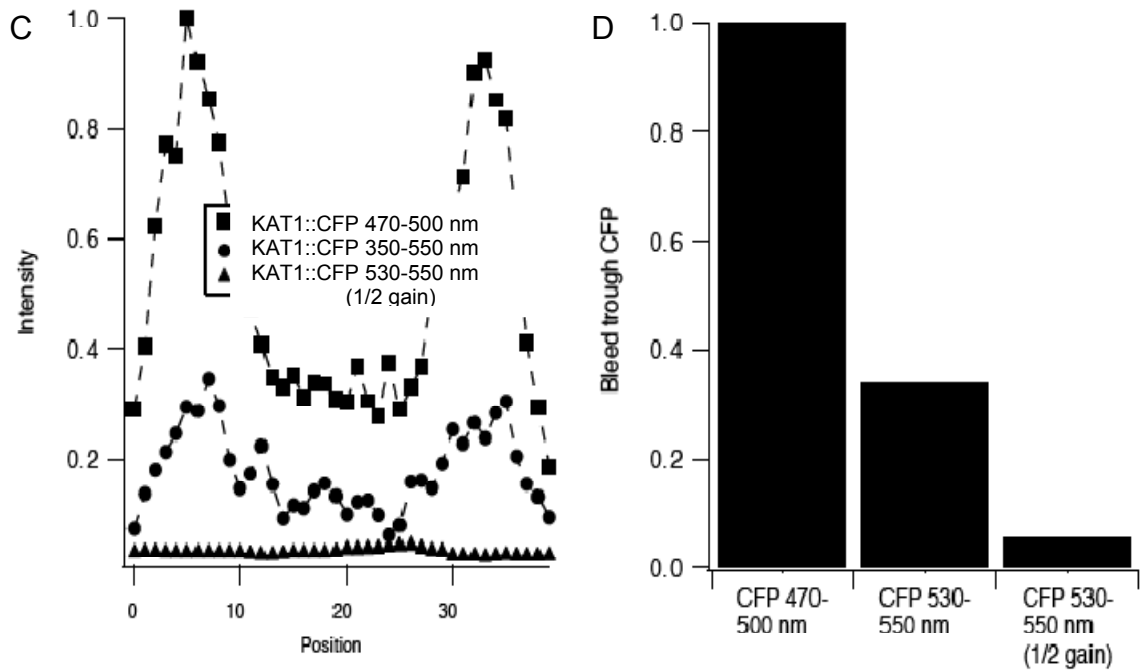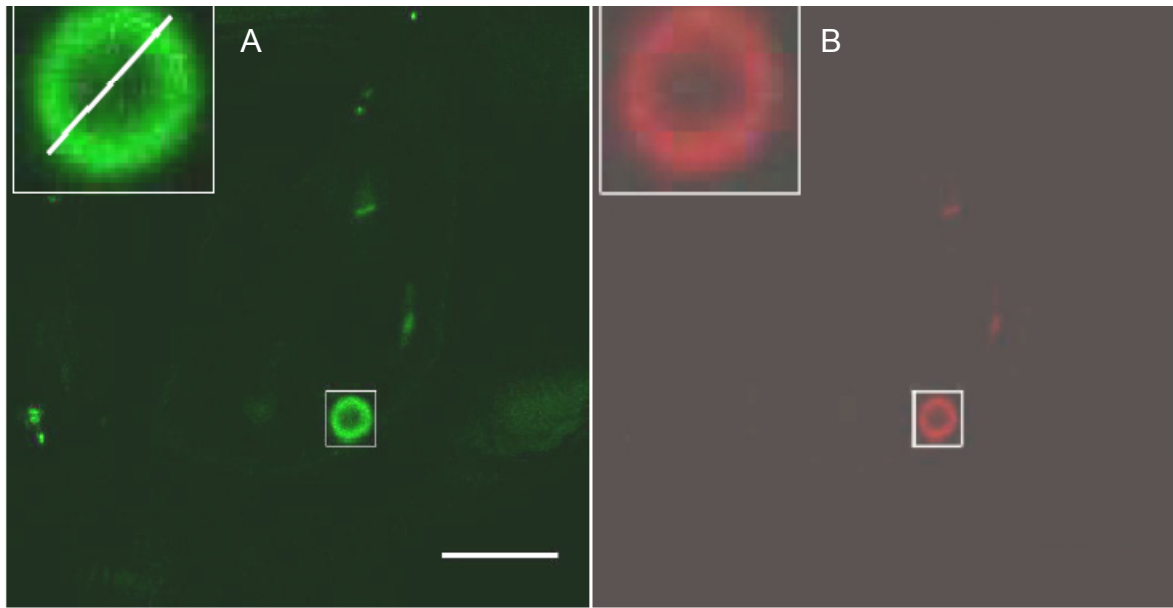
Figure 4.9: Bleed-through of CFP in *Vicia faba* guard cell expressing KAT1::CFP. The emission of KAT1::CFP was monitored in the CFP channel (A) and in the YFP channel (B) after excitation at 458nm. Boxed areas refer to image inserts. A line plot along the line shown in A and B (inserts) was performed to quantify the extent of bleed-through (C). D represents the sum of all intensity values along the line plot. Scale bar 10 μm.
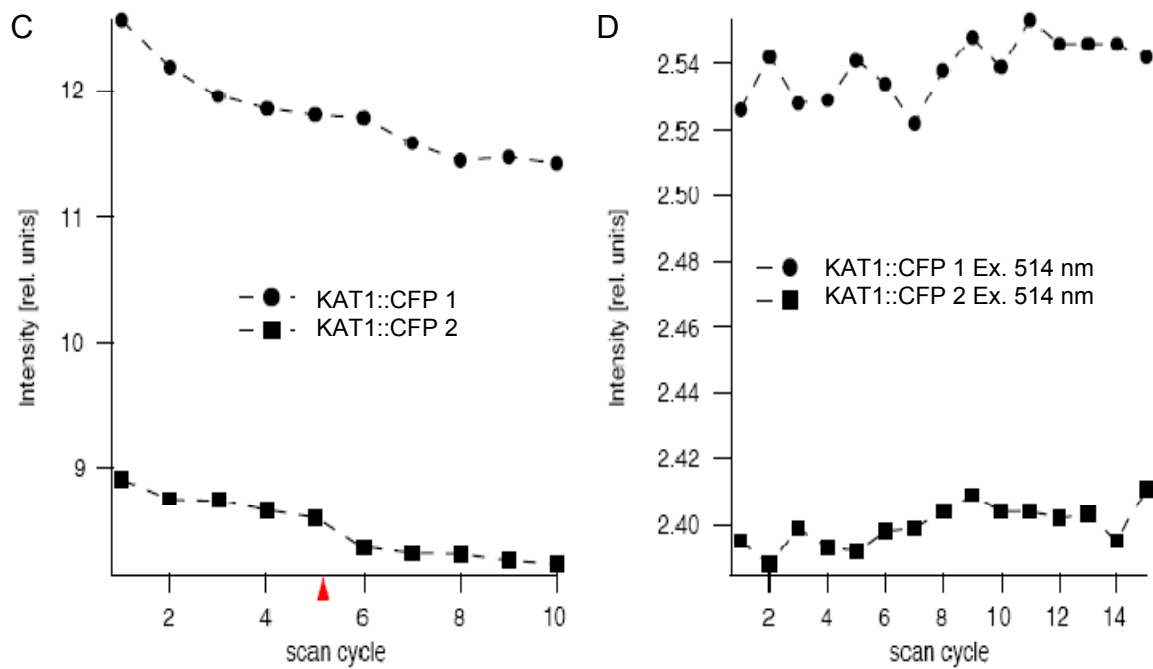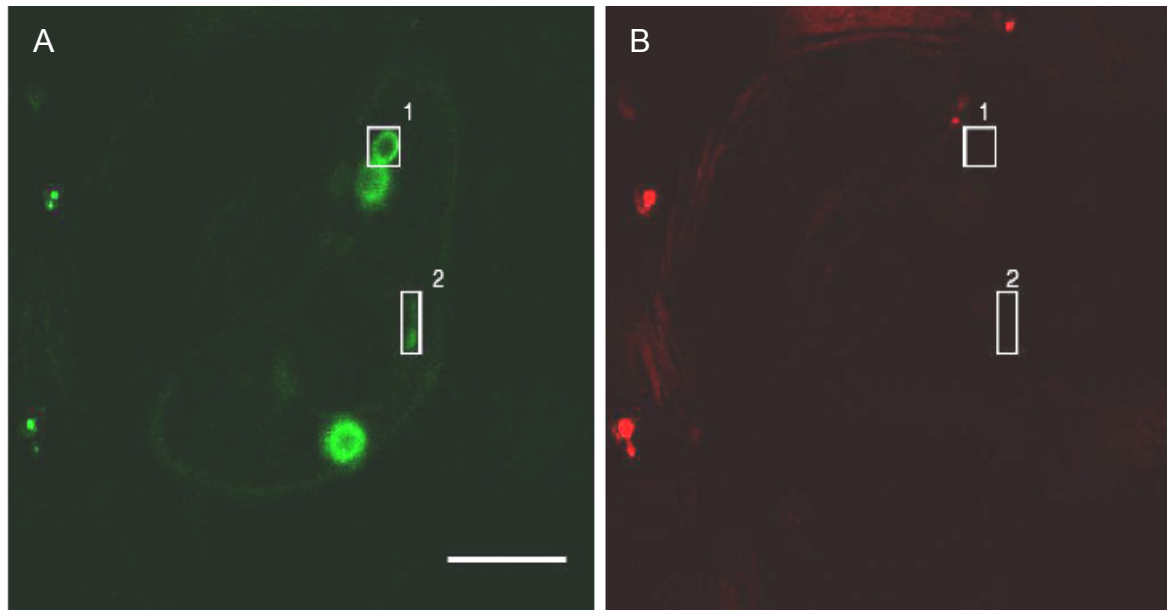
Figure 4.10: Pseudo-FRET as negative control. *Vicia faba* guard cell expressing KAT1::CFP (A). CFP emission (A) was measured for 5 scan cycles before and after bleaching (red arrowhead). The intensities were taken from ROI1 and 2 represented by the boxes in A and B. Bleaching was performed over 15 scan cycles with 514 nm laser line at maximum intensity. The progress was monitored between 470 and 500 nm (D). Scale bar 10 μm.

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Chapter 5 HOW DO DIACIDIC MOTIFS WORK ON ION CHANNEL

TRAFFICKING?
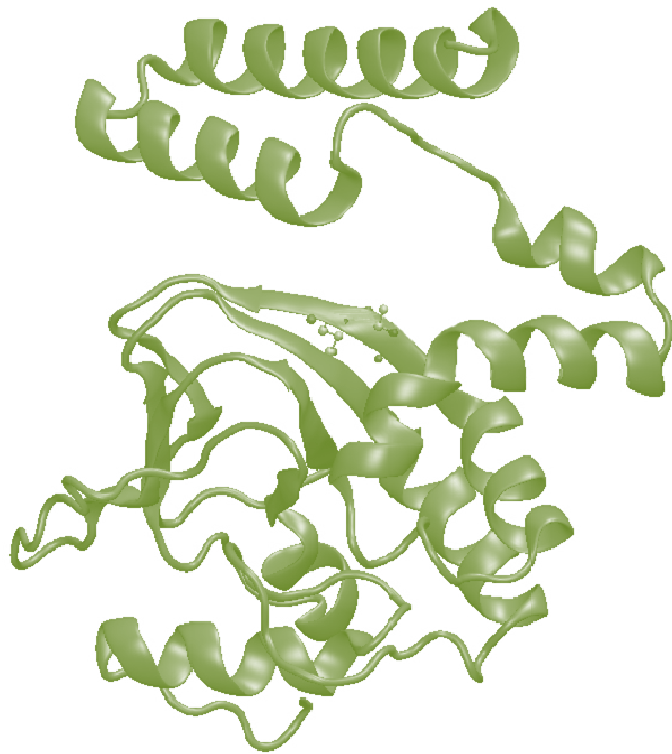
Structure model illustrating the C-terminus of the K$^+$ channel KAT1 according to the crystal structure of HCN2. A ribbon presentation with the two acidic amino acids D394 and E396 marked.

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5.1 Abstract

The function of cells is strongly affected by the type and number of ion channels in the plasma membrane. Recent investigations have highlighted the complexity of the regulation of ion channel trafficking and uncovered several trafficking determinants including diacidic motifs that influence surface expression of ion channels. The large number of ion channels for which functional diacidic motifs have already been identified underlines their general importance and has led to increasing research into the molecular function of these motifs. This chapter will summarise recent progress in identifying the molecular basis for recognition of ER export signals and the physiological relevance of regulated ER export of ion channels and its role in targeting of channel subunits.

5.2 Introduction

Ion channels are integral membrane proteins which allow the movement of ions across cell membranes. They play an important role in physiological functioning of cells and are key elements of multiple signal transduction pathways. The correct functioning of ion channels depends not only on the control of their activity but also on the number of channels in the membrane. The density of ion channels is determined jointly by the secretory and endocytic pathway. Recent investigations have uncovered the transport of ion channel from the endoplasmic reticulum (ER) to the Golgi apparatus as an important step for regulation of surface expression of ion channels (for review see for example Ma *et al*., 2002; Heusser and Schwappach, 2005; Jarvis and Zamponi, 2007). The ER is the first compartment of the secretory pathway. There processes like protein synthesis, folding, quality control and assembly into protein complexes take place. For further movement along the secretory pathway proteins exit the ER either by unspecific bulk flow or by selective recruitment into vesicles. The budding of these vesicles and the specific incorporation of cargo into the forming vesicles are both mediated by coat protein II (COPII) coats (Barlowe *et al*., 1994). For the efficient ER export of proteins their recruitment into COPII vesicles is essential. This process involves

specific amino acid motifs so called ER export signals which are recognised by components of the COPII coat (Otte and Barlowe, 2004).

Until now several classes of ER export motifs have been identified in the cytoplasmic part of transmembrane proteins: a terminal valine (V) at the cytoplasmic C-terminus as well as dihydrophobic, dibasic and diacidic motifs. In addition to these four classes of ER export signals a number of other motifs have been shown to be essential for recruitment of proteins into COPII vesicles. This chapter will focus on diacidic ER export signals and their importance for trafficking of plasma membrane proteins in particular ion channels. This chapter will summarise recent findings regarding the molecular mechanism of the interaction of the diacidic motif with COPII components and analyse the sometimes contradicting results on sequence specificity of diacidic ER export motifs. In addition possible physiological implications of regulated ER export are discussed.

5.3 Diacidic ER export signals of membrane proteins

Integral membrane proteins can be selected for incorporation into COPII vesicles by specific binding to the heterodimeric COPII component Sec23/Sec24 (Wendeler *et al.*, 2007). The first signal found to be involved in selection of cargo into COPII vesicles was the diacidic ER export motif. In 1997 it was identified in the vesicular stomatitis virus glycoprotein (VSVG) and consists of the two acidic amino acids aspartate (D) and glutamate (E) separated by any amino acid (x) (DxE) (Nishimura and Balch, 1997). Upon mutation of the two acidic amino acids to alanine (A) the protein remained largely in the ER with only about 10% still reaching the plasma membrane (Nishimura and Balch, 1997). This implies that sorting of proteins into COPII vesicles via the diacidic motif is very efficient. Over the past 10 years related diacidic ER export motifs have been identified in a number of proteins with ion channels and transporters accounting for the largest part of these proteins (Table 5.1). The importance of the signal for channel trafficking has first been demonstrated for human potassium (K^+) inward rectifying channels of the K_{ir} family (Ma *et al.*, 2001 and 2002). Subsequently, a diacidic ER export motif has also been identified for the cystic fibrosis transmembrane conductance regulator (CFTR). Malfunction of this channel causes the childhood

hereditary disease cystic fibrosis. The most common mutation among cystic fibrosis patients is a deletion of phenylalanine (F) at position 508 (ΔF508). This mutant likely fails to exit the ER because of improper folding that prevents exposure of a diacidic ER export motif towards the cytoplasm (Wang *et al.*, 2004).

Table 5.1: Proteins for which functional diacidic ER export motif have been identified. References are given as numbers in brackets.
[1]vesicular stomatitis virus glycoprotein; [2] type II Golgi matrix protein

protein	origin	motif	
Ion channels			
CFTR	human	DxD	Wang *et al.* (2004)
K_{ir} 1.1	human	ExD	Ma *et al.* (2001)
K_{ir} 2.1	human	ExE	Ma *et al.* (2001)
K_{ir} 3.2 a	human	DxE	Ma *et al.* (2002)
K_{ir} 3.4	human	DxE	Ma *et al.* (2002)
TASK 3	human	ExE	Zuzarte *et al.* (2007)
KAT1	plant	DxE	Mikosch *et al.* (2006)
Transporters			
GAP1	yeast	DxD	Malkus *et al.* (2002)
Sys1	yeast	DxE	Votsmeier and Gallwitz (2001)
Yor1	yeast	DxE	Pagant *et al* (2007)
GONST1	plant	DxE	Hanton *et al.* (2005)
Others			
VSVG[1]	virus	DxE	Nishimura and Balch (1997)
CASP[2]	plant	DxE	Hanton *et al.* (2005)

Recently, the functionality of diacidic ER export motifs has also been demonstrated for plant ion channels. Analysis of four diacidic motifs in the C-terminal tail of the K^+ inward rectifier KAT1 from *Arabidopsis thaliana* revealed that the first diacidic DxE motif is essential for efficient ER export of the functional channel (Mikosch *et al.*, 2006). Interestingly, mutation of the first diacidic motif did not only affect trafficking of KAT1 in plant cells but also in human cells suggesting that that ER export of plasma membrane ion channels is controlled via a conserved mechanism. Together with the identification of the diacidic motifs in a

variety of ion channels belonging to different families this underlines the relevance of the diacidic motif for efficient targeting of ion channels to the plasma membrane.

5.4 Interaction of the diacidic motif with the COPII component Sec24

In order to function as ER export signals diacidic motifs have to be recognised by Sec24, a component of the COPII coat which selects proteins for the anterograde transport from the ER to the Golgi apparatus.

The best characterised organism regarding the function of Sec24 and its homologues is the yeast *Saccharomyces cerevisiae*. In yeast three different isoforms of Sec24 have been identified (Pagano *et al*., 1999). The yeast Sec24 is an essential gene whereas the homologues Lst1 and Iss1 are not. Overexpression of Lst1 cannot compensate for loss of Sec24 function in yeast (Peng *et al*., 2000). Analysis of the cargo recognition in yeast revealed that different cargo molecules are selected by different Sec24 isoforms. Sec24 can bind to several proteins containing various ER export motifs including the LxxLE motif of the v-SNARE Bet1 which is not recognised by the Sec24 isoform Lst1. Lst1, on the other hand, specifically selects the plasma membrane H$^+$ ATPase Pma1 and a number of major secretory proteins into COPII vesicles (Pagano *et al*., 1999; Shimoni *et al*., 2000). Depending on the Sec24 isoforms involved in COPII formation the size of the vesicles can also differ. Vesicles containing both Sec24 and Lst1 are for example significantly larger then vesicles containing only Sec24 proteins (Miller *et al*., 2002).

Studies on the recognition of the yeast SNARE proteins Bet1, Sed5 and Sec22 by Sec24 revealed three independent cargo binding domains: The A-site which is the binding site for the YNNSNPF motif of Sed5, the B-site that recognises the LXXL/ME motif in Bet1 and Sed5 and the C-site found to bind to the NIE motif present in Sec22. The B-site is also the binding site of the diacidic DxE motif of Sys1 whereas the diacidic DID motif of the amino acid permease GAP1 does not use the B-site (Mossessova *et al*., 2003; Miller *et al*., 2003; Mancias and Goldberg, 2007). Recent investigations by Pagant *et al* (2007) demonstrate that recognition of the diacidic ER export motif of the yeast ATP-binding cassette transporter Yor1 also requires the B-site of Sec24.

Together the results imply that different Sec24 isoforms contain specific binding domains for selection of different cargo molecules. This is confirmed by investigations of the human Sec24 homologues. The human genome contains four isoforms of Sec24 which show different cargo recognition. The human SNARE protein Sec22 is for example only recognised by the human Sec24 isoforms A and B whereas the isoforms C and D failed to incorporate Sec22 into COPII vesicles (Mancias and Goldberg, 2007). Furthermore, studies of the ER export of the ERGIC53 protein suggests that Sec24A is responsible for export of proteins containing a dihydrophobic, dileucin ER export signal (Wendeler *et al*., 2007).

So far, information about cargo specificity of Sec 24 isoforms in plants is missing. However, sequence comparison of the three different Sec24 isoforms identified in *A. thaliana* (At3g07100, At3g44340 und At4g32640) with yeast and human Sec24 isoforms shows that the amino acids which have been demonstrated to be critical for binding of cargo to the B-site in yeast are highly conserved (Sieben *et al*., 2008). This suggests that the mechanism of cargo recognition by Sec24 is also conserved across species. Therefore recruitment of ion channels into COPII vesicles most likely also occurs via binding of the diacidic motif to the B-side of Sec24. Accordingly, diacidic ER export signals should be accessible for the binding pocket at the B-site of Sec24. This hypothesis is supported by the observation that for many proteins including the ion channels TASK3 and KAT1 and the transporter Yor1 the localization of the functional diacidic ER export motif is important and that the motif cannot be rescued by other diacidic motifs identified within the protein (Mikosch *et al*., 2006; Pagant *et al*., 2007; Zuzarte *et al*.,2007). Furthermore, homology modelling of the CFTR channel illustrates the ability of the diacidic motif in the nucleotide binding domain (NBD) of CFTR to insert directly into the diacidic binding pocket at the B-site of Sec24 (Wang *et al*., 2004). The modelling also implies that the localisation of the diacidic motif in the NBD ensures accessibility of the motif to Sec24. Indeed, the importance of the NBD for efficient trafficking has been demonstrated for a number of ion channels and also transporters. Deletion of the cNBD in mammalian channels like HCN2, HERG and ERG3 causes loss of functional channels in the plasma membrane (Proenza *et al*., 2002; Ayder and Palme, 2001; Akhavan *et al*., 2005). Similar results were found for plant transporters and channels. The plant plasma membrane H^+ ATPase from *Nicotiana plumbaginifolia* is retained in the ER after deletion of the whole putative

cNBD in the cytoplasmic C-terminus (Lefebvre *et al*., 2004). The K$^+$ channel KAT1 could only be functionally expressed in the plasma membrane when the deletion was behind the putative cNBD (Marten and Hoshi, 1997). A closer inspection of ER export signals of KAT1 shows that the functional diacidic ER export motif of KAT1 is indeed located in the cNBD of the channel (Mikosch *et al*., 2006).

The close relation (identity: 22.66 %, similarity: 37.47 %) between the C-terminus of KAT1 and the C-terminus of the mammalian HCN2 channel crystallised by Zagotta *et al*. (2003) allowed us to perform homology modelling of the cNBD of KAT1. The resulting model structure presented in Figure 5.1 shows that the functional diacidic ER export motif (marked red) is exposed on the surface and thus accessible for the Sec24 protein. This strongly suggests that the ER export of KAT1 is associated with binding of the channel to a specific site of Sec24. Recent studies on the interaction of KAT1 and Sec24 from *Arabidopsis thaliana* strongly support this hypothesis. FRET (fluorescence resonance energy transfer) measurements of guard cell cells co-expressing KAT1::CFP and YFP::Sec24 demonstrate that KAT1 interacts with Sec24 at distinct sites of the ER and that this interaction depends on the first diacidic ER export motif of KAT1 (Sieben *et al*., 2008). The results thus provide *in vivo* evidence for a Sec24 cargo interaction via a diacidic motif that leads to COPII-mediated ER export. Whether binding of KAT1 occurs indeed at the B-site of Sec24 remains to be shown.
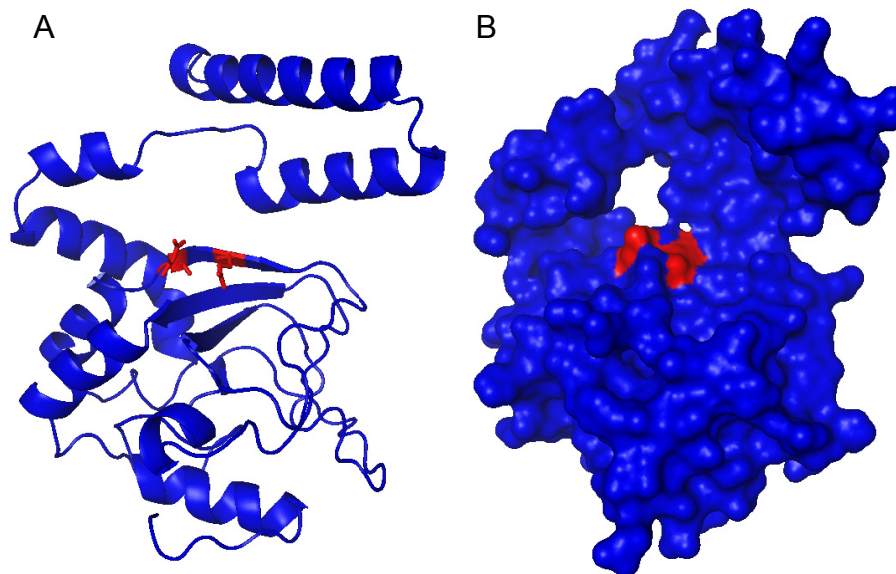
Figure 5.1: Structure model of the C-terminus of the potassium channel KAT1. A: Ribbon presentation with the two acidic amino acids D394 and E396 marked in red. B: Molecular surface representation of the monomer which shows that the diacidic motif is located at the surface of the protein. The sequence of the C-terminus of KAT1 was aligned with the HCN2 sequence with ClustalX. The model was generated using Modeller with the pdb of HCN2 C-terminus 1Q5O (http://salilab.org/modeller/modeller.html). The Figure was generated using PYMOL (http://www.pymol.org).

5.5 Sequence specificity of the diacidic ER export motif

The consensus motif of diacidic ER export motifs is generally believed to be D/ExD/E. However, a number of studies imply that D/ExE is more compatible than D/ExD whereas other investigations suggest that E at the final position can be substituted by D. In this paragraph the sometimes contradicting results on sequence specificity of the diacidic ER export motif are summarized.

Detailed investigations on the binding of different proteins and motifs to Sec24 in yeast imply that the binding domain for the diacidic motif is the B-site (Mossessova *et al.*, 2003) and that binding requires an E at the final position. Crystallization of the complex consisting of Sec24 together with peptides containing the ER export motif demonstrates that the C-terminal E of the ER export motif LxxLE of the v-SNARE Bet1 binds to the B-site of Sec24 in the same manner as the C-terminal E of the DxE motif of Sys1 (Mossessova *et al.*, 2003). The ER export motifs of Bet1 and Sys1 show some structural similarities: both form a helical structure at the C-terminal part of the motif so that the E fits into a conserved basic pocket. It

appears that a D cannot fit into this pocket. In accordance with this observation the C-terminal E is conserved in all the Bet1 proteins in yeast, humans and *Drosophila melanogaster*. The signal sequence of the ER export motif might thus be D/ExE (Mossessova *et al.*, 2003, Mancias and Goldberg, 2005). Indeed, most of the functional diacidic motifs identified so far contain an E at the third position (see also Table 5.1). However, for various proteins like the CFTR channel and the amino acid permease Gap1 a DxD motif was found to function as an ER export signal (see Table 5.1 for references). This suggests that both, D and E at the final position can be essential for functioning of the diacidic ER export signal. A possible explanation for the contradicting results on the importance of the final E may be that there is more than one binding site for diacidic motifs. This is supported by the observation that upon mutation of the B-side of yeast Sec24 the proteins Bet1 and Sys1 which both contain a DxE motif could not bind to Sec24 anymore. In contrast the amino acid permease Gap1 which contains the diacidic motif DxD was still recognised by the mutated Sec24 (Miller *et al.*, 2003). Binding studies carried out by Mossessova *et al.* (2003) led to the hypothesis that the A-site of Sec24 performs a shape-based recognition of the motif which might be much more tolerant regarding the signal sequence than the side chain chemistry based binding at the B-site.

The question of sequence specificity gets even more complicated when looking at the importance of the first amino acid of the diacidic motif. An alignment of the identified ER export motif DxE of the plant K^+ channel KAT1 with other closely related K^+ inward rectifiers from *Arabidopsis thaliana* shows that only the final E is conserved in all K^+ channels whereas the D is mostly substituted by lysine (K) or in the case of AtKC1 by glutamine (Q) (Figure 5.2). The presence of a K at the first position of the "diacidic" is likely to preclude binding to the Sec24 B-site, suggesting that in these plant ion channels either a different signal is at work or that a different site on Sec24 binds this signal. Interestingly, AtKC1, having a Q at the first position of the "diacidic" motif, are retained within the ER whereas all other K^+ inward rectifier are transported to the plasma membrane even as homotetramers (see also next section). This could suggest that only the last amino acid of the "diacidic" motif has to be acidic. However, mutation of both, the DxE motif of VSVG and the DxD motif of Gap1 to ExE let to retention of the proteins in the ER despite of the E at the final position (Nishimura and Balch, 1997; Malkus *et*

al., 2002). Furthermore, studies on the ER export of the $K_{ir}3.4$ channel clearly demonstrate that the first acidic amino acid is important for functioning. Mutation of D to N in the DxE motif of $K_{ir}3.4$ greatly reduced ER export of the channel similar to the mutation of E to Q (Ma *et al*., 2002). For most diacidic ER export motifs identified so far it has not been demonstrated as to whether mutation of only one of the acidic residues is sufficient to stop ER export. It thus remains to be shown if the diacidic ER export really requires two acidic amino acids.

When looking at the function of diacidic motifs one also has to keep in mind that the surrounding amino acids may have an impact on the signal sequence. An effect of amino acids in the neighbourhood of the diacidic motif on ER export has clearly been demonstrated for the VSVG protein (Nishimura *et al*., 1999). Such an effect may also explain the studies on ER export of the dopamine transporter DAT1. The transporter DAT1 has a putative diacidic ER export motif DxE in the cytoplasmic C-terminus but only mutation of the D results in inhibition of ER export. Interestingly, mutation of another not related residue G in the cytoplasmic C-terminus results in a strong reduction of ER export (Miranda *et al*., 2004). The authors reasoning is that the DxE motif is not a diacidic ER export motif. However, it may also imply that surrounding amino acids influence the function of the diacidic ER export motif.

Together the results imply that the diacidic motif itself may be much more complex than just two acidic amino acids. It is also possible, that ER export signals can be more variable than previously thought and that this variation may contribute to the regulation of trafficking (see also next paragraph).

```
KAT1        385FLFQLVSDIDAEYFPPKEDIIL
KAT2        385FLFQLVSDIDAEYFPPREDVIL
AKT5        404LLFQLVSEMKAEYFPPKEEVIL
SPIK        406LLFQLVTEMKAEYFPPKEDVIL
AKT1        380LLFQLVSEMKAEYFPPKEDVIL
AKT2/3      402ILLLLLVSKMKAEYIPPREDVIM
AtKC1       414LLVQLVSQIQAEYFPPKMEIIL
```

Figure 5.2: Alignment of ER export motif of KAT1 with the other identified *Arabidopsis thaliana* **inward potassium rectifier.**

5.6 Role of ER export signals in regulating ion channel density and composition

Properties of cells are strongly affected by the type and number of proteins expressed at the plasma membrane. In particular, only small changes in the density and/or composition of ion channels can have a large impact on membrane conductance and voltage and thus strongly alter the response of the cell to its environment. For mammalian ion channels it has been demonstrated that regulatory mechanisms at the side of ER export can affect both, the number and the subunit composition of ion channels in the plasma membrane.

Studies on trafficking of the mammalian ATP-sensitive K^+ channel $K_{ir}6.2$ demonstrated that transport of the channel to the plasma membrane is promoted by binding of 14-3-3 proteins to the channel (Heusser *et al*., 2006). Binding of 14-3-3 proteins was found to mask an ER retention signal of the channel thereby increasing the rate of ER export. An affect of 14-3-3 on the number of channels in the plasma membrane has also been described for the plant K^+ channel KAT1 although it is unknown how 14-3-3 acts on channel density of KAT1 (Sottocornola *et al*., 2008). In principle, efficiency of ER export and thus surface expression of ion channels may also be modified by interaction of proteins with ER export signals. So far such a regulatory mechanism remains to be identified.

In addition to the affect on the channel density ER export signals have also been implicated to be involved in the modulation of the subunit composition of channel heterotetramers. One conclusive example comes from investigations of the inwardly rectifying K^+ channels of the K_{ir} family which play an important role in neuronal signalling. These channels function as homo- or heterotetramer and tetramerization occurs already in the ER. Some but not all members of the K_{ir}

family contain diacidic ER export signals (Ma *et al*., 2008; Stockklausner *et al*., 2001). Homotetramers of subunits which do not contain an ER export signal like $K_{ir}3.1$ and $K_{ir}3.3$ are accumulating in the ER. In contrast, $K_{ir}2.1$ which does contain a diacidic ER export signal localises to the plasma membrane. Ma *et al*. (2002) have demonstrated that formation of heterotetramers containing both, K_{ir} subunits with and without ER export signals leads to expression of the heterotetramer in the plasma membrane. These results demonstrate that not all subunits of heterotetramers need to contain ER export signals in order to ensure efficient transport to the plasma membrane. More importantly, the ER export signal of one channel subunit can obviously affect targeting of other K_{ir} channels subunits thus leading to changes in the composition of heterotetramers in the plasma membrane. Changes in subunit composition alter the electrical characteristics of the functional K^+ channel and can therefore have a strong impact on the conductance of the plasma membrane and consequently on the function of the cell.

Heterotetramerisation is found for many members of different K^+ channel families and the impact of heterotetramerisation on the efficiency of ER export may well be of general importance for the control of membrane conductance.

This hypothesis is supported by studies on ER export mutants of both, mammalian and plant K^+ channels. Efficient trafficking of HCN channels to the plasma membrane has been demonstrated to depend on the cNBD (see previous paragraph). Deletion of the B-helix of the cNBD of HCN2 was found to disrupt ER export of the channel leading to retention of the channel in the ER (Proenza *et al*., 2002). However, coexpression of wildtype HCN1 together with the ER export mutant of HCN2 led to surface expression of the HCN1/HCN2 heterotetramer.

Similar results have been found for the plant K^+ channel KAT1. When KAT1 wildtype and the ER export mutant of KAT1 were coexpressed in HEK293 cells plasma membrane localisation of the ER export mutant could be rescued (Mikosch et al., 2009). This again implicates that those subunits which are retained in the ER as homotetramers can be targeted to the plasma membrane via heterotetramerisation with subunits that do contain an ER export signal. The physiological importance of such a mechanism is revealed by investigations of AtKC1, a member of the K^+ inward rectifying family from *Arabidopsis thaliana.* Several members of this family have been shown to form functional

heterotetramers (for review see Lebaudy et al., 2007). Interestingly, AtKC1 could only be detected in the plasma membrane when coexpressed with other members of the K$^+$ channel family (Reintanz et al., 2002; Pilot et al., 2003; Duby et al., 2008). When expressed on its own AtKC1 localised to the ER (Duby et al., 2008). This conditional targeting of AtKC1 most likely plays an important role in the down regulation of the physiological activity of other channel subunits in $Arabidopsis$ $thaliana$ (Duby et al., 2008). The mechanism responsible for the conditional targeting is not yet known. Duby et al. (2008) suggest that AtKC1 is not able to form homotetramers and that AtKC1 monomers contain ER retention signals. Heterotetramerisation may hide the ER retention signal and/or ER export signals of the other channel subunits may overwrite the ER retention signal.

For the human (h) ERG channel inactivation of ER retention signals in the course of heteromultimeric assembly has recently been described (Phartiyal et al., 2007). Phartiyal et al. (2007) demonstrated that heterotetramerisation of hERG channel subunits 1a and 1b overcomes an ER retention motif of hERG1b and thus regulates subunit composition of hERG surface channels.

Together this implicates that both ER-retention and ER export signals play an important role in regulation ion channel density and composition in the plasma membrane.
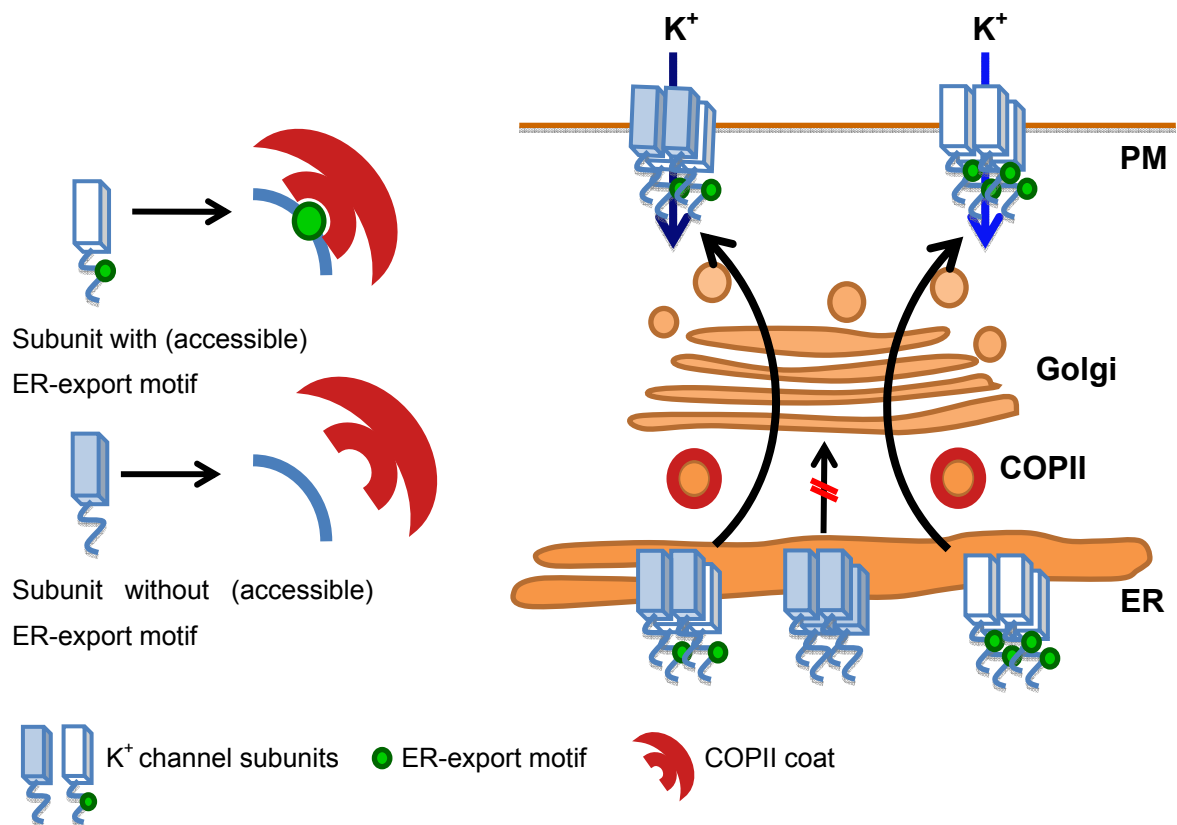
Figure 5.3: Model for the function of diacidic ER export motifs on trafficking of K$^+$ channel subunits. The C-terminus of some K$^+$ channel subunits contains a diacidic ER export motif. This motif can bind to the coat protein complex II (COPII) component Sec24. Subunits with or without ER export signals assemble as homo- or heterotetramers in the endoplasmic reticulum (ER). Only tetramers containing diacidic ER export signals are efficiently incorporated into COPII vesicles for further trafficking to the plasma membrane (PM). Incorporation requires binding of the diacidic signal to Sec24. The number ER export signals necessary for targeting of heterotetramers to the PM is not known.

5.7 Conclusions and perspectives

ER export signals have been identified in a variety ion channels and are now widely accepted as important determinants of ion channel trafficking. The mechanisms by which these signals act on ER to Golgi apparatus trafficking are still not fully understood but recent studies have provide new insights into the function of the diacidic signal sequence. The current understanding on how the diacidic motif affects channel density and composition is summarised in Figure 5.3 taking trafficking of K$^+$ channels subunits as an example. Accumulation of ion channels into COPII vesicles occurs via binding of the diacidic motif to a specific binding side of the COPII component Sec24. Channel subunits without such a motif are not sufficiently included into COPII vesicles and thus remain largely in

the ER. However, these subunits can be targeted to the plasma membrane via heterotetramerisation with subunits containing an ER export signal. As the electrical properties of heterotetramers may differ from that of homotetramers this in turn can lead to changes in the membrane conductance and consequently alter the function of the cell.

There is increasing evidence that the ER export signal itself is also subject to regulation which may involve phosphorylation or binding to scaffold proteins. These proteins may act via steric masking or unmasking of ER export signals. In order to fully understand the regulation of ion channel trafficking it will be important to uncover the mechanisms affecting the function of the ER export signal. For future research it will also be of major relevance to investigate the physiological significance of these mechanisms.

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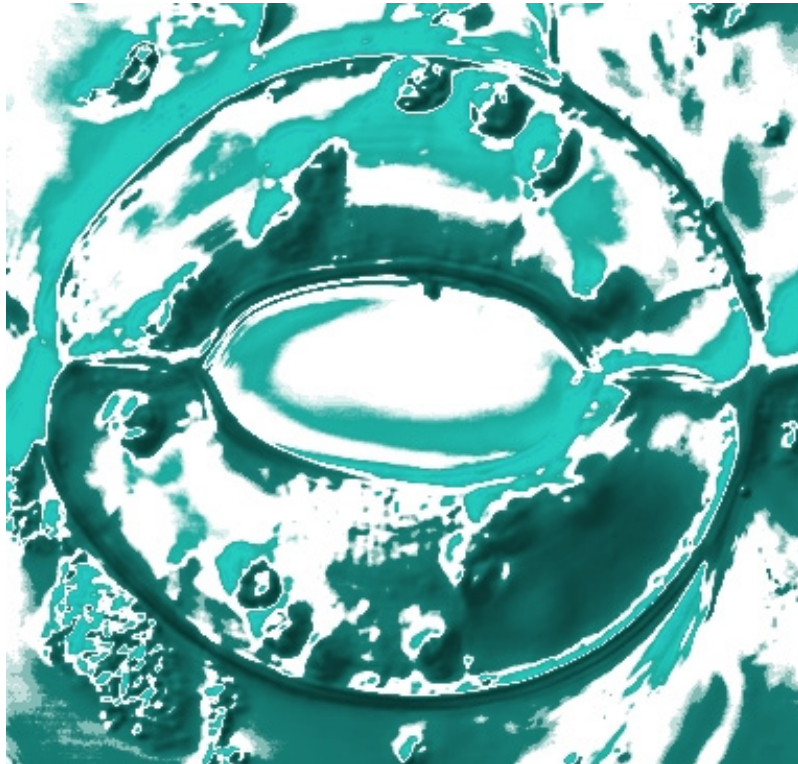
Modified transmission picture of an opened stomata from a *Vicia faba* leaf taken at the CLSM. The Chloroplasts are very nicely visible surrounding the nucleus in each guard cell.

6.1 Diacidic ER export motifs

6.1.1 ER export motifs regulate trafficking of membrane protein in plants

The current work shows for the first time that ER export motifs are functional in ion channel trafficking in plant cells. A diacidic ER export motif was identified in the plant potassium (K^+) channel KAT1 from *Arabidopsis thaliana* (Mikosch *et al*., 2006). Expression of the KAT1 mutant with aspartate (D394) and glutamate (E396) mutated to alanine (A) ($KAT1^{DiaAa}$) in guard cells as well as in HEK293 cells led to a strong reduction of the plasma membrane inward conductance compared to the KAT1 wildtype channel.

This reduction in plasma membrane conductance may occur due to several reasons incorporated in three parameters. The net plasma membrane current I can be described by Equation (1), where i is the single channel current, P_o the open probability of the channel and n the number of ion channels in the plasma membrane. A change in the net plasma membrane current I can be caused by a change in the single channel current i, a reduction of the number of channels in the plasma membrane, or by changes of the open probability P_o of the channel.

$$I = i * P_o * n \tag{1}$$

Rapid changes in I are very often accomplished by changes in the open probability P_o of the channel. For example, in case of KAT1 the open probability P_o is strongly affected by changes in membrane voltage. On the other hand, long time changes are more likely due to an altered number of ion channels in the plasma membrane. Kinetic analysis of the analysed ER export mutant revealed similar kinetics as the wildtype KAT1 K^+ channel. Therefore, the changes in plasma membrane conductance, observed in the ER export mutant, were most likely caused by a reduced number of ion channels.

This reduction of KAT1 K^+ channels in the plasma membrane was verified by confocal analysis of the localisation of the KAT1 K^+ channel and its mutants in *Vicia faba* guard cells as well as in HEK293 cells, which demonstrated that the analysed motif is truly functional as an ER export signal in both cell types. This

also implies that the mechanisms of ER export are very similar in plant and animal cells.

During this study a number of other ER export motifs have been discovered in plant membrane proteins which underlines the importance of the results. One of them is the diacidic ER export motif in the N-terminus of the maize plasma membrane aquaporins ZmPIP2;4 and ZmPIP2;5 (Zelazny *et al*., 2008). Schoberer *et al*. (2008) identified an arginine/lysine (RK) motif which is responsible for ER export of the Golgi resident glycosylation enzymes GnTI and GMII. ER export of the Golgi proteins GONST1 and CASP is also regulated by diacidic ER export motifs (Hanton *et al*., 2005). This shows that regulation of ER export of membrane proteins by intrinsic signal sequences is a highly conserved mechanism in all eukaryotic cells.

Further analysis of the diacidic motif and the surrounding amino acids of KAT1 revealed that the diacidic motif was not only diacidic but contained at least three acid residues at position D392, D394 and E396 (Mikosch *et al*., 2009). Single mutation of the amino acids led to the conclusion that all acidic residues have a similar influence on ER export. The amount of reduction in KAT1 conductance was clearly dependent on the number of mutated acidic residues. Mutants with only one of the residues mutated to alanine (A) had a slightly decreased inward conductance compared to the wildtype K^+ channel. Mutation of two of the residues results in a much stronger reduction of inward conductance. Only when all three acidic amino acids were mutated to A the ER export was completely inhibited and almost no KAT1 current could be measured.

6.1.2 ER export affects cargo selection by interaction with Sec24

The selection and concentration of cargo into vesicles at ER export sites (ERES) depends on interaction of the COPII coat with cargo transport signals. For most of the ER export signals identified so far an interaction with the Sec24 subunit of the COPII coat could be shown (Miller *et al*., 2002 and 2003; Mossessova *et al*., 2003). Sec24 contains at least three binding sites for several cargo proteins with different ER export motifs. The Sec24 A-site binds the NPF motif of the SNARE protein Sed5 (Miller *et al*., 2005; Mossessova *et al*., 2003). The B-site binds to the LxxLE motif of the SNARE proteins Sed5 and Bet1 as well as to several diacidic

ER export motifs like of the protein Sys1 (Nishimura *et al.*, 1999; Votsmeier and Gallwitz, 2001). The C-site binds the NIE motif of the SNARE Sec22 (Miller *et al.*, 2003; Mancias and Goldberg, 2007). An additional binding site was identified for binding of GAT1 and two other members of the SLC transporter family which exhibit an arginie/leucine (RL) ER export motif (Farhan *et al.*, 2007).

The first indication for the participation of the COPII coat in ER export of KAT1 came from coexpression analysis of wildtype KAT1 K$^+$ channel with Sar1^{T39N}, the dominant negative form of Sar1. In these cells ER export of KAT1 was inhibited and the channel was found in the same perinuclear structures as several ER export mutants with at least two acidic amino acids replaced by A. Binding of the K$^+$ channel KAT1 to the COPII subunit Sec24 could be demonstrated by FRET measurements. After mutation of the amino acids D394 and E396 to A the interaction between Sec24 and the KAT1^{DIaAa} channel at the ER was abolished (Sieben *et al.*, 2008). This reveals that KAT1 is recognised by Sec24 and selected for ER export with the acidic ER export sequence. As the B-site of Sec24 protein was identified as the binding site for diacidic ER export motifs in yeast, which is highly conserved between plant, yeast and human Sec24 homologues, it would be interesting to analyse the importance of the B-site for binding of the acidic ER export motif of KAT1.

In future studies it will be necessary to show the interaction of the acidic ER export motif of KAT1 with Sec24 in a more direct way. Regarding the complicated nature of the identified ER export motif of KAT1 it would also be interesting to analyse the importance of the single acidic residues for Sec24 binding and address the question whether they are functionally resistant to exchanges by the other acidic amino acid. Another aspect is the possibility that the amino acids, surrounding the acidic motif, may also be of importance for efficient ER export. Therefore, analysis of these amino acids would also be of major interest. In this context it is worth mentioning that cargo recognition may rely on multivalent interactions and the three dimensional structure of the signals which is only revealed upon proper folding and assembly of KAT1 (Mikosch and Homann, 2009). Further information on crystal structure would be needed to get an impression of the requirements for functional ER export motifs. Analysis of the crystal structures of the Sec22 NIE ER export motif which binds to the C-site of Sec24 demonstrated that the motif is most likely a folded epitope (Mancias and Goldberg, 2007).

The situation gets even more complex when considering the different Sec24 isoforms present in cells. For example in mammalian cells four different Sec24 isoforms (Sec24A-Sec24D) exist which can be further diversified by alternative splicing. The ER Golgi intermediate compartment protein 53 (ERGIC53) with the dihydrophobic diphenylalanine (FF) motif binds to all four Sec24 isoforms (Wendeler *et al.*, 2007). But the transporter GAT1 is only recognised by the Sec24D subunit (Farhan *et al.*, 2007). In *Arabidopsis thaliana* three isoforms of Sec24 have been identified but so far nothing is known about varying recognition of cargo by different Sec24 isoforms. Mancias and Goldberg (2008) showed by comparative analysis of crystal structures of the four human Sec24 isoforms that a conserved IxM ER export motif binds to a surface groove of mammalian Sec24C and Sec24D, but the groove is not accessible in the Sec24A and Sec24B subunits. On the other hand, the LxxLE ER export and the DxE signal of VSVG are selectively bound by Sec24A and Sec24B subunits. Sec22 is bound only by Sec24A and Sec24B with an NIE motif (Mancias and Goldberg, 2007).

Therefore, it is necessary to analyse the interaction of KAT1 with all three Sec24 isoforms present in *Arabidopsis thaliana* to get an insight into the regulation of ER export through the employment of different Sec24 receptors.

6.1.3 Restriction of the ER export mutant to the perinuclear area

ER export mutants of KAT1 with at least two of the acidic residues mutated to alanine show a special localization in HEK293 as well as in *Vicia faba* guard cells. The GFP fluorescence is restricted to parts of the ER in a perinuclear area.

This area may correspond to ER export sites (ERES). ERES are defined as ribosome free parts of the ER that label for the COPII coat complex. In mammalian cells it could be demonstrated that almost 50-60 % of ERES are actively positioned in the perinuclear area while others are distributed on the periphery of the cell (Bannykh *et al.*, 1997; Stephens, 2003). In plant cells the situation is quite different. The Golgi stacks in plants move along together with individual ERES but these sites are also very often located in the perinuclear area (daSilva *et al.*, 2004). Intense debate is going on about the nature of the ERES Golgi interface because several studies refer to this either as a tubular structure or as a more vesicular arrangement (Kirk and Ward, 2007). It seems likely that the sheet-like

structures, found in cells where ER export of KAT1 was prevented either by mutation of the ER export motif of the channel or by overexpression of Sar1^{T39N}, corresponds to ERES in the perinuclear area. Furthermore special arrangements of the ER may prevent free diffusion of KAT1 in the ER membrane and contribute to the restriction of the KAT1 K$^+$ channel to these ERES. A similar pattern of ER retention has been demonstrated for the G-protein coupled receptors α$_{2B}$-AR and AT1R when coexpressed with Sar1^{H79G}. Both receptors show a perinuclear staining (Dong *et al.*, 2008). In addition, mutants of the HERG K$^+$ channel which lack the cNBD domain which is important for ER export also showed a perinuclear staining (Akhavan *et al.*, 2005). In contrast, the G-protein coupled receptor β$_2$-AR displayed a totally different localisation upon coexpression with Sar1^{H79G}. The receptor was retained in the entire ER (Dong *et al.*, 2008). Experiments demonstrated that unlike KAT1, ER retention of proteins like the plasma membrane H$^+$ ATPase or the artificial plasma membrane marker TM23 by coexpression with Sar1^{T39N} led to a distribution of these proteins in the entire ER (unpublished results). The restriction of some membrane proteins to specialised ER subdomains can be due to special interaction with ER resident proteins.

The nature of the protein itself may also contribute to the ER localisation because it could be shown that length and hydrophobicity of the transmembrane domains of membrane proteins play an important role in protein sorting within the ER and throughout the secretory pathway (Ronchi *et al.*, 2008; Brandizzi *et al.*, 2002). Ronchi *et al.* (2008) expressed two proteins that differ in transmembrane domain length: FP-17 and FP-22. FP-17 was retained in the ER and FP-22 was located in the plasma membrane. Their respective distribution in the ER differs to a great extend although both of them are freely diffusible in the ER. It seems that FP-17 is excluded from ER export site and FP-22 not. As a result the authors assume that self organising principles are an important factor of the distribution within the ER.

In addition, the curvature of the membrane can influence the probability of protein localisation. To achieve a thermodynamically stable state, proteins tend to locate in plane or curved membranes according to the length and hydrophobicity of their transmembrane domains (dePlanque and Kilian, 2003).

The protein distribution in the ER can also be altered by ER residential proteins, by modification of lipids or direct protein-protein interaction. An interesting candidate conferring ER localisation is the ER resident transmembrane protein calnexin

which interacts with several neurotransmitter transporters like GAT1 in specialised regions of the ER. Korkhov *et al.* (2008) assumed that calnexin plays a role in the control of oligomeric assembly of these transporters. calnexin and the interacting proteins segregate into so called organised smooth ER membranes (OSER) which consist of multiple stacked ER membranes. They are supposed to act as a fence to prevent aggregation of membrane proteins in the OSER. These OSER are visible in confocal images as concentric bodies close to or associated with the perinuclear ER. Especially upon expression of a GAT1^{E101D} mutant, which cannot form oligomers anymore, the localisation in the OSER is dramatically increased (Korkhov *et al.*, 2008).

The ER export mutants of KAT1 are frequently found in concentric bodies in addition to the perinuclear area in both HEK293 cells and *Vicia faba* guard and epidermal cells. Therefore, it would be interesting to explore whether KAT1 also interacts with calnexin and if the observed concentric bodies are related to OSER.

6.1.4 mRNA localisation

There are indications that the localisation of mRNA within the cell is of general importance for cell function. The cotranslational insertion of locally synthesised proteins could be necessary especially for the formation of oligomeric protein complexes. The localisation of ion channel mRNA within the cell could have an enormous impact on the localisation of the channel within the ER and determine the availability of subunits for oligomerisation. This could be achieved by information in the noncoding regions of the mRNA. Targeting of mRNA to certain subdomains involves special mechanism like RNA binding proteins which interact with the cytoskeleton to ensure the localisation. In mammalian cells it could be shown that especially mRNA of membrane proteins is typically targeted to specific regions of the ER. For example the mRNA of the chloride channel ClC1 is directed to the perinuclear ER. Upon coexpression of the ClC1 channel with the dominant negative Sar1^{H79G} the channel is trapped in the perinuclear region of the ER (Papponen *et al.*, 2008). The ER region where the mRNA is localised may be connected to a kind of channel tetramerization domain in the ER. Also in plants the localisation of mRNA to special subdomains of the ER has been reported (Hamada *et al.*, 2003). Considering that the ER localisation pattern of the ClC1

protein resembles that of KAT1, it would be very interesting to analyse whether the specific localisation of KAT1 to the perinuclear region of the ER can be explained by the distribution of KAT1 mRNA in the cell.

6.2 Tetramerization of ion channels

6.2.1 Tetramerization of ion channels most likely occurs at the ER

The insight into ion channel tetramerization is based on overexpression experiments in cell culture or *Xenopus laevis* oocytes. However, nothing is known about the availability of subunits within the ER under normal conditions. It seems very likely that ion channel subunits are not very abundant in the ER because the number of ion channels in the plasma membrane is generally low. Accordingly, one may assume that mechanisms have been developed to support the formation of tetramers. These may either occur through the involvement of specialised proteins like chaperones or through the differential localisation of subunits within the ER. Maybe there are specific ion channel assembly domains in the ER where ion channel subunits are concentrated because tetramerization needs at least the close proximity of four subunits.

So far it has not been clearly revealed where and when ion channel tetramerization takes places in the ER. It is most likely that the tetramers are directly composed in the ER and only fully assembled ion channels can pass the quality control system of the ER. For a few oligomeric membrane proteins it has been shown that tetramerization is indeed a prerequisite for ER export (Farhan *et al*., 2007; Becker *et al*., 2007).

But how is the oligomeric state of a protein controlled in the ER? Several other proteins must be involved in the tetramerization control of ion channels in the ER. Their nature is quite often undetermined. Recent investigation revealed a very interesting protein in the ER, Rer1, which recognises polar residues facing the lipid bilayer (Sato *et al*., 2003 and 2004). Not yet assembled ion channel subunits are possible candidates for such a protein because they exhibit several polar as well as positively charged amino acids in the transmembrane and filter region. Also

calnexin, an ER resident membrane protein, can play a role in oligomeric assembly of proteins (Korkhov *et al*., 2008).

COPII can be involved in the control of proper oligomerisation, too. The kinetic stability of the prebudding complex consisting of Sar1, Sec24 and Sec23 bound to cargo molecules depends on interaction of the cargo with Sec24. Properly assembled cargo molecules enhance the stability of the prebudding complex. Unassembled cargo leads to Sar1-GTP hydrolysis and subsequently the prebudding complex collapses (Sato *et al*., 2005). Sato *et al.* (2004) concludes that by this process of Sar1-GTP hydrolysis unassembled cargo is actively excluded from forming COPII vesicles.

So far nothing is known about the tetramerization of KAT1 and related ion channels in plants. It is necessary to understand whether the KAT1 K$^+$ channel needs to tetramerise within the ER for efficient ER export and how the heterotetramerisation influences these processes.

6.2.2 ER export motifs can affect subunit composition of channel tetramers in the plasma membrane

The acidic amino acid sequence DxDxE (392-396) which is responsible for ER export of KAT1 is highly conserved among K$^+$ channels of *Arabidopsis thaliana* and several K$^+$ channels of other plant species. Nevertheless, there are major differences in the motif between the channels (Figure 3.1). The acidic nature of D392 is conserved among all depicted ion channel except AKT2/3 and AtKC1. D394 is replaced by lysine (K) in AKT5, SPIK, AKT1, AKT2/3 and AtKC1 from *Arabidopsis thaliana*. Residue E396 is totally conserved among all channels analysed. It seems that in many cases the motif is disrupted but the channel can reach the plasma membrane (AKT5, SPIK, AKT1 and especially AKT2/3). The differences in the ER export motifs of the various plant K$^+$ channels may result in different affinities of the channel to Sec24 and therefore may play a role in defining the rate of ER export of different subunits and subsequently render the composition of channel heterotetramers in the plasma membrane. In particular, this seems true for channel subunits which lack an ER export motif and are retained in the ER like AtKC1 from *Arabidopsis thaliana*. Expression of AtKC1 in different cell types like COS cells, *Xenopus laevis* oocytes, tobacco mesophyll

protoplasts and *Arabidopsis thaliana* root cells always led to a retention of the channel in the ER (Duby *et al*., 2008; Reintanz *et al*., 2001). Recent expression studies of AtKC1 in HEK293 cells confirmed these results. If the QIQAE motif of AtKC1 was mutated to DIDAE, the ER export motif of KAT1, the channel was still retained in the ER (unpublished results).

It could be possible that AtKC1 contains a yet unknown ER retention signal which can be masked through the heterotetramerisation with other K^+ channel subunits. It is also conceivable that an intrinsic ER export motif needs to be activated or a retrieval signal must be deactivated.

Plant aquaporins display a similar dilemma: the maize aquaporin ZmPIP1;2 is localised in the ER upon expression in mesophyll protoplasts in contrast to ZmPIP2;1 which is localised at the plasma membrane. Coexpression of both proteins results in plasma localisation for either of them because of heterooligomerisation. Zelazny *et al.* (2008) identified a diacidic DIE motif in the N-terminus of ZmPIP2;4 and ZmPIP2;5. But this diacidic motif could not rescue the plasma membrane localisation when introduced into ZmPIP1;2. The role of the diacidic motif is furthermore questioned by the fact that ZmPIP2;1 which is located in the plasma membrane does not contain a diacidic ER export motif. FRET-FLIM measurements showed that oligomerisation of the aquaporins already occurs in the ER and is not altered after mutation of the diacidic ER export motif. Another yet unknown factor for ER export of aquaporins is the methylation of the E in the potential diacidic ER export motif in AtPIP2;1 (Zelazny *et al*., 2008).

The role of heterotetramerisation for the targeting of K^+ channel subunits has been intensively investigated for members of mammalian K_v channels. These studies show that phosphorylation can also have an impact on K^+ channel composition and localisation. $K_v1.2$ lacks strong trafficking signals and exhibits several phosphorylation sites which are important for ER export and affect subunit assembly. Although it lacks ER export signals or ER retention signals $K_v1.2$ channel is differentially localised in many cell types. $K_v1.1$ is retained mostly in the ER as a homotetramer because of a retention signal. The channel is targeted to the plasma membrane as a heterotetramer with $K_v1.2$ but not $K_v1.4$ although $K_v1.4$ contains an ER export signal. The composition of the heterotetramers of $K_v1.1$, $K_v1.1$ and $K_v1.4$ confers the localisation of the channel and depends also on the phosphorylation of the $K_v1.2$ subunit (Yang *et al*., 2008). The phosphorylation sites

of K_v1.2 are located within a putative 14-3-3 binding site and phosphorylation of the channel may be linked to binding of 14-3-3. Phosphorylation-dependent binding of 14-3-3 may mask an intrinsic ER exit or retrieval signal. Such a regulatory effect of 14-3-3 binding on ER export has been demonstrated for KCNK3 and K2p3.1 channels (O'Kelly *et al.*, 2002 and 2008). In case of the K_{ir}4.2 channel phosphorylation has been proven to function as a negative regulator of trafficking (Wade *et al.*, 2006).

KAT1 contains several putative 14-3-3 binding sites and Sottocornola *et al.* (2008) demonstrated that 14-3-3 proteins not only control the voltage-dependency of the KAT1 channel but also the number of channels in the plasma membrane. Future studies have to identify the binding site for 14-3-3 in the sequence of KAT1 and to analyse the regulatory function of 14-3-3 for KAT1 trafficking. Phosphorylation and dephosphorylation is obviously an important parameter in the regulation of ion channel trafficking and necessitates a broadening of the investigative scope to regulatory factors such as kinases and phosphatases (Sehnke *et al.*, 2002).

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SUMMARY

Membrane proteins, like the K^+ channel, are transported to the plasma membrane through the secretory pathway. In this study it was shown for the first time that ER export motifs, which play an important role in the transport of membrane proteins, are functional in plant cells. A diacidic ER export motif was identified in the K^+ channel KAT1 from *Arabidopsis thaliana*. This motif was shown to be required for efficient transport of the protein to the plasma membrane. Mutation of the diacidic motif resulted in ER retention of KAT1 in *Vicia faba* guard cells and in HEK293 cells. Further analysis of the diacidic motif and the surrounding amino acids revealed that the diacidic motif was not only diacidic but contained at least three acid residues at position D392, D394 and E396. Patch clamp studies demonstrated that the ER export was almost completely inhibited only when all three acidic amino acids were mutated to alanine. Mutation of one or two of the three residues led to a reduction of conductance corresponding to the number of mutated residues. Confocal analysis of these mutants revealed an almost complete ER retention for K^+ channels with three and two mutated acidic amino acids but a clear plasma membrane staining for mutation of only one acidic amino acid. Plasma membrane localisation of the ER export mutant of KAT1 could be rescued upon heterotetramerisation with the wildtype KAT1 channel. Therefore, ER export motifs can affect also subunit composition of K^+ channel tetramers in the plasma membrane.

Functional analysis of the identified ER export motif by FRET measurements demonstrated that KAT1 binds to Sec24, a subunit of COPII. The interaction of the K^+ channel KAT1 with Sec24 was dependent on the acidic ER export motif. This implies that binding of the diacidic motif of KAT1 to Sec24 is essential for recruitment of the channel into COPII vesicles and efficient export out of the ER. Altogether, the experiments show that ER export signals are crucial for the regulation of ion channel density and composition. Analysis of ER export of KAT1 in plant and animal cells implies that the mechanism of ER export is highly conserved among eukaryotes.

ZUSAMMENFASSUNG

Membranproteine wie der Kaliumkanal KAT1 werden entlang des sekretorischen Weges zur Plasmamembran transportiert. Diese Studie konnte zum ersten Mal zeigen, dass auch in pflanzlichen Zellen ER Exportmotive an der Regulation dieses Transportes beteiligt sind. Im C-Terminus des Kaliumkanals KAT1 wurde eine Signalsequenz aus zwei sauren Aminosäuren identifiziert, die für den effizienten Transport des Kanals zur Plasmamembran verantwortlich ist. Eine Mutation des Motivs an den Positionen D394 und E396 zu Alanin führte zur Retention des Kanals im perinukleären ER. Eine genauere Analyse der Signalsequenz zeigte, dass eine weitere saure Aminosäure (D392) Einfluss auf den ER Export des Kaliumkanals hat. Damit entspricht die Signalsequenz einem Motiv aus drei sauren Aminosäuren. Durch *Patch Clamp* Messungen konnte gezeigt werden, dass nur die Mutation aller drei saurer Aminosäuren zu vollständiger Inhibierung des ER Exports führt. Die elektrophysiologischen Untersuchungen belegen außerdem, dass das Ausmaß der Reduktion der K^+ Leitfähigkeit von der Anzahl der mutierten sauren Aminosäuren abhängig ist.

Mit Hilfe von FRET Messungen konnte die Bindung des Kaliumkanals KAT1 an die COPII Untereinheit Sec24 nachgewiesen werden, wobei die Interaktion von KAT1 mit Sec24 abhängig vom ER Exportmotiv war. Die Signalsequenz ist somit verantwortlich für die Rekrutierung des Kanals in COPII Vesikel und damit für den effizienten Export aus dem ER.

Die Plasmamembranlokalisation der ER Exportmutante konnte in einem *Rescue* Experiment durch Heterotetramerisierung mit dem Wildtyp KAT1 wiederhergestellt werden. Dieses *Rescue* Experiment macht deutlich, dass nicht alle Untereinheiten in einem KAT1 Tetramer ein ER Exportmotiv tragen müssen, damit der Kanal an die Plasmamembran transportiert wird.

ER Exportmotive sind somit in der Lage, nicht nur die Dichte sondern auch die Zusammensetzung von Kanälen in der Plasmamembran zu beeinflussen. Ein Vergleich des ER Exports von KAT1 in tierischen und pflanzlichen Zellen zeigt, dass der Mechanismus des ER Exports in eukaryotischen Zellen hoch konserviert ist.

LIST OF PUBLICATIONS IN THIS THESIS

Chapter 2

Mikosch M, Hurst AC, Hertel B, Homann U (2006) Diacidic motif is required for efficient transport of the K$^+$ channel KAT1 to the plasma membrane. Plant Physiology 142: 923-930

Chapter 4

Sieben C, Mikosch M, Brandizzi F, Homann U (2008) Interaction of the K$^+$ channel KAT1 with the COPII coat component Sec24 depends on a diacidic ER export motif. The Plant Journal 56(6):997-1006

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Chapter 3

Mikosch M, Käberich K, Homann U (2009) Efficiency of ER export of the K$^+$ channel KAT1 depends on the number of acidic amino acids within a triacidic Motif. In preparation

Chapter 5

Mikosch M and Homann U (2009) How do diacidic motifs work on ion channel trafficking? In preparation

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Place of Birth	Cologne, Germany

Professional and Scientific Career:

08/2005-06/2009	Technical University of Darmstadt
	Dissertation in plant cell biology
	Supervisor: PD Dr. Ulrike Homann
10/1999-07/2005	Technical University of Darmstadt
	Diploma in biology
	(cell biology, microbiology and plant physiology)
07/1986-08/1999	Primary and Grammar School in Cologne

Grants and scholarship:

09/2003-12/2003	Scholarship of the DAAD
08/2005-06/2008	Associated member of the interdisciplinary DFG Graduate College 340 "Communication in Biological Systems"

List of publications, talks and poster presentations:

Publications:

Mikosch M and Homann U (2009) How do diacidic motifs work on ion channel trafficking? In preparation

Mikosch M, Käberich K, Homann U (2009) Efficiency of ER export of the K^+ channel KAT1 depends on the number of acidic amino acids within a triacidic Motif. In preparation

Sieben C, Mikosch M, Brandizzi F, Homann U (2008) Interaction of the K^+ channel KAT1 with the COPII coat component Sec24 depends on a diacidic ER export motif. The Plant Journal 56(6):997-1006

Mikosch M, Hurst AC, Hertel B, Homann U (2006) Diacidic motif is required for efficient transport of the K^+ channel KAT1 to the plasma membrane. Plant Physiology 142:923-930

Kumari N, Sharma V, Mikosch M, Unfried C, Gessler A, Fischer-Schliebs E, Luettge U (2005) Seasonal photosynthetic performance and nutrient relations of *Butea monosperma Taub*. in comparison to two other woody species of a seasonal deciduous forest in SE-Rajasthan and to planted trees in the area. Indian Journal of Forestry 28(2):116-126

Talks and poster presentation:

- Rhein-Main Colloquium (2005) Mainz, Germany, talk
- European plant endomembrane meeting (2005) Paris; France, talk
- Summer school of the interdisciplinary DFG Graduate College 340 "Communication in Biological Systems", (2005) Rizlern, Austria; talk
- European plant endomembrane Meeting (2006) Mosel; Germany, talk
- Summer school of the interdisciplinary DFG Graduate College 340 "Communication in Biological Systems" (2006) Rizlern, Austria; talk
- Plant transport group meeting (2006) Wye, Great Britain; talk
- DFG Schwerpunkttreffen (2006) Hirschberg, Germany, talk
- International workshop on plant membrane biology (2007) Valencia; Poster presentation
- Botaniker Tagung (2007) Hamburg, Germany, Poster presentation; best poster award
- European plant endomembrane meeting (2007) Oxford; Great Britain, talk
- 31. Jahrestagung der Deutschen Gesellschaft für Zellbiologie (2008) Marburg, Germany, Poster presentation
- DFG Schwerpunkttreffen (2008) Hirschberg, Germany
- European plant endomembrane meeting (2008) Lecce, Italy, talk

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt habe.
Ich habe bisher noch keinen Promotionsversuch unternommen.

Darmstadt, den 21.November 2008

Melanie Mikosch