SNARE Specificity in Membrane Protein Traffic

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Abstract

Recent findings indicate that there is a role for SNAREs in ion channel regulation. With the discovery of the vesicle trafficking SNARE protein NtSYP121 came the development of the predicted cytosolic domain fragment of the protein. This Sp2 fragment was found in studies to block the responses of guard cell K^+ and Cl^- channels to ABA. These observations raised questions about the role of SNAREs in ion channel control. In this study, the effects of several plasma membrane SNARE Sp2s and one PVC-localised SNARE Sp2 on the K^+ channel KAT1 were investigated. The experiments made use of GFP- and HA- tagged KAT1 to observe the traffic and localisation of KAT1 in tobacco cells. Acetylsalicylic acid was also used to combat the effects of the wound response activated during slide preparation. Coexpression of KAT1 with SYP121-Sp2, SYP122-Sp2 and SYP71-Sp2 resulted in a disruption of trafficking and in microdomains becoming diffuse and mobile at the plasma membrane. No disruption in trafficking or localisation of KAT1 was seen when coexpressed with the PVC-localised SNARE fragment SYP21-Sp2. None of the Sp2s used had any effect on the H^+-ATPase PMA2. These results offer evidence of a role for plasma membrane SNAREs in trafficking and anchoring of KAT1 to the plasma membrane.

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Declaration

All the work presented in this thesis is my own, with the exception of the Western Blots in Figure 5, which were carried out by Dr. Christopher Grefen. All analyses and interpretations contained in this thesis are my own.

Karen Deachon.

Abbreviations

13-HPLA	13-hydroxyperoxylinolenic acid
ABA	abscisic acid
APX	ascorbate peroxidase
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
BSA	bovine serum albumin
BY-2	bright yellow-2
CaMV	cauliflower mosaic virus
CAT	catalase
CFTR	cystic fibrosis transmembrane conductance regulator
DRM	detergent resistant membrane
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ET	ethylene
FRAP	fluorescence recovery after photobleaching
FWHM	full width at half maximum
GAP	GTPase activating protein
GFP	green fluorescent protein
GLUT4	glucose transporter 4
GPI	glucosyl phosphatidylinositol
GSV	GLUT4 storage vesicle
GTP	guanosine triphosphate
HA	hemagglutinin

JA	jasmonic acid
kDa	kiloDalton
Kv	voltage gated K^+ channel
MAPK	mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
mRNA	messenger RNA
NO	nitric oxide
NSF	N-ethylmaleimide-sensitive factor
OD	optical density
OPDA	12-oxo-phytodienoic acid
PAR	photosynthetically active radiation
PCR	polymerase chain reaction
PMA	plasma membrane H^+-ATPase
PMSF	phenylmethanesulfonyl fluoride
PR	pathogenesis related
PVC	prevacuolar compartment
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	salicylic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP $_{(1)}$	soluble NSF attachment protein
SNAP $_{(2)}$	synaptosomal-associated protein
SNARE	soluble NSF attachment protein receptor
SYP	syntaxin of plants
TBST	Tris-buffered saline Tween-20
TGN	trans-Golgi network

UV	ultra violet
VAMP	vesicle associated membrane protein
YFP	yellow fluorescent protein

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CHAPTER 1:

INTRODUCTION

It is a fundamental characteristic of all eukaryotic cells that they elaborate intracellular membrane structures, often referred to as organelles, in addition to the cell membrane or plasma membrane. These membrane structures serve a number of purposes by segregating cellular homeostatic and metabolic processes, and thereby enabling their efficient functioning within defined and, often widely varying conditions (Kurland *et al.,* 2006). Compartmentation raises the concentrations of metabolites as well as keeping some enzymes and various regulatory proteins in close proximity while separating others; it provides a structured membranous matrix on which to build many protein complexes; finally, it enables the separation of aqueous phases to allow the generation of pH and potential gradients that are essential for energy production.

Maintaining this compartmentation requires the coordinated traffic and upkeep of the individual membrane structures, the compartments they form and the integral membrane proteins that contribute to their functioning. Similarly cell growth and development depends on this same traffic. As part of this process, a targeted movement of membrane and membrane protein is essential (Jurgens, 2004). For example, different transmembrane solute transporters – pumps, coupled transporter and ion channels – are found at each of the membranes bounding different compartments. These distinct populations of transporters are important for appropriate concentration of solutes and communication between the different aqueous phases of the cell, and between the cell and its surrounding environment. As I will discuss later, well-defined populations of ion channels and pumps are found at the plasma membrane of plants that are important for K^+ transport and for its energisation. Thus, it is essential that, after their synthesis and folding, these (and many other) proteins are trafficked and accurately delivered to the correct membrane in order to maintain cellular activity.

Many aspects of the synthesis and traffic of membranes and of integral membrane proteins are conserved among eukaryotic cells (Elotza *et al.,* 2003; Paschen *et al.,* 2003;

Schmidt *et al.,* 2003). Membrane and protein synthesis takes place at the endoplasmic reticulum (ER) and these components are then generally moved by vesiculation and translocation to Golgi apparatus before relocation and targeting to different membrane structures, including the plasma membrane, endosomes and, in yeast and plants, the vacuole membrane (Rothman, 1994; ter Beest *et al.,* 2005). The machinery involved includes coat proteins and associated elements that form vesicles from the donor membrane and recruit specific protein components therein, as well as cytoskeletal motors that move the vesicles within the cell (Farsad and De Camilli 2003). Equally important is the fusion of these vesicles to their acceptor membrane and their correct targeting to this site. In this regard, one group of membrane proteins, the so-called SNAREs (soluble NSF [*N*-ethylmaleimide–sensitive factor] attachment protein receptors) are thought to play a key role by driving the later stages of membrane traffic and vesicle fusion. A very large body of literature now supports this model for SNARE function, based primarily on studies of the mammalian synapse and on yeast (Bhattacharya *et al.,* 2002; Rothman, 1994; Sollner *et al.,* 1993; Weber *et al.,* 1998). However, as I will explain, much less is known about the functions and specificity of SNAREs in plants, and several recent studies have uncovered additional roles for plant SNAREs that may not fit the mammalian and yeast models. The work summarised in this thesis explores one aspect of these issues by addressing the specificity among arabidopsis SNAREs for traffic of two well-defined marker proteins for the plasma membrane.

1.1 SNARE structure

1.1.1. Discovery of SNAREs

Studies in the late ‘80s and early ‘90s (Clary *et al.,* 1990; Weidman *et al.,* 1989; Whiteheart *et al.,* 1992) found that there were proteins which bound to the membranes and were needed for vesicle fusion. Two of these proteins are NSF (N-ethylmaleimide-

sensitive factor) and SNAP (soluble NSF attachment protein). Later work on signalling in the brain found that certain membrane proteins bound both NSF and SNAP, and these were named 'SNAP receptors' or SNARES (Rothman, 1994). The proteins that formed the SNARE complex comprised a vesicle associated membrane protein (VAMP), syntaxins, and a 25-kD protein associated with the synaptosome, also known as SNAP25 (Linial, 1997; Sollner *et al.,* 1993). The mechanism of membrane fusion remains highly conserved, and all fusion events have since been found to rely on these proteins, or versions of them.

1.1.2. Types of SNAREs

SNARE proteins can be divided functionally into vesicle-associated and target-membrane-associated SNAREs, or v- and t-SNAREs. In neuromuscular tissues VAMP is found on vesicle membranes and so is a v-SNARE while syntaxin and SNAP-25, both on the target membrane, are t-SNAREs (Rothman, 1994; Sollner *et al.,*1993; Weber *et al.,*1998). These definitions are empirical and more recently SNAREs have been classified as Qa, Qb or Qc SNAREs, and as R-SNAREs on the basis of the central amino-acid residue within the core coiled-coil domain of the proteins (Brunger, 2001; Fasshauer *et al.,*1998; Linial, 1997; Bock *et al.,* 2001). SNAREs which have a Q/Gln residue are classified as Q-SNAREs, and those with an R/Arg residue as R-SNAREs. Qa- Qb- and Qc-SNAREs are sub-divided on the basis of sequence similarity within this core domain to Syntaxin 1A and the N- and C-terminal halves of neuronal SNAP-25, respectively. VAMP proteins and syntaxins both contain very short C-terminal transmembrane domains, and a long N-terminus which includes one coiled-coil domain in the first and four coiled-coil domains in the second, also referred to as the Ha, Hb, Hc and H3 domains. The H3 domain, nearest the C-terminus of the syntaxin, includes the conserved glutamine residue and contributes to the formation of the SNARE complex. The coiled-coil domains of SNAP-25 and its homologues are situated one either side of a region composed of multiple

cysteine residues (Hess *et al.,*1992). These cysteines can undergo palmitoylation to anchor the protein at the target membrane (Salaun *et al.,* 2005). Interaction of these SNARE partners through their coiled-coil domains forms a ternary structure known as the SNARE core complex which is highly stable and resistant to detergent solubilisation. In the neuronal model, the core complex thus comprises domains from three different proteins, although in many instances the Qb- and Qc-SNARE domains reside on different proteins (Sutter *et al.,* 2006; Hong, 2005). In a general model, formation of the core complex draws and holds the vesicle and target membranes together for fusion, but can be dissociated by NSF (Hayashi *et al.,*1994; Sollner *et al.,*1993; Sutton *et al.,*1998).

1.1.3. SNARE complex structure and function

X-ray crystallography studies have provided a more detailed view of the SNARE core complex (Sutton *et al.,*1998; Tishgarten *et al.,*1999). Within the core complex, the helix bundle is hydrophobic, but the exposed surface contains different sites which are a mixture of hydrophobic, hydrophilic and charged residues. It is predicted that these sites, especially the hydrophobic sites, are important in the interaction of the complex with SNARE regulators, including Sec/Munc proteins (Latham *et al.,* 2006). The four helices associate in orientations that are parallel and antiparallel with one another, with syntaxin and VAMP each contributing one α-helix and SNAP-25 contributing two α-helices, one from each terminus (Poirier *et al.,* 1998; Sutton *et al.,* 1998). Within the helix bundle, the hydrophobic residues associate in a leucine-zipper-like fashion around the central ionic layer formed of the three conserved glutamine and one arginine residues. Residues of the hydrophobic layers are essential to the stability of the complex (McNew *et al.,* 2000; Sutton *et al.,* 1998).

Evidence that SNARE complexes function in vesicle traffic in vivo came with the discovery that, in nerve terminals, a number of toxins derived from *Clostridium botulinum*

act by cleaving different SNARE proteins. Each of the eight botulinum toxins (tetanus toxin and BotN/x, x=A-G) attack at specific peptide sequences – for example, BotN/C cleaves Syntaxin 1A adjacent a cysteine between the H3 and terminal transmembrane domains – and each was found to inhibit vesicle fusion (Montecucco and Schiavo, 1995) (Lonart and Sudhof, 2000). These observations, and biochemical evidence of the proteins contributing to the SNARE core complex and their localisations, led to the idea of SNARE-mediated fusion between target and vesicle membranes and has become known as the SNARE hypothesis (Rothman, 1994; Sollner *et al.,* 1993). This hypothesis has found support in studies of the secretory pathways in yeast and on SNAREs in mice, *C. elegans* and drosophila. For example, the complete sequencing of the drosophila genome has allowed identification of all of the drosophila SNAREs (Stewart *et al.,* 2001) and facilitated their study. Among others, drosophila mutants in neuronal VAMP or syntaxins have shown a complete lack of synaptic transmission, although vesicles still docked at the correct sites on the target membrane (Deitcher *et al.,*1998; Sweeney *et al.,*1995). Similar studies in *C. elegans* (Nonet *et al.,* 1998; Saifee *et al.,* 1998) backed up the evidence that SNAREs are vital for membrane fusion, and furthermore showed that a restricted mutation in the gene encoding for the coiled-coil domain of the syntaxin affected its ability to bind with VAMP (Kee *et al.,* 1995).

The complete sequencing of the yeast genome has also given important insights into the interactions and functions of the different SNAREs. The 21 yeast SNAREs include eight members of the syntaxin family (Pelham, 1999). Furthermore, analysing the complexes found when yeast are lysed has given a wealth of information about their interacting partners that have since been largely verified through a combination of mutational studies, crystallography studies and microscopy (Paumet *et al.,* 2004; Mcnew *et al.,* 2000; McNew *et al.,* 2000b; Bowen *et al.,* 2005). The number of known partners for each syntaxin varies – for example, Ufe1p interacts with Sec20p and Sec22p, Tlg2p, binds

Vti1p and Tlg1p while the Golgi SNARE Sed5p forms complexes with up to nine different partners (Tsui *et al.,* 2001) – however in each case the specificity of interactions appears to be encoded by the SNARE structure (Paumet *et al.,* 2004; Mcnew *et al.,* 2000). These observations show that the specificity of fusion of a vesicle can be related to SNARE partner binding, but they do not rule out roles for other proteins in the specificity of targeting to different membranes in vivo.

There is also evidence for a degree of overlap in the functions of many SNARE proteins. In yeast during targeting and fusion, Snc1p and Snc2p – yeast homologues of VAMP and synaptobrevin – form a heterotrimer with Sec9 and either Sso1p or Sso2p, similar to the VAMP/syntaxin/SNAP-25 core complex in neurons (Neiman *et al.,* 2000). Sso1 and Sso2p function as t-SNAREs at the plasma membrane. Snc1p and Snc2p, however, recycle to the Golgi and are involved in the targeting of both endocytic and exocytic vesicles (Pelham, 1999). Mutants lacking both Snc1p and Snc2p have been found to be impaired in secretion. However, the fact that there is secretion at all suggests either the presence of another SNARE which can substitute for Snc1p Snc2p, or that Sso1p and Sso2p can act as v-SNAREs and associate with other plasma membrane SNAREs (Pelham, 1999).

It is important to note that these observations conflict to an extent with earlier studies showing a promiscuity in the interactions between different SNAREs in vitro. Fasshauer *et al.,* 1998, Yang *et al.,* 1999, Jahn *et al.,* 2003, and others (Nichols and Pelham, 1998) found that many SNARE proteins would form complexes in vitro that were not always consistent with their localisation and interactions in vivo. Non-canonical complexes may also be formed that do not involve Qa:Qb:Qc:R pairing. For example, there are reports of complexes formed of two and four Qa-SNAREs as well as of two Qa-SNAREs with Qb- and Qc-SNAREs (Sutter *et al.,* 2006). Nonetheless, these findings may not reflect the normal situation, as the opportunities for these interactions are unlikely to

occur or are only transiently stable in vivo (Jahn *et al.,* 2003). Thus, the specificity of SNARE interactions appears to be encoded largely by the particular SNAREs and their unique structures.

1.1.4. SNAREs in arabidopsis

There are a large number of SNAREs in arabidopsis, and the functions of many are yet to be found. Indeed, in arabidopsis there are no less than 68 core SNARE proteins, roughly double the number found in humans and more than three times the number in drosophila. The arabidopsis SNAREs include VAMP and SNAP proteins (Gerst, 1999) and 24 syntaxins. The syntaxins are now classed as syntaxins of plants (SYPs; Blatt and Thiel, 2003; Sanderfoot *et al.,* 2000) and from hereafter I will use the term SYP to refer to those found in arabidopsis although a similar distribution of genes and subfamilies appears to be true also for other plant species (Sutter *et al.,* 2006). Syntaxins are comparatively large, at 200-400 residues in length, and belong to ten gene subfamilies; SYP11, 12, 13, 2, 3, 4, 5, 6, 7 and 8 that, with a few notable exceptions, parallel the distribution of syntaxin genes found in yeast and mammals (Sutter *et al.,* 2006; Hong, 2005). It is of interest that plants seem to have many more SNARE genes within each subfamily by comparison with yeast and mammals, and this difference does raise a question about their functions.

SYP121 and SYP122 are plasma membrane SNAREs (Leyman *et al.,* 1999; Collins *et al.,* 2003). It is probable that other members of the SYP12 subfamily localise to the same place. Knockouts of either gene do not have a great effect, however pathogen resistance is reduced with mutation or deletion of SYP121 (Collins *et al.,* 2003; Pratelli *et al.,* 2004). Less is known of the three other members of the family, SYP123, SYP124 and SYP125. SYP11- type syntaxins consist of SYP111 and SYP112. SYP111 is a syntaxin which is localized in the phragmoplast of dividing cells (Jurgens, 2005; Pratelli *et al.,* 2004). Studies have shown that gene disruption of SYP111 (the knolle mutation) will result in a

lethal phenotype, regardless of the plant producing SYP122, the second member of the gene family (Lukowitz *et al.*,1996). SYP71 has also been localised to the plasma membrane (Suwastika *et al.,* 2008) and recent studies using a dominant-negative strategy indicate that it also is functional at this site (Tyrrell *et al.,* 2007). There has not been much work to date attempting to show the function or localisation of the other proteins in this subfamily. However, there are three known members of the SYP7 family, and these have no apparent mammalian or yeast homologues to give clues to their function (Sanderfoot *et al.,* 2001).

In addition to those localized to the plasma membrane, there are also endomembrane syntaxins. Two members of the SYP2 syntaxins are known to localise to the prevacuolar compartment (SYP21 and SYP22) (Conceicao *et al.*,1997; Sanderfoot *et al.*,1998). Some studies have also shown SYP22 localising to the tonoplast in the shoot apical meristem (Sato *et al.*,1997). Disruption of either gene results in lethality (Sanderfoot *et al.,* 2001b). SYP23 is the last member of the family and not much is known about this syntaxin. More effort has gone into studying SYP4-like syntaxins (SYP41, SYP42 and SYP43). SYP41 and SYP42 both localise to distinct domains on the trans-golgi network (Bassham *et al.,* 2000), and disruption of either results in lethality at the gametophytic stage (Sanderfoot *et al.,* 2001b). SYP51 of the SYP5 syntaxins is found at several places in the late endosomal/secretory system, as is SYP61. Both have been found to localise to the prevacuolar membrane and to the trans-golgi network (Sanderfoot *et al.,* 2001). SYP52 is as yet uncharacterised. It is proposed that, due to their interactions, SYP51 and SYP61 are involved in TGN to PVC trafficking (Sanderfoot *et al.,* 2001). No data are yet to hand for the SYP3 and SYP8 subfamily gene products. However, sequence alignment shows that these genes share close homologies with yeast and mammalian syntaxins that are localised to the Golgi and the ER (Pratelli *et al.,* 2004).

Along with the 24 syntaxins, there exist three SNAP25-like Q-SNAREs, SNAP33, SNAP29, and SNAP30 (also known as SNP11, SNP12 and SNP13).

SNAP33 is localised to the plasma membrane in non-dividing cells, and at the phragmoplast during cytokinesis (Heese *et al.,* 2001). SNAP33 mutants show some difficulty with cytokinesis and have a dwarf phenotype which eventually results in lethality. Mutants display necrotic lesions, and SNAP33 is produced in response to certain bacteria. It is also linked to expression of PR-1, which interacts with SYP111 and has a pathogenesis related role (Heese *et al.,* 2001; Wick *et al.,* 2003). This suggests that SNAP33 is involved in the plant's defensive response to pathogens, and may be important in exocytosis. As mutation of SNAP33 is not immediately lethal, it is possible that its subfamily members, SNAP29 and SNAP30, may have some overlapping functions. Both, like SNAP33, interact with the syntaxin SYP111, which points to a role for each of these proteins in cytokinesis (Wick *et al.,* 2003).

There are, in addition to these proteins, fourteen VAMP-like SNAREs. Little is known about these SNAREs except that they show most homology with mammalian VAMP7 proteins (Pratelli *et al.* ,2004). These proteins are involved in endosomal trafficking (Prekeris *et al.,* 1999). There are no arabidopsis SNAREs with homology to mammalian VAMP1 proteins involved in secretory traffic to the plasma membrane (Chen and Scheller, 2001)

1.2 SNARE function in plants:

1.2.1. Defence

SNAREs have been found to have a key role in pathogen defence.

When plants are attacked by bacteria, fungi or a virus, they synthesise several pathogenesis related (PR) proteins, including PR1 (van Loon and von Strien, 1999). It has been found

that infection of arabidopsis by *P.syringae* causes the plant to secrete PR1, PR2 and PR5 (Uknes *et al.,* 1992). The plant will accumulate salicylic acid (SA) when PR proteins are expressed (Yalpani *et al.,* 1991). As the PR proteins are synthesised at the ER and are then transported either to the vacuole or outside of the cell, SNARE mediated secretory pathways are almost certainly involved as is vesicle fusion.

Studies on the role of SNAREs in secretion of PR proteins have focused on SNAP33, known to interact with the syntaxin KNOLLE (SYP111, involved in phragmoplast formation, see above) and SYP121 (Kargul *et al.,* 2001). The up-regulation of SNAP33 after inoculation with various pathogens (Wick *et al.,* 2003) is logical, as after attack, increased secretion of PR proteins would likely require increased vesicle fusion. Furthermore, other studies have shown that membrane repair requires up-regulation of SNAREs. For example, echinoderm SNAP25 (the SNAP33 homologue) plays a role in membrane resealing after damage in sea urchin eggs (Steinhardt *et al.,* 1994). Similarly, ROS (reactive oxygen species) produced after pathogen attack may cause damage to the plasma membrane, requiring repair by increased vesicle fusion (Park, 2006).

As well as SNAP33, the plasma membrane syntaxin from *Nicotiana benthamiana* NbSYP132 is involved in pathogen resistance. NbSYP132 silenced plants were found to be impaired in the accumulation of some PR proteins in the cell wall, pointing to the SNARE as an important target in the secretion of antimicrobial proteins (Kalde *et al.,* 2007). arabidopsis syntaxins SYP121 and SYP122 have also been found to play a role in plant defence. SYP121 has been observed to accumulate at sites of fungal attack (Asaad *et al.,* 2004). In knockout mutants, there was seen to be delayed growth in the thickness of the cell wall at areas of attack. This suggests that SYP121 may play a role in delivery of materials needed to form the cell wall (Schmelzer, 2002). Further studies (Zhang *et al.,* 2007) also reveal a post invasive defensive role for SYP121 and SYP122. Work with triple mutants defective in SYP121 and SYP122 and with mutant alleles of the salicylic acid

signalling genes point to SYP121 and SYP122 as negative regulators of SA, JA (jasmonic acid) and ethylene dependent defence pathways (Zhang *et al.,* 2007).

1.2.2. Gravitropism

Gravitropism is a characteristic exhibited by most plants and enables them to adapt to their environment. Gravitropic responses are essential for a plant to change its angle of growth in order to ensure leaves are exposed to sunlight and roots grow downwards to anchor the plant and obtain water and nutrients. So far, two agravitropic arabidopsis mutants have been found associated with lesions in SNARE encoding genes. These two mutants, *sgr3* and *sgr4* (also called *zig* or *zigzag*), display abnormal stem gravitropism (Kato *et al.,* 2002, Yano *et al.,* 2003). *SGR3* encodes VAM3/SYP22 (Yano *et al.,* 2003) and *SGR4* encodes VTI11 (Kato *et al.,* 2002). Both of these SNAREs are localised to vacuolar and prevacuolar compartments (PVC) (Sato *et al.,* 1997: Zheng *et al.,* 1999), and it is thought that they interact with SYP51 to form a complex involved in vesicular traffic to the vacuolar compartment or the PVC (Sanderfoot *et al.,* 2001). If this is so, then the mutants' agravitropism might be explained by improper vacuole formation, affecting the movement of statoliths. Statoliths are specialised amyloplasts which are denser than the surrounding cytoplasm, and can be found in statocytes, a subset of cells located in the root cap and shoot endoderm (Kato *et al.,* 2002b). Statoliths are connected to actin filaments, and when sedimentation occurs, tension in the actin network is thought to be transmitted to mechanoreceptors in the plasma membrane or affect related elements in cellular signalling (Blancaflor and Masson, 2003). Thus, the mutations in *VTI11* or *SYP22* may lead to a lack of proper transport or localisation of other proteins involved in gravitropism, such as ion channels or pumps involved in signalling during gravity sensing.

1.2.3. Hormone signalling

Stomatal movements in higher plants are subject to control by hormone signalling and the resultant changes in the activity of ion channels in the stomatal guard cells. Changes in turgor result from uptake or release of K^+ and anions by guard cells. When K^+ and anions accumulate, the water potential within the guard cells becomes more negative, water enters by osmosis, the guard cells become turgid, and the stomatal pore opens. Conversely, stomatal closure occurs when K^+ and anions are lost from the guard cells, leading to a loss in turgor. Among others, guard cells of arabidopsis express two K^+ channels, KAT1 and GORK, and both contribute to these fluxes of K^+ (Maser *et al.,* 2001). Both KAT1 and GORK are found at the plasma membrane of guard cells, and GORK is also found in root hairs. KAT1 is one of four inward-rectifying K^+ channels in arabidopsis that open on hyperpolarisation to permit K^+ entry into the cell; GORK is one of two outward-rectifying K^+ channels in arabidopsis that open on depolarisation to permit K^+ efflux from the cell. A unique feature of GORK is that its activity is also sensitive to K^+, thus compensating for the ion concentration outside. This same characteristic enables GORK to act as a potassium sensor in root hairs, and it is important in maintaining root hair polarity (Ivashikina *et al.,* 2001).

Most of the information available for guard cells relates to the plant hormone abscisic acid (ABA). ABA closes stomata by triggering changes in KAT1 and GORK activities, as well as anion channels, to permit the loss of K^+ and anions and reduce turgor. At least two signal cascades are involved in controlling these events. ABA activates Ca^{2+} channels, triggering a rise in cytosolic-free Ca^{2+} concentration. This rise in Ca^{2+} in turn activates anion channels, releasing anions from the cell and depolarising the membrane, and it also inactivates KAT1. Finally, membrane depolarisation activates GORK and further suppresses KAT1 activity by deactivation. [Both inactivation and deactivation relate to a decrease in channel activity. For voltage-dependent currents, inactivation relates

to a decrease in activity at the activating voltage; deactivation relates to a decrease in activity when the stimulus voltage is removed (Langer *et al.,* 2004; Becker *et al.,* 1996)]. ABA also alkalinises the cytosol, enhancing the outward-rectifying GORK current and further promoting K^+ efflux for stomatal closure.

1.3 KAT1 regulation:

1.3.1. Regulation by hormone signalling

Because several of the ABA triggered events associated with KAT1 occur very rapidly – within 30-60 s of its addition to epidermal peels (Sutter *et al.,* 2007) – much attention has focused on the regulation of this channel. KAT1 was the first channel shown to be expressed in guard cells (Nakamura *et al.,* 1995). It is activated by acidic pH outside (Brüggemann *et al.,* 1999), and experiments with KAT1 mutants have shown a decreased sensitivity in stomatal opening to Cs^+ (a channel blocker), indicating that it plays a key role in K^+ uptake (Ichida *et al.,* 1997). KAT1 subunits interact with AKT1, AKT2, KAT2 and KC1 subunits (Pilot *et al.,* 2003), although the significance of these associations are still unresolved. Like these other K^+ channels, KAT1 belongs to the shaker-like superfamily of channels characterised by six transmembrane domains S1-6 (Jan and Jan, 1997) one of which, the S4 helix, is positively charged and acts as a voltage sensor (Bezanilla, 2000; Papazain *et al.,* 1991). Two transmembrane domains, formed from the S5 and S6 helices, form the pore and channel selectivity filter when four channel subunits come together to form a functional unit (Heginbotham *et al.,* 1994).

Some aspects of KAT1 regulation appear to be intrinsic to the channel protein itself. In particular, KAT1 and its orthologues in tobacco and *Vicia* are sensitive to pH (Blatt, 1992). A decrease in extracellular pH has been found to activate KAT1 (Very *et al.,* 1995), and studies using site directed mutagenesis (Hoth *et al.,* 1997) identified two

histidine residues which affect pH sensitivity located on the extracellular loops of KST1, the KAT1 homologue of potato. Putative protonation sites (eg. histidines) have also been identified in the cytosolic C-terminus of the KAT1 protein (Marten and Hoshi, 1997). Other modes of regulation involve protein modification and interactions. KAT1 harbours a cyclic-nucleotide binding domain on its C-terminus (Kumar and Weber, 1992) as well as phosphorylation sites that may be phosphorylated by cAMP/cGMP- dependent protein kinases, Ca^{2+} calmodulin – dependent kinase II or protein kinase C (Pearson and Kemp, 1991; Tang and Hoshi, 1999; Li *et al.,* 1998). KAT1 may also be phosphorylated by an ABA-responsive protein kinase (Mori *et al.,* 2000). In fact, voltage clamp experiments have shown KAT1 currents to be inhibited by a calcium dependent protein kinase (Berkowitz *et al.,* 2000), and it has been suggested therefore that Ca^{2+} acts in vivo to regulate KAT1 by promoting its phosphorylation in this manner (Berkowitz *et al.,* 2000).

1.3.2. Regulation by membrane trafficking proteins

While the focus on KAT1 regulation has been dominated by investigations of these signalling pathways (above), several recent studies have indicated roles for membrane trafficking proteins as well. Interest in membrane traffic as a means to controlling K^+ (and other) ion channels in plants originated with the discovery of the vesicle trafficking (SNARE) protein NtSYP121 (=NtSyr1) and its function in guard cells by Leyman *et al.,* (1999). These researchers used an expression-cloning strategy to hunt for proteins involved in ABA signalling. They took advantage of Xenopus oocytes to express foreign mRNA and to incorporate exogenous proteins within the endogenous signal cascades that activate Cl^- channels at the oocyte plasma membrane. By sib-selection they isolated one protein (and its coding sequence), NtSYP121, that promoted Cl^- channel activity dependent on ABA. To obtain evidence of its function in the plant, Leyman, et al. made use of BotN/C toxin, which could be shown to cleave the SNARE-like protein, and they synthesized a

polypeptide corresponding to the predicted cytosolic domain of the protein, the so-called Sp2 fragment. They found that injection into guard cells of both BotN/C and the Sp2 fragment blocked the responses of the guard cell K^+ and Cl^- channels to ABA. Because the basal levels of activity of these ion channels were not affected, Leyman, *et al*. concluded that the toxin and Sp2 fragment must act upstream within the ABA signal cascade. SNAREs in animals are known to bind Ca^{2+} channels (Bezprozvanny *et al.,* 1995), CFTR Cl^- channels (Leyman *et al.,* 1999), as well as G-protein-coupled receptors and microfilament-associated proteins (Fujita *et al.,* 1998; Krasnoperov *et al.,* 1997). Not surprisingly, Leyman *et al.,* (1999) suggested that NtSYP121 might contribute as a scaffold protein of an ABA-receptor complex.

In fact, SYP121 is functional as a bone fide vesicle trafficking protein and the Sp2 fragment of SYP121 is known to block secretion and development. Geelen *et al.,* (2002) made use of the same Sp2 fragment, placing it under the control of a dexamethasone-inducible promoter to generate stable transgenic lines in tobacco. They also used soluble Green Fluorescent Protein (GFP) carrying a secretory leader sequence as a marker for traffic to the plasma membrane. They found that expressing
the Sp2 fragment resulted in a total cessation of growth in the transgenic tobacco and a block of GFP secretion leading to its accumulation in the endoplasmic reticulum and Golgi. They also observed that the effects on growth could be rescued by over-expressing the full-length SYP121. Subsequent work (Sutter *et al.,* 2006b) showed that the Sp2 fragment affected traffic of the KAT1 K^+ channel in a similar fashion. However, these studies also indicated an effect on the distribution of KAT1 at the plasma membrane. Finally, Sutter *et al.,* (2007) carried out experiments to determine whether KAT1 traffic is affected by ABA. These most recent studies showed that KAT1 undergoes endocytosis and recycling to the plasma membrane after treatment with ABA both in guard cells and in epidermal cells of tobacco. However, the time course for endocytosis and recycling was

too slow to account for the rapid response of the activity of the channel to ABA. From these data, therefore, it appears that SYP121 plays a role in the traffic of at least one ABA-sensitive K^+ channel. However, it also seems that the SNARE may have additional functions unrelated to vesicle trafficking which determine the localisation, and perhaps the protein associations, of the channel at the plasma membrane. Thus, one question raised by these studies is whether SYP121 is a protein with two separate functions.

1.4 Mechanisms for KAT1 regulation:

1.4.1. Population control

In general, there are two possible mechanisms that we might consider to account for SYP121 action in regulating KAT1 activity. First, the SNARE may function as a bone fide vesicle trafficking protein that is essential for the delivery of KAT1 to the plasma membrane. The second mechanism relies on its action through a direct interaction of the SNARE with KAT1 or with related proteins as part of a larger protein complex. Examples of both mechanisms are now found in the literature. The first model can account for changes in channel activity by affecting the population of channel proteins at the membrane. A good example of this model is the control of the GLUT4 glucose transporter in mammalian epithelia. GLUT4 is important for glucose uptake stimulated by insulin and has been studied extensively. In a basal state, less than 5% of GLUT4 in a cell will be located at the plasma membrane. Instead, it is contained within the endosomal system, the trans-Golgi network and specialised vesicles called GLUT4 storage vesicles (GSVs) (Kumudu *et al.,* 2003; Lampson *et al.,* 2001). GLUT4 is constantly recycled from the plasma membrane to the GSVs and back again. This internalisation occurs at a rapid pace, whereas delivery of GLUT4 to the plasma membrane is around 20-fold slower (Lampson *et al.,* 2001). In the presence of insulin, however, the delivery of GLUT4 to the plasma

membrane is accelerated. The effect is to shift the steady-state distribution of GLUT4, favouring their presence at the plasma membrane. With more GLUT4 at the plasma membrane the uptake of glucose by facilitated diffusion increases.

How do SNAREs contribute to these events? Studies have shown that the v- and t-SNAREs VAMP2 and SNAP23 (a homologue of SNAP25) are needed for translocation of GLUT4 to the plasma membrane (Foran *et al.,* 1999). From studies using brain extracts, it has been shown that in the presence of BotN/X toxins translocation of GLUT4 from GSVs to the plasma membrane is inhibited (Foran *et al.,* 1999). For the vesicle to fuse with the plasma membrane, it is also important that the syntaxin is in the correct conformation. When the syntaxin is 'closed', the N-terminus is folded back, preventing access to the core α-helix domain. The syntaxin must then flip over allowing it to interact with the other proteins in the SNARE complex (Dulubova *et al.,* 1999). For this change to happen, syntaxin-binding proteins must be present. Some of the proteins thought to be involved are the Sec/Munc proteins. It has been proposed that Munc18 is involved in the docking of GSVs and crystal studies have revealed that Munc18 fits snugly around Syntaxin 1A in its closed conformation (Misura *et al.,* 2000). However, other studies have shown that there was no effect on GLUT4 translocation or insertion into the plasma membrane in Munc18c null adipocytes (Kanda *et al.,* 2005). The interaction of Munc proteins with Syntaxin 4 seemed to be connected with the action of phosphoinositol-3 kinase. One substrate of this enzyme in adipose cells is AS160 which has homology with Rab GTPase-activating proteins (Kane *et al.,* 2002; Sano *et al.,* 2003). Rab GTPases are known to be involved in fusion and docking (Zeigerer *et al.,* 2002). On the basis of this information, it has been proposed that Munc18 stabilises the syntaxin in its closed conformation in the absence of insulin, and causes the slow rate of delivery of GLUT4 to the plasma membrane compared to the rate of internalisation. In the presence of insulin, phosphoinositol-3 kinase is activated and phosphorylates AS160, which in turn disables its GAP activity. The result is

a GTP loading of its related Rab GTPase. The Rab protein can now hydrolyse the bound GTP using the energy to bind to Munc18 and weakening its interaction with the syntaxin. The syntaxin is released to interact with the other SNARE proteins and docking and fusion occurs. In this way, the SNAREs have control over transport, by regulating the population of transporter proteins at the plasma membrane. The response to insulin takes place over ~10 min (Huang *et al.,* 2007), which is similar to the time course for KAT1 traffic evoked by ABA but is much slower than the 30-60 s required for ABA control of the KAT1 channel current (Sutter *et al.,* 2007).

1.4.2. Direct interaction

A direct interaction of the SNARE with the ion channel is a second mechanism by which control can be exerted. Currently, the best examples are found at the neuromuscular junction. In animals, Ca^{2+} channels are important for the final stages in synaptic vesicle docking and fusion that leads to neurotransmitter release at the presynaptic membrane (Mochida *et al.,* 1998). At the nerve terminal, membrane depolarisation during the action potential triggers Ca^{2+} entry through Ca^{2+} channels. The resulting rise in cytosolic-free Ca^{2+} concentration leads to Ca^{2+} binding with synaptotagmin, freeing VAMP2 for interaction with Syntaxin 1A and promoting the final stages of vesicle fusion (Chapman *et al.,* 1995). Structurally, these Ca^{2+} channels are closely associated with Syntaxin 1A at the plasma membrane and their activity has been shown to be affected by the SNARE and by BotN/C toxin which cleaves it (Stanley *et al.,* 1997). Among other observations, Bezprozvanny *et al.* (1995) found that co-expression with Syntaxin 1A in xenopus oocytes accelerates the inactivation of both N- and Q-type Ca^{2+} channels, and Degtiar *et al.* (2000) have noted that the effect is eliminated on BotN/C cleavage of the SNARE. Both channels and Syntaxin 1A can be shown to interact physically (Bezprozvanny *et al.,* 2000). Finally, most important, Stanley *et al.* (1997) reported that the regulation of the Ca^{2+} channel is

strongly affected by BotN/C cleavage of Syntaxin 1A in situ. These results indicate a very close interaction, and functionally a mutual regulation, between the SNARE and Ca^{2+} channels. Similar interactions may also occur between Syntaxin 1A and voltage-gated K^+ (Kv) channels (Leung *et al.,* 2007; Yamakawa *et al.,* 2007; Mohapatra *et al.,* 2007), although much less information is available about their physiological effects.

In fact, some questions have been raised about the validity of protein interactions with Syntaxin 1A. Fletcher, *et al.* (2003) noted that Syntaxin 1A is able to immunoprecipitate a number of different K^+ channels that normally do not reside at the same membrane, and they found that its binding was so indiscriminate that it interacted directly with sepharose and agarose resins. These observations are a concern in the context of the general models described above, because specificity of both interaction and function are implied in each case. For example, trafficking to the plasma membrane of GLUT4 has been associated with the SNAREs VAMP2, Syntaxin 4 and SNAP23, and is largely insensitive to experimental manipulations affecting other plasma membrane SNAREs (Foran *et al.,* 1999). Similarly, exocytosis of norepinephrine from PC-12 cells was found to be dependent on a small subset of plasma membrane SNAREs and insensitive to inhibition by cytosolic fragments of other plasma membrane and endomembrane SNAREs (Scales *et al.,* 2000).

The same questions apply to SYP121 and its function in the plant. As noted above, the Sp2 fragment of SYP121 has been observed to affect K^+ and Cl^- channels in guard cells and to block the traffic of a secretory GFP marker to the plasma membrane (Leyman *et al.,* 1999; Geelen *et al.,* 2002). It also affects the traffic of the KAT1 K^+ channel and its distribution at the plasma membrane (Sutter *et al.,* 2006). However, it could be argued that these are more general effects of introducing a soluble polypeptide that incorporates common elements of SNARE structure (if, indeed, these common elements are necessary at all) and that the effects are not specific to SYP121. There is some evidence to suggest a

degree of specificity; most important, Geelen, *et al.* (2002) reported that overexpressing the full-length SYP121 rescued wild-type growth in tobacco expressing the Sp2 fragment. These results suggest that the Sp2 action is limited to the function of SYP121 in the cell, but they do not address the question of specificity between different SNAREs. Recent evidence (Tyrrell *et al.,* 2007) has indicated that plasma membrane traffic of soluble cargo is selectively affected by Sp2 fragments of plasma membrane SNAREs, and not by Sp2 fragments of at least one endomembrane SNARE. Conversely, the Sp2 fragments of the plasma membrane SNAREs were found to be ineffective in blocking endomembrane traffic to the vacuole. These results indicate that the Sp2 fragments encode specificity that is at least consistent with the functional targets of the corresponding SNAREs. Nonetheless, the studies leave open several key questions. They do not address the traffic of integral membrane proteins, although Sutter, *et al.* (2006) did observe some important differences in the traffic of KAT1 and the H^+-ATPase affected by the SYP121 Sp2. Also, they do not indicate whether other syntaxin-like SNAREs may be involved in delivering KAT1 to the plasma membrane or whether these SNAREs may be important for KAT1 distribution within the plasma membrane.

1.5 Conclusion

In this study I outline experiments exploring the actions of Sp2 fragments for a selection of different plasma membrane and endomembrane SNAREs on the traffic and distribution of KAT1. The experiments make use of the KAT1 channel tagged with both GFP and an externally-exposed HA tag to observe its traffic and localisation when expressed. Much of the work takes advantage of transient expression in tobacco, because of its speed and ease of use. Results show that KAT1 is localised to the plasma membrane in positionally stable microdomains of 500-900nm. KAT1 traffic to the plasma membrane

was suppressed when coexpressed with the Sp2 fragments of SYP121, SYP122, SYP71 and SYP111. Microdomains at the plasma membrane also became diffuse and mobile when expressed with these SP2s, but no disruption of traffic or localisation of KAT1 was seen when coexpressed with the Sp2 fragment of PVC membrane localised SYP21.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Molecular constructs

Sp2 fragments of AtSYP121, AtSYP122, AtSYP21 and AtSYP71 were generated by M.Tyrrell and R.Pratelli, as described previously (Tyrrell *et al.,* 2007), from c-DNA clones kindly provided by A. Sanderfoot (Department of Plant Biology, University of Minnesota, Minneapolis) of *A.thaliana*. Stable transformants of *Nicotiana tabacum* SR1 tobacco carrying PMA2:GFP and PMA4:GFP were provided by B.Lefebvre and M.Boutry (Unité de Biochimie Physiologique, Institut des Sciences de la Vie, Université Catholique de Louvain). The KAT1 K^+ channel incorporating haemagluttinin (HA) epitopes in the extracellular loops and a C-terminal GFP tag was constructed as described in Sutter *et al.,* 2006. Plasmids were introduced by heat shock into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986) and the transformed agrobacterium stored as glycerol stocks.

2.2 Plant growth, protoplasts and transient expression

Wild type and PMA2:GFP tobacco (*N.tabacum*) were grown at 26°C and 60% relative humidity on a 16/8h day/night cycle with a photon fluence of 200 µmol m^{-2} s^{-1} PAR. Plants with 3-4 fully expanded leaves after 4-6 weeks of growth were selected for infiltration. Transient transfections were performed as described previously (Geelen *et al.,* 2002), and were carried out with agrobacterium suspended in infiltration buffer of 10mM $MgCl_2$ with 100µm acetosyringone, at a final OD_{600} of 0.1.

Protoplasts were obtained by enzymatic digestion of 6-8 cm^2 leaf tissue sections in a solution of 5mM $CaCl_2$, 1% BSA (Sigma-Aldrich), 3% Cellulase Onozuka RS (Duchefa), 1% Mazerocyme (Duchefa), 0.2% Pectoylase (Yakult), 500mM sorbitol, 10mM sodium ascorbate, and 10mM MES buffer titrated to pH5.5 with KOH. Digestions were performed at 30°C for 40 min. Debris was removed with forceps and protoplasts were passed through 200 µm nylon mesh before pelleting by gentle centrifugation at 50g for 5

min. Protoplast pellets were gently resuspended in a wash buffer of 10mM $CaCl_2$, 2mM $MgCl_2$, 100mM glycine and 2mM MES/Tris, pH5.5, with osmolarity adjusted to 545 mOsm with sorbitol. The centrifugation and wash steps were repeated before final resuspension of the pellet in wash buffer. A Wescor Vapour Pressure Osmometer (Wescor Model 5520, Logan, UT, USA) was used to measure osmolality and was calibrated following the manufacturer's instructions with sodium chloride solution at 100 mmol/kg, 290mmol/kg and 1000mmol/kg. Anti-HA antibody tagged with Alexafluor594 (Alexa594-α-HA, Molecular Probes) was used at a final dilution of 1:1000, and was added in 1:1 ratio with protoplast suspensions after first diluting 1:500 in wash buffer.

2.3 Confocal microscopy

Expression of fluorescence in lower leaf epidermal cells was assessed 3 days after infiltration as described previously (Sutter, *et al.* 2006). Pieces of leaf, of roughly 1cm by 0.5cm, were cut from previously agrobacterium-treated areas and immediately subjected to vacuum infiltration with distilled water prior to mounting to remove intercellular air pockets that give rise to false fluorescence background. Leaf pieces were then mounted in water for observation under the microscope. Observation began immediately after slide preparation, however most images were collected 30minutes to 1hr after slide preparation. At no time was the tissue allowed to dry out. For experiments with salicylic acid (ASA), leaves were treated in one of three ways. Controls were handled as described above, except for i8mage collection which began immediately after slide preparation. Control leaf tissue was compared with tissue infiltrated with 0.5mg/ml ASA immediately before cutting, and then mounted in water. A second set of experiments were designed to compare tissue with the upper epidermis removed by gently abrading with sandpaper before removing the tissue from the leaf. The tissue was infiltrated with ASA immediately before abrading,

cutting, and mounting in water. Image collection of ASA treated tissue began immediately after slide preparation. The tissue was kept mounted in water for the duration of observation and image collection, which began immediately after slide preparation and did not last longer than 3hrs.

Confocal images were obtained on a Zeiss CLSM510 microscope using 40x oil immersion objectives (Zeiss, Jena) for high resolution image collection. GFP fluorescence was excited using the 488nm line from a 30mW argon laser set at 6.1A, attenuated at 20%, and set for a scan speed of 1.6µsec per pixel. Fluorescent light was collected after passage through an HFT488 dichroic filter, and an NFT545 dichroic filter was used to split the emitted light between channels. Gains and pinholes were standardised along with laser power settings. Channel 2 was set with a 170µm pinhole and 505-530nm band-pass filter for GFP detection. To detect chloroplast fluorescence, Channel 3 was set with a 178µm pinhole and a 560nm long-pass filter. Detector gains were set at 960V (GFP) and 400V (chloroplasts). Bright field images were collected using the transmitted light detector, with detector gains set at 200-250V. For double-labeling experiments with Alexa594-tagged antibody, fluorescence was excited using 543nm light from a 1-mW helium-neon laser as a separate track, and fluorescent light was collected after passage through an HFT-UV/488/543/633 dichroic filter. Track 1 was configured with laser line, filters and detector gains as above. Track 2 was configured with the 543nm laser line attenuated to 50%, and for Alexa594 detection, and Channel 3 set with a 560-615nm band-pass filter, with a detector gain of 950V to collect the fluorescence emission.

2.4 Quantification and analysis

Kymographs were produced using Image J version 1.38, using the line selection tool to define the region for linescale analysis. Linewidth was set at 5 pixels, and the

Multiple Kymograph plugin used to extract kymographs. Drift in some images over time was corrected first using the translation feature of the Stackreg plugin. Colocalisation analysis in protoplasts with GFP and Alexa594 fluorescence was carried out using the profile feature of LSM510 software.

For analysis of domain widths and distributions, linescans were carried out using Metamorph V6.1r6 (Universal Imaging, MDS, Inc. Toronto, Canada) which offered some advantages over ImageJ. The multiline tool was used to select a region of the image. Using the Measure – linescan feature, data were converted into a 2D profile of fluorescence intensity, with line width set at 4 to ensure full capture of the cell periphery. The width at half height of each peak was recorded. These data were analysed using Sigma plot 10.0 (SPSS, Menlo Park USA). The widths of fluorescence domains, corresponding to KAT1 puncta, were sorted into bins using the transforms tool, where the maximum width was 15000nm, and intervals set at 300nm, to create 50 bins. Binned data were fitted to a Gausian function by non-linear least-squares using a Marquardt-Levenberg algorithm (Marquardt, 1963) provided by the software. Otherwise, Microsoft Excel 2003 was used to create final graph displays.

For analysis of KAT1 microdomain recovery after wounding, the time taken for KAT1 to reform punctate, non-mobile distributions at the plasma membrane was recorded in six independent experiments with tissues treated with or without ASA, and these data used for statistical comparisons.

2.5 Western blot analysis

Total protein was extracted from tobacco leaf tissue used for confocal analysis and previously infiltrated with KAT1 with and without the different SNARE Sp2 fragments by grinding leaf tissue frozen in liquid N_2 and resuspending in 1:1 (w/v) in extraction buffer

containing 100 mM Tris-HCl, pH 8.0; 1% sodium dodecylsulfate, 1% sodium deoxycholate; 20 mM EDTA, 1 mM DTT and 0.2 mM PMSF. Samples were centrifuged at 10,000 xg for 10 min at 4°C to remove debris. Aliquots corresponding to 10 μg of protein/sample were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hemel Hampstead UK). Protein was quantified by Bradford assay (Bio-Rad) and calibrated against bovine serum albumin according to the manufacturer's instructions.

Nitrocellulose membranes for Western Blot analysis were blocked with 5% non-fat milk in Tris-buffered saline solution containing 0.1% Tween (TBST) at 4 ^{o}C overnight, incubated at room temperature for 2 h with primary polyclonal rabbit antibodies prepared to the cytosolic domains of SYP121, SYP122, SYP71 and SYP21 at 1:2000 in TBST and 5% non-fat milk, washed 3 times for 15 min with TBST and incubated at room temperature for 1 h with a 1:10,000 dilution of goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Sigma-Aldrich , Poole, UK) in TBST and 5% non-fat milk. Filters were washed in TBST 4 times for 15 min. Bound antibodies were detected using ECL-plus chemiluminescent substrates (Amersham Biosciences, Little Chalfont, UK) as described previously (Sutter, *et al.* 2006; Leyman, *et al.* 1999).

2.6 Sources

Duchefa: Duchefa Biochemie B.V., P.O. box 809, 2003 Haarlem, The Netherlands.

Molecular Probes: http://www.probes.invitrogen.com

Sigma Aldrich; http://www.sigmaaldrich.com

Yakult: Yakult Honsha Co., Ltd., Minato-Ku, Tokyo, Japan.

CHAPTER 3:

RESULTS

SNAREs are thought to play a vital role in membrane traffic and vesicle fusion, and thus in the traffic and localisation of membrane proteins, such as KAT1. The specificity of these SNAREs is key to the correct delivery of proteins. There have been many SNAREs identified in *A.thaliana*, including ten subclasses of syntaxins, or SYPs (Sutter *et al.,* 2006b). Syntaxins are perhaps the best characterised subset of SNAREs, yet their sheer number hints at a more complicated role in the plant than simply assisting vesicle fusion. In fact SYPs are involved in many important processes in the plant, including defence, gravitropism and hormone signalling.

The number of SYPs found to be located at the plasma membrane raises questions about their role in the delivery of membrane proteins, and the specificity of each syntaxin involved. The number of known partners for syntaxins varies, but specificity appears to be encoded by SNARE structure. There also seems to be some functional overlap in those SYPs with a similar structure, for example plants lacking either SYP121 or SYP122 are not greatly affected, yet syntaxin double mutants display necrosis and dwarfism (Zhang *et al.,* 2007).

For all that syntaxins are the best characterised set of SNAREs, very little is known about the roles of individual SYPs in targeting proteins to the plasma membrane. Nevertheless, there are hints that different plasma membrane SNAREs have distinct functions in trafficking (Rehman et al., 2008). Previous work on K^+ channel trafficking and the effect of the SYP121-Sp2 fragment has shown that in the presence of SYP121-Sp2, control of K^+ and Cl^- channel response to abscisic acid is suppressed (Leyman *et al.,* 1999), and that traffic to the membrane is affected (Geelen *et al.,* 2002). Further work showed that SYP121-Sp2 contributed selectively to traffic of KAT1 to the plasma membrane, and that anchoring of the channel at the plasma membrane is also influenced by this Sp2 (Sutter *et al.,* 2006). With a wider range of tools at hand (Sp2 fragments of SYP71, SYP122 and

SYP21) it is possible to revisit the questions raised by these results about selectivity, namely: is the affect seen on KAT1 traffic and localisation produced by any Sp2, or is the effect selective to fragments of those SNAREs localised to the plasma membrane? It is now also possible to ask if the effect is selective for specific plasma membrane proteins, or do these Sp2s affect any plasma membrane protein?

I used transient, agrobacterium-mediated transfection, as before (Sutter *et al.,* 2006, Campanoni *et al*., 2007), to express the KAT1 K^+ channel tagged with heamagluttinin epitopes and Green Fluorescent Protein (HA-KAT1:GFP) and the PMA2 H^+-ATPase tagged with Green Fluorescent Protein (PMA2:GFP) in tobacco. Leaves transfected with HA-KAT1:GFP were also used for Western blot analysis. Expression was driven by the 35S promoter, notably because plant ion channels normally express at levels too low for detection by fluorescence and their distributions, and that of most SNAREs, commonly align with the native protein in vivo when driven by the constitutive promoter (Uemura, *et al.,* 2004; Sutter *et al.,* 2006; Sutter *et al.,* 2007). Analyses were carried out 2-3 d post-transfection, a time when both the K^+ channel and H^+-ATPase expression was near-maximal (Sutter *et al.,* 2006; Campanoni *et al.,* 2007). In leaves, expression of HA-KAT:GFP gave a fluorescence signal which, viewed using the laser-scanning confocal microscope, was distributed within discrete punctate microdomains. With the exception of co-expression studies using various Sp2 fragments (below), this fluorescence signal was generally restricted to the cell periphery as was judged by three-dimensional reconstructions of Z-axis image stacks (Figs. 1). By contrast, expressing PMA2:GFP gave a fluorescence signal that was uniformly distributed over the cell periphery (Fig. 2). These observations were consistent with previous findings (Sutter *et al.,* 2006; Campanoni *et al.,* 2007; Meckel *et al.,* 2004).

3.1 Treatment of cells with ASA suppresses the wound response.

After wounding, plants produce a number of defensive compounds in response. This type of defence has been studied for many years and is well documented. Recognition by a plant of pathogen or herbivore attack induces several changes within the plant, including the phosphorylation or dephosphorylation of plasma membrane proteins, an increase in cytosolic Ca^{2+}, and ion fluxes (Peck 2003). Other responses involve activation of mitogen-activated protein kinases (MAPK) and NADPH oxidase, as well as reactive oxygen species (ROS) (Zhao *et al.,* 2005). This leads to an increase in the production of certain signalling hormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). SA is mainly involved in a defence pathway which results in the accumulation of pathogenesis-related (PR) proteins in areas of the plant as-yet unaffected by the pathogen, a type of defence most effective against biotrophic pathogens (Glazebrook 2005). While the JA/ET defence pathway also involves the accumulation of PR proteins, it is mainly triggered by necrotrophic pathogens (Vidal *et al.,* 1998).

In arabidopsis, two defence signalling genes, EDS1 and PAD4, are necessary for the production of SA (Rustérucci *et al.,* 2001). Both genes are induced by both pathogen attack, and application of SA, which suggests that they are involved in a positive feedback loop (Feys *et al.,* 2001). ROS are also involved in an amplification loop with SA (Shirasu *et al.,* 1997) where SA is induced by ROS and then inhibits catalase (CAT) and ascorbate peroxidase (APX), hydrogen-peroxide scavenging enzymes. This in turn encourages ROS accumulation (Durrant and Dong 2004). There is also some evidence that accumulation of SA in plants triggers activation of NPR1, which normally exists in a non-active oligomeric form. Upon an SA induced redox change, the monomeric form activates the expression of PR genes (Després *et al.,* 2003).

It is clear that SA is involved in inducing a defence response, however there is also evidence that SA acts to inhibit the defence pathway in which JA and ABA are involved. JA is an intermediate in the signalling pathway which links production and accumulation of ABA to the resulting activation of genes involved in defence (Hildmann *et al.,* 1992). JA is synthesised from α-linolenic acid via 13-hydroxyperoxylinolenic acid (13-HPLA) and 12-oxo-phytodienoic acid (OPDA). Both SA and acetyl-salicylic acid (ASA) prevent the resulting defence gene activation by inhibiting hydroxyperoxide-dehydrase, the enzyme which is responsible for the conversion of 13-HPLA to OPDA (Pena-Cortés *et al.,* 1993).

Due to an observation that more tobacco epidermal cells expressing KAT1:GFP showed internal fluorescence only when viewed immediately after slide preparation, it was concluded that internalisation was due to the influence of ABA, caused by wounding. Internalisation of KAT1 caused by wounding posed serious problems to the collection of data on the effects of the Sp2s used, and it was impractical to wait for up to an hour to start the experiment. As aspirin (ASA) has been shown to block JA biosynthesis and thus the wound response in tobacco and tomato plants, ASA (0.5mg/ml) was injected into tobacco leaves prior to cutting the leaf.

Time taken for recovery was recorded for leaves cut and prepared normally, leaves injected with ASA and then prepared normally, leaves with most of the upper epidermis removed by sandpapering, and leaves injected with ASA immediately before removal of most of the upper epidermis. Localisation of KAT1:GFP was viewed and noted at regular intervals (0, 5, 10, 15, 20, 30, 45 and 60 minutes after slide preparation) using the laser-scanning confocal microscope. In each case, the leaf injected with ASA showed faster recovery, at 15-20 minutes as opposed to 45-60 minutes for the uninjected leaf tissue. Similar results were obtained in 10 separate experiments and are summarised in table 2.

3.2 KAT1 is non-mobile within the plasma membrane in arabidopsis

The K^+ channel is normally expressed primarily in aerial tissues and especially in guard cells (Pilot *et al.,* 2001). Nonetheless, even when expressed in epidermal cells the HA-KAT1:GFP and PMA2:GFP fluorescence were localised to the plasma membrane, much as described previously (Sutter *et al.,* 2006). In general, the K^+ channel was localised within discrete microdomains (Fig. 1), suggesting an anchoring similar to that observed by Sutter *et al* (2006). To quantify the mobility of the HA-KAT1:GFP construct, I made use of kymographic analysis, recording over the time course experiments the fluorescence signal from pixels along a line traced around the edge of the cells. A line width of 10 pixels (1.4 μm) was averaged in most cases, although similar results were obtained with line widths of 3, 5 and 8 pixels (0.5-1 μm). The data of Figure 1 are from such an experiment, and the analysis is shown in Figure 3 in kymographic format. Here position along the line (Fig. 3, *inset*) determines the horizontal axis, time progresses down along the vertical axis and fluorescence intensity is colour-coded (see Fig. 3, *scale inset*). Quantitatively equivalent results were obtained in each of 19 other experiments (not shown). The analysis shows stationary fronts of the punctate fluorescence distribution, evident as longitudinal striations in fluorescence intensity, confirming the absence of any measurable lateral displacement or relocation of the K^+ channel protein throughout the timecourse of the experiment.

For comparison, I examined the fluorescence distribution of PMA2:GFP, taking advantage of fluorescence recovery after photobleaching (FRAP) to test for lateral mobility of the H^+-ATPase. These experiments used the 488-nm Argon laser line to photobleach GFP fluorescence locally at the periphery of the cell while focusing on the cell in cross-section. Images collected before and after photobleaching in each of four separate experiments showed a loss of fluorescence within the photobleached regions that did not recover, even over periods of 8-10 min. The experiment of Fig. 2 shows one region

subjected to photobleaching and illustrates the lack of fluorescence recovery in this region. Much the same conclusion was drawn from kymographic analysis, shown in Fig. 2. The analysis yielded stationary fronts in fluorescence at the limits of the photobleached regions which showed no evidence of an appreciable displacement of the H^+-ATPase marker laterally around the cell over the time course of these measurements.

3.3 Plasma membrane delivery and microdomain distribution of KAT1 is selectively affected by dominant-negative fragments of plasma membrane SNAREs.

Previous work from this laboratory indicated that SYP121 is involved in the trafficking and localisation of KAT1 at the plasma membrane. When HA-KAT1:GFP was co-expressed with SYP121-Sp2 in tobacco epidermal cells, GFP fluorescence could be seen in cytoplasmic strands and in a ring around the nucleus, suggesting retention of the channel in the ER (Fig 4). This is similar to results observed for a secretory GFP cargo (Geelen *et al.,* 2002), but is markedly different from those for the PMA2 H^+-ATPase (Sutter *et al.,* 2006). A subsequent study (Tyrrell *et al.,* 2007) showed selectivity among SNARE Sp2 fragments, and with these new tools I addressed this question directly, examining whether trafficking and localisation of KAT1 is suppressed selectively by plasma membrane SNARE Sp2 fragments, and whether this effect can be seen on another plasma membrane protein, PMA2.

In each experiment, tobacco was co-transfected with constructs for each Sp2 fragment, driven by the 35S promoter. Sp2 fragment expression was verified by Western blot analysis in each case (Fig 5), and similar observations were obtained from each of at least 10 independent experiments for the different Sp2. As the specificity of each Sp2 is thought to be linked to its structure, the Sp2 fragment of SYP122, closely related to SYP121, was used first. When co-expressed with HA-KAT1:GFP in tobacco the effects

were remarkably similar to those of the SYP121-Sp2 fragment. Fluorescence was not confined to the cell periphery but could be seen in cytoplasmic strands and around the nucleus (Fig 6). Co-expression of HA-KAT1:GFP with another Sp2 fragment of a SNARE recently found to be present at the plasma membrane, SYP71, gave the same result (Fig 7). For comparison, I co-expressed the K^+ channel with another Sp2 fragment from the pre-vacuolar SNARE SYP21 (Sanderfoot *et al.,* 1998). Upon co-expression with SYP21-Sp2, KAT1 localisation remained the same as when expressed alone. GFP fluorescence was punctate at the cell periphery and no fluorescence in internal structures could be seen (Fig 8). The results suggested that the effect of the Sp2s is selective to those SNAREs located at the plasma membrane.

It has been indicated in past work that SYP121-Sp2 fragments had some effect on the positional anchoring of KAT1 at the plasma membrane (Sutter *et al.,* 2006). This appeared to be the case for SYP122-Sp2 and SYP71-Sp2 fragments also. These observations were supported by kymographic analysis of channel mobility, using the fluorescence signal from pixels along a line traced around the cell periphery as before. Figures 9-11 show this analysis, with position along the line (inset) determining the horizontal axis and time determining the vertical axis, with fluorescence intensity colour coded. Kymographic analysis of GFP fluorescence when HA-KAT1:GFP is co-expressed with each of SYP121-Sp2, SYP122-Sp2 and SYP71-Sp2 (Figs 9-11) clearly showed an absence of the stationary fronts seen in the analysis of FRAP experiments on HA-KAT1:GFP fluorescence, when expressed alone. Instead there was a definite ‘smearing’ of the signal, showing HA-KAT1:GFP to be mobile at the cell periphery under the influence of each Sp2. When coexpressed with SYP21-Sp2 fragments however, fluorescence of HA-KAT1:GFP remained immobile and kymographic analysis (Fig 12) showed the puncta to be positionally stable.

To confirm the effect suggested by the confocal images of plasma membrane SNARE Sp2 action on trafficking of KAT1, 100 images of HA-KAT1:GFP expressed alone and with each Sp2 were scored for fluorescence and presence of a nuclear ring (Fig 13). It is clear that the Sp2 fragments of SYP121, SYP122 and SYP71 had a major effect on the trafficking of KAT1, with over 70% of images in each case showing a nuclear ring, compared with under 30% for KAT1 expressed alone. Again, coexpression with SYP21-Sp2 showed no appreciable difference from expressing KAT1 on its own.

It was observed that when under the influence of the Sp2 fragments of SYP121, SYP122 and SYP71, fluorescence could still be seen at the periphery of the cell, indicating that some channels might still populate the plasma membrane. To test for HA-KAT1:GFP presence at the plasma membrane, I carried out dual labelling experiments using protoplasts from leaf tissue co-transfected with HA-KAT1:GFP and each Sp2, by labelling the Ha-epitope tag exposed on the outer surface of the protoplasts. Protoplasts from tobacco leaf tissue previously transfected with HA-KAT1:GFP and SYP121-Sp2 were incubated with Alexa-fluor594-tagged Ha monoclonal antibody (Alexa594-αHa) and fluorescence examined under the confocal microscope. Fluorescence intensity analysis confirmed co-localisation of Alexa-fluor594 and GFP fluorescence at the cell surface (Fig 14). Protoplasts expressing HA-KAT1:GFP together with the Sp2 fragment of SYP122, SYP71 or SYP21 gave similar results in each of 5 independent experiments, and are summarised in Figs 15-17. Each figure shows characteristic images from one tobacco protoplast expressing the K^+ channel and each Sp2 fragment, taken on a tangential (surface) plane. In each case, GFP and Alexa-fluor594 fluorescence colocalised, indicating that KAT1 traffic to the plasma membrane is not completely suppressed by the Sp2 fragments of SYP121, SYP122 or SYP71, and is unaffected by the Sp2 fragment of SYP21.

3.4 Microdomains of HA-KAT1:GFP are more diffuse when expressed with dominant-negative plasma membrane SNARE fragments.

Due to an observation that in protoplasts expressing both HA-KAT1:GFP and plasma membrane SNARE Sp2s, microdomains seemed more diffuse, puncta size was examined. Images with clear and distinct puncta from 10 different experiments for each Sp2 were used, and data was gathered for microdomain width of KAT1:GFP when expressed alone and with each Sp2. Size of channel clusters was measured using the full width at half-maximal (FWHM) peak height of fluorescence. Fig. 18 shows the results of the measurements, and the fitted parameters for frequency distributions of KAT1 cluster diameters are summarised in Table 1.

3.5 PMA2 is insensitive to secretory block by the SNARE Sp2 fragments.

For comparison, I examined the fluorescence distribution, traffic and mobility of the GFP-tagged PMA2 H^+-ATPase from *Nicotiana plumboginifolia*. In tobacco leaves of PMA2:GFP stable transformants, fluorescence was uniform around the cell periphery as reported previously (Lefebvre *et al.,* 2004; Sutter *et al*., 2006; Campanoni *et al.,* 2007). Kymographic analysis of fluorescence from FRAP experiments also showed no lateral mobility of PMA2:GFP (Fig. 2). Images collected from 4 separate FRAP experiments showed a loss of fluorescence within the photobleached area, which did not recover, even over a period of 7-8 minutes. Delivery and mobility of PMA2:GFP was unaffected by the Sp2 fragment of plasma membrane SNARE SYP122 (Fig. 19) or the other plasma membrane SNARE fragments of SYP121 and SYP71 (not shown), or the pre-vacuolar SNARE fragment SYP21-Sp2 (not shown). Kymographic analysis of each FRAP experiment showed no ‘smearing’ of fluorescence, indicating that there was no lateral

movement of PMA2:GFP. This suggests that the PMA2 H^+-ATPase is trafficked to the plasma membrane via a different pathway from that of KAT1.

To summarise, traffic to and anchoring of HA-KAT1:GFP at the plasma membrane are disrupted by the plasma membrane SNARE fragments SYP121-, SYP122- and SYP71-Sp2s. Co-expression with these Sp2s causes internal fluorescence and diffusion and mobility of microdomains, while expression of SYP21-Sp2 has no effect. None of the Sp2 fragments used affected PMA2. Thus, the effects of each Sp2 on KAT1 localisation and traffic lead to the conclusion that these plasma membrane Q-SNAREs contribute selectively to the trafficking of KAT1 to the plasma membrane, and are involved in anchoring the channel at the cell surface.

The effects of ASA, partly blocking the internalisation and speeding the return of KAT1 to the plasma membrane after wounding, are consistent with previous work which points to SA as an inhibitor of the defence pathway promoted by JA, and supports the evidence that ABA and JA operate in the same signalling pathway.

These points are discussed in more detail in the next chapter.

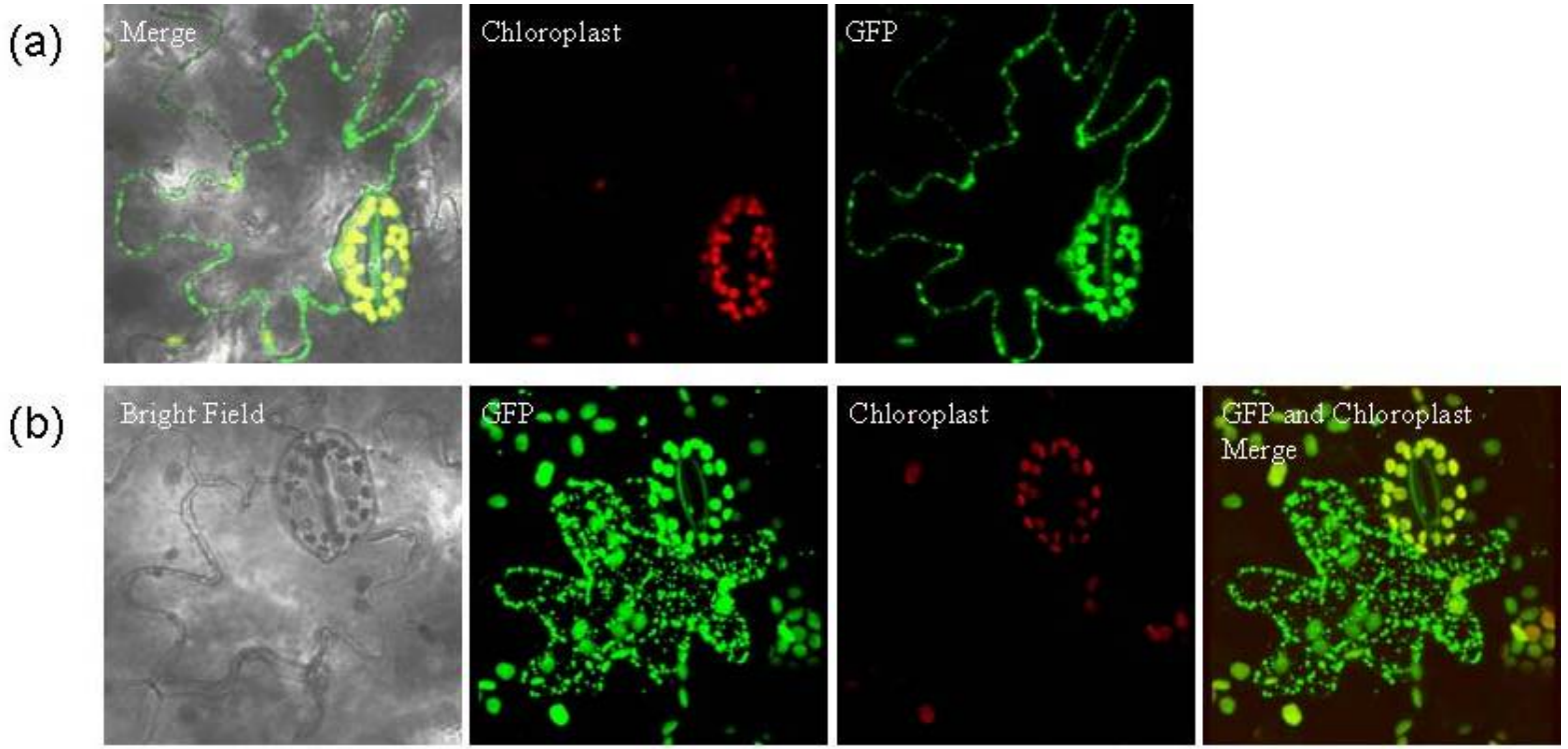
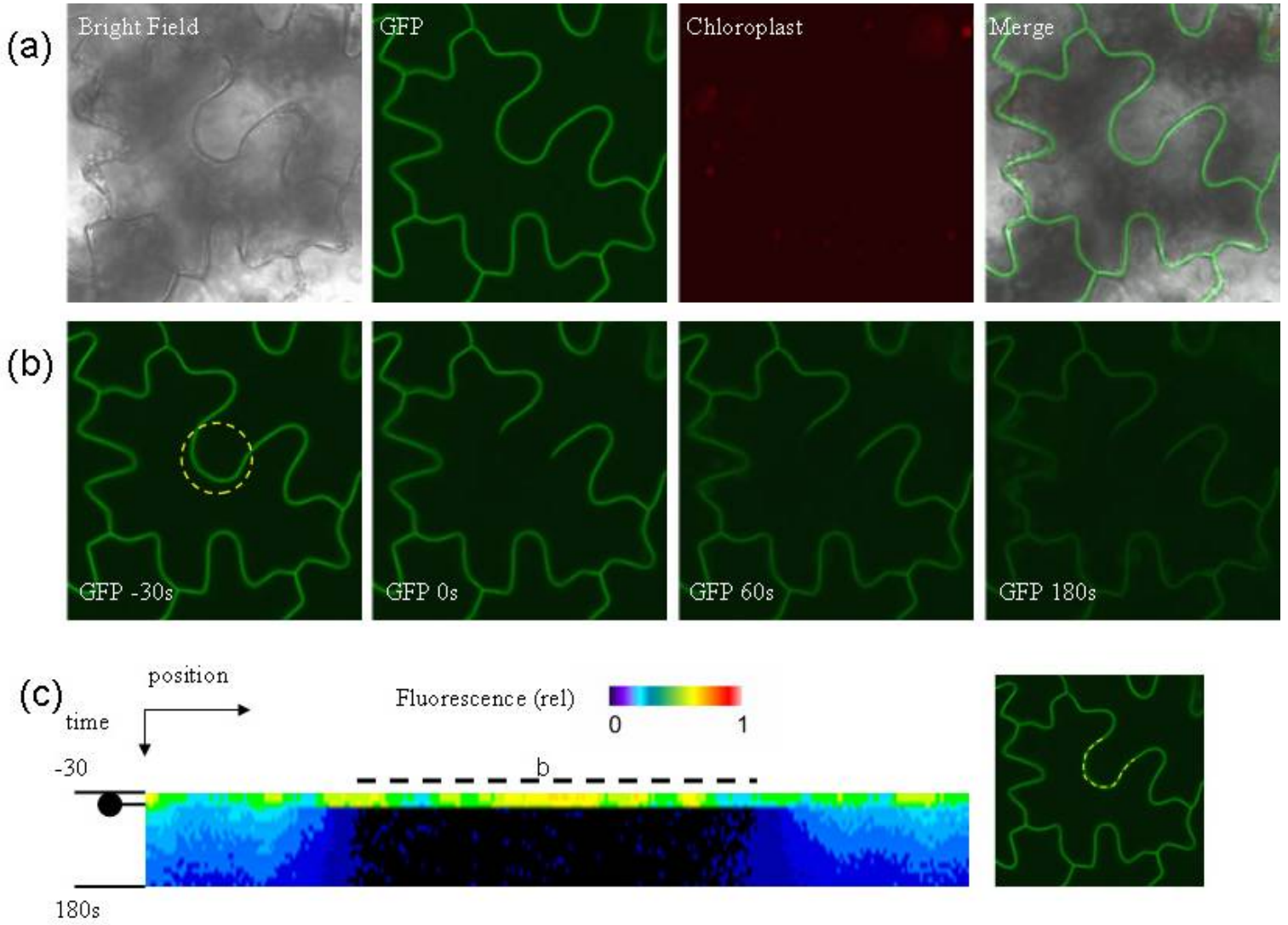


Figure 1. Tobacco leaves transfected with HA-KAT1:GFP yield a peripheral distribution of punctate GFP fluorescence.

Confocal images of tobacco epidermis scanned 3 days after transfection with HA-KAT1:GFP showing (a) 2D images and (b) 3D images (b) are (from left to right) a merged image single scan through the cell, the bright field merge, GFP fluorescence, chloroplast autofluorescence and the merged image stack. Distribution of HA-KAT1:GFP is punctate and localised to the periphery of the cell.

Figure 2. H^+-ATPase PMA2:GFP is non mobile at the plasma membrane. Confocal images showing (a) bright field, GFP chloroplast autofluorescence and merged images (left to right) of PMA2:GFP at the plasma membrane. Time series (b) images were collected before and after photobleaching with 488nm light within the area circled. Images are cross sections through the cell. Time in seconds relative to photoactivation is shown in each frame. Kymographic analysis (c) shows no recovery of fluorescence after photobleaching.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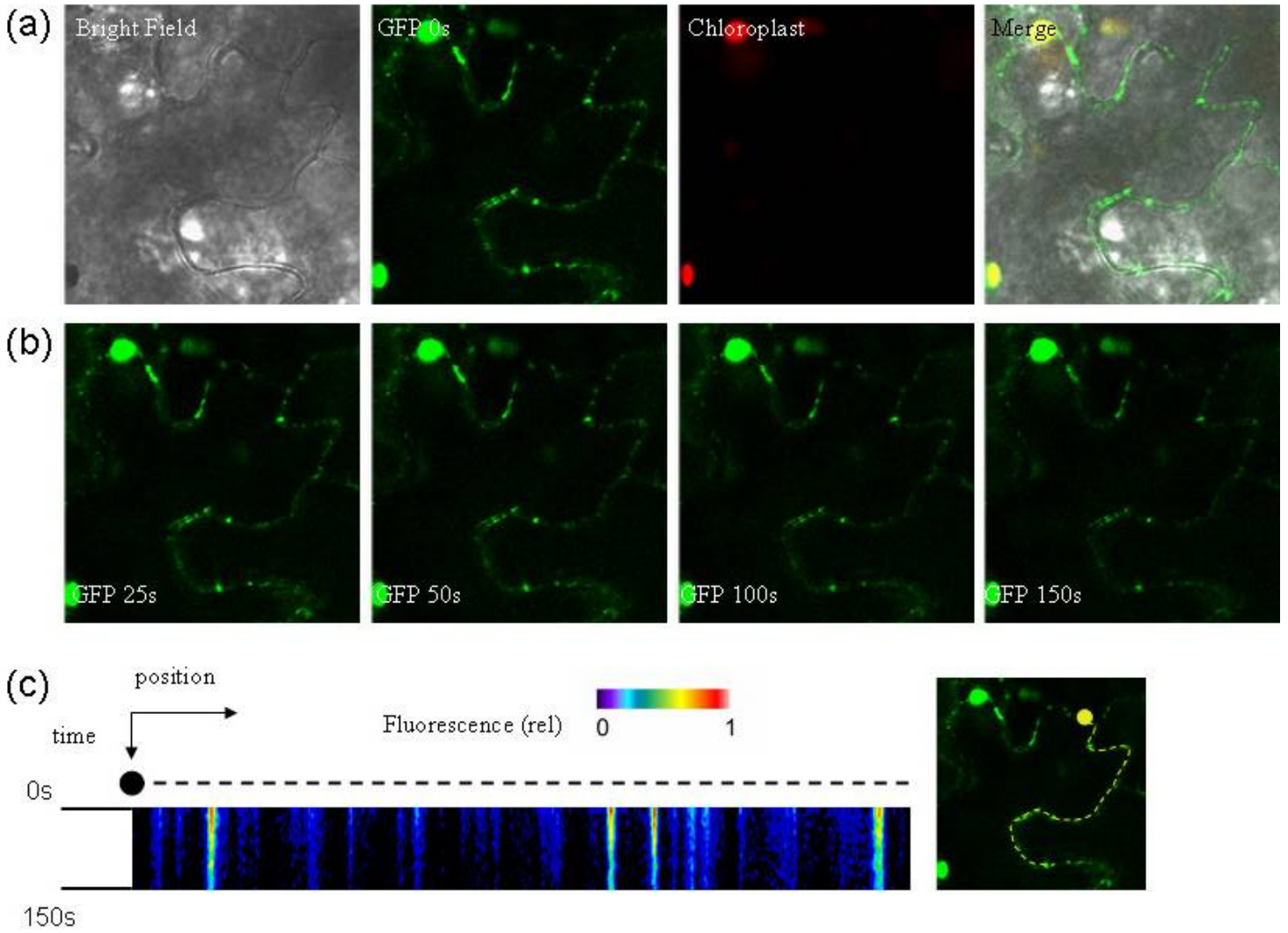
GFP
Chloroplast
Merge
(b)
GFP -30s
GFP 0s
GFP 60s
GFP 180s
(c)
position
time
Fluorescence (rel)
0
1
-30
b
180s

Figure 3. HA-KAT1:GFP is positionally stable at the cell periphery
Time series showing HA-KAT1:GFP localised to the cell periphery in puncta, positionally stable over time. Bright field, GFP, chloroplast and merge images (a, left to right) of tobacco epidermal cells and the time series (b) were collected by confocal microscopy. Images are cross sections through the cell, and time after first scan is indicated for each frame in (b). The fluorescent signal was taken over the time course of the experiment from pixels along a line drawn around the edge of the cell, averaging over a width of 5 pixels as shown in the GFP image by the dotted line. These data were converted into a kymograph (c), where position along the line is shown on the horizontal axis, time along the vertical axis, and fluorescence intensity is colour coded. No evidence of lateral movement can be seen.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Chloroplast
Merge
(b)
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GFP 50s
GFP 100s
GFP 150s
(c)
position
time
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0
1
0s
150s

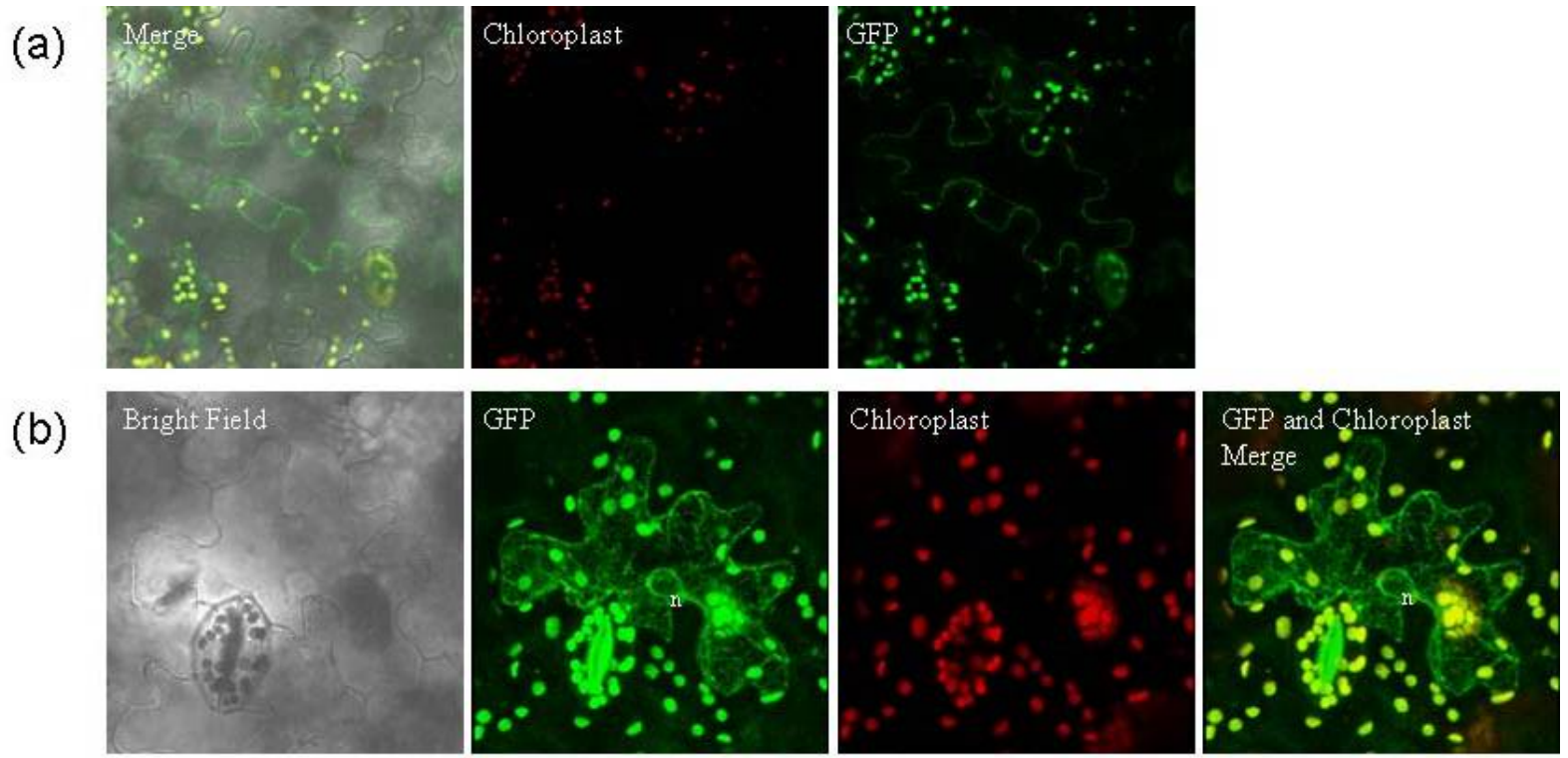


Figure 4. The Sp2 fragment AtSYP121-Sp2 suppresses traffic to, and affects the distribution of, KAT1 at the plasma membrane.

Confocal images of tobacco epidermis scanned 3 days after co-transfection with HA-KAT1:GFP and AtSYP121-Sp2 showing 2D images (a) and 3D images (b). Upon co-expression with AtSYP121-Sp2, distribution of HA-KAT1:GFP was no longer punctate but fluorescence was seen in a reticulate network at the cell periphery, in cytoplasmic strands, and in a nuclear ring (n).

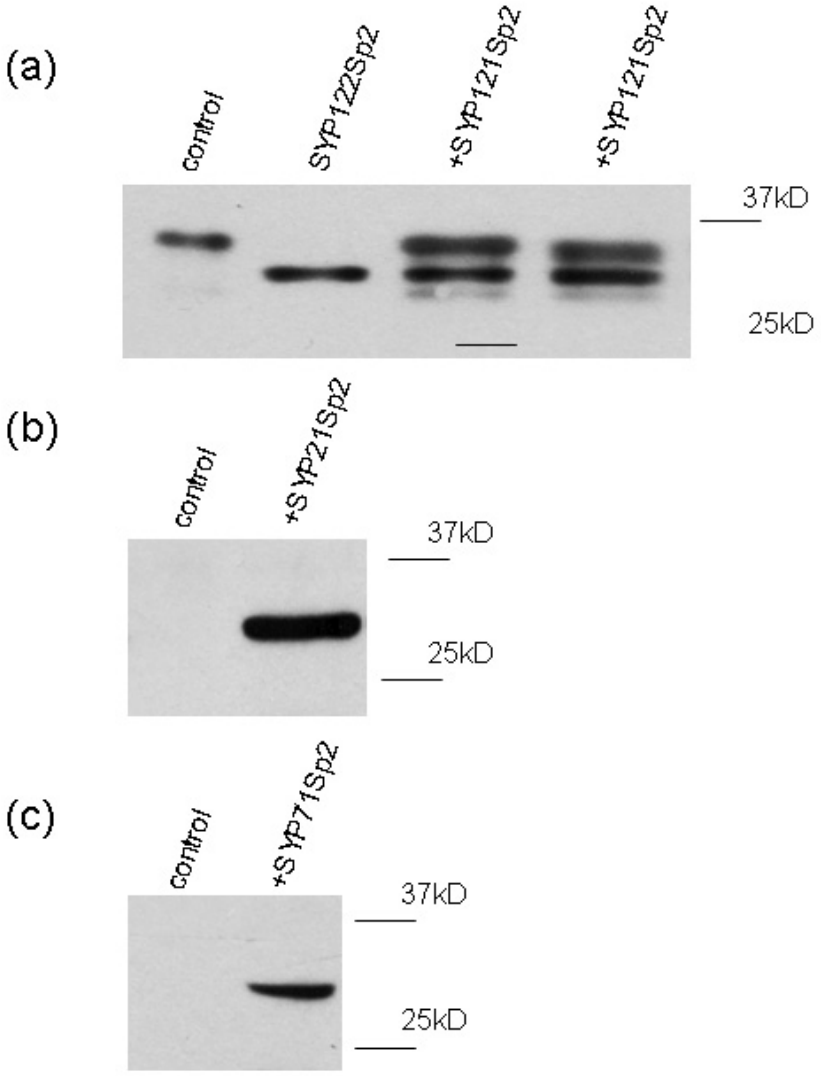


Figure 5. Western Blot analysis verifies expression of Sp2 fragments SYP121-Sp2, SYP122-SP2, SYP21-Sp2 and SYP71-Sp2.
Western blot analysis on protein from tobacco leaves transfected with SYP121-Sp2, SYP122-Sp2, SYP21-Sp2 and SYP71-Sp2.
(a) Western probed with anti AtSYP122 which recognises both SYP121 and SYP122 and clearly picks up a homologous band in tobacco (in the control); SYP122Sp2 protein included in lane 2 as control. (b) Western probed with antiAtSYP21 which shows no cross-reactivity with tobacco proteins. (c) Western probed with anti AtSyp71 which shows no cross-reactivity with tobacco proteins.

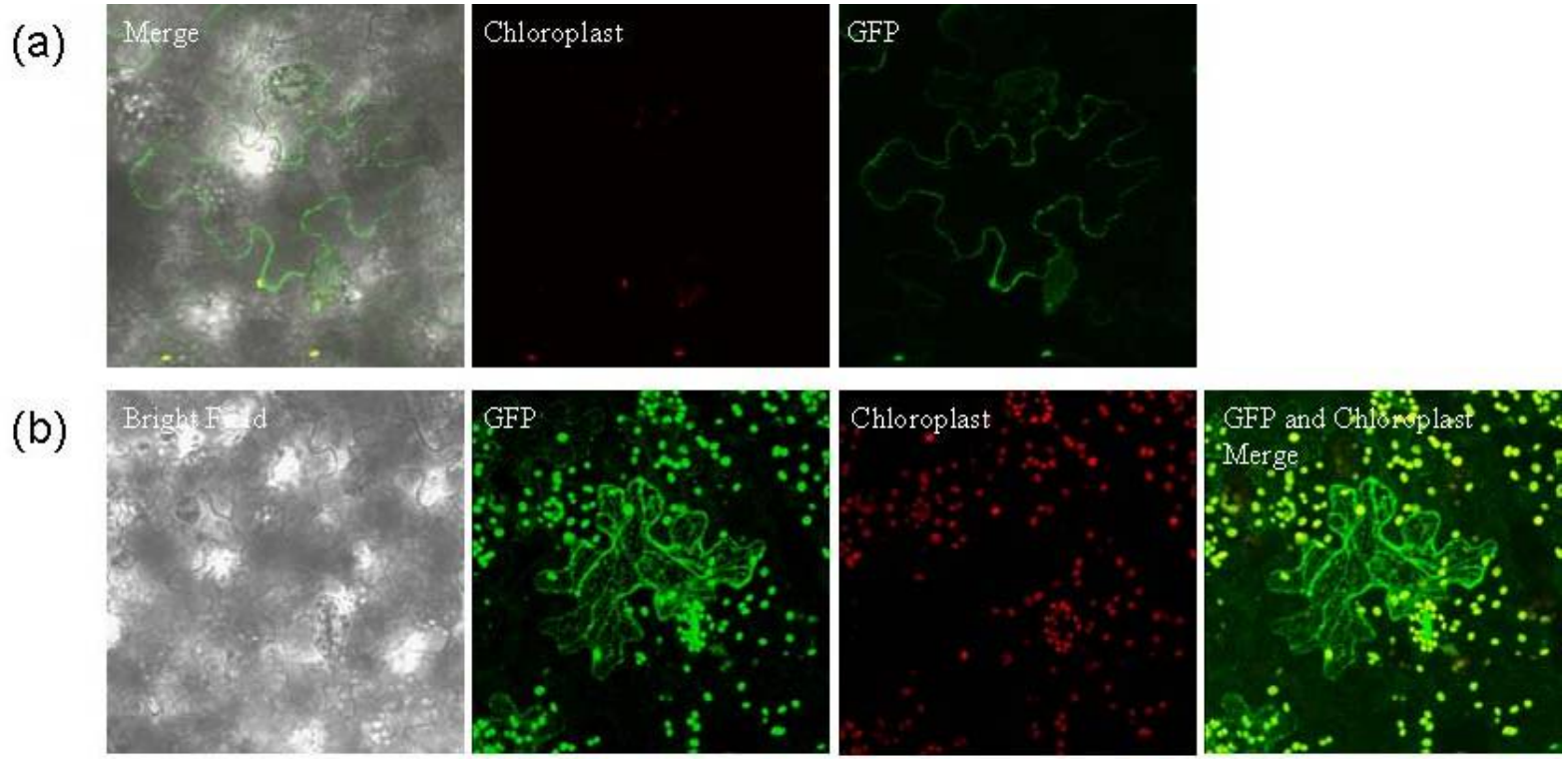


Figure 6. The Sp2 fragment AtSYP122-Sp2 suppresses traffic to, and affects the distribution of, KAT1 at the plasma membrane.

Confocal images of tobacco epidermis scanned 3 days after co-transfection with HA-KAT1:GFP and AtSYP122-Sp2 showing 2D images (a) and 3D images (b). Upon co-expression with AtSYP122-Sp2, distribution of HA-KAT1:GFP was no longer punctate and fluorescence was seen in a reticulate network at the cell periphery and in cytoplasmic strands.

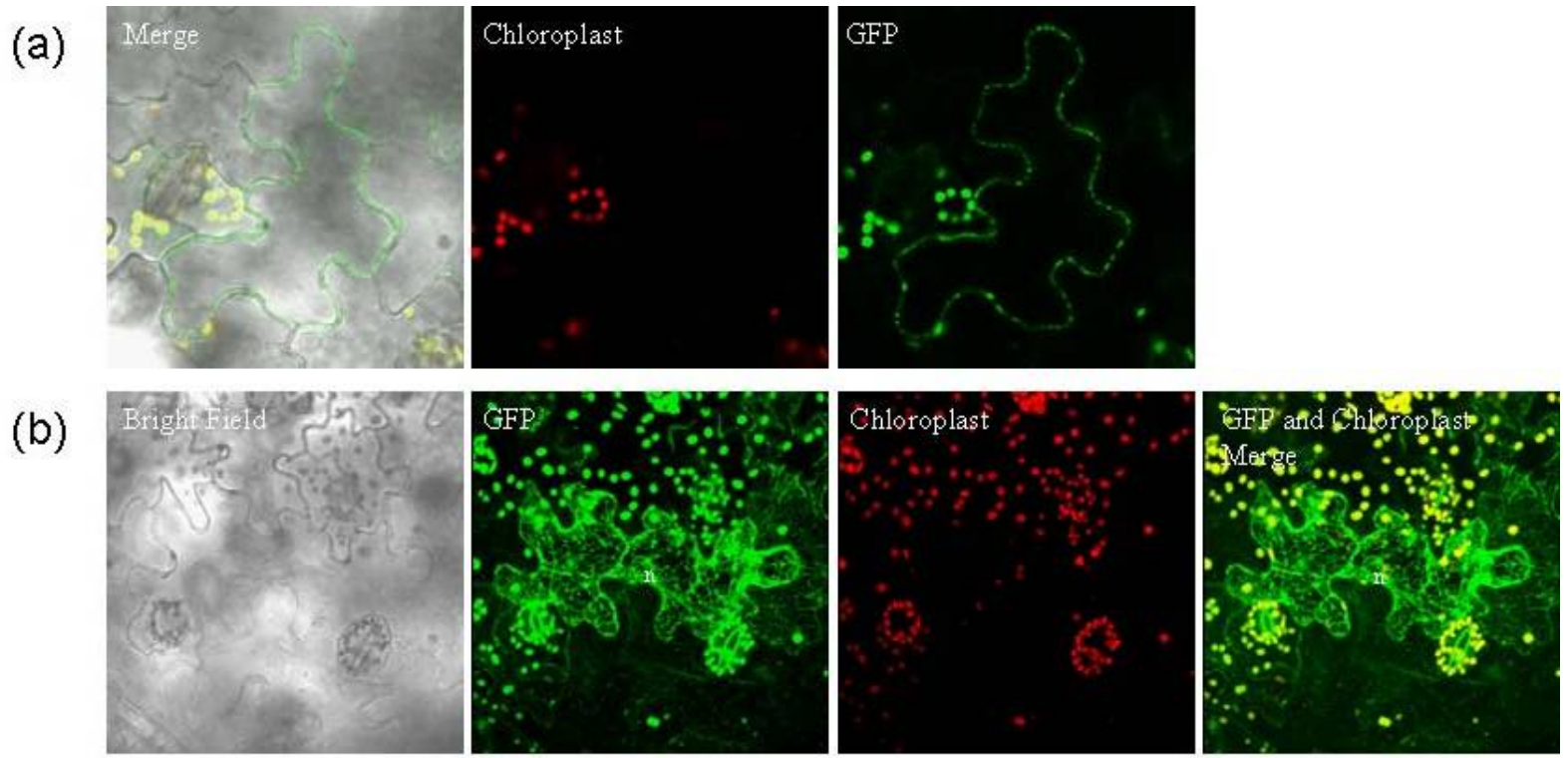


Figure 7. The Sp2 fragment AtSYP71-Sp2 suppresses traffic to, and affects the distribution of, KAT1 at the plasma membrane.

Confocal images of tobacco epidermis scanned 3 days after co-transfection with HA-KAT1:GFP and AtSYP71-Sp2 showing 2D images (a) and 3D images (b). Upon co-expression with AtSYP71-Sp2, distribution of HA-KAT1:GFP was no longer punctate and fluorescence was seen in a reticulate network at the cell periphery, in cytoplasmic strands, and in a nuclear ring (n).

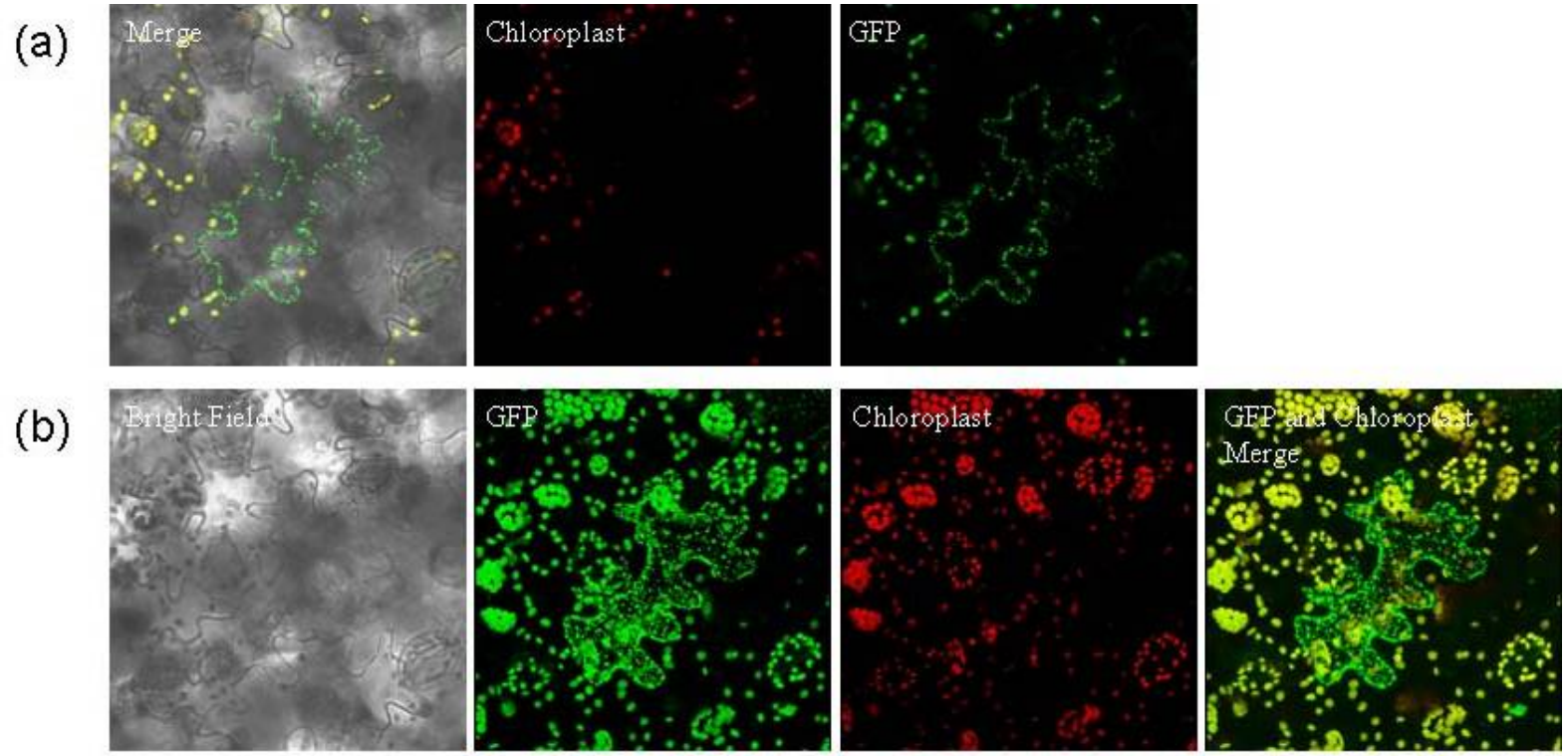
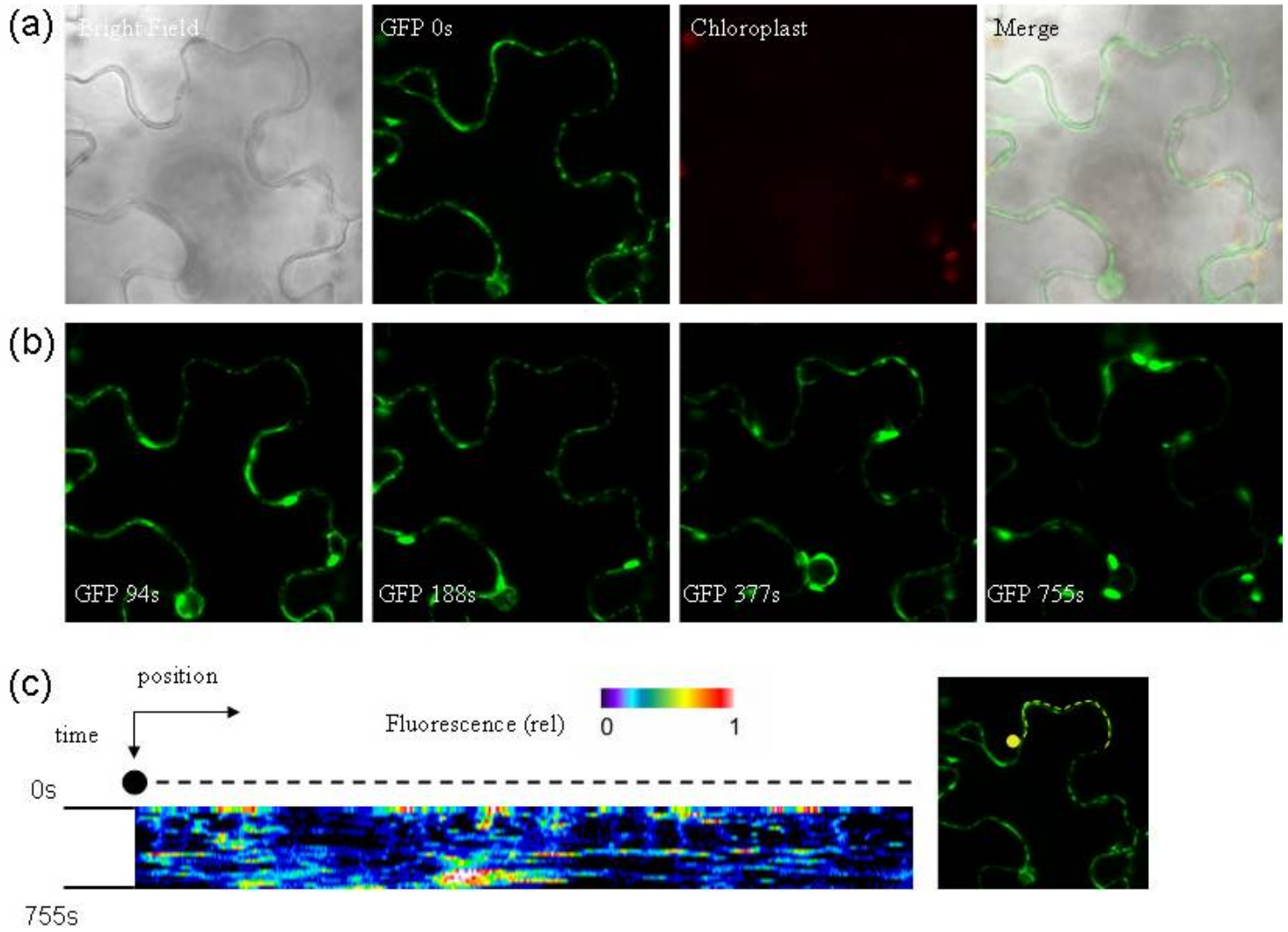


Figure 8. The Sp2 fragment of PVC localised AtSYP21 has no visible effect on the trafficking or distribution of KAT1.

Confocal images of tobacco epidermis scanned 3 days after co-transfection with HA-KAT1:GFP and AtSYP21-Sp2 showing 2D images (a) and 3D images (b). Upon co-expression with AtSYP21-Sp2, KAT1 was still distributed in puncta at the periphery of the cell.

Figure 9. HA-KAT1:GFP becomes mobile at the cell periphery when co-expressed with AtSYP121-Sp2

Bright field, GFP, chloroplast and merge images (a, left to right) of tobacco epidermal cells and the time series (b) were collected by confocal microscopy. Images are cross sections through the cell, and time after first scan is indicated for each frame in (b). The fluorescent signal was taken over the time course of the experiment from pixels along a line drawn around the edge of the cell, averaging over a width of 5 pixels as shown in the GFP image by the dotted line. These data were converted into a kymograph (c), where position along the line is shown on the horizontal axis, time along the vertical axis, and fluorescence intensity is colour coded. Movement of fluorescence is evident as diagonal and lateral fluorescence patterns.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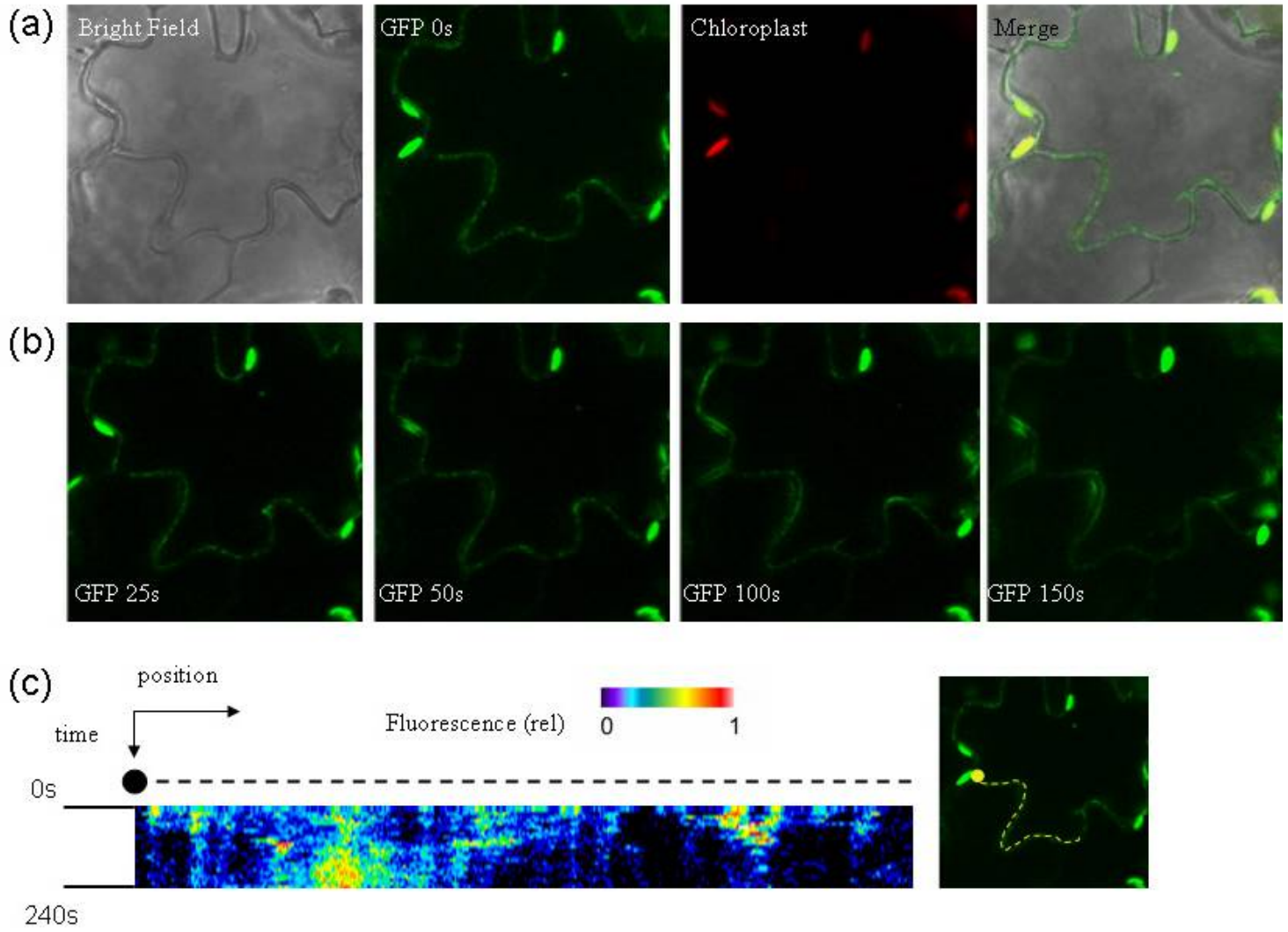
Chloroplast
Merge
(b)
GFP 94s
GFP 188s
GFP 377s
GFP 755s
(c)
position
time
Fluorescence (rel)
0
1
0s
755s

Figure 10. HA-KAT1:GFP becomes mobile at the cell periphery when co-expressed with AtSYP122-Sp2.
Bright field, GFP, chloroplast and merge images (a, left to right) of tobacco epidermal cells and the time series (b) were collected by confocal microscopy. Images are cross sections through the cell, and time after first scan is indicated for each frame in (b). The fluorescent signal was taken over the time course of the experiment from pixels along a line drawn around the edge of the cell, averaging over a width of 5 pixels as shown in the GFP image by the dotted line. These data were converted into a kymograph (c), where position along the line is shown on the horizontal axis, time along the vertical axis, and fluorescence intensity is colour coded. Movement of fluorescence is evident as diagonal and lateral fluorescence patterns.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Chloroplast
Merge
(b)
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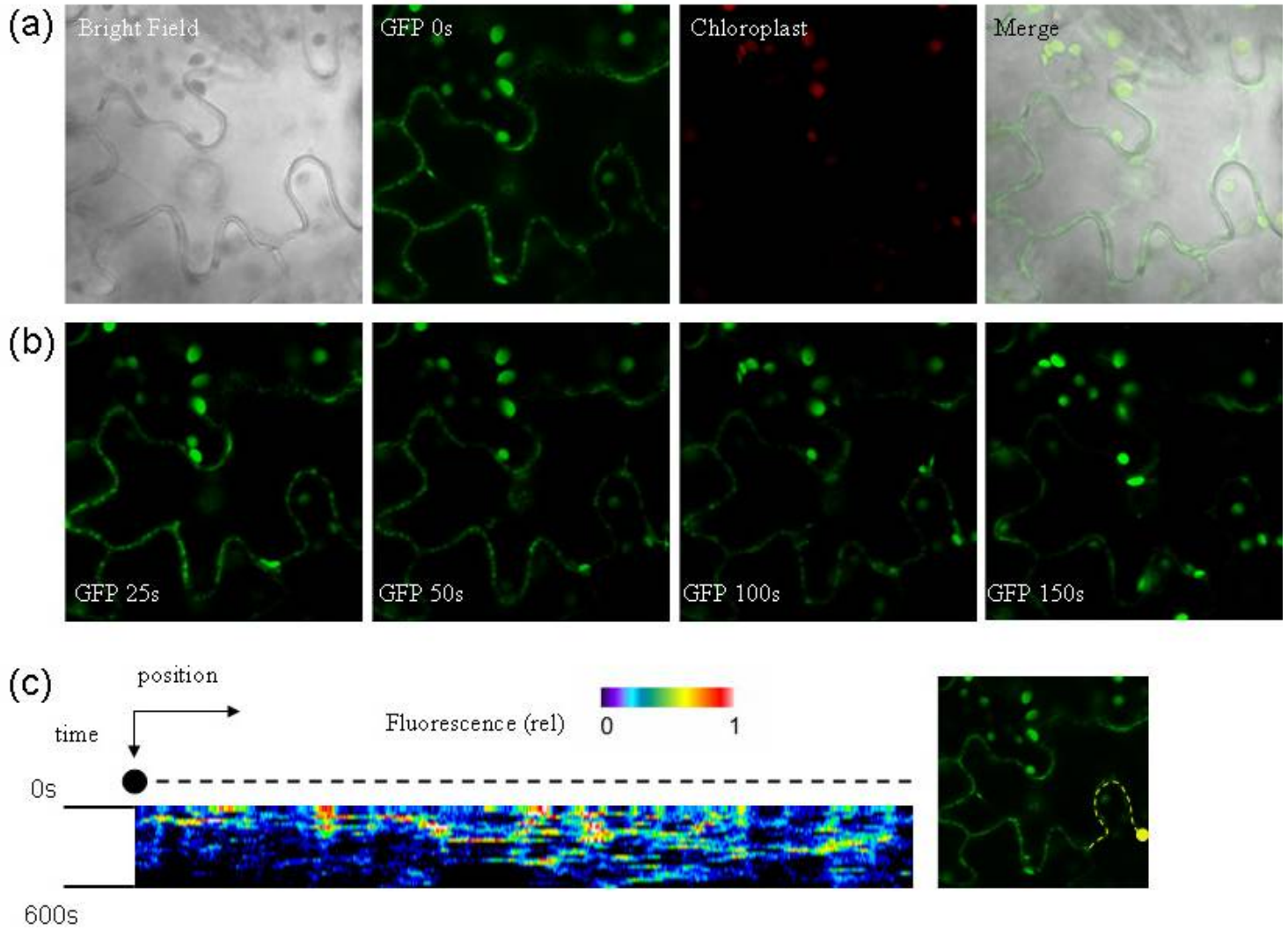
GFP 50s
GFP 100s
GFP 150s
(c)
position
time
Fluorescence (rel)
0
1
0s
240s

Figure 11. HA-KAT1:GFP becomes mobile at the cell periphery when co-expressed with AtSYP71-Sp2.
Bright field, GFP, chloroplast and merge images (a, left to right) of tobacco epidermal cells and the time series (b) were collected by confocal microscopy. Images are cross sections through the cell, and time after first scan is indicated for each frame in (b). The fluorescent signal was taken over the time course of the experiment from pixels along a line drawn around the edge of the cell, averaging over a width of 5 pixels as shown in the GFP image by the dotted line. These data were converted into a kymograph (c), where position along the line is shown on the horizontal axis, time along the vertical axis, and fluorescence intensity is colour coded. Movement of fluorescence is evident as diagonal and lateral fluorescence patterns.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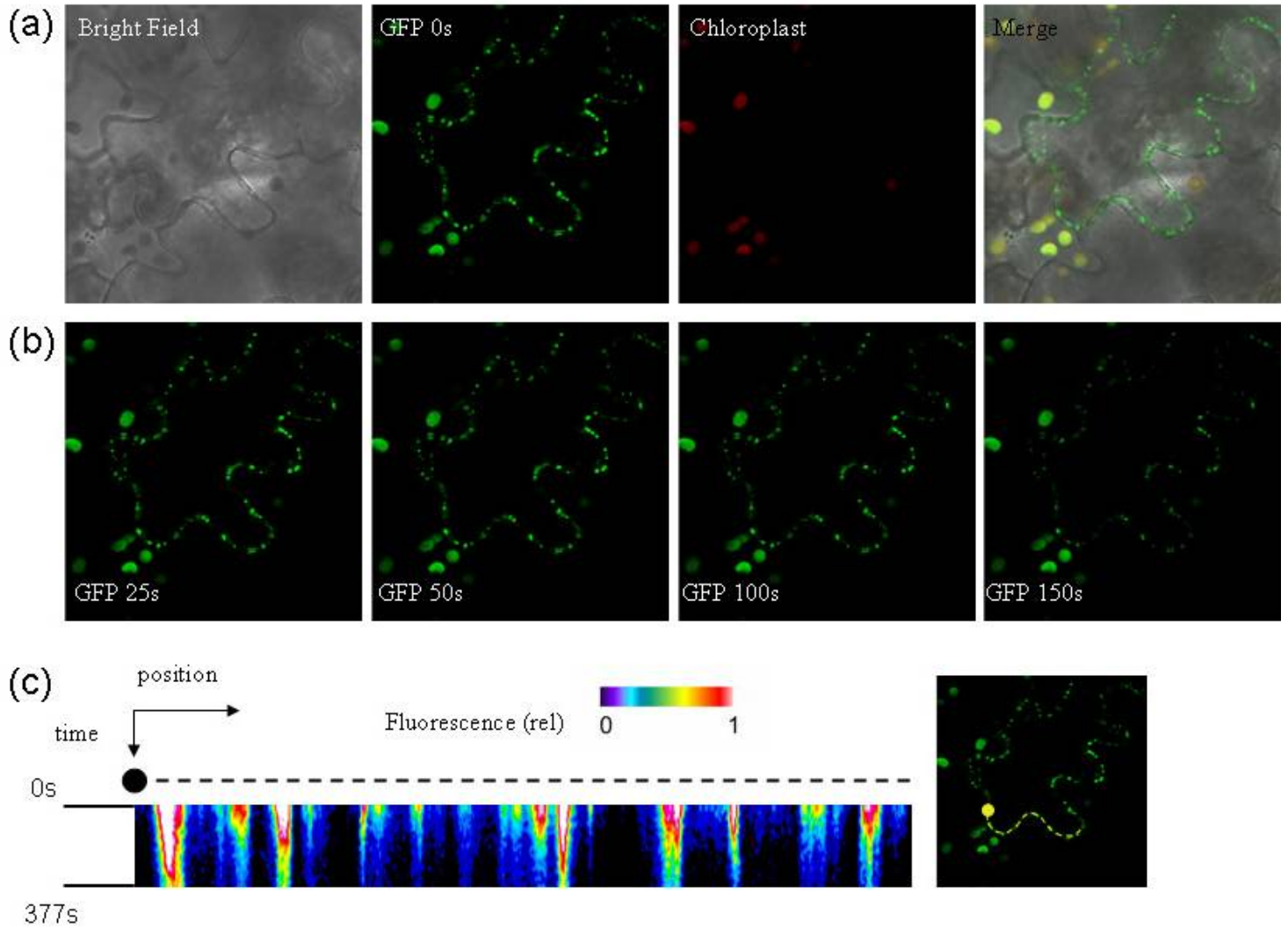
Chloroplast
Merge
(b)
GFP 25s
GFP 50s
GFP 100s
GFP 150s
(c)
position
time
Fluorescence (rel)
0
1
0s
600s

Figure 12. HA-KAT1:GFP is positionally stable at the cell periphery when co-expressed with the Sp2 fragment of PVC-localised AtSYP21
Time series (b) showing HA-KAT1:GFP localised to the cell periphery in puncta, positionally stable over time. Bright field, GFP, chloroplast and merge images (a, left to right) of tobacco epidermal cells and the time series (b) were collected by confocal microscopy. Images are cross sections through the cell, and time after first scan is indicated for each frame in (b). The fluorescent signal was taken over the time course of the experiment from pixels along a line drawn around the edge of the cell, averaging over a width of 5 pixels as shown in the GFP image by the dotted line. These data were converted into a kymograph (c), where position along the line is shown on the horizontal axis, time along the vertical axis, and fluorescence intensity is colour coded. No evidence of lateral movement can be seen, as evidenced by the vertical fluorescence intensity patterns.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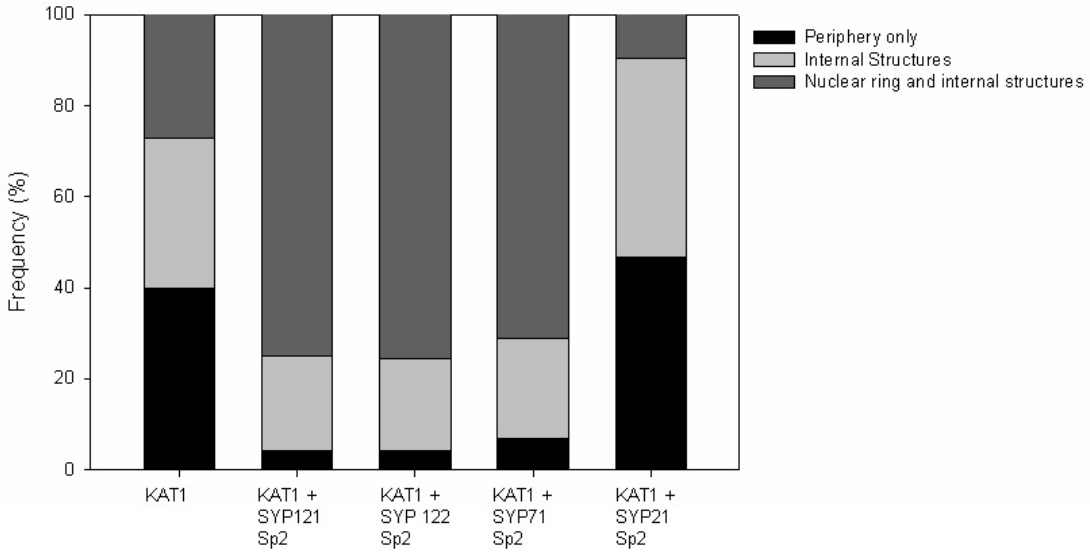

Figure 13. Sp2 fragments of SYP121, SYP122 and SYP71 affect trafficking to the plasma membrane.

Images of KAT1 expressed alone and with Sp2 fragments of SYP121, SYP122, SYP71 and SYP21 were scored for expression in the cell periphery only, expression in internal structures and for a visible nuclear ring.

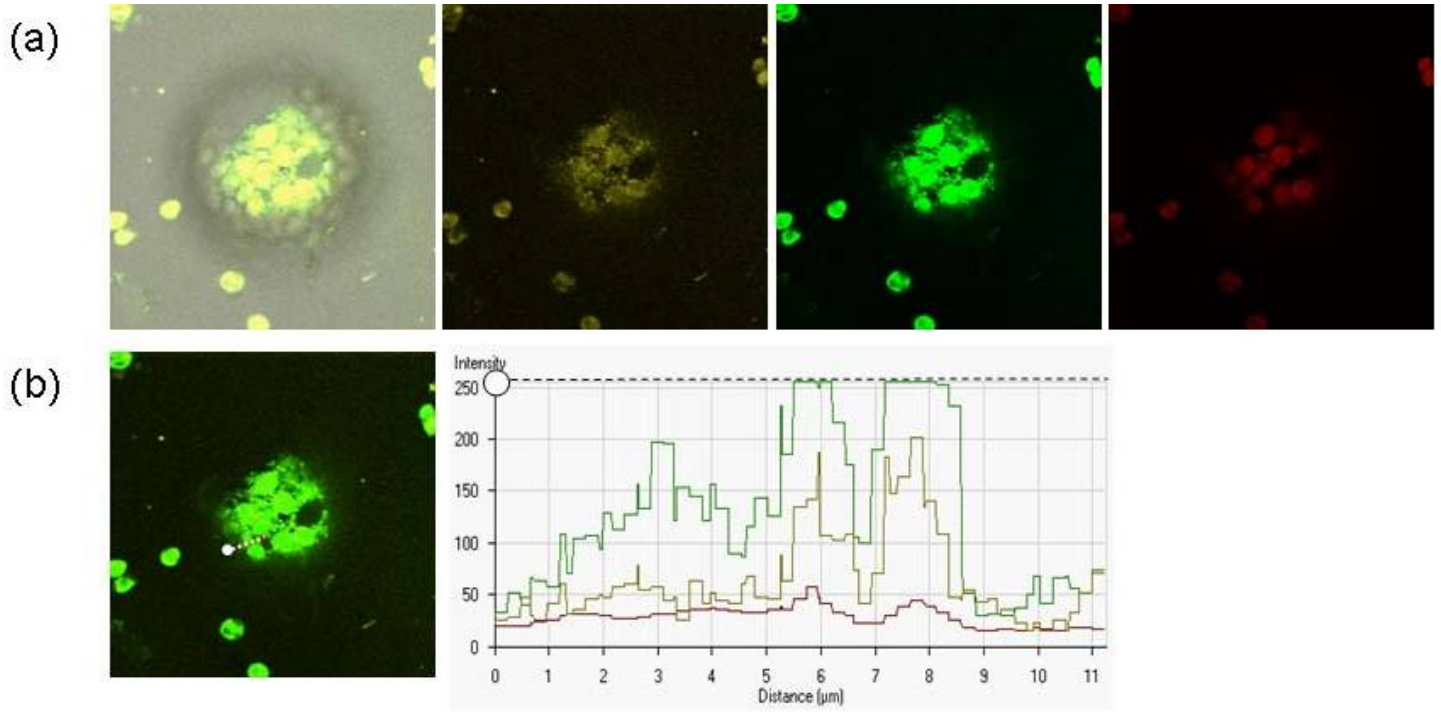

Figure 14. KAT1 traffic to the plasma membrane is not completely suppressed by the Sp2 fragment of AtSYP121.

Confocal images from a dual-labeling experiment using protoplasts from tobacco leaf tissue. Tobacco had been transfected 72 hours previously with both HA-KAT1:GFP and AtSYP21-Sp2. Protoplasts were bound with Alexa594-αHA for 40m prior to scan and are shown in (a) (left to right) as bright field image, Alexa594, GFP and chloroplast autofluorescence. In (b) co-localisation was analysed by comparing the fluorescence intensity of GFP and Alexa594 against that of chloroplast autofluorescence (red line) along the white dotted line. Fluorescence of Alexa594 overlaps with GFP, however GFP fluorescence does not always co-localise with that of Alexa594, as expected with some retention of KAT1 in the ER.

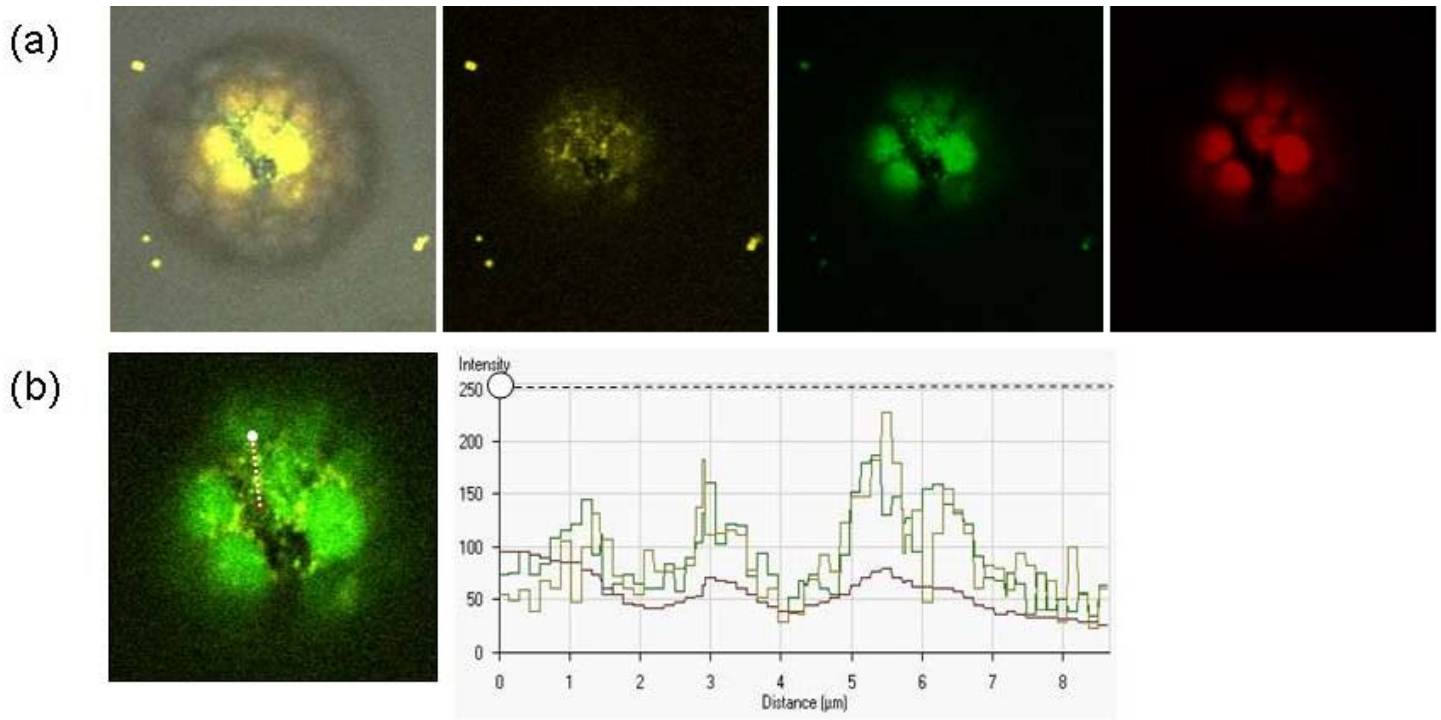

Figure 15. KAT1 traffic to the plasma membrane is not completely suppressed by the Sp2 fragment of AtSYP122.

Confocal images from a dual-labeling experiment using protoplasts from tobacco leaf tissue. Tobacco had been transfected 72 hours previously with both HA-KAT1:GFP and AtSYP122-Sp2. Protoplasts were bound with Alexa594-αHA for 30m prior to scan and are shown in (a) (left to right) as bright field image, Alexa594, GFP and chloroplast autofluorescence. In (b) co-localisation was analysed by comparing the fluorescence intensity of GFP and Alexa594 against that of chloroplast autofluorescence along the white dotted line. Fluorescence of GFP and Alexa594 overlaps, however chloroplast fluorescence (red line) peaks at the beginning of the dotted line where there is little GFP or Alexa594 fluorescence.

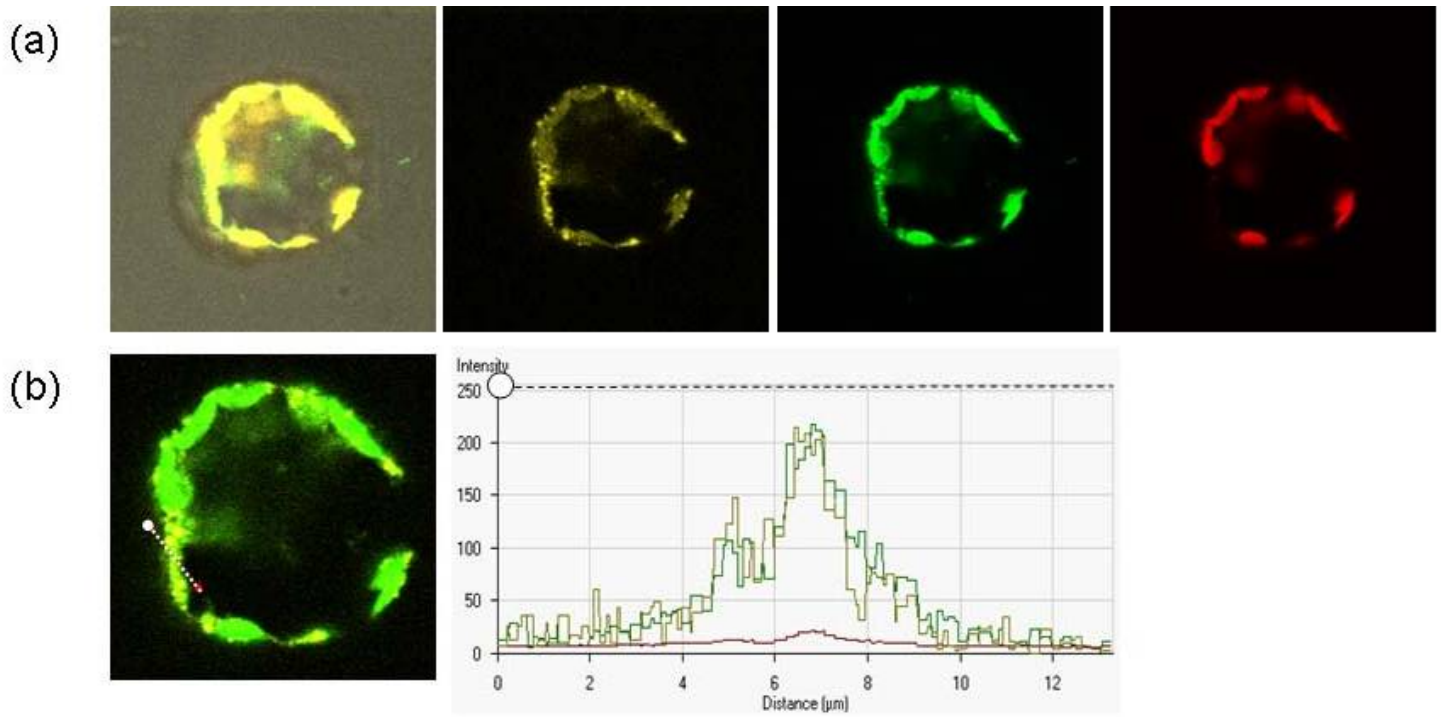

Figure 16. KAT1 traffic to the plasma membrane is not completely suppressed by the Sp2 fragment of AtSYP71.

Confocal images from a dual-labeling experiment using protoplasts from tobacco leaf tissue. Tobacco had been transfected 72 hours previously with both HA-KAT1:GFP and AtSYP71-Sp2. Protoplasts were bound with Alexa594-αHA for 20m prior to scan and are shown in (a) (left to right) as bright field image, Alexa594, GFP and chloroplast autofluorescence. In (b) co-localisation was analysed by comparing the fluorescence intensity of GFP and Alexa594 against that of chloroplast autofluorescence (red line) along the white dotted line. Fluorescence of GFP and Alexa594 overlaps.

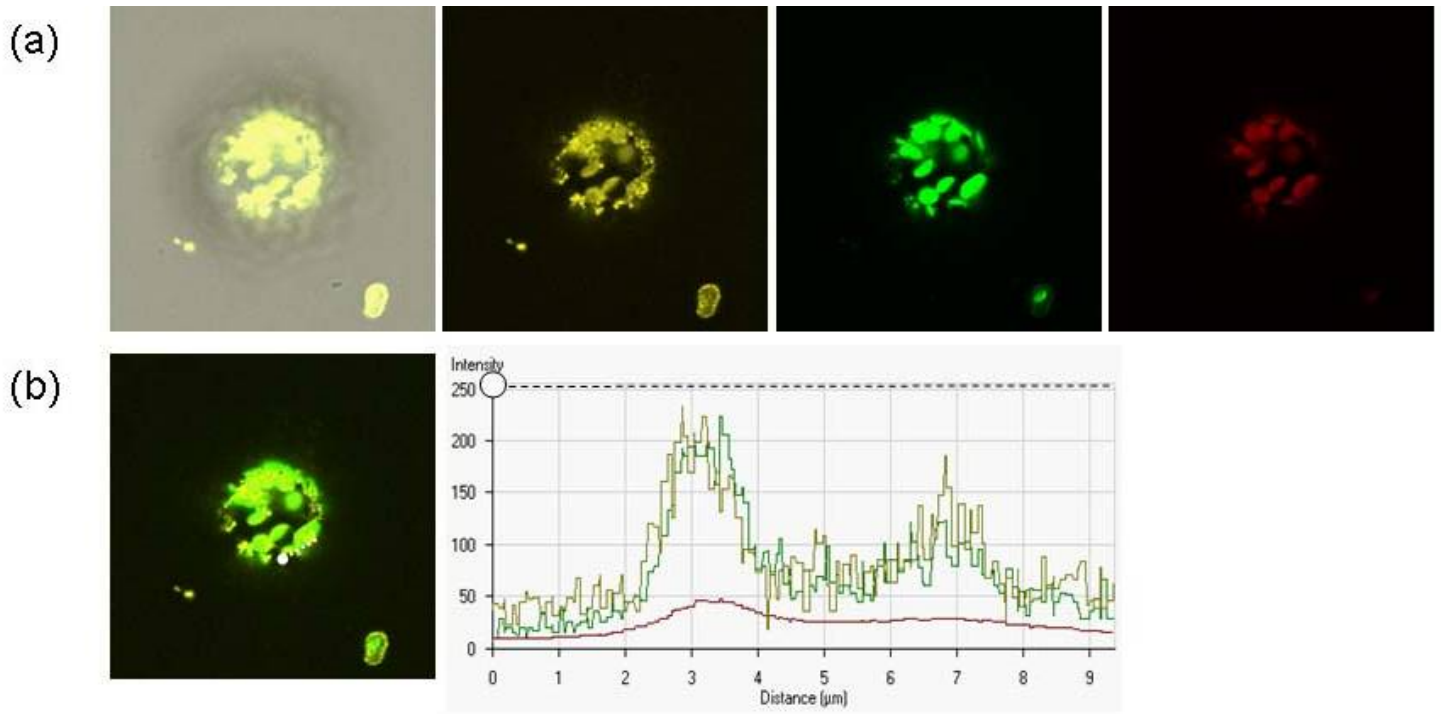

Figure 17. KAT1 traffic to the plasma membrane is unaffected by the Sp2 fragment of AtSYP21.

Confocal images from a dual-labeling experiment using protoplasts from tobacco leaf tissue. Tobacco had been transfected 72 hours previously with both HA-KAT1:GFP and AtSYP21-Sp2. Protoplasts were bound with Alexa594-αHA for 50m prior to scan and are shown in (a) (left to right) as bright field image, Alexa594, GFP and chloroplast autofluorescence. In (b) co-localisation was analysed by comparing the fluorescence intensity of GFP and Alexa594 against that of chloroplast autofluorescence (red line) along the white dotted line. Fluorescence of GFP and Alexa594 overlaps.

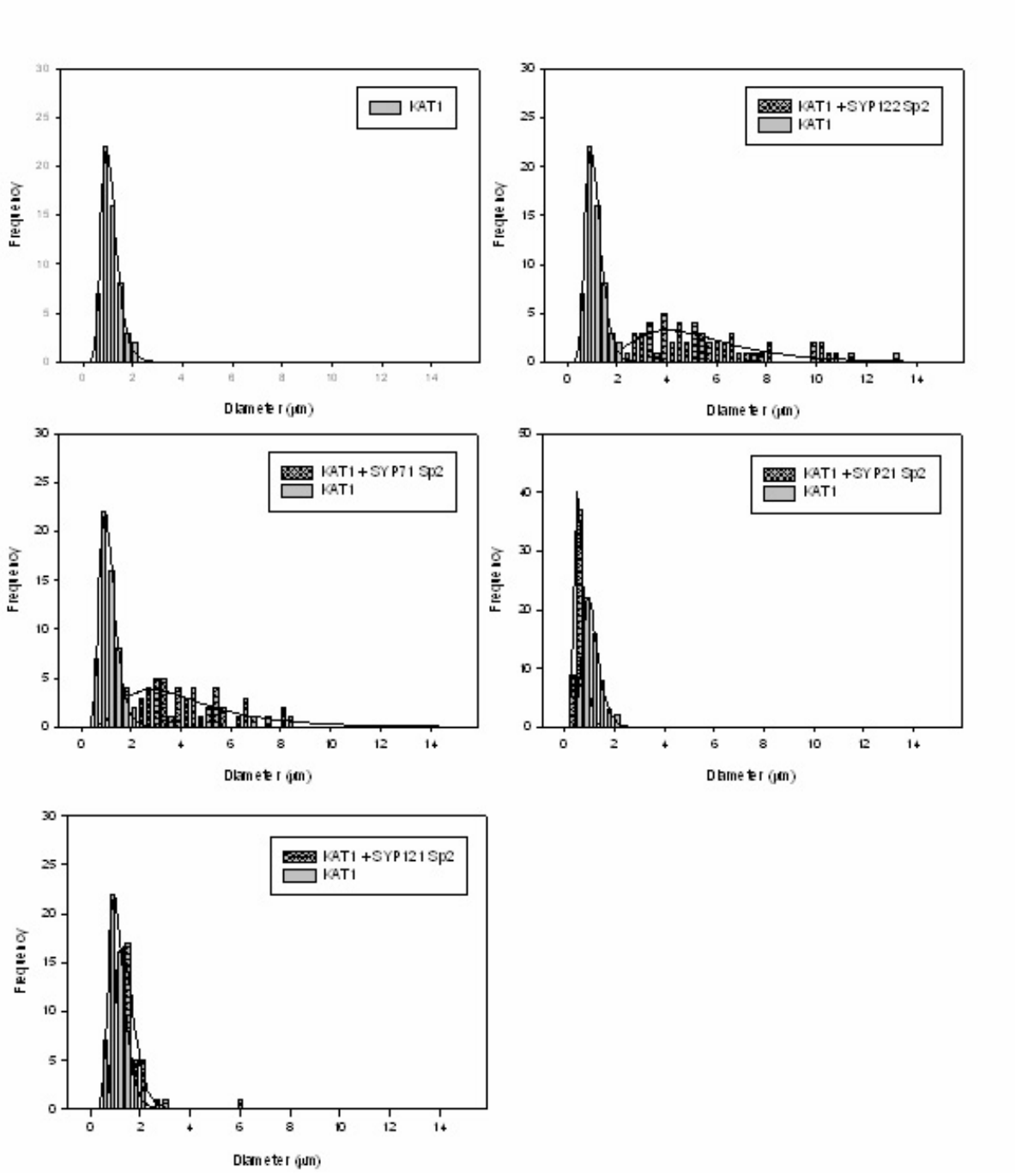

Figure 18. SYP122-, SYP121- and SYP71-Sp2 fragments, but not SYP21-Sp2 cause diffusion of KAT1 microdomains.
Comparison of KAT1:GFP microdomain width in tobacco epidermal cells. Data was gathered for microdomain width of KAT1 expressed alone and with the Sp2 fragments of SYP122, SYP121, SYP71 and SYP21. Data was taken for 120 microdomains from six independent experiments for each case. Width was measured using the full width at half-maximal (FWHM) peak height of fluorescence. The frequency distribution of cluster diameters in each case was well fitted to a single, log normal distribution function f=a*exp(-0.5*(ln(x/x0)/b)^2) (solid curves). Fitted parameters can be found in Table 1.

Table 1. Fitted parameters for frequency distributions of KAT1 cluster diameters

	Peak Maximum (nm)	Peak Spread Coefficient
Control (KAT1)	953 ± 3	0.314 ± 0.003
+Sp2 fragments		
SYP122	4005 ± 201	0.462 ± 0.046
SYP71	2786 ± 188	0.572 ± 0.059
SYP21	528 ± 2	0.326 ± 0.003
SYP121	1267 ± 21	0.290 ± 0.016

Figure 19. H^+-ATPase PMA2:GFP is non mobile at the plasma membrane when co-expressed with SYP122-Sp2.
Confocal images showing (a) bright field, GFP chloroplast autofluorescence and merged images (left to right) of PMA2:GFP co-expressed with SYP122-Sp2 at the plasma membrane. Time series (b) images were collected before and after photobleaching with 488nm light within the area circled. Images are cross sections through the cell. Time in seconds relative to photoactivation is shown in each frame. Kymographic analysis (c) is shown, with time on the vertical axis and position along the dotted line as horizontal. Photobleaching occurs at 0 seconds, marked by the lollipop. No recovery of fluorescence after photobleaching could be seen.

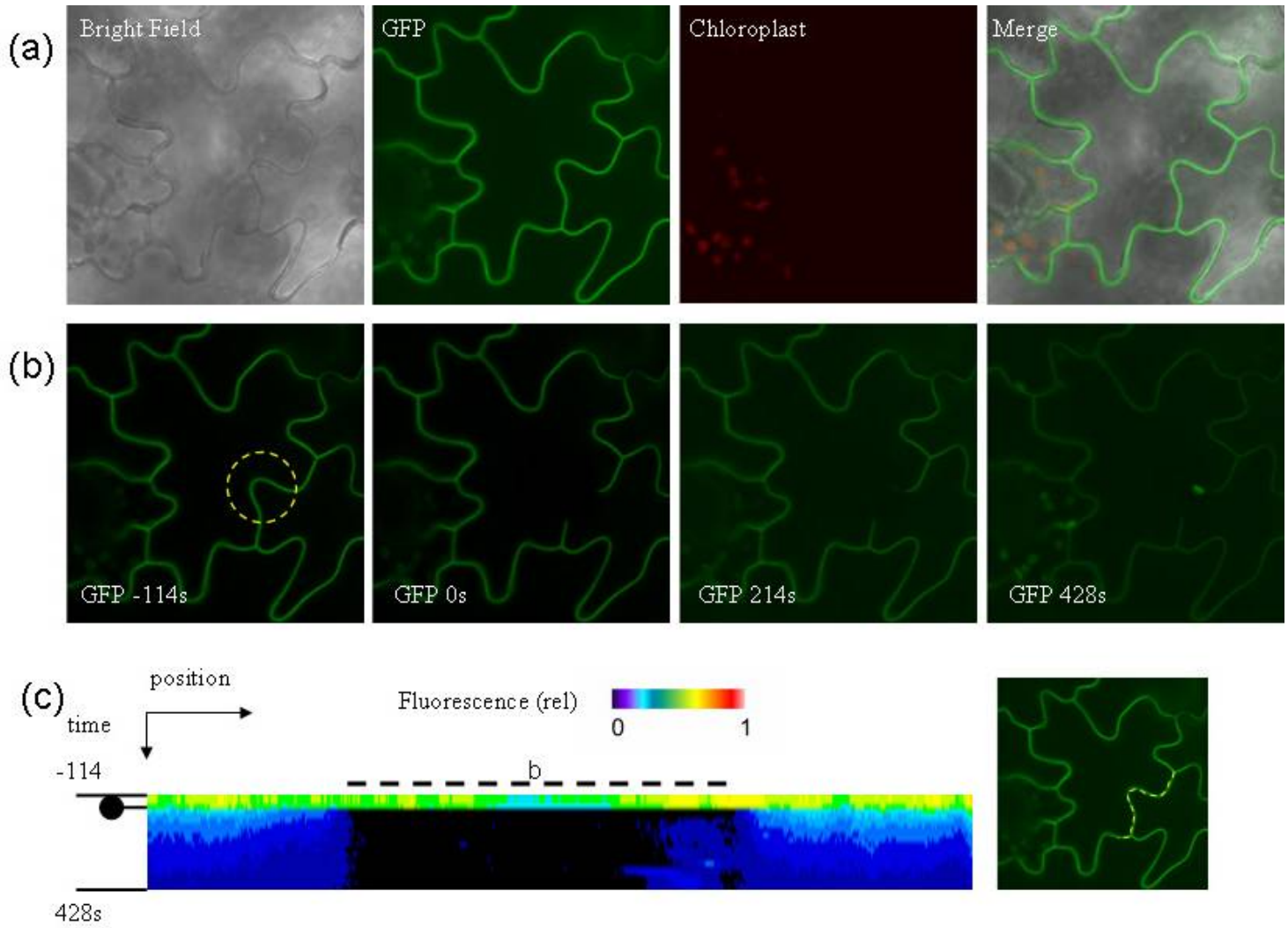
(a)
Bright Field
GFP
Chloroplast
Merge
(b)
GFP -114s
GFP 0s
GFP 214s
GFP 428s
(c)
position
time
Fluorescence (rel)
0
1
-114
b
428s

Table 2. ASA speeds recovery from the wound response.

Data showing time of first appearance of puncta at the plasma membrane for cells expressing KAT1:GFP. Four types of treatment were included (leaf tissue prepared normally, leaf tissue with the upper epidermis mostly removed with sandpaper, leaf tissue injected with ASA and then prepared normally and leaf tissue injected with ASA then sandpapered) and puncta were first seen at the plasma membrane at 25, 30, 20 and 15 minutes for each treatment type, respectively. Time taken for each treatment type to show a normal distribution of puncta (where all cells showed peripheral fluorescence) was then noted and marked as time taken for a complete recovery from the wound response. .

	time first puncta found in membrane (minutes)	time until normal distribution of puncta seen (minutes)
normal preparation	25	60
epidermis removed	30	45
ASA + normal preparation	20	20
ASA + epidermis removed	15	20

CHAPTER 4:

DISCUSSION

Vesicle trafficking is necessary for cell growth, homeostasis and development in all eukaryotes, including plants. Without vesicle trafficking maintenance of the cell membranes, both intracellular and delimiting plasma membrane, would be impossible. This traffic supports compartmentation within the cell, and is necessary for protein transport, in order to maintain cellular activity, and effects distribution of soluble proteins that are transported within vesicles as well as membrane proteins that are transported within the vesicle membrane targeted to different membranes within the cell. The superfamily of proteins known as SNAREs are, in turn, essential for vesicle trafficking, and act by guiding the docking and fusion of vesicles to their target membranes. There are four principal types of SNAREs, most recently classified as Qa, Qb, Qc SNAREs, and as R-SNAREs, as expanded upon in the **Introduction**. Molecular interactions of SNAREs through their coiled-coil domains gives rise to the SNARE core complex which is essential to overcome the dehydration energy barrier of lipid bilayers in free solution and to bring the target and vesicle membranes close enough for fusion. Specificity of SNARE interactions appears to be determined in large part by the structure of the SNARE proteins (Paumet 2004, McNew 2000), and thus specificity of vesicle fusion is related to pairing of these proteins that enables SNARE partner binding.

The trafficking of proteins to the plasma membrane is equally dependent upon SNARE proteins. SNARE proteins control the turnover of ion channels as well as other plasma membrane proteins, and thus they can be expected to affect the population of channels at the plasma membrane (Sutter, *et al*. 2006). Changes in the environment of the plant and in hormone levels is thought to have an effect on the turnover of these proteins, thus implying a role for SNAREs also in cellular signalling. For example, the Qa-SNARE NtSYP121 affects K^+ and Cl^- channel activity dependent on ABA (Leyman *et al.,* 1999). Yet, despite this important role for SNAREs in plant cell signalling, relatively little is known about the mechanisms of SNAREs in trafficking and targeting of specific vesicles

to the plasma membrane in plants. The great number of SNARE proteins to be found in plants generally – in arabidopsis there are at least 14 different SNAREs that are thought to act in plasma membrane trafficking (Sutter, *et al.* 2006) – does not make the task of characterising SNAREs any easier. Previous analysis has shown some SNAREs to be, to some degree, functionally redundant, while others are absolutely essential (Sanderfoot *et al.*, 2001b). Many different strategies have been employed so far to shed some light on the role played by SNAREs in trafficking, including the relatively new tactic utilised here of expressing dominant-negative fragments (so-called Sp2 protein fragments) of plasma membrane SNAREs to disrupt the traffic and anchoring of proteins to the plasma membrane in vivo (Tyrrell *et al.,* 2007; Scales *et al.,* 2000). Sequence analysis may also provide some clues to how SNAREs act in plasma membrane trafficking, and in figure 20 the amino acid sequences of the Sp2s used are compared, with the residues of the PVC localised SNARE SYP21-Sp2 highlighted where they differ from the residues of the plasma membrane Sp2s used.

My studies addressed important aspects of these questions on the role of different plasma membrane SNAREs in traffic and targeting of integral membrane marker proteins. I used two proteins, the GFP-tagged KAT1 K^+ channel of arabidopsis and the GFP-tagged PMA2 H^+-ATPase of tobacco, to track the localisation of these proteins under the influence of different dominant-negative Sp2 fragments and to test for differential specificity. The results showed (1) that KAT1 was sensitive to the plasma membrane SNARE Sp2s of SYP121, SYP122 and SYP71, but not to the Sp2 of the PVC SNARE SYP21; (2) that expressing the Sp2s of plasma membrane SNARE proteins affected the organisation of clusters of KAT1 at the plasma membrane, making them both more diffuse, and mobile, while the Sp2 of SYP21 had no effect on these KAT1 microdomains; (3) that the GPF-tagged H^+-ATPase PMA2 was insensitive to both the Sp2 fragments of plasma membrane SNAREs SYP121, SYP122, and SYP71, and to the Sp2 of PVC SNARE

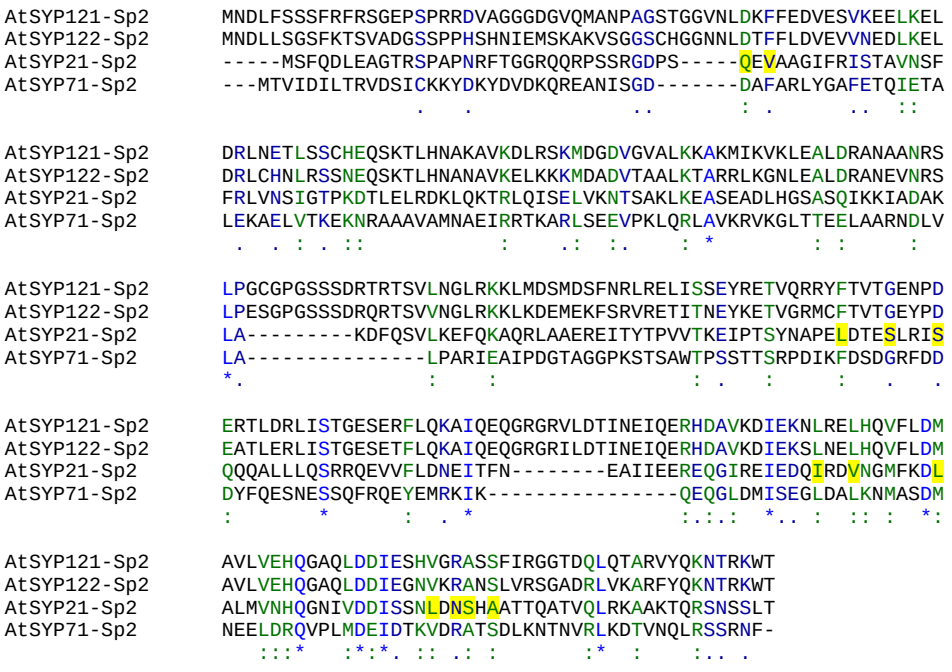

Figure 20. Sequence analysis shows the residues of SYP21-Sp2 which differ from the highly conserved residues of the plasma membrane Sp2s.
Sequence analysis of the Sp2s used. Highlighted in yellow are the residues in SYP21-Sp2 which vary from the highly conserved residues of all three of the the other Sp2s used. Some differences in amino acid sequence are to be expected between Sp2s of different groups , but those which are conserved between Sp2s of the plasma membrane SNAREs of both the SYP12 and SYP7 families and not in the PVC targeted SYP21 Sp2 provide a clue to the regions which may be responsible for the differential targeting.

SYP21. These findings indicate that SNAREs play a role in the selective trafficking of KAT1 and the H^+-ATPase protein to the plasma membrane, and suggest the SNAREs are involved in both the targeting of ion channels to a specific location at the plasma membrane, and in anchoring these channels, keeping them positionally stable within puncta.

4.1 Protein Clustering and Lipid Rafts

Previous work from this lab (Sutter *et al.,* 2006; Sutter *et al.,* 2007) has provided evidence for K^+ channel clustering, which supports the suggestion that ion channels and some other membrane proteins commonly form microdomains at the plasma membrane. One explanation for this phenomenon is the presence of raft-like subdomains or 'lipid rafts'.

The idea of lipid rafts arose from experiments on the formation of the two layers of polarised cell membranes in mammalian epithelial cells (Simons and van Meer, 1988). These studies showed that apical and basal plasma membranes varied in lipid composition, in addition to protein content, suggesting that proteins are not distributed randomly along the plasma membrane, but instead are organised into clusters within distinct areas of the membrane associated with the different lipids. Within the apical plasma membrane sphingolipid- and cholesterol-enriched microdomains were found. It is this enrichment of sphingolipids and cholesterol that results in a liquid-ordered phase within the membrane which can easily be isolated by detergent extraction, due to their nonionic detergent insolubility (Schroeder *et al*., 1994, Edidin *et al.,* 2003). In protein-free model membranes, sphingolipids form detergent resistant membranes (DRMs), however it is thought that interactions between proteins in the lipid rafts in vivo are also important for the formation of large clusters of lipid rafts in polarised or stimulated cells (Schroeder *et al.,* 1994,

Edidin *et al.,* 2003, Munro *et al.,* 2003). The biological significance becomes obvious when it is realised that animal lipid rafts commonly contain transmembrane and signalling proteins such as ion channels and kinases (Foster *et al.,* 2003). Thus, lipid rafts have come to be viewed as a mechanism to structurally order and localise signalling and other metabolic pathways in vivo.

It is not just animal cells which have been found to contain lipid rafts. Fungal lipids rafts have been found at the tips of mating projections in *Saccharomyces cerevisiae* and in hyphae in *Candida albicans* (Martin and Konopka, 2004; Bagnat and Simons, 2002). Since then, lipid rafts have been isolated as DRMs from the plasma membrane of plants, including tobacco leaf cells (Mongrande, *et al.* 2004). These DRMs contained proteins similar to those known to be found in animal lipid rafts (heterotrimeric G proteins and glycosylphosphatidylinositol (GPI) anchored proteins) (Peskan *et al.,* 2000). In 2004, Mongrande *et al* (2004) were the first to thoroughly characterise the lipid composition of plant DRMs from tobacco leaf cells, finding that plant DRMs are indeed similar to those of animals and yeast, containing sterols and sphingolipids. The plant DRMs were also found to be enriched in glucosylceramide and mostly lacking in phospholipids.

In the same year, Shahollari *et al* (2004) provided more evidence for plant lipid rafts, finding this time an enrichment of signalling proteins. Mongrande's observations were confirmed and taken further by Borner *et al* (2005). This group had used the fact that glycosylphosphatidyl inositol- (GPI-) anchored proteins were almost always identified in lipid rafts, and that GPI-anchored proteins have also been identified in arabidopsis (Sherrier *et al.,* 1999). Previously Borner *et al* predicted the GPI-proteome and confirmed most of the 250 predicted proteins using a proteomics approach (Borner *et al.,* 2002, 2003). Borner *et al* produced arabidopsis containing an immunodetectable GPI-anchored receptor protein which they could then track throughout the purification process. They found that in DRMs the GPI-anchored reporter protein was enriched relative to their starting material.

After isolating DRMs from various organelle membranes in arabidopsis root-callus tissue, they analysed the proteins found in the membranes. Most of the proteins were known plasma membrane proteins, suggesting that lipid rafts form only in the plasma membrane and not other intracellular membranes.

More recent work has used tobacco Bright Yellow-2 (BY-2) suspension cells, reasoning that undifferentiated cells would give results uncomplicated by tissue type or developmental stage. Proteins found in microdomains defined by DRMs in this case were largely involved in signalling and stress responses and cellular trafficking (Morel *et al.,* 2006). Many proteins also had post-translational modifications such as GPI-anchors or palmitoylation.

Results such as those outlined above suggest a role for lipid rafts in defining the targets for trafficking. This conclusion is strengthened by the evidence that plant and animal DRMs both are enriched for intracellular trafficking proteins, including proteins involved in SNARE complex function (Mongrande *et al.,* 2004, Morel *et al.,* 2006). The presence of plant lipid rafts can not be taken for granted, however. There is some controversy over whether lipid rafts actually exist in membranes or whether they are artefacts of detergent extraction (Simons and Ikonen, 1997; Simons and Van Meer, 1988).

4.2 K^+ Channel Clustering and Lipid Rafts

While it is possible that K^+ channel clustering can be explained by lipid rafts, it is worth noting that both PMA2 and possibly PMA4 H^+-ATPases are present in DRMs from arabidopsis and from leek (Borner *et al.,* 2005; Laloi *et al.,* 2007) and the homologue H^+-ATPase PMA1p is present in yeast DRMs (Malinska *et al.,* 2003, 2004). Despite this fact, at a microscopic level the H^+-ATPase proteins are uniformly distributed around the plasma membrane in each case, with the exception that in yeast exclusion zones are evident when

grown under certain conditions (Malinska *et al.,* 2003). It may be that the lipid rafts containing the H^+-ATPase proteins in plants are too small for microscopic resolution (Varma and Mayor, 1998; Pralle *et al.*, 2000), but this possibility still does not explain the difference in appearance between seemingly uniform distribution of the H^+-ATPase proteins at the plasma membrane, and the punctuate distribution of KAT1. It seems that if KAT1 is indeed contained within lipid rafts, then this is not necessarily enough to explain the organisation of KAT1 microdomains, or that the biochemical definition of lipid rafts as DRMs is too coarse a description.

Interestingly, previous work from this laboratory has shown that while the Sp2 fragment of SYP121 caused an accumulation of the KAT1 K^+ channel within endomembrane fractions and the endoplasmic reticulum, there was no effect on the PMA2 H^+-ATPase (Sutter *et al.,* 2006). I carried on this work, and upon expression of PMA2 with the Sp2 fragments of SYP122, SYP71 and SYP21, the same insensitivity could be seen. As none of the Sp2 fragments affected H^+-ATPase distribution, the findings strongly support previous observations that H^+-ATPase proteins must travel via a completely different pathway to KAT1. These findings also support the theory that SNAREs play a role in the organisation of KAT1 in microdomains.

4.3 KAT1 tagging and internalisation

While my results show that KAT1 is commonly seen in microdomains at the plasma membrane, the K^+ channel also appeared in internal structures on more occasions than in previous experiments (Sutter *et al.,* 2006) in this laboratory. Usually KAT1 is not expressed strongly enough, under its own promoter, to be seen clearly using GFP tagging and standard fluorescent confocal microscopy. Instead, KAT1 constructs driven by the 35S CaMV promoter were used, which may have led to overexpression of KAT1:GFP in many

cells, leading to a 'backlog' of the protein and accumulation in internal membrane structures. Problems with overexpressed GFP-tagged proteins are well documented. Previously, tagging a viral movement protein with GFP disrupted the proteins assembly into tubules (Thomas and Maule, 2000) and Persson *et al* (2002) found that a GFP fusion protein targeted to the ER was degraded in tobacco cells. With the knowledge that GFP tends to form dimers at high concentrations (Phillips *et al.,* 1997), Lisenbee *et al* (2003) studied the overexpression and mislocalisation of GFP-tagged peroxisomal ascorbate peroxidase (APX). These researchers suspected that dimerisation became a problem when GFP-tagged proteins are localised to more concentrated environments such as membrane microdomains. They found that overexpression of GFP-APX caused aggregation of peroxisomes and accumulation of circular membrane structures within the cytoplasm. With immunofluorescence labeling after permeabilisation of cells with digitonin, they were able to show that mislocalised GFP-tagged proteins had the same topological orientation where the GFP of the expressed protein faced the cytosol, suggesting that the organelle aggregation is caused by the oligomerisation of GFP proteins protruding into the cytosol. Lisenbee *et al* concluded that when overexpressed, membrane-targeted GFP fusion proteins can form organelle aggregates, causing misinterpretation of the sorting pathways of these proteins.

It is clear that the use of GFP to tag proteins can sometimes lead to problems in localisation, especially when used with a strong promoter like the 35S CaMV promoter. Overexpression and a 'backlog' of the protein, or dimerisation of GFP could both lead to mislocalisation, providing two possible reasons for the internal fluorescence seen in both my experiments and those previously conducted in this laboratory (Sutter *et al.,* 2006). I also observed that the occurrence of GFP fluorescence in internal structures was slightly more common when viewed immediately after slide preparation. When left for some time

after preparation of the slide, KAT1 was again seen in puncta. This effect may have been due to the internalisation of KAT1 as part of the wound response, a point I return to later.

4.4. KAT1 microdomains and internalisation

Previous work on KAT1 by this laboratory (Sutter *et al.,* 2007) has shown the KAT1 microdomains to be positionally stable unless KAT1 was expressed with the Sp2 of SYP121. When I co-expressed KAT1 with SYP122-Sp2 and SYP71-Sp2, the same result was found. KAT1 was observed to be mobile at the plasma membrane, by comparison with control expression of KAT1 alone, and the channel protein was clustered more loosely (Figs. 6 and 7). In the presence of SYP122-Sp2 I observed a mean microdomain width of 4000 ± 201nm as opposed to 950 ± 3nm, the size seen for KAT1 when expressed alone. Similarly SYP71-Sp2 expression caused the microdomains to become more diffuse (Fig. 18), with a width of 2786 ± 188nm (Table 1). The similarity in results is not so surprising as SYP121 and SYP122 are known to have overlapping roles in the plant. In genetic mutants lacking either one of these proteins, the phenotype is not obviously different from that of the wild-type plant, however in the *syp121-syp122* double knockout, severe effects on growth, and necrosis have been seen (Assaad *et al.,* 2004). As such, there is already evidence for a functional overlap *in vivo* of SYP121 and SYP122. Furthermore, as both these proteins are Qa SNAREs and SYP71 is a Qc SNARE, it is possible that SYP71 interacts with one or both of them to form functional SNARE complexes that are, hence, also sensitive to this SNARE Sp2.

The Sp2 of SYP21, by contrast, had no effect on the position or mobility of KAT1 (Fig. 8). The lack of effect on co-expressing SYP21-Sp2 is encouraging as it is in line with expectations based on the known functional target of the SNARE. SYP21 is localised to the PVC and involved in vacuolar traffic from the Golgi apparatus and trans-golgi network (Conceicao *et al.,* 1997; Foresti *et al*., 2006; Surpin and Raikhel, 2004), but not associated

with plasma membrane traffic. This supports previous work from this lab on SYP21-Sp2. When co-expressed with secreted green fluorescent protein (sec-GFP) and with the YFP-tagged aquaporin fusion protein TIP1;1-YFP, SYP21-Sp2 fragments blocked traffic of TIP1;1-YFP to the tonoplast, but had no effect on trafficking to the plasma membrane of sec-GFP (Tyrrell *et al.,* 2007). The lack of effect seen with SYP21-Sp2 expression is useful also in that SYP21-Sp2 acts as a control to show that any effect seen is not a consequence of infecting the plant with agrobacterium for transfections.

Given these results, how can we understand the effects of the Sp2 fragments on KAT1 trafficking and microdomain structure? There are three possible reasons for the effects seen with SYP121-, SYP122- and SYP71-Sp2 fragments. First, the Sp2 fragments may block direct interaction between the SNAREs and KAT1 which would otherwise hold the ion channel in place at the plasma membrane. It is already known that in mammals, some SNAREs interact directly with ion channels. For example, both SNAP-23 and syntaxin 1A have been found to regulate cystic fibrosis transmembrane conductance regulator (CFTR). SNAP-23 binds to the region that modulates channel gating, the amino terminal of CFTR and Syntaxin 1A binds to both CFTR and SNAP-23 (Cormet-Boyaka *et al.,* 2002). There are many other examples of direct SNARE-ion channel interaction (Arien *et al.,* 2003; Jarvis *et al.,* 2002; Ji *et al.,* 2002). Assuming that the plasma membrane SNAREs not only help traffic KAT1 to the right place on the plasma membrane, but hold it there, any interference in the interaction of KAT1 and the SNAREs would result in possible mislocalisation and a lack of anchoring of KAT1, leading to diffusion and mobility of microdomains.

The second reason for the observations could be that the Sp2 fragments interference is more general, with the trafficking of KAT1 to the plasma membrane resulting in mislocalisation of the channel failing to associate it with (as yet, unknown) anchoring proteins. This explanation implies both a basic role for the SNAREs in targeting and the

presence of discrete anchoring sites associated with the targets. Thus disrupting targeting leads to KAT1 being randomly distributed and mobile at the plasma membrane simply because it never reaches the anchor sites. There is some evidence that for vesicle trafficking to target the correct locations, specific SNARE interactions with other proteins are necessary (Scales *et al.,* 2000) and so mislocalisation is a possibility. As the final reason for the observations, the disruption caused by the Sp2 fragments may have affected the trafficking of other KAT1-associated proteins. In this case, the absence of these proteins would mean that they were no longer be in place to interact with KAT1. If such proteins were necessary to anchor KAT1 in the plasma membrane, and the trafficking of one or more of these proteins was also disrupted, then the effect might be seen as the diffuse microdomains of KAT1.

At present, there is no line of evidence which clearly distinguishes between these three possibilities. However, KAT1 has been expressed in other cell types with some interesting results. When expressed in *Vicia faba*, KAT1:GFP has been seen in clusters at the plasma membrane (Hurst *et al.,* 2004; Homann *et al.,* 2007). Patch clamp studies in oocytes have shown open current levels indicating the presence of a number of channels within the patch whereas other areas yield no current (see Sutter, *et al.* 2006 for discussion). These findings suggest the channels are grouped in clusters, however the areas with no current could be explained by non-active or non-functional channels. The localisation of KAT1 in clusters in other cell types means that KAT1 is still expressed in microdomains regardless of the absence of the other proteins it associates with in arabidopsis, which argues against the third reason given. The final reason can not be ruled out, however, as homologues of these associated proteins may be present in other cell types, forming the necessary interactions. Finally, in support of the first reason, while there is no evidence of the role of SNAREs in localisation at microscopic levels, at a macroscopic level there is plenty of literature on the subject as noted above. In many cases,

SNAREs are responsible for apical-basal polarity of protein distribution. In mammals, specific SNAREs cause an apical-basal distribution of Na^+/K^+-ATPase and gastric H^+-ATPase in cell membranes (Banerjee *et al.,* 1999; Ishiki and Klip, 2005). In plants, AUX1 is localised to the apical side in epidermal and root and stem cortex cells (Vieten *et al.,* 2007). This distribution is due to the arrangement of various subsets of SNAREs (Bassham and Blatt, 2008).

4.5. SNAREs, K^+ Channel Traffic and Signalling

My results are the latest from this laboratory to add to the understanding of the actions of SNAREs in ion channel control, and more generally in cellular signalling. They lend support to the data already gathered indicating that traffic of plasma membrane proteins, and specifically K^+ channels, is selective. In addition to this, the obvious diffusion and mobility of KAT1 puncta when expressed with plasma membrane SNARE Sp2 fragments raises a question about the role of SNAREs in anchoring KAT1, separate to their role in traffic. SNAREs have already been shown to be involved in targeting and spatial distribution in nerves, for example both SNAP-25 and Syntaxin1A are known to directly interact at the plasma membrane with Kv2.1 channels, and Syntaxin1A with neuronal Kv1.1 channels in rats (Leung *et al.,* 2003). This leads to the question: Does SNARE-dependent anchoring of K^+ channels enable them to associate with other regulatory proteins, forming part of a signalling protein scaffold?

It is interesting to note that other factors involved in signalling include the formation of lipid rafts, allowing the clustering of interacting proteins. More than this, it has been shown in mammalian cells that mechanosensitive ion channels are attached to the cytoskeleton as part of a signalling protein scaffold (Barritt and Rychkov, 2005). It has been suggested, too, that the cytoskeleton can play a part in anchoring proteins by ‘fencing

in' microdomains without any direct interaction with the proteins (Richie and Kusumi, 2004). Given that plant cells contain a cortical cytoskeleton, this function could well be the case in plants also. In animals, the extracellular matrix (ECM) can also determine the position of plasma membrane proteins (Arnold *et al.,* 2004). The plant cell wall has been shown in some cases to directly influence the distribution of KAT1 microdomains (Homann *et al.,* 2007). When expressed in the guard cells of *Vicia faba*, KAT1 was found to be present in a radially striped pattern in turgid cells. When turgor pressure decreased, the radial pattern disappeared. It was concluded that this striped arrangement is only produced when the plasma membrane is in close contact with the guard cell wall, which contains a radial arrangement of cellulose fibres. In epidermal cells, which contain no set arrangement of fibres, the clusters are randomly distributed, and are not seen in stripes. This data indicate that the cell wall also plays a part in the organisation of KAT1 at the plasma membrane, at least in guard cells.

At present, there is not enough known to say what the significance of this is, or what effect it has on the anchoring of KAT1 in cell types other than guard cells, but it will be interesting to see where this research leads, and its relevance to the anchoring of KAT1 in clusters at the plasma membrane. It is clear, however, that KAT1 plays an even more vital role in cellular signalling than was previously thought. It is already known that KAT1 is involved in signalling, in that stomatal opening in plants is known to be controlled by guard cell ion channel regulation, including regulation of KAT1 (Kwak *et al.,* 2001). It has been found that ABA causes inhibition of stomatal opening and internalisation of KAT1 (Sutter *et al.,* 2006). However, in the presence of Syp121 Sp2, K^+ channel responses to ABA are blocked (Leyman *et al.,* 1999). The effect of ABA on KAT1 is interesting, since ABA is also thought to be involved with the plant wound response. One of the earliest responses to wounding is a change in membrane permeability, which causes major changes in the flow of ions into and out of the cell. These changes in K^+, H^+ and Ca^{2+} concentration

are involved in the subsequent gene activation and triggering of defence responses (Zimmermann *et al.,* 1997; Loseva *et al.,* 2004).

The mechanism of these responses in plants has been studied extensively and yet the process is complex enough that only a fraction of the whole is known. It is understood that reactive oxygen species (ROS) such as H_2O_2 play a part (Wu *et al.,* 1997), as do various other chemical signals, notably salicylic acid (SA), jasmonic acid (JA), and nitric oxide (NO) (Huang *et al.,* 2004; Loake and Grant, 2007). It is difficult to say exactly what part each chemical signal plays as the plant defence signalling pathways are not linear, nor are they independent. Despite the conflicting research (Mur *et al.,* 2006; Laudert and Weiler, 1998; Norton *et al.,* 2007), it has been shown that NO does regulate inward rectifying K^+ channels through its action on Ca^{2+} release from intercellular Ca^{2+} stores (Garcia-Mata *et al.,* 2003). It is known that SA and aspirin (ASA) block JA biosynthesis, thereby blocking the wound response, as has been shown in tomato plants (Pena-Cortes *et al.,* 1993). This supports my findings that wounded leaves expressing KAT1 and injected with ASA recover much quicker than those not exposed to ASA.

As has been mentioned previously, ABA expression in response to wounding causes internalisation of KAT1 (Sutter *et al.,* 2006). If the wound response in cells expressing KAT1:GFP is suppressed by ASA then it is to be expected that recovery of KAT1:GFP fluorescence at the plasma membrane will happen at a faster rate, or that internalisation of KAT1 will halt upon injection of ASA. As can be seen from Table 2, KAT1 expression at the plasma membrane in discrete microdomains returns in around 15 minutes after wounding when treated with ASA, as opposed to around 45 minutes for untreated wounded leaves. This suggests that ASA does not prevent the internalisation of KAT1 but instead partially suppresses the reaction of the plant to wounding, causing it to return KAT1 to the plasma membrane. Interestingly, many of the cells showing KAT1 in puncta at the plasma membrane also retained internal fluorescence, suggesting that while

ASA may suppress the wound response and internalisation of KAT1, it has no effect on internal localisation of KAT1 caused by overexpression.

In conclusion, this study has supported previous findings that KAT1 is localised to the plasma membrane in positionally stable microdomains and that SYP121-Sp2 fragments suppress traffic and disrupt localisation of the K^+ channel. It has also demonstrated that the Sp2 fragments of plasma membrane SNAREs SYP122 and SYP71, in addition to that of SYP121, suppress traffic of KAT1 to the plasma membrane and cause diffusion and mobility of KAT1 microdomains. These results offer direct evidence that plasma membrane SNAREs SYP121, SYP122 and SYP71 are involved in both traffic to, and anchoring of KAT1 at the plasma membrane. Furthermore, data showing that ASA suppresses internalisation of KAT1 and increases the rate at which the cell recovers KAT1 in clusters at the plasma membrane offers evidence of a linking between possible roles for traffic of the K^+ channels and plant defence.

REFERENCES

Arien H., Wiser O., Arkin I.T., Leonov H., Atlas D. (2003) Syntaxin 1A modulates the voltage-gated L-type calcium channel (Ca(v)1.2) in a cooperative manner. J. Biol. Chem. 278(31), 29231-9

Arnold M., Cavalcanti-Adam E.A., Glass R., Blümmel J., Eck W., Kantlehner M., Kessler H., Spatz J.P. (2004) Activation of integrin function by nanopatterned adhesive interfaces. Chemphyschem. 5(3), 383-8

Assaad F.F., Qiu J.L., Youngs H., Ehrhardt D., Zimmerli L., Kalde M., Wanner G., Peck S.C., Edwards H., Ramonell K., Somerville C.R., Thordal-Christensen H. (2004) The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Mol. Biol. Cell. 15**,** 5118–5129.

Bagnat M., Simons K. (2002) Cell surface polarization during yeast mating Proc. Natl. Acad. Sci. USA 99, 14183–14188

Banerjee A., Shih T., Alexander E.A., Schwartz J.H. (1999) SNARE proteins regulate H^+-ATPase redistribution to the apical membrane in rat renal inner medullary collecting duct cells. J. Biol. Chem. 274, 26518–26522

Barritt G., Rychkov G. (2005) TRPs as mechanosensitive channels. Nat. Cell Biol. 7(2), 105-7

Bassham D.C., Blatt M.R. (2008) SNAREs—cogs or coordinators in signalling and development? Plant Physiol. 147, 1504–1515

Bassham D.C., Sanderfoot A.A., Kovaleva V., Zheng H., Raikhel N.V. (2000) AtVPS45 complex formation at the trans-Golgi network. Mol. Biol. Cell 11(7), 2251-65

Becker D., Dreyer I., Hoth S., Reid J.D., Busch H., Lehnen M., Palme K., Hedrich R. (1996) Changes in voltage activation, Cs^+ sensitivity and ion permeability in H5 mutants of the plant K^+ channel KAT1. Proc. Natl. Acad. Sci. USA 93, 8123–8128

Berkowitz G., Zhang X., Mercier R., Leng Q., Lawton M. (2000) Co-expression of calcium-dependent protein kinase with inward rectified guard cell K^+ channel KAT1 alters current parameters in Xenopus laevis oocytes. Plant Cell Physiol. 41, 785-790

Bezanilla F. (2000) The voltage sensor in voltage-dependent ion channels. Physiol. Rev. 80, 555-592

Bezprozvanny I., Scheller R.H., Tsien R.W. (1995) Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature 378, 623-626

Bezprozvanny I., Zhong P., Scheller R.H., Tsien R.W. (2000) Molecular determinants of the functional interaction between syntaxin and N-type Ca2+ channel gating. Proc. Natl. Acad. Sci. USA 97(25), 13943-8

Bhattacharya S., Stewart B.A., Niemeyer B.A., Burgess R.W., McCabe B.D., Lin P., Boulianne G., O'Kane C.J., Schwarz T.L. (2002) Members of the synaptobrevin/vesicle-associated membrane protein (VAMP) family in drosophila are functionally interchangeable in vivo for neurotransmitter release and cell viability. Proc. Natl. Acad. Sci. USA 99(21), 13867-13872

Blancaflor E.B., Masson P.H. (2003) Plant Gravitropism. Unraveling the Ups and Downs of a Complex Process. Plant Physiology 133, 1677-1690

Blatt M.R. (1992) K+ channels of stomatal guard cells. Characteristics of the inward rectifier and its control by pH. J. Gen. Physiol. 99(4), 615-44

Blatt M.R., Thiel G. (2003) SNARE components and mechanisms of exocytosis in plants In The Golgi apparatus and the plant secretory pathway. (Robinson D.G., ed) pp 208-237, Blackwell Publishing – CRC Press, Oxford.

Bock J.B., Matern H.T., Peden A.A., Scheller R.H. (2001) A genomic perspective on membrane compartment organisation. Nature 409, 839-841

Borner G.H.H., Lilley K.S., Stevens T.J., Dupree P. (2003) Identification of glycosylphosphatidylinositol-anchored proteins in arabidopsis. A proteomic and genomic analysis. Plant Physiol. 132(2), 568-77

Borner G.H.H., Sherrier D.J., Stevens T.J., Arkin I.T., Dupree P. (2002) Prediction of glycosylphosphatidylinositol-anchored proteins in arabidopsis. A genomic analysis. Plant Physiol. 129(2), 486-99

Borner G.H.H., Sherrier D.J., Weimar T., Michaelson L.V., Hawkins N.D., MacAskill A., Napier J.A., Beale M.H., Lilley K.S., Dupree P. (2005) Analysis of detergent-resistant membranes in arabidopsis. Evidence for plasma membrane lipid rafts. Plant Physiol. 137, 104–116

Bowen M.E., Weninger K., Ernst J., Chu S., Brunger A.T. (2005) Single-molecule studies of synaptotagmin and complexin binding to the SNARE complex. Biophysical Journal 89, 690-702

Brüggemann L., Dietrich P., Becker D., Dreyer I., Palme K., Hedrich R. (1999) Channel-mediated high-affinity K^+ uptake into guard cells from arabidopsis. Proc. Natl. Acad. Sci. USA 96(6), 3298–3302

Brunger A.T. (2001) Structure of Proteins Involved In Neurons. Annual Review of Biophysics and Biomolecular Structure 30, 157-171

Campanoni P., Blatt M.R. (2007) Membrane trafficking and polar growth in root hairs and pollen tubes. J. Exp. Bot. 58(1), 65-74

Chapman E.R., Hanson P.I., An S., Jahn R. (1995) Ca^{2+} regulates the interaction between synaptotagmin and syntaxin 1. J. Biol. Chem. 270, 23667–23671

Chen Y.A., Scheller R.H. (2001) SNARE-mediated membrane fusion. Nat. Rev. Mol. Cell Biol. 2, 98–106

Clary D.O., Griff I.C., Rothman J.E (1990) SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell. 61(4), 709-721.

Collins N.C., Thordal- Christensen H., Lipka V., Bau S., Kombrink E., Qiu J.L. Huckelhoven R., Stein M., Freialdenhoven A., Somerville S.C., Schulze-Lefert P. (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425(6961), 973-977

Conceicao A.S., Marty-Mazars D., Bassham D.C., Sanderfoot A.A., Marty F., Raikhel N.V. (1997) The syntaxin homologue AtPEP12p Resides on a Late Post-Golgi Compartment in Plants. Plant Cell 9, 571-582

Cormet-Boyaka E., Di A., Chang S.Y., Naren A.P., Tousson A., Nelson D.J., Kirk K.L. (2002) CFTR chloride channels are regulated by a SNAP-23/syntaxin 1A complex. Proc. Natl. Acad. Sci. USA 99(19), 12477-82

Degtiar V.E., Scheller R.H., Tsien R.W. (2000) Syntaxin modulation of slow inactivation of N-type calcium channels. J. Neurosci. 20(12), 4355-67

Deitcher D.L., Ueda A., Stewart B.A., Burgess R.W., Kidokoro Y., Schwarz T.L. (1998) Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the drosophila gene neuronal-synaptobrevin. J. Neurosci. 18, 2028-39

Després C., Chubak C., Rochon A., Clark R., Bethune T., Desveaux D., Fobert P.R. (2003) The arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell. 15(9), 2181-91.

Dulubova I., Sugita S., Hill S., Hosaka M., Fernandez I., Sudhof T.C., Rizo J. (1999) A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J. 18, 4372-4382

Durrant W.E., Dong X. (2004) Systemic aquired resistance. Annu. Rev. Phytopathol. 42, 185-209.

Edidin M. (2003) The state of lipid rafts: from model membranes to cells. Annu. Rev. Biophys. Biomol. Struct. 32, 257-83

Elotza F., Nuhse T.S., Foster L.J., Stensballe A., Peck S.C., Jensen O.N. (2003) Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. Molecular and Cellular Proteomics 2(12), 1261-1270

Farsad K., De Camilli P. (2003) Mechanisms of membrane deformation. Curr. Opin. Cell Biol. 15(4), 372-381

Fasshauer D., Sutton R.B., Brunger A.T., Jahn R. (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc. Natl. Acad. Sci. USA 95(26), 15781-6.

Feys B.J., Moisan L.J., Newman M.A., Parker J.E. (2001) Direct interaction between the arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J. 20(19), 5400-5411.

Fletcher S., Bowden S.E., Marrion N.V. (2003) False interaction of syntaxin 1A with a Ca^{2+}-activated K^+ channel revealed by co-immunoprecipitation and pull-down assays: implications for identification of protein-protein interactions. Neuropharmacology 44, 817-827

Foran P.G., Fletcher L.M., Oatey P.B., Mohammed N., Dolly J.O., Tavare J.M. (1999) Protein kinase B stimulates the translocation of GLUT4 but not GLUT1 or transferrin receptors in 3T3-L1 adipocytes by a pathway involving SNAP-23, synaptobrevin-2, and/or cellubrevin. J Biol Chem. 274(40), 28087-95

Foresti O., daSilva L.L., Denecke J. (2006) Overexpression of the arabidopsis syntaxin PEP12/SYP21 inhibits transport from the prevacuolar compartment to the lytic vacuole in vivo. Plant Cell 18(9), 2275-93.

Foster L.J., De Hoog C.L., Mann M. (2003) Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. Proc. Natl. Acad. Sci. USA 100(10), 5813-8

Fujita Y., Shirataki H., Sakisaka T., Asakura T., Ohya T., Kotani H., Yokoyama S., Nishioka H., Matsuura Y., Mizoguchi A. (1998) Tomosyn: a Syntaxin-1–Binding Protein that Forms a Novel Complex in the Neurotransmitter Release Process. Neuron 20(5), 905-915

Garcia-Mata C., Gay R., Sokolovski S., Hills A., Lamattina L., Blatt M.R. (2003) Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. Proc. Natl. Acad. Sci. USA 100, 11116–11121

Geelen D., Leyman B., Batoko H., Di Sansebastiano G.P., Moore I., and Blatt M.R. (2002) The abscisic acid-related SNARE homolog NtSyr1 contributes to secretion and growth: Evidence from competition with its cytosolic domain. Plant Cell 14, 387–406

Gerst J.E. (1999) SNAREs and SNARE regulators in membrane fusion and exocytosis. CMLS Cell. Mol. Life Sci. 55, 707-734

Glazebrook J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43, 205-227

Hayashi T., McMahon H., Yamasaki S., Binz T., Hata Y., Sudhof T.C., Niemann H. (1994) Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. EMBO J. 13(21), 5051-61

Heese M., Gansel X., Sticher L., Wick P., Grebe M., Granier F., Jurgens G. (2001) Functional characterization of the KNOLLE-interacting t-SNARE AtSNAP33 and its role in plant cytokinesis. J. Cell Biol. 155(2), 239-49.

Heginbotham L., Lu Z., Abramson T., Mackinnon R. (1994). Mutations in the K^+ channel signature sequence. Biophysical Journal 66, 1061-1067

Hess D.T., Slater T.M., Wilson M.C., Skene J.H.P. (1992) The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. J. Neurosci. 12, 4634-41

Hildmann T., Ebneth M., Peña-Cortés H., Sánchez-Serrano J.J., Willmitzer L., Prat S. (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell. 4(9), 1157-70

Homann U., Meckel T., Hewing J., Hütt M.T., Hurst A.C. (2007) Distinct fluorescent pattern of KAT1::GFP in the plasma membrane of Vicia faba guard cells. Eur. J.Cell Biol. 86(8), 489-500

Hong W. (2005) SNAREs and traffic. Biochim. Biophys. Acta. 1744(2), 120-144

Hoth S., Dreyer I., Dietrich P., Becker D., Müller-Röber B., Hedrich R. (1997) Molecular basis of plant-specific acid activation of K^+ uptake channels. Proc. Natl. Acad. Sci. USA 94, 4806-4810

Huang S., Lifshitz L.M., Jones C., Bellve K.D., Standley C., Fonseca S., Corvera S., Fogarty K.E., Czech M.P. (2007) Insulin stimulates membrane fusion and GLUT4 accumulation in clathrin coats on adipocyte plasma membranes. Mol. Cell Biol. 27(9), 3456-69

Huang X., Stettmaier K., Michel C., Hutzler P., Mueller M.J., Durner J. (2004) Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. Planta 218, 938–946

Hurst A.C., Meckel T., Tayefeh S., Thiel G., Homann, U. (2004). Trafficking of the plant potassium inward rectifier KAT1 in guard cell protoplasts of *Vicia faba.* Plant J. 37, 391–397

Ichida A.M., Pei Z.M., Baizabal-Aguirre V.M., Turner K.J., Schroeder J.I. (1997) Expression of a Cs^+-resistant guard cell K^+ channel confers Cs^+- resistant, light-induced stomatal opening in transgenic arabidopsis. The Plant Cell 9, 1843–1857

Ishiki M., Klip A. (2005) Minireview. Recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. Endocrinology 146, 5071–5078

Ivashikina N., Becker D., Ache P., Meyerhoff O., Felle H.H., Hedrich R. (2001) K(+) channel profile and electrical properties of arabidopsis root hairs. FEBS Lett. 508(3), 463-9

Jahn R., Lang T., Sudhof T. (2003) Membrane Fusion. Cell 112(4), 519-533

Jan L.Y., Jan Y.N. (1997) Cloned potassium channels from eukaryotes and prokaryotes. Annu. Rev. Neurosci. 20, 91-123

Jarvis S.E., Barr W., Feng Z.P., Hamid J., Zamponi G.W. (2002) Molecular determinants of syntaxin 1 modulation of N-type calcium channels. J. Biol. Chem. 277(46), 44399-407

Ji J., Tsuk S., Salapatek A.M., Huang X., Chikvashvili D., Pasyk E.A., Kang Y., Sheu L., Tsushima R.G., Diamant N.E., Trimble W.S., Lotan I., Gaisano H.Y. (2002) The 25-kDa synaptosome associated protein (SNAP-25) binds and inhibits delayed rectifier potassium channels in secretory cells. J. Biol. Chem. 277, 20195–20204

Jurgens G. (2004) Membrane trafficking in plants. Annu. Rev. Cell Dev. Biol. 20, 481-504

Jurgens G. (2005) Cytokinesis in higher plants. Annu. Rev. Plant Biol. 56, 281-299

Kalde M., Nuhse T.S., Findlay K., Peck S.C. (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. PNAS USA 104(28), 11850-11855

Kanda H., Tamori Y., Shinoda H., Yoshikawa M., Sakaue M., Udagawa J., Otani H., Tashiro F., Miyazaki J., Kasuga M. (2005) Adipocytes from Munc18c-null mice show increased sensitivity to insulin-stimulated GLUT4 externalization. J. Clin. Invest. 115, 291-301

Kane S., Sano H., Liu S.C.H., Asara J.M., Lane W.S., Garner C.C., Lienhard G.E. (2002) A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. J. Biol. Chem. 277, 22115-22118

Kargul J., Gansel X., Tyrrell M., Sticher L., Blatt M.R. (2001) Protein-binding partners of the tobacco syntaxin NtSyr1. FEBS Lett. 508, 253-258.

Kato T., Morita M.T., Fukaki H., Yamauchi Y., Uehara M., Niihama M., Tasaka M. (2002) SGR2, a phospholipase-like protein, and ZIG/SGR4, a SNARE, are involved in the shoot gravitropism of arabidopsis. Plant Cell 14(1), 33-46

b) Kato T., Moriko M., Tasaka M. (2002) Role of Endodermal Cell Vacuoles in Shoot Gravitropism. J. Plant Growth Regul. 21(2), 113-119

Kee Y., Lin R.C., Hsu S.C., Scheller R.H. (1995) Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. Neuron 14, 991–998

Koncz C., Schell J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of agrobacterium binary vector. Mol. Gen. Genet. 204, 383–396

Krasnoperov V.G., Bittner M.A., Beavis R., Kuang Y., Salnikow K.V., Chepurny O.G., Little A.R., Plotnikov A.N., Wu D., Holz R.W., Petrenko A.G. (1997) α-Latrotoxin Stimulates Exocytosis by the Interaction with a Neuronal G-Protein-Coupled Receptor. Neuron 18(6), 925-937

Kumar V.D., Weber I.T. (1992) Molecular model of the cyclic GMP-binding domain of the cyclic GMP-gated ion channel. Biochemistry. 31(19), 4643-9

Kumudu H., Perera I., Clarke M., Morris N.J., Hong W., Chamberlain L.H., Gould G.W. (2003) Syntaxin 6 Regulates Glut4 Trafficking in 3T3-L1 Adipocytes. Molecular Biology of the Cell 14, 2946-2958

Kurland C.G., Collins L.J., Penny D. (2006) Genomics and the Irreducible Nature of Eukaryote Cells. Science 312, 1011-1014

Kwak J.M., Murata Y., Baizabal-Aguirre V.M., Merrill J., Wang M., Kemper A., Hawke S.D., Tallman G., Schroeder J.I. (2001) Dominant negative guard cell K+ channel mutants reduce inward-rectifying K+ currents and light-induced stomatal opening in arabidopsis. Plant Physiol. 127(2), 473-85

Laloi M., Perret A.M., Chatre L., Melser S., Cantrel C., Vaultier M.N., Zachowski A., Bathany K., Schmitter J.M., Vallet M., Lessire R., Hartmann M.A., Moreau P. (2007) Insights into the role of specific lipids in the formation and delivery of lipid microdomains to the plasma membrane of plant cells. Plant Physiol. 143(1), 461-72

Lampson M.A., Schmoranzer J., Zeigerer A., Simon S.M., McGraw T.E. (2001) Insulin-regulated release from the endosomal recycling compartment is regulated by budding of specialized vesicles. Mol. Biol. Cell. 12(11), 3489-501

Langer K., Levchenko V., Fromm J., Geiger D., Steinmeyer R., Lautner S., Ache P., Hedrich R. (2004) The poplar K^+ channel KPT1 is associated with K^+ uptake during stomatal opening and bud development. Plant J 37, 828–838

Latham C.F., Lopez J.A., Hu S., Gee C.L., Westbury E., Blair D.H., Armishaw C.J., Alewood P.F., Bryant N.J., James D.E., Martin J.L. (2006) Molecular dissection of the Munc18c/syntaxin4 interaction: Implications for regulation of membrane trafficking. Traffic 7(10), 1408-1419

Laudert D., Weiler E.W. (1998) Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. Plant J. 15(5), 675-84

Lefebvre B., Batoko H., Duby G., Boutry M. (2004) Targeting of a *Nicotiana plumbaginifolia* H^+-ATPase to the plasma membrane is not by default and requires cytosolic structural determinants. Plant Cell 16, 1772–1789

Leung Y.M., Kang Y.H., Gao X.D., Xia F.Z., Xie H.L., Sheu L., Tsuk S., Lotan I., Tsushima R.G., Gaisano H.Y. (2003) Syntaxin 1A binds to the cytoplasmic C terminus of Kv2.1 to regulate channel gating and trafficking. J. Biol. Chem. 278, 17532–17538

Leung Y.M., Kwan E.P., Ng B., Kang Y., Gaisano H.Y. (2007) SNAREing voltage-gated K^+ and ATP-sensitive K^+ channels: tuning beta-cell excitability with syntaxin-1A and other exocytotic proteins. Endocr. Rev. 28(6), 653-63

Leyman B., Geelen D., Quintero F.J., Blatt M.R. (1999) A tobacco syntaxin with a role in hormonal control of guard cell ion channels. Science 283, 537-540

Li J., Lee Y.-R.J. Assmann S.M. (1998) Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel. Plant Physiol. 116, 785–795

Linial M. (1997) SNARE proteins--why so many, why so few?
J. Neurochem. 69(5), 1781-92

Lisenbee C.S., Karnik S.K., Trelease R.N. (2003) Overexpression and Mislocalization of a Tail-Anchored GFP Redefines the Identity of Peroxisomal ER. Traffic 4(7), 491-501

Loake G., Grant M. (2007) Salicylic acid in plant defence--the players and protagonists. Curr. Opin. Plant Biol. 10(5), 466-72

Lonart G., Sudhof T.C. (2000) Assembly of SNARE core complexes prior to neurotransmitter release sets the readily releasable pool of synaptic vesicles.
J Biol Chem. 275(36), 27703-7

Loseva N., Gordon L., Alyabyev A., Andreyeva I., Kolesnikov O., Chernov V., Ponomareva A., Kemp R.B. (2004) Effect of induced changes in membrane permeability on the defence response of *Chlorella vulgaris* to infection by *Acholeplasma laidlawii*. Thermochim. Acta 422, 95–100

Lukowitz W., Mayer U., Jurgens G. (1996) Cytokinesis in the Arabidopsis Embryo Involves the Syntaxin-Related KNOLLE Gene Product. Cell 84, 61-71

Malinska K., Malinsky J., Opekarova M., Tanner W. (2003) Visualization of protein compartmentation within the plasma membrane of living yeast cells. Mol. Biol. Cell 14, 4427–4436

Malinska K., Malinsky J., Opekarova M., Tanner W. (2004) Distribution of Can1p into stable domains reflects lateral protein segregation within the plasma membrane of living *S. cerevisiae* cells. J. Cell Sci. 117, 6031–6041

Marquardt D. (1963) An algorithm for fleast-squaires estimation of nonlinear parameters. J. Soc. Ind. Appl. Math. 11, 431–441

Marten I., Hoshi T. (1997) Voltage-dependent gating characteristics of the K^+ channel KAT1 depend on the N and C termini. Proceedings of the National Academy of Sciences, USA 94, 3448–3453

Martin S.W., Konopka J.B. (2004) Lipid raft polarization contributes to hyphal growth in *Candida albicans*. Eukaryot. Cell 3, 675–684

Maser P., Thomine S., Schroeder J.I., Ward J.M., Hirschi K., Sze H., Talke I.N., Amtmann A., Maathuis F.J., Sanders D., Harper J.F., Tchieu J., Gribskov M., Persans M.W., Salt D.E., Kim S.A., Guerinot M.L. (2001) Phylogenetic relationships within cation transporter families of arabidopsis. Plant Physiol 126(4), 1646-67

McNew J.A., Parlati F., Fukuda R., Johnston R.J., Paz K., Paumet F., Sollner T.H., Rothman J.E. (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature 407, 153-159

b) McNew J.A., Weber T., Parlati .F, Johnston R.J., Melia T.J., Sollner T.H., Rothman J.E. (2000) Close is not enough: SNARE-dependent membrane fusion requires an

active mechanism that transduces force to membrane anchors. J. Cell Biol. 50(1), 105-17

Meckel T., Hurst A.C., Thiel G., Homann U. (2004) Endocytosis against high turgor: intact guard cells of Vicia faba constitutively endocytose fluorescently labelled plasma membrane and GFP-tagged K-channel KAT1. Plant J. 39(2), 182-93.

Misura K.M., Scheller R.H., Weis W.I. (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature. 404, 355-362

Mochida S., Yokoyama C.T., Kim D.K., Itoh K., Catterall W.A. (1998) Evidence for a voltage-dependent enhancement of neurotransmitter release mediated via the synaptic protein interaction site of N-type Ca^{2+} channels. Proc. Natl. Acad. Sci. USA 95(24), 14523–14528

Mohapatra D.P., Vacher H., Trimmer J.S. (2007) The surprising catch of a voltage-gated potassium channel in a neuronal SNARE. Sci. STKE. 2007(393), 37

Mongrand S., Morel J., Laroche J., Claverol S., Carde J.P., Hartmann M.A., Bonneu M., Simon-Plas F., Lessire R., Bessoule J.J. (2004) Lipid rafts in higher plant cells: Purification and characterization of Triton X-100-insoluble microdomains from tobacco plasma membrane. J. Biol. Chem. 279, 36277–36286

Montecucco C., Schiavo G. (1995) Structure and function of tetanus and botulinum neurotoxins. Quarterly Reviews of Biophysics 28, 423-472

Morel J., Claverol S., Mongrand S., Furt F., Fromentin J., Bessoule J.J., Blein J.P., Simon-Plas F. (2006) Proteomics of plant detergent-resistant membranes. Mol. Cell Proteomics 5, 1396–1411

Mori I.C., Uozumi N., Muto S. (2000) Phosphorylation of the Inward-Rectifying Potassium Channel KAT1 by ABR Kinase in Vicia Guard Cells Plant Cell Physiol. 41(7), 850 – 856

Munro S. (2003) Lipid rafts: elusive or illusive? Cell 115, 377–388

Mur L.A., Kenton P., Atzorn R., Miersch O., Wasternack C. (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. Plant Physiol. 140(1), 249-62

Nakamura R.L., McKendree W.L. Jr., Hirsch R.E., Sedbrook J.C., Gaber R.F., Sussman M.R. (1995) Expression of an arabidopsis potassium channel gene in guard cells. Plant Physiol. 109, 371–374

Neiman A.M., Katz L., Brennwald P.J. (2000) Identification of domains required for developmentally regulated SNARE function in *Saccharomyces cerevisiae*. Genetics 155, 1643-1655

Nichols B.J., Pelham H.R. (1998) SNAREs and membrane fusion in the golgi apparatus. Biochim. Biophys. Acta. 1404(1-2), 9-31

Nonet M.L., Saifee O., Zhao H., Rand J.B., Wei L. (1998) Synaptic transmission deficits in Caenorhabditis elegans synaptobrevin mutants. J. Neurosci. 18, 70-80

Norton G., Pappusamy A., Yusof F., Pujade-Renaud V., Perkins M., Griffiths D., Jones H. (2007) Characterisation of recombinant Hevea brasiliensis allene oxide synthase: effects of cycloxygenase inhibitors, lipoxygenase inhibitors and salicylates on enzyme activity. Plant Physiol. Biochem. 45, 129–138

Papazian D.M., Timpe L.C., Jan Y.N., Jan L.Y. (1991) Alteration of voltage-dependence of Shaker potassium channel by mutations in the S4 sequence. Nature 349, 305-310

Park P. (2006) Ultrastructural analysis of cell responses of host cells to pathogen infection. J. Gen. Plant Pathol. 72(6), 404-407

Paschen S.A., Waizenegger T., Stan T., Preuss M., Cyrklaff M., Hell K., Rapaport D., Neupert W. (2003) Evolutionary conservation of biogenesis of β-barrel membrane proteins. Nature 426, 862-866

Paumet F., Rahimian V., Rothman J.E. (2004) The specificity of SNARE-dependent fusion is encoded in the SNARE motif. Proc. Natl. Acad. Sci. USA 101(10), 3376-3380

Pearson R.B., Kemp B.E. (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol. 200, 62-81

Peck S.C. (2003) Early phosphorylation events in biotic stress. Curr. Opin. Plant Biol. 6, 334-338

Pelham H.R.B. (1999) SNAREs and the secretory pathway - lessons from yeast. Experimental Cell Research 247(1), 1-8

Pena-Cortés H., Albrecht T., Prat S., Weiler E.W., Willmitzer L. (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta 191(1), 123-128

Persson S., Love J., Tsou P-L., Robertson D., Thompson W.F., Boss W.F. (2002) When a day makes a difference. Interpreting data from endoplasmic reticulum-targeted green fluorescent protein fusions in cells grown in suspension culture. Plant Physiol. 128, 341–344

Peskan T., Westermann M., Oelmüller R. (2000) Identification of low density Triton X-100-insoluble plasma membrane microdomains in higher plants Eur. J. Biochem. 267, 6989–6995

Phillips G.N. (1997) Structure and dynamics of green fluorescent protein. Curr. Opin. Struct. Biol. 7, 821–827

Pilot G., Lacombe B., Gaymard F., Chérel I., Boucherez J., Thibaud J-B., Sentenac H. (2001) Guard Cell Inward K^+ Channel Activity in arabidopsis Involves Expression of the Twin Channel Subunits KAT1 and KAT2. J. Biol. Chem. 276(5), 3215-3221

Pilot G., Pratelli R., Gaymard F., Meyer Y., Sentenac H. (2003) Five-group distribution of the Shaker-like K^+ channel family in higher plants. Journal of Molecular Evolution 56, 418–434

Poirier M.A., Xiao W., Macosko J.C., Chan C., Shin Y.K., Bennett M.K. (1998) The synaptic SNARE complex is a parallel four-stranded helical bundle. Nat. Struct. Biol. 5, 765-69

Pralle A., Keller P., Florin E.L., Simons K., Horber J.K.H. (2000) Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J. Cell Biol. 148, 997–1007

Pratelli R., Sutter J.U., Blatt M.R. (2004) A new catch in the SNARE. Trends Plant Sci. 9(4), 187-95.

Prekeris R., Yang B., Oorschot V., Klumperman J., Scheller R.H. (1999) Differential Roles of Syntaxin 7 and Syntaxin 8 in Endosomal Trafficking. Mol. Biol. Cell 10(11), 3891-3908

Rehman R.U., Stigliano E., Lycett G.W., Sticher L., Sbano F., Faraco M., Dalessandro G., Di Sansebastiano G.P. (2008) Tomato Rab11a characterization evidenced a difference between SYP121-dependent and SYP122-dependent exocytosis. Plant Cell Physiol. 49(5), 751-766

Ritchie K., Kusumi A. (2004) Role of the membrane skeleton in creation of microdomains. Subcellular Biochem. 37, 233-45

Rothman J.E. (1994) Intracellular membrane fusion. Adv. Second Messenger Phosphoprotein Res. 29, 81-96

b) Rothman J.E. (1994) Mechanisms of intracellular protein transport. Nature 372, 55–63

Rustérucci C., Aviv D.H., Holt B.F. 3rd, Dangl J.L., Parker J.E. (2001) The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in arabidopsis. Plant Cell. 13(10), 2211-24

Saifee O., Wei L., Nonet M.L. (1998) The Caenorhabditis elegans unc-64 locus encodes a syntaxin that interacts genetically with synaptobrevin. Mol. Biol. Cell 9, 1235-52

Salaün C., Gould G.W., Chamberlain L.H. (2005) The SNARE Proteins SNAP-25 and SNAP-23 Display Different Affinities for Lipid Rafts in PC12 Cells: Regulation by Distinct Cysteine-Rich Domains J. Biol. Chem., 280(2), 1236-1240

Sanderfoot A.A., Ahmed S.U., Marty-Mazars D., Rapoport I., Kirchhausen T., Marty F., Raikhel N.V. (1998) A putative vacuolar cargo receptor partially colocalizes with AtPEP12p on a prevacuolar compartment in arabidopsis roots. Proc. Natl. Acad. Sci. USA 95(17), 9920-5

Sanderfoot A.A., Assaad F.F., Raikhel N.V. (2000) The Arabidopsis Genome: an Abundance of Soluble N-ethylmaleimide Sensitive Factors. Plant Physiol. 121, 929-938

Sanderfoot A.A., Kovaleva V., Bassham D.C., Raikhel N.V. (2001) Interactions between syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the arabidopsis cell. Mol. Biol. Cell 12(12), 3733-43.

b) Sanderfoot A.A., Pilgrim M., Adam L., Raikhel N.V. (2001) Disruption of individual members of arabidopsis syntaxin gene families indicates each has essential functions. Plant Cell. 13(3), 659-66.

Sano H., Kane S., Sano E., Mîinea C.P., Asara J.M., Lane W.S., Garner C.W., Lienhard G.E. (2003) Insulin-stimulated phos-phorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J. Biol. Chem. 278, 14599-1460

Sato M.H., Nakamura N., Ohsumi Y., Kouchi H., Kondo M., Hara-Nishimura I., Nishimura M., Wada Y. (1997) The AtVAM3 encodes a syntaxin-related molecule implicated in the vacuolar assembly in *Arabidopsis thaliana*. J. Biol. Chem. 272(39), 24530-5.

Scales S., Chen Y., Yoo B., Patel S., Doung Y., Scheller R. (2000) SNAREs Contribute to the Specificity of Membrane Fusion. Neuron 26(2), 457-464

Schmelzer E. (2002) Cell polarization, a crucial process in fungal defence. Trends Plant Sci. 7, 411– 415.

Schmidt B., McCracken J., Ferguson-Miller S. (2003) A discreet water exit pathway in the membrane protein cytochrome *c* oxidase. Proc. Natl. Acad. Sci. USA 100(26), 15539–15542

Schroeder R., London E., Brown D. (1994) Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc. Natl. Acad. Sci. USA. 91(25), 12130-4

Shahollari B., Peskan-Berghöfer T., Oelmüller R. (2004) Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants. Physiol. Plant. 122, 397–403

Sherrier D.J., Prime T.A., Dupree P. (1999) Glycosylphosphatidylinositol-anchored cell-surface proteins from arabidopsis. Electrophoresis. 20, 2027-2035

Shirasu K., Nakajima H., Rajasekhar V.K., Dixon R.A., Lamb C. (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. Plant Cell. 9(2), 261-270

Simons K., Ikonen E. (1997) Functional rafts in cell membranes. Nature 387(6633), 569-72

Simons K., van Meer G. (1988) Lipid sorting in epithelial cells. Biochemistry. 27(17), 6197-202

Sollner T., Whiteheart S.W., Brunner M., Erdjument-Bromage H., Geromanos S., Tempst P., Rothman J.E. (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature. 362(6418), 318-24.

Stanley E.F., Mirotznik R.R. (1997) Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels. Nature 385, 340 - 343

Steinhardt R.A., Bi G., Alderton J.M. (1994) Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. Science 263(5145), 390-393

Stewart B.A., Mohtashami M., Zhou L., Trimble W.S., Boulianne G.L. (2001) SNARE dependant signalling at the drosophila wing margin. Developmental Biology 234, 13-23

Surpin M., Raikhel N. (2004) Traffic jams affect plant development and signal transduction. Nat. Rev. Mol. Cell Biol. 5, 100–109

Sutter J.U., Campanoni P., Blatt M.R., Paneque M. (2006) Setting SNAREs in a different wood. Traffic 7(6), 627-638

b) Sutter J.U., Campanoni P., Tyrrell M., Blatt M.R. (2006) Selective mobility and sensitivity to SNAREs is exhibited by the arabidopsis KAT1 K^+ channel at the plasma membrane. Plant Cell 18(4), 935-54.

Sutter J.U., Sieben C., Hartel A., Eisenach C., Thiel G., Blatt M.R. (2007) Abscisic Acid Triggers the Endocytosis of the Arabidopsis KAT1 K^+ Channel and Its Recycling to the Plasma Membrane. Current Biology 17(16), 1396-1402

Sutton R.B., Fasshauer D., Jahn R., Brunger A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. Nature. 395(6700), 347-53

Suwastika I.N., Uemura T., Shiina T., Sato N.H., Takeyasu K. (2008) SYP71, a Plant-specific Qc-SNARE Protein, Reveals Dual Localization to the Plasma Membrane and the Endoplasmic Reticulum in Arabidopsis. Cell Structure and Function 33(2), 185-192

Sweeney S.T., Broadie K., Keane J., Niemann H., O'Kane C.J. (1995) Targeted expression of tetanus toxin light chain in drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14, 341-51

Tang X.D., Hoshi T. (1999) Rundown of the Hyperpolarization-Activated KAT1 Channel Involves Slowing of the Opening Transitions Regulated by Phosphorylation. Biophys. J. 76(6), 3089 – 3098

ter Beest M.B.A., Chapin S.J., Avrahami D., Mostov K.E. (2005) The role of syntaxins in the specificity of vesicle targeting in polarized epithelial cells. Mol. Biol. Cell. 16, 5784–5792.

Thomas C.L., Maule A.J. (2000) Limitations on the use of fused green fluorescent protein to investigate structure-function relationships for the cauliflower mosaic virus movement protein. J. Gen. Virol. 81(7), 1851-5

Tishgarten T., Yin F.F., Faucher K.M., Dluhy R.A., Grant T.R., Fischer von Mollard G., Stevens T.H., Lipscomb L.A. (1999) Structures of yeast vesicle trafficking proteins. Protein Sci. 8(11), 2465-73.

Tsui M.M.K., Tai W.C.S., Banfield D.K. (2001) Selective formation of Sed5p-containing SNARE complexes is mediated by combinatorial binding interactions. Mol. Biol. Cell 12(3), 521-538

Tyrrell M., Campanoni P., Sutter J.U., Pratelli R., Paneque M., Sokolovski S., Blatt M.R. (2007) Selective targeting of plasma membrane and tonoplast traffic by inhibitory (dominant-negative) SNARE fragments. Plant J. 51(6), 1099-1115

Uemura T., Ueda T., Ohniwa R.L., Nakano A., Takeyasu K., Sato M.H. (2004) Systematic analysis of SNARE molecules in arabidopsis: dissection of the post-Golgi network in plant cells. Cell Struct. Funct. 29(2), 49-65

Uknes S., Mauch-Mani B., Moyer M., Potter S., Williams S., Dincher S., Chandler D., Slusarenko A., Ward E., Ryals J. (1992) Acquired resistance in arabidopsis. Plant Cell 4, 645-656

Van Loon L.C., van Strien E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55, 85-97.

Varma R., Mayor S. (1998) GPI-anchored proteins are organized in submicron domains at the cell surface. Nature 394, 798–801

Véry A.A., Gaymard F., Bosseux C., Sentenac H., Thibaud J.B. (1995) Expression of a cloned plant K+ channel in Xenopus oocytes: analysis of macroscopic currents. Plant J. 7(2), 321-32.

Vidal S., Eriksson A.R.B., Montesano M., Denecke J., Palva E.T. (1998) Cell Wall-Degrading Enzymes from *Erwinia carotovora* Cooperate in the Salicylic Acid-Independent Induction of a Plant Defense Response. MPMI 11(1), 23-32

Vieten A., Sauer M., Brewer P.B., Friml J. (2007) Molecular and cellular aspects of auxin-transport-mediated development. Trends Plant Sci. 12, 160–168

Weber T., Zemelman B.V., McNew J.A., Westermann B., Gmachl M., Parlati F., Söllner T.H., Rothman J.E. (1998) SNAREpins: minimal machinery for membrane fusion. Cell. 92, 759–772

Weidman P.J., Melancon P., Block M.R., Rothman J.E. (1989) Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. J. Cell Biol. 108(5), 1589-96.

Wick P., Gansel X., Oulevey C., Page V., Studer I., Durst M., Sticher L. (2003) The expression of the t-SNARE At SNAP33 is induced by pathogens and mechanical stimulation. Plant Physiology 132(1), 343 – 351

Whiteheart S.W., Brunner M., Wilson D.W., Wiedmann M., Rothman J.E. (1992) Soluble N-ethylmaleimide-sensitive fusion attachment proteins (SNAPs) bind to a multi-SNAP receptor complex in Golgi membranes. J. Biol. Chem. 267(17), 12239-43

Wu G., Shortt B.J., Lawrence E.B., Leon J., Fitzsimmons K.C., Levine E.B., Raskin I., Shah D.M. (1997) Activation of Host Defense Mechanisms by Elevated Production of H2O2 in Transgenic Plants. Plant Physiol. 115(2), 427-435

Yalpani N., Silverman P., Wilson T.M., Kleier D.A., Raskin I. (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell 3(8), 809-818

Yamakawa T., Saith S., Li Y., Gao X., Gaisano H.Y., Tsushima R.G. (2007) Interaction of syntaxin 1A with the N-terminus of Kv4.2 modulates channel surface expression and gating. Biochemistry 46(38), 10942-9

Yang B., Gonzalez L. Jr., Prekeris R., Steegmaier M., Advani R.J., Scheller R.H. (1999) SNARE interactions are not selective. Implications for membrane fusion specificity. J Biol. Chem. 274(9), 5649-5653

Yano D., Sato M., Saito C., Sato M.H., Morita M.T., Tasaka M. (2003) A SNARE complex containing SGR3/AtVAM3 and ZIG/VTI11 in gravity-sensing cells is important for arabidopsis shoot gravitropism. Proc. Natl. Acad. Sci. USA 100, 8589–8594

Zeigerer A., Lampson M.A., Karylowski O., Sabatini D.D., Adesnik M., Ren M., McGraw T.E. (2002) GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. Mol. Biol. Cell 13(7), 2421-35

Zhang Z., Feechan A., Pedersen C., Newman M.A., Qui J.L., Olesen K.L., Thordal-Christensen H. (2007) A SNARE-protein has opposing functions in penetration resistance and defence signalling pathways. Plant J. 49(2), 302-12

Zhao J., Davis L.C., Verpoorte R. (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. 23, 283-333

Zheng H., Fischer von Mollard G., Kovaleva V., Stevens T.H., Raikhel N.V. (1999) The plant v-SNARE AtVTI1a likely mediates vesicle transport from the TGN to the prevacuole. Mol. Biol. Cell 10, 2251–2264

Zimmermann S., Nürnberger T., Frachisse J.M., Wirtz W., Guern J., Hedrich R., Scheel D. (2007) Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defence. Proc. Natl. Acad. Sci. USA 94(6), 2751-5