A Novel Motif Essential for SNARE Interaction with the K+ Channel KC1 and Channel Gating in Arabidopsis

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The SNARE (for soluble N-ethylmaleimide-sensitive factor protein attachment protein receptor) protein SYP121 (=SYR1/PEN1) of Arabidopsis thaliana facilitates vesicle traffic, delivering ion channels and other cargo to the plasma membrane, and contributing to plant cell expansion and defense. Recently, we reported that SYP121 also interacts directly with the K+ channel subunit KC1 and forms a tripartite complex with a second K+ channel subunit, AKT1, to control channel gating and K+ transport. Here, we report isolating a minimal sequence motif of SYP121 prerequisite for its interaction with KC1. We made use of yeast mating-based split-ubiquitin and in vivo bimolecular fluorescence complementation assays for protein–protein interaction and of expression and electrophysiological analysis. The results show that interaction of SYP121 with KC1 is associated with a novel FxRF motif uniquely situated within the first 12 residues of the SNARE sequence, that this motif is the minimal requirement for SNARE-dependent alterations in K+ channel gating when heterologously expressed, and that rescue of KC1-associated K+ current of the root epidermis in syp121 mutant Arabidopsis plants depends on expression of SNARE constructs incorporating this motif. These results establish the FxRF sequence as a previously unidentified motif required for SNARE-ion channel interactions and lead us to suggest a mechanistic framework for understanding the coordination of vesicle traffic with transmembrane ion transport.

INTRODUCTION

The superfamily of SNARE (for soluble N-ethylmaleimide–sensitive factor protein attachment protein receptor) proteins, found in all eukaryotes, is essential in later stages of vesicle targeting and fusion. They help match vesicles with their target membranes for delivery of specific membrane proteins and cargo, and they overcome the dehydration forces associated with lipid bilayer fusion in an aqueous environment. These processes sustain cellular homeostasis in yeast (Ungar and Hughson, 2003), they are essential for synaptic transmission between nerves (Jahn et al., 2003), and they underpin growth and development in plants (Campanoni and Blatt, 2007; Grefen and Blatt, 2008). Complementary SNAREs, located at the vesicle and target membranes, interact to draw the membrane surfaces together for fusion. SNAREs comprise the minimal set of proteins required to accelerate fusion in reconstituted vesicle preparations (Weber et al., 1998; Parlati et al., 1999; Hu et al., 2003), although other components, including the N-ethylmaleimide–sensitive factor, Sec1, Munc18, and their homologs (Burgoine and Morgan, 2007; Sudhof and Rothman, 2009), affect SNARE conformations and their interactions (Brunger, 2005; Lipka et al., 2007; Bassham and Blatt, 2008).

In plants, SNAREs play important roles in vesicle traffic associated with defense against fungal pathogens (Collins et al., 2003; Pajonk et al., 2008) and the response to bacterial elicitors (Robatzek et al., 2006). In addition, they contribute to events beyond their canonical roles in membrane targeting and vesicle fusion (Grefen and Blatt, 2008). The vacuolar SNAREs SYT22 and VTI11, for example, have surfaced as components essential for gravitropism (Kato et al., 2002; Surpin et al., 2003; Yano et al., 2003), plausibly linking sensory processing to vacuolar membrane structure or composition (Saito et al., 2005). A few SNAREs are known also to interact with ion channels and affect their regulation. In neurovascular and neuroendocrine tissues, binding of the SNARE Syntaxin 1A to K+ and Ca2+ channels is thought to facilitate neurotransmission and hormone secretion (Leung et al., 2007). In the model plants tobacco (Nicotiana tabacum) and Arabidopsis thaliana, the SNARE SYP121 (=SYR1/PEN1) has been implicated in hormonal regulation of Ca2+, Cl−, and K+ channels in guard cells (Leyman et al., 1999; Sokolovski et al., 2008), in the latter case independent of its role in delivery and recycling of the ion channel proteins (Sutter et al., 2006, 2007).

Recently, we reported that SYP121 of Arabidopsis, originally identified with its tobacco homolog in a screen for signaling elements associated with abscisic acid and drought (Leyman et al., 1999; Geelen et al., 2002), interacts directly with the regulatory K+ channel subunit KC1 and forms a tripartite complex with a second K+ channel subunit, AKT1 (Honsbein et al., 2009). KC1 interaction proved highly selective for SYP121. We found the SYP121-KC1-AKT1 complex to be required for the activity of inward-rectifying K+ currents at the plasma membrane of root epidermal cells and for K+ nutrient acquisition and growth when channel-mediated K+ uptake was limiting. These results...
demonstrated an unexpected role for the SNARE analogous to SNARE-ion channel complexes of mammals but unrelated to signaling or its coupling to vesicle traffic. Thus, they raised fundamental questions about the mechanics of interaction, notably about the relationship of K⁺ channel binding to the domains required for SNARE core complex assembly that drive vesicle fusion. We have since identified the minimal sequence motif harbored by SYP121 and prerequisite for its interaction with KC1. We report here that the motif is unique to SYP121 and localized to a region of canonical SNARE structure not previously associated with ion channel interactions. The results show that the motif is essential for the interaction of SYP121 with KC1, and it is necessary for the SNARE to facilitate gating in the K⁺ channel when expressed heterologously and in the plant. Significantly, the identity and position of the motif within the primary SNARE sequence points to a novel mechanism for mutual control of SYP121-dependent membrane vesicle traffic and of the K⁺ channels. Thus, the findings lead us to suggest a novel framework for understanding the coordination of vesicle traffic with transmembrane ion transport.

RESULTS

SYP121/SYP122 Chimeras Identify the N Terminus of SYP121 as Essential for K⁺ Channel Interaction

We made use of a mating-based split-ubiquitin assay for interacting proteins (Grefen et al., 2007, 2009) employed previously to identify SYP121 interaction with KC1 and their formation of a tripartite complex with AKT1 (Honsbein et al., 2009). The mating-based assay gives Met-sensitive rescue of yeast growth on minimal medium. Because the assay relies on reassembly of the N- and C-terminal halves (Nub and Cub) of the ubiquitin moiety and cleavage of a LexA-VP16 transactivator, which then migrates to the nucleus, it permits work with full-length, integral membrane proteins. Our previous studies showed that KC1 bound selectively with SYP121 and not with SYP122, the closest homolog to SYP121, with 64% amino acid sequence identity with which it shares partial functional redundancy in vivo (Assaad et al., 2004; Zhang et al., 2007; Bassham and Blatt, 2008). To identify the binding domain responsible for SYP121 interaction, we therefore made initial use of complementary sets of constructs to generate chimeric proteins in which segments of the chimeric SNAREs corresponded to different combinations of the SYP121 or SYP122 residue sequences.

Figure 1 shows the yeast mating-based split-ubiquitin assay and supporting immunoblot data from one of three independent experiments, each of which yielded equivalent results. Like other Qa-SNAREs (Lipka et al., 2007; Grefen and Blatt, 2008; Sudhof and Rothman, 2009), both SYP121 and SYP122 are type-II integral membrane proteins comprising a C-terminal membrane anchor, an H3 coiled-coil domain that binds with its cognate SNARE partners to drive vesicle fusion, and an N-terminal set of coiled-coil domains (Ha, Hb, Hc) that folds back on the H3 domain and regulates its accessibility for binding. For purposes of preliminary analysis, we divided the SNAREs between four regions: N corresponding to the N-terminal 39–amino acid residues, the Ha-Hb-Hc α-helices, the H3 or Qαα-helix, and the transmembrane α-helix and C-terminal extension, respectively. Segment alignments (above) for the two SNAREs at the junction points are shown with arrows indicating the domain breaks. Yeast diploids created with NubG fusion constructs of each chimera (left) and with SYP121 (in gray font) and SYP122 (in black font) for reference together with controls (negative, NubG; positive, wild-type Nub) spotted (left to right) on CSM medium without Trp, Leu, and uracil (CSMwlu) to verify mating, CSM without Trp, Leu, uracil, adenine, His, and Met (CSMwluahm) to verify adenine- and His-independent growth (second panel), and on CSMwluahm with the addition of 0.2 mM Met to verify interaction at lower KC1-Cub expression levels (Obrdlik et al., 2004; Grefen et al., 2009). Diploid yeast was dropped at 1.0 and 0.1 OD 600 in each case. Immunoblot analysis (5 μg total protein/lane) of the haploid yeast used for mating is included (right) using commercial VP16 antibody for KC1 and both SYP121 and SYP122 polyclonal antibodies for the SNARE chimeras, the latter showing association of the principle epitopes with the Ha-Hb-Hc domains of the SNAREs.

Figure 1. SNARE Interaction with the KC1 K⁺ Channel Depends on the Presence of the N Terminus of SYP121.

Yeast mating-based split-ubiquitin assay for interaction of the SNARE chimeras with KC1-Cub as the bait. SNARE chimeras of SYP121 and SYP122 were constructed by exchange of four domains designated N, H, Q, and C, indicated above, corresponding to the N-terminal 39–amino acid residues, the Ha, Hb, and Hc α-helices, the H3 or Qαα-helix, and the transmembrane α-helix and C-terminal extension, respectively. Segment alignments (above) for the two SNAREs at the junction points are shown with arrows indicating the domain breaks. Yeast diploids created with NubG fusion constructs of each chimera (left) and with SYP121 (in gray font) and SYP122 (in black font) for reference together with controls (negative, NubG; positive, wild-type Nub) spotted (left to right) on CSM medium without Trp, Leu, and uracil (CSMwlu) to verify mating, CSM without Trp, Leu, uracil, adenine, His, and Met (CSMwluahm) to verify adenine- and His-independent growth (second panel), and on CSMwluahm with the addition of 0.2 mM Met to verify interaction at lower KC1-Cub expression levels (Obrdlik et al., 2004; Grefen et al., 2009). Diploid yeast was dropped at 1.0 and 0.1 OD 600 in each case. Immunoblot analysis (5 μg total protein/lane) of the haploid yeast used for mating is included (right) using commercial VP16 antibody for KC1 and both SYP121 and SYP122 polyclonal antibodies for the SNARE chimeras, the latter showing association of the principle epitopes with the Ha-Hb-Hc domains of the SNAREs.
residues preceding the Ha α-helix; H corresponding to the Ha, Hb, and Hc α-helices (residues 40 to 192); Q (residues 193 to 283) corresponding to the H3 α-helix terminating with the conserved Thr residue at position 283; and C corresponding to the remaining C-terminal sequence (residues 284 to 346) that incorporated the transmembrane α-helix and an extended stretch of residues that are predicted to reside outside the cell (Blatt et al., 1999; Leyman et al., 1999). As before (Honsbein et al., 2009), a readout of interaction was evidenced by growth of diploid yeast on selective media when they expressed both the KC1-Cub and Nub-SYP121 fusion proteins, and growth was retained in the presence of 0.2 mM Met to repress transcription of the KC1-Cub bait construct. Little or no growth was recovered on selective media and in the presence of Met when yeast carrying KC1-Cub and Nub-SYP122 were mated. Among the SYP121-SYP122 chimeras, growth was recovered in all matings incorporating fusion constructs with the N domain of SYP121, even when the H, Q, and C domains were derived from SYP122, and substitution with the SYP122 N domain virtually eliminated growth when mated with yeast carrying the KC1-Cub bait. Immunoblot analysis in every case showed expression of the chimeric SNAREs, although, as expected, the efficacy of the polyclonal antibodies depended on the relative distribution of epitopes between the two SNARE sequences in the chimeras. Expression of KC1 was verified by rescue of diploid yeast growth with the wild-type Nub and by immunoblots for the VP16 epitope of the fusion protein in the THY.AP4 yeast prior to mating. Thus, we concluded that the N-terminal 39 amino acids harbor a motif that is both sufficient and necessary for SNARE interaction with the KC1 K⁺ channel.

Alignment of SYP121 and SYP122 shows that the N-terminal amino acid sequences of the SNAREs diverge principally in three short segments of 4 to 10 residues each, denoted in the lowercase suffixes n1, n2, and n3 (Figure 2). We used these segments as the basis for constructing a second round of chimeras. In the first instance, we substituted the n1, n2, and n3 segments from SYP122 into the corresponding positions in the backbone of the Nub-SYP121 fusion construct. In a second set of experiments, we used as a backbone the fusion construct comprising the N domain of SYP121 and the H, Q, and C domains of SYP122 that retained the capacity to rescue yeast growth in conjunction with KC1-Cub (Figure 1). Figure 2 shows the readout from experiments using each of these backbones along with supporting immunoblot analyses. In both cases, an interaction with KC1 was indicated for constructs that incorporated the n1 segment of SYP121 by the rescue of yeast growth on selective media and in the presence of Met, whereas substitutions with the n1 segment of SYP122 showed a loss of yeast growth. Substitutions with either the n2 or n3 segment from SYP122 had no appreciable effect on the rescue of yeast growth. In short, these results indicated that residues within the N-terminal 20 amino acids were essential for SYP121 interaction with KC1.

The SYP121 N Terminus Is Essential for K⁺ Channel Gating When Heterologously Expressed

KC1 is a so-called silent K⁺ channel subunit; expressed on its own, it does not yield measurable K⁺ currents, but it interacts with different inward-rectifying K⁺ channel subunits including...
heterologously on its own or with KC1, AKT1 does yield an inward-rectifying K+ current (Gaymard et al., 1996; Duby et al., 2008). However, the gating of channels assembled as heteromers of AKT1 and KC1, like that of the homomeric AKT1 channels, differs fundamentally from that of the K+ channels in vivo, a difference evident in the midpoint for channel activation ($V_{1/2}$) and sensitivity to a change in voltage (the gating charge, $\delta$) (Dreyer and Blatt, 2009), unless coexpressed with SYP121 (Honsbein et al., 2009).

We used these characteristics to explore the functional consequences of selected SYP121 chimeras identified by the split-ubiquitin experiments. Electrophysiological recordings were performed under voltage clamp after coexpressing KC1 and AKT1 together with the SYP121 mutants in *Xenopus laevis* oocytes and verifying expression of the SNAREs. To ensure activation of AKT1 in the oocytes, all combinations of channel subunits and SNAREs were coexpressed with the protein kinase CIPK23 and calcineurin-like activator CBL1 (Li and Luan, 2006; Xu et al., 2006). We extracted the gating characteristics $V_{1/2}$ and $\delta$ in each case by jointly fitting the steady state K+ currents to a Boltzmann function of the form

$$I_K = g_{\text{max}} (V - E_K) / \left(1 + e^{(V - V_{1/2})/R T}\right)$$

where $g_{\text{max}}$ is the conductance maximum, $V$ is the membrane voltage, $E_K$ is the equilibrium voltage for K+, and $R$ and $T$ have their usual meanings.

As before (Honsbein et al., 2009), we found that expressed alone, AKT1 yielded an anomalous K+ current measurable at voltages negative of $\sim 50$ mV; expressing AKT1 together with KC1, with or without (data not shown) the noninteracting SNARE SYP122, gave a K+ current measurable only at voltages negative of $\sim 140$ mV (Figure 3). To avoid substantial indetermination in fitted parameters obtained from these data and from subsequent analyses (see also Figures 11 to 14), we applied standard methods of joint fittings with key parameters held in common between data sets (curves) (Press et al., 1992) and introduced the minimal assumption of parameters for known (control) data sets consistent with previous analyses and observations that KC1 coexpression does not affect significantly the saturation current or gating charge of the K+ channels, only the value for $V_{1/2}$ (Duby et al., 2008; Honsbein et al., 2009). On analysis, these currents were well-fitted jointly to the Boltzmann function in every case with a gating charge, $\delta$, near a value of unity and with only $V_{1/2}$ differing between the two circumstances (Figures 3 and 4). Fittings of currents were obtained with $g_{\text{max}}$ held in common ($g_{\text{max}}$ for the data shown, 48 ± 9 nS) and with separate, joint values for $\delta$ between SNARE chimeras that showed interaction with KC1 and those that did not (see Figures 1 and 2). Similar results were obtained in each of four independent experiments (>24 oocytes for each set of constructs). Both analysis and visual inspection showed an increase in $\delta$ and shift in $V_{1/2}$ on inclusion of SYP121 and the interacting chimeras with AKT1 and KC1.

### Verifiction of SNARE protein expression

Immunoblot analysis of total membrane protein (10 μg/lane) extracted from oocytes collected after electrical recordings and detected with αSYP121 and αSYP122 antibodies (Tyrrell et al., 2007; Honsbein et al., 2009).
recorded on coexpression of KC1 with AKT1 generally yielded $V_{1/2}$ values near the limit of clamp voltages achievable in oocytes. Coexpressing AKT1 and KC1 with wild-type SYP121 that interacts with KC1 (Figure 1; see also Honsbein et al., 2009), by contrast, yielded a K$^+$ current at voltages negative of $-100$ mV that was well-fitted to the same Boltzmann function, but with values for $\delta$ near 2 and $V_{1/2}$ close to $-150$ mV (Figures 3 and 4). These characteristics were similar to those reported previously on heterologous expression in oocytes and Sf9 insect cells and compare favorably with the characteristics of K$^+$ currents obtained in vivo (Gassmann and Schroeder, 1994; White and Lermtrichlieh, 1995; Buschmann et al., 2000; Honsbein et al., 2009).

We found a similar and strong divergence of gating characteristics that paralleled the SNARE–KC1 interactions when comparing the gating parameters of the currents recorded with the SNARE chimeras (Figures 3 and 4). Coexpressing AKT1 and KC1 with the SYP121-SYP122 chimera N1HQC2 yielded K$^+$ currents with $V_{1/2}$ and $\delta$ values similar to those associated with the wild-type SYP121, whereas coexpressing AKT1 and KC1 with the N2HQC1 chimera gave current characteristics that aligned closely with those obtained on coexpressing AKT1 and KC1 alone. Much the same separation of gating characteristics was observed on expressing the SYP121 incorporating the n1 segment substitution from SYP122. In this case, values for $V_{1/2}$ and $\delta$ aligned with those derived from currents on expressing AKT1 with KC1 alone or with SYP122. Thus, analysis of channel gating yielded unequivocal evidence of the functional requirement for the SYP121 N terminus to affect gating of the K$^+$ channels and paralleled the results of the yeast mating-based split-ubiquitin screen.

The SYP121 N Terminus Determines SYP121–KC1 Interaction in Vivo

Interactions on heterologous expression in yeast and in *Xenopus* oocytes do not rule out the possibility that additional components unique to the plant might be important in stabilizing or directing other domain interactions between SYP121 and KC1. We therefore made use of a bimolecular fluorescence complementation (BiFC) assay (Walter et al., 2004) to test these interactions in vivo. Constructs incorporating the open reading frames for KC1, SYP121, SYP122, and selected SYP121-SYP122 chimeras fused to the N- and C-terminal halves of yellow fluorescent protein (YFP) were used to transform *Agrobacterium tumefaciens* and were transiently expressed in *Arabidopsis* root epidermis by cocultivation (Grefen et al., 2010). We transformed both the wild type and the *syp121* (=*pen1-1/syp121-1*) mutant *Arabidopsis* as a check against possible interference by higher levels of expression of the native SNARE. The *syp121* mutation introduces a premature stop codon within the coding sequence of the gene that results in a loss of SNARE protein expression (Zhang et al., 2007; Pajonk et al., 2008). Because plant ion channels generally express at levels too low for detection by fluorescence microscopy, expression was driven by the constitutive *Arabidopsis Ubiquitin-10* gene promoter (Grefen et al., 2010). There is a potential for mistargeting when a protein is overexpressed. Nonetheless, ion channel distributions, and that of most SNAREs, generally align with the native

![Figure 4. Coexpression with SYP121-SYP122 Chimeras That Interact with KC1 Selectively Rescue the Gating Parameters Associated with SYP121 and the AKT1-KC1 K$^+$ Current in Xenopus Oocytes.](#)

Summary of K$^+$ channel gating parameters of gating charge (A) and $V_{1/2}$ (B) recorded from oocytes expressing AKT1 with KC1 in combinations with SYP121, SYP122, and their chimeras. Data are means ± SE obtained from joint fittings to Equation 1 of four or more independent data sets in each case, including the data of Figure 3. Fittings performed by nonlinear least squares minimization (Marquardt, 1963) with the conductance maximum $g_{\text{max}}$ held in common within each data set and the apparent gating charge ($\delta$) allowed to vary between SNAREs and chimeras that interact with KC1 and those that do not. Data for SYP121 and the chimera N1HQC2 are statistically different from the other data sets with $P < 0.01$. 

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protein in vivo when driven by the constitutive promoter (Uemura et al., 2004; Sutter et al., 2006, 2007; Grefen et al., 2010).

We used confocal laser scanning microscopy to quantify and compare fluorescence signals and their distributions obtained on expressing the KC1-cYFP fusion with different combinations of SNARE fusions. Confocal stacks were used to derive three-dimensional image projections, and these images were then analyzed for YFP fluorescence intensity after background subtraction. As before (Honsbein et al., 2009), we found pronounced YFP fluorescence over background in Arabidopsis epidermal cells, both in wild-type and syp121 plants, when transformed with complementary BiFC constructs fused to KC1 and to the wild-type SYP121, but not to SYP122 (Figures 5 and 6; see Supplemental Movies 1 to 3 online). In each of three, independent experiments, cotransformations of KC1-nYFP with the SYP121-SYP122 chimera N1HQC2 fused to cYFP yielded a fluorescence signal comparable with that of the wild-type SYP121-cYFP fusion; cotransformations of KC1-nYFP with cYFP fusions of the N2HQC1 chimera resulted in fluorescence signals close to background, although expression of the fusion constructs in these instances was confirmed by immunoblot analysis (see Figure 10). In principle, BiFC could yield false-positive interactions as a consequence of overexpression. However, moderate expression driven by the Ubiquitin-10 promoter (Grefen et al., 2010) and the absence of an interaction, among others with the close homolog of SYP121 and the noninteracting SYP121-SYP122 chimeras, militates against this idea. Additionally, we found the YFP fluorescence was restricted to the cell periphery and failed to recover after local photobleaching (Figure 7; see Supplemental Movie 4 online), indicating that, like the KC1 complex with wild-type SYP121, interacting assemblies with the SYP121 mutants were not mobile within the cytosol or within a circulating endomembrane compartment. Thus, we conclude that the N terminus of SYP121 is a primary determinant of the physical interaction between KC1 and SYP121 in vivo.
The FxRF Residues Define a Minimal Motif for SYP121–KC1 Interaction

To identify the residues critical for KC1 interaction, we sequentially mutated each of the eight amino acids between Ser-10 and Pro-17, corresponding to the n1 domain, of the wild-type Nub-SYP121 fusion to Ala before retransforming yeast with the site-mutated construct for mating and interaction analysis with KC1-Cub. Immediately preceding the N terminus of the n1 segment, the Phe at position 9 is highly conserved among Qa-SNAREs, not only those of Arabidopsis and other plants but also of yeast and mammals (see Figure 15 and Discussion). Therefore, we extended the analysis to include the Phe residue at position 9. Figure 8 summarizes the readouts for KC1–Cub interaction with these Nub-SYP121 single-site mutants and the corresponding immunoblot analysis verifying expression of the SNARE and KC1 fusion proteins. Of the nine SNARE mutants, yeast growth was lost following site substitutions with Ala at Phe-9, Arg-11, and Phe-12, indicating that substitutions at each of these residues interfered with the interaction between the SNARE and KC1. Significantly, an equivalent substitution of Arg-13 had no effect on yeast growth, nor was growth suppressed following replacements affecting the peptide backbone angle and negative charge with P17A and E16A, respectively.

To assess the requirement for the FxRF motif in vivo, we made use of BiFC as before by transient transformation with A. tumefaciens. Again, no differences in BiFC signal were observed in syp121 mutant Arabidopsis compared with the wild type, and these data were therefore pooled. Fluorescence signals were quantified by confocal laser scanning microscopy and their distributions obtained on expressing the KC1-nYFP fusion with different combinations of SNARE fusions was compared. The results (Figures 9 and 10) showed a pronounced YFP fluorescence over background in Arabidopsis epidermal cells when transformed with complementary BiFC constructs fused to KC1 and to the wild-type SYP121, but not to SYP122. In each of three independent experiments, cotransformations of KC1-nYFP with the SYP121-S10A mutant fused to cYFP yielded a fluorescence signal comparable with that of the wild-type SYP121-cYFP fusion, but cotransformations with cYFP fusions of the other SYP121 site mutants resulted in fluorescence signals that were statistically indistinguishable from background. Expression of the fusion constructs in each case was confirmed by immunoblot analysis. Thus, we conclude that the FxRF motif of SYP121 is a primary determinant of the physical interaction between KC1 and SYP121 in vivo.

The FxRF Motif Determines SNARE Control of Channel Gating and K+ Current Rescue in the Plant

We used the Ala substitution mutants in each of the four residues from F9 to F12 of SYP121 to assess their functional impact on K+ channel gating, quantified using the parameters of $V_{1/2}$ and the gating charge $\gamma$. As before, electrophysiological recordings were performed under voltage clamp after coexpressing KC1 and AKT1 together with the SYP121 mutants in Xenopus oocytes, including the protein kinase CIPK23 and calcineurin-like activator CBL1 (Li and Luan, 2006; Xu et al., 2006), and gating

![Figure 7. The Interacting Complex of SYP121-SYP122 Chimera N1HQC2 with the K+ Channel KC1 Is Localized to the Cell Periphery and Is Nonmobile.](image)

Fluorescence recovery after photobleaching in Arabidopsis syp121 mutant root epidermis expressing N1HQC2-cYFP and KC1-nYFP to yield a BiFC signal.

(A) Bright-field (left column), composite (middle column), and YFP fluorescence (right column) images taken at time points before and after local photobleaching (area indicated by dotted rectangle, center row, left) of the fluorophore. Images collected at times indicated in each frame relative to the time of photobleach. Bar = 10 μm. See Supplemental Movie 4 online for the complete time series.

(B) Fluorescence recovery after photobleaching analysis of the fluorescence signal taken from the regions indicated (inset) after normalization and correction for fluorescence decay. Solid curves are the results of nonlinear least squares fitting of the postbleach fluorescence signals to single exponential functions yielding an immobile fraction of 0.94. Similar results were obtained in each of four separate experiments and with BiFC fluorescence signals obtained with SYP121n2-cYFP and wild-type SYP121-cYFP paired with KC1-nYFP (data not shown; see Honsbein et al., 2009) yielding a mean immobile fraction of 0.92 ± 0.04.
characteristics were extracted from steady state K⁺ current–voltage curves by jointly fitting to a Boltzmann function (Equation 1). As with the SYP121-SYP122 chimeras, we found a strong divergence in gating characteristics of the SYP121 site mutants that paralleled their ability to interact with KC1. When expressed together with AKT1 and KC1, the SYP121-S10A mutant yielded a K⁺ current similar to that of the wild-type SNARE, showing an appreciable steady state current amplitude at voltages negative of −100 mV. Fitted to the Boltzmann function, these data gave values for $\delta$ near 2 and $V_{1/2}$ close to −150 mV (Figures 11 and 12), statistically equivalent to those for the wild-type SNARE. By contrast, coexpression with the SYP121 site mutants F9A, R11A, and F12A each yielded K⁺ currents similar to those recorded on expressing AKT1 and KC1 alone and gave corresponding values for $V_{1/2}$ negative of −200 mV and $\delta$ values close to unity. Analysis

Figure 8. SYP121 Interaction with the KC1 K⁺ Channel Depends on Residues Phe-9, Arg-11, and Phe-12.

Yeast mating-based split-ubiquitin assay for interaction between the KC1-Cub bait and Nub-SYP121 prey carrying substitutions with Ala at the sites indicated (left) and with controls (negative, NubG; positive, wild-type Nub [NubWt]) and with wild-type SYP121 and SYP122 for reference. The N-terminal amino acid sequences of SYP121 and SYP122 included with the n1 segment indicated (above) for reference. Diploid yeast spotted (left to right) on CSM medium without Trp, Leu, and uracil (CSMwlu) to verify crossing, CSM without Trp, Leu, uracil, adenine, His, and Met (CSMwluahm) to verify adenine- and His-independent growth, and with the addition of 0.2 mM Met to verify interaction on suppressing KC1-Cub expression levels (Obrdlik et al., 2004; Grefen et al., 2009). Diploid yeast was dropped at 1.0 and 0.1 OD₆₀₀ in each case. Immunoblot analysis (5 μg total protein/lane) of the haploid yeast used for mating is included on the right in each case using commercial VP16 antibody for KC1 and SYP121 polyclonal antibodies for the SNARE mutants. Note the presence of growth under Met suppression for the SYP121 site mutants S10A, R13A, S14A, G15A, E16A, and P17A.

Figure 9. Interaction of KC1 with SYP121 in Vivo Depends on Residues of the FxRF Motif of SNARE.

BiFC analysis of KC1 association with SYP121 site mutants expressed in Arabidopsis root epidermis as fusion constructs with the N- and C-terminal halves of YFP (nYFP and cYFP), respectively. Similar results obtained in each of four independent experiments (see Figure 10). Images shown for combinations of KC1-nYFP with the SYP121 site mutants F9A, S10A, and R11A are single-plane bright-field (A, E, and I), bright-field plus YFP fluorescence (B, F, and J), and YFP fluorescence only (C, G, and K), and three-dimensional reconstructions from fluorescence image stacks (D, H, and L). Bar = 50 μm.

See also Supplemental Movies 5 to 7 online.
clamp recordings from currents and channel gating in the plant, we made use of the voltage FxRF motif of SYP121 to alter the gating of the K+ channels. The functional requirement for the FxRF motif of SNARE thus showed the functional requirement for the SYP121 but, again, only when the clamp range was extended to voltages negative of −200 mV. By contrast, an inward-rectifying K+ current similar to that of the wild-type plants was observed in every case in recordings from syp121 mutant plants when previous studies (Honsbein et al., 2009) showed that the SNARE mutant, like the null mutants kc1-2 and akt1-1, resulted in a loss of inward-rectifying K+ current in the root epidermis; the K+ current was recovered in the syp121 mutant background when complemented with the wild-type transgene under control either of its own or a constitutive promoter. Therefore, we reasoned that the K+ current should similarly recover in the syp121 background when complemented with the SYP121 single-site mutant S10A, which retained the ability to interact with KC1, but not with the F9A, R11A, and F12A mutants that failed to interact with KC1. Measurements were performed using two-electrode voltage clamp methods on root epidermis of Arabidopsis. We compared K+ currents from wild-type and syp121 mutant plants, and syp121 mutant seedlings transformed with the four SYP121 single-site mutants under control of a constitutive promoter. We also used syp121 mutant seedlings transformed with wild-type SYP121 and SYP122 as positive and negative controls, respectively. To confirm transformations on a cell-by-cell basis, each of the SPRO constructs was cloned into a bicistronic binary vector with two, independent expression cassettes, both localized on the same T-DNA and each under the control of the Arabidopsis Ubiquitin-10 gene promoter, one of which was used to drive the expression of a cytosolic green fluorescent protein (GFP) marker. Epidermal cells expressing the GFP marker were identified visually under epifluorescence illumination and were impaled using multibarrelled microelectrodes for standard two-electrode voltage clamp recordings (Blatt, 1987; Blatt and Gradmann, 1997). Mature epidermal cells of Arabidopsis roots are not coupled through plasmodesmata (Duckett et al., 1994), thus enabling in situ recordings from these single cells. Nonetheless, root hairs can introduce a substantial and local current sink in electrical recordings (Mezargh et al., 1994). Therefore, to avoid problems of voltage control associated with nonhomogeneous current spread, we made use of epidermal cells in files lacking root hairs. Finally, to check against mistargeting of the mutant SNAREs, we also performed parallel transformations using fluorescently tagged constructs.

Figure 13 shows representative measurements from wild-type and syp121 mutant plants and from the various complementations of the syp121 mutant line. Similar results were obtained in at least six independent experiments in every case and are summarized in Figure 14. Wild-type plants showed currents under voltage clamp typical of the inward-rectifying K+ channels in the root epidermis (Honsbein et al., 2009). This current, relaxed with half-times of 300 to 400 ms, was evident principally at voltages near and negative of −120 mV and, on analysis, yielded the characteristic gating parameters for s near 2 and V1/2 close to −160 mV (Figure 14). The inward-rectifying K+ current was not evident in recordings from the syp121 mutant nor in syp121 mutant plants transformed with constructs encoding SYP122 (data not shown; see Honsbein et al., 2009) or the SYP121 single-site mutations F9A and F12A, except at voltages negative of −200 mV; a small but persistent current was recorded from syp121 mutant plants transformed with the R11A mutant of SYP121 but, again, only when the clamp range was extended to voltages negative of −200 mV. By contrast, an inward-rectifying K+ current similar to that of the wild-type plants was observed in every case in recordings from syp121 mutant plants when
The site mutants selectively rescue AKT1-KC1 K+ current in coexpression with SYP121 and interacting SYP121 single-site mutants. Least squares fitting of the K+ currents (I_K) to the Boltzmann function is essential for AKT1 function in oocytes, in 1:1:1 molar ratios. Measurements performed in coexpression with CBL1 and CIPK23, with SYP121 cRNA only gave results similar to those for KC1 alone. All referenced by symbol. Currents from oocytes injected with water and scale = 3 m. Inspection showed an increase in d and shift in V_1/2 on inclusion of cations (>12 oocytes for each set of constructs). Both analysis and visual inspection showed interaction with KC1 and those that did not (see also Figures 3 and 4). Similar results were obtained in each of three separate experiments (>12 oocytes for each set of constructs). Both analysis and visual inspection showed an increase in δ and shift in V_1/2 on inclusion of K+ currents (data not shown), much as previously described in mesophyll and guard cells (Very and Sentenac, 2003; Dreyer and Blatt, 2009) and in Arabidopsis root epidermal cells (Honsbein et al., 2009), indicating that effects of the experimental manipulations were restricted to the inward-rectifying current. Finally, parallel transformations using the fluorescently tagged SNARE mutants (see Supplemental Figure 1 online) showed their localization to the cell periphery, not the cytosol or tonoplast, with an accumulation around the tip of the root hair much as has been reported for the wild-type SYP121 (Enami et al., 2009; Grefen et al., 2010). Thus, we conclude that the FxRF motif is a primary determinant not only of the physical interaction between KC1 and SYP121 but of the functional expression of K+ current determined by channel assembly with SYP121 in vivo.

**DISCUSSION**

Membrane vesicle traffic and the SNARE proteins that drive it are increasingly recognized as important players in plant cell development and growth, as well as signaling and defense. Vesicle traffic affects the steady state complement of membrane proteins and their tissue distributions during development and at the cellular level it drives the turnover of ion channels, transporters, and receptor proteins, contributing to their modulation by hormones and environmental factors (Bassham and Blatt, 2008; Grefen and Blatt, 2008). These processes play a part in coordinating the ensemble of transport activities at the plasma membrane, although many details are only beginning to come to light. Additionally, there is now unequivocal evidence of other roles for SNAREs in the control of ion channels in plants. Our recent demonstration of a direct and selective interaction of the Arabidopsis SNARE SYP121 with the KC1 K+ channel established a role for the SNARE in mineral nutrition (Honsbein et al., 2009). This discovery and its seeming independence from signaling and vesicle traffic led us to propose its physiological function as a molecular governor coupling K+ transport with cell surface area in volume control (Grefen and Blatt, 2008). Thus, the discovery raised a fundamental question about the identity of the interacting domain on the SNARE and its relationship to the H3 coiled-coil that assembles in the SNARE core complex and to the SNARE-K+ Channel Interaction Motif.

SYP121 and the interacting mutants with AKT1 and KC1. (A) Current traces and steady state current-voltage curves recorded under voltage clamp in 96 mM K+ from oocytes expressing KC1 alone (diamonds), AKT1 alone (circles), AKT1 with KC1 (molar ratio 1:1) alone (closed squares) and with the SNARE, and SNARE mutants (molar ratio to KC1, 2:1; see Honsbein et al., 2009) SYP121 (upward-facing closed arrowheads), SYP121-F9A (downward-facing open arrowheads), SYP121-S10A (downward-facing closed arrowheads), SYP121-R11A (open squares), and SYP121-F12A (upward-facing open arrowheads). Clamp cycles: holding voltage, ~20 mV; voltage steps, 0 to ~180 mV. Scale = 3 μA and 4 s. Insets: Corresponding whole-cell currents cross-referenced by symbol. Currents from oocytes injected with water and with SYP121 cRNA only gave results similar to those for KC1 alone. All measurements performed in coexpression with CBL1 and CIPK23, which are essential for AKT1 function in oocytes, in 1:1:1 molar ratios with AKT1 (Xu et al., 2006). Solid curves are the results of joint, nonlinear least squares fitting of the K+ currents (I_K) to the Boltzmann function (Equation 1) and are summarized in Figure 13. Best fittings were obtained with g_max held in common (g_max for the data shown, 49 ± 3 nS) and with separate, joint values for δ between SYP121 and SNARE mutants that showed interaction with KC1 and those that did not (see also Figures 3 and 4). Similar results were obtained in each of three separate experiments (>12 oocytes for each set of constructs). Both analysis and visual inspection showed an increase in δ and shift in V_1/2 on inclusion of SYP121 and the interacting mutants with AKT1 and KC1. (B) Verification of SNARE protein expression. Immunoblot analysis of total membrane protein (10 μg/lane) extracted from oocytes collected after electrical recordings and detected with αSYP121 antibody (Tyrrell et al., 2007; Honsbein et al., 2009).
previously unidentified motif situated within the first 12 residues of the SNARE sequence, (2) that this same motif is the minimal requirement for SNARE-dependent alterations in the gating characteristics of K+ channels assembled with KC1, and (3) that rescue of the K+ current in syp121 mutant Arabidopsis depends on complementation with SYP121 that retains this motif. The KC1 binding site of SYP121 is situated immediately adjacent Ser residues thought to be phosphorylated in response to pathogen attack (Pajonk et al., 2008), and, as we note below, it overlaps with a consensus domain recognized to be important in regulating vesicle traffic. Thus, the site of KC1 interaction marks

Figure 12. Coexpression with SYP121 and Interacting SYP121 Single-Site Mutants Selectively Rescues AKT1-KC1 K+ Current in Xenopus Oocytes.

Summary of K+ channel parameters of gating charge (A) and $V_{1/2}$ (B) recorded from oocytes expressing AKT1 with KC1 in combinations with SYP121, SYP122, and the SYP121 site mutants F9A, S10A, R11A, and F12A. Data are means ± SE obtained from joint fittings to Equation 1 of four or more independent data sets in each case, including the data of Figure 3. Fittings were performed by nonlinear least squares minimization (Marquardt, 1963) with the conductance maximum $g_{max}$ held in common within each data set and the apparent gating charge ($\theta$) allowed to vary between SNAREs and chimeras that interact with KC1 and those that do not. Data for SYP121 and the S10A mutant are statistically different from the other data sets with P < 0.01.

Figure 13. Coexpression with Interacting SYP121 Single-Site Mutants Selectively Rescues AKT1-KC1 K+ Current in Vivo.

Current traces and steady state current-voltage curves recorded under voltage clamp from root epidermal cells of Arabidopsis wild-type plants (closed circles), syp121 mutant plants (open circles), and syp121 mutant plants expressing the transgenes for SYP121-F9A (upward-facing open arrowheads), SYP121-S10A (upward-facing closed arrowheads), SYP121-R11A (downward-facing open arrowheads), and SYP121-F12A (squares). Data for syp121 plants expressing SYP121 and SYP122 were omitted for clarity. Clamp cycles: holding voltage, $-20$ mV; voltage steps, 0 to $-220$ mV. Scale = 2 pA and 1 s. Insets: Corresponding whole-cell currents cross-referenced by symbol. Solid curves are the results of joint, nonlinear least squares fitting of the K+ currents ($I_K$) to the Boltzmann function (Equation 1) and are included in Figure 14. Similar results were obtained in each of five separate experiments (>12 seedlings for each construct). Both analysis and visual inspection showed a rescue of the K+ current with SYP121 and the SYP121-S10A mutant that retains SNARE-KC1 interaction and functionality in yeast and oocytes.
the N terminus of SYP121 as being of special importance: it suggests a close functional overlap between SYP121-dependent membrane vesicle traffic and control of the K⁺ channels, and it discounts a direct competition between SNARE core complex formation and channel binding. Furthermore, it offers a mechanistic framework from which to explore the coordinate control of vesicle traffic and transmembrane ion transport.

A Minimal Motif for K⁺ Channel Interaction and Control

Three observations point to the FxRF motif as an essential and unique determinant of KC1 binding with SYP121 and to its functional impact. The first and most important of these derives from chimeric substitutions with domains from the closely related SNARE SYP122. We found that replacing the N-terminal 39 amino acids of SYP121 with the corresponding sequence from SYP122 eliminated KC1 interaction in yeast and SNARE action on K⁺ channel gating in oocytes; conversely, SNARE–KC1 interaction and its impact on channel gating were recovered so long as this same sequence from SYP121 replaced the N-terminal domain of SYP122 (Figures 1, 3, and 4 to 6). Second, within this N-terminal domain a similar pattern of results was obtained on exchanging eight-residue segments between the two SNAREs (Figures 2 to 4 and 6), and Ala-scanning mutagenesis within and adjacent the critical segment identified the core FxRF sequence determining both KC1 binding and the action of SYP121 on gating of the K⁺ channel (Figures 8 to 12). Finally, the same N-terminal sequence proved essential for functional interaction of the SNARE with KC1 when expressed in vivo (Figures 13 and 14). These results do not rule out additional sites of interaction with the K⁺ channel subunit, but they demonstrate that the N-terminal FxRF motif is necessary for the physical and functional interaction of SYP121 with KC1.

A key to interpreting the impact of the SYP121 motif can be drawn from the electrophysiological analyses of SNARE expression in the syp121 Arabidopsis background that blocks the inward K⁺ channel current. We showed previously that SYP121, through its association with KC1, assembles with a third protein, the AKT1 K⁺ channel subunit, and that all three proteins are essential for functional expression of the inward K⁺ current in vivo (Honsbein et al., 2009): eliminating SYP121 expression, as in the syp121 mutant (Collins et al., 2003; Zhang et al., 2007), effectively suppressed the inward K⁺ current, and complementing the syp121 mutant with the full-length protein restored the current, both with SYP121 under control of its own promoter and when constitutively expressed. These effects were selective for the inward-rectifying K⁺ current and could be shown to arise from direct interaction with these channels rather than an effect mediated through channel protein traffic in vivo (Honsbein et al., 2009). It follows that SYP121–SYP122 chimeras and mutant SYP121 constructs that complement the syp121 Arabidopsis plants should retain a capacity to interact functionally with KC1 in order to affect channel gating. Thus, the parallel between the readout for interaction based on yeast growth (Figures 1, 2, and 8) and K⁺ current rescue (Figures 13 and 14), as well as the absence of any impact on the outward K⁺ currents in vivo, argues strongly that KC1 binding associated with the SYP121 FxRF motif is a prerequisite for its functional impact on gating.

Figure 14. Coexpression with Interacting SYP121 Single-Site Mutants Selectively Rescues AKT1-KC1 K⁺ Current in Vivo.

Summary of K⁺ current parameters of gating charge (A) and V₅₀ (B) recorded from Arabidopsis expressing AKT1 with KC1 in combinations with SYP121. Data are means ± SE obtained from joint fittings of at least six independent data sets for each construct, in each case with the K⁺ equilibrium voltage set to −30 mV and the conductance maximum g_max held in common (Equation 1). Parameter values for noninteracting constructs were determined assuming a g_max in common with the mean wild-type K⁺ current and 0.8 ≤ δ ≤ 1.2, consistent with results from heterologous expression studies (see above and Honsbein et al., 2009) and should therefore be viewed as estimates only. Data for wild-type Arabidopsis and for syp121 mutant plants complemented with the SYP121-S10A mutant are statistically different from the other data sets (for δ, P < 0.01; for V₅₀, P < 0.02).
The same conclusion can be drawn from the heterologous expression studies in Xenopus oocytes. Furthermore, analysis of channel gating in this case provides additional detail to the molecular consequences of the SYP121-KC1 association. As before (Honsbein et al., 2009), we found coexpression of AKT1 with KC1 only to give a small current near the negative voltage extreme, whereas coexpression of the channel subunits together with SYP121 yielded substantial K+ current near and negative of \(-100\) mV with values for \(V_{1/2}\) close to \(-150\) mV (Figure 4). Unlike the situation in the plant, expression of AKT1 alone also yields an inward K+ current in oocytes, but with anomalous gating characteristics (Duby et al., 2008; Geiger et al., 2009; Honsbein et al., 2009). Currents obtained with AKT1 alone and with KC1 were well-fitted to Boltzmann functions with \(\delta\) near unity. By contrast, coexpression of AKT1 and KC1 with SYP121 gave currents with \(\delta\) near 2, similar to values returned from analysis of the inward K+ current in the plant (see Figures 3, 4, and 11 to 13; Honsbein et al., 2009). The K+ currents obtained with the SNARE chimeras and SYP121 mutants followed this same dichotomous distribution, the pattern corresponding directly with SNARE–KC1 interaction in yeast: chimeras and mutants that failed to rescue yeast growth also showed currents similar to those recorded from oocytes expressing AKT1 and KC1 alone or with the noninteracting SNARE SYPI22; SNARE mutants and chimeras that rescued yeast growth also gave K+ currents with gating parameters that matched closely those on coexpression of AKT1 and KC1 with the wild-type SYP121. Because the gating parameters \(V_{1/2}\) and \(\delta\) together encapsulate the intrinsic voltage range and sensitivity for gating—in short, the capacity for membrane voltage to affect channel protein conformations (Dreyer and Blatt, 2009)—this dichotomy in gating characteristics underscores the functional importance of the FxRF motif in the conformational changes effected by the SNARE on the K+ channel.

A Unique N-Terminal Domain with Overlapping Functions?

A few examples of SNARE–ion channel interactions are known and have been associated with other cellular functions. Notably, the mammalian \(Q_{a}\)-SNARE Syntaxin 1A has been reported to bind a number of K+ and Ca\(^{2+}\) channels and to subtly affect their gating. Interactions of Syntaxin 1A with the SUR1-K\(_{aT P}\) complex of ATP-sensitive K+ channels and the voltage-gated Kv2.1 K+ channel are thought to help regulate insulin secretion (Michaellevski et al., 2003; Cui et al., 2004), and its associations with L- and N-type Ca\(^{2+}\) channels (Wiser et al., 1996; Bezprozvanny et al., 2000; Arien et al., 2003) have been suggested similarly to coordinate neurotransmission and neuroendocrine secretion (Leung et al., 2007). In all of these examples, the interacting domains of SNARE appear to be situated within the C-terminal \(\alpha\)-helix that anchors the protein in the plasma membrane and the adjacent H3 \(\alpha\)-helix that normally assembles as part of the SNARE core complex during vesicle fusion. The H3 \(\alpha\)-helix also binds the CFTR Cl\(^{-}\) channel (Naren et al., 1997; Ganeshan et al., 2003), although the association with vesicle fusion and possible roles for this interaction are less obvious. One difficulty in each of these examples rests with the amphipathic properties of the \(Q_{a}\)-SNARE H3 \(\alpha\)-helix: its seemingly promiscuous capacity for protein interaction has raised concerns about interpreting the physiological significance of SNARE binding with the channel proteins (Fletcher et al., 2003). It remains an unprecedented feature of SYP121, therefore, that its binding with KC1 depends not on the H3 or membrane-spanning \(\alpha\)-helices, but on a previously unidentified motif isolated at the cytosolic N terminus of the SNARE and unique to SYP121. Indeed, the FxRF motif appears unique to plants and, among Arabidopsis \(Q_{a}\)-SNAREs, to SYP121 (Figure 15).

KCl interaction with the SYP121 N terminus now offers a structural framework to explore its regulation and association with other physiological phenomena in vivo. There is good evidence that Ser residues near the N terminus of SYP121 and its close homolog SYPI22 are phosphorylated in response to pathogen challenge (Nuhse et al., 2003; Heese et al., 2005), although their significance for any underpinning mechanisms remains unknown. In SYP121, phosphorylation is thought to occur at the Ser-7 position, while in SYP122, the adjacent Ser-6 and Ser-8 residues appear the major targets for kinase action (Benschop et al., 2007). Furthermore, we note that residues within and adjacent to the FxRF motif of SYP121 are likely to be important in regulating SNARE core complex formation and vesicle traffic. The N terminal of several \(Q_{a}\)-SNAREs, including mammalian Syntaxin 1A and Syntaxin 5, and the yeast SNAREs Sed5p, Tlg1p, and Tlg2p, are now recognized to form binding sites for so-called Sec1/Munc18 (SM) proteins that facilitate assembly of the SNARE core complex in yeast and mammalian tissues (Burgoyne and Morgan, 2007; Sudhof and Rothman, 2009). Many details of SM protein binding are still unresolved at this time. Nonetheless, it is clear that their interaction with the N-terminal domains of the cognate SNAREs greatly accelerates fusion, probably by stabilizing protein conformations and vesicle positioning during core complex formation (Sudhof and Rothman, 2009). At the heart of the binding site on the SNAREs is a motif comprising highly conserved Asp and Phe residues separated by four to five amino acids, roughly one \(\alpha\)-helical turn, Syx1A[D_d melanogaster] ----- MTXR1LAAZTWAACRDEEEFY
Syx1A[H_sapiens] ----- NSDQFST-ESDAYKEDDDD-EDY
Syx5[H_sapiens] ----- MSCKDRT-ORELSAC-KSIQEBR-
Syx5[C_elegans] ----- MSCKDRT-ORELSAC-KSIQEBR-
Sed5p[S_cerevisiae] ----- MN1RSFSEEQSVLSWYKRRKRK
Ufelp[S_cerevisiae] ----- MDRSTL-FIPKYVATIDARAKRE
Tlg2p[S_cerevisiae] ----- MFQPRTMLFLGTFPPRHTFSS
SYPI21_AT3G11820 ----- NDFFSSEASEKSGSSPFRD
SYPI12_AT3G02400 ----- NDLLGSQHTSTVADGS6PPP8
SYPI21_AT3G161290 ----- NDFFSSEASEKSGSSPFRD
SYPI12_AT3G11250 ----- NDFFSSEASEKSGSSPFRD
SYPI31_AT3G03500 ----- NDLLKQGSRFDRKSNRSDEIE
SYPI32_AT5G08080 ----- NDLLKQGSRFDRKSNRSDEIE
SYPI11_AT3G05650 ----- NDFFSSEASEKSGSSPFRD
SYPI12_AT2G18260 ----- NDLLQYKSWLVPKQARQTD
SYPI31_AT5G05760 ----- MGSFTRKVEWNLSLQYPKRCIGA
SYPI12_AT3G24350 ----- MSAFAQGQDSTKDEDEKTVETLAKARISAF

Figure 15. Conserved Phe Residue Associated with Sec1/Munc18 Protein Binding Overlaps the FxRF Motif of SYP121.

Alignment of representative \(Q_{a}\)-SNAREs from Drosophila, Homo sapiens, Caenorhabditis elegans, Saccharomyces cerevisiae, and Arabidopsis showing the highly conserved Asp and Phe residues associated with Sec1/Munc18 protein binding (dark gray), adjacent and largely conserved residues (light gray), and the overlap with the KC1 interaction motif of SYP121 (boxed).
close to the N terminus of the protein. These residues fit within a minor groove on the surface of the SM proteins (Bracher and Weissenhorn, 2002) when the SNARE is in one or more open (active) conformations, and binding of the SNARE at this site on the SM proteins is thought to facilitate SM binding with the core complex (Carpp et al., 2006; Sudhof and Rothman, 2009).Aligning the corresponding Arabidopsis Qa-SNAREs (Figure 15) shows that the conserved Asp and Phe residues critical for SM binding are found in SYP121 and, furthermore, that the Phe residue is incorporated within the FxRF motif of SYP121, implying an overlap and, plausibly, competition for binding between KC1 and one or more Arabidopsis SM proteins. The observation thus raises entirely new questions that will bear future exploration, notably whether KC1 competes for SYP121 binding with Arabidopsis SM proteins or KC1 interaction with SYP121 might substitute in the role of an SM partner and whether KC1 binding affects SYP121-mediated vesicle traffic to the plasma membrane. Addressing these questions will require knowledge of the SNARE binding domain of KC1. Regardless of the answers, the identity of the SYP121 FxRF motif defines a unique site for K+ channel interaction and gating control, and it presents important and novel perspectives on the concept of the SNARE-K+ channel association as a molecular governor (Grefen and Blatt, 2008; Honsbein et al., 2009) to coordinate membrane traffic with osmotically active solute transport in the plant.

METHODS

Molecular Biology

Open reading frames for AKT1, KC1, SYP121, and SYP122 were amplified with gene-specific primers including Gateway attachment sites (attB1/attB2). A subsequent BP reaction in pDONR207 (Invitrogen) yielded Entry clones that were verified via sequencing. The AKT1 and KC1 sequences were obtained without stop codon to allow C-terminal fusions, whereas SYP121 and SYP122 were amplified to include their native stop codons and facilitate correct localization as type-II membrane proteins. Chimeric clones were constructed using restriction endonucleases, site-directed mutagenesis, PCR amplification, and DNA ligation. For the N2HQC1 chimera, SYP121-pDONR207 was cut by sequential digestion with ApaI and AgeI. The 227-bp fragment incorporating the attL1 site and the first 39 codons of SYP121 was discarded. A PCR product was amplified using SYP122-pDONR207 as template to contain the attL1-site and the first 38 codons of SYP122 with ApaI and AgeI restriction sites and was inserted at the ApaI and AgeI restriction site. For the N2HQC1 chimera, codon 190 of SYP122 in pDONR207 was mutated to create an AgeI site through the translationally silent point substitution of ACA with ACT. Sequences corresponding to the H3 and transmembrane domains of SYP122 were excised using AgeI and PvuII and replaced with the corresponding PCR fragment of SYP121 including the same restriction sites. The NHQC1 chimera was constructed by transcriptionally silent, site-directed mutagenesis at codons 282 and 283 of SYP122 in pDONR207 to generate a MluI site (mutation of ACACGG to ACGCGT). Sequences coding for the transmembrane domain of SYP122 were then excised by digestions with MluI and PvuII, and the corresponding PCR fragment of SYP121 was inserted. The N1HQC2 chimera was generated by AgeI and PvuII digestion of SYP121-pDONR207, and the excised 1035-bp fragment was discarded and replaced with a corresponding PCR fragment of SYP122. The N1HQC2 chimera was constructed by mutation of SYP121-pDONR207 to eliminate an AgeI site at codons 39 to 40. Thereafter, sequences corresponding to the N-terminal and Habc domains of SYP121 were PCR amplified with primers to add 5’ Apal and 3’ AgeI sites. The PCR product was used to replace the corresponding domains in SYP122-pDONR207. The NHQC2 chimera was created by Apal and MluI digestion to excise a fragment containing the attL1 site, N, Habc, and H3 domains of SYP122 from the MluI mutagenized SYP121-pDONR207 (see above). This site was then replaced by ligation with the corresponding PCR product from SYP121. Point mutants were generated by site-directed mutagenesis as described by Qi and Schothof (2008) using primer sequences listed in Supplemental Table 1 online. Finally, Gateway Destination clones were generated using LR Clonase II (Invitrogen) by LR reaction according to the manufacturer’s instructions. For BiFC and split-ubiquitin assays, coding sequences for AKT1 and KC1 were cloned in pMetYC-Dest and pUBC-cYFP, and coding sequences for the SNAREs SYP121 and SYP122, the chimeras, and point mutants were cloned in pNX32-Dest and pUBN-cYFP (Grefen et al., 2009, 2010). For electrophysiological analysis in oocytes, these constructs were subcloned into pGTDest (see below), and for subcellular localization analyses, single residue mutants of SYP121 were cloned in pUBN-EOS (Grefen et al., 2010).

The bicistronic vector was prepared by digesting pUB-Dest (Grefen et al., 2010) using the unique SbfI restriction site, which is situated between the 35S terminator and the Basta resistance gene. A PCR reaction was performed with pUBN-GFP-Dest as template to create a P_UBQ10-GFP fragment flanked by SbfI sites using the primers TTCTCTGCAGGTACCAGAGTCAG5’ to 3’ and TTCTCTGCAGGTACCTGTACAGCTGCTGTCATGC3’ to 5’). This product was introduced in the linearized vector to create the bicistronic vector pUB-Bic-Dest, pUB-Bic-Dest was maintained and amplified using ccdB survival cells (Invitrogen) grown in the presence of the selection markers chloramphenicol (30 mg/L) and spectinomycin (100 mg/L), and the Gateway Entry and Destination clones were amplified using Top10 cells (Invitrogen) and the appropriate antibiotic (gentamycin [20 mg/L] for Entry clones and spectinomycin [100 mg/L] for Destination clones).

Split-ubiquitin Assays

For yeast mating-based split-ubiquitin assays, the haploid yeast strains THY.AP4 and THY.AP5 (Obrdlik et al., 2004; Grefen et al., 2007) were transformed as described previously (Grefen et al., 2009). Pools of 10 to 15 single colonies were selected after 3 d and were inoculated in selective media for overnight growth. The liquid cultures were harvested at OD_600 2 to 3 and were resuspended in YPD. Matings were performed by mixing equal aliquots of cultures containing KC1-Cub in THY.AP4 with the appropriate NubG-SNARE in THY.AP5. Aliquots of 4 mL from each mixture were dropped on YPD plates and incubated at 30°C overnight. Diploid colonies were selected and inoculated in vector-selective media lacking Leu, Trp, and uracil (CSM渭渭) and were grown to OD_600 of 2 to 3. Thereafter, the yeast was harvested and resuspended in sterile water. Serial dilutions at OD_600 1.0 and 0.1 in water were dropped, 7 μL per spot, onto plates without and with additions of 200 μM Met on interaction-selective media additionally lacking His, Met, and adenine (CSM渭渭). Growth was monitored at 2, 3, and 4 d, and images used were taken on the final day. Yeast was also dropped on CSM渭渭 media as a control for mating and cell density, and images were taken after 24 h. To verify expression, yeast was harvested in aliquots equal to those used for the dilution series and was extracted for protein gel blot analysis using polyclonal antibodies against SYP121 and SYP122 as before (Grefen et al., 2009; Honsbein et al., 2009). KC1-Cub expression was verified in THY.AP4 yeast prior to mating using the VP16 antibody (Abcam).

Electrophysiology

For electrical recordings using Xenopus laevis oocytes, constructs with AKT1, KC1, CiPK23, CBL1, SYP121, and SYP122 were used as
described previously (Honsbein et al., 2009). The SNARE chimeras and point mutants were cloned in pGTDest, which we created using the oocyte expression vector pBSXG1 (Groves and Tanner, 1992) and introducing a Gateway cassette with the Gateway conversion kit (Invitrogen). Plasmids were linearized and capped cRNA was synthesized in vitro using T7 mMessage mMachine (Ambion). cRNA quality as a single trogen). Plasmids were linearized and capped cRNA was synthesized in introducing a Gateway cassette with the Gateway conversion kit (Invi-

Stage V and VI oocytes were isolated from mature Xenopus, and the follicular cell layer was digested with 2 mg/ml collagenase (type 1A; Sigma-Aldrich) for 1 h. Injected oocytes were incubated in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 10 mM HEPES-NaOH, pH 7.4) supplemented with gentamycin (5 μg/ml) at 18°C for 3 d before electrophysiological recordings. Whole-cell currents were recorded under voltage clamp using an Axoclamp 2B two-electrode clamp circuit (Axon Instruments) as described previously (Leyman et al., 1999; Sutter et al., 2006). Measurements were performed under continuous perfusion either with 30 mM KCl and 6 mM NaCl or with 96 mM KCl, in each case with the addition of 1.8 mM MgCl2, 1.8 mM CaCl2, and 10 mM HEPES-NaOH, pH 7.2. Oocytes yielding currents were collected and total membrane protein isolated according to Sottocornola et al. (2006) using 20 μl of extraction buffer per oocyte. Polyclonal antibodies against SY121 and SY122 were used at dilutions of 1:5000 in combination with either with 30 mM KCl and 66 mM NaCl or with 96 mM KCl, in each case with the addition of 1.8 mM MgCl2, 1.8 mM CaCl2, and 10 mM HEPES-NaOH, pH 7.2. Oocysts yielding currents were collected and total membrane protein isolated according to Sottocornola et al. (2006) using 20 μl of extraction buffer per oocyte. Polyclonal antibodies against SY121 and SY122 were used at dilutions of 1:5000 in combination with the ECL Advance Detection Kit (GE Healthcare). Immunoblots were quantified and normalized to the Ponceau S stain using ImageJ (http://rsweb.nih.gov/ij/).

Recordings from Arabidopsis thaliana root epidermal cells were carried out on wild-type and syp121 mutant seedlings 6 to 8 d postgermination following transformation by Agrobacterium tumefaciens cocultivation (Grefen et al., 2010) with the SNAREs and SNARE mutant constructs in pUB-Bic-Dest. Seedlings were bathed in solutions of 10 and 30 mM KCl (Grefen et al., 2010) with the SNAREs and SNARE mutant constructs in following transformation by Agrobacterium tumefaciens (A. tumefaciens GV3101 (Grefen et al., 2010), methods developed from those previously described for Agrobacterium rhizogenes (Campanoni et al., 2007) and including 0.003% Sylwet (Duchefa) in the cocultivation medium to aid transformation (Li et al., 2009). Confocal images were obtained as before (Sutter et al., 2006) on a Zeiss LSM510-UV microscope. GFP fluorescence was excited with the 514-nm laser line. Emitted light was collected through a NFT515 dichroic and 505- to 530-nm (GFP) and 535- to 590-nm (YFP) band-pass filters. Pinholes were set to 1 airy unit. Bright-field images were collected with a transmitted light detector. Laser intensity, photomultiplier gain, and offset were standardized.

Confocal Microscopy
Arabidopsis seedlings were grown in 0.5 × Murashige and Skoog, pH 7.2, for 3 to 5 d and transformed by cocultivation with A. tumefaciens GV3101 (Grefen et al., 2010), methods developed from those previously described for Agrobacterium rhizogenes (Campanoni et al., 2007) and including 0.003% Sylwet (Duchefa) in the cocultivation medium to aid transformation (Li et al., 2009). Confocal images were obtained as before (Sutter et al., 2006) on a Zeiss LSM510-UV microscope. GFP fluorescence was excited with the 458- or 488-nm argon laser lines; YFP fluorescence was excited with the 514-nm laser line. Emitted light was collected through a NFT515 dichroic and 505- to 530-nm (GFP) and 535- to 590-nm (YFP) band-pass filters. Pinholes were set to 1 airy unit. Bright-field images were collected with a transmitted light detector. Laser intensity, photomultiplier gain, and offset were standardized.

Statistics
Statistical analysis of independent experiments is reported as means ± se as appropriate with significance determined by Student’s t test or analysis of variance. Joint nonlinear least squares fittings were performed using a Marquardt-Levenberg algorithm (Marquardt, 1963) implemented in SigmaPlot v.11 (SPSS).

Sequences, Vectors, and Maps
Sequences and maps for all vectors described above are available at www.psrg.org.uk, and vectors are available on request.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AKT1 (At2g26650), KC1 (At4g32650), SY121 (At3g11820), and SY122 (At3g52400).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. The Cellular Distribution in Vivo of SY121 Is Unaffected by the SY121-R11A Mutation.

Supplemental Table 1. Oligonucleotides That Were Designed to Construct the Clones Used in This Study.

Supplemental Movie 1. 3D Projection of Transient Expression in Arabidopsis Root Epidermis of the BiFC Pair cYFP-SY121 and KC1-nYFP.

Supplemental Movie 2. 3D Projection of Transient Expression in Arabidopsis Root Epidermis of the BiFC Pair cYFP-N1HQC2 and KC1-nYFP.

Supplemental Movie 3. 3D Projection of Transient Expression in Arabidopsis Root Epidermis of the BiFC Pair cYFP-N2HQC1 and KC1-nYFP.

Supplemental Movie 4. FRAP Time Series of Transient Expression in Arabidopsis Root Epidermis of the BiFC Pair cYFP-N1HQC2 and KC1-nYFP.

Supplemental Movie 5. 3D Projection of Transient Expression in sy121 Mutant Arabidopsis Root Epidermis of the BiFC Pair cYFP-SYP121-F9A and KC1-nYFP.

Supplemental Movie 6. 3D Projection of Transient Expression in sy121 Mutant Arabidopsis Root Epidermis of the BiFC Pair cYFP-SYP121-S10A and KC1-nYFP.

Supplemental Movie 7. 3D Projection of Transient Expression in sy121 Mutant Arabidopsis Root Epidermis of the BiFC Pair cYFP-SYP121-R11A and KC1-nYFP.

Supplemental Movie Legends.

ACKNOWLEDGMENTS
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A Novel Motif Essential for SNARE Interaction with the K⁺ Channel KC1 and Channel Gating in *Arabidopsis*

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