RESEARCH ARTICLE

The C2-domain protein QUIRKY and the receptor-like kinase STRUBBELIG localize to plasmodesmata and mediate tissue morphogenesis in Arabidopsis thaliana

Prasad Vaddepalli1, Anja Herrmann1, Lynette Fulton1, Maxi Oelschneider1, Stefan Hillmer2, Thomas F. Stratil3, Astrid Fastner4, Ulrich Z. Hammes4, Thomas Ott3, David G. Robinson2 and Kay Schneitz1

ABSTRACT

Tissue morphogenesis in plants requires communication between cells, a process involving the trafficking of molecules through plasmodesmata (PD). PD conductivity is regulated by endogenous and exogenous signals. However, the underlying signaling mechanisms remain enigmatic. In Arabidopsis, signal transduction mediated by the receptor-like kinase STRUBBELIG (SUB) contributes to inter-cell layer signaling during tissue morphogenesis. Previous analysis has revealed that SUB acts non-cell-autonomously suggesting that SUB controls tissue morphogenesis by participating in the formation or propagation of a downstream mobile signal. A genetic screen identified QUIRKY (QKY), encoding a predicted membrane-anchored C2-domain protein, as a component of SUB signaling. Here, we provide further insight into the role of QKY in this process. We show that like SUB, QKY exhibits non-cell-autonomy when expressed in a tissue-specific manner and that non-autonomy of QKY extends across several cells. In addition, we report on localization studies indicating that QKY and SUB localize to PD but independently of each other. FRET-FLIM analysis suggests that SUB and QKY are in close contact at PD in vivo. We propose a model where SUB and QKY interact at PD to promote tissue morphogenesis, thereby linking RLK-dependent signal transduction and intercellular communication mediated by PD.

KEY WORDS: Arabidopsis, Plasmodesmata, Signal transduction, Tissue morphogenesis, Receptor-like kinase, STRUBBELIG, QUIRKY

INTRODUCTION

Tissue morphogenesis in plants depends on cell-cell communication as cells and groups of cells divide and expand in a coordinated fashion. One complication in this process arises from the fact that plant cells are encased by a semi-rigid cell wall that constitutes a barrier to the communication between plant cells. During evolution, this obstacle has been overcome by at least two types of chemical signaling mechanisms (Lucas et al., 2009). The first involves plasmodesmata (PD), plasma membrane-lined channels that traverse the cell wall interconnecting the cytoplasm of neighboring cells, thereby enabling the intercellular movement of molecules. The second encompasses combinations of small, cell wall-penetrating ligands, e.g. peptides or phytohormones, and their receptors.

Inter-cell layer communication during tissue morphogenesis in Arabidopsis relies on signal transduction mediated by the atypical leucine-rich repeat receptor-like kinase (RLK) STRUBBELIG (SUB) (Chevalier et al., 2005; Vaddepalli et al., 2011; Lin et al., 2012). Plants lacking SUB activity exhibit defects in integument initiation and outgrowth, altered floral organ shape, stem morphology and reduced plant height, and asymmetric leaf shape. At the cellular level, SUB participates in division plane control in cells of the L2 layer of floral meristems. In addition, SUB also known as SCRAMBLED (SCM), affects root hair patterning (Kwak et al., 2005). Interestingly, SUB acts in a non-cell-autonomous fashion across several cells in the shoot apex, floral meristem, ovule and root (Kwak and Schiefelbein, 2008; Yadav et al., 2008). SUB is present at the plasma membrane (PM) and does not appear to traffic between cells. This indicates that signal perception by SUB results in the formation or propagation of a SUB-dependent mobile signal (SMS) that modifies the behavior of cells located several cells away.

Recent efforts have resulted in the identification of additional genetic components of SUB signaling, including QUIRKY (QKY) (Fulton et al., 2009; Trehin et al., 2013). Plants lacking SUB or QKY activities display very similar phenotypes at the gross morphological and cellular levels. In addition, flowers of these mutants exhibit a significant overlap in mis-regulated gene expression. These results indicate that QKY represents a central component of SUB signaling. Sequence analysis predicts that QKY encodes a 121.4 kDa membrane protein with four C2 domains and a phosphoribosyltransferase (SMS) domain (PT_C) containing two putative transmembrane domains (Fulton et al., 2009). As a rule, C2 domains represent autonomously folding modules that bind phospholipids in a Ca2+-dependent manner (Rizo and Südhof, 1998). In addition, QKY is the founding member of a 17-member gene family that includes FT-INTERACTING PROTEIN 1 (FTIP1) a regulator of flowering under long-day conditions (Liu et al., 2012).

Data presented in this work address the functional connection between QKY and SUB. They reveal that QKY acts in a non-cell-autonomous fashion, similar to SUB. They further show that functional SUB:EGFP and mCherry-QKY fusion proteins colocalize to PD and that association with PD occurs independently of each other. In addition, the data reveal that PD localization requires the extracellular and transmembrane domains of SUB and the C2C/C2D and the PT_C domains of QKY, respectively. Finally, the data show that SUB:EGFP and mCherry-QKY can physically interact in vivo at PD.
RESULTS
QKY acts in a non-cell-autonomous fashion

Previous genetic data indicated that QKY contributes to SUB-mediated signaling (Fulton et al., 2009; Trehin et al., 2013). Supporting this notion, we found that the expression patterns of QKY and SUB largely overlap in young flowers and ovules (supplementary material Fig. S1) (Chevalier et al., 2005). Moreover, both genes share similar synergistic interactions with ERECTA in the regulation of plant height (supplementary material Fig. S2) (Vaddepalli et al., 2011). Non-cell autonomy of SUB (Kwak and Schiefelbein, 2008; Yadav et al., 2008) is another central feature of the SUB-dependent signaling pathway. Formally, QKY could either contribute to the non-cell autonomy aspect of SUB signaling or participate in the perception of the mobile signal. If the former scenario were true, QKY should also function in a non-cell-autonomous fashion. To address this issue, we generated qky-8 plants that carried an EGFP:QKY reporter gene under the control of either the WUSCHEL (WUS) or MERISTEM LAYER 1 (ML1) promoters, two well-characterized tissue-specific promoters (Sessions et al., 1999; Bäurle and Laux, 2005), and assayed for rescue of the mutant phenotype in aerial tissues. A translational fusion of EGFP to the N-terminus of QKY results in a functional rescue of the mutant phenotype in aerial tissues. A translational fusion of EGFP to the N-terminus of QKY results in a functional rescue of the mutant phenotype in aerial tissues. A translational fusion of EGFP to the N-terminus of QKY results in a functional rescue of the mutant phenotype in aerial tissues. A translational fusion of EGFP to the N-terminus of QKY results in a functional rescue of the mutant phenotype in aerial tissues. A translational fusion of EGFP to the N-terminus of QKY results in a functional rescue of the mutant phenotype in aerial tissues. A translational fusion of EGFP to the N-terminus of QKY results in a functional rescue of the mutant phenotype in aerial tissues. A translational fusion of EGFP to the N-terminus of QKY results in a functional rescue of the mutant phenotype in aerial tissues.

QKY is expressed in inflorescence apices and broadly in floral meristems and flowers (supplementary material Fig. S1). WUS expression in the inflorescence meristem is limited to a few cells beneath the L3 layer (Mayer et al., 1998). In floral meristems, WUS expression can be monitored up to stage 6 (stages according to Smyth et al., 1990), where its expression is primarily restricted to cells below the L2 layer but is occasionally detected in the L2 layer showed partial and 14 displayed no phenotypic rescue. With respect to pML1::EGFP:QKY qky-8, we generated 22 independent lines that all developed as wild-type individuals. Phenotypes were validated in the T2 generation.

In Arabidopsis ovules, the initiating outer integument is separated from the nucellus by five to seven cells (Schneitz et al., 1995). QKY is expressed throughout young ovules (supplementary material Fig. S1A). WUS expression is restricted to the nucellus during stages 1 and 2 (stages according to Schneitz et al., 1995) and is no longer detectable around stage 3 (Gross-Hardt et al., 2002). Thus, we tested whether restricting EGFP:QKY expression to the distal region of the ovule primordium can rescue the outer integument defects of qky-8. Interestingly, the outer integument of pWUS::EGFP:QKY qky-8 plants, expressing EGFP:QKY solely in the distal region of the ovule primordium, showed a wild-type appearance (Fig. 1A-D), indicating rescue. This result suggests that QKY can influence the development of cells, several cells distant from where it is expressed.

QKY is expressed in inflorescence apices and broadly in floral meristems and flowers (supplementary material Fig. S1). WUS expression in the inflorescence meristem is limited to a few cells beneath the L3 layer (Mayer et al., 1998). In floral meristems, WUS expression can be monitored up to stage 6 (stages according to Smyth et al., 1990), where its expression is primarily restricted to cells below the L2 layer but is occasionally detected in the L2 layer.

Fig. 1. Non-cell autonomy of QKY function. (A,B,E-H,I-L″) Longitudinal confocal sections. (A,E,G) GFP channel. (B,F,H) Differential interference contrast (DIC) channel overlaid with GFP channel to outline tissue. (A,B) pWUS::EGFP:QKY reporter in a stage 2-III ovule. Signal is restricted to the nucellus. (B) Arrow indicates early outer integument. Inset shows a scanning electron micrograph of a wild-type ovule at a similar stage (for details, see Schneitz et al., 1995). (C,D) Scanning electron micrographs of stage 4 ovules. Inset in C shows a wild-type ovule. (D) The qky-8 phenotype is rescued by pWUS::EGFP:QKY. (E,F) Signal of the pML1::EGFP:QKY reporter in a stage 3 flower. Signal is restricted to the outermost layer of the tissue. (G,H) Signal of the pWUS::EGFP:QKY reporter in a stage 3 flower. (I-L″) Morphology of flowers. (K,L″) The qky-8 phenotype is rescued in qky-8 plants carrying the respective reporters. (L) Partial rescue of floral morphology. (I-L″) Morphology of siliques (upper panels) and stems (lower panels). (I-L″) Central region of stage 3 floral meristems stained with pseudo-Schiff propidium iodide (mPS-PI). (I) Note the regular anticlinal cell divisions in the L1 and L2 layers. (J) Arrows indicate two periclinal cell divisions in the L2 layer of a qky-8 mutant. (M,N) Above-ground overall morphology of qky-8 plants carrying different transgenes. (M) TG: pML1::EGFP:QKY. (N) TG: pWUS::EGFP:QKY.

Abbreviations: ii, inner integument; nu, nucellus; oi, outer integument. Scale bars: 5 μm in A,B; 10 μm in E-H,I-L″; 50 μm in C,D; 0.5 mm in I-L″.
Table 1. Number of periclinal cell divisions in the L2 layer of stage 3 floral meristems

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NPCD</th>
<th>Percentage</th>
<th>NFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ler</td>
<td>3</td>
<td>7.3</td>
<td>41</td>
</tr>
<tr>
<td>qky-8</td>
<td>19</td>
<td>15.9</td>
<td>34</td>
</tr>
<tr>
<td>ProML1::EGFP:QKY qky-8</td>
<td>3</td>
<td>8.6</td>
<td>34</td>
</tr>
<tr>
<td>ProWUS:EGFP:QKY qky-8</td>
<td>5</td>
<td>14.7</td>
<td>34</td>
</tr>
</tbody>
</table>

*Number of periclinal cell divisions observed *

**Rescue, floral development of stem and floral development of qky-8**

Development and plant height was rescued in due to occasional L2 expression of the reporter. Furthermore, stem development and plant height was rescued in transgenic lines showing full or partial rescue, QKY qky-8 promoter (Fig. S3). Despite rescue, the signal could not be detected indicating the presence of a functional reporter (supplementary material Fig. S4). To test whether the punctate EGFP:QKY pattern relates to PD, we investigated the root tip and cotyledon epidermis of EGFP:QKY qky-8 seedlings stained with aniline blue (AB), a standard stain for callose associated with PD (Bell and Oparka, 2011). As shown in Fig. 2E-H, M-P EGFP:QKY coincides with AB-derived spots, indicating that EGFP:QKY localizes close to or at PD. Using software-based image analysis we determined that 89% of EGFP:QKY spots overlapped with AB punctae in root epidermis cells (Pearson’s correlation coefficient=0.79).

Preceding work using conventional confocal microscopy revealed that SUB resides at the plasma membrane (PM) (Kwak and Schiefelbein, 2008; Yadav et al., 2008). In the light of a possible PD localization of QKY, we re-examined the subcellular signal distribution of a functional SUB:EGFP reporter driven by the endogenous promoter (pSUB:SUB:EGFP, gSUB:EGFP), whose expression fully overlaps with the reported SUB expression in flowers and roots (Vaddepalli et al., 2011), using a HSD-equipped confocal microscope. In addition to a visible PM localization, we also detected punctate signal at the circumference of cells in all tissues tested (Fig. 2I-L,Q-T) (supplementary material Fig. S5). Interestingly, those spots colocalized with signals derived from AB staining. In root epidermal cells, image analysis revealed that 88 percent of the SUB:EGFP spots overlapped with AB punctae (Pearson’s correlation coefficient=0.76). These results indicate that SUB:EGFP not only localizes to the PM but is also enriched at PD.

To confirm that EGFP:QKY and SUB:EGFP are positioned at PD, we performed immunogold electron microscopy. Depending on the section EGFP:QKY-derived signals were seen in either the neck region or to central domain of a plasmodesma (Fig. 2U-V). The results reveal the presence of EGFP:QKY at PD and suggest a broad distribution in individual PD. Likewise, SUB:EGFP signal was also seen in PD (Fig. 2W,X). Thus, EGFP:QKY and SUB:EGFP were both detected at PD by immunogold microscopy.

EGFP:QKY and SUB:EGFP localize to plasmodesmata

To address the subcellular localization of QKY, we fused EGFP to the N terminus of QKY under the control of the endogenous QKY promoter (pQKY:EGFP:QKY). Thirty out of 33 pQKY:EGFP:QKY qky-8 transgenic lines showed full or partial rescue, indicating the presence of a functional reporter (supplementary material Fig. S3). Despite rescue, the signal could not be detected in floral organs, indicating its very low expression in those tissues. However, signal could be observed in the root using a confocal microscope equipped with high sensitivity detectors (HSD). We also selected the UBQUITIN 10 (UBQ10) promoter to drive expression of the EGFP:QKY reporter and generated 26 qky-8 and 11 Ler lines transgenic for the pUBQ:EGFP:QKY (EGFP:QKY) reporter. EGFP:QKY qky-8 (24/26) lines showed full rescue of the qky-8 phenotype (supplementary material Fig. S3). In addition, we found no evidence that enhanced expression of EGFP:QKY in qky-8 or Ler backgrounds caused abnormal morphology or development. Although signal intensity varied the spatial distribution of the signal did not differ across transgenic lines. Both reporters exhibited identical subcellular signal distribution patterns.

Using the pQKY::EGFP:QKY reporter, we detected a broad signal distribution throughout the root (Fig. 2A,B). At the subcellular level, a punctate pattern around the circumference of cells was observed suggesting labeling of specific sites, such as vesicle docking sites or PD (Fig. 2C-D). This EGFP:QKY signal distribution looked similar in all tissues tested (Fig. 2E-G,M-O) (supplementary material Fig. S4). To test whether the punctate EGFP:QKY pattern relates to PD, we investigated the root tip and cotyledon epidermis of EGFP:QKY qky-8 seeds stained with aniline blue (AB), a standard stain for callose associated with PD (Bell and Oparka, 2011). As shown in Fig. 2E-H, M-P EGFP:QKY coincides with AB-derived spots, indicating that EGFP:QKY localizes close to or at PD. Using software-based image analysis we determined that 89% of EGFP:QKY spots overlapped with AB punctae in root epidermis cells (Pearson’s correlation coefficient=0.79).

Previous work using conventional confocal microscopy revealed that SUB resides at the plasma membrane (PM) (Kwak and Schiefelbein, 2008; Yadav et al., 2008). In the light of a possible PD localization of QKY, we re-examined the subcellular signal distribution of a functional SUB:EGFP reporter driven by the endogenous promoter (pSUB:SUB:EGFP, gSUB:EGFP), whose expression fully overlaps with the reported SUB expression in flowers and roots (Vaddepalli et al., 2011), using a HSD-equipped confocal microscope. In addition to a visible PM localization, we also detected punctate signal at the circumference of cells in all tissues tested (Fig. 2I-L,Q-T) (supplementary material Fig. S5). Interestingly, those spots colocalized with signals derived from AB staining. In root epidermal cells, image analysis revealed that 88 percent of the SUB:EGFP spots overlapped with AB punctae (Pearson’s correlation coefficient=0.76). These results indicate that SUB:EGFP not only localizes to the PM but is also enriched at PD.

To confirm that EGFP:QKY and SUB:EGFP are positioned at PD, we performed immunogold electron microscopy. Depending on the section EGFP:QKY-derived signals were seen in either the neck region or to central domain of a plasmodesma (Fig. 2U-V). The results reveal the presence of EGFP:QKY at PD and suggest a broad distribution in individual PD. Likewise, SUB:EGFP signal was also seen in PD (Fig. 2W,X). Thus, EGFP:QKY and SUB:EGFP were both detected at PD by immunogold microscopy.

QKY does not affect the subcellular localization of SUB:EGFP

The data so far presented further strengthen a functional connection between SUB and QKY. Both genes are components of SUB signaling that contribute to the formation or relay of a non-cell-autonomous mobile signal and the two proteins localize to PD. Next, we investigated at what level SUB and QKY interact. The two genes do not appear to regulate each other at the transcriptional level (Fulton et al., 2009) (supplementary material Fig. S6). Thus, we tested whether QKY affects the subcellular localization of SUB:EGFP. We generated 42 gSUB:EGFP qky-8 transgenic lines and showed normal tissue-specific and PM localization of reporter signal in roots and young ovules (Fig. 3A-F). We also examined the...
subcellular SUB:EGFP signal in root epidermal cells and confirmed an unaltered punctate pattern (Fig. 3G,H). QKY is therefore not required for localizing SUB:EGFP to the PM or PD.

**SUB is not an upstream regulator of QKY during SUB-dependent signaling**

During the course of investigating gSUB:EGFP expression in qky-8, we noticed an interesting effect of the gSUB:EGFP transgene on the qky-8 phenotype. Thirty-seven out of 42 of the gSUB:EGFP qky-8 T1 plants exhibited a mild but reproducible reduction of the qky-8 mutant phenotype. Increasing transgene copy number by generating T2 lines slightly improved this effect, although the qky-8 phenotype was never fully rescued (Fig. 3I-K). Only 5/42 lines showed no rescue. Furthermore, expression of the nonfunctional reporter gSUBR599C:EGFP, which carries a mutated SUB kinase domain (Vaddepalli et al., 2011), ablated the observed rescue of the qky-8 phenotype (0/18 T1 lines) (Fig. 3L). These genetic results imply that QKY acts upstream of SUB or that QKY and SUB act in parallel on a common downstream target (see also below).

**SUB does not influence plasmodesmatal localization of EGFP:QKY**

On the basis of the previous findings, one would expect that SUB does not affect the subcellular localization of QKY. Indeed, no changes were detected in the EGFP:QKY signal in roots or carpel walls of sub-1 mutants (Fig. 4A-C) (12 transgenic lines). Moreover, the EGFP:QKY transgene did not rescue the sub-1 phenotype (Fig. 4D-F). These results suggest that the subcellular localization of EGFP:QKY occurs independently of SUB.

**Targeting of SUB:EGFP to PD requires the extracellular domain in combination with the transmembrane domain**

A number of functionally characterized RLKs have been reported to be present at PD, including CRINKLY 4 (CR4) (Tian et al., 2007), ARABIDOPSIS CRINKLY 4 (ACR4) and CLAVATA 1 (CLV1) (Stahl et al., 2013), FLAGELLIN-SENSITIVE 2 (FLS2) (Faulkner et al., 2013), and SUB (this study). In addition, RLKs are frequently found in catalogues of PD proteomes of *Arabidopsis* and rice (Fernandez-Calvino et al., 2011; Jo et al., 2011). It is unknown,
however, how RLKs are targeted to PD. To obtain insights into the mechanism, we sought to identify domains of SUB that are involved in this process. We investigated the subcellular localization of a set of mutant cDNA-based SUB:EGFP reporters (cSUBdel:EGFP) in stably transformed plants (Fig. 5). All the tested mutations result in the absence of SUB activity (Vaddepalli et al., 2011). We observed normal signal distribution in lines expressing reporters lacking either the kinase domain or the entire intracellular domain of SUB (Fig. 5A,B). This result indicates that the intracellular domain is dispensable for PD localization of SUB. In lines carrying a reporter lacking the extracellular domain (ECD) of SUB, but including the transmembrane domain (TMD) (cSUBΔECD:EGFP), we observed a stronger signal around the circumference of the cell. However, no punctate spots were detected. Furthermore, a weak signal distributed in an endoplasmic reticulum (ER)-like pattern was detected (Fig. 5C). The result is compatible with plasma membrane (PM) localization of the reporter and suggests that the ECD is required for localization of SUB to PD. It further indicates that absence of the ECD renders transport through the secretory pathway inefficient. To test whether the ECD is sufficient to target SUB to PD we analyzed the signal distribution of a reporter carrying only the ECD fused to EGFP (cSUBΔTM-Intra:EGFP) (Fig. 5D). We observed aggregates inside the cell, indicating that the ECD is insufficient for passage through the secretory pathway. The targeting of SUB to PD thus requires the ECD in combination with the TMD.

Identification of domains targeting EGFP:QKY to PD

The QKY homolog FTIP1 was reported to reside at PD of phloem cells, where it interacts with the long-day flowering signal FLOWERING LOCUS T (FT) (Liu et al., 2012). Moreover, an additional five QKY family members have been identified in the Arabidopsis PD proteome (Fernandez-Calvino et al., 2011). These findings indicate that the QKY family represents a novel class of PD-localized proteins. It is unknown how members of this family get targeted to PD. We investigated whether specific domains of QKY are required for this process. We generated stable transgenic qky-8 plants carrying a set of reporters encoding progressive N-terminal deletions of QKY under the control of the endogenous QKY promoter (pQKY::EGFP:QKYdel). None of the mutant reporters was able to rescue the qky-8 phenotype and the signal was very weak (not shown). Thus, we used equivalent reporters driven by the UBQ10 promoter and analyzed the corresponding subcellular signal patterns in root epidermal cells (Fig. 6). We observed normal punctate subcellular signal in lines carrying the EGFP:QKYΔC2A, EGFP:QKYΔC2AL and EGFP:QKYAA-B reporters (Fig. 6A-C). However, a faint ER-like pattern also became
apparent. Those fusion proteins feature progressive deletions of the N-terminal C2 domains (Fig. 6H). Interestingly, in lines carrying the EGFP:QKYΔC2A-D fusion proteins, we could still discern a PM-like staining but no punctate pattern (Fig. 6D,E). The ER-like pattern became even more obvious. The results indicate that the C2C, and possibly the C2D, domains are important for targeting EGFP:QKY to PD. By contrast, the N-terminal region up to and including the C2B domain is not essential for this process. Moreover, the C2 domains appear important for efficient transport of QKY to the PM. The results further suggest that the PRT_C domain is necessary and sufficient for PM localization. To test this idea, we analyzed lines expressing a EGFP:QKYΔPRT_C reporter that lacks the PRT_C domain. We observed both ER-like and cytoplasmic signal distribution, and a reduced PM localization (Fig. 6F). This result suggests that the PRT_C domain is necessary for PM localization of EGFP:QKY. The ER-like signal accumulation further indicates that, in the absence of the PRT_C domain, the remaining fusion protein can associate with the ER. Finally, we detected a weak and diffuse cytoplasmic signal for gEGFP:QKY-L (Fig. 6G). In summary, the correct targeting of QKY to PD requires the C2C and/or C2D domains in combination with the PRT_C domain.

**SUB:EGFP and mCherry:QKY interact physically in planta**

So far the data suggest specific localization of EGFP:QKY to PD, whereas SUB:EGFP is found both at the PM and PD. To test whether QKY and SUB colocalize to the same PD, we analyzed the signal distribution in transgenic lines carrying a functional pQKY::mCherry:QKY (mCherry:QKY) reporter (32/35 pQKY::mCherry:QKY gky-8 transgenic lines showed rescue, supplementary material Fig. S3), as well as the gSUB:EGFP construct. We detected signal overlap in a punctate pattern (Fig. 7A-C), indicating that SUB:EGFP and mCherry:QKY are present at the same PD. This finding raised the possibility that the two proteins are in close contact.

To test whether SUB and QKY proteins can interact directly, we performed a yeast two-hybrid (Y2H) assay using the C2-domain-containing region of QKY (QKYΔPRT_C, lacking the PRT_C domain) as bait and either the ECD or the intracellular domain (ICD) of SUB as prey. On selective medium, yeast growth was observed when QKYΔPRT_C was combined with the ICD of SUB (Fig. 7D), demonstrating that QKY can interact with the ICD of SUB in this assay, extending previous results (Trehin et al., 2013).

To investigate whether SUB:EGFP and mCherry:QKY can interact physically in planta, we performed Förster Resonance Energy Transfer – Fluorescence Lifetime Imaging Microscopy (FRET–FLIM) assays using root epidermis cells of 7-day Arabidopsis seedlings stably transformed with the gSUB:EGFP reporter or with gSUB:EGFP and functional pUBQ::mCherry:QKY (mCherry:QKY) reporter constructs (38/40 pUBQ::mCherry:QKY gky-8 transgenic lines showed rescue). Using a pulsed multi-photon laser, FLIM allows determination of the fluorescence lifetime (τ) of the donor fluorophore in a time-resolved manner. In this intensity-independent assay, an interaction between two proteins (distance <10 nm) is characterized by a reduction in the lifetime of the donor fluorophore (EGFP) owing to an energy transfer to the acceptor (mCherry). It should be noted that the lifetime of the donor greatly depends on the cellular environment and compartment (Nakabayashi et al., 2008; van Manen et al., 2008).

To restrict measurements to plasma membrane-derived fluorescent signals, we first measured mean fluorescence lifetime values along the plasma membranes of multiple root epidermal cells and determined a mean τ value of 2263.6±9.1 ps (mean±s.e.m., n=41) in plants that only express SUB:EGFP under control of its native promoter. In the presence of mCherry:QKY, an overall τ value of 2243.3±6.8 ps (n=42) was found, revealing no significant reduction in fluorescence lifetime (Student’s t-test, P<0.04; FRET efficiency: 0.9%). However, inspection of the corresponding FLIM CLSM images clearly suggested a non-uniform reduction in fluorescence lifetime restricted to specific punctae (Fig. 7E). These punctae colocalized with the brighter spots labeled by SUB:EGFP and therefore likely represent PD (Fig. 7F). We re-analyzed the
fluorescence lifetime of SUB:EGFP within these spots. We determined a $\tau$ value of 2123.8±6.05 ps ($n=205$), indicating that the fluorescence lifetime of SUB:EGFP alone in these spots was different compared with the overall fluorescence lifetime ($P=6.8e^{-20}$). Importantly, however, in the presence of mCherry:QKY, we measured a $\tau$ value of 2075.5±4.98 ps ($n=210$). Thus, the PD-confined fluorescence lifetime of SUB:EGFP was reduced in a statistically significant manner ($P=7.9e^{-10}$), revealing a FRET efficiency of 2.3% when compared with 0.9% in the overall plasma membrane control. Taken together, the data indicate that SUB:EGFP and mCherry:QKY interact in epidermal cells of intact Arabidopsis roots and that the interaction occurs at PD.

**DISCUSSION**

The available data support a functional connection between $SUB$ and $QKY$. How do $SUB$ and $QKY$ relate in the context of $SUB$ signaling? Increasing the copy number of $SUB$ partially bypasses the requirement for $QKY$, indicating that $QKY$ acts upstream or in parallel to $SUB$. The result further suggests that $QKY$ enables $SUB$ function in a more quantitative rather than a strictly qualitative manner. Transcription of the two genes is regulated independently of each other, as is the subcellular localization of $SUB$ and $QKY$. Our data further indicate that the two proteins are in close contact in vivo. Thus, one way to explain the genetic observation is that a physical interaction between $QKY$ and $SUB$ renders $SUB$ more active. In the absence of $QKY$ function, this condition can be partially circumvented by increasing $SUB$ dose.

Our data suggest that $QKY$ and $SUB$ colocalize to and physically interact at PD. Although the presence of $SUB$ is not constrained to PD, localization of $QKY$ appears to be restricted to PD. With respect to the subcellular localization of $QKY$, our results differ from a recent study, that reported $QKY$ to be present at the PM (Trehin et al., 2013). Trehin et al., however, used a nonfunctional reporter carrying EGFP at the C terminus of $QKY$. Here, we fused EGFP to the N terminus of $QKY$, which resulted in a functional EGFP:QKY reporter. This discrepancy suggests that a C-terminal fusion of EGFP to $QKY$ disrupts $QKY$ function and impairs PD localization. It indicates that PD localization of $QKY$ is essential for its function. Our results are also in agreement with findings of a recent proteomic study, which identified $QKY$ and $SUB$ as components of PDs (Fernandez-Calvino et al., 2011).

How PD-localized RLKs are targeted to PD is unknown. Our results indicate that the ECD, in combination with the TMD, is sufficient to target the protein to PD (Thomas et al., 2008). In the case of PDLP1α, the TMD was sufficient to target the protein to PD (Thomas et al., 2008). These results indicate that targeting different types of type I membrane proteins to PD involves different mechanisms. Regarding $SUB$, our findings are in accordance with at least two models. In one scenario, the TMD anchors the $SUB$ RLK to the plasma membrane (PM) while the ECD, through interactions with as yet unknown PD-targeting factors, is instrumental for enriching $SUB$ at PD. Alternatively, PD localization of $SUB$ requires interaction of...
PD-targeting factors with both the ECD and TMD domains. QKY is likely not among those factors, as our genetic results revealed that SUB localizes to PD independently of QKY. In future studies, it will be interesting to identify the PD-targeting factors involved in positioning SUB at PD.

The mechanism that targets QKY family members to PD is also unknown. With respect to QKY this study could not assign the role of PD-targeting to any one domain. Rather, like for SUB, a more complex scenario is implicated. Thus, the PRT_C domain, which includes the putative TMDs, may first anchor QKY to the PM, and interaction of a PD-targeting factor with either C2C alone or a combination of C2C and C2D results in localizing QKY to PD. Alternatively, the hypothetical PD-targeting factor must interact with both, PRT_C and the C2C/C2D domains. The latter may explain the PM localization observed for a nonfunctional reporter carrying a fusion of EGFP to the C terminus of PRT_C (QKY:EGFP) (Trehin et al., 2013).

The non-cell autonomy of SUB and QKY indicates that both genes function in the production and/or propagation of an unknown SUB-dependent mobile signal (SMS). It is curious that the two genes act non-autonomously, although they are broadly expressed in tissues, such as floral meristems or ovule primordia. There is precedence for this observation, however, because BRRII and LFTY (for example) also function in a non-autonomous fashion notwithstanding their broad expression (Sessions et al., 2000; Wu et al., 2003; Savaldi-Goldstein et al., 2007). Regarding SUB signaling, one possible explanation is that SUB and QKY may exert a homeostatic function, ensuring distribution throughout the tissue of important factors that potentially occur in very low amounts in a given cell. This notion may also explain the lack of polarity in SUB/QKY non-cell autonomy.

Several scenarios could principally account for the presence of SUB and QKY at PD. For example, the specific colocalization of QKY and SUB at PD is compatible with the notion that SUB signaling influences trafficking of molecules through PD, as was suggested for other RLKs (Tian et al., 2007; Stahl et al., 2013). The non-cell autonomy of QKY and SUB indicates that SUB signaling would support passage of the SMS signal through PD, rather than blocking it. In support of this, there is genetic evidence that the QKY family member FTIP1 promotes movement of FT through PD (Liu et al., 2012). The nature of the proposed SMS signal is unknown. The transcription factors CAPRICE (CPC) and GLABRA3 (GL3) would be obvious candidates, as the two proteins are known to travel laterally between root epidermal cells during root hair patterning (Wada et al., 2002; Bernhardt et al., 2005; Kurata et al., 2005; Savage et al., 2008). However, SUB does not affect the movement and function of CPC or GL3 (Kwak and Schiefelbein, 2007). SUB signaling is unlikely to render PD generally more penetrable, as movement of GFP, known to pass through PD by diffusion (Oparka et al., 1999; Crawford and Zambrisky, 2000), is not altered in sub or qky mutants (supplementary material Figs S7-S9). Thus, if this hypothesis is valid, SUB signaling must promote selective passage of a factor yet to be identified. Alternatively, PD could simply provide convenient structural platforms for functional clustering of SUB and QKY, as the PM within PD exhibits special characteristics, similar to membrane rafts (Maule et al., 2011; Tilsner et al., 2011). In this view, the microenvironment at PD would foster physical interaction between SUB and QKY, resulting in the subsequent initiation of further downstream processes. One conceivable response could comprise secretion of the SMS factor to neighboring cells via vesicular transport and passage across the cell wall. Another feasible response could involve alteration of cell wall mechanics (Fulton et al., 2009). It will thus be interesting to explore the exact mechanism by which SUB signaling orchestrates tissue morphogenesis in future studies.

MATERIALS AND METHODS

Plant work

Arabidopsis thaliana (L.) Heynh. var. Columbia (Col-0) and var. Landsberg erecta (erecta mutant) (Ler) were used as wild-type strains. Plants were grown essentially as described previously (Fulton et al., 2009). The EMS-induced sub-1 and qky-8 mutations (Ler) and the T-DNA induced qky-11 (SALK_043901, Col) mutations have been described previously (Chevalier et al., 2005; Fulton et al., 2009). Wild-type, sub-1 and qky-8 plants were transformed with different constructs using Agrobacterium strain GV3101/pMP90 (Koncz and Schell, 1986) and the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were selected on either kanamycin (50 μg/mL) or hygromycin (20 μg/mL) plates and transferred to soil for further inspection.

Recombinant DNA technology
For DNA and RNA work, standard molecular biology techniques were used. PCR fragments used for cloning were obtained using Phusion high-fidelity DNA polymerase (New England Biolabs, Frankfurt, Germany) or TaKaRa PrimeSTAR HS DNA polymerase (Lonza, Basel, Switzerland). PCR fragments were subcloned into pJET1.2 using the CloneJET PCR cloning kit (Fermentas) or into pCR-TOPO (Invitrogen). All PCR-based constructs were sequenced. The plasmid pCAMBIA2300 (www.cambia.org) and the Gateway-based (Invitrogen) destination vector pMDC32 (Curtis and Grossniklaus, 2003) were used as binary vectors. Primer sequences used in this work are listed in supplementary material Table S1.
**ProUBQ10:EGFP:QKY.** All clones were verified by sequencing. PCR using primers QKY linker/Spe1_F and QKY/Pst1/R and cloned into QKY 3 EGFP:QKY. For EGFP:QKY-L, the linker fragment was amplified with the following primers: C2A/Del_F, C2AB/Del_F, C2B/ala/Spe1_F, C2ABC/DEL_F, C2ABCD/DEL_F and qky/Pst1/R. Amplified PCR products were digested with SpeI/PstI and subcloned into ProUBQ10:EGFP:QKY. For EGFP:QKY-L, the linker fragment was amplified with QKY linker/Spe1_F, QKY linker_R and the 3′ UTR was amplified with QKY 3′UTR_F and QKY/Pst1/R. Fragments were fused by overlapping PCR using primers QKY linker/Spe1_F and QKY/Pst1/R and cloned into ProUBQ10:EGFP:QKY. All clones were verified by sequencing.

**Yeast two-hybrid assay**

pGBKTT7 plasmids (Matchmaker, Clontech) containing either the SUB extracellular (ECD) or intracellular (ICD) domain (Bai et al., 2013) were co-transformed with pGADT7-QKYAPRT_C plasmid [obtained by PCR-based cloning using primers NdeI/QKY(-TM)_F and QKY(-TM)Xmal_R] into the yeast strain AH109. After 3 days, transformants were selected on synthetic complete (SC) medium lacking leucine and tryptophan (-LW) at 30°C. To examine Y2H interactions, the transformants were grown on solid SC medium with a spatial resolution of 256x256 pixel. Fluorophore lifetimes at the plasma membranes were determined using SPCImage software (Becker & Hickl) and image-adapted settings. A total of 40 images from a minimum of 12 independent roots were acquired and evaluated. For PD-confined lifetime analysis, five individual spots were analyzed per image, resulting in over 200 measurements per genotype. Data were statistically analyzed using Student’s t-test. In situ hybridization with digoxigenin-labeled probes have been described previously (Sieber et al., 2004). To generate the QKY probe, a 2.1 kb fragment, spanning the 5′ part of the coding sequence, was amplified from genomic DNA using primers P-UBQ (Kpn1)_For/P-UBQ (Asc1)_rev and Ala_Qky_F/(At1g74720) coding sequence was amplified from L SUB:EGFP (Yadav et al., 2008) using primers pWUS::SUB:3xmyc (Yadav et al., 2008) and pML1 [3.4 kb, amplified from plasmid gSUB:EGFP].

**Microscopy, in situ hybridization and art work**

Confocal laser scanning microscopy using staining with Aniline Blue or with pseudo-Schiff propidium iodide (mPS-PI), or detection of EGFP and FM4-64 was performed primarily as described (Schneitz et al., 1997; Sagi et al., 2005; Truernit et al., 2008; Yadav et al., 2008; Fulton et al., 2009). In some instances, confocal high sensitivity detection (HSD) was employed involving two gallium arsenide phosphide photomultipliers (GaAsP PMTs) mounted equidistantly to the probe. Colocalization studies were carried out using the respective module of the confocal microscope software (Olympus, Fluoview 4.1a). Approximately 14 ROIs (rectangle selection tool, length of 5-8 μm) were randomly placed across different image areas depicting plasma membrane regions. Histochemical localization of β-glucuronidase (GUS) activity in whole-mount tissue and scanning electron microscopy were performed as reported previously (Jefferson et al., 1988; Neitzet et al., 1997; Sieburth and Meyerowitz, 1997). High-pressure freezing and immersion electron microscopy have also been described (Schneitz et al., 2011; Hillmer et al., 2012). Immunogold labeling was performed as described previously (Schneitz et al., 2011) using a GFP antiserum at a dilution of 1:600 or 1:1000 in PBS. Sections were observed in a JEOL JEM4100 transmission electron microscope operating at 80 kV and images were taken with a Fastscan-F214 CCD camera (TVIPS, Gauting, Germany). Contrast and brightness were adjusted in 16-bit images using the software of the camera. Procedures for FRET-FLIM analysis and measurements were adapted from (Toth et al., 2012). FLIM was performed using a Leica SP5 confocal microscope with an integrated pulsed (80 MHz) Ti:Sapphire laser (Mai Tai) for multi-photon excitation and a fast FLIM photomultiplier (Becker & Hickl, Berlin, Germany). Two-photon excitation of the EGFP donor fluorophore was achieved at 900 nm and fluorescence was detected at 500-580 nm. Time correlated spectral counting was performed for a maximum of 5 min and 64 scanning cycles with a spatial resolution of 256x256 pixel. Fluorophore lifetimes at the plasma membranes were determined using SPCImage software (Becker & Hickl) and image-adapted settings. A total of 40 images from a minimum of 12 independent roots were acquired and evaluated. For PD-confined lifetime analysis, five individual spots were analyzed per image, resulting in over 200 measurements per genotype. Data were statistically analyzed using Student’s t-test. In situ hybridization with digoxigenin-labeled probes have been described previously (Sieber et al., 2004). To generate the QKY probe, a 2.1 kb fragment, spanning the 5′ part of the coding sequence, was amplified from genomic DNA using primers P-UBQ (Kpn1)_For/P-UBQ (Asc1)_rev and Ala_Qky_F/(At1g74720) coding sequence was amplified from L SUB:EGFP (Yadav et al., 2008) using primers pWUS::SUB:3xmyc (Yadav et al., 2008) and pML1 [3.4 kb, amplified from plasmid gSUB:EGFP].

**Author contributions**


**Acknowledgements**

We thank Keko Tori for the pKUT196 construct, Xuelin Wu and Detlef Weigel for the pML1-2xGFP and pML1::NLS:GFP plasmids, Christine Faulkner and Andrew Maule for the pPDLP3-PLP3:GFP construct, and Ruth Stadler for the pSUC2::GFP and pSUC2::tmGFP9 plasmids. We further acknowledge Ruth Stadler for helpful comments on the manuscript and members of the Schneitz lab for stimulating discussions.

**Funding**

This work was funded by the German Research Council (DFG) [SCHN 723/6-1 and SFB924 (TP A2) to K.S., SFB924 (TP B4) to T.O. and SFB924 (TP A8) to U.Z.H.] and by the Free State of Bavaria.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.113878/-/DC1

**References**


mediated by the receptor-like kinase STRUBBELIG in Arabidopsis thaliana
Plant Cell 23, 3463-3481.

in Arabidopsis thaliana: a light microscope study of cleared whole-mount tissue.
Plant J. 7, 739-747.

sexual organ ontogeny: a genetic analysis of ovule development in Arabidopsis

MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and

Sessions, A., Yanofsky, M. F. and Weigel, D. (2000). Cell-cell signaling and
movement by the floral transcription factors LEAFY and APETALA1. Science 289, 779-781.

Sieber, P., Gheyeselinck, J., Gross-Hardt, R., Laux, T., Grossniklaus, U. and


Stahl, Y., Grabowski, S., Bleckmann, A., Kühnemuth, R., Weidtkamp-Peters, S.,


Tian, Q., Olsen, L., Sun, B., Lid, S. E., Brown, R. C., Fosnes, K.,


Tóth, K., Strátl, T. F., Madsen, E. B., Ye, J., Popp, C., Antolin-Llovera, M.,

Treinh, C., Schremp, S., Chauvet, A., Berne-Dedieu, A., Thoerry, A.-M., Faure,

Truemn, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthelemay,


van Manen, H.-J., Verkruist, P., Wittendorp, P., Subramaniam, V., van

Wada, T., Kurata, T., Tominaiga, R., Koshino-Kimura, Y., Tachibana, T., Goto, K.,
