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The role of QUIRKY in inter-cellular signaling mediated by the *Arabidopsis* receptor-like kinase STRUBBELIG

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Summary

Tissue morphogenesis in plants requires the coordination of cellular behavior within and across clonally distinct histogenic layers. The underlying extensive signaling mechanisms are presently being unraveled. Determining the molecular basis of inter- and intracellular communication is crucial to understand organogenesis in plants.

In Arabidopsis, the cell surface leucine-rich repeat receptor-like kinase STRUBBELIG (SUB) is involved in tissue morphogenesis of many organs. To understand better its mode of action an extensive structure-function analysis of *SUB* was performed. The phenotypes of 16 EMS -induced *sub* alleles were assessed and homology modeling was applied to rationalize their possible effects on SUB protein structure. The analysis was complemented by phenotypic, cell biological, and pharmacological investigations of a *sub* null allele carrying genomic rescue constructs encoding fusions between various mutated SUB proteins and GFP. The results indicate that *SUB* accepts quite some sequence variation, reveal the biological importance for the SUB N-capping domain, and reinforce the notion that kinase activity is not essential for its function in vivo. Furthermore, individual protein domains of SUB cannot be related to specific *SUB* -dependent biological processes suggesting that process specificity is mediated by factors acting together with or downstream of *SUB*. In addition, the evidence indicates that biogenesis of a functional SUB receptor is subject to endoplasmic reticulum-mediated quality control, and that MG132 and MLN4924 -sensitive processes regulate its stability. The data provide genetic and molecular insights indicating multiple mechanisms contribute for proper *SUB* activity in tissue morphogenesis.

QUIRKY (QKY), ZERZAUST (ZET) and *DETORQUEO (DOQ)* belong to *STRUBBELIG-LIKE MUTANT (SLM)* class of genes, proposed to contribute to *SUB*-dependent signal transduction. In the present work, molecular characterization of *QKY* was undertaken using a combination of genetic, cell biological and biochemical approaches with the main focus on its role in *SUB* mediated signaling. Mapping and molecular identification of *QKY* revealed that it encodes a novel multiple C2 domain containing transmembrane protein (MCTP). Biochemical studies imply that QKY

binds to phospholipids in a Ca^{+2} dependent manner. Gene expression suggests that *QKY* is expressed broadly and has a similar expression domain as *SUB*. Protein localization experiments indicate that QKY is specifically associated with plasmodesmata (PD). Using a deletion analysis, it is shown that the two transmembrane domains (TMDs) of QKY contain all the information necessary for intracellular targeting of QKY to PD. Immunogold electron microscopy results confirm the PD localization of QKY and also reveal the previously unknown PD localization of SUB. Genetic and yeast-two-hybrid data indicate that SUB and QKY can interact directly. However, SUB and QKY do not appear to be involved in the non-selective movement of GFP proteins through PD. Furthermore, localization data also indicate that QKY and SUB are present at the growing cell plate during cytokinesis. Thus, the data imply that SUB signaling mediates tissue morphogenesis either by influencing selective transport of molecules through plasmodesmata or by regulating cytokinesis.

Zusammenfassung

Die Gewebemorphogenese bei Pflanzen benötigt ein koordiniertes zelluläres Verhalten innerhalb und zwischen den histogenen Zellschichten. Die dafür verantwortlichen Signalkaskaden werden zur Zeit intensiv erforscht, um ein molekulares Verständnis der pflanzlichen Organogenese zu erhalten.

In Arabidopsis reguliert die Zelloberflächen-Rezeptorkinase STRUBBELIG (SUB) die Gewebemorphogenese in vielen Organen. Um die Funktion von SUB besser zu verstehen wurde eine extensive Struktur-Funktions-Analyse durchgeführt. Die Phänotypen von 16 EMS-induzierten *sub*-Allelen wurden analysiert. Mit Hilfe des „homology-modeling“ wurde versucht die entsprechenden Effekte auf die Proteinstruktur zu rationalisieren. Die Analyse wurde durch phänotypische, zellbiologische und pharmakologische Untersuchungen von *sub* Nullmutanten, die verschiedene mutierte SUB:GFP Fusionsproteine exprimierten komplementiert. Die Resultate legen nahe, dass SUB eine erstaunliche Menge an Sequenzvariabilität akzeptiert, belegen die biologische Wichtigkeit der N-capping Domäne von SUB und vertiefen die Hypothese, dass Kinaseaktivität von SUB nicht von essentieller Bedeutung für seine in vivo Funktion ist. Des Weiteren konnten keine SUB-Domänen mit speziellen biologischen Prozessen assoziiert werden. Das legt den Schluss nahe, dass Prozessspezifität von zusätzlichen Faktoren bestimmt wird, die entweder zusammen mit SUB oder unterhalb von SUB in der Signalkaskade agieren. Zusätzlich konnte gezeigt werden, dass die Biogenese eines funktionellen SUB-Rezeptors durch die endoplasmatische Retikulum-vermittelte Qualitätskontrolle reguliert wird und dass MG132 und MLN4924-sensitive Prozesse die Stabilität des SUB Proteins regulieren. Die Resultate liefern genetische und molekulare Einblicke und schlagen vor, dass mehrere unterschiedliche Prozesse die SUB-Aktivität während der Gewebemorphogenese steuern.

QUIRKY (*QKY*), *ZERZAUST* (*ZET*) und *DETORQUEO* (*DOQ*) gehören zur *STRUBBELIG-LIKE MUTANT* (*SLM*) Klasse von Genen die wohl in der *SUB*-abhängigen Signalvermittlung eine Rolle spielen. In dieser Arbeit wurde mittels einer Kombination von genetischen, zellbiologischen und biochemischen Ansätzen eine

molekulare Charakterisierung von *QKY* durchgeführt. Der Hauptfokus lag auf einer Untersuchung der Rolle von QKY in der SUB-Signalkaskade. Die Kartierung und die molekulare Identifizierung zeigte, dass *QKY* für ein neuartiges Protein kodiert, das vier C2- und zwei Transmembrandomänen trägt (ein MCTP-Klassen Protein). Biochemische Studien zeigten, dass QKY Phospholipide in einer Kalzium-abhängigen Art und Weise bindet. Genexpressionsstudien zeigten, dass *QKY* eine breite Expressionsdomäne besitzt, die mit der von *SUB* überlappt. Proteinlokalisationexperimente zeigten, dass QKY spezifisch mit Plasmodesmata (PD) assoziiert ist. Mittels einer Deletionsanalyse konnte gezeigt werden, dass die beiden Transmembrandomänen die gesamte Information für diese Lokalisation tragen. Transmissionselektronenmikroskopie gekoppelt mit Immunogold-Markierungsverfahren bestätigten eine Lokalisation von QKY an PD und zeigten auch eine vorher nicht bekannte Lokalisation von SUB an PD. Genetische und „yeast two-hybrid“ Methoden legen den Schluss nahe, dass SUB und QKY direkt interagieren. Jedoch spielt SUB keine Rolle in der freien Diffusion von GFP-Proteinen durch PD. Des Weiteren deuten zusätzliche Studien auch auf eine Lokalisation dieser beiden Proteine im wachsenden Phragmoplast hin. Die erhaltenen Daten deuten daraufhin, dass die *SUB*-Signalkaskade entweder den spezifischen regulierten Transport von Molekülen durch PD oder die Zytokinese reguliert.

Table of contents

1 Introduction

In order to achieve complex adult body comprised of different tissues and organs multicellular eukaryotes require organized cell divisions and cell specification. Inter and intra cellular communication plays a crucial role in organogenesis. In plant cells, the situation is further complicated because of the presence of cell wall. At the same time the task should be easier in plants compared to animals as cell-division patterns are readily traced and plant cells do not move relative to each other (Meyerowitz, 1997). Organogenesis in plants is a post-embryonic event. In higher plants, shoot apical meristem is the ultimate source for all the aboveground organs like leaves, flowers and floral organs (Taylor A. Steeves, 1989).

1.1 Meristems, cell-to-cell communication and tissue morphogenesis

Meristems, consisting of undifferentiated cells, are organized into three clonally distinct histogenic or meristem layers (Satina et al., 1940; Tilney-Bassett, 1986). The L1/ L2 and the L3 layers are referred to as tunica and corpus, respectively (Figure 1.1.1). Cells of the outer epidermal L1 and subepidermal L2 divide anticlinally (divide perpendicularly to the surface), thereby maintaining separate layers. The outermost L1 layer will form epidermis, and an underlying L2 layer will produce the subepidermal tissues of the stem and lateral organs. Cells of the L3 divide in an apparently random fashion and make up the inner core of the meristem. L3 layer cells differentiate into the pith of the stem and interior tissues of organs.

Despite the fact that the histogenic layers are clonally separated in wild-type development, there is sufficient evidence from chimeric plants to show that cells develop according to their actual position but not by their origin. For example, an L1 cell relocated into the subepidermal layer will eventually develop into a subepidermal cell type (Stewart and Dermen, 1970). Apart from this, there must be additional co-ordination between the meristematic layers during their growth and differentiation so that they maintain the shape of meristem and produce normal organs. For instance, the

integuments of Arabidopsis ovules that eventually develop into the seed coat are entirely made up of L1-derived cells, while L2 cells generate the inner tissue (Satina et al., 1940). Taken together, these data imply that cells of meristematic tissue communicate extensively to coordinate their behavior (Haecker and Laux, 2001). Genetic and molecular investigations have revealed a complex network of signaling between cells within the meristems and between meristems and other parts of the plant (Gallagher and Benfey, 2005; Ingram and Waites, 2006).

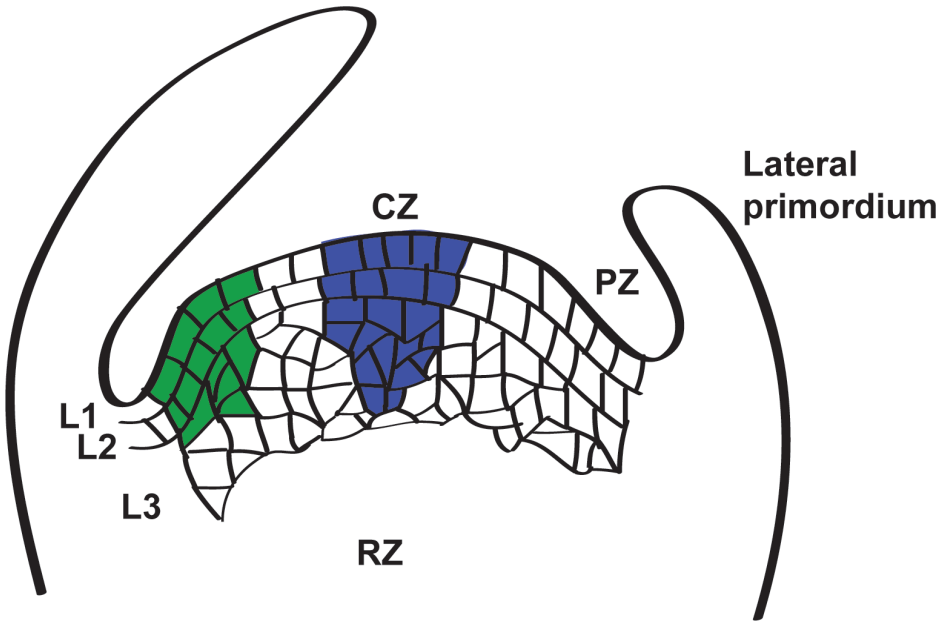


Figure 1.1.1 Organization of apical meristem

The epidermal (L1) and subepidermal (L2) layers form the outer layers called the tunica. The inner L3 layer is called the corpus. Cells in the L1 and L2 layers divide anticlinally, which keeps these layers distinct, whereas the L3 layer divides in a more random fashion and make up the inner core of the meristem. CZ: Central zone, PZ: Peripheral zone, RZ: Rib zone.

In animals two major types of cell-to-cell communication play important role in cell fate specification and differentiation: ligand-receptor mediate systems on the cell surface (Hubbard and Miller, 2007; Lemmon and Schlessinger, 2010) and gradients of signaling molecules that play crucial role during patterning and organ development (Bhalerao and Bennett, 2003; Smith et al., 2008). Plants share not only these mechanisms but also a third mechanism of intercellular communication, because of the presence of cellulosic walls surrounding each individual cell, the symplastic pathway via channels that span the walls between adjacent cells called plasmodesmata.

1.2 Role of plasmodesmata in cell-to-cell communication of plants

Extensive studies in plants have established that cell-to-cell communication involves the intercellular trafficking of regulatory proteins, RNAs and protein-RNA complexes through the plasmodesmata (PD) and allows non-cell autonomous regulation of plant development (Lucas et al., 2001; Lucas and Lee, 2004; Oparka, 2004; Kim, 2005; Gallagher and Benfey, 2005). PD can be considered as two concentric cylinders, which connect the plasma membrane, cytoplasm, and endoplasmic reticulum (ER) of neighboring cells. The plasma membrane lines the outer cylinder of the PD, whereas the ER forms an internal cylinder (desmotubule) and links the ER of two adjacent cells. Thus, the PDs provide multiple routes for intercellular communication (Roberts and Oparka, 2003; Blackman and Overall, 2001). Non-selective or non-targeted cell-to-cell movement of macromolecules through the PD can be achieved by simple diffusion. Targeted trafficking of macromolecules, however, requires the interaction of proteins with PD or associated proteins to increase the size exclusion limit (SEL) of PD for their movement. The factors, which are involved in movement of macromolecules through plasmodesmata, are under investigation. PDs are classified as primary, if formed during cytokinesis, or secondary, when synthesized through an existing cell wall. The secondary PDs are important for connecting cells that do not share a recent division wall. The size of PD aperture fluctuates from closed to open to dilate during different stages of post-embryonic development (Kim et al., 2005; Kim et al., 2005).

1.2.1 Non-selective movement of GFP and LEAFY

Examples of simple diffusion (non-targeted) are illustrated with cytoplasmically localized GFP (Crawford and Zambryski, 2000) and the transcription factor, LEAFY (LFY) (Wu et al., 2003). In transgenic Arabidopsis, 27-kDa soluble green fluorescent protein (1×GFP) moves between cells throughout the entire embryos while 2×GFP movement becomes more restricted as development proceeds. This indicates that PD in younger tissues are more dilated and less obstructive than PD in older tissues (Kim and Zambryski, 2005). Moreover cells in seedling shoot apical

meristems (SAM) have a higher size exclusion limit (SEL) and allow 2×GFP movements. The cells surrounding the SAM that differentiates into specific cell types show more restricted movement of 2×GFP than 1×GFP. This indicates that the PD aperture is regulated spatially and temporally throughout development (Kim et al., 2005). Cells safeguard the cell-specific regulation of diffusible proteins by the localization of proteins to specific subcellular sites or by multimerization. GFP movement is restricted in Nicotiana leaf when an ER specific signal was added to GFP. It reduces the extent of protein movement from source cell. Addition of a nuclear localization signal (NLS) signal to 2×GFP almost blocked the protein movement (Crawford and Zambryski, 2000).

LFY is a plant-specific transcription factor required for SAM transition from vegetative to reproductive development (Weigel et al., 1992). Normally, *LFY* mRNA and protein both are expressed in all three layers of floral primordia (L1, L2, and L3). Surprisingly, expression of *LFY* in just L1 layer (epidermis) was sufficient for its function indicating LFY protein is able to travel into the L2 and L3 layers and rescue the *lfy* phenotype (Sessions et al., 2000). Thus, it is possible that movement of LFY protein provides only a redundant mechanism to ensure complete conversion of a meristem into a flower. Moreover the movement of LFY (47kDa) is limited in the shoot apical meristem of Arabidopsis similar to 2×GFP. Deletion studies spanning to LFY, N-terminus, C-terminus or in between N and C-terminus domains, indicates that the cytoplasmic localization of truncated protein was sufficient for protein movement. There is no specific domain required for LFY movement suggesting LFY cell-to-cell movement is non-targeted (Wu et al., 2003).

1.2.2 Selective movement of transcription factors

The targeted trafficking of endogenous transcription factors plays an important role in cell fate specification. For example, KNOTTED1 (KN1), a homeodomain protein of maize, was the first transcription factor found to traffic from cell-to-cell selectively through PD (Lucas et al., 1995; Kim et al., 2002). Mosaic analysis of a dominant KN1 allele showed that it acts non-autonomously during maize leaf

development (Hake and Freeling, 1986). Further studies with a loss-of-function mutant suggested that KN1 controls the maintenance of shoot meristem (Vollbrecht et al., 1991; Kerstetter et al., 1997). GFP:KN1 fusion protein expressed in the leaf perivascular bundles was able to traffic through the mesophyll to epidermis. But when GFP:KN1 was expressed in leaf epidermis, it was unable to move to mesophyll cells, indicating a directional regulation of protein trafficking (Kim et al., 2003). However, GFP:KN1 showed bidirectional protein trafficking in SAM. Thus, the movement of KN1 is regulated in a tissue-specific manner. Deletion studies of KN1 revealed a homeodomain in the protein, which is required for cell-to-cell trafficking of KN1 protein in planta (Kim et al., 2005). A recent study showed that chaperonins are essential for the cell-to-cell trafficking of KN1 and demonstrates the importance of chaperonin-dependent protein trafficking for plant stem cell function (Xu et al., 2011). The Arabidopsis orthologs of KN1, KNOTTED 1-like homeobox protein 1/BREVIPEDICELLUS (KNAT1/BP) and SHOOTMERISTEMLESS (STM) are also shown to traffic from cell-to-cell (Kim et al., 2002; Kim et al., 2003). STM and KN1 are involved in shoot apical meristem (SAM) initiation and maintenance whereas KNAT1/BP is required for regulation of inflorescence architecture.

The Arabidopsis root cell pattern is maintained by a group of pluripotent cells in the root meristem that contacts the quiescent center cells (QC) (Dolan et al., 1993; van den Berg et al., 1997). Mobile signals move from the stele to the QC to maintain stem cell fate; for example, the SHORT-ROOT (SHR) transcription factor (Nakajima et al., 2001) moves from the stele into the neighboring QC and endodermis where it serves as a cell fate determinant. *SHR* mRNA expresses in the stele cells of the Arabidopsis root. Protein localization studies revealed the presence of SHR in both nucleus and cytoplasm in these cells. Surprisingly, localization was also observed in the nucleus of neighboring endodermal cells. Further, studies have shown that SHR moves from stele to the neighboring endodermis and localizes in the nucleus (Nakajima et al., 2001). When the SHR:GFP fusion protein was expressed under the control of epidermal or phloem specific promoters, it was unable to move, suggesting tissue-specificity of SHR movement (Sena et al., 2004). But when expressed in the root epidermis of *scarecrow* (*scr*) mutant, SHR was able to traverse to adjacent cell

layers indicating possible role of SCR in limiting SHR movement (Sena et al., 2004; Gallagher et al., 2004). It was also demonstrated that SHR movement requires cytoplasmic localization as SHR:GFP with NLS was unable to move from stele to endodermis when expressed under *SHR* promoter. Though, a novel missense allele of *shr*, *shr-5*, shows cytoplasmic localization of the protein, it was unable to move to endodermis cells, suggesting that the cytoplasmic localization is required but not sufficient for movement and also indicates that SHR is not diffusible (Gallagher et al., 2004).

1.3 Receptor kinases in communication

In general protein kinases are molecular switches, which activate or inactivate a protein by their phosphotransfer activity. There are different types of protein kinases that play many important roles in the development and physiological aspects of a plant, which include organogenesis, patterning, hormone signaling, stress responses and disease resistance.

1.3.1 Receptor Like Kinases

The receptor-like-kinase (RLK/Pelle) family of kinases in plants are the largest class of protein kinases among several classes of protein kinases, which are important mediators of cell-to-cell signaling in multicellular organisms. Evolutionarily, the genes encoding the plant RLK proteins are more closely related to many kinase-encoding genes in animals than to those encoding plant protein kinases in other classes such as MAPKs, CDPKs, GSK3s and others (Gish and Clark, 2011).

1.3.2 RLKs are involved in various signaling pathways

Plant receptor-like kinases (RLKs) are transmembrane proteins with a predicted signal sequence, single transmembrane region and cytoplasmic kinase domain. The Arabidopsis genome sequence revealed more than 610 RLKs that represent 2.5% of the protein coding genes (Shiu and Bleecker, 2003; Morris and

Walker, 2003). The large number of diverse RLKs in the Arabidopsis genome suggests that RLKs may function in perception of a wide-range of signals.

Most of the known plant receptor-like kinases have serine/threonine kinase specificity in contrast to the many animal receptor tyrosine kinases (Shiu and Bleecker, 2001). However, recent advances indicate that tyrosine phosphorylation is as extensive in plants as it is in animals (de la Fuente van Bentem and Hirt, 2009). Tyrosine autophosphorylation of leucine-rich repeat receptor-like kinase, such as BRI1 (BRASSINOSTEROID INSENSITIVE1), has been reported recently (Oh et al., 2009).

The very large family of Arabidopsis RLKs has been divided into several subclasses depending on their domain organization (Shiu and Bleecker, 2001). Presently, the biological roles of only a handful of RLKs in each subclass have been analyzed in significant detail.

EGF class: The wall-associated kinases (WAKs) share similarity to the epidermal growth factor receptor family in animals. They have extracellular domains that are cysteine-rich with potential to serve both as linkers of the cell wall to the plasma membrane and as signaling molecules (He et al., 1999; Kohorn et al., 2006; Kohorn et al., 2009). WAKs are linked to cell growth based on mutant and co-suppression phenotypes, which exhibit defects in cell elongation and expansion (Wagner and Kohorn, 2001; Kohorn et al., 2006).

CrRLK1L class: The plant specific Catharanthus roseus-like RLKs (CrRLK1L) family of proteins emerged as a candidate group for sensing changes at the cell wall and translating this information to cellular responses during both the reproductive and vegetative phases of the plant life cycle (Boisson-Dernier et al., 2011). Evidence from members such as FERONIA (FER), THESEUS1, HERKULES1 and HERKULES2 suggest a role in monitoring the integrity of the cell wall and providing response to challenges to integrity (Hématy and Höfte, 2008). FERONIA was shown to mediate male-female interactions during pollen tube reception (Escobar-Restrepo et al., 2007).

LysM class: Originally identified in bacterial lysins, the repeats have been termed LysM domains for lysin motif (Ponting et al., 1999). The LysM domain is conserved among prokaryotes and eukaryotes. The combination of LysM and receptor kinase domains is present exclusively in plants (Zhang et al., 2007). Legume isoforms of RLKs with LysM-containing extracellular domains recognize symbiotic bacterial signals that trigger plant responses to facilitate the formation of nodules for nitrogen fixation (Mulder et al., 2006; Arrighi et al., 2006).

TNFR class: CRINKLY4 (CR4) possesses tumor necrosis factor receptor (TNFR)-like repeats. The original protein identified from maize was shown to be important for normal cell differentiation of epidermis (Becraft et al., 1996). ACR4, encoded by the Arabidopsis ortholog of CR4, is an epidermal-specific protein that mediates several aspects of epidermal patterning, in addition to integument development in ovules (Gifford et al., 2003; Gifford et al., 2005).

S-domain Class: The cysteine-rich S-domain is present in the extracellular domains of approximately 40 different Arabidopsis RLKs. The function of this domain is best characterized in the Brassica S-receptor kinase (SRK), which is critical for pollen recognition during self-incompatibility (Stein et al., 1991; Goring and Rothstein, 1992; Takasaki et al., 2000). The broad expression of many S-domain RLKs in many tissues and their induction linked to pathogenesis suggest possible roles in both developmental control and disease responses (Dwyer et al., 1994; Pastuglia et al., 1997; Pastuglia et al., 2002).

Thaumatin class: This is a relatively small class of RLK family. The *Arabidopsis* PR5K (PR5-like receptor kinase) is the known example of this class. It resembles to PR5 (pathogenesis related protein 5), whose expression is induced upon pathogen attack (Wang et al., 1996). Thaumatin domains possess antifungal activity and in vitro chitinase activity (Fritig et al., 1998).

LRR (Leucine-Rich Repat) class: LRR-RLKs comprise the largest class of plant RLKs with over 200 members in Arabidopsis. LRRs are tandem repeats of approximately 24 amino acids with leucine at conserved positions. So far, only a small number of the receptors have been functionally characterized and few ligands are identified (Butenko et al., 2009).

1.4 Leucine-rich repeat receptor-like kinases

LRR-RLKs, as might be expected from their large numbers, have been shown to regulate a wide range of biological functions such as pathogen recognition and disease resistance (Afzal et al., 2008), symbiosis (Parniske, 2008) Brassinosteroid signaling (Clouse, 2011), epidermal patterning (Shpak et al., 2004) and stem cell control (Clark et al., 1997).

1.4.1 Shoot apical meristem maintenance

The CLAVATA (CLV) pathway is the best-known plant receptor-like kinase pathway that plays a central role in meristem regulation maintaining the balance between stem cell production and cellular differentiation (Clark et al., 1993). CLV1 is the first component of the pathway identified that encodes a receptor-kinase protein with 21 leucine-rich repeats (LRRs) in its predicted extracellular domain, a single pass transmembrane domain, and a cytoplasmic serine/ threonine kinase domain (Clark et al., 1997). Mutant *clv1* plants show an enlargement of the shoot and flower meristems, resulting in more rapid production of leaves and flowers, thicker stems, and larger fruits (Clark et al., 1993). CLV1 acts to repress transcription of the meristem-promoting *WUSCHEL* transcription factor (Schoof et al., 2000). In this pathway, the receptor-like kinase CLV1 and the receptor-like protein CLV2 are proposed to heterodimerize (Jeong et al., 1999). Biochemical evidence demonstrated that the small secreted dodecapeptide CLV3 functions as the ligand by directly binding to the CLV1 ectodomain (Ogawa et al., 2008). Additional LRR-RLKs have been identified in the regulation of shoot stem cells, which belong to the *CLV1* clade: *BAM1, BAM2, BAM3* (DeYoung et al., 2006). In contrast to CLV1, which promotes differentiation, the

CLV1-related BAM receptors are involved in stem cell maintenance. Apart from the *CLV1* expressing meristem center *BAM* genes also show expression in the flanks of meristem (DeYoung et al., 2006). It was proposed that BAM1-BAM2 would sequester CLE peptide ligands in the flanks of the meristem, keeping them away from the more centrally located CLV-receptor complexes and thereby controlling the delicate balance required for stem cell maintenance (Deyoung and Clark, 2008).

1.4.2 Epidermal maintenance and cell specification

Brassinosteroids (BRs) are plant steroid hormones that regulate a variety of plant growth and developmental processes. The brassinosteroid receptor, BRI1, was found in a genetic screen for BR insensitivity in Arabidopsis. The *bri1* mutants show dwarfed height, male sterility and de-etiolation in the dark, among other phenotypes (Clouse et al., 1996). BRI1 was later identified as an LRR X RLK and confirmed as a transmembrane BR receptor by direct binding of a plant brassinosteroid, brassinolide to BRI1 (Li and Chory, 1997; Wang et al., 2001; Kinoshita et al., 2005). BRI1-like1 (BRL1) and BRL3 are the most homologous RLKs to BRI1 that bind BR and were shown to be involved in vascular differentiation (Caño-Delgado et al., 2004; Zhou et al., 2004)

The single cell layered epidermis both promotes and restricts growth of the inner tissues via BRI1 mediated signaling. Targeted expression of BRI1 in the L1 layer of the shoot apical meristem was sufficient to restore normal growth (Savaldi-Goldstein et al., 2007). BAK1 (also known as AtSERK3), an LRR II RLK, was identified as a genetic and physical interactor with BRI1 (Wang et al., 2001; Nam and Li, 2002). A current model suggests that BRI1 forms homodimers upon hormone binding, and then activates BAK1 by phosphorylation (Wang et al., 2005). Then the kinase domains of BRI1 and BAK1 trans-phosphorylate each other on multiple sites (Wang et al., 2008) and the fully activated receptor triggers downstream signaling events, which include the activation of a small family of transcription factors that regulate the expression of more than a thousand genes in a BR-dependent manner (Kim and Wang, 2010; Clouse, 2011).

Crystallographic studies revealed that the ectodomain of BRI1 folds into a superhelical assembly of 25 LRRs, an architecture that is strikingly different from the assembly of LRRs in animal Toll-like receptors (Hothorn et al., 2011). The hormone-binding region is a 70-amino acid island domain (ID) located between LRR21 and LRR22 in the extracellular domain of BRI1 (Kinoshita et al., 2005) that folds back into the interior of the superhelix to create a surface pocket for binding the plant hormone brassinolide (Hothorn et al., 2011).

1.4.3 ERECTA, a pleiotropic developmental regulator

The Arabidopsis ERECTA (ER) gene is required for the elongation of inflorescence. *er* plants exhibit a corymb-like inflorescence inflorescence with short internodes and short pedicels (Torii et al., 1996). Along with inflorescence development, ER regulates multiple developmental processes as well as environmental and biotic responses (van Zanten et al., 2009). ERECTA LIKE1 (ERL1) and ERL2 are paralogs of ER with similar roles and the *er* dwarfing phenotypes are severely enhanced in an *er erl1 erl2* triple mutant background. Actually, their phenotype is more similar to that of plants with defective cell-cycle genes. Accordingly, in triple mutant ovules misregulation of CYCLIN A2 genes was identified suggesting that ER-signaling pathway might regulate core cell-cycle genes thus stimulating cell division (Pillitteri et al., 2007). They also control stomatal patterning by regulating the specification of stomatal stem cell fate and the differentiation of guard cells (Shpak et al., 2005). An LRR receptor-like protein, TOO MANY MOUTHS (TMM), modulates stomatal patterning, by associating with ER-family RLKs (Nadeau and Sack, 2002; Shpak et al., 2005; Lee et al., 2012). The ligands for these receptors, EPF1 and EPF2 are secreted from neighboring stomatal precursors (Hara et al., 2007; Hunt and Gray, 2009; Hara et al., 2009; Lee et al., 2012).

Recently, it was shown that the expression of ER in the phloem is sufficient to rescue compact *er* inflorescences (Uchida et al., 2012). Two EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) secreted peptide genes were identified, EPFL4 and EPFL6/CHALLAH (CHAL), as redundant, upstream components of ER-

mediated inflorescence growth. The endodermal expression of EPFL4 or EPFL6 and the phloem expression of ER are sufficient for proper inflorescence growth. EPFL4 and EPFL6 were also shown to physically associate with ER. Thus proper inflorescence architecture in Arabidopsis can be specified by inter-tissue layer communication between the phloem and the endodermis mediated by ERECTA (Uchida et al., 2012).

1.5 The atypical LRR-RLK, STRUBBELIG regulates tissue morphogenesis

Inter-cellular communication during floral morphogenesis in Arabidopsis also depends on signaling mediated by the leucine-rich repeat transmembrane receptor-like kinase (LRR-RLK) STRUBBELIG (SUB). It is identified based on ovule phenotype (Schneitz et al., 1997; Chevalier et al., 2005). Analysis of *sub* mutants indicated that *SUB* is required for the shape of floral organs and for the control of cell shape and/or the orientation of the cell division plane.

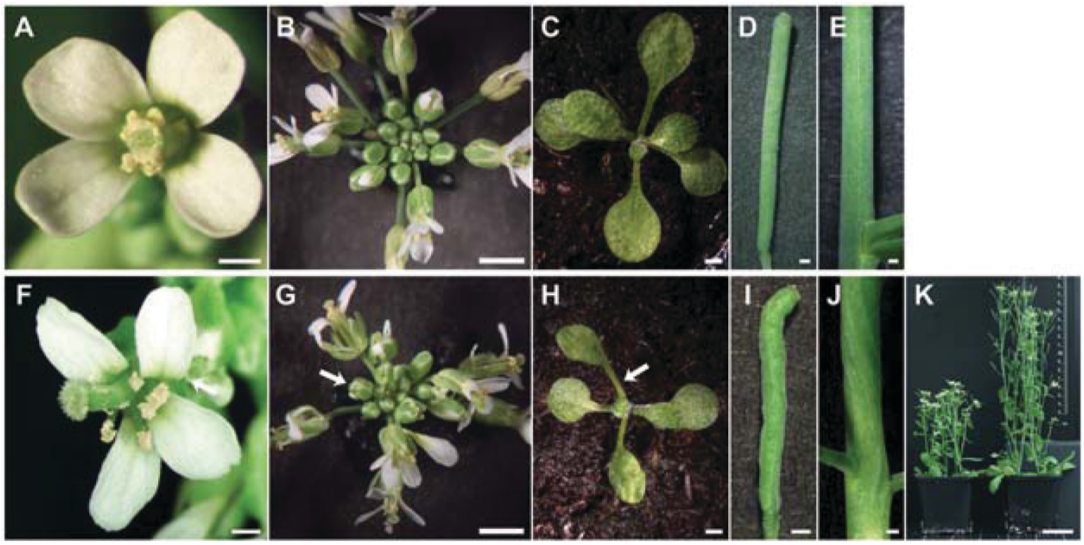


Figure 1.5.1 *sub-1* above-ground morphology
(A-E) Wild-type L*er*. (F-K) *sub-1*. (A, F) An open stage 13 flower from a 30-day old plant. (F) Petals can also show small notches. (B, G) Top view of a 30-day inflorescence. (G) Flower phyllotaxis is irregular. Arrows mark prematurely opened flower buds. (C, H) Top view of a 12-day rosette. (H) Leaf petioles can be twisted (arrow). (D, I) Morphology of mature siliques. (E, J) A lateral view of a section of stems from a 30-day plant. (K) Plant height *sub-1* (left) in comparison to L*er* (right). (Modified from Fulton et al., 2009).

At the macroscopic level, *sub* mutants show a pleiotropic phenotype. 4-week old *sub* plants exhibit reduced plant height compared to wild type and display irregular twisting of stems (Figure 1.5.1J, K). Flowers open prematurely and often show a large percentage of twisted petals (Figure 1.5.1F, G). The mutant carpels show twisting and ovules display aberrant initiation of the outer integument. This results in outer integuments with gaps that often resemble ‘‘multifingered clamps’’ or ‘‘scoops’’

(Figure 1.5.2B, C). The aberrant outer integument phenotype becomes visible in late stage 2-III/stage 2-IV of ovule development (Chevalier et al., 2005; Fulton et al., 2009).

At the cellular level, robustly scorable numbers of periclinal divisions in stage-3 floral meristem were observed. Occasionally, periclinal divisions were also observed in the L1 layer (Figure 1.5.3F, G) (Fulton et al., 2009). The horizontal stem sections of 30-day old *sub* stem revealed reduced number of epidermal, cortex, and pith cells (Chevalier et al., 2005). In addition, an independent study found that, *SUB*, also known as *SCRAMBLED* (*SCM*), affects specification of hair cells in the root epidermis (Kwak and Schiefelbein, 2007; Kwak et al., 2005).

1.5.1 SUB can act in non-cell-autonomous fashion

Although SUB is expressed in a broad fashion in floral meristems and young ovules (Chevalier et al., 2005), expression of a functional SUB:EGFP fusion protein to the L1 layer is sufficient to rescue the L2 division plane defects in floral meristems (Yadav et al., 2008). In addition, SUB:EGFP expression in the distal nucellus of ovule primordia can rescue to a large extent defects in the integuments, tissue that originates from the central chalaza. Thus, it was proposed that SUB can act in a non cell-autonomous fashion and mediates inter-cell-layer signaling during floral development (Yadav et al., 2008). In this respect SUB may relate to BRI1 that has a broad expression pattern and acts in a non-cell-autonomous fashion (Savaldi-Goldstein et al., 2007; Hacham et al., 2011).

SUB is a member of the LRRV/STRUBBELIG-RECEPTOR FAMILY (SRF) family of receptor-like kinases (Shiu and Bleecker, 2001; Eyüboglu et al., 2007). Another member, SRF4 acts as a positive regulator of leaf size (Eyüboglu et al., 2007) while SRF3 plays a role in plant pathogen response and potentially in speciation (Alcázar et al., 2010). For other SRF genes, such as SRF4 or SRF7, a role in cell wall biology was proposed (Eyüboglu et al., 2007).

1.5.2 Signaling through atypical kinases in plants

SUB is predicted to carry an extracellular domain with six leucine-rich repeats, a transmembrane domain, and a cytoplasmic intracellular domain with the juxtramembrane and kinase domains. Interestingly, phosphotransfer activity of the kinase domain is not essential for its function *in vivo* (Chevalier et al., 2005) and thus SUB seems to belong to the family of atypical or "dead" receptor kinases (Kroiher et al., 2001; Boudeau et al., 2006; Castells and Casacuberta, 2007). Other examples from plants include maize atypical receptor kinase (MARK) from corn (Llompart et al., 2003), Arabidopsis CRR1 (CRINKLY4-RELATED1) and CRR2 (Gifford et al., 2003; Cao et al., 2005).

The knowledge about signaling by atypical kinases is less, particularly in plants. Generally, the corresponding mechanisms are believed to rely on regulated protein-protein interactions (Kroiher et al., 2001; Boudeau et al., 2006; Castells and Casacuberta, 2007). Known mechanisms potentially depend on the phosphorylation of the atypical RLK by other kinases or on the stimulation of functional kinases by the atypical RLK. For example, AtCRR2 can be phosphorylated in vitro by its homologue ACR4, indicating that these two receptors may form a heterodimer involved in ACR4 signaling (Cao et al., 2005). In contrast, the atypical RLK, MARK was found to interact with the functional GCN (general control non-derepressible)-like MIK (MARK-interacting kinase) in vitro and in COS-7 cells (Llompart et al., 2003), but apparently the MARK- IK interaction did not result in the phosphorylation of MARK. Interestingly, it brought about a several fold stimulation of MIK kinase activity

1.5.3 Novel components in SUB mediated signaling

Further understanding of the molecular basis of SUB mediated signaling is of great interest to understand how cellular morphogenesis is coordinated by SUB across cell layers. The apparent lack of kinase activity raises intriguing questions about its signaling mechanism. Through a forward genetic approach three additional genetic factors were identified, *QUIRKY (QKY), ZERZAUST (ZET),* and *DETORQUEO*

(DOQ), mutations in which result in a *sub*-like phenotype. Plants with a defect in these genes show corresponding defects in outer integument development (Figure 1.5.2), floral organ shape, and stem twisting. The mutants also show *sub*-like cellular defects in the floral meristem and in root hair patterning (Figure 1.5.3). These three genes together with *SUB* define the *STRUBBELIG-LIKE MUTANT (SLM)* class of genes (Fulton et al., 2009).

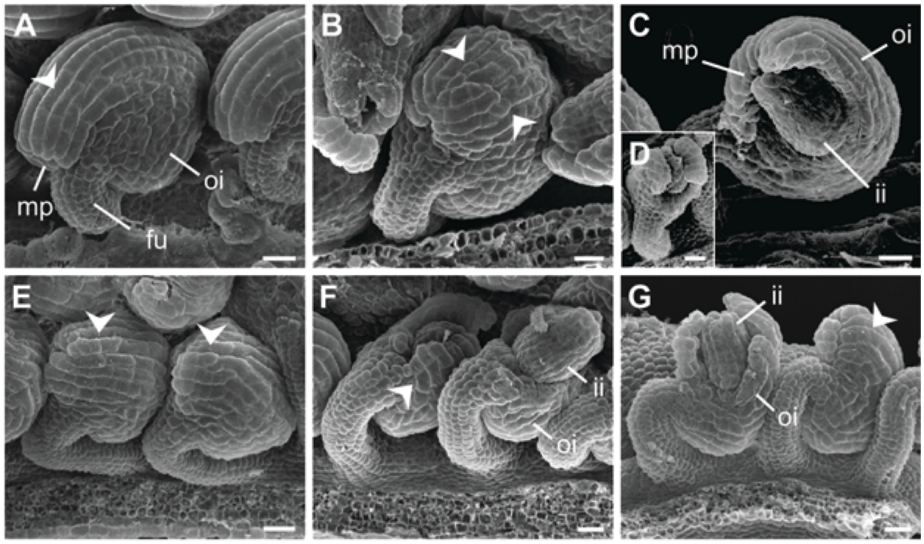


Figure 1.5.2 Comparison of ovule morphology in *sub-1, doq-1, qky-8,* and *zet-2* mutants. (A) Wild-type L*er*. The arrow marks one of the elongated cells of the distal outer integument. (B) *sub-1*. A mild phenotype is shown. Note the irregular size and shape of cells at the distal outer integument (arrow heads, compare to (A)). (C, D) *sub-1*. Strong phenotypes are depicted. Note the half-formed outer integument. (D) shows an example where the outer integument shows several gaps. (E) *doq-1*. This mutant shows a mild ovule phenotype comparable to the one depicted in (B). The arrow heads highlight the disruption of the regular cell files of the distal outer integument. (F) *qky-8*. Note the variability of the phenotype. The specimen to the left shows only a mild disorganisation of the cell shape (arrow head). The one to the right shows a strong phenotype (compare to (C)). (G) *zet-2*. This mutant shows a variable phenotype. The ovule to the left shows gaps in the outer integument while the one to the right exhibits only mild alterations in cell shape and size (arrow head). Abbreviations: fu, funiculus; ii, inner integument; mp, micropyle; oi, outer integument. Scale bars: 20 µm. (From Fulton et al., 2009).

Morphological analysis of *SLM* single and pair-wise double mutants, as well as whole-genome level investigation of *SLM*-responsive gene activity by transcriptomics, revealed a highly significant overlap between *SLM* gene function, but also suggested that individual *SLM* genes have distinct functions as well (Fulton et al., 2009). The results indicated that *SLM* genes contribute to *SUB*-dependent processes, but may not

act in a simple linear pathway. Moreover, the genes identified represent general components of the *SLM*-dependent mechanism that are reused in different biological contexts. Further analysis indicated that many *SLM*-responsive genes have functions in cell wall biology, hormone signaling, and various stress responses (Fulton et al., 2009). Thus *DOQ*, *QKY*, and *ZET* contribute to *SUB*-dependent organogenesis and shed light on the mechanisms, which are dependent on signaling through the atypical receptor-like kinase SUB.

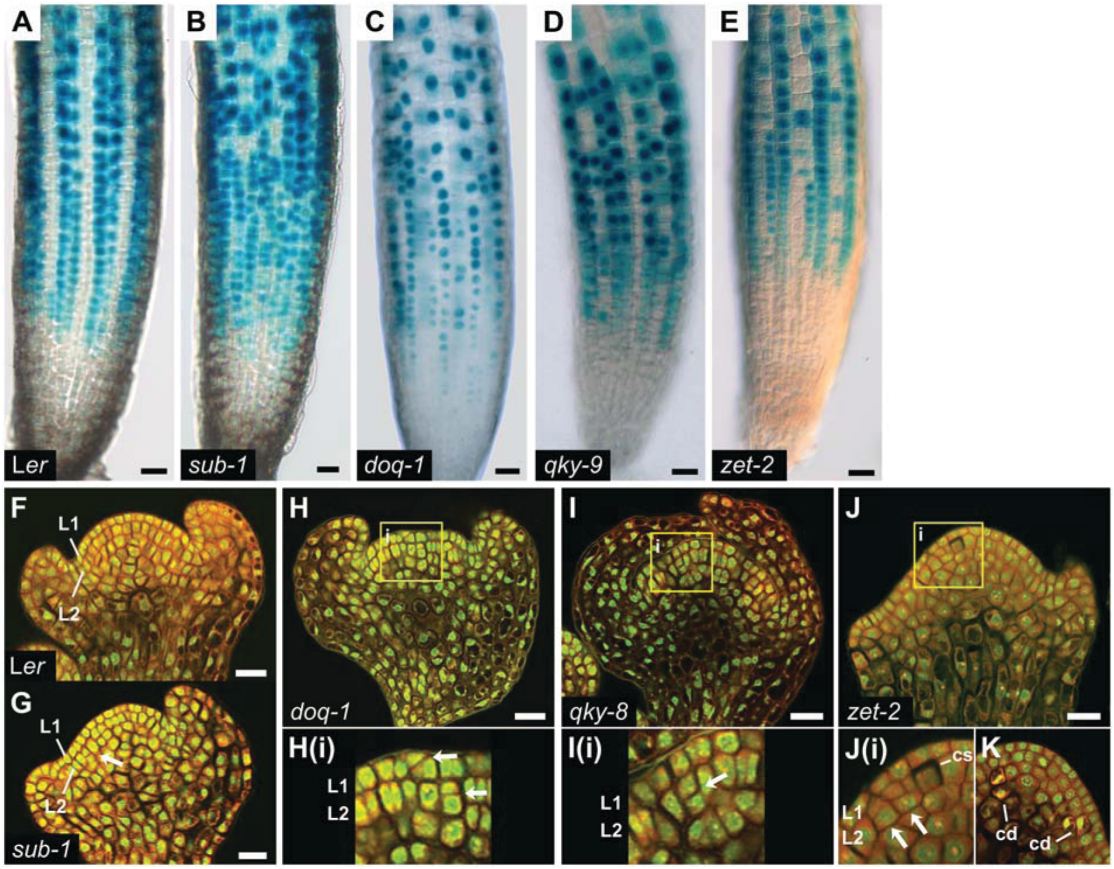


Figure 1.5.3 Analysis of cellular defects in 4-day old main roots and stage 3 floral meristems of *sub-1, doq-1, qky* and *zet-2* mutants.
(A- E) Expression of the GL2::GUS reporter in whole-mount main roots. (A) WT L*er* root. The reporter is detected in regular files of non-hair cells. (B- E) GL2::GUS reporter expression is patchy. (F-K) Mid-optical sections through propidium-iodide-stained stage 3 floral meristems. (F) WT L*er*. Note the regular arrangement of cells in the L1 and L2. (G) *sub-1*. The arrow marks a periclinal cell division event. (H) *doq-1,* (I) *qky-8,* (J-K) *zet-2*. The regions marked by the square (i) are shown at higher magnification in (Hi), (Ii), (Ji). (Hi) The arrows highlight aberrant oblique and periclinal cell divisions in the L1 and L2, respectively. (Ii) The arrow labels a periclinal cell division in the L2. (Ji) The arrows highlight periclinal cell divisions. A cell undergoing cell separation is indicated. (K) Disintegrating cells are marked. Abbreviations: cd, cell disintegration; cs, cell separation; L1, L1 cell layer; L2, L2 cell layer. Scale bars: (A-E) 25 mm, (F-K) 20 µm. (Modified from Fulton et al., 2009).

1.5.4 QUIRKY, a STRUBBELIG-LIKE MUTANT (SLM)

Many aspects of the phenotypes of *sub-1* and *qky-8* at the cellular level were comparable though the overall plant morphology of *qky-8* seems slightly less affected compared to *sub-1*. The *sub-l qky-8* double mutants display petal and carpel twisting similar to that shown by each single mutant. In contrast, twisting of siliques, stems, and leaf petioles was more pronounced as was the reduction in plant height. In addition, ovule development was more heavily affected compared to the single mutants with 13% of ovules showing inner integument defects and a corresponding reduction in fertility. About 5% of ovules were hardly recognizable as such, but rather resembled a mass of cells with integument-like outgrowths. Thus, an exaggerated phenotype was observed in a double mutant combination making it difficult to decide whether the displayed phenotype is additive or synergistic, or whether a particular mutation was epistatic to another. Whole-genome level investigation by transcriptomics, revealed a highly significant overlap between SUB and QKY responsive genes. Interestingly, *qky* mutant showed higher number of mis-expressed genes when compared to *sub* suggesting *QKY* might be involved in wide array of biological processes (Fulton et al., 2009). As a first step to molecularly define the *SLM* pathway, it will be an exciting challenge to further dissect the nature and biological role of *QKY* in *SUB* mediated signaling.

1.6 Objectives

In this study a structure-function analysis was performed to gain at a better molecular understanding of how the atypical RLK SUB regulates its various downstream signaling processes. Using a combination of genetic, cell biological and pharmacological approaches, I provide evidence that SUB principally accepts sequence variability and that the N-capping domain in the extracellular domain of the SUB protein is important for its biological activity. In addition, the data indicate that delivery of functional SUB receptor to the plasma membrane is monitored by endoplasmic reticulum-mediated quality control. It was also shown that SUB requires multiple introns for its broad expression pattern. Furthermore, tissue-specific or cell-specific SUB-dependent processes do not appear to be integrated into the SUB mechanism by the receptor itself, through functionally differentiated protein domains, but likely via other components acting together with or downstream of SUB.

The later part of the thesis deals with the characterization of QKY; a potential component in SUB mediated signaling. Mapping and molecular identification studies revealed that QKY is a Multiple C2 domain containing Transmembrane Protein (MCTP). Later, molecular characterization of QKY was done using a combination of genetic, cell biological and biochemical approaches. Gene and protein expression studies were carried out. It was shown that QKY binds to phospholipids in a Ca^{+2} dependent manner. SUB and QKY interaction was confirmed via yeast-two-hybrid. In addition, data indicate that QKY and SUB are present at the PD and cell plate. Furthermore, SUB and QKY does not appear to be involved in the movement of non-selective proteins through PD. The data implies that SUB mediated inter-cellular signaling via QKY is likely involved in selective transport of components through PD or by regulating cytokinesis.

2 Materials and Methods

2.1 Plant work

Arabidopsis thaliana (L.) Heynh. var. Columbia (Col-0) and var. Landsberg (*erecta* mutant) (L*er*) were used as wild-type strains. The *sub-1* to *sub-5* mutants (Ler background) were described previously (Chevalier et al., 2005) as was *sub-6* (Col background) (Kwak et al., 2005). Plants were grown in a greenhouse under Philips SON-T Plus 400 Watt fluorescent bulbs on a long day cycle (16 hrs light). Dry seeds were sown on soil (Patzer Einheitserde, extra-gesiebt, Typ T, Patzer GmbH & Co. KG, Sinntal-Jossa, Germany) situated above a layer of perlite, stratified for 4 days at 4^0C and then placed in the greenhouse. The plants were kept under a lid for 7–8 days to increase humidity and support equal germination.

2.2 SUB tilling

The EMS-induced mutations *sub-10* to *sub-20* were identified in conjunction with the Seattle Arabidopsis TILLING facility (http://tilling.fhcrc.org/files/Welcome_to_ATP.html/) (Till et al., 2003). Tilling was performed in a Col line that carries the fast-neutron-induced er-105 mutation (Torii et al., 1996). Three different 0.8 to 1 kb genomic regions spanning the SUB/LRR, PRR, and kinase domains were screened. The mutations in homozygous form were confirmed in M3 plants by sequencing. Mutant plants were outcrossed to L*er* before analysis.

2.3 Map-Based Cloning of QKY

To map the QKY locus at high resolution, an F2-mapping population was generated. F1 plants from *qky/qky* (L*er*) and QKY/QKY (Col) crosses were allowed to self-pollinate, and the F2 progeny were screened for *qky* individuals based on twisted inflorescence morphology. DNA was isolated and used for PCR-based amplification of molecular markers. Indel polymorphism data was derived from the Monsanto L*er* sequence database at TAIR (Jander et al., 2002). Primer sequences for indel and CAPS markers are shown in supporting table of Fulton et al., 2009. Marker amplifications from 598 mutant individuals restricted the *qky* map interval to 103 kb, as defined by

markers F25A4(BglII) and 27.99(RsaI). Candidate genes were analysed via T-DNA insertion mutant analysis and/ or sequence determination revealing that At1g74720 carried mutations in various *qky* alleles. To confirm *qky* allelic mutations, nucleotide sequences were obtained from both strands of PCR-amplified fragments. In all three EMS-induced *qky* alleles transitions result in stop codons. The *qky-7* allele carries a G to A transition at position 2229 (genomic DNA, relative to the start ATG triplet (+1)) resulting in a shorter predicted protein (W743*). The *qky-8* allele carries another G to A transition at position 2706 (W902*), and in *qky-9* a C to T transition was found at position 649 (Q217*). I also determined the genomic integration sites of two T-DNA insertions (Alonso et al., 2003) in At1g74720 obtained from the ABRC (http:// www.arabidopsis.org). SALK_140123 is located at position 2576 and SALK_043901 at position 3056. Both lines exhibit a *qky* phenotype. SALK_140123 is predicted to carry a shorter QKY protein of 878 residues with the 29 last residues (RSHKGSHVMTPAD-DAGQAVLRLELTEPQR*) being encoded by the T-DNA. SALK_043901 results in a predicted shorter protein of 1022 residues with residues 1019-1022 (LFVV*) being encoded by the T-DNA. Near full-length QKY cDNA sequence was assembled from sequences derived from four publicly available RIKEN RAFL cDNA clones (Seki et al., 2002). The cDNA clones partially overlapped (RAFL16-35-G10, RAFL22-02-B15, RAFL22-66-H20, RAFL22-96-F19) with one clone (RAFL22-96-F19) containing the 3' poly(A) stretch. Additional 5' RACE experiments (Frohman et al., 1988) did not result in more extended 5' cDNA sequences and comparisons of the available QKY genomic and cDNA sequences did not reveal introns.

2.4 Recombinant DNA work

For DNA and RNA work standard molecular biology techniques were used (Sambrook et al., 1989, Molecular Cloning). PCR-fragments used for cloning were obtained using either PfuUltra high-fidelity DNA polymerase (Stratagene) or TaKaRa PrimeSTAR HS DNA polymerase (Lonza, Basel, Switzerland) or Phusion High-Fidelity DNA polymerase (NEB GmbH). The plasmid pCAMBIA2300 was used as binary vector (www.cambia.org). All PCR-based constructs were sequenced. Information regarding the primers is given in Table S2.

2.5 Generation of various SUB Constructs

2.5.1 Wild-type SUB::c/gSUB:EGFP reporter constructs

The SUB::cSUB:EGFP reporter construct was described previously (Yadav et al., 2008). To generate the SUB::gSUB:EGFP construct L*er* genomic DNA was used as template and amplified with primers SUB-Genomic2/F and SUB-Genomic2/R. The PCR fragment was reamplified by using primers SUB_cmyc_F, SUB_cmyc_R and cloned into pJET1.2 by blunt end cloning generating pJET1.2gSUB. The insert was released by an *Asc*I/*Aat*II restriction digestion and subcloned into *Asc*I/*Aat*II digested SUB::cSUB:EGFP (in pCAMBIA2300), thereby replacing cSUB with gSUB and generating SUB::gSUB:EGFP. The vector 35S::SUB:3xmyc pCAMBIA2300 was generated as follows. To clone the 35S promoter adjacent to SUB:3×myc plasmid SUB:3×myc pCAMBIA2300 was used. The 35S fragment was obtained by digesting vector pART-7 first with *Not*I and, then blunt ending using T4 DNA polymerase followed by digestion after gel purification with *Xba*I. To generate compatible end for the 35S insert vector SUB:3×myc pCAMBIA2300 was digested first with *Bam*HI, made blunt with T4 DNA polymerase, and subsequently gel purified and digested with *Spe*I generating 35S::SUB:3×myc pCAMBIA2300.

2.5.2 Wild-type N-terminal tagged SUB::EGFP:cSUB fusion construct

The DNA fragments representing the signal peptide (SP) sequence of SUB and the coding sequence of EGFP were fused via overlapping PCR. The resulting SP:EGFP fragment was cloned into cSUB:3×myc (lacking the SP) in pCRII-TOPO by *Bam*HI digestion resulting in SP:EGFP:cSUB:3×myc pCRII-TOPO. The SP:EGFP:cSUB fragment was amplified using primers SUB_cmyc_F and Sig:SUB_Xba1_R and subcloned into binary vector cSUB:EGFP pCAMBIA2300 (Yadav et al., 2008) replacing SUB:EGFP by *Asc*I/*Xba*I restriction digestion. Then the 3.5 kb *SUB* promoter fragment was subcloned from SUB::cSUB:EGFP by *Kpn*I/*Asc*I digestion resulting in SUB::SP:EGFP:cSUB in pCAMBA2300 (SUB::EGFP:cSUB).

2.5.3 Mutant SUB::c/gSUBmut:EGFP reporter constructs

To design the five truncated versions of *SUB*, a PCR amplification based approach was used. The plasmid pCRII SUB:3×myc (Yadav et al., 2008) served as a template. The 35S::SUB:3×myc pCAMBIA2300 (Yadav et al., 2008) plasmid used as a backbone. Full length *SUB* was replaced by truncated versions of *SUB* using *Asc*I and *Aat*II sites. For the SUBDTM-Intra primers SUB-Cmyc-F, and 35S-extra-myc-rev were used. To construct 35S::SUBDIntra:3×myc primers SUB-Cmyc-F and 35S-TMmyc-rev were used. The 35S::SUBDCD:3×myc plasmid was constructed using primers SUB:3×myc-F and JuxtraAatII-R. PCR fragments were treated with *Asc*I and *Aat*II and cloned into correspondingly digested 35S::SUB:3×myc pCAMBIA2300. To generate SUBDECD:3×myc primers SUB-Cmyc-F and Alalinksignal-rev were used to amplify the signal sequence of *SUB*. Primers Alalink-TM-intra-for and SUB-Cmyc-R were used to amplify the TM-intracellular domain fragment. After gel purification an overlap PCR was setup to generate a fragment carrying the signal peptide and the TM-intracellular domain but lacking the ECD. This fragment was digested with *Asc*I and *Aat*II and cloned into 35S::SUB:3×myc pCAMBIA 2300. To generate SUBDECD-TM:3xCmyc the entire intracellular region was amplified using primers AscIntra-F and SUB-Cmyc-R pair, digested with *Asc*I and *Aat*II, and cloned into 35S::SUB:3×myc pCAMBIA 2300. To clone the truncated SUB versions into a SUB promoter plasmid, the five truncations were digested with *Asc*I/*Aat*II respectively and cloned into *Asc*I/*Aat*II digested vector pSUB::SUB:EGFP (Yadav et al., 2008).

All point mutations were generated using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer's recommendations (Agilent Technologies). For the cDNA-based cSUBmut versions 35S::SUB:3×myc pART7 was used as template (Chevalier et al., 2005) while for the genomic gSUBmut versions, pJET 1.2 gSUB was employed as template. The sequence of the mutagenized constructs was verified by sequence analysis. The cSUBmut variants were amplified from in vitro mutagenized 35S::SUB:3xmyc pART7 plasmids using primers SUB_cmyc_F, SUB_cmyc_R and subcloned into SUB::cSUBΔECD:EGFP (in pCAMBIA 2300 binary vector), thereby replacing the cSUBΔECD fragment, by

*Asc*I/*Aat*II restriction digestion. The gSUBmut variants were subcloned from in vitro mutagenized pJET1.2gSUB into SUB::cSUB:EGFP using *Asc*I/*Aat*II restriction digestion, replacing cSUB with gSUBmut.

2.5.4 Generation of SUB intron deletion constructs

SUB with 10 introns: For this clone SUB::gSUB:EGFP was digested by *Asc*I/*Xma*I and sub-cloned into SUB::cSUB:EGFP (Yadav et al., 2008) resulting SUB::SUB (10introns):EGFP.

SUB with 6 introns: Primers 354 and 2350 were used to amplify SUB with first six introns from SUB::gSUB:EGFP and primers 2349 and 355 were used to amplify the rest of the fragment without introns from SUB::cSUB:EGFP (Yadav et al., 2008). Overlapping PCR using primers 354 and 355 fused both the fragments.

SUB with 3 introns: Primers 354 and 2348 were used to amplify SUB with first six introns from SUB::gSUB:EGFP and primers 2347 and 355 were used to amplify the rest of the fragment without introns from SUB::cSUB:EGFP (Yadav et al., 2008). Overlapping PCR using primers 354 and 355 fused both the fragments.

All the above fragments were digested with *Asc*I/*Aat*II and cloned into SUB::EGFP pCambia 2300 resulting into respective clones.

2.6 Generation of various QKY constructs

2.6.1 QKY pJET1

For cloning of QKY, genomic DNA of *Arabidopsis thaliana* (L.) Heynh. Landsberg (*erecta* mutant) (L*er*) was used as template. The coding sequence of QKY including 3'UTR was amplified using primers 1531 and 1719 and cloned into pJET1 by blunt end cloning using the CloneJET PCR cloning kit (Fermentas) resulting pJET1 QKY. *Kpn*I restriction site in the coding sequence is changed to *Bci*VI by introducing a silent mutation using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer's recommendations (Agilent Technologies) with primers 1698 and 1699. This new clone is named pJET1 QKY.1.

2.6.2 QKY::QKY pCAMBIA 2300.

The promoter of QKY was amplified using primers 1724 and 1529 from genomic DNA. The PCR product was reamplified using primers 1714 and 1529. The coding sequence of QKY was amplified from pJET1 QKY.1 using primers 1531 and 1719. Overlapping PCR using primers 1714 and 1719 fused these two PCR products. The resulting PCR product was digested using *Kpn*I/*Xba*I and cloned into pCAMBIA 2300 binary vector resulting pCAMBIA 2300 QKY::QKY.

2.6.3 mCherry:QKY pJET1

mCherry was amplified from pGEM-T mCherryNLS rev using primers 1716 and 1717. QKY was amplified from pJET1 QKY.1 using primers 1718 and 1719. Overlapping PCR using primers 1716 and 1719 fused both PCR products. The resulting PCR product was cloned into pJET1 by blunt end cloning generating pJET1 mCherry:QKY.

2.6.4 QKY::mCherry:QKY pCambia 2300

QKY promoter was amplified using primers 1714 and 1715 from pCAMBIA 2300 QKY::QKY. The PCR product was digested with *Kpn*I/*Asc*I and cloned into SUB::cSUB:EGFP:pCambia 2300 (Yadav et al., 2008) replacing SUB promoter resulting QKY::cSUB:EGFP:pCambia 2300. mCherry:QKY from pJET1 mCherry:QKY was released with *Asc*I/*Xba*I and cloned into QKY::cSUB:EGFP:pCambia 2300 replacing cSUB:EGFP generating QKY::mCherry:QKY pCambia 2300.

2.6.5 QKY::EGFP:QKY pCambia 2300

EGFP sequence was amplified using primers 2011 and 2012 from SUB::cSUB:EGFP:pCambia 2300 (Yadav et al., 2008). QKY was amplified from pJET1 QKY.1using primers 2013 and 2014. Overlapping PCR using primers 2011 and 2014 fused both these PCR products. The resulting PCR product was digested with *Asc*I/*Pst*I and subcloned into QKY::mCherry:QKY pCambia 2300 replacing mCherry:QKY resulting finally into QKY::EGFP:QKY pCambia 2300.

2.6.6 QKY::T-Sapphire:QKY pCambia 2300

T-Sapphire CDS was amplified from ER-T-Sapphire pRSETB (Zapata-Hommer and Griesbeck, 2003) using primers 2011 and 2386. The resulting PCR product was digested with *Asc*I/*Spe*I and subcloned into QKY::EGFP:QKY pCambia 2300 replacing EGFP resulting finally into QKY::T-Sapphire:QKY pCambia 2300

2.6.7 QKY::QKY:mCherry pCambia 2300

QKY and mCherry sequences were amplified from QKY::mCherry:QKY pCambia 2300 using primers 1732, 1742 and 1743, 1814 respectively. The two PCR products were fused by overlapping PCR using primers 1732 and 1814 and digested with *Asc*I/*Xba*I and subcloned into QKY::mCherry:QKY pCambia 2300 by replacing mCherry:QKY by *Asc*I/*Xba*I resulting into QKY:: QKY:mCherry pCambia 2300. QKY 3' UTR was amplified with 1733 and 1719 and cloned into QKY:: QKY:mCherry pCambia 2300 *Xba*I restriction digestion.

2.6.8 QKY::GUS pCambia 2300

GUS CDS was amplified from pCambia 2301 using primers 2017 and 2018. The 3' UTR of QKY was amplified using primers 2016 and 2014. Both the PCR products were fused by overlapping PCR using primers 2017 and 2014 and cloned into QKY::EGFP:QKY pCambia 2300 by *Asc*I/*Pst*I digestion resulting into QKY::GUS pCambia 2300.

2.6.9 Generation of QKY yeast two hybrid clones

QKY Coding sequence without TM domains was amplified using primers 2148 and 2149 from pJET1 QKY.1 and cloned into pGBKT7 by *Nde*I/*Xma*I restriction digestion giving rise to QKY-TM pGBKT7. Then the same fragment was subcloned into pGADT7 by same restriction sites resulting into QKY-TM pGADT7.

2.6.10 Generation of recombinant protein constructs

Individual C2 domain recombinant proteins are fusions of a glutathione-S-transferase (GST) tag to the amino-terminus. For QKY C2 domains QKY pJET1 was used as template and for SYT1 C2A *Rattus rattus* cDNA was used. The primers used

for amplifying the respective domains are summarized in Table S2 (From 2169 to 2176). The amplified PCR products were digested with *EcoR*I/*Not*I and cloned into *EcoR*I/*Not*I digested vector pGEX-6P-1 (GE Healthcare Europe, Munich, Germany).

2.7 Generation of transgenic plants

Wild type and *sub-1* plants were transformed with different constructs using Agrobacterium strain GV3101/pMP90 (Sambrook et al., 1989) and the floral dip method (Koncz and Schell, 1986). Transgenic T1 plants were selected on Kanamycin plates (50 µg/ml) and subsequently transferred to soil for further inspection.

2.8 Quantitative real-time PCR analysis

Tissue for quantitative real-time PCR was harvested from 25-day plants grown under long day conditions. Tissue was harvested in Eppendorf tubes pre-cooled on dry ice for 30 minutes and stored at -80°C. With minor changes, RNA extraction and quality control was performed as described previously (Box et al., 2011). DNase treatment was performed using rDNase (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1.0 µg of total RNA via reverse transcription, using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed on a Roche LightCycler480 using the iQ SYBR Green Super mix (Bio-Rad, Hercules, USA) according to the manufacturer's recommendations. Using the ΔΔ-Ct method, *SUB* and *QKY* gene expression levels were normalized against At5g25760, At4g33380 and At2g28390 expression (Czechowski et al., 2005). The primer pair used for SUB and QKY are 2922-2923 and 2959-2960 respectively. Expression levels are depicted as -fold changes compared to wild type expression in the respective tissues.

2.9 Expression and purification of recombinant proteins

The various constructs were transformed into *E. coli* BL21 (DE3) cells (Invitrogen, Karlsruhe, Germany) and grown to an OD600 of 0.5-0.8. Recombinant protein expression was then induced by 0.25 mM isopropyl-β-d-thiogalactoside (IPTG, FLUKA, Germany) for 4-8 hours at 30º C. Cells were pelleted and subjected to

solubilisation and recombinant protein purification using the Protino® Glutathione Agarose 4B kits (GE Healthcare, Germany) according to the manufacturer's recommendations.

2.10 Phospholipid binding assay

The phospholipid binding property of QKY single C2 domains was assessed by a centrifugation assay as described previously (Fernandez et al., 2001; Fernández-Chacón et al., 2002; Shin et al., 2003). The phospholipids (Phosphatidylserine 25%/ Phosphatidylcholine 75% from Avanti Polar Lipids) in chloroform were dried by overnight evaporation and vacuum drying for 2 h. Phospholipids were then resuspended in Buffer A (50mM HEPES/NaOH pH 6.8, 100mM NaCl, 4mM EGTA). Phospholipid vesicles were formed by water bath sonication for 10 minutes. The vesicles were pelleted down by centrifugation at 20800g, 4 °C for 20 minutes. The pellet was either resuspended in buffer A or buffer A with 100 µM free Ca^{2+}. The free Ca^{2+} concentration was calculated using WEBMAXC program (http://www.stanford.edu/~cpatton/maxc.html,NISTdatabase) and the needed amount was added from a concentrated $CaCl_2$ stock solution. For one experiment, 100µg of phospholipids were incubated with 8 µg of a GST C2 domain protein (total volume 1ml) for 15 min in a shaker (27 °C, 250 rev/min). Afterwards vesicles and bound protein were spun down (10 min, 20 800 g, 4 °C). The vesicles were washed three times with 500µl of corresponding buffer. All fractions collected during the experiment were further analyzed with SDS-PAGE and Coomassie blue staining.

2.11 Bioinformatic Analysis

Protein domain searches were conducted using the PFAM database (Punta et al., 2012). Transmembrane topology was predicted using the TMHMM webserver (Kahsay et al., 2005).

2.12 Analysis of natural variation at the STRUBBELIG protein level

We downloaded the TAIR10 genome matrix containing 80 Arabidopsis thaliana accessions (MPICao2010) from the http:// 1001genomes.org/ website. The Weigel

laboratory at the Max Planck Institute for Developmental Biology produced these sequence data. We extracted and translated the corresponding STRUBBELIG (At1g11130) sequences by loci using in- house software. ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for the protein alignment.

2.13 Homology modeling

Homology modeling was made by submitting the entire SUB protein sequence to the web-based Swiss-Model workspace (http://swissmodel.expasy.org/workspace/) (Bordoli et al., 2008) using default settings. The algorithms generated two models, one for the LRRs and one for the kinase domain. The templates were 1ogqA and 2qkwB for the LRRs and the kinase domain, respectively. The 1ogqA protein data bank (PDB, http://www.rcsb.org/pdb/home/home.do) entry corresponds to the structure of polygalacturonase-inhibiting protein 2 (PGIP2), a leucine-rich repeat protein involved in plant defense (Di Matteo et al., 2003). The 2qkwB entry relates to tomato Pto kinase (Xing et al., 2007). Identical results were obtained by submitting just the LRR and kinase domain sequences to the Swiss-Model website. Models were saved as protein data bank (.pdb) files and molecular graphics images were produced using the UCSF Chimera package (Calderon-Villalobos et al., 2006).

2.14 *in situ* hybridization

in situ hybridization with digoxigenin-labelled probes was done essentially as described earlier (Sieber et al., 2004). A detailed protocol can be found at http://plantdev.bio.wzw.tum.de/index.php?id=69. A 2.076 kb *QKY* antisense probe was obtained from pSK:QKY construct and the sense control was obtained from pRSET-C:QKY. A 2.657 kb *SUB* antisense probe was obtained by PCR using a full-length cDNA clone as template and the primer pair 2631/2632. The sense control was obtained using primer pair 2629/2630. Slides were viewed with an Olympus BX61 upright microscope using DIC optics.

2.15 GUS histo-chemistry

For GUS staining all the tissues were collected in 90% acetone and processed according to (Gross-Hardt et al., 2002).

2.16 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy was performed with an Olympus FV1000 setup using an inverted IX81 stand and FluoView software (FV10-ASW version 01.04.00.09) (Olympus Europa GmbH, Hamburg, Germany). After excitation at 488 nm with a multi-line argon laser EGFP fluorescence (502-536 nm slit width) and FM4-64 fluorescence (610-672 nm slit width) was detected. One-way scan images (scan rate 12.5µs/pixel, 512x512 pixels, Kahlman frame, average of four scans) were obtained using an Olympus 40x water objective (PlanApo 40x/0.90, WLSM). Samples were dissected under a dissection scope, either in H_2O or directly in FM4-64 (4 µM in H_2O; Molecular Probes/Invitrogen, Karlsruhe, Germany), and covered with a 18 x 18 mm glass cover slip of 0.17 mm thickness (#1, Menzel-Gläser, Braun- schweig, Germany. T-Sapphire and mCherry were excited using 405nm and 559nm laser line respectively.

2.17 Callose Staining

Callose was stained by incubating 4-day old seedlings for 30 min in a mixture of 0.1% aniline blue in double distilled water and 1 M glycine, pH 9.5, at a volume ratio of 2:3, respectively.

2.18 Drug treatments

Transgenic seeds containing various *SUB::SUB:EGFP* or *SUB::SUBmut:EGFP* transgenes were germinated on vertical minimal media plates. After five days whole seedlings were transferred to 24-well suspension-culture-plates (Cellstar®, Greiner Bio-one GmbH, Frickenhausen, Germany), the bottom of the wells coated with full-strength MS agar containing 50 µM kifunensine (Enzo Life Sciences, Lausen, Switzerland) or 25 µM MLN4924, and incubated at standard growth conditions for 24 or 48 hours respectively. Reporter expression was subsequently assayed as described

below. For Mg132 treatments seedlings were placed in 24-well culture plates and treated for 24 hours with 50 μM MG132 in liquid full-strength MS medium as outlined previously (Yadav et al., 2008). The *RGA::RGA:GFP* (Czechowski et al., 2005) transgenic line was used as control for Mg132 and MLN4924 treatments.

2.19 Accession Numbers

The QKY cDNA sequence was deposited at GenBank under the accession number FJ209045. The expression profile data have been deposited to the EMBL-EBI ArrayExpress repository under the accession E-MEXP-1592.

3 Results

3.1 Structure-function analysis of STRUBBELIG

3.1.1 SUB structure prediction by homology modelling

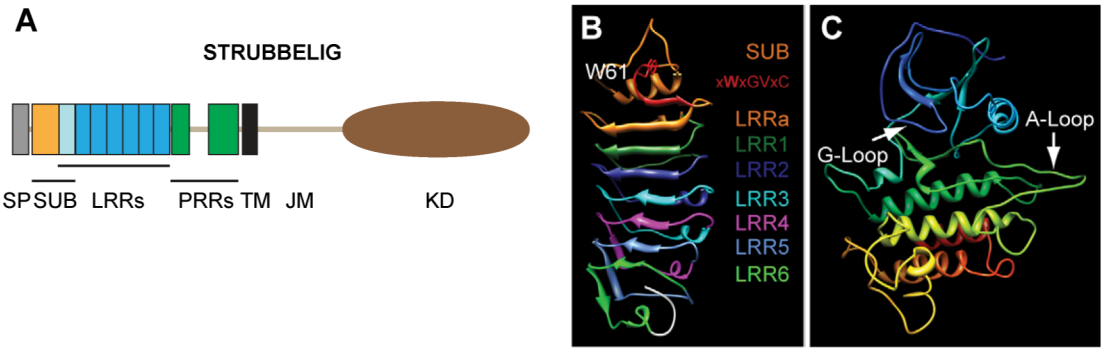


Figure 3.1.1 Predicted structure of the SUB protein.
(A) Overview of the domain architecture of SUB. The SUB-domain is indicated by orange color. The imperfect CxWxGVxC motif with the conserved tryptophan is highlighted in red. Individual LRRs are marked by the respective colors. (C) Model of the kinase domain. Different colors arbitrarily denote distinct secondary structures to aid in visualization. The ATP-binding (G-loop) and substrate binding (A-loop) regions are marked. Abbreviations: JM, juxtamembrane domain; KD, kinase domain; LRR, leucine-rich repeat; PRR, proline-rich repeat; SP, signal peptide; SUB, SUB-domain; TM, transmembrane domain. Length of SUB protein: 768 amino acids. M.W:84.5kDa

The SUB protein was predicted to contain an extracellular domain (ECD) with a 24-aa signal peptide, an amino-terminal region of about 59 residues that is conserved between the LRRV/SRF members (termed SUB domain), six LRRs and a proline-rich region. The ECD is followed by a transmembrane domain (TM) and the intracellular juxtamembrane (JM) and the carboxy-terminally-located kinase domain (KD) (Chevalier et al., 2005; Shiu and Bleecker, 2001) (Figure 3.1.1). Crystallographic information about the structure of SUB is presently lacking. To gain insights regarding the possible structure of SUB, which might help to rationalize the effect of some sub mutations (see below), homology modeling was applied using the Swiss-Model workspace (Bordoli et al., 2008). The suggested template for the SUB/LRR region (Figure 3.1.1B) turned out to be polygalacturonase-inhibiting protein (PGIP2) from Phaseolus vulgaris, a LRR protein involved in plant defense (Di Matteo et al., 2003). The kinase domain (Figure 3.1.1C) was modelled after the tomato Pto kinase (Xing et al., 2007).

The model for the SUB/LRR region predicts that the SUB domain consists of a short amino-terminal α-helix, a structurally ill-defined region, a loop that is formed by a very highly conserved stretch of amino acids, a second loop carrying a strictly conserved tryptophan, a small β-strand, and finally a single imperfect LRR (termed LRRa) (Figure 3.1.1A, B; Figure S1). The SUB domain is then followed by another six LRRs (termed LRR1 to LRR6). Thus, it is likely that the ECD of SUB contains seven rather than the six LRRs originally identified. An imperfect CxWxGVxC motif is located just before the LRRa region (Figure S1). This motif precedes the first LRR in many plant LRR-containing ECDs (Diévart and Clark, 2003; van der Hoorn et al., 2005). The first half of the SUB domain thus likely represents an N-terminal capping domain thought to protect the hydrophobic core of the LRR in many plant extracellular LRR proteins (Kobe and Deisenhofer, 1994; Di Matteo et al., 2003; Choe et al., 2005; van der Hoorn et al., 2005; Kolade et al., 2006). The model predicts that the LRRs form a curved structure with a slight right-handed twist, which carries eight β-strands, located at its inner or concave side. In analogy to resolved structures of LRR proteins the β-strands are presumed to form an interface that can interact with other proteins (Kobe and Kajava, 2001) (sheet B1 in PGIP2 (Di Matteo et al., 2003)). In addition, three additional β-strands form a second small β-sheet located at the bottom and to one side of the curved structure (Figure 1B). For PGIP2 it was proposed that this second β-sheet (sheet B2) might represent an additional protein-protein interaction site (Di Matteo et al., 2003).

The model of the SUB kinase domain resembles a standard kinase structure with the smaller N-terminal and the bigger C-terminal lobes and shows no obvious structural peculiarities (Figure 3.1.1C, Figure S1) (Huse and Kuriyan, 2002).

3.1.2 Identification and analysis of novel *sub* alleles

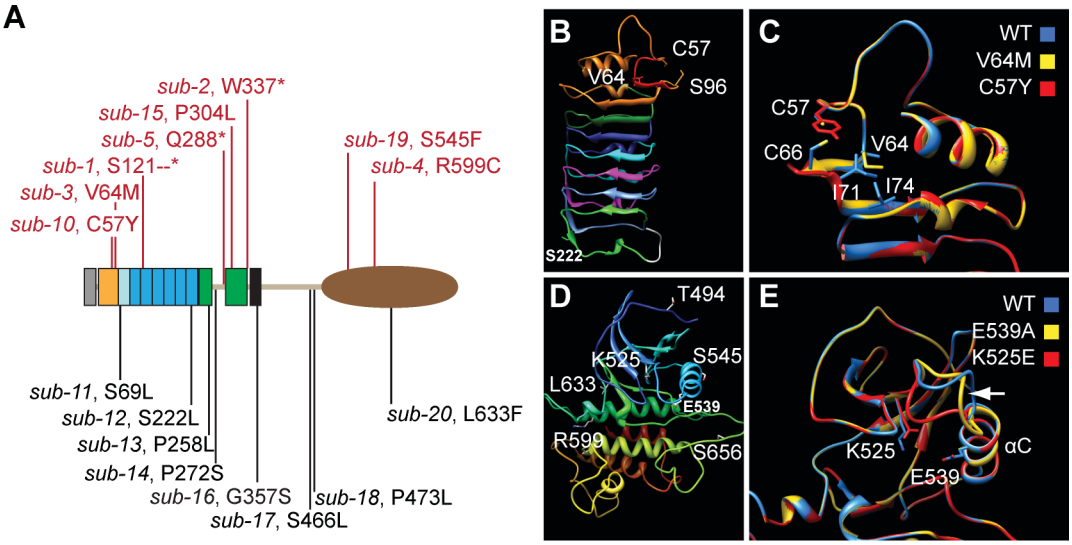


Figure 3.1.2 Molecular nature of mutations affecting SUB and homology models of mutant SUB variants.
(A) Position of different SUB alleles. Phenotypic mutations are listed above the protein and depicted in red while aphenotypic mutations are listed below the protein. Stars denote premature stops. Length of SUB protein: 768 amino acids. (B-E) Homology models of SUB variants. (B, C) SUB-domain plus leucine-rich repeats. Residues affected by mutation are highlighted. (B) Wild-type. (C) Overlay of wild-type and two mutant models. Focus resides on the region encompassing the SUB-domain and the first leucine-rich repeat. (D, E) Kinase domain. (D) Wild-type. (E) Overlay of wild-type and two mutant models. Focus is on the region encompassing the G-loop and the αC helix. The arrow marks the predicted structural variation in the loop that connects the β3 sheet with the αC helix.

In previous work we identified five EMS-induced *sub* alleles (*sub-1* to *sub-5*) in L*er* background (Schneitz et al., 1997; Chevalier et al., 2005) (Figure 3.1.2A, Table 1, Figure S1). To further elucidate structure-function relations of *SUB* additional EMS-induced alleles were identified in the Col *er-105* background using targeted-induced local lesions in genomes (TILLING) (Till et al., 2003). Of the 26 mutations with altered nucleotides identified in the *SUB* locus 11 mutations, named *sub-10* to *sub-20*, reside in exons and were predicted to cause amino acid alterations in the SUB protein (Figure 3.1.2, Table 1, Figure S1). Interestingly, only three of these alleles resulted in a *sub* phenotype (*sub-10* (C57Y), *sub-15* (P304L), and *sub-19* (S545F)) (Figure 3.1.3). The phenotypes of several *sub* mutations have been described extensively (see introduction). In short, *sub* mutants in the L*er* background show characteristic defects such as impaired integument development, twisted siliques,

misshaped floral organs, and short and twisted stems (Figure 3.1.3) (Chevalier et al., 2005; Fulton et al., 2009).

Allele	Mutagen	Mutation#	AA change	Background
sub-1[§]	EMS	G>A, 999	S121--*	L*er*
sub-2[§]	EMS	G>A, 2157	W337*	L*er*
sub-3[§]	EMS	G>A, 567	V64M	L*er*
sub-4[§]	EMS	C>T, 3127	R599C	L*er*
sub-5[§]	EMS	C>T, 2008	Q288*	L*er*
sub-10	EMS	G>A, 547	C57Y	Col *er-105*
sub-11[+]	EMS	C>T, 583	S69L	Col *er-105*
sub-12[+]	EMS	C>T, 1728	S222L	Col *er-105*
sub-13[+]	EMS	C>T, 1916	P258L	Col *er-105*
sub-14[+]	EMS	C>T, 1960	P272S	Col *er-105*
sub-15	EMS	C>T, 2057	P304L	Col *er-105*
sub-16[+]	EMS	G>A, 2215	G357S	Col *er-105*
sub-17[+]	EMS	C>T, 2639	S466L	Col *er-105*
sub-18[+]	EMS	C>T, 2660	P473L	Col *er-105*
sub-19	EMS	C>T, 2966	S545F	Col *er-105*
sub-20[+]	EMS	C>T, 3302	L633F	Col *er-105*

Table 1. Summary of *sub* alleles.
[§]previously described in Schneitz et.al. 1997 and/or Chevalier et.al. 2005
[+]aphenotypic mutations
[#]the coordinates refer to the genomic sequence and relate to the ATG of *SUB* (At1g11130) of BAC T19D16 (see Chevalier et.al. 2005).

I compared the aboveground morphology of all 16 EMS-induced *sub* alleles (Figure 3.1.2A, Table 1, Figure S1) among which eight alleles, all carrying amino acid substitutions, were aphenotypic. Eight alleles exhibited a mutant phenotype with three predicted to be null alleles, the *sub-1*, *sub-2*, and *sub-5*. These mutants are likely devoid of any *SUB* function as the mutations result in shorter proteins that comprise part of the extracellular domain and the mutant proteins will not be able to transmit a signal across the plasma membrane (Chevalier et al., 2005) (Figure 3.1.2).

The remaining five phenotypic alleles carry amino acid substitutions (*sub-3* (V64M), *sub-4* (R599C), *sub-10* (C57Y), *sub-15* (P304L), and *sub-19* (S545F). Morphological characteristics of the null allele *sub-1* and *sub-3* or *sub-4* (all in L*er*) were essentially identical suggesting that the latter two mutations result in amino acid changes that cause complete loss of SUB function (Chevalier et al., 2005). The three phenotypic TILLING alleles *sub-10*, *sub-15*, and *sub-19* (in Col *er-105*) demonstrated *sub-1*-like phenotypes, although the alterations in floral morphology and stem shape of *sub-10* and *sub-15* were slightly less marked (Figure 3.1.3). Potentially, these two alleles could be hypomorphic or the somewhat milder phenotypes may relate to the presence of modifiers in the Col *er-105* background (Vaddepalli et al., 2011). Overall, the analysis of the available EMS-induced mutations indicated that irrespective of their nature the phenotypic mutations all result in the loss of SUB function.

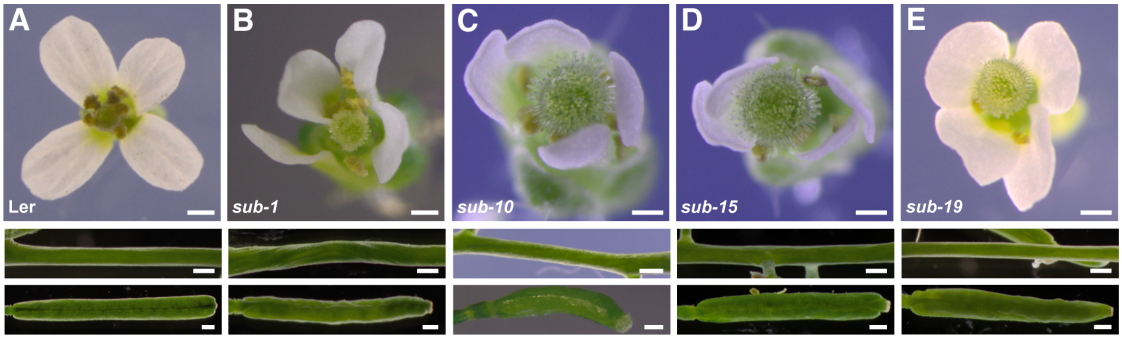


Figure 3.1.3 Comparative analysis of the *sub-10*, *sub-15* and *sub-19* phenotypes. Flower morphology and stem and silique shape.
(A) Wild type (L*er*). (B) *sub-1*. (C) *sub-10*. Siliques are a bit shorter compared to *sub-1*. (D) *sub-15*. Stem twisting is not quite as strong as in *sub-1*. (E) *sub-19*. Resembles *sub-1*. Scale bars: 0.5 mm.

3.1.3 Homology modeling of mutant SUB/LRR and kinase domains

The biochemical and/or structural functions of the altered residues in the non-synonymous phenotypic *sub* alleles are presently unknown. SUB homology models were used to predict how different *sub* mutations may affect the protein. Genetic analysis suggests that the conformation of the N-terminal capping domain appears to be critical for SUB activity as two phenotypic mutations; *sub-3* (V64M) and *sub-10* (C57Y) affect this domain. V64 resides towards the top and at the beginning of the first small β-strand that is part of LRRa and that likely contributes to the potential ligand-binding interface (Figure 3.1.2B, C). However, the residue's side-chain points away from this interface, suggesting that V64 does not directly contribute to protein-protein interaction through this surface. In *sub-3* (V64M) the long side chain of the methionine may interfere with formation of a hydrophobic region that is generated by isoleucines 71 and 74 (Figure 3.1.2C), and affect the relative orientation of the first small β-strand and adjacent large β-strand of LRRa, and thus the architecture of LRRa per se. The *sub-10* (C57Y) mutation affects the first cysteine in the imperfect CxWxGVxC motif. The wild-type SUB model suggests that the strictly conserved C57 may contribute to folding or stabilization of the N-terminal capping domain via an intramolecular disulphide bond formation with C66 (Figure 3.1.2C).

Two aphenotypic mutations also reside within the SUB/LRR domain, *sub-11* (S69L) affects a strictly conserved serine at position 69 that localizes close to the two nearby cysteines C57 and C66 in the loop between the first and the second β-sheets of LRRa and *sub-12* (S222L) is located towards the end of the third small β-strand in sheet B2. The side-chains of both these amino acids are facing outwards and to the side of the protein (Figure 3.1.2B). This architecture and the nature of the side-chain exchange may explain the lack of a mutant phenotype.

The phenotypic *sub-4* and *sub-19* alleles hint at the importance of the kinase domain for SUB function. In *sub-4* a cysteine replaces the arginine at position 599. However, a KD model (Figure 3.1.2D) of SUB_{R599C} did not reveal obvious structural changes (not shown) and the exact role of this residue remains to be elucidated. The

sub-19 (S545F) mutation resides within the conserved αC helix, a mediator of conformational changes in the catalytic center (Huse and Kuriyan, 2002), principally explaining its loss of function. Interestingly, however, another mutation in the αC helix (E539A) did not affect SUB activity (see below). Furthermore, *sub-20* (L633F), also situated in the kinase domain, was aphenotypic, indicating that the KD of SUB tolerates some sequence variability. The reason for this property of SUB awaits further investigation, as KD models of SUBS545F and SUBL633F were uninformative (not shown).

3.1.4 Kinase activity is not essential for SUB function

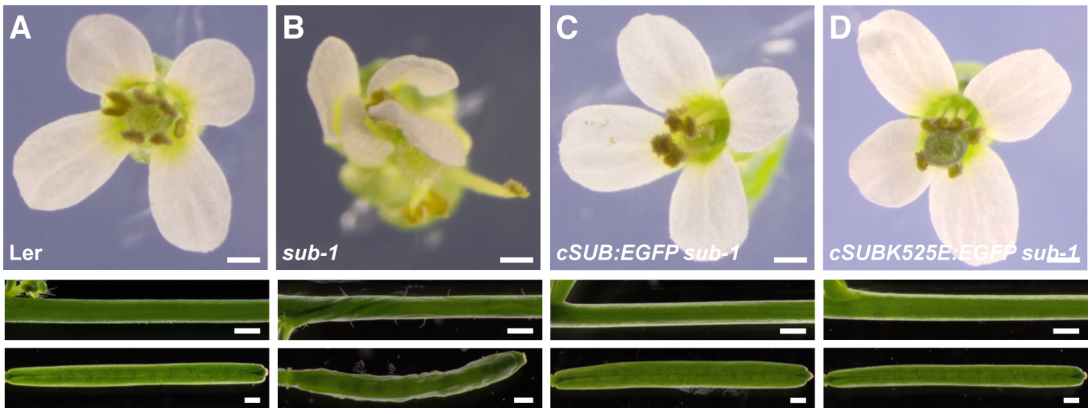


Figure 3.1.4 Genetic evidence that kinase activity is not essential for SUB function in vivo.
Flower morphology and stem and silique shape of *sub-1* plants carrying *SUB::cSUB:EGFP* and *SUB::cSUB$_{K525E}$:EGFP* reporter constructs. (A) Wild type (L*er*). (B) *sub-1*. (C) *SUB::cSUB:EGFP sub-1*. (D) *SUB::cSUB$_{K525E}$:EGFP sub-1*. A functional construct. Plant appears wild type. Scale bars: 0.5 mm.

SUB is likely an atypical or dead kinase as several substitutions in the small lobe known to eliminate kinase activity, such as the well-known K525E substitution or the E539A alteration (Hanks et al., 1988) (Carrera et al., 1993), are tolerated in vivo. This was demonstrated by the rescued wild-type phenotype of *sub-1* plants expressing *35S::cSUBK525E* or *35S::cSUBE539A* transgenes (Chevalier et al., 2005). The K525 resides in a b-strand (normally classified as β3) preceding a loop connecting β3 with the conserved aC helix, a mediator of conformational changes in the catalytic center (Huse and Kuriyan, 2002), while residue E539 is part of the αC helix (Figure 3.1.2E). Interestingly, homology modeling predicts that both mutations result in different conformations for the loop that connects β3 and the αC helix (Figure 3.1.2E).

However, our previous genetic results indicate that these conformational changes either do not occur or are irrelevant in vivo. To exclude that the use of the 35S promoter weights these results, I generated *sub-1* plants that carried constructs in which the mutated *SUB* cDNA-based constructs were under control of endogenous *SUB* promoter and UTR elements established previously (Kwak et al., 2005; Yadav et al., 2008). Using in vitro mutagenesis, I introduced K525E and E539A mutations into the reporter *SUB::cSUBmut:EGFP*. The *SUB::cSUBK525E:EGFP sub-1* and the *SUB::cSUBE539A:EGFP sub-1* plants exhibited a wild-type phenotype as well (Figure 3.1.4). These findings reinforce the notion that SUB is an atypical or dead kinase.

Although phosphorylation activity of the SUB kinase domain is not essential for its function in vivo it is possible that phosphorylation of SUB by as yet unknown kinases is important. To test this possibility, I altered two semi-conserved threonines (T486A/E and T494A) in the juxtamembrane and kinase domains, respectively. In addition, I changed the single serine in the activation loop (S656A) (Figure S1). Correspondingly, all three *35S::cSUBmut* constructs rescued the *sub-1* phenotype (not shown) indicating that phosphorylation of these residues is not required for SUB function.

3.1.5 Nonfunctional *SUB::cSUBmut:EGFP* reporters fail to express detectable signals

In order to understand the biological importance of various mutations described above, I generated a set of reporters encoding mutant SUB:EGFP fusion proteins that carried either deletions or individual point mutations (Figure 3.1.5), and were tested for their capability to restore *SUB* function in *sub-1* plants by analyzing the phenotype of *SUB::cSUBmut:EGFP sub-1* plants. The subcellular distribution of the mutant fusion proteins was also assayed.

As expected, in vitro generated mutant constructs recapitulating the *sub-3* (V64M), *sub-4* (R599C) and *sub-10* (C57Y) mutations failed to rescue the *sub-1*

mutant phenotype (Chevalier et al., 2005) (Figure 3.1.6D). Furthermore, *sub-1* plants carrying different deletion constructs (Figure 3.1.5) all remained *sub-1* in appearance, indicating that each deletion eliminates *SUB* function (not shown). As discussed above C57 affected in *sub-10* and C66 in the SUB domain might form a disulphide bridge important for N-capping domain tertiary structure. In accordance with this notion C66Y mutant construct failed to rescue *sub-1* plants (Figure 3.1.6E). Similarly, simultaneous alteration of both cysteines (C57Y/C66Y) did not result in a functional construct either (Figure 3.1.6F).

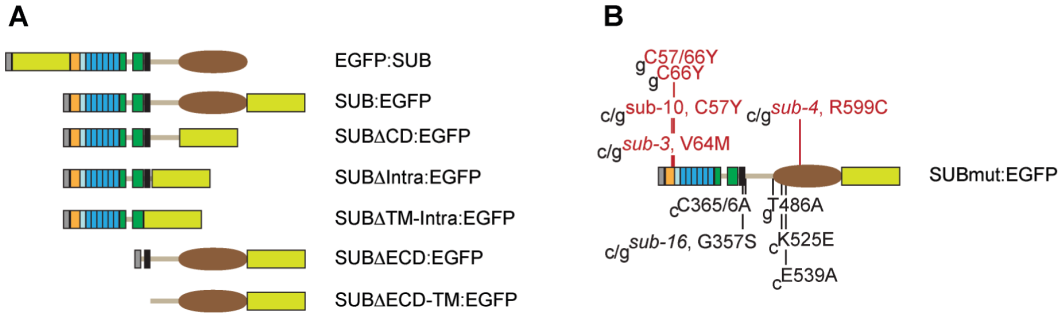


Figure 3.1.5 Synopsis of SUB:EGFP variants generated by in vitro mutagenesis.
The domain architecture of SUB is depicted as in Figure 1. The EGFP tag is indicated by a yellow/green box. (A) N- and C-terminal fusions of EGFP to wild-type SUB and C-terminal fusions of EGFP to various SUB deletions. All constructs included endogenous *SUB* promoter elements with *SUB* cDNA (c) or genomic DNA (g), including all *SUB* introns. All the deletions were unable to rescue the *sub-1* phenotype. (B) Point mutations. Mutations resulting in a failure to rescue the *sub-1* phenotype are listed above the protein in red while mutations that rescued are listed below in black. Abbreviations: CD, kinase domain; ECD, extra-cellular domain; Intra, intracellular domain; TM, transmembrane domain.

In the ECDs of many LRR-RLKs, a cysteine pair is found proximal to the LRRs that appear to be functionally relevant, possibly for heterodimerization or as a component of a C-terminal capping domain involved in structural stabilization of the LRR domain (Kobe and Deisenhofer, 1994; Diévart and Clark, 2003; Kolade et al., 2006). While SUB lacks such a cysteine pair in its ECD it carries two neighboring cysteines just proximal to the transmembrane domain (C365/6) (Figure 3.1.5B). Transgenic *cSUB$_{C365/6A}$:EGFP sub-1* plants, however, appeared wild type, indicating these cysteines do not contribute to SUB function (not shown).

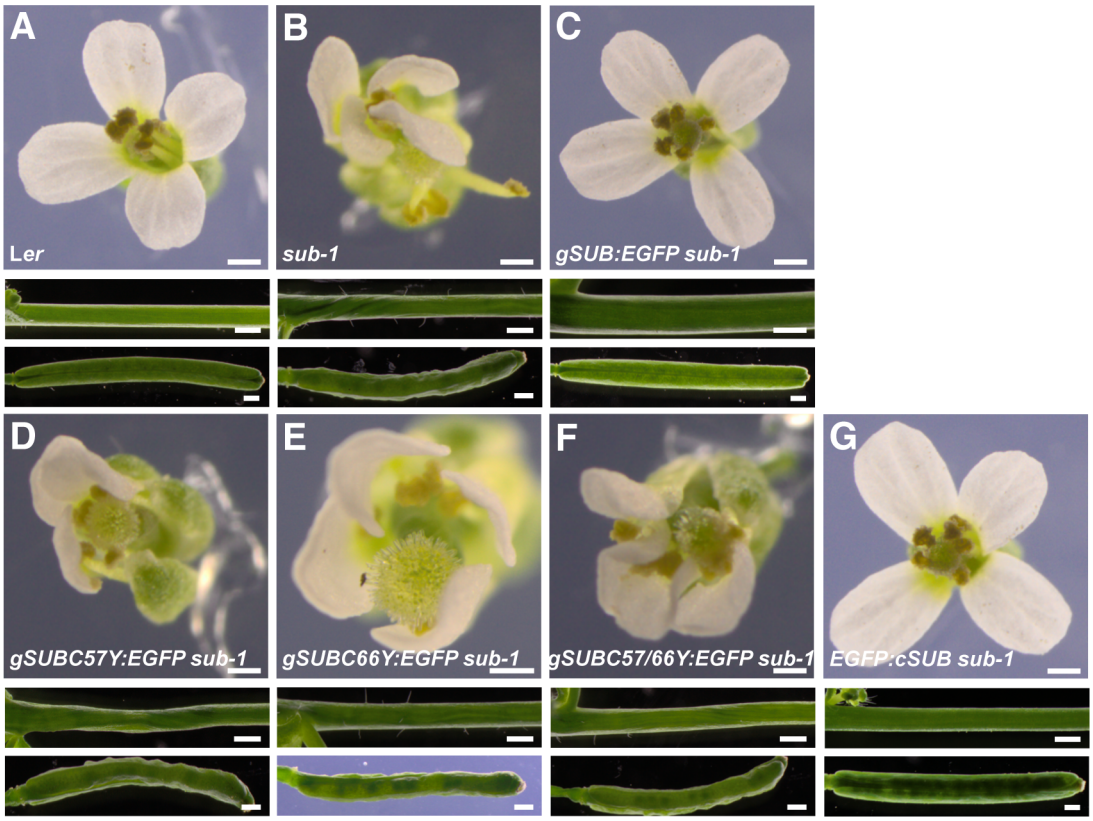


Figure 3.1.6 Functional analysis of different *SUB::SUB:EGFP*-based constructs.
Flower morphology and stem and silique shape of *sub-1* plants carrying various reporter constructs. (A) Wild type (L*er*). (B) *sub-1*. (C) *SUB::gSUB:EGFP sub-1*. The plant appears wild type. (D) *SUB::gSUB*$_{C57Y}$*:EGFP sub-1*, (E) *SUB::gSUB*$_{C66Y}$*:EGFP sub-1*. and (F) *SUB::gSUB*$_{C57Y/C66Y}$*:EGFP sub-1*. No rescue of the *sub-1* phenotype. (G) *SUB::EGFP:cSUB sub-1*. A cDNA-based construct encoding a fusion of EGFP to the N-terminus of SUB. The plant looks wild type. Scale bars: 0.5 mm.

All functional *cSUBmut:EGFP* reporters exhibited the expected signal strength and distribution (Figure 3.1.7A-L). Surprisingly, although I screened at least 100 primary transformants for each construct, I was unable to detect an EGFP signal in nonfunctional *cSUBmut:EGFP sub-1* plants (Figure 3.1.7M-P). This interesting finding could principally provide a coherent explanation for the observed homogeneity of the mutant phenotypes among the different *sub* alleles. In all tested alleles no mutant SUB protein would be present and thus all would exhibit a null phenotype. Further analysis, however, did not support this notion.

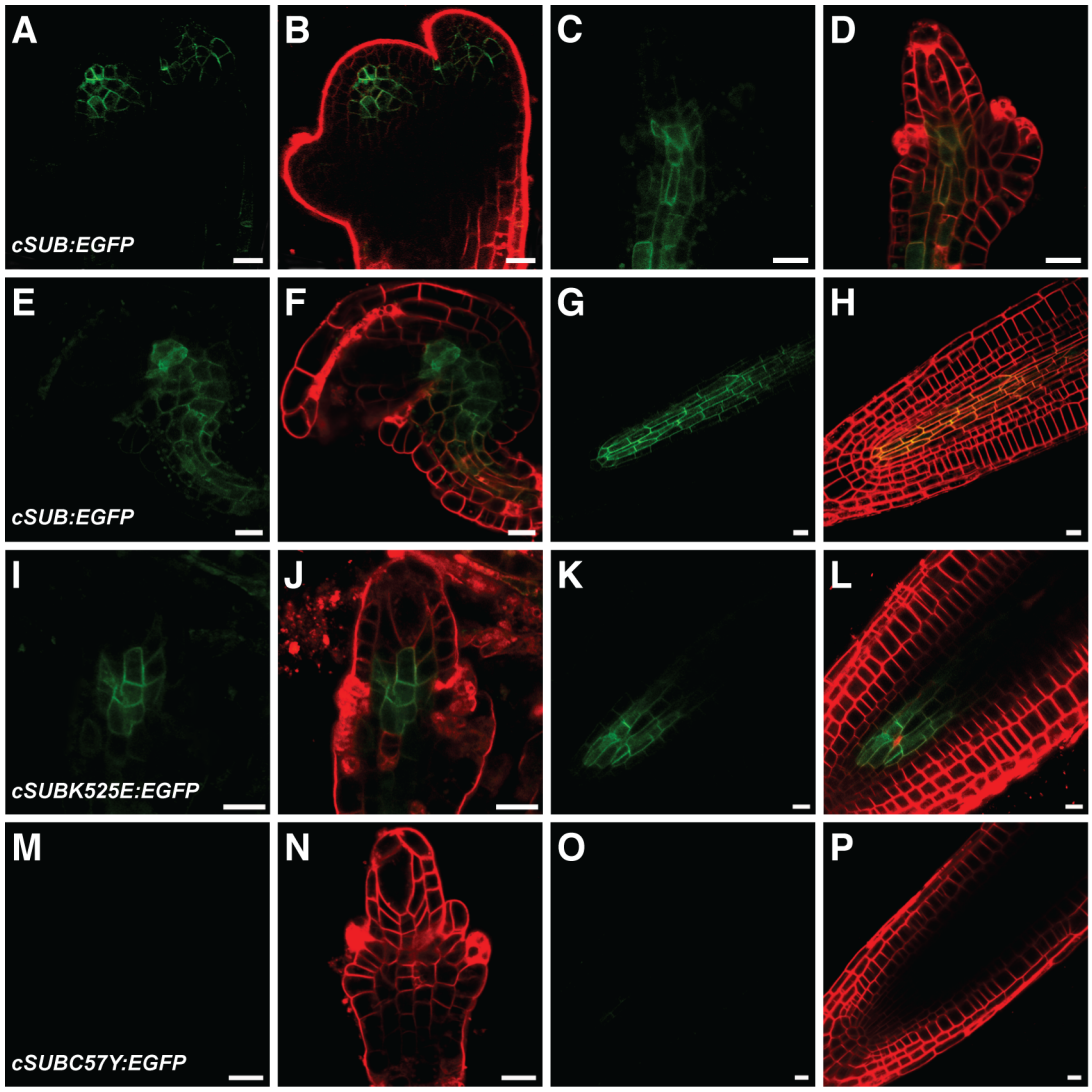


Figure 3.1.7 Expression analysis of different *SUB::cSUB:EGFP*-based reporters.
Live confocal microscopy images obtained from L*er* plants carrying various cDNA-derived SUB:EGFP reporters. The FM4-64 stain was used to mark the outline of all cells in a tissue. Signals from the EGFP and FM4-64 channels are shown in green and red, respectively. Stage 3 floral meristems (A-B), stage 2-III (C-D, I-J, M-N) and 3-V/VI ovules (E-F), and roots from 4-day old seedlings (G-H, K-L, O-P) are depicted. (A-H) *SUB::cSUB:EGFP*. Weak signals are only detected in the center of the different organs. (I-L) *SUB::cSUB*$_{K525E}$*:EGFP*. Weak signal that is noticeably restricted to the interior part of ovules and roots. (M-P) *SUB::cSUB*$_{C57Y}$*:EGFP*. No detectable signal in ovules or roots. Scale bars: 10 µm.

3.1.6 *SUB* intronic sequences positively influence *SUB::SUB:EGFP* signal strength

Previous studies indicated that the spatial expression domain of *SUB* transcription extends to the periphery of several organs, such as ovules, floral meristems, and roots (Chevalier et al., 2005; Kwak et al., 2005) whereas the *SUB::cSUB:EGFP* reporter only exhibits a weak signal in interior cells of those organs (Yadav et al., 2008) (Figure 3.1.7A-H). In addition, a similar construct failed to rescue the root phenotype of *sub* mutants (Kwak and Schiefelbein, 2008). Thus, as previously suggested, *SUB* expression may be subject to complex control (Yadav et al., 2008) and the findings raise the possibility that intronic sequences of *SUB* influence transcriptional or post-transcriptional processes. Therefore, I generated a genomic *SUB* DNA construct that shares identical promoter elements with the cDNA-based reporter (Yadav et al., 2008) but included all *SUB* introns (*SUB::gSUB:EGFP*). Similar to its cDNA-based variant this construct could also rescue the *sub-1* phenotype (Figure 3.1.6C). Moreover, the new reporter indeed exhibited a broad signal that was detectable in the center and at the periphery of ovules, floral meristems and roots (Figure 3.1.8A-H). The *gSUB:EGFP* reporter expression thus mimicked the *SUB* expression pattern as observed by *in situ* hybridization (Chevalier et al., 2005) and *SUB::GUS* studies (Kwak et al., 2005; Yadav et al., 2008). The signal tended to be somewhat stronger in internal tissues compared to more peripheral cell layers.

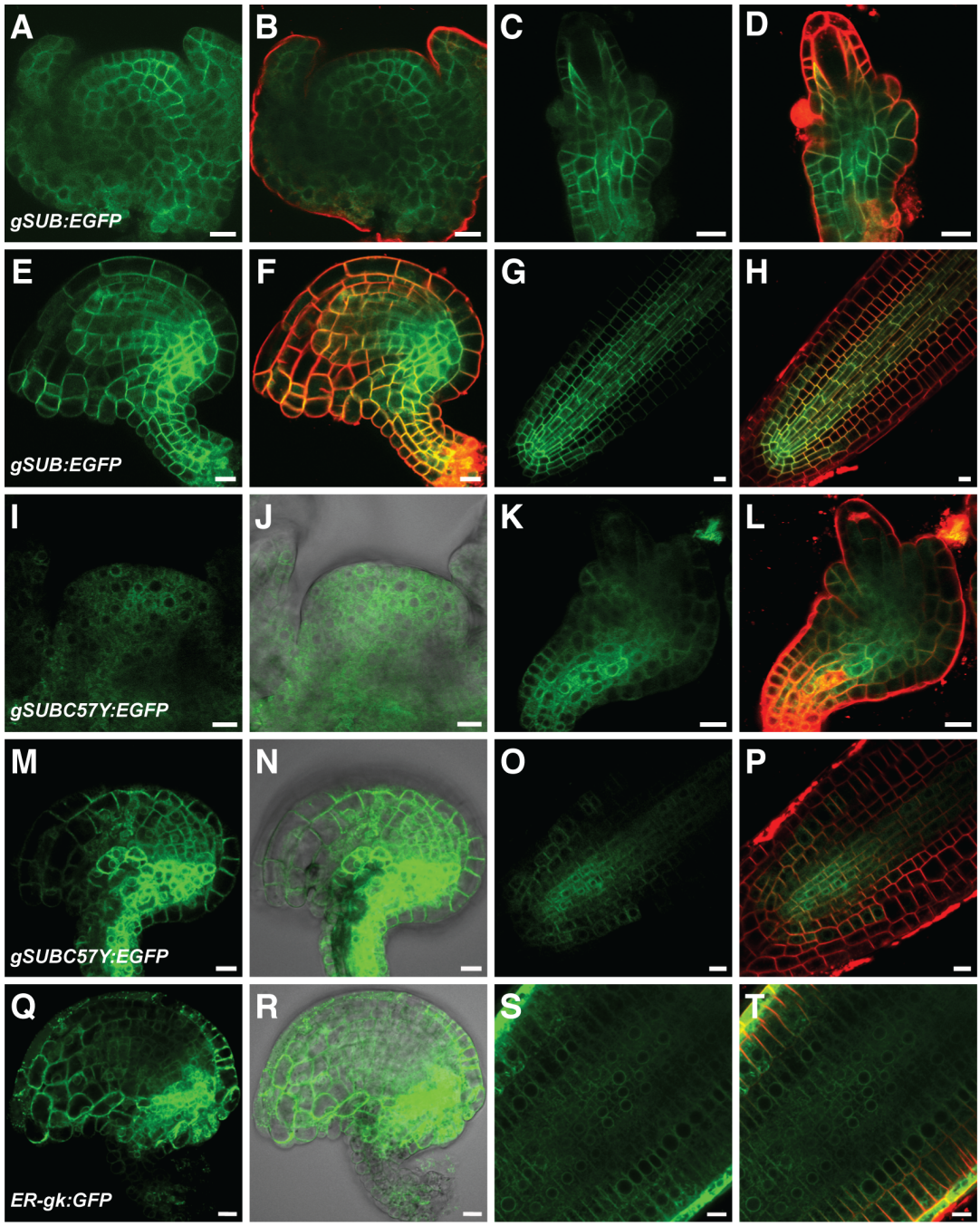


Figure 3.1.8 Expression analysis of *SUB::gSUB:EGFP*-based reporters.
Live confocal microscopy images obtained from L*er* plants carrying various genomic DNA-derived SUB:EGFP reporters. The FM4-64 stain (red) was used to mark the outline of all cells in a tissue. Differential interference contrast (DIC) photomicrographs are shown to outline the tissue (J, N, R). Stage 3 floral meristems (A-B, I-J), stage 2-III (C-D) stage 2-IV (K-L) and 3-V/VI ovules (E-F, M-N, Q-R), and roots from 4-day old seedlings (G-H, O-P, S-T) are depicted. Note the broad expression pattern, which includes the epidermis, in all examined tissues and with all tested reporter constructs. Signal tends to be stronger in interior tissues. (A-H) *SUB::gSUB:EGFP*. (I-P) *SUB::gSUB$_{C57Y}$:EGFP*. Signal remains broadly detectable in tested tissues. Note the ER-like sub-cellular distribution (compare with Q-T). (Q-T) Line ER-gk CS16251 (Col) carrying plasmid ER-gk CD3-955. Control reporter exhibiting expression in the ER (Pettersen et al., 2004). Scale bars: 10 µm.

3.1.7 The presence of multiple introns is essential for SUB broad expression

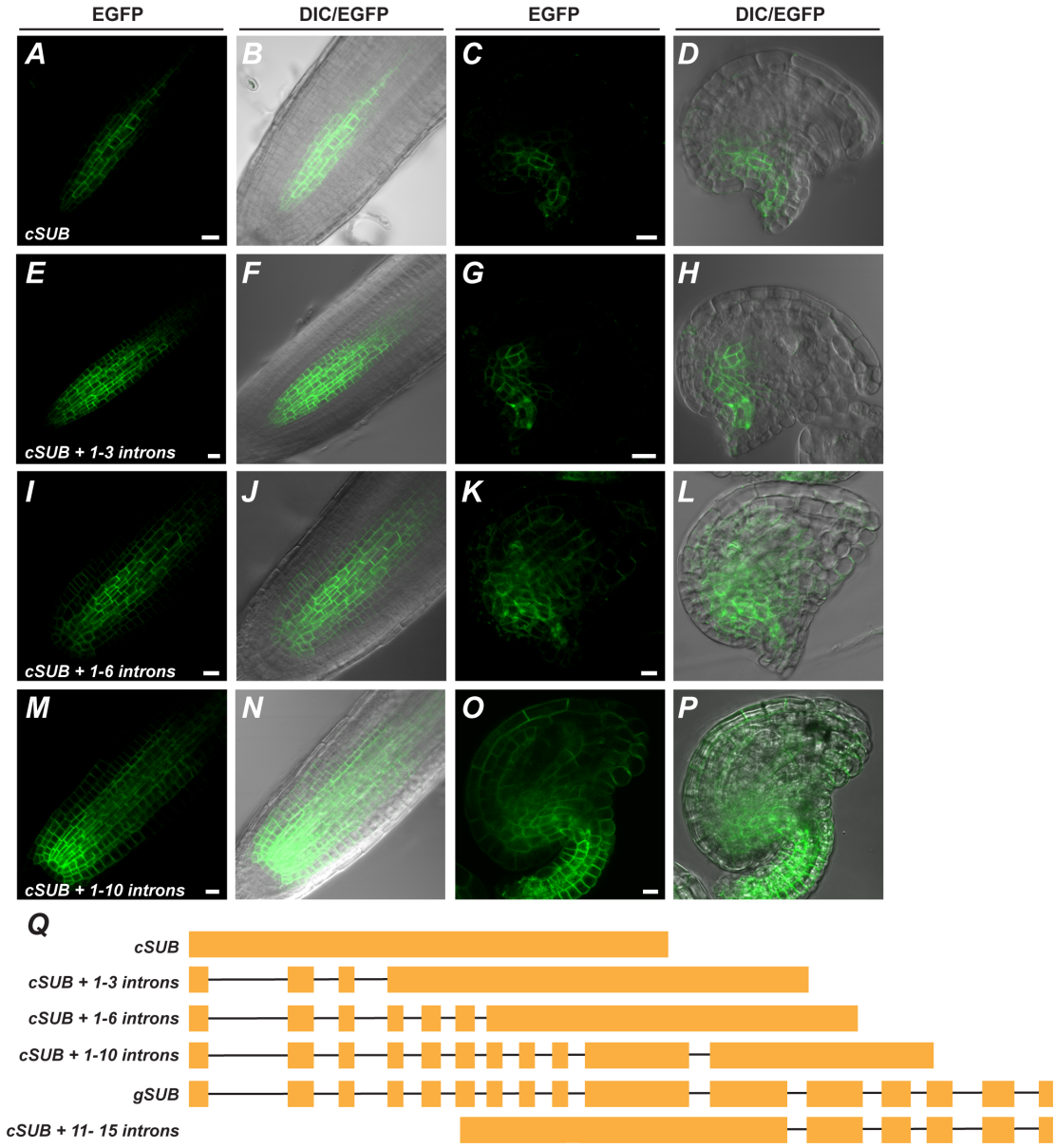


Figure 3.1.9 *SUB* intron mapping.
Live confocal microscopy images obtained from L*er* plants carrying various intron deletion SUB:EGFP reporters (A-P). Left panel shows the expression in roots. Right panel shows expression in ovules. Scale bars: 10 µm. The intron and exon organization of the various constructs (Q).

Introns have the ability to promote gene expression. For example, maize alcohol dehydrogenase-1 expression in a transient assay is increased 50-100 times when introns are included (Callis et al., 1987) and floral homeotic gene AGAMOUS

(AG) requires the second intron for its proper native expression pattern (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000). *SUB* locus has 15 introns (Figure 3.1.9Q). To know whether single or multiple introns are necessary for the *SUB* broad expression, three different *SUB::SUB:EGFP* reporter constructs were made each having 3, 6 or 10 introns respectively (Figure 3.1.9Q). As shown in Figure 3.1.9, in root when compared to intronless *cSUB* (Figure 3.1.9A and B), expression of *SUB* with first 3 introns (Figure 3.1.9E and F) extends to one more peripheral cell layer whereas for *SUB* with first 6 introns expression can be seen up to epidermal layer but is inconsistent (Figure 3.1.9I-J). Finally, *SUB* with 10 introns shows the broad expression comparable to *gSUB* (Figure 3.1.9M-N). Similar type of trend can also be observed in ovules (Figure 3.1.9 right panel). In summary, the expression pattern of *SUB* becomes stronger and broader as the number of introns increase from 1 to 10 indicating introns contribute to the stability of *SUB* transcript. Interestingly, when a construct was made with the last 5 introns only (Figure 3.1.9Q), no signal was observed and also it couldn't rescue the *sub-1* phenotype (not shown).

3.1.8 Equivalent *SUB::cSUBmut:EGFP* and *SUB::gSUBmut:EGFP* constructs behave in a genetically identical manner

To examine if the observed differences in SUB:EGFP signal strengths between the cDNA and genomic based reporters could influence the genetic analysis, I introduced into the *SUB::gSUB:EGFP* reporter by in vitro mutagenesis many of the different point mutations that are predicted to allow the translation of a full-length SUB protein but to result in either a functional or nonfunctional *SUB::cSUBmut:EGFP* constructs (Figure 3.1.5B). In summary, it was found that mutations rendering the cDNA-based construct nonfunctional also resulted in nonfunctional genomic versions, as corresponding *SUB::gSUBmut:EGFP sub-1* plants showed no rescue of the *sub* mutant phenotype (an example is given in Figure 3.1.6D). A similarly coherent relationship was observed for mutations that retained functionality (not shown). Thus, in terms of genetic complementation of *sub-1* plants the cDNA and genomic versions yielded identical results demonstrating that choice of construct did not influence the functional analysis in a noticeable manner.

Next I assayed the signal strength and distribution of different *SUB::gSUBmut:EGFP* reporters. Though the spatial distribution at the organ level was similar to wild type (Figure 3.1.8I-P) fewer lines exhibited detectable signal when compared to the wild-type *SUB::gSUB:EGFP* reporter (about 10/50 independent T1 lines vs 30/50) indicating overall weaker expression of the mutant reporters. These findings indicate that absence of an EGFP signal in cDNA-based reporter lines indeed relates to the weaker overall expression levels because of lack of introns. Interestingly, not all *sub-1* T1 lines carrying the *SUB::gSUB:EGFP* reporter exhibited detectable expression (20/50). Of these 20 T1 lines without apparent expression 15 still showed partial to complete rescue of the *sub-1* phenotype (not shown). This result indicates that very low levels of *SUB* expression provide sufficient *SUB* activity (see also below).

3.1.9 Various mutant SUB variants are retained in the endoplasmic reticulum and degraded by ERAD

The detectable *SUB::gSUBmut:EGFP* reporter signals allowed the ready analysis of the subcellular distribution of the SUBmut:EGFP fusion proteins. In all instances, and irrespective of mutations in the extracellular or intracellular domains of SUB, a reticulated signal distribution typical for an ER-like pattern was observed, although minor signal was present at the plasma membrane as well (Figure 3.1.8I-T and Figure 3.1.10). Subcellular signal distribution was essentially identical to a functional reporter carrying an N-terminal fusion of EGFP to SUB (Figure 3.1.6G and Figure 3.1.10Q-T) or to a bri1-5-GFP reporter (Hong et al., 2008). The ER-related signal was never observed in wild-type *SUB::gSUB:EGFP* reporter lines (Figure 3.1.8A-H). The lack of signal in *SUB::cSUBmut:EGFP* reporters and their ER-like subcellular signal distribution of the genomic versions (*SUB::gSUBmut:EGFP)* suggest that nonfunctional mutant SUB variants are partially retained in the ER by endoplasmic reticulum-mediated quality control (ERQC) system which disposes of misfolded and/or unassembled proteins by endoplasmic reticulum-associated degradation (ERAD) (Ellgaard and Helenius, 2003; Römisch, 2005; Vembar and Brodsky, 2008; Vitale and Boston, 2008). Similar scenario has been proposed for

mutant variants of BRI1 and EFR (Jin et al., 2007; Nekrasov et al., 2009; Li et al., 2009).

To corroborate the notion that SUB receptors can be subject to ERQC/ERAD, I treated wild-type or *sub-1* seedlings carrying the *sub-3* and *sub-10* mutations in the ECD of SUB:EGFP with kifunensine (Kif). The investigated reporters include *SUB::cSUB$_{V64M}$:EGFP*, *SUB::gSUB$_{V64M}$:EGFP*, *SUB::cSUB$_{C57Y}$:EGFP*, and *SUB::gSUB$_{C57Y}$:EGFP*. Furthermore, I included in our analysis a reporter corresponding to the *sub-4* mutation in the intracellular kinase domain (*SUB::cSUB$_{R599C}$:EGFP*, *SUB::gSUB$_{R599C}$:EGFP*). Kif is a potent inhibitor of glycoprotein processing mannosidase I in the ER and prevents ERAD of many terminally misfolded proteins (Elbein et al., 1990; Tokunaga et al., 2000). Expression analysis of the mutated SUB$_{mut}$:EGFP fusion proteins in roots (three independent lines, 10 individual seedlings each) revealed that indeed signals could be observed for the cDNA-based ECD mutational variants *SUB::cSUB$_{V64M}$:EGFP* and *SUB::cSUB$_{C57Y}$:EGFP* upon Kif treatment (Figure 3.1.10A-D). The expression patterns were comparable to the related wild-type *SUB::cSUB:EGFP* reporter (Figure 3.1.7G and H) (Yadav et al., 2008) and were irrespective of the genetic background (wild-type versus *sub-1*). The data suggest that *SUB::cSUBmut:EGFP* transgenes are principally transcribed (despite the absence of an EGFP-signal), that mutant variants of SUB:EGFP fusion proteins are subject to ERAD and that this process contributes to undetectable levels of fusion proteins derived from *SUB::cSUBmut:EGFP* reporters. I also examined the roots of seedlings carrying genomic reporter variants (*SUB::gSUB$_{V64M}$:EGFP*, *SUB::gSUB$_{C57Y}$:EGFP*). The addition of Kif to seedling growth medium resulted in increased signal intensity in root tips (Figure 3.1.10E-H) substantiating the results obtained with the cDNA-based reporters.

As described, the C57Y and V64M variants carry alterations in the ECD of SUB. What happens to variants with a mutation in the intracellular domain? To address this question, I assessed reporter lines carrying either *SUB::cSUB$_{R599C}$:EGFP* or *SUB::gSUB$_{R599C}$:EGFP* (*sub-4*-derived) reporters. No signal could be detected in roots of *sub-1* or wild-type plants carrying the *SUB::cSUB$_{R599C}$:EGFP* reporter,

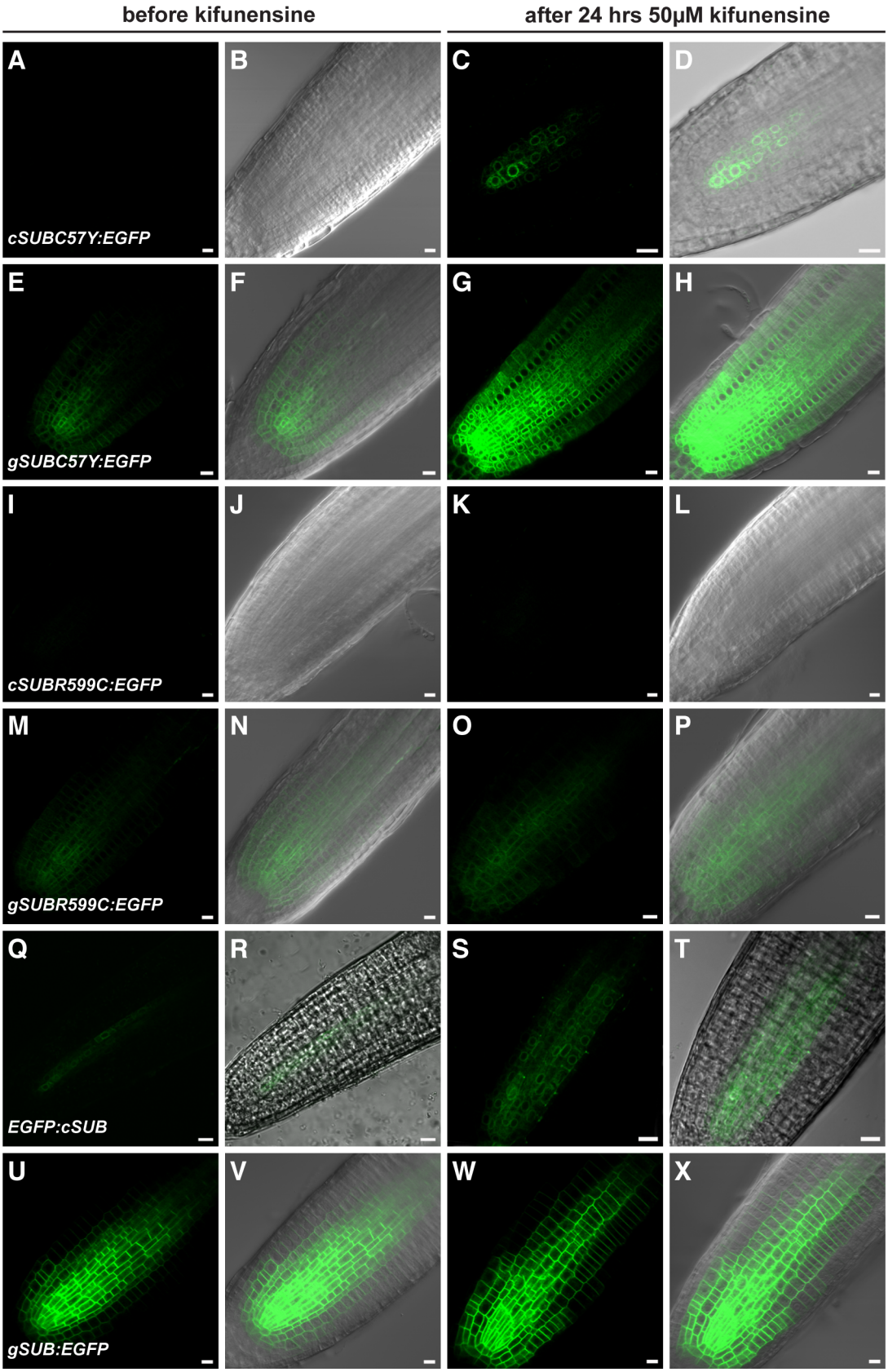


Figure 3.1.10 Effects of kifunensine treatments on the expression of different SUB::c/gSUB:EGFP reporters.

Live confocal microscopy images from roots were generated using 4-day old Arabidopsis seedlings (Ler) carrying different SUB:EGFP reporters. Signals from the EGFP channel are shown in green. DIC photomicrographs are shown to outline root tissue (B, D, F, H, J, L, N, P, R, T, V, X). The same root before (A-B, E-F, I-J, M-N, Q-R, U-V) and after (C-D, G-H, K-L, O-P, S-T, W-X) 24-hrs treatment with 50 μM kifunensine. (A-D) SUB::cSUBC57Y:EGFP. Signal becomes detectable upon kifunensine treatment. Note ER-like pattern (compare with Figure 9S-T). (E-H) SUB::gSUBC57Y:EGFP. Signal becomes stronger upon kifunensine treatment. (I-L) SUB::cSUBR599C:EGFP. Absence of signal, irrespective of kifunensine treatment. (M-P) SUB::gSUBR599C:EGFP. Signal is easily detectable and not noticeably influenced by kifunensine treatment. (Q-T) SUB::EGFP:cSUB. Note the ER-like pattern (compare with Figure 9S-T). No change in signal intensity was observed upon kifunensine treatment. (U-X) SUB::gSUB:EGFP. The reporter signal does not change detectably upon treatment with kifunensine. Scale bars: 10 μm.

irrespective of the addition of Kif (Figure 3.1.10I-L) (8 independent T1 lines tested). Individual seedlings of three independent lines (10 seedlings per line) carrying the genomic *SUB::gSUB$_{R599C}$:EGFP* variant exhibited a signal in root tips that stayed constant upon Kif treatment (Figure 3.1.10M-P). Interestingly, signals of the *SUB::c/gSUB$_{R599C}$:EGFP* reporters exhibited a similar sub-cellular distribution to the one exhibited by SUBmut:EGFP fusion proteins with defects in their ECDs. The results suggest that a Kif-dependent process does not measurably affect the *sub-4* variant of SUB, which carries an altered cytoplasmic kinase domain.

3.1.10 SUB undergoes MLN4924-sensitive degradation

Mg132 is a common 26S proteasome inhibitor that prevents degradation of a wide range of ERAD substrates (Schmitz and Herzog, 2004). Unfortunately, I could not test the involvement of the proteasome in SUB-related ERAD as *SUB* undergoes MG132-sensitive posttranscriptional regulation in root tips (Yadav et al., 2008). Seedlings carrying *cSUB:EGFP* reporters start to lose detectable SUB:EGFP signal after three hours of treatment with MG132 (Yadav et al., 2008). This phenomenon was also observed for *gSUB:EGFP*-based reporter constructs and was irrespective of whether wild-type or mutant SUB:EGFP fusion proteins are examined (Figure 3.1.11).

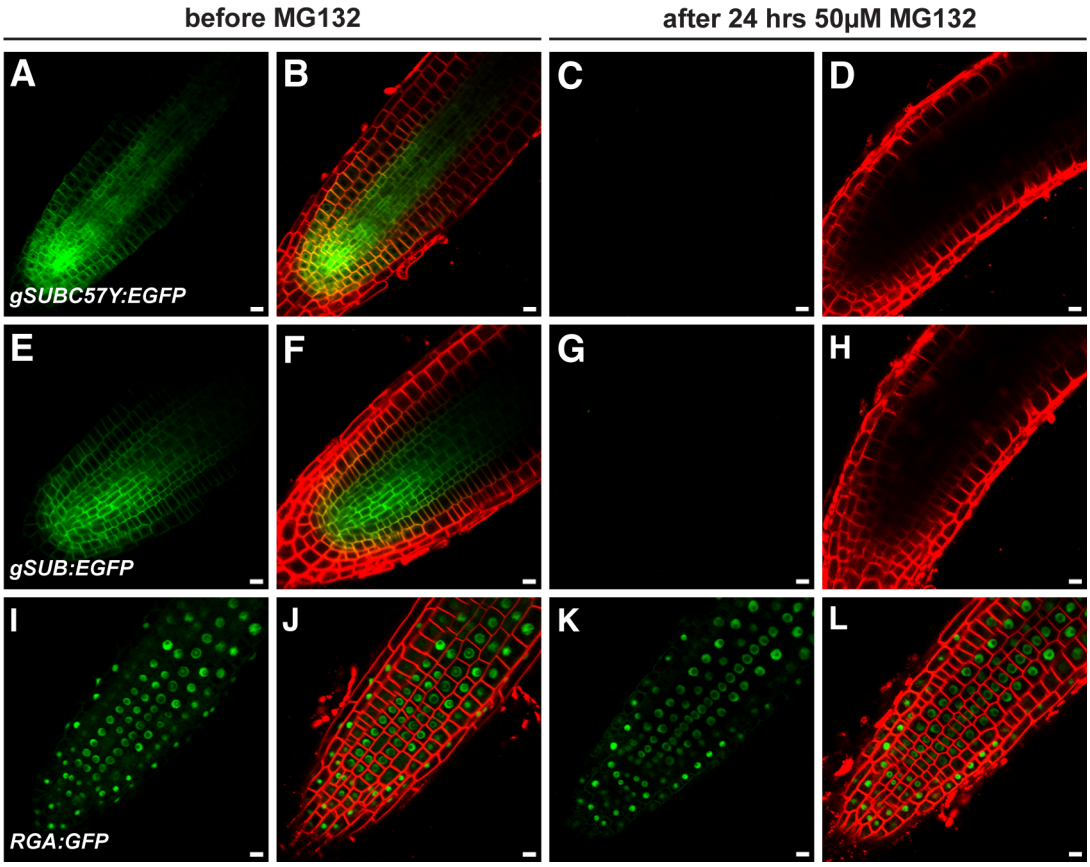


Figure 3.1.11 Effects of MG132 treatments on the expression of different *SUB::gSUB:EGFP* reporters.
Live confocal microscopy images from roots were generated using 4-day old Arabidopsis seedlings (L*er*) carrying different SUB:EGFP reporters. The same root is shown before (A-B, E-F, I-J) and after (C-D, G-H, K-L) 24-hrs treatment with 50 µM MG132. The signals from the EGFP and FM4-64 channels are shown in green and red, respectively. (A-D) *SUB::gSUB$_{C57Y}$:EGFP*. (E-H) *SUB::gSUB:EGFP*. (I-L) A *RGA::RGA:GFP* reporter that served as control (Czechowski et al., 2005). Note that signal persisted after MG132 treatment (K-L). Scale bars: 10 µm.

Neddylation is an important post-translational modification of the cullin subunits of cullin-RING-type E3 ubiquitin ligases (CRLs), which leads to the degradation of CRL substrates. MLN4924 has been described as a neddylation inhibitor (Soucy et al., 2009; Bennett et al., 2010). Recently, MLN4924 efficiency was also demonstrated in Arabidopsis (Hakenjos et al., 2011). I asked if MLN4924-sensitive process could degrade SUB:EGFP in cells of the main root. Transgenic *SUB::gSUB:EGFP* seedlings were treated with MLN4924 which resulted in loss of reporter signal (Figure 3.1.12) indicating SUB undergoes MLN4924-sensitive post-transcriptional regulation.

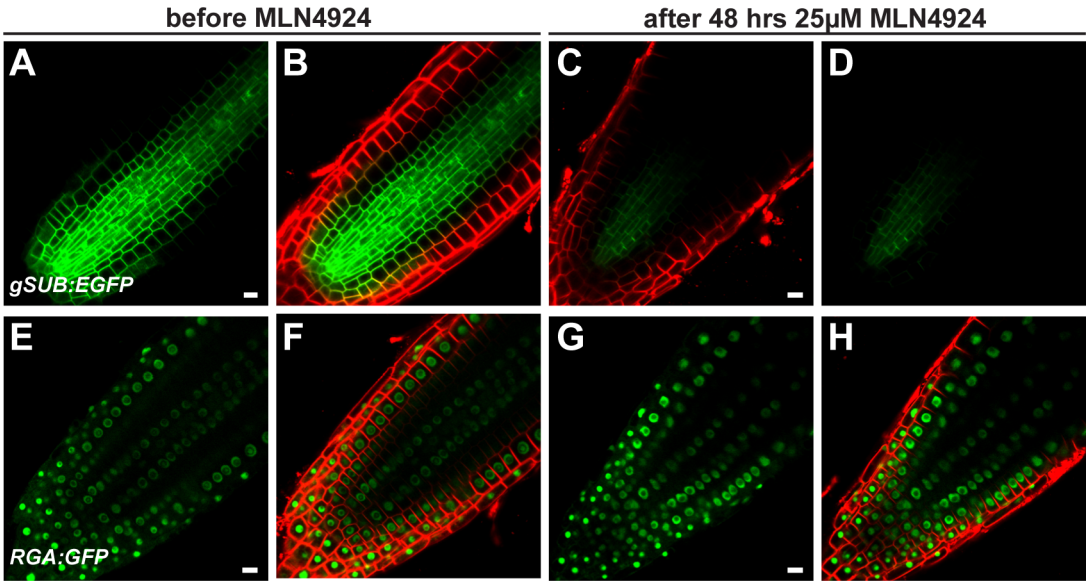


Figure 3.1.12 Effects of MLN4924 treatment on the expression of *SUB::gSUB:EGFP* reporters.
Live confocal microscopy images from roots were generated using 4-day old Arabidopsis seedlings. The same root is shown before (A-B, E-F) and after (C-D, G-H) 24-hrs treatment with 25 µM MLN4924. (A-D) *SUB::gSUB:EGFP*. (E-H) *RGA::RGA:GFP* reporter control (Czechowski et al., 2005). Scale bars: 10 µm.

3.2 Molecular characterization of QUIRKY

3.2.1 Mapping and Molecular Identification of *QUIRKY*

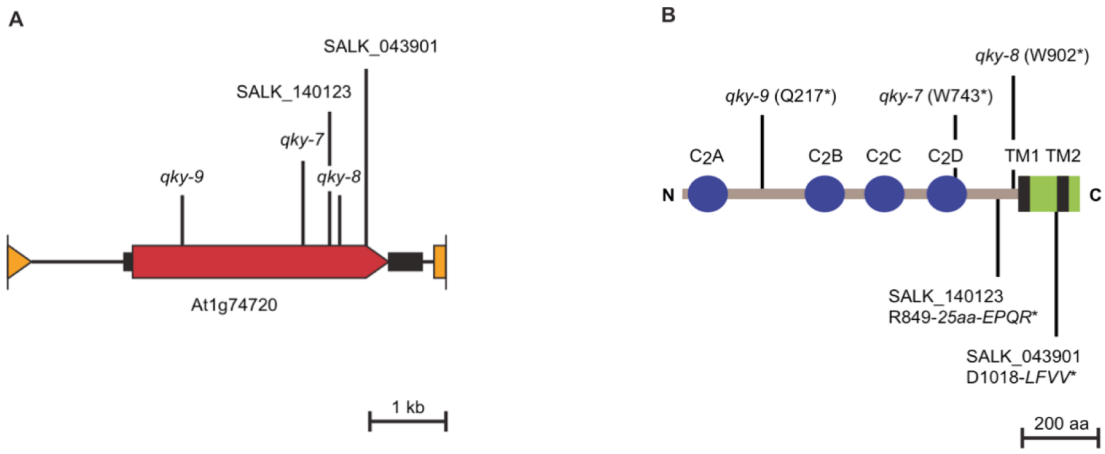


Figure 3.2.1 Molecular characterization of *QKY*.
(A) Molecular organization of the *QKY* locus on chromosome 1. The horizontal black line highlights genomic DNA. The red arrow indicates the orientation and extent of the *QKY* open reading frame. The small black boxes mark the 5' and 3' untranslated regions, respectively. The orange arrow and box denote the end of the preceding and the start of the next annotated open reading frames, respectively. The positions of the various point mutations or T-DNA insertion sites are marked. (B) Predicted domain topology of the QKY protein. The sequence and numbering of the C2 domains, and the altered C-termini of the mutant protein variants, are given. The italic capital letters represent the de novo residues present in the predicted mutant QKY proteins from the two T-DNA lines. Stars mark the premature stops in the predicted mutant proteins. The green box denotes the plant phosphoribosyl-transferase C-terminal region (PRT_C). Abbreviations: aa, amino acids; N, amino terminus; C, carboxy terminus; TM, transmembrane domain.

To map the *QKY* locus at high resolution, an F2-mapping population of q*ky* (L*er*)/Col was generated. Preliminary mapping genetically linked *QKY* to a single region on the lower arm of chromosome 1. Marker amplifications from 598 mutant individuals restricted the *qky* map interval to 103 kb, as defined by markers F25A4(BglII) and 27.99(RsaI). Candidate genes within this interval were analyzed via T-DNA insertion mutant analysis and/ or sequence determination revealing that At1g74720 carried mutations in various *qky* alleles. In all three EMS-induced *qky* alleles single point mutations could be identified in At1g74720 (Figure 3.2.1). Further, two distinct T-DNA insertions (Figure 3.2.1) in this locus result in *qky* mutant phenocopies as shown in Figure 3.2.2. Similar to *SUB*, the *QKY* T-DNAs show subtle phenotype in Columbia background with normal stem morphology and subtle floral

twisting when compared to the *qky-8* in L*er* background (Vaddepalli et al., 2011) (Figure 3.2.2).

Figure 3.2.2 Morphology of mutant *qky* in Columbia and L*er* backgound
(A) Wild type (Col). (B) *salk-140123* (Col). (C) salk-*043901* (Col) (D) Wild type (L*er*), (E) *qky-8* (L*er)*. In T-DNAs stem twisting is absent, flowers show mild twisting, silique twisting is prominent. In comparison twisted morphology is clear in L*er* background (*qky-8)* with severe floral, stem and silique twisting. Scale bars: 0.5 mm.

3.2.2 QUIRKY, a novel multiple C2 domain containing transmembrane Protein (MCTP)

Sequence analysis predicted that *QKY* contains no introns and encodes a transmembrane protein of 1081 amino acids with a calculated molecular mass of 121.4 kDa harboring four C2 domains (Figure 3.2.1B). In addition, two transmembrane domains are embedded in a plant-specific phosphoribosyltransferase C-terminal region (PRT_C, PFAM identifier PF08372), which according to the PFAM database occurs characteristically at the carboxy-terminus of phosphoribosyltransferases and

phosphoribosyltransferase-like proteins (Punta et al., 2012). Interestingly, this domain often appears together with C2-domains, as in QKY. A related domain topology, albeit with only three C2 domains, is found in several proteins present in humans, *Drosophila melanogaster* and *Caenorhabditis elegans* (*C. elegans*), collectively referred to as multiple C2 domain and transmembrane region proteins (MCTPs) (Shin et al., 2005). The role of animal MCTPs is not well defined despite the fact that in *C. elegans* a single MCTP gene is present and essential for embryo viability (Maeda et al., 2001). QKY represents the first described plant MCTP-class gene. In accordance with proposed transmembrane topography for MCTPs the QKY C2 domains most likely have an intracellular localization as QKY lacks a distinguishable N-terminal signal peptide and all known C2 domains are cytoplasmic. C2 domains are autonomously folding modules and form Ca^{2+}-dependent phospholipid complexes, although some exceptions are known that do not bind Ca^{2+} or phospholipids or both (Rizo and Südhof, 1998; Südhof, 2002; Bai and Chapman, 2004). Other animal proteins with multiple C2 domains, but only one transmembrane region, include the synaptotagmins (Südhof, 2002), the extended synaptotagmins (Min et al., 2007), and the ferlins (Bansal and Campbell, 2004). Synaptotagmins and ferlins are known to play a role in membrane trafficking. Although there is general resemblance in domain topology, very little primary sequence conservation is observed between QKY and the animal MCTPs, synaptotagmins, and ferlins. The *qky-7*, *qky-8*, and *qky-9* alleles likely cause a complete loss of QKY function. All three mutations are predicted to introduce stop codons leading to shorter proteins with variable numbers of C2 domains but always lacking the two transmembrane domains (TMDs) (Figure 3.2.1). Thus, the three mutants likely contain truncated QKY variants that are not properly located to the membrane. Membrane localization, however, seems essential for QKY function as all three mutants show identical phenotypes, regardless of the number of C2 domains still present in the predicted truncated proteins. In summary, the results suggest that QKY is a membrane-bound Ca^{2+} signaling factor.

3.2.3 Biochemical characterization of QUIRKY C2 domains

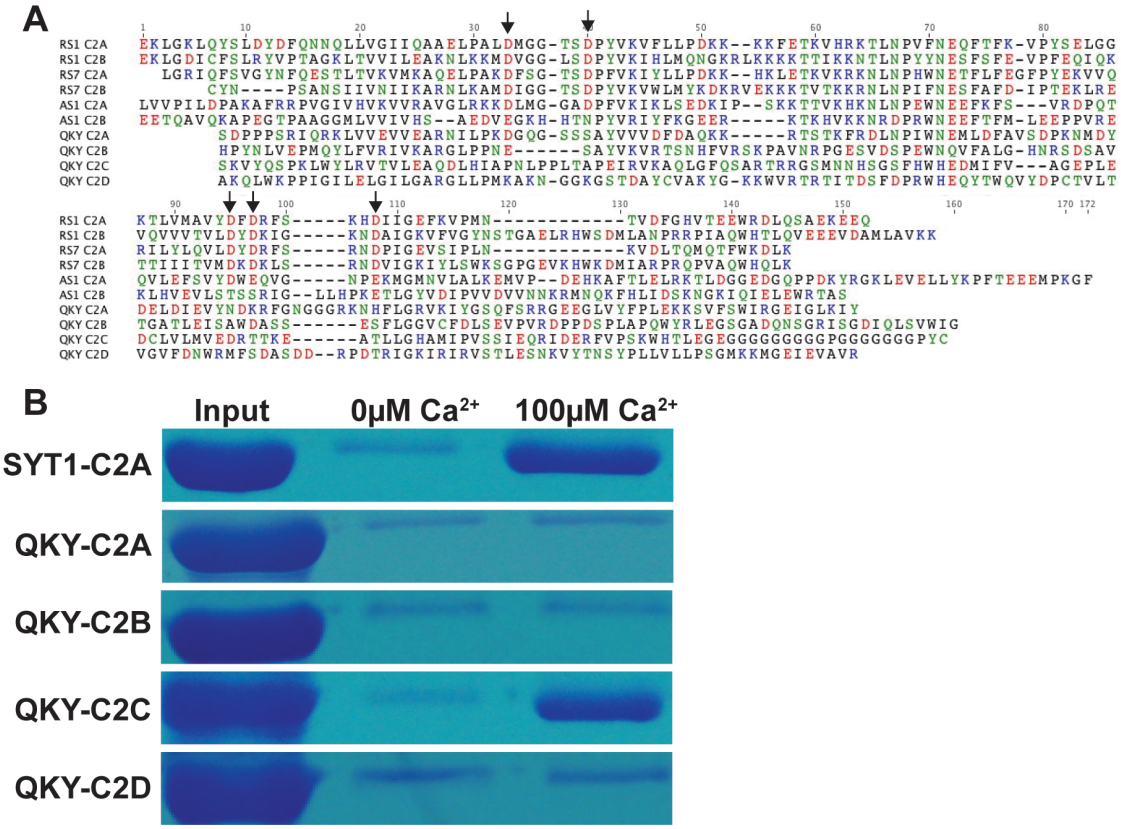


Figure 3.2.3 Phospholipid binding assay of QKY C2 domains
(A) Sequence alignment of the C2 domains from rat synaptotagmin 1 (RS1), rat synaptotagmin 7 (RS7), Arabidopsis synaptotagmin 1 (AS1) and QKY. Similar colors indicate similar polarity of the residues. Amino acid residues that form the Ca2+ binding sites of the C2 domains in rat synaptotagmin 1 and 7 are indicated by arrowheads. (B) Recombinant GST fusion proteins of the individual C2 domains were used in phospholipid binding assays. Proteins were incubated in the absence or the presence of 100 µM Ca^{2+} with liposomes composed of 25% PS/75% PC. Liposomes were precipitated by centrifugation, and bound proteins were analyzed by SDS-PAGE. C2A domain of SYT1 was used as control. QKY C2C domain shows calcium dependent phospholipid binding. Weak background bands could be due to unspecific binding of denatured protein to the liposomes.

The Ca^{2+}-dependent binding to phospholipids is the best characterized property of C2 domains. Among all the C2 domains studied so far the Ca^{2+} binding property is shown to be dependent on five aspartate, glutamate or asparagine residues at conserved positions. Interestingly, these conserved amino acids are absent in QKY (Figure 3.2.3A). Nevertheless, analyses of animal synaptotagmins indicate that Ca^{2+} and phospholipid binding properties cannot be reliably predicted from sequence analysis alone (Dai et al., 2004). Therefore, I studied whether the C2 domains of QKY

are capable of forming Ca^{2+}-dependent complexes with negatively charged liposomes (25% PS/75% PC). For this purpose, I used a centrifugation assay to investigate Ca^{2+} -dependent phospholipid binding of C2 domains to liposomes (Fernandez et al., 2001; Fernández-Chacón et al., 2002; Shin et al., 2003). Four recombinant C2 fragments of QKY and C2A from rat-syt1 (used as a positive control) were purified as glutathione S-transferase (GST) fusion proteins and incubated with liposomes in the presence of free Ca^{2+} or 100µM Ca^{2+}. After isolation (see materials and methods), liposome-bound proteins were analyzed using SDS-PAGE and Coomassie blue staining. Interestingly, of all QKY C2 domains only C2C showed capability of binding to phospholipids in a Ca^{2+}-dependent manner (Figure 3.2.3B). Unfortunately, I was not able to assay full-length QKY protein as it has shown degradation fragments after purification (not shown).

3.2.4 *QUIRKY* Expresses in various plant organs

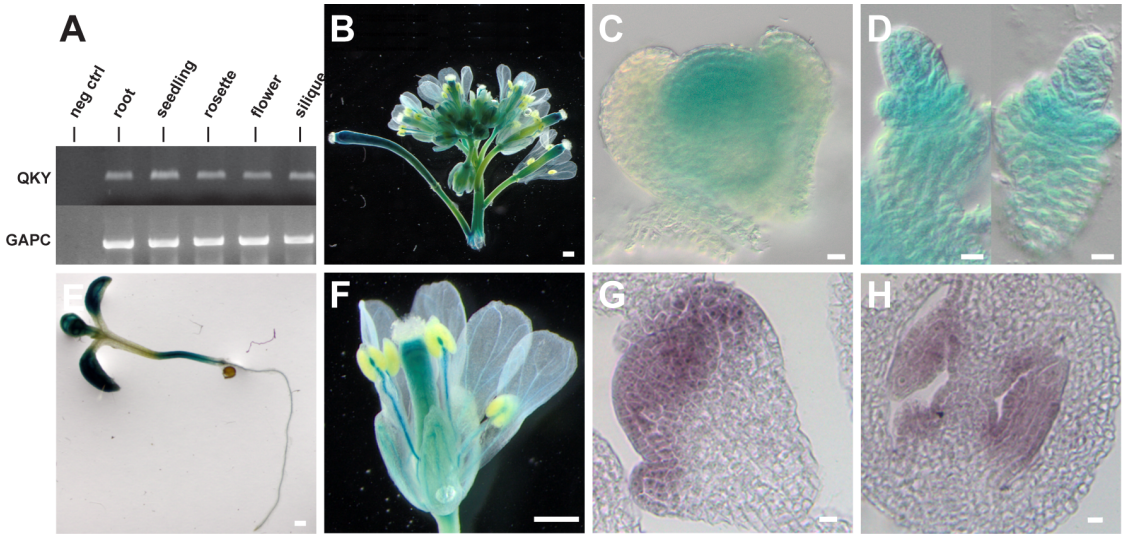


Figure 3.2.4 *QKY* gene expression pattern.
Tissue distribution of QKY expression as revealed by semi-quantitative reverse transcription PCR (A). The result obtained after 28 cycles for all tested genes is shown. GAPC served as control (Shih et al., 1991). QKY::GUS activity (B-F). *In situ* hybridization (G and H). Inflorescence (B). 6-day old seedling (E). flower (F). Stage 3 floral meristems (C, G). Stage 2-3 ovules(D, H). Scale bars: 0.5mm(B, E and F); Scale bars: 10 µm (C, D, G and H).

To investigate *QKY* gene expression pattern, first data was compiled using the Genevestigator and AtGenExpress databases, which showed a constitutive expression

in all tissues analyzed (Figure S2) (Zimmermann et al., 2004; Schmid et al., 2005). Semi-quantitative RT-PCR experiments using RNA extracted from roots, seedlings, rosette leaves, inflorescences, and siliques also suggested a ubiquitous expression for *QKY* (Figure 3.2.4A). In order to study the in planta expression pattern of *QKY*, a construct was generated wherein GUS (β-glucuronidase) reporter was driven under *QKY* cis-regulatory sequences located upstream and downstream of the open reading frame (Figure 3.2.1A) (see materials and methods). In the transgenic *QKY::GUS* plants, *QKY* expression can be seen in most tissues and in general, the reporter expression was strong in vascular tissues. At the seedling stage, reporter expression can be seen in root, hypocotyl, cotyledons and young leaves (Figure 3.2.4E). In inflorescence, GUS activity was observed in most tissues of inflorescence stems, sepals, and anther filaments (Figure 3.2.4B). These results of GUS activity are in agreement with the *QKY* expression determined by semi-quantitative RT-PCR and the Genevestigator database (Zimmermann et al., 2004).

Further, *QKY::GUS* expression analysis was done at tissue level using ovules and floral meristems. The *QKY::GUS* reporter labeled all cell layers of the floral meristem and of the developing ovule (Figure 3.2.4C and D). To further establish the spatial expression pattern of *QKY* in situ hybridization was carried out. As shown in Figure 3.2.4G and H, the expression pattern was comparable to *QKY::GUS* in floral meristem and young ovules. In summary, in all tissues assayed the spatial and temporal expression pattern of *QKY* gene was essentially identical to the reported *SUB* expression pattern (Chevalier et al., 2005).

3.2.5 QUIRKY is localized to Plasmodesmata

Though sequence analysis predicts QKY to be a membrane protein insight about its cellular localization and expression pattern is imperative to further understand its involvement in SUB signaling pathway. In this direction transgenic plants were generated that carried N-terminal translational fusion of EGFP and QKY. The *QKY::EGFP:QKY* construct includes promoter fragments identical to those in the *QKY::GUS* construct described. Unfortunately, though more than 100 independent

transgenic lines were analyzed none of them showed any detectable EGFP signal. The *qky-8* plants carrying *QKY::EGFP:QKY* exhibited a WT phenotype indicating that the EGFP:QKY fusion protein is biologically functional as shown in Figure 3.2.8C.

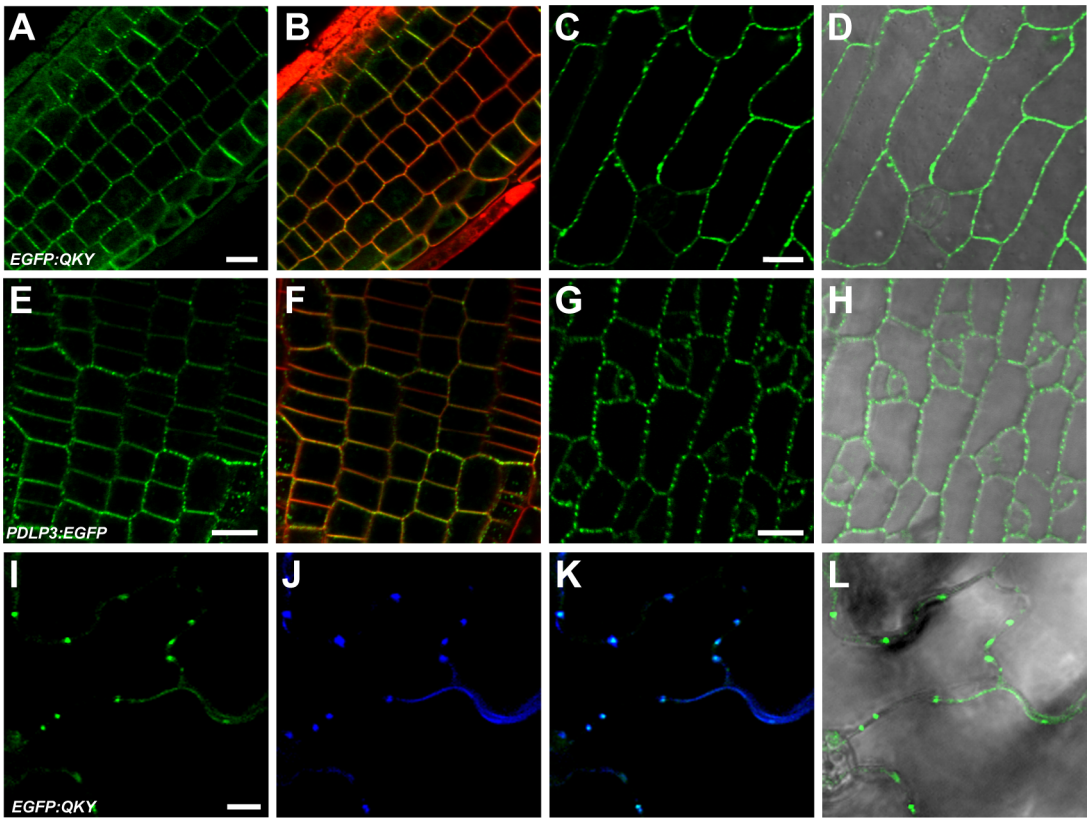


Figure 3.2.5 Localization of QUIRKY to PD
Live confocal microscopy images obtained from WT Arabidopsis carrying *UBQ::EGFP:QKY* or *PDLP3::PDLP3:EGFP*. The FM4-64 stain was used to mark the outline of all cells in a tissue. Signals from the EGFP, FM4-64 and aniline blue channels are shown in green, red and blue respectively. Differential interference contrast (DIC) photomicrographs are shown to outline the tissue (D, H, L). Optical sections of root epidermis (A, B, E, F), and carpel epidermis (C, D, G, H). Notice the punctate pattern of EGFP:QKY is similar to PDLP3:EGFP. In the carpel epidermis the punctate EGFP signal clearly appear in the gap between the cells (C, D, G, H). Aniline blue staining (I-L). Epidermal sections of cotyledons following chemical staining of callose using aniline blue. 4-day old seedlings were used. Scale bars: 10 µm.

Nevertheless, transgene expression could be detected by RT-PCR in seedlings (not shown). I have also used fluorescent tags like mCherry and T-Sapphire, which are considered to be pH and photostable (Zapata-Hommer and Griesbeck, 2003; Shaner et al., 2005; Shaner et al., 2007; Bayle et al., 2008), but with no success. This result is in contrast to the easily detectable *QKY* gene expression results indicating that *QKY* is subjected to intricate regulation at post-transcriptional level or the protein undergoes

rapid recycling. To test if ERAD or proteasomal degradation contributes to the lack of signal the roots of transgenic seedlings were treated with chemical inhibitors Kifunensine and Mg132 respectively. Incubation of seedlings with these chemicals has not shown any significant effect (not shown).

The absence of signal with native promoter hampered the possibility to analyze the spatial expression pattern of QKY at the organ and tissue level and to compare it with gene expression pattern. Nevertheless, to examine QKY localization, the native promoter was replaced with the *UBQ10* promoter and stable transgenic plants carrying *UBQ::EGFP:QKY* were generated. Transgenic *qky-8* lines carrying this construct were rescued and WT-L*er* plants carrying the construct did not display any noticeable alterations in phenotype. This indicates that EGFP:QKY is functional and that over expression of QKY does not negatively affect the plant. Under the constitutively expressing *UBQ* promoter, EGFP:QKY signal was detectable in punctate manner, presumably at plasmodesmata (PD) as shown in Figure 3.2.5A-D. The localization pattern was comparable to previously reported PD localized PDLP3:EGFP (Figure 3.2.5E-H) (Thomas et al., 2008). To confirm that these punctate sites were PD, callose staining was performed using the fluorescent dye aniline blue. Callose is an integral component of PD deposited at the neck of the aperture and controls its permeability (Zavaliev et al., 2011). As shown in Figure 3.2.5I-L, EGFP signal co-localizes to the blue staining of callose confirming that QKY is localized to PD.

Apart from PD localization, QKY also exhibits peculiar localization patterns in roots. For example, ER-like pattern can be easily seen in lateral roots and punctate signal was also observed within the cell (Figure 3.2.6A and B). This type of pattern is quite common in developing lateral roots. Expression of the *UBQ::EGFP:QKY* reporter was monitored in several tissues, including the inflorescence meristem, young flowers, developing ovules, and roots. Interestingly, in all the organs analyzed the signal can be seen only in the outer layers but not in the inner layers (Figure 3.2.6E and F) further providing evidence that QKY is regulated post transcriptionally.

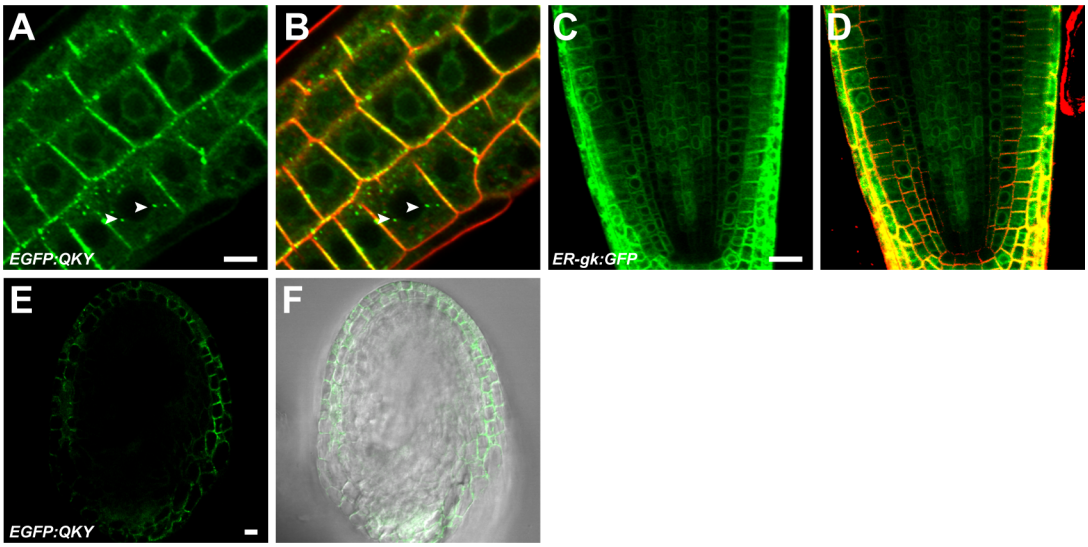


Figure 3.2.6 Peculiar localization patterns of QKY
The FM4-64 stain was used to mark the outline of all cells in a tissue. Signals from the EGFP and FM4-64 channels are shown in green and red respectively. DIC photomicrographs are shown to outline the tissue (D). (A and B) Notice the EGFP:QKY dots inside the cell (arrowheads) and ER-like pattern. Compare the pattern with ER marker line (C and D) (Pettersen et al., 2004). Mid-optical section of seed at early stage (E, F). Scale bars: 10 µm.

3.2.6 TM domains of QKY are important for Plasmodesmata localization

All the three *qky* alleles identified lack the two TM domains embedded in the PRT_C (Figure 3.2.1). Further a C- terminal tag renders QKY nonfunctional as *QKY::QKY:mCherry* construct can't rescue the *qky-8* phenotype (not shown) implying PRT_C with two TM domains is important for QKY activity and could be important for its subcellular PD localization. In order to test this a deletion construct (*UBQ::EGFP::QKYΔTM*) was made such that it mimics the *qky-8* allele which lacks

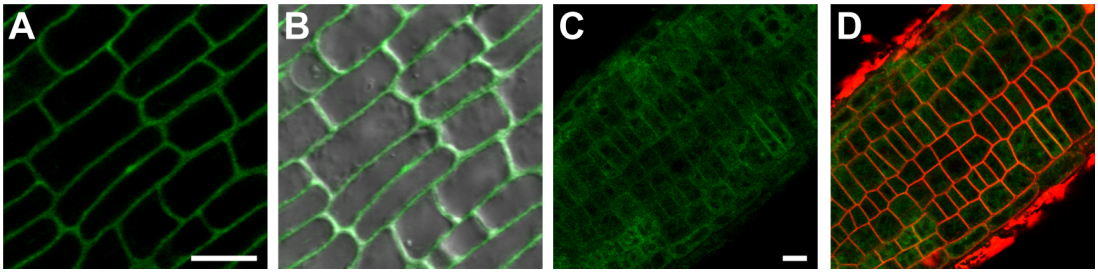


Figure 3.2.7 Expression analysis of EGFP::QKYΔTM reporter.
Live confocal microscopy images obtained from Ler plants carrying *UBQ::EGFP::QKYΔTM*. The FM4-64 stain was used to mark the outline of all cells in a tissue. Signals from the EGFP and FM4-64 channels are shown in green and red, respectively. Differential interference contrast (DIC) photomicrograph is shown to outline the tissue (B). Epidermal sections of carpel (A and B), root meristematic zone (C and D). Scale bars: 10 µm.

the PRT_C because of a premature stop codon (Figure 3.2.1). In effect, only the four C2 domains will be translated. As shown in Figure 3.2.7, the EGFP signal loses the punctate pattern. The epidermal cells of root meristematic zone show indiscriminate cytoplasmic and ER-like pattern of the EGFP signal (Figure 3.2.7C-D). In the epidermis of carpel wall also the punctate pattern is completely lost and EGFP signal can be seen at the periphery of the cell, which could be because of the large vacuole (Figure 3.2.7A-B). Overall, these results suggest that the two TM domains at the C-terminus are important for QKY PD localization. As expected, the transgenic *UBQ::EGFP::QKYΔTM*, *qky-8* plants still showed a *qky* phenotype. But, interestingly the WT plants carrying this construct showed *qky* mutant phenotype (Figure 3.2.8D) indicating that this construct acts in a dominant negative manner. One possible reason could be that the truncated version of QKY dimerizes with the WT version of QKY and obstructs its functionality. In order to test this, I proposed yeast two-hybrid test. As shown in the Figure 3.3.5, QKYΔTM can self-associate in yeast. In conclusion, QKY may form homodimers and could be part of a protein complex.

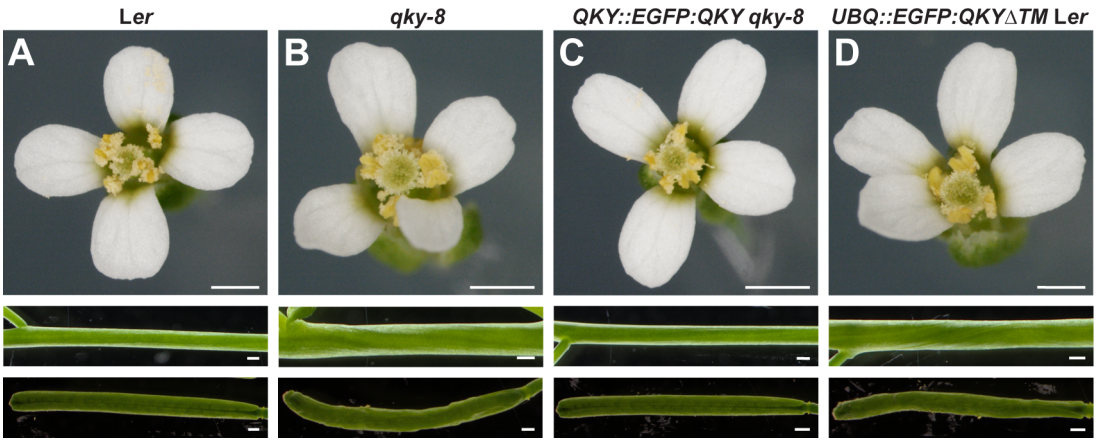


Figure 3.2.8 Functional analysis of different *EGFP:QKY*-based constructs
QKY::EGFP:QKY; qky-8 morphology appears wild type (C). *UBQ::EGFP:QKYΔTM;* L*er* shows mutant phenotype (D). Scale bars: 0.5 mm.

3.2.7 Quirky is not sensitive to BFA treatment

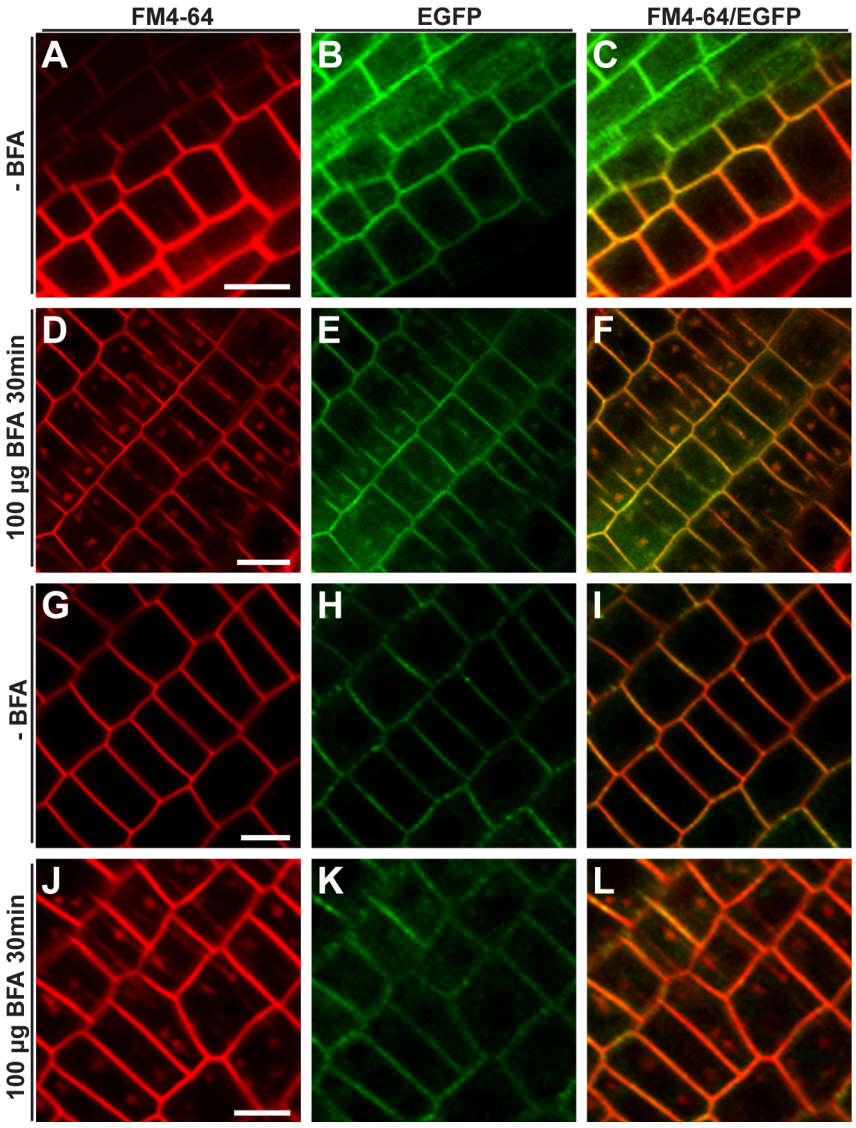


Figure 3.2.9 Effect of BFA on SUB:EGFP and EGFP:QKY reporters
Live confocal microscopy images from root were generated from 4-day old Arabidopsis seedlings. Expression profile of the SUB:EGFP (A-F) and EGFP:QKY (G-L) in untreated roots (A-C, G-I) and after a 30-minute treatment with 100 µg/ml of BFA (D-F, J-L). Scale bars: 10 µm.

Previous studies have shown that SUB is sensitive to BFA treatment and can be found in BFA compartments (Figure 3.2.9A-F) (Yadav et al., 2008). Brefeldin A (BFA), an inhibitor of specific ADP ribosylation factor (ARF) GTPase exchange factors (GEFs), arrests vesicle trafficking at various points along the secretory pathway (Nebenführ et al., 2002). To test EGFP:QKY internalization roots of 4 day-old *UBQ::EGFP:QKY* transgenic seedlings were treated with brefeldin A (BFA). BFA

treatment results in the aggregation of endosomal and trans-Golgi network vesicles, so-called BFA compartments that are also labeled by the dye FM4-64. As shown in Figure 3.2.9G-L EGFP:QKY was not detected in FM4-64 labeled BFA compartments and the PD localization of QKY was not disturbed by BFA treatment. These results indicate that QKY doesn't undergo BFA-sensitive recycling like SUB (Yadav et al., 2008) (Figure 3.2.9A-F).

3.3 The role of QKY in SUB mediated inter-cellular signaling

3.3.1 An extra copy of *SUB* can partially rescue *qky* phenotype

The *sub-1 qky-8* double mutants showed exaggerated phenotype with pronounced twisting morphology and reduced height compared to single mutants (Fulton et al., 2009). It was difficult to decide whether a double mutant displayed an additive or synergistic phenotype, or whether a particular mutation was epistatic to another. This genetic analysis raises question whether *SUB* and *QKY* act in a single linear pathway or separate pathways with both overlapping and distinct functions. To address this question further investigations were done at the molecular level. First it was tested whether the individual gene expressions were responsive to each other. Expression analysis was done using stage 8-11 flowers from *sub-1* and *qky-8* mutants by quantitative real time PCR (qRT-PCR). As can be seen in Figure 3.3.1, only very moderate change in *QKY* expression was detected in *sub-1* background whereas no difference was observed for *SUB* expression in *qky-8* background, which is consistent with our transcriptome analysis where *SUB* transcript levels were unaltered in *qky-8* background (Fulton et al., 2009).

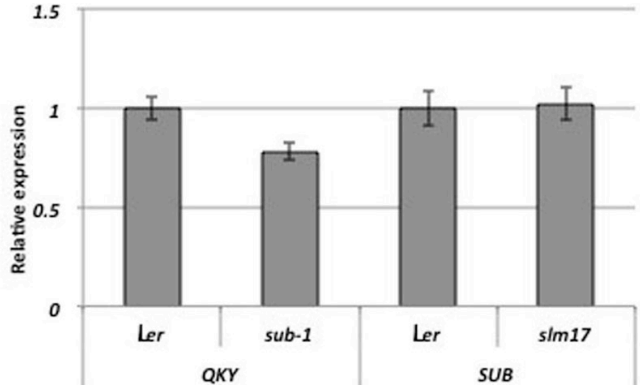


Figure 3.3.1 SUB and QKY expression profiles in stage 8-11 flowers.
A qRT-PCR-based comparison in wild type and mutant backgrounds.

Next, It was tested if the loss of functional QKY affects proper expression and localization patterns of SUB and vice versa. To test this *SUB::gSUB:EGFP* and *QKY::EGFP:QKY* were introduced into *qky-8* and *sub-1* backgrounds respectively. Interestingly, though the expression domains and localization pattern of SUB::EGFP has not changed in *qky* mutant background (Figure 3.3.2A-H), the majority of

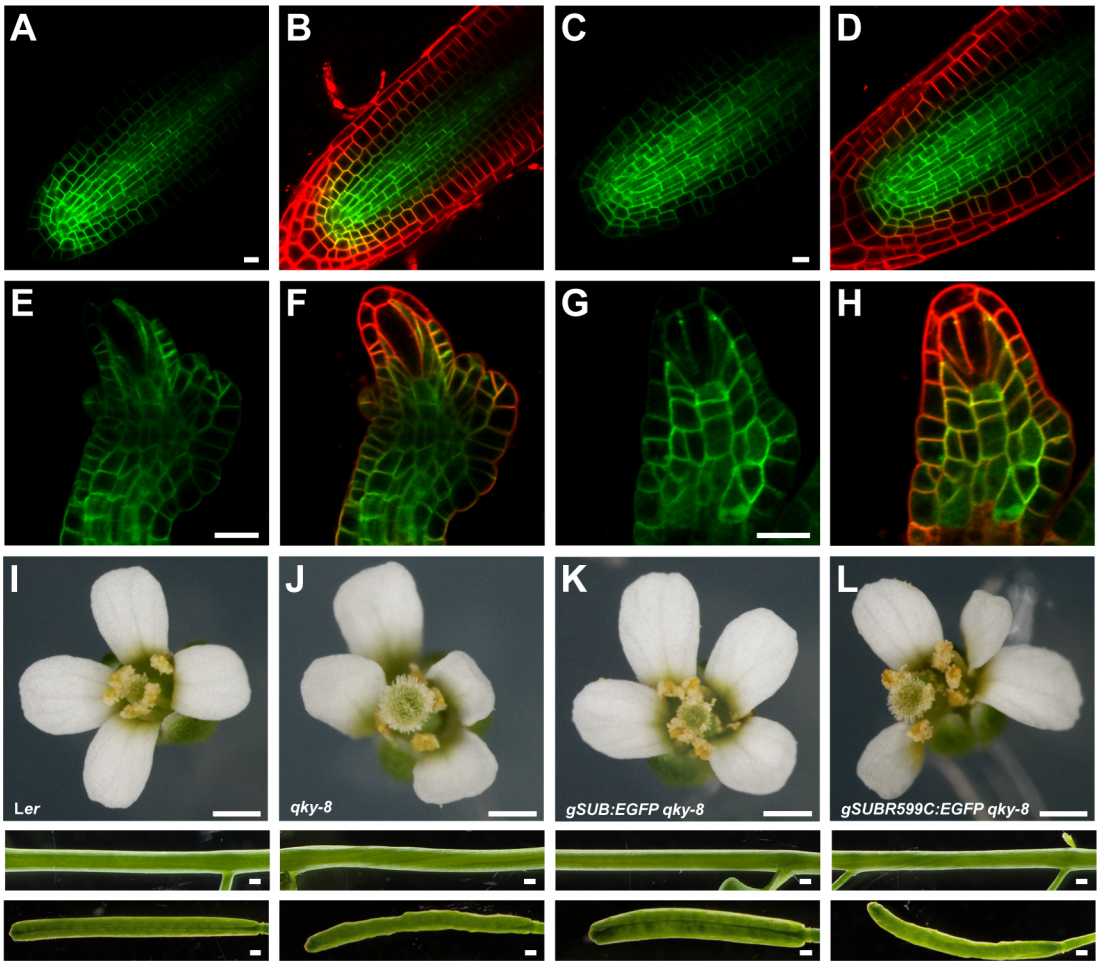


Figure 3.3.2 Expression and rescue pattern of *SUB::gSUB:EGFP* in *qky-8*
SUB.EGFP expression (A-H). The FM4-64 stain was used to mark the outline of all cells in a tissue. Signals from the EGFP and FM4-64 channels are shown in green, and red respectively. Optical sections of root epidermis (A-D), and young ovules (E-H). SUB:EGFP expression in *qky-8* (A, B, E, F). SUB:EGFP expression in WT for comparison (C, D, G, H).
Morphological analysis (I-L). (I) L*er*. (J) *qky-8*. (K) *SUB::gSUB:EGFP qky-8*. Flowers show mild twisting, stem and silique twisting is absent but the length of the silique is not rescued. (L) *SUB::gSUB (R599C):EGFP qky-8* is not rescued. (A-H) Scale bars: 10 µm. (I-L) Scale bars: 0.5 mm.

independent *SUB::SUB:EGFP;qky-8* lines were partially rescued while the degree of rescue varied with the line (Figure 3.3.2I-L). But none of the mutant plants were rescued completely although I checked approximately 100 independent primary transformants. The partially rescued plants showed lesser degree of flower twisting and silique twisting but the length of the silique remained the same. The stem twisting was completely rescued consequently the height (Figure 3.3.2K). In summary, this result demonstrates that QKY is involved in SUB mediated signaling pathway and is acting upstream to SUB. In similar lines when *QKY::EGFP:QKY* was introduced into

sub-1, I have not seen any dramatic effects as the phenotype was unchanged and I couldn't detect any signal. Nevertheless, I used *UBQ::EGFP:QKY:sub-1* transgenic plants to check if absence of SUB has any effect on localization of EGFP:QKY and apparently no change was observed (Figure 3.3.3).

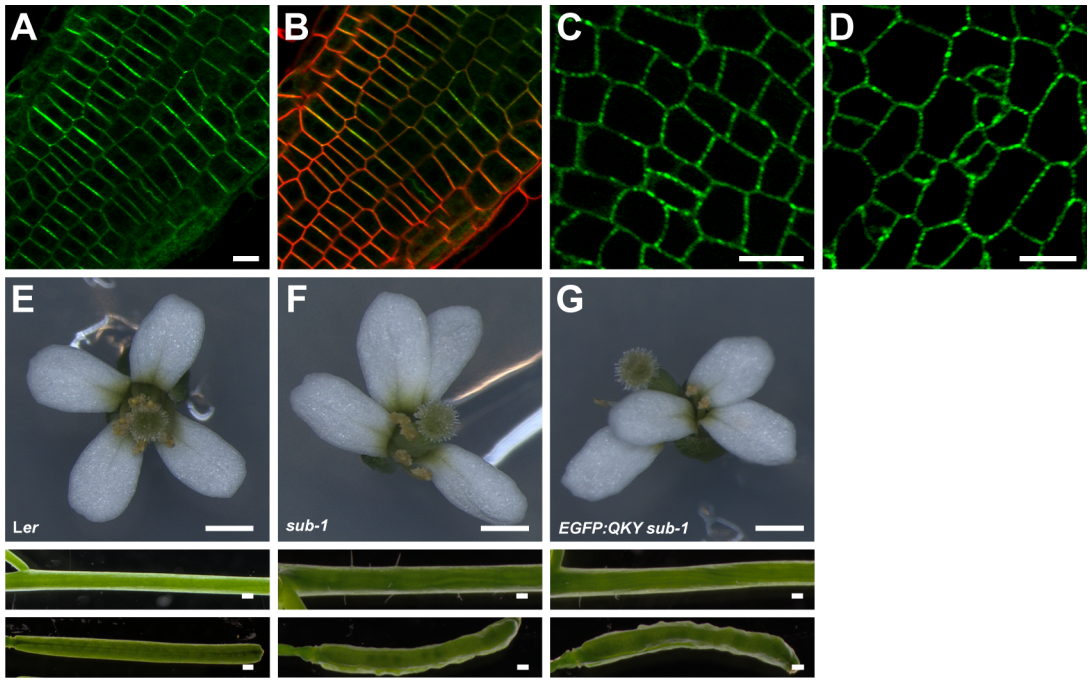


Figure 3.3.3 Expression and rescue pattern of *UBQ::EGFP:QKY* in *sub-1*
EGFP:QKY localization (A-D). The FM4-64 stain was used to mark the outline of all cells in a tissue. Signals from the EGFP and FM4-64 channels are shown in green, and red respectively. Optical sections of root epidermis (A, B), and carpel epidermis (C, D). EGFP:QKY localization in *sub-1* (A-C). EGFP:QKY expression in WT for comparison (D). Morphological analysis (E-G). (E) L*er*. (F) *sub-1*. (G) *UBQ::EGFP:QKY sub-1*. (A-D) Scale bars: 10 µm. (E-G) Scale bars: 0.5 mm.

3.3.2 Internalization and BFA sensitive recycling of SUB is independent of QKY

In the light of the well-described role of C2 domain containing proteins in the control of endo-exocytosis, it was proposed that QKY might also affect vesicle trafficking. In this context, it is plausible to check if absence of QKY has any effect on internalization and recycling of SUB. To test this the *SUB::SUB:EGFP;qky-8* seedlings were treated with BFA and root tips were analyzed. As shown in Figure 3.3.4, SUB:EGFP behaved normally and was found in FM4-64 labeled BFA compartments suggesting that QKY has no effect on internalization and BFA sensitive recycling of SUB.

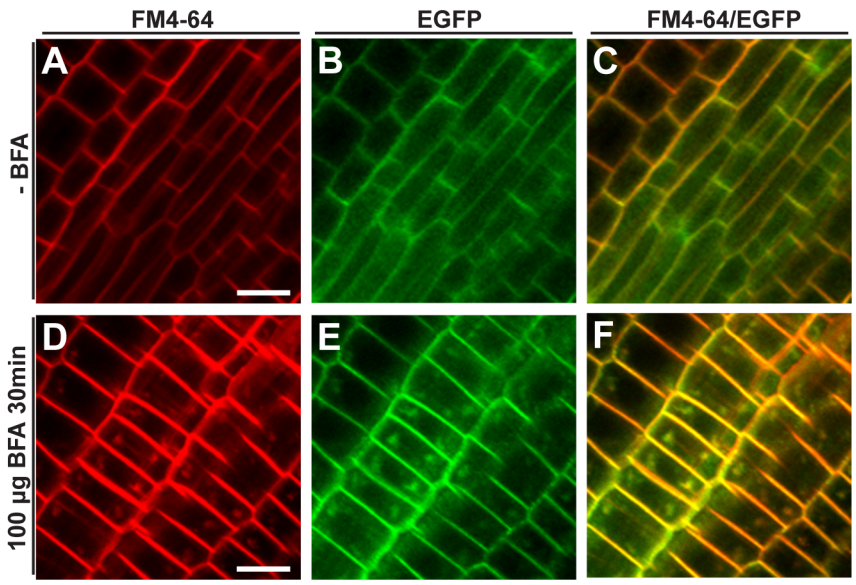


Figure 3.3.4 Effect of BFA treatment on *SUB::SUB:EGFP* reporter in *qky-8* background. Root images were generated from 4-day old Arabidopsis seedlings. Expression profile of the SUB:EGFP fusion protein in an untreated root (A, C), and after a 30-minute treatment with 100 µg/ml of BFA (D, F). Scale bars: 10 µm.

3.3.3 SUB and QKY interact in yeast

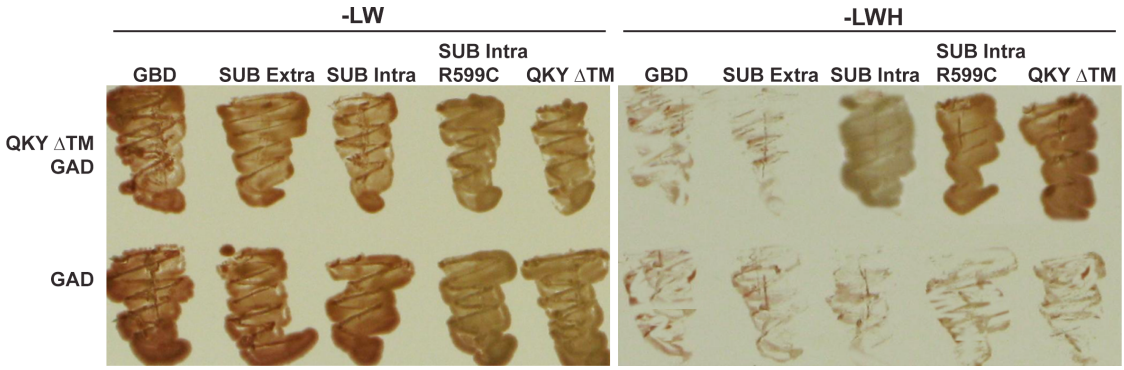


Figure 3.3.5 QKYΔTM interacts with SUB intra
Transformants were tested for growth on SC-LW or SC-LWH plates to test their auxotrophic growth.

Genetic and molecular analysis so far support that QKY is involved in SUB mediated intercellular signaling. To further investigate the direct physical interaction between SUB and QKY targeted yeast-two-hybrid (Y2H) assay was performed. The results indicate that QKY without the c-terminal PRT_C region (QKYΔTM) interacts with SUB intracellular domain but not with the SUB extracellular domain (Figure

3.3.5). The interaction can be also seen with R599C mutation that mimics the *sub-4* allele implying that this mutation would not interfere with SUB-QKY interaction.

3.3.4 Immuno-electron microscopy reveals PD localization of SUB

SUB-QKY Y2H interaction and PD localization of QKY raises question whether SUB is also present in PD along with plasma membrane localization. In order to confirm this we took advantage of Immuno-electron microscopy in collaboration with S. Hillmer and D. Robinson (U. Heidelberg). Immuno-gold labeling, using an anti-EGFP antibody on sections of root cells of an *SUB::SUB:EGFP* transgenic line revealed the PD localization of SUB along with membrane localization pattern (Figure 3.3.6B). We applied the same technique on a *UBQ::EGFP:QKY* transgenic line which further confirmed the PD localization of QKY (Figure 3.3.6A). Thus, this data reveals the PD localization of SUB and also confirms the PD localization of QKY.

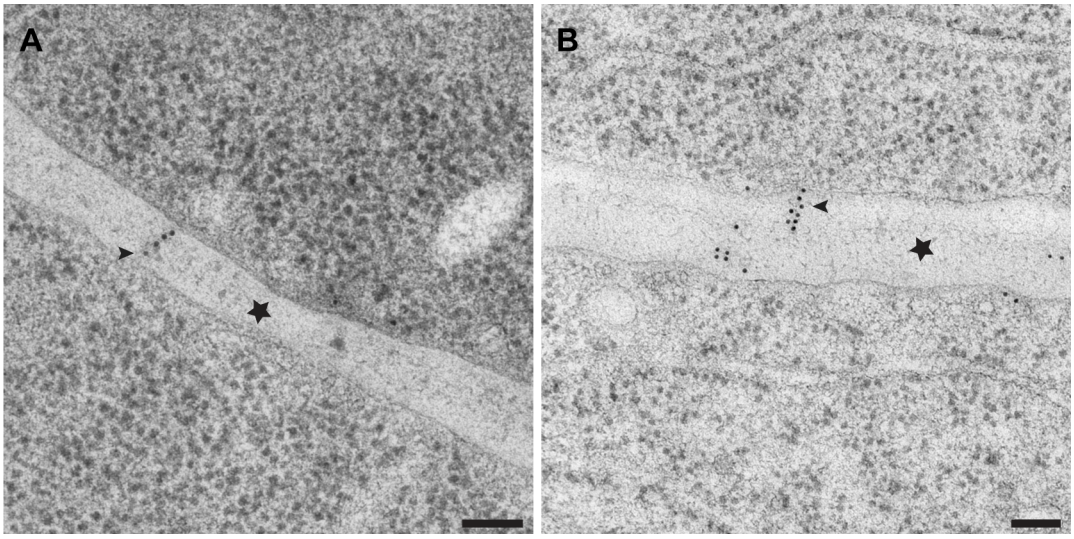


Figure 3.3.6 Localization of SUB and QKY by Immunogold labelling
Root sections of transgenic *UBQ::EGFP:QKY* (A) and *SUB::SUB:EGFP* (B). Note QKY-positive gold particles along PD (A) and aggregates of SUB-positive gold particles at PD (B). PD (arrowheads) cell wall (star). Scale bars = 100 nm.

3.3.5 QKY does not affect non-targeted movement of molecules

The localization of QKY to the PD raises an interesting prospect regarding its involvement in the SUB mediated inter-cellular signaling, as PD are symplastic channels between adjacent cells, which provide intercellular communication. In this regard, *qky* mutation may affect size exclusion limit (SEL) of PD. Thus, SUB signaling via QKY might have an effect on targeted or non-targeted movement of molecules through PD. Therefore; First I wanted to check if simple diffusion of non-targeted molecules is disturbed in the mutant background. To test this GFP fusion proteins were introduced into the mutant background and their movement was compared to the wild type. GFP has been widely used to study non-targeted intercellular movement of molecules (Crawford and Zambryski, 2000). First movement of molecules from epidermis to internal tissues was tested by using *ML1:: 2×GFP* reporter (Wu et al., 2003). *ML1* is a L1 specific promoter and its specificity has been demonstrated several times before. Previous studies with this construct have shown that 2×GFP, with a molecular weight of approximately 54 kDa, could move from L1 to internal layers forming a gradient with the highest levels in the L1 (Wu et al., 2003) (Figure 3.3.7). The GFP signal in floral meristems, ovules and root tissues were compared between *ML1:: 2×GFP* transgenic WT and *qky-8* plants (Figure 3.3.7). In all the tissues tested identical expression pattern was observed in both backgrounds. The fluorescence was detected in all the cells and a gradient in intensity is observed from epidermis to internal layers of each tissue tested (Figure 3.3.7). Reporter constructs, *ML1::NLS: 2×GFP,* which blocks the movement of GFP because of the addition of a nuclear localization signal (NLS), and *ML1::LFY:GFP,* where GFP was tagged to the floral identity protein *LEAFY,* were also tested which too have shown identical expression pattern in both WT and *qky-8* backgrounds (not shown).

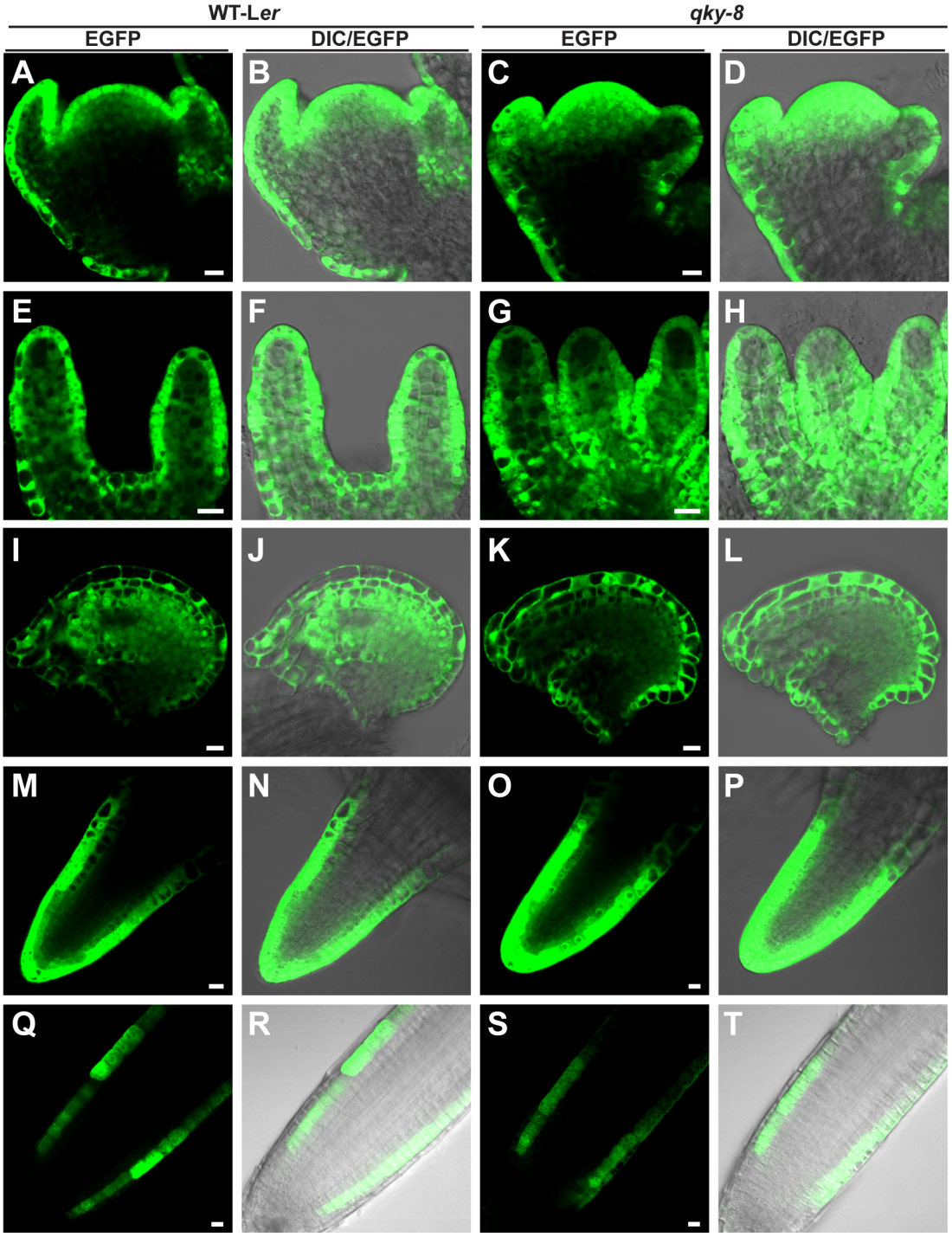


Figure 3.3.7 *ML1::2×GFP* reporter expression
Live confocal microscopy images from were generated from various Arabidopsis tissues. Stage 3 floral meristems (A-D), stage 2-3 (E-H) and 3-V (I-L) ovules, lateral roots (M-P) and main root (Q-T). Scale bars: 10 µm.

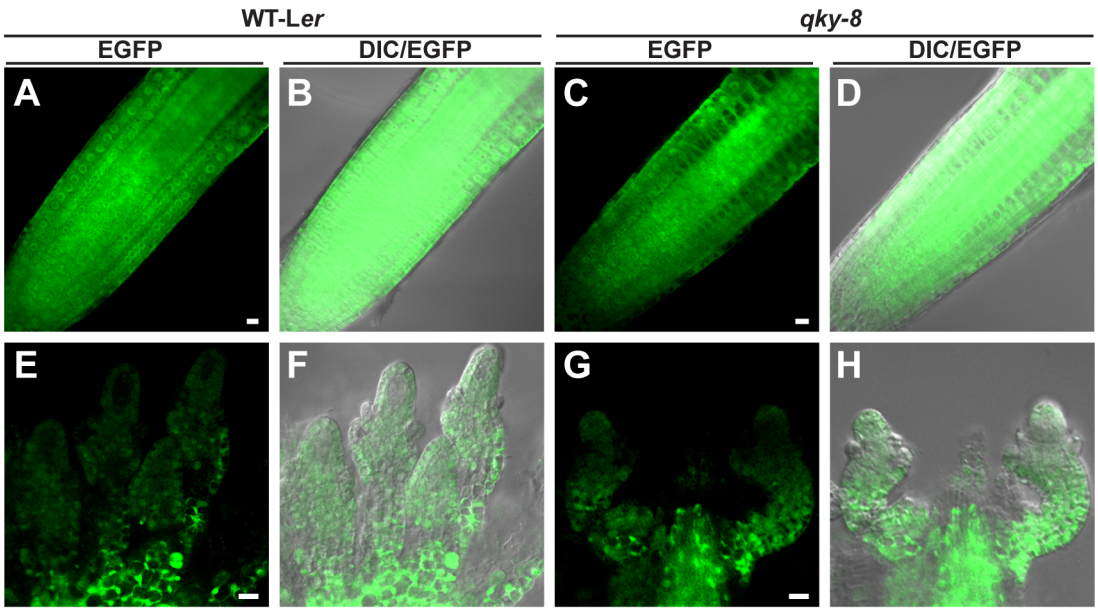


Figure 3.3.8 *AtSUC2::GFP* reporter expression
Live confocal microscopy images were generated from transgenic Arabidopsis main root (A-D) and stage 2-3 ovules (E-H). Scale bars: 10 µm.

Next, I asked if movement of molecules via PD is disturbed from inner layers to outer layers in *qky-8*. To test this phloem specific *AtSUC2::GFP* reporter was used. The expression of *AtSUC2* was shown to be specific to phloem companion cells (Stadler and Sauer, 1996; Truernit et al., 1999). In wild-type plants, GFP driven by the *AtSUC2* promoter traffic through phloem strands in the gynoecium wall to the ovule primordia via symplastic pathway, which is called phloem unloading (Imlau et al., 1999; Werner et al., 2011). To test, if this movement is disturbed in *qky-8,* the GFP signal in carpel and root tissues were compared between WT and *qky-8* plants transgenic to *AtSUC2::GFP* (Figure 3.3.8). In both the tissues tested identical expression pattern was observed in both backgrounds. Thus the movement from inside to outside also seems to be not affected in *qky-8* background.

The above reporter experiments demonstrate that the symplastic movement between clonally distinct layers, which are connected through the secondary PD, is not perturbed in *qky* mutant background. To further examine whether the movement of molecules within a layer is affected in *qky-8* plants, I took the advantage of the *AG intron*::2×GFP* reporter (Wu et al., 2003). This construct drives reporter gene

expression in the center of floral and inflorescence meristems (Hong et al., 2003). Previous studies with this construct have demonstrated that compared with movement from the L1 to internal layers, lateral movement of 2×GFP within tissue layers is much more limited, or possibly even absent suggesting that the PD SEL within L1 and L2 layers is lower than between layers (Wu et al., 2003) (Figure 3.3.9A and B). Similarly, As shown in Figure 3.3.9C and D the GFP signal was not observed in the lateral domains of the floral primordia indicating that the SEL of PD within a layer has not increased in *qky-8* background. However, a gradient of 2×GFP could be seen extending into deeper cell layers in L3 in stage 3 flowers (Figure 3.3.9), which is consistent with our earlier observation (Figure 3.3.7).

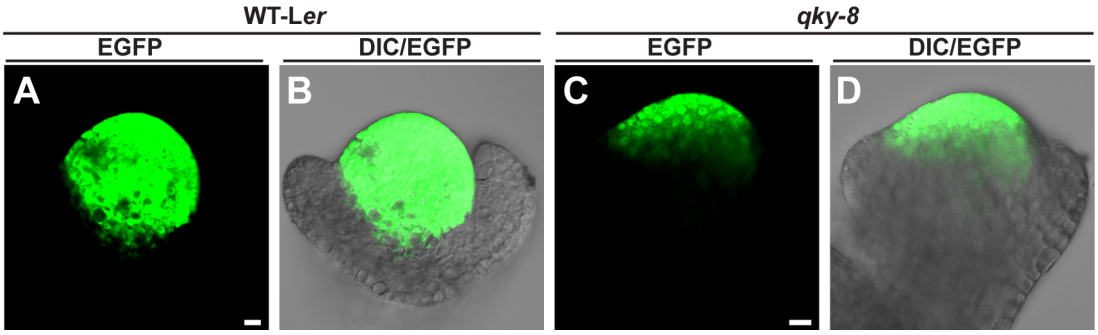


Figure 3.3.9 *AG intron*::2×GFP* reporter expression
Live confocal microscopy images were generated from transgenic Arabidopsis floral meristems. Scale bars: 10 µm.

Similar analysis was done in *sub-1* background using *ML1::2×GFP, ML1::NLS:2×GFP* and *AtSUC2::GFP* and similar results were obtained with no difference in expression pattern compared to wild-type background (not shown).

3.3.6 SUB and QKY are located at the cell plate

Cytokinesis is a process during which the cells separate physically after duplication of their genetic material. It is a complex process, which involves trafficking of components at the phragmoplast to build the cell plate, which eventually matures into cell wall (Jürgens, 2005). Interestingly, during the cell biological investigations both SUB:EGFP and EGFP:QKY displayed signal at the growing cell

plate (Figure 3.3.10). It appears that both are present from the early stages of cell plate expansion. Thus the results indicate a role for SUB and QKY during cytokinesis.

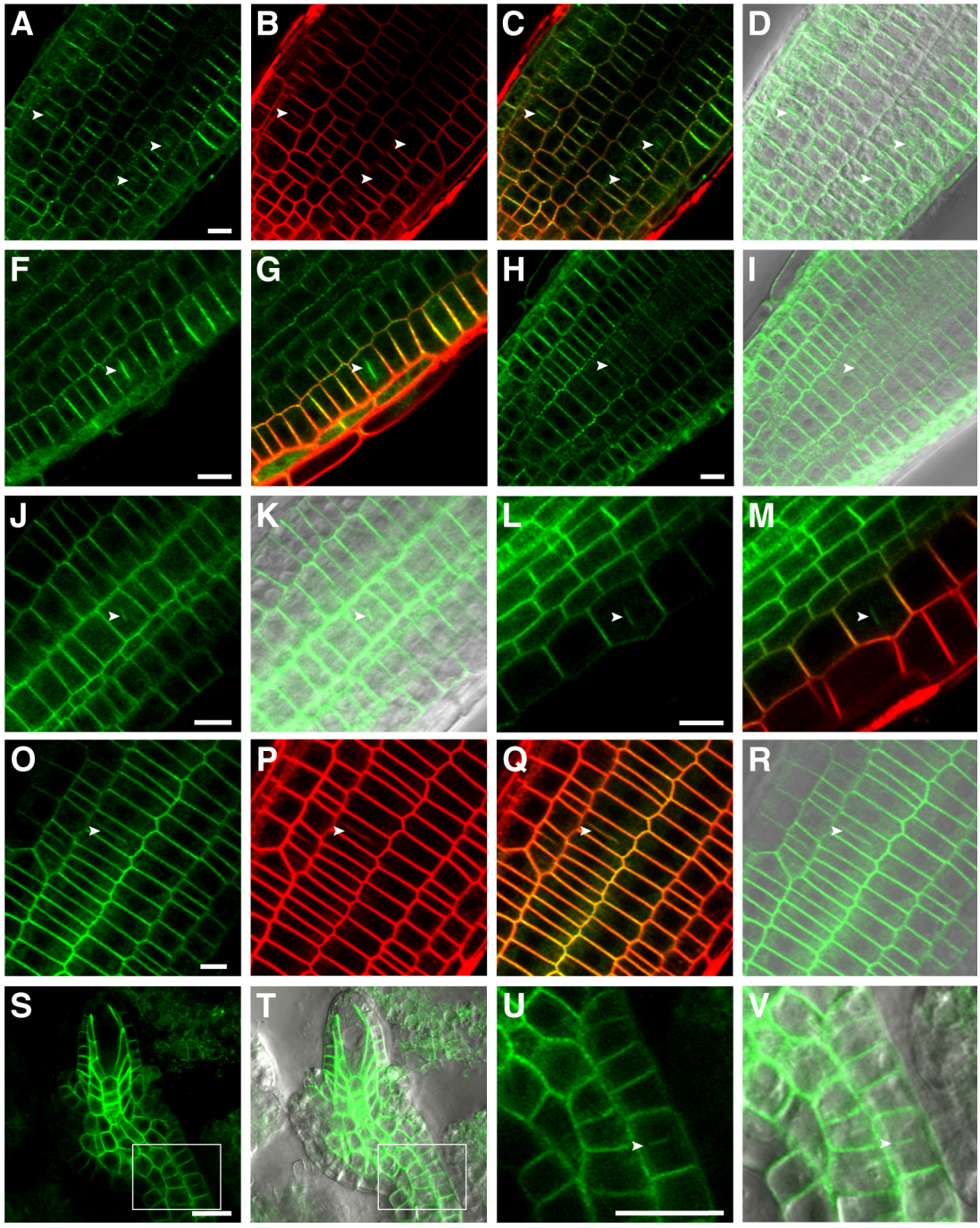


Figure 3.3.10 Localization of the SUB and QKY at the cell plate
The FM4-64 stain was used to mark the outline of all cells in a tissue. Signals from the EGFP and FM4-64 channels are shown in green and red respectively. DIC photomicrographs are shown to outline the tissue (D, I, K, R, T, V). *UBQ::EGFP:QKY* (A-I). *SUB::gSUB:EGFP* (J-V). Different root sections (A-R). Young ovule (S, T). Magnification of marked area in S and T (U, V). Arrows indicate cell plate. Scale bars: 10 µm.

4 Discussion

Organogenesis requires co-ordination of cellular behavior. The corresponding mechanisms are extensive (Gallagher and Benfey, 2005; Ingram and Waites, 2006) and cell-surface-localized RLKs (receptor-like kinases) are natural candidates to mediate intercellular communication. The leucine-rich repeat transmembrane receptor-like kinase (LRR-RLK) *STRUBBELIG (SUB)* is involved in cell-to-cell communication during morphogenesis (Chevalier et al., 2005). Analysis of *sub* mutants indicated that *SUB* is required for proper shaping of organs such as stem, carpels, petals and ovules. In addition, *SUB*, also known as *SCRAMBLED (SCM)*, affects specification of hair cells in the root epidermis (Kwak et al., 2005; Kwak and Schiefelbein, 2008). In the quest of finding new components involved in *SUB* mediated signaling three genes were discovered. They are *DETORQEO (DOQ), QUIRKY (QKY), and ZERZAUST (ZET),* which were grouped into *STRUBBELIG-LIKE MUTANT (SLM)* class of genes (Fulton et al., 2009). In the present study, I performed a structure-function analysis of *SUB* to understand how *SUB* regulates its various downstream signaling processes. I have also focused on the molecular characterization of *QKY*, one of the *SLM* mutants and tried to comprehend its role in morphogenesis via signaling through the atypical receptor-like kinase *SUB*.

4.1 Structure-function analysis of STRUBBELIG

4.1.1 SUB accepts a surprising amount of sequence variation

In this study, I set out to genetically identify important amino acids or domains of SUB, which might confer to a specific aspect of SUB activity. Overall, the analysis of the available EMS-induced mutations indicated that *SUB* is able to accommodate a perhaps astonishing level of sequence variability even at conserved positions. Alternatively, aphenotypic alleles may affect a SUB function not revealed by our morphological analysis. Irrespective of their nature phenotypic mutations all result in the complete loss of *SUB* function. *SUB* exhibits a different genetic behavior when

compared to the *CLAVATA1* (*CLV1*) RLK gene. Interestingly, *clv1* null alleles show a weak phenotype whereas many *clv1* missense mutations lead to a strong phenotype (Diévart et al., 2003). It was reasoned that missense *clv1* alleles interfere with redundantly acting receptors, such as CLV2/CORYNE (CRN) and BAM1/2 (Bleckmann et al., 2010; DeYoung et al., 2006; Deyoung and Clark, 2008; Guo et al., 2010; Müller et al., 2008; Zhu et al., 2010). A similar scenario does not seem to be the case for *SUB*. In addition, the results preclude the mapping of particular SUB domains to individual biological processes, such as stem or integument development. This suggests that organ or cell-specific aspects of *SUB* signaling may not be integrated at the level of the SUB receptor itself but involve other components that act together with or downstream of SUB. This notion is substantiated by genetic evidence involving *ERECTA* (Vaddepalli et al., 2011). It was previously assumed that the reduction in plant height of *sub* mutants was at least in part due to stem twisting. But the *sub-1* plants carrying the ER transgene showed essentially normal internode length and accordingly, plant height (Vaddepalli et al., 2011). This result shows that the strong reduction in plant height of *sub* alleles in the L*er* background is caused by a synergistic effect between the *sub* and *er* mutations. Furthermore, the milder phenotype of *sub-9* (Col) compared to *sub-1* (L*er*) implies that Col carries additional modifiers affecting the SUB-dependent regulation of stem, flower, and silique form (Vaddepalli et al., 2011). Future genetic and molecular analysis of these modifiers will likely identify interesting novel components involved in SUB signaling.

Surprisingly, there was no strict correlation between degree of conservation of the altered residue throughout the Arabidopsis SRF family and presence of the *sub* phenotype. The five phenotypic amino acid substitutions affected either strictly conserved (*sub-4*, R599C; *sub-10*, C57Y), structurally conserved (*sub-3*, V64M), semi-conserved (*sub-19*, S545F), or nonconserved amino acids (*sub-15*, P304L). Furthermore, while many of the aphenotypic changes affect nonconserved residues (*sub-12* - *sub-14*, *sub-16* - *sub-18*), two aphenotypic mutations, *sub-11* (S69L) and *sub-20* (L633F), result in changes at amino acid positions that are strictly conserved (Shiu and Bleecker, 2001; Eyüboglu et al., 2007) (Figure S1).

Further assessment of natural variation at the level of the SUB protein using 57 Arabidopsis accessions revealed no overlap between our set of artificially induced mutations and the naturally occurring alleles (Table S1). For the most part natural variation was observed at semi- or nonconserved residues. One notable exception was the T595N polymorphism in the kinase domain, which occurred in several accessions. I speculate that the type and position of the alteration may not noticeably interfere with the kinase domain structure. Alternatively, this polymorphism, as with some of the other naturally occurring polymorphisms, may be balanced by second-site mutations. For example, an altered site may have a biologically relevant negative effect on SUB conformation. However, it is conceivable that a second-site mutation in for example, a direct interactor of SUB may result in a protein that can still interact with the altered SUB protein and thus compensate for the principally deleterious effect. It is presently unclear if, and how often, this possibility actually occurs in the case of SUB in wild-type accessions. A different scenario, where accumulation of genetic incompatibilities between accessions can lead to reproductive isolation, has been described for SRF3 (Alcázar et al., 2010).

4.1.2 Loss-of-function mutations in *SUB* leads to ERAD

Endoplasmic reticulum-mediated quality control (ERQC) tries to rectify or dispose of misfolded and/or unassembled proteins by endoplasmic reticulum-associated degradation (ERAD) (Ellgaard and Helenius, 2003; Römisch, 2005; Vembar and Brodsky, 2008; Vitale and Boston, 2008). Additional reports provided compelling evidence that the *bri1-5* and *bri1-9* variants of the brassinosteroid receptor BRI1, carrying substitutions in their ECD domains, are retained in the ER and degraded by the ERAD system (Su et al., 2011; Jin et al., 2007; Hong et al., 2008). Another well-characterized example is the LRR-RLK EFR, a plant innate immune receptor involved in the perception of the bacterial translation elongation factor EF-Tu (Li et al., 2009; Nekrasov et al., 2009).

It is perceived that in LRR proteins, the N-terminal Cysteine pair (C57 and C66 in SUB) forms a disulfide bond that plays a structural role in shielding

hydrophobic residues of the first LRR (van der Hoorn et al., 2005). It is thus quite possible that the mutation of Cys-57 to Tyr in *sub-10* creates a free thiol group at Cys-66 that can be recognized by ER retention mechanism. BRI1, for example, seems to carry such a disulphide bond at a related position (Hong et al., 2008) and a similar disulphide bond is critical for Cf-9 activity (van der Hoorn et al., 2005). In *sub-10* (C57Y) this disulphide bond would not occur. In accordance with this notion the results suggest that C57 and C66 participate in a critical disulphide bridge required for proper N-capping domain architecture. Hence the mutant proteins are subjected to ERQC similar to other plant receptor kinases, such as BRI1 or ERF and the close proximity of C57 and C66 in the homology model favors their importance of such cysteine pairs for the stabilization of LRR domains (Kobe and Kajava, 2001).

SUB behaves similar to BRI1 at structural level but behaves differently at the functional level. Structurally defective *bri1-5* (C69Y) mutant, which is analogous to *sub-10* (C57Y), is a functionally active allele. Further analysis on *bri1-5* mutant demonstrated that the observed phenotype was because of low BRI1 protein at the cell surface (Hong et al., 2008). Simultaneous mutations of Cys-69 and its partner Cys-62 can mitigate ERQC, resulting in significant suppression of the *bri1-5* phenotype, which is not the case for SUB (C57Y-C66Y). It was proposed that an overvigilant ERQC system keeps a structurally defective, yet functionally competent, BR receptor in the folding compartment (Hong et al., 2008). Intriguingly, the N- terminally tagged EGFP:SUB protein, which is also subjected to ERQC was able to rescue the *sub-1* phenotype indicating that ERAD doesn't have deleterious effects on SUB. Thus EGFP:SUB is a functionally active protein though it is structurally defective. In addition, I have observed effective rescue of transgenic *sub-1* plants carrying alternatively 35S::SUB:EGFP, SUB::c/gSUB:EGFP or functional SUB::c/gSUBmut:EGFP constructs, which showed no apparent signal (not shown, see above). These findings indicate that several types of functional transgenes with either likely altered ECDs or undetectable expression levels can provide sufficient SUB activity. Thus, the data suggests that the cysteine pair is not only important for proper structural confirmation of SUB but also important for its function. In this regard SUB appears to behave similarly to Cf-9, where equivalent mutations caused absence of Cf-

9 activity (van der Hoorn et al., 2005). It could be that the imperfect domain containing these two cysteines is important for ligand perception of SUB or for protein-protein interactions.

R599 residue in the kinase domain is strictly conserved among the SRF members and is affected in, for example, the *bri1-8* and *bri1-108* alleles of BRI1 (Noguchi et al., 1999; Hong et al., 2008). Conservation of an arginine at the equivalent position across many plant kinases implies an important function for this residue (Chevalier et al., 2005). The model of the SUB kinase domain suggests that R599 is situated at end of the long alpha helix of subdomain VIa that runs through the back of the C-terminal lobe. R599 may thus have a structural role hence the ER-like distribution with $gSUB_{R599C}$:EGFP reporter. But the undetectable signal of the $cSUB_{R599C}$:EGFP reporter in the absence of Kif and the ER-like distribution of the $gSUB_{R599C}$:EGFP signal suggests that a Kif-independent mechanism of ERQC/ERAD is involved in limiting the amount of *sub-4*-like SUB variants. The process likely depends on the recognition of the misfolded kinase domain by cytoplasmic chaperones involved in ERQC/ERAD, such as certain 70 kDa heat-shock proteins (Hsp70s), and associated factors (Vembar and Brodsky, 2008).

Taken together, the results indicate that the phenotypic similarity of different *sub* alleles is not due to absence of mutant SUB protein from cells. Rather, different tested phenotypic mutations all result in mutant SUB proteins that are likely present at the plasma membrane but lack SUB activity. In addition, the results provide indirect evidence that mature SUB receptor is glycosylated at the ECD.

4.1.3 Introns mediate broad expression of SUB

It is known that introns can influence protein expression levels (Evans and Scarpulla, 1989; Mascarenhas et al., 1990; Bourdon et al., 2001). Historically, stimulation of plant gene expression by introns has been called intron-mediated enhancement (IME) (Mascarenhas et al., 1990). The reduced ability of *SUB cDNA* constructs to express broadly is analogous to the reduced ability of intronless *ERECTA*

gene to complement its mutant phenotype compared to intron containing *ERECTA* (Karve et al., 2011). Similar to *ERECTA, SUB* needs multiple introns at specific locations in additive manner for the overall high expression and there is no particular intron, which is absolutely important for its accumulation. *ERECTA cDNA* produces about 500-900 times less protein compared with the identical construct containing introns (Karve et al., 2011).

The *SUB* promoter is active across all the cell layers though expression seems to be strong in the internal layers compared to the periphery (Kwak et al., 2005; Yadav et al., 2008), which is also reflected in its protein expression pattern (this study). The absence of introns affects stability of the *SUB* mRNA in all cell layers, which leads to the complete absence of SUB protein in the outer layers where the activity of *SUB* promoter is already less. Since mRNA produced by intronless *ERECTA* is degraded at the 3' end, it was speculated that introns increase mRNA accumulation through increasing its stability. As the 3' end influences mRNA stability, transport, and translation, the splicing-induced changes to its structure should alter the level of gene expression (Huang and Gorman, 1990; Niwa et al., 1990; Lu and Cullen, 2003). The importance of splicing is further supported by the SUB construct where only last 5 introns were included without the first 10 introns. One could speculate that the absence of SUB activity with this transgene could be because of the non-splicing of the 5 introns present at the 3' end leading to premature stop codon. The absence of introns at the 5' end might have affected the initiation of splicing process.

Apart from stability, another explanation put forward suggests that upon splicing of an intron some factors remain bound to the exon-exon junction of the mRNA and the composition of such an mRNP may influence translation (Bourdon et al., 2001). In contrast to above examples, some genes carry one or several cis-acting elements positively regulating overall transcript levels. The most well understood mechanism of gene expression regulation by introns is through the presence of cis-regulatory elements in intronic sequences. For example, the second intron of the Arabidopsis gene *AGAMOUS* contains binding sites for several transcription factors, including LEAFY and WUSCHEL (Sieburth and Meyerowitz, 1997; Hong et al., 2003).

It was previously shown that *SUB* acts in a non-cell-autonomous manner and regulates inter-cell-layer communication (Yadav et al., 2008). For example, specifically expressing *cSUB:EGFP* under the control of the epidermis-specific *ML1* promoter rescued the sub-epidermal defects in floral meristems of *sub-1* mutants. In addition, restricting *cSUB:EGFP* expression to the nucellus still allowed partial development of the integuments. The broad expression pattern of the *SUB::gSUB:EGFP* reporter was perhaps not to be expected in the light of those results. However, *BRI1* represents another example for a broadly expressed gene with a histogenic-layer-specific role in the regulation of cellular behaviour at the tissue level (Savaldi-Goldstein et al., 2007). Moreover, the *SUB::gSUB:EGFP* expression pattern provides a convenient explanation for the previously puzzling observation that *ML1::cSUB:EGFP* could also rescue the integument defects of *sub-1* (Yadav et al., 2008). Thus, an easy explanation for all observations is to propose that *SUB* regulates the behavior of cells within an L1-derived cell layer, such as the integuments of ovules (Jenik and Irish, 2000), and between histogenic cell layers. One way this could be achieved is through the regulation of cell wall biology (Fulton et al., 2009).

4.1.4 Multiple mechanisms control SUB protein levels

The data discussed above suggest a complex control of SUB protein levels. First, a mechanism is in place that regulates the spatial and temporal transcription pattern of *SUB*. The different results obtained with various *SUB:EGFP* and *SUBmut:EGFP* reporter constructs imply that additional processes regulate overall SUB protein accumulation. One mechanism depends on the presence of *SUB* intronic sequences and regulates SUB levels either in a transcriptional or post-transcriptional fashion, by for example influencing *SUB* mRNA stability and/or translation. During their passage through the secretory pathway SUB proteins are subject to ERQC. Finally, in roots there is evidence for a feedback mechanism regulating differential cell-type-specific SUB accumulation in the root epidermis (Kwak and Schiefelbein, 2008). I could confirm cell-type specific differences in SUB:EGFP accumulation in the root epidermis (not shown), however, in all investigated lines *SUB::gSUB:EGFP*-

derived signals appeared uniform across cells within cell layers in floral meristems and ovules. Furthermore, assessment of overall *SUB* expression levels in *sub* flowers via qRT-PCR did not provide evidence for a feedback loop regulation of *SUB* transcription (Vaddepalli et al., 2011). The combined results indicate that cell-type-specific feedback mechanisms regulating SUB accumulation may be specific to the root.

4.2 Molecular characterization of QUIRKY

4.2.1 QKY, a novel MCTP involved in SUB signaling

The later part of the thesis deals with QKY, a putative transmembrane protein involved in Ca^{2+}-Mediated Signaling. Sequence analysis of QKY suggests that QKY is the first described plant representative of the previously described MCTP family (Shin et al., 2005; Fulton et al., 2009) and that it might act as a membrane-bound protein involved in a process regulated by Ca^{2+} and phospholipids. While the function of animal MCTPs is unknown, human MCTP2 is a membrane protein located to intracellular vesicular structures (Shin et al., 2005). Other membrane-bound proteins with multiple C2 domains are the synaptotagmins and ferlins (Südhof, 2002; Bansal and Campbell, 2004). Synaptotagmins contain two C2 domains and an amino-terminal transmembrane domain while most ferlins carry between four and six C2 domains and a carboxy-terminal transmembrane domain. Members of these two protein families function during regulated exocytosis, a process in which specific vesicles are signaled to fuse with the plasma membrane. Processes that rely on regulated exocytosis include neurotransmitter release at synapses and plasma membrane repair, a basic cellular process that mends physical injuries inflicted upon the plasma membrane (Andrews, 2002; McNeil and Steinhardt, 2003; Chieregatti and Meldolesi, 2005). The synaptotagmins, Syt 1 and 2 are required for Ca^{2+}-regulated synaptic vesicle exocytosis during neurotransmitter release into the synaptic cleft (Geppert et al., 1994; Fernández-Chacón et al., 2001; Südhof, 2002) while Syt VII promotes lysosomal exocytosis during plasma membrane repair in fibroblasts (Reddy et al., 2001). The *C. elegans* ferlin FER-1 is localized to the membranes of membranous organelles (MOs) and promotes the fusion of MOs with the plasma membrane during the development of crawling spermatozoa (Washington and Ward, 2006; Achanzar and Ward, 1997). Mutations in dysferlin result in progressive muscular dystrophies (Liu et al., 1998; Bansal et al., 2003; Bashir et al., 1998) and dysferlin appears to be required for Ca2+-dependent sarcolemma resealing during membrane repair in skeletal muscle fibres (Bansal et al., 2003; Lennon et al., 2003).

SYTs were thought to be exclusive to animals but recently they have been characterized in plants. SYT1 is the first plant synaptotagmin investigated that belongs to a five-member family in Arabidopsis (Lewis and Lazarowitz, 2010). Freezing tolerance involves a Ca^{2+}-dependent membrane resealing process that depends on SYT1 (Yamazaki et al., 2008). An independent study identified SYT1 from a genetic screen for Arabidopsis mutants showing hypersensitivity to salt stress (Schapire et al., 2008). Both these studies established that SYT1 is essential for plasma membrane repair under abiotic stresses such as salt, osmotic stress and freezing (Schapire et al., 2008; Yamazaki et al., 2008).

4.2.2 QKY is a PD associated protein

In this study it was shown that QKY, a novel multi C2 domain containing protein is localized to PD. Several PD associated proteins have signal peptide at the N-terminus (Simpson et al., 2009; Thomas et al., 2008). QKY lacks any signal peptide which could regulate its sorting to PD. Deletion of the two putative TM domains completely disrupted QKYs PD localization. Thus it seems the C-terminal PRT_C domain with two TM domains has all the information for its PD localization. But not much is known about the function of PRT_C domain in which these TM domains are embedded. Single transmembrane domain of PDLP1a contains all the information necessary for its intracellular targeting to plasmodesmata (Thomas et al., 2008). Further experiments are needed to investigate if the TM domains of QKY are sufficient for its PD targeting without the assistance of any C2 domains and either one or both of the TM domains are needed to do the job.

The peculiar localization pattern of QKY in developing root cells is intriguing. For example QKY shows ER like localization pattern. The appearance of dotted signal within the cell along with phragmoplast localization indicates that QKY might have a role to play in cell wall formation. The primary PD that form during cytokinesis are nothing but strands of ER that are trapped in the new cell wall deposition resulting in desmotubule of the PD (Maule, 2008; Burch-Smith et al., 2011; Burch-Smith and Zambryski, 2011). Thus it could be that the QKY is trapped in PD during cytokinesis

and has a regulatory role in the deposition of cell wall specific components. Consequently, plants lacking functional QKY show aberrant periclinal divisions in L1 and L2 layers of floral meristems and other morphological defects (Fulton et al., 2009). The appearance of large number of genes related to cell wall biology during our transcriptome analysis of *QKY*-responsive genes further supports this notion (Fulton et al., 2009).

The lack of detectable signal under its native promoter suggests that QKY might be subjected to intrinsic post-transcriptional regulation. Even under *UBQ* promoter the signal is lacking in developing ovules and floral primordia. In the organs that showed visible signal, the PD localization is restricted to the epidermis or outermost layer. It could be either because QKY is subjected to rapid recycling or there is a QKY specific translation suppressor. On the other hand expression of *UBQ::EGFP:QKY* in the outermost layer also indicates there is tissue specific regulation of *QKY*. Interestingly, our observations from several lines of evidence indicate that detectable level of SUB protein is not needed for the complementation of *sub-1* phenotype. Accordingly, one explanation could be that the native protein expression of SUB and QKY itself is very low and is sufficient for their activity.

4.2.3 QKY might be involved in Calcium mediated vesicle trafficking

In earlier investigations, evidence was presented showing that transport through plasmodesmata is reduced in the presence of high calcium concentrations. Moreover, plasmodesmata of maize cells rapidly closed, either when injected with calcium or as a result of cold, a treatment that induces an increase in cytoplasmic calcium (Holdaway-Clarke et al., 2000). Investigations on Aluminum-Induced 1-->3-beta-D-glucan indicate that local alterations in calcium levels may trigger callose formation preferentially at PD (Sivaguru et al., 2000).

Biochemical assays determined that the C2C domain of QKY binds to phospholipids in a Ca^{2+} dependent manner. Interestingly, the C2 domains of human MCTPs were found to bind Ca^{2+} with high affinity but lacked any phospholipid

binding capacity (Shin et al., 2005). The Ca^{+2} affinity of the other three QKY C2 domains needs investigation though they don't bind to phospholipids in a Ca^{+2} dependent manner. Thus, QKY protein might be involved in gating the PD by sensing the intracellular Ca^{+2} levels. In animals, a Ca^{+2}-dependent interaction between SYT1 and phospholipids is proposed to be critical in mediating Ca^{+2}-triggered vesicle fusion for the neurotransmitter release (Sudhof, 2004; Chapman, 2008; Rizo and Rosenmund, 2008). In similar lines, one could postulate that Ca^{+2} dependent interaction of QKY with phospholipids is also crucial for fusion of vesicles at PD or phragmoplast, as deposition of cell wall related components requires extensive vesicle trafficking (Lerouxel et al., 2006).

The C2A domain of Arabidopsis SYT1 is a canonical C2 domain in terms of phospholipid binding, whereas the C2B domain shows phospholipid binding in a Ca^{2+} -independent manner (Schapire et al., 2008). SYT1 is demonstrated to participate in Ca^{2+}-dependent repair of membranes (Schapire et al., 2009). Along with plasma membrane localization SYT1 is also abundantly localized within plasmodesmata (Schapire et al., 2008). Additionally, SYT1 was also identified in a screen for Arabidopsis proteins interacting with the movement protein (MP) from Cabbage leaf curl virus (Lewis and Lazarowitz, 2010). SYT1 regulates endocytosis, and suggest that distinct virus movement proteins transport their cargos to plasmodesmata for cell-to-cell spread via an endocytic recycling pathway (Lewis and Lazarowitz, 2010). SYT1 was also found in Arabidopsis PD-proteome analysis (Fernandez-Calvino et al., 2011).

4.2.4 The PD localized QKY might be involved in the callose regulation

Intercellular movement of several macromolecules like transcription factors via PD in a selective or non-selective manner has been reported in plant developmental events such as root radial or epidermal cell patterning and shoot organogenesis (Kurata et al., 2005). But the factors involved in this movement are largely unknown.

In developing tissues of *qky* mutant the free movement of GFP between the layers and within the layer is not perturbed indicating that QKY has no effect on

endogenous SEL of PD. In hindsight this result is expected, as most of the mutants disrupted in symplastic connectivity and non-selective movement are shown to be lethal (Kim et al., 2002).

Various genetic screens have led to identification of genes, which are not directly localized to PD but involved in their regulation. Known examples are *increased size exclusion limit 1* and *2*, mutants of RNA helicases leading to de novo production of plasmodesmata increasing the plasmodesmata-mediated intercellular transport (Kim et al., 2002; Kobayashi et al., 2007; Stonebloom et al., 2009; Burch-Smith and Zambryski, 2010; Xu et al., 2012). In contrast *green fluorescent protein arrested trafficking1*, a mutant of m-type thioredoxin leads to callose accumulation, which would in turn affect plasmodesmal aperture resulting in decreased intercellular transport (Benitez-Alfonso et al., 2009; Zavaliev et al., 2011). *ise1* and *gat1* mutants produce increased levels of reactive oxygen species (ROS) suggesting ROS as critical signaling molecules for PD development and/or function (Stonebloom et al., 2009; Benitez-Alfonso et al., 2009). But the levels of ROS produced in *gat1* are higher than those produced in *ise1*. Thus, the site and amount of ROS production seems to differentially regulate associated specific targets that in turn affect PD function.

Callose, a β-glucan polysaccharide is an important component of plasmodesmata, which accumulates in the cell wall regulating the size of the aperture and thus plasmodesmal transport. It is deposited at the neck regions of plasmodesmata during wounding and pathogen attack (Zavaliev et al., 2011). Biochemical and molecular studies undertaken to isolate proteins that are directly associated with PDs have contributed to the identification of multiple proteins that affect PD permeability. These include plasmodesmata-associated b-1,3-glucanase (At-BG_ppap) (Levy et al., 2007), PD-located protein 1 (PDLP1) (Thomas et al., 2008), PD-callose binding proteins (PDCBs) Chorus (CHS) or Glucan synthase-like 8 (GSL8) (Simpson et al., 2009; Guseman et al., 2010), giberellic acid-inducible β-1,3-glucanases (Rinne et al., 2011), PDLP5 increases callose (Lee et al., 2011) and Callose Synthase 3 (CALS3) (Vatén et al., 2011). All the above-mentioned genes act as either positive or negative regulators of callose. Research on genes controlling the selective movement of

proteins by regulating the callose trafficking at PD is in its infancy. Given the nature of the protein QKY might be involved in the selective movement of protein via exo-endocytosis of callose at the PD. But preliminary aniline blue staining experiments failed to show differential callose accumulation in *qky-8* and *sub-1* mutants when compared to WT (not shown). Nevertheless, further investigations are needed to know if application of biotic or abiotic stresses will have any affect on callose accumulation in theses mutants.

4.3 The role of QKY in SUB mediated inter-cellular signaling

4.3.1 The mechanism of SUB-QKY interaction

The gene expression data suggests that *SUB* expression is not responsive to *qky-8* whereas *QKY* has shown slight reduction in *sub-1* background, which could be a consequence of indirect influences due to tissue sampling or the altered morphology of the mutant rather than the result of direct effect. However, this observation needs confirmation at the protein level, as it would indicate a positive feedback loop mechanism. Nonetheless, their respective cellular localization patterns were not affected in each other's absence.

Intriguingly, extra SUB protein can partially compensate the loss of QKY. This result can be interpreted in four different ways. From the genetics point of view, QKY acts upstream to SUB. In *qky-8* mutants extra SUB protein helps to do the downstream work in a limited way. The second possibility is that QKY directly interacts with SUB and assists SUB mediated signaling, for example for the selective trafficking of components. In the absence of QKY extra molecules of SUB can do a limited job. Third possibility is QKY along with another component act downstream to SUB and in the absence of QKY, the hypothetical component is forced to do QKY's job in a limited manner. Fourth possibility would be QKY acts as a recruiter of SUB and could be involved in trafficking of SUB at PD. But when the SUB molecules are increased by default the need for QKY becomes less. In all the four scenarios interaction between SUB and QKY is necessary either directly or indirectly and none of them are mutually exclusive. Interestingly, extra QKY protein did not had any influence in *sub-1* mutant, which suggests that SUB is the central regulator and its function is irreplaceable by QKY protein.

The physical interaction of SUB and QKY in yeast further strengthens the genetic interaction suggesting they act in a linear pathway. The data implies that the observed exaggerated phenotype of the *qky-8 sub-1* double mutants phenotype (Fulton et al., 2009) may be because of a synergistic genetic interaction rather than additive. At the

same time partial complementation of *qky* by *SUB* indicates that *QKY* has separate functions, which is reasonable given that transcriptome profiling has shown mis-expression of several genes in *qky-8* background, which do not overlap with *sub-1* responsive genes (Fulton et al., 2009).

In principle, the predicted membrane localization of QKY allows for the possibility that the SUB and QKY proteins interact directly which was further supported by our Y2H results. But the ER-like localization pattern and the lack of BFA sensitivity suggests that QKY might take the route of ER to plasmodesmata and might lead to its anchoring in desmotubule membrane. In contrast SUB is a PM localized protein and was shown to be BFA sensitive. In this context it will be interesting to know whether QKY is anchored to the PM at the plasmodesmata or membranes of ER derived desmotubule. Though the interaction of QKY with SUB intracellular domain means that the topology of QKY C2 domains is cytoplasmic further investigations are needed to confirm this. This investigation becomes more interesting in the context of suggestive QKY localization to membranes of desmotubule.

4.3.2 SUB-QKY complex might be involved in the selective trafficking of components

The function of several plant proteins depends on their selective trafficking between cells. Cytoplasm localized CCT8 a subunit of the type II chaperonin complex is required for trafficking of the structurally unrelated KN1 and TTG1 proteins, but not for SHR, suggesting that distinct pathways exist for proteins to traffic between cells. The KNOTTED1 (KN1) homeodomain transcription factor functions in SAM initiation and/or maintenance (Kerstetter et al., 1997), TRANSPARENT TESTA GLABROUS1 (TTG1) is a WD40-repeat protein whose movement is involved in trichome spacing (Bouyer et al., 2008), and SHORTROOT (SHR) is a plant-specific transcription factor whose movement is essential in specifying root endodermal identity (Nakajima et al., 2001). The mutant version of ER localized *Nicotiana tabacum* NON-CELL-AUTONOMOUS PATHWAY PROTEIN1 (NtNCAPP1)

inhibits the cell-cell trafficking of CmPP16 and tobacco mosaic virus (TMV) MP as well as their PD gating ability. This effect was specific, as the PD-gating activity of KN1 was not blocked. Plants overexpressing a dominant negative mutation of NCAPP1 showed a loss of organ symmetry, fusion of organs and altered cell-fates in the leaf epidermis, highlighting the developmental significance of the NCAPP1-associated trafficking pathway (Lee et al., 2003). These studies support the presence of selective trafficking pathways, and suggest that multiple networks mediate movement through PD.

Arabidopsis plasmodesmal proteome analysis identified several proteins along with SUB and QKY (Fernandez-Calvino et al., 2011). Among those more than 30 belong to RLKs and the plasmodesmal localization of 3 of these candidates was confirmed by fluorescence microscopy (Fernandez-Calvino et al., 2011). Six rice RLKs have recently been identified and confirmed as plasmodesmata-localized proteins (Jo et al., 2011). Maize CR4, a protein receptor-like Ser/Thr kinase, has been shown to preferentially accumulate in plasma membranes associated with the plasmodesmata promoting the lateral movement of aleurone signalling molecules between aleurone cells (Tian et al., 2007). Very recently FT-INTERACTING PROTEIN 1 (FTIP1), another Arabidopsis MCTP was shown to be required for FT export from companion cells to sieve elements, thus affecting FT transport through the phloem to the SAM (Liu et al., 2012). This study provided a mechanistic understanding of florigen transport, demonstrating that FT moves in a regulated manner and that FTIP1 mediates FT transport to induce flowering (Liu et al., 2012).

The localization of SUB and QKY at the plasmodesmata may help in understanding the non-cell-autonomous function of SUB (Yadav et al., 2008). *sub-1* and *slm* mutants also show root hair patterning defects (Fulton et al., 2009) (Kwak et al., 2005). Root hair patterning involves several putative transcription factors (TFs) to specify the root epidermal cell types through the general mechanism of lateral inhibition with feedback (Ishida et al., 2008; Grebe, 2012) and several of them show mis-expression in *scrambled-2 (scm-2)* background (Kwak et al., 2005; Kwak and Schiefelbein, 2007). One assumption would be that SUB-QKY complex and possibly

other SLM genes could participate in the control of the size exclusion limit (SEL) of PD and facilitates the selective movement of transcription factors mediating the lateral inhibition. But from the results of Kwak et al., 2007 one could learn that the trafficking of CAPRICE and GLABRA3, which show lateral movement, is not disturbed in *scm-2* mutants. Thus, the role of SUB and QKY at PD needs to be determined.

4.3.3 SUB mediated signaling may have a role to play during cell plate formation

Transcriptome analysis done previously has indicated that many *SLM*-responsive genes have functions in cell wall biology, hormone signaling, and various stress responses (Fulton et al., 2009). For example, the list includes inositol oxygenase family enzymes MIOX2 and MIOX4 required for biosynthesis of uridine-diphospho-glucuronic acid (UDP-GlcA), a precursor for various cell-wall matrix polysaccharides (Kanter et al., 2005), and a gene from the GERMIN-LIKE protein family, members of which are shown to play a role in biotic stress responses and cell wall biology (Carter et al., 1998). Other genes encode for cell wall proteins such as Glycine-rich proteins, lectin family proteins, and chitinases.

The *slm* phenotype suggests that SUB signaling affects the formation and shape of several organs by influencing cell morphogenesis, the orientation of the division plane, and cell proliferation (Fulton et al., 2009). The cell number in epidermis, cortex, and pith tissues of stem are also reduced in *sub-1* (Chevalier et al., 2005). Gene expression studies of *SUB* and *QKY* point out their role in proliferating tissues (Chevalier et al., 2005; Yadav et al., 2008). The apparent localization of SUB and QKY at the growing cell plate indicates a possible role in cell plate expansion during cytokinesis.

Two plant-specific cytoskeletal arrays, the preprophase band (PPB) and the phragmoplast assist somatic cytokinesis (Lloyd and Hussey, 2001). The preprophase band (PPB), which marks the future cortical-division site (CDS), appears in the cell cortex late in G2 phase and disappears with the breakdown of the nuclear envelope

during prometaphase (Jürgens, 2005). The phragmoplast originates in late anaphase between daughter nuclei and expands laterally. Golgi-derived vesicles deliver membranes and non-cellulosic polysaccharides to the phragmoplast midzone, where they fuse to form the cell plate (Jürgens, 2005). Thus, the phragmoplast guides the growing cell plate to the former location of the PPB (the CDS), where it attaches to the parental cell wall (Müller et al., 2009). Defect at any of these steps could lead to improper cell shape or division plane (Van Damme, 2009), which could explain the altered cell shape and/or the orientation of the cell division plane in *slm* mutants. The protein localization of SUB and QKY at the cell plate, and the mis-expression of genes involved in cell wall biosynthesis further validate this notion (Fulton et al., 2009).

Interestingly, root hair density in *scm-2* is unaffected but it shows mis-expression of several TFs involved in root hair patterning (Kwak et al., 2005; Kwak and Schiefelbein, 2007). *SCM* was postulated to affect an upstream signaling pathway to influence the entire cell-fate transcriptional network (Kwak and Schiefelbein, 2008). However, interestingly, a significant reduction in the relative division rate in the hair and non-root hair positions was also reported (Kwak et al., 2005) and a cross section of mutant root shows mild distortion of cell shapes. It would be interesting to see if the division plane in the epidermal cells is also altered in *scm* or *sub* mutants that could lead to misplacement of root hair and non-rot hair cells. In which case, according to the current hypothesis the mis-expression of root hair patterning TFs could be just a consequence of problems during cytokinesis.

4.3.4 Does SUB/QKY regulate cytokinesis or plasmodesmata or both

Interestingly, free diffusion of molecules is not affected in proliferating tissues of *sub* and *qky* mutants. Primary PDs are formed at cytokinesis in the developing phragmoplast. Thus, the presence of SUB and QKY at PD might be just a consequence of their role in cell wall biosynthesis or at PD they might be involved in selective trafficking and/or stress signaling (Fulton et al., 2009). On the other hand, the observed mis-expression of genes related to biotic stress could be an outcome of system interpreting changes in the cell wall as wounding stress.

Targeted vesicle trafficking and fusion at phragmoplast contribute to cell plate formation at the division site (Jürgens, 2005; Van Damme et al., 2008). The C2 domain containing protein QKY would make an ideal candidate for this process. Therefore, apart from the proposed symplastic regulation, SUB-QKY complex might be involved in proper deposition of cell wall components at the growing cell plate affecting proper cell shape and division plane. This would finally result in precise patterning of cells in different tissues.

The SUB mediated signaling does not seem to be essential for cytokinesis that affects the various steps of cell plate formation in major fashion but rather it assists the process, which affects the cellular shape or division plane. Thus, the results raise an interesting question whether a defect in SUB mediated intercellular signaling at the PD influences the overall cellular defects in mutants or SUB mediated signaling influences the cellular morphology directly with a role in cell wall biosynthesis. Both of the arguments are not mutually exclusive. Further research in this direction would help in understanding the biological functions of *SLM* genes in tissue morphogenesis.

5 Conclusion

Plant organs like stem, leaves, inflorescences and flowers are derived from a group of undifferentiated cells called shoot apical meristem. Cell-to-cell communication in meristematic tissue is essential for proper organogenesis.

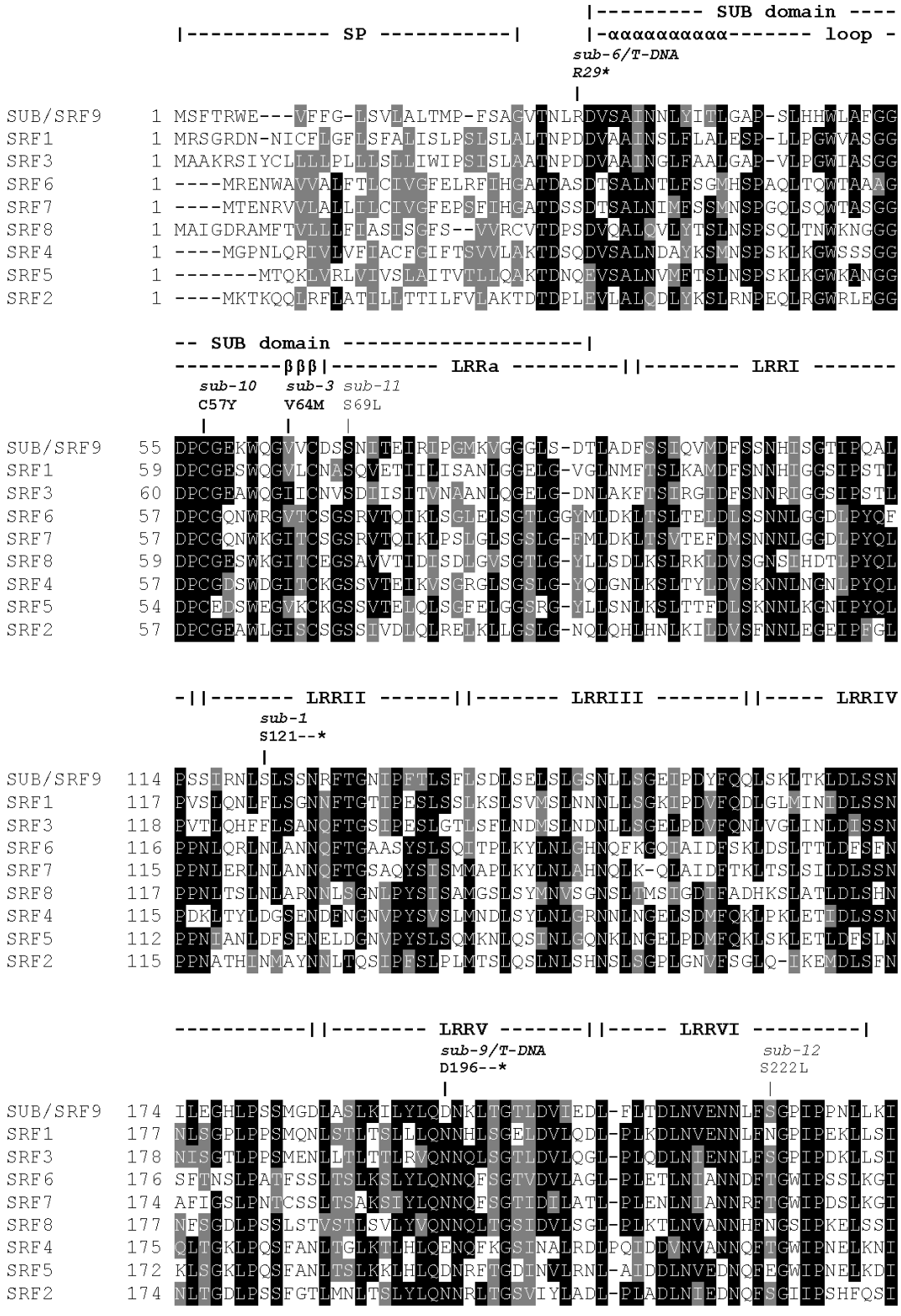
STRUBBELIG (SUB) is an atypical LRR-RLK gene with a role in tissue morphogenesis of many plant organs. Further understanding the signaling mechanism of SUB is of great interest. In this context, I asked whether the different SUB domains carry information for specific SUB-dependent biological processes. The data indicate that SUB process specificity is mediated by factors acting together with or downstream of SUB. The results reveal the importance of the N-capping domain of SUB and suggest that biogenesis of a functional SUB receptor is subjected to endoplasmic reticulum-mediated quality control. Multiple introns are needed for the *SUB* broad expression pattern. However, it still needs to be determined whether the introns affect splicing of *SUB* mRNA and stability or they have a role at the translation level. Further, the data suggest that SUB accepts a surprising level of sequence variation in its protein sequence but when a few essential amino acids are mutated the SUB function is lost completely.

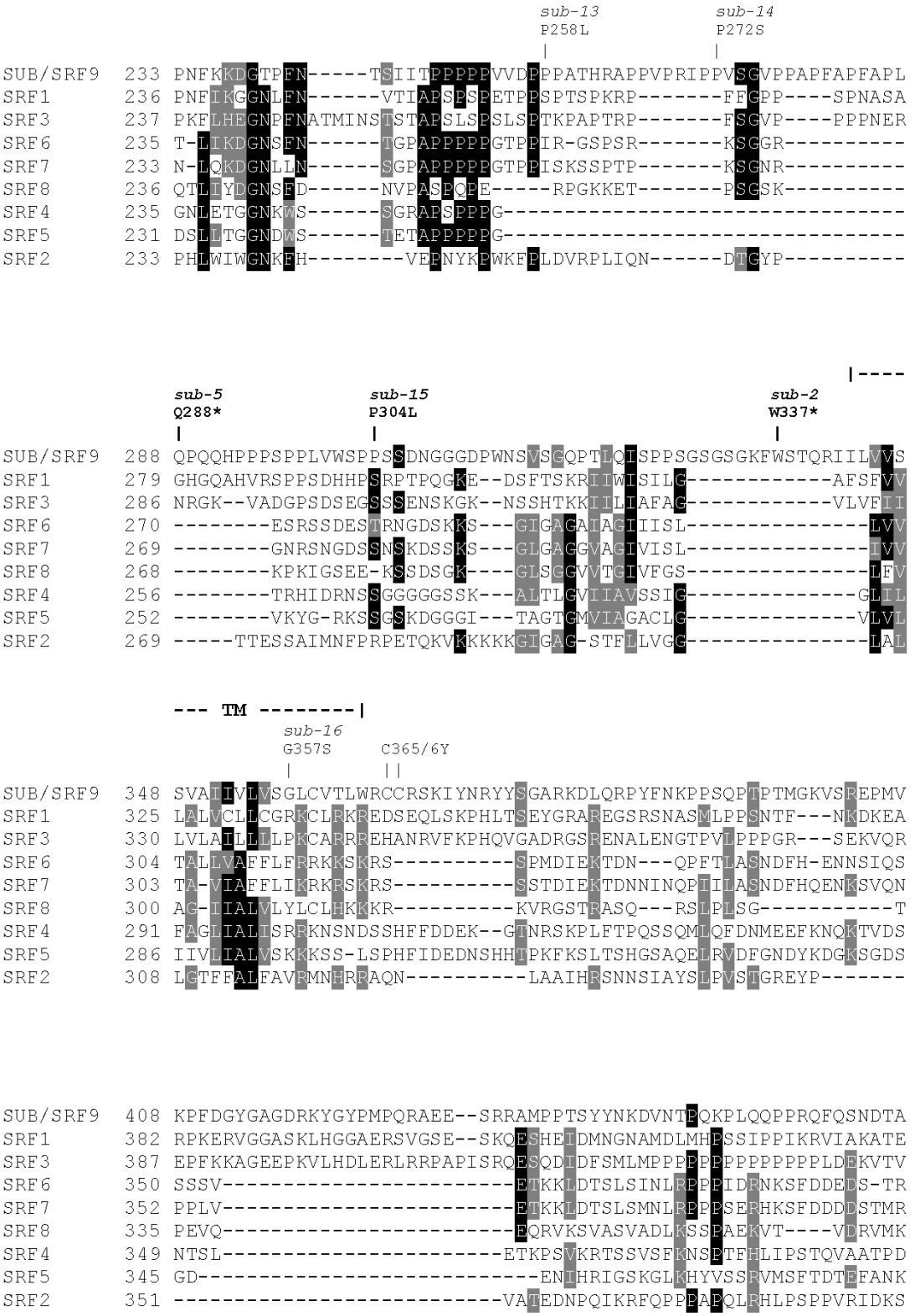
To understand the signaling mechanism of components involved in SUB mediated signaling, I went on to characterize *QUIRKY (QKY)* one of the *STRUBBELIG-LIKE-MUTANTS*. QKY is a multiple C2 domain containing transmembrane protein (MCTP) and comparison of QKY domain structure with related C2 domain containing proteins suggests a role in Ca^{2+}-dependent signalling and membrane trafficking. Accordingly, QKY was shown to exhibit phospholipid binding capacity in a Ca^{2+} dependent manner via its C2C domain. However, the Ca^{2+} binding capacity of individual C2 domains needs to be investigated to further understand the biochemical nature of QKY. Extensive microscopic analysis suggests that QKY is localized to plasmodesmata (PD) and also revealed the PD localization of SUB. The C-terminal transmembrane domains (TMDs) seems to be important for the PD localization of QKY but the crucial amino acids in the TMDs which are necessary for the PD localization needs to be determined.

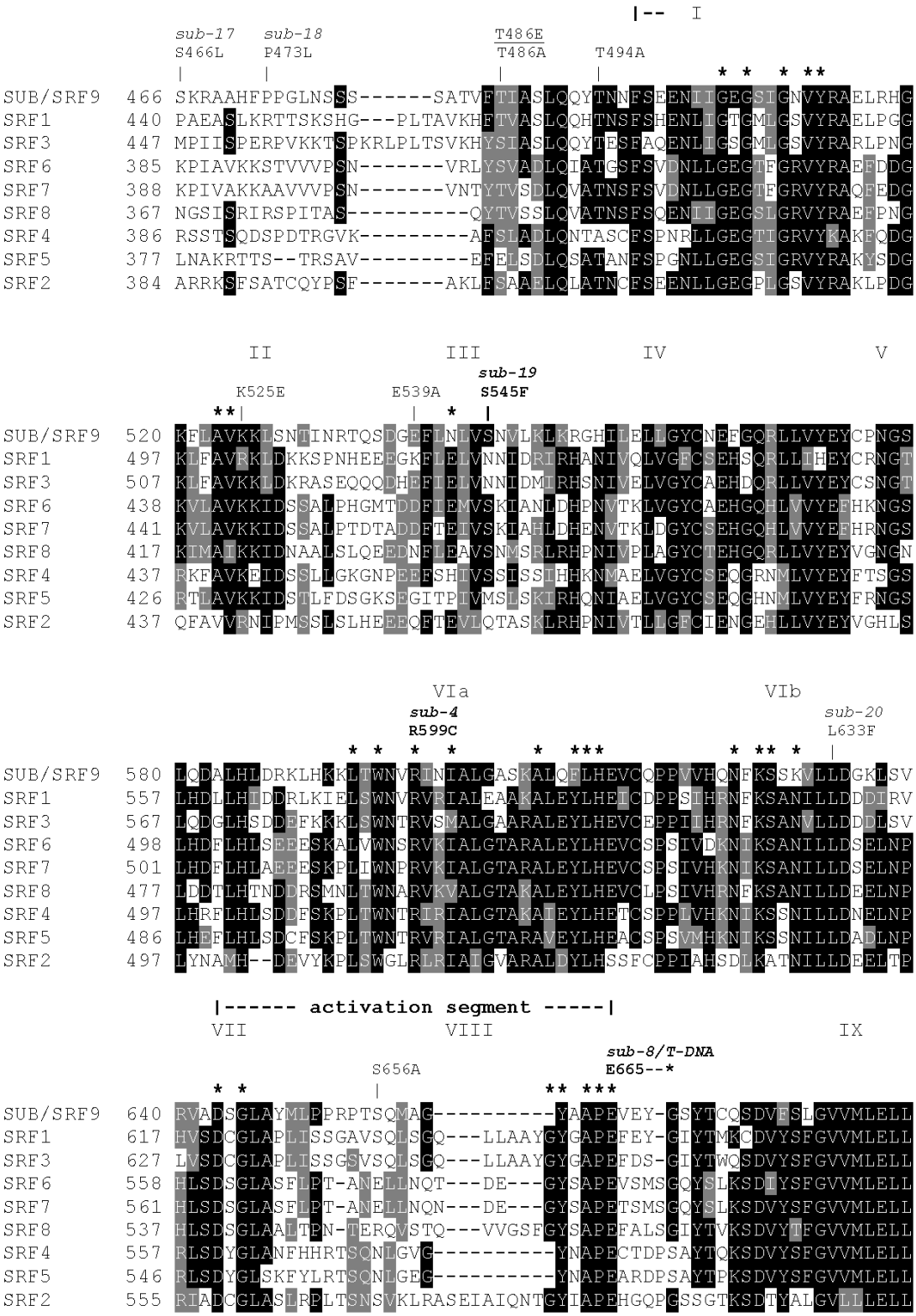
Furthermore, genetics and yeast-two-hybrid data indicate a direct interaction between SUB and QKY. Nevertheless, SUB-QKY interaction needs to be confirmed in vivo. In addition, data reveals the localization of SUB and QKY at the cell plate. Nevertheless, how this influences the cellular shape needs more attention. On the other hand, the symplastic free diffusion of molecules via PD is not disturbed in *sub* and *qky* mutants which leads to the challenging task of finding their role in symplastic trafficking, if any.

The overall analysis raises question whether the observed morphological defects in *sub* and *qky* result from improper deposition of cell wall components or due to improper symplastic trafficking at the plasmodesmata. Further analysis at the cellular and molecular level is needed to resolve this conflict.

6 Supplement




sub-13
P258L
sub-14
P272S
SUB/SRF9 233 PNFKKDGTPFN-----TSIITPPPPPVVDPPPATHRAPPVPRIPPVSGVPPAPFAPFAPL
SRF1 236 PNFIKGGNLFN------VTIAPSPSPETPPSPTSPKRP-------FFGPP----SPNASA
SRF3 237 PKFLHEGNPFNATMINSTSTAPSLSPSLSPTKPAPTRP-------FSGVP----PPPNER
SRF6 235 T-LIKDGNSFN------TGPAPPPPPGTPPIR-GSPSR-------KSGGR----------
SRF7 233 N-LQKDGNLLN------SGPAPPPPPGTPPISKSSPTP-------KSGNR----------
SRF8 236 QTLIYDGNSFD------NVPASPQPE------RPGKKET-------PSGSK----------
SRF4 235 GNLETGGNKWS------SGRAPSPPPG----------
SRF5 231 DSLLTGGNDWS------TETAPPPPPG----------
SRF2 233 PHLWIWGNKFH--------VEPNYKPWKFPLDVRPLIQN------DTGYP----------
sub-5
Q288*
sub-15
P304L
sub-2
W337*
SUB/SRF9 288 QPQQHPPPSPPLVWSPPSSDNGGGDPWNSVSGQPTLQISPPSGSGSGKFWSTQRIILVVS
SRF1 279 GHGQAHVRSPPSDHHPSRPTPQGKE--DSFTSKRIIWISILG-----------AFSFVV
SRF3 286 NRGK--VADGPSDSEGSSSENSKGK--NSSHTKKIILIAFAG-----------VLVFII
SRF6 270 --------ESRSSDESTRNGDSKKS---GIGAGAIAGIIISL----------LVV
SRF7 269 --------GNRSNGDSSNSKDSSKS---GLGAGGVAGIVISL----------IVV
SRF8 268 --------KPKIGSEE-KSSDSGK----GLSGGVVTGIVFGS----------LFV
SRF4 256 --------TRHIDRNSSGGGGGSSK---ALTLGVIIAVSSIG----------GLIL
SRF5 252 --------VKYG-RKSSGSKDGGGI---TAGTGMVIAGACLG----------VLVL
SRF2 269 -----TTESSAIMNFPRPETQKVKKKKKGIGAG-STFLLVGG----------LAL
--- TM --------|
sub-16
G357S
C365/6Y
SUB/SRF9 348 SVAIIVLVSGLCVTLWRCCRSKIYNRYYSGARKDLQRPYFNKPPSQPTPTMGKVSREPMV
SRF1 325 LALVCLLCGRKCLRKREDSEQLSKPHLTSEYGRAFEGSRSNASMLPPSNTF---NKDKEA
SRF3 330 LVLAILLLLPKCARRREHANRVFKPHQVGADRGSRENALENGTPVLPPPGR---SEKVQR
SRF6 304 TALLVAFFLFRRKKSKRS----------SPMDIEKTDN---QPFTLASNDFH-ENNSIQS
SRF7 303 TA-VIAFFLIKRKESKRS----------SSTDIEKTDNNINQPIILASNDFHQENKSVQN
SRF8 300 AG-IIALVLYLCLEKKKR----------KVRGSTRASQ---RSLPLSG----------T
SRF4 291 FAGLIALISRPKNSNDSSHFFDDEK--GTNRSKPLFTPQSSQMLQFDNMEEFKNQKTVDS
SRF5 286 IIVLIALVSKKKSS-LSPHFIDEDNSHHTPKFKSLTSHGSAQELRVDFGNDYKDGKSGDS
SRF2 308 LGTFFALFAVRMNHRRAQN----------LAAIHRSNNSIAYSLPVSTGREYP-------
SUB/SRF9 408 KPFDGYGAGDRKYGYPMPQRAEE--SRRAMPPTSYYNKDVNTPQKPLQQPPRQFQSNDTA
SRF1 382 RPKERVGGASKLHGGAERSVGSE--SKQESHEIDMNGNAMDLMHPSSIPPIKRVIAKATE
SRF3 387 EPFKKAGEEPKVLHDLERLRRPAPISRQESQDIDFSMLMPPPPPPPPPPPPLDEKVTV
SRF6 350 SSSV----------ETKKIDTSLSINLRPPPIDRNKSFDDELS-TR
SRF7 352 PPLV----------ETKKIDTSLSMNLRPPPSERHKSFDDDLSTMR
SRF8 335 PEVQ----------PQRVKSVASVADLKSSPAEKVT----VLRVMK
SRF4 349 NTSL----------ETKPSVKRTSSVSFKNSPTFHLIPSTQVAATPD
SRF5 345 GD----------ENIHRIGSKGLKHYVSSRVMSFTDTEFANK
SRF2 351 ----------VATEDNPQIKRFQPPPAPQLRHLPSPPVRIDKS


|-- I
sub-17 S466L
sub-18 P473L
T486E T486A
T494A
SUB/SRF9 466 SKRAAHFPPGLNSSS------SATVFTIASLQQYTNNFSEENIIGEGSIGNVYRAELRHG
SRF1 440 PAEASLKRTTSKSHG---PLTAVKHFTVASLQQHTNSFSHENLIGTGMLGSVYRAELPGG
SRF3 447 MPIISPERPVKKTSPKRLPLTSVKHYSIASLQQYTESFAQENLIGSGMLGSVYRARLPNG
SRF6 385 KPIAVKKSTVVVPSN-------VRLYSVADLQIATGSFSVDNLLGEGTFGRVYRAEFDDG
SRF7 388 KPIVAKKAAVVVPSN-------VNTYTVSDLQVATNSFSVDNLLGEGTFGRVYRAQFEDG
SRF8 367 NGSISRIRSPITAS----------QYTVSSLQVATNSFSQENIIGEGSLGRVYRAEFPNG
SRF4 386 RSSTSQDSPDTRGVK---------AFSLADLQNTASCFSPNRLLGEGTIGRVYKAKFQDG
SRF5 377 LNAKRTTS--TRSAV---------EFELSDLQSATANFSPGNLLGEGSIGRVYRAKYSDG
SRF2 384 ARRKSFSATCQYPSF-------AKLFSAAELQLATNCFSEENLLGEGPLGSVYRAKLPDG
II III IV V
sub-19 S545F
K525E E539A
SUB/SRF9 520 KFLAVKKLSNTINRTQSDGEFLNLVSNVLKLKRGHILELLGYCNEFGQRLLVYEYCPNGS
SRF1 497 KLFAVRKLDKKSPNHEEEGKFLELVNNIDRIRHANIVQLVGFCSEHSQRLLIHEYCRNGT
SRF3 507 KLFAVKKLDKRASEQQQDHEFIELVNNIDMIRHSNIVELVGYCAEHDQRLLVYEYCSNGT
SRF6 438 KVLAVKKIDSSALPHGMTDDFIEMVSKIANLDHPNVTKLVGYCAEHGQHLVVYEPHKNGS
SRF7 441 KVLAVKKIDSSALPTDTADDFTEIVSKIAHLDHENVTKLDGYCSEHGQHLVVYEPHRNGS
SRF8 417 KIMAIKKIDNAALSLQEEDNFLEAVSNMSRLRHPNIVPLAGYCTEHGQRLLVYEYVGNGN
SRF4 437 RKFAVKEIDSSLLGKGNPEEFSHIVSSISSIHHKNVAELVGYCSEQGRNMLVYEYFTSGS
SRF5 426 RTLAVKKIDSTLFDSGKSEGITPIVMSLSKIRHQNIAELVGYCSEQGHNMLVYEYFRNGS
SRF2 437 QFAVVRNIPMSSLSLHEEEQFTEVLQTASKLRHPNIVTLLGFCIENGEHLLVYEYVGHLS
VIa VIb
sub-4 R599C
sub-20 L633F
SUB/SRF9 580 LQDALHLDRKLHKKLTWNVRINIALGASKALQFLHEVCQPPVVHQNFKSSKVLLDGKLSV
SRF1 557 LHDLLHIDDRLKIELSWNVRVRIALEAAKALEYLHEICDPPSIHRNFKSANILLDDDIRV
SRF3 567 LQDGLHSDDEFKKKLSWNTRVSMALGAARALEYLHEVCEPPIIHRNFKSANVLLDDDLSV
SRF6 498 LHDFLHLSEEESKALVWNSRVKIALGTARALEYLHEVCSPSIVDKNIKSANILLDSELNP
SRF7 501 LHDFLHLAEEESKPLTWNPRVKIALGTARALEYLHEVCSPSIVHKNIKSANILLDSELNP
SRF8 477 LDDTLHTNDDRSMNLTWNARVKVALGTAKALEYLHEVCLPSIVHRNFKSANILLDEELNP
SRF4 497 LHRFLHLSDDFSKPLTWNTRIRIALGTAKAIEYLHETCSPPLVHKNIKSSNILLDNELNP
SRF5 486 LHEFLHLSDCFSKPLTWNTRVRIALGTARAVEYLHEACSPSVMHKNIKSSNILLDADLNP
SRF2 497 LYNAMH--DEVYKPLSWGLRLRIAIGVARALDYLHSSFCPPIAHSDLKATNILLDEELTP
|------ activation segment -----|
VII VIII IX
sub-8/T-DNA E665--*
S656A
SUB/SRF9 640 RVADSGLAYMLPPRPTSQMAG----------YAAPEVEY-GSYTCQSDVFSLGVVMLELL
SRF1 617 HVSDCGLAPLISSGAVSQLSGQ---LLAAYGYGAPEFEY-GIYTMKCDVYSFGVVMLELL
SRF3 627 LVSDCGLAPLISSGSVSQLSGQ---LLAAYGYGAPEFDS-GIYTWQSDVYSFGVVMLELL
SRF6 558 HLSDSGLASFLPT-ANELLNQT---DE---GYSAPEVSMSGQYSLKSDIYSFGVVMLELL
SRF7 561 HLSDSGLASFLPT-ANELLNQN---DE---GYSAPETSMSGQYSLKSDVYSFGVVMLELL
SRF8 537 HLSDSGLAALTPN-TERQVSTQ---VVGSFGYSAPEFALSGIYTVKSDVYTFGVVMLELL
SRF4 557 RLSDYGLANFHHRTSQNLGVG----------YNAPECTDPSAYTQKSDVYSFGVVMLELL
SRF5 546 RLSDYGLSKFYLRTSQNLGEG----------YNAPEARDPSAYTPKSDVYSFGVVMLELL
SRF2 555 RIADCGLASLRPLTSNSVKLRASEIAIQNTGYIAPEHGQPGSSGTKSDTYALGVLILELL

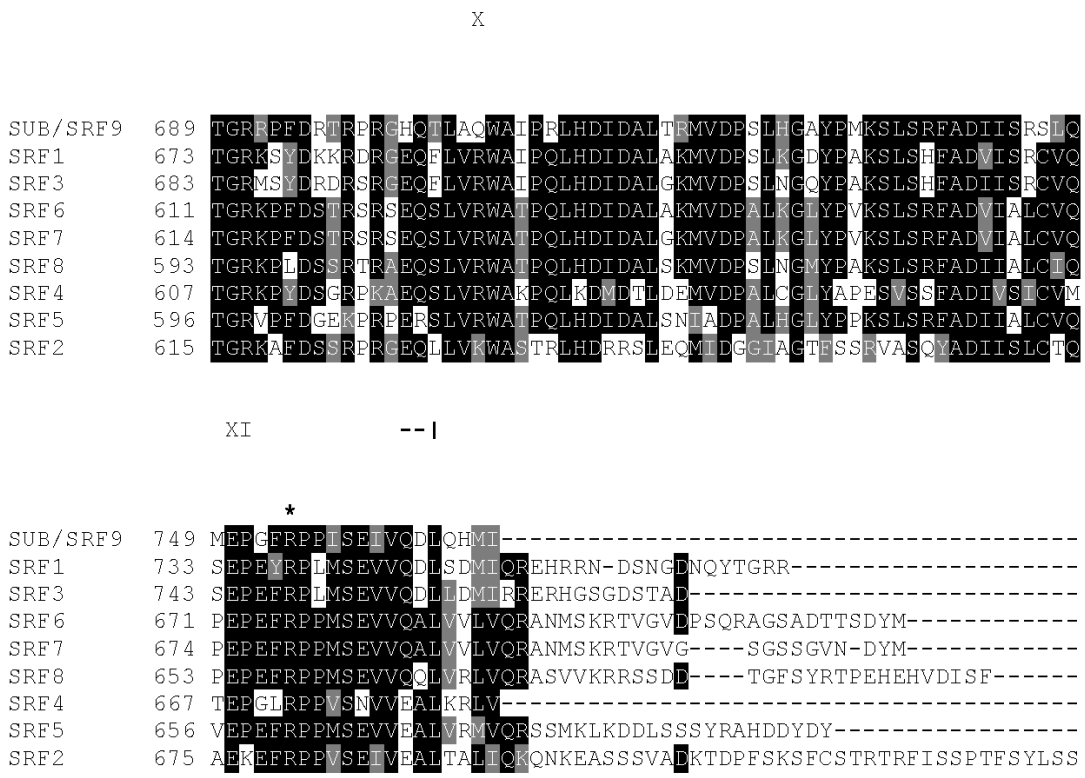


Figure S1 Protein sequence alignment of the Arabidopsis SRF family.

Highlights the different predicted structural motifs of SUB, the positions of the sub mutations described in this paper

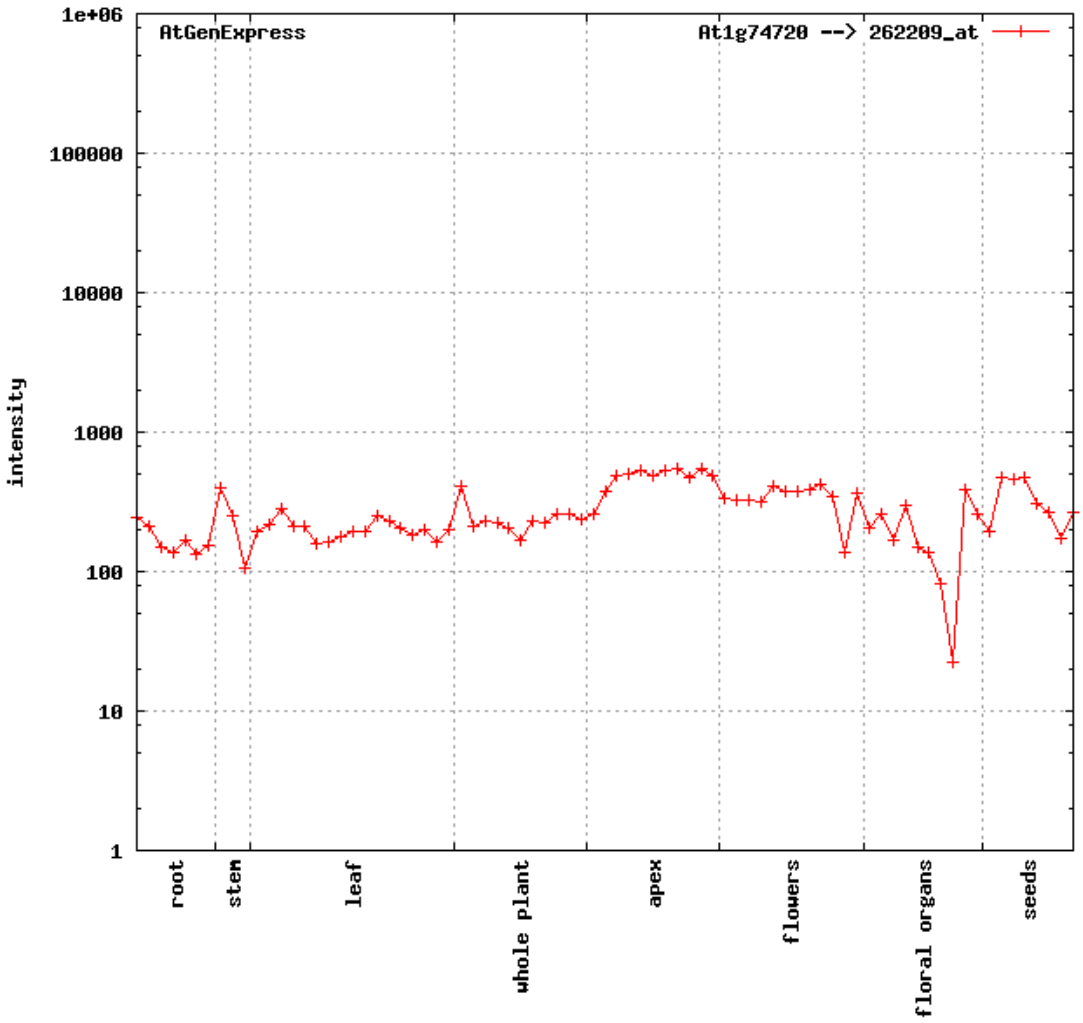


Figure S2 Gene expression map for *QKY*

AtGenExpress data showing gene expression of *QKY* in different organs (Schmid et al., 2005)

Position[+]	Change	Accession
2	S2T	ICE102, Qui-0, ICE61
9	F9L	ICE102, Qui-0
11	G11V	ICE102, Qui-0, ICE61
22	S22T	ICE102, Qui-0, ICE61
284	F284L	Pra-6
285	A285D	TueWa1-2, Vash-1
349	V349M	ICE120
369	K369E	Koch-1
426	Q426R	ICE61
456	P456L	ICE72
465	A465G	ICE120, Tuescha-9
595	T595N	Del-10, ICE107, Nie1-2, Ped-0, Koch-1
667	E667D	ICE72

Table S1 Summary of SUB amino acid polymorphisms in different Arabidopsis accessions.

Primer name	Sequence (5' to 3')
SUB_cmyc_F	CCTAGGAGGCGCGCCATGAGCTTTACAAGATGGGAAGTGTTC
SUB_cmyc_R	GAGACCGACGTCAGGGCCCCGATCATATGTTGAAGATCTTGG
SUB-Genomic2_F	GGCAAGTTTTCTCTGTTTCACACTTTGGAGACG
SUB-Genomic2_R	GCCCTCTGTGGAGCAGATTGATTTGAAGTTTA
Sig:SUB_Xba1_R	GCTCTAGACCGACGTCAGGGC
SUBC57Yf	GCTTTTGGAGGAGACCCTTATGGAGAAAAGTGGCAAGGTG
SUBC57Yr	CACCTTGCCACTTTTCTCCATAAGGGTCTCCTCCAAAACG
35SsubV64Mf	GAGAAAAGTGGCAAGGTATGGTGTGTGACTCCTCA
35SsubV64Mr	TGAGGAGTCACACACCATACCTTGCCACTTTTCTC
SUB_C66Y_F	GGCAAGGTGTGGTGTATGACTCCTCAAACATC
SUB_C66Y_R	GATGTTTGAGGAGTCATACACCACACCTTGCC
SUB_C57-66Y_F	GGAGGAGACCCTTATGGAGAAAAGTGGCAAGGTGTGGTGTATGACTCCTCAAAC
SUB_C57-66Y_R	GTTTGAGGAGTCATACACCACACCTTGCCACTTTTCTCCATAAGGGTCTCCTCC
SUBC365-6Af	GTGTGTTACACTTTGGAGATATTACAGAAGTAAAATAT

	ATAACCG
SUBC365-6Ar	CGGTTATATATTTTACTTCTGCAACATCTCCAAAGTGTAACAC
G357S_SP	GCCATAATTGTTCTTGTATCCAGCTTGTGTGTTACACTTTGGAG
G357S_ASP	CTCCAAAGTGTAACACACAAGCTGGATACAAGAACAATTATGGC
SUBT486Af	CCTCATCTTCTGCTACTGTTTTCGCCATTGCTTCACTTCAGC
SUBT486Ar	GCTGAAGTGAAGCAATGGCGAAAACAGTAGCAGAAGATGA GG
SUBT486Ef	CCTCATCTTCTGCTACTGTTTTCGAAATTGCTTCACTTCAGC
SUBT486Er	GCTGAAGTGAAGCAATTTCGAAAACAGTAGCAGAAGATGA GG
SUBK525Ef	GGAAAGTTTCTTGCGGTGGAGAAGCTGAGCAATACCATCAAC
SUBK525Er	GTTGATGGTATTGCTCAGCTTCTCCACCGCAAGAAACTTTCC
SUBE539A35Sf	AGAACACAGAGTGACGGCGCATTCCTCAATCTAGTCTCC
SUBE539A35Sr	GGAGACTAGATTGAGGAATGCGCCGTCACTCTGTGTTCTG
35SsubR599Cf	AAGAAGCTCACTTGGAATGTATGTATAAATATTGCATTAGG AGCTTC
35SsubR599Cr	GAAGCTCCTAATGCAATATTTATACATACATTCCAAGTGAG CTTCTT
SUBT494Af	GCTTCACTTCAGCAATACGCAAATAATTTCTCAGAAGAG
SUBT494Ar	CTCTTCTGAGAAATTATTTGCGTATTGCTGAAGTGAAGC
SUBT494Ef	GCTTCACTTCAGCAATACGAAAATAATTTCTCAGAAGAG
SUBT494Er	CTCTTCTGAGAAATTATTTTCGTATTGCTGAAGTGAAGC
35S-extra-myc-rev	CCG ACG TCA GGG CCC CGA ATT TTC CTG ATC CTG AAC CTG A
35S-TMmyc-rev	CCG ACG TCA GGG CCC CTC GGT TAT ATA TTT TAC TTC TGC A
JuxtraAatII-R	AAG ACG TCA GGG CCC CAT TAT TTG TGT ATT GCT GA
Alalinksignal-rev	GGC GGC GGC GGC GGC AAC TCC AGC TGA GAA AGG CAT TG
Alalink-TM-intra-for	GCC GCC GCC GCC GCC GCC TCA GGT TCA GGA TCA GGA AAA T-
AscIntra-F	TGG CGC GCC ATG AGA TGT TGC AGA A
1529(720(P1)R1)	GCTAGAGCTTCCTTGACCATCTT
1531(720(P2)F1)	CTACCACTACGATGAACACGAC

1698 Kpn1/Qky/Bci6/F	GGTTCTTGTTTGGTATCCTGATTTGGTAGTCC
1699 Kpn1/Qky/Bci6/R	GGACTACCAAATCAGGATACCAAACAAGAACC
1714 Kpn1/Qky::/F	CGGGGTACCAGAAAGAGTAACATTTGTATTTGATTGT
1715 Qky::/Asc1/R	GCCCTTGCTCACCATTTGGCGCGCCAATCGTAGTGGTAGTCTTCT
1716 Asc1/mCherry/F	GGCGCGCCAAATGGTGAGCAAGGGCGAGGAGGA
1717 mcherry/ala/R	AAACGGCGTCGTGTTGGCAGCCGCAGCGGCAGCGGCAGCAGCCTTGTACAGCT
1718 (Ala/Qky/F)	GCTGCCAACACGACGCCGTTTCACTCGGATC
1719 Qky/Xba1/R	GCTCTAGATGAAGAAAAATCGAAGCGAAGCGATGA
1724 5'UP:Qky::/F	ATGAAGCAACAACATCTCTACAGGCG
1732 Asc1:Qky:/F	AAGGCGCGCCATGAACACGACGCCGTTTCA
1733(Xba1:3'UTR (Qky)/F)	GCTCTAGAGGAGAGAAAGATGTCAAGATAGTAAAT
1742(Qky/ala/R)	CTTGCTCACAGCCGCAGCGGCAGCGGCAGCGATGAGTCGATCGGA
1743 ala/mCherry/F	GCTGCCGCTGCCGCTGCGGCTGTGAGCAAGGGCGAG
1814(mcherry/xba1/R.3)	GCTCTAGATTACTTGTACAGCTCGTCCATGCCG
2011 Asc1/EGFP/F	AGGCGCGCCATGGTGAGCA
2012 EGFP/Spe1/R	AGCACTAGTCTTGTACAGCTCGTCCAT
2013(ala/Qky/F)	AAGACTAGTGCTGCTGCCGCTGCCGCTGCGG
2014(qky/Pst1/R)	AACCAACTGCAGTGAAGAAAAATCGAAGCGAAGC
2016(Spe1/Qky_3'UTR/F)	TGAACTAGTGAGAGAAAGATGTCAAGATAGTAAATG
2017(Asc1/Gus-plus/F)	AGGCGCGCCATGGTAGATCTGA
2018(Gus-plus/spe1/R)	CTCTCACTAGTTCACACGTGATGGTG
2148(Nde1/QKY (-TM)_F)	CGCTCATATGAACACGACGCCGTTTCACTCGGATCCTCCGCCG
2149(QKY (-TM)/Xma1_R)	CCCACCCGGGATGGACTAGCACCGTCGT
2169 C2A (pGEX)/F)	AGAATTCTCGGATCCTCCGCCGTCG
2170 C2A (pGEX)/R	TAGCGGCCGCGTAGATTTTGAGTCCAATCTCGCCGCGAAT

2171 C2B (pGEX)/F	AGAATTCCATCCTTACAATCTTGTTGAGCC
2172 C2B (pGEX)/R	TAGCGGCCGCACCAATCCAAACAGAGAGCTG
2173 C2C (pGEX)/F	AGAATTCTCTAAGGTGTATCAATCGCCG
2174 C2C (pGEX)/R	TAGCGGCCGCTCCACAATAAGGTCCACCACC
2175 C2D (pGEX)/F	AGAATTCGCTAAGCAGCTATGGAAACCG
2176 C2D (pGEX)/R	TAGCGGCCGCCCGGACTGCCACTTCAAT
2347 (SUB_Ex4_F)	ATTCCGCAGGCTTTGCCTTCTTCCATCC
2348 (SUB_Ex4_R)	GGATGGAAGAAGGCAAAGCCTGCGGAAT
2349 (SUB_Ex7_F)	ACCTTCTTCCATGGGAGACTTAGCTTCTC
2350 (SUB_Ex7_R)	GAGAAGCTAAGTCTCCCATGGAAGAAGGT
2386 EGFP/Spe1_R	CCACTAGTTTACTTGTACAGCTCGTCCATGCC
2630 SUBsens_2657_R	CAGGTTGGTGAATAATGTTTGAGC
2631 SUBas_2657_F	GGAATATCCCTGTTTCACCCTTCT
2632 SUBas_2657_R	TAATACGACTCACTATAGGGCAGGTTGGTGAATAATGT TTGAGC
2922(SUB_RT_F)	CAACGAGTCAGATGGCGGGT
2923(SUB_RT_R)	ATGGTCTGCGTCCAGTGAGC
2959 QKY_RT_Fnew	GCTCCTAATCTCCCGCCG
2960 QKY_RT_Rnew	GCCAATGAAACGAACCACTGT

Table S2 Primers used in this study

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