Inter-cell-layer signalling during *Arabidopsis* ovule development mediated by the receptor-like kinase STRUBBELIG

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Abstract

Plant organs, such as ovules and flowers, arise through cellular events that are precisely co-ordinated between cells within and across clonally distinct cell layers. Receptor-like kinases are cell-surface receptors that perceive and relay intercellular information. In *Arabidopsis* the leucine-rich repeat receptor-like kinase STRUBBELIG (SUB) is required for integument initiation and outgrowth during ovule development, floral organ shape and the control of the cell division plane in the first subepidermal cell layer of floral meristems, among other functions. A major goal is to understand SUB-mediated signal transduction at the molecular level. Present evidence suggests that SUB affects neighbouring cells in a non-cell-autonomous fashion. In addition, our results indicate that SUB is an atypical, or kinase-dead, kinase. Forward genetics identified three genes, *QUIRKY* (*QKY*), *ZERZAUST* and *DETORQUEO*, that are thought to contribute to *SUB*-dependent signal transduction. *QKY* encodes a predicted membrane-bound protein with four cytoplasmic C_2 domains. By analogy to animal proteins with related domain topology, we speculate that QKY may be involved in Ca^{2+}-dependent signalling and membrane trafficking. Studying SUB-dependent signalling will contribute to our understanding of how atypical kinases mediate signal transduction and how cells co-ordinate their behaviour to allow organs, such as ovules, to develop their three-dimensional architecture.

Introduction

Organogenesis requires the co-ordination of cellular behaviour. Individual cells and groups of cells divide and undergo changes in size and shape during morphogenesis and thus cells need to repeatedly assess and communicate their morphogenetic status to allow an organ to attain its characteristic size and shape. In plants, an additional level of complexity is encountered, as plant cells are encased in a cell wall that allows only limited relative movement of cells. Therefore plant cellular behaviour must be intrinsically intertwined with cell wall biogenesis and dynamics. It is a major current challenge in plant biology to elucidate the intercellular communication mechanisms underlying plant morphogenesis.

Arabidopsis ovules provide an excellent model system to study organogenesis in plants [1,2]. They are the progenitors of the seed and represent the major female reproductive organ in higher plants. Ovules originate from the placenta of the carpel and rapidly develop into finger-like protrusions. Soon after, three distinct elements can be recognized along the proximal–distal axis. Distally, the nucellus generates the megaspore mother cell and eventually the embryo sac with the egg cell proper. Centrally, the chalaza initiates an inner and outer integument. The integuments eventually encapsulate the nucellus and develop into the seed coat. Proximally, a stalk-like structure, the funiculus, connects the ovule to the placenta and enables nourishment of the ovule through its vascular strand [3,4].

With respect to their radial dimension, ovules, like other organs and the meristems, are composites of clonally distinct histogenic cell layers [5]. In principle, the L1 layer gives rise to the epidermis, whereas the directly subjacent L2 layer and the inner L3 layer contribute to the internal tissues of plant organs. This is also the case for *Arabidopsis* ovules [6].

Communication between the histogenic layers is extensive [7], and the corresponding mechanisms are under investigation [8,9]. Cell-surface-localized RLKs (receptor-like kinases) are natural candidates to mediate intercellular communication. *Arabidopsis* carries over 600 such RLKs [10], and one of them, BRI1 (brassinosteroid-insensitive 1), has recently been implicated in organ growth control mediated by the epidermis [11]. Several RLKs are known to be important for ovule development. *ACR4*, a homologue of maize *CRINKLY4* [12], is specifically expressed in the epidermis and may thus receive signals from the cells beneath. Defects in *ACR4* result in aberrant integument initiation and in abnormal epidermal differentiation in ovules and other parts of the plant [13,14]. Recently, a novel RLK,

Key words: *Arabidopsis*, atypical kinase, organogenesis, receptor-like kinase, signal transduction, STRUBBELIG.

Abbreviations used: ACR4, *Arabidopsis* homologue of maize *CRINKLY4*; AtCRR, *Arabidopsis thaliana* CRINKLY4-RELATED; BRI1, brassinosteroid-insensitive 1; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; MARK, maize atypical receptor kinase; MCTP, multiple C_2 domain and transmembrane region protein; MIK, MARK-interacting kinase; *ML1*, *MERISTEM LAYER 1*; *QKY*, *QUIRKY*; RLK, receptor-like kinase; *SLM*, STRUBBELIG-like mutant; *SUB*, *STRUBBELIG*; *WUS*, *WUSCHEL*.

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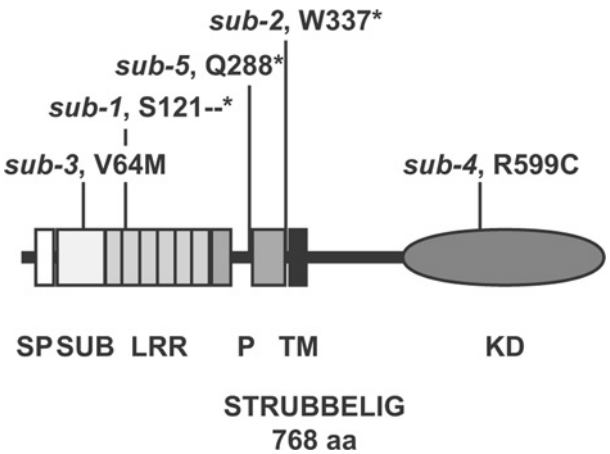
Figure 1 | Schematic representation of SUB domain architecture

The N-terminus is to the left. The EMS (ethyl methanesulfonate)-induced point mutations and associated amino acid exchanges are indicated. Abbreviations: aa, amino acids; KD, kinase domain; LRR, leucine-rich repeats; P, proline-rich region; SP, signal peptide; SUB, SUB domain; TM, transmembrane domain.



ALE2, was identified that may participate in the ACR4 pathway [15]. The role of the ERECTA (ER) family of RLKs in ovule development differs from ACR4. This family, and particularly ERL2, is required for the progression of integument growth by the regulation of cell proliferation [16].

In the present article, we focus on signalling in ovule development that is mediated by the RLK STRUBBELIG (SUB) [17]. SUB is required for integument development, is likely to function as an atypical kinase, and influences neighbouring cells in a non-cell-autonomous fashion. Recent evidence suggests that *SUB* exerts its function in part through the regulation of membrane trafficking.

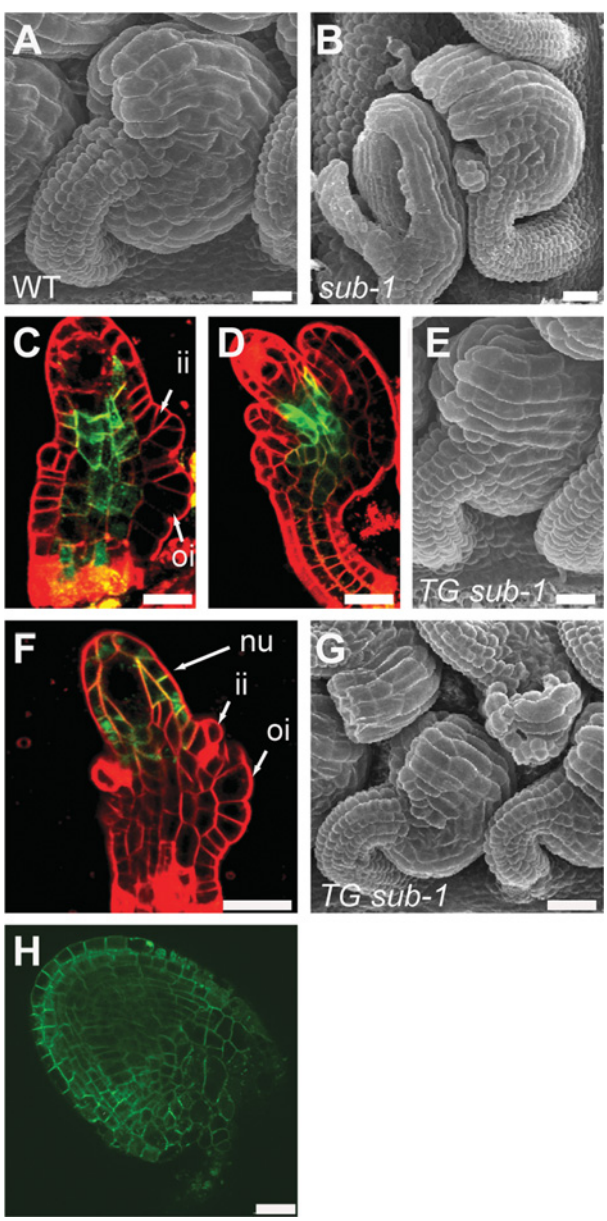
The RLK SUB regulates integument initiation and outgrowth

SUB encodes a predicted RLK with six leucine-rich repeats in its extracellular domain [17] (Figure 1). Ovules of *sub*-null mutants show a variable phenotype [18]; however, outer integuments often exhibit initiation defects, resulting in prominent gaps of different sizes and thus resembling 'multifingered clamps' or 'scoops' (Figures 2A and 2B). Apart from defects in integument development, *sub* plants are also characterized by aberrations in floral organ shape, such as twisted carpels or twisted and notched petals, and defects in other parts of the plant [19,20].

At the cellular level, *SUB* appears to affect cell division patterns. Integument initiation in *Arabidopsis* occurs in the epidermis and is accompanied by a change in the orientation of the cell division plane in integument progenitor cells [4]. The outer integument initiation defects in *sub* mutants point to a role for *SUB* in the regulation of this process. Furthermore, the L2 cells of stage 3 floral meristems of *sub* plants show irregular shapes and frequently

Figure 2 | Non-cell autonomy of *SUB*

(**A**, **B**) Scanning electron micrographs of mature ovules of wild-type and *sub-1* respectively. (**C**) A stage 2-III ovule of a *SUB::SUB:EGFP sub-1* plant (staging according to [4]). Reporter expression is only detectable in interior tissue, but not in the young developing integuments. Green signal relates to GFP and red signal to the background stain FM4-64. (**D**) A stage 3-I ovule of a *SUB::SUB:EGFP sub-1* plant. (**E**) Scanning electron micrographs of a mature ovule of a *SUB::SUB:EGFP sub-1* (*TG sub-1*) plant. Note the wild-type appearance. (**F**) A stage 2-III ovule of a *WUS::SUB:GFP sub-1* plant. Reporter signal is only detected in the nucellus. (**G**) Scanning electron micrographs of mature ovules of a *WUS::SUB:GFP sub-1* (*TG sub-1*) plant. Growth of the outer integument is largely restored to wild-type. (**H**) Mature ovule of a plant carrying a *SUB::SUB:EGFP* reporter that includes introns. Note the broad signal (compare with **D**). Abbreviations: ii, inner integument; nu, nucellus; oi, outer integument. Scale bars, 20 μm (**A**, **B**, **E**, **G** and **H**); 10 μm (**C**, **D** and **F**).



undergo periclinal, rather than the typical anticlinal, cell divisions [17,19]. These data indicate that *SUB* is required for correct orientation of the cell division plane, at least during integument development and in floral meristems. *SUB* also affects cell division, as reduced cell numbers in outer integuments and stems of *sub* mutants are observed [17].

SUB acts in a non-cell-autonomous fashion

Recent results indicate that *SUB* contributes to the control of ovule and floral morphogenesis by regulating intercellular communication across cell layers [21]. To study the cellular and subcellular distribution of the SUB protein, reporter assays were performed using a cDNA-based translational fusion between SUB and an enhanced version of GFP (green fluorescent protein) (SUB:EGFP), driven by an endogenous *SUB* promoter fragment that reproduces the *SUB* expression pattern as monitored by *in situ* hybridization. As expected, *sub* plants carrying the *SUB::SUB:EGFP* reporter exhibited wild-type ovule, flower and stem development, indicating that the reporter construct is functional. Interestingly, however, the SUB:EGFP fusion protein was not detected in cells that exhibit a mutant phenotype in non-transgenic *sub* plants. In particular, reporter activity was observed in the inner L2-derived tissue of the ovule, but not in the neighbouring L1-derived integuments (Figures 2C–2E). In floral meristems, the reporter was detected in the L3 layer, but not in the L2 or L1 layers. These results indicate that *SUB* may affect development of neighbouring cells in a non-cell-autonomous fashion. Further evidence was obtained by clonal analysis. Expression of two SUB:GFP fusion proteins was driven by tissue-specific promoters and the ability of these two constructs to rescue the above-ground *sub* phenotype was scored. In ovules, the *WUSCHEL* (*WUS*) promoter governs expression specifically in the nucellus, a tissue distal to the integuments [22]. *MERISTEM LAYER 1* (*ML1*) promoter activity is exclusively detected in the epidermis throughout much of plant development [23,24]. *WUS::SUB:GFP* could rescue the *sub* ovule phenotype to a large extent and the *ML1::SUB:GFP* transgene could amend scored aspects of the *sub* phenotype, although some cell division problems in the stem remained [21] (Figures 2F–2G). Taken together, the evidence strongly indicates that *SUB* acts in a non-cell-autonomous fashion. Furthermore, SUB function does not depend on a distinct polarity, as L1-specific expression of SUB:GFP rescued the L2 defects in floral meristems and the ovule phenotype. Why then the apparent L2- or L3-specific expression in ovules and floral meristems? Insights may be provided by the analysis of an alternative *SUB::SUB:GFP* reporter construct [25]. This construct incorporated *SUB* introns and exhibited a broad radial expression, including the epidermis, in the root tip. This also holds true for ovules (Figure 2H). Since normal spatial expression of *SUB* does not depend on intronic sequences, this result indicates that one or several *SUB* introns influence expression levels of SUB protein in a post-transcriptional manner. Effects of introns on the level of protein expression have been described previously in animals and plants [26–28]. One 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 (messenger ribonucleoprotein) may influence translation [26]. The observed differences in SUB:GFP expression of the two reporters highlights the need to corroborate SUB distribution in tissues by complementary means. In any case, the clonal analysis outlined above [21], and similar experiments performed in roots [25], strongly indicate that *SUB* acts in a non-cell-autonomous fashion. *BRI1* constitutes another example of a gene that has a broad expression pattern and acts in a non-cell-autonomous fashion [11].

SUB, an atypical RLK?

Interestingly, there is evidence that the SUB kinase domain is essential for SUB function; enzymatic phosphotransfer activity, however, is not [17]. SUB may thus belong to the class of atypical or kinase-dead kinases [29–31]. Animal examples include members of the RYK (receptor tyrosine kinase) family of receptor kinases [32,33]. In plants, AtCRR (*Arabidopsis thaliana* CRINKLY4-RELATED) 1 and 2 [34] or MARK (maize atypical receptor kinase) [35] fall into this class. Sequence comparisons revealed that SUB features two atypical residues at conserved positions within the catalytic loop of the kinase domain, suggesting that SUB may have diminished kinase activity. Indeed, *in vitro* kinase assays did not result in detectable kinase activity. Furthermore, several *sub* lines that carried different *SUB* cDNA variants with point mutations, predicted to result in critical amino acid exchanges, showed a wild-type phenotype. Nevertheless, *sub-4*, a missense mutation in the kinase domain, results in a *sub* phenotype [17] as does a deletion of the kinase domain (R.K. Yadav and K. Schneitz, unpublished work) findings that highlight the importance of the kinase domain for SUB function. Taken together, the biochemical and genetic results strongly suggest that SUB kinase activity is not required *in vivo*. At the same time, the kinase domain appears to be essential for SUB function. It was therefore speculated that this domain acts as a scaffold where downstream effectors could still bind and mediate SUB signalling.

Despite the evidence discussed above, the small possibility remains that SUB is a functional kinase. For example, the observed alterations in the catalytic loop do not always result in loss of kinase activity [36,37]. In addition, there are reports where kinase activity of functional kinases was found to be functionally irrelevant. For example, the ACR4 sequence suggests it to be a functional kinase and corresponding kinase activity could be demonstrated *in vitro* [13,34]. However, genetic experiments similar to the ones described above for *SUB* indicated that intrinsic kinase activity is not required *in vivo* [38]. To explain this intriguing result, it was speculated that ACR4 is part of a multiprotein receptor complex with other components of this complex being able to substitute for an absence of ACR4 kinase activity. Alternatively, at least part of ACR4 signalling may be independent of ACR4 kinase

activity [38]. Similar scenarios may relate to the RLK FEI1, although genetic evidence indicates that, although FEI kinase activity is not essential, it is required for optimal function [39].

Signalling through atypical kinases in plants

Little is known about signalling by atypical kinases, particularly in plants. Generally, the corresponding mechanisms are believed to rely on regulated protein–protein interactions [29–31]. 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 signalling [34]. SUB may also be part of a multiprotein complex and, in this context, become phosphorylated by an active kinase as there is genetic evidence that phosphorylation of SUB is essential for its function (M. Batoux, L. Fulton, P. Vaddepalli and K. Schneitz, unpublished work). 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 [35], but apparently the MARK–IK interaction did not result in the phosphorylation of MARK. Interestingly, however, it brought about a severalfold stimulation of MIK kinase activity.

Novel components in SUB signalling

Several aspects of SUB function, such as its non-cell autonomy and its apparent lack of kinase activity, raise intriguing questions as to its signalling mechanism. To address this issue, a genetic screen for *sub*-like mutants (*SLM*) was performed [19]. The screen yielded additional *sub* alleles and three novel complementation groups, *ZERZAUST* (*ZET*), *QUIRKY* (*QKY*) and *DETORQUEO* (*DOQ*), bringing the present *SLM* gene count to four. 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 [19]. Taken together, the results indicated that *SLM* genes contribute to *SUB*-dependent processes, but do 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.

To advance our understanding of the *SLM*-dependent mechanism, it is necessary to clone and characterize the *SLM* genes. To this end, *QKY* was identified, and sequence analysis suggested QKY to be anchored to a membrane by its C-terminus and to carry four cytoplasmically localized C_2 domains [19]. C_2 domains were originally identified in PKC (protein kinase C), frequently act as Ca^{2+}-binding modules, usually form Ca^{2+}-dependent phospholipid complexes, and are required for protein–protein interactions [40]. Although there is little sequence conservation, the predicted QKY protein has a domain architecture related to human and animal MCTPs (multiple C_2 domain and transmembrane region proteins), which carry three C_2 domains [41]. Not much is known about the function of animal MCTPs. In contrast, other proteins with only one transmembrane domain and multiple C_2 modules include the well characterized synaptotagmins [42], ferlins [43] and the extended synaptotagmins [44]. Some synaptotagmins, such as Syts 1 and Syts 2, are membrane-trafficking proteins involved in synaptic vesicle exocytosis, whereas Syts VII and members of the ferlins promote membrane trafficking during plasma membrane repair [45–47]. Synaptotagmins also exist in plants [48], and a role in exocytosis and membrane repair seems to be conserved across kingdoms [49,50].

In the light of the well-described function of synaptotagmins and ferlins in the control of exocytosis, it was proposed that *QKY* might also affect vesicle trafficking. This model could also conveniently explain the non-cell autonomy of *SUB*. In support of this notion, QKY has been found to locate at the plasma membrane (P. Vaddepalli and K. Schneitz, unpublished work). We therefore currently speculate that SUB and QKY may be closely connected and that SUB could somehow influence QKY activity. This influence might subsequently affect exocytosis of factors mediating non-cell autonomy of *SUB* signalling. Such factors could directly influence neighbouring cells, or could impinge on the cell wall, thereby affecting close-by cells in an indirect fashion.

Perspective

Work on the *SUB* pathway has just begun and there remain many exciting challenges. Future work will reveal how *SUB*-expressing cells influence their neighbours in a *SUB*-dependent fashion and whether the model sketched above proves true. Further studies on this pathway will also contribute to our understanding of how atypical RLKs transduce signals in plants. Although the *SUB* pathway represents but one jigsaw piece in the big puzzle, its elucidation will contribute to the dissection of the intricate intercellular communication network that orchestrates cellular behaviour during plant organogenesis.

Funding

This work was funded by the German Research Council (DFG) [grant numbers SCHN 723/1-3 and SCHN 723/6-1 (to K.S.)] and by the Free State of Bavaria.

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Received 17 August 2009
doi:10.1042/BST0380583