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To preserve or to destroy, that is the question: the role of the cell wall integrity pathway in pollen tube growth

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Abstract

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Keywords	RAPID ALKALINIZATION FACTORS; CrRLK1L receptor kinases; LEUCIN-RICH REPEAT EXTENSIN proteins; pollen tube growth; cell wall integrity pathway; plant reproduction; signal transduction; mechanosensing
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June 21st, 2019

Dear Alison,

in the meantime, Hannes sent you and Eva a copy of our manuscript. I arrived in China this morning and will now upload all the files on the online system.

Best wishes,
Ueli

June 19th, 2019

Dear Alison, dear Eva,

our Current Opinion article has now finally been completed. I am really sorry for this delay but I think it came out quite nicely. Hannes is just adding some citations and finalizing the reference list and will then send you the manuscript as I will leave for China tomorrow. I asked him to send it to you also by email, in order to prevent further delays.

Best wishes and thank you for your patience,
Ueli

Highlights

- *CrRLK1L* receptor-like kinases control many processes including pollen tube growth
- *CrRLK1L*s transmit information from the cell wall to the cell's interior
- RALF4/19 control pollen tube growth as autocrine ligands activating *CrRLK1L*s
- RALF4/19 also interact with LRX cell wall proteins in regulating pollen tube growth

To preserve or to destroy, that is the question: the role of the cell wall integrity pathway in pollen tube growth

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Short title: RALF-CrRLK1L signaling module controls pollen tube growth

Abstract

In plants, cell-shape is defined by the cell wall, a complex network of polymers located outside the plasma membrane. During cell growth, cell wall properties have to be adjusted, assuring cell expansion without compromising cell integrity. Plasma membrane-located receptors sense cell wall properties, transducing extracellular signals into intracellular cascades through the cell wall integrity (CWI) pathway that, in turn, leads to adjustments in the regulation and composition of the cell wall. Using pollen tube growth as single cell model system, we describe the importance of RAPID ALKALINIZATION FACTOR (RALF) peptides as sensors of cell wall integrity. RALF peptides can mediate the communication between cell wall components and plasma membrane-localized receptor-like kinases (RLKs) of the CrRLK1L family. The subsequent activation of intracellular pathways regulates H^+ , Ca^{2+} , and ROS levels in the cell and apoplast, thereby modulating cell wall integrity. Interestingly, the RALF-CrRLK1L module and some of the components working up- and downstream of the RLK, is conserved in many other developmental and physiological signaling processes.

Introduction

A pollen tube's life is stressful. On the way to deliver its cargo, the two sperm cells, to the ovule, it has to find the right direction while dealing with many obstacles. Therefore, the perception and processing of extracellular signals, chemical and mechanical, is of great importance for pollen tubes to successfully perform their tasks. In about half the flowering plant taxa, the challenge starts right after the pollen grain lands on the stigma. Molecularly diverse self-incompatibility (SI) systems preclude 'self' pollen from fertilization by preventing germination or by inhibiting pollen tube growth in the pistil [1, 2]. Once a pollen grain has been accepted as suitable, the pollen tube germinates and starts growing through the pistil into the stylar tissue towards the ovary. In the ovary, the pollen tube grows within a specialized tissue, the transmitting tract, from which it eventually exits and finds its way to the micropylar opening of a receptive ovule. A crosstalk between the pollen tube and the synergid cells ensures that, under normal conditions, only one pollen tube enters the ovule [3]. As the pollen tube enters the micropyle it encounters the synergid cells of the female gametophyte, and an extended cross-talk involving receptor-like kinase-mediated signaling and calcium (Ca^{2+}) oscillations ensues during pollen tube reception, eventually leading to successful double fertilization [4–6].

Several peptide signaling factors have been identified that are crucial for guiding the pollen tube through the different tissues and to actively modulate its growth. The most prominent pollen tube guidance molecules are the LURE proteins [7, 8]. Rapid ALkalinization Factor (RALF) is the new kid on the block. The RALFs comprise a large family of peptides that lead to the alkalinization of the growth medium and modulate cell expansion through the cell wall integrity (CWI) pathway [9]. The pollen tube cell wall with its astonishing properties that allow extremely rapid tip growth plays a central role for successful fertilization. Not only does it act as a barrier between the cell and the extracellular environment to control the exchange of molecules, its mechanical properties also determine the growth behavior of the pollen tube via the CWI pathway. Here, we discuss selected, recent findings that shed light onto the mechanisms underlying the CWI pathway in pollen tubes.

The cell wall acts as an interface between extra- and intracellular signaling

Plant cell walls are mainly composed of the polysaccharides cellulose, hemicelluloses, and pectins, resulting in a high tensile strength but also providing enough flexibility for cell expansion. The cell wall needs to be flexible enough to permit rapid growth but also stay intact and preserve its integrity while defying mechanical adversities. During the rapid growth of pollen tubes, which elongate exclusively at their tip, the cell wall needs to remain intact while navigating through the different female tissues. However, at the end of its journey upon successful pollen tube reception, it must burst to release the two sperm cells. Without rupture, double fertilization will not take place. Therefore, a growing pollen tube lives on the edge with the risk of premature bursting. Turgor pressure drives cell expansion at the tip by putting the cell wall under stress. As a consequence, there is a wall thinning phase, which needs to be compensated by the delivery of new cell wall material via the exocytotic pathway. If these processes are out of phase, the pollen tube would either lyse due to over-thinning or experience growth arrest due to over-thickening of the cell wall. In the yeast *Saccharomyces pombe*, Davì and colleagues used an original approach to measure the dynamics of growth-induced cell wall thinning and could show that thickening negatively influenced growth [10]. To avoid this, a fine-tuned balance between cell wall synthesis and turgor-driven expansion is crucial. Such a mechanism must constantly sense the status of the cell wall and trigger intracellular physiological and biochemical responses that signal back to the cell wall. A CWI pathway has been postulated a while ago [11, 12] and the cytoplasmic components have been identified, but the ligand and the link between cell wall and plasma membrane stayed elusive until very recently.

In addition to the polysaccharide network, the cell wall contains cell wall proteins that were thought to have a largely structural function, like rebars used to give concrete a higher tensile strength. However, it recently became clear that they are much more than that and play an important role in the perception and transduction of extracellular signals. Among these proteins, the LEUCINE-RICH REPEAT EXTENSINS (LRXs) recently attracted much attention, because they were identified as a central element of the CWI pathway [13]. Pollen tube-expressed LRX8, LRX10, and LRX11 have been shown to directly interact with RALF4 *in planta* (and RALF9 in a yeast two-hybrid system) [14]. Both LRXs and RALFs are secreted by the pollen tube, indicating autocrine signaling to explore the extracellular environment. Genetic and biochemical evidence implies a connection of RALF4/19 with

ANXUR1 (ANX1), ANX2, BUDDHA'S PAPER SEAL1 (BUPS1), and BUPS2, which are all members of the *Catharanthus roseus* RECEPTOR-LIKE KINASE1-LIKE (*CrRLK1L*) family, thereby providing a link between extra- and intracellular signaling [14, 15] (Figure 1).

Transducing signals across the membrane

During pollen tube growth, LRX proteins are required for the sensing of RALF peptides to control pollen tube integrity [14]. Mutations in pollen-expressed LRX proteins lead to premature bursting of the pollen tubes [13, 16, 17], similar to what is observed when down-regulating the pollen-specific RALF4/19 peptides [14, 15]. Recently, it was shown that LRX-RALF interactions are also relevant for abiotic stress and plant growth [18, 19], pointing out the importance of RALF peptides as signaling molecules that sense changes in the cell wall. RALFs are small peptides that alkalize the culture medium of cells in suspension culture and repress growth if added to the growth medium [20]. Being present throughout the plant kingdom [20], they coordinate cell-wall related processes, like polarized cell growth, leaf and root growth, and innate immunity [14, 15, 18, 19, 21, 22]. RALFs are secreted as proRALF polypeptides and processed by SITE-1 PROTEASE (S1P), releasing mature 5 kDa RALF peptides into the apoplast [23]. Once in the apoplast, RALF peptides can act as an autocrine signal, like the pollen tube-expressed RALF4/19 peptides [14, 15], or as a paracrine signal, like ovule-expressed RALF34, which regulates pollen tube integrity [15]. It was reported that RALF1 binds the extracellular domain of FERONIA (FER) and activates a phosphorylation cascade that represses H⁺-ATPase activity, thereby increasing the extracellular (apoplastic) pH and reducing cell elongation in the root [21, 24]. FER belongs to the *CrRLK1L* subfamily of receptor-like kinases (RLKs), key regulators involved in sensing cell wall changes occurring during development or induced by environmental stimuli [21, 25–30]. *CrRLK1L*s are transmembrane receptors with an extracellular domain, consisting of one or two lectin-like domains [31], a transmembrane domain, and a cytoplasmic Ser/Thr kinase domain [26, 32] (Figure 1). FER can also directly interact with LRX4, controlling cell expansion, suggesting the formation of an LRX4/RALF1/FER complex, linking the cell wall with components of signal transduction across the membrane [18] (Table 1). Similar to FER, THESEUS1 (THE1) interacts with RALF34 to control pericycle divisions during lateral root initiation [26, 33], and during pollen tube growth, several RALF and *CrRLK1L* members are implied in controlling cell wall stiffness and integrity. Pollen-expressed RALF4/19 peptides interact with the pollen-specific *CrRLK1L* receptors ANX1, ANX2, BUPS1, and BUPS2

[15, 25, 27], assuring proper pollen tube elongation at the tip. However, when a pollen tube enters the micropyle to reach the female gametophyte, ovule-expressed RALF34 was reported to compete with RALF4/19 for binding to the ANX1/2 and BUPS1/2 RLKs, inducing pollen tube rupture and the release of the two sperm cells for fertilization [15]. Although FER is expressed in all tissues analyzed except for pollen, it also regulates pollen tube integrity during fertilization [32]. When a pollen tube approaches the synergid cells of the female gametophyte, the accumulation of reactive oxygen species (ROS) and Ca^{2+} oscillations are induced in a FER-dependent manner, which is important for the dialogue between the receptive synergid and the pollen tube, eventually leading to pollen tube rupture [5, 32, 34]. LORELEI (LRE), a small glycosylphosphatidylinositol (GPI)-anchored protein, is also involved in pollen tube reception and physically interacts with FER, possibly acting as chaperon during transport through the endomembrane system, but also, importantly, as a co-receptor of FER [35–37]. Similarly, during vegetative development LORELEI-LIKE GPI-ANCHORED PROTEIN 1 (LLG1), the closest relative of LRE, collaborates with the FER RLK [36, 38] (Figure 1). However, it is currently unclear whether LRE/LLG co-receptors interact with all *CrRLK1L* members or are required for all their diverse functions. Finally, a functionally redundant set of five ovule-expressed EARLY NODULIN-LIKE PROTEINs (ENODLs), encompassing a GPI anchor motif, a plastocyanin-like domain, and an arabinogalactan glycomodule, are required for pollen tube reception. In a yeast two-hybrid system and GST pull-down assays, ENODL14 interacts with the juxta-membrane region of FER [39] but it is unknown whether ENODLs also play a role in other *CrRLK1L*-mediated processes (Figure 1).

Figure 1. Schematic representation of cell wall sensing by RAPID ALKALINIZATION FACTORS (RALFs) and downstream signaling components. Cell-wall LRX proteins are recognized by RALF peptides, sensing alterations in the cell wall. To activate intracellular signaling pathways, they form a complex with *CrRLK1L* receptors and LRE/LLG co-receptors, inducing autophosphorylation of the *CrRLK1L* intracellular domain. Through signaling via ROPGEFs and RAC/ROPs, H^+ -ATPases (AHA), Ca^{2+} channels, and NADPH oxidases (RBOHs) are activated, which can modify cell wall properties by controlling H^+ , Ca^{2+} and ROS levels, respectively. Moreover, *CrRLK1L* can activate intracellular phosphatases of the ATUNIS (AUN) family and cytoplasmic receptor-like kinases like MARIS (MRI).

Intracellular signaling events involve second messengers and protein (de)phosphorylation

Upon reception of the signals in the CWI pathway, intracellular events are triggered involving downstream effectors that are slowly being uncovered. In eukaryotes, small GTPases of the Ras and Rho subfamilies form molecular switches in many signal transduction cascades. In plants, there are no small G-proteins of the Ras family but the Rho family is represented by Rho of Plants (ROP) proteins also referred to RACs [40, 41]. In tip-growing root hairs, RAC/ROP proteins interact with ROP-GUANINE NUCLEOTIDE EXCHANGE FACTORS (GEFs), which switch between an inactive GDP-bound and an active GTP-bound state, to regulate the tip growth [42]. ROP-GEF1, in turn, was shown to

bind to FER in yeast two-hybrid and bimolecular fluorescence complementation assays [43] (Figure 1). In roots, this eventually results in activating plasma membrane H^+ -ATPases of the AHA family [44] (Table 1), which are essential to control the membrane potential and to power ion transport across the plasma membrane in order to maintain polar growth of tip growing cells [45, 46].

Signal transduction processes usually rely on second messengers, small chemical like nitric oxide, ROS, and Ca^{2+} , whose levels and spatiotemporal patterns are specifically altered by external signals. These so-called signatures are detected and interpreted by the cell to elicit specific downstream responses. Both Ca^{2+} and ROS have been implicated in CrRLK1L-mediated signaling; while the Ca^{2+} -channels involved have not yet been identified, ROS-producing NADPH oxidases known as RESPIRATORY BURST OXIDASE HOMOLOGUES (Rbohs) [47], are direct targets of RAC/ROP G-proteins [36, 48, 49]. The genome of the model plant *Arabidopsis thaliana* contains 10 Rboh genes with versatile functions, with RbohH and RbohJ having been implicated in the ANX1/2 pathway regulating pollen tube growth [50–52] (Figure 1). Double mutant *rbohH rbohJ* pollen tubes show reduced ROS levels and an unsteady tip-focused Ca^{2+} gradient [50, 52]. While this indicates that Ca^{2+} channels may be regulated by ROS levels, the cross-regulatory interactions between ROS, Ca^{2+} , and H^+ in controlling pollen tube growth are complex and need to be dissected further [50–54].

Plant RLKs often function as homo- or hetero-dimers and can interact with receptor-like cytoplasmic kinases (RLCKs), which lack extracellular domains, to induce distinct responses in various processes during pollen tube growth. [55]. On the one hand, the RLCKs LOST IN POLLEN TUBE GUIDANCE1 (LIP1) and LIP2 are involved in the pollen tube's response to AtLURE1, a cysteine-rich peptide involved in pollen tube guidance [56, 57]. LIP1/2 interact with the tip-localized POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 6 (PRK6), one of the AtLURE1 receptors [58, 59], and thus potentially underlies receptor complex activation during pollen tube guidance to the micropyle. On the other hand, the RLCK MARIS (MRI) is directly involved in the control of CWI (Figure 1). A constructively active form of MRI (MRI^{R240C}, *mri-3D*), which was identified in a suppressor screen of the *anx1/2* double mutant, acts as a positive regulator of RALF4/19-ANX1/2-dependent pollen tube growth and can suppress male sterility caused by RALF4/19 down-regulation [14] as well as the bursting phenotype observed in *rhbohj/h* double mutant pollen tubes [60]. In contrast, *mri-3D* fails to suppress the reduced fertility of *lrx8/9/10/11* quadruple mutants [61], suggesting that the

intracellular response mediated by the ANX1/2 RLKs splits into distinct pathways in the regulation of pollen tube growth. Interestingly, expression of the mutant MRI^{R240C} protein can also suppress the bursting root hair phenotype observed in *fer* mutants, demonstrating that MRI acts downstream of diverse members of the *CrRLK1L* family in the CWI pathway [60].

In the same *anx1/2* suppressor screen, the *atunis1 (aun1)* mutant was recovered, rendering a member of the TYPE ONE PROTEIN PHOSPHATASE (TOPP) family inactive. Together with its closest homolog, AUN1/2 function redundantly as negative regulators of the ANX1/2-dependent CWI pathway [61]. Genetic experiments showed that the mutant form of AUN1, AUN1^{D94N}, can suppress diverse mutants of the pollen tube CWI pathway, placing AUN1/2 downstream of LRX 8/9/10/11, RALF4/19, ANX1/2, and RbohH/J, but not of MRI [61] (Figure 1). Thus, it was proposed that the AUN1/2 phosphatases may counterbalance the kinase activity of MRI and/or regulate the phosphorylation status of common or different target proteins to regulate pollen tube growth [61]. However, how exactly AUN1/2 are activated and which target proteins they dephosphorylate requires further investigations. Moreover, AUN1/2 are localized to both the cytoplasm and the vegetative nucleus of growing pollen tubes, where they may have distinct functions. Indeed, TOPP4, another family member involved in gibberellic acid and auxin signaling, directly interacts with proteins at the plasma membrane and in the nucleus [62, 63]. Although many of the players involved in *CrRLK1L* signaling were initially identified through their role in plant reproduction, they or their homologs were found to play similar roles in diverse processes, including root growth, root hair development, cell expansion, innate immunity and a variety of biotic and abiotic stress responses (Table 1). It is thus likely just a matter of time until other members of the ENODL and TOPP family will also be implicated in other *CrRLK1L*-mediated signaling transduction processes.

Table 1. Conservation of the RALF-*CrRLK1L* signaling module in different contexts of plant development and physiology.

Gene family	Reproduction	Root development	Cell expansion	Bio
LRX	LRX8-11 [13, 14, 16, 17]	LRX1/2 [64, 65]	LRX2-4 [18, 66]	LRX3/4/5 [19]
RALF	RALF4/19 [14, 15] RALF34 [15]	RALF1 [21, 67–69] RALF34 [33]	RALF1 [18] RALF17/23/32/33 [22]	RALF22/23 [19, 22, 23] F-RALF [70]
CrRLK1L	ANX1/2 [15, 25, 27]	FER [21, 43]	FER [18, 36, 74, 75]	FER [19, 21, 22, 24, 70,

	BUPS1/2 [15] FER [32, 71, 72]	ERU [21, 73] THE [33]	THE [26, 76] HERK1 [74] CVY [77]	MEDOS1/2/3/4 [28] ANX1/2 [81]
LRE	LRE [35, 38]	LLG1 [36]	LLG1 [36]	LLG1 [82]
ROP	ROP1 [83]	ROP2/4 [43, 48]	ROP2/4/6 [84, 85]	
RBOH	RBOHH/J [50, 52]	RBOHC [43, 86, 87]	RBOHD/F [88]	RBOHD/F [89–91]
MARIS	MRI [60, 92]	MRI [60, 92]		
ENODL	EN14 [39]			
ATUNIS	AUN1/2 [61]	AUN1/2 [61]		

Signaling back to the cell wall

Although our knowledge about the regulation of the CWI pathway has increased a lot over the past few years, many interesting questions are still open. For example, it is still unclear how pollen tube rupture is induced once it reaches the embryo sac. The FER-mediated Ca^{2+} -dialogue between the pollen tube and the receptive synergid cell is important for the timely rupture of the the pollen tube as in mutants affecting the *FER* pathway in synergids, most pollen tubes do not burst [5]. In maize, the synergid-expressed cysteine-rich protein ZmES4 induces the opening of the intrinsic rectifying K^{+} -channel KZM1 and it was proposed that K^{+} influx leads to water uptake and osmotic burst [93]. However, it is currently unknown how the opening of KZM1 or similar channels is precisely timed during the rapid growth phase of the pollen tube across the receptive synergid cell [5] and whether the CWI pathway of the pollen tube is also affected by other mechanisms leading to pollen tube rupture and the release of the sperm cells.

As mentioned above, the addition of RALF34 to growing pollen tubes in culture leads to instantaneous rupture. This indicates that the CWI pathway of the pollen tube is hijacked by ovule-produced RALF34 which competes for the RALF4/19 binding sites on pollen-expressed LRXs and/or *CrRLK1*Ls, thus inducing rupture at a precise time and place [15]. Several questions are still open. For instance, how does RALF34, which accumulates in the inner integuments of the ovules, end up on the pollen tube surface and how does the replacement of RALF4/19 with RALF34 disrupt the signaling cascade?

In fact, RALF4/19 being autocrine signals, they may have functions both in signaling the state of the cell wall back to the pollen tube via the ANX1/2 *CrRLK1*Ls in conjunction with pollen-expressed LRXs, but also signal extracellularly via the LRXs, leading to changes in the pollen tube cell wall (Figure 1). The fact that *lrx8/9/10/11* mutant can be suppressed by expression of $\text{MRI}^{\text{R240C}}$ but not $\text{AUN1}^{\text{D94N}}$ [14, 61], further indicates a complex CWI pathway that also branches intracellularly.

Finally, it will be interesting to connect mechanosensing with the CWI sensing. Several cues point towards a direct connection of mechanosensing and growth control. MSL8 is a mechanosensitive anion channel localized in the plasma membrane, where it has a proposed function as an osmotic safety valve that regulates turgor pressure. Although it is not required for normal pollen development, it is necessary to respond to osmotic stress [94, 95]. In *Arabidopsis* and lily pollen tubes, the concept of a perceptive force has been established. When growing into an obstacle, the pollen tubes push until they reach a force threshold at which growth stops. After a lag phase, during which the pollen tubes reorient themselves, they resume growth in another direction [96]. The mechanosensor underlying this behavior is currently unknown. Davì and colleagues found that, in fission yeast, the strain in the cell wall is perceived as a mechanical signal. Rapid cell elongation leads to an increase in the strain rate, which activates cell wall synthesis via the CWI pathway and vice versa. Interestingly, they found the transmembrane protein Wsc1, which via its physical interaction with a Rho-GEF activates a Rho G-protein, to be implicated in cell wall thickness homeostasis, suggesting that it could act as a direct cell wall mechanical sensor [10].

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Gene family	Reproduction	Root development	Cell expansion	Biotic/abiotic stress
LRX	LRX8-11 [13, 14, 16, 17]	LRX1/2 [64, 65]	LRX2-4 [18, 66]	LRX3/4/5 [19]
RALF	RALF4/19 [14, 15] RALF34 [15]	RALF1 [21, 67–69] RALF34 [33]	RALF1 [18] RALF17/23/32/33 [22]	RALF22/23 [19, 22, 23] F-RALF [70]
CrRLK1L	ANX1/2 [15, 25, 27] BUPS1/2 [15] FER [32, 71, 72]	FER [21, 43] ERU [21, 73] THE [33]	FER [18, 36, 74, 75] THE [26, 76] HERK1 [74] CVY [77]	FER [19, 21, 22, 24, 70, 75, 78–80] MEDOS1/2/3/4 [28] ANX1/2 [81]
LRE	LRE [35, 38]	LLG1 [36]	LLG1 [36]	LLG1 [82]
ROP	ROP1 [83]	ROP2/4 [43, 48]	ROP2/4/6 [84, 85]	
RBOH	RBOHH/J [50, 52]	RBOHC [43, 86, 87]	RBOHD/F [88]	RBOHD/F [89–91]
MARIS	MRI [60, 92]	MRI [60, 92]		
ENODL	EN14 [39]			
ATUNIS	AUN1/2 [61]	AUN1/2 [61]		

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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