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Receptor-like kinases as surface regulators for RAC/ROP-mediated pollen tube growth and interaction with the pistil

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Abstract

Background

RAC/ROPs are RHO-type GTPases and are known to play diverse signalling roles in plants. Cytoplasmic RAC/ROPs are recruited to the cell membrane and activated in response to extracellular signals perceived and mediated by cell surface-located signalling assemblies, transducing the signals to regulate cellular processes. More than any other cell types in plants, pollen tubes depend on continuous interactions with an extracellular environment produced by their surrounding tissues as they grow within the female organ pistil to deliver sperm to the female gametophyte for fertilization.

Scope

We review studies on pollen tube growth that provide compelling evidence indicating that RAC/ROPs are crucial for regulating the cellular processes that underlie the polarized cell growth process. Efforts to identify cell surface regulators that mediate extracellular signals also point to RAC/ROPs being the molecular switches targeted by growth-regulating female factors for modulation to mediate pollination and fertilization. We discuss a large volume of work spanning more than two decades on a family of pollen-specific receptor kinases and some recent studies on members of the FERONIA family of receptor-like kinases (RLKs).

Significance

The research described shows the crucial roles that two RLK families play in transducing signals from growth regulatory factors to the RAC/ROP switch at the pollen tube apex to mediate and target pollen tube growth to the female gametophyte and signal its disintegration to achieve fertilization once inside the female chamber.

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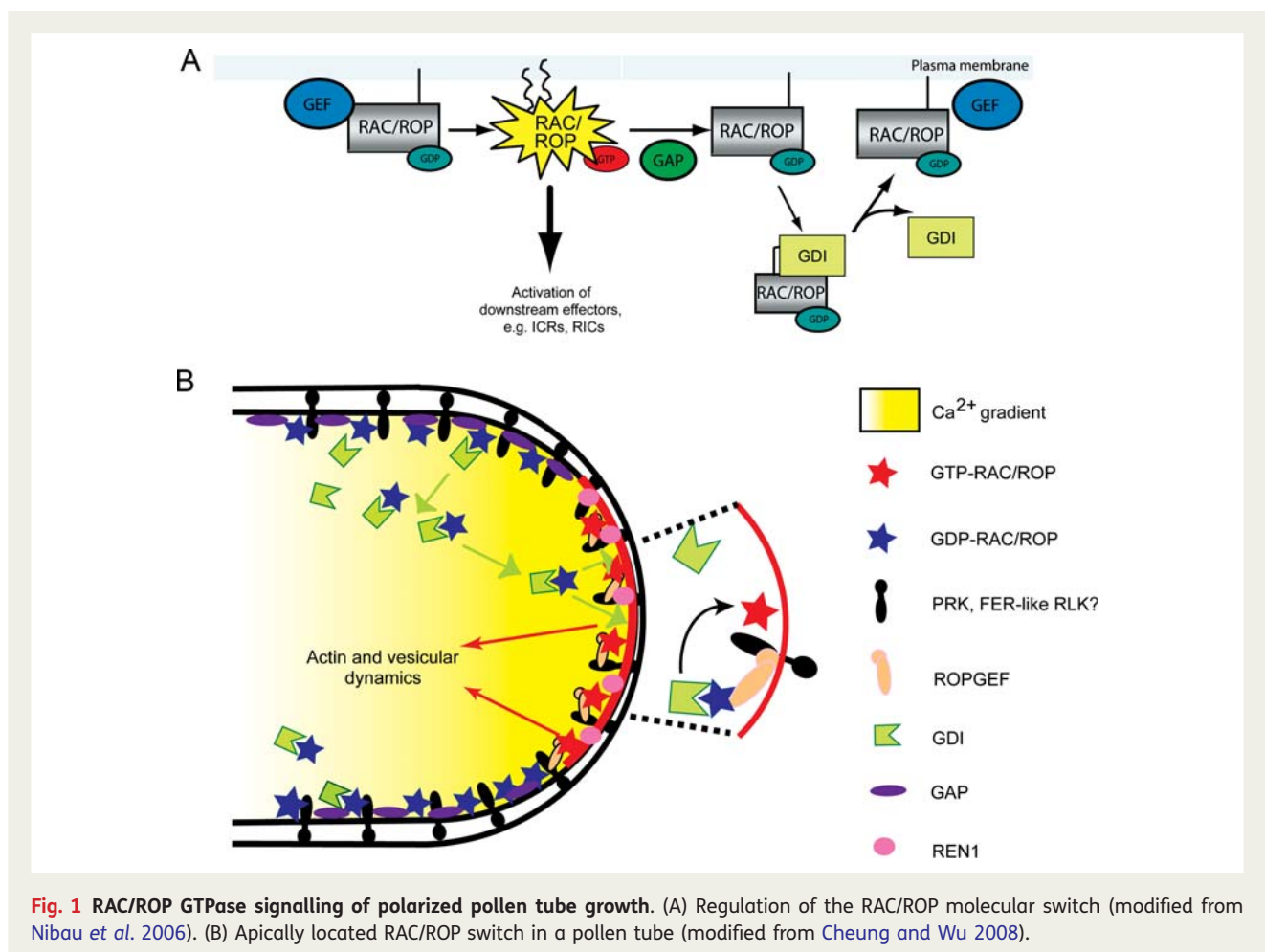
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Introduction

G proteins are key components in many eukaryotic signalling pathways. They act as molecular signalling switches by shuttling between an inactive GDP-bound form and an active GTP-bound form (Fig. 1). In the GTP-bound form the GTPase interacts with target proteins to affect cellular changes, until GTP hydrolysis returns the protein to the inactive, GDP-bound state. Of the two main types of G proteins, the RAS-related family of monomeric small GTPases and the heterotrimeric G proteins comprised of an α and a dimeric $\beta\gamma$ complex, plants rely considerably more on plant-specific RHO subfamily of RAS-related GTPases, referred to as RAC/ROPs (for RAC-like/RHOs of plants), for transduction of a broad array of signals (see Yalovsky et al. 2010; Wu et al. 2011). For instance, *Arabidopsis* and a number of other higher plants, including maize, rice and poplar, have close to 10 or more RAC/ROPs, while the moss *Physcomitrella patens* has 4 (Eklund et al. 2010; Fowler 2010). The most extensively

examined functional role for RAC/ROP GTPases is their control of polarized cell growth, exemplified by tip growth in pollen tubes and root hairs (Kost 2008; Yang 2008). The function of the pollen tube is to deliver sperm, transported as cargoes in its cytoplasm, to the female gametophyte for fertilization. Research over the last 25 years and originating from different perspectives related to the pollination process led to the broadly accepted view that RAC/ROPs located at the pollen tube apical membrane (Fig. 1B) represent a major molecular switch regulating the tip growth process. Recent research also implicates potential roles for these small GTPases in regulating pollen tube growth via their actions in female tissues. Several reviews have comprehensively discussed the cellular processes regulated by RAC/ROPs to mediate polarized cell growth (Cheung and Wu 2008; Kost 2008; Yalovsky et al. 2008; Yang 2008). We focus here on the discussion of upstream regulators, in particular receptor-like kinases (RLKs) that may control



the ON/OFF state of the RAC/ROP molecular switch to mediate pollen tube growth and pollen–pistil interaction.

Regulated RAC/ROP signalling is crucial for pollen tube growth

Regulation of the RAC/ROP molecular switch

Similar to other RAS-related small G proteins, RAC/ROP activity shuttles between the active GTP-bound form and the inactive GDP-bound form of RAC/ROPs (Fig. 1A). Guanine nucleotide exchange factors (GEFs) catalyse the exchange of GDP for GTP and activate RAC/ROPs (Rossman *et al.* 2005). In elongating pollen tubes, RAC/ROPs are typically located at the apical membrane domain. Several GEFs, referred to as ROPGEFs, have been located at the apical membrane of elongating pollen tubes, where they serve the functional role of activating apically located RAC/ROPs (Gu *et al.* 2006; Zhang and McCormick 2007). In their activated state, RAC/ROPs interact with effectors (Wu *et al.* 2001; Lavy *et al.* 2007) to mediate cellular target systems. In pollen tubes, two CRIB-domain-containing proteins, referred to as RICs (Wu *et al.* 2001), have been shown to interact with activated RAC/ROPs and to mediate actin and membrane dynamics (Hwang *et al.* 2005), processes that are well established to be crucial for maintaining pollen tube growth (Fig. 1B) (Cheung and Wu 2008).

RAC/ROP signalling is terminated by the activity of the GTPase-activating proteins (GAPs) that stimulate the GTPase activity inherent to G proteins, rendering them inactive (Fig. 1A). Studies of a GAP from tobacco, Nt-RhoGAP1, showed that it is located at the pollen tube apical flank cell membrane, just distal to where RAC/ROPs are located (Fig. 1B). The lateral location of Nt-RhoGAP1 to the apically located RAC/ROPs suggests a role in restricting active RAC/ROPs from spreading beyond the apical dome membrane (Klahre and Kost 2006), a model that is quite easy to conceptualize. On the other hand, a novel RhoGAP REN1 from *Arabidopsis* co-localizes with RAC/ROPs at the apical membrane (Hwang *et al.* 2008). The overlapping localization domain of RAC/ROPs and a negative regulator to the same membrane domain led to the proposal of a global inhibition model that suggests an elaborate regulatory loop involving ‘alternating and interlinking positive and negative’ feedback interactions between REN1 and RAC/ROPs (Hwang *et al.* 2008). It is possible that both local and lateral inhibitory mechanisms co-exist to ensure tip growth. It is reasonable to envision that regulated inactivation of RAC/ROPs at the tube apex is crucial for locally modulating their activity while lateral

inhibition ensures inactivation of those GTPases that are displaced to the apical flank as the apex migrates forward, ensuring that active signalling is restricted to the tip. Once inactivated, the inactive RAC/ROPs are extracted from the cell membrane by guanine nucleotide dissociation inhibitor (GDI), which also sequesters them in the GDP-bound form in the cytoplasm and recycles them back to the apical dome membrane for activation (Klahre *et al.* 2006).

Pollen tube growth is dependent on properly regulated RAC/ROP signalling

Directly perturbing the normal level of RAC/ROP signalling activity results in defects in tip growth characteristics. Pollen tubes with elevated RAC/ROP activity, e.g. by the over-expression of constitutively active (CA) RAC/ROPs, are typically highly depolarized, often expanding isotropically resulting in a balloon-tipped or spoon-shaped tube and growth arrest (Kost *et al.* 1999; Chen *et al.* 2003; Fu *et al.* 2001). On the other hand, down-regulating RAC/ROP activity, e.g. by over-expression of dominant-negative (DN) RAC/ROPs, reduces tube elongation rates and pollen tubes are typically broader than rapidly elongating pollen tubes, reflecting that cell expansion has occurred over a broader apical dome region while still capable of maintaining highly asymmetric growth and thus giving rise to a wider tube.

Indirectly altering the endogenous RAC/ROP regulatory capacity in a pollen tube also results in pollen tube growth defects mimicking those induced by over-expressing CA or DN RAC/ROPs. For instance, over-expressing ROPGEFs induced ballooned pollen tubes (Kaothien *et al.* 2005; Gu *et al.* 2006; Cheung *et al.* 2008) and knocking out endogenous RhoGAP in *ren1* null mutants resulted in bulbous pollen tubes, mimicking the result of over-expressing wild-type or CA RAC/ROPs, and reduced male transmission (Hwang *et al.* 2008). On the other hand, over-expression of a GDI, presumably increasing RAC/ROP sequestration in the cytoplasm, also resulted in reduced pollen tube growth (Klahre *et al.* 2006), similar to the effect of DN RAC/ROP over-expression. In maize and when competing with co-pollinated wild-type pollen, *rop2::Mu* pollen grains in which the ROP2 mRNA level was significantly reduced as a result of Mu insertion showed compromised transmission capacity (Arthur *et al.* 2003). These observations provide strong support for properly regulated RAC/ROP signalling being crucial for polarity maintenance at the pollen tube tip and important for optimum reproductive success.

RAC/ROPs as mediators for exogenous pollen tube growth regulators

Pollen tube growth in the pistil is responsive to regulatory factors secreted by female tissues acting e.g. as nutrients and directional guidance molecules. *In vitro*, pollen tubes are responsive to exogenously applied biological growth regulators originating from female tissues or from pollen themselves. These include native proteins isolated from pistil tissues (Cheung *et al.* 1995; Wu *et al.* 1995; Mollet *et al.* 2000; Kim *et al.* 2003; Zhang *et al.* 2008), recombinant proteins expressed in *Escherichia coli* corresponding to female cell-expressed proteins (Okuda *et al.* 2009) or synthetic peptides corresponding to pollen-expressed proteins (Covey *et al.* 2010). Besides the well-known pistil controls that inhibit incompatible pollination in self-incompatible plants (McClure and Franklin-tong 2006; Tantikanjana *et al.* 2010), negative pollen tube growth regulators from female tissues are also known to play important roles in ensuring successful reproduction. For instance, they are needed to mediate growth arrest and rupture upon pollen tube penetration of the female gametophyte inside the ovule, enabling sperm discharge and fertilization (Huck *et al.* 2003, Rotman *et al.* 2003; Escobar-Restrepo *et al.* 2007; Amien *et al.* 2010). Late-arriving pollen tubes approaching already penetrated ovules are believed to be repelled by signals emanating from within the ovules (Palanivelu and Preuss 2006), causing them to re-route to receptive, not yet visited ovules, thus preventing polyspermy, ensuring progeny health and maximizing reproductive yield (Shimizu and Okada 2000).

Given the importance of the RAC/ROP GTPases to pollen tube growth, it is easy to envision them as key intracellular signal mediators to convey the signal from some of these growth regulators to the cellular machinery for growth and directionality. Presumably the extracellular to intracellular signalling linkage would be provided by cell surface-located signal perception molecules that will recruit and impact the activity of intracellular ROPGEFs, which in turn regulate the activity of the RAC/ROP switch (Fig. 1B). Thus far, RLKs have been implicated as upstream cell surface regulators for RAC/ROP GTPases and potential mediators for extracellular signals to the RAC/ROP switch. Receptor-like kinases are transmembrane proteins with an extracellular domain (ECD), a single membrane-spanning domain and a cytoplasmic kinase domain. Members of the plant RLK family (Shiu and Bleeker 2003; De Smet *et al.* 2009; Lehti-Shiu *et al.* 2009) share conserved catalytic kinase domains and predominantly phosphorylate serine and threonine residues, although some have

also been found to phosphorylate tyrosine residues. They perceive signals via specific ligand–ECD interactions and subsequently activate signalling cascades via their cytoplasmic domain (CD). The plant RLK family is enormous; for instance, the *Arabidopsis* RLK family is comprised of more than 600 members. The ECDs of these RLKs are quite divergent, allowing them to be grouped into 15 subfamilies, one being a leucine-rich repeat (LRR)-containing family (Torii 2004) and the other a *Catharanthus roseus* RLK1 (CrRLK1)-like family (Hématy and Höfte 2008; Boisson-Dernier *et al.* 2011; Nibau and Cheung 2011). We focus our discussion here on several members of these two sub-RLK families and the signalling pathways that they regulate as they have been implicated to be cell surface regulators for RAC/ROP signalling for polarized cell growth.

Pollen-specific receptor kinases interact with ROPGEFs and regulate RAC/ROP-signalled pollen tube tip growth

Localization of pollen-specific receptor kinases suggests a cell surface signalling function in pollen tubes

Pollen-specific receptor kinases (PRKs) share features allowing their identification as a family of pollen-specific receptor kinases among a much larger LRR subtype of receptor kinases in plants (Kim *et al.* 2002). Much of the functional understanding related to PRKs has been derived from two of the earlier identified members of the family, LePRK1 and LePRK2 from tomato, and more recently from At-PRK2a from *Arabidopsis*. Immunodetection (Muschietti *et al.* 1998; Kim *et al.* 2002) and fluorescent protein (FP)-tagged localization of PRKs showed that these receptor kinases are localized along the entire pollen tube cell surface (Fig. 2A) (Cheung *et al.* 2002; Zhang and McCormick 2007). Immunolocalization analysis using antibodies specific to their respective ECD shows that LePRK1, LePRK2 and a more distantly related homologue LePRK3 occupy overlapping domains along the pollen tube surface but each displaying some noticeable distinction from the others (Kim *et al.* 2002). Although it is not clear yet whether these localization differences signify functional specialization, biochemical studies to be discussed below suggest that even the closely related LePRK1 and LePRK2 are likely to be differentially involved in their capacity to interact with signals from the female environment. Since dimerization is often associated with receptor kinase activation and LePRK1 and LePRK2 are known to form heterodimers (Wengier *et al.* 2003), it is conceivable that interactions between the three related LePRKs provide subtle

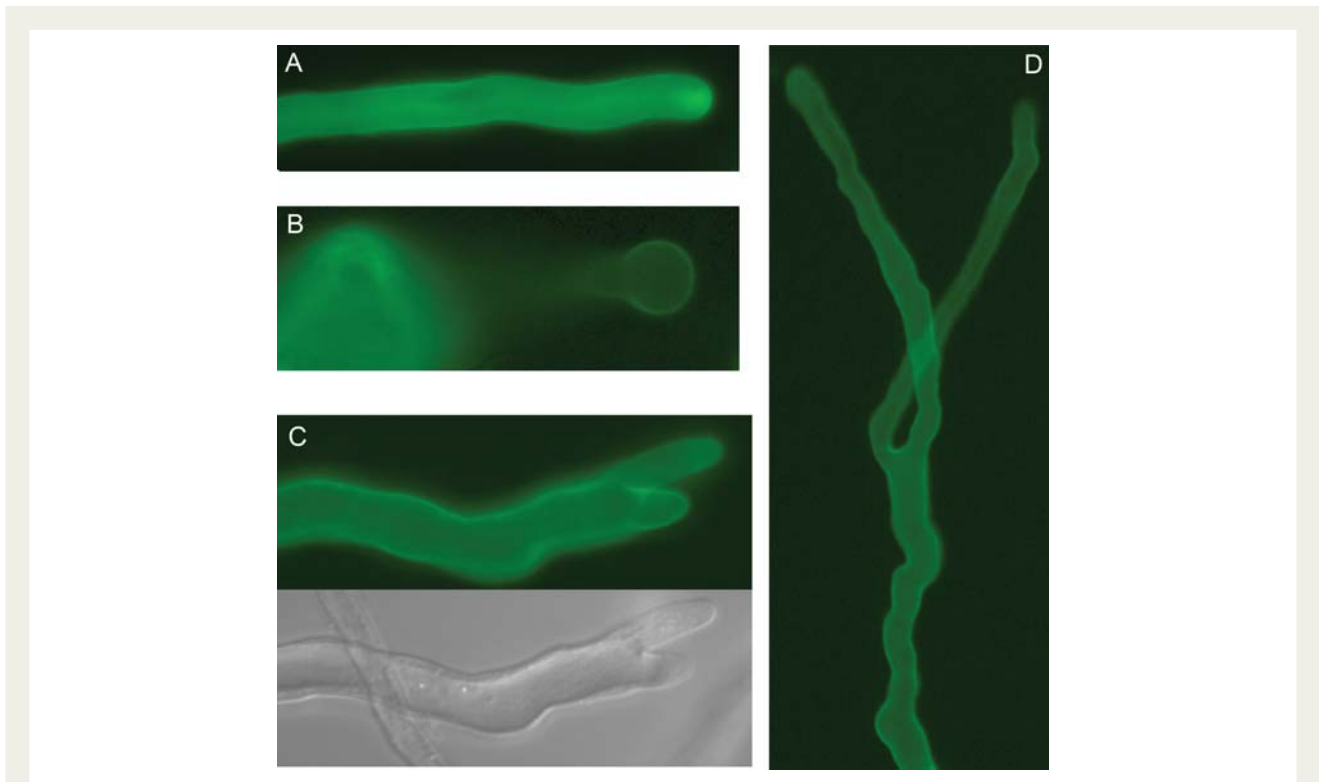


Fig. 2 Cell membrane-located PRK regulates pollen tube tip growth. LAT52-GFP-Nt-PRK-transformed tobacco pollen tubes are shown. (A) Cell surface localization of GFP-Nt-PRK in elongating pollen tubes. Typical of pollen tube cell membrane-associated proteins, vesicles loaded with FP-PRKs also concentrate in and cycle rapidly in and out of the apical cytoplasm (see also Cheung et al. 2002), which is known to be occupied almost exclusively by transport vesicles. (B–D) Prevalent tip growth defects induced by expression of GFP-Nt-PRK. (B) Balloon tipped and growth arrested. (C) Bifurcated tip; the vacuole-occupied branch (lower, DIC image) was either already growth arrested or likely to cease growth shortly after the image was acquired. (D) A bifurcated GFP-Nt-GFP-expressing pollen tube where growth at both tips had been sustained for some time. Balloon-tipped but not bifurcated tubes were observed previously in LePRK2-transformed pollen tubes (Zhang et al. 2008).

differences expanding their extracellular perception capability and intracellular signalling capacity.

PRKs interact with ROPGEFs

Over-expression of GFP-PRKs in pollen tubes results in growth defects indicative of compromised tip growth (Fig. 2B–D) (Zhang et al. 2008). A long series of molecular genetic studies, which we shall discuss below, implicated the LePRKs as playing important roles in mediating reproductive success. The observation revealing their potential participation in RAC/ROP-regulated pollen tube growth was reported in 2005 when the CDs of LePRK1 and LePRK2 were shown to interact directly with a highly phosphorylated pollen-specific peripheral membrane protein LeKPP (for tomato kinase partner protein) (Kaothien et al. 2005). Over-expression of LeKPP in transformed pollen tubes resulted in ballooned tips and other severely malformed apices, accompanied

by disrupted actin organization and vesicle dynamics, reminiscent of phenotypes induced by up-regulating RAC/ROP signalling. Moreover, a family of 14 KPP-like proteins was also identified in *Arabidopsis*. KPP and the 14 KPP-like *Arabidopsis* proteins turned out to be the long-sought GEFs for RAC/ROP GTPases (Berken et al. 2005), which had until then eluded identification from direct homology searches. Therefore, the discovery of KPP as a PRK-interacting protein establishes a functional linkage between PRKs and RAC/ROPs in regulating pollen tube tip growth. Similar to RAC/ROPs constituting a plant-specific clade of the RHO GTPase protein family, KPP and ROPGEFs from *Arabidopsis* and other plant species are also unique to plants. ROPGEFs share a conserved central catalytic domain for GDP to GTP exchange but diverge on their N- and C-terminal regions, presumably providing plasticity in their signalling interactions and thus the range of their signalling capacity.

PRK regulates RAC/ROP-mediated pollen tube tip growth

Over-expression of the *Arabidopsis* ROPGEF1 in tobacco pollen tubes induces a highly ballooned apical morphology and disorganized actin organization (Gu *et al.* 2006; Cheung *et al.* 2008), similar to the effect of over-expressing CA RAC/ROPs. GFP-ROPGEF1 is localized throughout the ballooned apical region (Gu *et al.* 2006), consistent with the induced isotropic growth having resulted from the spread of the RAC/ROP activation to beyond the apical membrane domain. ROPGEF1 is broadly expressed in *Arabidopsis* (Zimmermann *et al.* 2004), but its transcripts are much less abundant than those of other ROPGEFs identified as pollen-specific or pollen-enriched (Gu *et al.* 2006; Zhang and McCormick 2007). Unlike ROPGEF1, the GFP-labelled pollen-specific or -enriched ROPGEFs were located in a limited membrane region around the apical dome when they were over-expressed in pollen tubes and induced only slightly depolarized growth resulting in broader pollen tubes. This was consistent with increased RAC/ROP activation over a broader apical membrane area but nonetheless still confined to the extending tip region, therefore sustaining asymmetric growth albeit over a broader apical membrane domain, yielding the wider tubes. The more restricted apical localization for these pollen-specific or -enriched ROPGEFs suggests the presence of endogenous mechanisms to preclude their activating RAC/ROP to beyond the apical flank region. Such mechanisms apparently do not apply to the constitutively expressed ROPGEF1, which is not highly pollen prevalent.

To directly establish a signalling linkage between PRKs and RAC/ROPs in pollen tube growth, Zhang and McCormick (2007) used bimolecular fluorescence complementation to establish that At-PRK2a and ROPGEF12 directly interact along the pollen tube cell membrane. When At-PRK2a and full-length ROPGEF12, which is auto-inhibited by its C-terminal domain, were over-expressed individually in transformed tobacco pollen tubes, they induced the relatively weak phenotype of wider tubes. Their co-overexpression, however, resulted in the severe phenotype of isotropic growth. Similarly, co-expression of LePRK2 and LeKPP in transiently transformed tobacco pollen tubes also resulted in a similar synergistic effect (Zhang *et al.* 2008). Furthermore, biochemical studies showed that LePRK1 and LePRK2 associate with a high-molecular-weight (~400 kDa) protein complex in germinating tomato pollen (Wengier *et al.* 2003). RAC/ROPs and LeKPP were reportedly also detected in the same column fractions (see Wengier *et al.* 2003), suggesting that the 400-kDa

complex might represent a PRK-signalling complex that initiates RAC/ROP signalling in pollen tubes. Taken together, these observations show that PRKs function as cell surface regulators interacting with ROPGEFs to mediate a level of spatially restricted RAC/ROP signalling activities at the pollen tube apex to ensure tip-focused growth.

PRKs interact with pollen tube growth regulators to mediate reproductive success

Biochemical and molecular genetic studies show that the LePRKs interact with molecules secreted either from female tissues or from pollen (Tang *et al.* 2002, 2004). Results from studies based on antisense suppression of LePRK2 (Zhang *et al.* 2008) and of two LePRK2-interacting pollen proteins, LAT52 (Tang *et al.* 2002) and a homologue of SHY (Tang *et al.* 2004) from *Petunia* (Guyon *et al.* 2004), and analysis of pollen tube growth regulatory activities of two LePRK2-interacting female molecules, STIL (Zhang *et al.* 2008; Wengier *et al.* 2010) and STIG1 (Tang *et al.* 2004), are consistent with the PRK-mediated signalling pathway being important for pollen germination and tube growth.

LePRK2 interacts with a stylar-secreted pollen germination and tube growth promoting molecule, STIL

LePRK1 and 2 are closely related and their *E. coli*-produced kinase domains are active kinases and autophosphorylate (Muschietti *et al.* 1998). Immunoprecipitation studies showed that they form heterodimers in the cell membrane of pollen and of yeast when co-expressed in the heterologous system (Wengier *et al.* 2003). However, LePRK1 and 2 are not functionally equivalent. At the gene expression level, LePRK1 and 2 are expressed late in pollen development but only the level of LePRK2 increases further when pollen germinates. Phosphorylation assays using pollen membrane fractions showed that LePRK2, but not LePRK1, is phosphorylated. Furthermore, heterodimerization apparently relies on LePRK2 being an active kinase as dimer formation in yeast between the wild-type LePRK1 and a kinase-inactive form of LePRK2 was prohibited, whereas that between the wild-type LePRK2 and kinase-inactive LePRK1 was not affected (Wengier *et al.* 2003).

Phosphorylation of LePRK2 is apparently regulated *in vivo* as pollen germinates and during tube growth. A low-molecular-weight species from the tomato stylar exudates, STIL (stylar interactor for LePRKs),

dephosphorylates LePRK2 when added to *in vitro* phosphorylation assays by pollen membrane fractions (Muschietti *et al.* 1998; Wengier *et al.* 2010). Furthermore, stylar fractions, when added to germinating pollen grains, disrupted the LePRK-containing 400-kDa protein complexes and rendered the majority of these PRKs to molecular weight fractions closer to their monomeric molecular mass (Wengier *et al.* 2003). Moreover, the LePRK1 and LePRK2 heterodimers formed in yeast were disrupted when stylar fractions enriched in STIL were added to membrane fractions from the LePRK1 and LePRK2 co-expressing yeast, presumably as a result of STIL dephosphorylation of LePRK2.

STIL, described as a ‘peculiar’ molecule from the style, is a low-molecular-weight species of 3350 Da (Wengier *et al.* 2010). Its activity to dephosphorylate LePRK2 is resistant to heat, base, dithiothreitol and protease treatment. STIL is also resistant to traditional acid treatment (1 N HCl at 100 °C for 20 h) but sensitive to microwave-assisted acid treatment (1.5 N HCl for 10 min at 900 W), which apparently mediated more extensive hydrolysis of STIL than acid treatment under normal conditions. The exact chemical nature of STIL remains to be determined, although it is likely to be at least partially peptidic as it absorbs at 280 nm and has amino acid content. When added to wild-type pollen, STIL promoted germination and enhanced tube growth rates (Zhang *et al.* 2008). Transformed tomato pollen tubes with antisense-suppressed LePRK2 levels failed to respond to STIL-stimulated growth, providing evidence for a LePRK-regulated pathway underlying the STIL-stimulated growth responses (Zhang *et al.* 2008).

Taking into account the molecular genetics, biochemical and cellular observations surrounding the LePRKs, a picture of a LePRK2-mediated signalling pathway emerges whereby the stylar factor STIL promotes pollen germination and tube growth by dephosphorylating LePRK2. This in turn disrupts the LePRK1 and LePRK2 heterodimer and its association with a high-molecular-weight protein complex, potentially impacting interaction with KPP, and modulates downstream RAC/ROP signalling activity to promote tip growth.

LePRK2 interacts with pollen tube growth regulatory molecules from pollen or pistil

The signalling capacity of LePRK1 and LePRK2 can apparently be modulated by proteins from both pollen and pistil through their interactions with their ECDs. Yeast two-hybrid screens and biochemical protein–protein interaction assays showed that the ECD of LePRK2 interacts with at least two pollen glycoproteins, the secreted cysteine-rich LAT52 (Tang *et al.* 2002) and the cell wall-located SHY (Guyon *et al.* 2004; Tang *et al.* 2004).

Moreover, LePRK2 interacts with LAT52 before but not after pollen germination, hinting that the pistillate environment encountered by the germinating pollen must have resulted in conditions that disrupt their interaction. Both LAT52 and SHY apparently contribute to ensure productive pollination resulting in fertilization. Antisense suppression of LAT52 resulted in the inability of pollen grains to hydrate properly *in vitro*, abortive tube growth in the style and reduced male transmission (Muschietti *et al.* 1994). Antisense-mediated reduction of the SHY homologue in *Petunia* severely inhibited pollen germination and for those SHY-suppressed pollen grains that managed to germinate, their tubes were arrested just above the ovary, so overall seed yields in these plants were poor (Guyon *et al.* 2004).

The ECDs of LePRK1 and LePRK2 both interact physically with a female partner LeSTIG1, a cysteine-rich protein secreted by the stigma (Tang *et al.* 2004) where pollen grains are deposited and germinate. *Escherichia coli*-produced recombinant STIG1 stimulated *in vitro* pollen tube growth when added to pollen germination cultures. *In vitro*, STIG1 bound to the ECD of LePRK2 and displaced binding by the pollen-produced LAT52. Displacement of LAT52 from complexing with LePRK2 by STIG1 observed *in vitro* provided a plausible explanation for the observation that LAT52 binds to LePRK2 before but not after pollen germination, and led to the proposition that LePRK2 changes interacting partners when on the surface of pollen grains and upon tube germination (Tang *et al.* 2004). How the pollen partners LAT52 and SHY and the pistil partner LeSTIG1 impact the signalling capacity of LePRKs remains to be established. Observations that each of these pollen- and pistil-secreted proteins has a biological impact on pollen germination and tube growth *in vitro* or for the reproductive process *in vivo* provide strong implications for the PRK-regulated signalling pathway being crucial for reproduction.

Finely tuned phosphoregulation of LePRK2 underlies its activity in regulating pollen tube growth

Studies from STIL and the other LePRK-interacting pollen and pistil proteins show that PRK-mediated signalling capacity could be modulated in several ways and during different stages of pollination. That dephosphorylation of LePRK2 by STIL disrupts its complex formation with other potential signalling partners implies important functional significance for LePRK2 phosphorylation. It was shown recently that LePRK2 exists as multiple isoforms in the pollen cell membrane, apparently phosphorylated on two phosphorylatable motifs located in the cytoplasmic

juxtamembrane region (Salem *et al.* 2011). Over-expression of wild-type and site-directed mutant forms of LePRK2 showed that these two motifs act antagonistically in affecting how LePRK2 impacts pollen tube growth. When transiently expressed in tobacco pollen tubes, mutations rendering the more N-terminally located motif non-phosphorylatable (serines to alanines) resulted in longer pollen tubes, suggesting that phosphorylation negatively impacts growth. Conversely, similar mutations in the more distally located motif resulted in shorter pollen tubes, suggesting that phosphorylation stimulates growth. Furthermore, the results obtained by using phospho-mimicking mutations in these motifs led to inverse observations and therefore similar conclusions on the antagonistic effects of these LePRK2 phosphorylatable motifs. Thus it is possible to envision that the output signalling activity from the PRK signalling module is maintained by varying the level of phosphorylation on these two motifs. The multiple interacting male and female partners and the two antagonistic phosphorylated motifs argue for the presence of mechanisms capable of fine-tuning the PRK signalling capacity to facilitate interactions with different partners and modulate RAC/ROP GTPase activity to mediate polarized growth throughout the long pollen tube growth journey in the pistil.

The FERONIA family of RLKs as cell surface regulators for pollen tube growth and pollen–pistil interaction

The FERONIA (FER) family of RLKs (more frequently referred to as the CrRLK family after its founding member found in *C. roseus*) is emerging as an important subfamily of plant RLKs regulating plant growth and development. CrRLKs are conserved among plants; in *Arabidopsis* they comprise a 17-membered family (Shiu and Bleeker 2003; Lehti-Shiu *et al.* 2009). Studies on five of these, including FER (Huck *et al.* 2003; Rotman *et al.* 2003; Escobar-Restrepo *et al.* 2007; Guo *et al.* 2009; Deslauriers and Larsen 2010; Duan *et al.* 2010), THESEUS1 (THE1) (Hématy *et al.* 2007), HERCULES1 (Guo *et al.* 2009), ANXUR1 and ANXUR2 (ANX1 and ANX2) (Boisson-Dernier *et al.* 2009; Miyazaki *et al.* 2009), have already revealed important roles for these RLKs in regulating cell growth processes in different tissues and under different development conditions. Recent reviews have discussed the structure and emerging knowledge about the biological roles of these FER family RLKs (Hématy and Höfte 2008; Boisson-Dernier

et al. 2011; Nibau and Cheung 2011). The ECDs of these FER RLKs are distinguishable from other RLKs in having two domains with recognizable homology with malectin, a conserved animal endoplasmic reticulum-anchored membrane protein that binds di-glucose (Schallus *et al.* 2008). Although the functional significance of these potential sugar-binding domains is unclear, their presence is intriguing considering that FER, ANX1 and ANX2 control pollen tube integrity, and that THE1 regulates cell growth in response to the cell wall environment and suppresses plant growth under cellulose deficiency. Here, we focus on the FER-related RLKs known to impact the reproductive process, FER, ANX1 and ANX2 and their potential roles in RAC/ROP-regulated processes in reproduction.

FER regulates pollen tube–ovule interaction and induces pollen tube disintegration

The *Arabidopsis Feronia (Fer)/Sirène (Srn)* (for simplicity, *Fer* will be used from here on unless the *srn* mutant is referred to specifically) was initially identified through genetic screens as an important regulator for female gametophytic function (Huck *et al.* 2003; Rotman *et al.* 2003). Molecular analysis of wild-type and mutant alleles of *fer* showed that it encodes an RLK belonging to the broader CrRLK family (Escobar-Restrepo *et al.* 2007). In wild-type plants, after being guided by attractants that emanate from the female gametophyte to enter the ovule, the pollen tube and ovule engage in a series of pollen tube–ovule interactive events referred to as pollen tube reception whereby the first-arriving pollen tube enters one of the paired synergid cells at the entrance to the female gametophyte and ruptures, resulting in sperm discharge and fertilization (see Dresselhaus and Marton 2009). Late-arriving pollen tubes are prevented from entering an already penetrated ovule (Palanivelu and Preuss 2006; Sandaklie-Nikolova *et al.* 2007). In the ovules, FER is expressed most predominantly in the synergid cells, and GFP-labelled FER localizes to synergid cell membrane and accumulates prominently in the filiform apparatus (Escobar-Restrepo *et al.* 2007), a thickened cell wall region at the entrance to the female gametophyte secreted by the synergid cells and with a high density of criss-crossing synergid cell membrane (Punwani *et al.* 2007). *fer* female gametophytes attract pollen tubes normally but fail to induce rupture of the penetrated pollen tube and prevent multiple pollen tube penetration. This results in supernumerary pollen tube entrance yet female sterility as sperm release and therefore fertilization fail. Because of multiple pollen tube entry and their overgrowth inside

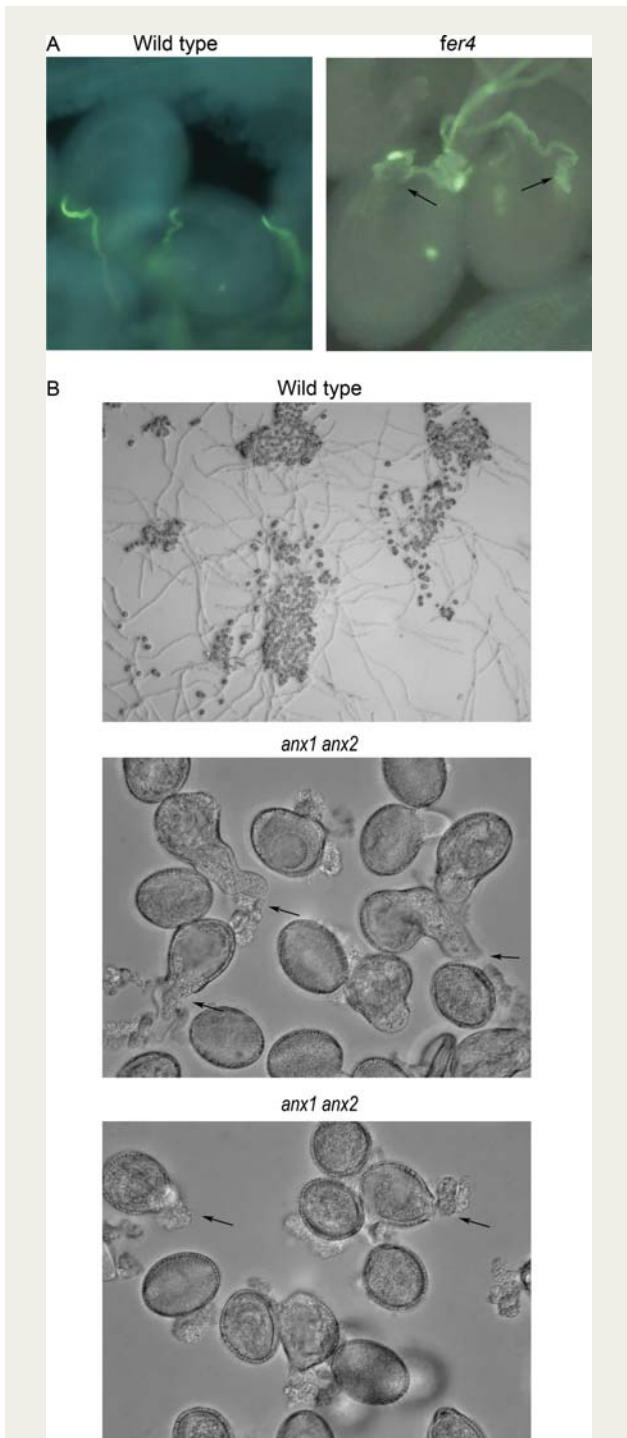


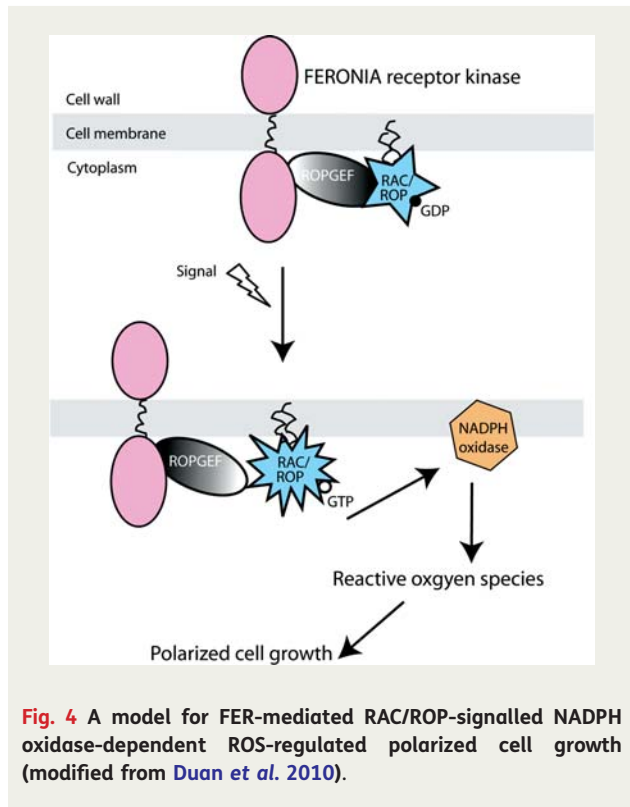
Fig. 3 FER-family RLKs regulating pollen and pistil functions. (A) Single pollen tube entrance in wild-type (left) and supernumerary pollen tube entrance and overgrowth (arrows) in *fer* ovules (right). (B) As reported in Miyazaki et al. (2009) and Boisson-Dernier et al. (2009), pollen from double *anx1 anx2* mutant rupture (arrows, middle panel) immediately or shortly after tube emergence (arrows, lower panel), while wild-type pollen tubes (top panel) elongate efficiently under similar culture conditions.

the female gametophyte, *fer* ovules show the dramatic phenotype of pollen tube pile-up inside the female gametophyte and sometimes bundles of pollen tubes gather at the entrance to the ovule (Fig. 3A) (Huck et al. 2003; Rotman et al. 2003; Escobar-Restrepo et al. 2007).

ANX1 and ANX2 maintain pollen tube integrity

ANX1 and ANX2 are two of four pollen-specific FER-like RLKs in *Arabidopsis* and their transcripts accumulate to very high levels in mature pollen (Zimmermann et al. 2004). GFP-labelled ANXs are localized to the apical and subapical membrane (Boisson-Dernier et al. 2009; Miyazaki et al. 2009), a region where spatially and temporally regulated cell expansion is crucial for maintaining tip-focused growth. Mutant analyses showed that ANX1 and ANX2 act redundantly to maintain pollen tube integrity. Plants with homozygous mutant alleles in one of the ANXs and heterozygous ones in the other are normal, whereas double *anx1 anx2* mutants are male sterile and their pollen tubes rupture *in vitro* (Fig. 3B) and burst precociously while growing in the pistil, never reaching the ovules (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). Therefore, the activity from ANXs is apparently needed to maintain cell integrity while pollen tubes are growing within the maternal tissue but must somehow be suppressed upon arrival at the female gametophyte to permit tube disintegration, enabling sperm discharge and therefore fertilization.

Taken together with the function of FER as a facilitator of pollen tube disintegration in the female gametophyte, we cannot avoid speculating that somehow male–female interaction at the point of pollen tube contact with and entrance into the female gametophyte underlies the activation of FER-mediated activity and inactivation of the ANX-regulated pathway. Models have been proposed (Escobar-Restrepo et al. 2007; Boisson-Dernier et al. 2009; Miyazaki et al. 2009; Kanaoka and Torii 2010). For instance, male arrival may trigger activation of FER in the female gametophyte, which in turn inhibits pollen tube growth followed by tube rupture. Alternatively, FER and ANXs may share the same ligand and that in the female gametophyte FER sequesters the ligand, thus preventing ANX-mediated signalling in the pollen tube, resulting in its growth arrest and rupture. Furthermore, it is also possible that as the pollen tube penetrates the female gametophyte, direct interaction between the ANX ECDs on the pollen tip and the ECD of FER along the synergid cell surface may trigger downstream events in both cells. Determining if and how



the FER- and ANX-regulated pathways interact allowing the female-controlled pathway to induce male disintegration and the male-operating pathway to permit its own destruction enabling sperm release and fertilization should provide important insight into pollen tube-ovule interaction.

A multifunctional FER is a cell surface regulator for RAC/ROP signalling

FER is broadly expressed except in pollen (Zimmermann et al. 2004). Besides its female gametophytic function in regulating pollen tube growth, FER is important for a broad range of growth and development-related processes, including polarized root hair growth, maintenance of normal trichome morphology and mediation of hormone responses including those of brassinolides, ethylene and auxin (Guo et al. 2009; Deslauriers and Larsen 2010; Duan et al. 2010). Homozygous *fer* and RNAi-suppressed *fer* mutants are noticeably growth inhibited throughout development. We discuss here studies that reveal FER as an upstream regulator for RAC/ROP-signalled NADPH oxidase-dependent polarized cell growth (Fig. 4) (Duan et al. 2010).

A FER RLK linkage to RAC/ROP signalling

FER was identified as an interacting protein with several *Arabidopsis* ROPGEFs in a direct effort to identify potential upstream regulators for RAC/ROP signalling by yeast two-hybrid analysis, followed by bimolecular fluorescence complementation assays in plant cells (Duan et al. 2010). Biochemically, an epitope-tagged FER expressed in transformed seedlings and protoplasts was pulled down by RAC/ROPs, which also interact with activator ROPGEFs (Berken et al. 2005; Gu et al. 2006), suggesting at least a tripartite complex with RAC/ROP, FER and ROPGEF providing the linkage between its upstream regulator and downstream effector. Furthermore, RAC/ROP pull-down of FER was considerably more favoured by GDP-saturated RAC/ROPs, consistent with the bridging ROPGEFs interacting preferentially with the inactive form of the GTPases to stimulate GDP/GTP exchange, as has been shown previously in the biochemical characterization of ROPGEFs (Berken et al. 2005). Presumably, upon activation the GTP-bound RAC/ROPs would interact with effectors to activate downstream target pathways, weakening RAC/ROP association with the upstream regulators (Fig. 4).

FER as a cell surface regulator for RAC/ROP-mediated polarized cell growth

Analysis of a T-DNA-induced knockout mutant *fer4* and one of the originally described *fer* mutants, *srn* (Rotman et al. 2003), showed that they are severely defective in root hair development, along with other growth-suppressed phenotypes (Duan et al. 2010). Like pollen tubes, root hairs grow exclusively at the tip and it is also well established that RAC/ROP signalling at the root hair tip is crucial for proper hair cell formation, starting from the emergence from hair-forming cells, trichoblasts, on the root epidermis to maintaining tip growth. Up-regulating RAC/ROP signalling most prevalently led to highly depolarized growth resulting in ballooned hairs, whereas down-regulating these small GTPases resulted in shorter and wavier root hairs (Molendijk et al. 2001; Jones et al. 2002; Tao et al. 2002). GFP-labelled RAC/ROPs locate to the bulges on trichoblasts that mark the sites of root hair emergence and to the root hair apical membrane throughout its development (Molendijk et al. 2001; Jones et al. 2002). Up-regulating RAC/ROP signalling by over-expressing At-ROP2 also induced more and ectopic emergence of root hairs, some of them developing split tips (Jones et al. 2002), consistent with ectopic activation of RAC/ROP signalling as underlying ectopic growth fronts.

In a series of molecular genetics and physiological studies involving *Arabidopsis* mutants defective in *Root*

hair defective2 [*Rhd2/RbohC* encoding an NADPH oxidase that produces reactive oxygen species (ROS)] and *Supercentipede1* (*Scn1/AtRhoGdi* encoding a GDI for RAC/ROPs, thus resulting in up-regulated RAC/ROP signalling), Dolan and colleagues demonstrated a RAC/ROP-dependent pathway whereby active RAC/ROPs are crucial for focusing NADPH oxidase-dependent ROS production at the root hair apical region mediating tip growth (Foreman *et al.* 2003; Carol *et al.* 2005; Carol and Dolan 2006). *fer* root hairs morphologically mimic those observed in *rhd2* (Duan *et al.* 2010) and lack NADPH oxidase-dependent ROS. Although the manner in which ROS regulate cell growth is complex (Knight 2007; Monshausen *et al.* 2007; Swanson and Gilroy 2010), the *fer* phenotypes nonetheless implicate FER as functioning in the RAC/ROP-regulated pathway to mediate polarized root hair growth. Moreover, relative to wild-type plants, *fer* seedlings accumulate significantly lower levels of activated RAC/ROPs while over-expressing RAC/ROPs in *fer* restores root hair morphology, ROS level and auxin-regulated ROS production and root hair development to normal (Duan *et al.* 2010). Taken together with results showing physical interactions between FER and ROPGEFs, these functional studies provide strong evidence supporting FER as an upstream regulator for RAC/ROP signalling, specifically that of NADPH oxidase-dependent ROS-mediated polarized root hair growth during seedling development (Fig. 4).

Conclusions and forward look

The discovery of FER acting as an upstream regulator of a well-established RAC/ROP-regulated pathway and the fact that FER, ANX1 and ANX2 play key roles in regulating pollen tube integrity during growth in the pistil suggest potentially that a subset of FER family members could act as surface regulators for RAC/ROP-regulated processes in pollen tube growth. Tip-located ROS and extracellular superoxide to the pollen tubes apex have been detected in growing pollen tubes (Potocký *et al.* 2007). Reducing ROS by antisense oligonucleotide-mediated suppression of NADPH oxidase expression, application of inhibitors for ROS production or ROS scavengers all inhibited tube growth whereas growth in these NADPH oxidase-suppressed pollen tube could be restored by H₂O₂ application. Together these observations provide support for ROS playing an important role in regulating pollen tube growth. Given the linkage between FER and RAC/ROP-regulated NADPH oxidase-dependent ROS production in root hairs, it is reasonable to envisage that the role of ANX1 and ANX2 is to regulate a similar pathway in pollen tubes to sustain tip growth. Furthermore, Ca²⁺ augments apical ROS accumulation in

pollen tubes (Potocký *et al.* 2007). The Ca²⁺-augmented apical superoxide production apparently requires proper PRK signalling as it could not be induced in LePRK2-suppressed tomato pollen tubes (Zhang *et al.* 2008). Therefore, it is plausible to suggest convergence of PRK-mediated and the potentially ANX1- and ANX2-regulated pathways in yet unknown crosstalk interactions between these two RLKs.

FER, ANX1 and ANX2 are not the only FER family RLKs expressed in reproductive tissues in *Arabidopsis*. There are two other closely related pollen-specific family members (At4g39110 and At2g21480) that clearly are functionally non-redundant with ANX1 and ANX2. Furthermore, FER is also prominently expressed in the stigma (Zimmermann *et al.* 2004; Duan *et al.* 2010), along with another family member (At5g59700), which is almost exclusively expressed in the stigma (Zimmermann *et al.* 2004). Determining the biological roles for these yet to be characterized FER family RLKs in the pollination and fertilization processes, and mechanistically dissecting their signalling pathways, will reveal whether they represent additional cell surface regulators for the reproductive process and whether their signalling activity is also mediated by the RAC/ROP switch.

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Contributions by the authors

All authors contributed to the preparation of the manuscript. The first author contributed to images shown in Figs 2 and 3. The second author contributed to some background studies related to images shown in Fig. 3 and contents in the introductory sections of the manuscripts. The third author contributed to plant materials used for Fig. 3. The fourth and fifth authors contributed to all aspects of the presented work.

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Conflicts of interest statement

None declared.

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