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Arabidopsis BUPS-ANX Receptor Complex Coordinates with RALF Peptides to Regulate Pollen Tube Integrity and Sperm Release

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Arabidopsis BUPS-ANX Receptor Complex Coordinates with RALF Peptides to Regulate Pollen Tube Integrity and Sperm Release

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To my grandparents and parents

Supervisor's Foreword

In angiosperms, fertilization relies on extensive cellular coordination to deliver the two immotile sperm cells as passive cargo by the pollen tube that grows from the papilla cells of the stigma through the various pistil tissues toward the female gametophyte deeply imbedded in ovules. During the long journey of pollen tubes, a number of interesting but important scientific questions caught the attentions of scientists in plant reproduction field, such as how the pollen tubes are recognized by the compatible stigma, how pollen tubes are guided to target the ovule, and so on. Among these scientific questions, how a pollen tube maintains its integrity when it grows and when and how it ruptures to release the two sperm cells to realize fertilization are among the top questions that people have been eager to answer for long. Dr. Zengxiang Ge's thesis was to try to answer this long-pursuing question. In his thesis, Dr. Ge provides evidence to demonstrate that a pollen tube-localized BUPS1/2–ANX1/2 heteromer receptor complex coordinates with pollen tube-secreted autocrine peptide signals RALF4/19 and female tissue-secreted paracrine peptide signal RALF34 to control the integrity maintenance and rupture of pollen tubes for sperm release in Arabidopsis ovules. This model has solved not only the problem that pollen tubes maintain its integrity when they grow by identifying the two autocrine signals and their receptor complex but also the question of when and how the pollen tubes rupture to release sperm cells by introducing another signal player, a female tissue-secreted paracrine signal.

Dr. Ge's research results, as presented here, have established a basic framework for regulatory mechanism of pollen tube integrity maintenance and sperm release control, which further promotes the comprehensive understanding of the multilayer signaling during plant fertilization process.

Beijing, Republic of China
September 2018

Prof. Li-Jia Qu, Ph.D.

Abstract

In flowering plants, the pollen tube is emerged from pollen grains and grows to deliver the immotile sperms to the egg, during the journey of which it communicates with several types of the female reproductive tissues and cells. The integrity of a pollen tube needs to be maintained during its long journey to the ovule but also needs to be timely ruptured in the female gametophyte to release the two sperm cells to achieve successful fertilization. This process to maintain or not to maintain the pollen tube integrity is controlled by complex cell-to-cell communication mediated by extracellular signaling molecules interacting with receptors at the pollen tube surface. However, up-to-date only two CrRLK1L (*Catharanthus roseus* receptor-like kinase) receptors, ANX1 and ANX2, are reported to be involved in pollen tube integrity control in Arabidopsis.

In this book, through whole-genome transcriptome analysis of Arabidopsis CrRLK1L family members in different tissues, we found two CrRLK1L members, which are designated as *BUPS1* (Buddha's Paper Seal l) and *BUPS2* that showed pollen grains/tubes-intensive expression patterns. Both BUPS1 and BUPS2 proteins were expressed in pollen grains/tubes and localized at the apical plasma membrane of pollen tubes. Both the *bups1* single mutants and *bups1 bups2* double mutants, generated by CRISPR/Cas9 technology, exhibited severely compromised male transmission. Pollen tubes of *bups1* burst at short length while those of *bups1 bups2* ruptured immediately upon germination *in vitro*. Most mutant tubes could not penetrate beyond the style *in vivo*; some burst in the style or at the top of the ovary locule. Interestingly, we detected physical interactions between the ectodomains of BUPS1/2 and those of ANX1/2 in a yeast two-hybrid assay and a Co-IP assay.

Meanwhile, we provided evidences that two pollen-specific Rapid Alkalinization Factor (RALF) peptides, RALF4 and RALF19, serve as the signal ligands for BUPS1/2 and ANX1/2. The *ralf4 ralf19* double mutant exhibits similar pollen tube phenotypes to those of *bups1 bups2* and *anx1 anx2* both *in vitro* and *in vivo*. RALF4/19 directly interacted with the ectodomains of both BUPS1/2 and ANX1/2 in the pull-down assays. The Microscale Thermophoresis (MST) analysis also

indicated that RALF4/19 exhibited high affinity to the ectodomains of these receptors.

Furthermore, we found that a female tissue-derived RALF peptide, RALF34, that could induce pollen tube burst at nanomolar concentrations. RALF34 peptide is the closest homolog of RALF4/19, but it shows high expression level in ovules. Interestingly, we found that RALF34 peptide was able to compete with RALF4/19 for the interaction with BUPS1/2–ANX1/2 receptor complex.

Therefore, we propose that BUPS1/2 and ANX1/2 form heteromer receptor complex in the membrane of pollen tube tips to perceive the autocrine signals, RALF4 and RALF19, to maintain the integrity of the pollen tube during its long journey to the ovule. When the pollen tube reaches the female gametophyte, paracrine signal RALF34 is secreted to compete with and replace RALF4/19 at the interface of pollen tube–female gametophyte contact, thereby deregulating BUPS–ANX signaling pathway and leading to pollen tube rupture and sperm release. This book illustrated a precise spatiotemporal regulation mechanism underlying the control of pollen tube integrity and sperm release and filled a major gap in our understanding of plant reproductive process.

Parts of this book have been published in the following articles:

Ge, Z., Bergonci, T., Zhao, Y.L., Zou, Y.J., Du, S., Liu, M.C., Luo, X.J., Ruan, H., Garcia-Valencia, L.E., Zhong, S., *et al*. (2017). *Arabidopsis* pollen tube integrity and sperm release are regulated by RALF-mediated signaling. ***Science*** 358, 1596–1599.

Ge, Z., Cheung, A.Y., and Qu, L-J. (2019). Pollen tube integrity regulation in flowering plants: insights from molecular assemblies on the pollen tube surface. ***New Phytol***. 222, 687–693.

Ge, Z., Dresselhaus T., and Qu, L-J. (2019). How CrRLK1L receptor complexes perceive RALF signals. ***Trends Plant Sci.*** 24, 978–981.

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Finally, I want to express my special thanks to my family, for their understanding and love in life.

Contents

Abbreviations

3-AT	3-amino-1,2,4-triazole
Ade	Adenine
AS	Acetosyringone
bp	base pair
CA-630	Octylphenoxypolyethoxyethanol
cDNA	Complementary DNA
CTAB	Cetyl trimethyl ammonium bromide
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Distilled deionized water
DMSO	Dimethylsulfoxide
dNTP	Denucleotide triphosphate
DTT	DL-Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
GFP	Green fluorescent protein
GUS	β-glucuronidase
His	Histidine
LB	Luria-Bertani media
hr	Hour
Leu	Leucine
MES	2-(*N*-Morpholino) ethanesulfonic acid
min	Minute
mL	Milliliter
mm	Millimeter
MS	Murashige–Skoog
OD	Optical density
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	1,4-piperazine ethyl sulfonic acid
PMSF	Phenylmethanesulfonyl fluoride

rpm	Round per minute
SDS	Sodium dodecyl sulfonate
sec	Second
TEMED	N,N,N′,N′-Tetramethylethylenediamine
Tris	Trihydroxymethyl aminomethane
RT-PCR	Reverse transcriptase polymerase chain reaction
X-gluc	5-Bromo-4-chloro-3-indolyl-gulucuronide
μL	Microliter
μm	Micrometer

Chapter 1
Review of Cell–Cell Communication in Plant Reproduction

Abstract Unlike animals, the angiosperm gametes are emerged in the multicellular gametophytes, which are produced from meiotic products through multiple mitotic divisions. The non-motile sperm cell should be delivered to the female gamete via a pollen tube during the process of sexual reproduction. During this journey of the pollen tube to the female gametophyte, a series of events happen, including activation and germination of pollen grains on the stigma, polar tip growth of pollen tubes in the transmitting tract, guidance of pollen tubes to the ovules, perception of pollen tubes by the female gametophyte, and pollen tubes rupture to release the sperm cells for the double fertilization. In general, the tube cell interacts and communicates with more than seven types of tissues/cells; thereby the smooth going of this long journey depends on complex signal exchanges and coordinated communications between male and female tissues/cells in each step. In particular, peptide signals and their corresponding receptor-like kinases are found to play important roles in mediating the male/female communication. In recent years, more and more molecular mechanisms of the male/female interactions have been uncovered and a number of important advances have been made, which will be mainly introduced and discussed in this review.

1.1 Overview of Plant Reproduction

Life cycle of angiosperm includes a diploid generation and a haploid generation, which alternates with each other to promote the evolution of life. In the haploid generation, male and female gametophytes are generated from the microspore and megaspore, respectively, both of which are developed from the sporophyte via meiosis. After fertilization process when the male gamete and the female gamete meet and fuse with each other, the diploid generation is initiated. Therefore, the fertilization process is the critical step for the switch from the haploid generation to the diploid generation. Flowering plants adopt a characteristic fertilization manner; that is, double fertilization, in which one sperm cell fuses with an egg cell to produce embryo while the other sperm cell with a central cell produces the triploid endosperm [1, 2].

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In animal fertilization process, sperm could swim on their own to find and target eggs after being released from the father. However, compared with animals, the male gametes (i.e., sperm cells) and female gametes (i.e., the egg cell and the central cell) of higher plants are deeply emerged in pollen grains and ovules, respectively, which are spatially separated. Neither the sperm cell nor the female gamete could move autonomously. Therefore, after a long period of evolution, flowering plants invent the pollen tube that germinated from the pollen grain as a vehicle to deliver the two sperm cells as its cargoes. To achieve the delivery of the sperm cells, pollen undergoes a series of events, including pollen adhesion, hydration, and germination on the stigma, pollen tube growth in the female tissues, pollen tube guidance to the ovules, and pollen tube reception. After arriving at the ovule, the growth of the pollen tube is arrested followed by pollen tubes discharge to release the cargo, sperm cells, for double fertilization with female gametes (Fig. 1.1) [3–5]. In general, during this long journey, the tube cell interacts and communicates with more than seven types of tissues and cells, that is, the papilla cells on the stigma, the style tissue, the transmitting tract, the septum, funiculus and micropyle of ovules, and synergid cells [6]. In recent years, the molecular mechanism of the female/male interactions has been extensively studied and understood, and a series of important advances have been made, which will be introduced and discussed in the following context according to the biological phase involved.

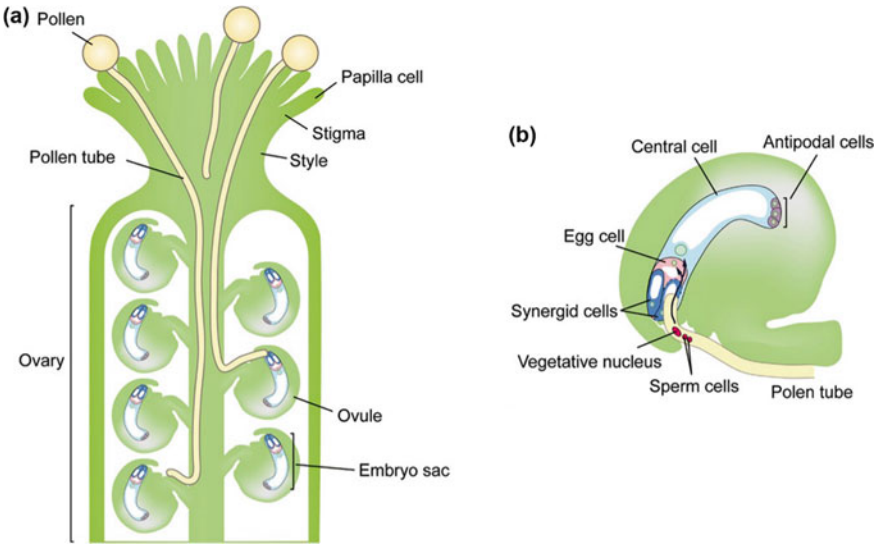

Fig. 1.1 The fertilization process of Arabidopsis. Pollen tubes are generated from mature pollen grains after being released and landed on the stigma, and undergo tube cell growth, guidance, and reception in/by the female tissues/cells. The pollen tube finally reaches to the female gametophyte and burst to release two sperm cells for double fertilization. Reprinted with modifications from Kurihara et al. [3] @ John Wiley and Sons. Reprinted by permission

1.1.1 Interaction Between Pollen and the Stigma

After landing on the stigma, pollen grains could germinate to form pollen tubes only when appropriate interactions take place between pollen grains and the stigma during pollen adhesion, hydration, and germination. According to the secretion on the stigma surface, flowering plants can be divided into two groups, plants with wet or dry stigma. In the wet stigma plants, some types of lipids, proteins, and carbohydrates are reported to be enriched on the stigma, which could generally promote the adhesion of pollen grains to the stigma [7, 8]. However, the stigma of Arabidopsis is a typical dry stigma in which pollen adhesion depends on the outer wall of pollen grains. In the later stage of pollen adhesion, pollen grains interact and communicate with papilla cells, and this communication is very important for the connection between pollen and the stigma. For example, the crosstalk during pollen adhesion determines whether the self-pollen grains are recognized in Arabidopsis and Brassica. In some species from Brassicaceae, it even initiates the self-incompatibility process [9].

In general, mature pollen is often dehydrated, with the water content from 15 to 35%. For this reason, pollen germination occurs only when pollen hydration is initiated. During the hydration process, the overlay on pollen surface flows upon pollen grains land on the stigma, inducing the formation of a special area with enriched proteins and lipids, which is further beneficial for pollen hydration.

Several proteins have been reported to control pollen adhesion, hydration, and germination. For example, ECERIFERUM 1/3/6 (CER1/3/6) from CER family are mainly involved in the synthesis of long-chain fatty acids which are the main components of the pollen outer wall. In Arabidopsis, *cer1*, *cer3*, and *cer6* mutants produce wax-free pollen grains which could be properly adhered to the stigma but fail in pollen hydration and germination [10, 11]. Moreover, glycine-rich protein 17 (GRP17) is also reported to coordinate with extracellular lipase 4 (EXL4) in the control of pollen hydration via affecting the lipid composition in the pollen/stigma contacting area, which further promotes the transfer of moisture from the stigma to pollen grains [12, 13].

1.1.2 Pollen Tube Growth in the Transmitting Tract

After interacting with the stigma, pollen grains germinate to form pollen tubes which will penetrate the style and then enter the transmitting tract. After receiving the attraction signal, the pollen tube will change its direction of growth and be guided to the female gametophyte (Fig. 1.1). Similar to root growth, the growth of pollen tubes in maternal tissues is a typical kind of the polarized growth. It depends on the gradient distribution of certain proteins or small molecules in female tissues/cells. Up to now, some substances, such as TTS glycoprotein, SCA peptide, chemocyanin peptide, GABA, Ca^{2+}, and D-serine, have been identified as regulators during pollen tube growth.

TTS gene is specifically expressed in transmitting tract and encodes a proline-rich glycoprotein which belongs to the arabinogalactan protein (AGP) family in tobacco. In sexual reproduction, TTS serves as an adhesive substrate and could bind to the surface of the pollen tube tip to stimulate its growth from the stigma to the ovary. Besides, TTS was shown to have the ability to attract pollen tubes. When *TTS* was knocked down through RNAi technology, the rate of pollen tube growth slows down, thus leading to a decrease in seed production. Moreover, it is found that the glycosylation of TTS increases from the tip of the stigma to the end of the ovary in the transmitting tract, which is consistent with the direction of tube growth, and pollen tubes could directly de-glycosylase TTS. Therefore, the gradient of TTS glycosylation is supposed to participate in mediating the pollen tube growth in vivo [14, 15].

Stigma/stylar cysteine-rich adhesin (SCA) is a small peptide containing eight conserved cysteine residues and involved in the adhesion of pollen tubes to the transmitting tracts. Immunolabeling assay has uncovered that SCA could bind to macromolecular polysaccharides in a pH-dependent manner, which in turn induces pollen tubes to adhere to the artificial style matrix [16, 17]. Chemocyanin is a 9.9 kDa small peptide accumulated in the lily stigma and is able to induce pollen tube growth. Although SCA has no activity in pollen tube attraction, it could significantly improve the attraction efficiency when mixed together with chemocyanin [18]. Moreover, Arabidopsis plantacyanin is the homologous protein of chemocyanin and is also thought as important for the pollen tube growth and pollen tube guidance [19].

The γ-aminobutyric acid (GABA) is a signal molecule to mediate the pollen tube growth and pollen tube guidance. In Arabidopsis pistil, the concentration of GABA exhibits an increasing gradient from the style to the micropyle. In the *pop2* mutant, GABA could not be degraded, and pollen tubes show defect in tubes growth and guidance [20]. Further transcriptome analysis found that POP2 and GABA control pollen tube growth by affecting the expression of cell wall-related proteins and secreted proteins [21]. In addition, calcium concentration has also been revealed to manipulate the pollen tube growth, and the concentration of Ca^{2+} is closely related to D-serine which is a key activator of glutamate receptor-like channels (GLRs). D-serine can activate GLRs to promote Ca^{2+} influx, which consequently maintains the normal growth of Arabidopsis pollen tubes [22, 23].

1.1.3 Pollen Tube Guidance

After finishing tip growth in the transmitting tract, pollen tubes turn to and climb onto the funiculus, and then enter the embryo sac through the micropyle. This process is called pollen tube guidance, which can be divided into two stages: the funicular guidance and the micropylar guidance. Funicular guidance refers to the process of pollen tubes emerging from septum surface and moving onto the funicular surface, while micropylar guidance means that the pollen tube penetrates micropyle after finishing its growth on the funicular surface [24].

Pollen tube guidance is coordinately mediated by many tissues or cells, such as the sporophyte, pollen tubes, and the female gametophyte. For example, in the funicular guidance, plants lacking inner no outer (INO) protein was defective in ovule development and pollen tubes targeting to the funicular, indicating diploid sporophytes play crucial roles in the pollen tube guidance [25]. MPK3/6 kinases also function in the funicular guidance in that *mpk3 mpk6* pollen tubes fail to climb to the funicular in vivo, but could target ovules normally in the semi-in vitro ovule targeting assay [26]. CHX21/23 belong to the K^+ transporter protein family and are localized to the endoplasmic reticulum in the pollen tube. Pollen tubes of *chx21 chx*23 double mutant are incapable of turning toward ovules in Arabidopsis [27]. Furthermore, COBL10 is a glycosylphosphatidylinositol (GPI)-anchored protein (GPI-AP) expressed in pollen tubes and is critical for pollen tube growth in the funicular guidance [28, 29].

After the funicular guidance, the pollen tube climbs up the funicular, moves along the surface toward the micropyle, and finally enters the ovule. This is the micropylar guidance. In recent years, molecular mechanism of the micropylar guidance has been well studied and many key components have been identified. Synergid cells are assumed to play a major role in the micropylar guidance since that the ovule without synergid cells which were removed simultaneously by laser completely lost its ability to attract pollen tubes in *Torenia fournieri* [30]. In the synergid, a R2R3-MYB-type transcription factor MYB98 is exclusively expressed and directly involved in regulating micropylar guidance. In the *myb98* mutant, the ovules show defect in filiform apparatus development, resulting in that the pollen tube fails to enter the micropyle. Further studies uncovered that MYB98 regulates pollen tube guidance via regulating the expression of pollen tube attractants [31].

Egg Apparatus 1 (ZmEA1) peptide, which is the first attractant identified to be responsible for micropylar guidance in maize, is highly expressed in the female gametophyte but drastically decreases after fertilization. Ovules with down-regulation of *ZmEA1* lost the function to attract pollen tubes [32]. In *Torenia fournieri*, defensin-like cysteine-rich peptide (CRP) LUREs function as synergid cell-secreted attractants, which show activity in pollen tubes attraction in vitro. Another group of peptides AtLURE1 are pollen tube attracts in *Arabidopsis thaliana*, and are specifically expressed in the filiform apparatus of synergid cells and could be secreted to the micropyle [33, 34]. The receptor of AtLURE1 was recently identified as PRK6, which is a plasma membrane-localized receptor-like kinase. The genetic experiment demonstrated that the pollen tube of *prk6* completely lost the ability to response to AtLURE1 peptides. It is proposed that PRK6 receptor interacts with AtLURE1 via the extracellular domain, and with LIP1/2, a receptor-like cytoplasmic kinase (RLCK) in the pollen tube, via its intracellular domain to coordinate the pollen tube guidance [35–37]. In addition, MDIS1/2 and MIK1/2 in pollen tubes have also been reported to be involved in the perception of AtLURE1 signals [24, 38].

In addition to the components above, pollen tubes endoplasmic reticulum protein POD1 and calcium ion channel CNGC18 are also involved in the process of pollen tube guidance [24, 39–42]. Besides, central cell guidance (CCG) protein is a transcription factor in central cells and 41% of *ccg* ovules exhibit defects in pollen tube

attraction, suggesting that the central cell is directly involved in the pollen tube guidance. Recent studies further showed that CCG interacts with CCG binding protein 1 (CBP1), forming a transcription complex, to regulate the expression of some peptides, thereby controlling the micropylar guidance [43, 44].

1.1.4 Pollen Tube Reception

After the funicular and micropylar guidance, the pollen tube successfully arrives at and enters the micropylar. Subsequently, the pollen tube stops its growing and bursts to release two sperm cells. This process is called pollen tube reception, in which the pollen tube interacts with many female tissues and cells. To date, regulators involved in the pollen tube reception include FERONIA/SIRENE (FER/SRN), ENODL14 (EN14), EN15, LOELEEI (LRE), NORTIA (NTA), ANXUR1 (ANX1), ANX2, and MYB97/101/120.

FER/SRN is a receptor-like kinase belonging to CrRLK1L family and is widely expressed in *Arabidopsis*. In reproductive tissues, FER/SCN accumulates asymmetrically in the filiform apparatus of synergid cells and mainly mediates pollen tube reception as an important receptor in the ovule. In *fer* mutant, the pollen tube could be normally guided to the ovule but failed to arrest its growth, resulting in overgrowing pollen tubes in the embryo sac [45, 46]. Given that FER is a membrane-localized receptor, it is likely to recognize a certain signal released from the pollen tube to activate the downstream signaling in the female gametophyte to cease pollen tube growth. Unfortunately, this signal has not been identified yet. Similar to the phenotype of *fer* mutant, *en14 en15*, *lre* and *nta* mutants are defective in pollen tube reception as well. GPI-APs EN14/15 and LRE accumulate at the filiform apparatus of synergid cells and are thought to regulate pollen tube reception through interacting with FER [47–49]. NTA, belonging to the plant-specific Mildew resistance Locus O (MLO) family, is mainly localized in the cytoplasm of synergid cells before fertilization, but the distribution of NTA changes to the filiform apparatus, which is dependent on FER [50].

In pollen tubes, MYB97, MYB101, and MYB120, three R2R3-MYB transcription factors, have been found to participate in the regulation of pollen tube reception as well. *myb97 myb101 myb120* triple mutant is defective in pollen tube reception, with the pollen tubes fail to stop and burst to release sperm at the synergids, which is similar to the phenotype of *fer* mutant [45, 46, 51, 52]. Besides, MYB97/101/120 are assumed to participate in pollen tube reception through regulating the expression of some downstream signaling molecules [51]. In addition, ANX1/2 are the homologs of FER but specifically accumulate on the cell membrane of pollen tubes. *anx1 anx2* pollen grains germinate normally both in vitro and in vivo, but the pollen tubes burst immediately after germination, leading to severe defect in plant fertility [53, 54]. Given that ANX1/2 belong to the receptor-like kinase family, they are believed to act as the receptors in the pollen tube to perceive some signal cues to maintain the

cell integrity of pollen tubes or trigger pollen tube rupture, the mechanism of which is not clear yet.

1.1.5 Male and Female Gametophyte Fusion

After entering the ovule and penetrating the synergid, the pollen tube stops and bursts to release two sperm cells, which will fuse with the egg and the central cell, respectively, to produce the zygote and the endosperm. This process mainly includes cell migration, gamete recognition and attachment, gamete activation and fusion. Until recently, it is unclear what is happening to the gametes during this process. In *Arabidopsis*, a group of cysteine-rich peptides EC1 could be secreted from the egg cell and have been predicated to control gamete fusion by triggering sperm cells activation. After knocking down of the expression of *EC1*, the fusion between the male/female gamete was severely disturbed, leading to the abolishment of seed production. The delivery of sperm is able to induce EC1-GFP to distribute extracellularly, suggesting that exocytosis of EC1 is actively triggered. Besides, exogenously synthesized EC1 peptides could change the distribution of HAPLESS 2 (HAP2) from the endomembrane system to the cell membrane of sperm cells [55]. HAP2 is a membrane protein and predominantly localized to the sperm endomembrane. In the *hap2* mutant, the sperm cell can be normally delivered to the ovule, but the fertilization with the egg/central cell was completely blocked [56, 57]. Therefore, it is thought that EC1 peptides coordinate with HAP2 to regulate the gametes interaction after being recognized on the sperm cell surface.

Furthermore, GEX2 that is mainly localized to the plasma membrane of sperm cell and GLC that is expressed in the female gametophyte are also involved in the male/female gamete fusion process. It was demonstrated that *gex2* showed defects in the cell adhesion between the sperm cell and the egg cell or the central cell, further leading to the abnormal gamete fusion. In contrast, in the *glc* mutant, sperm cells could fuse with the egg cell successfully but failed to fuse with the central cell, which is caused by the absence of acyltransferases in the central cell [58, 59].

1.2 Plant Peptides and RLKs-Mediated Signaling in Plant Fertilization

Small peptide, which is derived from the Greek "πεπτο" and means "digestible", is the smallest protein with a size between 2 and 100 amino acids in plants [60]. Plant peptides can be divided into secreted peptides and non-secreted peptides, of which secreted peptides are further divided into cysteine-rich peptides (CRPs) and non-cysteine-rich peptides (NCRPs). Most NCRPs are post-translationally modified peptides with the sizes of 5–20 amino acids, which are mainly released from the

precursor through protease-mediated cleavage and require the processing of protein modification. In contrast, although some CRPs can also be processed from the precursors, almost all CRPs do not require post-translational modification, and their size is generally 20–100 amino acids. The typical characteristic of CRPs is that mature peptides generally contain 4–16 relatively conserved cysteine residues, and disulfide bonds will be formed between them to keep the correct protein conformation [5, 60, 61].

During the signal transduction, small peptides are secreted extracellularly and can bind to the extracellular domain of a specific receptor which is localized on the membrane of the neighboring cell. Most receptors identified up to now are receptor-like kinases which are transmembrane proteins with an extracellular domain, transmembrane domain, and intracellular kinase domain [60, 62].

Plant peptides and their corresponding receptors often work as important signal regulators to mediate cell–cell communication and are widely involved in many biological processes, such as growth, development, reproduction, and response to stresses [63–66]. In particular, some small peptides and receptor-like kinases have been found to play important roles in the male/female crosstalk during fertilization. In addition to AtLURE1-PRK6-LIP1/2 and AtLURE1-MDIS/MIK complexes mentioned in Sect. 1.1.3, some other receptor-ligand pairs have been identified as shown in Fig. 1.2.

During the interaction between pollen and the stigma, S-locus protein 11 (SP11) peptide is secreted from pollen and acts as a signal molecule to initiate the response of self-incompatibility. SP11 peptide is a CRP with eight conserved cysteine residues and can bind to S-locus receptor-like kinase (SRK) specifically [67, 68].

LAT52 is a tomato pollen-specific peptide and identified as an important signaling molecule to regulate pollen germination and pollen tube growth. It could bind to the extracellular domains of LePRK1/2 that are specifically expressed in pollen. In addition, STIG1 peptide that is specifically expressed in the stigma and style can compete with LAT52 for the binding of receptor LePRK1/2 to promote the elongation of pollen tubes [69–71].

Owing to the limitation of current technologies, only a few receptor/ligand pairs were identified. Some peptides or RLKs have been reported to function importantly in plant fertilization, but their corresponding receptors or ligands remain unclear. For example, ZmES1–4 peptides and EA1 peptide function as the signals for pollen tubes burst and pollen tube guidance, respectively, in maize while EC1 for gamete activation/fusion in Arabidopsis [32, 55, 72]. As for receptors, CrRLK1L member ANX1/2 and FER function importantly in pollen tube growth and reception. As mentioned in Sect. 1.1.4, ANX1/2 might perceive the signals from pollen to maintain the cell integrity of pollen tubes or from the female tissues to trigger the burst of pollen tubes, and the signal of FER during pollen tube reception might be from pollen [45, 46, 54, 73]. Although the signals of CrRLK1L receptor in the pollen tube reception remains elusive, it is showed that RALF peptides could act as signal ligands of FER receptor to regulate root cells development and plant immunity [74, 75]. Among 40 RALF peptides which are ubiquitously expressed in Arabidopsis [62, 76–78], are there any RALF peptides that act as signals for ANX1/2 to regulate the cell integrity

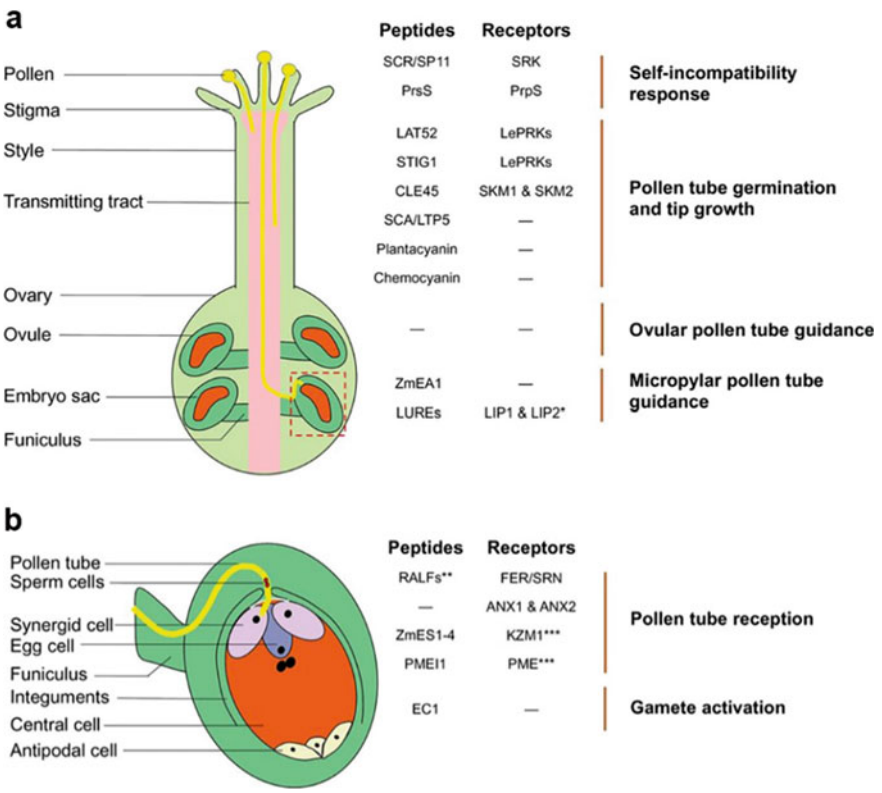

Fig. 1.2 Small peptides and receptors during plant fertilization. Reprinted from Qu et al. [5] @ Oxford University Press. Reprinted by permission

of pollen tubes? Is there a RALF that interacts with FER to regulate the growth of pollen tubes? These questions need to be solved in further studies.

1.3 Summary and Perspective

As an important process in the life cycle of angiosperm, plant fertilization has been well studied during the past decade, and significant processes have been made in understanding the signal communication and crosstalk between male and female tissues/cells. A number of key regulators, such important membrane-bound receptors or secreted signaling molecules, are uncovered. However, some gaps still exist and some important work should be done in the future, such as: (1) screening the ligands or receptors of known receptors or signals, (2) exploring the downstream signaling of key ligand/receptor modules, (3) identification of novel peptide/receptor pairs-mediated signaling pathway, and (4) exploring more components involved

in plant fertilization. In addition, introduction of advanced technologies, such as CRISPR/Cas9, single-cell sequencing, live imaging, and in vivo protein tagging into the plant reproduction field will greatly promote the understanding of molecular mechanisms controlling fertilization in the long term.

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Chapter 2
Receptor-Like Kinases BUPS1/2 are Involved in Pollen Tubes Integrity Maintenance in Arabidopsis

Abstract Receptor-like kinases have been reported as important components in the cell–cell communications during plant reproduction. In this chapter, we mainly focused on two previously unreported receptors BUPS1 and 2 from CrRLK1L family and uncovered their novel functions in controlling pollen tubes integrity. Through the RNA-seq analysis and the GUS staining, *BUPS1/2* genes were found to be highly expressed in mature pollen grains/tubes, the encoded proteins of which were further confirmed in pollen tubes by GFP-fused reporter assays. To explore the biological function of BUPS1/2, CRISPR/Cas9 technology was used to knock out *BUPS1/2* and a series of mutants of different combinations were generated. Phenotypical analysis revealed that *bups1* single mutants and *bups1 bups2* double mutants showed severe defects in plant fertility. By performing alternative crosses and reciprocal crosses, we found that the fertility defects of *bups1* and *bups1 bups2* were caused by the aborted male transmission efficiency. Pollen grains of these mutants developed normally based on SEM observations and Alexander staining; however, the pollen tubes completely arrested their growth and lost the cell integrity both in the in vitro pollen germination and Aniline blue staining assays, which resembles the phenotype of *anx1 anx2* double mutant. Yeast two hybrid assays and Co-IP experiments were thus conducted, showing that BUPS1/2 directly interacted with ANX1/2 via the ectodomains. Taken together, these results indicate that BUPS1/2 possibly form a receptor complex with ANX1/2 in the pollen tubes to control the integrity of tube cells in Arabidopsis.

Keywords Receptor-like kinases · CrRLK1L receptors · Pollen tubes integrity · Polar tip growth

2.1 Introduction

In order to deliver the sperm cells for fertilization in angiosperms, a pollen tube makes a long journey to the female gametophyte by interacting and communicating with several types of tissues/cells [1, 2]. Therefore, maintaining the cell integrity of pollen tubes during the tip growth in maternal tissues until the pollen tubes arrive at

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the female gametophyte to trigger the tube discharge sperm is essential for successful sperm delivery and fertilization. The entire process is mediated by complex signal dialogues.

Plant peptides and receptor-like kinases are key components of cell–cell communications during plant growth, development, and reproduction processes [3–8]. Up to now, many plant peptides and receptors have been found to play a leading role in the communications between male and female tissues [2, 6, 9]. For example, FER and ANX1/2 receptors have been well reported to regulate pollen tube reception and rupture (see Chap. 1). FER is ubiquitously expressed except in pollen and is critical for plant growth in that *fer* mutant displays premature bursting of root tips and disordered stomata distribution in the leaf. FER is also essential for successful fertilization because wild-type pollen tubes could be guided to the ovules but fail to stop and burst in *fer* mutant ovules [10–12]. ANX1 and ANX2 are intensively localized on the membrane and function redundantly in pollen tube. *anx1 anx2* double mutant is male sterile, with the pollen tubes burst upon germination, which leads to the premature release of sperm cells before pollen tubes reach the female gametophyte [13, 14].

Both ANX1/2 and FER belong to the *Catharanthus roseus* RLK1-like (CrRLK1L) receptor family, which have been well established as key membrane-localized receptors to control the cell integrity and cell growth in various tissues [8, 15, 16]. In addition to these three receptors, ERULUS was found to control the tip growth of root hairs by maintaining cytoplasmic Ca^{2+} levels and pH gradients, HERCULUS1 (HERK1), HERCULUS2 (HERK2), and THESEUS (THE1) to function in the cell elongation control in adult leaves, and CURVY1 (CVY1) to regulate pavement cells morphogenesis, flowering time, and seed production [8, 17–19].

There are 17 members in CrRLK1L family in Arabidopsis and five members are highly expressed in male or female tissues, of which FER, ANX1, and ANX2 have been well studied [8, 15, 16, 20]. However, little is known about the other two members AT4G39110 and AT2G21480 (named as BUPS1 and BUPS2, respectively, in this book), the genes of which are also highly expressed in pollen grains and tubes in Arabidopsis [15, 20]. Here, we mainly focused on these two receptors and determined their functions in pollen tubes integrity control.

2.2 Materials and Methods

2.2.1 Plant Material

Arabidopsis thaliana (Columbia-0) was used as wild-type. All transgenic lines used in this study were in the Columbia ecotype;
Nicotiana benthamiana was used for transient expression of the ectodomains.

2.2.2 Strains and Plasmids

2.2.2.1 Strains

Agrobacterium tumefaciens GV3101: used for Arabidopsis and tobacco transformation;
E. coli: used for plasmid transformation in this laboratory;
Yeast NMY51: used for dual membrane Y2H.

2.2.2.2 Plasmids

pK7FWG0: modified from pK7FWG2 (Department of Plant Systems Biology, VIB-Ghent University, Ghent, Belgium) and used for GFP-tagging;
pB7GUSWG0: modified from pB7WGR2 (Department of Plant Systems Biology, VIB-Ghent University, Ghent, Belgium) and used for GUS report system;
pCBC-DT1T2 and pHEE401E: gifts from Prof. Qijun Chen and used for CRISPR/Cas9 construction;
pMetycgate-GW and pPR3N-GW: GATEWAY-compatible destination vectors in this laboratory for dual membrane Y2H;
BA-MYC and pB7FLAGWG2: GATEWAY-compatible destination vectors in this laboratory for Co-IP construction.

2.2.3 Chemicals and Reagents

Anti-Flag-HRP: Sigma-Aldrich, A8592-2MG;
Goat Anti-Mouse IgG-HRP: CWBIO, CW0102S;
Anti-Myc: CMCTAG, AT0023;
3xFlag Peptide: Sigma, F4799-4 MG;
SuperSignal@West Femto Maximum Sensitivity Substrate: Thermo Fisher, 34095;
Copmplete Protease Inhib. Cocktail: Roche, 11836145001;
Anti-c-Myc affinity gel: Sigma, E6654-1 ML;
Anti-Flag affinity gel: Bimake, B23101.

2.2.4 Methods

2.2.4.1 Plant Growth Condition and Plant Transformation

Arabidopsis thaliana and tobacco plants were grown under long-day conditions (16 h light/8 h dark cycles) at 22 ± 2 °C with a light intensity of ~170 μmol/m^2/s using LED bulbs (Philips F17T8/TL841 17 W) [21].

2.2.4.2 Phylogenic Analysis and Transcription Assay

The heat-map of transcription profile was drawn based on the RNA-seq data. RNA isolation, high-throughput RNA sequencing, bioinformatics, and statistical analysis of the RNA-seq data were performed as previously described [22, 23]. The phylogenetic analysis was conducted using MEGA 5.0 (http://www.megasoftware.net/).

2.2.4.3 Plasmids Construction

Genomic sequences of *BUPS1/2* containing 2 kb promoter sequence and these with the coding sequence were cloned into pB7GUSWG0 and pK7FWG0 for GUS staining assays and GFP reporter assays, respectively.

To knock out *BUPS1* and *BUPS2*, the EPC CRISPR/Cas9 technology was used in this book as previously reported [24, 25]. sgRNA sequences mentioned above are as followed:

BUPS_sgRNA1: TAC ACA CAG GAA CAG CTC ANG G
BUPS_sgRNA2: TAT GCT CTT CAG CTT CAA GNG G

The constructs were transformed into *Agrobacterium tumefaciens* GV3101 and transformed into wild-type (Col-0).

2.2.4.4 Pollen Assays

As for the in vitro pollen germination experiment, pollen germination medium (PGM) (18% sucrose, 0.01% boric acid, 2 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 1 mM $MgSO_4$, pH 7.0, 1.5% low-melting point agarose) was prepared [26, 27]. After pollen grains were dispersed on the medium, the PGM was kept in a humid box for germination at 22 °C for 7 h. As for Alexander staining, mature pollen grains, which were collected from freshly opened flower, were subjected to the Alexander solution directly [28]. The observation was made using a microscope (Olympus BX51, Japan) or a spinning disc confocal microscopy (Zeiss Cell Observer SD, Zeiss, Germany). In the SEM, a scanning electron microscope (Hitachi S3000N, JOEL JSM-6610) was used for the observation directly.

Aniline blue staining was used for in vivo PT growth assays [29]. To be more specific, siliques after 20 h pollination were collected and fixed in acetic acid/ethanol 1:3 for 2 h, then washed in 70, 50, 30% ethanol, and ddH_2O for 10 min each time. Subsequently, the samples were treated with 8 M NaOH overnight, washed with ddH_2O for twice, and stained with Aniline blue solution (0.1% aniline blue, 108 mM K_3PO_4) for more than 2 h. Finally, the observation was taken under a fluorescence microscope quipped with an ultraviolet filter set (Olympus BX51, Japan).

2.2.4.5 Dual-Membrane Yeast Two-Hybrid

Ectodomains of BUPS1/2 and ANX1/2 were cloned into pMetycgate-GW bait vector and pPR3N-GW prey vector, respectively, through TOPO and LR reaction. Different combinations of bait and prey constructs were co-transformed into yeast NMY51 strain by the lithium acetate transformation method according to the manufacturer's procedure (DualmembraneBiotech) and grown in SD-Trp/Leu medium for 48 h. The positive clones were transferred to the medium-stringency selective medium (-Trp/Leu) and high-stringency medium (-His/Trp/Leu/Ade).

2.2.4.6 Co-IP Experiments

For the generation of Co-IP constructs, the coding sequences of ANX1/2 and BUPS1/2 ectodomain with no stop codon were cloned into pBA-MYC and pB7FLAGWG2, transformed into *Agrobacterium tumefaciens* GV3101, and co-infiltrated into the leaves of *N. benthamiana* with different combinations. After incubation for 60 h, the infiltrated leaves were lysed with the lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 5 mM DTT, 1 mM PMSF, 0.5% NP-40, 1xC-complete protease inhibitor) on ice. The extracts were centrifuged at top speed (17,000–19,000 *g*) for 15 min; the supernatant was transferred into a new tube and centrifuged at top speed for another 15 min. Then the extract was incubated with anti-c-Myc affinity gel (Sigma, E6654-1 ML) at 4°C for 3 h and washed five times with the lysis buffer. Finally, western blot was adopted using anti-Myc (CMCTAG, AT0023) and anti-Flag-HRP (Sigma-Aldrich, A8592-2 MG).

2.3 Results

2.3.1 Expression Pattern and Subcellular Localization of BUPS1/2

In the previous work, our laboratory has performed the RNA-seq analysis of various tissues, including vegetative tissues (seeds, seedlings, and roots) and reproductive tissues (pollen grains/tubes and ovules at different developmental stages) [22]. According to the RNA-seq data, we found that five members in CrRLK1L family were specifically or highly expressed in pollen grains/tubes (Fig. 2.1), of which ANX1 (At3g04690), ANX2 (At5g28680), and CAP1 (At5g61350) have been reported [12, 14, 30, 31], while At4g39110 (named BUPS1 in this book) and At2g21480 (named BUPS2 in this book) have not been well studied yet. RNA-seq analysis showed that *BUPS1* and *BUPS2* were abundantly expressed in mature pollen grains, and the expression level of these two receptors was very high in germinating pollen tubes in vivo. BUPS1 and BUPS2 shared high similarity in both amino acid or nucleic

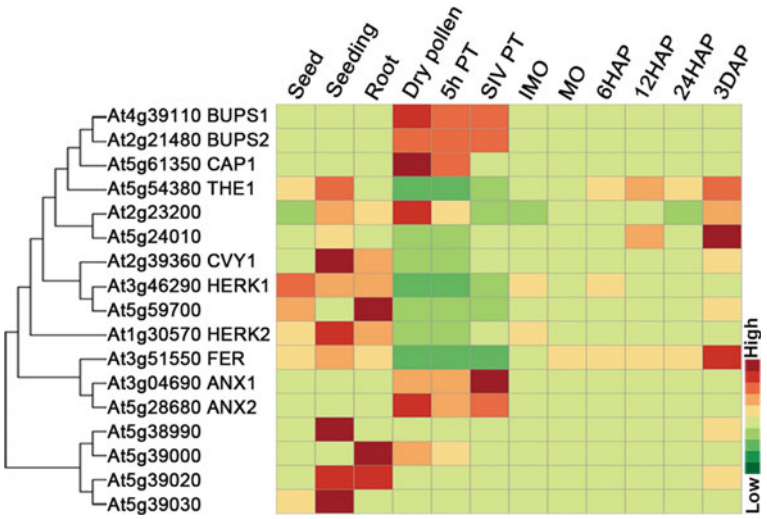

Fig. 2.1 Transcription analysis of CrRLK1L family in different tissues. On the left is the phylogenetic tree of CrRLK1L family and on the right shows the heat-map analysis of the gene expression levels of the CrRLK1L family members based on the RNA-seq data. 5 h PT, pollen tubes germinated in vitro for 5 h; SIV PT, semi-in vivo germinated pollen tubes; IMO, immature ovules; MO, mature ovules; 6HAP, ovules pollinated for 6 h; 12HAP, ovules pollinated for 12 h; 24HAP, ovules pollinated for 24 h; 3DAP, ovules pollinated for 3 days. Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

acid sequences, and they were clustered on the same clade in the phylogenetic tree (Fig. 2.1), implying that these two receptors might function redundantly during pollen tubes growth or in female/male interactions.

In order to verify the expression patterns of *BUPS1/2*, we constructed *BUPS1p::GUS* and *BUPS2p::GUS* expression vectors and generated the corresponding transgenic plants. GUS staining assays revealed that *BUPS1* and *BUPS2* were mainly expressed in flowers at the later stages, especially in mature pollen grains and in vivo germinating pollen tubes (Fig. 2.2). At the same time, we generated transgenic plants harboring *BUPS1p::BUPS1-GFP* or *BUPS2p::BUPS2-GFP* to analyze the subcellular localization of BUPS1 or BUPS2 protein. We found that the fluorescence of BUPS1-GFP fusion protein was mainly localized in mature pollen grains (Fig. 2.3a). In the in vitro germinating pollen tubes, the GFP signal was intensive on the cell membrane (Fig. 2.3b). Consistent with this result, BUPS2 protein was also highly accumulated in pollen grains/tubes (Fig. 2.3c, d). Taken together, these results showed both BUPS1/2 were pollen expressed receptors and mainly localized on the cell membrane of pollen tubes, implying that they might have important biological functions in pollen development or the male/female crosstalk.

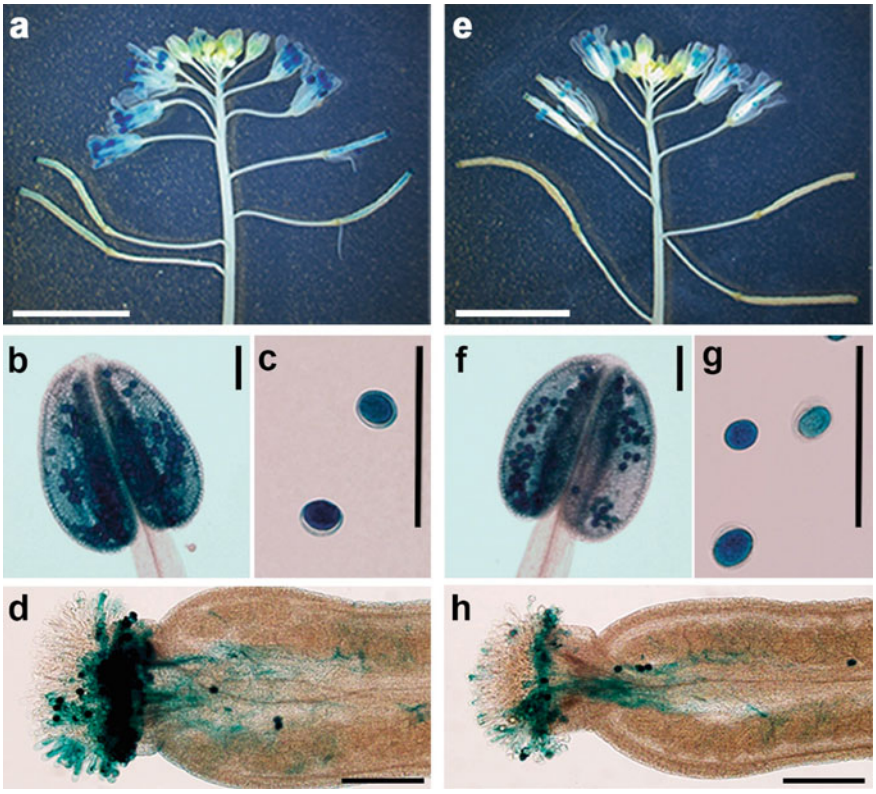

Fig. 2.2 Gene expression patterns of *BUPS1/2* via GUS staining assays. GUS staining was used to study the expression pattern of *BUPS1* (**a**–**d**) and *BUPS2* (**e**–**h**). Different tissues or cells from *BUPS1p::GUS* and *BUPS2p::GUS* transgenic plants, including inflorescences (**a**, **e**), anthers (**b**, **f**), mature pollen grains (**c**, **g**), and pollen tubes in the pistil (**d**, **h**), were observed. Scale bar = 10 mm (**a**, **e**) or 100 μm (**b–d**, **f–h**). Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

2.3.2 Using CRISPR/Cas9 Technology to Knock Out BUPS1/2

Given that BUPS1/2 are highly similar in nucleic acid/amino acid sequences, these two receptors may have similar or redundant function in vivo. To overcome the gene redundancy, we adopted CRISPR/Cas9 technology [24, 25], designed two single-guide RNAs (sgRNAs), and constructed them into one expression vector to knock out the two genes. In more detail, each sgRNA could directly target *BUPS1* and *BUPS2* at the same time, and all the sgRNAs were located in the corresponding coding sequence of the protein kinase domain (Fig. 2.4a, b). This design has two advantages as follows:

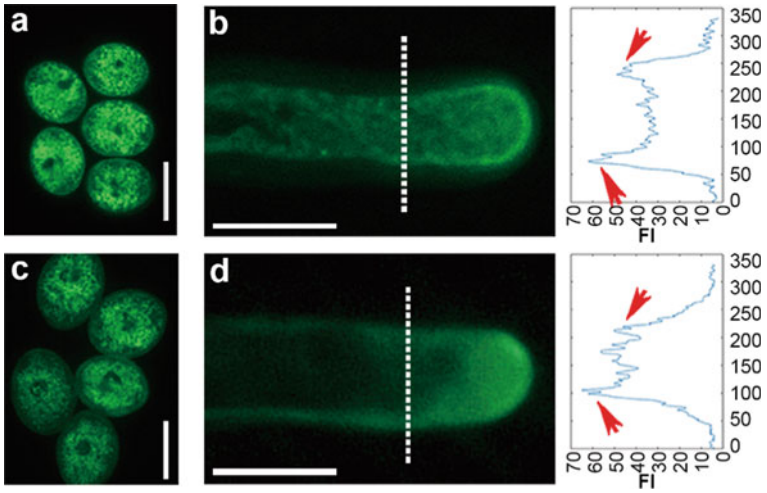

Fig. 2.3 Subcellular localization of BUPS1/2. The subcellular localizations of BUPS1 (**a**, **b**) and BUPS2 (**c**, **d**) were determined by the GFP fuse proteins in mature pollen grains (**a**, **c**) or pollen tubes (**b**, **d**), respectively. Fluorescence intensity (FI) along the dotted line in the pollen tube was shown on the left and the red arrow indicates where the fluorescence signal was enriched. Scale bar = 20 μm (**a**, **c**) or 10 μm (**b**, **d**). Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

1. If these sgRNAs work effectively, double mutants with *BUPS1* and *BUPS2* mutated at the same time could be generated.
2. If two sets of sgRNAs work at the same time, mutations with gene fragments deleted are able to be induced, which would greatly facilitate the identification and screening of mutants in the offspring.

After obtaining the transgenic plants, we amplified the genomic DNA, cloned the DNA fragment harboring the CRISPR/Cas9 targets, and performed Sanger-sequencing to screen the mutant. Several lines with *BUPS1* or/and *BUPS2* mutated at genomic level by the CRISPR/Cas9 technology were obtained. The mutation types include insertion/deletion and large fragment deletions between the two sgRNAs, all of which could result in the disruption of the original transcript and the protein function. To facilitate the genotypic screening and phenotypic analysis in the offspring, we used the mutants with five types of mutation in each gene for further phenotypic analysis (Fig. 2.4c, d).

2.3.3 bups1 *and* bups1 bups2 *Mutant Show Fertility Defect*

After both *BUPS1* and *BUPS2* were knocked out via CRISPR/Cas9 technology, we first analyzed phenotypes of all the single and double mutants during vegetative

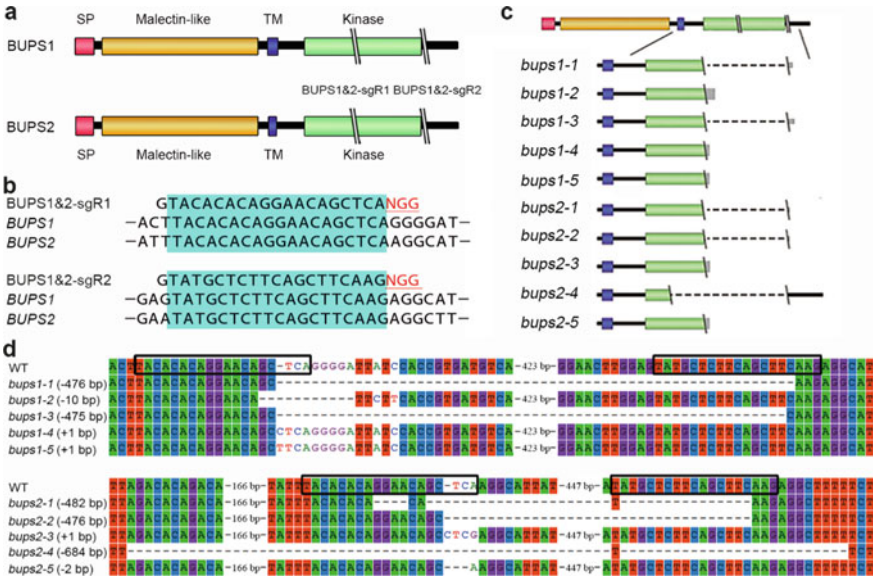

Fig. 2.4 Knocking out *BUPS1*/*BUPS2* via CRISPR/Cas9 technology. **a** The protein structure of BUPS1 and BUPS2. "\\" indicates the position of the sgRNA sequence on the protein structure. SP, signal peptide; TM, transmembrane domain. **b** The sequence alignment of BUPS1 & 2-sgR1 and BUPS1 & 2-sgR2 targets. **c**, **d** The detailed mutations in protein structure (**c**) and nucleic acid sequence (**d**) of *bups1* and *bups2* mutants. The dashed line indicates the deleted domain, and the gray color indicates the new protein generated from a novel transcript with frameshift mutation and pre-termination in (**c**). The black box in (**d**) indicates the position of the sgRNA. Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

growth. The results showed that *bups1* single mutant, *bups2* single mutant, and *bups1 bups2* double mutant showed no obvious defects in vegetative growth compared to the wild-type plants (Fig. 2.5a and c, the data of *bups2* was not provided). As for the reproduction, however, the siliques of *bups1* single mutant and *bups1 bups2* double mutant plants are significantly shorter than those of wild-type plants, in which almost no seeds were produced (Fig. 2.5a–d). To be more specific, 46.73 seeds per silique were produced in wild-type plants on average, but only 0.84 seeds were produced in *bups1* single mutant plants and 0.08 seeds in the *bups1 bups2* double mutant plants. In addition, *bups2* single mutant showed no significant difference with the WT in terms of the silique length and the seed number (Fig. 2.5e, f).

Taken together, these results suggest that BUPS1 receptor is important for Arabidopsis fertility control and BUPS2 receptor has redundant function with BUPS1, since *bups1 bups2* show more severe defects than *bups1* in plant fertility (Fig. 2.5a–f). Nevertheless, the reason why the fertility defect is caused needs to be investigated further.

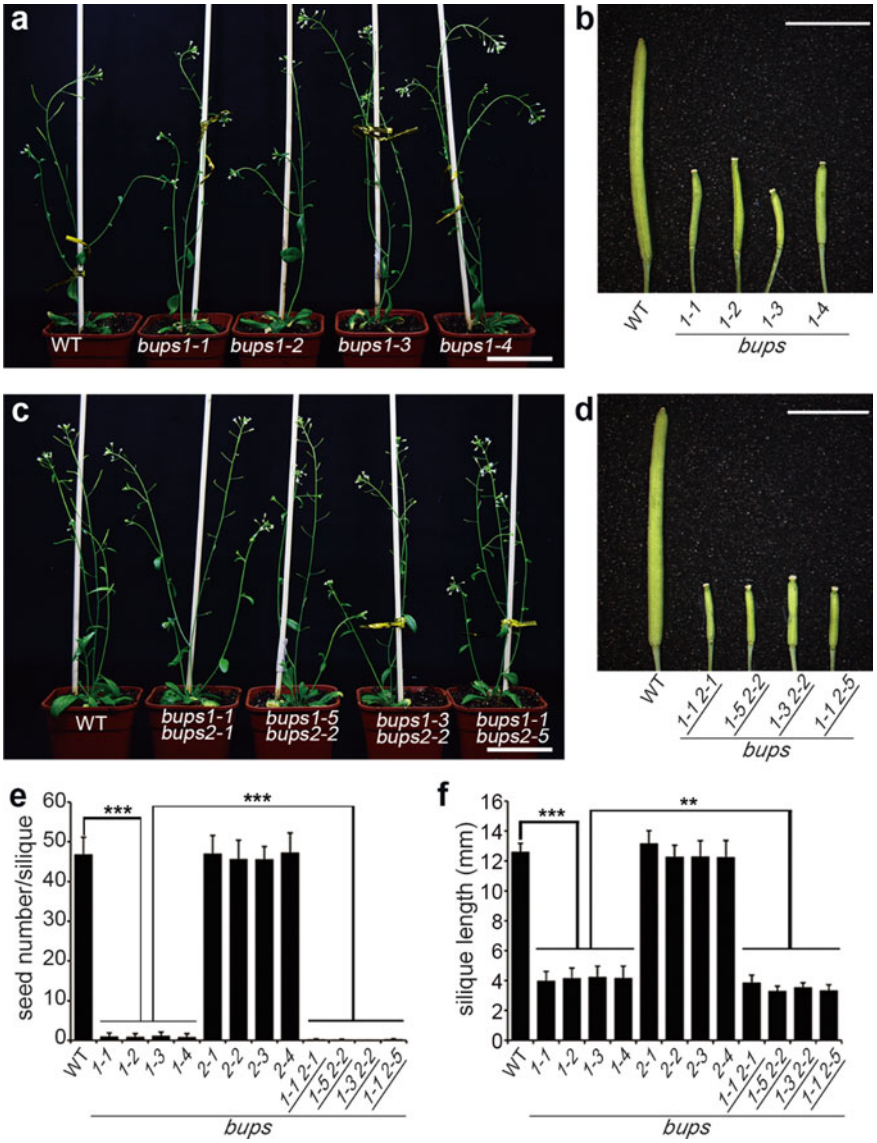

Fig. 2.5 *bups1* and *bups1 bups2* mutant plants have fertility defects. **a**–**d** The vegetative growth (**a**, **c**) and siliques (**b**, **d**) of wild-type, *bups1* and *bups1 bups2* mutants. At least 20 plants and 15 siliques are observed in each group. **e**, **f** The seed number and the silique length of the wild-type, *bups1*, *bups2* and *bups1 bups2* mutants. At least 20 siliques were analyzed in each group, $^{**}P < 0.005$, $^{***}P < 0.001$, T-test. Scare bar = 5 cm (**a**, **c**) or 5 mm (**b**, **d**). Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

2.3.4 bups1 *and* bups1 bups2 *are Male Sterile*

To test whether the fertility defects of *bups1* and *bups1 bups2* mutants are caused by factors from the male or the female, we performed alternative crosses between homozygous mutants and wild-type plants. First, we pollinated the pistils of WT, *bups1*, and *bups1 bups2* with wild-type pollen grains, respectively, and found no obvious defects in the fertility (Fig. 2.6a, c). However, when the WT stigmas were pollinated with pollen grains of *bups1* and *bups1 bups2* homozygous mutants, the resulted silique length was significantly shorter than those of the self-pollinated wild-type, indicating fertility defects of *bups1* and *bups1 bups2* mutants are caused by factors from the male (Fig. 2.6b, d).

In addition, reciprocal crosses were performed between WT and Cas9-free *bups1* or *bups1 bups2* heterozygous mutants. When the *bups1* −/+ was pollinated with WT pollen grains, the proportion of plants carrying mutation in the offspring was 50.2%

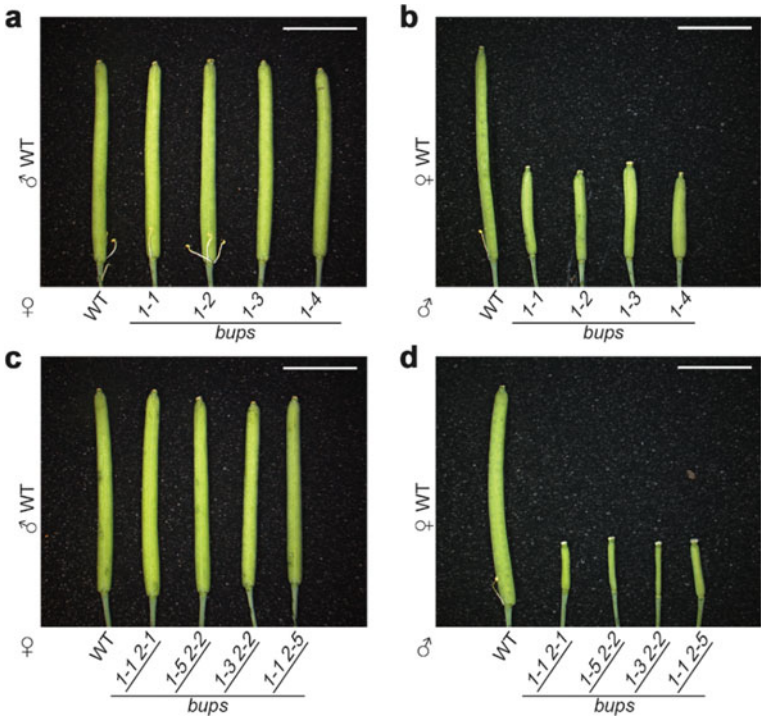

Fig. 2.6 *bups1* or *bups1 bups2* homozygous mutants were male sterile. Alternative crosses were performed between wild-type and *bups1* homozygous mutants (**a**, **b**) or *bups1 bups2* homozygous mutants (**c**, **d**), respectively. After 48 h of pollination, the siliques ($n > 10$) were taken for photos. Scale bar = 5 mm. Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

Table 2.1 Segregation analysis of CRISPR/Cas9-free *bups1*, *bups2*, *bups1 bups2* by PCR-based genotyping in reciprocal crosses

Female	Male	Number of progeny of each genotype		TE^f (%)	TE^m (%)	*p* value
		BUPS1/BUPS1	*bups1/BUPS1*			
bups1–1/BUPS1	WT	149	150	99.3	NA	1.00
WT	*bups1–1/BUPS1*	229	3	NA	1.3	2.22E-49
		BUPS2/BUPS2	*bups2/BUPS2*			
bups2–1/BUPS2	WT	128	125	102.4	NA	0.90
WT	*bups2–1/BUPS2*	124	102	NA	82.3	0.16
		BUPS1/BUPS1	*bups1/BUPS1*			
		bups2–1/BUPS2	*bups2–1/BUPS2*			
bups1–1/BUPS1 bups2–1/bups2–1	WT	149	143	104.2	NA	0.77
WT	*bups1–1/BUPS1 bups2–1/bups2– 1*	245	0	NA	0.0	8.70E-55

NA, not applicable; TE^f, transmission efficiency of the female gametophyte; TE^m, transmission efficiency of the male gametophyte. X^2 were calculated based on the segregation ratio 1:1 in reciprocal crosses between mutants and wild-type plants.
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and that of WT plants was 49.8% (Table 2.1). In other words, the female transmission efficiency of *bups1* was 100.7%, indicating that the female gametophytes of the mutants are normal. However, the male transmission efficiency of *bups1* severely decreased to 1.3% when *bups1* heterozygous plants were used as the pollen donors, suggesting that the male transmission defects of *bups1* almost account for the fertilization defects. With the same strategy, we found that the male transmission efficiency of *bups1–1 bups2–1* was completely lost, while the female transmission efficiency was 96.0% (Table 2.1). However, both the transmission efficiency of male and female gametophyte of *bups2* showed no obvious defects in the reciprocal crosses, consistent with the results of alternative crosses.

To sum up, we demonstrate that the fertility defect of *bups1–1* single mutant or *bups1–1 bups2–1* double mutant is caused by the loss of the male gametophyte transmission. Therefore, these pollen tubes-expressed BUPS1 and BUPS2 receptors play critical roles either in the development of male gametophyte or in the behavior/interaction of pollen tubes with female tissues/cells.

2.3.5 *Pollen Development of* bups1 *or* bups1 bups2 *is Normal*

Because the loss of male gametophyte transmission could be possibly caused by the abnormal development of pollen grains or abnormal behavior of pollen tubes, such as the defects in pollen viability, pollen germination, pollen tube growth, guidance, or reception, we performed the scanning electron microscope (SEM) experiments to examine the morphology of *bups1* and *bups1 bups2* mature pollen grains. The results showed both *bups1* and *bups1 bups2* could produce normal pollen grains as wild-type plants (Fig. 2.7a, c). We also conducted Alexander staining assays to test whether those *bups1* and *bups1 bups2* mutant pollen is viable. The result showed that the pollen grains of *bups1* or *bups1 bups2* were stained similarly to those of WT, suggesting that the pollen is viable (Fig. 2.7b, d). Taken together, the results above indicate that pollen grains of *bups1* and *bups1 bups2* develop normally and there are no obvious defects in pollen viability either. Therefore, the sterility of these mutants might be caused by the defects in the other processes, such as pollen germination, pollen tube growth, and guidance.

2.3.6 *The Pollen Tube of* bups1 *and* bups1 bups2 *Rupture Prematurely*

Since that pollen grains of *bups1* and *bups1 bups2* develop normally in SEM observation and Alexander staining assays, we next decided to examine pollen germination and pollen tube growth through the in vitro pollen germination assays. WT pollen

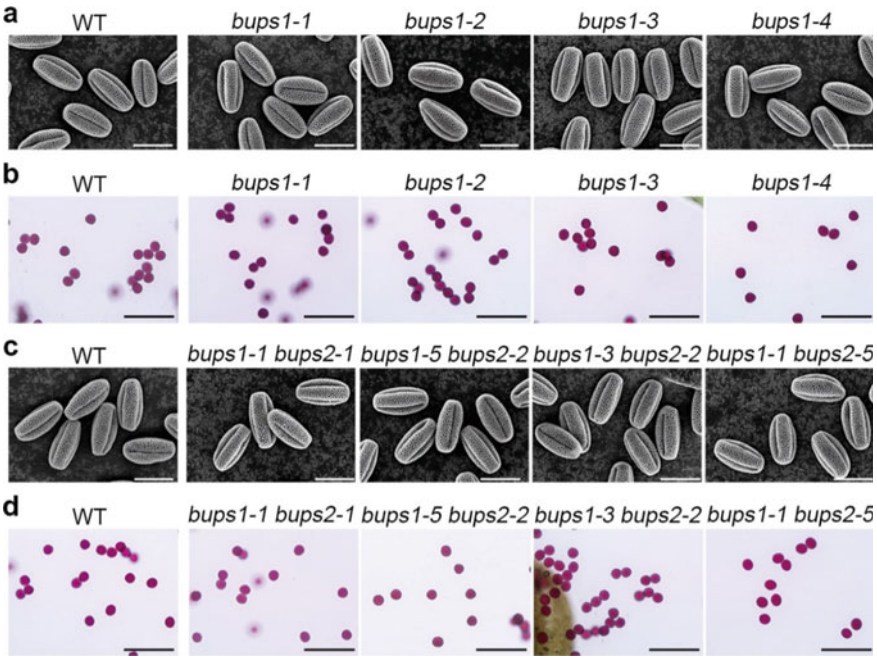

Fig. 2.7 Pollen development of *bups1* and *bups1 bups2* homozygous mutants. SEM analyses were performed to observe the morphology of mature pollen grains of wild-type plant, *bups1* (**a**) and *bups1 bups2* (**c**), and Alexander staining of wild-type plants, *bups1* (**b**), and *bups1 bups2* (**d**) were conducted as well to investigate pollen viability. Scale bar = 20 μm (**a**, **c**) or 100 μm (**b**, **d**). Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

grains could germinate normally with a germination rate of 73.7% after 7 h incubation on the PGM (Fig. 2.8a, Table 2.2). Consistently, the pollen germination rate of *bups1* and *bups1 bups2* were 72.2–75.0% and 77.7–80.7%, respectively (Table 2.2). However, *bups1* pollen tubes did not grow normally; they ruptured quickly after a short period of growth. The tube length was from 87 to 93 μm and the rupture ratio was higher than 94.9% (Fig. 2.8a, Table 2.2), which was significantly different from those of WT which was 268 μm at length and ruptured at the percentage of 7.3%. Similarly, all of *bups1 bups2* pollen tubes ruptured prematurely and the tube length was too short to be measured, since they burst immediately after germination (Fig. 2.8b, Table 2.2). Compared to *bups1* and *bups1 bups2*, however, *bups2* single mutant behaved as wild-type in terms of the pollen germination and the pollen tube burst (Table 2.2). Therefore, these data indicate that BUPS1 and BUPS2 function importantly as receptors in pollen tubes to maintain cell integrity during the pollen tube growth.

To explore the pollen tube behavior of *bups1* and *bups1 bups2* in vivo, we carried out Aniline blue staining assays. To be specific, abundant pollen grains of *bups1*, *bups1 bups2*, and WT were pollinated to wild-type pistils, respectively, which have

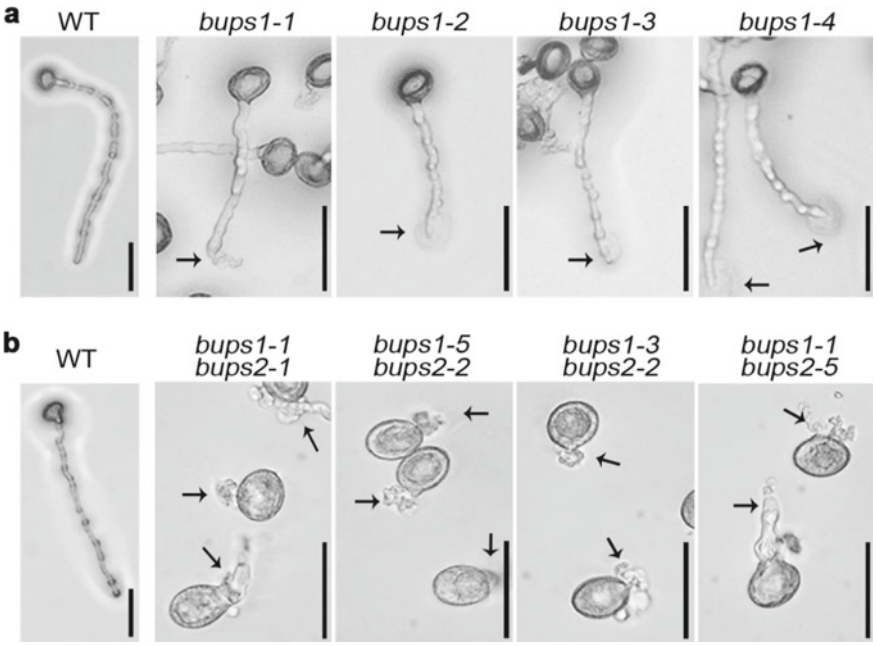

Fig. 2.8 In vitro pollen germination of *bups1* and *bups1 bups2*. The pollen germination of wild-type, *bups1* (**a**), and *bups1 bups2* (**b**) were observed after 7 h of incubation. The black arrow indicates the bursting pollen tube. Scale bar = 50 μm. Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

been castrated in advance. Twenty hours after pollination, siliques were collected for Aniline blue staining. The results showed that WT pollen grains could germinate on the papilla cells and penetrate the stigma and style. After growing in the transmitting tract, the pollen tubes were guided to the ovules (Fig. 2.9a). Although *bups1* and *bups1 bups2* pollen germinated normally on stigmas, the growth of the majority of *bups1* pollen tubes was arrested in the style or between style and ovary locule while that of pollen tubes of *bups1 bups2* double mutant was mainly stopped in papilla cells (Fig. 2.9a). Compared to the wild-type, the pollen tube tips of *bups1* and *bups1 bups2* mutants showed a "bubble" structure (Fig. 2.9b, c), resembling *anx1 anx2* double mutant [14, 30]. Occasionally, only a few pollen tubes were observed to target the ovule normally and succeeded in double fertilization ultimately (Fig. 2.9a). That's the reason why a very small number of seeds could be obtained from *bups1* and *bups1 bups2* self-pollinated plants.

In summary, our results show that *bups1* and *bups1 bups2* pollen tubes are defective in cell integrity in vitro and tube growth in vivo. Therefore, our data support that BUPS1 and BUPS2 are key receptors controlling the cell integrity of Arabidopsis pollen tubes and tube growth during double fertilization.

Table 2.2 Pollen germination and tube growth properties of *bups1*, *bups2*, *bups1 bups2* mutant of pollen germination after 7 h growth in vitro

Genotype	Germinated pollen	Non-germinated pollen	Intact PT	PT bursting	PT length (μm)	Pollen germination (%)	PT bursting (%)
WT	547	195	507	40	268	73.7	7.3
bups1–1	468	180	24	444	87	72.2	94.9
bups1–2	468	156	10	458	87	75.0	97.9
bups1–3	530	202	6	524	93	72.4	98.9
bups1–4	504	180	4	500	90	73.7	99.2
bups2–1	450	155	416	34	272	74.4	7.6
bups2–2	550	210	495	55	253	72.4	10.0
bups2–3	528	199	497	31	267	72.6	5.9
bups2–4	546	189	518	28	281	74.3	5.1
bups1–1	585	168	0	585	NA	77.7	100.0
bups2–1							
bups1–5	524	122	0	524		81.1	100.0
bups2–2							
bups1–3	517	136	0	517		79.2	100.0
bups2–2							
bups1–1	527	126	0	527		80.7	100.0
bups2–5							

PT, pollen tube; NA, not applicable.
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2.3.7 *BUPS1/2 Interact with ANX1/2 to Form a Receptor Complex*

The genetic experiments have revealed that plant receptor-like kinases BUPS1/2 are highly expressed in pollen tubes, with proteins enriched on the cell membrane. When we used CRSIPR/Cas9 technology to knock out *BUPS1* with/without *BUPS2*, the pollen tubes showed defects in cell integrity, with precocious bursting in vivo and in vitro, which finally leaded to the failure of plant fertility. These phenotypes of *bups1 bups2* highly resemble those of *anx1 anx2* mutant [14, 30]. Given that both BUPS1/2 and ANX1/2 belong to the CrRLK1L receptor family and are proposed as membrane-localized receptors, we speculate that these two groups of receptors might work together as a receptor complex to control pollen tube integrity. Therefore, we decided to test the interaction between ANX1/2 and BUPS1/2.

First, we adopted the membrane system of yeast two-hybrid assays. We cloned the ectodomains of BUPS1/2 and ANX1/2 (denoted as ectoBUPS1/2 and ectoANX1/2) into pMetYCgate vector (as bait) and pPR3N-ccdB vector (as prey), respectively. We

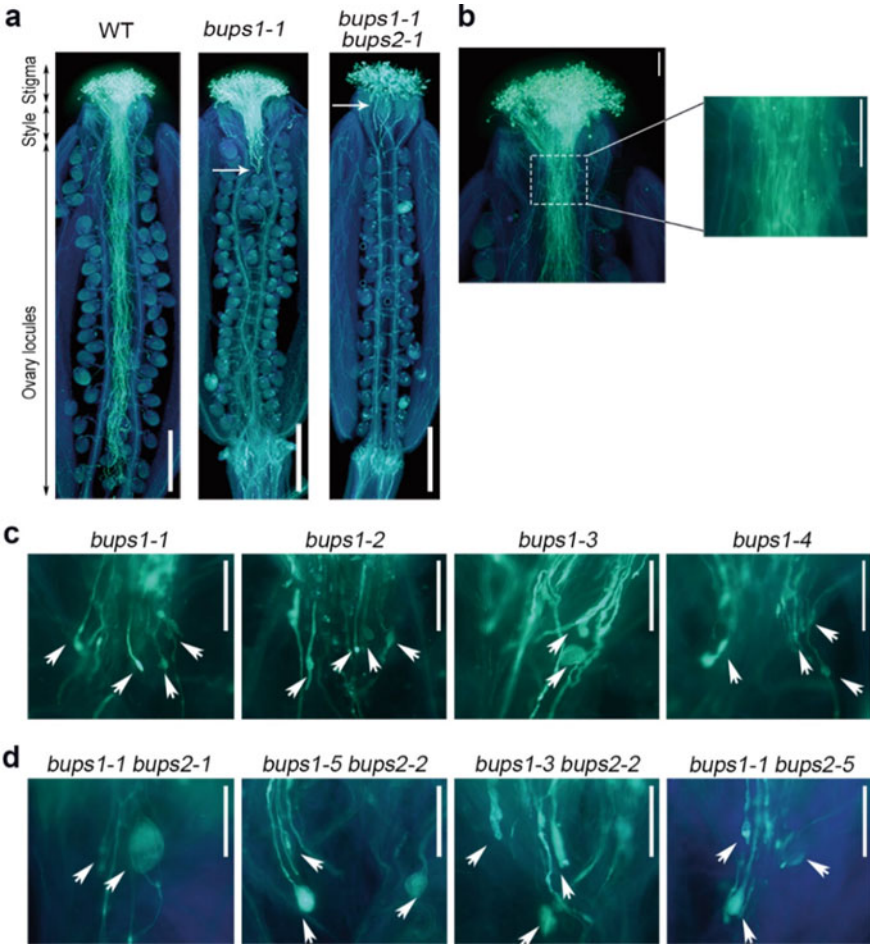

Fig. 2.9 *bups1* and *bups1 bups2* show defects in tubes growth in vivo. **a–d** The pollen tube growth of wild-type, *bups1*, and *bups1 bups2* pollinated to the wild-type pistil, respectively. The white arrow in (**a**) indicates the area where most pollen tubes reach in the ovary. At least 10 pistils in each group were observed. The white arrows in (**b–d**) indicate the pollen tube tip of wild-type (**b**), *bups1* (**c**) and *bups1 bups2* (**d**) in the style. Scale bar = 500 μm (**a**) or 100 μm (**b–d**). Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

found that yeast cells, co-transfected with different combinations of ectoBUPS1/2-Cub and NubG-ectoANX1/2, could normally grow on the selective medium (SD-Trp-Leu-His-Ade), suggesting that BUPS1/2 and ANX1/2 could directly interact with each other (Fig. 2.10a).

To further confirm the interaction between the ectodomains of BUPS1/2 and ANX1/2, co-immunoprecipitation (Co-IP) experiments were performed using flag-tagged or/and myc-tagged proteins which were transient-expressed in tobacco leaf

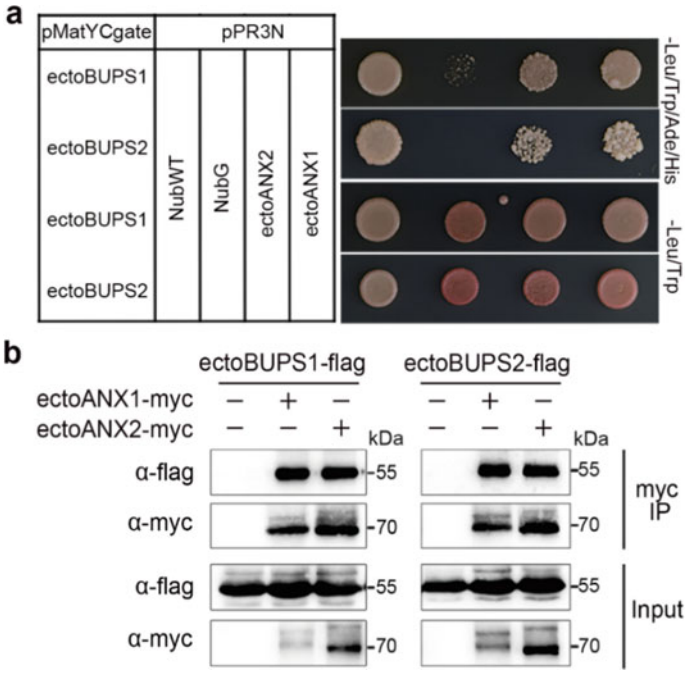

Fig. 2.10 Interaction between ectodomains of BUPS1/2 and ANX1/2. Yeast two-hybrid assays (**a**) and Co-IP assays (**b**) were performed to test the interaction between ectodomains of BUPS1/2 and ANX1/2. Co-immunoprecipitation experiments were conducted using tobacco cells. The extract of the total proteins from the leaves that injected with different combinations of Agrobacterium was enriched in anti-Myc beads. Western blot analysis was performed with α-myc and α-flag antibodies, respectively. Modified from Ge et al. [32] @ The American Association for the Advancement of Science. Reprinted with permission

cells (Fig. 2.10b). After tobacco total proteins were extracted, the tagged proteins were enriched with anti-Myc beads and analyzed by western blot with α-myc and α-flag antibodies, respectively. The result showed that both the ectodomains of BUPS1 and BUPS2 could be pulled down together with the ectodomains of ANX1/2 (Fig. 2.10b), indicating that the ectodomains of BUPS1/2 and ANX1/2 indeed could interact with each other together.

Therefore, our data indicate that BUPS1/2 and ANX1/2 are localized at the plasma membrane of pollen tubes, interact with other through the ectodomains, and form a receptor complex. This receptor complex thereby will perceive the unknown signal molecules to maintain the cell integrity during tip growth of pollen tubes in the maternal tissues.

2.4 Discussion

In Arabidopsis, cell–cell communications between male and female tissues play a critical role in fertilization. Many membrane-localized receptor-like kinases or/and secreted peptides have been well demonstrated to function in pollen germination and pollen tube growth, pollen tube guidance, pollen tube reception, and gamete activation/fusion [6, 16, 33]. For example, FER and ANX1/2 from CrRLK1L receptor family coordinate the reception and burst of pollen tubes, since that pollen tubes show overgrowing phenotype in the ovules of *fer* mutant whereas *anx1 anx2* pollen tubes rupture prematurely [8, 16]. There are 17 receptors in CrRLK1L family in Arabidopsis, some of which have been identified to regulate the cell expansion and cell integrity [8, 15, 20]. Here, we demonstrated that two receptors from the same family, BUPS1 and BUPS2, intensively accumulate on the plasma membrane of pollen tubes and are critical for pollen tube integrity control during the pollen tube growth.

BUPS1/2 are clustered in different clades with ANX1/2 and FER in the phylogenetic tree, and they are highly expressed in mature pollen grains and pollen tubes. After pollen germination, both BUPS1-GFP and BUPS2-GFP are mainly localized on the plasma membrane of pollen tubes. When we knocked out *BUPS1* using CRISPR/Cas9 technology, we found *bups1* mutant lines were male sterile and produced few seeds in the siliques. Further phenotypic analyses revealed that *bups1* pollen tubes showed premature rupture both in vivo and in vitro, while no obvious defects were observed in *bups2* single mutant lines. Nonetheless, *bups1 bups2* showed a more severe defect in male fertility and *bups1 bups2* pollen tubes burst immediately after germination in vitro. Therefore, BUPS1/2 are possibly the key membrane-localized receptors to monitor the extracellular environment and maintain the pollen tube integrity during its growth.

Among 600 receptor-like kinases in Arabidopsis, some of them have been reported to function at different stages of vegetative and reproductive growth [8, 34, 35]. For example, in Brassinosteroids (BRs) signaling pathway, BRI1 serves as a receptor to perceive BRs, whereas BAK1/SERK3 from SERK family is thought as a co-receptor of BRI1. Hetero-oligomerization between BRI1 and BAK1 is triggered by the binding of BRs. Similarly, flg22 peptide (a 22-amino acid peptide) is perceived by a membrane-localized FLS2 receptor, which results in the oligomerization between FLS2 and the co-receptor BAK1 [34–36]. Our current knowledge about the role of RLKs in reproduction is still very limited, so are the molecular mechanisms of these receptors. In this chapter, we found the expression pattern and protein subcellular localization of BUPS1/2 are similar to those of ANX1/2, and the phenotypes of *bups1 bups2* resemble those of *anx1 anx2* [14, 30]. Given that BUPS1/2 and ANX1/2 belong to the same family and are typical receptor-like kinases, it is reasonable to think that they might work together as a receptor complex in pollen tubes. Indeed, we further found that BUPS1/2 could directly interact with ANX1/2 through their ectodomains. This interaction might be essential for the peptide recognition and

binding even though the ligand is unknown. Taken together, we propose that a BUPS–ANX heteromeric receptor complex controls pollen tube integrity, which promotes our understanding of signaling dialogues between male and female tissues/cells in plant reproduction.

As membrane-localized receptor-like kinases, BUPS1/2 and ANX1/2 might perceive some signals secreted from pollen tubes itself or female tissues. However, signal cues of these CrRLK1L receptors in pollen tube reception remain elusive. Recently, RALF peptides, belonging to the cysteine-rich peptide subfamily, were shown to function as the ligand of a CrRLK1L receptor FER to coordinate root cell expansion and plant immunity [12, 16, 37]. There are 37 RALF members in Arabidopsis, which are widely expressed in various tissues, including pollen grains/tubes and female tissues [4, 38–40]. Therefore, it is worth investigating the relationship between RALF peptides and BUPS1/2–ANX1/2 in the pollen tube reception in the future.

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Chapter 3
RALF4/19 are Autocrine Signals to Maintain Pollen Tubes Integrity

Abstract Receptor-like kinases BUPS1/2 coordinate with ANX1/2 to control pollen tubes integrity possibly via depending on ligands from pollen tubes, and RALF peptides are included as the candidates. To screen the ligand of BUPS1/2–ANX1/2 complex, we first performed transcriptome analysis of RALF gene family and found 17 RALF peptides, of which *RALF4* and *RALF19* were focused on in this chapter, showing high or specific transcript in pollen. *RALF4/19* were mainly expressed in pollen grains and pollen tubes via GUS staining, and their coding proteins accumulated in pollen grains/tubes. We adopted CRISPR/Cas9 technology to knock out *RALF4/19* and successfully generated a series of mutants with *RALF4* and *RALF19* mutated separately/simultaneously. Through the phenotypical analyses, *ralf4 ralf19* double mutants, other than any single mutants, showed defects in plant fertility. These defects were male-specific and caused by the abolished cell integrity of pollen tubes. *ralf4 ralf19* pollen tubes ruptured prematurely in vitro. Aniline blue staining determined that the pollen grains of *ralf4 ralf19* could normally germinate on the stigma, but the pollen tubes arrested their growth shortly after penetrating the transmitting tracts. Given that this phenotype highly resembles that of *bups1 bups2* or *anx1 anx2*, pull-down experiments and MST assays were performed and uncovered that RALF4/19 could not only bind to BUPS1/2 but also show high affinity to ANX1/2. Therefore, we believe that pollen tube-secreted RALF4/19 peptides are the ligands of BUPS1/2–ANX1/2 complex on the membrane of pollen tubes, to maintain pollen tubes integrity during the polar growth to the ovules in Arabidopsis.

Keywords RALF peptides · Pollen tubes integrity · Autocrine signals

3.1 Introduction

During plant developmental and reproductive processes, plant receptor-like kinases are key components of cell–cell communications. These membrane-localized receptors generally contain an extracellular domain for the signal ligands perception, an intracellular kinase domain for the downstream signaling activation, and a transmembrane domain. They make cells to adapt to various cues from the internal matrix or

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the external environment via monitoring the extracellular signals of growth, development, environment, and microbe interaction [1–3]. Among 600 receptor-like kinases in Arabidopsis, some of them have been well studied at different stages of vegetative development and reproductive development, such as, SGN3 receptor functioning in Casparian strip diffusion barrier formation, TMM receptor playing a significant role in the stomatal development, PXY involved in the vascular development, and EFR mediating the initiation of plant immunity [1, 4–7]. In general, plant peptides are the signal ligands of these receptor-like kinases. For example, in plant reproductive processes, SCR peptide binds to SRK receptor to initiate self-incompatibility, LAT52 and STIG1 peptides compete the binding to LePRK1/2 receptor to coordinate pollen germination and pollen tube growth, and pollen tube-localized PRK6 mediates pollen tube guidance by perceiving AtLURE1 attractants from the ovules [8–14]. Although FER, ANX1/2, and the newly identified BUPS1/2 receptors from CrRLK1L family play critical roles in regulating pollen tube reception and pollen tube burst, the signal ligands of these receptors in plant reproduction remain elusive [3, 15, 16].

Recently, it was reported that RALF peptide serves as the ligand of CrRLK1L receptor in that FER binds to RALF1 and RALF23, respectively, to coordinate different signaling pathways in root cells and leaves [16–19]. RALFs are the cysteine-rich peptides, accumulate ubiquitously in various tissues and have emerged as key signals to regulate roots elongation, stress response, pollen germination, and female tissues development [17, 20–26]. Given that BUPS1/2 and ANX1/2 belong to the same receptor family as FER, it is likely that RALF peptides might function as the ligands of BUPS–ANX complex as well. With the purpose of screening BUPS–ANX ligands, we first performed transcriptome analysis of RALF gene family and specifically studied two pollen-expressed RALF4/19 in this chapter. We further generated evidences from cell biology, genetic experiments, and in vitro protein binding assays to prove that RALF4/19 were indeed the ligands of BUPS–ANX receptor complex. We thereby conclude that RALF4/19 function as autocrine signals from pollen to maintain cell integrity of pollen tubes when pollen tubes are on their way to deliver the sperm.

3.2 Materials and Methods

3.2.1 Plant Material

Arabidopsis thaliana (Columbia-0) was used as wild-type. All transgenic lines used in this study were in the Columbia ecotype;

Nicotiana benthamiana was used for transient expression of the ectodomains.

3.2.2 Strains and Plasmids

3.2.2.1 Strains

Agrobacterium tumefaciens GV3101: used for Arabidopsis and tobacco transformation;
E. coli: for plasmid transformation.

3.2.2.2 Plasmids

pK7FWG0: modified from pK7FWG2 (Department of Plant Systems Biology, VIB-Ghent University, Ghent, Belgium) and used for GFP-tagging;
pB7GUSWG0: modified from pB7WGR2 (Department of Plant Systems Biology, VIB-Ghent University, Ghent, Belgium) and used for GUS report system;
pCBC-DT1T2 and pHEE401E: gifts from Prof. Qijun Chen (China Agriculture University) and used for CRISPR/Cas9 construction;
pENTR-MSR: entry vector in this laboratory for multi-sgRNAs construction;
pB7FLAGWG2: GATEWAY-compatible destination vector in this laboratory for flag-tagged protein expression;
pFastBac-Dual vector: from Invitrogen for protein expression in insect cells.

3.2.3 Chemicals and Reagents

ProteinFind Anti-His Mouse Monoclonal Antibody: TransGenBiotech, HT501-02;
Anti-Flag-HRP: Sigma-Aldrich, A8592-2MG;
Goat Anti-Mouse IgG-HRP: CWBIO, CW0102S;
3xFlag Peptide: Sigma, F4799-4 MG;
SuperSignal@West Femto Maximum Sensitivity Substrate: Thermo Fisher, 34095;
Copmplete Protease Inhib. Cocktail: Roche, 11836145001;
Ni Sepharose 6 FF: GE Healthcare, 17531801;
Streptavidin Magnetic Particles: Spherotech, SVMS-30-10;
Anti-Flag Affinity Gel: Bimake, B23101.

3.2.4 Methods

3.2.4.1 Plant Growth Condition and Plant Transformation

Arabidopsis thaliana and tobacco plants were grown under long-day conditions (16 h light/8 h dark cycles) at 22 ± 2 °C with a light intensity of 170 μmol/m^2/s using LED bulbs (Philips F17T8/TL841 17 W) [27].

3.2.4.2 Phylogenic Analysis and Transcription Assay

Phylogenic analysis and transcription assay were performed as described in Chap. 2. The sequences of RALF family in Arabidopsis can be found in the Arabidopsis Information Resource (http://www.arabidopsis.org/). Accession numbers are as follows: RALF1, At1g02900; RALF2, At1g23145; RALF3, At1g23147; RALF4, At1g28270; RALF5, At1g35467; RALF6, At1g60625; RALF7, At1g60815; RALF8, At1g61563; RALF9, At1g61566; RALF10, At2g19020; RALF11, At2g19030; RALF12, At2g19040; RALF13, At2g19045; RALF14, At2g20660; RALF15, At2g22055; RALF16, At2g32835; RALF17, At2g32890; RALF18, At2g33130; RALF19, At2g33775; RALF20, At2g34825; RALF21, At3g04735; RALF22, At3g05490; RALF23, At3g16570; RALF24, At3g23805; RALF25, At3g25165; RALF26, At3g25170; RALF27, At3g29780; RALF28, At4g11510; RALF29, At4g11653; RALF30, At4g13075; RALF31, At4g13950; RALF32, At4g14010; RALF33, At4g15800; RALF34, At5g67070; RALF35, At1g60913; RALF36, At2g32885; RALF37, At4g14020.

3.2.4.3 Plasmids Construction

To knock out *RALF4* and *RALF19*, we developed pENTR-MSR vector for multi-sgRNAs construction. The fragments containing two sgRNA1s or sgRNA2s were ligated into pENTR-MSR, respectively, leading to the generation of pENTR-R4R19V1 and pENTR-R4R19V2. Through the isocaudamer enzyme-ligation from these two vectors, pENTR-R4R19V1_V2 was constructed and subsequently cloned into pHEE401E by Golden-Gate Cloning to generate pHEE401E-R4R19V1_V2 construct. sgRNA sequences mentioned above are as follows:

RALF4_sgRNA1: AAC GGG CAA GGT TGC ATC GNG G
RALF4_sgRNA2: ACC GTC GTC AAC TCG CAA GNG G
RALF19_sgRNA1: CCG GCG CCA ACT AGC CGC GNG G
RALF19_sgRNA2: TGA CTC GGC GAC TAC AGC CNG G

3.2.4.4 Flag-Tagged Ectodomains Expression and Purification in Tobacco Leaves

To express the flag-tagged ectodomains, the coding sequences of ectodomain with no stop codon were cloned into pB7FLAGWG2, respectively, to generate *p35S::BUPS1-ECD-3* × *Flag*, *p35S::BUPS2/-ECD-3* × *Flag*, *p35S::ANX1-ECD-3* × *Flag*, and *p35S::ANX2-ECD-3* × *Flag* constructs. The expression vectors were then transformed into *Agrobacterium tumefaciens GV3101*, infiltrated into *N. benthamiana* leaves, and purified as described in Chap. 2. The ectodomains purified by anti-Flag gel (Bimake, B23102) were eluted with 3xFlag Peptide (Sigma, F4799-4 MG) for three times at room temperature and the washes were collected for pull-down assays.

3.2.4.5 Expression and Purification of His-Tagged Ectodomains

The constructs of ectoBUPS1/BUPS2/ANX1/ANX2 with a C-terminal 6 × His tag were generated by standard PCR-based cloning strategy and then were cloned into the pFastBac-Dual vector (Invitrogen) with a modified N-terminal melittin signal peptide. Recombinant baculoviruses were generated and amplified using the Sf21 insect cells maintained in the SIM SF medium. 1 L High Five insect cells (1.5 × 106 cells mL^{-1} cultured in SIM HF medium) was infected with 5 mL recombinant baculoviruses. After 48 h, the medium was harvested and concentrated using a Hydrosart Ultrafilter and exchanged into the binding buffer (25 mM Tris–HCl, pH 8.0, 150 mM NaCl). The proteins were purified with Ni-NTA resin (GE Healthcare). Then the bound protein was eluted in buffer containing 25 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 500 mM imidazole.

3.2.4.6 In Vitro Protein Binding Assays

Pull-down assays were conducted to test the interaction between RALF peptides and flag-tagged ectodomains as described previously [18]. In more detail, 1 ng/μL flag-tagged proteins and 10 nM biotin-RALF peptide were mixed in 500 μL buffer (20 mM Tris–HCl, pH 7.5, 1% IGEPAL). 50 μL streptavidin magnetic particles (Spherotech, SVMS-30-10) were mixed with the sample, and the mixture was rotated for 3 h at 4 °C, washed 4–5 times with the buffer, and subjected to the western blot by using anti-Flag-HRP (Sigma-Aldrich, A8592-2MG). As for the His-tagged ectodomains, similar procedures were followed except that α-His antibody (TransGen Biotech, HT501-02) was used in the western blot.

3.2.4.7 MST Assays

MST assay was conducted to test the binding affinity of RALF peptides to the ectodomains in this chapter by using Monolish NT.115 (NanoTemper Technologies). His-tagged ectodomains purified from insect cells were labeled according to the manufacturer's procedure. First, the His-tagged proteins were exchanged into a labeling buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.5, 200 mM NaCl, 5% Glycerol, 0.01% Tween 20). The protein concentration was then adjusted to 200 nM with the buffer. Finally, a volume of 90 μL sample was mixed with 90 μL dye solution (100 nM) diluted from the labeling kit (Monolith™ His-Tag Labeling Kit RED-Tris-NTA, MO-L008) for 30 min at room temperature. For each assay, the labeled protein mixed with 1 mg/mL BSA was incubated with a series of peptide concentration gradients. The samples were loaded into the silica capillaries (Monolith™ NT.115 Standard Treated Capillaries, MO-K002; Monolith™ NT.115 MST Premium Coated Capillaries, MO-K005) and measured by using Monolish NT.115 (NanoTemper Technologies) at medium MST power, 50 or 90% LED power. The data was further analyzed by MO. Affinity Analysis software (V2.2.4).

3.2.4.8 Peptides Synthesis and in Vitro Treatment of Pollen Tubes

All RALF peptides used in this study were synthesized by Scilight Biotechnology LLC (Beijing, China) with a purity higher than 95% and diluted into 2 mM with sterile pure water. For in vitro peptide treatment, the dissolved peptide was diluted with PGM into 2 μM and subjected to the treatment of pollen tubes at 7 h after germination.

3.3 Results

3.3.1 Expression Pattern and Subcellular Localization of RALF4/19

In Chap. 2, we have identified that the pollen tubes of *bups1 bups2* showed a similar phenotype as *anx1 anx2*: the pollen tubes ruptured immediately after germination in vitro and arrested the tip growth in vivo. Besides, these two groups of receptor-like kinases could interact with each other to form a receptor complex, which might involve the perception of unknown signals from pollen tubes. Recently, Arabidopsis RALF peptides have been reported as signaling molecules of FER receptor, another CrRLK1L receptor, to regulate root cells development and plant immunity [18, 19]. Given that BUPS1/2 and ANX1/2 belong to the CrRLK1L receptor family, we assumed that RALF peptide might serve as the signal ligand of BUPS–ANX receptor complex as well. We believed that these signal molecules should be expressed highly

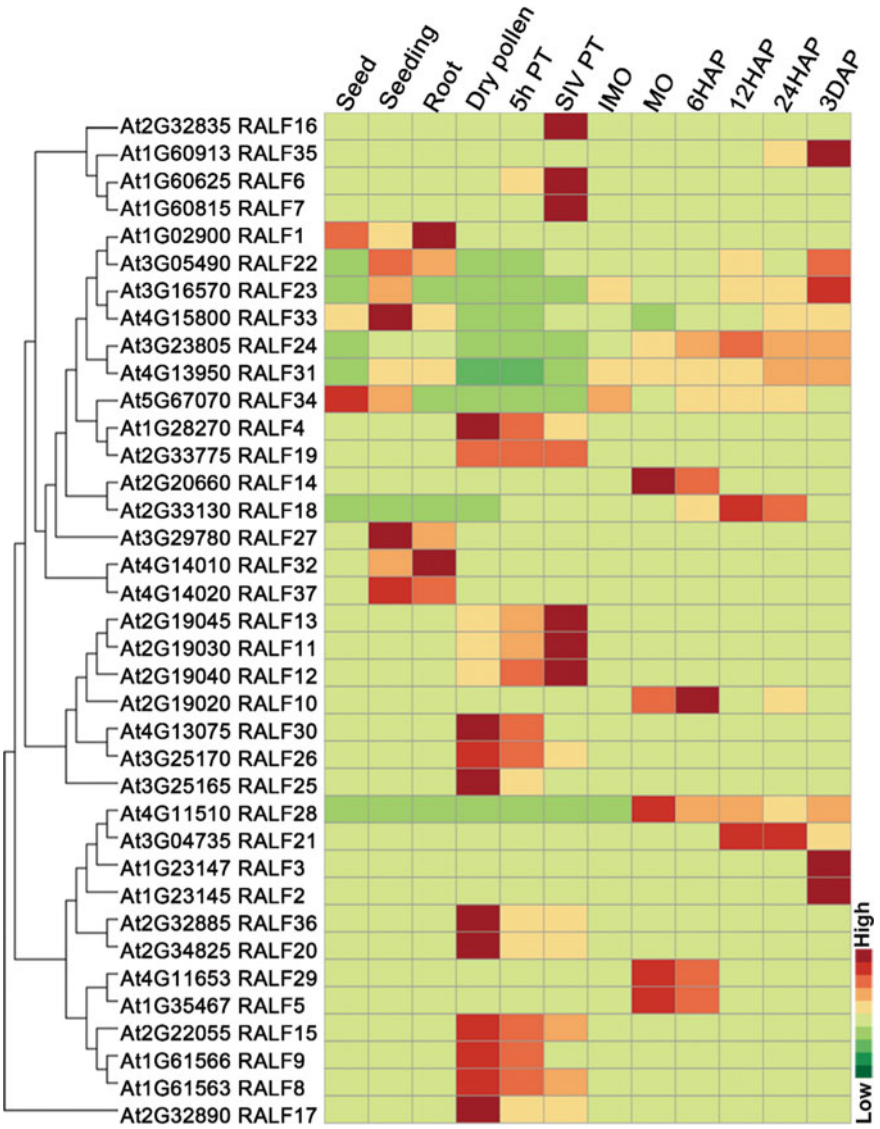

Fig. 3.1 Transcription analysis of RALF family in different tissues. On the left side is the phylogenetic tree of the RALF family and on the right shows the heat-map analysis of the gene expression levels of RALF family members based on the RNA-seq data. 5 h PT, pollen tubes germinated in vitro for 5 h; SIV PT, semi-*in vivo* germinating pollen tubes; IMO, immature ovules; MO, mature ovules; 6HAP, ovules pollinated for 6 h; 12HAP, ovules pollinated for 12 h; 24HAP, ovules pollinated for 24 h; 3DAP, ovules pollinated for 3 days. Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

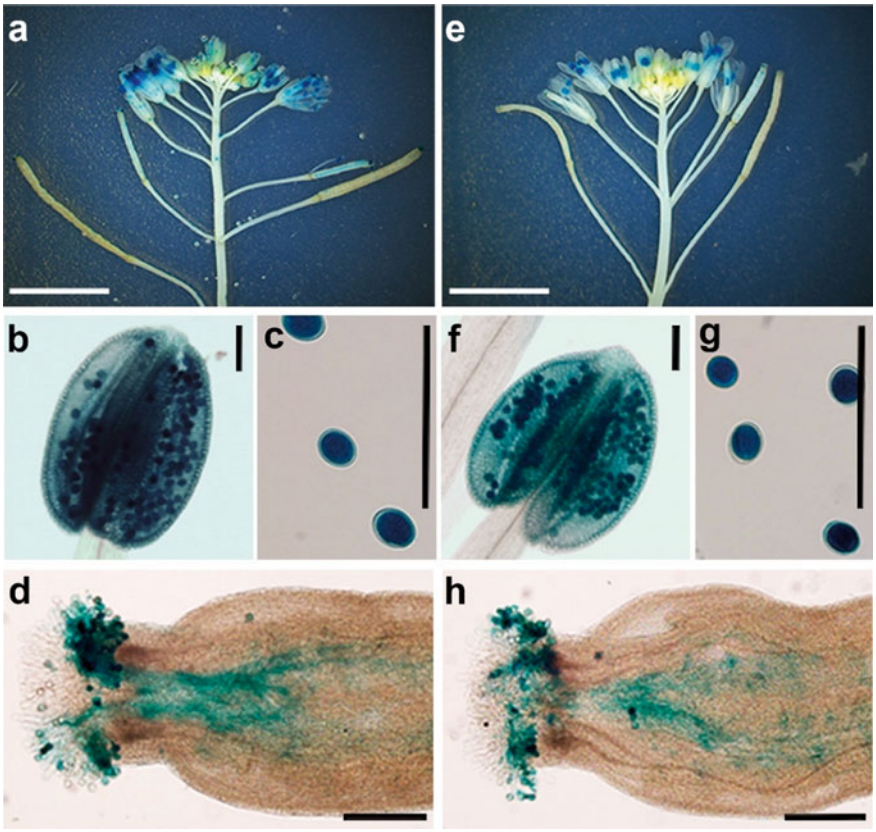

Fig. 3.2 Gene expression patterns of *RALF4/19* in inflorescences. The GUS staining of *RALF4p::GUS* (**a–d**) and *RALF19p::GUS* (**e–h**) in the inflorescence (**a**, **e**), anthers (**b**, **f**), mature pollen grains (**c**, **g**), and pistils (**d**, **h**). Scale bar = 10 mm (**a**, **e**) or 100 μm (**b–d**, **f–h**). Modified from Ge et al. [28] @ The American Association for the Advancement of Science. Reprinted with permission

or specifically in pollen in that the cell integrity of WT pollen tubes could be maintained but *bups1 bups2* or *anx1 anx2* pollen tubes rupture prematurely in vitro.

To test this hypothesis, we did the transcriptome analysis of all the RALF gene family in vegetative tissues and reproductive tissues based on the RNA-seq data in this laboratory. From this result, four groups of RALF peptides were found to be specifically or highly expressed in pollen grains and tubes, of which *RALF4* and *RALF19* were specifically focused in this chapter (Fig. 3.1).

In order to explore the gene expression pattern and protein subcellular localization of RALF4 and RALF19 peptides in Arabidopsis, we constructed *RALF4p::GUS*, *RALF19p::GUS*, *RALF4p::RALF4-GFP*, and *RALF19p::RALF19-GFP* constructions and generated the transgenic plants, respectively. The results of GUS staining showed that the promoter activity of *RALF4* or *RALF19* was mainly in mature flowers

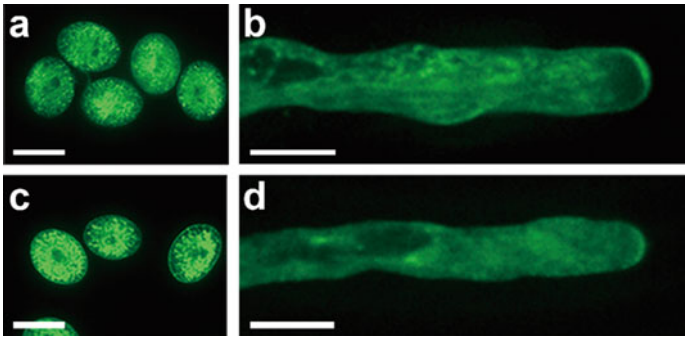

Fig. 3.3 Subcellular localization of RALF4/19 peptides. The protein distribution of RALF4 (**a**, **b**) and RALF19 (**c**, **d**) in pollen grains and pollen tubes. Scale bar = 20 μm (**a**, **c**) or 10 μm (**b**, **d**). Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

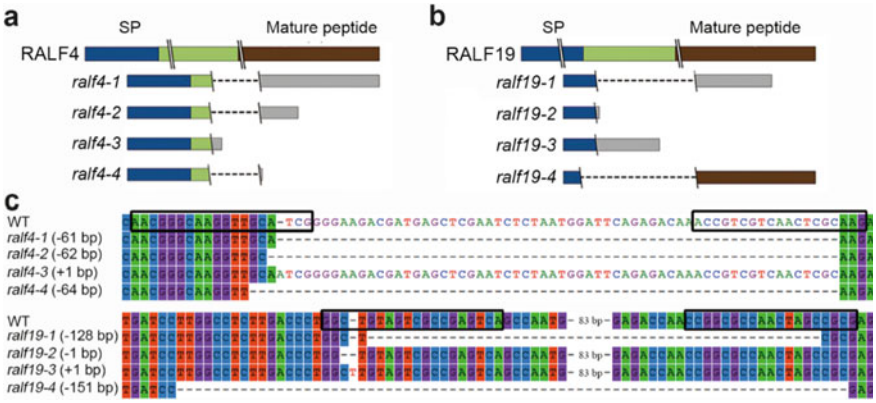

Fig. 3.4 Knocking out *RALF4* and *RALF19* via CRISPR/Cas9 technology. **a**, **b** The protein structure of RALF4 and RALF19, and the identification of *ralf4* and *ralf19* mutant. "\\" indicates the position of the sgRNA target on the protein structure. The dashed line indicates the deleted domain, and the gray color indicates the new protein generated from a novel transcript with frameshift mutation and pre-termination. SP, signal peptide. **c** The detailed mutation of *RALF4* or *RALF19* at the nucleic acid level. The black box indicates the position of the sgRNA. Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

(Fig. 3.2a, e). Further observations revealed that the GUS signal appeared mainly in mature pollen grains and pollen tubes (Fig. 3.2b–d, f–h). At the same time, GFP fluorescence analyses showed that RALF4-GFP and RALF19-GFP were mainly distributed in the cytoplasm of mature pollen grains and pollen tubes (Fig. 3.3a–d). Therefore, these results indicated that *RALF4/19* were mainly expressed in mature pollen grains/tubes, and RALF4/19 proteins accumulated in the cytoplasm of pollen tubes. Considering that RALF4/19 are small peptides, we speculate that they might

function as "signaling molecules" to participate in the crosstalk with pollen cell itself or between male and female tissues/cells.

3.3.2 *Using CRISPR/Cas9 Technology to Knock Out RALF4/19*

Given that RALF4 and RALF19 shared high identity in nucleic acid or amino acid sequences, and their expression patterns and subcellular localizations are very similar, we think that they may function redundantly in pollen. At the same time, as small peptide, *RALF4/19* have relatively short gene structure and thereby are very difficult to be targeted simultaneously by the T-DNA insertion method. Therefore, we used CRISPR/Cas9 technology, which works with more target specificities and flexibilities, to knock out *RALF4* and *RALF19*. In order to knock out *RALF4/19* at the same time, two sgRNAs for two target sites were designed for each gene. Therefore, a total of four sgRNAs were ligated into the pENTR-MSR vector, and then into the pHEE401E expression vector for transgenic experiments.

After generating the antibiotic-positive transgenic plants, we did genotyping by combining PCR and Sanger-sequencing to test whether there was a mutation in the target. The results showed that CRISPR/Cas9 technology could induce mutations in *RALF4* and *RALF19* successfully. The mutation types mainly included base pairs insertion/deletion and large fragment deletions which were consistent with these in *BUPS1* and *BUPS2*. Finally, four types of mutation in each gene were retained for further phenotypic analyses and genetic experiments (Fig. 3.4a–c).

3.3.3 ralf4 ralf19 *Double Mutants are Male Sterile*

After successfully knocking out *RALF4* and *RALF19* using CRISPR/Cas9 technology, we did phenotypic analyses from aspects of the vegetative growth and reproductive development. The results showed that *ralf4 ralf19* double mutants grew normally with no difference in vegetative growth from the wild-type plants (Fig. 3.5a). Nevertheless, *ralf4 ralf19* double mutants showed serious defects in plants fertility and the silique length was significantly shorter than that of the wild-type plants (Fig. 3.5b, c). To be more specific, the length of WT silique was 13.14 mm, with each silique producing 49.13 seeds on average, while the silique length of *ralf4 ralf19* double mutant was 3.90 mm and the seeds number in each silique is 0.26 (Fig. 3.5c, d). At the same time, this result also showed that the silique produced by *ralf4* single mutant and *ralf19* single mutant were identical both in the silique length and seeds number with WT (Fig. 3.5c, d). Therefore, these results indicated that the pollen-expressed RALF4/19 peptides function redundantly in vivo for regulating plant fertility in the reproductive development in plant.

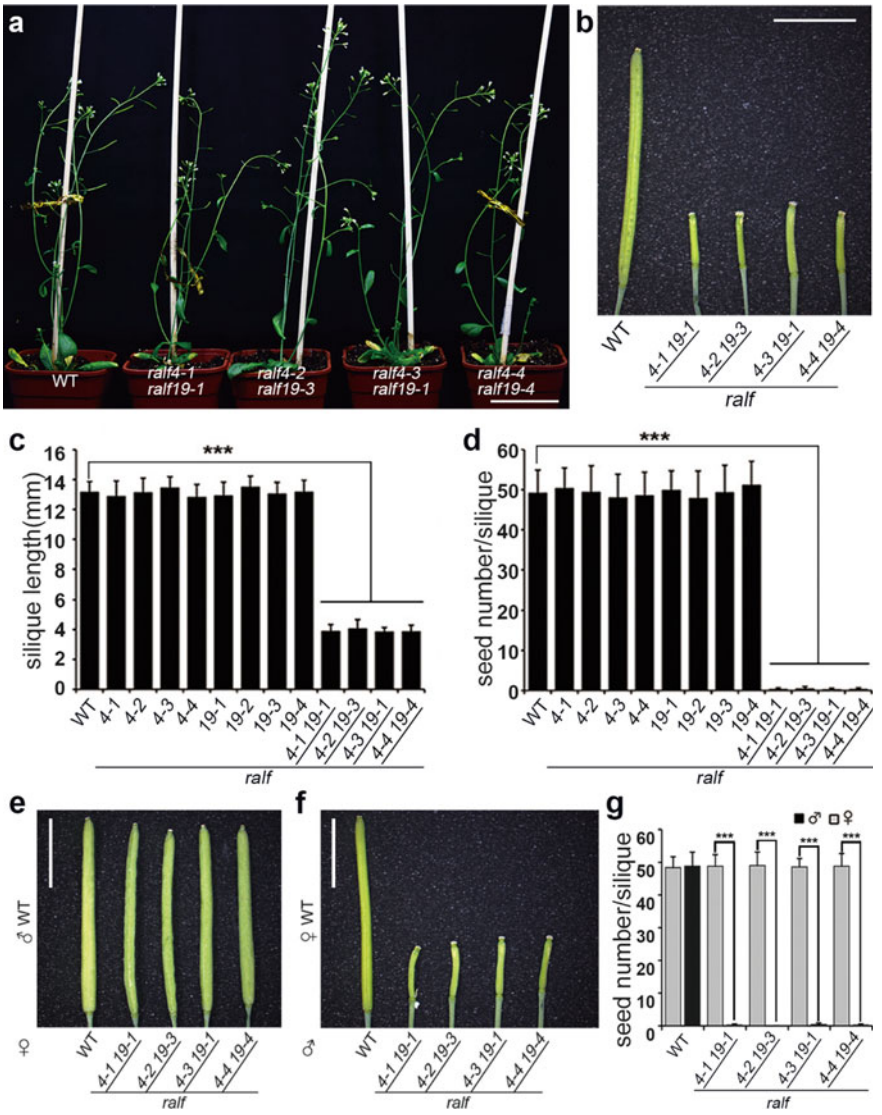

Fig. 3.5 *ralf4 ralf19* double mutants show male-specific sterility. **a** The vegetative growth of the wild-type and *ralf4 ralf19* mutants. At least 20 plants in each group were observed. **b** The siliques produced by WT and *ralf4 ralf19* double mutant. 15 siliques were observed in each group. **c**, **d** The statistical results of the silique length (**c**) and the seeds number (**d**) of the wild-type and *ralf4 ralf19* double mutants. At least 20 siliques were analyzed in each group, $***P < 0.001$, T test. (**e–g**) The alternative crosses between WT and *ralf4 ralf19* homozygous mutants. 10 siliques were observed in each group. Scale bar = 5 cm (**a**) or 5 mm (**b**, **e**, **f**). Modified from Ge et al. [28] @ The American Association for the Advancement of Science. Reprinted with permission

To reveal whether the fertility defects of *ralf4 ralf19* mutant are caused by factors from the male or the female, alternative-cross experiments between WT and *ralf4 ralf19* homozygous mutants were conducted. The results showed that either the wild-type pistils or the *ralf4 ralf19* pistils could produce normal siliques after being pollinated with wild-type pollen grains (Fig. 3.5e, g). In contrast, when *ralf4 ralf19* homozygous mutants were used as the pollen donors to the wild-type pistils, the siliques developed abnormally with nearly no seeds produced inside, and the siliques were significantly shorter than these of the self-pollinated WT (Fig. 3.5f, g). It indicated that the fertility defect of *ralf4 ralf19* mutant was caused by factors from the male.

Therefore, our results show that RALF4 and RALF19 peptides accumulate mainly in pollen. *ralf4 ralf19* mutant has fertility defect, and it is caused by the male-side. It implies that these two peptides may serve as important signal molecules which control the transmission of male gametophytes, to participate in the regulation of sexual reproduction in *Arabidopsis thaliana.*

3.3.4 ralf4 ralf19 *Exhibits* bups1 bups2*-like Phenotypes*

It is revealed that *ralf4 ralf19* double mutant has fertility defects and these defects are caused by the aborted transmission of pollen grains, however, it is still unclear that which process of the male-side is affected. With the aim to determine the male defects of *ralf4 ralf19* mutant, we subsequently used the SEM observation and Alexander staining assays to check the development of pollen grains in mutants. We found that pollen grains of *ralf4 ralf19* double mutants were full in shape and no obvious abnormality was found in the SEM observation, and pollen grains of the mutant could be stained similarly to those of WT by Alexander staining. These results were consistent with these of WT, thus indicating that the morphology of *ralf4 ralf19* pollen grains was normal and there were no obvious defects in pollen viability as well (Fig. 3.6a, b). Therefore, we conclude that RALF4/19 might have no function in pollen development or pollen viability, which suggests that the fertility defect of *ralf4 ralf19* might be caused by other processes, such as pollen grains germination, pollen tube growth, pollen tube guidance, or reception.

Next, we did the in vitro pollen germination experiments to investigate whether *ralf4 ralf19* double mutant had defects in pollen germination, pollen tube growth, and pollen tubes morphology. We found that pollen grains of WT and *ralf4 ralf19* germinated normally in that the pollen germination rate of WT was 74.8% while *ralf4 ralf19* germinated with the percentage ranging from 78.7 to 81.5% (Table 3.1). The pollen tubes length of WT could reach to 261 μm at length with only 8.6% showing premature discharge. However, *ralf4 ralf19* pollen tubes ruptured immediately after germination under the same condition, the rupture ratio was 100%, and the tube length was too short to be measured (Table 3.1; Fig. 3.6c). Thereby, our results showed that, similar to the role of BUPS1/2 or ANX1/2 receptors, pollen-expressed

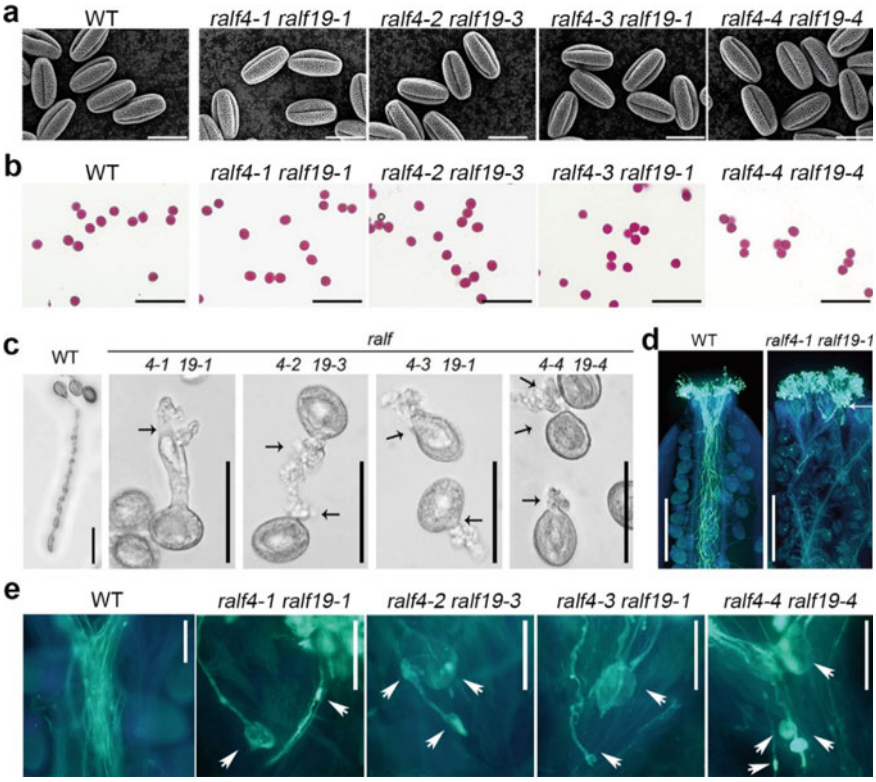

Fig. 3.6 Phenotypic analyses of *ralf4 ralf19* double mutants (**a**, **b**). The SEM observation (**a**) and Alexander staining (**b**) of WT and *ralf4 ralf19* pollen grains. **c** In vitro germinating pollen tubes of WT and *ralf4 ralf19*. Pollen grains were incubated for 7 h on the PGM before the observation. The black arrows indicate the bursting pollen tubes. **d**, **e** The in vivo growth of WT and *ralf4 ralf19* by Aniline blue staining. The white arrow in Figure **d** indicates the area where most of the pollen tubes reach and these in Figure **e** indicate the tip of pollen tubes with abnormal shape. Scale bar = 20 μm (**a**), 100 μm (**b**), 50 μm (**c**, **e**), or 500 μm (**d**). Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

RALF4/19 peptides are also involved in maintaining the cell integrity of Arabidopsis pollen tubes.

To analyze the tube growth of *ralf4 ralf19* in vivo, we pollinated pollen grains from wild-type plants or *ralf4 ralf19* mutants to the wild-type pistils, respectively, to do Aniline blue staining experiments. The staining results showed that pollen tubes of the wild-type could grow in the maternal tissues and target the ovules with successful fertilization finally (Fig. 3.6d). Compared with the wild-type, although the pollen grains of *ralf4 ralf19* double mutant could germinate normally on the stigma, the pollen tubes failed to penetrate the style normally and stopped in the style untimely (Fig. 3.6d, e). Besides, the tip of *ralf4 ralf19* pollen tubes showed a

Table 3.1 Pollen germination and tubes growth properties of *ralf4 ralf19* mutant in vitro

Genotype	Germinated pollen	Non-germinated pollen	Intact PTs	PT bursting	PT length (μm)	Pollen germination (%)	PT bursting (%)
WT	548	185	501	47	261.0	74.8	8.6
ralf4-1 ralf19-1	622	145	0	622	NA	81.1	100.0
ralf4-2 ralf19-3	649	164	0	649		79.8	100.0
ralf4-3 ralf19-1	537	122	0	537		81.5	100.0
ralf4-4 ralf19-4	589	159	0	589		78.7	100.0

PT, pollen tube; NA, not applicable. Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

bubble-like structure (Fig. 3.6e); this abnormal shape was also reported in the *anx1 anx2* mutant and thought as the tip of the bursting pollen tubes [29, 30].

Based on these results, we found *ralf4 ralf19* double mutants showed premature pollen tubes rupture in vitro, arrested tubes growth in maternal tissues, and abnormal shape of the pollen tube tip in vivo, which leads to the abolishment of plant fertility. Our findings suggest that the pollen-expressed RALF4 and RALF19 peptides function redundantly and importantly to control pollen tubes integrity and the tubes growth in vivo. Besides, these phenotypes of *ralf4 ralf19* are very similar to these of *bups1 bups2* and *anx1 anx2* double mutants [29, 30]; thereby we believe that the function of RALF4/19 peptides might be coupled with BUPS1/2–ANX1/2 receptor complex in Arabidopsis reproduction.

3.3.5 *RALF4/19 Interact with BUPS1/2 Ectodomains*

We have shown that CrRLK1L receptors BUPS1/2, similar to ANX1/2, are mainly localized on the plasma membrane of pollen tubes and function coordinately with ANX1/2 as a receptor complex in controlling pollen tubes integrity. RALF4/19 are also pollen tube accumulated peptides and share similar function with BUPS–ANX complex. Therefore, we assume that RALF4/19 peptides are likely to be the pollen-secreted signal molecules and could be perceived by BUPS–ANX receptor complex, thereby maintaining the cell integrity of pollen tubes. To test this hypothesis, we performed biochemical experiments to explore whether RALF4/19 peptides could interact with the ectodomains of BUPS1/2 or ANX1/2.

To generate RALF4 and RALF19 proteins, we chemically synthesized RALF4/19 peptides with/without biotinylation modification and tested their biological activities via pollen germination assays. The PGM with a certain concentration of small peptide

Table 3.2 Inhibition of RALF peptides on the WT pollen germination

Peptide concentration	Germinated pollen	Non-germinated pollen	Pollen germination (%)
0	131	35	79
2 μM RALF4	0	141	0.00
2 μM biotin-RALF4	0	121	0.00
2 μM RALF19	0	171	0.00
2 μM biotin-RALF19	0	153	0.00

In this experiment, chemically synthesized RALF4/19 peptides were directly mixed into the PGM and pollen germination experiments were performed in vitro to detect the effect of these peptides on the pollen germination of WT. After 7 h of incubation, the pollen germination was observed and statistical analysis was performed. Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

was subjected to in vitro pollen germination experiments, and pollen germination of WT was statistically analyzed. Finally, it was found that the pollen grains of WT with no peptide treatment could germinate normally with a germination rate of 79.9%. However, 2 μM RALF4 or biotin-RALF4 could completely block the pollen germination and similar inhibition effect was observed when RALF19 or biotin-RALF19 was added into the medium (Table 3.2). This result was consistent with the previous finding [26], indicating that biotin-RALF4/19 as well as RALF4/19 synthesized in this chapter have similar biological activities to inhibit pollen germination.

After confirming that the chemically synthesized RALF peptides maintain their biological activities, we subjected these peptides to pull-down assays to investigate whether RALF4/19 peptides could interact with BUPS1/2 and ANX1/2. We first adopted tobacco leaf-expression system to express and purify ectoBUPS1/2-Flag, respectively. We incubated the ectodomains with biotin-RALF4 or biotin-RALF19 for 2–4 h, then used streptavidin magnetic particles for purification, and finally did western blot assays. The results showed that both ectoBUPS1-Flag and ectoBUPS2-Flag can be captured by biotin-RALF4 or biotin-RALF19 (Fig. 3.7a), indicating that RALF4/19 peptides could interact with the ectodomains of BUPS1/2 directly. However, no signal of output was detected when RALF8, another peptide expressed in pollen, was incubated with ectoBUPS1-Flag or ectoBUPS2-Flag as a control, respectively (Fig. 3.7a), thus indicating that the interaction between RALF4/19 peptides and BUPS1/2 ectodomains has specificity. Besides, similar interactions between RALF4/19 and BUPS1/2 were also observed when insect cells-expressed ectoBUPS1-His or ectoBUPS2-His was used in the pull-down assays (Fig. 3.7b). Therefore, our results show that RALF4/19 peptides are indeed able to interact with the ectodomains of BUPS1/2 and this interaction is specific. We thereby conclude that RALF4/19 are the ligands of BUPS1/2 in pollen.

To quantitatively analyze the binding affinity between RALF4/19 and BUPS1/2, the microscale thermophoresis (MST) was performed. We first used the RALF23-FER combination as a positive control for a pre-test and found the dissociation

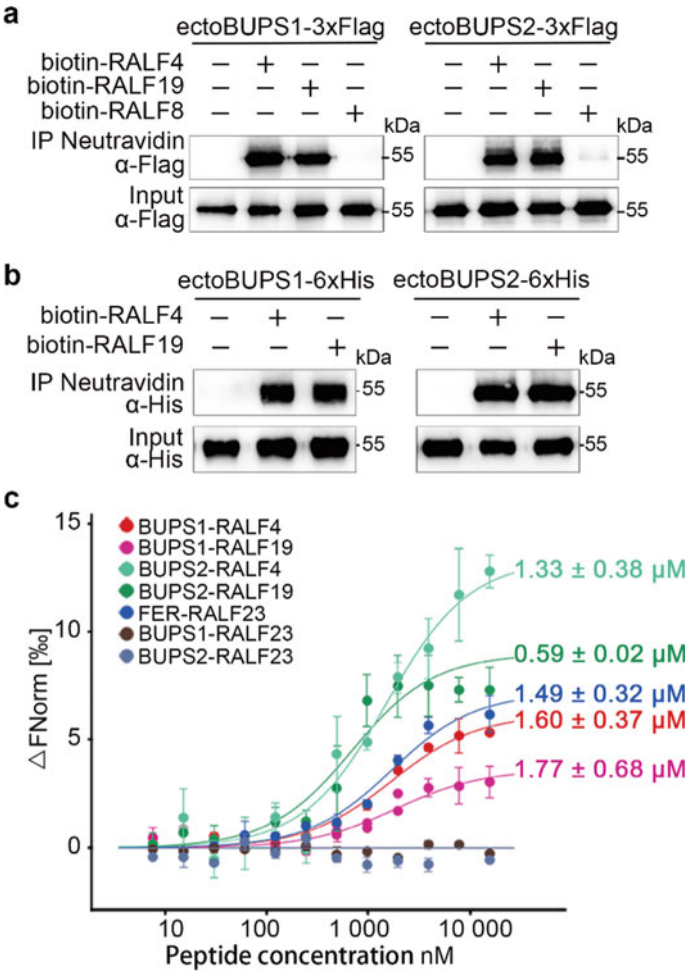

Fig. 3.7 Interaction between the RALF4/19 peptides and the ectodomains of BUPS1/2. **a**, **b** In vitro pull-down experiments to detect the interaction between biotinylated RALF4/19 peptides and the ectodomains of BUPS1/2. Ectodomains of BUPS1/2 (**a**) were expressed in tobacco leaf cells. After incubation for 2–4 h in each combination, streptavidin magnetic particles were added for purification and then subjected to the western blot analysis. The combination with biotin-RALF8 peptide served as a negative control. Ectodomains of BUPS1/2 (**b**) were expressed in insect cells and the pull-down assays were conducted as (**a**). **c** The MST experiments between ectoBUPS1/2-His and RALF4/19 peptides. ΔFNorm represents a relative change in fluorescence. The RALF23-FER combination was used as a positive control and the RALF23-BUPS1/2 were used as a negative control. Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

constant (Kd value) as 1.49 μM (Fig. 3.7c), which was consistent with the previously reported result [18]. Under the same condition with the pre-test, we detected the binding affinities between RALF4/19 peptides and BUPS1/2 ectodomains and found that the dissociation constants were 1.60 μM for BUPS1-RALF4, 1.33 μM for BUPS2-RALF4, 1.77 μM for BUPS1-RALF19, and 0.59 μM for BUPS2-RALF19, respectively (Fig. 3.7c). These data were consistent with the result of RALF23-FER combination, suggesting that the binding affinity of RALF4/19 to BUPS1/2 ectodomain were comparable to that of RALF23-FER. In addition, the MST results also showed that ectodomains of BUPS1/2 receptor had no binding affinity to RALF23 (Fig. 3.7c), suggesting the interaction between RALF4/19 and BUPS1/2 was specific.

Taken together, we found that RALF4/19 peptides could directly bind to the ectodomains of BUPS1/2 with high affinity and specificity. We thereby conclude that RALF4/19 peptides indeed function as signal molecules to be perceived by BUPS1/2 receptors to maintain pollen tubes integrity in Arabidopsis.

3.3.6 RALF4/19 Show High Affinity to ANX1/2

Given that ANX1/2 participate in the regulation of pollen tubes integrity by interacting with BUPS1/2 as a receptor complex and we have demonstrated that BUPS1/2 are the receptors of RALF4/19 peptides, we decide to further investigate whether RALF4/19 peptides could interact with ANX1/2 ectodomains using pull-down experiments and MST assays.

At first, we conducted the pull-down experiments and the results showed that either Flag-tagged ectodomains of ANX1/2 expressed in tobacco leaf cells or His-tagged ectodomains of ANX1/2 purified from insect cells could be pulled down together with the biotinylated RALF4 or RALF19 peptide (Fig. 3.8a, b), suggesting that both RALF4 and RALF19 could interact with ectodomains of ANX1/2. Meanwhile, our protein binding results also showed that the interactions between RALF4/19 and ANX1/2 were specific because neither ectoANX1-Flag nor ectoANX2-Flag could be captured by the biotinylated RALF8 peptide (Fig. 3.8a). Based on these results, we believe that RALF4 and RALF19 peptides are able to interact with the ectodomains of ANX1/2 and this binding is high specific.

In addition, the results of MST experiments also showed RALF4/19 have high affinity to the ectodomains of ANX1/2. To be more specific, RALF4/19 could bind to the ectodomains of ANX1/2 with the dissociation constants ranging from 0.31 to 1.59 μM (0.31 μM for ANX1-RALF4; 1.28 μM for ANX2-RALF4; 1.34 μM for ANX1-RALF19; 1.59 μM for ANX2-RALF19) (Fig. 3.8c), which were comparable to these between RALF4/19 and BUPS1/2 or RALF23 and FER (Fig. 3.7c). Besides, we also found ANX2 showed no binding to RALF23. Therefore, this experimental

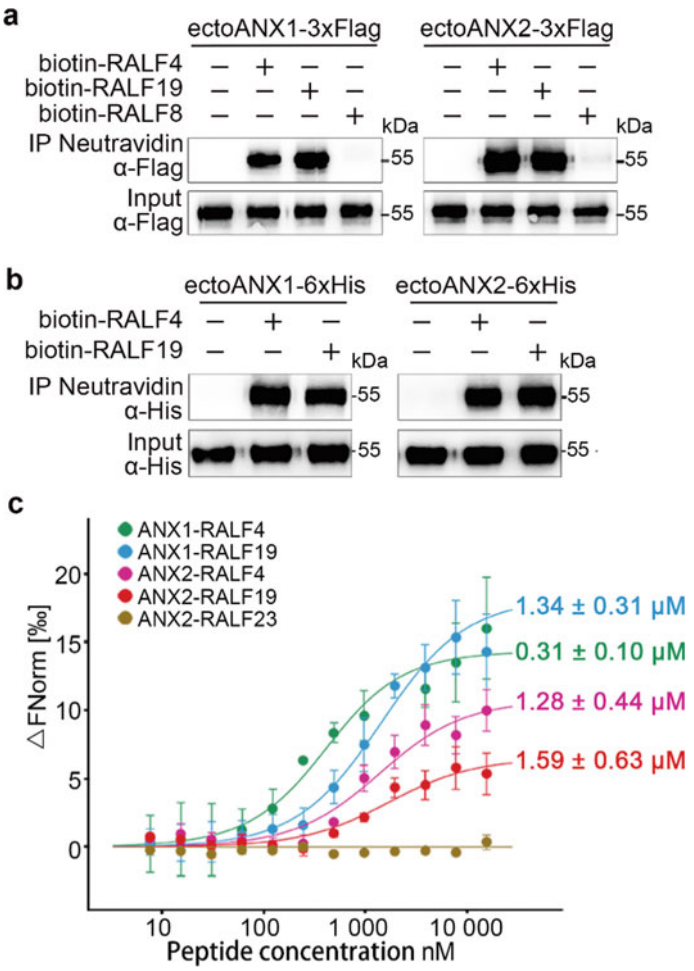

Fig. 3.8 Interaction between the RALF4/19 peptides and the ectodomains of ANX1/2. **a**, **b** In vitro pull-down experiments to detect the interactions between biotinylated RALF4/19 peptides and ANX1/2 ectodomains. Ectodomains of ANX1/2 were expressed in tobacco leaf cells (**a**) and in insect cells (**b**). Pull-down assays were conducted as Fig. 3.7a, b. **c** MST experiments between ectoANX1/2-His and RALF4/19 peptides. ΔFNorm represents a relative change in fluorescence. The RALF23–ANX2 combination served as a negative control. Modified from Ge et al. [28] @The American Association for the Advancement of Science. Reprinted with permission

data suggested that RALF4/19 peptides could specifically bind to ectoANX1/2 with high affinity.

To sum up, during the pollen tube integrity maintenance, BUPS1/2 are able to interact with ANX1/2 to form a receptor complex at the plasma membrane. Here, we

show RALF4/19 could bind to BUPS1/2 and ANX1/2 ectodomains specifically with high affinities, suggesting RALF4/19 are the ligands of BUPS1/2–ANX1/2. Therefore, we conclude that pollen-secreted RALF4/19 peptides function as the autocrine signals to bind to BUPS1/2–ANX1/2 receptor complex on the cell membrane, thereby stabilizing the signaling pathway for the tube cell integrity maintenance during the growth of pollen tubes.

3.4 Discussion

As membrane localized receptors, plant receptor-like kinases should perceive secreted signals from neighboring cells over short distance or long distance [31–33]. However, only few receptor-signal pairs involved in the male/female crosstalk in Arabidopsis have been identified till today, such as PRK6/AtLURE1 pair that is critical for pollen tube guidance [10, 15, 34, 35]. CrRLK1L receptors BUPS1/2 and ANX1/2 are found to interact with each other through the ectodomains to form a receptor complex in pollen tubes, which is critical for pollen tubes integrity during pollen tubes growth. However, the ligands of BUPS–ANX receptor complex remain unclear.

In this chapter, we demonstrate that two cysteine-rich peptides RALF4 and RALF19, which are highly expressed in pollen grains/tubes, participate in maintaining the cell integrity of pollen tubes. *ralf4 ralf19* double mutants show silique abortion and it is the male-specific defect. *ralf4 ralf19* pollen grains show normal morphology and could germinate as the wild-type; however, the pollen tubes discharge at 100% rate right away after germination and the tube growth is arrested prematurely in the style. This phenotype is similar to this of *anx1 anx2* [29, 30] or *bups1 bups2* double mutant. In vitro protein binding assays show that RALF4/19 could bind to BUPS1/2 ectodomains, as well as ANX1/2 ectodomains, with high affinity and specificity, suggesting that RALF4/19 indeed function as the ligands of BUPS1/2–ANX1/2 heteromeric complex, to maintain pollen tube integrity.

Identification of BUPS–ANX receptor complex along with their ligands RALF4/19 have substantially advanced our knowledge of pollen tubes growth and cell integrity maintenance, however, many immediately pertinent questions remain. For example, it is still unclear now how this signaling complex works. To answer this question, the crystal structure of RALF4/19-BUPS/ANX needs to be solved in the future. Moreover, when a pollen tube arrives in the female gametophyte where the sperm cells need to be released, the cell integrity of the pollen tubes should be abolished. What is the signal in female side to trigger pollen tube burst and sperm discharge? Whether BUPS/ANX receptor complex is involved in this process? What will happen in the pollen tubes during this time? These important questions will furnish a fuller mechanistic understanding of plant reproduction and must be addressed in the new future.

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Chapter 4
RALF34 is a Paracrine Signal to Trigger Pollen Tubes Burst and Sperm Release

Abstract During the delivery of sperm cells, pollen tubes maintain the cell integrity via RALF4/19-BUPS/ANX signaling complex when they grow in the maternal tissues. After arriving at the female gametophyte, pollen tubes should rupture timely to release the sperm cells for successful double fertilization, the mechanism of which remains unknown. Here, we show that RALF34 could induce pollen tubes burst in a paracrine manner. *RALF34* shares high sequence identity with *RALF4* and *RALF19*, but it shows distinct expression pattern from *RALF4/19* in that *RALF34* is mainly expressed in the ovule while *RALF4/19* are pollen-expressed. RALF34 is distributed in the inner integument cells. Furthermore, RALF34 peptide could not only interact with BUPS1/2 but also be able to bind to ANX1/2, which is consistent with the pull-down results of RALF4/19 and BUPS1/2–ANX1/2, suggesting RALF34 is another ligand of BUPS–ANX receptor complex. In the competition assay, RALF34 peptide could compete with RALF4 for BUPS/ANX binding. Therefore, our results suggest that RALF34 peptide might act as a paracrine signal to bind BUPS1/2–ANX1/2 complex and participates in regulating pollen tubes rupture and sperm cell release in the female tissues.

Keywords RALF peptides · Paracrine signals · Pollen tubes burst · Sperm release

4.1 Introduction

In cell–cell communications, plant peptides could serve as signal cues and bind to the corresponding receptors which are mainly localized on the cell membrane [1–3]. In general, one peptide delivering a kind of developmental or environmental signal is usually perceived by one receptor or a receptor complex, such as flg22–FLS2 in plant immunity, IDA–HAE/HSL2 for the floral organ abscission and RALF1–FER in root development [3–8]. However, several signal cues could be perceived by a receptor unit in some cases, which often results in different downstream outputs and responses. For instance, EPF2 peptide could inhibit stomata development while EPFL9 peptide mainly promotes the formation of stomata. Both these peptides are the signal ligands of the ER–TMM receptor complex, but EPFL9 could destroy the

Z. Ge, *Arabidopsis BUPS-ANX Receptor Complex Coordinates with RALF Peptides to Regulate Pollen Tube Integrity and Sperm Release*, Springer Theses,
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binding of EPF2 to the receptor complex [9–12]. Besides, LePRK1/2 are pollen specifically expressed receptors and could perceive LAT52 and STIG1 peptides to regulate pollen germination and pollen tubes growth. To be more specific, LAT52 is a pollen-expressed CRP and could bind to LePRK1/2 to control pollen germination. However, STIG1 is mainly accumulated in stigmas/styles and is able to promote pollen tubes growth via binding to LePRK1/2. Interestingly, STIG1 can compete with the LAT52 peptide that has already bound to LePRK1/2 receptor [13–16]. Therefore, these antagonistic peptides of one receptor might be involved in regulating some related but opposite processes.

In plant reproduction, maintaining the pollen tubes integrity during the polar growth and rupture of pollen tubes for sperm release are also related and opposite processes [1, 16–18]. It is known that autocrine signals RALF4/19 coordinate with BUPS1/2–ANX1/2 to maintain the cell integrity of pollen tubes when pollen tubes grow in the maternal tissues. After being guided into the female gametophyte, the pollen tubes will stop growing and rupture to release the sperm cells. However, it remains unclear whether this process is also controlled by the antagonistic signals. In this chapter, with the aim to solve this question, we synthesized RALF peptides that accumulate in ovules but not in pollen tubes, applied them to pollen tubes, and found that RALF34 could trigger pollen tubes rupture in vitro. We further demonstrated that RALF34 peptide could not only bind to BUPS1/2 and ANX1/2 but also is able to compete with RALF4/19 for the interaction with BUPS1/ANX1. Thereby, we believe that RALF34 is an ovule-derived paracrine signal to induce pollen tubes rupture and sperm cell discharge for fertilization; it binds to BUPS–ANX receptor complex by replacing the autocrine signals RALF4/19 which are required for cell integrity maintenance.

4.2 Materials and Methods

4.2.1 Plant Material

Arabidopsis thaliana (Columbia-0) was used as wild-type. All transgenic lines used in this study were in the Columbia ecotype;
fer-4 and *ralf34-1* mutants were ordered from ABRC;
Nicotiana benthamiana was used for transient expression of the ectodomains.

4.2.2 Strains and Plasmids

4.2.2.1 Strains

Agrobacterium tumefaciens GV3101: used for Arabidopsis and tobacco transformation;

E. coli: used for plasmid transformation.

4.2.2.2 Plasmids

pK7FWG0: modified from pK7FWG2 (Department of Plant Systems Biology, VIB-Ghent University, Ghent, Belgium) and used for GFP-tagging;
pB7GUSWG0, modified from pB7WGR2 (Department of Plant Systems Biology, VIB-Ghent University, Ghent, Belgium) and used for GUS report system;
pB7FLAGWG2: GATEWAY-compatible destination vector in this laboratory for flag-tagged protein expression;
pFastBac-Dual vector: bought from Invitrogen for protein expression in insect cells.

4.2.3 Chemicals and Reagents

ProteinFind Anti-His Mouse Monoclonal Antibody: TransGenBiotech, HT501-02;
Anti-Flag-HRP: Sigma-Aldrich, A8592-2MG;
Goat Anti-Mouse IgG-HRP: CWBIO, CW0102S;
3xFlag Peptide: Sigma, F4799-4 MG;
SuperSignal@West Femto Maximum Sensitivity Substrate: Thermo Fisher, 34095;
Copmplete Protease Inhib. Cocktail: Roche, 11836145001;
Ni Sepharose 6 FF: GE Healthcare, 17531801;
Streptavidin Magnetic Particles: Spherotech, SVMS-30-10;
Anti-Flag Affinity Gel: Bimake, B23101.

4.2.4 Methods

4.2.4.1 Plant Growth Condition and Plant Transformation

Arabidopsis thaliana and tobacco plants were grown under long-day conditions (16 h light/8 h dark cycles) at 22 ± 2 °C with a light intensity of ~170 μmol/m^2/s using LED bulbs (Philips F17T8/TL841 17 W) [19].

4.2.4.2 Peptides Synthesis and in Vitro Treatment on Pollen Tubes

All RALF peptides were synthesized by Scilight Biotechnology LLC (Beijing, China) with a purity higher than 95% used in this study and diluted into 2 mM with sterile pure water. For in vitro peptide treatment, the dissolved RALF4/23/34

peptide was diluted with PGM into 2, 20, 200, 2000, and 20,000 nM, and then subjected to the treatment of pollen tubes at 7 h after germination.

4.2.4.3 Peptide Competition Assays

Peptide competition assays in this chapter were conducted based on the in vitro protein binding assay (Chap. 3) [20]. In particular, 20 nM biotinylated peptide and 1 ng/μL flag-tagged ectodomain were mixed in 500 μL binding buffer (20 mM Tris–HCl, pH 7.5, 1% IGEPAL), rotated for 2 h at 4 °C. Then the samples were mixed with 50 μL streptavidin magnetic particles (Spherotech, SVMS-30-10) and 5 μM unlabeled RALF peptide, which was rotated at 4 °C in further for 2–4 h for the elution. Finally, the mixture was washed 4–5 times using the binding buffer and subjected to the western blot by using anti-Flag-HRP (Sigma-Aldrich, A8592-2MG) antibody.

4.3 Results

4.3.1 Screening Female-Secreted Signals Inducing Pollen Tubes Rupture

In Chaps. 2 and 3, we have shown that RALF4/19–BUPS–ANX signaling complex is responsible for the pollen tubes integrity maintenance during the polar growth of the pollen tubes. However, it is still unclear how pollen tubes burst to release sperm after being guided to the female gametophytes. We speculated that there might be a signaling molecule secreted from the female part to block the signaling pathway in pollen tubes, thereby inducing the rupture of pollen tubes. This signal molecule is likely to be the RALF peptide and it might bind the BUPS–ANX receptor complex as well.

In order to test the hypothesis above, we first did transcriptome analysis of RALF gene family to screen the candidates showing high or specific transcript in ovules but not in pollen. In the end, RALF5, RALF14, RALF18, RALF24, RALF28, RALF29, RALF31, RALF33, and RALF34 were successfully selected (Fig. 3.1). We chemically synthesized these peptides, treated wild-type pollen tubes that germinated for 7 h in vitro, and finally identified a peptide, RALF34, that could induce pollen tubes rupture efficiently (Fig. 4.1a–f). Results of peptides treatment showed that 2 nM concentration of RALF34 was able to induce 23% of pollen tubes burst, and the response time was less than 2 min (Fig. 4.1a–g). When the peptide concentration was increased, the proportion of bursting pollen tubes increased. 70% of the pollen tubes were induced to rupture when the peptide concentration reached 20 mM (Fig. 4.1g). However, neither RALF4 peptide (expressed in pollen) nor RALF23

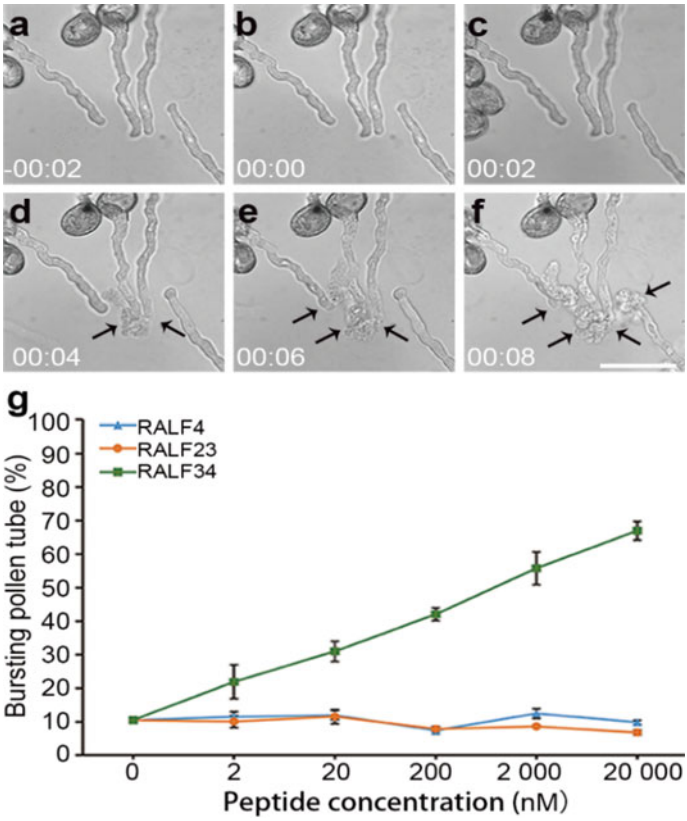

Fig. 4.1 Pollen tubes rupture induced by RALF peptides. **a**–**f** Pollen tubes burst triggered by 20 mM RALF34. The black arrows indicate the bursting pollen tubes and the time format is (minutes: seconds). **g** The effect on pollen tubes rupture of different concentrations of RALF4, RALF23, and RALF34. The observation was taken 2 min after the treatment. Scale bar = 50 μm (**a**–**f**). Modified from Ge et al. [21] @ The American Association for the Advancement of Science. Reprinted with permission

peptide (expressed in leaves) could trigger pollen tubes rupture efficiently at any concentration (Fig. 4.1g), thus indicating that the effect of RALF34 was specific.

The above results show RALF34 peptide is able to specifically induce pollen tubes rupture in vitro. It implies that RALF34 peptide is likely to serve as a signal molecule that is secreted from the female part, target the cell integrity of pollen tubes, and induce the pollen tubes rupture for sperm release.

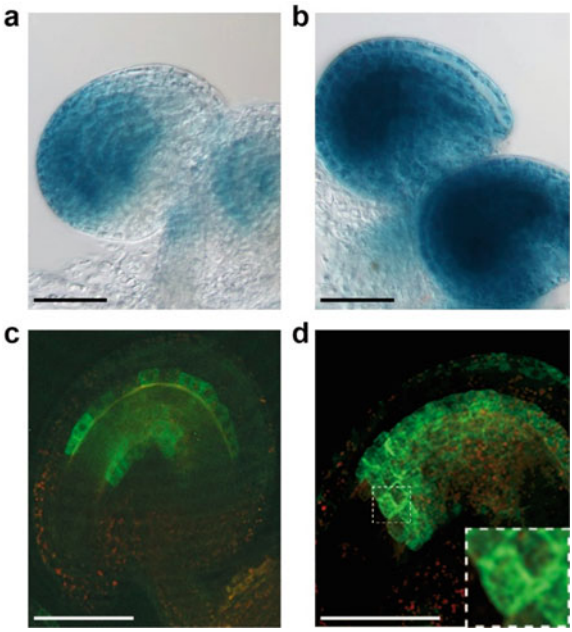

Fig. 4.2 Expression pattern and protein localization of RALF34 peptide. **a**, **b** GUS staining results of *RALF34p::GUS*. **c**, **d** Subcellular localization of RALF34 determined by the GFP observation of *RALF34p::RALF34-GFP*. The scanning Z axis of (**c**) and (**d**) are different. Scale bar = 100 μm (**a**, **b**) or 50 μm (**c**, **d**). Modified from Ge et al. [21] @ The American Association for the Advancement of Science. Reprinted with permission

4.3.2 *Expression Pattern and Subcellular Localization of RALF34*

To reveal the expression pattern of *RALF34*, we constructed *RALF34p::GUS* expression vector and performed the transgene experiment. After obtaining transgenic plants, GUS staining experiment was conducted and revealed that the GUS signal was intensive in the integument of mature ovules (Fig. 4.2a, b), suggesting *RALF34* was mainly expressed in integument cells. Moreover, we also generated transgenic plants of *RALF34p::RALF34-GFP* to analyze the subcellular localization of RALF34 protein. The result showed that the fluorescence signal of RALF34-GFP mainly appeared in unfertilized ovules, and the GFP signal was relatively accumulated in the inner integument cell (Fig. 4.2c, d). Based on these results, we believe that RALF34 peptide is mainly expressed in mature ovules and the coding protein is accumulated in the integument cell.

4.3.3 *RALF34 is Another Ligand of BUPS1/2–ANX1/2*

It has been revealed that BUPS1/2–ANX1/2 receptor complex is involved in maintaining the cell integrity of pollen tubes by perceiving RALF4/19 signals. In the other CrRLK1L modules, such as RALF1-FER and RALF23-FER, RALF is also a

general ligand of CrRLK1L receptor. We presumed that RALF34 might function as the ligand of the pollen-expressed BUPS–ANX receptor complex as well.

To test this hypothesis, pull-down assays were first performed by testing whether RALF34 could interact with the ectodomains of BUPS1/2. The results showed that both ectoBUPS1-Flag and ectoBUPS2-Flag purified from tobacco leaf cells could be pulled down together with the biotinylated RALF34 peptide, suggesting that BUPS1/2 might have direct interaction with RALF34 (Fig. 4.3a). Similar results were also found when insect-cell expressed BUPS1/2 were used in the pull-down assays (Fig. 4.3 b), further verifying the interaction between RALF34 and BUPS1/2.

Besides, we also investigated the interaction between ANX1/2 and RALF34 with the same strategy and the results showed that both the ectoANX1/2-Flag protein expressed in tobacco leaf cells and the ectoANX1/2-His protein expressed in insect cells could be captured by the biotinylated RALF34. It suggested that RALF34 peptide could interact with the extracellular domains of ANX1/2 (Fig. 4.3c, d). At the same time, RALF8 showed no binding to BUPS1/2 or ANX1/2 (Fig. 4.3a–d), suggesting that RALF34 has specific interaction with ANX1/2 and BUPS1/2.

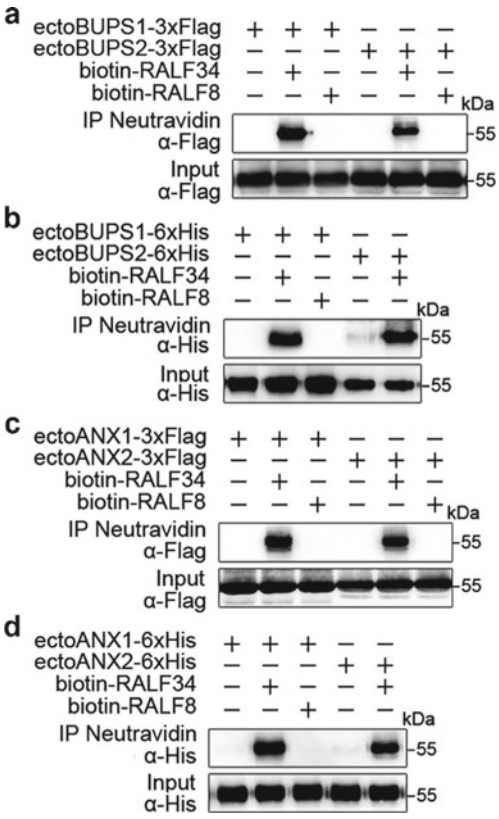

Fig. 4.3 Interaction between RALF34 and ectodomains of BUPS1/2–ANX1/2. In vitro pull-down experiments were used to detect the interaction between BUPS1/2–ANX1/2 and RALF34. **a**, **b** Pull-down assays between RALF34 and BUPS1/2 expressed in tobacco leaf cells (**a**) and in insect cells (**b**). **c**, **d** In vitro binding assays between RALF34 and ANX1/2 expressed in tobacco leaf cells (**c**) and in insect cells (**d**). Modified from Ge et al. [21] @ The American Association for the Advancement of Science. Reprinted with permission

Taken together, our biochemical data showed RALF34 could not only interact with BUPS1/2 but also is able to bind to ANX1/2, which is consistent with the pull-down results of RALF4/19 and BUPS1/2–ANX1/2. Therefore, we believe that RALF34 peptide is another signal ligand of BUPS1/2–ANX1/2 complex and participates in regulating pollen tube rupture and sperm cells release.

4.3.4 RALF34 Competes with RALF4/19 to Bind BUPS1/2–ANX1/2

According to the phylogenetic tree, ovule-expressed RALF34 shares high sequence identity with the pollen-expressed RALF4/19. They are clustered together in the same branch and could directly bind to BUPS1/2–ANX1/2. However, RALF4/19 are involved in maintaining the cell integrity of pollen tubes while RALF34 can induce pollen tube rupture. It is very similar to the working model of LAT52-STIG1-LePRK for pollen germination and tubes growth control, or EPF2/EPFL9-ER/TMM for the regulation of stomatal development in Arabidopsis [1, 11, 12, 15, 22]. Therefore, we speculated that RALF34 might compete with RALF4/19 for the binding of BUPS1/2–ANX1/2 receptor in pollen tubes.

To test this hypothesis, we conducted competition assays via pull-down assays as described previously [20]. First, we incubated the biotinylated RALF4 peptide with ectoBUPS1-Flag or ectoANX1-Flag for 2–4 h and used high concentration of unlabeled RALF4 to elution for competition. The results showed that biotin-RALF4 was able to directly bind to BUPS1 or ANX1, but this interaction was destroyed by the addition of 5 μM RALF4 (Fig. 4.4a). Similar result that 5 μM RALF19 could compete biotinylated RALF19 for the binding to BUPS1 or ANX1 was also observed (Fig. 4.4b). Therefore, it indicated that RALF4/19 peptide indeed could directly bind to BUPS–ANX with high specificity. In the competition assays, we used RALF34 peptide to elute RALF4 and found 5 μM unlabeled RALF34 peptide could disrupt the interaction between the biotinylated RALF4 and ectoBUPS1-Flag or ectoANX1-Flag (Fig. 4.4a). Consistent result was observed when RALF34 was used to elute biotin-RALF19 (Fig. 4.4b). However, unlabeled elf24, a peptide involved in plant immunity [23], showed no effect on the interaction. Therefore, it suggests that RALF34 is able to specifically compete with RALF4/19 that has bound to the BUPS1 or ANX1 receptor.

Taken together, we conclude that RALF34 peptide could not only directly interact with BUPS1/2–ANX1/2, but is also able to compete with RALF4/19 for the receptor binding. We think that RALF4/19–BUPS/ANX functions in pollen tubes to maintain cell integrity and RALF34 serves as the signal switch to turn off this signaling and induce pollen tube burst and sperm release.

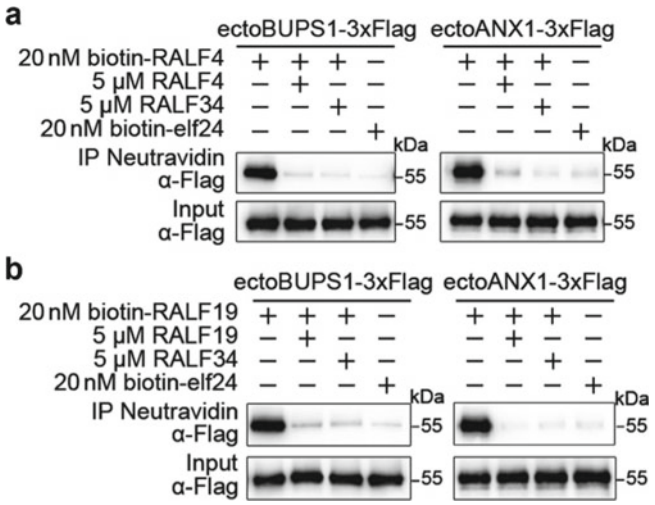

Fig. 4.4 RALF34 competes with RALF4/19 to bind BUPS1/2–ANX1/2. **a**, **b** The binding of RALF4 (**a**) and RALF19 (**b**) to BUPS1 and ANX1 disturbed by RALF34 peptide. Biotinylated RALF4/19 was incubated with ectoBUPS1-Flag or ectoANX1-Flag expressed in tobacco leaf cells, eluted with unlabeled RALF4/19 or RALF34, and subjected to the western blot analysis. Biotinylated elf24 served as a negative control. Modified from Ge et al. [21] @ The American Association for the Advancement of Science. Reprinted with permission

4.3.5 Phenotypical Analysis of RALF34 Loss-of-Function Mutant

We have revealed that RALF34 peptide could induce pollen tube rupture, thus we speculated that RALF34 loss-of-function mutant might have defects in the pollen tube rupture, with pollen tubes overgrowing in ovules. We then did phenotypic analysis of *ralf34-1* mutant [24] and found that, compared with wild-type, *ralf34-1* had no obvious fertility defects with 99.41% seed set (Fig. 4.5a, b). Further Aniline blue staining revealed that the pollen tube in the *fer-4* mutant [25] failed to stop, which led to the abolishment of fertilization. However, the pollen tubes in the *ralf34-1* ovules, similar to these in the wild-type ovules, could normally stop and rupture to release sperm, and the fertilization was achieved successfully (Fig. 4.5c). Therefore, these results show that *ralf34* mutant seems to have no obvious defects in plant fertilization (Fig. 4.5).

4.4 Discussion

Plant peptides and their receptors function as important regulators to mediate cell–cell signal communications, and they are widely involved in many biological processes,

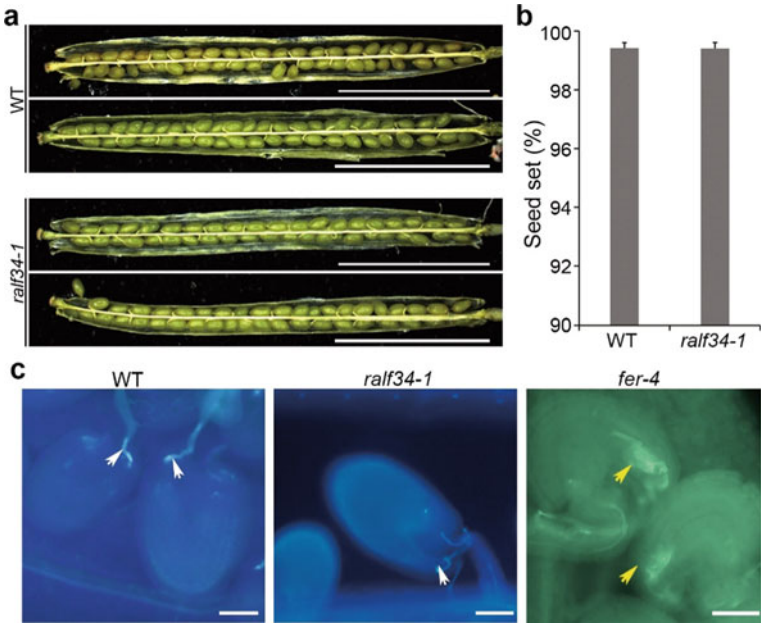

Fig. 4.5 Phenotypical analyses of *ralf34-1*. **a**, **b** The seed production of WT and *ralf34-1* mutant. **c** Aniline blue staining results of WT, *ralf34-1* and *fer-4* after 20 h pollination. White arrows indicate normally fertilized pollen tubes and yellow arrows indicate overgrowing pollen tubes. Scale bar = 5 mm (**a**) or 50 μm (**c**). Modified from Ge et al. [21] @ The American Association for the Advancement of Science. Reprinted with permission

such as plant growth, development, and reproduction [1, 2, 8, 26, 27]. There are approximately 756 genes encoding CRP peptides in Arabidopsis, however, about 205 CRPs show genes transcript in female gametophytes revealed by transcriptome profiling [28]. It implies that CRP peptides play important roles in the communications between male and female tissues, such as pollen tube growth, pollen tube guidance and reception.

In this chapter, we show that the ovule-secreted RALF34 peptide functions as a paracrine signal to induce pollen tube discharge and sperm release in the female gametophyte. RALF34 peptide can compete with RALF4/19 for the binding to ectodomains of ANX and BUPS. We hypothesize that RALF4/19-ANX/BUPS complex is crucial for pollen tubes integrity maintenance during tip growth and RALF34-ANX/BUPS for the pollen tube rupture and sperm discharge (Fig. 4.6).

With the discovery of RALF4/19/34-ANX/BUPS signaling complex, it becomes clear that how the pollen tube integrity is spatiotemporally controlled, which further sheds the light to understand the signaling communication in plant reproduction.

Nevertheless, it is still unclear how the integument distributed RALF34 triggers the rupture of pollen tubes in that the pollen tube stops and bursts in the degraded synergid cell where it is separated from the inner integument cell. It is possible that, on the one

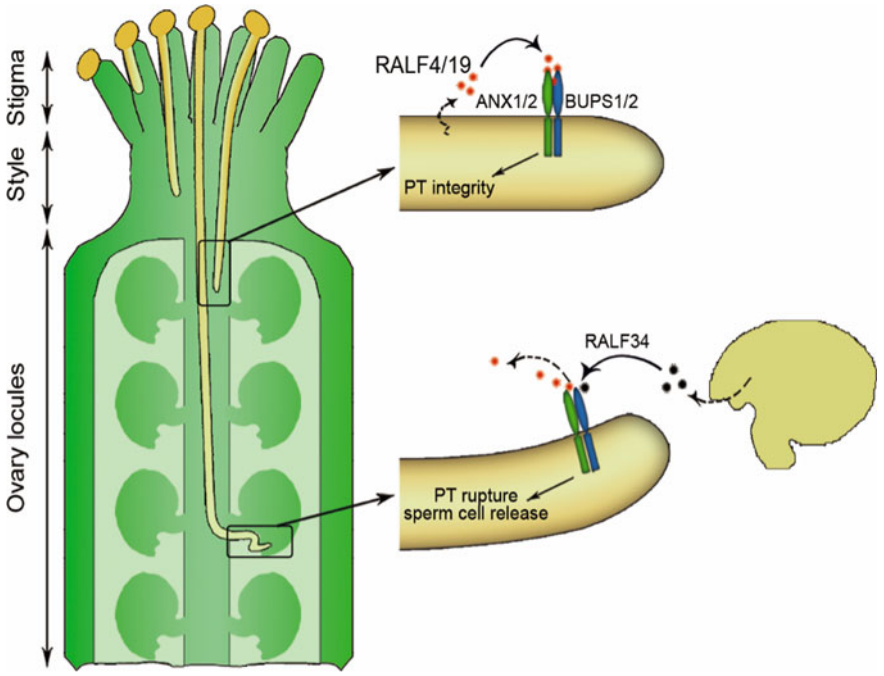

Fig. 4.6 Working model for BUPS1/2–ANX1/2-RALF regulation of pollen tube integrity and sperm cell release. During the tip growth in the maternal tissue, pollen-localized BUPS1/2–ANX1/2 receptor heteromer positively regulate PT integrity by perceiving pollen tube-secreted autocrine signal peptides RALF4 and RALF19. Once arriving at the synergid cell surface, female tissue generated peptides, e.g., RALF34, displace the pollen RALFs and bind to the BUPS–ANXUR receptor heteromer in a paracrine fashion leading to pollen tube rupture to release sperm cells for fertilization. PT, pollen tube. Reprinted from Ge et al. [21] @ The American Association for the Advancement of Science. Reprinted with permission

hand, the pollen tube rupture is an extremely rapid process and the transport/secretion of RALF34 is very dynamic, which is so difficult to be captured by the conventional imaging methods. On the other hand, whether RALF34-GFP fusion is functional needs to be investigated. Moreover, the mutant lacking *RALF34* shows no defects in pollen tube reception and fertilization, which might be resulted from the remaining expression of *RALF34* in that *ralf34-1* is a knock-down mutant, or the redundant roles of other ovule-expressed RALFs. Besides, cell wall components, downstream signal mediators and cellular targets, such as MRI, ROS, Ca^{2+} and K^{+} homeostasis, are also involved in the pollen tube integrity [29–35], but the output of RALF-BUPS/ANX signaling complex and the relationship with these cues remain elusive and should be answered in further. In addition, although RAFL34 and RALF4/19 are believed as the antagonistic ligands of BUPS–ANX receptor complex, how the antagonistic roles of RALF34 and RALF4/19 are mediated needs to be determined by the structure-based strategies and high-resolution imaging as well.

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