Arabidopsis Ras-group LRR PIRL6 functions in male & female gametophyte
development and is regulated by alternative splicing

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Genes essential in both male and female gametophyte development pose a
challenge for the comprehensive functional characterization of plant genomes,
because dual gametophyte lethality can prevent establishment of null alleles,
hampering both forward and reverse genetic analysis. PIRLs are leucine-rich
repeat proteins related to Ras-group LRRs that take part in transcriptional
regulation and developmental cell signaling in animals. Systematic knockout
analysis of the Arabidopsis thaliana PIRL family has implicated four of the genes
in pollen differentiation. However, for one locus, PIRL6, no bona fide knockout
alleles have been recovered. We therefore hypothesized that PIRL6 functions in
both male and female gametophytes, and investigated its structure, expression,
and the effects of RNAi knockdown. RT-PCR in wild type plants and spl mutant
homozygotes, which do not carry out meiosis, indicated that functional PIRL6
mRNA is expressed primarily in gametophytes. A genomic PIRL6-GFP reporter
construct confirmed expression in both pollen and the embryo sac. Leaves, roots,
and spl mutant flowers expressed multiple PIRL6 transcripts, and
characterization of 21 cDNAs identified five alternatively spliced transcripts, all of
which contained premature termination codons, suggesting negative regulation
by unproductive splicing in sporophyte tissues. RNAi-inducing constructs
introduced into wild-type plants triggered high frequencies of both aborted ovules
and abnormal pollen. As expected with reduced gametophyte transmission, the
constructs exhibited segregation distortion, but they were heritable due to
incomplete lethality. Gametophyte defects were also present in subsequent
generations. Together, these results strongly support a role for PIRL6 in
development of both male and female gametophytes. The non-lethal knockdown
approach employed here may provide a promising strategy for functional
annotation of other candidate gametophyte-essential genes in Arabidopsis.
The Magnesium Transportation during Male Gametophyte Development in Arabidopsis

Zhong-Nan Yang

During anther development, male gametophyte develops inside the locule and the tapetal cell provides all nutrients including Mg for its development. Magnesium Transporter 5 (MGT5), a member of MGT family, has dual functions in Mg export and import. Here we show the male gametophyte mitosis and intine formation are defect in mgt5 mutant. The transient expression of GFP-MGT5 revealed that MGT5 is localized in plasma membrane. These suggest that MGT5 in male gametophyte play a role to import Mg from the locule and Mg is essential for male gametophyte development. The expression of MGT5 in the knockout mutant of AMS, an essential regulator of tapetum, is tremendously reduced. Chromatin immunoprecipitation and mobility shift assay experiments demonstrate that AMS can directly bind the promoter of MGT5. Immuno-electron microscopy assay shows MGT5-His localizes in the tapetum plasma membrane. These suggest that AMS directly regulates MGT5 in tapetum for Mg export into locule. The mgt5 plant shows severe male sterility. The expression of MGT5 under tapetum-specific promoter A9 can partly rescue mgt5 fertility. Under high Mg condition, mgt5 fertility can also be restored. These suggest that mgt5 tapetum still has the ability to export Mg, and sufficient Mg supply from tapetum can improve the Mg import of mgt5 male gametophyte. Therefore, MGT5 plays an important role in Mg transport from tapetum to microspore.
**Gametogenesis session**

**MS188 directly regulates the expression of CYP703A2 for sporopollenin biosynthesis in Arabidopsis Thaliana**

Jun Zhu

The major composition of sexine layer of pollen grain has been considered to be the sporopollenin, the biosynthesis of which is controlled by the sporophytic secretory tapetum. Several enzymes involved in sporopollenin biosynthesis have been reported, but the mechanism how these enzymes are regulated by tapetum remains elusive. In this study, we report that a sporopollenin biosynthesis gene, CYP703A2, is positively regulated by two tapetal transcription factors, MALE STERILE 188 (MS188) and ABORTED MICROSPORES (AMS) in Arabidopsis thaliana. The distribution of AMS-GFP, MS188-GFP and CYP703A2-GFP exhibited a similar expression pattern among these proteins. The transcripts of CYP703A2 were down-regulated in ams and ms188. Chromatin immunoprecipitation (ChIP) and a luciferase-inducible assay validated that MS188 can directly bind to the promoter of CYP703A2 and activate its expression. The genetic analysis revealed that the expression of CYP703A2 required the normal function of both AMS and MS188. Biochemical evidence is also provided to demonstrate that MS188 interacted with AMS to trigger the expression of the downstream enzyme for sexine formation.
Male germ-line specification in flowering plants

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The pollen grain is responsible for the production and delivery of sperm cells to the female gametes in flowering plants. Early in pollen development there is an asymmetric division that is critical for the specification of the male germ line. In the germ cell, shortly after the division, the transcription factor DUO1 is expressed. DUO1 controls the differentiation of the germ line through activating a suite of genes required for cell cycle progression and sperm cell function (Borg et al, 2011). Thus, the early germ-cell specific expression of DUO1 is a key step in the specification of the male germ-line. We are investigating the mechanisms that control DUO1 expression and how they relate to the asymmetric division. As a DUO1 promoter:reporter shows the same expression pattern as DUO1, we are focussing on transcriptional regulation. Bioinformatic approaches using DUO1 promoter sequences from a range of flowering plants identified a region showing a surprising level of conservation for a regulatory region. We will report on our functional analysis of this region, and show that a small fragment is sufficient for germ-line specific expression in Arabidopsis thaliana. Next we aim to identify the transcription factors that bind to this region.
Cell fate specification in Arabidopsis female gametophyte development

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The angiosperm female gametophyte contains each specified cell with distinct functions such as pollen tube attraction and fertilization for plant reproduction. In Arabidopsis thaliana, one megaspore undergoes three round of mitosis without cytokinesis as coenocyte. So-called the Polygonum-type female gametophyte contains one egg cell, one central cell, two synergid cells, and three antipodal cells after cellularization. Although the cell fate is thought to be specified via cell-cell communication, it is remains unknown when and how the cell fate is determined during female gametophyte development.

Here, we established the in vitro female gametophyte development system and optical manipulation for disruption of a single nuclear within the Arabidopsis ovule to analyze the cell fate specification in real time. First, we succeeded in time-lapse recordings of nuclear dynamics and division from one-nucleate stage to seven-cell stage after cellularization using the in vitro ovule culture system. These movies showed that nuclear divisions are occurred along the micropylar-chalazal axis as previously observed in the fixed Arabidopsis ovules. During cellularization, the polar nuclei migrate along the cell plate formation. The onset of a synergid-specific marker MYB98pro::GFP expression was detected 2.5 hours after the end of the third mitosis. Considering that cell plate formation was completed 1.5 ~ 2.0 hours after the end of the third mitosis, cell fate are specified relatively immediately after cellularization.

Moreover, we are currently performing optical manipulation of a single nuclear before cellularization. Laser irradiation of a micropylar nuclear, which should divide to form the egg and polar nuclei, at the four-nucleate stage demonstrated that remnant micropylar nuclear divided into the egg and synergid cells after cellularization. We will discuss the cell fate specification of female gametophyte around cellularization in Arabidopsis.
Pollen tubes perceive gravity stress

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Plants are able to perceive gravity stimulation through statoliths present in specialized cells such as the root columella and the endodermis. The pollen tube is not equipped with statoliths and does not display a gravitropic response. However, it is a metabolically extremely active cell with a rapid growth rate and represents an ideal model system to investigate whether processes such as intracellular transport or cell wall assembly are affected non-specifically by altered gravity conditions. We exposed growing pollen tubes to hyper-gravity and simulated micro-gravity conditions using a large diameter centrifuge and a random positioning machine at the European Space Agency. Pollen germination and pollen tube growth were affected to very different degrees by these conditions. Surprisingly, although cell wall assembly was clearly affected, pollen tube growth proceeded almost unhampered once germination was successful. The fact that intracellular transport is affected by altered gravity conditions raises questions about cellular functioning in all eukaryotic organisms including humans.
BINUCLEAR POLLEN1 is a novel regulator of DNA replication through interaction with MCMs

Dong-Qiao Shi

Pollen development or microgametogenesis in higher plant, is a simple and coordinated developmental process covering cell division, cell specification, and cell polarity. We try to combine the study on male gametogenesis and cell cycle, so we performed screening of T-DNA or EMS mutants with cell cycle defect during pollen development. Mutants of pollen mitosis category often show 1N, or 2N pollen grains, instead of 3N in mature wild type pollen. We named mutants with only 2N pollen grains at anthesis as binucleate pollen (bnp). In heterozygous mutant bnp1/BNP1, about 62% of mutant pollen cannot finish PMII and contain only two nuclei, which results in low penetration of mutation (about 6%, n=1026) through male gametophyte. BNP1 is an evolutionally conserved protein from yeast, to plant and animal. It is reported that yeast homologue of BNP1 is involved in cell cycle through interaction with subunit of eIF2, a central regulator of protein translation initiation. However, we found that DNA replication is slowed down during pollen development or root growth when BNP1 is mutated or down-regulated. Further results show that BNP1 play roles in DNA synthesis through interaction with MCM members in planta. Very similar phenotype in pollen development is also observed in mcm mutants, and there are about 25% binucleate mcm pollen at anthesis. We propose that BNP1 is a novel regulatory protein of DNA replication and cell cycle. It is likely that BNP1 participates in DNA replication or elongation through interaction with MCM members.
Toward understanding the regulatory network of antherozoid formation using a liverwort *Marchantia polymorpha*

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In land plants, there are two types of male gamete: one is a non-motile sperm cell which is delivered to an egg cell by a pollen tube, the other is a motile sperm cell with flagella called antherozoid. The genes essential for male germ cell differentiation in flowering plants are identified by genetic and transcriptomic analysis with model angiosperm plants, but little is known about the regulators of antherozoid formation. A liverwort *Marchantia polymorpha* is an emerging model basal land plant, which generates bi-flagellated antherozoids for sexual reproduction. To understand the regulatory network of antherozoid formation, we examined the genes preferentially expressed in developing antheridia (male sexual organs) of *Marchantia* by RNA-sequencing analysis. Among 23,498 predicted genes in *Marchantia* draft genome, we found 731 genes were preferentially expressed in developing antheridia among tested gametophyte organs. In haploid-dominating *Marchantia*, gamete formation does not involve meiosis, which is the distinct feature of gametogenesis in animals and flowering plants. Despite this difference, we found that the gene expression profile of developing antheridia has some common features with that of male gametogenesis in animals and flowering plants. We identified homologs of key genes involved in male gamete formation in Arabidopsis such as DUO1. In Arabidopsis, DUO1 integrates the cell division and expression of sperm cell specific genes during male gametogenesis. *Marchantia* ortholog, MpDUO1 is specifically expressed in sperm cells of *Marchantia*. In *Mpwdou1-1ko*, the final cell division during the male gametogenesis was observed, but the sperm cells of *Mpwdou1-1ko* did not undergo normal spermiogenesis. Neither flagella formation nor nuclear condensation were observed in *Mpwdou1-1ko*. These results will be presented.
A Protein S-Acyl Transferase (PAT) is essential for both male and female fertility in Arabidopsis thaliana

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S-acylation, commonly known as S-palmitoylation, is a reversible posttranslational lipid modification in which fatty acid, usually palmitic acid, covalently attaches to specific cysteine residues of proteins. Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association. It plays roles in protein trafficking and signaling. A family of Protein S-acyl Transferases (PATs) is responsible for this reaction. PATs are multi-pass transmembrane proteins that possess a catalytic Asp-His-His-Cys (DHHC) cystine rich domain. In Arabidopsis there are at least 24 such PATs, four of which have been characterised, revealing their important roles in growth, development, stress responses and early senescence. Here, we reported the characterisation of another novel PAT and the diverse roles it plays in Arabidopsis sexual reproduction. Loss-of-function mutation by T-DNA insertion in this PAT results in failure of seed production in the Arabidopsis mutant. Further studies confirmed that the sterility of the mutant is caused by defects in both male and female sporophytes and gametophytes. This highlights the importance of protein lipid modification by S-palmitoylation in the development and specification of male and female sporophytes and gametophytes.
Flavonols affect the development and heat stress response of tomato pollen through their antioxidant activity

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Climate change is predicted to negatively impact crop yields, partly due to reduced pollen viability and germination at increased temperatures. However, the molecular mechanisms leading to impaired fertility at elevated temperatures are largely unknown. One potential mechanism for this reduced viability is heat-induced accumulation of reactive oxygen species (ROS), which cause damage to macromolecules. By inhibiting ROS production in wild type tomato pollen using diphenyleneiodonium, we showed that basal levels of ROS are necessary for pollen germination and tube growth. Using the ROS sensor DCF and real-time confocal imaging of pollen, we visualized increasing ROS upon transfer to elevated temperatures that impair pollen germination and tube growth, suggesting a biphasic response to ROS. One mechanism by which ROS levels can be maintained at optimal levels is the presence of ROS scavengers in pollen. We used the dye DPBA to demonstrate that flavonols, a class of plant special metabolites with antioxidant properties, accumulate in pollen. The are tomato mutant, which displays reduced levels of flavonols, has impaired pollen viability and germination and accumulates higher levels of ROS in pollen grains and tubes. Strikingly, the are mutant exhibits higher sensitivity to heat stress during pollen germination than wild type tomato. It also accumulates higher levels of temperature-induced ROS. Taken together, this evidence suggests that flavonols act as modulators of pollen development and are involved in the heat stress adaptability of pollen through their ROS scavenging activity.
SNAIL1 plays an essential role in ribosome biogenesis during Arabidopsis gametogenesis and embryogenesis

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Gametogenesis and embryogenesis are two pivotal steps for alternation of generations in flowering plants. Previous research show that ribosome biogenesis play important roles in these two processes. Here, we identified an essential gene SNAIL1, which encodes a protein function in ribosome biogenesis. Loss-of-function of SNAIL1 causes defective transmission efficiency of both gametes. The female gametophyte development in snail1 ovules is delayed and abnormal. Although the male gametogenesis of snail1 is normal, the pollen tube growth is affected. After introduce SNAIL1 gene driven by a female gametophyte-specific promoter to the mutant, we found that SNAIL1 is also essential for embryogenesis. Further analysis show that, SNAIL1 is involved in pre-rRNA processing in Arabidopsis. These data suggest that SNAIL1 plays a crucial role in ribosome biogenesis during gametogenesis and embryogenesis in plants.
Functional Conservation and Evolution of Duo Pollen 1

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DUO1 is a transcription factor that controls cell division as well as cell differentiation in male gametophyte. It belongs to the R2R3 MYB protein family. Although some studies put DUO1 into the GAMYB family, our phylogenetic study showed otherwise, making it a distinct clade on its own. DUO1 homologs have been found in all land plants but not any algae sequenced up to date. The most divergent DUO1 homologs were found in bryophytes like Physcomitrella patens and Marchantia polymorpha. The sequences of DUO1 homologs MYB domains are conserved through all species, while the C-termini bear a hydrophobic/acidic feature in all angiosperms. In functional tests, the monocot Oryza sativa DUO1 was able to fully rescue the phenotypes of Arabidopsis thaliana duo1 mutant in planta and in vivo. While the bryophyte DUO1 MYB domain seems to recognise the same targets in Arabidopsis thaliana, the activation capabilities between the two have a significant difference. Furthermore, the deletion of the hydrophobic/acidic C-terminal end hugely compromised its activation ability in vivo. The effect of C-terminus absence can be partially restored through attaching another activation domain. This suggests the importance of the C-terminus in the activation of its targets and potentially reflects the evolutionary adaptation to the scale of its regulatory network in angiosperms.
Serine as a crucial element for proper pollen development

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A proper pollen development is an essential process for the reproduction of higher plants. Previous works on the implication of the plastidial glyceraldehyde-3-phosphate dehydrogenase (GAPCp) in pollen development showed that mutants on this enzyme produced male sterility (Muñoz-Bertomeu et al., Plant physiology 2010). We suggested that GAPCp was required to provide the precursor 3-phosphoglycerate for the phosphorylated pathway of serine biosynthesis (PPSB). This pathway is one of the three serine biosynthetic pathways described in plants. We further demonstrated that mutations in the first enzyme (phosphoglycerate dehydrogenase, EDA9) (Toujani et al., Plant physiology 2013) and the last enzyme (3-phosphoserine phosphatase, PSP1) (Cascales-Miñana et al., The Plant Cell, 2013) of the PPSB also produced male sterility. Concretely mutations in PSP1 produced not only non-viable-shrunken pollen stopped at polarized microspore, but also lack of pollen coat and absence of oil bodies in the tapetal cell tapetosomes (Flores-Tornero et al. 2015). A co-expression analysis of genes related to PSP1 and EDA9 performed with the CressExpress tool revealed some genes whose mutations affect male gametophyte development and that could be probably related with the availability of serine produced by the PPSB. Here in this work we want to underline not only the important role of the GAPCp on providing substrate for the PPSB, but also the key role of the PPSB on providing serine in those heterotrophic cells where photorespiration does not take place, that is, tapetal cells and pollen grains, among others.
Evolution of the germline specific transcriptional regulators DAZ1/DAZ2 in the plant lineage

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Sexual reproduction of flowering plants comprises the formation of two sperm cells, which are necessary for angiosperms specific double fertilisation. The male germline specific transcription factor DUO POLLEN 1 (DUO1) is an R2R3 myb transcription factor that is required for male gamete formation, specifically for entry of the generative cell into mitosis and for gamete differentiation. Among the DUO1 downstream target genes, two ERF-associated amphiphilic repression (EAR) motif-containing zinc finger proteins (DAZ1 and DAZ2) are essential for germline development and fertility. DAZ1/DAZ2 form together with DUO1 a regulatory module, which is crucial for pollen development and mutation of either DUO1 or both DAZ1 and DAZ2 genes leads to a arrest of male germline development in Arabidopsis. Intriguingly DUO1 and DAZ1/DAZ2 orthologs were identified in a wide spectrum of plants, representing not only angiosperms, but also bryophytes, lycophytes and charophycean green algae, indicating the evolution of this important regulatory module in heterogamous species possessing motile sperm. In contrast to the relatively conserved arrangement of functional domains of DUO1 proteins, DAZ1/DAZ2 homologs show significant variation especially with respect to their Zn finger DNA binding motifs. Consequently DAZ1/DAZ2 proteins from different species differ in their capability to modulate Arabidopsis target gene expression and to complement Arabidopsis mutants, underpinning the adaptation of DAZ1/DAZ2 proteins during plant evolution.
Mitochondrial functions in plant reproduction: insights from S male-sterile maize

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Mitochondria-encoded pollen collapse conditioned by S-type cytoplasmic male sterility (CMS-S) in maize can be reversed by multiple nuclear restorer-of-fertility lethal (rfl) mutations, which also condition a homozygous-lethal seed phenotype. The molecular and cellular features of these phenotypes were investigated to understand mitochondrial contributions to maize reproduction. CMS-S pollen collapse at the young bicellular stage was characterized by DNA and nuclear fragmentation typical of mitochondria-signaled programmed cell death in animals. Immunoblotting studies demonstrated a regulated program of mitochondrial gene expression during pollen development. Cytochrome respiratory pathway proteins were absent or weakly detected and the alternative oxidase was abundant in both CMS-S and normal (N)-cytoplasm uninucleate microspores. Cytochrome pathway components accumulated during starch fill of N-cytoplasm bicellular pollen and remained abundant thereafter. Mitochondrial ribosomal proteins were abundant in microspores and starch-filling pollen, but absent in late bicellular and mature tricellular pollen. In restored CMS-S pollen, the accumulation of cytochrome pathway proteins was reduced by 75% compared to wild-type (+) pollen. CMS-S rfl pollen can effect fertilization in the absence of competition from + pollen, but 75-95% of pollinations were accomplished by pollen carrying the + allele in crosses of N-cytoplasm rfl /+ pollen parents. Maize pollen function is therefore flexible with respect to requirements for cytochrome pathway respiration, but fertility is enhanced in the presence of a wild-type cytochrome pathway. Light microscopy revealed early developmental arrest of rfl mutant embryos and endosperms compared to wild-type siblings, consistent with more stringent requirements for cytochrome pathway respiration during maize seed development.
TGN/EE acidification is required for exocytosis and recycling in Arabidopsis

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In plants, vacuolar H⁺-ATPase (V-ATPase) activity acidifies both the trans-Golgi network/early endosome (TGN/EE) and the vacuole. This dual V-ATPase function has impeded our understanding of how the pH homeostasis within the plant TGN/EE controls exo- and endocytosis. Here, we show that the weak V-ATPase mutant deetiolated3 (det3) displayed a pH increase in the TGN/EE, but not in the vacuole, strongly impairing secretion and recycling of the brassinosteroid (BR) receptor BRI1 to the plasma membrane, in contrast to mutants lacking tonoplast-localized V-ATPase activity only. The brassinosteroid insensitivity defects in det3 were tightly correlated with reduced Golgi and TGN/EE motility. Thus, our results provide strong evidence that acidification of the TGN/EE is indispensable for functional secretion and recycling in plants.