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Update

A wave of specific transcript and protein accumulation accompanies pollen dehydration

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Abstract

In flowering plants, male gametes are immotile and carried by dry pollen grains to the female organ. Dehydrated pollen is thought to withstand abiotic stress when grains are dispersed from the anther to the pistil, after which sperm cells are delivered via pollen tube growth for fertilization and seed set. Yet, the underlying molecular changes accompanying dehydration and the impact on pollen development are poorly understood. To gain a systems perspective, we analyzed published transcriptomes and proteomes of developing *Arabidopsis thaliana* pollen. Waves of transcripts are evident as microspores develop to bicellular, tricellular, and mature pollen. Between the "early"- and "late"-pollen-expressed genes, an unrecognized cluster of transcripts accumulated, including those encoding late-embryogenesis abundant (LEA), desiccation-related protein, transporters, lipid-droplet associated proteins, pectin modifiers, cysteine-rich proteins, and mRNA-binding proteins. Results suggest dehydration onset initiates after bicellular pollen is formed. Proteins accumulating in mature pollen like ribosomal proteins, initiation factors, and chaperones are likely components of mRNA-protein condensates resembling "stress" granules. Our analysis has revealed many new transcripts and proteins that accompany dehydration in developing pollen. Together with published functional studies, our results point to multiple processes, including (1) protect developing pollen from hyperosmotic stress, (2) remodel the endomembrane system and walls, (3) maintain energy metabolism, (4) stabilize presynthesized mRNA and proteins in condensates of dry pollen, and (5) equip pollen for compatibility determination at the stigma and for recovery at rehydration. These findings offer novel models and molecular candidates to further determine the mechanistic basis of dehydration and desiccation tolerance in plants.

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ADVANCES

1) The normal development of the male gametophyte in many flowering plants undergoes stress-related changes. Pollen dehydrates before grain dispersal, then rehydrates to enable pollen function. The bases of dehydration and its impact on pollen development are not understood.
2) Accumulation of late-embryogenesis-abundant mRNAs in bicellular pollen (BCP) suggests dehydration onset after pollen mitosis I. Transcriptome and proteome datasets revealed a new wave of transcripts that peaks in late BCP.
3) Transcripts coexpressed in late BCP show dehydration is accompanied by changes to transporters, pectin modifiers, membrane trafficking, and cysteine-rich proteins.
4) Proteins required for respiration and energy production are abundant in developing and mature pollen.
5) Some pollen-expressed RNA-binding proteins bind mRNA and form mRNA-protein granules.
6) The protein translation machinery accumulating in dry pollen is likely stored in mRNA-protein stress granules until rehydration.
7) Uncovering transcripts and proteins that accompany pollen dehydration is revealing new working models and genes to study dehydration and desiccation tolerance in plant parts.

Introduction

The reproductive adaptations of land plants have played a key role in their successful colonization on land. Dependence on water and motile male gametes for sexual reproduction was retained by moss and basal vascular plants but overcome in seed plants by pollen dispersal and guided-delivery of nonmotile sperms to the female gametes (Hackenberg and Twell 2019). In many flowering plants, male gametophytes undergo programmed dehydration before pollen grains mature. A major purpose of dehydration in pollen grains is to better withstand and survive atmospheric conditions when grains are dispersed from the male organ and transported to the female pistil (Firon et al. 2012). After landing on a compatible pistil, pollen grains adhere, hydrate, and extend a pollen tube (PT) to deliver 2 sperm cells to the embryo sac for union with the egg and the central cell. Successful double fertilization is followed by embryo and endosperm development to produce a seed. Development of the haploid uninucleate pollen to a mature tricellular pollen grain in *Arabidopsis thaliana* has been reviewed mostly from a genetic and genomic perspective (Borg et al. 2009; Hackenberg and Twell 2019). However, the physiological, biochemical, and cellular changes associated with dehydration in developing pollen and the impact on pollen fitness remain poorly understood at the molecular level (Firon et al. 2012; Sze et al. 2021). Integrating these changes during pollen development is critical for understanding subsequent functions, like germination, sperm delivery, and ultimately male fertility for seed and fruit production.

In flowering plants, male gametophyte development inside the anther chamber begins with meiosis of the diploid microspore mother cell to produce 4 haploid microspores, followed by microgametogenesis that ends with the formation of a mature pollen grain (MP) (Borg et al. 2009) (Fig. 1A). Each microspore divides to give a bicellular pollen (BCP) containing a large vegetative cell that engulfs a small generative cell. The generative cell divides later to produce 2 sperm cells. In some plants, as in *A. thaliana*, tricellular pollen (TCP) grains are formed before they are released from the anther. In other plants, such as tobacco (*Nicotiana tabacum*), BCP grains are released and the generative cell divides later within the elongating PT (Hafidh and Honys 2021). In one perspective, "pollen function" refers to post-pollination events that start when the mature grain lands on a compatible female pistil and terminates with discharge of the sperm cells for double fertilization in the female gametophyte (Johnson et al. 2019). Fertilization of the egg and the central cell by the 2 sperms is followed by embryo and endosperm development to produce a seed (Johnson et al. 2019).

Published transcriptomes of developing pollen (Honys and Twell 2004; Qin et al. 2009; Julca et al. 2021) remain largely unmined, though their analyses can reveal new insights and ideas. By comparing and integrating transcriptomes across pollen experiments, we recently showed that the normal development and function of the 1- to 3-celled male gametophyte of flowering plants includes abiotic stress responses (Sze et al. 2021). We postulated that mRNA-protein particles are formed in maturing pollen in response to programmed dehydration similar to stress granules induced by heat or other stresses in vegetative cells. Moreover, we predicted the identity of stored mRNAs in pollen grains based on their developmental expression pattern and reasoned that heat-unresponsive transcripts in germinating pollen are likely stabilized in MP (Sze et al. 2021).

Pollen is categorized as desiccation tolerant (orthodox) or desiccation sensitive (recalcitrant) depending on their relative water content at dispersal and wall properties (Franchi et al. 2011; Firon et al. 2012; Pacini and Dolferus 2019). BCP, like tobacco and lily, are orthodox as they contain less water (<30% relative water content [RWC]) at dispersal and are relatively tolerant to dry periods. Some self-pollinating plants produce recalcitrant pollen with high water content (>30% to 70% RWC) and are short lived, such as rice (5 minutes) (Pacini and Dolferus 2019; Moon and Jung 2020). Like other plant parts, "dehydration" in pollen is considered as a steady water loss that progresses from

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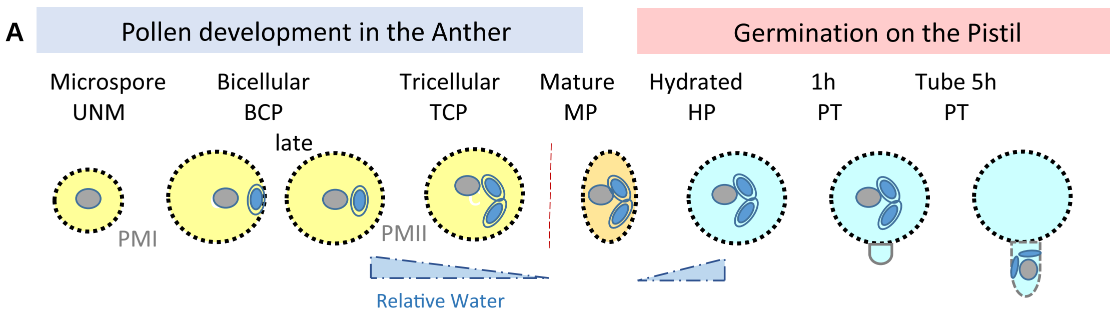

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Gene	UNM	BCP	L-BCP	TCP	MP	PT	Sperr	ID
	mRNA Abundance (Normalized TPM)							
STP2	0.62	0.39	0.07	0	0	0	0	At1G07340
SWEET8	0.84	0.55	0.11	0	0	0	0	At5G40260
COPT3	0.96	0.77	0.17	0	0	0	0	At5G59040
STP9	0.02	0.27	1	0.1	0	0	0	At1G50310
SUC1	0.02	0.09	1	1	0	0	0	At1G71880
SWEET5	0.01	0.04	0.53	1	0	0	0	At5G62850
TIP1;3	0.0	0.2	1.0	1.0	0.1	0.3	0	At4G01470
SPIK1	0.06	0.05	0.07	0.3	1	0.1	0	At2G25600
AHA8	0.01	0.01	0.04	0.5	0.9	0.6	0	At3G42640
ACA9	0.01	0.07	0.24	0.6	1	0.4	0	At3G21180
SUC9	0	0	0	0	0	1	0	At5G06170
LHT5	0	0	0	0	0	1	0	At1G67640
AVP1	0.13	0.15	0.18	0.2	0.1	0.1	0	At1G15690
VHA-a3	0.15	0.2	0.28	0.3	0.3	0.2	0	At4G39080

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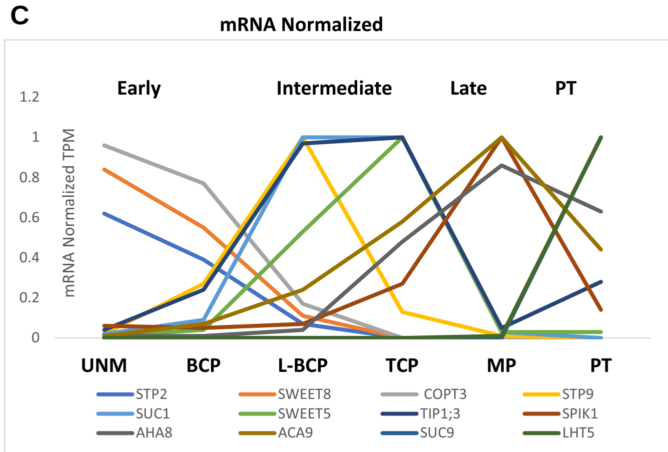

Figure 1. Waves of gene expression in developing pollen reveal a novel intermediate cluster. **A)** Stages of pollen development and function. Inside the anther, the uninucleate microspore (UNM) develops to bicellular pollen (BCP), tricellular pollen (TCP), and mature/dry pollen (MP). PMI and PMII refer to Pollen Mitosis I and II, respectively. After dry pollen is transferred to a compatible stigma, pollen hydrates (HP) and extends to a pollen tube (PT). The vegetative nucleus is shown grey. Generative and sperm cells are dark blue. Transcriptome data of pollen stages are available from ATH1 microarray (Honys and Twell 2004; Wang et al. 2008; Qin et al. 2009) and RNA-Seq (Julca et al. 2021). Relative water content is shown in blue dashes. **B)** Heat map of mRNA abundance reveals 4 clusters as pollen develops, matures, and extends a tube. Representative examples of mRNA abundance selected from transcript clusters determined by RNA-Seq (Julca et al. 2021). Four mRNA clusters peak at UNM-BCP, late BCP-TCP, MP, and in vitro germinated PT (5 hours). The cell/tissue-type with the highest transcript per million (TPM) is scaled to a normalized read of 1.0. Transporter genes are identified by their Atg ID # and names. AVP1 and VHA-a3 represent transcripts with constitutive expression. All transcripts shown here are not detected in sperm cells (Sper). **C)** A novel transcript wave peaks between the early- and late-pollen expressed genes. A cluster of mRNAs rises after BCP formation, accumulates at the late BCP or TCP stage, and drops at TCP and MP (late). This "intermediate" wave of mRNAs is distinct from late-pollen expressed transcripts which increase at TCP, peak in the MP and can persist in the pollen tube (PT). Early pollen-expressed genes show maximal mRNA in UNM that is reduced in BCP. (Data from **B**).

mild stress (80% to 65% RWC) to moderate (65% to 50%) and severe stress (40% RWC), and desiccation (<35%) is the final result of dehydration (Zhang and Bartels 2018). Although *A. thaliana* self-pollinates and produces tricellular pollen, its pollen is considered orthodox. Orthodox pollen is considered desiccation tolerant because (1) they are viable for up to 3 days after anthesis, (2) the pollen wall contains furrows that allow pollen to change shape from spherical when hydrated to ovoid when partially dehydrated (Franchi et al. 2011), and (3) they recover and are competent for germination after hydration. In contrast, desiccation-sensitive pollen lacks strategies to maintain osmotic balance. Thus, desiccation tolerance depends on the interplay of multiple processes in pollen with the environment (Franchi et al. 2011).

Pollen grains reach maturity when they are dehydrated through a process called developmentally programmed dehydration, though the molecular and physiological basis of programmed dehydration is poorly understood. After a haploid microspore divides to form a BCP containing a vegetative and a generative cell, as in tobacco, pollen enters a metabolically "quiescent" state as the anther becomes desiccated, dehisces, and disperses MP (Firon et al. 2012). The regulation and physiological basis of anther dehydration are unclear, it probably includes 1 or a combination of factors, including prevention of water flow in the stamen filament to the anther, diversion of water in the anther to the stamen filament, loss of water by transpiration via anther stomata (Wei et al. 2018), and evaporation after anthers dehisce, exposing pollen to the environment (Sze et al. 2021). In *A. thaliana*, stamen maturation is regulated by jasmonic acid (JA) as mutants of JA biosynthesis or perception are male sterile (Jewell and Browse 2016; Acosta and Przybyl 2019). Mutants of DAD1 lipase or DDE1/OPR3, 2 enzymes required for JA biosynthesis, show delayed anther dehiscence

(Sanders et al. 2000; Ishiguro et al. 2001). Transcripts or protein of DAD1 or DDE1/OPR3 localize to the stamen filament particularly at maturation. The site of JA perception was shown to reside in the epidermis of the stamen filament and the anther, as epidermis-expressed COI1 coreceptor restored male fertility of *coi1* mutant (Jewell and Browse 2016). How JA influences water distribution is unknown. Two possibilities are that either water flow into the filament is blocked, or water flows out of the anther to the stamen filament (Ishiguro et al. 2001) due to solute accumulation (Stadler et al. 1999) followed by water flux down a water potential gradient. As anther cells dehydrate, they likely draw liquid from the locules causing pollen to dehydrate. Anthers fail to split open if cells with secondary thickening at the endothecium layer do not dehydrate and shrink. Thus, programmed dehydration starts in sporophytic tissues surrounding the gametophyte, in response to JA perception in stamen filament and anther epidermis in *A. thaliana* (Jewell and Browse 2016).

To begin understanding the molecular basis of dehydration and desiccation tolerance in pollen, we analyzed transcriptome and proteome results (Julca et al. 2021; Klodova et al. 2023) of *A. thaliana*. Waves of coexpressed transcripts corresponding to early and late pollen-expressed genes (Fig. 1, B and C) were observed before (Honys and Twell 2004). Here we reveal a temporal wave of transcripts that peak at the late BCP stage between the early and late pollen-expressed genes. Previously unnoticed, this intermediate wave includes mRNAs encoding multiple LEA proteins, which are thought to protect proteins and membranes from aggregation due to desiccation or osmotic stress (Goyal et al. 2005). Combined with published functional studies, our analysis revealed that genes coexpressed with LEAs are not only involved in osmotic stress adjustment but also in membrane and wall remodeling, in protection of transcripts in "stress" granules, and in recovery at rehydration after pollen lands on the stigma. As water deficit becomes more prevalent around the globe (Mancosu et al. 2015), the normal development of orthodox pollen provides a simple model to understand the basis of dehydration and desiccation in vegetative and reproductive plant parts, and in the evolution of sexual reproduction.

Expression waves reveal a distinct "intermediate" gene cluster in late BCP

Clustering analysis revealed that waves of coexpressed transcripts correspond with pollen development stages (Fig. 1). Previously, analysis of *A. thaliana* pollen transcriptome (Honys and Twell 2004) had shown that genes expressed early in development in microspores (UNM) and BCP became repressed in tricellular (TCP) and MP, whereas late pollen-expressed genes were low or absent in UNM and BCP but increased in TCP and in MP (Fig. 1C). Importantly, the recent RNA-seq study included an additional substage, the *late* BCP. A pilot test of the new results revealed a novel cluster of transcripts, peaking at the late BCP to TCP stage, between the early and late pollen-expressed genes (Fig. 1C). Transcripts encoding transporters accumulating in UMN-BCP (early), TCP, and TCP-MP (late), shown by microarray (Bock et al. 2006), are confirmed by RNA-seq (Julca et al. 2021), except for a distinct "intermediate" peak. The early pollen-expressed transcripts encode proteins enriched for function in protein translation, nucleic acid metabolism, protein-modifying enzymes, and metabolite interconversion enzymes (Supplementary Fig. S1). The late-expressed transcripts encode proteins enriched in characterized or predicted roles in cell wall synthesis and remodeling, signaling proteins, and receptors that support pollen germination and tube growth (Supplementary Fig. S1) (Sze et al. 2021). The role of the intermediate cluster is unknown.

To identify genes expressed preferentially at the intermediate (late BCP-TCP) stage, we collected cluster 1 and 2 derived by k-means clustering of male-specific genes (from Fig. S14 of Julca et al. 2021). This study had normalized gene expression in transcript per million (TPM) to compare relative expression of 48 samples and scaled gene expression from 0 to 1.0. The clusters revealed transcripts peaking at 4 stages: UNM-BCP, late BCP-TCP, MP, and PT stages similar to Fig. 1B. This analysis confirms that specific transcripts are preferentially coexpressed at a particular pollen developmental stage(s) (Fig. 1). Notably, other transcripts are constitutively expressed at all stages, as shown for vacuolar proton pumps, VHA and AVP1 (Fig. 1B).

For simplicity, we define transcripts of the "intermediate" cluster (Supplementary Tables S1 and S2) as those that rise after BCP is formed, peak at late BCP or TCP, and drop by MP (Fig. 1). To focus on transcripts mainly coexpressed in the vegetative cell, sperm-preferential genes (evorepro.sbs.ntu.edu.sg) were removed. The "intermediate" cluster of 427 transcripts was further analyzed here. We verified that transcripts peaking at the TCP stage from microarray results overlapped with those obtained in the intermediate stage by k-means clustering from RNA-seq results. Taking the LEA family as an example, 13 (array) of 14 (RNA-seq) (92%) members were expressed in developing pollen (late BCP-TCP), and 24 (array) of 26 (RNA-seq) showed elevated mRNA in developing seeds (Supplementary Fig. S2). Even though LEA transcript abundance using 2 methods are not quantitatively similar, the temporal and spatial distribution in pollen or seeds were strikingly consistent (Supplementary Fig. S2).

Genes expressed in late BCP point to the onset of dehydration

Panther v17 (Thomas et al. 2022) classified at least one-half of the query genes (e.g. 427), and the remaining genes were manually categorized using TAIR, Araport, Aramemnon, and UniProt. Top protein classes expressed in BCP and TCP were metabolite interconversion enzyme, transporter, defensin, and protein-modifying enzyme. Plant-specific genes like

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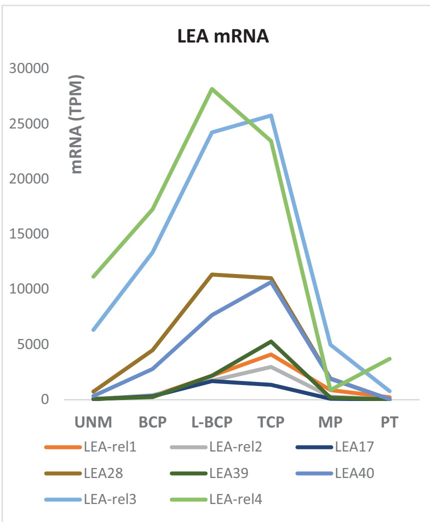

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ID	Name	i. RNA (TPM) L-BCP	i. RNA (TPM) TCP	ii. RNA Normalized L-BCP	ii. RNA Normalized TCP	ii. RNA Normalized Endo	ii. RNA Normalized Seed	B. Protein AA	B. Protein IDR	B. Protein Early	B. Protein Late	B. Protein MP
At1g02820	LEA2	4	0	0.0	0.0	0.0	0.0	91	0.3	nd	nd	nd
At1g03120	LEA3	100	62	0.9	0.6	0.0	1.0	135	0.4	4	0	6
At1g15415	(LEA-rel1)	2153	4084	0.4	0.8	0.0	0.0	96	0.8	30	1	49
At1g52680	(LEA-rel2)	1725	2953	0.6	1.0	0.0	0.0	114	1	63	2	29
At1g54410	LEA8	830	721	0.1	0.1	0.2	0.0	98	1	1	0	3
At2g18340	LEA13_er	10	121	0.1	0.8	0.1	1.0	417	0.1	9	0	28
At2g23120	LEA16	221	84	0.1	0.0	0.0	0.3	83	1	nd	nd	3
At2g33690	LEA17	1671	1342	1.0	0.8	0.0	0.0	75	1	13	0	9
At2g44060	LEA26	168	49	0.1	0.0	0.1	0.0	324	n/a	83	37	107
At2g46140	LEA27	413	704	0.4	0.6	1.0	0.0	166	n/a	36	2	69
At3g02480	LEA28	11331	11009	1.0	1.0	0.0	0.2	68	1	18	1	42
At4g02380	LEA38_mt	132	2526	0.0	0.5	0.0	0.0	192	n/a	nd	nd	nd
At4g13230	LEA39	2149	5258	0.4	1.0	0.0	0.0	120	0.5	50	4	25
At4g13560	LEA40	7647	10648	0.7	1.0	0.0	0.0	109	0.8	203	16	194
At4g36600	LEA43_er	89	206	0.1	0.2	0.0	1.0	353	0.1	7	1	25
At5g06760	LEA46	222	494	0.1	0.1	0.0	1.0	158	0.9	14	0	15
At5g27980	LEA47	29	443	0.0	0.7	0.0	0.0	192	0.2	20	0	65
At5g38760	(LEA-rel3)	24229	25749	0.9	1.0	0.0	0.0	67	1	19	2	6
At5g53820	(LEA-rel4)	28155	23441	1.0	0.8	0.0	0.0	67	1	25	4	13

Figure 2. LEA mRNAs and proteins accumulate in late bicellular and tricellular pollen. **A)** Expression of LEA mRNAs in developing pollen. Eleven of 19 LEA transcripts are specifically or preferentially expressed in pollen. Eight of 11 accumulate in late BCP and TCP. Transcripts expressed as raw TPM (1) or normalized TPM (2) in late BCP, TCP, endosperm (Endo) or seed. Relative mRNA abundance (TPM) is shown as high (red), medium (orange-yellow) and low (green). Normalized RNA of 1.0 indicates the maximum expression across 48 tissues or developmental stages. High to low abundance is shown as dark to light red. Four genes with high expression are unnamed. Here we tentatively call them LEA-related1 to LEA-related4 (LEA-rel). **B)** LEA proteins in pollen. AA and IDR refer to amino acid residues, and predicted intrinsically disordered region, respectively. High to low IDR is shown from dark to light brown. Right, Relative protein abundance from 2 studies: UNM-BCP (early), TCP-MP (late) (Klodova et al. 2023) and mature pollen (MP) (Grobei et al. 2009). Relative scale from high (blue), medium (yellow), to low (grey).

LEAs are often poorly annotated (Supplementary Table S1) and were manually identified from publications.

LEAs

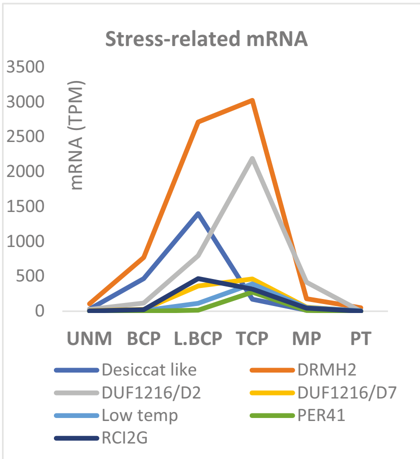
A. thaliana has over 50 LEA genes (Hundertmark and Hincha 2008; Candat et al. 2014), and 41 show preferential expression either in developing seeds (23 LEAs), in pollen (9 LEAs), or both (5 LEAs) (Supplementary Fig. S2). Some LEA proteins are localized to organelles, such as ER (LEA43-er) and mitochondria (LEA38-mt), and thus are present in many tissues (Hundertmark and Hincha 2008; Candat et al. 2014). The remaining 9 to 11 LEA transcripts accumulate in other tissues, such as stamen filament (LEA8), sepals, petals, root quiescent center (LEA5, LEA44), and root differentiating zone (evorepro.sbs.ntu.edu.sg).

Our analysis of RNA-seq results (Julca et al. 2021) showed that 19 of 50 LEA genes are expressed during *A. thaliana* pollen development (Fig. 2) (Honys and Twell 2004; Qin et al. 2009; Sze et al. 2021). Named for their abundance in seeds, LEA protein accumulation to high concentration coincides with the acquisition of desiccation tolerance. Thus, LEA proteins are thought to play a primary role in desiccation tolerance, as in protecting the viability of desiccated seeds (Hoekstra et al. 2001). LEA proteins are hydrophilic, heat-stable, and are thought to protect membranes and proteins from aggregation due to osmotic stress from desiccation (Goyal et al. 2005). They are induced in response to programmed dehydration in developing seeds (Olvera-Carrillo et al. 2010), and some are ABA inducible (Hundertmark and Hincha 2008). Many LEAs contain predicted intrinsically disordered regions (IDR) (Fig. 2B), and LEA46 might serve as a potential osmosensor in plant cells (Cuevas-Velazquez et al. 2021). Of 19 pollen-expressed LEA genes in *A. thaliana*, most peak at TCP and/or late BCP (Fig. 2A) and drop in the MP. Nine LEAs are specific to pollen. Most pollen-expressed LEAs (e.g. 15–17) are translated, based on peptides detected in developing or MP (Grobei et al. 2009; Klodova et al. 2023) (Fig. 2B). Together, the results suggest that the onset of dehydration occurs at the BCP to late BCP stage.

Other stress- related proteins

Several other stress-responsive transcripts accompany LEA mRNAs at the late BCP or TCP stage, including a desiccation-related protein (DRP), a dormancy-associated protein homolog or stress response regulatory protein (DRMH2), a low-temperature–induced protein (AT3G46750), 2 low-temperature and salt-responsive proteins (RCI2C/RCI2G), and an ABA-related protein (CAR5) (Fig. 3).

A DRP was named originally in a resurrection plant, *Craterostigma plantagineum*, after leaves were desiccated or in callus treated with ABA (Bartels et al. 1990; Piatkowski et al. 1990). Resurrection plants recover after complete desiccation (<2% water content) and are viable after rehydration. Interestingly, homologs of the CpDRP [PCC13-62] are found in many nonresurrection plants. In *A thaliana*, 2 homologs are expressed in developing pollen, anther, or seed [mined from (Julca et al. 2021)], suggesting this protein



ID	Name	i. RNA L.BCP	i. RNA TCP	ii. RNA Normalized L.BCP	ii. RNA Normalized TCP	Anth	Pod	B. Protein Dev	B. Protein MP
At1G47980	Desiccation	1399	173	0.8	0.1	1.0	0.1	208	281
At5G44300	DRMH2	2711	3022	0.9	1.0	0.2	0.0	9	5
At3G28780	DUF1216/D2	797	2189	0.4	1.0	0.5	0.0	4	300
At3G28840	DUF1216/D7	360	463	0.7	0.9	1.0	0.0	22	364
At3G46750	(Low temp)	114	392	0.3	1.0	0.2	0.0	19	67
At4G17690	PER41	14	273	0.0	0.9	0.1	0.0	16	68
At4G28088	RCI2G	467	319	0.1	0.1	0.0	1.0	nd	nd
At1G66360	CAR2	16	288	0.1	1.0	0.0	0.0	nd	nd
At1G70810	CAR7	13	188	0.0	0.4	0.1	1.0	3	12
At4g17870	PYR1	104	61	0.5	0.3	0.3	0.6	nd	nd
At3G28980	DUF1216/D8	3718	5522	0.7	1.0	0.5	0.0	40	1331

Figure 3. mRNA and protein accumulation of stress-related proteins in late BCP and TCP. RNA abundance in TPM at various pollen stages is plotted (left) and values from late BCP and TCP stages are tabulated (1). Red, yellow, and green shadings refer to high, medium, and low abundance, respectively. Normalized RNA (2) abundance in pollen, anther (Anth) or seed pod (Pod) is scaled from 0 to 1. Atg gene ID# and abbreviated names of genes are shown if available. "Desiccation" and "Low temp." are uncharacterized genes annotated as "Desiccation-related protein" and "low temperature-induced protein" in Araport, respectively.

has a role in desiccation tolerance in angiosperms. Although AtDRP transcript declined in MP, the protein level was relatively high (Fig. 3), suggesting the protein is translated and likely functional before maturation.

Although genes in Fig. 3 are largely uncharacterized, their expression in response to salt, cold, and desiccation in non-pollen cells point to hyperosmotic stress in late BCP to TCP stage. Whether ABA, the stress hormone, regulates pollen dehydration is unclear, though transcriptome analysis infers participation of ABA biosynthesis and signaling (Supplementary Fig. S3). Together, our results reveal gene expression changes that point to the onset of stress, such as dehydration, in late BCP.

mRNAs coexpressed in the intermediate phase provide clues to the dehydration process

If LEA transcripts are induced in BCP at the onset of dehydration, then transcripts coexpressed with LEAs are potentially enriched in functions to adjust and protect cells from osmotic stress. To this end, we surveyed genes induced at the intermediate phase. Our analysis reveals that dehydration is accompanied by multiple changes in the single vegetative cell of the male gametophyte. Changes include ion and metabolite fluxes, endomembrane dynamics, cell wall remodeling, and strategies to protect transcripts, proteins, and the translation machinery. Transcripts with potential roles in pollen dehydration and adaptation are highlighted.

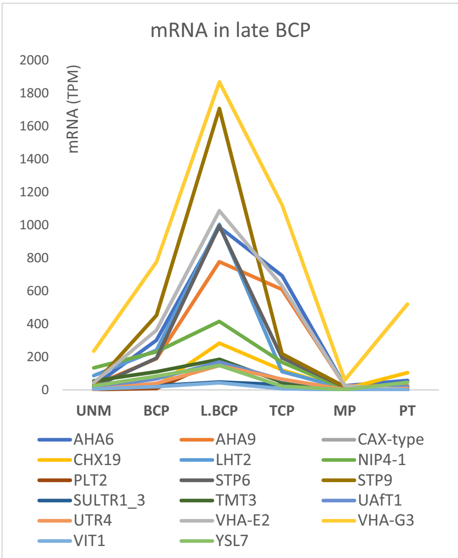
Transporters are abundant and point to dynamic cellular functions

Transporters represent one of the most abundant protein classes (18%–20%) expressed in the "intermediate" cluster (Fig. 4, A and B, Supplementary Fig. S4) and provide glimpses into their cellular functions. Most transcripts in developing pollen (late BCP and TCP) are translated based on the proteome results (Grobei et al. 2009; Klodova et al. 2023). Proteins were detected for 14 to 17 out of 20 transcripts (70%–85%) peaking at late BCP and 10 to 15 of 27 (37%–55%) transcripts in TCP (Fig. 4). These are likely underestimated, as detection of peptides is less sensitive than transcripts. The absence of a spectral count (indicated as n.d.) indicates the peptide level is low, undetectable, or absent. Thus, at least one-half to three-quarters of the transcripts are translated before pollen desiccates. Protein level is frequently high when transcript level is high, as seen for AHA6, AHA9, VHA-G3, VHA-E2, STP6, and STP9 (Fig. 4). However, some abundant transcripts yielded little or undetectable protein, like LHT8 and SWEET5. Based on the patterns, 4 outcomes are considered: (1) the protein is translated and functional during pollen development (late BCP-TCP); (2) protein is synthesized, stable, and stored in the MP for use later in germination and PT growth; (3) a combination of both (1) and (2); or (4) mRNA is stored and not translated until germination. Multiple transmembrane-spanning or low-abundance proteins, like channels, are often not detected (e.g. CNGC18; OSCA1.7) (Supplementary Fig. S4). As many transporters have been experimentally characterized, we provide a working model integrating transport activities (characterized or predicted) within a single cell of developing pollen (Fig. 4B).

Proton pumps

As H^+-pumps provide the central driving force for all co-transport and channel fluxes (Taiz et al. 2015), PM-localized H^+ pumps are postulated to be functional in developing pollen before the cell becomes metabolically quiescent in MP. Similarly, the vacuolar-type H^+ pump is required to energize transport across the vacuolar

A



		i. RNA		ii. Normal		iii. Protein		iv. Function	
ID	Name	L-BCP	TCP	L-BCP	TCP	Dev	MP	Memb	Activity
At3g57330	ACA11	122	37	1	0.3	48	96	vac	Ca pump
At2G07560	AHA6	990	694	1	0.7	1166	1055	pm	H+ pump
At1G80660	AHA9	778	611	1	0.8	149	211	pm	H+ pump
At2G34030	CAX-type C	46	9	1	0.2	0	3	nd	(Cation 2+)
At3G17630	CHX19	284	121	1	0.4	37	86	pm/endo	K+/H+
At4g16480	INT4	393	129	0.7	0.2	42	164	pm	Inositol
At1G24400	LHT2	1005	112	1	0.1	4	19	pm	Amino acid
At5G37810	NIP4-1	416	168	1	0.4	1	4	pm	Water, urea
At2G16130	PLT2	179	50	1	0.3	3	16	pm	Fru, xylitol
At3g19930	STP4	520	45	1	0.1	5	231	pm	Glu, Gal
At3G05960	STP6	997	195	1	0.2	12	70	pm	Glu, Man
At1G50310	STP9	1708	220	1	0.1	39	282	pm	Glu,
At1G22150	SULTR1_3	48	32	1	0.7	nd	nd	(pm)	Sulfate
At3G51490	TMT3	186	47	1	0.3	15	73	vac	Glu, Fru
At5G25400	UAfT1	170	62	1	0.4	1	4	Golgi	UDP-sugar
At1G12600	UTR4	148	66	1	0.4	nd	nd	Golgi	UDP-sugar
At3G08560	VHA-E2	1088	633	1	0.6	80	64	endo	V H+ pump
At4G25950	VHA-G3	1870	1123	1	0.6	9	21	endo	V H+ pump
At2G01770	VIT1	44	8	0.9	0.2	nd	nd	vac	Fe/H+ influx
At1G65730	YSL7	149	23	1	0.2	4	60	pm	Fe-sideroph
At3G54700	PHT1-7	116	107	0.7	0.6	4	37	pm	Pi/H+
At2G22950	ACA7	92	102	0.9	1.0	102	114	er	Ca pump
At3G54510	OSCA2.5	16	15	1.0	1.0	nd	nd	(pm)	(Ca channel)
At1G71880	SUC1	1061	1059	1.0	1.0	6	69	pm	Suc
At5G19600	SULTR3_5	120	146	0.8	1.0	12	59	plastid	Sulfate
At4G01470	TIP1-3	974	999	1.0	1.0	1	20	vac	Water

B

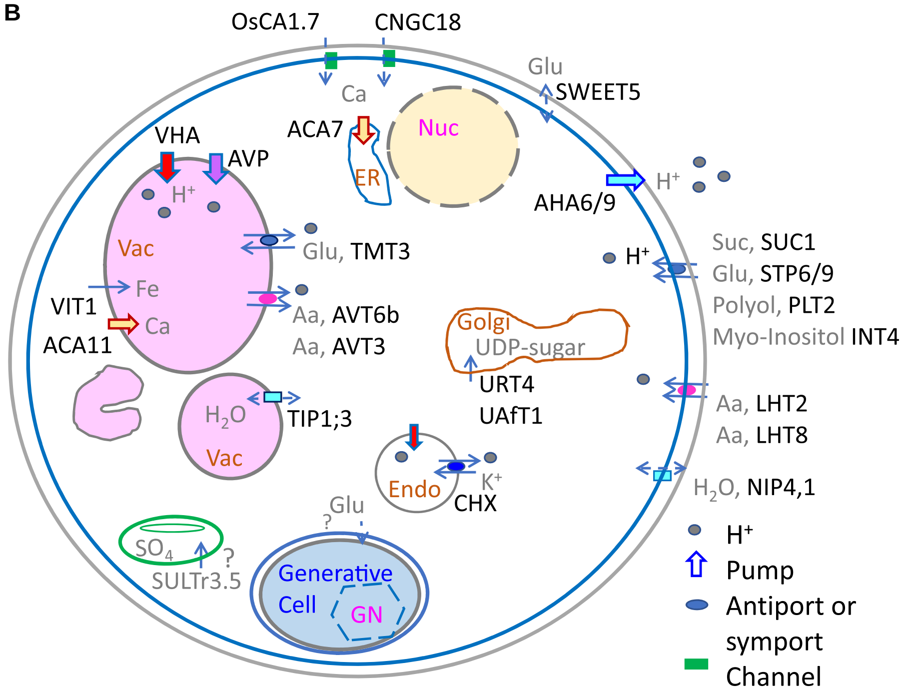


Figure 4. mRNA and protein abundance of diverse transporters in BCP and TCP. **A.i)** and **ii)** Transcripts peaking at late BCP. Abundance determined by RNA-Seq are shown as raw TPM (i) or normalized value (ii). Transcripts that peak (1.0) at the late BCP or TCP are preferentially-expressed in pollen. **A.iii)** Relative protein abundance in developing (early, late) and mature pollen (MP). About 77% (16 of 20) of the transcripts are translated based on proteome data (Grobei et al. 2009; Klodova et al. 2023). **A.iv)** Function refer to membrane localization (Memb) and transport mode (Activity) which are characterized or predicted (parenthesis). vac, vacuole; pm, plasma membrane; endo, endomembrane; Golgi, Golgi apparatus; nd, not determined. (see Supplementary Fig. S4 for transporters expressed in TCP). **B)** Working model of transport activities in BCP and TCP. The transcripts accumulated in late BCP and/or TCP, are largely translated and likely functional in TCP. The transport mode and membrane localization of previously characterized transporters are shown with key substrates (**A**). Pumps, H^+-coupled symporters (2 parallel arrows), and antiporters (2 opposite arrows) are defined as in the box. H^+ (grey ball) are extruded at the PM or into the vacuole. Flux through channels (rectangle) are shown as dashed arrows.

membrane and post-Golgi endomembrane. Consistent with this idea, transcripts of most VHA-V_1 and V_o subunits are constitutively expressed in MP (Supplementary Fig. S5), except for VHA-E2 and VHA-G3, which are specifically induced in the intermediate cluster (Fig. 4).

AHA6, 9, or 8 is localized to the PM of the PT shank. The triple mutant *aha6/8/9*, but not double mutant (*aha6/9* or *aha8/9*), pollen showed the most severe defects in germination, PT length, and seed set (Hoffmann et al. 2020). Triple mutants showed reduced cytosolic pH, decreased tip to shank proton gradients, and less negative membrane potentials, supporting their critical roles in PT growth (Hoffmann et al. 2020). However, because AHA6 and AHA9 transcripts accumulated in late BCP and their proteins were present before pollen maturation (Fig. 4), we propose that AHA6 and AHA9 pumps support BCP development to TCP. In contrast, AHA8 mRNA, but not protein, is abundant in MP and PT (Supplementary Fig. S5), suggesting its transcript is largely stored in MP for later use in tube growth.

Sugar transporters

Transcripts of many sugar transporters peak in late BCP and TCP with relatively high protein in developing and mature pollen (Fig. 4), including PM-localized, STP9, SWEET5, INT4, and vacuolar TMT3. STP4, STP6, and STP9 are expressed in late BCP (Fig. 4; Supplementary Fig. S5), whereas STP2 and STP11 are "early" and "late"-pollen expressed genes, respectively. Single *stp* mutants show no obvious defects in pollen development or function, possibly due to redundancy (Rottmann et al. 2018). However, wild-type PT length is reduced in a sucrose medium supplemented with glucose. Hexokinase/sugar sensor mutants *gin2-1* or *hxk1.3* are insensitive to glucose. Knockout *stp* mutant (*stp4,6,8,9,10,11*) showed decreased glucose uptake but 2-fold increase in PT length on medium containing glucose and sucrose. These results indicate that high glucose inhibits PT growth via the glucose sensor (HXK1) present in developing pollen and MP. We postulate that bicellular and tricellular pollen depend on high-affinity sugar uptake via STP9, STP6, and STP4 (Supplementary Fig. S5) for nutrition and osmolyte balance as pollen dehydrates.

Strikingly, SUC1 mRNAs peak sharply at late BCP and TCP and is very abundant even though the protein is relatively low (Fig. 4). Single *Atsuc1* mutant pollen shows low germination in vitro (Sivitz et al. 2008), suggesting sucrose accumulation by a high-affinity sugar/H^+ symporter at the PM in germination. Whether SUC1 has other roles in sugar signaling or pollen development is not known.

Sugar storage and homeostasis is coordinated by vacuoles in plant cells (Hedrich et al. 2015) and possibly in developing pollen. For instance, TMT3/TST3, expressed preferentially in BCP (Fig. 4), is predicted to be a tonoplast monosaccharide/H^+ antiporter based on studies of TMT1 and TMT2. Intriguingly, both TMT1 and TMT2 are induced by cold, drought, and salt stress in vegetative tissues (Wormit et al. 2006). T-DNA knockout lines had much lower levels of glucose and fructose especially after cold stress. These results suggest that TMT3/TST3 mediates glucose uptake into vacuoles perhaps in response to stress, like dehydration onset in pollen.

Amino acid cotransporters

Multiple amino-acid/H^+ cotransporters localized to the PM (LHT2, LHT8) and the vacuole (AVT) are expressed in pollen. LHT2 and LHT8 mRNAs peak at BCP and TCP, and the proteins are present in MP suggesting they mediate H^+-amino-acid uptake into TCP (Fig. 4, A and B; Supplementary Fig. S4). Less is known about vacuolar amino acid transporters in plants. Vacuoles usually degrade proteins and the amino acids produced must be exported to the cytosol for new protein synthesis. The AVT family is thought to mediate amino acid export from vacuoles based on studies of AVT3 (Fujiki et al. 2017). Six of 12 pollen-expressed AVT transcripts (AVT1c, AVT6b, ANT1, AVT1a, AVT1d, AVT3a) accumulate with low abundance in developing pollen (Supplementary Fig. S4), though the protein was undetectable except for AVT1c. Curiously, AVT6d is induced by ABA, osmotic, or drought stress (Dhatterwal et al. 2022) and strongly expressed in siliques, suggesting its promoter is responsive to abiotic stress. Whether other AVT-like amino acid transporters in pollen are stress responsive with roles in osmolyte balance and nutrient acquisition is not yet known.

Nucleotide-sugar transport

Nucleotide-sugar transporters (NSTs) translocate nucleotide-sugars from the cytosol to the lumen of the Golgi apparatus where glycosyltransferases mediate synthesis of noncellulosic polysaccharides and glycoproteins (Temple et al. 2016). The products inside Golgi compartments are then transferred to the extracellular matrix or cell wall via exocytosis of secretory vesicles. Developing pollen expresses multiple NSTs. Increase in transcripts of nucleotide-sugar transporters (e.g. UTR4, UAfT1) localized to the ER or Golgi is consistent with active cell wall synthesis, remodeling, and glycosylation. UTr1 and UTr3 transport UDP-Glu into the ER and Golgi (Reyes et al. 2010) for glucosylation of glycoproteins. UTr1 transcripts increase in late BCP and persist in MP (Fig. 4). The double mutant is lethal, probably due to their functions in a wide range of vegetative and reproductive organs. In contrast, UTr4 is preferentially expressed in pollen at late BCP suggesting UDP-Glu/Gal transport into the ER lumen (and Golgi) serve roles specific for developing pollen. UAfT1-UAfT4 transport UDP-Arabinose as a furanose ring (Araf) from the cytosol into the Golgi lumen (Rautengarten et al. 2017), where Araf is used by glycosyltransferases to synthesize non-cellulosic polysaccharides, like Rhamnogalacturan I pectin, and Arabinogalactan proteins (AGP). Three UAfT are expressed in pollen as well as other tissues, like root and seeds. Only UAfT1 expression is pollen specific, peaking at late BCP. Transcripts of additional nucleotide-sugar transporters are induced at late BCP-TCP stage though they are not pollen specific. These examples illustrate that developing pollen has an active endomembrane system, where ER and Golgi are engaged in cell wall synthesis, glycosylation, and signaling.

Ca^{2+} dynamics: pumps and channels

Transient increases in cytosolic $[Ca^{2+}]_{cyt}$ levels often follow stress cues, though little is known about dynamic Ca^{2+} responses in developing pollen. The induction of OsCA1.7 and OsCA2.5, members of the hyperosmolality-gated cation channel, initially named ERD4 (early-response to dehydration family 4) (Yuan et al. 2014) would suggest a Ca^{2+} response to dehydration in late BCP to TCP (Fig. 4; Supplementary Fig. S4). Furthermore, several cyclic nucleotide-gated Ca^{2+}-permeable channels (CNGC) are induced in TCP (Supplementary Fig. S4), though like late-expressed genes, their mRNAs persist in MP. CNGC18 is specifically expressed in pollen. Mutant *cngc18* pollen appears to be sterile due to defects in PT growth (Frietsch et al. 2007). CNGC7 and CNGC8 are also critical for male fertility, and *cngc7/8* mutant pollen bursts at germination (Tunc-Ozdemir et al. 2013). GFP-CNGC7 localizes to the PM at the tip of emerging PTs and to the shank of growing PTs. Thus, these CNGC channels function in pollen germination and tube growth, their role in developing pollen remains untested.

Four ACA-type Ca^{2+} pumps localized to the PM, vacuole, or ER show differential expression during pollen development (Supplementary Fig. S5). mRNA of PM-localized ACA9 peaks late in pollen development and is critical for PT functions as *aca9* mutants are defective in tube growth and fertility (Schiott et al. 2004). ACA11 mRNA is expressed early in pollen development, and the protein is localized to the vacuole. In contrast, ACA7 and ACA2 mRNA are expressed in BCP and translated. ACA2, ACA7, and ACA1 are ER-localized, and triple knockout *aca1/2/7* mutants showed reduced transmission of the mutation through pollen and plant growth defects (Rahmati Ishka et al. 2021). Thus, in developing pollen, ER-localized ACAs likely pump Ca^{2+} into the lumen for ER functions and modulate Ca^{2+} signaling.

Vacuolar aquaporins

Curiously, transcripts of a vacuolar, but not PM (PIP#), aquaporins are induced at late BCP in *A. thaliana* (Fig. 4). AtTIP1; 3 is pollen specific and is the major vacuolar aquaporin in the pollen vegetative cell. The protein is translated in BCP as shown by AtTIP1; 3-GFP–labeled membranes and in vacuolar membranes of MP and the PT (Wudick et al. 2014). Another aquaporin, TIP1,5 localized to small vacuoles, is specifically expressed in sperm cells. Single loss-of-function mutants are fertile; however, double mutants produce short seed pods with unfertilized ovules (Wudick et al. 2014). Thus, TIP1; 3 or TIP1; 5 can replace one another functionally in single loss-of-function mutants. The *tip1; 3/tip1; 5* double mutant is more susceptible to water, nutrient or heat stress, indicating that the transporters participate in stress tolerance. Based on characterized functions of aquaporins in pollen and other cells (Li et al. 2014), we hypothesize that TIP1,3 plays multiple roles: (1) TIP1,3 protein is translated and function in vacuole dynamics of BCP and TCP before pollen maturation; (2) part of TIP1; 3 protein is stored in dry pollen and then activated to mediate rapid water balance and vacuolar dynamics during rehydration when pollen lands on a compatible stigma; and (3) some TIP1; 3 mRNA is newly transcribed and translated at recovery for osmoregulation, membrane trafficking, and vacuolar morphology to support germination and tube elongation.

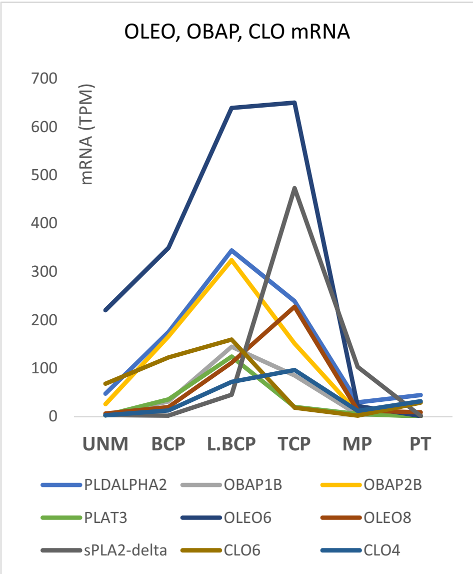
Cation/H^+ antiporters in endomembrane trafficking and cargo sorting

Over 20 members of the CHX family in *A. thaliana* are preferentially and developmentally expressed in pollen (Bock et al. 2006). *CHX19* accumulates in late BCP (Fig. 4), 9 other CHXs at TCP, 4 in MP, and 9 are induced in PTs (Supplementary Fig. S5). mRNAs peaking at BCP or TCP are probably active before pollen maturation based on protein expression. Functional studies using yeast mutants and structure-guided mutagenesis support the idea that CHX17(and homologs) exchanges K^+ for H^+ providing a leak pathway to modulate pH and K^+ homeostasis across membranes (Sze and Chanroj 2018). A current model is CHXs responds to the microenvironment (osmotic) and modulate local pH and ion balance of endomembrane compartments and/or the plasma membrane (Fig. 4B), and so affect membrane trafficking, cargo sorting, and cell wall remodeling (Sze and Chanroj 2018).

mRNAs encoding lipid droplet proteins

The large class of metabolic interconversion enzymes expressed during pollen dehydration and development (BCP-TCP) includes genes predicted to function in carbon metabolism, cell wall synthesis and remodeling, and oxidative stress. Here, we spotlight selected genes involved in lipid droplet and pollen wall formation.

Multiple studies have reported that oil bodies/droplets, common in seeds, increase in plants and algae during stress. One study (de Vries and Ischebeck 2020) proposed that formation of oil bodies is an evolutionarily conserved mechanism to tolerate desiccation and that the required proteins have been co-opted in seed plants. Here, we reveal that the transcripts of lipid droplet proteins accumulate in developing pollen of *A thaliana*. Examples include oleosin (OLEO), oil-body associated protein (OBAP), caleosin (CLO), and seipins (Fig. 5). Oleosin, a protein on the surface of lipid bodies, is specific to plants and expressed mainly in tissues that become desiccated, like seeds and pollen (Chapman et al. 2012; Kim et al. 2002). Mutant analysis suggests oleosins and seipins keep lipid droplets small by preventing their aggregation during seed desiccation (Chapman et al. 2012) or in pollen (Taurino et al. 2018). Figure 5 shows that 2 AtOLEO (AtOLEO6 and AtOLEO8) transcripts are preferentially expressed in pollen, while 5 (AtOLEO1,2,4,5, and 7) are transcribed in seeds. Moreover, 2 AtOBAP (1b and 2b) are expressed in developing pollen and 3 other AtOBAP (1a, 2a, and 2c) are highly expressed in seeds or pods. In tobacco cells, transiently expressed maize OBAP1 (like AtOBAP1a) and maize Oleosin1 protein colocalize to the same compartments that were stained by a neutral lipid dye (Lopez-Ribera



		i. mRNA (TPM)			ii. RNA Normalized			iii.Protein	
Oleosin	Name	L.BCP	TCP	Seed	L.BCP	TCP	Seed	Dev	MP
At2g25890	OLEO6	639	650	926	0.7	0.7	1.0	nd	nd
At3g18570	OLEO8*	111	227	205	0.5	1.0	0.9	8	10
At4g25140	OLEO1	0	0	15041	0.0	0.0	0.9	nd	nd
At3g27660	OLEO4*	0	0	5898	0.0	0.0	1.0	3	15
At5g40420	OLEO2	0	0	4839	0.0	0.0	1.0	nd	nd
At3g01570	OLEO7	1	0	3249	0.0	0.0	1.0	nd	nd
At1g48990	OLEO5	0	0	203	0.0	0.0	1.0	nd	nd
At5g07530	GRP17	5	0	0	0.0	0.0	0.0	75	1181
Oil-body assoc. protein									
At4G18920	OBAP2b*	324	151	138	1.0	0.5	0.42	42	28
At2G31985	OBAP1b*	144	85	43	1.0	0.6	0.3	47	13
At1G05510	OBAP1a	3	1	4664	0	0.0	1.0	nd	nd
At5G45690	OBAP2a	0	0	4514	0	0.0	1.0	nd	nd
At1G29680	OBAP2c	0	0	480	0	0.0	1.0	nd	nd
Seipin									
At1G29760	SEIPIN2	9	12	29	0.1	0.2	0.4	nd	nd
At2G34380	SEIPIN3	5	2	9	0.2	0.1	0.3	nd	nd
At5G16460	SEIPIN1	0	0	65	0	0	1.0	nd	nd
Caleosin									
At5G29560	CLO6	159	18	0	1.0	0.1	0.0	nd	nd
At1G70670	CLO4*	72	96	6	0.2	0.2	0.0	10	6
At2G33380	CLO3	0	0	2	0.0	0.0	0.0	nd	nd
At1G70680	CLO5	0	2	12	0.0	0.0	0.0	nd	nd
At4G26740	CLO1	0	0	962	0.0	0.0	0.9	nd	nd
At5G55240	CLO2	0	0	1049	0.0	0.0	1.0	nd	nd

Figure 5. mRNAs encoding lipid body proteins are expressed in bicellular and tricellular pollen. Preferential expression of oleosins (OLEO), caleosin (CLO), and oil-body associated genes (OBAP) in developing pollen (late BCP and MP) and seeds. Columns refer to (1) RNA abundance in TPM, (2) normalized mRNA in pollen or in seeds, and (3) relative protein level in developing (early and late) or mature pollen. Examples in graph (left) include At1g52570 (PLDalpha), At4g29470 (sPLA2-delta) and At5g65158 (PLAT3).

et al. 2014). Together, the results imply that OLEO- and OBAP-containing lipid droplets form in developing pollen of *A. thaliana* perhaps in response to dehydration. This idea is supported by ultrastructural studies where lipid bodies surround the generative cell in *A. thaliana* BCP (Owen and Makaroff 1995; Yamamoto et al. 2003) as large vacuoles remodel, split into small ones, and disappear.

A subset of oleosins (Mayfield and Preuss 2000) and extracellular lipases are abundant in the pollen coat of the exine wall, which protects the pollen from stress and dehydration. For instance, the oleosin-domain containing Glycine Rich Protein17 (GRP17) is associated with tapetosomes that are discharged from the anther tapetal cells (sporophyte) to form the pollen coat of developing pollen. Homozygous *grp17(-/-)* mutant is male sterile, and pollen grains show delayed hydration on the stigma relative to wild type. Moreover, double mutant pollen of *grp17/exl4* shows enhanced delay in hydration in vivo. EXL4 belongs to a group of extracellular lipases that originates from the tapetal cells and are abundant in the pollen coat (Updegraff et al. 2009). Thus, the pollen coat, containing GRPs mixed with other proteins and lipids, form a complex hydrophobic barrier to protect grains from dehydration until the pollen lands on a compatible pistil and extracellular lipase action promotes hydration.

Pectin wall synthesis and remodeling

Because of the unique physical and chemical properties, pectins influence wall hydration, porosity, mechanics, and ion binding to alter wall structure and dynamics (Hofte and Voxeur 2017; Du et al. 2020). The importance of pectin and its dynamics in plants is illustrated by the multiplicity of pectin-modifying enzymes (Senechal et al. 2014). In dicots, pectin is the major structural component of the cell wall in pollen, which undergoes major changes during its development and PT growth. According to the expression of multiple galacturonosyltransferases (e.g. *GAUT1,6,7,8,9*), pectin biosynthesis in the Golgi lumen (Atmodjo et al. 2013) is predicted early in pollen development from UNM and BCP, whereas *GAUT13, 14* are expressed later in TCP (Fig. 6A).

For simplicity, we illustrate pectin dynamics using mainly homogalacturonan (HG), which is synthesized in the Golgi by GAUT and then methyl esterified. About 19 pectin methyltransferases (PMTs) from a family of 29 are expressed in pollen. Six transcripts peaked in late BCP and TCP, and others peaked at the early or late stages (Fig. 6B). Functional studies showed 1 PMT (QUA2) is a methyltransferase, localized to the Golgi membrane with the catalytic methyltransferase domain inside the lumen (Mouille et al. 2007; Du et al. 2020). Furthermore, cellulose content was reduced and less organized in *qua2/tsd2* mutants, suggesting

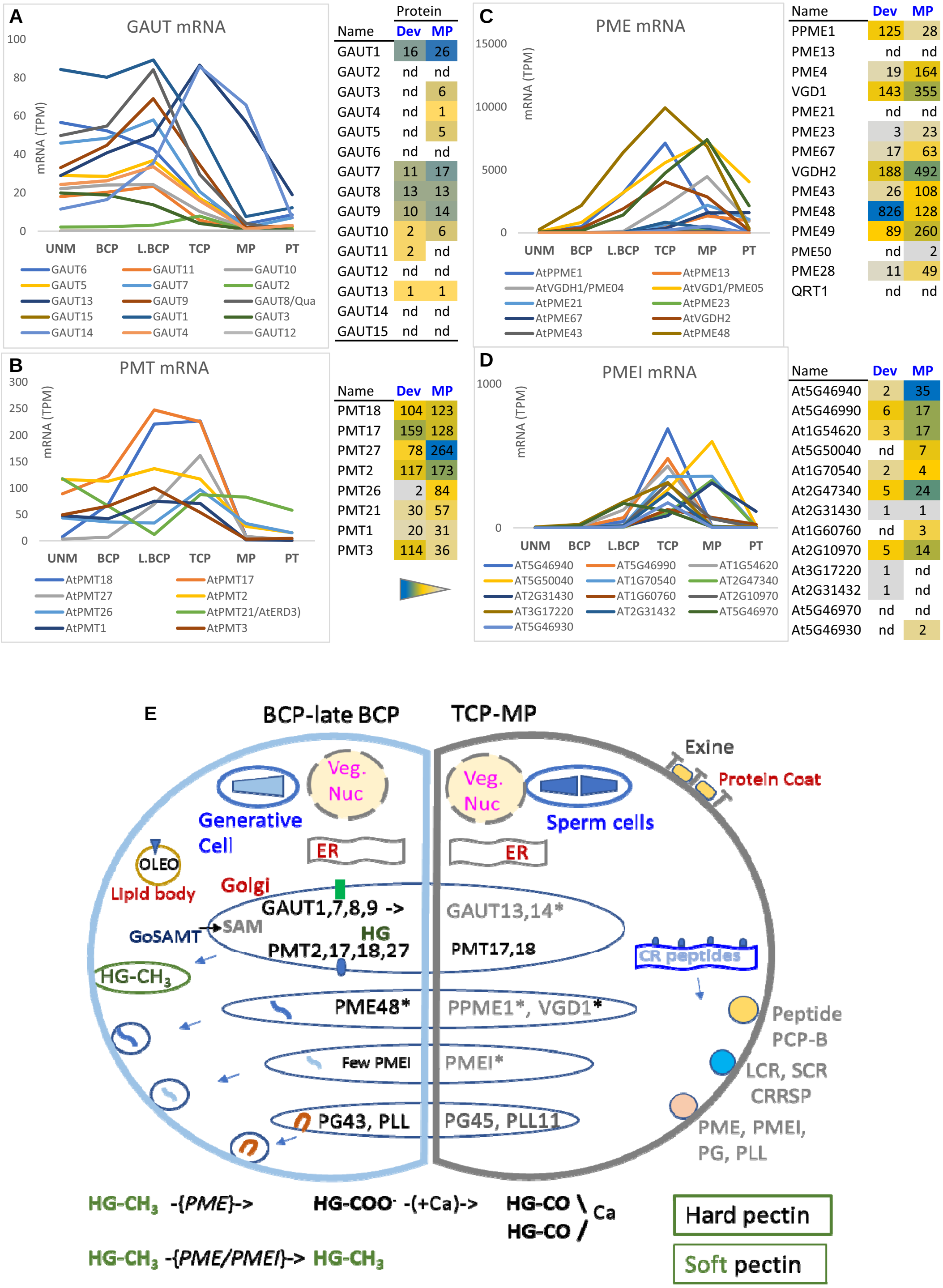


Figure 6. Differential expression of pectin remodelers in developing pollen. Transcripts (TPM) of gene families expressed at different pollen stages: **A)** GAUT, Galacturonosyltransferase; **B)** PMT, Pectin Methyltransferase; **C)** PME, Pectin methylesterase; **D)** PMEI, Pectin Methylesterase inhibitors. Pollen stages were defined in Fig. 1A. Relative protein (right) in developing pollen (early-late) (Dev. Klodova) or mature pollen (MP, Grobei) is scored as high (blue), medium (orange/yellow) or low (grey), respectively. nd, no data. (see Supplementary Fig. S6 for PG, polygalacturonase and PLL, pectate lyase.) **E)** Working model showing synthesis and secretion of pectin, pectin remodelers, and Cys-rich proteins in a bicellular (BCP, left) and a tricellular pollen (TCP, right). Late BCP show transcripts and proteins associated with pectin synthesis and remodeling (see **A** to **D**). Pectin HG is synthesized in the Golgi using GAUT and methyl-esterified by PMT. Pectin released in the wall is modified by enzymes (PME, PMEI, PG, PLL) previously synthesized in the ER and Golgi, and then secreted to the wall. Other pectin synthesis transcripts accumulating in TCP (GAUT13/14) may be stored in MP for use at germination. Transcripts encoding scores of secreted CRP peptides (e.g. PCP-B, LCR, SCR) are postulated to be translated in developing pollen (late BCP-early TCP) and the protein is stored either in secretory vesicles or bound to the wall before pollen reaches maturity and desiccates.

pectin interacts with cellulose synthesis and assembly and influences cell wall integrity (Du et al. 2020). Expression of PMT17, PMT18, and PMT27 mRNA and protein in late BCP would infer they methyl-esterify pectin in developing pollen similarly to QUA2 in the vegetative plant. Results suggest that homogalacturonan is methyl-esterified in the Golgi before being secreted to the pollen wall.

After export to the cell wall, methyl-esterified pectin that produces a soft wall can be modified in multiple ways spatially and temporally, depending on the microenvironment (Hofte and Voxeur 2017). Esterified pectin can be de-esterified by pectin methylesterase (PME) to yield HG that upon cross-linking with Ca^{2+} gives a stiff wall. Twelve out of 66 *A. thaliana* PME are highly expressed in pollen. Transcripts peak late in development in TCP and MP (Fig. 6C), and protein was detected for most except for 3 transcripts accumulating at MP. In contrast, all 4 transcripts (PPME1, PME48, PME49, and VGDH2) peaking at TCP showed relatively abundant protein. VGD1/PME5 protein is distributed in MP and PT, including the PM and cell wall. *vgd1* mutants show decreased PME activity and PT growth in the pistil and reduced male fertility. Moreover, mutant tubes burst more often than wild type when germinated in vitro (Jiang et al. 2005). PME48 transcript and protein are abundant in developing pollen (Fig. 6C), and fluorescent-tagged protein was detected in dry and imbibed pollen (Leroux et al. 2015). Homozygous *pme48* mutant showed a delay in pollen grain imbibition and drastic reduction in germination, and they had reduced PME activity and higher levels of methyl-esterification. The PTs were less stable and burst more than wild-type tubes. PME48, likely modulates the mechanical properties of the intine wall when pollen grains mature and the hydration rate at imbibition. Together, the examples suggest that certain PMEs could protect developing pollen from osmotic stress by strengthening the wall.

PME activities can be targeted and inhibited by specific pectin methylesterase inhibitors (PMEI) in a spatial and temporal manner (Levesque-Tremblay et al. 2015). PMEI are secreted small proteins (average 194 aa) that bind to the cleft of PME and prevent substrate access (Hothorn et al. 2004; Di Matteo et al. 2005). Individual PMEI can pair with a PME, some in a pH-dependent manner (Hocq et al. 2017), though the specificity of pairing and their functional diversity remain poorly understood. Of 76 PME-inhibitor genes in *A. thaliana*, 26 are expressed in pollen in a developmentally regulated manner. Eleven PMEI genes show abundant transcripts (>2 k TPM) in MP or in PTs and are postulated to participate in PT growth. In contrast, 9 PMEI transcripts peaking in TCP with low expression (<1 k TPM) and low protein level (Fig. 6D; Supplementary Fig. S6) infer potential roles in BCP-TCP. Interestingly a PME and its corresponding PMEI protein can be spatially separated. Accumulation of PMEI2, but not PPME1, in endosomal compartments suggests that endocytosis of extracellular PMEI at the lateral walls resulted in inhibitor accumulation at the apex. Thus, polar growth is regulated in part by the spatial and temporal coordination of anterograde and retrograde membrane trafficking of secreted pectin remodelers. The differential distribution of PMEIs suggests wall mechanics and properties can be finely tuned in space and time.

How pectin modification by enzymes, like PME, polygalacturonase (PG), pectate lyases (PLL) (Supplementary Fig. S6), and regulators (e.g. PMEI), mediate pollen dehydration, hydration, cell expansion, or tube navigation, has yet to be determined. An emerging model is that pectin modification can drive phase transitions in the wall to regulate wall assembly and growth (Haas et al. 2021). Such phase transitions could elicit physical and biochemical responses to small changes (like osmotic stress) in the microenvironment of a cell.

Proteins abundant in developing and mature pollen: energy metabolism and translation machinery

The first proteome of *A. thaliana* at 4 pollen stages (Klodova et al. 2023) reveals proteins abundant in developing and mature pollen. The total proteins detected previously in MP (Grobei et al. 2009) and recently in developing pollen was estimated at 3,467 and 4,964, respectively. Proteins in the top 10% of the proteome (350–500) are over-represented by metabolic interconversion enzymes (32%) and translation proteins (22%) followed by chaperones (6.3%), protein modifying enzymes (6.1%), transporters (5.4%) and RNA metabolism (3.6%).

Proteins in respiration and energy production are abundant and stable

Previous physiological studies of plants had shown respiration overall is less sensitive to water deficit than photosynthesis (Mittler 2006; Taiz et al. 2015). For example, desiccation-tolerant resurrection plants are able to preserve respiration even when water content is very low. In desiccation-tolerant seeds, the ability to sustain respiration during dehydration and then to resume respiration shortly after seed imbibition, indicates mitochondria function is preserved during desiccation (Atkin and Macherel 2009).

Analysis of highly-abundant proteins (top 500) with a robust positive signal (>35) in the pollen proteome, showed that most mitochondrial proteins high in the early stages, remained steady in MP, while only 22% of proteins dropped in TCP and MP. In contrast, transcripts of mitochondrial proteins high in the early stages (UNM, BCP) drop in the TCP and mature pollen (Fig. 7A). Transporters are among the most abundant mitochondrial proteins (Fig. 7A), including F-ATP synthase subunits, dicarboxylate/tricarboxylate carrier (DTC), phosphate transporter (PHT1,3), and ADP/ATP carrier (AAC1/2) of the inner mitochondria membrane, and VDAC channels at the outer mitochondrial membrane. All ions and metabolites (ADP or ATP) entering or leaving the mitochondrion cross the outer membrane via

A **Mitochondria**

Gene	Name	RNA (TPM) UNM	BCP	L.BCP	TCP	MP	PT	Relative Protein Early	Late	MP
At3G08580	AAC1	1418	1284	1021	1463	258	87	244	62	401
At5G13490	AAC2	175	148	110	621	51	99	66	9	137
At4G28390	AAC3	27	16	3	0	0	0	nd	nd	2
At5G19760	DTC	323	352	355	206	49	6	10	6	206
AtMG01190	F1-ATP-alpha	579	1098	1015	219	12	0	376	147	nd
At5G13450	F1-ATP-delta	624	609	568	275	14	9	41	6	169
At2G33040	F1-ATP-gamma	600	534	417	223	15	21	46	8	208
At2G21870	Fo-ATP	358	362	372	177	19	12	167	108	96
At3G52300	Fo-ATP-d	634	668	647	287	33	34	126	49	35
At5G14040	PHT3;1/MPT3	401	377	363	275	74	33	47	31	379
At3G01280	VDAC1	929	753	449	48	10	13	197	291	113
At5G67500	VDAC2	256	242	215	113	7	15	29	9	193
At5G15090	VDAC3	512	482	278	37	5	3	163	295	141
At5G08690	F1-ATP-beta	234	228	189	83	6	3	1568	1149	nd
Gene	Name	UNM	BCP	L.BCP	TCP	MP	PT	Early	Late	MP
At2G05710	ACO3	185	148	109	66	5	5	481	449	403
AT2G30970	ASP1	20	96	209	347	12	4	304	49	288
At2G47510	FUM1	280	244	189	101	23	10	131	123	119
At1G53240	mMDH1	265	284	250	53	4	0	102	169	70
AT5G66760	SDH1-1	294	265	249	176	13	38	230	243	121

B **Glycolysis**

Gene	Name	RNA (TPM) UNM	BCP	L.BCP	TCP	MP	PT	Relative Protein Early	Late	MP
At2G36530	ENO2/LOS2	1068	752	418	167	10	1	684	668	819
At3G52930	FBA8	780	493	177	98	6	8	593	538	123
At4G10260	FRK4	1877	2005	1136	18	16	1	347	101	320
At3G04120	GAPC1	1391	1249	1062	614	26	20	1689	1608	87
At4G29130	HXK1	78	184	308	115	20	16	284	108	408
At1G23190	PGM3_cyt	98	153	232	216	7	1	524	114	151

C

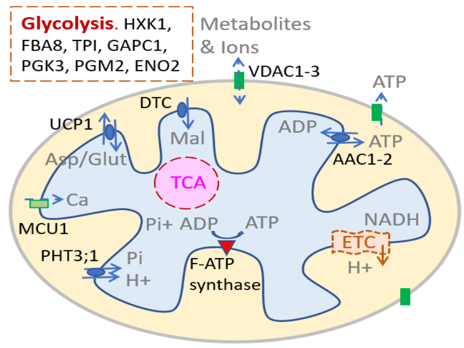


Figure 7. Energy and C metabolism proteins are abundant and stable in developing pollen. **A)** Mitochondria transporters and enzymes. Transcripts of transporters and enzymes high in UNM to TCP drop in mature pollen. Relative abundance of proteins in early developing (UNM-BCP), late (TCP-MP) or mature pollen (MP) as Fig. 2. Selected mitochondria enzymes are ACO3, aconitase; FUM1, fumarase; MDH1, malate dehydrogenase; SDH1, succinate dehydrogenase. **B)** Transcripts encoding selected glycolytic enzymes are high in developing pollen but drop in MP. Proteins remain abundant from developing to mature pollen. (see Supplementary Fig. S6 for additional mitochondria and glycolysis enzymes). **C)** Map of transporters and proteins in the mitochondria and cytosol. Transporters on the inner mitochondria membrane include F-ATP synthase subunits, ADP/ATP carrier (AAC), phosphate transporter (PHT3; 1/MPT3), and carboxylic acid carrier (DTC). Most metabolites and ions cross the outer mitochondria membrane via porins (VDAC1-3). TCA and ETC refer to proteins of the Tricarboxylic Acid (Kreb) Cycle and the oxidative Electron Transport Chain, respectively. Glycolysis enzymes include hexokinase1 (HXK1), fructose bis-phosphate aldolase (FBA8), triose phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPC1), phosphoglycerate kinase (PDK3), phosphoglucomutase (PGM2), and enolase (ENO2).

VDAC1/VDAC3 (Fig. 7B). Similarly, proteins of the Krebs cycle and oxidative phosphorylation remain steady until MP, though transcripts are also abundant early in pollen development (Fig. 7). Cytoplasmic glycolytic enzymes are also present early in pollen development and persist in MP, especially Glyceraldehyde-3-phosphate dehydrogenase GAPC1, enolase ENO2, fructose-bis phosphate aldolase FBA8, phosphofructokinase FRK4, phosphoenolpyruvate carboxykinase PCK2 and phosphoglucomutase PGM2/3 (Fig. 7B). Intriguingly, GAPC and ENO2/LOS2, are bifunctional proteins with roles in heat or freezing stress protection in *A. thaliana* (Lee et al. 2002; Kim et al. 2020). Together the respiration-related proteome (Grobei et al. 2009; Klodova et al. 2023) indicates the proteins are translated in developing pollen and stabilized in mature dry pollen. Thus, MP is well-equipped for respiration and immediate energy production when grains reach a compatible stigma and rehydrate.

Proteins of the translation machinery and chaperones accumulate in mature pollen

Proteins of the translation machinery are abundant in TCP and MP, though mRNA levels maximal early in pollen development (UNM and BCP) drop sharply by TCP and MP (Fig. 8). The translation machinery includes at least 50 60S ribosomal proteins, 37 40S ribosomal proteins 20 translation initiation factors, and over 25 amino acid-tRNA ligases (Fig. 8; Supplementary Fig. S7). Initiation factors included TIF4A-2, TIF3A-1, TIF3C-1, ELF5A-2, TIF3B-1, and elongation factors, EF1-alpha2 and LOS1/EF2 (Supplementary Fig. S7).

Combined with the mRNA expression pattern, the results indicate (1) the translation machinery is synthesized and active when pollen is metabolically active (BCP-early TCP); and (2) these proteins are stable, or protected as TCP dehydrates, matures, and enters developmental arrest (Grobei et al. 2009; Honys et al. 2009). Identified as components of stress granules (Kosmacz et al. 2019; Sze et al. 2021), proteins of the translation machinery are likely protected, stored in mRNP aggregates/granules found in pollen, and are fully prepared to begin translation when pollen reaches a compatible stigma. This conclusion is consistent with studies that initial pollen germination and tube growth depends largely on protein translation and not transcription (Mascarenhas 1993).

Molecular chaperones are involved in processes like folding of newly-synthesized proteins, transport of proteins across membranes, refolding of proteins after stress, preventing aggregation of misfolded proteins, and degradation of unfolded proteins. Chaperone proteins rise late in pollen development when their corresponding transcripts have dropped to minimal levels. Abundant are HSP70- and

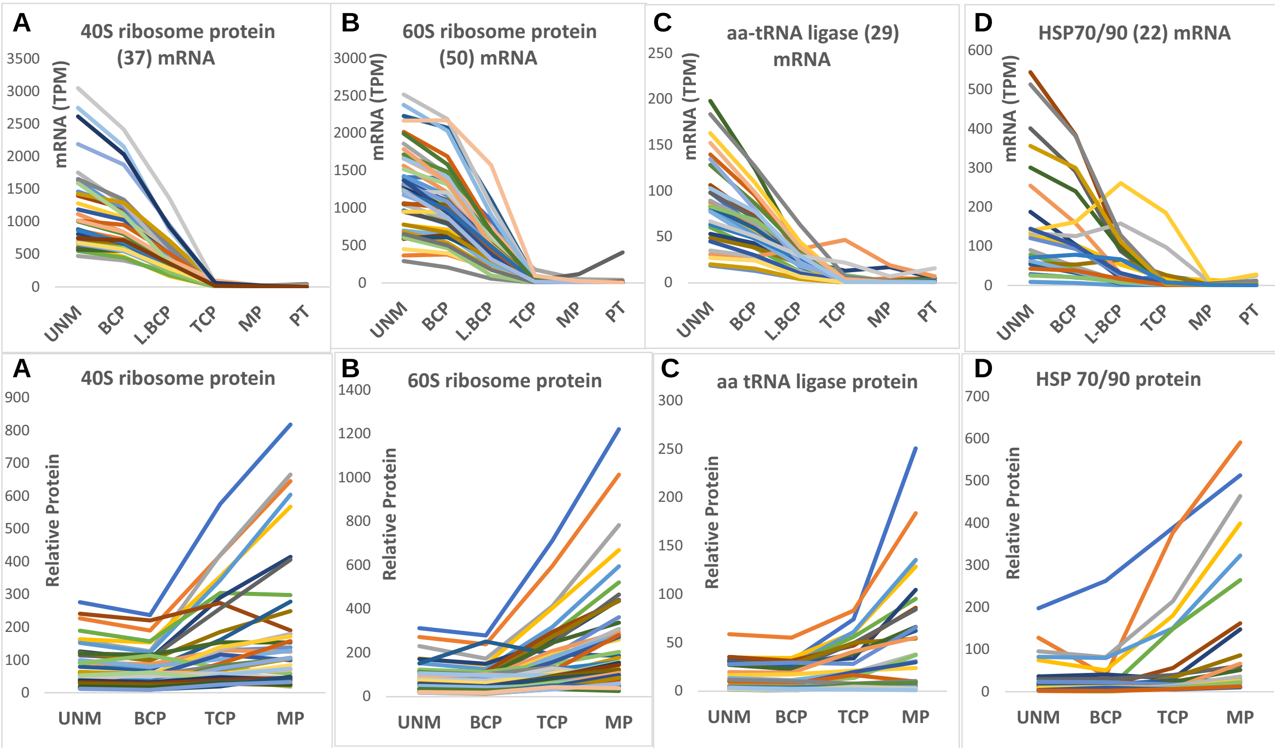


Figure 8. mRNAs of the translation machinery are abundant early in UNM and BCP but proteins accumulate later in mature pollen. Examples of mRNA (top) and protein (bottom) abundance in developing pollen are shown for selected families. See Supplementary Tables S3 and S7 for gene ID#. **A)** 40S ribosomal proteins. Selected 37 with relatively high protein abundance. **B)** 60S ribosomal proteins. Examples of 50 with relatively abundant proteins. **C)** Amino-acid tRNA ligases. Examples of 29 relatively abundant proteins. **D)** Molecular chaperones detected in the proteome. Examples include HSP70, HSP90, and BiP from the cytosol, ER, plastids or mitochondria.

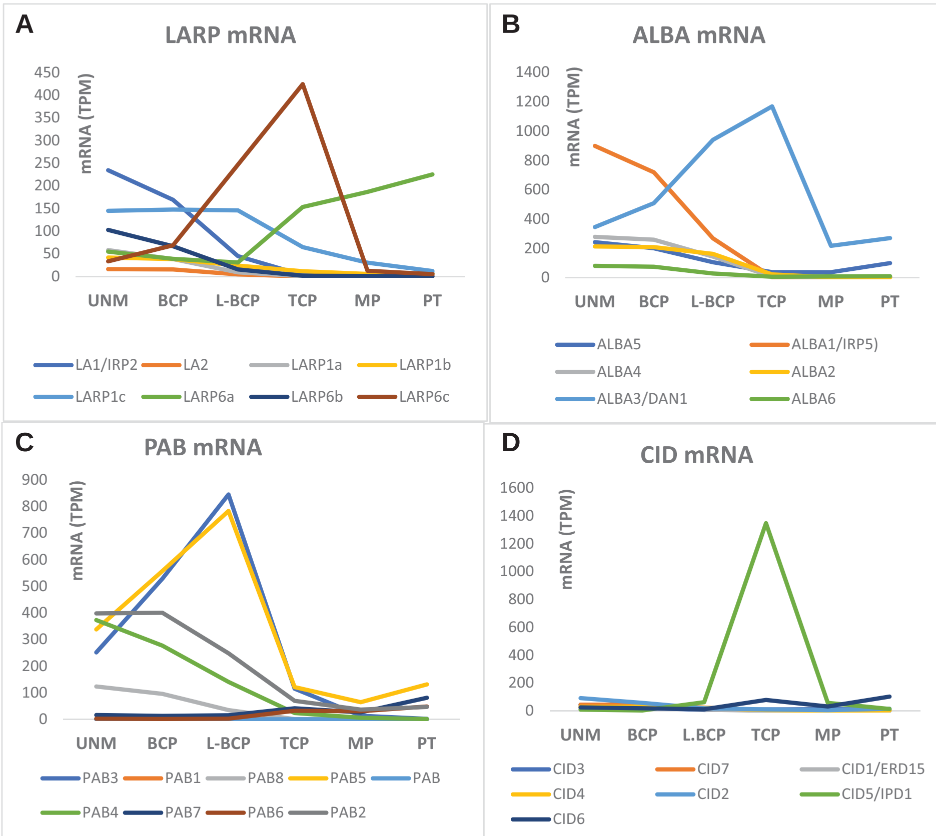
HSP90-type proteins localized in the cytosol, mitochondria, plastids; along with CPN60 subunits in plastids (Fig. 8D, Supplementary Fig. S9). Small HSP20 proteins were not detected. The cytoplasmic chaperonin complex (CCT) and ER-associated chaperones also rise late in pollen development (Supplementary Fig. S9) when transcript levels are reduced. ER chaperones include BIP1/2, SHD, calnexin CNX, calreticulin, and protein disulfide isomerase (PDI). Together, these results indicate MP is equipped with many types of chaperones to promote protein synthesis, protect proteins under osmotic or salt stress and aid stress recovery at rehydration on the stigma and during rapid tip growth.

RNA-binding proteins associate with mRNAs in stress granules

RNA-binding proteins control development of organisms and response to environmental stress by controlling mRNA fate, including stability, storage, transport, and translation (Chantarachot and Bailey-Serres 2018; Wiedner and Giudice 2021), though, their roles in pollen development remain largely unexplored. We had postulated that mRNP particles are formed in developing pollen in response to dehydration (Sze et al. 2021), though direct evidence was lacking. The transcriptome analysis here shows that some RNA-binding proteins are preferentially expressed in the intermediate phase (BCP-TCP), and recent reports indicate they bind transcripts and are associated with stress granules.

Transcripts of RNA-binding proteins rising in late BCP or TCP, include poly(A)-binding protein (PAB3 and PAB5), poly(A)-binding protein-interacting protein (CID5), DNA/RNA binding protein (ALBA3), and La-related protein (LARP6c) (Fig. 9). Furthermore, presence of protein products for most transcripts in late BCP to TCP, suggest they are functional before pollen grains enter the quiescent state. Additional RNA-binding proteins are expressed early in pollen development (e.g. Rbp45/47; RNA helicases), though their functions are unknown.

ALBA transcripts are expressed early in pollen development, and GFP-tagged fusion proteins (e.g. ALBA6, ALBA3) are abundant in MP and localized in cytoplasmic foci of the vegetative cell (Naprstkova et al. 2021). Furthermore, ALBA4 and ALBA6 co-localized with pollen-specific PABP3-RFP, implying these ALBAs participate in storage of mRNA in stress granules. In seedlings, ALBA4,5,6 phase separates into stress granules and processing bodies (P-bodies) under heat stress (Tong et al. 2022). In vivo targets of ALBA5 are mostly mRNAs, including heat-responsive transcription factors, like DREB2A, MBF1C, HSFB2A, and



ID	Name	MP Prot	Pred. IDR
At4g32720	LA1/IRP2	3	0.31
At1g79880	LA2	nd	0.08
At5g21160	LARP1a	nd	0.38
At5g66100	LARP1b	1	0.631
At4g35890	LARP1c	6	0.614
At5g46250	LARP6a	nd	0.547
At2g43970	LARP6b	nd	0.593
At3g19090	LARP6c	11	0.264
At1g22760	PAB3	162	nd
At1g34140	PAB1	nd	
At1g49760	PAB8	26	0.089
At1g71770	PAB5	70	nd
At2g15500	PAB	nd	nd
At2g23350	PAB4	36	0.137
At2g36660	PAB7	3	nd
At3g16380	PAB6	nd	0.065
At4g34110	PAB2	15	0.08
At1g20220	ALBA5	19	0.55
At1g29250	ALBA1	6	nd
AT1G76010	ALBA4	55	0.6
AT2G34160	ALBA2	2	nd
AT3G04620	ALBA3/DAN	72	0.213
At3g07030	ALBA6	nd	0.528
At1g54170	CID3	nd	0.14
At2g26280	CID7	nd	0.11
At2g41430	CID1	nd	0.28
At3g14010	CID4	2	0.48
At4g14270	CID2	nd	nd
At5g11440	CID5	2	0.27
At5g25540	CID6	2	0.18

Figure 9. Transcripts that affect RNA fate increase in late BCP and TCP and their proteins persist in mature pollen. Examples of genes encoding RNA-binding proteins are shown in **A to D**. Relative protein abundance in mature pollen (MP) and predicted intrinsically disordered regions (IDR) of the genes in **A** to **D** are shown at the right. **A)** LARP family (La-related protein). Most LARPs are expressed early in pollen development except for LARP6c which accumulate in late BCP and TCP. **B)** ALBA family (Acetylation lowers binding affinity). ALBA4, 5, 6 are expressed early in UNM and BCP. Only ALBA3 show peak transcripts at late BCP and TCP. **C)** PAB (poly-A binding protein). PAB3 and PAB5 transcripts peak in late BCP. **D)** CID. PAB-binding interacting protein. CID5 mRNA peaks at TCP.

HSFA2. Triple *alba4,5,6* mutants showed reduced thermotolerance. Tong et al. (2022) demonstrated that ALBA proteins are critical to stress granule formation which store mRNAs and protect them from heat and degradation.

La-related proteins (LARP) are conserved RNA-binding proteins but largely uncharacterized in plants (Merret et al. 2013; Bayfield et al. 2021). Billey et al. (2021) showed that LARP6c expression is pollen specific and necessary for post-transcriptional regulation during the transition of dry pollen to PT. LARP6c is physically associated with hundreds of transcripts that function in PT growth. In dry pollen, LARP6c is associated with cytoplasmic granules containing poly(A)-binding protein, suggesting LARP6c is localized with stored mRNA and granules in pollen.

We suggest LARP6c performs at least 2 major roles in pollen development. One, LARP6c binds transcripts as condensates form to protect translationally silent mRNA when pollen desiccates. Second, LARP6c activates translation as stored transcripts are released from granules upon pollen germination on a compatible stigma. Results support this idea include:

1) Temporally, only LARP6c transcript increases at the late BCP-TCP, and drop sharply by MP stage (Fig. 9A).
2) The fluorescent protein-tagged LARP6c is localized to the nucleolus of the microspore, then appears diffused in the cytoplasm of the BCP and TCP, where this pattern transitions to granular aggregates in the MP (Billey et al. 2021). Furthermore, in MP, LARP6c aggregates concentrate near the perinuclear membrane and around the endo-PM membrane surrounding the sperms.
3) Tagged PAB5, but not DCP1, colocalized with LARP6c, suggesting the LARP6c-associated aggregates are stress

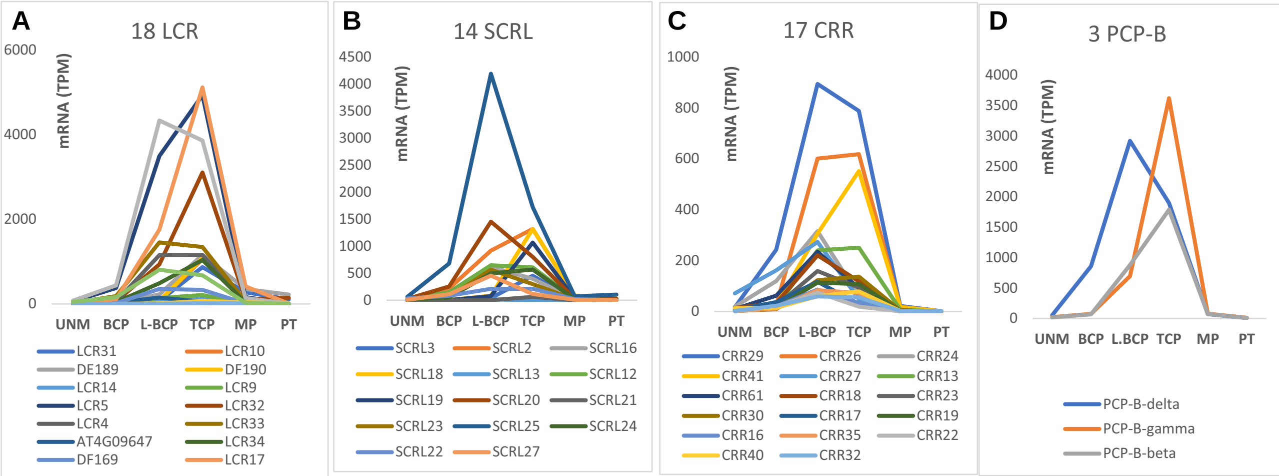


Figure 10. Scores of transcripts encoding cysteine-rich proteins accumulate in late BCP and TCP. At least 54 of 105 pollen-expressed genes are induced at the intermediate stage. **A)** LCR (Low molecular weight CRP) family: 18 of 25 LCR pollen-expressed genes accumulate in late BCP and TCP. Protein detected for 3 of 18. **B)** SCRL (S-locus CRP-like) family: 14 of 16 transcripts are expressed in late BCP and TCP. **C)** CRR (Cysteine rich repeat) (DUF26) family: 17 of 21 transcripts in late BCP or TCP show TPM reads of 60 or above. Protein was detected for 12 of 21 genes (average 254 residues). **D)** ESF-like (Embryo surrounding factor) family includes PCP-b (pollen coat protein): Of 4 PCP-b genes, 3 are expressed in developing pollen. Only PCP-b delta was detected in the mature pollen proteome. Supplementary Tables S4 and S5 show summary and gene ID#. Supplementary Fig. S11 shows pollen expressed SPH (self-incompatibility protein homologue) and RALF (Rapid Alkalinization Factor) gene families.

granules that include the translational machinery but are not P-bodies that turnover mRNA. PABP and DCP1 are markers of stress granules and P-bodies, respectively.

4) Hundreds of transcripts are physically associated with LARP6c as shown by RNA immunoprecipitation and RNA sequencing (Billey et al. 2021). At least 112 transcripts were shown as clients of LARP6c. Some of these transcripts were predicted to be stored in granules, based on their mRNA abundance late in pollen development and on their resistance to heat stress in germinating pollen (Poidevin et al. 2021; Sze et al. 2021).

As LARP6c binds hundreds of transcripts that encode proteins with varied biochemical roles in MP; it is likely that *larp6c* mutants would be compromised in multiple pollen functions (Billey et al. 2021). Indeed, it was shown that ∼10% of the pollen in *larp6c* T-DNA insertional mutants failed to develop to maturity and were arrested at the BCP stage compared with only 3% in wild type. Thus, LARP6c is critical for pollen development from the BCP to MP. Based on these reports, we propose that TCP without a functional LARP6c form defective mRNP aggregates containing non-translating mRNAs as pollen dehydrates. Aggregates with altered mRNA composition, binding or architecture due to loss of function in LARP6c could result in pollen with less vigor/fitness that result in impaired functions, including reduced germination, tube burst, and failed guidance as shown in Billey et al. (2021). LARP6c function is likely conserved across flowering plants, as pollen germination and tube growth from maize mutants of LARP6c were less competitive than wild type (Zhou et al. 2021).

Importantly, LARP6 and ALBA proteins are predicted to have disordered domains (Fig. 9) suggesting their conformation, properties, like phase separation and RNA-binding affinity, may be responsive to physio-chemical changes in cells undergoing osmotic stress (Alberti and Hyman 2021) in TCP, hydrating pollen or both. Additional RNA-binding proteins and their roles in pollen development have yet to be determined.

Scores of cysteine-rich protein mRNAs induced in late BCP have unknown roles

Over 100 transcripts encoding cysteine-rich proteins (CRP) are expressed in pollen, and 60 are specifically induced at the late BCP-TCP stage. The remaining CRP mRNAs appear in MP or in germinating tubes (Supplementary Table S3; Fig. 10). In addition, a large fraction (46%) of CRP transcripts (total 270) is expressed in seeds, siliques, and endosperm, and a small fraction (15%) is expressed in vegetative tissues, like roots and stem (Supplementary Table S4). Often called defensin-like proteins (DEFL) or anti-microbial peptides (Silverstein et al. 2005; Tesfaye et al. 2013), we refer to the pollen-expressed peptides as pollen CRP. The roles of CRPs from different families (e.g. LCR, SCR, SPH, CRRSP, or PCP-B) are largely unexplored (Fig. 10; Supplementary Fig. S11). CRPs are secreted ligands and emerging studies show they are important for cell-cell communication and cell-cell recognition to prevent self-fertilization and inbreeding. Previous studies have largely focused on CRPs produced by female cells, like stigma papilla, pistil, or synergid (Qu et al. 2015), or in developing embryo (Costa et al. 2014). Much less is known about pollen CRPs.

A recent study revealed a pollen CRP in regulating pollen-stigma compatibility and subsequent hydration. *A. thaliana* PCP-B (Protein coat protein-type B) genes identified by homology to Brassica PCP-B are expressed in late BCP and TCP (Wang et al. 2017; Fig. 10D). Mutants of *A. thaliana* PCP-B displayed delayed rehydration scored as change in pollen diameter and delayed tube growth on the stigma. A receptor-like kinase complex of ANJ-FER on the stigma was essential as the receptor mutant also showed delayed rehydration. Furthermore, before pollen landing on the stigma, RALF binding to the receptor-complex stimulated ROS production; however, pollen PCP-B competes with RALF binding, which leads to suppression of ROS levels at the stigma (Liu et al. 2021). These results demonstrate that after pollen lands on a stigma, PCP-B class of CRPs is sensed by a stigma receptor complex that signals to promote pollen adhesion and rehydration. This gating mechanism is probably a key first step to determine pollen-stigma compatibility. Most pollen-expressed CRPs remain uncharacterized, we postulate that some CRP mRNA are translated and the protein secreted before pollen desiccates in preparation for recognition, rehydration, and germination.

Summary and perspectives

To our knowledge, this study reports the first analysis of transcripts and proteins accompanying normal dehydration in developing male gametophyte. This analysis is relevant to major questions in biology:

1) What regulates pollen development/microgametogenesis when a haploid microspore divides to produce 2 cells with distinct fates: a vegetative and a generative cell; and
2) As dehydration onset occurs in BCP, what is the cellular and molecular basis of intrinsic dehydration and desiccation tolerance?

We distinguished an "intermediate" wave of transcripts that accumulate between the "early-" and "late"-pollen expressed genes. The intermediate cluster detected at late BCP-TCP in *A. thaliana* included multiple LEA transcripts and stress-related proteins. Most genes coexpressed at the late BCP are pollen specific or preferential and translated before pollen maturation, suggesting the proteins are functional. What regulates the "intermediate" wave of transcripts seen in late BCP and TCP after pollen mitosis I is largely unknown. Most studies have focused on male germline development (Twell 2011; Hackenberg and Twell 2019); however, events controlling vegetative cell differentiation are unexplored. The "early" pollen-expressed genes in UNM after meiosis reflect those passed on from the sporophyte-transcribed mRNA. The repression of early-pollen genes and the activation of the "intermediate" transcript cluster likely marks the developmental time (Fig. 1C) when the transition of the sporophyte-to-gametophyte program takes place. Our analysis suggests the transition happens after mitosis I in *A. thaliana*, similar to that in maize. In maize, transcripts synthesized in the sporophyte persist in the male gametophyte days after meiosis, and the transition to the haploid genome was observed after mitosis I (Nelms and Walbot 2022). Whether the transcription program specific to the vegetative cell (Supplementary Fig. S10) is governed by chromatin remodeling (Borg and Berger 2015), developmental cues, abiotic cues (like dehydration), or a combination is largely unknown and awaits future studies.

The onset of dehydration after mitosis I is supported by several molecular indicators. First, the transcripts and proteins of LEAs and other desiccation-related proteins peaking in BCP or TCP are homologous to those accumulating in seed maturation. Second, increase in lipid droplets and expression of lipid-droplet associated proteins in BCP are also observed in dehydrating seeds and in plants and algae under stress. Third, many transcripts in BCP are also induced by salt, osmotic, or cold stress in other tissues, including RNA-binding proteins that bind mRNAs and associate with mRNA-protein granules.

Genes coexpressed with LEAs suggests the BCP is actively modifying the vegetative cell as it dehydrates and differentiates to a mature pollen grain. First, many transporters with known functions are expressed in late BCP, including H^+ and Ca^{2+} pumps, sugar and amino acid co-transporters, aquaporins, nucleotide-sugar porters, and Ca^{2+}-permeable channels. Together they mediate not only nutrient uptake, but also osmolyte balance, wall synthesis/remodeling and possibly sugar and Ca^{2+} signaling. Second, an active endomembrane trafficking system is central to the coordination of transport activities, wall formation, and pectin remodeling in dehydrating and developing pollen. GAUT and PMT expression suggests homogalacturonan synthesis and methyl-esterification in the Golgi started early in pollen development. Pectin released to the wall is then modified by secreted PME and PMEI perhaps in a spatial and temporal manner. Third, scores of CRP transcripts accumulate at the intermediate stage. Largely uncharacterized, they may be signaling cues for cell-cell communication and cell-cell recognition when the pollen interacts with the stigma and pistil. Fourth, mRNAs encoding RNA-binding proteins accumulate in late BCP-TCP, and functional studies support their roles in binding mRNAs as part of condensates and stress granules in MP. Finally, proteins of the translation machinery and molecular chaperones accumulate in MP. Moreover, proteins required for carbon metabolism and energy production are abundant and stabilized in the cytosol, mitochondria, and plastids. Together, these results support the idea that MP tolerates desiccation at dispersal after remodeling membranes and walls, protecting mRNAs and the translation machinery in stress granules, and preparing pollen for recognition and compatibility at the stigma, for adhesion and rehydration.

This first survey of genes coexpressed at the "intermediate" stage expands our perspective about processes accompanying dehydration and pollen development. Analysis of more transcript clusters and protein classes will reveal additional relationships and insights. This integrated omics approach to combine large datasets with development and functional studies reveals new models and strategies to answer major questions in dehydration stress and plant reproduction.

OUTSTANDING QUESTIONS BOX

1) What is the basis of programmed dehydration in anther and pollen development?
2) What regulates the "intermediate" wave of transcripts peaking in late BCP? Is it epigenetic changes, regulatory networks related to osmotic stress, or both?
3) What regulates the stabilization of proteins required for energy production and protein translation?
4) What cellular roles do genes coexpressed at dehydration (transporters, pectin modifiers, pollen CRP) serve?
5) What protective strategies are critical for desiccation tolerance? What controls the formation of mRNA-protein granules?
6) What cellular processes mediate the recovery from desiccation at rehydration?

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Author contributions

H.S. conceived the project after discussions with all authors. B.K. and D.H. provided the proteome datasets. D.H. laboratory generated the transcriptome datasets. H.S. analyzed the data with J.M.W., R.P., J.F.H., M.A.J., B.K., and D.H. All authors contributed to writing the manuscript and approved the final version. H.S. agrees to serve as the author responsible for contact and communication.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Panther classified protein classes of transcripts expressed in 4 pollen stages.

Supplementary Figure S2. AtLEA gene expression in developing pollen and seed.

Supplementary Figure S3. ABA biosynthesis and signaling genes in pollen.

Supplementary Figure S4. mRNA and protein abundance of transporters peaking in TCP.

Supplementary Figure S5. Developmentally-regulated expression of transporters.

Supplementary Figure S6. Expression of pectin remodelers in developing pollen.

Supplementary Figure S8. Translation machinery accumulates in mature pollen.

Supplementary Figure S9. Chaperone proteins accumulate in TCP and mature pollen.

Supplementary Figure S10. Transcripts of transcription regulators accumulated in BCP and TCP.

Supplementary Figure S11. Transcripts encoding Cys-rich peptides from the SPH and RALF families.

Supplementary Table S1. Transcripts accumulating in late BCP of *Arabidopsis thaliana*.

Supplementary Table S2. Transcripts accumulating in TCP of *Arabidopsis thaliana*.

Supplementary Table S3. Genes, transcripts and proteins of the translation machinery expressed in pollen in Fig. 8 and Supplementary Fig. S8.

Supplementary Table S4. Most transcripts of Cysteine-rich proteins are expressed in pollen and seeds of *A. thaliana*.

Supplementary Table S5. Atg genes with ID # encoding cysteine-rich proteins in developing pollen in Fig. 10.

Supplementary Table S6. Transcripts and proteins of the mitochondria and glycolysis in developing pollen (Fig. 7).

Supplementary Table S7. Translation initiation and elongation factors in developing pollen from Supplementary Fig. S8.

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Conflict of interest statement. None declared.

Data availability

The RNA-seq data underlying this article are available in the EvoRepro database at https://evorepro.sbs.ntu.edu.sg/, and in the article and in its online supplementary material. The Proteomics data is obtained from Klodova et al. 2023 and Grobei et al. 2009.

References

Acosta IF, Przybyl M. Jasmonate signaling during Arabidopsis stamen maturation. Plant Cell Physiol. 2019:**60**(12):2648–2659. https://doi.org/10.1093/pcp/pcz201

Alberti S, Hyman AA. Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing. Nat Rev Mol Cell Biol. 2021:**22**(3):196–213. https://doi.org/10.1038/s41580-020-00326-6

Atkin OK, Macherel D. The crucial role of plant mitochondria in orchestrating drought tolerance. Ann Bot. 2009:**103**(4):581–597. https://doi.org/10.1093/aob/mcn094

Atmodjo MA, Hao Z, Mohnen D. Evolving views of pectin biosynthesis. Annu Rev Plant Biol. 2013:**64**(1):747–779. https://doi.org/10.1146/annurev-arplant-042811-105534

Bartels D, Schneider K, Terstappen G, Piatkowski D, Salamini F. Molecular cloning of abscisic acid-modulated genes which are induced during desiccation of the resurrection plant *Craterostigma plantagineum*. Planta. 1990:**181**(1):27–34. https://doi.org/10.1007/BF00202321

Bayfield MA, Vinayak J, Kerkhofs K, Mansouri-Noori F. La proteins couple use of sequence-specific and non-specific binding modes to engage RNA substrates. RNA Biol. 2021:**18**(2):168–177. https://doi.org/10.1080/15476286.2019.1582955

Billey E, Hafidh S, Cruz-Gallardo I, Litholdo CG, Jean V, Carpentier MC, Picart C, Kumar V, Kulichova K, Marechal E, et al. LARP6C orchestrates posttranscriptional reprogramming of gene expression during hydration to promote pollen tube guidance. Plant Cell. 2021:**33**(8):2637–2661. https://doi.org/10.1093/plcell/koab131

Bock KW, Honys D, Ward JM, Padmanaban S, Nawrocki EP, Hirschi KD, Twell D, Sze H. Integrating membrane transport with male gametophyte development and function through transcriptomics. Plant Physiol. 2006:**140**(4):1151–1168. https://doi.org/10.1104/pp.105.074708

Borg M, Berger F. Chromatin remodelling during male gametophyte development. Plant J. 2015:**83**(1):177–188. https://doi.org/10.1111/tpj.12856

Borg M, Brownfield L, Twell D. Male gametophyte development: a molecular perspective. J Exp Bot. 2009:**60**(5):1465–1478. https://doi.org/10.1093/jxb/ern355

Candat A, Paszkiewicz G, Neveu M, Gautier R, Logan DC, Avelange-Macherel MH, Macherel D. The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in Arabidopsis offers tailored protection against abiotic stress. Plant Cell. 2014:**26**(7):3148–3166. https://doi.org/10.1105/tpc.114.127316

Chantarachot T, Bailey-Serres J. Polysomes, stress granules, and processing bodies: a dynamic triumvirate controlling cytoplasmic mRNA fate and function. Plant Physiol. 2018:**176**(1):254–269. https://doi.org/10.1104/pp.17.01468

Chapman KD, Dyer JM, Mullen RT. Biogenesis and functions of lipid droplets in plants: thematic review series: lipid droplet synthesis and metabolism: from yeast to man. J Lipid Res. 2012:**53**(2):215–226. https://doi.org/10.1194/jlr.R021436

Costa LM, Marshall E, Tesfaye M, Silverstein KA, Mori M, Umetsu Y, Otterbach SL, Papareddy R, Dickinson HG, Boutiller K, et al. Central cell-derived peptides regulate early embryo patterning in flowering plants. Science. 2014:**344**(6180):168–172. https://doi.org/10.1126/science.1243005

Cuevas-Velazquez CL, Vellosillo T, Schmidt GK, Yu HB, Moses F, Brophy D, Cosio-Acosta JAN, Das D, Wang A, Jones L, et al. Intrinsically disordered protein biosensor tracks the physical-chemical effects of osmotic stress on cells. Nat Commun. 2021:**12**(1):5438. https://doi.org/10.1038/s41467-021-25736-8

de Vries J, Ischebeck T. Ties between stress and lipid droplets pre-date seeds. Trends Plant Sci. 2020:**25**(12):1203–1214. https://doi.org/10.1016/j.tplants.2020.07.017

Dhatterwal P, Mehrotra S, Miller AJ, Aduri R, Mehrotra R. Effect of ACGT motif in spatiotemporal regulation of AtAVT6D, which improves tolerance to osmotic stress and nitrogen-starvation. Plant Mol Biol. 2022:**109**(1–2):67–82. https://doi.org/10.1007/s11103-022-01256-x

Di Matteo A, Giovane A, Raiola A, Camardella L, Bonivento D, De Lorenzo G, Cervone F, Bellincampi D, Tsernoglou D. Structural basis for the interaction between pectin methylesterase and a specific inhibitor protein. Plant Cell. 2005:**17**(3):849–858. https://doi.org/10.1105/tpc.104.028886

Du J, Kirui A, Huang S, Wang L, Barnes WJ, Kiemle SN, Zheng Y, Rui Y, Ruan M, Qi S, et al. Mutations in the pectin methyltransferase QUASIMODO2 influence cellulose biosynthesis and wall integrity in Arabidopsis. Plant Cell. 2020:**32**(11):3576–3597. https://doi.org/10.1105/tpc.20.00252

Firon N, Nepi M, Pacini E. Water status and associated processes mark critical stages in pollen development and functioning. Ann Bot. 2012:**109**(7):1201–1214. https://doi.org/10.1093/aob/mcs070

Franchi GG, Piotto B, Nepi M, Baskin CC, Baskin JM, Pacini E. Pollen and seed desiccation tolerance in relation to degree of developmental arrest, dispersal, and survival. J Exp Bot. 2011:**62**(15):5267–5281. https://doi.org/10.1093/jxb/err154

Frietsch S, Wang YF, Sladek C, Poulsen LR, Romanowsky SM, Schroeder JI, Harper JF. A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. Proc Natl Acad Sci U S A. 2007:**104**(36):14531–14536. https://doi.org/10.1073/pnas.0701781104

Fujiki Y, Teshima H, Kashiwao S, Kawano-Kawada M, Ohsumi Y, Kakinuma Y, Sekito T. Functional identification of AtAVT3, a family of vacuolar amino acid transporters, in Arabidopsis. FEBS Lett. 2017:**591**(1):5–15. https://doi.org/10.1002/1873-3468.12507

Goyal K, Walton LJ, Tunnacliffe A. LEA proteins prevent protein aggregation due to water stress. Biochem J. 2005:**388**(1):151–157. https://doi.org/10.1042/BJ20041931

Grobei MA, Qeli E, Brunner E, Rehrauer H, Zhang R, Roschitzki B, Basler K, Ahrens CH, Grossniklaus U. Deterministic protein inference for shotgun proteomics data provides new insights into Arabidopsis pollen development and function. Genome Res. 2009:**19**(10):1786–1800. https://doi.org/10.1101/gr.089060.108

Haas KT, Wightman R, Peaucelle A, Hofte H. The role of pectin phase separation in plant cell wall assembly and growth. Cell Surf. 2021:**7**:100054. https://doi.org/10.1016/j.tcsw.2021.100054

Hackenberg D, Twell D. The evolution and patterning of male gametophyte development. Curr Top Dev Biol. 2019:**131**:257–298. https://doi.org/10.1016/bs.ctdb.2018.10.008

Hafidh S, Honys D. Reproduction multitasking: the male gametophyte. Annu Rev Plant Biol. 2021:**72**(1):581–614. https://doi.org/10.1146/annurev-arplant-080620-021907

Hedrich R, Sauer N, Neuhaus HE. Sugar transport across the plant vacuolar membrane: nature and regulation of carrier proteins. Curr Opin Plant Biol. 2015:**25**:63–70. https://doi.org/10.1016/j.pbi.2015.04.008

Hocq L, Pelloux J, Lefebvre V. Connecting homogalacturonan-type pectin remodeling to acid growth. Trends Plant Sci. 2017:**22**(1):20–29. https://doi.org/10.1016/j.tplants.2016.10.009

Hoekstra FA, Golovina EA, Buitink J. Mechanisms of plant desiccation tolerance. Trends Plant Sci. 2001:**6**(9):431–438. https://doi.org/10.1016/S1360-1385(01)02052-0

Hoffmann RD, Portes MT, Olsen LI, Damineli DSC, Hayashi M, Nunes CO, Pedersen JT, Lima PT, Campos C, Feijo JA, et al. Plasma membrane H^+-ATPases sustain pollen tube growth and fertilization. Nat Commun. 2020:**11**(1):2395. https://doi.org/10.1038/s41467-020-16253-1

Hofte H, Voxeur A. Plant cell walls. Curr Biol. 2017:**27**(17):R865–R870. https://doi.org/10.1016/j.cub.2017.05.025

Honys D, Renak D, Fecikova J, Jedelsky PL, Nebesarova J, Dobrev P, Capkova V. Cytoskeleton-associated large RNP complexes in tobacco male gametophyte (EPPs) are associated with ribosomes and are involved in protein synthesis, processing, and localization. J Proteome Res. 2009:**8**(4):2015–2031. https://doi.org/10.1021/pr8009897

Honys D, Twell D. Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol. 2004:**5**(11):R85. https://doi.org/10.1186/gb-2004-5-11-r85

Hothorn M, Wolf S, Aloy P, Greiner S, Scheffzek K. Structural insights into the target specificity of plant invertase and pectin methylesterase inhibitory proteins. Plant Cell. 2004:**16**(12):3437–3447. https://doi.org/10.1105/tpc.104.025684

Hundertmark M, Hincha DK. LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. BMC Genomics. 2008:**9**(1):118. https://doi.org/10.1186/1471-2164-9-118

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K. The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell. 2001:**13**(10):2191–2209. https://doi.org/10.1105/tpc.010192

Jewell JB, Browse J. Epidermal jasmonate perception is sufficient for all aspects of jasmonate-mediated male fertility in Arabidopsis. Plant J. 2016:**85**(5):634–647. https://doi.org/10.1111/tpj.13131

Jiang LX, Yang SL, Xie LF, Puah CS, Zhang XQ, Yang WC, Sundaresan V, Ye D. VANGUARD1 encodes a pectin methylesterase that enhances pollen tube growth in the Arabidopsis style and transmitting tract. Plant Cell. 2005:**17**(2):584–596. https://doi.org/10.1105/tpc.104.027631

Johnson MA, Harper JF, Palanivelu R. A fruitful journey: pollen tube navigation from germination to fertilization. Annu Rev Plant Biol. 2019:**70**(1):809–837. https://doi.org/10.1146/annurev-arplant-050718-100133

Julca I, Ferrari C, Flores-Tornero M, Proost S, Lindner AC, Hackenberg D, Steinbachova L, Michaelidis C, Gomes Pereira S, Misra CS, et al. Comparative transcriptomic analysis reveals conserved programmes underpinning organogenesis and reproduction in land plants. Nat Plants. 2021:**7**(8):1143–1159. https://doi.org/10.1038/s41477-021-00958-2

Kim HU, Hsieh K, Ratnayake C, Huang AH. A novel group of oleosins is present inside the pollen of Arabidopsis. J Biol Chem. 2002:**277**(25):22677–22684. https://doi.org/10.1074/jbc.M109298200

Kim SC, Guo L, Wang X. Nuclear moonlighting of cytosolic glyceraldehyde-3-phosphate dehydrogenase regulates Arabidopsis response to heat stress. Nat Commun. 2020:**11**(1):3439. https://doi.org/10.1038/s41467-020-17311-4

Klodova B, Potesil D, Steinbachova L, Michailidis C, Lindner AC, Hackenberg D, Becker JD, Zdrahal Z, Twell D, Honys D. Regulatory dynamics of gene expression in the developing male gametophyte of Arabidopsis. Plant Reprod. 2023:**36**(3):213–241. https://doi.org/10.1007/s00497-022-00452-5

Kosmacz M, Gorka M, Schmidt S, Luzarowski M, Moreno JC, Szlachetko J, Leniak E, Sokolowska EM, Sofroni K, Schnittger A, et al. Protein and metabolite composition of Arabidopsis stress granules. New Phytol. 2019:**222**(3):1420–1433. https://doi.org/10.1111/nph.15690

Lee H, Guo Y, Ohta M, Xiong L, Stevenson B, Zhu JK. LOS2, a genetic locus required for cold-responsive gene transcription encodes a bifunctional enolase. EMBO J. 2002:**21**(11):2692–2702. https://doi.org/10.1093/emboj/21.11.2692

Leroux C, Bouton S, Kiefer-Meyer MC, Fabrice TN, Mareck A, Guenin S, Fournet F, Ringli C, Pelloux J, Driouich A, et al. PECTIN METHYLESTERASE48 is involved in Arabidopsis pollen grain germination. Plant Physiol. 2015:**167**(2):367–380. https://doi.org/10.1104/pp.114.250928

Levesque-Tremblay G, Pelloux J, Braybrook SA, Muller K. Tuning of pectin methylesterification: consequences for cell wall biomechanics and development. Planta. 2015:**242**(4):791–811. https://doi.org/10.1007/s00425-015-2358-5

Li G, Santoni V, Maurel C. Plant aquaporins: roles in plant physiology. Biochim Biophys Acta. 2014:**1840**(5):1574–1582. https://doi.org/10.1016/j.bbagen.2013.11.004

Liu C, Shen L, Xiao Y, Vyshedsky D, Peng C, Sun X, Liu Z, Cheng L, Zhang H, Han Z, et al. Pollen PCP-B peptides unlock a stigma peptide-receptor kinase gating mechanism for pollination. Science. 2021:**372**(6538):171–175. https://doi.org/10.1126/science.abc6107

Lopez-Ribera I, Paz L, Repiso JL, Garcia C, Miquel N, Hernandez M, Martinez-Rivas ML, Vicient JM, M C. The evolutionary conserved oil body associated protein OBAP1 participates in the regulation of oil body size. Plant Physiol. 2014:**164**(3):1237–1249. https://doi.org/10.1104/pp.113.233221

Mancosu N, Snyder RL, Kyriakakis G, Spano D. Water scarcity and future challenges for food production. Water (Basel). 2015:**7**(12):975–992. https://doi.org/10.3390/w7030975

Mascarenhas JP. Molecular mechanisms of pollen tube growth and differentiation. Plant Cell. 1993:**5**(10):1303–1314. https://doi.org/10.2307/3869783

Mayfield JA, Preuss D. Rapid initiation of Arabidopsis pollination requires the oleosin-domain protein GRP17. Nat Cell Biol. 2000:**2**(2):128–130. https://doi.org/10.1038/35000084

Merret R, Martino L, Bousquet-Antonelli C, Fneich S, Descombin J, Billey E, Conte MR, Deragon JM. The association of a La module with the PABP-interacting motif PAM2 is a recurrent evolutionary process that led to the neofunctionalization of La-related proteins. RNA. 2013:**19**(1):36–50. https://doi.org/10.1261/rna.035469.112

Mittler R. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 2006:**11**(1):15–19. https://doi.org/10.1016/j.tplants.2005.11.002

Moon S, Jung KH. First steps in the successful fertilization of rice and Arabidopsis: pollen longevity, adhesion and hydration. Plants (Basel). 2020:**9**(8):956. https://doi.org/10.3390/plants9080956

Mouille G, Ralet MC, Cavelier C, Eland C, Effroy D, Hematy K, McCartney L, Truong HN, Gaudon V, Thibault JF, et al. Homogalacturonan synthesis in *Arabidopsis thaliana* requires a Golgi-localized protein with a putative methyltransferase domain. Plant J. 2007:**50**(4):605–614. https://doi.org/10.1111/j.1365-313X.2007.03086.x

Naprstkova A, Malinska K, Zaveska Drabkova L, Billey E, Naprstkova D, Sykorova E, Bousquet-Antonelli C, Honys D. Characterization of ALBA family expression and localization in *Arabidopsis thaliana* generative organs. Int J Mol Sci. 2021:**22**(4):1652. https://doi.org/10.3390/ijms22041652

Nelms B, Walbot V. Gametophyte genome activation occurs at pollen mitosis I in maize. Science. 2022:**375**(6579):424–429. https://doi.org/10.1126/science.abl7392

Olvera-Carrillo Y, Campos F, Reyes JL, Garciarrubio A, Covarrubias AA. Functional analysis of the group 4 late embryogenesis abundant proteins reveals their relevance in the adaptive response during water deficit in Arabidopsis. Plant Physiol. 2010:**154**(1):373–390. https://doi.org/10.1104/pp.110.158964

Owen HA, Makaroff CA. Ultrastructure of microsporogenesis and microgametogenesis in *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (Brassicaceae). Protoplasma. 1995:**185**(1–2):7–21. https://doi.org/10.1007/BF01272749

Pacini E, Dolferus R. Pollen developmental arrest: maintaining pollen fertility in a world with a changing climate. Front Plant Sci. 2019:**10**:679. https://doi.org/10.3389/fpls.2019.00679

Piatkowski D, Schneider K, Salamini F, Bartels D. Characterization of five abscisic acid-responsive cDNA clones isolated from the desiccation-tolerant plant *Craterostigma plantagineum* and their relationship to other water-stress genes. Plant Physiol. 1990:**94**(4):1682–1688. https://doi.org/10.1104/pp.94.4.1682

Poidevin L, Forment J, Unal D, Ferrando A. Transcriptome and translatome changes in germinated pollen under heat stress uncover roles of transporter genes involved in pollen tube growth. Plant Cell Environ. 2021:**44**(7):2167–2184. https://doi.org/10.1111/pce.13972

Qin Y, Leydon AR, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R. Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil. PLoS Genet. 2009:**5**(8):e1000621. https://doi.org/10.1371/journal.pgen.1000621

Qu LJ, Li L, Lan Z, Dresselhaus T. Peptide signalling during the pollen tube journey and double fertilization. J Exp Bot. 2015:**66**(17):5139–5150. https://doi.org/10.1093/jxb/erv275

Rahmati Ishka M, Brown E, Rosenberg A, Romanowsky S, Davis JA, Choi WG, Harper JF. Arabidopsis Ca^{2+}-ATPases 1, 2, and 7 in the endoplasmic reticulum contribute to growth and pollen fitness. Plant Physiol. 2021:**185**(4):1966–1985. https://doi.org/10.1093/plphys/kiab021

Rautengarten C, Birdseye D, Pattathil S, McFarlane HE, Saez-Aguayo S, Orellana A, Persson S, Hahn MG, Scheller HV, Heazlewood JL, et al. The elaborate route for UDP-arabinose delivery into the Golgi of plants. Proc Natl Acad Sci U S A. 2017:**114**(16): 4261–4266. https://doi.org/10.1073/pnas.1701894114

Reyes F, Leon G, Donoso M, Brandizzi F, Weber AP, Orellana A. The nucleotide sugar transporters AtUTr1 and AtUTr3 are required for the incorporation of UDP-glucose into the endoplasmic reticulum, are essential for pollen development and are needed for embryo sac progress in *Arabidopsis thaliana*. Plant J. 2010:**61**(3):423–435. https://doi.org/10.1111/j.1365-313X.2009.04066.x

Rottmann T, Fritz C, Sauer N, Stadler R. Glucose uptake via STP transporters inhibits in vitro pollen tube growth in a HEXOKINASE1-dependent manner in *Arabidopsis thaliana*. Plant Cell. 2018:**30**(9): 2057–2081. https://doi.org/10.1105/tpc.18.00356

Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB. The Arabidopsis DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell. 2000:**12**(7):1041–1061. https://doi.org/10.1105/tpc.12.7.1041

Schiott M, Romanowsky SM, Baekgaard L, Jakobsen MK, Palmgren MG, Harper JF. A plant plasma membrane Ca2+ pump is required for normal pollen tube growth and fertilization. Proc Natl Acad Sci U S A. 2004:**101**(25):9502–9507. https://doi.org/10.1073/pnas.0401542101

Senechal F, Wattier C, Rusterucci C, Pelloux J. Homogalacturonan-modifying enzymes: structure, expression, and roles in plants. J Exp Bot. 2014:**65**(18):5125–5160. https://doi.org/10.1093/jxb/eru272

Silverstein KA, Graham MA, Paape TD, VandenBosch KA. Genome organization of more than 300 defensin-like genes in Arabidopsis. Plant Physiol. 2005:**138**(2):600–610. https://doi.org/10.1104/pp.105.060079

Sivitz AB, Reinders A, Ward JM. Arabidopsis sucrose transporter AtSUC1 is important for pollen germination and sucrose-induced anthocyanin accumulation. Plant Physiol. 2008:**147**(1):92–100. https://doi.org/10.1104/pp.108.118992

Stadler R, Truernit E, Gahrtz M, Sauer N. The AtSUC1 sucrose carrier may represent the osmotic driving force for anther dehiscence and pollen tube growth in Arabidopsis. Plant J. 1999:**19**(3):269–278. https://doi.org/10.1046/j.1365-313X.1999.00527.x

Sze H, Chanroj S. Plant endomembrane dynamics: studies of K(+)/H(+) antiporters provide insights on the effects of pH and ion homeostasis. Plant Physiol. 2018:**177**(3):875–895. https://doi.org/10.1104/pp.18.00142

Sze H, Palanivelu R, Harper JF, Johnson MA. Holistic insights from pollen omics: co-opting stress-responsive genes and ER-mediated proteostasis for male fertility. Plant Physiol. 2021:**187**(4): 2361–2380. https://doi.org/10.1093/plphys/kiab463

Taiz L, Zeiger E, Moller IM, Murphy AS. Plant physiology and development. 6th ed. Sunderland (CT): Sinauer Associates; 2015.

Taurino M, Costantini S, De Domenico S, Stefanelli F, Ruano G, Delgadillo MO, Sanchez-Serrano JJ, Sanmartin M, Santino A, Rojo E. SEIPIN proteins mediate lipid droplet biogenesis to promote pollen transmission and reduce seed dormancy. Plant Physiol. 2018:**176**(2):1531–1546. https://doi.org/10.1104/pp.17.01430

Temple H, Saez-Aguayo S, Reyes FC, Orellana A. The inside and outside: topological issues in plant cell wall biosynthesis and the roles of nucleotide sugar transporters. Glycobiology. 2016:**26**(9):913–925. https://doi.org/10.1093/glycob/cww054

Tesfaye M, Silverstein KA, Nallu S, Wang L, Botanga CJ, Gomez SK, Costa LM, Harrison MJ, Samac DA, Glazebrook J, et al. Spatio-temporal expression patterns of *Arabidopsis thaliana* and Medicago truncatula defensin-like genes. PLoS One. 2013:**8**(3): e58992. https://doi.org/10.1371/journal.pone.0058992

Thomas PD, Ebert D, Muruganujan A, Mushayahama T, Albou LP, Mi H. PANTHER: making genome-scale phylogenetics accessible to all. Protein Sci. 2022:**31**(1):8–22. https://doi.org/10.1002/pro.4218

Tong J, Ren Z, Sun L, Zhou S, Yuan W, Hui Y, Ci D, Wang W, Fan LM, Wu Z, et al. ALBA proteins confer thermotolerance through stabilizing HSF messenger RNAs in cytoplasmic granules. Nat Plants. 2022:**8**(7):778–791. https://doi.org/10.1038/s41477-022-01175-1

Tunc-Ozdemir M, Rato C, Brown E, Rogers S, Mooneyham A, Frietsch S, Myers CT, Poulsen LR, Malho R, Harper JF. Cyclic nucleotide gated channels 7 and 8 are essential for male reproductive fertility. PLoS One. 2013:**8**(2):e55277. https://doi.org/10.1371/journal.pone.0055277

Twell D. Male gametogenesis and germline specification in flowering plants. Sex Plant Reprod. 2011:**24**(2):149–160. https://doi.org/10.1007/s00497-010-0157-5

Updegraff EP, Zhao F, Preuss D. The extracellular lipase EXL4 is required for efficient hydration of Arabidopsis pollen. Sex Plant Reprod. 2009:**22**(3):197–204. https://doi.org/10.1007/s00497-009-0104-5

Wang L, Clarke LA, Eason RJ, Parker CC, Qi B, Scott RJ, Doughty J. PCP-B class pollen coat proteins are key regulators of the hydration checkpoint in *Arabidopsis thaliana* pollen-stigma interactions. New Phytol. 2017:**213**(2):764–777. https://doi.org/10.1111/nph.14162

Wang Y, Zhang WZ, Song LF, Zou JJ, Su Z, Wu WH. Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in Arabidopsis. Plant Physiol. 2008:**148**(3):1201–1211. https://doi.org/ 10.1104/pp.108.126375.

Wei D, Liu M, Chen H, Zheng Y, Liu Y, Wang X, Yang S, Zhou M, Lin J. INDUCER OF CBF EXPRESSION 1 is a male fertility regulator impacting anther dehydration in Arabidopsis. PLoS Genet. 2018:**14**(10): e1007695. https://doi.org/10.1371/journal.pgen.1007695

Wiedner HJ, Giudice J. It's not just a phase: function and characteristics of RNA-binding proteins in phase separation. Nat Struct Mol Biol. 2021:**28**(6):465–473. https://doi.org/10.1038/s41594-021-00601-w

Wormit A, Trentmann O, Feifer I, Lohr C, Tjaden J, Meyer S, Schmidt U, Martinoia E, Neuhaus HE. Molecular identification and physiological characterization of a novel monosaccharide transporter from Arabidopsis involved in vacuolar sugar transport. Plant Cell. 2006:**18**(12):3476–3490. https://doi.org/10.1105/tpc.106.047290

Wudick MM, Luu DT, Tournaire-Roux C, Sakamoto W, Maurel C. Vegetative and sperm cell-specific aquaporins of Arabidopsis highlight the vacuolar equipment of pollen and contribute to plant reproduction. Plant Physiol. 2014:**164**(4):1697–1706. https://doi.org/10.1104/pp.113.228700

Yamamoto Y, Nishimura M, Hara-Nishimura I, Noguchi T. Behavior of vacuoles during microspore and pollen development in *Arabidopsis thaliana*. Plant Cell Physiol. 2003:**44**(11):1192–1201. https://doi.org/10.1093/pcp/pcg147

Yuan F, Yang H, Xue Y, Kong D, Ye R, Li C, Zhang J, Theprungsirikul L, Shrift T, Krichilsky B, et al. OSCA1 mediates osmotic-stress-evoked Ca^{2+} increases vital for osmosensing in Arabidopsis. Nature. 2014:**514**(7522):367–371. https://doi.org/10.1038/nature13593

Zhang Q, Bartels D. Molecular responses to dehydration and desiccation in desiccation-tolerant angiosperm plants. J Exp Bot. 2018:**69**(13):3211–3222. https://doi.org/10.1093/jxb/erx489

Zhou L, Vejlupkova Z, Warman C, Fowler JE. A maize male gametophyte-specific gene encodes ZmLARP6c1, a potential RNA-binding protein required for competitive pollen tube growth. Front Plant Sci. 2021:**12**: 635244. https://doi.org/10.3389/fpls.2021.635244