OVULE DEFECTS IN *ARABIDOPSIS THALIANA* PIN LOCALIZATION PATHWAY MUTANTS LEAD TO REDUCED SEED SET

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ABSTRACT

Chapter 1:

Auxin is a plant hormone that is essential for a wide range of growth and developmental processes in plants. The coordinated, polar transport of auxin through plant tissues is crucial for proper development and function. The asymmetric localization of PIN-FORMED (PIN) proteins determines the directionality of auxin transport between cells and across a tissue, creating an auxin gradient that can influence gene expression and development. It is well known that auxin gradient establishment is vital for vegetative development, including leaf and root formation, but the involvement of auxin in reproductive development is less-well studied. The plasma-membrane localization of PIN proteins to direct auxin transport between cells is a critical process that involves a complex vesicle transport pathway and is regulated by a diverse set of genes. This review provides an overview of the current state of knowledge regarding the roles of PIN proteins during the development of the reproductive structures of a flower, the gynoecium and androecium, and describes some of the genes that have been proposed to function in this PIN localization pathway. Mainly, the importance of PIN1 localization and PIN1-mediated auxin transport for the development of the gynoecium, including the carpel tissue, ovary, ovules, and female gametophyte, is described in detail, emphasizing the importance of these processes during reproductive development in plants.

Chapter 2:

The formation of the gynoecium, including the stigma, style, transmitting tract, ovary, ovules, and female gametophytes requires the correct establishment of auxin gradients across a tissue directed by polar transport. PIN-FORMED (PIN) proteins are crucial for establishing local cellular auxin gradients that establish spatially organized auxin maxima and minima. PIN1 vesicle transport is tightly regulated by various genes that play a role in vesicle formation, transport, uncoating, and membrane localization. *SCARFACE/VASCULAR NETWORK DEFECTIVE 3* (*SFC/VAN3*), *FORKED1* (*FKD1*) & *FORKED-LIKE* (*FL*) Group 1 genes, *COTYLEDON VASCULAR PATTERN 2* (*CVP2*) and *CVP2-LIKE 1* (*CVL1*), *PINOID* (*PID*), and *ROTUNDA3* (*RON3*) have all been proposed to be key players in the regulation of PIN1 vesicle trafficking. Mutations to these genes have been shown to disrupt PIN1 localization and lead to developmental defects such as impaired root development and vein formation. However, the role of these genes has not been previously characterized in gynoecium development, despite mutants showing severe reductions to seed set. This study examines the underlying causes of the seed set reductions present in *fkd1/fl1-2/fl2/fl3*, *sfc-40*, and *cvp2/cvl1* mutants by analyzing reproductive development. Through manual pollination experiments, observation of pollen tube growth, and the analysis of ovule defects and *pPIN1:PIN1-GFP* expression during ovule development, I identified that reduced seed set in these mutants results from a gynoecium defect. Specifically, a reduced seed set involves altered ovule development, and I suggest that the observed morphological defects are consistent with alterations to PIN1 localization during early ovule development stages (2 ii-iii) in these mutants. This study suggests, for the first time, that *SFC*, *FKD1*, *FL1*, *FL2*, *FL3*, *CVP2*, and *CVL1* play an important role in reproduction during ovule development.

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LIST OF NOTATION & ABBREVIATIONS

Notation

SFC-40 – wild type gene is capitalized and italicized
sfc-40 – mutant gene is italicized
SFC-40 – protein is capitalized

Abbreviations

35S	Cauliflower Mosaic Virus (CaMV) 35S promoter
ANT	AINTEGUMENTA
ARF	AUXIN RESPONSE FACTOR
ARF	ADP ribosylation factor GTPase
ARF-GAP	ARF-GTPase activating protein
ARF-GEF	ADP-ribosylation factor (ARF) guanine nucleotide exchange factor
At / AT	*Arabidopsis thaliana*
AUX/IAA	Auxin/Indole-3-Acetic Acid
AUX1	AUXIN RESISTANT 1
AUX1/LAX	AUXIN RESISTANT 1/ LIKE AUX1
AuxRE	Auxin response element
BAR	Bin-Amphiphysin-RVS domain
BEL1	BELL1
BIG	BREFELDIN A INHIBITED ARF-GUANINE EXCHANGE FACTOR
CMM	Carpel margin meristem
CVL1	CVP2-LIKE 1
CVP2	COTYLEDON VASCULAR PATTERN 2
DR5	DR5 synthetic AuxRE reporter system
DUF	Domain of unknown function
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FG	Female gametophyte
FKD1	FORKED-1
FL	FORKED-LIKE
flQ	*fkd1/fl1-2/fl2/fl3* quadruple mutant
FS	Floral stage
GA	Golgi apparatus
GAP	GTPase-activating protein
GBF	GOLGI BREFELDIN A RESISTANCE FACTOR
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green Fluorescent Protein
GN	GNOM
GNL1	GNOM-LIKE1
GNL2	GNOM-LIKE2
GTP	Guanosine triphosphate

GTPase	Guanosine triphosphatase
GUS	β-glucuronidase
IAA	Indole-3-acetic acid
INO	INNER NO OUTER
LAX1	LAX PANICLE 1
MMC	Megaspore mother cell
NPA	N-1-naphthylphthalamic acid
NZZ	NOZZLE
PAT	Polar auxin transport
PH	Pleckstin homology domain
PI	Phosphoinositide
PI4,5P2	Phosphatidylinositol 4,5-bisphosphate
PI4P	Phosphatidylinositol-4-phosphate
PID	PINOID
PIN	PIN-FORMED
PL	Pleckstrin-like domain
PM	Plasma membrane
PMI	Pollen Mitosis I
PMII	Pollen Mitosis II
PP2A	Protein Phosphatase 2A
RAB	Ras-related brain GTPase
RABA	Ras-related brain GTPase (RAB) subgroup A
RAN	Ras-related nuclear GTPase
RGTB1	RAB geranylgeranyl transferase beta subunit 1
RON3	ROTUNDA3
ROP	Rho (Rhodopsin) of plant GTPase
SFC *(=VAN3 =FKD2)*	SCARFACE (= VASCULAR NETWORK DEFECTIVE 3 = FORKED 2)
SIN1	SHORT INTEGUMENTS
SPL	SPOROCYTELESS
TAA	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS
TAR2	TRYPTOPHAN AMINOTRANSFERASE RELATED 2
TGN	Trans-Golgi Network
TIR1/AFB	TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX
WT	wild type
YUC	YUCCA

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CHAPTER 1

CHAPTER 2

CHAPTER 1: LITERATURE REVIEW: The Role of Auxin and PIN-FORMED Proteins in *Arabidopsis thaliana* Gynoecium Development

ABSTRACT

Auxin is a plant hormone that is essential for a wide range of growth and developmental processes in plants. The coordinated, polar transport of auxin through plant tissues is crucial for proper development and function. The asymmetric localization of PIN-FORMED (PIN) proteins determines the directionality of auxin transport between cells and across a tissue, creating an auxin gradient that can influence gene expression and development. It is well known that auxin gradient establishment is vital for vegetative development, including leaf and root formation, but the involvement of auxin in reproductive development is less-well studied. The plasma-membrane localization of PIN proteins to direct auxin transport between cells is a critical process that involves a complex vesicle transport pathway and is regulated by a diverse set of genes. This review provides an overview of the current state of knowledge regarding the roles of PIN proteins during the development of the reproductive structures of a flower, the gynoecium and androecium, and describes some of the genes that have been proposed to function in this PIN localization pathway. Mainly, the importance of PIN1 localization and PIN1-mediated auxin transport for the development of the gynoecium, including the carpel tissue, ovary, ovules, and female gametophyte, is described in detail, emphasizing the importance of these processes during reproductive development in plants.

1.0 Auxin Transport & PIN-FORMED (PIN) Proteins

1.1 Overview of Auxin & PIN

Auxins are an abundant class of plant growth regulators that coordinate development across all life stages. The predominant form synthesized in land plants is Indole-3-acetic acid (IAA) (Zhao, 2010). It is well established that auxin is involved in a comprehensive range of plant growth processes including the regulation of root, leaf, flower, fruit, embryo, seed, and vascular tissue development, as well as tropic responses such as gravitropism and phototropism (Zhao, 2010; Teale et al., 2005; Xi et al., 2016). Auxin synthesis occurs predominantly in the shoot apex and leaf primordia regions, primarily from a tryptophan precursor, and is then initially transported primarily basipetally (tip to base) in a directional manner known as polar auxin transport (PAT) (Dettmer & Friml, 2011; Křeček et al., 2009; Pan & Chen, 2015). PAT in

multiple directions through the plant is crucial for establishing local cellular auxin gradients that establish spatially organized auxin maxima and minima that drive developmental processes primarily by influencing the expression of auxin-inducible genes (Zhao, 2010; Teale et al., 2005; Křeček et al., 2009; Pan & Chen, 2015; Ganguly et al., 2012).

Auxin movement into a cell (influx) is driven by both active and passive transport mechanisms. Passive auxin influx operates based on the pH differences in the apoplast space outside of the plasma membrane (pH ~5.5) and inside the cell cytoplasm (pH ~7). In the apoplast, auxin exists in its uncharged, protonated state (IAAH) which can move into the cell across the plasma membrane via diffusion. Once auxin enters the cytoplasm (pH ~7), it is deprotonated and converted to an ionized form (IAA^-) and consequently can no longer passively exit across the plasma membrane. Together with passive influx, active auxin import also occurs, and is mediated by membrane transport proteins, mainly of the *AUXIN1/LIKE-AUX1* (*AUX/LAX*) gene family (Swarup & Péret, 2012; Buchanan et al., 2015).

Auxin efflux and intracellular auxin transport are actively facilitated by the localization of the eight-membered *PIN-FORMED* (*PIN*) gene family that is found exclusively in land plants (Superdivision: Embryophyta) (Křeček et al., 2009). The diversification of PIN proteins is believed to have contributed to the diversity of land plant structure, function, and development (Viaene et al., 2013). PIN proteins are transmembrane proteins that are dynamically localized to specific membrane faces of a plant cell to consequently direct the transport of auxin within and between cells (Dettmer & Friml, 2011; Pan & Chen, 2015). PIN proteins were named after the "pin-like" floral phenotype of the first loss-of-function mutant examined in *Arabidopsis thaliana* (*AtPINFORMED1*). *pin1* mutants display organ initiation defects at the shoot apical meristem resulting in naked inflorescences with a "pin-like" structure (Okada et al., 1991). PIN proteins

ultimately play a key role in the regulation of developmental events including embryogenesis, morphogenesis, organogenesis, growth responses, and responses to environmental stimuli, mainly owing to their influence on auxin transport (Křeček et al., 2009; Pan & Chen, 2015; Aloni et al., 2006; Larsson et al., 2013; Sundberg & Ostergaard, 2009).

The overall structure of all PIN proteins includes a central hydrophilic loop connected on either side to five hydrophobic transmembrane helices (Ganguly et al., 2012; Křeček et al., 2009). The eight PIN proteins are classified into two main groups based on the length of this central hydrophilic loop; “long-looped” or canonical PINs (PIN1, PIN2, PIN3, PIN4, PIN6, PIN7) and “short-looped” or non-canonical PINs (PIN5 & PIN8). The non-canonical PIN proteins lack phosphorylation sites and are localized mainly to the endoplasmic reticulum (ER), where they mediate auxin compartmentalization and homeostasis within the cell (Mravec et al., 2009; Ganguly et al., 2012; Seifu et al., 2024). In contrast, the general role of all canonical PIN proteins involves auxin efflux from the cell, resulting from localization to the plasma membrane (PM) (Ganguly et al., 2012; Křeček et al., 2009). Canonical PINs mainly differ from one another by the sequence of the hydrophilic loop which contains many conserved phosphorylation motifs targeted by kinases, likely driving the trafficking of these canonical proteins to the membrane (Ganguly et al., 2012). The canonical PIN protein structure has recently been suggested to be ancestral as it is present in all land plants including the liverworts (Bennett, 2015).

Following synthesis in the endoplasmic reticulum (ER), most canonical PIN proteins are initially secreted from the trans-Golgi (TG) to the plasma membrane in a non-polar fashion (equally to all cell faces) through vesicular transport (see *Section 2.2*) (Ganguly et al., 2012). Subsequently, phosphorylation of the central loop of PIN proteins by a serine/threonine kinase (i.e. AGC VIII group kinases including PINOID (PID)) results in apical localization, whereas

dephosphorylation by protein phosphatases such as protein phosphatase 2A (PP2A) promotes basal localization (Michniewicz et al., 2007; Pan & Chen, 2015; Saini et al., 2017). Additionally, PIN proteins also undergo continuous "PIN-cycling", or cycles of endocytosis and exocytosis driven by feedback from auxin signalling, indicating that positive and negative regulation by auxin also affects PIN localization along with phosphorylation and dephosphorylation processes (Křeček et al., 2009; Michniewicz et al., 2007; Ganguly et al., 2012). Through these dynamic and highly regulated synthesis, transport, and phosphorylation/de-phosphorylation processes, the localization of PIN proteins in a plant cell is tightly controlled and continually remodelled to direct auxin transport during various development processes.

1.2 Auxin Regulates Transcription

Auxin transport and gradient establishment by PIN proteins are critical for various downstream processes that drive gene expression. The critical role of auxin in a comprehensive range of cellular processes and developmental pathways is largely explained by the effects that auxin has on gene expression. Inside a cell, auxin perception occurs through the binding of auxin to *TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN* (*TIR1/AFB*) auxin receptors. The main protein families involved in auxin signalling include the F-box auxin receptors *TIR1/AFB*, the transcriptional repressors *Auxin/Indole-3-Acetic Acid* (*AUX/IAA*), and the *AUXIN RESPONSE FACTOR* (*ARF*) transcription factors (Lavy & Estelle, 2016; Buchanan et al., 2015).

The presence or absence of auxin in the cell results in a transcriptional switch that can activate or repress gene expression pathways **(Figure 1).** ARFs bind to Auxin Response Elements (AuxREs) in the promoter regions of auxin-responsive genes. When cellular auxin concentrations are low (**Fig.1a**) Aux/IAA form a heterodimer complex with ARFs causing the

transcriptional repression of the auxin-inducible gene. Aux/IAAs lack a DNA-binding region and therefore must bind to an ARF to repress gene expression (Lavy & Estelle, 2016; Buchanan et al., 2015; Dharmasiri & Estelle, 2002; Tiwari et al., 2003). Auxin binds to TIR1/AFB proteins and causes an interaction between TIR1 and AUX/IAA that promotes ubiquitination, marking AUX/IAA for degradation (Kepinski & Leyser, 2002; Lavy & Estelle, 2016; Dharmasiri & Estelle, 2002). Therefore, in the presence of high auxin concentrations, the AUX/IAA repressor is ubiquitinated and subsequently degraded by the 26*S* proteasome. This allows ARFs to activate the expression of the auxin-inducible gene (Lavy & Estelle, 2016; Buchanan et al., 2015).

It is noteworthy that the twenty-nine identified AUX/IAA gene family members show varying affinities for and interactions with other related co-repressors and the 22 unique ARFs that have been identified in *Arabidopsis* (Li et al., 2016). This has a profound effect on the concentration of cellular auxin required to generate an auxin-induced gene expression response. The different Aux/IAA-ARF interactions also dictate how fast an expression response may occur (Nemhauser, 2018; Luo et al., 2018). Ultimately, this helps to explain the wide array of differences that have been characterized in the amount, timing, and tissue-specific requirements of auxin in various auxin-dependent developmental and growth processes.

2.0 PIN1-Localization Pathway Genes

2.1 PIN1 in Developmental Processes

PIN1 is the founding member of the *PIN* gene family and is known to play a key role in auxin-dependent processes in several organs. In the root, PIN1 is required for gravitropism and curvature responses (Xi et al., 2016) and in the root apical meristem for primary root growth and lateral root branching (Fernandez-Marcos et al., 2011; Li et al., 2015). *PIN1* function is also well studied in the shoot apical meristem during leaf initiation (Vernoux et al., 2000; Banasiak et al.,

2019) and in the leaf vasculature for auxin canalization in midvein and vascular network establishment (Sawchuk et al., 2013; Hou et al., 2010; Wenzel et al., 2007; Govindaraju et al., 2020; Scarpella et al., 2006). PIN1-mediated PAT is also critical for many reproductive processes, particularly involving the development of female (gynoecium) structures (Nemhauser et al., 2000; Barro-Trastoy et al., 2020; Ceccato et al., 2013). *pin1* mutants produce a sterile pin-like stem in place of flowers or may produce an occasional infertile flower lacking reproductive parts (Okada et al., 1991; Bennett et al., 2015; see *Sections 5.3 & 5.5*).

2.2 Overview of PIN1 Localization Pathway Genes & Apical vs. Basal Localization

The PIN1 localization pathway (summarized in **Figure 2**) involves multiple key genes, including *PINOID* (*PID*), *ROTUNDA3* (*RON3*), the *FORKED-LIKE (FL)* gene family, *SCARFACE/VASCULAR NETWORK DEFECTIVE 3* (*SFC/VAN3*), *COTYLEDON VASCULAR PATTERN 2* (*CVP2*), *CVP2-LIKE 1* (*CVL1*), *ADP Ribosylation Factors* (*ARFs*), *ARF Guanine Exchange Factors* (*ARF-GEFs*), and *ARF-GTPase Activating Proteins* (*ARF-GAPs*). These genes play a role in the localization of PIN1 proteins through vesicle trafficking and membrane localization processes. Consequently, disrupting the function of these genes can lead to developmental defects by altering auxin gradients and the expression of auxin-responsive genes. This has potentially significant consequences for developmental and fertility processes that require proper PIN1 localization and auxin transport.

As previously described, the asymmetric localization of PIN proteins to the apical or basal side of a cell is dependent upon their phosphorylation status (Křeček et al., 2009; Michniewicz et al., 2007; Ganguly et al., 2012). The phosphorylation status of PIN is controlled by the protein kinase PINOID (PID) and protein phosphatase 2A (PP2A), both members of gene families with known redundancy. PID acts as a positive regulator of apical auxin efflux by acting

as a binary switch for PIN polarity (Karampelias et al., 2016). Increasing PID kinase activity results in the hyperphosphorylation of PIN proteins and an apical shift in PIN localization (Michniewicz et al., 2007; Li et al., 2011). Decreasing PID activity to levels lower than PP2A results in the dephosphorylation of PIN proteins and the targeting of PIN to the basal end of these cells (Michniewicz et al., 2007). *pid* mutants show a similar phenotype to *pin1* mutants, with a sterile pin-like inflorescence, as basal localization prevents auxin from being transported into the shoot meristem (Roeder & Yanofsky, 2006). The *ROTUNDA3* (*RON3*) gene is known to control PP2A phosphatase activity. PP2A functions in auxin-associated processes in all plant organs by regulating the recycling of PIN1 and PIN2 (Karampelias et al., 2016). *ron3-2* mutants show decreased PP2A activity and consequently PIN hyperphosphorylation which mimics the effects of PID overexpression, causing an apical PIN localization shift (Karampelias et al., 2016; Michniewicz et al., 2007). Together with the establishment of an apical or basal trajectory of PIN localization by PID and PP2A-influenced phosphorylation status, vesicular transport is also critical for polar PIN localization and involves various complex components to ensure tight regulation.

2.3 Vesicle Trafficking (ARF-GTPases; ARF-GEFs, ARF-GAPs, and RABs)

2.3.1 Vesicle Trafficking Pathways & Endomembrane System

Vesicle trafficking is a critical process required for the transport of secretory molecules from the endomembrane system (endoplasmic reticulum (ER), Golgi apparatus (GA), endosomes, and vacuoles) to various locations in and beyond the cell, including the PM and extracellular matrix (ECM). Endomembrane system transport is a highly regulated, bi-directional process that requires vesicular protein packaging, transport, recognition, membrane tethering, and ultimately fusion with the recipient membrane (Bonifacio & Glick, 2004; Jürgens, 2004; Bassham et al., 2008).

The bi-directionality of this process involves two main transport pathways: the secretory (anterograde) and endocytic (retrograde) pathways. The secretory pathway transports newly synthesized proteins from their site of synthesis to the PM, ECM, or to a vacuole (Bonifacio & Glick, 2004; Jürgens, 2004; Bassham et al., 2008). In contrast, the endocytic pathway plays an important role in the recycling of cellular proteins, including PM localized proteins, back to the endomembrane system through a series of early and late endosomes for subsequent degradation, modification, and/or re-localization (Jurgens, 2004; Bassham et al., 2008). In the context of membrane transport proteins like PIN1, this retrieval of localized proteins is important for subsequent re-localization of PIN1 to a different position on the PM. This is required to alter the direction of auxin flux and for the dynamic re-establishment of auxin gradients in a tissue during a developmental process (Luschnig & Vert, 2014).

The vesicle trafficking process in all eukaryotes, from the formation of a vesicle at the donor membrane budding to the delivery and fusion with the target membrane, relies on an incredibly complex network of molecular regulation (Singh & Jurgens, 2018; Nielson, 2020; Saito & Ueda, 2009). A major conserved regulatory module involved in this process is the GTPase molecular switch (Nielson, 2020). A particular group of regulatory proteins that utilize this mechanism are small GTPases that share a common mechanism of action involving cycling between GDP- (inactive) and GTP-bound (active) states (Asaoka et al., 2012; Saito & Ueda, 2009; Nielson, 2020). Once activated, these small GTPases can bind to effector proteins which can regulate various cell processes.

In plants, there are four main groups of small GTPases with names derived from the organism in which they were first discovered: Ras-related nuclear (RAN) GTPases, Ras-related brain (RAB) GTPases, ADP-ribosylation factor (ARF) GTPases, and Rho (Rhodopsin) of plant

(ROP) GTPases. The activation/inactivation cycle of these small GTPases is controlled by guanine nucleotide exchange factors (GEFs), which activate small-GTPases by exchanging GDP for GTP, and GTPase-activating proteins (GAPs) that inactivate small-GTPases by hydrolysing GTP to GDP. In plant vesicle trafficking, ARFs and RABs are recognized as being particularly important small GTPases (Nielson, 2020). It is suggested that PIN protein transport from the TGN to the PM requires both ARF and RAB family members and their regulators (GAFs and GEFs) (Rojek et al. 2020; Feraru et al., 2012).

2.3.2 ADP Ribosylation Factors (ARFs)

As described previously in *Section 1.1*, the polar localization of PIN proteins to the PM is critical for auxin transport which is required for various developmental processes. Vesicle packaging and trafficking involves cycles of phosphorylation and dephosphorylation controlled by the activity of small GTPases called ADP ribosylation factors (ARFs) (Summarized in **Figure 3**). ARFs are guanine nucleotide-binding proteins (G-proteins) that cyclically bind to GTP and GDP, essentially functioning as molecular switches (Cevher-Keskin, 2020; Bonifacio & Glick, 2004). ARFs bound to GTP form an active ARF-GTP complex responsible for the formation of transport vesicles. Conversion to the inactive ARF-GDP is necessary for vesicle uncoating which is required for vesicle fusion to a target membrane and cargo sorting (Donaldson & Jackson, 2000; Bonifacino & Jackson, 2003; Cevher-Keskin, 2020). The interconversion of ARF-GTP/ARF-GDP is mainly regulated by two classes of proteins; ARF-GEFs (ARF-guanine nucleotide exchange factors), and ARF-GAPs (ARF-GTPase activating proteins) (Sieburth et al., 2006; Cevher-Keskin, 2020). ARF-GEFs activate ARF-GTPases by exchanging GDP for GTP, promoting the recruitment of various coat proteins required for vesicle formation and protein trafficking. ARF-GAPs inactivate ARF-GTPases by hydrolysing GTP to GDP and causing

dissociation of coat proteins to promote vesicle uncoating and docking and are therefore important for regulating the timing and localization of vesicle trafficking (Sieburth et al., 2006; Luschnig & Vert, 2014; Bonifacio & Glick, 2004). Consequently, ARF-GEFs and ARF-GAPs play a key role in the initial and concluding stages of vesicle trafficking. Most of the current knowledge of protein sorting to the PM in plants has been elucidated from studies involving PIN protein trafficking (Luschnig & Vert, 2014)

2.3.3 ARF-GEFs (ARF-Guanine nucleotide Exchange Factors): GBFs & BIGs

ARF-GEFs promote the exchange of GDP for GTP during vesicle formation (Bonifacino & Jackson, 2003; Sieburth et al., 2006). In *Arabidopsis*, there are two subfamilies of ARF-GEF proteins (Richter et al., 2007); the GBF (GOLGI BREFELDIN A RESISTANCE FACTOR) and BIG (BREFELDIN A INHIBITED ARF GUANINE EXCHANGE FACTOR) subfamilies. The GBF subfamily (containing GNOM (GN), GNOM-LIKE1 (GNL1), and GNOM-LIKE2 (GNL2)) plays a key role in the PIN1 recycling pathway, from the endosome to the basal PM (Teh & Moore, 2007; Richter et al., 2009) *gnom* mutants have been well-characterized and display apical-basal axis defects in developing embryos and seedlings due to improper PIN1 endocytic recycling and subsequent auxin transport (Steinmann et al. 1999; Mayer et al., 1991). Various *gnom* alleles display a range of PIN-auxin-related phenotypes including those related to polarity establishment in the embryo (complete loss of the root, aberrant or eliminated cotyledons, discontinuous vascular tissue development) (Mayer et al., 1991), as well as reduced lateral root formation and root gravitropism defects (Okumura et al., 2013). GN is required for endocytic recycling of PIN1 from the TGN or endosomes to the basal PM, and in *gnom* mutants PIN proteins therefore remain in the endosome which inhibits PAT (Richter et al., 2014; Geldner et al., 2003; Naramoto et al., 2010). Overall, GN has been established as essential for PIN protein

recycling and auxin gradient establishment (Adamowski & Friml, 2015) and highlights the importance of vesicle trafficking regulation to PIN and auxin-dependent development processes.

The second ARF-GEF subfamily, the BIG subfamily (containing BIG1, BIG2, BIG3, BIG4, and BIG5), is required in the secretory pathway from the TGN to the PM and vacuole (Richter et al., 2014; Xue et al., 2019). BIGs are suggested to act redundantly in vesicle trafficking and to play a key role in PIN protein localization to the PM (Suo et al., 2021; Richter et al., 2014; Xue et al., 2019; Suo et al., 2021). Various *big* mutants show root development defects including reduced primary root length (Suo et al., 2021) and gravitropic defects (Xue et al., 2019). Overall, ARF-GEFs have been established to play a key role in vesicle trafficking processes involving PIN1, with various mutants displaying improper PIN localization, altered auxin transport, and subsequent developmental defects.

2.3.4 ARF-GAPs (ARF-GTPase Activating Proteins): SFC

In addition to ARF-GEFs, vesicle trafficking also involves ARF-GAPs. ARF-GAPs convert ARF-GTP to ARF-GDP (the inactive form), which is necessary for vesicle uncoating required for fusion to a target membrane and cargo sorting (Stefano et al., 2010; Sieburth et al., 2006). *SCARFACE* (*SFC*) (also known as *FORKED2* (*FKD2*) or *VASCULAR NETWORK DEFECTIVE 3* (*VAN3*)) is involved in vesicle trafficking and unloading. The SFC protein contains an ARF-GAP domain believed to regulate PIN1 vesicle trafficking (Sieburth et al., 2006). The ARF-GAP protein encoded by *SFC* contains four domains: (i) a BIN/ amphiphysin / RVS (BAR) domain, (ii) a pleckstrin homology (PH) domain, (iii) an ARF-GAP (ADP ribosylation factor GTPase activating protein) domain and (iv) two Anykrin repeats that bind actin (Sieburth et al., 2006). The ARF-GAP encoded by SFC negatively regulates ARF-GTPase activity and is likely responsible for the localization of PIN1 to the PM by driving the uncoating

of PIN1-containing vesicles (Sieburth et al., 2006). *sfc* mutants have a reduced root length and show altered auxin transport (Sieburth et al., 2006).

2.3.5 RAB Proteins

In addition to ARFs, RAB proteins are another family of small GTPases required for vesicle trafficking that are also regulated by GEFs and GAPs. In *Arabidopsis*, 57 RAB members classified into eight groups are known, and are each proposed to control specific steps in membrane trafficking (Asaoka et al., 2012). RABs mainly play a role in vesicle docking at the recipient membrane (Saito & Ueda, 2009). RABA family members have been shown to be involved in the transport of PIN proteins from the TGN to the PM (Feraru et al., 2012) The *rgtb1* mutant, for example, shows improper RAB membrane anchoring. In *rgtb1*, PIN1 and PIN3 RAB-dependent vesicle transport is inhibited leading to arrest in ovule development (Rojek et al., 2020).

2.4 Plasma Membrane Localization of Protein-Containing Vesicles

2.4.1 FORKED-LIKE (FL) Gene Family

Once a PIN1-containing vesicle arrives at the PM, regulatory membrane lipids called phosphoinositides (PI) are required to localize the vesicle to the membrane (Heilmann, 2016). PIs play a crucial role in auxin distribution by directing membrane trafficking and sorting of transport proteins, specifically at the apical and basal domains of the PM where PIs are differentially enriched (Tejos et al., 2014). PIN1 vesicle transport involves members of the *FORKED-LIKE (FL)* gene family, although their role is less well-understood than ARF-GAPs and ARF-GEFs. In *Arabidopsis*, the *FORKED-LIKE (FL)* gene family consists of nine members in three distinct groups; Group 1 (*FKD1, FL1, FL2, FL3*), Group 2 (*FL4* and *FL8*), and Group 3 (*FL5, FL6, FL7*). Multiple copies of *FL* genes are present in vascular plant genomes and are

expanded in angiosperms (Mariyamma et al., 2018). The proteins of the FL family are defined by their Domain of Unknown Function (DUF828) and PH or PH_2 domains that bind inositol phosphates (IP) at specific membranes (Mariyamma et al., 2018; Hou et al. 2010). Group 1 of the *FL* gene family includes *FKD1*, *FL1*, *FL2*, and *FL3*. Group 1 *FL* genes play an important role in the asymmetric localization of PIN1-containing vesicles to the plasma membrane. By controlling auxin flux via PIN1 localization, group 1 *FL* genes are necessary for initiating the auxin response that directs vascular tissue development and the formation of closed vein loops (Steynen and Schultz, 2003; Hou et al., 2010). *fkd1* mutants also have a lack of distal vein meeting and show erratic PIN localization and a lower auxin response suggesting irregular auxin transport (Steynen and Schultz, 2003; Hou et al., 2010). FKD1 contains a pleckstrin-like (PL) domain and was found to strongly localize with SFC, and this complex is believed to interact with a phosphoinositide membrane lipid (PI4P) that is important for vesicle docking at the PM (Mariyamma et al., 2017). FKD2, an allele of *SFC*, is also proposed to act redundantly with FKD1 and either protein's pleckstrin domain is sufficient to localize the FDK1-SFC complex to the TGN (Naramoto et al., 2009). This suggests that FKD1 and SFC (or FKD2) act together in PIN1 exocytic localization involving PI binding at the PM (Mariyamma et al., 2017). Additionally, FKD1 and SFC have been found to co-localize with members of the RABA group of RAB-GTPases, suggesting that FKD1, SFC, and RABA members are all important to the post-Golgi secretory pathway (Mariyamma et al., 2017).

Other Group 1 *FL* Gene Family mutants (*fl1*, *fl2*, and *fl3*) show no obvious characterized phenotype when single genes are mutated. As more group 1 genes are mutated within the *fkd1* background, however, the disconnected vein phenotype becomes more severe (Mariyamma et al., 2018). In group 1 triple and quadruple mutants (*fkd1/fl2/fl3* and *fkd1/fl1-2/fl2/fl3*), PIN1

localization is less polarized, and the leaves fail to form a reticulate vein pattern, indicating that these genes may have a redundant function in establishing PIN1 polarity during leaf vein development (Mariyamma et al., 2018). The *fkd1/fl1-2/fl2/fl3* quadruple mutant (*flQ*) also shows novel phenotypes that are absent in single mutants including root elongation defects and a reduction in gravitropic response. Leaf and root development defects in *fkd1/fl1-2/fl2/fl3* suggest that group 1 *FL* genes may control PIN localization in tissue types other than vascular tissue. *fkd1/fl2/fl3* and *fkd1/fl1-2/fl2/fl3* mutants show cell polarity defects in leaf epidermal cells causing leaf deformities such as undulations and indentations (Mariyamma et al., 2018). Overall, *FL1*, *FL2*, and *FL3* are believed to act redundantly with *FKD1* in the vesicle secretory pathway that drives the asymmetric localization of PIN1 at the membrane through their interaction with PIs. Group 2 and Group 3 members of the *FL* gene family have been less extensively characterized but do not show phenotypic characteristics associated with PIN1 localization defects (Mariyamma et al., 2018). Because Group 1 *FL* gene family mutants show alterations to PIN1 polarization, reproductive processes which rely on PIN1 for proper auxin transport may also be affected, as supported by a lower seed set present in the *fkd1/fl1-2/fl2/fl3* mutant (Reiter & Schultz, unpublished).

2.4.2 COTYLEDON VASCULAR PATTERN 2 (CVP2) and CVP2-LIKE 1 (CVL1).

As previously described, *SFC*, *FKD1*, *FL1*, *FL2*, and *FL3* are proposed to control vesicle transport from the TGN to the PM (Sieburth et al., 2006; Mariyamma et al., 2018), which are then received at the membrane by PIs (Heilmann, 2016). It is suggested that the PH domain of SFC plays a role in docking of the vesicular cargo at the PM following dephosphorylation and the selective binding to PIs in the membrane to localize PIN to the membrane (Koizumi et al., 2005; Sieburth et al., 2006). Two specific membrane phosphoinositides (PI4P and PI4,5P2) play

a key role in asymmetric PIN localization (Tejos et al., 2014). *CVP2* and its closest homolog *CVL1* encode a phosphatase (5PTase) required for the dephosphorylation of the inositol ring of PI4,5P_2 to yield PI4P (Carland & Nelson, 2009). This dephosphorylation process is important for the localization of vesicular cargo (ex. PIN1) to the PM. *CVP2* and *CVL1* act redundantly to regulate the ARF-GAP function of the SFC protein, with CVP2 producing the phosphoinositide (PI4P) that is bound by SFC (Carland & Nelson, 2009). The redundancy of SFC PH and FKD1 PL domains in complex localization indicates that FKD1 also recognizes PI4P (Mariyamma et al., 2017). Therefore, CVP2 and CVL1 play a key role in the binding of SFC's PH domain and FKD1's PL domain to the membrane to localize PIN1. Interestingly, *cvp2/cvl1* double mutants show increased cytosolic expression of FKD1 and SFC suggesting that mutations in *CVP2* and *CVL1* may also alter PIN localization at the PM secondarily by interfering with membrane localization of FKD1 and SFC (Mariyamma et al., 2017; Carland & Nelson, 2009; Naramoto et al., 2009).

2.5 Summary & Importance of PIN Vesicle Trafficking to Plant Development

In summary, the secretory and endocytic vesicular transport pathways are important for the localization and re-distribution of proteins to various faces of the PM. When considering PIN1 proteins, these initial protein localization and re-distribution processes mediated through vesicular transport are critical for the dynamic establishment of auxin gradients during the developmental process. Vesicle transport is tightly regulated by various classes of small GTPases, including ADP ribosylation factors (ARFs) and RAB-like GTPases (RABs). ARFs cycle between active (ARF-GTP) and inactive (ARF-GDP) forms to control vesicle formation, uncoating, and fusion with target membranes. The ARF activation cycle is mediated by ARF-GEFs (GTPase guanine nucleotide exchange factors), which promote ARF activation and vesicle

formation, and ARF-GAPs (GTPase activating proteins), which inactivate ARFs for vesicle uncoating. RABs mainly play a role in vesicle tethering and docking at the recipient membrane. Various ARF-GEFs, ARF-GAPs, and RABs have been well studied in the context of PIN proteins and are required for proper PIN localization to ensure that auxin-related developmental processes proceed correctly. The role of various ARF-GEFs and ARF-GAPs is particularly well-studied in root development and root growth responses. *SFC* encodes an ARF-GAP and plays a role in the uncoating of PIN1-containing vesicles, thus facilitating PIN1 localization to the PM. *FKD1*, a member of the Group 1 *FORKED-LIKE* (*FL*) gene family, interacts with SFC to regulate the polar localization of PIN1 vesicles. Both FKD1 and SFC interact at the membrane with phosphoinositides (PIs) which is essential for vesicle docking and unloading. By catalyzing PI4P formation, CVP2 and CVL1 enable binding of SFC's PH domain and FKD1's PL domain to the membrane to localize PIN1. Therefore, *SFC*, *FKD1* & *FL* Group 1 genes, *CVP2*, and *CVL1* are important for the regulation of PIN1 vesicle trafficking and mutations in these genes disrupt PIN1 localization and lead to developmental defects such as impaired root development and vein formation, owing to their influence on auxin transport.

3.0 Reproduction in Angiosperms

3.1 Overview of Angiosperm Reproduction

Angiosperm reproduction is a highly regulated and complex process involving various structures and developmental processes. The reproductive structures of angiosperms are contained within the flower (**Figure 4A**). A flower is a modified shoot that produces several organ whorls that are each highly evolved leaves specialized for reproduction. The male reproductive organs, the stamens, are each comprised of an anther in which pollen is produced and a supporting stalk-like filament that structurally supports and nourishes the developing pollen. Collectively, the stamens are referred to as the androecium (**Fig. 4B-D**). The female

reproductive organs are called the carpels (or collectively, the pistil). A carpel consists of an apical stigma connected to a column of specialized connective tissue called the style, at the base of which is an ovary that contains the ovules. Inside each ovule, the embryo sac is the site where an egg will be produced. Collectively, the carpel(s) and these component structures are termed the gynoecium (**Fig. 4D-G**). Although carpel and stamen number and structure vary greatly, the overall structure and core components are highly conserved. Pollination, the delivery of the pollen grains to the carpel, marks the beginning of the male-female interaction that will ultimately lead to fertilization and the development of a seed.

Pollination occurs after mature pollen grains are released from the anthers (a process termed anthesis) and are transferred to the receptive stigma of the carpel. Once mature pollen lands on the stigmatic surface, stigma exudate hydrates the pollen grain, degrading the external exine layer and promoting pollen germination and the division of the tube cell to form the pollen tube (Sundberg & Ostergaard, 2009). The pollen tube enters through the cell wall of stigma tissue and grows along the stylar transmitting tract towards the unfertilized ovules. Mature pollen grains also contain a generative cell which divides to form two sperm either prior to pollen tube germination (as in *Arabidopsis*) or following pollen tube germination. In either case, the sperm (or generative cell) are contained within the growing pollen tube (Borg et al., 2009). Once the pollen tube reaches the ovule, it continues to grow across the ovule and enters through a small opening called the micropyle. At this point, the pollen tube has reached the mature egg cell and subsequently delivers one sperm cell to the egg (to form the zygote) and a second sperm cell to the polar nuclei of the embryo sac to form a nutritive tissue (the endosperm). This process of "double fertilization" is a unique synapomorphy for angiosperms, and the evolutionary significance of this process has been long debated (Li, 1957; Baroux et al., 2002; reviewed in

Friedman, 2001). Double fertilization may have been a process that arose as a mechanism to meet the nutritional requirements of the greatly reduced female gametophyte in angiosperms, as this process enables synchronization of both zygote development and the endosperm formation (Takhtajan, 1991). Given the initial separation of these reproductive stages, it is crucial to consider male and female reproductive development both individually as well as in the context of each other following their interaction.

4.0 The Androecium

4.1 Microsporogenesis & Microgametogenesis

Within the pollen sacs of an anther, the development of pollen grains (and ultimately sperm) occurs in two sequential phases: microsporogenesis and microgametogenesis. Microsporogenesis forms tetrads of haploid microspores, the pollen-grain precursor cells, from diploid sporogenous cells. During *Arabidopsis* microgametogenesis, these microspores divide mitotically to form a two-celled immature microgametophyte during the Pollen Mitosis I (PMI) stage. This bicellular pollen grain consists of one generative cell and one tube cell, and this PMI stage is critical for correct microgametophyte cellular patterning (Borg et al., 2009; Simpson, 2019; Furness et al., 2002). Following the migration of the generative cell to the interior of the immature pollen grain, the generative cell undergoes mitosis to form two sperm cells during the Pollen Mitosis II (PMII) stage. This results in the formation of a three-celled mature microgametophyte or pollen grain (Borg et al., 2009; Simpson, 2019). Developing microgametophyte tissue requires nutrients, hormones, and other signalling molecules from the sporophytic tissue to direct development. It is well-established that both pollen development and maturation require high auxin levels and auxin transport in microgametophyte tissue (Aloni et al., 2006; Salinas-Grenet et al., 2018; Sundberg & Ostergaard, 2009).

4.2 PIN Proteins & Auxin in Pollen Development

PIN3, *PIN4*, *PIN5*, *PIN6*, and *PIN8* are all expressed in the male gametophyte with *PIN4* and *PIN6* only expressed at low levels (Dal Bosco et al., 2012). *PIN8* is the only PIN family member that strongly accumulates in pollen and is highly expressed during the late stages of microgametogenesis from the tricellular pollen stage to dehiscence. *PIN8* is also expressed during pollen germination in the pollen tube (Dal Bosco et al., 2012; Pina et al., 2005; Wang et al. 2008). PIN5 co-localizes with PIN8 to the ER, indirectly affecting intracellular auxin homeostasis regulation (Dal Bosco et al, 2012). *pin8* and *pin5* pollen grains showed misplaced germ cell units, and mitosis defects, or were collapsed and morphologically distinct from WT pollen. Therefore, *PIN5* and *PIN8* have been shown to play a key role in male gametophyte development regulating intracellular auxin homoeostasis to maintain auxin levels which promote pollen development, morphology, and growth (Ding et al., 2012).

In contrast, the role of PIN1 during pollen and androecium development is less clear. PIN1 expression has been detected in *Arabidopsis* anthers and filaments during stages 10-14 of floral development which, together with observations of polar auxin transport, suggests that PIN1 may play a role in establishing auxin transport during stamen development (Feng et al., 2006). PIN1 has also been suggested to play a possible role in stamen formation and early pollen development stages, based on findings about a similar gene (*BnPIN1-like*) in *Brassica napus* (Pérez-Pérez et al., 2023). Additionally, *pid* mutants with defective PIN1 localization sometimes show reductions in stamen number, and high *PIN1* expression levels have been detected at the site of incipient stamens in developing flowers (Cardarelli & Cecchetti, 2014; Reinhardt et al., 2003). Overall, the role of and requirement for PIN1 during stamen development is believed to be less important than other PIN proteins. Additionally, a preliminary screen of PIN1

localization pathway mutants (*fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40*) found that pollen fertility, germination, pollen tube growth, and overall androecium development were occurring normally, suggesting that PIN1 is likely not critical for androecium development and pollen growth (Reiter & Schultz, unpublished).

4.3 PIN Proteins & Auxin in Pollen Tube Growth

It has been well established that high levels of auxin accumulate in pollen, regulating pollen viability, pollen and anther maturation, and anther dehiscence (Cecchetti et al, 2008; Salinas-Grenet et al, 2018). In fact, auxin levels in mature pollen grains have been found to be greater than in developing leaves, the sites of auxin biosynthesis (Dal Bosco et al., 2012). Germinating pollen has also been found to have very high internal auxin levels (Ding et al., 2012). Auxin plays a key role in pollen germination and directs the pollen tube's growth through the transmitting tract and stigmatic tissue of the carpel. Once pollen germinates and begins growth through the carpel, both internal (pollen tube) and external (carpel) auxin are required to direct this process. Pollen tube growth occurs via polarized tip growth, a rapid process that is dependent on polar vesicle transport to deliver cell wall and membrane components to the actively growing tip (Qin & Yang, 2011). As pollen tubes grow, their cytoplasm is restricted to a small area near the actively growing tip and older regions of the pollen tube become blocked off with callose plugs (Buchanan et al., 2015). In the carpel, auxin levels are greatest in the stigma, the site of pollen tube entry to the transmitting tissue and decrease in a gradient in the direction of pollen tube growth towards the base of the carpel. External auxin application stimulates pollen tube growth in *Nicotiana tabacum* and *Torenia fournieri* also suggesting an important role of auxin in the growth of the pollen tube tip and elongation (Chen & Zhao, 2008; Wu et al., 2008). PIN8 is the main PIN protein that has been identified to play a role in pollen germination

and pollen tube growth, with *pin8* mutants showing a decrease in germination and pollen tube length (Gao et al., 2021; Ding et al., 2012), suggesting that ER-localized PIN transporters (PIN8/PIN5) may regulate the release of auxin from the ER to drive pollen tube elongation. Overall, the role of auxin in pollen tube growth has been acknowledged, although PIN8 has been suggested to be the main PIN protein required for pollen development, maturation, and germination processes.

5.0 The Gynoecium

5.1 Components of the Gynoecium

The gynoecium of a flower has three main functions; development of megagametophytes; reception of pollen grains; and facilitating the growth and direction of pollen tubes to the ovules. The components of the *Arabidopsis* gynoecium are illustrated in **Figure 4E-G**. In *Arabidopsis*, two fused carpels comprise the gynoecium and together form the pistil which contains the ovules in the ovary. The megagametophyte, or embryo sac, is the haploid gametophytic tissue that is enclosed within the gynoecium (Hawkins & Liu, 2014). The embryo sac contains the egg cell and endosperm precursors and is formed within the ovule through the processes of megasporogenesis and megagametogenesis. Development of the gynoecium also requires the overall formation of the diploid sporophytic pistil structures, including the transmitting tissue. In a gynoecium comprised of two fused carpels, the apical portion of the pistil consists of the stigma and stigmatic papillae, and the basal portion consists of the ovary which is divided into chambers by a septum (or several septa). The tissue of the transmitting tract develops along the length of the style and into the septum and functions as the route of pollen tube growth towards unfertilized ovules (Drews & Koltunow, 2011; Cresti et al., 1992; Mangla et al., 2022; Lersten, 2008; Di Marzo et al., 2020).

5.2 Arabidopsis Floral & Ovule Development Stages

The authoritative description of floral development stages in *Arabidopsis* was presented by Smyth *et al*. (1990) and expanded on by Schneitz *et al*. (1995) and Christensen *et al.* (1997) to provide descriptions of ovule development and female gametogenesis stages, respectively. These floral and ovule stages, along with detailed descriptions of floral development events at each progressive stage, are summarized in **Table 1** and serve as a reference point for mutant characterization.

5.3 Auxin in Gynoecium Development

Development of the *Arabidopsis* gynoecium (**Table 1**) begins at floral stage 6 when the floral meristem broadens to form a bilaterally flattened plate, followed by central invagination and the initiation of the medial and lateral domains (replum and valve precursors, respectively) (Larsson et al., 2014). During stage 7, two medial ridges called the carpel margin meristems (CMMs) emerge to give rise to the placentae and ovule primordia in stage 8. At stage 9, the morphological specification of most mature ovary tissue occurs, including the transmitting tract tissue, and shortly afterwards both the style and stigmatic papillae begin differentiation. Final cell differentiation and expansion proceed through stages 10 to 12 until the gynoecium is fully mature around stage 13 when the flower is fully open (Larsson et al., 2014; Smyth et al., 1990).

Auxin is important for establishing the apical-basal axis of the gynoecium, which requires high auxin levels in the apical region to promote stigma and style development, intermediate levels in the central region to control ovary and ovule formation, and low levels at the base for gynophore development as shown in **Figure 5** (Nemhauser et al., 2000). PIN1-mediated PAT plays a key role in the formation of the carpel primordia and tissues along the pistil axis, as well as ovule development and megagametogenesis (Ceccato et al., 2013; Hawkins

& Liu, 2014). Application of the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) to developing flowers results in a shift in the auxin gradient within the developing carpel. This results in increased elongation of stigma and style tissue in the apical regions causing a basalized style/ovary boundary and increased elongation of the gynophore. NPA treatment also causes a reduction in ovary production, and decreased production of carpel valves (Nemhauser et al., 2000). Overall, many models which explain gynoecium development highlight the importance of polar auxin transport and PIN proteins in the development and maintenance of the stigma, style, transmitting tract, ovary, and ovules (Larsson et al., 2014; Hawkins & Lieu, 2014; Moubayidin & Ostergaard, 2017; Robert et al., 2015; Cucinotta et al., 2014; Nemhauser et al., 2000). Proper gynoecium development is required for subsequent developmental events and processes that will ultimately give rise to a mature seed.

5.4 Ovule & Embryo Sac Development

Ovules are sporophytic structures contained within the ovary of the gynoecium and are the site of megaspore and embryo sac (or megagametophyte) formation. A structural overview of an ovule is presented in **Figure 4 F-G**. The ontogeny of the ovule has been described in detail by Schneitz *et al*. (1995) with reference to Smyth *et al.'s* (1990) floral developmental stages. Christensen *et al.* (1997) provide descriptions of female gametophyte (FG) stages. These models have been integrated and summarized in **Table 1**. An angiosperm ovule consists of three main components (Baker et al., 1996; Lersten, 2008; Cresti et al., 1992; Mangla et al., 2022; Rojek et al., 2021; Truernit & Haseloff, 2008):

a) The **Nucellus** (= Megasporangium), the tissue where megasporogenesis and megagametogenesis occur representing the boundary between the sporophytic and gametophytic generations; the nucellus begins to degenerate following embryo sac formation and maturation (during ovule stage 3-ii).

b) **Two Integuments**, the protective, outer-most layers of the ovule, will elongate and close around the mature embryo sac. Integuments are the precursors of the seedcoat which differentiates after fertilization. Integuments are the only lateral organs produced by the ovule, and their development closely follows that of other determinate lateral plant organs like the leaves.
c) The **Funiculus**, is the stalk that connects an ovule to the placental tissue of the ovary wall enabling direct transport, via the vascular tissue, of hormones and nutrients between sporophytic and gametophytic tissues.

A concise description of ovule development is presented in **Table 1** (Mangla et al. 2022; Lersten, 2008; Johri, 1984; Maheshwari, 1950; Christensen et al., 1997; Smyth et al. 1990; Schneitz et al. 1995; Christensen et al. 1997). Ovules initially arise within the gynoecium as protrusions of the placental tissue during floral stage 8 (stage 1-i ovules). An early ovule is a very simple structure that consists of only a few cell layers. Placental protrusions begin to elongate during floral stage 9 (stage 1-ii ovules) and patterning of the primordium begins to specify the nucellar, chalazal, and funicular regions. In stage 10 flowers, the initiation of megasporogenesis occurs within the nucellus. Megasporogenesis begins with the enlargement of the megasporocyte in stage 2-i ovules. Shortly afterwards, the inner integument begins to initiate (stage 2-ii ovules) which is quickly followed by the initiation of the outer integument and division of the chalazal nucleus (stage 2-iii ovules). In stage 11 flowers, the enlarged megasporocyte undergoes meiosis to form a tetrad of megaspores and the chalazal pole of the nucellus enlarges (stage 2-iv ovules). Megasporogenesis concludes with the extension of the integuments toward the apex (distal or micropylar pole) of the nucellus (stage 2-v ovules). By the end of megasporogenesis, each immature ovule has four megaspores and partially extended integuments.

Near the end of floral stage 11, megagametogenesis begins which will ultimately lead to the formation of a mature embryo sac. Embryo sac formation most commonly occurs following

the *Polygonum* pattern, which is observed in over 70% of angiosperms, including *Arabidopsis* (Summarized in **Figure 4H**) (Maheshwari, 1950). Megagametogenesis begins with the degeneration of three megaspores and the retention of a single functional megaspore. The outer integument continues to envelop the nucellus and surpass the inner integument as the funiculus and nucellus begin to curve back towards the placental tissue (stage 3-i ovules). During floral stage 12, the functional megaspore undergoes the first mitotic division to form a two-nucleate embryo sac and the outer integument completely extends around the nucellus leaving a small opening where the integuments meet called the micropyle (stage 3-ii ovules). Stage 3-ii marks the initiation of the nucellus degeneration processes, and a large vacuole arises in the embryo sac (stage 3-iii ovules). A second mitotic event follows, producing a four-nucleate embryo sac. The inner integument also grows to surround the nucellus, and the nutritive and protective endothelium tissue layer of the inner integument differentiates (stage 3-iv ovules). The final mitotic division then occurs, forming an eight-nucleate syncytial embryo sac which undergoes cellularization to form a seven-celled, eight-nucleate embryo sac (stage 3-v ovules), consisting of an egg cell and two synergid cells at the micropylar pole, the central cell with two polar nuclei, and three antipodals at the chalazal pole. Megagametogenesis concludes with the fusion of the polar nuclei and degeneration of the antipodal cells. An additional cell layer in the endothelium of the inner integument also initiates (stage 3-vi ovules). Throughout the development of the ovule and embryo sac, the micropylar end of the ovule is successively brought closer to the funiculus, ultimately positioning the micropyle next to the placental tissue for entry of the pollen tube during fertilization. Within an ovule, the embryo sac coordinates the development of the egg cell internally and is ultimately the site where fertilization will occur to form the embryo and nutritive endosperm tissue (Baker et al., 1996; Lersten, 2008; Cresti et al., 1992; Mangla et al.,

2022; Maheshwari, 1950). Because *Arabidopsis* pollen is produced in excess, the number of ovules produced typically limits the maximum seed number per flower (Yuan, 2019). Therefore, ovule initiation, patterning, and maturation play a central role in sexual reproduction and the coordination of female reproductive development with an ovule ultimately functioning as the progenitor of a seed.

5.5 Auxin in Ovule Initiation and Development

Many studies support the importance of auxin transport from the sporophytic tissues of a plant into the tissues directly surrounding the developing ovule. PIN1 localization has been characterized in the newly formed ovule primordia, developing nucellus, and the funiculus. The role of auxin in the developing embryo sac itself, however, remains less clear.

PIN1-mediated PAT has been most well-studied during ovule initiation and nucellus development (Aloni et al., 2006; Larsson et al., 2013; Sundberg & Ostergaard, 2009; Ceccato et al., 2013). Auxin is known to play a key role in the initiation, patterning, and morphogenesis of the ovule (Barro-Trastoy et al., 2020). During the development of the gynoecium, ovule primordia initiation (floral stage 8) occurs asynchronously along the placental tissue at auxin maxima, determining the spatial distribution of ovules along the placenta (Cucinotta et al., 2014; Yu et al., 2022; Hu et al., 2022). Although many *PIN* family members, including *PIN1*, act redundantly with other members in various developmental processes (Vieten et al., 2005; Paponov et al., 2005), this does not seem to be the case in the gynoecium and the ovules (Ceccato et al., 2013). *PIN1* is required for the formation of early ovule primordia at floral stage 9 whereas *PIN3* promotes the formation of late-stage ovules and elongation of the pistil (Galbiati et al., 2013; Bencivenga et al., 2012; Benkova et al., 2003; Hu et al., 2022). Experimental evidence supports the importance of PIN1 proteins and auxin transport during ovule initiation.

For example, the application of the auxin transport inhibitor NPA to developing carpels induces ovule loss with ovules reported to be the most sensitive of all gynoecium tissue to altered polar auxin transport (Nemhauser et al., 2000). Furthermore, *pin1* mutants show a high reduction (*pin1-5*) or complete elimination (*pin1-1*) of ovules (Bennett et al., 1995; Okada et al., 1991; Barro-Trastoy et al., 2020; Galbiati et al. 2013). *pin1-5* ovules that do form often arrest early in gametogenesis (FG1 stage) or have embryo sac defects (Bencivenga et al., 2012).

PIN1 expression has been observed in the developing nucellus tissue from ovule stages 1-ii to 3-iii. These stages correspond with ovule primordium elongation, megasporogenesis, and megagametogenesis. Early ovule development is characterized by auxin flux from placental tissue into initiating primordia and developing nucellus, and later development involves auxin flux from the developing ovules back towards the placenta. PIN1-induced auxin transport has been proposed to drive the development of the female gametophyte (megagametogenesis), and nucellus degeneration which begins shortly afterwards (Ceccato et al., 2013; Wang et al., 2021; Yang et al. 1999). PIN1 down-regulation during gametophyte development has been shown to cause the arrest of embryo sac development (Ceccato et al., 2013). Notably, PIN1 is detectable in the funiculus (or its precursor region) throughout ovule development, supporting its dual role in auxin transport into the ovule at early stages and out to the sporophytic tissue later in development (Ceccato et al., 2013; Bencivenga et al., 2012). Following initiation of an ovule primordium (ovule stage 1-i) (**Figure 6A**), PIN1 is first present in the nucellus pole with its polarity towards the primordium tip, directing maternal auxin from the sporophytic tissue towards the apex of the nucellus (the distal pole) (Ceccato et al., 2013; Wang et al., 2021; Rojek et al., 2021). Later in stage 2-ii and 2-iv ovules, PIN1 is present in the inner integument (**6B**) and the funiculus (**6B-D**), corresponding to the areas of auxin response (Barro-Trastoy et al., 2020;

Panoli et al., 2015; Hu et al., 2021; Yu et al., 2020; Rojek et al., 2021). Expression of *PIN1* occurs in the inner integument from stage 2-ii to stage 3-ii but is not clearly observed in the outer integument. As the inner and outer integuments begin to close around the embryo sac in stage 3-ii and stage 3-iii ovules, PIN1 continues to direct some auxin to the apex of the nucellus tissue (**6E**) and localization is later observed in the funiculus directing auxin out of the ovule as the nucellus continues to degenerate in 3-iv ovules (**6F**) (Ceccato et al., 2013). During the final stages of integument closure and degeneration of the nucellus, PIN1 expression persists in the funiculus, playing a key role in auxin canalization and transport out of the mature ovule to the placenta (**6F-G**) (Ceccato et al., 2013; Larsson et al., 2017; Wang et al. 2021). Auxin transport out of the ovule is required for the final stages of megagametogenesis (Ceccato et al. 2012). For example, it has been shown that when vesicle trafficking is disrupted in late-stage ovules of *rgtb1* mutants that lack proper RAB membrane anchoring during vesicle docking, PIN1 (and, to a lesser degree, PIN3) endosome recycling is affected in the funiculus preventing auxin transport out of the ovule (Rojek et al., 2021). Altered PIN1 and PIN3 localization reduces auxin efflux from the nucellus, causing a sustained auxin maximum around the female gametophyte which inhibits embryo sac development and the subsequent degeneration of the nucellus (Rojek et al. 2021) which may lead to embryo sac arrest and abortion of the ovule. These findings highlight the importance of PIN1 in the initial process of auxin movement into an early-stage ovule that initiates female gametophyte development, followed by PIN1 and PIN3 redistribution and subsequent auxin transport out of the late-stage ovule. Transport of auxin out of late-stage ovules is necessary for the continued progression of female gametophyte development and the degeneration of the nucellus. Without correct PIN localization patterns to direct auxin movement

both into and out of a developing ovule, development may be compromised leading to ovule abortion.

Other PIN proteins have also been recognized to be important for ovule development. PIN3 has been observed in the placental tissue (Hu et al. 2021; Larsson et al., 2017), the developing nucellus following the appearance of the ovule primordia, and in the funiculus during late ovule stages (Ceccato et al., 2013). However, *PIN3* is not believed to be critical for proper ovule development, as *pin3-4* plants lack ovule defects, and *PIN3* silenced *pin1-5* plants do not differ significantly from *PIN1* silenced *pin1-5* plants (Ceccato et al., 2013). PIN3, along with PIN4 and PIN7, has been suggested to act redundantly to deliver auxin to late nucellar cells, promoting nucellar degeneration while concurrently controlling the expansion of the female gametophyte's central vacuole. Additionally, *PIN6* is expressed in the funicular vascular bundles and may assist auxin flux in and out of the mature female gametophyte and early embryo during late ovule development (Larsson et al., 2017; Rojek et al., 2021; Wang et al., 2021).

Finally, the role of auxin in the developing embryo sac itself is not entirely clear. It has been suggested that the presence and location of PIN1 in the nucellus may facilitate the transport of auxin into the developing embryo sac (Ceccato et al., 2013; Pagnussat et al. 2009). Pagnussat *et al.* (2009) proposed that an auxin gradient, with a maximum at the micropyle, determines the fate of each embryo sac cell; the gradient is formed in the micropylar region of the nucellus through local auxin synthesis occurring within the embryo sac. Consistent with this proposal, Panoli *et al.* (2015) found that *YUCCA/TAA* auxin biosynthetic genes are expressed asymmetrically (near the micropylar region) in the ovule and embryo sac from stage FG2 to FG6 of embryo sac development, and that the auxin influx carriers *AUX1* and *LAX1* are expressed in the micropylar region and adjacent cells around the embryo sac. Moreover, auxin synthesis

mutants *yuc1* and *yuc2* showed cell specification defects, and *yuc8*, *taa1*, and *tar2* mutants displayed defects in nuclear proliferation and vacuole formation in the embryo sac (Panoli et al., 2015). Other findings suggest that both local auxin synthesis in developing ovules together with transport of auxin into the ovule from the maternal tissues are both required for the development of the female gametophyte (Wang et al., 2021). In contrast, other studies have been unable to detect auxin accumulation or production within the embryo sac during any stage of development (Ceccato et al., 2013; Lituiev et al., 2013). Instead, Ceccato *et al.'s* (2013) findings indicate the presence of an auxin gradient in the surrounding sporophyte tissue (nucellus, integuments, and funiculus) that may affect the development of the embryo sac indirectly through an uncharacterized interaction. The degeneration of the nucellus has been suggested to be initiated by a signal from the female gametophyte, as orderly degeneration begins in nucellus cells immediately adjacent to the female gametophyte (Wang et al., 2021). Furthermore, accumulated auxin in the surrounding nucellus tissue may amplify the cell degeneration signal as the nucellar cells at the micropylar end undergo cell death later than those cells that directly surround the female gametophyte (Wang et al., 2021). Nucellar degeneration is also disrupted when female gametophyte (megasporocyte) formation is impaired, suggesting the importance of the female gametophyte to the nucellus degeneration process (Yang et al. 1999; Rojek et al., 2021). Wang *et al.* (2021) also found that the *PIN* mutant haplotype is transmitted from the female gametophyte to the next generation at the same rate as the WT haplotype, indicating that *PIN1* (and *PIN3*, *PIN4*, and *PIN7*) do not directly influence female gametophyte development but rather affect its development via the maternal genotype. PIN1 expression is also reduced in *sporocyteless/nozzle* (*spl/nzz*) ovule tissues, inhibiting the formation of the megaspore mother cell (MMC) and arresting the embryo sac (Bencivenga et al., 2012; Balasubramanian & Schneitz, 2000).

However, *pin-1-5* mutants show normal MMC development, suggesting that while PIN1 is important for megagametogenesis, it may not be explicitly required for MMC formation (Schneitz et al., 1995; Ceccato et al., 2013). Overall, the proper localization of PIN1, and to a lesser extent other PIN proteins (PIN3, PIN4, PIN7), are crucial for regulating auxin flux that directs ovule development, progression of gametophyte development, and nucellus degeneration, with disruptions in these processes leading to ovule and embryo sac defects.

6.0 Auxin in Embryo Patterning & Seed Formation

The importance of auxin to various aspects of embryo development and seed formation has been strongly established. Auxin accumulation has been suggested to be a prerequisite for reprogramming the development of ovules into seeds (Larsson et al., 2017). Following fertilization, auxin drives the formation of the endosperm, patterning of the embryo, and seed coat development, processes required for fruit initiation and seed development (Figueiredo & Köhler, 2018; Matilla 2020). Once double fertilization occurs, the resulting endosperm becomes the primary site of auxin synthesis. The auxin is then transferred to regions around the ovule, including the integuments, where it directs seed coat formation (Figueiredo et al., 2016). Auxin levels have also been suggested to regulate the transition from cell division to expansion in both the developing embryo and endosperm, playing an important role in establishing seed size (Cao et al., 2020). Post-fertilization auxin production has also been shown to induce fruit formation, and both exogenous auxin and endosperm and seed coat extracts can induce fruit development without fertilization, supporting the importance of the endosperm and seed coat as auxin sources that drive fruit development (Figueiredo & Kohler, 2018).

Auxin also plays a key role in defining the polarity of the embryo through the establishment and maintenance of the apical-basal axis by PIN1, PIN4 and PIN7 (Figueiredo &

Kohler, 2018; Michniewicz et al., 2017; Friml et al., 2003). After the initial asymmetric division of the zygote, sporophytic auxin accumulates in the apical cell of the two-celled embryo, directed by PIN7. Auxin is then equally re-distributed by PIN1 throughout the two apical cells of the four-celled embryo proper. During the early globular stage, PIN1, PIN4, and PIN7 are dynamically reorganized and localized to the basal membrane of the uppermost cells of the embryo in the protoderm (epidermis) precursor region. This causes basipetal transport to the uppermost suspensor cell, the hypophysis, which is important for the organization of the embryonic root meristem (Friml et al., 2003; Michniewicz et al., 2017). As in other processes, PIN1, PIN4, and PIN7 likely act redundantly as single *pin* mutants show many early embryogenesis defects but are typically able to recover and complete embryogenesis (Friml et al., 2003), whereas quadruple mutants (*pin1/pin3/pin4/pin7*) show strong defects to apical-basal axis patterning in the embryo that affect the mature plant (Petrasek & Friml, 2009). Together, the roles of auxin in ovule development and post-fertilization events suggest that auxin is a key component in these processes that ultimately promote fruit and seed production.

7.0 *pin* Mutants vs. PIN Localization Pathway Mutants

Mutations to genes involved in the PIN localization pathway allow for the detailed exploration of various processes involved in PIN localization including vesicle trafficking, membrane localization, PIN cycling, and the establishment of PIN polarity. Studying these processes provides a more complete understanding of PIN protein roles, functions, and cellular transport mechanisms that cannot be determined by examining *pin* mutants alone. Additionally, the redundancy of PIN proteins in many processes makes the characterization of individual *PIN* gene functions more difficult. Particularly in reproductive processes, *pin* mutants present a unique set of challenges because many *pin* mutants typically show altered floral and meristem development. For example, various allelic *pin1* mutants rarely produce floral buds and instead

form a pin-like stem from the shoot. If flowers are formed, however, they typically are infertile due to a loss of stamens and develop a pistil-like structure lacking ovules (Okada et al., 1991; Bennett et al., 2005). Also, when studying *pin* mutants with floral defects, lines must typically be maintained as heterozygotes due to their sterility which increases the complexity of, and time required for, maintaining and examining multiple lines. Mutations that affect PIN localization are also advantageous over *pin* loss of function mutants since altered PIN localization as opposed to a complete elimination of PIN function allows a different set of experimental questions to be addressed, including the importance of directed polar transport of auxin.

8.0 Conclusion

Overall, models for gynoecium, ovule, embryo sac, and embryo development highlight the importance of precise auxin distribution patterns, generated by polar auxin transport and PIN proteins (commonly PIN1) in the development and maintenance of various female reproductive structures including the stigma, style, transmitting tract, ovary, ovules, seed, and fruit. However, the function of *SFC*, *CVP2*, *CVL1*, and *FORKED-LIKE* Group 1 (*FKD1*, *FL1*, *FL2*, *FL3*) genes that are proposed to be involved in PIN1 vesicle trafficking have never been characterized in gynoecium development.

Interestingly, *fkd1/fl1-2/fl2/fl3*, *sfc-40*, and *cvp2/cvl1* mutants all show significantly reduced seed set and a flowering delay compared to wild-type plants. Pollen fertility, pollen tube growth, and overall androecium development appear unaffected in these lines, however (Reiter & Schultz, unpublished). Furthermore, manual cross-pollination experiments resulted in drastically reduced seed sets when mutant carpels were fertilized with wild-type pollen, together suggesting that the reduced fertility of these PIN1 localization pathway mutants is likely due to a gynoecium defect. This is consistent with the previous review that illustrates the many crucial

roles that PIN1 plays in the development of the gynoecium. Ultimately, the preliminary identification of a fertility defect in these PIN1 localization pathway lines sets the stage for the further characterization of this defect and a better understanding of the role of PIN1 localization in the female stages of floral development.

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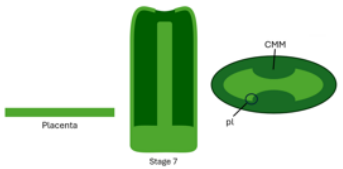
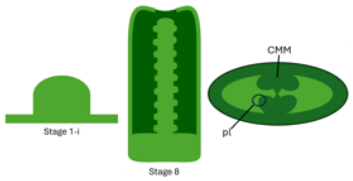
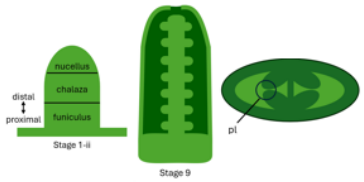
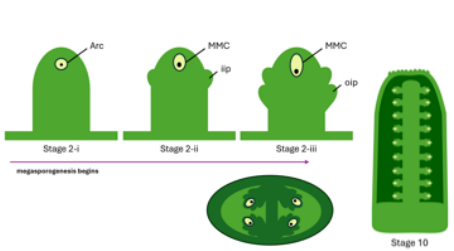
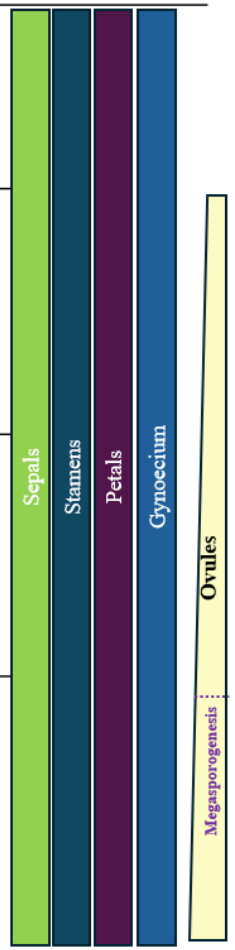
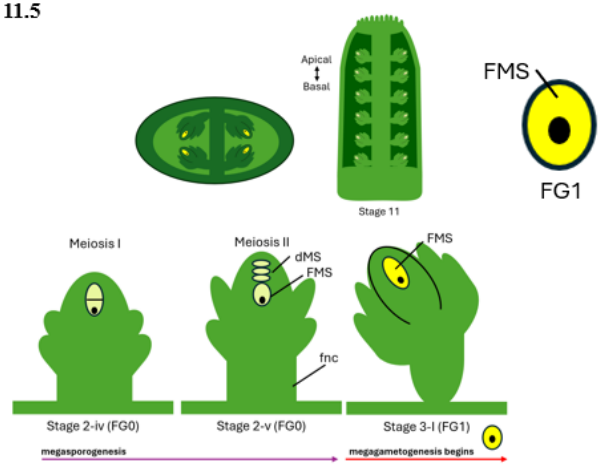
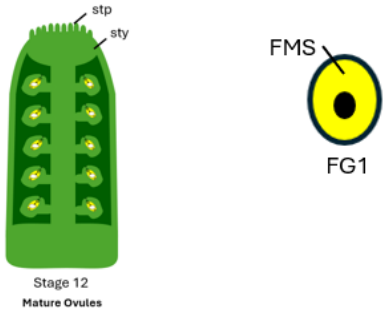
10.0 Tables & Figures

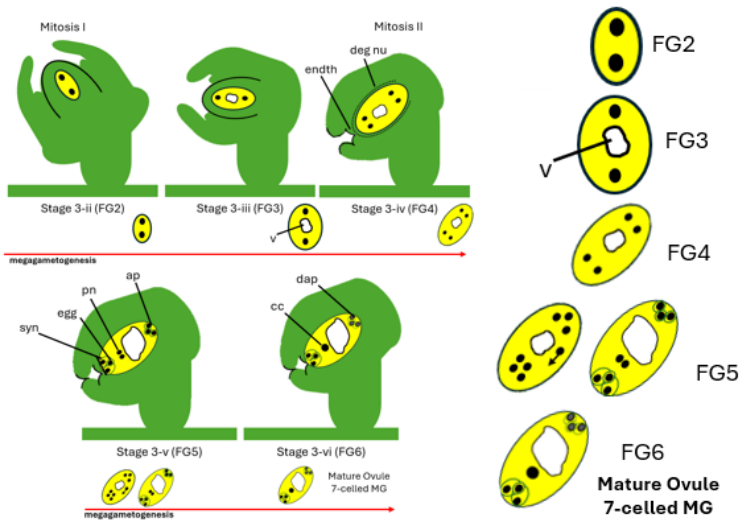
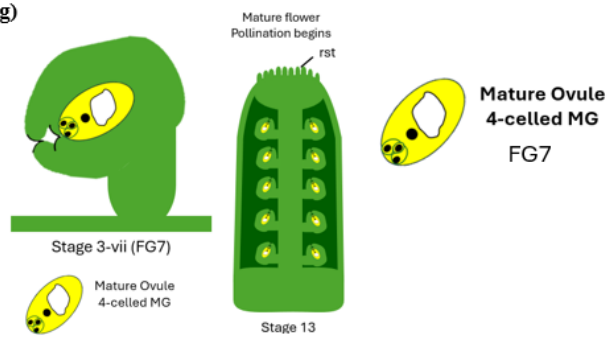
Table 1. Smyth *et al.'s* (1990) authoritative stages of *Arabidopsis* (Ler-0 ecotype) floral development from floral initiation (FS1) to shortly after fertilization (FS15). Reference to key floral hallmarks and approximate stage durations and flower ages (under continuous lighting at 25°C) are provided. Floral stages are presented with reference to Schneitz *et al.'s* (1995) ovule stages and Christensen *et al.'s* (1997) megagametophyte stages. Illustrations modelled from Barro-Trastoy *et al.* (2020) and Ma & Sundaresan (2010).

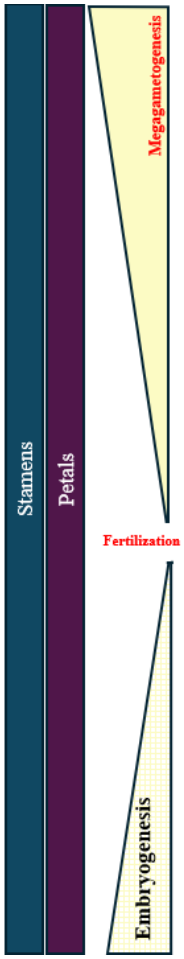
Floral Stage (FS)	Floral Landmark at Beginning of Stage	Description of Developmental Events **Ovule development stages according to Schneitz et al. (1995) and Megagametophyte development stages according to Christensen et al. (1997) indicated in brackets from FS 8 to FS 15.*	Duration of Stage (hrs)	Flower Age at Stage End (days)	Gynoecium & Ovule Development	Female Gametophyte Development	Developmental Timeline
1	Floral buttress appears	*Floral Development*: Flower buttress initiates on flank of the apical meristem and grows laterally from the meristem apex.	24	1	—	—	
2	Floral primordium separates from meristem	*Floral Development*: The primordium lengthens and widens at the point of attachment of the bud.	30	2.25	—	—	
3	Sepal primordia arise	*Floral Development*: Flower primordium is now stalked, and vertical growth begins. Abaxial sepal primordium arises first, followed by the adaxial and the two lateral primordia.	18	3	—	—	
4	Sepals overlie the meristem	*Floral Development*: Pedicel elongates, and the bud diameter increases.	18	3.75	—	—	
5	Petal and stamen primordia appear	*Floral Development*: Four long (medial) stamen primordia appear first, followed by four small petal primordia amongst the sepal bases, ending with two lateral (short) stamen primordia.	6	4	—	—	
6	Sepals enclose the petals and stamens	*Floral Development:* Long (medial) stamen primordia become distinct; Short (lateral) stamen primordia develop later. Petal primordia remain small. *Gynoecium:* Primordium forms a hollow tube; initiation of the medial and lateral domains.	30	5.25	 Stage 6	—	

7	Long (medial) stamen primordia are stalked at the base	*Floral Development*: Stalked bases will form the stamen filament; petal primordia hemispherical (but still small). *Gynoecium*: Gynoecial tube continues to develop; carpel margin meristems (CMMs) emerge.	**24**	**6.25**		—
8	Locules visible on the inner (adaxial) surface of the long (medial) stamens.	*Floral Development*: Majority of the long (medial) stamens have developing anthers; Locules appear in the lateral (short) stamens. Petal growth begins to accelerate. *Gynoecium:* Open cylinder morphology; CMMs form placental protrusions. *Ovules*: Protrusions arise on placentae. (*Stage 1-i*)	**24**	**7.25**		—
9	Petal primordia stalked at the base	*Floral Development:* All organs lengthen rapidly; petals increase in length four- to five-fold; stamens grow rapidly, still mainly in anther region. Sepals elongate with organs to keep bud enclosed. *Gynoecium:* Cylinder becomes constricted at the apex but remains open. *Ovules:* Protrusions elongate; patterning of primordium begins (*Stage 1-ii*)	**60**	**9.75**		—
10	Petals become level with the short (lateral) stamens	*Floral Development*: Stamen growth continues. *Gynoecium:* Cylinder apex is closed; most mature gynoecium tissue types are now morphologically distinct, including the transmitting tract. *Ovules:* **Megasporogenesis begins**. Archesporial cell (Arc) enlarges (*Stage 2-i*). Megaspore mother cell (MMC) enlarges and inner integument primordium (iip) initiates (*Stage 2-ii*); outer integument initiates (oip) and chalazal nucellus prepares to divide (*Stage 2-iii*)	**12**	**10.25**		—

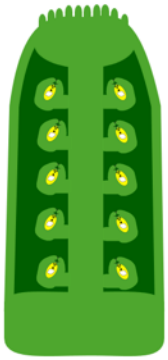


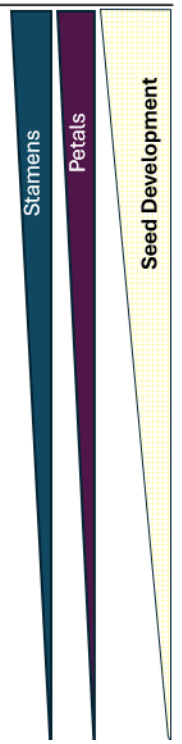
11	Stigmatic papillae appear	*Floral Development:* Filament elongation begins; anthers are green. *Gynoecium:* Immature papillae cells elongate first in the regions not in contact with overlapping sepals, but do not completely cover the stigma. Lateral carpel vasculature appears lighter in color. *Ovule:* MMC undergoes meiosis I (*Stage 2-iv*). MMC undergoes meiosis II to form the tetrad of megaspores; chalazal end of the nucellus enlarges. Integuments extend towards the apex of the nucellus (*Stage 2-v*). **Megagametogenesis begins**. Near the end of stage 11, the megaspore tetrad begins to degenerate leaving the functional megaspore (FM). The outer integument nearly envelops the nucellus and inner integument and the funiculus and nucellus begin to curve (*Stage 3-i* or *FG1*).	**30**	**11.5**	
12	Petals level with long (medial) stamens	*Floral Development*: Petals continue to lengthen rapidly; lateral sepal growth slows; anthers near mature length and turn yellow, filaments rapidly elongate. *Gynoecium:* Gynoecium differentiates to form a distinct short style, separated sharply from the now differentiated stigmatic papillae; carpel valves become increasingly distinct and are prominent by the end of the stage. *Ovule:* FM undergoes first mitosis to form the two-nuclear embryo sac. Outer integument fully surrounds the nucellus, integuments continue to differentially grow. Micropyle end of ovule points >90° from the funiculus, nucellus initiates process of degeneration (*Stage 3-ii* or *FG2*).	**42**	**13.25**	

		Vacuole appears in the embryo sac; micropyle points 90° (perpendicular) to the funiculus (*Stage 3-iii* or *FG3*). Second mitotic event occurs forming four-nucleate embryo sac. Inner integument surrounds the degenerating nucellus, nutritive endothelium differentiates and will ultimately replace the nucellus. Outer integument is nearly closed. Micropyle begins recurving towards the funiculus (*Stage 3-iv* or *FG4*). Final mitotic division occurs to form the eight-nucleate syncytial embryo sac. Cellularization quickly follows marking formation of the mature 8-nucleate, 7-celled embryo sac (*Stage 3-v* or *FG5*). Nucellus has fully degenerated. Immediately prior to fertilization, the polar nuclei fuse and antipodals begin to degenerate. Additional cell layer initiates in the endothelium marking the end of megagametogenesis (*Stage 3-vi* or *FG6*).		
13	Sepals (bud) open; petals beginning to emerge	*Floral Development:* Petals begin to rapidly elongate and become visible; anthesis occurs shortly after and long stamen filaments quickly elongate. Pollination occurs and fertilization process begins *Gynoecium*: stigmatic papillae are long; stigma is receptive; gynoecium reaches maturity. *Ovule:* **Ovules are mature**. Antipodals have degenerated (*Stage 3-viii* or *FG7*). *Double fertilization occurs.*	**6**	**0.5 (post-opening)**	



NOTE: *From FS13 onwards, floral development and ovule development do not remain synchronous. Therefore, Stage 4 ovules are also present in S13 and S14 flowers (Schneitz et al. 1995).*

14	Anthers elevated above the gynoecium; flower fully open; pistil green.	*Floral Development:* Anthers extend above the mature gynoecium and petals are fully open. *Gynoecium:* Gynoecium mature. *Ovule:* Fertilization has occurred. Egg cell vacuole begins to degenerate. Micropyle is positioned next to the funiculus (campylotropous orientation) (*Stage 4-i* or *FG8*). *Embryogenesis:* First nuclear division occurs in the endosperm; Additional cell layer in the endothelium continues growth (*Stage 4-ii*) One endosperm nucleus migrates to the chalazal end (*Stage 4-iii*); Second endosperm nuclear division occurs (*Stage 4-iv*). Third nuclear division occurs; zygote elongates; ovule is now in amphitropous orientation (strongly recurved; horseshoe shaped) (*Stage 4-v*)	**18**	**1 (post-opening)**	 Stage 4-i (FG8)	Post-fertilization  Stage 14  **Mature Ovule** **3-celled MG** Synergid degenerates post-fertilization
15	Stigma overtops the long anthers and sepals	*Floral Development:* Long anthers and sepals surpassed by the stigma. *Gynoecium:* Gynoecium mature. *Embryogenesis:* Further divisions of nuclei occur in the endosperm. Single-celled embryo-proper present (*Stage 4-vi*)	**24**	**2 (post-opening)**	 **Developing Seed**	—



Abbreviations: *FS* = floral stage; *CMM* = carpel margin meristem; *pl* = placenta(e); *Arc* = archesporial cell; *MMC* = megaspore mother cell; *iip* = inner integument primordium; *oip* = outer integument primordium; *dMS* = degenerating megaspore; *FMS* = functional megaspore; *FG* = female gametophyte / female gametophyte stage; *fnc* = funiculus; *endth* = endothelium; *deg nu* = degenerating nucellus; *v* = vacuole; *syn* = synergids; *egg* = egg cell; *pn* = polar nuclei; *ap* = antipodal cells; *cc* = central cell; *dap* = degenerating antipodal cells; *rst* = receptive stigma.

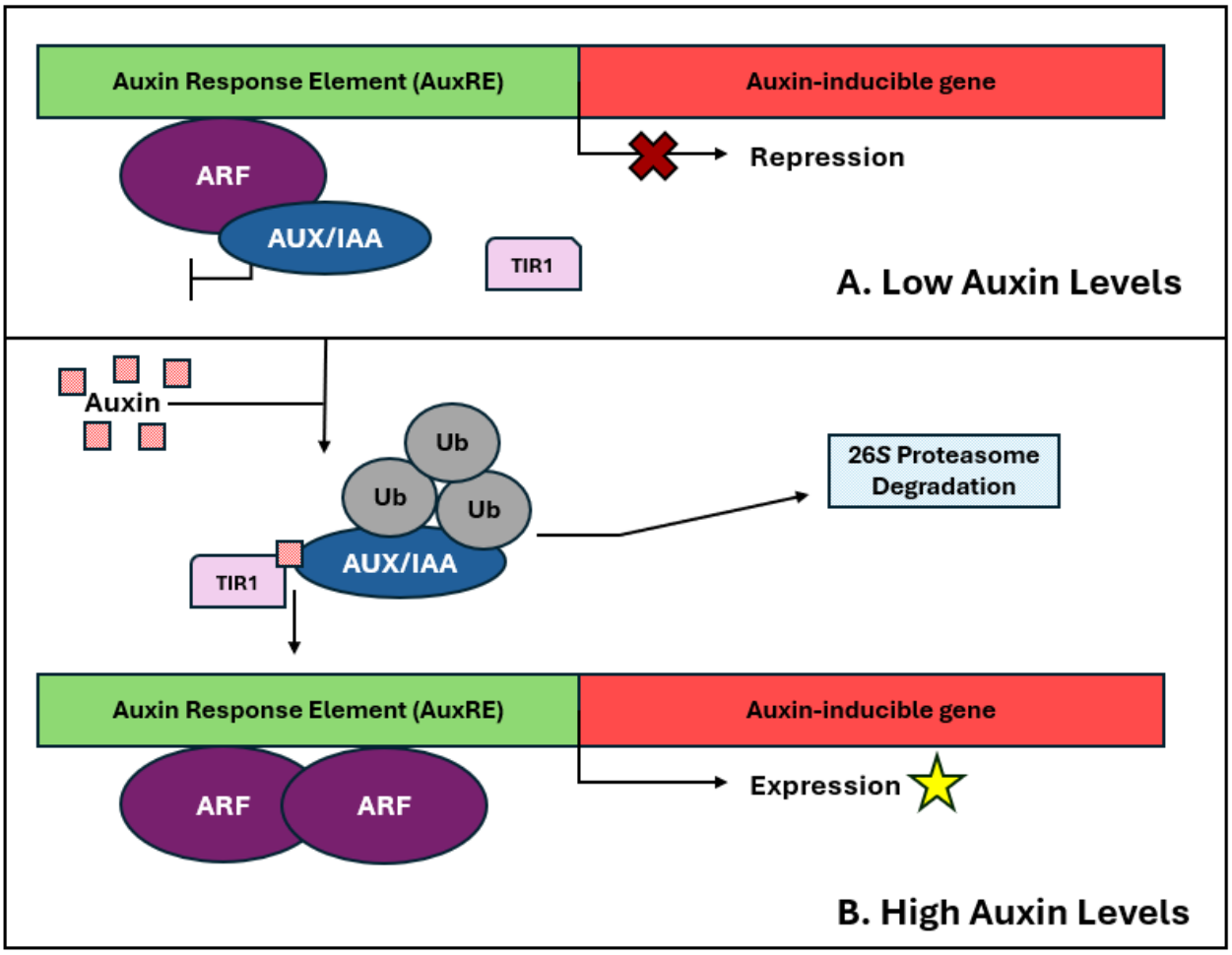


Figure 1. ***Key components of auxin perception, signalling, and gene expression pathways in a plant cell***. In the absence of auxin (**Panel A**), Aux/IAAs form a heterodimer complex with ARFs causing the transcriptional repression of the auxin inducible gene. When auxin levels are high (**Panel B**), auxin molecules bind to a TIR1/AFB auxin receptor complex. This binding enables an interaction between TIR1 and the AUX/IAA repressor promoting ubiquitination and release of AUX/IAA from the AuxRE for degradation in the 26*S* proteosome. The ARFs then form dimers and can activate expression of the auxin-inducible gene.

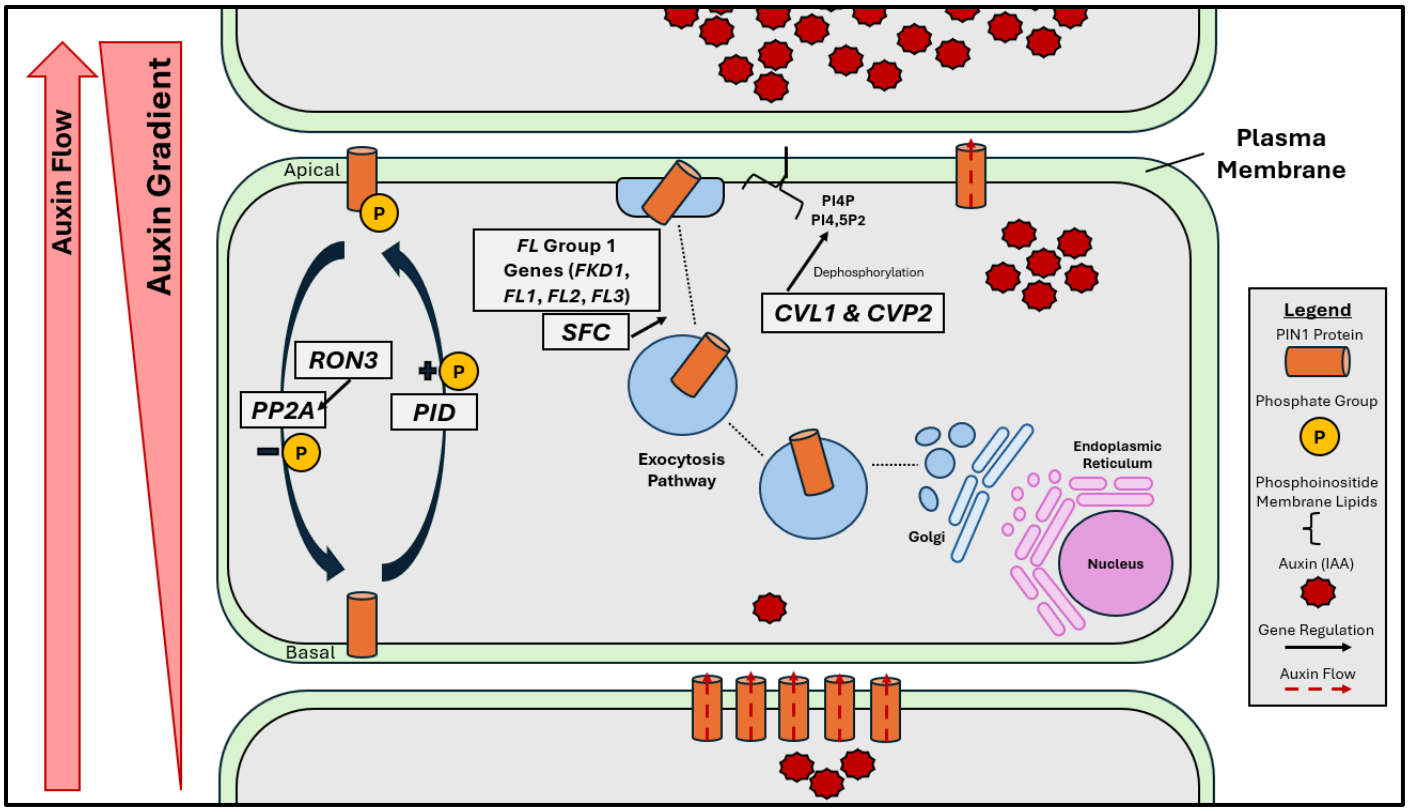
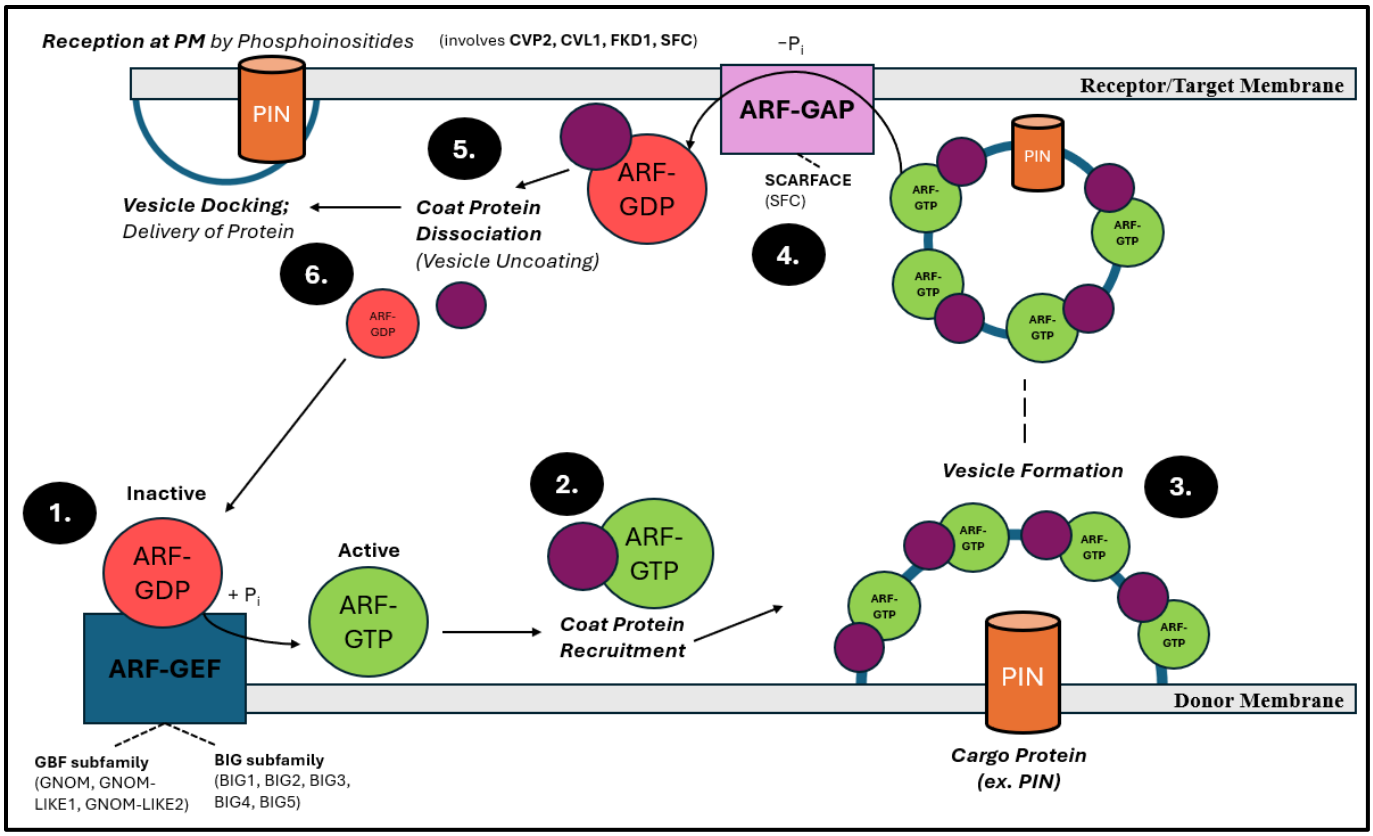


Figure 2. ***Overview of some important genes involved in the PIN1 localization pathway***. PIN1 proteins are synthesized in the endoplasmic reticulum and initially transferred to the Golgi. FL Group 1 genes play a redundant role in PIN1 vesicle localization from the trans-Golgi to the plasma membrane (PM). SFC regulates vesicle trafficking and is required for normal PIN1 transport from the plasma membrane to the endosome. Together, SFC and FKD1 regulate vesicle transport from the TGN to the PM. At the PM, phosphoinositides (PI4P and PI4,5P_2) are dephosphorylated by a phosphatase encoded by CVP2 and CVL1, which is important for the localization of vesicular cargo at the PM. PIN1 proteins are initially secreted to the plasma membrane in a non-polar fashion. Subsequent phosphorylation of the central loop of PIN proteins by PINOID (PID) results in apical localization, whereas dephosphorylation by protein phosphatases such as protein phosphatase 2A (PP2A) promotes basal localization. PP2A phosphatase activity is controlled by ROTUNDA3 (RON3). The asymmetric localization of PIN proteins to the PM directs polar auxin efflux within a cell file/tissue, ultimately promoting the formation of an auxin gradient that influences gene expression to drive various developmental processes.



<u>Figure 3.</u> ***Overview of vesicle trafficking in plants.*** The vesicle trafficking process in plants, from the formation of a vesicle at the donor membrane budding to the delivery and fusion with the target membrane, relies on a complex network of molecular regulation, including by the GTPase molecular switch. (**1.)** At the donor membrane, inactive ARF-GDP interacts with an ARF-GEF protein causing activation and conversion to ARF-GTP. (**2-3.)** This activation promotes recruitment of various coat proteins required for vesicle formation around the membrane cargo protein. (**4.)** At the target membrane, ARF-GTP in the vesicle coat-protein complex interacts with an ARF-GAP in or around membrane. This causes conversion of the ARF-GTP back to the inactive ARF-GDP, which is necessary for **(5)** the promotion of vesicle coat protein dissociation **(6).** After coat protein dissociation, vesicle fusion proceeds at the target membrane integrating the cargo protein. Figure based on Nielsen (2020) and Jürgens Research Group (2021).

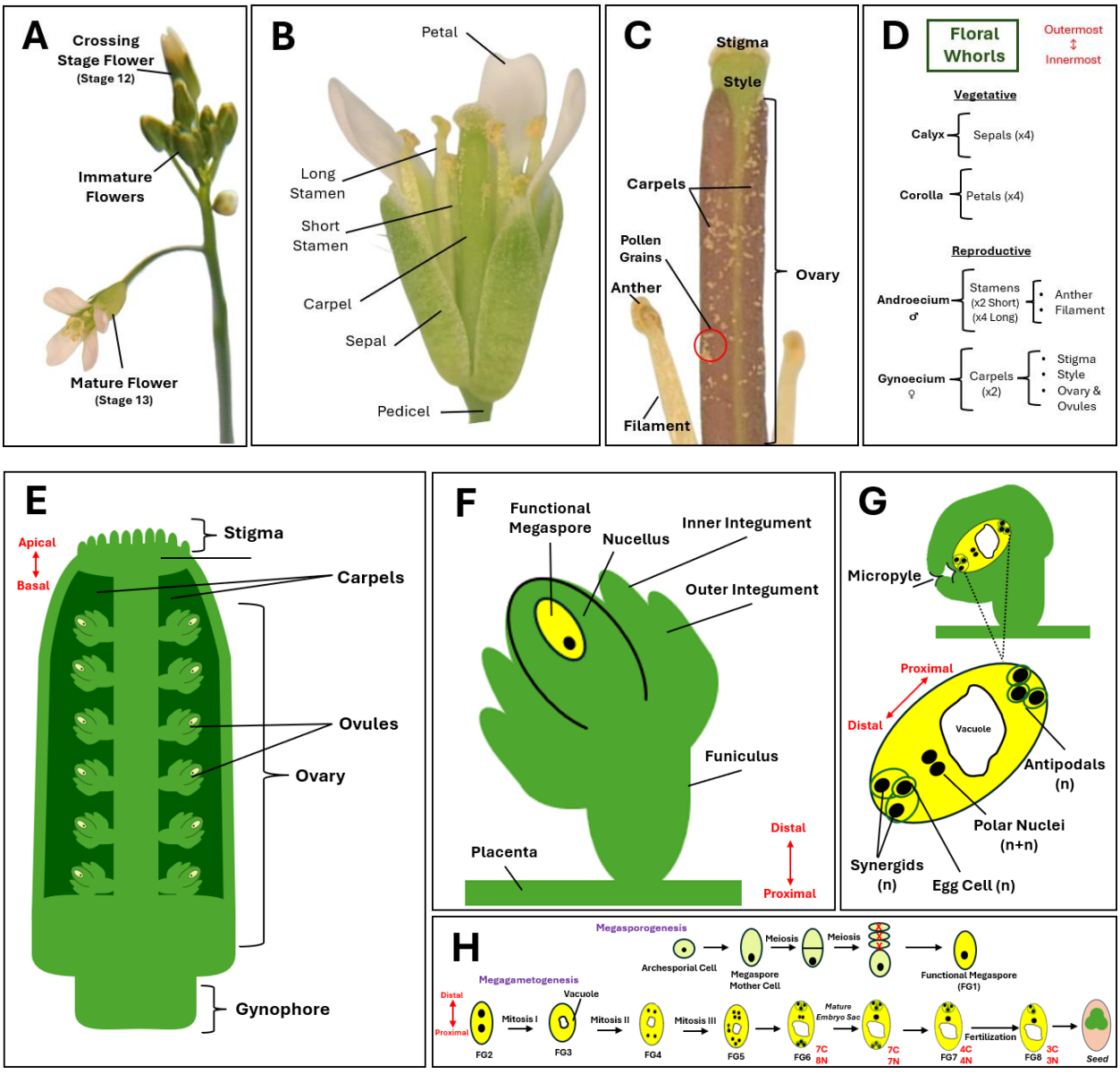


Figure 4. ***Overview of Arabidopsis thaliana anatomy***. (**A**) Mature plant with mature (post-anthesis) and immature terminal flowers. (**B**) Anatomy of a stage 14 flower showing the main floral organs. (**C**) A close-up view of the carpels (stigma, style, and ovary) and the stamens (anther and filament). (**D**) Components of each *Arabidopsis* floral whorl from outside to inside (calyx, corolla, androecium, and gynoecium). (**E**) The gynoecium (female floral whorl) is composed of two fused carpels containing the ovaries and ovules. (**F**) An individual ovule (*Stage 3-i*) connected to the placental tissue by the funiculus. The functional megaspore is surrounded and nourished by the nucellus, which are enveloped by the growing inner and outer integuments. (**G**) A mature ovule (*Stage 3-v*) following formation of the eight-nucleate 7-celled embryo sac containing the synergids, egg cell, central cell, and antipodals. The integuments surround the mature embryo sac and form the seed coat following double fertilization. The pollen tube will enter through the micropyle to fertilize the egg cell and the polar nuclei of the central cell. (**H**) An overview of Megasporogenesis (top) and Megagametogenesis (bottom) as described in detail in the text and Table 1 with reference to Christensen *et al.'s* (1997) stages of female gametogenesis (FG1 to FG8). C = number of cells, N = number of nuclei.

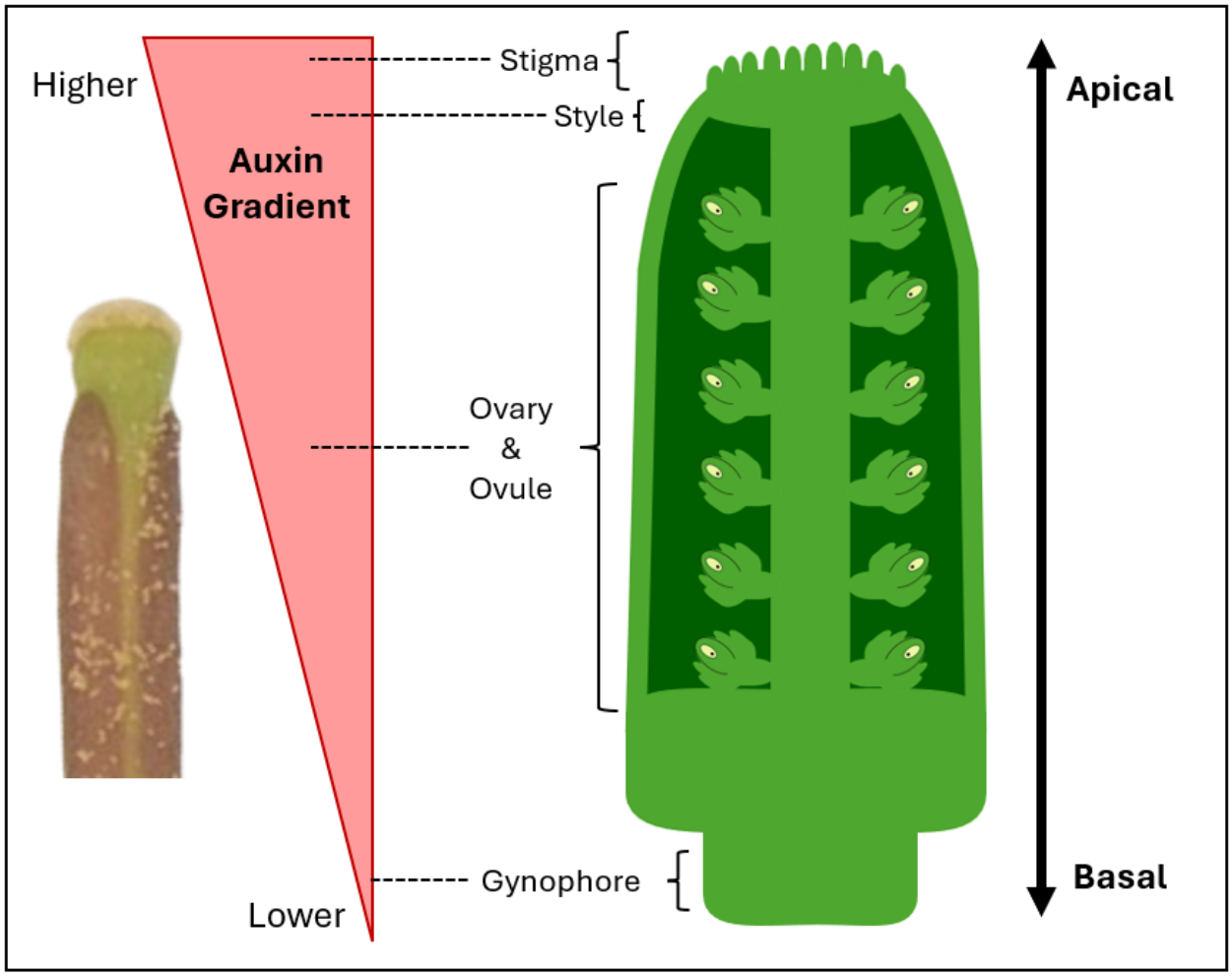
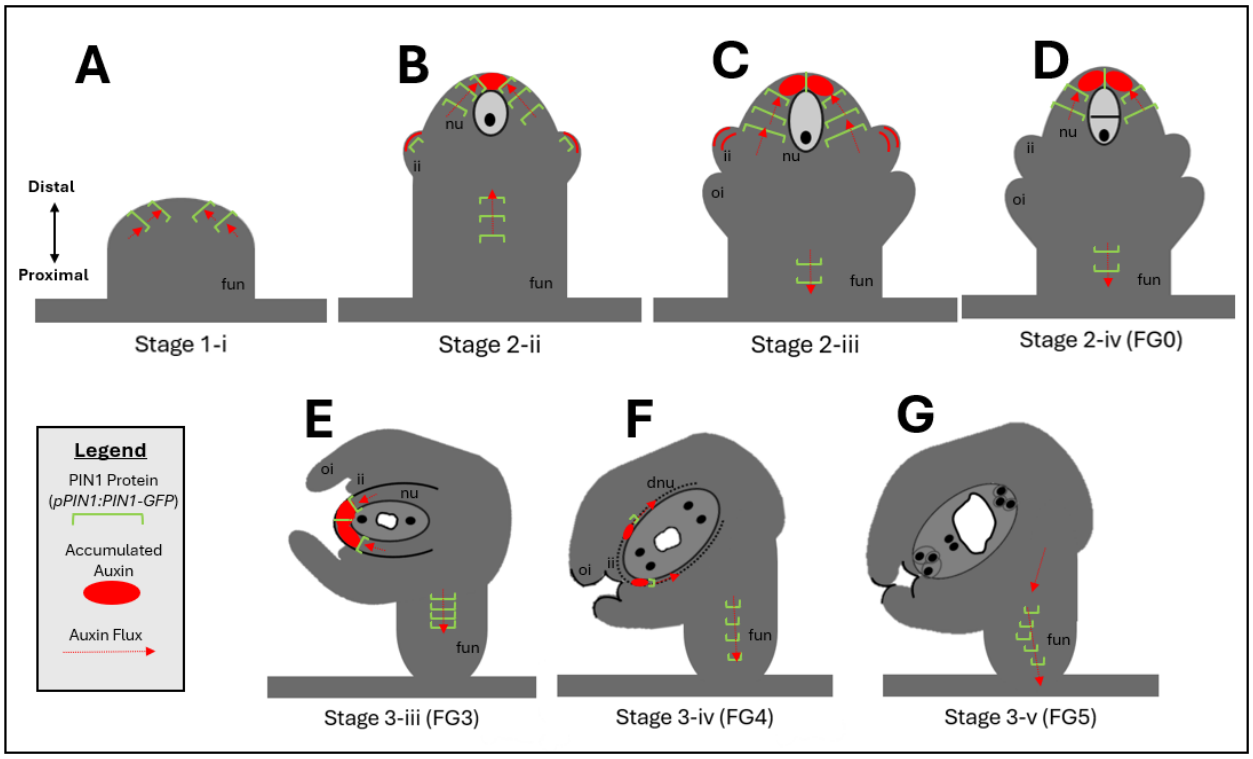


Figure 5. ***The development of the female floral organs requires the formation of a PIN-mediated auxin gradient along the apical-basal axis of the gynoecium***. High auxin levels are present in the apical regions where they promote stigma and style development. Intermediate levels in the central region control ovary and ovule formation. Low levels at the carpel base direct gynophore development. Proper development of the gynoecium is critical for succeeding reproductive development events. Figure modelled from Nemhauser *et al.* (2000).



<u>Figure 6.</u> ***PIN1 localization and auxin transport during ovule patterning and development in Arabidopsis thaliana.*** Initially, PIN1 is localized apically in the nucellus cells (nu) to direct auxin towards the distal tip of the developing ovule (**A**). Auxin begins to accumulate at the distal tip of the ovule and in the inner integuments (ii) (**B-D**) and PIN1 localization is then reversed to begin directing auxin out of the ovule (**C-D**). PIN1 continues to direct some auxin to the apex of the nucellus tissue (**E**) but is later notably observed in the funiculus (fun) directing auxin out of the ovule as the nucellus continues to degenerate (dnu) (**F**). During the final stages of integument closure and degeneration of the nucellus, PIN1 expression persists in the funiculus, playing a key role in auxin canalization and efflux from the mature ovule. Model based on Ceccato *et al.'s* (2013) description of *pPIN1:PIN1-GFP* and *DR5rev:GFP* expression in developing ovules.

CHAPTER 2: Ovule Defects in *Arabidopsis thaliana* PIN Localization Pathway Mutants Lead to Reduced Seed Set

ABSTRACT

The formation of the gynoecium, including the stigma, style, transmitting tract, ovary, ovules, and female gametophytes requires the correct establishment of auxin gradients across a tissue directed by polar transport. PIN-FORMED (PIN) proteins are crucial for establishing local cellular auxin gradients that establish spatially organized auxin maxima and minima. PIN1 vesicle transport is tightly regulated by various genes that play a role in vesicle formation, transport, uncoating, and membrane localization. *SCARFACE/VASCULAR NETWORK DEFECTIVE 3* (*SFC/VAN3*), *FORKED1* (*FKD1*) & *FORKED-LIKE* (*FL*) Group 1 genes, *COTYLEDON VASCULAR PATTERN 2* (*CVP2*) and *CVP2-LIKE 1* (*CVL1*), *PINOID* (*PID*), and *ROTUNDA3* (*RON3*) have all been proposed to be key players in the regulation of PIN1 vesicle trafficking. Mutations to these genes have been shown to disrupt PIN1 localization and lead to developmental defects such as impaired root development and vein formation. However, the role of these genes has not been previously characterized in gynoecium development, despite mutants showing severe reductions to seed set. This study examines the underlying causes of the seed set reductions present in *fkd1/fl1-2/fl2/fl3*, *sfc-40*, and *cvp2/cvl1* mutants by analyzing reproductive development. Through manual pollination experiments, observation of pollen tube growth, and the analysis of ovule defects and *pPIN1:PIN1-GFP* expression during ovule development, I identified that reduced seed set in these mutants results from a gynoecium defect. Specifically, a reduced seed set involves altered ovule development, and I suggest that the observed morphological defects are consistent with alterations to PIN1 localization during early ovule development stages (2 ii-iii) in these mutants. This study suggests, for the first time, that *SFC*, *FKD1*, *FL1*, *FL2*, *FL3*, *CVP2*, and *CVL1* play an important role in reproduction during ovule development.

1.0 Introduction

In flowering plants, the reproductive structures are contained within the flower and the development of the highly reduced gametophytes occurs enclosed within diploid sporophytic tissue. Enclosed within the gynoecium, the megagametophyte, or embryo sac, is the haploid gametophytic tissue that is formed within the ovule through the processes of megasporogenesis and megagametogenesis (Schneitz et al., 1995; Christensen et al., 1997; Hawkins & Liu, 2014). The formation of the gynoecium has been shown to involve the phytohormone auxin (Larsson et al., 2014; Hawkins & Lieu, 2014; Moubayidin & Ostergaard, 2017; Robert et al., 2015; Cucinotta et al., 2014; Nemhauser et al., 2000), which is also critical to a comprehensive range

of plant growth processes including the regulation of root, leaf, flower, fruit, embryo, seed, and vascular tissue development, as well as tropic responses such as gravitropism and phototropism (Zhao, 2010; Teale et al., 2005; Xi et al., 2016).

Polar auxin transport (PAT) is crucial for establishing local cellular auxin gradients that establish spatially organized auxin maxima and minima that drive developmental processes primarily by influencing the expression of auxin-inducible genes (Zhao, 2010; Teale et al., 2005; Křeček et al., 2009; Pan & Chen, 2015; Ganguly et al., 2012). PAT involves the *PIN-FORMED (PIN)* gene family found exclusively in land plants (Superdivision: Embryophyta) (Křeček et al., 2009). Auxin efflux from a cell is actively facilitated by the localization of the "long-looped" or canonical PIN proteins (PIN1, PIN2, PIN3, PIN4, PIN6, PIN7) (Mravec et al., 2009; Ganguly et al., 2012; Seifu et al., 2024). To direct auxin efflux, the precise localization of PIN1 proteins through vesicle trafficking and membrane localization processes is required. Endomembrane system transport is a highly regulated, bi-directional process that requires vesicular protein packaging, transport, recognition, membrane tethering, and ultimately fusion with the recipient membrane (Bonifacio & Glick, 2004; Jürgens, 2004; Bassham et al., 2008).

PIN1 vesicle transport is tightly regulated by various classes of small GTPases, including ADP ribosylation factors (ARFs). ARFs cycle between active (ARF-GTP) and inactive (ARF-GDP) forms to control vesicle formation, uncoating, and fusion with target membranes (Cevher-Keskin, 2020; Bonifacio & Glick, 2004). The ARF activation cycle is mediated by ARF-GEFs (guanine nucleotide exchange factors), which promote ARF activation and vesicle formation, and ARF-GAPs (GTPase activating proteins), which inactivate ARFs for vesicle uncoating (Sieburth et al., 2006; Cevher-Keskin, 2020). Various ARF-GEFs and ARF-GAPs have been well studied in the context of PIN protein trafficking and are required for proper PIN localization

to ensure that auxin-related developmental processes proceed correctly (Luschnig & Vert, 2014). The role of various ARF-GEFs and ARF-GAPs is particularly well-studied in root development and root growth responses (Okumura et al., 2013; Suo et al., 2021; Xue et al., 2019; Sieburth et al., 2006).

Several genes have been identified to play a role in vesicle transport and membrane localization of PIN1. *SCARFACE/VASCULAR NETWORK DEFECTIVE 3* (*SFC/VAN3*) encodes an ARF-GAP that plays a role in the uncoating of PIN1-containing vesicles and facilitates their localization to the PM (Sieburth et al., 2006). *FORKED1* (*FKD1*), a member of the Group 1 *FORKED-LIKE* (*FL*) gene family (including *FKD1*, *FL1*, *FL2*, *FL3*), interacts with SFC to regulate polar localization of PIN1 vesicles through interaction with phosphoinositides (PIs) at the PM which are essential for vesicle docking and unloading (Mariyamma et al., 2018). *COTYLEDON VASCULAR PATTERN 2* (*CVP2*) and *CVP2-LIKE 1* (*CVL1*) encode a phosphatase (5PTase) required for the dephosphorylation of the inositol ring of PI4,5P_2 to PI4P important for the localization of vesicular cargo to the PM (Carland & Nelson, 2009). *CVP2* and *CVL1* act redundantly to regulate the ARF-GAP function of the SFC protein, with CVP2 producing the phosphoinositide (PI4P) that is bound by SFC (Carland & Nelson, 2009). The redundancy of SFC PH and FKD1 PL domains in complex localization indicates that FKD1 also recognizes PI4P (Mariyamma et al., 2017). Consequently, CVP2 and CVL1 play a key role in the binding of SFC's PH domain and FKD1's PL domain to the membrane to localize PIN1 (Carland & Nelson, 2009). Therefore, *SFC*, *FKD1* & *FL* Group 1 genes, *CVP2*, and *CVL1* have all been identified to be key players in the regulation of PIN1 vesicle trafficking. Mutations in these genes have been shown to disrupt PIN1 localization and lead to developmental defects such

as impaired root development and vein formation, owing to their influence on auxin transport (Sieburth et al., 2006; Mariyamma et al., 2018; Steynen and Schultz, 2003; Hou et al., 2010).

Additionally, the phosphorylation status of PIN is controlled by the protein kinase *PINOID* (*PID*) and protein phosphatase 2A (PP2A) whose activity is controlled by *ROTUNDA3* (*RON3*). PID acts as a positive regulator of apical auxin efflux by acting as a binary switch for PIN polarity (Karampelias et al., 2016). Increasing PID kinase activity results in the hyperphosphorylation of PIN proteins and an apical shift in PIN localization (Michniewicz et al., 2007; Li et al., 2011) and *pid* mutants show a similar phenotype to *pin1* mutants, with a sterile pin-like inflorescence, as basal localization prevents auxin from being transported into the shoot meristem (Roeder & Yanofsky, 2006). The *ROTUNDA3* (*RON3*) gene is known to control PP2A phosphatase activity. PP2A functions in auxin-associated processes in all plant organs by regulating the recycling of PIN1 and PIN2 (Karampelias et al., 2016). *ron3-2* mutants show decreased PP2A activity and consequently PIN hyperphosphorylation which mimics the effects of PID overexpression, causing an apical PIN localization shift (Karampelias et al., 2016; Michniewicz et al., 2007). Therefore, the establishment of an apical or basal trajectory of PIN localization by PID and PP2A-influenced phosphorylation status is also important for the regulation of auxin transport during development.

PIN1-mediated PAT is critical for many reproductive processes, particularly involving the development of gynoecium structures (Nemhauser et al., 2000; Barro-Trastoy et al., 2020; Ceccato et al., 2013). *pin1* mutants produce a sterile pin-like stem in place of flowers or may produce an occasional infertile flower lacking reproductive parts (Okada et al., 1991; Bennett et al., 2015). It has been shown that PIN1-mediated PAT plays a key role in the formation of the carpel primordia and tissues along the pistil axis, as well as ovule development and

megagametogenesis, and more generally that auxin is important for establishing the apical-basal axis of the gynoecium (Ceccato et al., 2013; Hawkins & Liu, 2014; Nemhauser et al., 2000). Recently, many studies have explored the role of PIN1 localization and recognized it as critical to ovule development (Cucinotta et al., 2014; Yu et al., 2022; Hu et al., 2022; Ceccato et al., 2013; Panoli et al., 2015).

During the development of the gynoecium, ovule primordia initiation during floral stage 8 occurs asynchronously along the placental tissue at auxin maxima, determining the spatial distribution of ovules along the placenta (Smyth et al., 1990; Cucinotta et al., 2014; Yu et al., 2022; Hu et al., 2022). *PIN1* is required for the formation of early ovule primordia at floral stage 9 and *PIN3* promotes the formation of late-stage ovules and elongation of the pistil (Smyth et al., 1990; Galbiati et al., 2013; Bencivenga et al., 2012; Benkova et al., 2003; Hu et al., 2022). Early ovule development is characterized by auxin flux from placental tissue into initiating primordia and developing nucellus, and later development involves auxin flux from the developing ovules back towards the placenta. PIN1-induced auxin transport has been proposed to drive the development of the female gametophyte (megagametogenesis), and nucellus degeneration which begins shortly afterwards (Ceccato et al., 2013; Wang et al., 2021; Yang et al. 1999). PIN1 down-regulation during gametophyte development has been shown to cause the arrest of embryo sac development (Ceccato et al., 2013). Notably, PIN1 is detectable in the funiculus (or its precursor region) during all ovule development stages, supporting its dual role in auxin transport into the ovule at early stages and out to the sporophytic tissue later in development (Ceccato et al., 2013; Bencivenga et al., 2012).

The importance of PIN1 vesicle trafficking to ovule development has also been shown. *rgtb1* mutants show improper RAB membrane anchoring during vesicle docking, disrupted

vesicle trafficking in late-stage ovules, and inhibited PIN1 (and, to a lesser degree, PIN3) endosome recycling in the funiculus preventing auxin transport out of the ovule (Rojek et al., 2021). Altered PIN1 and PIN3 localization is proposed to reduce auxin efflux from the nucellus, causing a sustained auxin maximum around the female gametophyte which inhibits embryo sac development and the subsequent degeneration of the nucellus (Rojek et al. 2021) which may lead to embryo sac arrest and abortion of the ovule. These findings reiterate the importance of PIN1 localization in the initial process of auxin movement into an early-stage ovule that initiates female gametophyte development, followed by PIN1 and PIN3 redistribution and subsequent auxin transport out of the late-stage ovule.

Overall, models for gynoecium, ovule, and embryo sac development convey the importance of precise auxin distribution patterns, generated by polar auxin transport and PIN proteins (commonly PIN1) in the development and maintenance of various female reproductive structures including the stigma, style, transmitting tract, ovary, and ovules. However, the function of *SFC*, *CVP2*, *CVL1*, and *FORKED-LIKE* Group 1 (*FKD1*, *FL1*, *FL2*, *FL3*) genes that are proposed to be involved in PIN1 vesicle trafficking have never before been characterized in gynoecium development. Interestingly, *fkd1/fl1-2/fl2/fl3*, *sfc-40*, and *cvp2/cvl1* mutants all show significantly reduced seed set and a flowering delay compared to wild-type plants. Pollen fertility, pollen tube growth, and overall androecium development appear unaffected in these lines (Reiter & Schultz, unpublished). Furthermore, manual, reciprocal cross-pollination experiments resulted in drastically reduced seed sets when mutant carpels were fertilized with wild-type pollen, suggesting that the reduced fertility of these PIN1 localization pathway mutants is due to a gynoecium defect. This is consistent with the established roles of PIN1 in the development of the gynoecium. We hypothesize that the seed set reductions in *fkd1/fl1-2/fl2/fl3*,

sfc-40, and *cvp2/cvl1* mutants result from altered PIN1 localization during gynoecium development that ultimately leads to reduced fertility. We predict that these mutants will display disruptions to ovule development that correlate with altered PIN1 localization, leading to the observed reductions to the seed set.

Our study examines the underlying cause of seed set reductions by analyzing gynoecium development in *fkd1/fl1-2/fl2/fl3, sfc-40*, and *cvp2/cvl1* mutants. We characterize the male vs female nature of the reproductive defect through manual pollination experiments and observation of pollen tube growth, along with the analysis of ovule defects and *pPIN1:PIN1-GFP* expression during ovule development. Our findings indicate that the seed set reductions in *fkd1/fl1-2/fl2/fl3*, *sfc-40*, and *cvp2/cvl1* mutants are due to a gynoecium defect involving early stage (2 ii-iii) ovule development and we suggest that these defects are consistent with observed alterations to PIN1 localization during early ovule development in these mutants.

2.0 Methodology

2.1 Plant Material

The Columbia (Col-0) ecotype was used as a wild-type (WT) control in all experiments. PIN1–GFP (green fluorescent protein) seeds were obtained from the Arabidopsis Biological Resource Centre at Ohio State University, USA. *35S::PID* was obtained from Dr. Joanne Chory at The Salk Institute for Biological Studies, USA. *ron3-2* originated from Dr. Mieke Van Lijsebettens at Ghent University, Belgium. *cvp2/cvl1*, *sfc-40*, *pSFC:GUS, pCVP2:GUS,* and *pCVL1:GUS* were provided by Dr. Francine Carland, Yale University, USA. DR5::GUS (Ulmasov et al., 1997) was obtained from Dr. Jane Murfett, University of Missouri. *fkd1* and *fkd2* mutants were generated by Steynen & Schultz (2003). The *fkd1/fl1-2/fl2/fl3 (flQ)* quadruple mutant was previously generated by crossing the *fkd1//fl2/fl3* triple mutant with the *fkd1/fl1-2*

double mutant (Mariyamma et al., 2018). The generation of *pFKD1:GUS* and *pFL1:GUS* is described in Hou *et al*. (2010) and Clarke (2018), respectively.

pPIN1:PIN1-GFP fusion protein was introgressed into the different mutant lines (*fkd1/fl1-2/fl2/fl3, ron3-2*, and *35S::PID*) as previously described (Pageni, 2021). All *pPIN1:PIN1-GFP* lines were screened via confocal microscopy (as described below) to confirm the homozygosity of the transgene. Heterozygous lines were replanted and re-screened until homozygous lines were obtained. Homozygous lines were not obtained for *sfc-40* x *pPIN1:PIN1-GFP* and *cvp2/cvl1* x *pPIN1:PIN1-GFP* in time for confocal analysis.

2.2 Sowing & Growth Conditions

As previously described (Pahari et al., 2014), seeds were grown on either soil or media plates according to the type of analysis. For soil plantings, seeds were surface sown on a pre-moistened 3:1 volume of soilless mix (Professional Gardener, Calgary, AB) to vermiculite (Holiday, Montreal, QB) with nine seeds per 4-inch pot. Seeds were misted with a spray bottle and pots were placed in a 1020 growing tray (SunBlaster Quad Thick) covered with plastic domes to be removed following germination. For plate plantings, seeds were first sterilized using a series of 70% ethanol, 30% bleach solution, and distilled water, and then sowed on *Arabidopsis thaliana* growth medium (AT) plates (Mariyamma et al., 2018). Seeds sown on either soil or media were cold stratified at 4˚C for 3 days to promote germination before being transferred to a 22˚C growth chamber. Plants were grown in growth chambers at 60% humidity under continuous light intensity of ~130 μmol photons m^{-2} s^{-1} from either Sylvania Cool White, Grow Lux and 60 W frosted incandescent bulbs (Osram Sylvania Inc, Danvers, MA, USA) or Byingo T5 LED full-spectrum (10W, 4000K) and Barrina LED red/blue grow lights (24W) (Mariyamma et al., 2018). Plants were bottom watered with RO water when the soil surface began to dry.

2.3 Cross-Pollination Procedure

Crosses were completed for manual cross pollinations in three ♀ x ♂ combinations: i). Mutant ♀ x WT ♂ to assess female fertility of mutant; ii). WT ♀ x Mutant ♂ to assess male fertility of mutant; iii). WT ♀ x WT ♂ and Mutant ♀ x Mutant ♂ as manual self-pollination controls. Mature floral stage 13 flowers (Smyth et al., 1990) from each primary inflorescence were selected for crosses based on observed floral landmarks, including opening sepals, emerging petals, receptive stigmatic papillae, and pre-anthesis stamens. To conduct each pollination, sepals, petals, and anthers were removed from the flower of the female genotype with fine-tipped forceps (Dumont-Inox #4 or #5) sterilized in 70% EtOH leaving the unfertilized, receptive gynoecium exposed. 5-7 mature anthers (visibly shedding pollen) from a floral stage 14 flower of the male genotype were brushed several times across the emasculated stigma with forceps to ensure successful pollen transfer. Pollinated carpels were covered with polyethylene plastic wrap to prevent additional pollination and carpel desiccation. The polyethylene wrap was removed when the enlargement of the carpel was clearly visible in the days following pollination. Siliques for seed counts were collected for clearing (as described in *Silique Clearing, Measurements, and Seed Counts*) after reaching a green-mature stage once elongation had halted and prior to dehiscence and seed dispersal (~1-2 weeks post-fertilization). For each genotype, a minimum of 10 crosses of the three types were performed. Seed number and silique length were measured and compared among the paired reciprocal crosses and the manual and natural self-pollination controls.

2.4 Morphological Characterization of Mutants

2.4.1 Floral Dissections

The gross floral morphology of all mutant lines was qualitatively assessed to determine if any visible defects were present in floral structures including the carpels (stigma, style) and

stamens. A random sample of flowers collected from the primary inflorescence from plants at the same stage of inflorescence development was dissected with fine-tipped forceps (Dumont-Inox #4 or #5). Double-sided sticky tape was used to position the flowers for delicate manipulation and imaging when required. Pistil measurements were made from the gynophore to the stigma. Androecium measurements included lengths of the short and long filaments and the anthers. The gynoecium was dissected using methods previously detailed (Hedhly et al., 2018; Enugutti et al., 2013) by making longitudinal slits in the carpel valves with stainless steel dissecting needles (0.15 to 0.4mm diameter). A scalpel or razor blade was used to remove the stigma, style, and peduncle from the carpels. When required, ovules were removed from the carpel walls also using the stainless-steel dissecting needles. Ovule dissections were performed on slides in a small volume of dH_2O or 67% glycerol to prevent desiccation. Total ovule counts were recorded from floral stage 13 carpels. All quantitative measurements (seeds, pistils, stamens) were completed on images of dissected flowers using NIH ImageJ (Version 1.47v).

2.4.2 Silique Clearing and Measurements

After reaching a green-mature stage, a random sample of both properly and improperly elongated siliques was collected from the primary inflorescence. For seed counts via light microscopy, siliques were cleared in Carnoy's I solution (3:1 mixture of ethanol: glacial acetic acid) for 2 to 3 days before being transferred to 70% ethanol. Siliques were mounted on a microscope slide and photographed through a dissecting scope (Leica) using a white backlight and a Nikon COOLPIX 995 camera. Seed counts and measurements, and silique measurements were completed using NIH ImageJ (Version 1.47v). For silique length, measurements were taken from the gynophore to the tip of the style. Filled siliques were examined within each genotype and scored as improperly elongated according to the phenotypes described in Sun *et al.* (2004) and Lauxmann *et al.* (2016). Seeds were scored as "normal" or "aberrant" based on size, colour,

and morphology; unfertilized ovules were also counted. Discrimination between “normal” or “aberrant” seeds and unfertilized ovules for scoring purposes is detailed in **Figure A**.

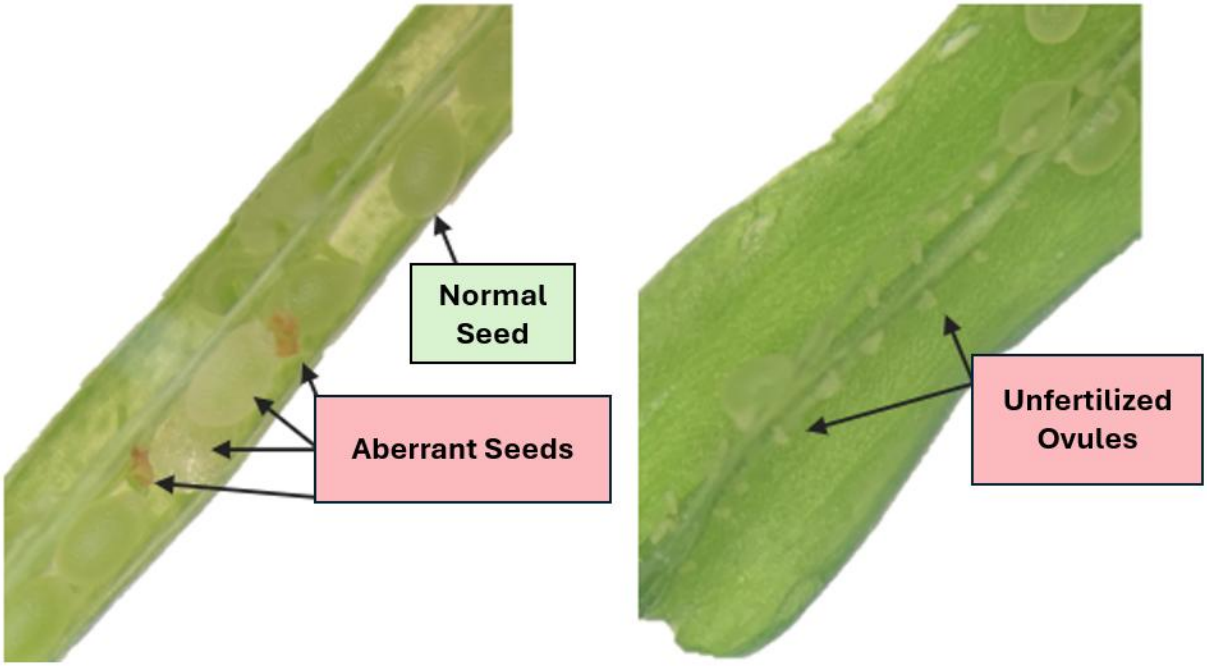


Figure A. ***Morphological discrimination between “normal” or “aberrant” seeds and unfertilized ovules.*** “Normal” seed morphology is relatively large, inflated, whitish/green, and uniform in shape. “Aberrant” seeds are often misshapen or pale/miscoloured and may begin desiccation and seed coat darkening prematurely. Unfertilized ovules appear as small, shrivelled, white-yellow protrusions. Ovules do not turn brown following desiccation and can be differentiated from aborted seeds based on their smaller size and lack of pigmentation. Classification of “normal” or “aberrant” seeds and unfertilized ovules follows Meinke *et al.* (2008) and the SeedGenes project database (http://www.seedgenes.org).

2.4.3 Pollen-Tube Growth

Naturally self-pollinated flowers of each genotype were examined for pollen tube growth through the carpel. Flowers were first marked when immature and lacking petal emergence (floral stage 12) and were then collected after natural pollination (floral stage 13) indicated by dehiscing anthers and petal emergence. Following collection, flowers were covered with 500µL of 3:1 EtOH: glacial acetic acid and incubated for 30 minutes in a 60°C water bath. Following

incubation, the EtOH: glacial acetic acid was replaced with 500µL of 1M NaOH, after which flowers were left overnight and subsequently rinsed twice with dH2O. To visualize pollen tubes, fixed flowers were mounted on a slide with decolourized aniline blue (0.1% in dH_2O, pH 11.0) for two minutes and viewed with UV epifluorescence generated by a mercury lamp (NIKON Eclipse E600 microscope) (Qin et al., 2012). Images were acquired using QCapture Pro 7 software and a Q Imaging RETIGA EXi Fast 1394 camera. Pollen tube growth was quantitatively analyzed by examining callose plug deposition (indicating pollen tubes) across the funiculus with a particular focus on the basal-most region of the carpel indicative of successful pollen tube growth through the entire transmitting tract.

2.4.4 Ovule Morphology

To observe ovule development, flowers were decolourized using a graded ethanol series (30%, 50%, 60%, 70%; minimum of one hour for each stage, left overnight in 70%). Flowers were then cleared in a chloral hydrate solution (8:2:1 of chloral hydrate:glycerol:water) for one week prior to mounting in 67% glycerol for visualization. After dissection from fixed flowers (described under *Floral Dissections*), ovules at various developmental stages were examined using DIC microscopy (NIKON Eclipse E600 microscope) to identify any structural or developmental abnormalities relative to wild-type ovules of the same stage (n= ≥ 50 per genotype) (Hedhly et al., 2018; Enugutti et al., 2013). Ovules were scored as i). normal; ii). misshapen (elongated, irregular in shape, etc) with normal integument closure; or iii). misshapen (elongated, irregular in shape, etc) with improper integument closure (integuments open; nucellus exposed). Images were captured with a Nikon Coolpix 995 camera.

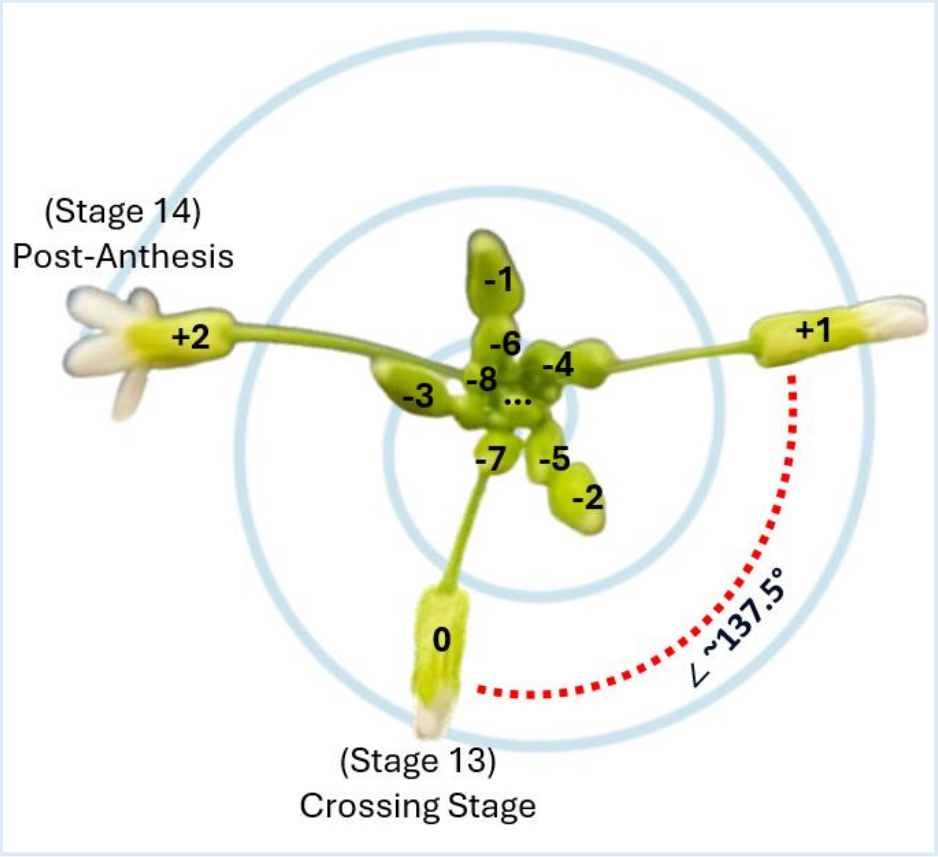
2.4.5 GUS Staining

Flowers of Promoter-GUS reporter gene lines were analysed for GUS expression. Flowers (n ≥20) were incubated for 20 minutes on ice in ~0.5 mL of 90% acetone and were then

washed two times with 50 mM $NaPO_4$ buffer for 5 minutes on ice. NaPO4 buffer was removed and replaced with X-Gluc solution (50 mM $NaPO_4$, 3 mM $K_3Fe_3(CN)_6$, 10 mM Ethylenediaminetetraacetic acid, 0.1% (v/v) Nonidet P_{40}, 0.75 mg/ mL X-gluc). Flowers were vacuum infiltrated at 9 PSI overnight at room temperature and then rinsed four times in 70% EtOH and cleared in an 8:2:1 chloral hydrate:glycerol:distilled water solution for one week. Following clearing, flowers were dissected to remove sepals, petals, and stamens, and the carpels were mounted on slides in 67% glycerol and photographed under a Nikon Eclipse E600 microscope with a Nikon Coolpix 995 camera. Collected flowers were assigned a "Position on Inflorescence" between 0 and -8. This number refers to the order of the flower on the inflorescence relative to a Stage 13 flower at which stage the gynoecium is fully mature (Position 0), with increasingly negative positions indicating younger flowers (i.e. more recently differentiated from the floral meristem). The corresponding floral stage according to Smyth *et al.* (1990) was determined for each "Position on Inflorescence" under the growing conditions used here through manual dissections and is summarized in **Table 1**. GUS expression was then qualitatively scored in flowers of each position on the inflorescence.

Table 1. ***"Position on Inflorescence" classification with the corresponding floral stage approximation according to Smyth et al. (1990) based on observed floral landmarks.*** Image shows a top-down view of the spiral phyllotaxis of the *Arabidopsis* inflorescence with "Position on Inflorescence" indicated. Each flower arises in a spiral, separated by a divergence angle close to the golden angle (137.5°) (Palaugui & Laufs, 2011).

	"Position on Inflorescence"	**Approximate Floral Stage** following Smyth *et al.* (1990)
Younger Flowers	-8 -7 -6	10 to 11
	-5 -4	11 to 12
	-3 -2 -1	12 to 13
	0	13 (Crossing Stage)
Older Flowers	+1 +2 …	14 + (post-Anthesis)



2.4.6 Confocal Microscopy

For *pPIN1:PIN1-GFP* expression analysis in ovules, whole flowers (youngest ovule stages) or dissected carpels (older stages) were mounted in water and gently squashed by applying pressure to the cover slip. Tissues were viewed under ×60 magnification oil-immersion objectives with an Olympus Fluoview FV1000 confocal microscope. GFP was excited using a 488 nm laser and observed using a detection window from 505-540nm. Identical image acquisition parameters were used for all genotypes and ovule stages.

2.4.8 PIN1:GFP Quantification

Images were processed with ImageJ with the same channel adjustments for all images. For GFP localization, at least 25 ovules were analysed for each genotype. The images presented are representative of localization patterns and all samples were processed in NIH ImageJ (Version 1.47v) with the same channel adjustments for all images. PIN1 localization during ovule development in late stage 2 ovules (following initiation of the outer integument primordium but preceding the formation of the function megaspore; Stages *2-iii* to *2-v*) was quantified from pixel intensity as described in **Figure B**. For each genotype, a minimum of 75 nucellus cells were measured from 15 ovules from 5 flowers.

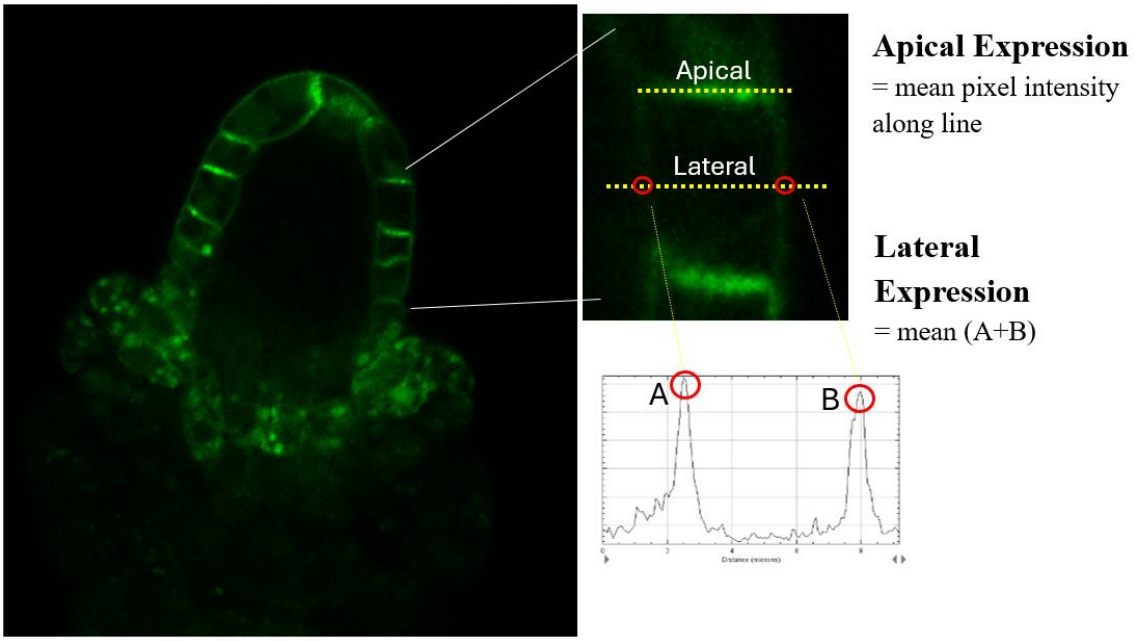


Figure B. ***Quantification of PIN1:GFP localization in apical and lateral membranes of the ovule nucellar cells.*** Mean pixel intensity was measured in the apical region of nucellar cells from a horizontal transect through the entire length of the apical membrane. To approximate localization in lateral cell membranes, a horizontal transect was drawn at the halfway point of each nucellar cell and the maximum intensity values at the two regions where the line intersected the membrane were recorded from a histogram and averaged. To compare PIN1:GFP localization intensity in each mutant line to the wild type control, the mean pixel intensity was transformed to a proportion with reference to the mean apical localization in wild type.

2.5 Data Analysis

All statistical analyses were completed using R version 4.0.3 (R Core Team, 2020). For seed (**Figure 1**) and ovule counts (**Figure 7**), silique lengths and proportion of elongated siliques (**Figure 2C-D**), reciprocal cross pollinations (**Figure 3**), and silique composition analysis (**Figure 8**), one-way ANOVAs were conducted and significant differences in seed number and silique length were identified with a Tukey's HSD post-hoc test using the Bonferroni adjustment for multiple comparisons. Data from all genotypes were compared to wild type (and other genotypes in all possible combinations) and differences were considered significant at an α of 0.05 ($p < 0.05$). Seed counts in the top and bottom halves of naturally self-pollinated, elongated siliques were examined to determine if the distribution of seeds differed in these regions from an

expected equal (50-50) frequency, which may occur if ovule development or fertilization success varied between the top and bottom of the carpel. Pearson's Chi-squared tests were conducted to compare the number of seeds in the top and bottom halves of the silique within each genotype, as well as across genotypes (**Figure 6**). Ovule defects were scored and examined with Fischer's Exact Tests for pairwise comparisons between WT and each genotype for each of the three ovule categories (**Figure 8**). For PIN1:GFP localization analysis (**Figure 11 I-J**), a two-way ANOVA was conducted with Genotype (levels: WT, *fkd1/fl1-2/fl2/fl3*, *ron3-2*, *35S::PID*) and Cell Region (Apical vs. Lateral) as factors. The model also included an interaction term (Genotype:Cell Region) to examine whether the difference between Apical and Lateral intensity measurements varied across genotypes. For tests with multiple comparisons, p-values were adjusted using either the False Discovery Rate (FDR) method (>10 comparisons) or the Bonferroni Correction (<10 comparisons).

3. Results

3.1 Seed Set Reductions

The natural seed set was compared between the wild-type and the PIN1 localization pathway mutants. On average, wild type plants produce 56 (± 8) seeds per silique (**Figure 1**). The most severe seed set reductions, of between 57% to 85% relative to WT, were observed in *cvp2/cvl1* (8.4 ± 4.8), *sfc40* (15.4 ± 6.5), *fkd1/fl1-2/fl2/fl3* (23.8 ± 10.8), and *cvp2* (20 ± 6.3) mutants (One-Way ANOVA and Tukey's HSD post-hoc). Group 1 *FL* gene family single mutants (*fl3*, *fl2*, *fl1-2*, and *fkd1*) all display less severe reductions than the quadruple mutant (*fkd1/fl1-2/fl2/fl3*), with only *fl1-2*, and *fkd1* displaying significant reductions relative to wild type (51 and 48 seeds on average, respectively). Both PIN phosphorylation mutants *ron3-2* and *35S::PID* also display significant reductions to seed set (33 & 43 seeds per silique, respectively).

fkd1/fl1-2/fl2/fl3, *sfc-40*, and *cvp2/cvl1* seeds are also significantly larger in mean length and width than WT (**Figure S1**).

fkd1/fl1-2/fl2/fl3, *sfc-40*, and *cvp2/cvl1* show the most severe seed set defects and were further examined. All these mutants also have a significantly increased proportion of improperly elongated siliques per plant (**Figure 2D-E**). When considering only the properly elongated siliques of *fkd1/fl1-2/fl2/fl3*, *sfc-40*, and *cvp2/cvl1*, there are significant reductions to average seed per silique (**Figures 1 & 2A**) despite no differences in silique length (**2C**) or the total number of siliques produced per plant (**2B**).

3.2 Manual Pollination Experiments to Assess Male and Female Fertility

Manual pollination experiments were conducted to determine if the seed set defects were a result of female (gynoecium) or male (androecium) defects. For each mutant, seed counts from three combinations of crosses were conducted: manual self-pollination (to control for crossing technique), mutant carpel x wild-type pollen (to assess female fertility), and wild-type carpel x mutant pollen (to assess male fertility). In addition, seed counts from naturally self-pollinated flowers were completed to establish a control for seed set values with no manual manipulations.

Manual pollination experiments produced results consistent with a female defect and provided no evidence suggesting altered male fertility (**Figure 3).** Seed counts from manual self pollinations for all genotypes did not significantly differ from their corresponding natural self pollination seed counts. Additionally, seed counts for all manual crosses using wild-type carpels and mutant pollen (WT ♀ x Mutant ♂) did not differ significantly from manual or natural wild-type self-pollinations.

Finally, crosses using mutant carpels and wild-type pollen (Mutant ♀ x WT ♂) produced significantly fewer seeds than crosses using wild-type carpels and mutant pollen (WT ♀ x Mutant ♂), consistent with a female defect in all mutant lines. Mutant ♀ x WT ♂ seed sets also did not vary significantly from the respective natural or manual self pollinations within each genotype.

3.3 Expression of Promoter-GUS Reporter Gene Lines in the Gynoecium

Analysis of Promoter-GUS lines suggests that *SFC, FKD1, FL1,* and *CVP2* are all expressed at varying strengths in several regions of the developing gynoecium from floral stage 10 to 12 (n ≥20 flowers per genotype) (**Figure 4**). *SFC*, *FKD1*, and *FL1* are strongly expressed in the vasculature of the developing carpels from the carpel base to the style in the flowers from floral stages 10 to 12 (**4A-I**). *SFC* and *FKD1* are also strongly expressed in the developing ovules (**4S-Z**) mainly in the funicular region in stage 2-ii to stage 2-iv ovules (floral stage 10 and 11), and more weakly in the nucellar cells until ovules reach late stage 3 (floral stage 12). Expression ceases in the ovules of stage 13 flowers. Strong *FL1* expression in the carpel vasculature and stylar region persists from floral stages 10 to 12 (**4G-I**), although no expression was observed in the developing ovules. Similarly, *CVP2* is weakly expressed in the carpel vascular tissue during these same stages (**4J-L)** and is also absent in the ovules**.** No expression in the carpel tissue or ovules was observed for *CVL1* or *DR5:GUS* (**4M-R**). None of the genes examined were clearly expressed in the stigmatic region, with the boundary for apical expression ending in the stylar region.

3.4 Pollen Tube Growth

Qualitative analysis of pollen tube growth through the carpel tissue towards unfertilized ovules was examined by visualizing callose plug deposition, which occurs as pollen tubes

successfully grow through the carpel tissue to reach the ovules (**Figure 5A-L**). Pollen tube growth was found to progress normally through the transmitting tract tissue to reach the basal-most ovules in *fkd1/fl1-2/fl2/fl3, sfc40,* and *cvp2/cvl1*. Additionally, the number of pollen tubes observed in all genotypes was similar. Callose plug deposition was observed throughout the entire length of the carpel, indicating successful pollen tube advancement through the entirety of the transmitting tract. Additionally, callose plug deposition was observed over the funiculus and ovule regions of all mutants (indicated by arrowheads in Figure **5A-L**).

3.5 Within-Silique Seed Distribution

A Pearson's Chi-squared test was conducted to compare the number of seeds in the top and bottom halves of the silique within four genotypes (WT, *cvp2/cvl1*, *sfc40,* and *fkd1/fl1-2/fl2/fl3*). This comparison found that seed number was not significantly different in the top and bottom halves within genotypes (**Figure 6**). For all genotypes, the frequency of seeds in the top and bottom halves was not significantly different from an equal (50-50) proportion with $p > 0.05$ for all genotypes {For WT, X^2 (1, N=30) = 1.88, p=0.1702; For *cvp2/cvl1*, X^2 (1, N=30) = 1.30, p=0.2545; For *sfc40*, X^2 (1, N=30) = 2.25, p=0.1333; and for *fkd1/fl1-2/fl2/fl3*, X^2 (1, N=30) = 2.83, p=0.0924}.

An additional Pearson's Chi-squared test was conducted to compare the proportion of seeds in the top and bottom halves of the silique for each genotype compared to the wild-type. For both the top and bottom halves of the silique, there was no significant difference in the proportion of seeds in each half between all genotypes and wild type {X^2 (3, N=120) = 6.33, p=0.097 for both halves}.

3.6 Ovule Production & Silique Composition

fkd1/fl1-2/fl2/fl3, sfc40, and *cvp2/cvl1* flowers produce significantly fewer ovules than wild-type plants **(Figure 7**) determined by a One-Way ANOVA and Tukey's HSD. Mean ovule production in WT plants is 58 ± 13, while *fkd1/fl1-2/fl2/fl3, sfc40,* and *cvp2/cvl1* produce an average of 46 ± 11, 41 ± 7, and 45 ± 5 ovules, respectively.

Of the total number of ovules produced, 95% of WT ovules successfully develop into seeds, while 2% are aberrant and remain unfertilized, and 3% abort post-fertilization (**Figure 8**). In contrast, *fkd1/fl1-2/fl2/fl3, sfc40,* and *cvp2/cvl1* ovules develop into seeds at a significantly lower rate than WT plants (58%, 66%, and 57%, respectively), with a much greater proportion of these ovules developing improperly and remaining unfertilized (14%, 14%, and 24%) or appearing aberrant post-fertilization (28%, 20%, and 19%).

3.7 Ovule Morphology Defects

Ovule morphology was scored for wild-type, *fkd1/fl1-2/fl2/fl3, sfc40, cvp2/cvl1, ron3-2,* and *35S::PID*. Ovules were scored as normal, misshapen with normal integument closure (elongated, irregular in shape, etc.), or misshapen with improper integument closure (integuments open; nucellus exposed). Improper integument closure was always correlated with changes to shape, whereas shape changes could also occur even when integument closure was occurring properly. *fkd1/fl1-2/fl2/fl3, sfc40,* and *cvp2/cvl1* ovules display integument closure defects and misshapen, elongated ovules (**Figures 9 & 10**). For each of the three ovule categories, significant differences from wild type were determined by Fischer's Exact Tests for pairwise comparisons between WT and each genotype and adjusted using the False Discovery Rate (FDR) method for multiple comparisons.

All mutants had many mature ovules that displayed a characteristic exposed nucellus (**Figure 9; middle columns; red arrows**), that failed to be surrounded by the inner and outer

integuments. Ovules with an exposed nucellus generally also appeared elongated compared to normal ovules or improperly recurved towards the funiculus. The defects observed in *fkd1/fl1-2/fl2/fl3*, *sfc40*, and *cvp2/cvl1* appear to be more severe on average than those displayed in *ron3-2* and *35S::PID* mutants (**Figures 9 & 10**). Additionally, all mutants formed ovules with proper integument closure but aberrant shapes, including flattened, elongated, or asymmetric development (**Figure 9; right columns).** The total proportions of ovules with defective integument closure or shape defects were approximately 62% in *fkd1/fl1-2/fl2/fl3*, 60% in *sfc-40*, 23% in *cvp2/cvl1*, 25% in *ron3-2*, and 28% in *35S::PID*, closely corresponding with total seed set reductions observed in these mutants (*Section 3.1*; **Figure 1**). *cvp2/cvl1* shows an approximately similar proportion of defective ovules as *ron3-2* and *35S::PID* (**Figure 10**), but the defects are generally more severe in *cvp2/cvl1* (as shown in **Figure 9**). No integument closure defects or noticeably misshapen ovules were observed in WT, wherein only ~5% are aberrant and remain unfertilized or abort post-fertilization (**Figure 8**).

3.8 PIN1:GFP Localization During Ovule Development

PIN1:GFP localization in late ovule stage 2 nucellus cells of *fkd1/fl1-2/fl2/fl3, ron3-2*, and *35S::PID* was quantified from pixel intensity and compared to wild-type plants (**Figure 11 A-H**). PIN1:GFP localization in all mutant *pPIN1:PIN1-GFP* lines is significantly reduced in the apical membrane region of the nucellar cells, as well as in the lateral membrane regions (except *35S::PID*) compared to localization in wild type (**Figure 11 I-J**). The two-way ANOVA testing for an interaction between cell region and genotype indicated a significant difference ($F(3, 659) = 43.49$, $p < .001$) indicating that the effect of cell region on PIN1:GFP intensity differs across genotypes. Within each genotype, relative mean apical membrane localization was significantly greater than lateral membrane localization (**Figure 11 K**). Notably, the relative proportion of

fluorescence in the apical compared to the lateral region of the nucellus is more greatly reduced in the mutants compared to wild type (**Figure 11 J**). In *flQ* and *ron3-2*, the relative proportion of fluorescence in the apical versus the lateral membrane is much more similar than in wild-type and *35S::PID*, where a much larger proportion of fluorescence is observed in the apical membrane (**Figure 11 J**). Together, these patterns suggest that the overall level of PIN1:GFP localization (as measured by pixel intensity) in all mutant lines examined is reduced compared to PIN1:GFP expression in WT plants at both the apical and lateral membranes.

4. Discussion

4.1 SFC, FKD1, FL1, and CVP2 are Expressed in the Developing Gynoecium

My analysis of promoter GUS expression patterns suggests that *SFC, FKD1, FL1,* and *CVP2* are all expressed during gynoecium development. *SFC*, *FKD1*, *FL1*, *FL2*, *FL3*, *CVP2*, and *CVL1* genes have been previously studied in the context of their role in PIN1 localization during vascular tissue development in the leaves and cotyledons and during root development (Steynen & Schultz, 2003; Mariyamma et al, 2017; Mariyamma et al, 2018; Scarpella et al., 2006; Carland & Nelson, 2009; Hou et al. 2010). For example, *SFC* maintains PIN1 polarity in leaf pre-procambial cells, with *sfc* mutants displaying a severe vascular discontinuity phenotype (Scarpella et al., 2006). *sfc* mutants also display reduced root length and altered auxin transport (Sieburth et al., 2006). Similarly, *cvp2/cvl1* double mutants display disrupted primary root protophloem development and a discontinuous vein phenotype like *sfc* mutants and these genes have been suggested to regulate vein continuity and are required for PIN1 protein reception at the PM (Rodriguez-Villalon et al., 2015; Carland & Nelson, 2009).

Furthermore, Group 1 *FL* gene family members (*FKD1*, *FL1*, *FL2*, and *FL3*) have been implicated in the establishment and maintenance of asymmetric localization of PIN1-containing

vesicles to the plasma membrane. *FKD1* is also necessary for initiating the auxin response that directs vascular tissue development and the formation of closed vein loops, likely involving its role in PIN1 localization (Steynen and Schultz, 2003; Hou et al., 2010)**.** *fkd1* mutants have a lack of distal vein meeting and show erratic PIN localization and a lower auxin response suggesting irregular auxin transport (Steynen and Schultz, 2003; Hou et al., 2010).

My results show that *SFC*, *FKD1*, *FL1*, and *CVP2* are also all expressed in several regions of the developing gynoecium (**Figure 4**). *SFC, FKD1,* and *FL1* are strongly expressed in the vasculature of the developing carpels from the carpel base to the style, consistent with the expression of these genes in the leaf vasculature, suggesting a possible role in auxin transport and canalization during gynoecium development. The presence of *SFC* and *FKD1* expression in the funiculus and nucellus regions of late-stage ovules (**Figure 4; A to H**) also supports the involvement of these genes in ovule development.

4.2 Female Developmental Defects Drive Seed Set Reduction

The considerable reductions to seed set observed in the examined set of PIN1 localization pathway mutants suggest, for the first time, that these genes play an important role during reproductive development. *cvp2/cvl1*, *sfc40,* and *fkd1/fl1-2/fl2/fl3* mutants show significant reductions to seed set, with a total reduction between 57% to 85% relative to WT (**Figure 1**). Interestingly, these reductions do not appear to simply result from fewer total siliques produced or shorter siliques, as no significant differences in these traits were identified (**Figure 2B-C**). In addition to the overall seed set decrease from mature siliques compared to wild type, *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40* also produce significantly more siliques that fail to elongate (**Figure 2D**). Previous studies have identified silique abortion in plants displaying reduced polarization of PIN1 and decreased auxin signal from DII-VENUS, suggesting that silique

abortion can result from improper PIN localization (Lauxmann et al., 2016). This is consistent with the ovule arrest observed in *pin1-5*, which could drive silique abortion if it occurs at a high enough frequency in a developing silique (Bencivenga et al., 2012).

Manual pollination experiments were first conducted to determine if the seed set defects were a result of gynoecium or androecium defects (**Figure 3**). Crosses using a mutant carpel and wild-type pollen (Mutant ♀ x WT ♂) produced significantly fewer seeds than crosses using a wild-type carpel and mutant pollen (WT ♀ x Mutant ♂), consistent with a female defect in all mutant lines. Seed counts for all manual crosses using a wild-type carpel and mutant pollen (WT ♀ x Mutant ♂) did not differ significantly from manual or natural wild-type self-pollinations, suggesting that pollen development and pollen tube growth are occurring normally in all mutant lines. Additionally, the similarity among seed production in Mutant ♀ x WT ♂ crosses compared to the respective natural or manual self pollinations for each genotype suggests that WT and mutant pollen do not differ in the ability to form pollen tubes and fertilize a receptive ovule. Finally, the absence of significant differences between the manual and natural self-pollinations supports the consistency of the manual pollination technique with the natural pollination process.

Additional evidence further supports the normal formation of the androecium in *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40*. Previous data from fertility characterization experiments of *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40* showed normal pollen fertility, germination, pollen tube growth, and overall androecium development, suggesting that aberrant PIN1 localization may not significantly affect androecium development and pollen growth (Reiter & Schultz, unpublished). Furthermore, pollen tube germination and growth and carpel patterning appear to be occurring normally in *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40* (**Figure 5**). The pollen tube growth patterns described here further support pollen fertility in the mutants. Pollen tube growth

was observed to occur normally through the entirety of the transmitting tissue to the basal-most ovules in *fkd1/fl1-2/fl2/fl3*, *sfc40*, and *cvp2/cvl1* (**Figure 5**), suggesting that mutant pollen tube growth is occurring properly. Additionally, proper growth through the transmitting tract tissue supports normal patterning and development of the carpel tissue. The observations of callose plug deposition in the funicular and ovule region of all mutants also suggest that at least some of the ovules are receptive and signalling pollen tube growth normally. Together, the manual pollination experiments, previous androecium fertility analysis (Reiter & Schultz, unpublished), and pollen tube growth patterns (**Figure 5**) strongly suggest that the reduced fertility of *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40* is due to a gynoecium defect.

Furthermore, I found that seed proportions in the top and bottom halves of mutant siliques were approximately equivalent, as in wild-type (**Figure 6)**. These findings also support the observed pollen tube growth patterns, suggesting that pollen is indeed growing through the entire length of the transmitting tract tissue and successfully reaching the basal-most ovules. It has been established that auxin levels in the carpel are typically greatest in the stigma and decrease in a gradient fashion towards the base of the carpel, which is important for both pollen tube germination and establishment of the apical-basal axis of the gynoecium (Nemhauser et al., 2000; Chen & Zhao, 2008). Previous studies have shown that the application of the auxin transport inhibitor NPA to developing flowers results in a shift in the auxin gradient within the developing carpel that results in increased elongation of stigma and style tissue in the apical regions causing a basalized style/ovary boundary and increased elongation of the gynophore. NPA treatment also causes a reduction in ovary production, and decreased production of carpel valves (Nemhauser et al., 2000). Interestingly, the seed frequency patterns observed in the mutant carpels do not support a positional bias occurring with regard to successful fertilization,

likely indicating that no significant overall shift in the auxin gradient along the length of the carpel is occurring. The lack of clear carpel patterning defects in these auxin mutants suggests that PIN1-mediated PAT is not severely disrupted in the process of carpel formation (which would be expected to show similar defects as NPA treatment) and is therefore not significantly affecting the carpel primordia and tissues along the pistil axis. These findings collectively suggest that these differences are instead likely the result of ovule development defects, as both pollen tube growth and carpel patterning appear to be progressing normally.

4.3 Decreased Ovule Production Contributes to Seed Set Reductions

My findings also indicate that *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40* produce fewer ovules overall (**Figure 7**). The importance of PIN1 localization during ovule initiation has been well established, with early ovule primordia initiation occurring asynchronously along the placental tissue at PIN1-defined auxin maxima, determining the spatial distribution of ovules along the placenta (Cucinotta et al., 2014; Yu et al., 2022; Hu et al., 2022). Additionally, NPA application to developing carpels also inhibits ovule formation, and ovules have been reported to be the most sensitive of all gynoecium tissue to altered polar auxin transport (Nemhauser et al., 2000). *pin* mutants also show a high reduction (*pin1-5*) or complete elimination (*pin1-1*) of ovules (Bennett et al., 1995; Okada et al., 1991; Barro-Trastoy et al., 2020; Galbiati et al. 2013). The reduction in ovule number observed in *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40* may result from improper PIN1 localization during ovule initiation in the placenta. Examination of PIN1 expression in the placenta during early carpel development would help to confirm this hypothesis.

4.4 Improper Integument Closure Appears to Drive Pre- and Post-Fertilization Defects

In addition to reduced ovule production in *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40*, all mutants also display siliques with a significantly greater proportion of aberrant seeds and unfertilized ovules than wild-type plants (**Figure 8**). I suggest that a combination of both reduced ovule production and subsequent ovule development defects are driving the reductions to seed sets that are observed in these PIN1 localization pathway mutants. Alterations in PIN1 localization within ovules of *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1* and *sfc-40* leading to aberrant ovule development are also supported by similar changes to PIN1 localization and ovule defects in *ron3-2* and *35S::PID*, where defective PIN localization is better understood at the molecular level than in the set of examined PIN1-localization pathway genes.

All mutants displayed a significant subset of ovules with an exposed nucellus that was incompletely enclosed by the inner and outer integuments (except *cvp2/cvl1*, which displayed defects that were not statistically significantly different from wild-type) (**Figures 9 & 10**). The nucellus of these ovules also appeared both more elongated and improperly recurved towards the funiculus. The integument closure and elongation phenotype was more severe in *fkd1/fl1-2/fl2/fl3*, *sfc40*, and *cvp2/cvl1* than in *ron3-2* and *35S::PID* mutants, suggesting that integument and nucellus development may be more greatly impacted in these mutants. Integuments are the only lateral organs produced by the ovule, with both the outer and inner integuments being of epidermal and sub-epidermal origin, and their development has been shown to closely follow that of other determinate lateral plant organs like the leaves (Robinson-Beers et al., 1992; Schneitz et al., 1995; Truernit & Haseloff, 2008). Leaves and integuments have been proposed to be analogous structures (Kelley & Gasser, 2009). Specifically, the outer ovule integument requires precise regulation of the direction and rate of cell division, and improper closure or curvature leaves the embryo unprotected (Truernit & Haseloff, 2008). Several genes important

for integument development have been characterized in *Arabidopsis*, including *AINTEGUMENTA* (*ANT*), *BELL1* (*BEL1*), *SHORT INTEGUMENTS* (*SIN1*), and *INNER NO OUTER* (*INO*). Many of these integument patterning genes are also critical for lateral organ development, and the commonalities between the morphology, development, and genetic control of integuments and leaves have been well established (as summarized in Kelley & Gasser, 2009). The role that *SFC*, *FKD1*, *FL1*, *FL2*, *FL3*, *CVP2*, and *CVL1* play in PIN1 localization during the development of vascular tissue and the formation of vein patterns in the leaves aligns with a possible function in the formation of the integuments, like other integument patterning genes that are also important for vascular development (reviewed in Kelley & Gasser, 2009). Interestingly, *PIN1* expression has been observed in the inner integument from stage 2-ii to stage 3-ii ovules but is not clearly expressed in the outer integument (Ceccato et al., 2013). I also did not observe strong expression in the outer integument during these stages, suggesting that these genes could be driving outer-integument differentiation and growth through an indirect process, possibly involving coordinated development with the inner integument which has been shown to be important for proper closure (Truernit & Haseloff, 2008). A more detailed analysis of *fkd1/fl1-2/fl2/fl3*, *sfc40*, and *cvp2/cvl1* ovules is required to determine the sequence of the defect and the extent of inner and outer integument involvement to the observed integument closure phenotypes observed in the mature ovules of these mutants.

In addition to the observed integument closure defects, all mutants also displayed ovules that had proper integument closure but an overall aberrant shape, including mature ovules that were flattened, elongated, or asymmetric compared to normal morphology (**Figures 9 & 10**). These findings may also relate to the regulation of cell division required for the formation of the integuments and nucellus being affected in the mutants. Interestingly, the presence of leaf cell

polarity defects in *fkd1/fl1-2/fl2/fl3*, *sfc40*, and *cvp2/cvl1* has also been documented (Mariyamma et al., 2018; Clarke, 2018; Reiter, unpublished data) suggesting that a similar polarity defect could be present in the integument of the developing ovules. If the integument can develop sufficiently to enclose the nucellus, but the overall patterning and elongation of the ovule are still greatly affected, this could contribute to the aberrant shapes observed and possibly explain the high proportion of aberrant seeds that are present after fertilization. Additionally, cell polarity defects that prevent integument closure could also be driving aberrant ovule formation where fertilization is inhibited. It is worth noting that the aberrant seeds observed were not analyzed for viability, and it is therefore not clear exactly how many of these seeds are "aborted". Therefore, some of these aberrant seeds may be inviable, while others may be misshapen but still able to reach maturity and produce a viable embryo. In conclusion, I suggest that the observed post-fertilization seed defects are likely being driven by earlier ovule patterning and development defects which inhibit either successful fertilization or the progression of seed development post-fertilization.

4.5 pPIN1::PIN1GFP Localization Patterns Support Ovule Development Defects

My findings suggest that *flQ*, *ron3-2*, and *35S::PID* all display reduced PIN1:GFP localization in the apical region of late ovule stage 2 nucellus cells compared to wild-type plants (**Figure 11**). This decreased intensity of PIN1:GFP expression in all mutant lines examined in both the apical and lateral plasma membranes supports that alterations to PIN1 localization are likely driving the characterized ovule defects and the large reductions to the seed set. The combination of both reduced PIN1 expression and less asymmetric localization is likely to reduce directed auxin flux towards the tip of the ovule, which would cause reduced auxin efflux in the nucellus (**Figure 12**). Furthermore, the relative proportion of fluorescence in the apical

versus the lateral membrane is more similar in *flQ* and *ron3-2* than in wild type and *35S::PID*, where a greater proportion of fluorescence is observed in the apical membrane (**Figure 11 J**). When PIN1 localization is more symmetric, reduced polar flux likely results causing more auxin to move laterally instead of towards the apex of the nucellus, ultimately reducing the auxin accumulation at the nucellus tip (**Figure 12**). Additionally, **Figure 11 J** shows a larger variation in the proportion of PIN1 localization in the apical and lateral membranes in *flQ* and *ron3-2*. In nucellus cells with a greater proportion of apically localized PIN1, sufficient auxin transport may still occur through the nucellus cell files of certain ovules. Conversely, polar transport may be inhibited when PIN1 localization is greater in lateral membranes and reduced in the apical region. This variation in the proportion of PIN1 localization in the apical and lateral membranes in *flQ* and *ron3-2* may explain why only some of the developing ovules arrest, while others can seemingly complete development normally.

Significantly, the altered PIN1:GFP localization patterns observed here are most clearly occurring during early ovule developmental stages (mainly in stage 2 ii-iii ovules) during which megasporogenesis, nucellus enlargement, and integument initiation and growth occur. These initial processes are critical for subsequent ovule developmental processes (megagametogenesis, embryo sac maturation, and integument closure). Likewise, *flQ*, *ron3-2*, and *35S::PID* also show decreased PIN1 intensity in the inner integument primordia in stage 2 ii-iii ovules, which could reduce auxin levels in developing integument leading to the integument closure defects observed here. Together these observations fit with previous findings regarding the importance of PIN1 to various ovule development processes, from initiation to nucellus formation, embryo sac patterning, and integument closure.

Although the development of the embryo sac was not directly examined, the formation of the embryo sac in the mutant lines could also be contribute to the large proportion of the aberrant ovules that remain unfertilized. Auxin is known to play a key role in the patterning and morphogenesis of the ovule (Barro-Trastoy et al., 2020). *PIN1* expression has been observed in the developing nucellus tissue from ovule stages 1-ii to 3-iii and these stages correspond with ovule primordium elongation, megasporogenesis, and megagametogenesis. Early ovule development is characterized by auxin flux from placental tissue into initiating primordia and developing nucellus, and later development involves auxin flux from the developing ovules back towards the placenta. PIN1-induced auxin transport in stage 2 and 3 ovules has been proposed to drive the development of the female gametophyte (megagametogenesis), and nucellus degeneration which begins shortly afterwards (Ceccato et al., 2013; Wang et al., 2021; Yang et al. 1999). The reduced expression of PIN1 in *flQ*, *ron3-2*, and *35S::PID*, together with reduced asymmetry in localization, is expected to reduce auxin flux through the nucellus and inhibit the establishment of an auxin maximum at the distal tip.

PIN1 down-regulation during gametophyte development has been shown to cause the arrest of embryo sac development (Ceccato et al., 2013). *pin1-5* ovules characteristically arrest early in gametogenesis (FG1 stage) or have embryo sac defects (Bencivenga et al., 2012), highlighting the importance of proper PIN1 localization during early ovule development. Additionally, PIN1 has been identified to be critical for the accumulation of auxin in the nucellus and megaspore mother cell specification (Huang et al., 2022). It is plausible that alterations to PIN1 localization in the nucellus of *fkd1/fl1-2/fl2/fl3*, *sfc40*, and *cvp2/cvl1* could be affecting the progression of nucellus development and embryo sac maturation, as the importance of proper PIN1 localization and auxin maximum establishment at the distal tip of the nucellus for these

processes is well established (Barro-Trastoy et al., 2020; Yang et al. 1999; Ceccato et al., 2013; Larsson et al., 2017; Wang et al. 2021; Bencivenga et al., 2012; Huang et al., 2022). It is also noteworthy that the elongated nucellus phenotype evident in *fkd1/fl1-2/fl2/fl3, cvp2/cvl1* and *sfc-40* resembles the phenotype of the *sporocyteless* mutant which forms a "finger-like" nucellus. In *sporocyteless/nozzle (spl/nzz)* ovule tissues, PIN1 expression is greatly reduced, inhibiting the formation of the megaspore mother cell (MMC) and arresting the embryo sac (Bencivenga et al., 2012; Balasubramanian & Schneitz, 2000). This "finger-like" nucellus phenotype has been suggested to result from the megasporocyte (and subsequent embryo sac) failing to form, which causes the nucellus to elongate rather than degenerate, and the lack of embryo sac formation leads to ovule abortion (Yang et al., 1999). Overall, further analysis of embryo sac formation in *fkd1/fl1-2/fl2/fl3, cvp2/cvl1* and *sfc-40* at each stage of megagametogenesis would provide evidence to support this observation.

During the final stages of integument closure and degeneration of the nucellus, *PIN1* expression also persists in the funiculus, playing a key role in auxin canalization and efflux from the mature ovule to the placenta, which also affects the final stages of megagametogenesis (Ceccato et al., 2013; Larsson et al., 2017; Wang et al. 2021). The importance of PIN1 vesicle trafficking to the formation of the embryo sac and degeneration of the nucellus has also been established in mutants with vesicle trafficking defects. For example, *rgtb1* mutants that show improper RAB membrane anchoring have altered PIN1 endosome recycling in the funiculus which prevents auxin efflux from the late-stage ovule (Rojek et al., 2021). Altered PIN1 localization reduces auxin efflux from the nucellus, causing a sustained auxin maximum around the female gametophyte which inhibits embryo sac development and the subsequent degeneration of the nucellus (Rojek et al. 2021) which has been suggested to lead to embryo sac

arrest and abortion of the ovule. *SFC-40* and *FKD1* are strongly expressed in the funiculus region during late ovule development stages (**Figure 4, T, U, X, Y)** possibly indicating an important role in auxin transport out of the late-stage ovule which has been shown to be important for nucellus degeneration and embryo sac development.

These findings highlight the importance of vesicle-mediated PIN1 localization to the initial process of auxin movement into the ovule during early development that initiates female gametophyte development, followed by PIN1 redistribution and subsequent auxin transport out of the late-stage ovule. This movement of auxin out of the ovule is necessary for the continued progression of female gametophyte development and the degeneration of the nucellus. Without correct PIN localization patterns to direct this flow of auxin in and out of the ovule, these processes are compromised and can lead to ovule abortion. Because of the roles of *SFC*, *FKD1*, *FL1*, *FL2*, *FL3*, *CVP2*, and *CVL1* in PIN1 vesicle trafficking, it is plausible that the altered PIN1 localization patterns in the mutants may be contributing to the aberrant ovule and seed development observed that are driving large reductions to seed set.

5.0 Results Figures

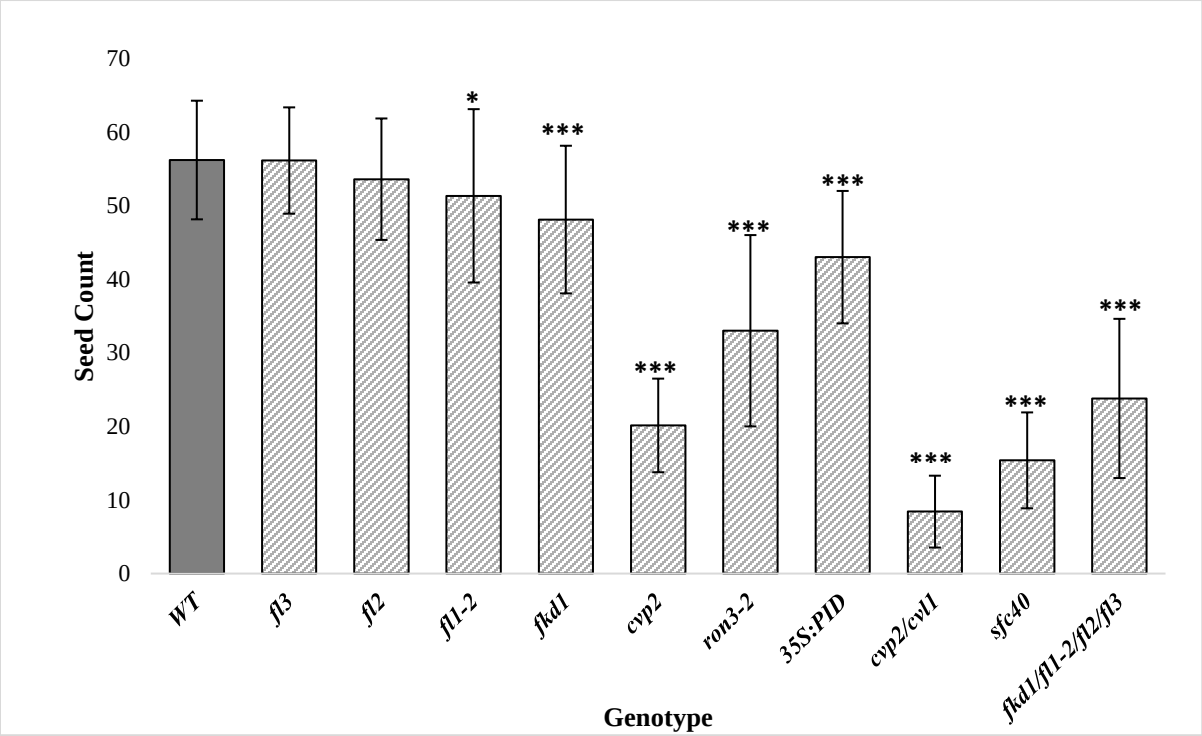
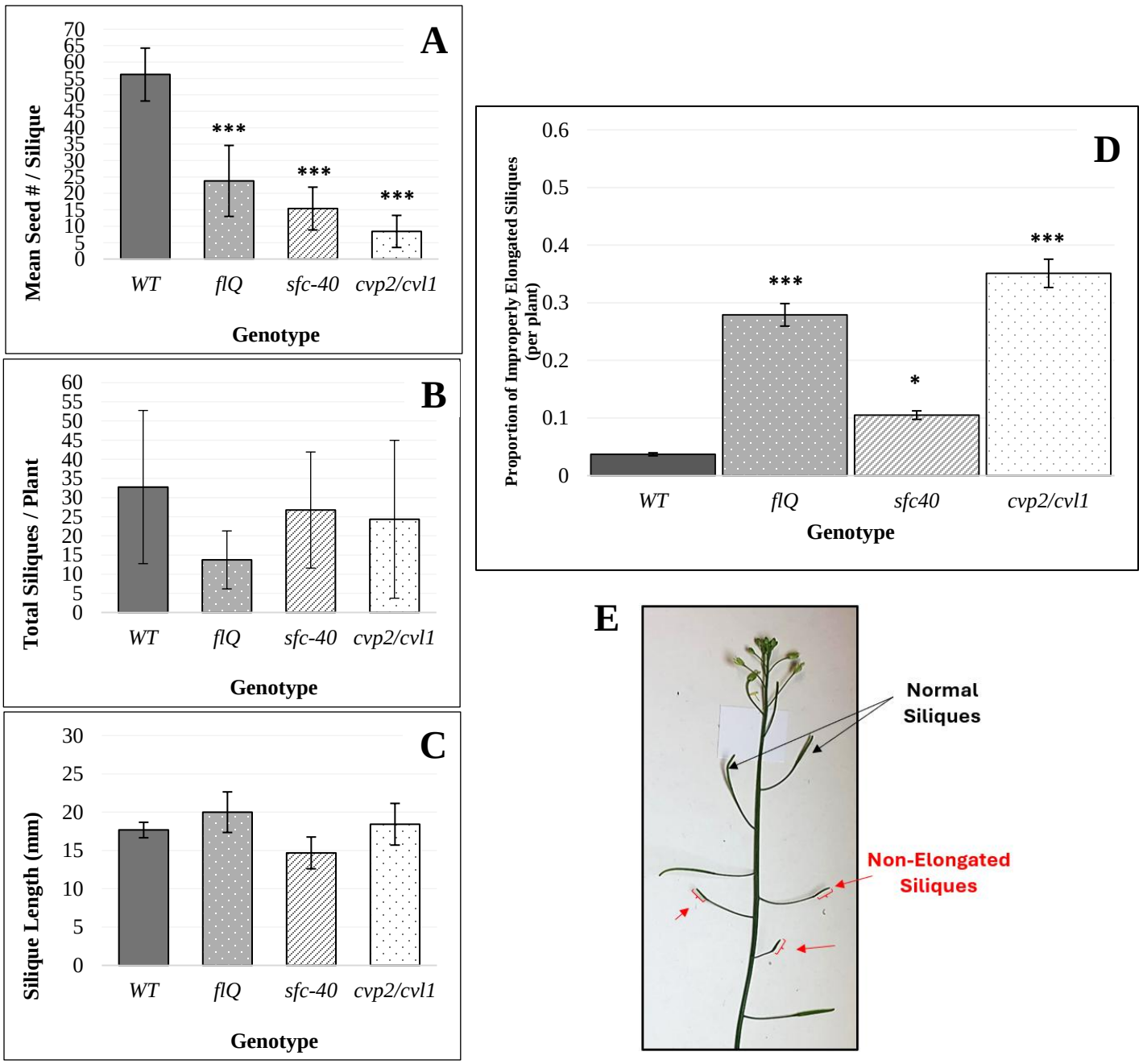


Figure 1. ***Several PIN1 localization pathway mutants produce significantly fewer seed than WT plants***. Mean number of seeds produced per silique for various PIN1 localization pathway mutants. Values are presented as the mean ± standard deviation. Significant differences from wild type are indicated by asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) as determined by a One-Way ANOVA and Tukey's HSD post-hoc. Seeds were counted for a minimum of 40 randomly selected elongated siliques per genotype.



<u>Figure 2.</u> ***fkd1/fl1-2/fl2/fl3, sfc40, and cvp2/cvl1 produce significantly fewer seed than WT and a greater number of siliques with improper elongation despite no differences in elongated silique length or total number of siliques produced***. Seed counts (**A**), number of siliques produced (**B**), and silique length (**C**) of naturally self-pollinated, elongated siliques for wild type and mutant plants. (**D**-**E**) Siliques were scored according to phenotype descriptions in Sun *et al.* (2004) and Lauxmann *et al.* (2016). Values are presented as the mean ± standard deviation. Significant differences from wild type are indicated by asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) as determined by a One-Way ANOVA and Tukey's HSD post-hoc (sample sizes = (A) ≥40 siliques; (B) ≥15 plants/genotype; (C) ≥20 siliques; (D) ≥10 plants/genotype.

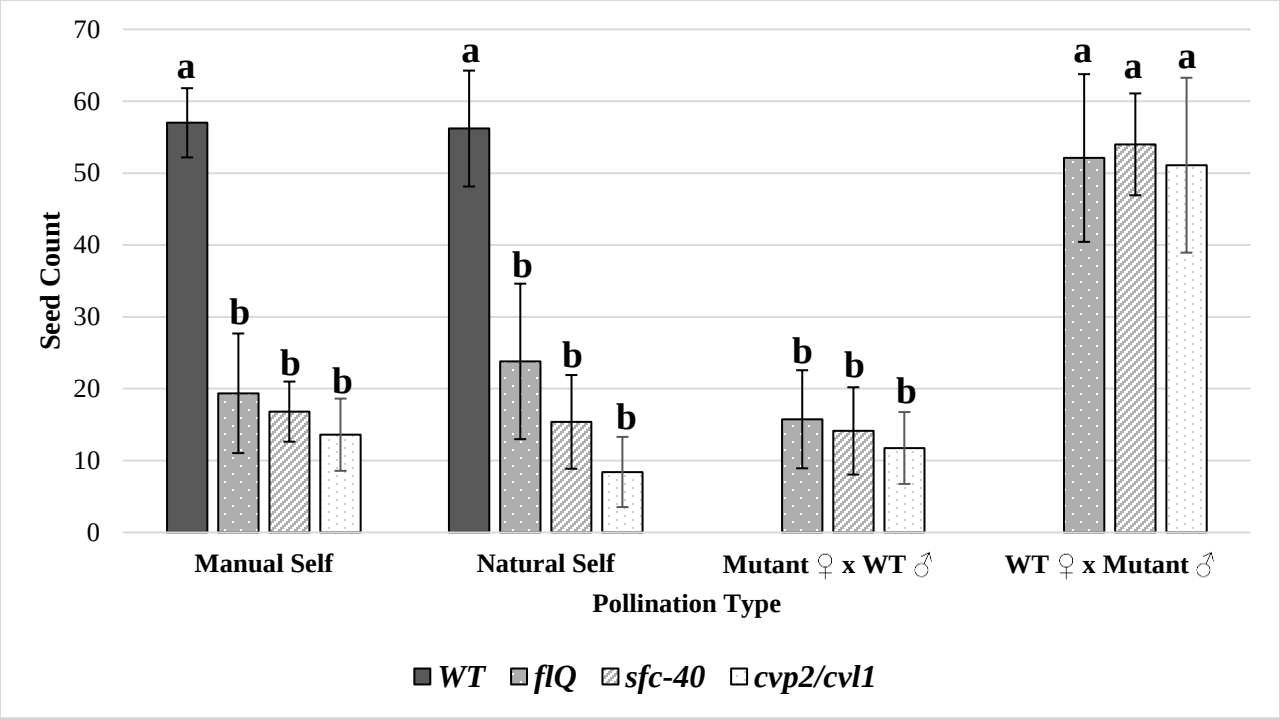
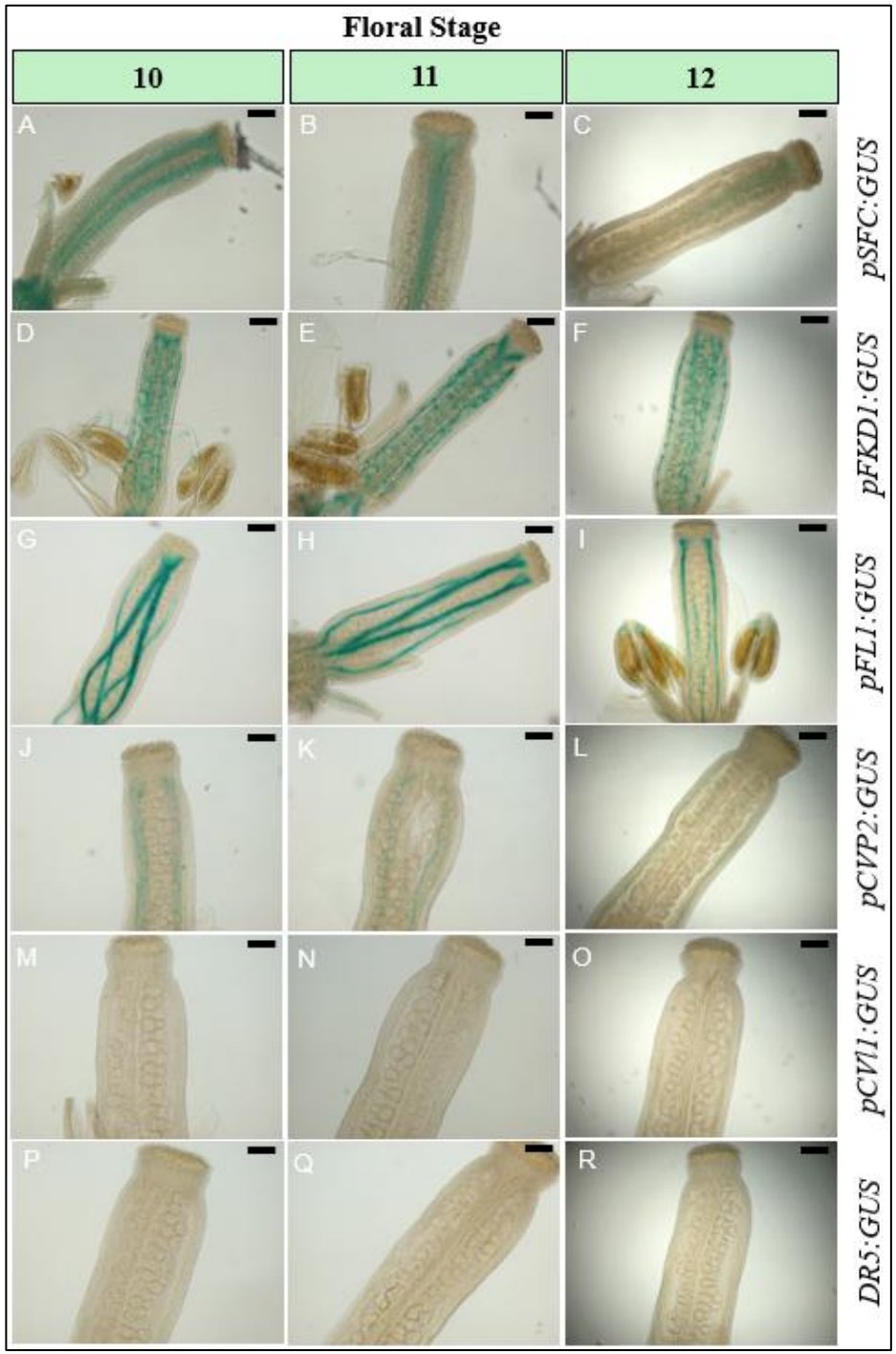
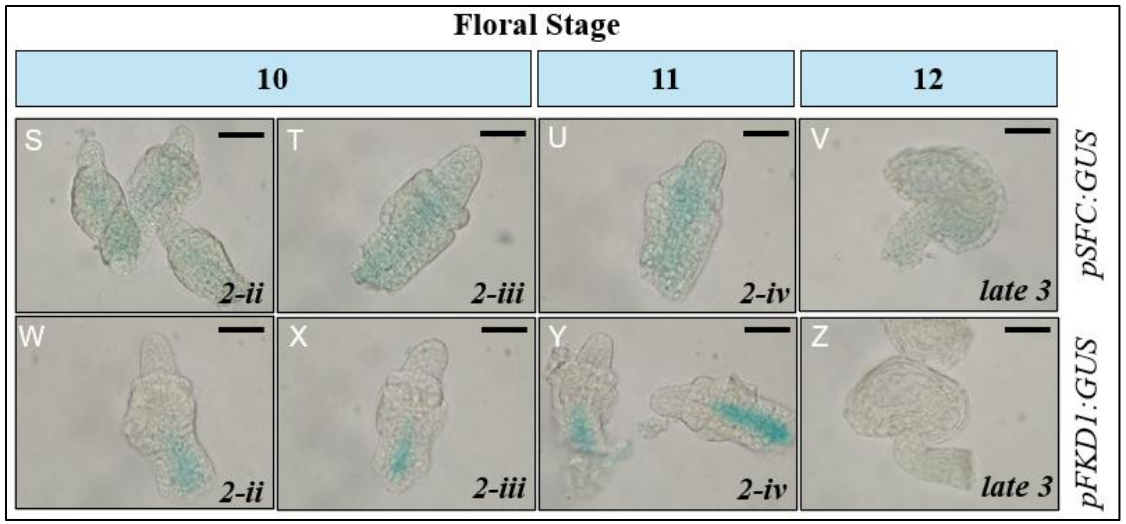


Figure 3. ***Manual pollination experiments indicate that seed set is significantly reduced when mutant carpels are used in any combination, but not when wild type carpels are used, supporting that a gynoecium defect is responsible for seed set reductions in fkd1/fl1-2/fl2/fl3, sfc40, and cvp2/cvl1.*** Values are presented as the mean seed count ± standard deviation. Significant differences ($p < 0.05$) were determined by a One-Way ANOVA and Tukey's HSD post-hoc and are indicated by letters (a, b). (For each genotype, $n \geq 40$ siliques for natural self pollinations, and $n \geq 10$ for manual self pollinations, WT ♀ x Mutant ♂, and Mutant ♀ x WT ♂).





<u>Figure 4.</u> ***Expression pattern of SFC, FKD1, FL1, CVP2, CVL1 and DR5 in carpels and ovules (flower stages 10 to 12) in GUS reporter gene lines***. GUS expression was not observed in the ovules of *FL1, CVP2, CVL1 and DR5* from floral stages 10 to 12. Ovule images (S-Z) include the ovule development stages according to Schneitz *et al.* (1995) in the bottom right corner of each image. Scale bars indicate 100 μm.

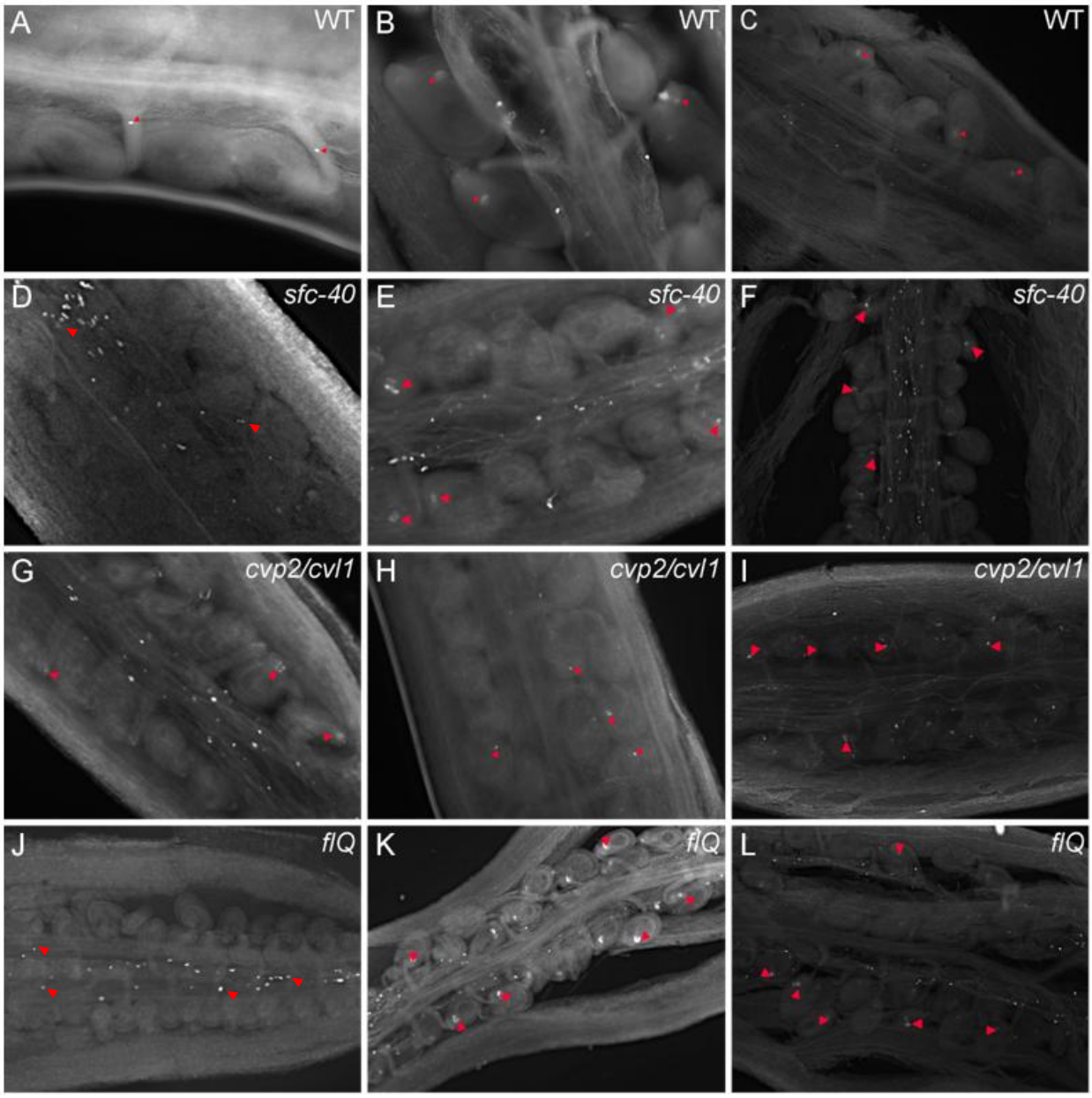


Figure 5. ***fkd1/fl1-2/fl2/fl3, sfc40, and cvp2/cvl1 pollen tube growth does not appear to differ from wild type.*** Representative images of fixed Stage 13 flowers mounted on a slide and stained with decolourized aniline blue, viewed with UV epifluorescence (≥15 siliques per genotype). Callose plug deposition on the ovule and funiculus, indicating the progression of pollen tube growth into the ovule, is indicated by red arrowheads in representative photos from each genotype. Pollen tubes were observed reaching the carpel bases and growing across the funiculus for all genotypes.

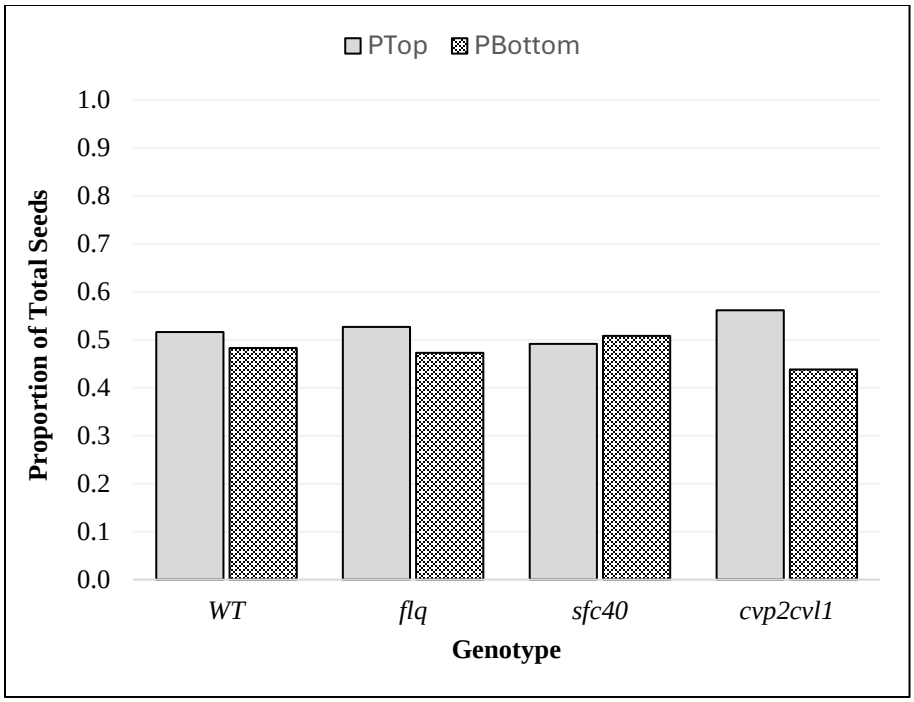


Figure 6. ***Distribution of seeds within the top and bottom halves of a silique is not significantly different from and expected value of 0.5 within and between genotypes***. Values are presented as the mean proportion of total seeds in each half of the silique. Count data was used to conduct Pearson's Chi-squared tests. The number of seeds in the top and bottom halves of a silique were counted from 30 siliques per genotype.

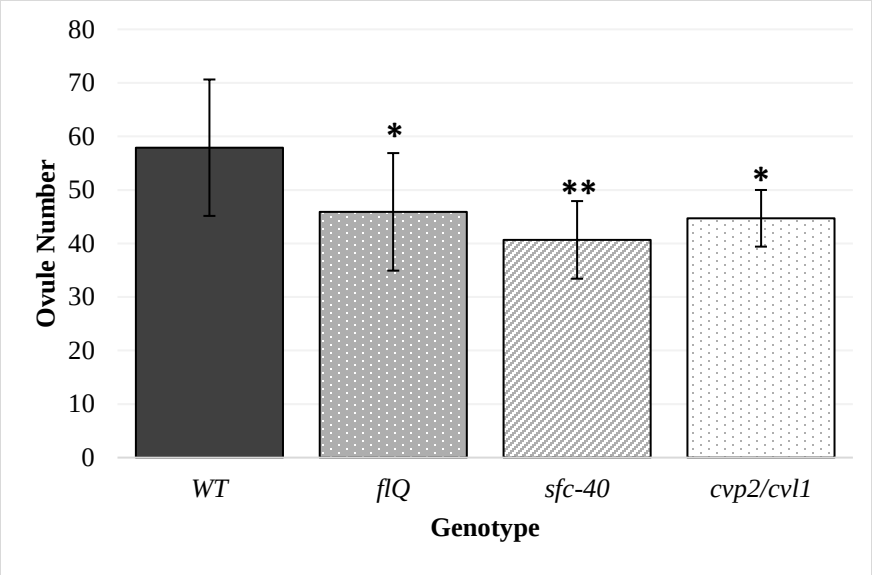


Figure 7. ***fkd1/fl1-2/fl2/fl3, sfc40, and cvp2/cvl1 plants produce a significantly fewer number of ovules than wild type plants.*** Values are presented as the mean ± standard deviation. Significant differences from wild type are indicated by asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) as determined by a One-Way ANOVA and Tukey's HSD. Counts were taken from a minimum of 10 elongated siliques per genotype.

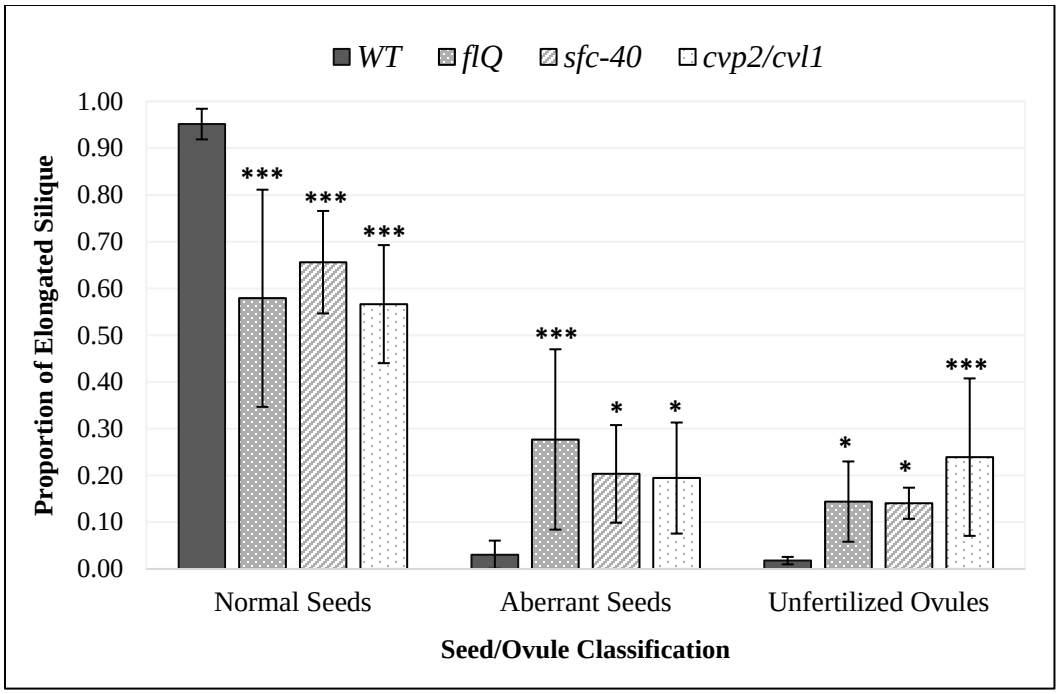


Figure 8. ***fkd1/fl1-2/fl2/fl3, sfc40, and cvp2/cvl1 siliques contain significantly more aberrant seeds and unfertilized ovules than wild type plants.*** Values are presented as the mean proportion ± standard deviation. Significant differences from wild type are indicated by asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) as determined by a One-Way ANOVA and Tukey's HSD post-hoc using count data. Counts were taken from a minimum of 10 elongated siliques per genotype. Classification of "normal" or "aberrant" seeds and unfertilized ovules follows Meinke *et al.* (2008) and the SeedGenes project database (http://www.seedgenes.org) (see **Figure A** in *Methods*).

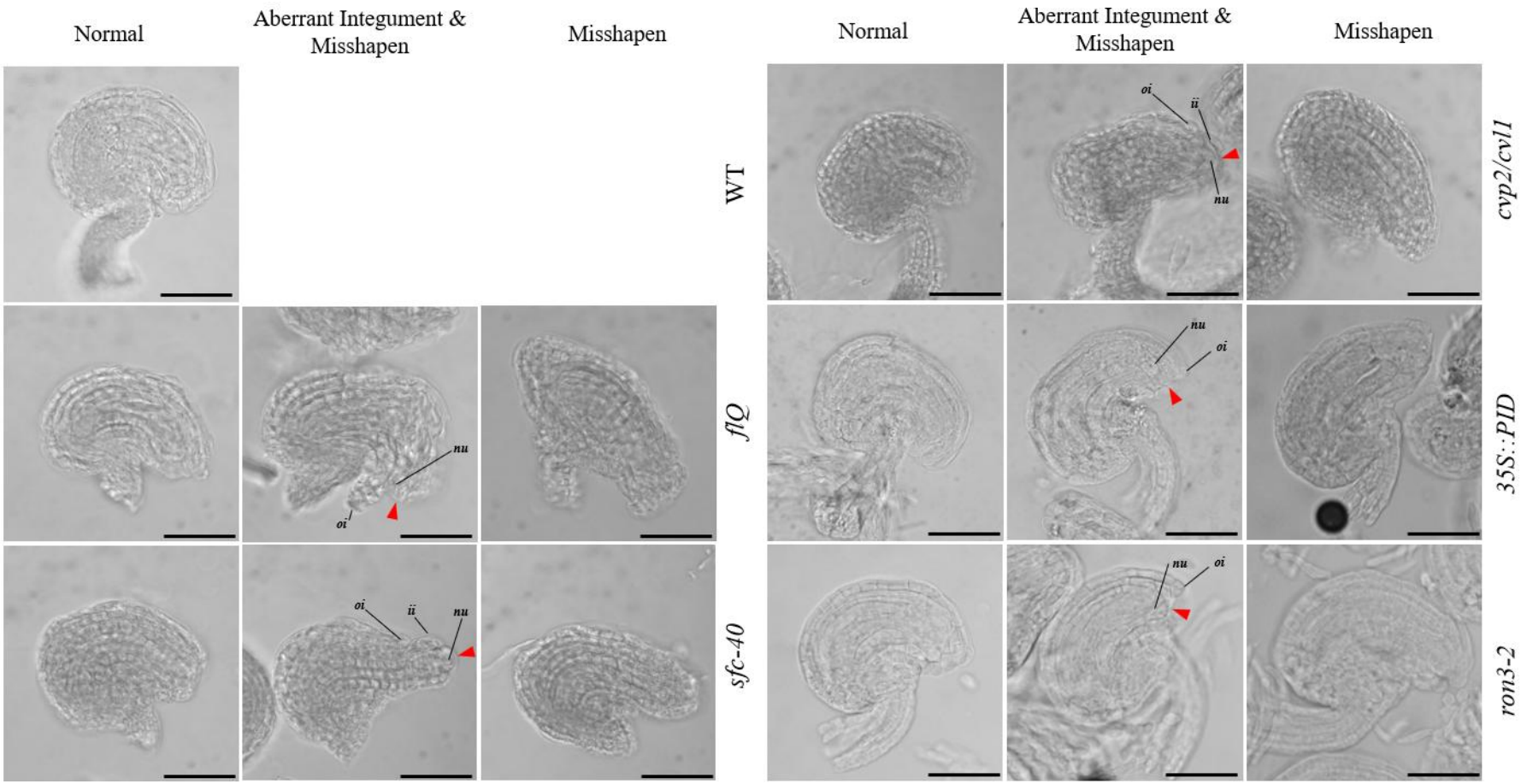


Figure 9. ***fkd1/fl1-2/fl2/fl3, sfc40, and cvp2/cvl1 ovules display integument closure defects and misshapen elongated ovules more severe than in ron3-2 and 35S::PID mutants and not present in wild type plants.*** Representative images of ovule defects were taken from ≥30 scored ovules. Red arrows indicate an exposed nucellus (nu) and outer and inner integuments (oi and ii, respectively) are labelled. Scale bars = 50 µm.

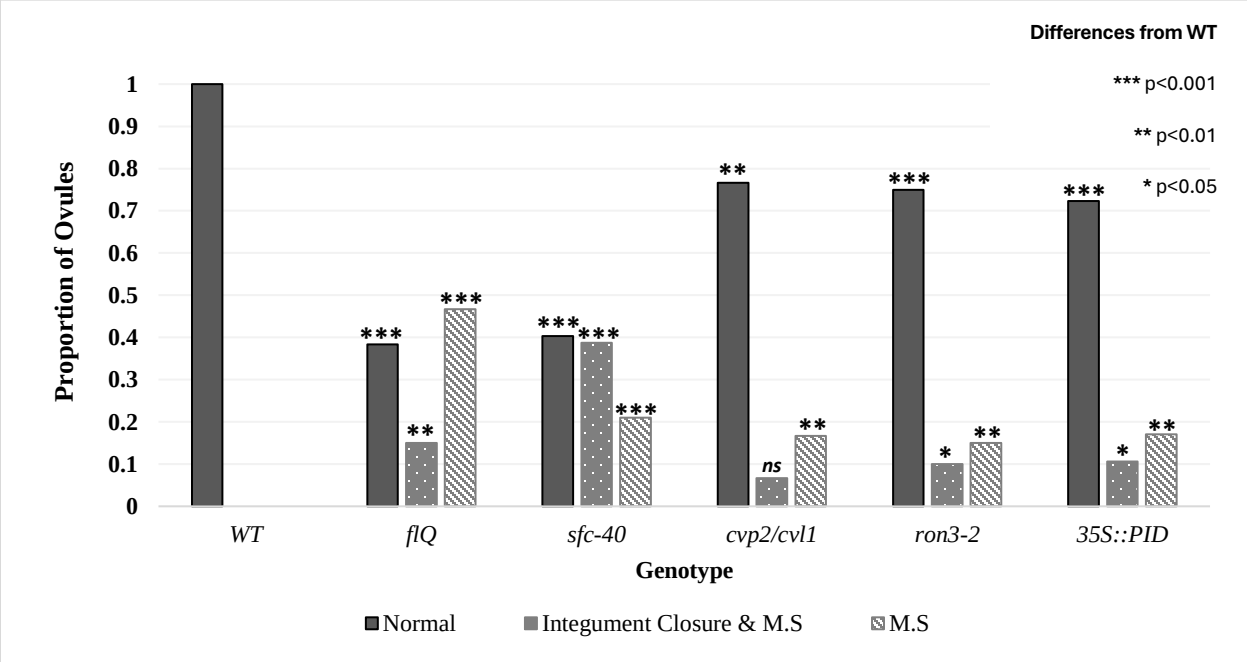
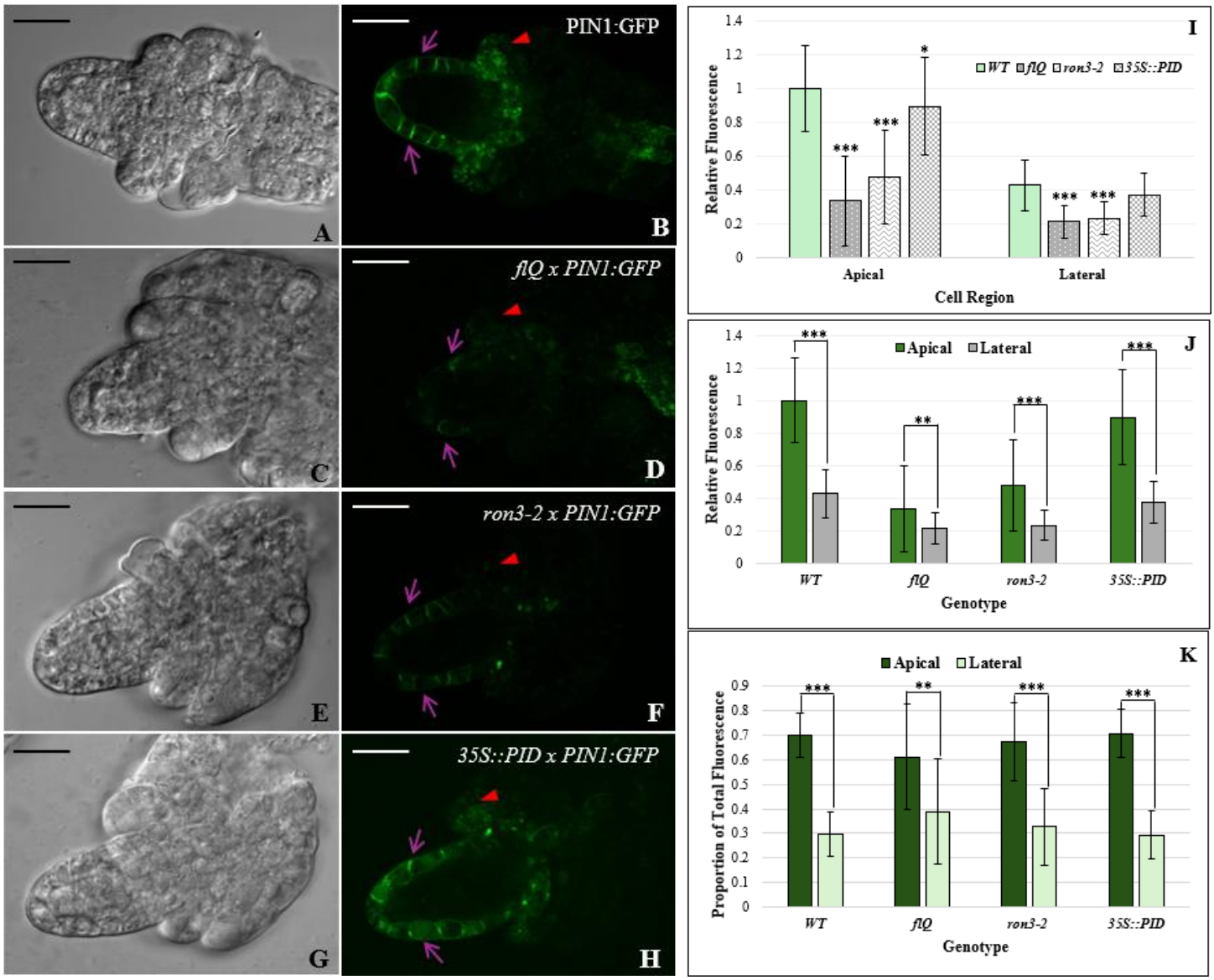


Figure 10. ***All mutants show a significantly greater proportion of ovules with integument closure defects or misshapen elongated ovules than wild type plants.*** Values are presented as the total proportion of all scored ovules. Significant differences from wild type are indicated by asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) as determined by Fischer's Exact Tests for 15 pairwise comparisons between WT and each genotype for each of the three ovule categories. p-values were adjusted using the False Discovery Rate (FDR) method for multiple comparisons. A minimum of 30 ovules per genotype from five flowers were scored. Significance was determined using a two-way ANOVA with Genotype (levels: WT, *fkd1/fl1-2/fl2/fl3, ron3-2, 35S::PID*) and Cell Region (Apical vs. Lateral) as factors.



<u>Figure 11.</u> ***fkd1/fl1-2/fl2/fl3, ron3-2, and 35S::PID display reduced PIN1:GFP localization in the apical region of nucellus cells compared to wild type plants.*** **(A-H).** Representative ovule (late stage 2) images are shown for each *pPIN1:PIN1-GFP* line (Scale bars = 15 µm). Red arrow heads indicate PIN1 localization in the inner integument, and purple arrows highlight PIN1 localization in nucellus cells **(I).** PIN1:GFP localization in all mutant lines is significantly reduced in the apical region of the nucellar cells, as well as in the lateral regions (excluding *35S::PID*) (purple arrows). **(J).** Within each genotype, apical localization was significantly greater than lateral localization. (**K**). Within each genotype, the average proportion of total flourescence localized to the apical membrane was significantly greater than the average proportion localized to the lateral membrane.. Significant differences in I-K are indicated by asterisks (* = $p<0.05$, **= $p<0.01$, ***= $p<0.001$). PIN1:GFP quantification is described in the *Methods* (See *Figure B*).

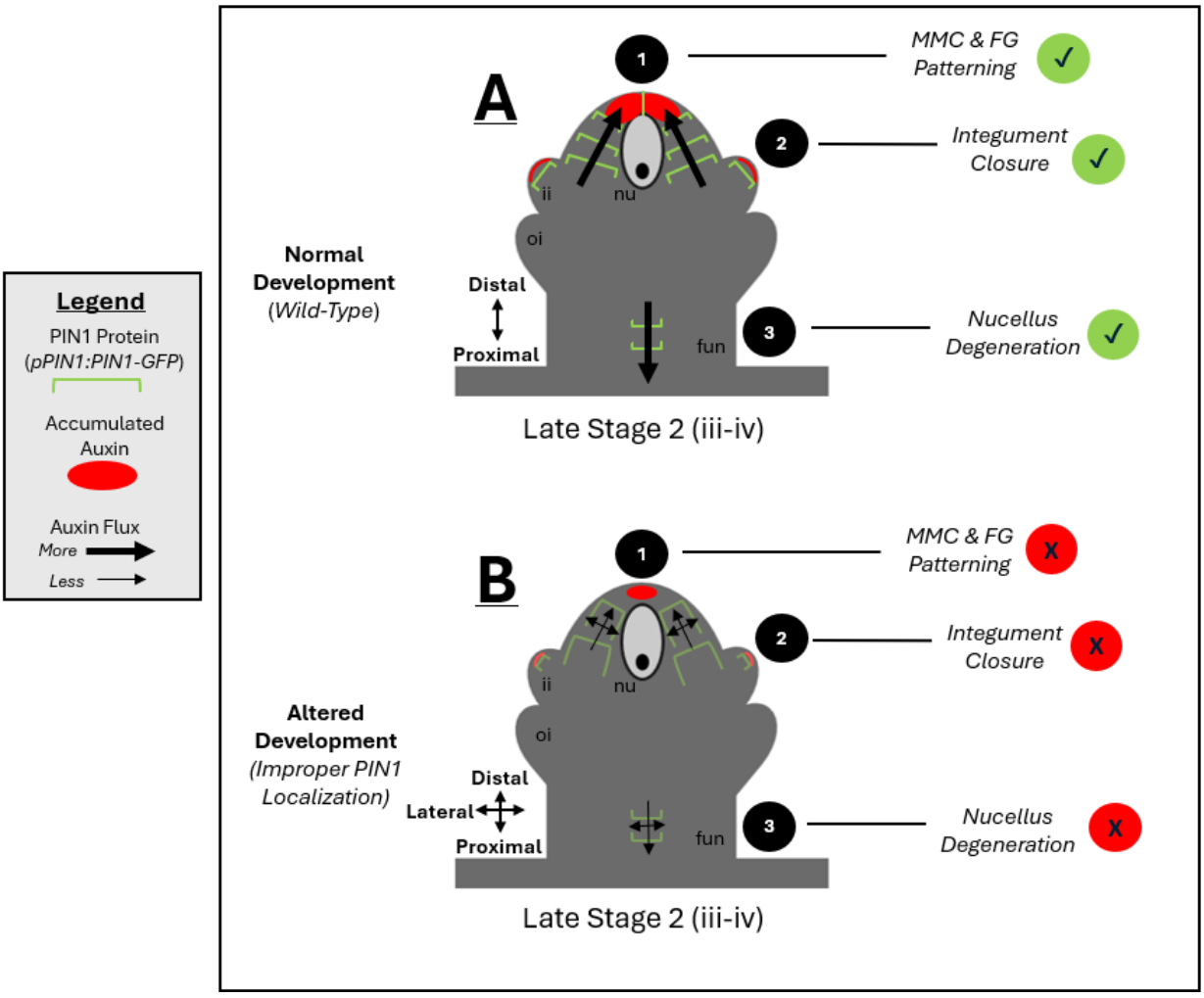
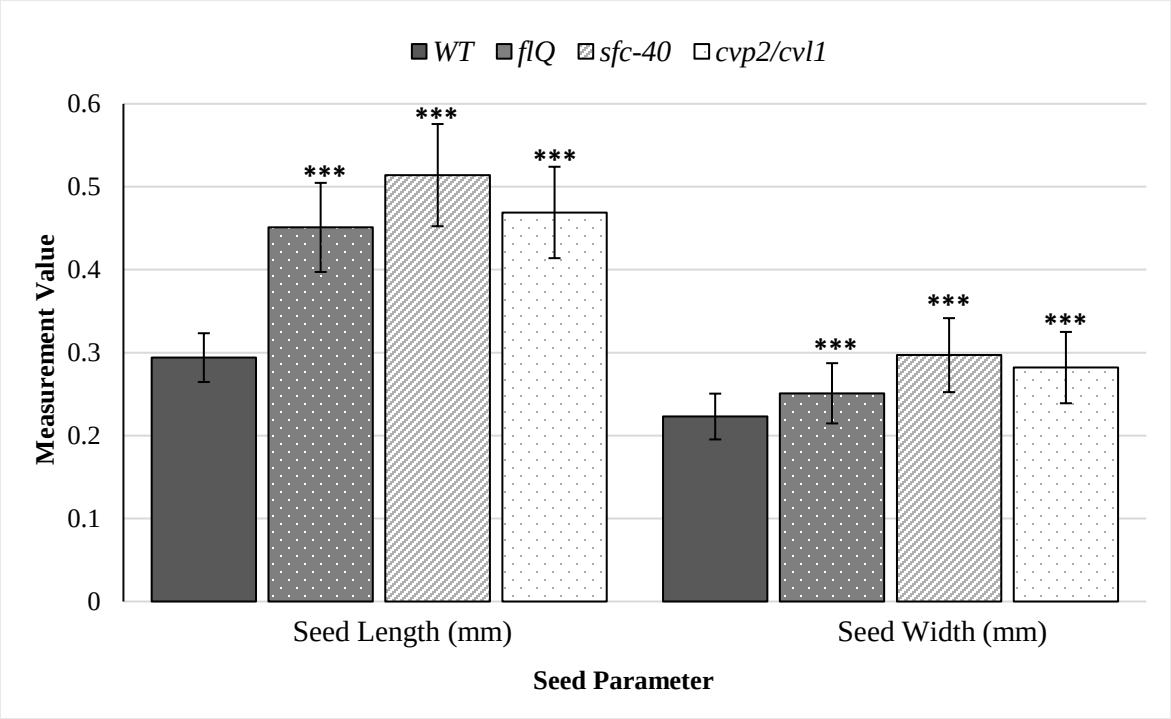


Figure 12. ***Proposed model showing altered PIN1 localization patterns in late stage ii ovules of fkd1/fl1-2/fl2/fl3, sfc40, and cvp2/cvl1, ron3-2, and 35S::PID driving downstream developmental effects resulting in reduced seed set.*** **(A)** *1.* In wild type ovules, strong apical PIN1 localization drives the transport of auxin towards the distal tip of the nucellus to establish a maximum at the apex. *2.* PIN1 localization is detectable in the inner integument primordia, which may play an important role in directing inner integument development and syncronizing the closure of inner and outer integuments. *3.* PIN1 localization in the funiculus (fun) promotes auxin drainage from late-stage ovules, important for nucellus degeneration and progression of female gametopyte (FG) development. **(B)** *1.* In ovules with altered PIN1 localization, decreased localization to the apical membrane and increased localization in the lateral membrane of nucellar cells may cause reduced polar auxin transport to the distal apex of the nucellus, and an increase in lateral diffusion of auxin. This may ultimately decrease the auxin accumulation at the apex affecting the normal progression of megaspore mother cell (MMC) and female gametophyte (FG) development. *2-3*. Decreased PIN1 localization in the inner integuments and the funiculus may drive integument closure defects and inhibit auxin drainage from a late stage ovule required for nucellus degeneration. These altered PIN1 localization patterns may ultimately be driving ovule development defects resulting in reduced seed set.

5.1 Supplemental Figures



<u>Figure S1.</u> ***fkd1/fl1-2/fl2/fl3 (flQ), sfc-40,*** **and** ***cvp2/cvl1*** **produce seeds with a significantly greater length and width than wild type seeds**. Values are presented as the mean ± standard deviation. Significant differences from wild type are indicated by asterisks (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) as determined by a One-Way ANOVA and Tukey's HSD post-hoc. A minimum of 30 seeds were measure per genotype.

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CONCLUSION

The formation of the gynoecium, including the stigma, style, transmitting tract, ovary, ovules, and female gametophytes requires the correct establishment of auxin gradients across a tissue directed by polar transport. PIN-FORMED (PIN) proteins are crucial for establishing local cellular auxin gradients that establish spatially organized auxin maxima and minima. PIN1 vesicle transport is tightly regulated by various genes that play a role in vesicle formation, transport, uncoating, and membrane localization. *SCARFACE/VASCULAR NETWORK DEFECTIVE 3* (*SFC/VAN3*), *FORKED1* (*FKD1*) & *FORKED-LIKE* (*FL*) Group 1 genes, *COTYLEDON VASCULAR PATTERN 2* (*CVP2*) and *CVP2-LIKE 1* (*CVL1*), *PINOID* (*PID*), and *ROTUNDA3* (*RON3*) have all been proposed to be key players in the regulation of PIN1 vesicle trafficking. Mutations to these genes have been shown to disrupt PIN1 localization and lead to developmental defects such as impaired root development and vein formation. However, the role of these genes has not been previously characterized in gynoecium development, despite mutants showing severe reductions to seed set.

My study found that several of these genes that have previously only been known to be important for leaf development are also expressed in the developing carpel tissue and ovules, particularly in the funiculus during early ovule development stages. Additionally, *fkd1/fl1-2/fl2/fl3*, *cvp2/cvl1*, *sfc-40* mutants all show significant decreases to seed production, and my findings suggest that these seed set reductions are due to gynoecium defects including decreased ovule production, integument closure defects, and improper nucellus elongation. In summary, my findings suggest that PIN1 localization and auxin transport is especially important during early ovule development stages to direct proper development in later stages, with defects in these early stages causing downstream “cascading” effects (**Figure 1**). Specifically, I suggest that PIN

localization is affected during early stages of ovule development in these mutants which alters the initial establishment of an auxin gradient in the developing ovule. This early disruption may ultimately be compromising subsequent development processes and preventing proper synchronized development in the ovule, leading to the observed developmental defects in ovule morphology, integument closure, and nucellus elongation. The incidence and severity of these observed ovule defects are also supported by altered localization of PIN1 in developing nucellus of late stage-ii ovules of a subset of the mutants that were examined. My study suggests, for the first time, that *SFC*, *FKD1*, *FL1*, *FL2*, *FL3*, *CVP2*, and *CVL1* play an important role during reproductive development and I suggest that this is due to the role these genes have in PIN1 localization and subsequent polar auxin transport during early ovule development

Further study of megagametogenesis and integument formation in *fkd1/fl1-2/fl2/fl3, cvp2/cvl1, sfc-40* will help to clarify the specific stages at which these defects arise to ultimately contribute to the large reductions in seed set that I observed in these PIN1 localization pathway mutants. Additionally, assessing specification of the megaspore mother cell in the mutants will help to determine if altered PIN1 localization in the developing nucellus is affecting auxin maximum establishment at the distal tip sufficiently enough to inhibit megaspore mother cell patterning and formation. The use of an active auxin-responsive promoter, such as DR5:GFP, to visualize auxin response in the developing nucellus would help to confirm if the observed PIN1 localization patterns are indeed affecting auxin response as predicted. This analysis would involve the introgression of the DR5:GFP marker into the mutant lines, similar to the process by which the PIN1:GFP lines were generated for this study. Overall, my research further contributes to the body of literature addressing the importance of PIN1 localization and auxin transport to

reproductive development in plants and identifies a set of novel genes that are implicated in these processes.

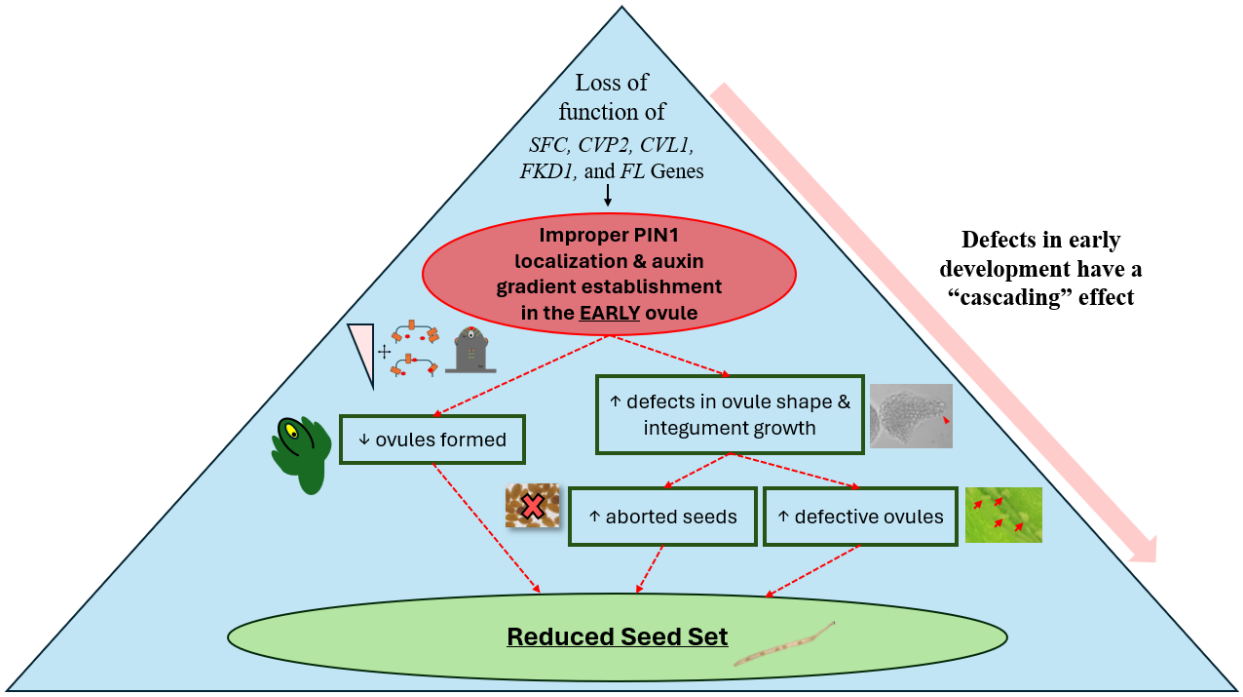

Figure 1. ***Defects in early ovule development have a "cascading" effect resulting in reduced seed set.*** The main findings of my research, summarized here, provide novel experimental evidence to support and expand on previous models of PIN1 localization and auxin transport in ovule development, and suggest that correct PIN1 localization and auxin transport is particularly important during early ovule development stages to direct proper development in later stages.