Understanding the Molecular Basis of Differential Growth during Apical Hook Development

Kristoffer Jonsson
Faculty of Forest Sciences
Department of Forest Genetics and Plant Physiology
Umeå

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Abstract

Plants' adaptation to their environment often involves change in development, which in many cases involves the establishment of differential growth rates across organs, for instance during phototropic and gravitropic responses. A striking example of differential growth is the formation of the apical hook, a structure that forms to protect the apical meristem as seedlings penetrate through soil. Coordination of differential growth across tissues is a multilayered process involving the combined effect of spatiotemporally controlled events such as gene expression, biosynthesis of proteins and polymers, transport and incorporation of biosynthetic products to their sites of participation, regulation of expansion driven by vacuolar turgor and control of cell mechanical properties via cell wall modifications.

This thesis addresses mechanisms that underlie differential growth, using the apical hook as a model. Particularly, this work focuses on the role of two distinct but interrelated processes; transport of components to the cell surface, and regulation of composition of components at the cell surface in apical hook development. This work demonstrates that secretion of different auxin carriers follow distinct routes from the *trans*-Golgi network (TGN) to the plasma membrane, where delivery of AUX1 but not PIN3 relies the TGN-localized protein ECHIDNA (ECH). Data show that the ECH-dependent secretory pathway is essential for ethylene-mediated differential growth of the apical hook in *Arabidopsis*. Moreover, this work investigates the mechanism by which ECH operates, and shows that ECH is required for the localization of the GTPase ARF1 and its activator GEFs BIG1-4, which are key components of a vesicle formation machinery at the TGN. ARF1 members and BIG1-4 are, like ECH, required for AUX1 delivery to the PM and for ethylene-mediated hook development. Finally, the thesis explores the role of the cell wall in differential growth, particularly, that of homogalacturonan pectin and its modification by methylesterification. This thesis demonstrates that differential cell elongation during hook development relies on establishing asymmetric cell wall mechanical properties across the hypocotyl via pectin methylesterification modifications in an auxin-dependent manner, and that a mechanochemical component provides feedback to the auxin machinery.

Taken together, this thesis demonstrates the multilayered regulation of growth asymmetry which facilitates shape generation.

Keywords: *Arabidopsis*, differential growth, apical hook, secretion, TGN, auxin transport, ECHIDNA, cell wall, pectin, methylesterification

Author's address: Kristoffer Jonsson, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, SE-901 83 Umeå, Sweden
E-mail: Kristoffer.jonsson@slu.se

Till Karla, Kjell och Klint, mina älsklingar!

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I Yohann Boutté*, **Kristoffer Jonsson***, Heather McFarlane, Delphine Gendre, Ranjan Swarup, Jiri Friml, Lacey Samuels, Stephanie Robert, Rishikesh Bhalerao (2013). ECHIDNA-mediated post-Golgi trafficking of auxin carriers for differential cell elongation. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*, 100(40):16259-64

II **Kristoffer Jonsson**, Yohann Boutté, Rajesh Singh, Delphine Gendre, Rishikesh Bhalerao (2017). Ethylene Regulates Differential Growth via BIG ARF-GEF-Dependent Post-Golgi Secretory Trafficking in Arabidopsis. *The Plant Cell*, 29:1039-1052

III **Kristoffer Jonsson**, Rahul Lathe, Daniel Kierzkowski, Anne-Lise Routier-Kierzkowska, Olivier Hamant, Rishikesh Bhalerao. Mechanochemical feedback between auxin and pectin modification mediates the control of apical hook development. *Manuscript*.

* Authors contributed equally to this work

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The contribution of Kristoffer Jonsson to the papers included in this thesis was as follows

I Performing experiments, analysing data, contributions to writing and formatting the manuscript

II Planning and performing experiments, analysing data, writing and formatting the manuscript.

III Planning and performing experiments, analysing data, writing and formatting the manuscript

Abbreviations

1-NOA	1-naphthoxyacetic acid
ABCB	ATP-binding cassette, sub-family B protein
ABCG	ATP-binding cassette, sub-family G protein
ACC	1-aminocyclopropane-1-carboxylic acid
AFB	Auxin signaling F box protein
AFM	Atomic force microscopy
ALA	Aminophospholipid ATPase
ARF (1)	ADP-Ribosylation factor
ARF (2)	Auxin response factor
ARF-GEF	ARF Guanine Exchange Factor (See ARF (1))
AUX	Auxin resistant
Aux/IAA	Auxin/Indole-3-Acetic Acid
BAK	BRI1-associated receptor kinase (See BRI1)
BDL	Bodenlos
BFA	Brefeldin A
BIG	BFA-inhibited GEF
BRI1	Brassinosteroid insensitive 1
CCV	Clathrin-coated vesicle
CESA	Cellulose synthase
CGR	Cotton Golgi-related
CHX	Cycloheximide
CIN	Cytokinin insensitive
CMT	Cortical microtubule
ConcA	Concanamycin A
COP	Coat protein
CTR	Constitutive triple response
Cyc1B	Cyclin 1B
D6PK	D6 protein kinase

EBS	EIN3 binding site
ECH	Echidna
EE	Early endosome
EIN	Ethylene insensitive
ER	Endoplasmic reticulum
ERS	Ethylene response sensor
ESCRT	Endosomal sorting complexes required for transport
ETR	Ethylene resistant
FAM18B	Family with sequence similarity 18 member B
FRAP	Fluorescence recovery after photobleaching
GA-TGN	Golgi-associated TGN (See TGN)
GBF	Golgi-Specific Brefeldin A Resistance Factor
GFP	Green fluorescent protein
GI-TGN	Golgi-independent TGN (See TGN)
GNL	GNOM-Like
GSL	Glucan synthase-like
GUS	β-glucuronidase
HLS	Hookless
IAA	Indole-3-acetic acid
IAMT	IAA carboxyl methyltransferase
KEG	Keep on going
KN	Knolle
LAX	Like-auxin resistant
LE	Late endosome
MASC	Microtubule-associated CESA compartment (See CESA)
mRNA	messenger ribonucleic acid
MT	Methyltransferase
MVB	Multivesicular body
NPA	1-N-naphthylphtalamic acid
OG	Oligogalacturonide
PA	Phosphatidic acid
PC	Phosphatidylcholine
PG	Polygalacturonase
PHOT	Phototropin
PI4K	Phosphatidylinositol-4-phosphate kinase
PID	Pinoid
PILS	Pin-likes
PIN	Pin-formed
pKa	Acid dissociation constant
PL	Pectate lyase

PLD	Phospholipase D
PM	Plasma membrane
PME	Pectin methylesterase
PMEI	PME inhibitor (See PME)
QUA	Quasimodo
RAB	Ras-associated binding
RALF	Rapid alkalinisation factor
RG	Rhamnogalacturonan
RLK	Receptor-like kinase
RLP	Receptor-like protein
SAM	Shoot apical meristem
SCAMP	Secretory carrier membrane protein
SEC	Secretory
SecGFP	Secretory GFP (See GFP)
SHY	Short hypocotyl
SmaCC	Small CESA compartment (See CESA)
SNX	Sortin nexin
SUR	Superroot
SV	Secretory vesicle
SVC	SCAMP2-marked mobile secretory vesicle compartment (See SCAMP)
SYP	Syntaxin of plants
TAA	Tryptophan aminotransferase
TAR	Tryptophan aminotransferase related
TGN	*trans*-Golgi network
TIR	Transport inhibitor response
TRAPPII	Transport protein particle II
TVP	TLG2 compartment vesicle protein
USP	Unconventional secretory pathway
VHA	Vacuolar H^+ ATPase
WAK	Wall-associated kinase
XGA	Xylogalacturonan
YIP	YPT/RAP GTPase interacting protein
YUC	Yucca

1 Introduction

In all organisms, growth and development involves changes in body plan (Gilbert, 2000). In multicellular organisms, these processes must be coordinated at multiple levels. In animals, cellular migration plays important roles, particularly in embryonic development, immune responses and wound healing (Trepat et al., 2012). In stark contrast, cells of plants cannot migrate, and development of their body plans and associated structures involves tightly coordinated changes in the size and structure of cells, tissues and organs. This involves multilayered, spatiotemporally integrated processes such as gene expression, biosynthesis of proteins and polymers, transport and incorporation of biosynthetic products to their functional sites, regulation of expansion forces through vacuolar turgor and control of cells' mechanical properties through cell wall modifications. In this thesis, I address how differential growth contributes to plant development, focusing particularly on roles of two distinct but interrelated processes: transport of substances to plant cells' surfaces, and regulation of the composition of materials at their surfaces.

1.1 Differential growth in plants

Plant growth involves increases in the number of cells via cell division and changes in cells' size via elongation and expansion (Sablowski, 2016). The relative importance of cell division and expansion varies among developmental processes. Division is extremely important during plant embryogenesis, in which series of asymmetric cell divisions, together with cell differentiation, transforms a zygote into a mature embryo with a basal body plan and strictly defined shape (van Dop et al., 2015). Other striking examples of morphogenetic processes involving sequences of asymmetric cell divisions include the formation of stomata and lateral root primordia (Torii, 2015; Du and Scheres, 2018).

While asymmetric cell divisions play key roles in plant development, as noted above, plants' adaptation to their environment often involves differential growth at the organ level, driven by differential rates of elongation in cells of a tissue or organ rather than asymmetric cell divisions (Dunser and Kleine-Vehn, 2015). I briefly describe some extensively studied systems for studying differential growth, and then provide a more detailed description of apical hook development, in the following sections.

Tropisms – Models for studying differential growth

The most thoroughly studied example of differential growth may be the gravitropic response of roots. The main root of a plant generally exhibits positive gravitropism, that is, grows downward along the gravity vector, and if it is tilted so its angle deviates from this vector, a machinery is set in motion that re-orients growth (Figure 1A). Starch-filled amyloplasts (organelles within cells) called statholiths sediment to the bottom of columella cells, the sites of graviperception in roots (Sack, 1991; Blancaflor et al., 1998). When the root is tilted, statholiths move to the new bottom within 5 minutes, causing basal polarization of several PIN-FORMED (PIN) auxin efflux carriers, and hence re-direction of transport of the growth hormone auxin towards the lower part of the root (Kleine-Vehn et al., 2010). Auxin is subsequently transported shootward by polarly localized PIN2 in the epidermis towards the elongation zone (Muller et al., 1998; Wisniewska et al., 2006). The resulting increase in auxin levels on the lower side causes a transient increase in pH in the apoplast (the space outside plant cells' outer membranes, described in more detail later) that is thought to inhibit elongation, while the lower auxin levels on the upper side are growth-permissive, sparking elongation (Barbez et al., 2017).

Another well-studied differential growth process is the phototropic bending of the hypocotyl in response to blue light (Figure 1B). Since the seminal examination of coleoptile bending in response to unilateral light by Charles and Francis Darwin (Darwin, 1880), abundant information has been obtained on the mechanisms involved in this process. Direction of light is perceived mainly by blue light receptors PHOTOTROPIN1 (PHOT1) and PHOT2 (Briggs et al., 2001), which inhibit activity of the protein kinase PINOID upon illumination, thereby limiting PIN3 phosphorylation. This directs PIN3 trafficking via the ARF-GEF GNOM recycling pathway towards cells' inner lateral membrane (Ding et al., 2011). Furthermore, PHOT1 directly phosphorylates the auxin efflux carrier ABCB19, inhibiting its auxin transport capacity (Christie et al., 2011). Unilateral light thus triggers polarization of auxin transport, directing

auxin towards the shaded side of the plant. The resulting auxin gradient causes asymmetric elongation, re-aligning the hypocotyl towards the light source.

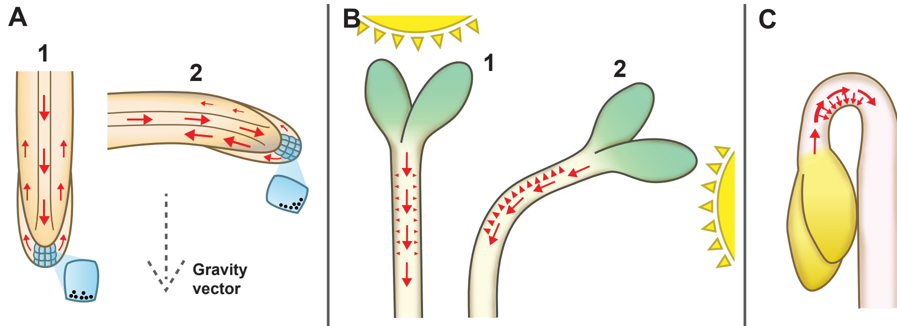

Figure 1. **Auxin gradients mediate differential growth processes. (A)** Root gravitropic response, (1) Auxin is transported rootward via the central cylinder. When the root is aligned with the gravity vector, statoliths sediment to the bottom of columella cells (blue), and shootward transport of auxin (red arrows) is symmetric. (2) When the root is tilted, statoliths sediment to the new bottom, causing redirection of auxin transport towards the lower side of the root, where high auxin levels inhibit elongation. Conversely, low auxin levels on the upper side of the root promotes growth. **(B)** Hypocotyl phototropism. (1) When light illuminates the plant uniformly, auxin is transported rootward via the central cylinder (red arrows), and is radially directed uniformly to the outer cell layers. When light illumination is non-uniform, radial auxin transport is inhibited on the illuminated side, causing preferrential auxin transport towards the shaded side, where high auxin levels promote elongation. **(C)** Apical hook development. In darkness, auxin is transported rootward from the cotyledons and/or apical meristem via the central cylinder (red arrows). In the hook, auxin is transported towards the inner side of the hook, inhibiting cell elongation.

1.2 Apical hook development

This thesis focuses primarily on another striking example of differential growth, which leads to formation of an apical hook during the germination of dicotyledonous plants (Figure 1C). In *Arabidopsis*, this occurs 8-12 hours after germination through bending of the shoot apical meristem (SAM)-proximal part of the hypocotyl until it becomes semitoroidal, i.e., a cylinder with approximately 180° curvature (Mazzella et al., 2014) (Figure 1C, Figure 2). Formation of this structure is part of a developmental process called skotomorphogenesis, which involves a suite of adaptive responses to the challenges posed by germination of seedlings buried in dark, hypoxic soil (Josse and Halliday, 2008). Skotomorphogenesis also involves rapid growth of the hypocotyl and folded cotyledons. The hook structure provides protection for the fragile cotyledons and apical meristem from physical trauma by folding them

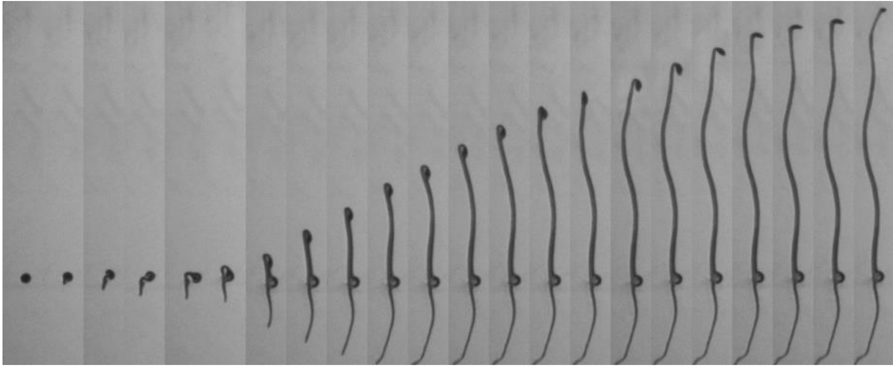

Figure 2. Time-lapse imaging of dark-grown *Arabidopsis* wild-type seedling, imaged at 6-hr intervals

away and causing the cuticle-clad hypocotyl to lead the upward penetration of the buried seedling's shoot through the soil (Briggs, 2016). The hook remains closed (usually for several days) as the seedling grows through soil, and upon perception of light it rapidly uncurls and the plant transitions to a photomorphogenic lifestyle (Liscum and Hangarter, 1993). In contrast to tropic responses, hook development is an intrinsic developmental program that is initiated even in the absence of external stimuli, although external factors such as light and oxygen levels may modulate their timing (Powell and Morgan, 1970; Liscum and Hangarter, 1993; Abbas et al., 2015).

The apical hook is an outcome of differential growth that is largely if not exclusively due to asymmetric cell elongation on opposing sides of the post-embryonic hypocotyl (Silk and Erickson, 1978; Raz and Ecker, 1999). Thus, understanding the regulation of differential cell elongation is crucial for elucidating the intricate mechanisms of hook development.

The regulation of skotomorphogenic hook development is addressed in detail in the following sections. Photomorphogenetic processes, such as light-induced hook opening, are beyond the scope of the thesis, and thus are not further considered here.

1.2.1 Hormonal regulation of apical hook development

Like most developmental processes, apical hook development is tightly controlled by a complex hormonal network or 'cross-talk', which allows fine-tuning of every phase. Roles of the hormones ethylene and auxin in the process have received the most attention. Thus, in this section I focus on the involvement of these key players in hook development.

1.2.2 Auxin

Auxins are a class of phytohormones (often collectively called auxin) that participate in regulation of many aspects of plant growth and development, *inter alia*, embryo patterning, cell expansion and division, organ development, tropic responses and senescence (Khan et al., 2014; Rakusova et al., 2015; Smit and Weijers, 2015; Taylor-Teeples et al., 2016). Auxin was originally identified in study of plant tropisms, i.e., the differential growth of plant organs in response to an external cue, which were predicted to involve unequal distribution of auxin, as formalized in Cholodny-Went theory (Went and Thimann, 1937). This prediction has proved largely correct. Examples are tropic responses of young roots and shoots, in which re-direction of asymmetric auxin transport causes unequal growth of organs. There are several forms of auxin, but the most important active form is indole-3-acetic acid (IAA), which is also simply (and rather confusingly) called auxin sometimes (Simon and Petrasek, 2011). This was also the first discovered plant hormone (Went, 1926). Auxin has been known to modulate curvature of the apical hook for more than 50 years (Kang et al., 1967). However, the earliest experimental clues that hook development shared a central feature of tropic responses – an auxin distribution gradient – were much more recent demonstrations of the preferential accumulation of auxin in the inner side of the hook in etiolated *Phaseolus vulgaris* (bean) seedlings (Schwark and Schierle, 1992). The presence of an asymmetric auxin gradient in the hook has been subsequently confirmed in several studies, mainly through use of synthetic auxin-responsive promoter DR5 reporter constructs in experiments with *Arabidopsis* (Friml et al., 2002; Li et al., 2004; Zadnikova et al., 2010) (Figure 3). Subsequent work has established a genetic framework for the involvement of auxin during hook development. The

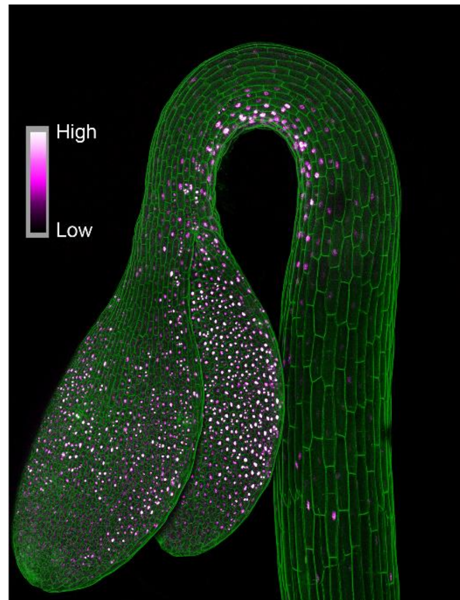

***Figure 3*. Auxin is asymmetrically distributed during hook development.** Confocal image of the apical hook, depicting the auxin response pattern, as reported by the synthetic auxin responsive reporter promoter DR5-Venus (magenta heat), counterstained with propidium iodide (green).

mechanism and factors involved in generation of an auxin gradient are described below.

Auxin metabolism

Mutations to genes involved in auxin biosynthesis, including YUCCA Flavin monooxygenases (Zhao et al., 2001), *TRYPTOPHAN AMINOTRANSFERASE 1*/*TRYPTOPHAN AMINOTRANSFERASE RELATED 2* (*TAA1*/*TAR2*) (Stepanova et al., 2008; Vandenbussche et al., 2010) and *SUPERROOT 1/2* (*SUR1/SUR2*) (Boerjan et al., 1995; Delarue et al., 1998), severely perturb hook development. Moreover, hook formation is impaired in *yuc1/2/4/6* and *wei8 tar2* mutants, highlighting the requirement for proper auxin biosynthesis in hook development (Stepanova et al., 2008; Vandenbussche et al., 2010; Stepanova et al., 2011). Interestingly, although some auxin biosynthesis genes are expressed in the hook, their expression pattern is generally not asymmetric, except for upregulation of *YUC1* on the outer side during hook opening and *TAR2* on the inner side during the exaggerated curvature response to ethylene (Stepanova et al., 2008; Vandenbussche et al., 2010) which is further discussed below. Furthermore, upon treatment with the auxin transport inhibitor 1-N-naphthylphtalamic acid (NPA), activity of the auxin signaling reporter *DR5::GUS* is strongly elevated in the cotyledons, implying that cotyledons are the main sources of auxin in the process, and asymmetric biosynthesis in the hook itself is not the main generator of the auxin gradient in the hook (Zadnikova et al., 2010). The involvement of auxin catabolism in hook development is scarcely investigated. IAA CARBOXYL METHYLTRANSFERASE1 (IAMT1) catalyzes IAA methylation (Zubieta et al., 2003). Methylated auxin (Me-IAA) is considered an inactive form of auxin (Qin et al., 2005). *iamt1* mutants prematurely open their hooks, suggesting that auxin metabolic pathways beyond biosynthesis operate to regulate hook development (Abbas et al., 2018).

Auxin transport

Although auxin biosynthesis is required for hook development, active carrier-mediated transport of auxin is thought to be the main mechanism responsible for asymmetric auxin distributions in the hook. IAA is a weak acid (pKa 4.75), and in the apoplast, which is mildly acidic, a small percentage of IAA can diffuse passively in protonated form across the plasma membrane. However, most is in polar IAA^- form, requiring active carrier-mediated transport to enter cells (Swarup and Peret, 2012). Inside the largely neutral cytoplasm, IAA almost exclusively exists in its dissociated IAA^- form, requiring active transport to exit cells. The movement of auxin across the plasma membrane is facilitated by auxin

influx carriers of the AUXIN RESISTANT 1 (AUX1)/ LIKE-AUXIN RESISTANT1 (LAX) family (Peret et al., 2012; Swarup and Peret, 2012) and efflux carriers of the PIN-FORMED (PIN) (Adamowski and Friml, 2015) and P-glycoprotein (ABCB/PGP) families (Geisler et al., 2017), as well as PIN-LIKES (PILS; putative auxin carriers) (Barbez et al., 2012). The activity of PIN and AUX/LAX proteins can be blocked by NPA and 1-naphthoxyacetic acid (1-NOA), respectively, and treatment with either drug perturbs establishment of auxin response asymmetry and hook formation (Vandenbussche et al., 2010; Zadnikova et al., 2010). Two of the influx carriers, AUX1 and LAX3, exhibit dramatically differing expression patterns in aerial tissues of etiolated seedlings, but appear to have overlapping functions. AUX1 is expressed mainly in the epidermis in the apical hook, localizing to the plasma membrane in a non-polar fashion. Its expression is strongly elevated on the inner side of the hook upon ethylene treatment. In contrast, LAX3 expression in the hook is restricted to the stele, but it is also expressed in epidermis close to the hypocotyl-root junction, in both cases non-polarly localizing to the plasma membrane (Vandenbussche et al., 2010). The *aux1* mutant does not exhibit any hook defects under control conditions, but its hook curvature is not exaggerated in responses to ethylene (discussed below). However, the *lax3* mutant cannot fully form a closed hook, and *aux1 lax3* double mutants have severe phenotypic perturbations, indicating synergistic effects (Vandenbussche et al., 2010). The localization patterns indicate that AUX1 may recruit auxin to the hook from its presumed source in the cotyledons, while LAX3 might participate in fine-tuning of auxin depletion from the hook region.

Of the efflux carrier families, PINs have been most thoroughly investigated in the context of hook development. Four (PIN1, PIN3, PIN4 and PIN7) are expressed during, and contribute to, the process. Correspondingly, mutations to these PIN genes cause hook developmental defects (Zadnikova et al., 2010). During hook formation, PIN3 and PIN4 are expressed in the central cylinder, cortex and epidermis of the developing hook, while PIN1 is solely expressed in the central cylinder (Zadnikova et al., 2010; Zadnikova et al., 2016). As formation is completed, PIN1 expression and plasma membrane localization is restricted to epidermis on the inner side. PIN4 expression also increases on the inner side of the hook during the maintenance phase, while PIN3 is expressed somewhat more strongly on the outer side. Cortical PIN3 localizes preferentially toward the outer-facing longitudinal membrane, while PIN7 expression is restricted to lower parts of the hypocotyl during hook formation, and gradually increases close to the meristem as its development proceeds (Zadnikova et al., 2010; Zadnikova et al., 2016).

The genetic and cell biological data clearly show that concerted action of auxin transport machinery plays a key role in hook development. However the individual roles of the auxin transporters and how their expression patterns and localizations result in precise generation of auxin gradient are far from fully elucidated. Based on the localization of auxin transporters, several models have been suggested for the regulation of auxin gradients during hook development. One posits that cotyledon- or meristem-derived auxin is transported through the stele, and subsequently released towards epidermis on the outer side in a PIN-dependent manner (Zadnikova et al., 2016; Beziat and Kleine-Vehn, 2018). Auxin is then radially transported through epidermis towards the inner side by the joint function of PINs and AUX1. However, this model ignores factors that regulate transporter subcellular targeting and activity. Additional studies of PIN regulators, such as AGCVIII kinases of the PINOID (PID)/WAG and D6PK subfamilies (Willige and Chory, 2015), are probably needed for full understanding of the intricacies of transport-dependent establishment of auxin gradients during hook development.

PID/WAGs are known to regulate PIN polarity by phosphorylating PINs at conserved sites (Christensen et al., 2000; Friml et al., 2004), while the D6PK family, comprising four members, may regulate PINs by polarly controlling their activity through phosphorylation (Weller et al., 2017). In support of this hypothesis, *wag2* mutants exhibit apical hook defects and reduced DR5 expression on the inner side of the hook (Willige et al., 2012). Various D6PK double and higher-order mutants exhibit strong differential growth defects during phototropism of the hypocotyl, thought to be caused by reductions in PIN phosphorylation that impair the plants' ability to establish asymmetric auxin gradients (Willige et al., 2013). Thus, it seems fair to assume that tight regulation of PIN phosphorylation might also be needed during hook development.

There is also some evidence of the involvement of other transporter families, although their potential roles have received little attention. For example, the timing and rate of hook opening partly depend on depletion of auxin on the inner side via ABCB19-mediated auxin efflux (Wu et al., 2010) and reduction on the inner side of nuclear auxin signaling through PILS-mediated transport of auxin into the endoplasmic reticulum (ER) (Beziat et al., 2017).

In summary, joint activities of both auxin efflux and influx carriers are essential in establishment of an asymmetric auxin gradient toward the inner side of the hook.

Auxin signaling

The asymmetric auxin gradient established during hook formation must be translated via a signaling pathway to result in asymmetric growth. In canonical

auxin signaling, auxin is perceived by co-receptor complexes comprising TIR/ABF F-box and AUX/IAA proteins (Peer, 2013). Upon auxin binding, AUX/IAAs become ubiquitinated, destining them for degradation by 26S proteasomes (Santner and Estelle, 2010). Since AUX/IAAs function as repressors of auxin response factors (ARFs), their degradation following auxin perception results in release of ARFs' repression, which activates downstream gene expression (De Smet et al., 2010).

A plethora of studies have demonstrated the involvement of several components of the auxin signaling pathway in apical hook development. Quadruple mutations of TIR/ABF receptors (Dharmasiri et al., 2005), gain-of-function mutations of SHY2/IAA3, BDL/IAA12 or IAA13 (Tian and Reed, 1999; Zadnikova et al., 2010), IAA19 (Tatematsu et al., 2004), as well as ARF7 and ARF19 loss-of-function mutations cause strong apical hook developmental defects (Stowe-Evans et al., 1998; Harper et al., 2000; Zadnikova et al., 2010). Furthermore, mutations to ARF1 and ARF2, negative regulators of auxin-induced gene expression, cause hook exaggeration (Li et al., 2004). In addition, certain auxin signaling elements, such as IAA3, IAA12 and IAA13 exhibit asymmetric expression patterns (Zadnikova et al., 2010), but neither ARF2 nor ARF7 exhibit any discernible expression asymmetry, according to experiments with β-glucuronidase (GUS) reporter constructs (Zadnikova et al., 2010). The overlapping and symmetric transcriptional profiles of *ARF2* and *ARF7*, acting repressive and promotive on hook development respectively, imply that understanding their modes of action in generating growth asymmetry lies beyond their expression patterns, for instance, by differences in protein accumulation in response to auxin.

However, auxin operates in concert with several other plant hormones, such as ethylene, gibberellins and brassinosteroids. In this thesis, I studied the role of ethylene in hook-related phenomena, so its roles are described in the following section.

1.2.3 Ethylene

Ethylene is a gaseous hormone that was found to be a modifier of plant growth more than a century ago (Neljubow, 1901). Its connection to apical hook development was established in a number of studies in the 1960s, which showed that hook opening in bean is inhibited by low levels of exogenous ethylene. This was corroborated by the finding that ethylene production gradually decreases concurrently with hook opening in both bean (Kang et al., 1967) and pea (Goeschl et al., 1966) seedlings. Subsequent genetic studies, involving *Arabidopsis* ethylene biosynthesis and signaling mutants, confirmed its role in

control of hook development. Exogenous treatment with ethylene gas or its biosynthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC) delays transition from formation to the maintenance phase, and induces exaggerated hook curvature (Ecker, 1995) (Figure 3C). This response is mimicked in ethylene-overproducing mutants, such as *eto1*, *eto2* and *eto3* (Guzman and Ecker, 1990; Vogel et al., 1998a; Woeste et al., 1999). In contrast, mutants with reduced ethylene biosynthesis, such as *cytokinin insensitive* (*cin1*, *cin2*, *cin3* and *cin4*) single mutants exhibit defective hook development (Vogel et al., 1998b). Ethylene is perceived by five receptors: ETR1, ETR2 (ETHYLENE RESISTANT 1 and 2), ERS1, ERS2 (ETHYLENE RESPONSE SENSOR 1 and 2) and EIN4 (ETHYLENE INSENSITIVE 4) (Light et al., 2016). The receptors are active in the absence of ethylene, and dominant mutations of the receptors cause ethylene insensitivity and pronounced apical hook defects, while higher-order loss-of-function mutants exhibit a constitutive ethylene response phenotype (Hua and Meyerowitz, 1998). The receptors bind to and activate CTR1 (CONSTITUTIVE TRIPLE RESPONSE), which functions as a negative regulator of EIN2 (ETHYLENE INSENSITIVE 2), which in turn recruits transcription factors EIN3 and EIL1 (Light et al., 2016). Loss of CTR1 function causes strong exaggeration of hook curvature, while *ein2* mutants exhibit ethylene insensitivity and perturbed hook development (Kieber et al., 1993).

A notable role of ethylene during hook development is its participation in control of cell division. Cell divisions do not occur at high frequency in the hook under standard growth conditions, according to Cyc1B-GUS and KN-GFP expression analyses, but ethylene treatment increases division frequencies and cell numbers in the hook region during its formation (Raz and Koornneef, 2001; Zadnikova et al., 2016). Conversely, frequencies of divisions are strongly reduced in ethylene-insensitive mutants, and ethylene treatment does not induce hook exaggeration in them (Raz and Koornneef, 2001). Interestingly, however, pharmacological or genetic interference with cell division only somewhat reduces ethylene-induced hook exaggeration, and has no apparent effect on hook formation under standard growth conditions (Raz and Koornneef, 2001; Zadnikova et al., 2016). Thus, while ethylene controls cell division during hook development, this mechanism seems to make a minor contribution to the apical hook's differential growth.

While ethylene promotes growth asymmetry in the hook, its biosynthesis, perception and signaling do not exhibit any easily discernible asymmetries in the apical hook. There are reports of preferential ethylene biosynthesis gene expression on both the outer side (Raz and Ecker, 1999) and inner side (Peck et al., 1998) of the hook. However, the ethylene response appears to be

symmetrical, according to experiments with *EBS::GUS* response reporter constructs (Zadnikova et al., 2010)

Instead, ethylene may strongly influence hook development indirectly, by regulating establishment of the auxin gradient that determines growth asymmetry. This control may occur at multiple levels, including auxin biosynthesis, auxin-induced gene expression and auxin transport. For example, ethylene promotes auxin biosynthesis specifically on the inner side of the hook via upregulation of TAR2 expression (Vandenbussche et al., 2010). It also induces expression of auxin transporters PIN1, PIN3, and PIN7 (Zadnikova et al., 2010; Zadnikova et al., 2016), AUX1 and LAX3 in the hook, and AUX1 turnover on the inner side is enhanced by ethylene treatment (Vandenbussche et al., 2010). Accordingly, auxin transporter mutants *aux1* and *pin3* exhibit reduced responsiveness to ethylene (Vandenbussche et al., 2010; Zadnikova et al., 2010), while treatment with the auxin efflux carrier inhibitor NPA completely abolishes the effect of ethylene treatment.

Ethylene also promotes expression of *HOOKLESS 1* (HLS1) (Lehman et al., 1996). *hls1* mutants fail to form an apical hook, while HLS1 overexpressors produce exaggerated hooks even in the absence of ethylene (Lehman et al., 1996; Gallego-Bartolome et al., 2011). Interestingly, DR5 asymmetry is also abolished in *hls1* mutants, possibly because HLS1 negatively regulates the abundance of ARF2, which represses hook development by negatively regulating ARF7, adding another layer of ethylene action via auxin-dependent processes in regulation of hook development.

1.3 The endomembrane system

The location and functional site of many auxin transport components involved in hook development is the plasma membrane (PM). Their delivery to and subcellular localization in the PM is controlled by a complex intracellular trafficking machinery. Numerous studies in recent decades have revealed the importance of this trafficking for myriads of processes, *inter alia*, proper auxin transport, and thus plant development. Hence, endomembrane trafficking is described in the following sections.

The PM is a lipid bilayer that encloses cells of almost all living organisms, thereby separating their interior from the external environment. Composed of amphiphilic lipids such as phospholipids, glycolipids and sterols, the PM also contains numerous proteins with diverse biological activities. Unlike prokaryotes, eukaryotic cells also contain internal membrane-enclosed organelles: specialized intracellular endomembrane compartments with distinct functions (such as the previously mentioned amyloplasts and statoliths). Two

types of organelles, chloroplasts and mitochondria, are thought to have originated from endosymbiotic acquisition (Sagan, 1967; Jensen and Leister, 2014; Roger et al., 2017). However, there are also endomembrane compartments, including the nuclear envelope, endoplasmic reticulum (ER), Golgi apparatus (often simply called the Golgi) and post-Golgi compartments, with origins that are still debated (Gould et al., 2016; Dacks and Field, 2018). While their origin is still unclear, the benefits of compartmentalization are more obvious and there are plenty of examples. For example, the nuclear envelope allows separation of pre-mRNA splicing and protein synthesis, while peroxisomes and vacuoles provide micro-environments with evolutionarily tailored pH and chemical characteristics for functions such as catabolism of various cellular components, thereby boosting energy efficiency. In addition, transporting cargo in membrane-enclosed vesicles allows selective intracellular transport, internalization of extracellular material and secretion.

The endomembrane system compartments ER, Golgi and *trans*-Golgi network jointly function as a system for synthesis and delivery of molecules to targeted sites within cells, in the PM or the outer environment. At the ER, most proteins traffic to the Golgi, while a subset exit directly for targeting to the PM or the vacuole (Viotti et al., 2013). In the Golgi, which is composed of a series of fused disc-like membrane sacs called cisternae, proteins destined for secretion may undergo post-translational modifications such as glycosylation (Strasser, 2016). Furthermore, several components of the cell wall, such as xyloglucans and pectin, are synthesized in the Golgi (Zhang and Staehelin, 1992). The Golgi is commonly divided into *cis*, *medial* and *trans* compartments. The *cis* compartment sits adjacent to the ER. On the opposite side, the Golgi is thought to mature into the *trans*-Golgi network (TGN) (De Matteis and Luini, 2008; Kang, 2011), the main exit site of secretory vesicles. Due to its many specific functions and morphological distinctness, the TGN is considered a discrete compartment (Viotti et al., 2010; Kang et al., 2011). From the TGN, vesicles may be delivered to the vacuole or cell surface, and hence secreted (Figure 4). The TGN also serves as the receiver of cargos taken up from the PM by a process known as endocytosis. These cargos may then be recycled to the PM or the vacuole. Thus, the TGN acts as a sorting station for several trafficking pathways in plant cells.

In the following sections I provide an introduction to vesicle trafficking, particularly secretory trafficking mediated by the TGN, and its role in cell expansion, the driving force for growth processes such as apical hook development.

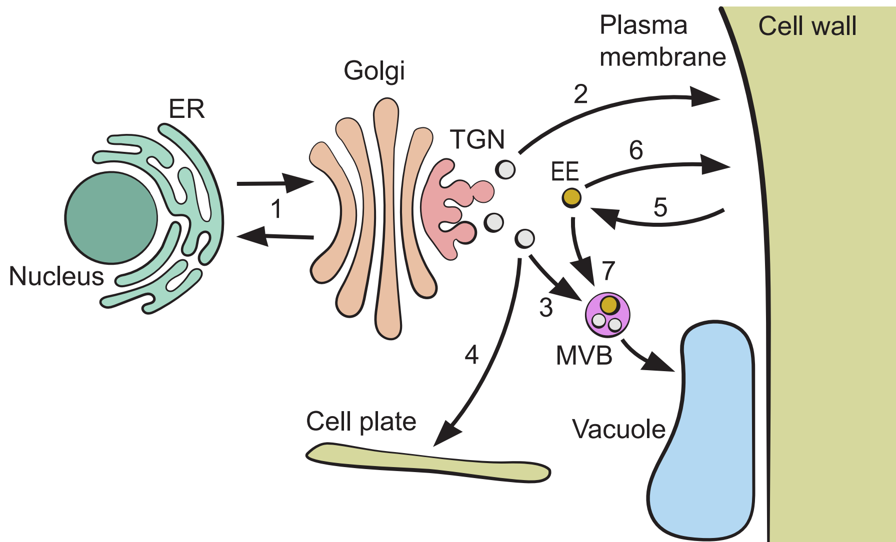

Figure 4. **Secretory pathways via the TGN.** (1) Proteins synthesized at ER traffic to the Golgi, and may be retrieved back to the ER. When reaching the TGN, cargos may be secreted to the cell surface via secretory vesicles (2), sent to the vacuole via MVBs (3) or redirected to the forming cell plate during cytokinesis (4). The plant TGN also serves as an early endosome (EE) receiving endocytosed material (5), which may either be recycled back to the cell surface (6) or directed towards the vacuole via MVBs (7).

1.3.1 Secretory trafficking

Secretion in plants encompasses the synthesis, delivery and release of material to the cell surface and the vacuole. For plant cells, delivery to the cell surface entails secretion to the PM or the apoplast (comprising a continuum of cell wall and extracellular space). In dividing cells, secretory trafficking is also directed to the cell plate separating incipient daughter cells. Trafficking to the vacuole can be regarded as secretory trafficking too, since its initial delivery pathway largely follows the same route as for cargos destined for the cell surface. Recent findings have also revealed an unconventional secretory pathway (USP), through which leaderless secretory proteins lacking a signal peptide bypass the Golgi route (Robinson et al., 2016). However, most secreted proteins carry a signal peptide and enter the conventional secretory pathway through vesicle trafficking via the Golgi and TGN (Gendre et al., 2015). This thesis focuses on secretory trafficking at the TGN. As mentioned above, the TGN is a major 'sorting station' and point of intersection of secretory pathways. Thus, understanding the molecular basis for divergence of secretory pathways at the TGN is highly relevant for understanding how organism complexity may arise, given that secretory trafficking could play a key role in processes such as cell

polarity that in turn could modulate organ development. The following sections introduce the TGN compartment, its role in secretion, the key players in vesicle formation in the TGN, and the role of TGN-dependent secretion in plant development, particularly cell expansion.

1.3.2 The *trans*-Golgi network

The Golgi apparatus was first described at the end of the 19th century (Golgi, 1898), but the *trans*-most appendage we now identify as the TGN was only described as a distinct structure more than 70 years later (Novikoff, 1964). Early observations from studies of rat neurons identified it as a tubular extension of ER, referring to it as Golgi endoplasmic reticulum lysosome, or GERL. It was subsequently given many names – examples include Boulevard Peripherique (Morre and Ovtracht, 1977), *trans* Tubular Network (Rambourg et al., 1979), and *trans* Golgi Reticulum (Willingham and Pastan, 1984) – before the community settled on TGN (Griffiths and Simons, 1986).

As in animals, the plant TGN is believed to mature from the *trans*-most face of the Golgi and appears as a mesh of tubulo-vesicular membrane structures (De Matteis and Luini, 2008; Kang, 2011) (Figure 5). While the mammalian TGN acts in sorting of cargos to the PM or endosomes, endocytic cargos are initially received by the Early Endosome (EE) compartment for recycling back to the PM or sent to late endosomes (LEs)/multivesicular bodies (MVBs) (Scott et al., 2014). The mammalian EE is considered distinct from the TGN both spatially and functionally. In contrast, the plant TGN may exist in direct proximity to the Golgi, i.e. Golgi-associated TGN (GA-TGN) or may release from the Golgi to exist as Golgi-independent TGN (GI-TGN) (Uemura et al., 2014; Uemura et al., 2019). Moreover, the endocytic tracer FM4-64 rapidly labels the plant TGN (Dettmer et al., 2006), prior to FM4-64 reaching LE/MVBs. Plant cells appear to lack an EE compartment, with the plant TGN instead fulfilling the role of the EE, serving as a recipient and sorter of endocytic cargo. Cargos which are not recycled back to the PM and instead destined for the vacuole are transferred to the LE/MVB, which is suggested to mature from EE/TGN (Scheuring et al., 2011). Vacuolar delivery via EE/TGN is subsequently completed by fusion of the LE/MVB with the vacuole (Singh et al., 2014).

Although the TGN is derived from the Golgi, the TGN harbors a distinct proteome (Parsons et al., 2013), with hallmark resident proteins, such as the Vacuolar H^+-ATPase (VHA) subunit VHA-a1, Syntaxin of Plants (SYP) proteins SYP61 and SYP43 or the RAB-GTPase RabA2a, distinguishing it from the Golgi. It bears consideration that despite the "TGN proteome", there appears to be considerable complexity to TGN, and in a single plant cell, several

morphologically heterogeneous TGN structures may thus exist. The complexity of the TGN may be a reflection of its multiple functions. For example, the TGN is the site of several distinct secretory trafficking pathways, and this is reflected in observation that, perhaps in line with its morphological heterogeneity, it appears to have discrete subdomains (Kang et al., 2011). Three types of vesicles have been associated with the TGN: (1) Clathrin-coated vesicles (CCV), approximately 35 nm in diameter and characterized by their clathrin triskelion polyhedral lattice surrounding the vesicle membrane, (2) Secretory vesicles (SV), which are comparatively large and variable in size (70-150 nm in diameter) bearing only a thin coat or potentially coatless, and (3) COPIb vesicles exhibiting a two-layer coat (Donohoe et al., 2007). Sites of CCVs and SVs often exist on the same TGN structure, but may be separated in space (Figure 5). This spatial separation is also reflected by a partial separation of associated proteins

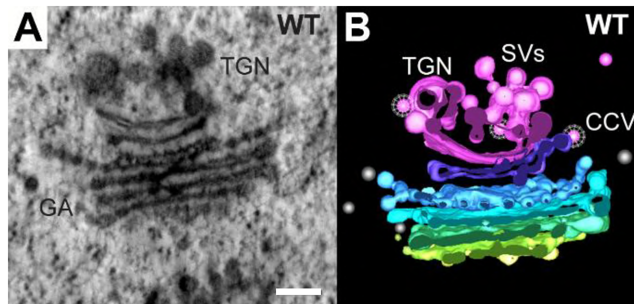

Figure 5. **Electon microscopy tomogram of Golgi and TGN.** **(A)** Tomograph of Golgi apparatus (GA) and TGN. **(B)** 3D model of tomogram in **(A)**, showing the Golgi and the tubulo-vesiculated TGN structure with distinct regions harboring SVs and CCVs. Adapted from Boutté et al. 2013). Scale bar 200 nm

as observed in both electron microscopy and confocal microscopy studies. For example, sites of SVs overlap with localization of the syntaxin SYP61, while CCV domains preferentially coincide with RAB-GTPase RAB-A2a labeling (Chow et al., 2008; Gendre et al., 2011; Wattelet-Boyer et al., 2016). The numerical ratio of CCVs to SVs in a TGN structure also varies (from 5:1 to 1:4) (Staehelin and Kang, 2008), and both amounts and types of TGN-associated vesicles in cell types might reflect particular secretory needs. For instance, in meristematic cells the TGN is typically CCV-rich while highly cell wall-secreting cells generally have SV-dense TGNs (Young et al., 2008; Kang, 2011). The partitioning of the TGN into subdomains may depend on local lipid compositional variations. A recent study revealed marked differences between SV and CCV domains in *Arabidopsis* in the abundance of sphingolipids with α-hydroxylated acyl-chains of at least 24 carbon atoms, and showed that these lipid

components are specifically required for PIN2 but not AUX1 secretion (Wattelet-Boyer et al., 2016). While evidence for lipid rafts at the plant TGN is still lacking, in animal systems, sphingolipid-rich rafts at the TGN are thought to specifically mark sites for apical sorting of cargos exhibiting affinity for glycosphingolipids in epithelial cells (Surma et al., 2012).

1.3.3 Secretory routes at the *trans*-Golgi network

Secretory trafficking to the cell surface via the TGN appears to be the major secretory route. Although there are unconventional pathways bypassing the TGN (Crowell et al., 2009), the studies this thesis is based upon investigated conventional secretion via the TGN. Thus, the following section focuses on secretory trafficking via the TGN. Soluble cargos are delivered inside the lumen of vesicles, while hydrophobic cargos are embedded in their membranes. As vesicles reach and fuse with their destination membrane, soluble cargos are released to the cell exterior while embedded proteins become components of the PM. In this way, vesicle secretion provides the PM with lipids and proteins required for its functions, and increases the membrane surface area, which is essential for growth. Growth also entails incorporation of new cell wall material such as polysaccharides and cell wall biosynthetic and modifying enzymes. Using polysaccharide-binding antibodies, xyloglucan and pectin have been observed in the TGN and SVs (Sherrier and Vandenbosch, 1994; Vicre et al., 1998; Stierhof and El Kasmi, 2010; Viotti et al., 2010). Their delivery to the wall is perturbed in plants lacking the TGN-resident proteins ECHIDNA or its interactors YPT/RAB GTPase Interacting Protein 4a (YIP4a) and YIP4b, deficient in SV trafficking from the TGN (Gendre et al., 2013; McFarlane et al., 2013).

Furthermore, several cell wall biosynthetic enzymes that are active at the PM, such as cellulose synthase (CESA) complex subunits and glucan synthase-like1 (GSL1) have been observed in the TGN (Brownfield et al., 2008; Gutierrez et al., 2009). A proteomic analysis of the TGN-resident syntaxin of plants 61 (SYP61)-compartment identified several CESAs (Drakakaki et al., 2012). Other associated proteins identified in the cited analysis have been linked to endocytosis or recycling, but SYP61 has been implicated (together with SYP121) in secretion of cell wall material to the papillae in response to pathogen attack. Interestingly, while CESAs have been found to co-localize with both SYP61 (Drakakaki et al., 2012) and the endosomal TGN marker VHA-a1 (Crowell et al., 2009), CESAs have also been observed in vesicles not overlapping with any known secretory trafficking marker (Crowell et al., 2009; Gutierrez et al., 2009). These compartments have been called small CESA

compartments (SmaCCs) or microtubule-associated CESA compartments (MASCs). The exact nature of SmaCCs/MASCs is obscure, and it is not clear if or where this potentially distinct pathway diverges from conventional secretory routes.

The level of secretory pathway convergence has long been debated. SV trafficking was historically viewed as an indiscriminate bulk-flow route carrying most secretory cargos (Hawes and Satiat-Jeunemaitre, 2005; Foresti and Denecke, 2008). However, this view is incomplete at best, and instead secretion appears to entail a complex network of overlapping and discrete routes. For instance, delivery of the secretory protein marker secGFP is hampered by overexpression of the syntaxin SP2 in tobacco, while integration of radiolabeled glucose to the wall remains unaffected, implying that polysaccharide and protein secretion may follow separate routes (Leucci et al., 2007). Protein secretion has also been shown to diverge at the TGN, as exemplified by KEEP ON GOING (KEG), a TGN-localized protein required for secretion of apoplastic defense proteins but not the PM-localized proteins BRASINOSTEROID INSENSITIVE1 (BRI1), AUX1or PIN1 (Gu and Innes, 2012).

1.3.4 Secretory vesicles and their formation

Vesicles are membrane-enclosed spheres derived from a donor membrane, carrying soluble cargo in their lumens and hydrophobic cargos embedded in their membranes. After budding from their donor compartments, vesicles travel to and fuse with an acceptor membrane to release their cargo. Vesicle formation involves cargo recruitment, assembly of factors required to form the vesicle structure, and pinching off the structure from its donor membrane (Bard and Malhotra, 2006). Of the three types of vesicles observed in the TGN, only SVs have a verified role in secretion to the plasma membrane. COPI vesicles likely recycle material back from the TGN to Golgi (Donohoe et al., 2007; Kang et al., 2011), while CCVs account for the major endocytic route, while the potential involvement of CCVs in TGN-to-vacuole transport is still a matter of debate. The formation of vesicles such as CCVs or COPI vesicles through recruitment of coat proteins that force membrane curvature has been described in detail (Paul and Frigerio, 2007; Jackson, 2014; Kirchhausen et al., 2014). Briefly, initially small GTPases of the ADP Ribosylation Factor (ARF) family are activated by ARF Guanine Exchange Factors (GEF)s (for more information on ARF GTPases and ARF-GEFs, see the following sections). Upon activation, ARF proteins associate with endomembranes and recruit coat proteins and other factors involved in cargo assembly. In contrast to the abundant information on CCV and COP vesicle formation, much less is known about the formation of SVs, which

may form without the aid of a coat protein skeleton (Donohoe et al., 2007). Indeed, no study has identified a coat surrounding SVs, and instead there may be a machinery involving lipid composition modifications to induce membrane curvature, a mechanism not yet described in plants but suggested for mammalian constitutive secretory vesicles (CSV) (Bard and Malhotra, 2006).

In rat adrenal medulla pheochromocytoma-derived cell-free systems, CSV formation in the TGN has been shown to require ADP Ribosylation Factor 1 (ARF1), but not COPI coat protein (Barr and Huttner, 1996). In mammalian cells, Active ARF1, which localizes to Golgi and TGN in *Arabidopsis*, binds to membranes upon myristoylation, and can recruit PItdIns4-kinases (PI4Ks), which catalyze production of phosphatidylinositol-4-phosphate (PtdIns4P) (De Matteis and Godi, 2004). Notably, PI4K mutants of *Arabidopsis* exhibit severely perturbed TGN and SV morphology (Preuss et al., 2006; Kang et al., 2011). PtdIns4P is thought to mark an endomembrane subdomain site, and it can recruit several proteins in mammalian cells (Wang et al., 2007; Dumaresq-Doiron et al., 2010), and notably sortin nexins (SNX) (Xu et al., 2001). SNX proteins contribute to retromer complexes (which mediate recycling from sorting compartments in animals and yeasts) and are thought to induce membrane deformation and cargo recruitment. These proteins, which have been localized to the TGN in plants (Stierhof et al., 2013), may enhance membrane curvature in domains that are already curved due to membrane bilayer lipid asymmetry (Thomas and Poznansky, 1989). Such asymmetry occurs through unequal incorporation and rearrangement of conical lipids, including phosphatidic acid (PA), into the bilayer (Kooijman et al., 2003). The formation of vesicles is also aided by phospholipid flippases flipping phospholipids across the bilayer, thereby creating structural membrane asymmetry (Sebastian et al., 2012). In *Arabidopsis,* a mutant with impairments in the Golgi-localized flippase P4-ATPase aminophospholipid ATPase3 (ALA3) exhibits severe defects in vesicle production in root cap cells (Poulsen et al., 2008). The conical PA can be converted from the cylindrical phosphatidylcholine (PC) by phospholipase D (PLD) (Pappan et al., 1998). Interestingly, mammalian PLD has been shown to stimulate SV budding from TGN *in vitro*, and this PLD-dependent action is reportedly stimulated by myristoylated ARF1 (Chen et al., 1997). Thus, although this mechanism is yet to be demonstrated in plants, homologs of components of the machinery are present in plants. Other vesicle formation mechanisms, such as clathrin-mediated endocytosis, COPI vesicle formation and ESCRT-mediated vesicle formation are mechanistically highly similar among eukaryotic kingdoms. It seems fair to hypothesize that the SV machinery in plants could exhibit similar conservation, but confirmation in future studies is needed.

ARF/SAR1 proteins and their accompanying ARF-GEF activators are highly interesting due to their conserved and integral roles as master switches for initiation of both coated and potentially coatless vesicle formation. In the next sections I describe the ARF/SAR1 subfamily ARF1 as well as ARF-GEFs (particularly the BIG ARF-GEF subfamily), which are major players in the focal phenomena of this thesis.

1.3.5 ARF GTPases

Across eukaryotic kingdoms, GTPases exhibit a remarkable level of conservation, highlighting their importance in various cellular processes (Jekely, 2003). Small GTPases act as molecular switches by shifting between GTP- and GDP-bound states (Bourne et al., 1990). Members of the small GTPase subclass ARFs regulate organelle structure and membrane trafficking in eukaryotic organisms. ARFs are activated by ARF-GEFs that catalyze release of GDP nucleotides from them (Casanova, 2007). In a mechanism demonstrated in great detail, upon binding of GTP, mammalian ARFs undergo a conformational change allowing them to anchor to membranes and interact with various effector proteins, such as coat proteins and lipid-modifying enzymes (Goldberg, 1998; Yorimitsu et al., 2014; Karandur et al., 2017). Thus, ARFs operate in the initiation of vesicle formation.

ARFs in mammalian systems are divided into three functional classes, designated Classes I-III. In contrast, plants host only Class I ARF1, lacking Class I sub-classes ARF2 and ARF3, as well as both Class II and III ARFs (Singh and Jurgens, 2018). In plants, the Class I ARF1 subfamily, comprising six closely related members (ARFA1a-f) instead operate in a wider spectrum of trafficking pathways than their mammalian counterparts, such as ER-Golgi trafficking (Lee et al., 2002; Takeuchi et al., 2002), vacuolar trafficking (Pimpl et al., 2003), endocytosis and/or recycling (Xu and Scheres, 2005; Naramoto et al., 2010; Tanaka et al., 2014). Additionally, the *Arabidopsis* genome encodes members of two plant-specific ARF classes, A and B, which have unknown biological roles (Singh et al., 2018). Due to the high level of sequence similarity and expected redundancy between the six ARF1 members in *Arabidopsis*, ARF1 functions have been dissected by studying effects of dominant-negative and constitutively active variants, as well as indirect targeting using the fungal toxin brefeldin A (BFA), which interferes with functions of ARF1 effectors ARF-GEFs (as discussed in the next section) (Dascher and Balch, 1994; Nebenfuhr et al., 2002). ARF1^{T31N}, carrying an asparagine instead of threonine at amino acid position 31, locks ARF1 in a GDP-bound state (Dascher and Balch, 1994), preventing its integration with membranes and blocking downstream steps, causing strong

morphological aberrations of Golgi systems in animal cells (Garcia-Mata et al., 2003). Upon induction of ARF1^{T31N} expression in *Arabidopsis*, root elongation and root hair growth are severely perturbed (Xu and Scheres, 2005). Similarly, a mutation causing lockage of ARF1 in a GTP-bound state (ARF1^{Q71L}) strongly perturbs root growth, underscoring the crucial roles of ARF1 members in plant growth (Xu and Scheres, 2005).

As described above, in *Arabidopsis*, ARF1 members act in diverse trafficking pathways. Intriguingly, activities of these pathways, such as secretion and vacuolar transport, may dynamically shift depending on cellular requirements. Pathway specificity for ARF-mediated processes is instead thought to be conferred by their ARF-GEF activators, which are described in the next section.

1.3.6 ARF-GEFs

ARFs shuttle between inactive GDP-bound and active GTP-bound states, and their activation is catalyzed by ARF-GEFs. ARF-GEFs have a characteristic SEC7 domain, which catalyzes GDP/GTP exchange, named after the yeast protein Sec7p, the first ARF-GEF described (Franzusoff and Schekman, 1989). Additional features are used to further divide ARF-GEFs into subclasses. Based on their size, in humans ARF-GEFs are grouped into small, medium and large subclasses (Cox et al., 2004; Mouratou et al., 2005). Plants lack both small and medium ARF-GEFs, but the *Arabidopsis* genome harbors eight large ARF-GEFs (150-220 kDa in size), while humans and yeasts have three members (Anders and Jurgens, 2008). The *Arabidopsis* ARF-GEFs are separated into two subclades, one comprising GNOM, GNOM-LIKE1 (GNL1) and GNL2, which are homologous to the human GBF1 (Anders and Jurgens, 2008). The other subclade consists of BFA-Inhibited-GEF (BIG) 1-5, which are analogous to the human BIG ARF-GEF subclade (Anders and Jurgens, 2008). GNOM is the best-studied ARF-GEF in *Arabidopsis*. Seminal studies in the 1990s and early 2000s demonstrated a requirement for GNOM in PIN1 recycling from EE to the PM (Steinmann et al., 1999; Geldner et al., 2001; Geldner et al., 2003). The fungal toxin brefeldin A (BFA) causes agglomeration of PIN1 in intracellular structures called BFA bodies even in presence of the protein synthesis inhibitor cycloheximide (CHX) (Geldner et al., 2001). Following BFA washout, the PIN1-labelled BFA bodies disappear. BFA interferes with ARF-GEF-dependent activation of ARFs by binding to ARF-GDP/ARF-GEF complex at particular sites of the SEC7 domain (Cherfils and Melancon, 2005; Zeeh et al., 2006). This prevents the hydrolysis of GDP required for GDP/GTP exchange, locking the complex in a GDP-bound inactive state (Cherfils and Melancon, 2005). An amino acid substitution at position 696 in the Sec7 domain may render ARF-

GEFs insensitive to BFA inhibition. Accordingly, expression of an engineered BFA-resistant GNOM ($GNOM^{M696L}$) can alleviate BFA-induced PIN1 agglomeration (Geldner et al., 2003). In humans, all large ARF-GEFs exhibit sensitivity to BFA (Anders and Jurgens, 2008). In *Arabidopsis* however, GNL1 carries a leucine at amino acid position 696 (L696), rendering its activity in ER-Golgi trafficking BFA-resistant (Teh and Moore, 2007).

Until recently, the functions of BIG ARF-GEFs in *Arabidopsis* were unknown, except for evidence that BIG5/BEN1 participates in endocytic trafficking (Tanaka et al., 2009). However, a recent study showed that BIG1-4 localize to the TGN, like their counterparts in humans, and operate redundantly (Richter et al., 2014). The SEC7 catalytic domain of one of the BIG ARF-GEFs, BIG3 (previously denoted BIG2), can catalyze ARF1 GDP/GTP exchange even in the presence of BFA *in vitro* (Nielsen et al., 2006). Moreover, like GNL1, BIG3 is resistant to BFA and, accordingly, *big3* is reportedly the only BIG single mutant that is hypersensitive to BFA treatment (Richter et al., 2014). In *Arabidopsis*, BIG3 shares, with GNL1, the L696 in the Sec7 domain thought to confer this BFA insensitivity. BIGs play a distinct role in trafficking from GNOM, since neither of the defects observed in BFA-treated *big3* seedlings can be rescued by introduction of a BFA-resistant variant of GNOM (Richter et al., 2014). Recent studies have clearly demonstrated that BIG1-4 function in secretion of both soluble and membrane-associated cargos to the PM and trafficking to the vacuole, and furthermore, their activities are required during cytokinesis for redirection of trafficking of the *de novo* synthesized material to nascent cell plates (Richter et al., 2014). Hence, cell division is perturbed when BIG function is disrupted. *Inter alia*, lateral root initiation fails, implying that BIG-dependent secretory trafficking during cytokinesis is required for lateral root initiation. In contrast, during root gravitropic responses, which require PIN-mediated re-direction of auxin transport, BIGs do not play a major role, but GNOM activity is essential (Kleine-Vehn et al., 2010; Richter et al., 2014). This suggests that recycling rather than secretory trafficking is the major pathway for dynamic PIN traffic redirection during gravitropic responses.

In conclusion, the two ARF-GEF subclasses in plants have distinct trafficking roles that vary depending on the specific growth processes their host cells are participating in.

1.3.7 ECHIDNA

In recent years, several factors that reside at the TGN, like ARFs and ARF-GEFs, have been identified and proposed to play a role in TGN function and trafficking (Dettmer et al., 2006; Drakakaki et al., 2012; Richter et al., 2014). Of particular

interest to this work is the protein ECHIDNA (ECH). Initially, ECH was identified in microarray analyses, as a highly expressed gene in cell types exhibiting rapid expansion from *Arabidopsis* and hybrid aspen (*Populus tremula* x *P. tremuloides*). ECH was found to mediate cell elongation in both root and hypocotyl epidermal cells in *Arabidopsis* (Gendre et al., 2011). Named Echidna due to resemblance of the *ech* mutant's bushy midflowering phenotype to the monotreme (egg-laying mammal) *Echidna*, the ECH protein is predicted to harbor three to four transmembrane domains, and is evolutionarily conserved among eukaryotes. This is exemplified by ability of the *Arabidopsis* ECH protein to partially rescue growth defects of the *Saccharomyces cerevisiae* mutant *tvp23Δ ypt6Δ*, which lacks the yeast ECH homolog TVP23 and its putative interactor RAB-GTPase YPT6 (Gendre et al., 2011). In yeast, TVP23 has been suggested to mediate retrograde transport from EE to Golgi (Stein et al., 2009). A human homolog TV23B/FAM18B has suggested association with diabetic retinopathy, and RNAi-mediated FAM18B knockdown in primary human retinal microvascular endothelial cells reduces their viability in hyperglycemic conditions (Wang et al., 2014). However, no specific cellular function has been ascribed to the human ECH homologs.

In *Arabidopsis*, examination of ECH-YFP revealed that ECH localizes to the TGN, overlapping strongly with TGN-resident SNAREs SYP41 and SYP61 as well as V-ATPase subunit VHA-a1 (Gendre et al., 2011). The *ech* mutant was shown to exhibit reduced Golgi-TGN association compared to wild-type plants, while Golgi stack morphology was largely unaffected (Gendre et al., 2011). Several TGN-residing proteins mislocalize in *ech* mutants, e.g. VHA-a1 and RabA2a, to the vacuole and cell plate, respectively. The morphological aberrations and mislocalization of VHA-a1 and RabA2a observed in *ech* mutants are mimicked by pharmacological inhibition of V-ATPases with concanamycin A (ConcA) (Gendre et al., 2011), implying that ECH might participate in maintenance of proper TGN V-ATPase localization.

Early endocytic trafficking, indicated by FM4-64 uptake, uptake operates largely independently of ECH (Gendre et al., 2011), with only minor effects on endocytosis as revealed by a mild delay of FM4-64 internalization in *ech* compared to WT (Ravikumar et al., 2018). In contrast, delivery of various secretory cargos to the cell surface is strongly dependent on ECH. Several wall polymers, such as xyloglucan and pectin, are secreted to the wall in an ECH-dependent manner (Gendre et al., 2013). In *Arabidopsis* hypocotyls, both of these polysaccharides agglomerate intracellularly in *ech* mutants in addition to localizing in the wall, as revealed by CCRC-M1 (xyloglucan), LM5 (homogalacturonan pectin) and JIM7 (rhamnogalacturonan pectin) labelling (Gendre et al., 2013). In addition, ECH is required for secretion of pectin-rich

seed mucilage in the seed coat epidermis. Unsurprisingly, since wall polymers must be continuously supplied to the wall of growing cells, *ech* mutants exhibit severe elongation defects (Gendre et al., 2011), and other phenotypic deviations potentially associated with cell wall perturbations, such as male infertility due to reductions in anther length and abnormal pollen tapetum walls (Fan et al., 2014).

In addition to wall polysaccharides, ECH is required for delivery of components of the cuticle deposited in extracellular space. The cuticle, which is composed mainly of cutin (composed of C16 and C18 fatty acid derivatives) and subherin (composed of very-long-chain fatty acids, fatty alcohols, α,ω-dicarboxylic acids and 2-hydroxy fatty acids), provides a barrier between plant cells and their environment, and protects plants from non-stomatal water loss. Cuticular components are synthesized in ER and secreted to the cell exterior. In the absence of ECH, wax accumulation on the cell surface is severely reduced (McFarlane et al., 2014).

The secretory reporter secGFP, which is normally secreted to the apoplast where its fluorescence is quenched by the low apoplastic pH (Zheng et al., 2004), is retained intracellularly and clearly observable in *ech* mutants (Gendre et al., 2011). The secGFP secretory pathway is thought to represent the route of numerous cargos trafficked to the cell surface. ECH also mediates secretion of proteins to the PM. Accordingly, the receptor kinase BRI1 exhibits partial mislocalization to the vacuole in *ech* (Gendre et al., 2011). In contrast, the auxin efflux carrier PIN2, which localizes polarly at the PM in root epidermal cells, is unaffected in *ech* (Gendre et al., 2011). Similarly, ABCG11, required for wax export at the PM in the *Arabidopsis* stem, does not require ECH for delivery to the PM (McFarlane et al., 2014). Since mutations in ECH affect PM delivery of only a subset of proteins, ECH displays functional specificity that highlights the complexity of secretory trafficking from TGN.

The exact molecular function of ECH still remains an enigma, but its molecular interactors provide some hints. Two, identified by a yeast two-hybrid screen using ECH as bait, are YIP4a and YIP4b, members of the YIP family (Gendre et al., 2013). Homologous yeast and mammalian YIPs are known interactors of RAB GTPases, which are core players in vesicle trafficking (Li and Segev, 2018). ECH genetically interacts, and co-localizes strongly and interdependently, with YIP4a/YIP4b in the TGN (Gendre et al., 2013). Additionally, a *yip4a yip4b* double mutant partially phenocopies *ech* mutants, and exhibits reduced root and hypocotyl growth, mislocalization of TGN-resident VHA-a1 and SYP61, weakened Golgi-TGN association, and intracellular retention of wall polysaccharides (Gendre et al., 2013). A recent study identified YIP4a/YIP4b as interactors of TGNap1 in *Arabidopsis*, a TGN-resident protein furthermore interacting with Rab6 and microtubules (MT)

(Renna et al., 2018). TGNap1 putatively functions in MT-dependent TGN biogenesis or remodeling, which by extension hints at a potential role of ECH in maintenance of Golgi/TGN morphology, since *ech* mutants exhibit Golgi/TGN morphological defects (McFarlane et al., 2013). Interestingly, while ECH operates in a distinct pathway from that of the TRAPPII complex, which is a crucial component of trafficking to the cell plate, ECH and TRAPPII can apparently compensate for one another in critical TGN roles such as secretion (Ravikumar et al., 2018).

To probe the elusive mechanism of ECH, in the study described in Paper II, I explored the potential involvement of ECH in vesicle formation in *Arabidopsis*.

In summary, ECH is an evolutionarily conserved TGN-resident protein involved in both root and hypocotyl growth. It is essential for the retention of several crucial proteins in the TGN, such as V-ATPase subunit VHA-a1 and SYP61. Furthermore, ECH is crucial for the proper secretion of cell wall material and PM-resident proteins to the cell surface.

1.4 The cell wall

ECH functions in delivery of cell wall material such as pectin to the cell surface. Paper III describes an investigation of the role of the cell wall component pectin in differential growth of the apical hook. Hence, I here provide an introduction to plants' cell walls.

1.4.1 General structure

Plants' cell walls are complex composites of fibers and associated substances that surround plant cells. They have numerous biological roles, such as provision of mechanical support for the plant body, protection against biotic and abiotic stresses, facilitation of intercellular communication and provision of shape to the various types of cells required to form plants' tissues and organs (Hoson and Wakabayashi, 2015; Braybrook and Jonsson, 2016; Bacete et al., 2018; Kierzkowski and Routier-Kierzkowska, 2019). Classically plant walls are divided into two categories: primary walls that encase growing cells or cells capable of growth, and secondary walls which are thicker and contain lignin, such as fiber cells or vessel elements (Keegstra, 2010). In this thesis, I focus on the primary cell wall that encloses rapidly growing cells.

The primary cell wall of dicot species is comprised of polysaccharides classified into cellulose, hemicellulose and pectin (Figure 6), together with various structural and wall-modifying proteins. Cellulose is composed of β-1,4-

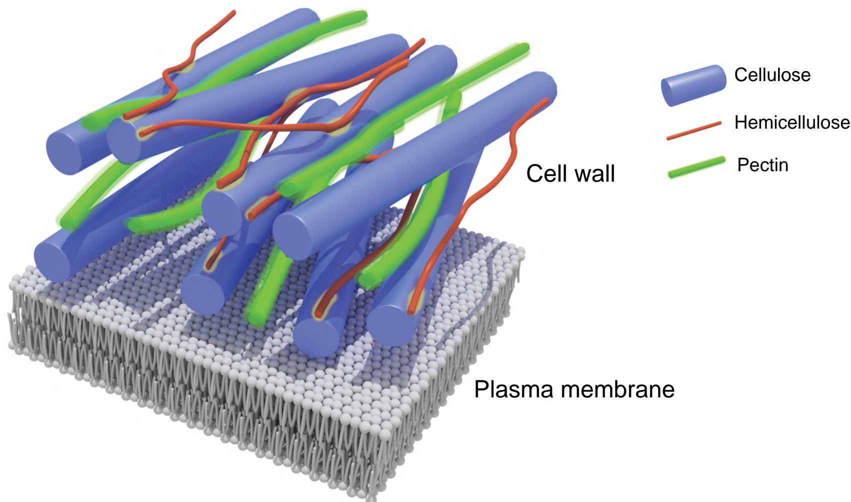

Figure 6. Structure of the primary cell wall. Cellulose depicted as blue rods. Hemicellulose (orange) binds cellulose at sites of interaction (highlighted in yellow). Pectin (Green) fills the space between cellulose and hemicelluloses and interacts with cellulose.

linked glucan chains organized in more or less crystalline microfibrils, and is thought to be the major load-bearing constituent (Lampugnani et al., 2018). Hemicelluloses comprise a class of different polymers, such as xylans, xyloglucans and glucomannans, that cross-link cellulose fibrils and modulate their strength (Park and Cosgrove, 2015). Pectins surround the aforementioned polymers and form a gel-like glue that provides stiffness or flexibility, depending on its chemical modifications.(Saffer, 2018). In *Arabidopsis* leaves, pectin comprises approximately 40-50% of the cell wall (Zablackis et al., 1995; Albersheim et al., 2011; Atmodjo et al., 2013). Pectins comprise a diverse group of polysaccharides commonly divided into homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan, rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Mohnen, 2008). Although ratios of the pectin constituents vary among species and tissues, HG is generally the most abundant pectic polysaccharide, often accounting for more than 50% of the total pectin content (Mohnen, 2008).

Recent studies have identified important roles for HG in wall mechanical properties and cellular growth (as described below). This, together with the involvement of ECH and its interactors in secretion of pectin to the wall, prompted me to investigate pectin’s involvement in one of the developmental processes crucially regulated by ECH – differential growth during hook development (Paper III). Therefore, here I focus on the roles of HG and its chemical modifications in both mechanical properties of the cell wall and cells’ growth.

1.4.2 Homogalacturonan Biosynthesis

HG is a linear polymer of α-1,4-linked galacturonic acid residues that are partially methyl-esterified at C-6 and *O*-acetylated at O-2/O-3 (Mohnen, 2008). It is thought to be synthesized in the Golgi along with other classes of pectin (Sterling et al., 2001). Unsurprisingly, given pectins' structural complexity, their synthesis hypothetically requires more than 65 distinct enzymes (Anderson, 2016). Furthermore, several pectin classes may have overlapping biosynthetic pathways. The HG backbone is thought to be assembled by galacturonosyltransferases, which catalyze transfer of GalA from UDP-GalA to

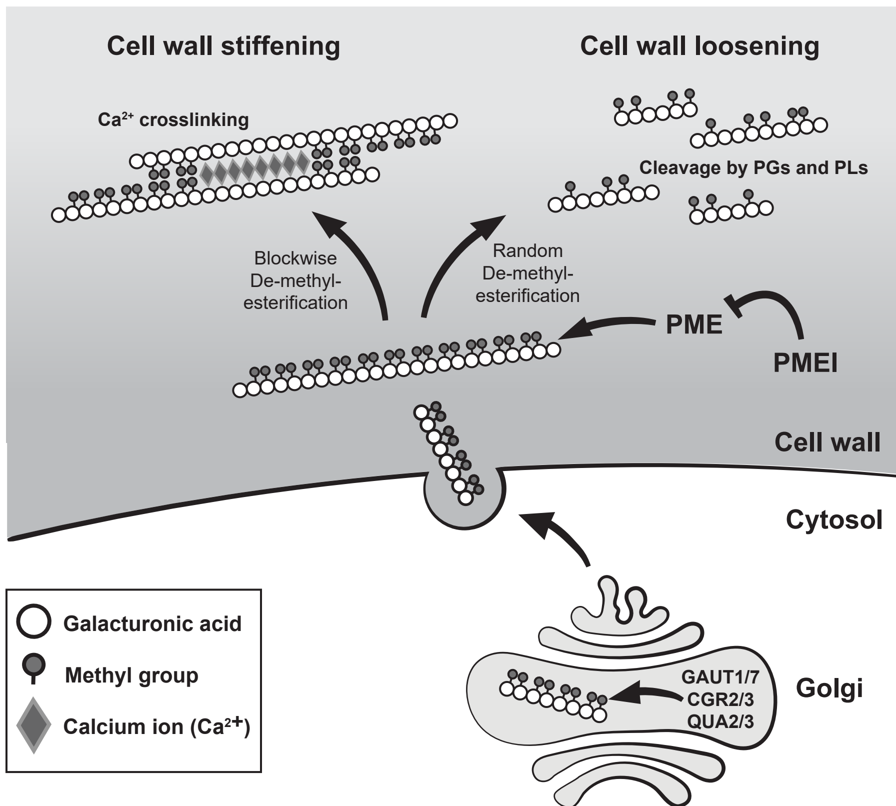

***Figure 7.* Homogalacturonan biosynthesis and modification.** In the Golgi, galacturonosyltransferase1 (GAUT1) and 7 contribute to HG biosynthesis. Putative methyl transferases (CGR2/3 and QUA2/3) add methylesters to the HG backbone. HG is then secreted to the cell wall, where it may be de-methylesterified by PMEs, the action of which may be inhibited by PMEIs. The fate of HG is thought to depend on the mode of de-methylesterification; Blockwise de-esterification allows for Ca^{2+}-crosslinking of HG chains, leading to wall stiffening. Alternatively, randomly de-methylesterified HG may be targeted by polygalacturonases (PG) and pectate lyases (PL), which cleave HG into oligogalacturonides, thought to lead to wall softening. (Figure inspired by Levesque-Tremblay et al. 2015).

an oligo-GalA acceptor (Sterling et al., 2006). In the Golgi, HG is thought to be methylesterified by putative methyltransferases QUASIMODO2 (QUA2), QUASIMODO3 (QUA3), COTTON GOLGI-RELATED2 (CGR2) and/or COTTON GOLGI-RELATED3 (CGR3) (Mouille et al., 2007; Miao et al., 2011; Kim et al., 2015) (Figure 7). In *Arabidopsis*, *cgr2 cgr3* double mutants have reductions in the degree of methylesterification of HG and lengths of both roots and hypocotyl (Kim et al., 2015). QUA2 carries a putative MT domain. Surprisingly, a *qua2* mutant reportedly exhibited 50% reduction in HG, but no alteration to HG methylesterification compared with wild-type plants (Mouille et al., 2007), which may reflect a compensatory response to reduced methyltransferase activity.

1.4.3 Homogalacturonan delivery

While the view that HG synthesis occurs in the Golgi is generally accepted (Mohnen, 2008), the exact routes of its delivery to the cell surface are less certain. ECH and its interactors YIP4a and YIP4b appear to function in delivery of pectin from the TGN, since wall polymers are mis-sorted and accumulate in the vacuole in mutants lacking these genes (Gendre et al., 2013). SECRETORY CARRIER MEMBRANE PROTEIN2 (SCAMP2)-marked Mobile Secretory Vesicle Compartments (SVCs) have been shown to be marked by JIM7 antibodies, which label pectin with high degree of methylesterification, implying their involvement in pectin delivery to the apoplast (Toyooka et al., 2009). The human SCAMP2 protein mediates secretion in concert with the GTPase Arf6 and phospholipase D1 (PLD1) (Liu et al., 2005). Analogously, a similar mechanism involving a plant SCAMP2 pathway may facilitate HG delivery to the wall. Additionally, the Exocyst complex may be required for pectin delivery. Several mutants with defects in Exocyst subunits exhibit reduced pectin accumulation in seed coat epidermal cells (Kulich et al., 2010). Thus, HG secretion might occur through multiple pathways, potentially reflecting a requirement to dynamically regulate incorporation of pectin in the wall depending on the developmental stage or growth response.

1.4.4 Homogalacturonan modifications by PMEs and PMEIs

HG polymers are delivered to the wall in a highly methylesterified state (Staehelin and Moore, 1995; Sterling et al., 2001; Driouich et al., 2012). *In muro*, HG may then be selectively de-esterified through the action of pectin methylesterases (PMEs), the activity of which in turn is regulated by pectin methylesterase inhibitors (PMEIs) (Levesque-Tremblay et al., 2015). The

Arabidopsis genome reportedly harbors 66 PMEs and 69 PMEIs, indicating the importance and complexity of HG biosynthesis and modification (Wolf et al., 2009; Senechal et al., 2014). The specific spatial pattern of de-esterification reportedly yields contrasting outcomes regarding the fate of HG and the wall's mechanical properties (Figure 7). In one scenario, blockwise de-esterification of large sections of HG by PMEs would produce negatively charged Gal-A chains that interact with calcium ions (Ca^{2+}). Ca^{2+}-dependent crosslinking causes formation of so-called "egg-box" structures underlying the formation of pectin gels (Braccini and Perez, 2001), and has been demonstrated *in vitro* to promote cell wall rigidification (Ngouemazong et al., 2012). In contrast, through another suggested mode of action, PME-mediated random de-esterification of HGs would prevent egg-box formation. Instead, non-blockwise de-methylesterified HGs would be substrates of HG-modifying enzymes, such as polygalacturonases (PGs) and pectate lyases (PLs) that cleave HG polymers into shorter oligogalacturonides (OG), thereby promoting cell wall loosening (Wakabayashi et al., 2003; Pelloux et al., 2007). PG overexpression in *Arabidopsis* results in a longer hypocotyl, supporting the notion that PGs mediate increased wall loosening (Xiao et al., 2014). Correspondingly, PME and PMEI overexpression studies have connected various members of the two families to both promotion and inhibition of growth (Marc et al., 1998; Bosch and Hepler, 2006; Lionetti et al., 2007; Pelletier et al., 2010; Braybrook and Peaucelle, 2013). In many cases, putative PMEs or PMEIs have been assessed by testing their ability to modify pectin methyl esterification status using pectin-binding antibodies with affinity for highly esterified or de-esterified pectin, which do not discriminate between random and blockwise de-esterification patterns. Furthermore, PMEs exhibit differential activity depending on the pH, e.g., alkaline pI PMEs catalyze blockwise de-esterification while acidic pI PMEs randomly de-esterify pectin (Jolie et al., 2010; Hocq et al., 2017).

Nonetheless, several recent studies support a promotive role of PME5 in cell wall loosening. When new organs are formed by the shoot apical meristem, local de-esterification occurs at sites of incipient primordia (Peaucelle et al., 2008). Overexpression of PMEI3, which globally inhibits de-esterification, prevents primordia formation, while PME5 overexpression leads to increased de-esterification and ectopic organ initiation. The sites of incipient primordia have higher elastic modulus than peripheral zones of the apical meristem, according to atomic force microscopy (AFM) measurements (Peaucelle et al., 2011). The inhibition of organ initiation in PMEI3-overexpressing plants also corresponds with reduction in elasticity (Peaucelle et al., 2011). This indicates that local softening via HG de-methylesterification could be required for organ initiation in the apical meristem.

Subcellular regulation of HG methylesterification may also be crucial in controlling cell anisotropic growth. It has long been thought that longitudinal growth of epidermal cells in the hypocotyl is determined by the orientation of organized cellulose microfibrils (Baskin, 2005; Lloyd, 2011). CESAs synthesize cellulose microfibrils as they travel along the PM, guided by cortical microtubules (CMT) oriented perpendicularly to the growth axis (Paredez et al., 2008). Disruption of either microtubule organization or cellulose synthesis causes perturbations to longitudinal growth, supporting this hypothesis (Paredez et al., 2008; Li et al., 2012). However, recent findings suggest that before cells have initiated longitudinal growth, and before microtubules acquire transverse organization, prospective longitudinal walls of isodiametric cells are preferentially HG de-esterified while transverse walls retain high degree of HG methylesterification (Peaucelle et al., 2015). This suggests that asymmetric HG de-esterification triggers the transition to directional growth in the hypocotyl, whereas the CMT-guided cellulose network reinforces the aforementioned asymmetry (Peaucelle et al., 2015). Regulation of HG modification may therefore play an essential role not only in modification of growth properties but also in fundamental growth decisions throughout plant development.

1.4.5 Auxin regulation of PME/PMEI

As HG modifications sometimes occur in highly spatially restricted regions, for instance during organ initiation in the apical meristem (Peaucelle et al., 2008), HG modulation seems likely to be tightly regulated. Hence, HG methylesterification is putatively subject to multi-level control in the modulation of growth patterns, and the influence of auxin in this control has been extensively studied.

As already noted, the plant hormone auxin is a master regulator of growth, and may context-dependently either promote or inhibit growth of plant organs. Its growth-promotive activity is largely linked to acidification of the wall, as postulated in the acid growth theory (Rayle and Cleland, 1992). Upon auxin perception, PM-localized H^+ ATPase proton pumps pump protons into the wall matrix, acidifying the wall (Takahashi et al., 2012). The consequent reduction in pH activates potassium channels that transport K^+ into the cytosol, driving H_2O uptake and maintaining turgor (Philippar et al., 2004). In the wall, low pH activates wall-modifying enzymes such as expansins that sever cell wall polysaccharide connections, loosening the wall (Cosgrove, 2000). The pH reduction also activates acidic PMEs, thought to mainly execute random de-esterification, thus further promoting wall loosening as described in the previous section (Duvetter et al., 2006; Cameron et al., 2008). A demonstrative example

comes from studies of organ initiation in the apical meristem. New organs form at sites of high auxin concentration in a PIN auxin efflux-dependent manner (Braybrook and Peaucelle, 2013). Local application of auxin is sufficient to restore organ initiation in the apical meristem of the auxin transporter mutant *pin1*, which cannot initiate organs under control conditions (Reinhardt et al., 2000). This coincides with local de-esterification and loosening of the wall. Overexpression of PMEI blocks auxin-induced outgrowth, while local PME application promotes bulging of *pin1* apices (Braybrook and Peaucelle, 2013).

Notably, exogenous auxin application rapidly inhibits root elongation, in stark contrast to its elongation-promoting effect in shoots (Bonner and Koepfli, 1939). Furthermore, upon gravistimulation, auxin accumulates in and inhibits growth of cells on the lower side, allowing reorientation of growth (Band et al., 2012). Auxin thus plays a complex role in growth regulation, potentially mediating both growth promotion and inhibition. A recent study demonstrated that upon auxin application or gravistimulation, the apoplast is rapidly and transiently alkalized. This auxin-mediated alkalization involves the receptor-like kinase FERONIA (Barbez et al., 2017), which upon binding to its ligand RALF phosphorylates and inhibits the PM H^+-ATPase AHA2, required for proton pumping into the apoplast (Haruta et al., 2014). Interestingly, the auxin response factor ARF7 positively regulates the expression of ERULUS, an RLK hypothesized to function to negatively regulate PME activity via the FER-PM H^+-ATPase mechanism (Schoenaers et al., 2018).
Auxin furthermore modulates the cell wall biosynthesis and modification transcriptome (Nemhauser et al., 2006). However, the transcriptomic effect of auxin may be highly context-specific. For instance, auxin has been reported to both up- and downregulate expression of various PMEs and PMEIs in *Arabidopsis* (Goda et al., 2004; Nemhauser et al., 2006; Chapman et al., 2012).

1.4.6 Cell wall sensing via homogalacturonan

Plant growth is a highly dynamic process, in which plants must continuously assess their environment and adjust their growth accordingly. For such dynamic responses, a machinery is presumably required to sense not only external conditions, but also endogenous states. Recent advances have begun illuminating mechanisms involved in cell wall integrity sensing. Several pathways have been identified through which changes in wall status trigger cellular homeostatic responses, and multiple mechanisms that may participate in sensing of HG modification have been proposed (Hematy et al., 2007; Voxeur and Hofte, 2016; Feng et al., 2018).

As noted above, pectin is secreted to the wall with high degree of methylesterification. Inhibition of PME by PMEI activates brassinosteroid signaling via the receptor BRI1 and its co-receptor BAK1 and the BAK1 interactor RLP44, which enhances expression of wall loosening enzymes such as PMEs and expansins (Wolf et al., 2012; Wolf et al., 2014). As described above, randomly de-esterified HG may be cleaved into OGs, as part of a wall loosening mechanism. OGs are also products of wall damage, for instance during pathogen attack. Wall-associated kinases (WAKs) have been demonstrated to bind to HG with higher affinity to smaller fragments such as OGs (Kohorn et al., 2014), and are involved in stress responses via OG perception (Brutus et al., 2010; Kohorn et al., 2012). WAKs are also required for normal cell expansion, and thought to operate by enhancing turgor through sensing of pectin status (Kohorn et al., 2006). Recently, increasing attention has been paid to the *Catharanthus roseus* receptor-like kinase (CrRLK1L) subfamily as potential wall integrity sensors (Voxeur and Hofte, 2016). Several lines of evidence indicate that the CrRLK1L member FERONIA may be a regulator of wall homeostasis, potentially by probing HG-Ca^{2+} crosslinking status, and modulating alkalinization of the apoplast, thus controlling PME/PMEI activity (Haruta et al., 2014; Feng et al., 2018; Schoenaers et al., 2018).

2 Objectives

The objective of this study was to understand the mechanisms involved in differential cell elongation, using the apical hook as a model. The following questions were addressed in this work:

- How does ECHIDNA mediate in the regulation of apical hook development?
  (PAPER I)

- What is the mechanism underlying ECHIDNA-mediated secretion of auxin carriers at the *trans*-Golgi network?
  (PAPER II)

- How do auxin and cell wall modifications control differential growth during apical hook development?
  (PAPER III)

3 Results and Discussion

3.1 ECHIDNA-mediated post-Golgi trafficking of auxin carriers for differential cell elongation **(Paper I)**

3.1.1 ECHIDNA is required for ethylene-mediated apical hook development and interacts genetically with AUX1

In a previous study, ECHIDNA (ECH) was characterized as a novel regulator of secretory trafficking from the TGN to the cell periphery, and shown to be required for cell elongation (Gendre et al., 2011). In Paper I, I examined the apical hook development of the *ech* mutant by time-lapse imaging, and found that compared to WT, *ech* hook development was severely perturbed and insensitive to exogenously applied ACC, a precursor of the plant hormone ethylene (Figure 8A, D-E). A major role of ethylene during hook development is to promote the establishment of an asymmetric auxin distribution (Lehman et al., 1996; Zadnikova et al., 2010; Zadnikova et al., 2016). The *ech* mutant exhibited disrupted auxin asymmetry establishment compared to WT, as revealed by examination of the synthetic auxin reporter DR5 (Figure 8B-C). Auxin asymmetry establishment relies polar auxin transport mediated by the combinatorial action of auxin transporters of the AUX/LAX influx and PIN efflux carrier families (Vandenbussche et al., 2010; Zadnikova et al., 2010). Of these, I examined the expression patterns of the central players AUX1 and PIN3, and found them both to be expressing in the epidermis (with PIN3 additionally expressing in cortex), overlapping with the epidermal expression pattern of ECH-YFP. Like *ech*, I observed that the *aux1-21* mutant, in agreement with

previous studies (Vandenbussche et al., 2010), but not the *pin3-4* mutant exhibited insensitivity to ACC treatment. Furthermore, an *ech aux1-21* double mutant exhibited a strongly enhanced phenotype, suggesting that ECH and AUX1 operate in a common pathway and are required for ethylene-mediated control of hook development.

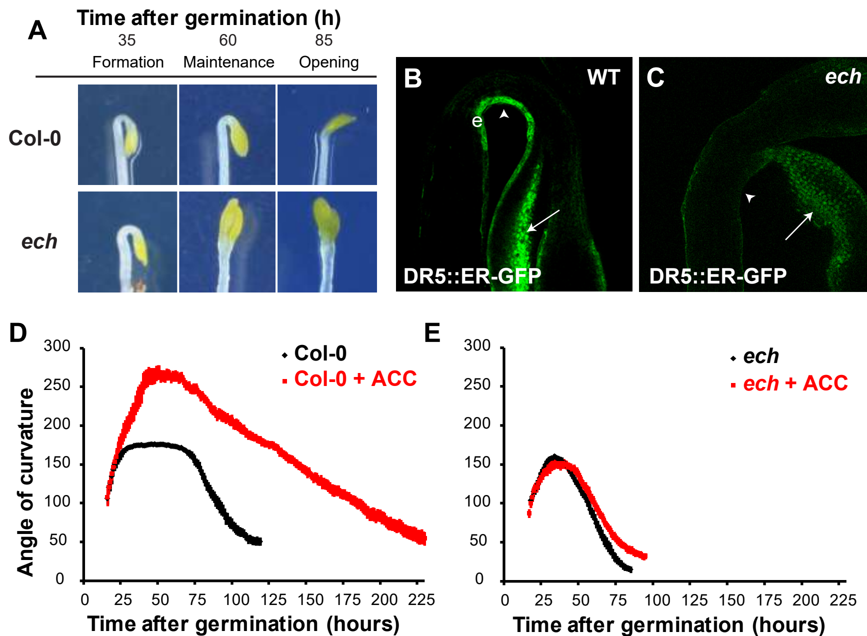

***Figure 8.* ECH is required for ethylene-mediated hook development and auxin asymmetry establishment. (A, D-E).** WT seedlings form a closed hook under control conditions, and respond to ACC treatment. In contrast, *ech* mutant seedlings fail to form a closed hook, and exhibit ACC insensitivity. **(B-C)** DR5 is strongly expressed on the inner side of the hook in WT, while *ech* mutants have severely perturbed DR5 expression pattern.

3.1.2 ECHIDNA mediates secretion of AUX1 from the TGN

I observed that AUX1-YFP but not PIN3-GFP levels were strongly reduced at the PM in *ech* compared to WT, with AUX1-YFP additionally agglomerating in intracellular compartments overlapping with that of Lysotracker Red, a low-pH-associated dye which labels vacuolar structures. Fluorescence recovery after photobleaching (FRAP) experiments revealed that *de novo* secretion of AUX1-YFP was strongly reduced in *ech* compared to WT (Figure 9), while delivery of PIN3-GFP and LAX3-YFP, an AUX1 paralog, was only marginally affected in *ech*. These findings highlight the specificity of post-Golgi secretory trafficking

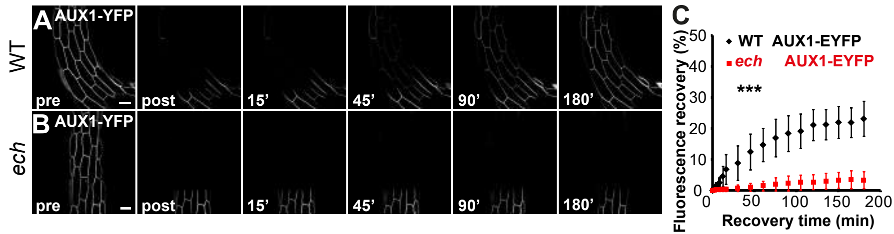

***Figure 9*. ECHIDNA is required for delivery of de novo synthesized AUX1 to the PM.** Upon photobleaching of AUX1-YFP, it recovered to 25% of initial intensity in WT after 180 minutes (**A** and **C**), while AUX1-YFP recovery was severely reduced in *ech* (**B**). Scale bars 5 µm.

of auxin carriers from the TGN, where AUX1 would follow an ECH-dependent pathway distinct from that of PIN3 and LAX3 trafficking.

3.1.3 TGN-Mediated Trafficking of AUX1 and PIN3 to the Plasma Membrane Is Independent of V-ATPases

The TGN-localized V-ATPase is crucial for both endocytic and secretory trafficking (Dettmer et al., 2006). In this study I observed that the TGN-specific V-ATPase subunit VHA-a1 mislocalized to Lysotracker Red-positive compartments in the hook of *ech* mutants. However, I found that pharmacological inhibition of V-ATPase function using Concanamycin A (ConcA) only marginally affected WT apical hook development and *de novo* delivery of AUX1-YFP and PIN3-GFP to the PM, compared to the severe hook developmental and AUX1-YFP secretion defects observed in *ech*. VHA-a1 is essential for secretion of other PM-resident cargos, such as BRI1 (Dettmer et al., 2006). Thus, the results in Paper I indicate potentially multiple secretory pathways from the TGN and some of these are independent of VHA-a1 function.

3.1.4 ECHIDNA Resides Predominantly with SVs at the TGN

The plant TGN is a hub where multiple pathways intersect (Viotti et al., 2010). This is reflected by the observation that it comprises several subdomains, and produces several distinct vesicle structures, such as secretory vesicles (SVs) and Clathrin-coated vesicles (CCVs) (Kang et al., 2011). Co-localization analysis revealed that while ECH and VHA-a1 co-localize strongly, ECH and VHA-a1 only partly overlaps with CLATHRIN HEAVY CHAIN-positive structures, suggesting that ECH resides at sites of SVs rather than CCV sites at the TGN. Moreover, high-resolution 3D electron tomography of Golgi/TGN showed that the *ech* TGN appears more tubulated with fewer SV than WT, while the number

of CCVs per TGN is unaffected compared to WT. Taken together these findings suggest that ECH has a role in SV genesis at the TGN.

Altogether, the data presented in Paper I indicate that auxin carriers are delivered via differential trafficking routes at the TGN, where AUX1 appears to rely on a pathway distinct from that of PIN3 or LAX3 trafficking. AUX1 and LAX3 are highly similar at the amino acid level (Peret et al., 2012). However, LAX3 is unable to functionally replace AUX1. Potentially, part of their distinction lies at the level of sorting at the TGN. It is not clear how such pathway separations are established within a subdomain of the TGN. Possibly ECH may function to recruit specific cargos or vesicle formation components required for a defined subset of cargos, allowing fine-tuning of secretory trafficking from the TGN.

3.2 Ethylene Regulates Differential Growth via BIG ARF-GEF-Dependent Post-Golgi Secretory Trafficking in *Arabidopsis* **(Paper II)**

3.2.1 BIG1-4 function during apical hook maintenance and genetically interact with ECH

In Paper I, the TGN-localized protein ECH was shown to be crucial for ethylene-mediated hook development and secretion of AUX1 to the PM. The *ech* mutant exhibited TGN morphological defects, with a strong reduction in SV number, while the number of CCVs was unaffected compared to WT. This suggests that ECH might play a role in formation of SVs at the TGN. In Paper II, I investigated whether ECH function involved players of a vesicle formation machinery.

A common mechanism for vesicle formation involves GTPases of the ARF family, which act to recruit cargos and vesicle components (Bourne et al., 1990; Singh and Jurgens, 2018). The activity of ARFs is regulated by ARF-GEFs, which activate ARFs by catalysing GDP/GTP exchange (Anders and Jurgens, 2008). I dissected the involvement of ARF-GEFs during apical hook development. Taking advantage of the sensitivity of ARF-GEFs to the fungal toxin BFA, and using genetically modified BFA-resistant ARF-GEFs, I identified that the ARF-GEF GNOM is required early in hook development, during hook formation, while BIG ARF-GEFs operate redundantly and independently of GNOM to mediate hook maintenance. While BIG single mutants exhibited no discernible hook defects, higher order mutants were defective in hook maintenance, indicating functional redundancy between them. The BIG3 protein is the sole BIG ARF-GEF member resistant to BFA (Richter

et al., 2014). Treating *big3* with BFA thus allows simultaneous targeting and inhibition of BIG1-4 function. When *big3* is treated with BFA, hook development and ethylene response are strongly perturbed, phenocopying the *ech* mutant (Figure 10). Similar phenotypes and potential role of ECH in SV genesis prompted me to examine if ECH and BIGs operated in a common pathway during hook development. Like *big3*, the *ech* mutant exhibited hypersensitivity to BFA compared to WT, suggesting compromised ARF-GEF function in *ech*. Furthermore, an *ech big2 big3* triple mutant displayed a strongly enhanced hook phenotype compared with *ech* or *big2 big3* mutants. Taken together, these results indicate that ECH and BIG1-4 operate in a common pathway downstream of ethylene.

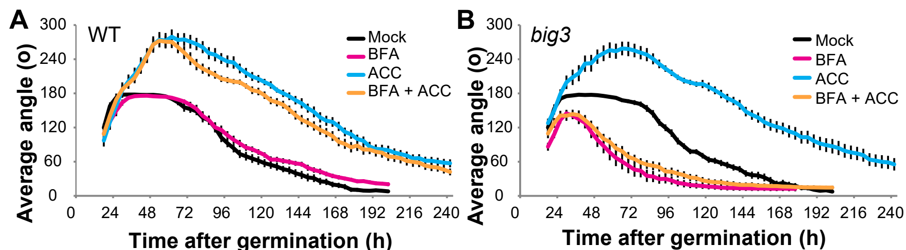

***Figure 10.* BIG ARF-GEF function is required for ethylene-mediated apical hook development.** Compared to WT **(A)**, BFA-treated *big3* seedlings fail to form a closed apical hook, and exhibit insensitivity to ACC treatment when grown on BFA **(B)**.

3.2.2 BIG1-4 are required for AUX1 trafficking to the PM

The results in Paper I demonstrated that ECH is essential for secretion of AUX1 to the PM. When germinated upon BFA, *big3* mutants exhibited strongly reduced AUX1-YFP levels at the PM compared to WT. Concurrently, AUX1 transcript levels were only mildly reduced in *big3* upon BFA, pointing towards a post-transcriptional effect of blocking BIG function on AUX1 levels at the PM. In agreement, short-term BFA treatment caused strong AUX1-YFP agglomeration in *big3* mutants but not in WT (Figure 11). AUX1-YFP agglomerations in *big3* +BFA overlapped with the TGN-localized VHA-a1-RFP. The AUX1-YFP agglomeration in *big3* disappeared upon pre-treatment with the protein synthesis inhibitor CHX followed by CHX + BFA treatment. Furthermore, the AUX1-YFP agglomerations upon BFA treatment in *big3* disappeared when similar experiments were performed in *Arabidopsis* lines expressing BFA-resistant BIG4 but not BFA-resistant GNOM. Taken together,

these observations indicate that BIGs operate redundantly in delivery of newly synthesized AUX1 to the PM independently of GNOM at the TGN.

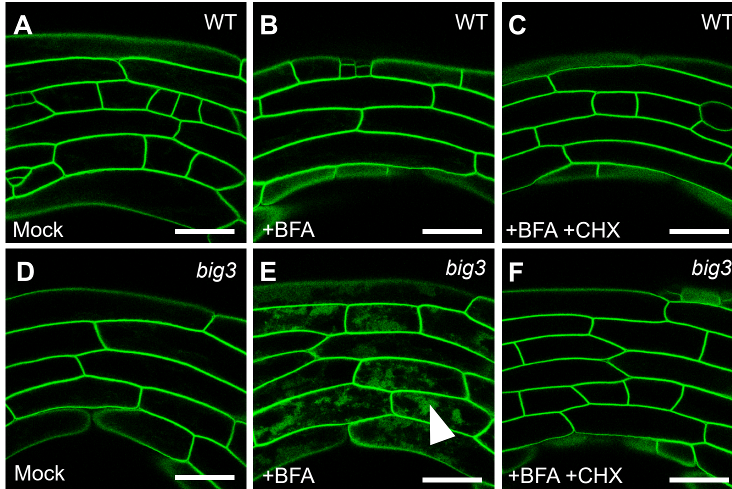

Figure 11. **BIG1-4 Are Required for *de novo* Delivery of AUX1-YFP. (A)** to **(F)** AUX1-YFP agglomerates in *big3* upon BFA treatment. While under mock conditions, AUX1-YFP remains exclusively at the PM in both the wild type **(A)** and *big3* **(D)**; upon 3 h of 50 µM BFA, AUX1-YFP agglomerates intracellularly in *big3* **(E)** (white arrowhead) but not wild-type **(B)**. Agglomerations are blocked in *big3* when seedlings are pretreated with 50 µM CHX for 1 h followed by 3 h of 50 µM CHX + 50 µM BFA treatment **(F)**, while AUX1-YFP remains unaffected in the wild type **(C)**. Scale bars 20 µm.

3.2.3 BIG4 and ECH Colocalize at the TGN and Their Localization Is Interdependent

With ECH and BIG1-4 operating in overlapping pathways, I investigated whether ECH and BIG1-4 co-localize at the TGN. I observed strong co-localization between ECH-YFP, BIG4-RFP and VHA-a1-GFP/VHA-a1-RFP, suggesting that ECH and BIG ARF-GEFs operate at the same TGN domain. I furthermore observed that in the absence of ECH, BIG4^{R}-YFP was strongly mislocalized, exhibiting a diffuse labelling compared to the strictly punctate labelling in WT. Similarly, ECH localization was strongly perturbed upon disruption of BIG function, with ECH-YFP exhibiting a diffuse pattern compared to the punctate ECH-YFP pattern in WT. In the *ech* mutant, VHA-a1 is mislocalized. Disruption of VHA-a1 function results in trafficking defects from the TGN (Luo et al., 2015). Therefore, it was previously hypothesized that the defects observed in *ech* were due to perturbed V-ATPase function at the TGN (Gendre et al., 2011). However, pharmacological inhibition of V-ATPase function using ConcA did not affect BIG3-YFP nor BIG4R-YFP localization

upon 2 hr treatment, in contrast to the strong observed agglomeration of VHA-a1-GFP. This is in accordance with findings in Paper I, that ECH-dependent AUX1 trafficking is independent of VHA-a1 function, and supports the suggestion that multiple secretory pathways operate even within a subdomain at the TGN.

3.2.4 BIG ARF-GEFs and ECH Are Required for ARF1 Localization at the TGN

BIG3 was previously demonstrated to function as an ARF-GEF for ARF1 GTPase in vitro (Nielsen 2006). I observed that, while ARF1-GFP exhibited a largely punctate pattern in WT seedlings, in *ech* the ARF1-GFP signal was largely diffuse with few punctate structures (Figure 12A and F). Similarly, when disrupting BIG1-4 by BFA-treatment of *big3*, ARF1-GFP exhibited an almost exclusively diffuse labelling, in contrast to BFA-treated WT seedlings (Figure 12B-C, G-H). In comparison, VHA-a1-GFP did not exhibit agglomeration in WT or *big3* upon identical BFA-treatment (Figure 12D-E, I-J). Thus, the data suggest that ARF1 relies on ECH and BIG1-4 for its localization independently of VHA-a1 function.

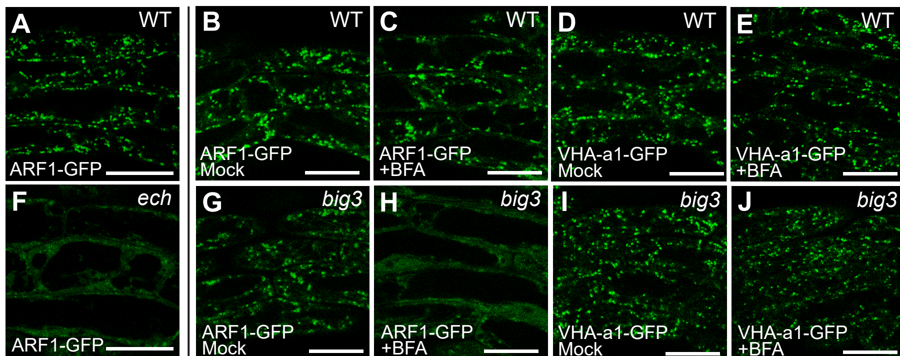

Figure 12. **ECH and BIGs Are Required for Proper ARF1-GFP Localization.** ARF1-GFP localization is punctate in the wild type **(A)** while strongly disrupted in *ech* **(F)**. While 15 min of 50 µM BFA has no effect on ARF1-GFP localization in the wild type **(C)** compared with mock **(B)**, punctate ARF1-GFP in *big3* under mock conditions **(G)** becomes strongly mislocalized in *big3* upon 15 min of 50 µM BFA treatment **(H)**. By contrast, VHA-a1-GFP localization under mock conditions in either the wild type **(D)** or *big3* **(I)** remains unaffected upon 15 min of 50 µM BFA treatment in both the wild type **(E)** and *big3* **(J)**. Scale bars = 20 µm.

3.2.5 ARF1 Members Are Essential for Ethylene-Mediated Apical Hook Development and AUX1 Trafficking

ARF1 localization relies on ECH and BIG1-4. Therefore I investigated if ARF1 also plays a role during apical hook development. Due to expected high redundancy among the six ARF1 members (Xu and Scheres, 2005), I generated a dominant negative GDP-locked ARF1 variant ($ARF1^{T31N}$) under control of β-estradiol inducible UBQ10 promoter to interfere with ARF1 function. Upon induction of $ARF1^{T31N}$ expression, hook development was severely perturbed, and $ARF1^{T31N}$-expressing seedlings did not respond to ACC treatment. Thus, ARF1, like ECH and BIG1-4, is required for ethylene-mediated hook development. I therefore examined whether ARF1, like ECH and BIG1-4, is required for AUX1 trafficking to the PM. Upon induction of $ARF1^{T31N}$ expression, AUX1-YFP PM levels were reduced to 25% of WT levels. Additionally, in contrast to the exclusively PM-localized AUX1-YFP observed in WT, AUX1-YFP agglomerated strongly in intracellular structures in $ARF1^{T31N}$.

In conclusion, Paper II revealed additional components acting in concert with ECH in the AUX1 secretory pathway essential for ethylene-mediated hook development. This pathway comprises BIG ARF-GEFs and their target ARF1 and functions in AUX1 delivery independently of VHA-a1, as shown for ECH in Paper I. It is interesting to note that, while ECH, BIG ARF-GEFs and ARF1 converge in mediating trafficking of AUX1 at the TGN, both ARF1 and BIG ARF-GEFs have broad functions in trafficking compared to those revealed so far for ECH. For instance, ARF1 operates independently of ECH to facilitate COPI-mediated retrograde trafficking from the Golgi (Stefano et al., 2006). BIG1-4 also function in pathways not involving ECH, such as delivery of cargo to the vacuole (Richter et al., 2014). One may therefore hypothesize that ECH acts as an element of a machinery that, although requiring ARF1 and BIG ARF-GEFs, might provide compartment and/or pathway specificity for trafficking via the TGN.

3.3 Mechanochemical Feedback Between Auxin and Pectin Modification Mediates the Control of Apical Hook Development **(Paper III)**

As described in Paper I and Paper II, ECH mediates secretion of the auxin carrier AUX1. However, ECH was also shown to mediate delivery of cell wall material, and in agreement, the *ech* mutant exhibits altered wall composition (Gendre et al., 2013). Additionally, as demonstrated in Paper I, the *ech* mutant has severe

defects in hook development, a process involving differential cell elongation in which cell wall would be expected to play a critical role. Therefore I chose to investigate the role of cell wall regulation during differential growth in apical hook development in Paper III.

3.3.1 Cell elongation rates display stage specific differences during apical hook development

Apical hook development largely relies on asymmetric cell elongation (Silk and Erickson, 1978; Raz and Ecker, 1999). Control of cell expansion, such as elongation, involves an interplay between the force exerted by vacuolar turgor, and the capacity of the wall to resist or yield turgor (Kierzkowski and Routier-Kierzkowska, 2019). The cell wall composition largely determines its mechanical strength (Cosgrove, 2018). To identify regions of growth asymmetry during hook development, I initially mapped cell elongation rates across the hook by time-lapse confocal imaging. I observed that during hook formation, growth asymmetry is most pronounced close to the shoot apical meristem (SAM) with cells on the outer side exhibiting 3-fold higher growth rates than cells on the inner side. As hook formation is completed, growth rates dynamically change where growth asymmetry is reduced compared to during hook formation (Figure 13).

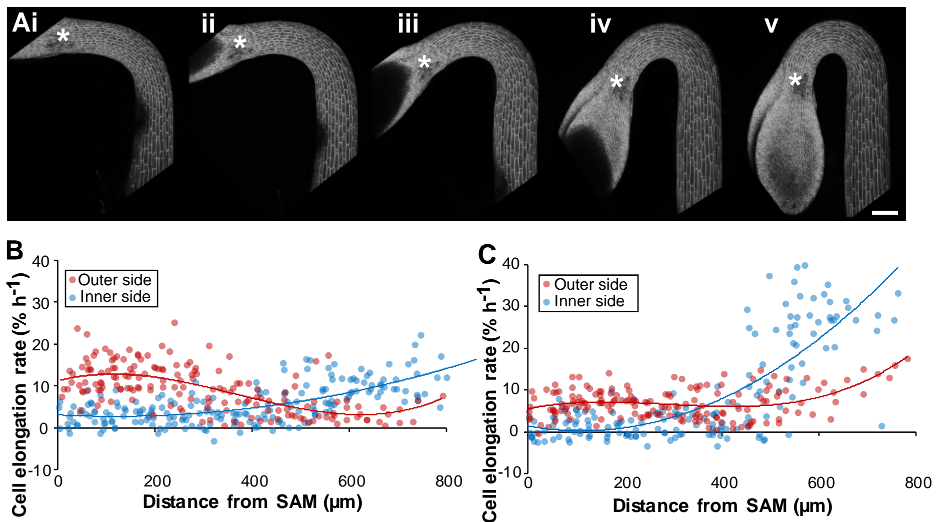

Figure 13. **Cell elongation is asymmetric during hook development (A i-v)** Time-lapse macro-confocal images of hook formation in WT, obtained at 2 hr intervals from 90° **(Ai)** to 180° angle **(Av)**. **(B and C)** Cell elongation rates of epidermal cells at 90° **(B)** and 180° **(C)**. In all figures, asterisks mark positions of SAM. Continuous lines in red and blue represent trendlines for outer side and inner side cells, respectively. All scale bars 100 µm

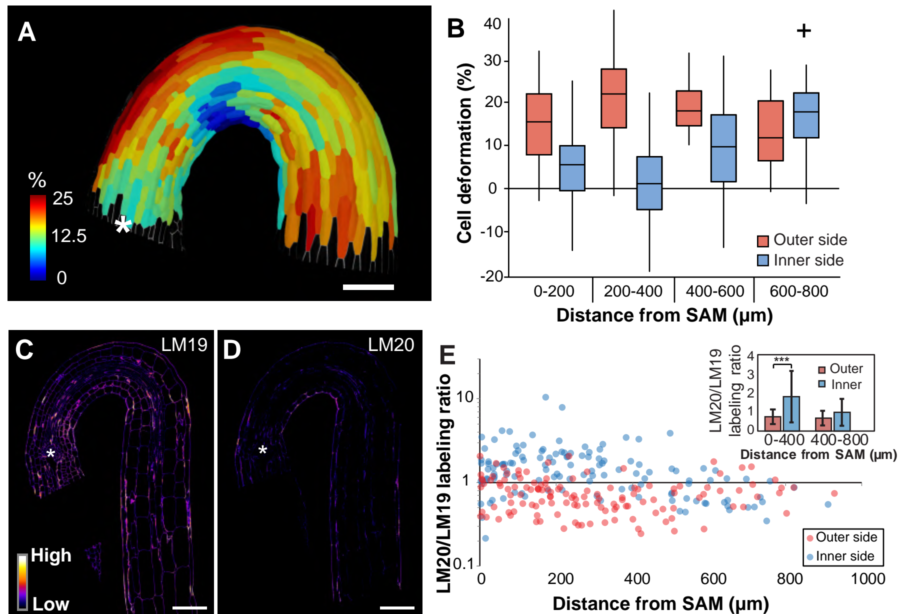

***Figure 14*. The apical hook exhibits mechanical and wall compositional asymmetry**. **(A)** Averaged heat map of surface area deformation in epidermal cells upon osmotic treatment in WT. **(B)** Boxplot of cell deformation levels for epidermal cells on the outer and inner sides in 200 µm longitudinal zones starting from SAM, based on **(A)**. **(C-D)** Heat maps of immunolabeling with LM20 **(C)** and LM19 **(D)** in 2.5 µm sections of WT seedlings during early hook maintenance. **(E)** Scatterplot calculated ratio of LM20 and LM19 labeling fluorescence intensity for individual cells in WT seedlings based on **(C)** and **(D)**. Graph inset in **(E)** showing data pooled into 400 µm zones for statistical analysis. In **(A)**, **(C)** and **(D)**, white asterisks mark the position of SAM. In **(E)** inset, black asterisks mark T-test p-value < 0.0001. In **(B)** + represents outlier. Scale bars 100 µm.

3.3.2 Cell mechanochemical property differences correlate with differential cell elongation and are required for hook development

Using osmotic treatments to modulate turgor pressure, I observed that cell deformation levels that are governed by cell wall mechanics largely matched cell elongation patterns, with cells on the outer side close to SAM exhibiting high deformation, while cells on the inner side close to SAM exhibited low deformation levels (Figure 14A and B). Cell wall composition contributes to cell mechanical properties. Recent studies have demonstated that methylesterification of homogalacturonan (HG) pectin affects wall mechanical properties (Peaucelle et al., 2011; Peaucelle et al., 2015). I this study (Paper III) I found that HG was highly methylesterified in cells on the inner side close to SAM, corresponding to cells which exhibited low elongation rate and level of

deformation. In contrast, cells on the outer side close to SAM had comparatively lower levels of HG methylesterification.

HG is delivered to the cell wall in a highly methylesterified state (Staehelin and Moore, 1995; Driouich et al., 2012). *In muro* it may be de-esterified by pectin methylesterases (PMEs), a process that can be inhibited by PME inhibitors (PMEIs) (Wolf et al., 2009). Overexpression of PMEI5 caused a global increase in HG methylesterification, and disruption of methylesterification asymmetry observed in WT. This lead to markedly reduced cell deformation levels and elongation rates in PMEI5oe seedlings, effects that were most pronounced on the outer side of the hook compared to WT. I observed that compared to WT, hook development in PMEI5oe seedlings was severely perturbed. Taken together, these observations suggest that asymmetric HG methylesterification is required for proper hook development.

3.3.3 Auxin promotes methylesterification and pattterns wall compositional asymmetry during hook development

Auxin crucially regulates apical hook development (Zhao et al., 2001; Dharmasiri et al., 2005; Zadnikova et al., 2010). As the hook forms, auxin becomes asymmetrically distributed, with an auxin response maximum being established on the inner side of the hook (Zadnikova et al., 2010). Auxin is thought to inhibit elongation during hook development. However, the downstream events are not as well understood. The auxin maximum on the inner side overlaps with the region exhibiting high levels of HG methylesterification. To test whether high auxin mediates HG methylesterification, I used *yuc1D* mutant that has high expression of YUCCA1 that encodes a rate-limiting enzyme in IAA/auxin biosynthesis pathway (Zhao et al., 2001). *yuc1D* seedlings exhibited a strongly expanded region of auxin response maxima compared to WT, as revealed by the synthetic auxin reporter DR5, with cells on the outer side exhibiting strong DR5 expression, in contrast with the WT. *yuc1D* seedlings exhibited elevated HG methylesterification levels and disrupted methylesterification asymmetry compared to WT. Furthermore, cells on the outer side in *yuc1D* seedlings were greatly reduced in size compared with WT, and *yuc1D* seedlings were unable to form an apical hook. These data suggest that auxin may promote HG methylesterification, and that the asymmetric auxin distribution may pattern wall compositional asymmetry required for proper hook development.

3.3.4 PMEI5oe exhibit altered auxin response pattern

The defects in hook development in PMEI5oe seedlings led me to examine whether this impacted auxin response. Using time-lapse confocal imaging I observed that in WT, auxin response asymmetry as reported by DR5 was established only as bending reached 90°. This asymmetry gradually became more prominent as WT seedlings approached 180°. In contrast, PMEI5oe seedlings failed to establish DR5 asymmetry. Instead, PMEI5oe seedlings exhibited an extended pattern of DR5 expression towards the outer side of the hook. The transcriptional response of auxin responsive Aux/IAA genes *IAA5, IAA19* and *IAA29* upon 2 hr treatment with IAA did not show any discernible trend of alteration in PMEI5oe compared to WT, suggesting that the altered DR5 pattern is not due to a simple enhancement in auxin sensitivity. These data suggest that while auxin may promote HG methylesterification, HG methylesterification may in turn also feedback to impact the auxin response pattern, and the interplay between these two factors (wall modifications and auxin) is essential for proper hook development.

4 Conclusions and Future perspectives

4.1 Paper I

I showed that ECH regulates ethylene-mediated differential growth during apical hook development by mediating secretion of the auxin influx carrier AUX1 but not the auxin efflux carrier PIN3 at the TGN, independently of VHA-a1 function. These results indicate divergence and selectivity of secretory trafficking at the TGN, the molecular basis of which remain unknown. The selectivity of defects in trafficking in the *ech* mutant may indicate that ECH either aids in establishing TGN sub-compartmentalization essential for selectivity in cargo, or alternatively ECH is recruited to specific lipid micro-domains at the TGN, where it may mediate downstream steps e. g. in recruitment of a distinct vesicle formation machinery.

Thus, it is worth noting that in another publication (which I co-authored, not included in this thesis) (Wattelet-Boyer et al., 2016), it was shown that the lipid composition of TGN sub-compartments appears crucial for recruitment of cargo into distinct secretory pathways and this may maybe the mechanism for selectivity in cargo at the TGN.

4.2 Paper II

In Paper II, I demonstrated that secretion of AUX1, a crucial mediator of ethylene action during hook development, relies on a secretory module involving ECH, BIG ARF-GEFs and ARF1 at the TGN, independently of VHA-a1 function. Furthermore, I showed that the retention of ECH and BIGs at the TGN is interdependent. Since both BIGs and ARF1 operate in additional pathways

beyond TGN-to-PM secretion (Takeuchi et al., 2002; Pimpl et al., 2003; Richter et al., 2014), the data presented here demonstrates how multiple pathway components give rise to a complex network of distinct and overlapping endomembrane trafficking routes. ARF-GEFs are thought to confer pathway specificity to ARF action by recruiting them to distinct compartments (Anders and Jurgens, 2008). ARF-GEFs carry lipid-binding domains, such as the pleckstrin homology (PH) domain, that provide affinity for specific membrane lipids (Lemmon, 2007; Anders et al., 2008). As discussed for Paper I above, ECH may function in defining lipid environments favouring BIG recruitment. Alternatively, ECH and BIGs may interact directly at distinct TGN sites. Future studies could address the molecular mechanisms of how the interdependent module comprising ECH and BIGs is recruited to the TGN.

4.3 Paper III

Herein, I demonstrated that differential elongation during apical hook development relies on the establishment of asymmetric cell wall mechanical properties across the hook, through modification of pectin methylesterification, in an auxin-dependent manner. Furthermore, the data presented in Paper III suggests the existence a of mechanochemical component providing feeback to the auxin machinery. This study presents numerous outstanding questions: How does auxin regulate pectin methylesterification? The answer may lie in auxin mediated control of PME and PMEI genes. Furthermore, the activity of PME and PMEIs is influenced by apoplastic pH, which is known to be modulated by auxin providing a connection between auxin and pectin modification. Growth asymmetry during hook formation appears to precede establishment of the auxin response asymmetry, as reported by DR5 reporter. Since auxin and a mechanochemical component are interinfluential during hook development, it is tempting to hypothesize that auxin is not the sole trigger, but that initiation of hook formation results from initial mechanochemical asymmetries that are amplified via feedback mechanisms involving auxin. Exploring the earliest stages of hook formation could address such questions.

References

Abbas, M., Hernandez-Garcia, J., Pollmann, S., Samodelov, S.L., Kolb, M., Friml, J., Hammes, U.Z., Zurbriggen, M.D., Blazquez, M.A., and Alabadi, D. (2018). Auxin methylation is required for differential growth in Arabidopsis. P Natl Acad Sci USA **115,** 6864-6869.

Abbas, M., Berckhan, S., Rooney, D.J., Gibbs, D.J., Conde, J.V., Correia, C.S., Bassel, G.W., Marin-de la Rosa, N., Leon, J., Alabadi, D., Blazquez, M.A., and Holdsworth, M.J. (2015). Oxygen Sensing Coordinates Photomorphogenesis to Facilitate Seedling Survival. Current Biology **25,** 1483-1488.

Adamowski, M., and Friml, J. (2015). PIN-Dependent Auxin Transport: Action, Regulation, and Evolution. Plant Cell **27,** 20-32.

Albersheim, P., Darvill, A., Roberts, K., Sederoff, R., and Staehelin, A. (2011). Plant Cell Walls: From Chemistry to Biology. Plant Cell Walls: From Chemistry to Biology**,** 1-430.

Anders, N., and Jurgens, G. (2008). Large ARF guanine nucleotide exchange factors in membrane trafficking. Cell Mol Life Sci **65,** 3433-3445.

Anders, N., Nielsen, M., Keicher, J., Stierhof, Y.D., Furutani, M., Tasaka, M., Skriver, K., and Jurgens, G. (2008). Membrane association of the Arabidopsis ARF exchange factor GNOM involves interaction of conserved domains. Plant Cell **20,** 142-151.

Anderson, C.T. (2016). We be jammin': an update on pectin biosynthesis, trafficking and dynamics. J Exp Bot **67,** 495-502.

Atmodjo, M.A., Hao, Z.Y., and Mohnen, D. (2013). Evolving Views of Pectin Biosynthesis. Annu Rev Plant Biol **64,** 747-+.

Bacete, L., Melida, H., Miedes, E., and Molina, A. (2018). Plant cell wall-mediated immunity: cell wall changes trigger disease resistance responses. Plant J **93,** 614-636.

Band, L.R., Wells, D.M., Larrieu, A., Sun, J.Y., Middleton, A.M., French, A.P., Brunoud, G., Sato, E.M., Wilson, M.H., Peret, B., Oliva, M., Swarup, R., Sairanen, I., Parry, G., Ljung, K., Beeckman, T., Garibaldi, J.M., Estelle, M., Owen, M.R., Vissenberg, K., Hodgman, T.C., Pridmore, T.P., King, J.R., Vernoux, T., and Bennett, M.J. (2012). Root gravitropism is regulated by a transient lateral auxin gradient controlled by a tipping-point mechanism. P Natl Acad Sci USA **109,** 4668-4673.

Barbez, E., Dunser, K., Gaidora, A., Lendl, T., and Busch, W. (2017). Auxin steers root cell expansion via apoplastic pH regulation in Arabidopsis thaliana. Proc Natl Acad Sci U S A **114,** E4884-E4893.

Barbez, E., Kubes, M., Rolcik, J., Beziat, C., Pencik, A., Wang, B.J., Rosquete, M.R., Zhu, J.S., Dobrev, P.I., Lee, Y., Zazimalova, E., Petrasek, J., Geisler, M., Friml, J., and Kleine-Vehn, J. (2012). A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. Nature **485,** 119-U155.

Bard, F., and Malhotra, V. (2006). The formation of TGN-to-plasma-membrane transport carriers. Annu Rev Cell Dev Bi **22,** 439-455.

Barr, F.A., and Huttner, W.B. (1996). A role for ADP-ribosylation factor 1, but not COP I, in secretory vesicle biogenesis from the trans-Golgi network. Febs Lett **384,** 65-70.

Baskin, T.I. (2005). Anisotropic expansion of the plant cell wall. Annu Rev Cell Dev Bi **21,** 203-222.

Beziat, C., and Kleine-Vehn, J. (2018). The Road to Auxin-Dependent Growth Repression and Promotion in Apical Hooks. Current Biology **28,** R519-R525.

Beziat, C., Barbez, E., Feraru, M.I., Lucyshyn, D., and Kleine-Vehn, J. (2017). Light triggers PILS-dependent reduction in nuclear auxin signalling for growth transition. Nature Plants **3**.

Blancaflor, E.B., Fasano, J.M., and Gilroy, S. (1998). Mapping the functional roles of cap cells in the response of Arabidopsis primary roots to gravity. Plant Physiology **116,** 213-222.

Boerjan, W., Cervera, M.T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., Van Onckelen, H., Van Montagu, M., and Inze, D. (1995). Superroot, a recessive mutation in Arabidopsis, confers auxin overproduction. Plant Cell **7,** 1405-1419.

Bonner, J., and Koepfli, J. (1939). The Inhibition of Root Growth by Auxins. Am J Bot **26,** 557-566.

Bosch, M., and Hepler, P.K. (2006). Silencing of the tobacco pollen pectin methylesterase NtPPME1 results in retarded in vivo pollen tube growth. Planta **223,** 736-745.

Bourne, H.R., Sanders, D.A., and Mccormick, F. (1990). The Gtpase Superfamily - a Conserved Switch for Diverse Cell Functions. Nature **348,** 125-132.

Braccini, I., and Perez, S. (2001). Molecular basis of Ca2+-induced gelation in alginates and pectins: The egg-box model revisited. Biomacromolecules **2,** 1089-1096.

Braybrook, S.A., and Peaucelle, A. (2013). Mechano-chemical aspects of organ formation in Arabidopsis thaliana: the relationship between auxin and pectin. PLoS One **8,** e57813.

Braybrook, S.A., and Jonsson, H. (2016). Shifting foundations: the mechanical cell wall and development. Current Opinion in Plant Biology **29,** 115-120.

Briggs, W.R. (2016). Plant Biology: Seedling Emergence through Soil. Curr Biol **26,** R68-R70.

Briggs, W.R., Beck, C.F., Cashmore, A.R., Christie, J.M., Hughes, J., Jarillo, J.A., Kagawa, T., Kanegae, H., Liscum, E., Nagatani, A., Okada, K., Salomon, M., Rudiger, W., Sakai, T., Takano, M., Wada, M., and Watson, J.C. (2001). The phototropin family of photoreceptors. Plant Cell **13,** 993-997.

Brownfield, L., Wilson, S., Newbigin, E., Bacic, A., and Read, S. (2008). Molecular control of the glucan synthase-like protein NaGSL1 and callose synthesis during growth of Nicotiana alata pollen tubes. Biochem J **414,** 43-52.

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. P Natl Acad Sci USA **107,** 9452-9457.

Cameron, R.G., Luzio, G.A., Goodner, K., and Williams, M.A.K. (2008). Demethylation of a model homogalacturonan with a salt-independent pectin methylesterase from citrus: I. Effect of pH on demethylated block size, block number and enzyme mode of action. Carbohyd Polym **71,** 287-299.

Casanova, J.E. (2007). Regulation of arf activation: the sec7 family of guanine nucleotide exchange factors. Traffic **8,** 1476-1485.

Chapman, E.J., Greenham, K., Castillejo, C., Sartor, R., Bialy, A., Sun, T.P., and Estelle, M. (2012). Hypocotyl transcriptome reveals auxin regulation of growth-promoting genes through GA-dependent and -independent pathways. PLoS One **7,** e36210.

Chen, Y.G., Siddhanta, A., Austin, C.D., Hammond, S.M., Sung, T.C., Frohman, M.A., Morris, A.J., and Shields, D. (1997). Phospholipase D stimulates release of nascent secretory vesicles from the trans-Golgi network. J Cell Biol **138,** 495-504.

Cherfils, J., and Melancon, P. (2005). On the action of Brefeldin A on Sec7-stimulated membrane-recruitment and GDP/GTP exchange of Arf proteins. Biochem Soc T **33,** 635-638.

Chow, C.M., Neto, H., Foucart, C., and Moore, I. (2008). Rab-A2 and Rab-A3 GTPases define a trans-Golgi endosomal membrane domain in Arabidopsis that contributes substantially to the cell plate. Plant Cell **20,** 101-123.

Christensen, S.K., Dagenais, N., Chory, J., and Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. Cell **100,** 469-478.

Christie, J.M., Yang, H.B., Richter, G.L., Sullivan, S., Thomson, C.E., Lin, J.S., Titapiwatanakun, B., Ennis, M., Kaiserli, E., Lee, O.R., Adamec, J., Peer, W.A., and Murphy, A.S. (2011). phot1 Inhibition of ABCB19 Primes Lateral Auxin Fluxes in the Shoot Apex Required For Phototropism. Plos Biology **9**.

Cosgrove, D.J. (2000). Loosening of plant cell walls by expansins. Nature **407,** 321-326.

Cosgrove, D.J. (2018). Diffuse Growth of Plant Cell Walls. Plant Physiol **176,** 16-27.

Cox, R., Mason-Gamer, R.J., Jackson, C.L., and Segev, N. (2004). Phylogenetic analysis of Sec7-domain-containing Arf nucleotide exchangers. Mol Biol Cell **15,** 1487-1505.

Crowell, E.F., Bischoff, V., Desprez, T., Rolland, A., Stierhof, Y.D., Schumacher, K., Gonneau, M., Hofte, H., and Vernhettes, S. (2009). Pausing of Golgi Bodies on Microtubules Regulates Secretion of Cellulose Synthase Complexes in Arabidopsis. Plant Cell **21,** 1141-1154.
Dacks, J.B., and Field, M.C. (2018). Evolutionary origins and specialisation of membrane transport. Curr Opin Cell Biol **53,** 70-76.
Darwin, C. (1880). The Power of Movement in Plants. (London: John Murray).
Dascher, C., and Balch, W.E. (1994). Dominant Inhibitory Mutants of Arf1 Block Endoplasmic-Reticulum to Golgi Transport and Trigger Disassembly of the Golgi-Apparatus. J Biol Chem **269,** 1437-1448.
De Matteis, M.A., and Godi, A. (2004). PI-loting membrane traffic. Nat Cell Biol **6,** 487-492.
De Matteis, M.A., and Luini, A. (2008). Exiting the Golgi complex. Nat Rev Mol Cell Biol **9,** 273-284.
De Smet, I., Lau, S., Voss, U., Vanneste, S., Benjamins, R., Rademacher, E.H., Schlereth, A., De Rybel, B., Vassileva, V., Grunewald, W., Naudts, M., Levesque, M.P., Ehrismann, J.S., Inze, D., Luschnig, C., Benfey, P.N., Weijers, D., Van Montagu, M.C.E., Bennett, M.J., Jurgens, G., and Beeckman, T. (2010). Bimodular auxin response controls organogenesis in Arabidopsis. P Natl Acad Sci USA **107,** 2705-2710.
Delarue, M., Prinsen, E., Van Onckelen, H., Caboche, M., and Bellini, C. (1998). Sur2 mutations of Arabidopsis thaliana define a new locus involved in the control of auxin homeostasis. Plant J **14,** 603-611.
Dettmer, J., Hong-Hermesdorf, A., Stierhof, Y.D., and Schumacher, K. (2006). Vacuolar H+-ATPase activity is required for Endocytic and secretory trafficking in Arabidopsis. Plant Cell **18,** 715-730.
Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jurgens, G., and Estelle, M. (2005). Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell **9,** 109-119.
Ding, Z.J., Galvan-Ampudia, C.S., Demarsy, E., Langowski, L., Kleine-Vehn, J., Fan, Y.W., Morita, M.T., Tasaka, M., Fankhauser, C., Offringa, R., and Friml, J. (2011). Light-mediated polarization of the PIN3 auxin transporter for the phototropic response in Arabidopsis. Nat Cell Biol **13,** 447-U222.
Donohoe, B.S., Kang, B.H., and Staehelin, L.A. (2007). Identification and characterization of COPIa- and COPIb-type vesicle classes associated with plant and algal Golgi. P Natl Acad Sci USA **104,** 163-168.
Drakakaki, G., van de Ven, W., Pan, S.Q., Miao, Y.S., Wang, J.Q., Keinath, N.F., Weatherly, B., Jiang, L.W., Schumacher, K., Hicks, G., and Raikhel, N. (2012). Isolation and proteomic analysis of the SYP61 compartment reveal its role in exocytic trafficking in Arabidopsis. Cell Res **22,** 413-424.
Driouich, A., Follet-Gueye, M.L., Bernard, S., Kousar, S., Chevalier, L., Vicre-Gibouin, M., and Lerouxel, O. (2012). Golgi-mediated synthesis and secretion of matrix polysaccharides of the primary cell wall of higher plants. Frontiers in Plant Science **3**.
Du, Y.J., and Scheres, B. (2018). Lateral root formation and the multiple roles of auxin. Journal of Experimental Botany **69,** 155-167.
Dumaresq-Doiron, K., Savard, M.F., Akam, S., Costantino, S., and Lefrancois, S. (2010). The phosphatidylinositol 4-kinase PI4KIII alpha is required for the recruitment of GBF1 to Golgi membranes. J Cell Sci **123,** 2273-2280.
Dunser, K., and Kleine-Vehn, J. (2015). Differential growth regulation in plants - the acid growth balloon theory. Current Opinion in Plant Biology **28,** 55-59.
Duvetter, T., Fraeye, I., Sila, D.N., Verlent, I., Smout, C., Hendrickx, M., and Van Loey, A. (2006). Mode of de-esterification of alkaline and acidic pectin methyl esterases at different pH conditions. J Agr Food Chem **54,** 7825-7831.
Ecker, J.R. (1995). The Ethylene Signal-Transduction Pathway in Plants. Science **268,** 667-675.
Fan, X.P., Yang, C.Y., Klisch, D., Ferguson, A., Bhaellero, R.P., Niu, X.W., and Wilson, Z.A. (2014). ECHIDNA Protein Impacts on Male Fertility in Arabidopsis by Mediating trans-Golgi Network Secretory Trafficking during Anther and Pollen Development. Plant Physiol **164,** 1338-1349.
Feng, W., Kita, D., Peaucelle, A., Cartwright, H.N., Doan, V., Duan, Q.H., Liu, M.C., Maman, J., Steinhorst, L., Schmitz-Thom, I., Yvon, R., Kudla, J., Wu, H.M., Cheung, A.Y., and

Dinneny, J.R. (2018). The FERONIA Receptor Kinase Maintains Cell-Wall Integrity during Salt Stress through Ca2+ Signaling. Curr Biol **28,** 666-+.
Foresti, O., and Denecke, J. (2008). Intermediate organelles of the plant secretory pathway: Identity and function. Traffic **9,** 1599-1612.
Franzusoff, A., and Schekman, R. (1989). Functional Compartments of the Yeast Golgi-Apparatus Are Defined by the Sec7 Mutation. Embo J **8,** 2695-2702.
Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K. (2002). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. Nature **415,** 806-809.
Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B.F., Ljung, K., Sandberg, G., Hooykaas, P.J.J., Palme, K., and Offringa, R. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science **306,** 862-865.
Gallego-Bartolome, J., Arana, M.V., Vandenbussche, F., Zadnikova, P., Minguet, E.G., Guardiola, V., Van Der Straeten, D., Benkova, E., Alabadi, D., and Blazquez, M.A. (2011). Hierarchy of hormone action controlling apical hook development in Arabidopsis. Plant J **67,** 622-634.
Garcia-Mata, R., Szul, T., Alvarez, C., and Elizabeth, S. (2003). ADP-ribosylation factor/COPI-dependent events at the endoplasmic reticulum-Golgi interface are regulated by the guanine nucleotide exchange factor GBF1. Mol Biol Cell **14,** 2250-2261.
Geisler, M., Aryal, B., di Donato, M., and Hao, P.C. (2017). A Critical View on ABC Transporters and Their Interacting Partners in Auxin Transport. Plant and Cell Physiology **58,** 1601-1614.
Geldner, N., Friml, J., Stierhof, Y.D., Jurgens, G., and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle traficking. Nature **413,** 425-428.
Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003). The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell **112,** 219-230.
Gendre, D., Jonsson, K., Boutte, Y., and Bhalerao, R.P. (2015). Journey to the cell surface-the central role of the trans-Golgi network in plants. Protoplasma **252,** 385-398.
Gendre, D., McFarlane, H.E., Johnson, E., Mouille, G., Sjodin, A., Oh, J., Levesque-Tremblay, G., Watanabe, Y., Samuels, L., and Bhalerao, R.P. (2013). Trans-Golgi Network Localized ECHIDNA/Ypt Interacting Protein Complex Is Required for the Secretion of Cell Wall Polysaccharides in Arabidopsis. Plant Cell **25,** 2633-2646.
Gendre, D., Oh, J., Boutte, Y., Best, J.G., Samuels, L., Nilsson, R., Uemura, T., Marchant, A., Bennett, M.J., Grebe, M., and Bhalerao, R.P. (2011). Conserved Arabidopsis ECHIDNA protein mediates trans-Golgi-network trafficking and cell elongation. P Natl Acad Sci USA **108,** 8048-8053.
Gilbert, S.F. (2000). Developmental biology. (Sunderland, Mass.: Sinauer Associates ; Basingstoke : Macmillan).
Goda, H., Sawa, S., Asami, T., Fujioka, S., Shimada, Y., and Yoshida, S. (2004). Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in Arabidopsis. Plant Physiol **134,** 1555-1573.
Goeschl, J.D., Rappaport, L., and Pratt, H.K. (1966). Ethylene as a Factor Regulating Growth of Pea Epicotyls Subjected to Physical Stress. Plant Physiology **41,** 877-+.
Goldberg, J. (1998). Structural basis for activation of ARF GTPase: Mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. Cell **95,** 237-248.
Golgi, C. (1898). Intorno alla struttura delle cellule nervose. Bollettino della Società Medico Chirurgica di Pavia **13,** 3-16.
Gould, S.B., Garg, S.G., and Martin, W.F. (2016). Bacterial Vesicle Secretion and the Evolutionary Origin of the Eukaryotic Endomembrane System. Trends Microbiol **24,** 525-534.
Griffiths, G., and Simons, K. (1986). The trans Golgi network: sorting at the exit site of the Golgi complex. Science **234,** 438-443.
Gu, Y.N., and Innes, R.W. (2012). The KEEP ON GOING Protein of Arabidopsis Regulates Intracellular Protein Trafficking and Is Degraded during Fungal Infection. Plant Cell **24,** 4717-4730.
Gutierrez, R., Lindeboom, J.J., Paredez, A.R., Emons, A.M.C., and Ehrhardt, D.W. (2009). Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat Cell Biol **11,** 797-U743.

Guzman, P., and Ecker, J.R. (1990). Exploiting the Triple Response of Arabidopsis to Identify Ethylene-Related Mutants. Plant Cell **2,** 513-523.

Harper, R.M., Stowe-Evans, E.L., Luesse, D.R., Muto, H., Tatematsu, K., Watahiki, M.K., Yamamoto, K., and Liscum, E. (2000). The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial Arabidopsis tissue. Plant Cell **12,** 757-770.

Haruta, M., Sabat, G., Stecker, K., Minkoff, B.B., and Sussman, M.R. (2014). A Peptide Hormone and Its Receptor Protein Kinase Regulate Plant Cell Expansion. Science **343,** 408-411.

Hawes, C., and Satiat-Jeunemaitre, B. (2005). The plant Golgi apparatus - Going with the flow. Bba-Mol Cell Res **1744,** 93-107.

Hematy, K., Sado, P.E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J.P., and Hofte, H. (2007). A receptor-like kinase mediates the response of Arabidopsis cells to the inhibition of cellulose synthesis. Curr Biol **17,** 922-931.

Hocq, L., Pelloux, J., and Lefebvre, V. (2017). Connecting Homogalacturonan-Type Pectin Remodeling to Acid Growth. Trends Plant Sci **22,** 20-29.

Hoson, T., and Wakabayashi, K. (2015). Role of the plant cell wall in gravity resistance. Phytochemistry **112,** 84-90.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell **94,** 261-271.

Jackson, L.P. (2014). Structure and mechanism of COPI vesicle biogenesis. Curr Opin Cell Biol **29,** 67-73.

Jekely, G. (2003). Small GTPases and the evolution of the eukaryotic cell. Bioessays **25,** 1129-1138.

Jensen, P.E., and Leister, D. (2014). Chloroplast evolution, structure and functions. F1000Prime Rep **6,** 40.

Jolie, R.P., Duvetter, T., Van Loey, A.M., and Hendrickx, M.E. (2010). Pectin methylesterase and its proteinaceous inhibitor: a review. Carbohyd Res **345,** 2583-2595.

Josse, E.M., and Halliday, K.J. (2008). Skotomorphogenesis: The Dark Side of Light Signalling. Curr Biol **18,** R1144-R1146.

Kang, B.G., Yocum, C.S., Burg, S.P., and Ray, P.M. (1967). Ethylene and Carbon Dioxide - Mediation of Hypocotyl Hook-Opening Response. Science **156,** 958-&.

Kang, B.H. (2011). Shrinkage and fragmentation of the trans-Golgi network in non-meristematic plant cells. Plant Signal Behav **6,** 884-886.

Kang, B.H., Nielsen, E., Preuss, M.L., Mastronarde, D., and Staehelin, L.A. (2011). Electron Tomography of RabA4b-and PI-4K beta 1-Labeled Trans Golgi Network Compartments in Arabidopsis. Traffic **12,** 313-329.

Karandur, D., Nawrotek, A., Kuriyan, J., and Cherfils, J. (2017). Multiple interactions between an Arf/GEF complex and charged lipids determine activation kinetics on the membrane. P Natl Acad Sci USA **114,** 11416-11421.

Keegstra, K. (2010). Plant Cell Walls. Plant Physiol **154,** 483-486.

Khan, M., Rozhon, W., and Poppenberger, B. (2014). The Role of Hormones in the Aging of Plants - A Mini-Review. Gerontology **60,** 49-55.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). Ctr1, a Negative Regulator of the Ethylene Response Pathway in Arabidopsis, Encodes a Member of the Raf Family of Protein-Kinases. Cell **72,** 427-441.

Kierzkowski, D., and Routier-Kierzkowska, A.L. (2019). Cellular basis of growth in plants: geometry matters. Curr Opin Plant Biol **47,** 56-63.

Kim, S.J., Held, M.A., Zemelis, S., Wilkerson, C., and Brandizzi, F. (2015). CGR2 and CGR3 have critical overlapping roles in pectin methylesterification and plant growth in Arabidopsis thaliana. Plant J **82,** 208-220.

Kirchhausen, T., Owen, D., and Harrison, S.C. (2014). Molecular Structure, Function, and Dynamics of Clathrin-Mediated Membrane Traffic. Csh Perspect Biol **6**.

Kleine-Vehn, J., Ding, Z.J., Jones, A.R., Tasaka, M., Morita, M.T., and Friml, J. (2010). Gravity-induced PIN transcytosis for polarization of auxin fluxes in gravity-sensing root cells. P Natl Acad Sci USA **107,** 22344-22349.

Kohorn, B.D., Kohorn, S.L., Saba, N.J., and Martinez, V.M. (2014). Requirement for Pectin Methyl Esterase and Preference for Fragmented over Native Pectins for Wall-associated Kinase-activated, EDS1/PAD4-dependent Stress Response in Arabidopsis. J Biol Chem **289,** 18978-18986.

Kohorn, B.D., Kohorn, S.L., Todorova, T., Baptiste, G., Stansky, K., and McCullough, M. (2012). A Dominant Allele of Arabidopsis Pectin-Binding Wall-Associated Kinase Induces a Stress Response Suppressed by MPK6 but Not MPK3 Mutations. Molecular Plant **5,** 841-851.

Kohorn, B.D., Kobayashi, M., Johansen, S., Riese, J., Huang, L.F., Koch, K., Fu, S., Dotson, A., and Byers, N. (2006). An Arabidopsis cell wall-associated kinase required for invertase activity and cell growth. Plant J **46,** 307-316.

Kooijman, E.E., Chupin, V., de Kruijff, B., and Burger, K.N.J. (2003). Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. Traffic **4,** 162-174.

Kulich, I., Cole, R., Drdova, E., Cvrckova, F., Soukup, A., Fowler, J., and Zarsky, V. (2010). Arabidopsis exocyst subunits SEC8 and EXO70A1 and exocyst interactor ROH1 are involved in the localized deposition of seed coat pectin. New Phytol **188,** 615-625.

Lampugnani, E.R., Khan, G.A., Somssich, M., and Persson, S. (2018). Building a plant cell wall at a glance. J Cell Sci **131**.

Lee, M.H., Min, M.K., Lee, Y.J., Jin, J.B., Shin, D.H., Kim, D.H., Lee, K.H., and Hwang, I. (2002). ADP-ribosylation factor 1 of arabidopsis plays a critical role in intracellular trafficking and maintenance of endoplasmic reticulum morphology in Arabidopsis. Plant Physiol **129,** 1507-1520.

Lehman, A., Black, R., and Ecker, J.R. (1996). HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. Cell **85,** 183-194.

Lemmon, M.A. (2007). Pleckstrin homology (PH) domains and phosphoinositides. Biochem Soc Symp **74,** 81-93.

Leucci, M.R., Di Sansebastiano, G.P., Gigante, M., Dalessandro, G., and Piro, G. (2007). Secretion marker proteins and cell-wall polysaccharides move through different secretory pathways. Planta **225,** 1001-1017.

Levesque-Tremblay, G., Pelloux, J., Braybrook, S.A., and Muller, K. (2015). Tuning of pectin methylesterification: consequences for cell wall biomechanics and development. Planta **242,** 791-811.

Li, G., and Segev, N. (2018). Rab GTPases and Membrane Trafficking. (Bentham Science Publishers).

Li, H., Johnson, P., Stepanova, A., Alonso, J.M., and Ecker, J.R. (2004). Convergence of signaling of differential cell growth pathways in the control in Arabidopsis. Dev Cell **7,** 193-204.

Li, S.D., Lei, L., Somerville, C.R., and Gu, Y. (2012). Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes. P Natl Acad Sci USA **109,** 185-190.

Light, K.M., Wisniewski, J.A., Vinyard, W.A., and Kieber-Emmons, M.T. (2016). Perception of the plant hormone ethylene: known-knowns and known-unknowns. J Biol Inorg Chem **21,** 715-728.

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., Favaron, F., Cervone, F., and Bellincampi, D. (2007). Overexpression of pectin methylesterase inhibitors in Arabidopsis restricts fungal infection by Botrytis cinerea. Plant Physiol **143,** 1871-1880.

Liscum, E., and Hangarter, R.P. (1993). Light-Stimulated Apical Hook Opening in Wild-Type Arabidopsis-Thaliana Seedlings. Plant Physiology **101,** 567-572.

Liu, L.X., Liao, H.N., Castle, A., Zhang, J., Casanova, J., Szabo, G., and Castle, D. (2005). SCAMP2 interacts with Arf6 and phospholipase D1 and links their function to exocytotic fusion pore formation in PC12 cells. Mol Biol Cell **16,** 4463-4472.

Lloyd, C. (2011). Dynamic Microtubules and the Texture of Plant Cell Walls. Int Rev Cel Mol Bio **287,** 287-329.

Luo, Y., Scholl, S., Doering, A., Zhang, Y., Irani, N.G., Di Rubbo, S., Neumetzler, L., Krishnamoorthy, P., Van Houtte, I., Mylle, E., Bischoff, V., Vernhettes, S., Winne, J., Friml, J., Stierhof, Y.D., Schumacher, K., Persson, S., and Russinova, E. (2015). V-ATPase activity in the TGN/EE is required for exocytosis and recycling in Arabidopsis. Nat Plants **1**.

Marc, J., Granger, C.L., Brincat, J., Fisher, D.D., Kao, T.H., McCubbin, A.G., and Cyr, R.J. (1998). A GFP-MAP4 reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. Plant Cell **10,** 1927-1939.

Mazzella, M.A., Casal, J.J., Muschietti, J.P., and Fox, A.R. (2014). Hormonal networks involved in apical hook development in darkness and their response to light. Front Plant Sci **5,** 52.

McFarlane, H.E., Watanabe, Y., Yang, W.L., Huang, Y., Ohlrogge, J., and Samuels, A.L. (2014). Golgi- and Trans-Golgi Network-Mediated Vesicle Trafficking Is Required for Wax Secretion from Epidermal Cells. Plant Physiol **164,** 1250-1260.

McFarlane, H.E., Watanabe, Y., Gendre, D., Carruthers, K., Levesque-Tremblay, G., Haughn, G.W., Bhalerao, R.P., and Samuels, L. (2013). Cell Wall Polysaccharides are Mislocalized to the Vacuole in echidna Mutants. Plant Cell Physiol **54,** 1867-1880.
Miao, Y.S., Li, H.Y., Shen, J.B., Wang, J.Q., and Jiang, L.W. (2011). QUASIMODO 3 (QUA3) is a putative homogalacturonan methyltransferase regulating cell wall biosynthesis in Arabidopsis suspension-cultured cells. J Exp Bot **62,** 5063-5078.
Mohnen, D. (2008). Pectin structure and biosynthesis. Current Opinion in Plant Biology **11,** 266-277.
Morre, D.J., and Ovtracht, L. (1977). Dynamics of the Golgi apparatus: membrane differentiation and membrane flow. Int Rev Cytol Suppl**,** 61-188.
Mouille, G., Ralet, M.C., Cavelier, C., Eland, C., Effroy, D., Hematy, K., McCartney, L., Truong, H.N., Gaudon, V., Thibault, J.F., Marchant, A., and Hofte, H. (2007). Homogalacturonan synthesis in Arabidopsis thaliana requires a Golgi-localized protein with a putative methyltransferase domain. Plant J **50,** 605-614.
Mouratou, B., Biou, V., Joubert, A., Cohen, J., Shields, D.J., Geldner, N., Jurgens, G., Melancon, P., and Cherfils, J. (2005). The domain architecture of large guanine nucleotide exchange factors for the small GTP-binding protein Arf. Bmc Genomics **6**.
Muller, A., Guan, C.H., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K. (1998). AtPIN2 defines a locus of Arabidopsis for root gravitropism control. Embo J **17,** 6903-6911.
Naramoto, S., Kleine-Vehn, J., Robert, S., Fujimoto, M., Dainobu, T., Paciorek, T., Ueda, T., Nakano, A., Van Montagu, M.C.E., Fukuda, H., and Friml, J. (2010). ADP-ribosylation factor machinery mediates endocytosis in plant cells. P Natl Acad Sci USA **107,** 21890-21895.
Nebenfuhr, A., Ritzenthaler, C., and Robinson, D.G. (2002). Brefeldin A: Deciphering an enigmatic inhibitor of secretion. Plant Physiol **130,** 1102-1108.
Neljubow, D. (1901). Ueber die horizontale Nutation der Stengel von Pisum sativum und einiger anderen Pflanzen. Botanisches Centralblatt **10,** 128-139.
Nemhauser, J.L., Hong, F.X., and Chory, J. (2006). Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell **126,** 467-475.
Ngouemazong, D.E., Jolei, R.P., Cardinaels, R., Fraeye, I., Van Loey, A., Moldenaers, P., and Hendrickx, M. (2012). Stiffness of Ca2+-pectin gels: combined effects of degree and pattern of methylesterification for various Ca2+ concentrations. Carbohyd Res **348,** 69-76.
Nielsen, M., Albrethsen, J., Larsen, F.H., and Skriver, K. (2006). The Arabidopsis ADP-ribosylation factor (ARF) and ARF-like (ARL) system and its regulation by BIG2, a large ARF-GEF. Plant Sci **171,** 707-717.
Novikoff, A.B. (1964). GERL, its form and function in the neurons of rat spinal ganglia. Biological Bulletin **127,** 358A.
Pappan, K., Austin-Brown, S., Chapman, K.D., and Wang, X.M. (1998). Substrate selectivities and lipid modulation of plant phospholipase D alpha, -beta, and -gamma. Arch Biochem Biophys **353,** 131-140.
Paredez, A.R., Persson, S., Ehrhardt, D.W., and Somerville, C.R. (2008). Genetic evidence that cellulose synthase activity influences microtubule cortical array organization. Plant Physiol **147,** 1723-1734.
Park, Y.B., and Cosgrove, D.J. (2015). Xyloglucan and its Interactions with Other Components of the Growing Cell Wall. Plant Cell Physiol **56,** 180-194.
Parsons, H.T., Drakakaki, G., and Heazlewood, J.L. (2013). Proteomic dissection of the Arabidopsis Golgi and trans-Golgi network. Frontiers in Plant Science **3**.
Paul, M.J., and Frigerio, L. (2007). Coated vesicles in plant cells. Semin Cell Dev Biol **18,** 471-478.
Peaucelle, A., Wightman, R., and Hofte, H. (2015). The Control of Growth Symmetry Breaking in the Arabidopsis Hypocotyl. Curr Biol **25,** 1746-1752.
Peaucelle, A., Braybrook, S.A., Le Guillou, L., Bron, E., Kuhlemeier, C., and Hofte, H. (2011). Pectin-induced changes in cell wall mechanics underlie organ initiation in Arabidopsis. Curr Biol **21,** 1720-1726.
Peaucelle, A., Louvet, R., Johansen, J.N., Hofte, H., Laufs, P., Pelloux, J., and Mouille, G. (2008). Arabidopsis phyllotaxis is controlled by the methyl-esterification status of cell-wall pectins. Curr Biol **18,** 1943-1948.
Peck, S.C., Pawlowski, K., and Kende, H. (1998). Asymmetric responsiveness to ethylene mediates cell elongation in the apical hook of peas. Plant Cell **10,** 713-719.

Peer, W.A. (2013). From perception to attenuation: auxin signalling and responses. Current Opinion in Plant Biology **16,** 561-568.

Pelletier, S., Van Orden, J., Wolf, S., Vissenberg, K., Delacourt, J., Ndong, Y.A., Pelloux, J., Bischoff, V., Urbain, A., Mouille, G., Lemonnier, G., Renou, J.P., and Hofte, H. (2010). A role for pectin de-methylesterification in a developmentally regulated growth acceleration in dark-grown Arabidopsis hypocotyls. New Phytol **188,** 726-739.

Pelloux, J., Rusterucci, C., and Mellerowicz, E.J. (2007). New insights into pectin methylesterase structure and function. Trends Plant Sci **12,** 267-277.

Peret, B., Swarup, K., Ferguson, A., Seth, M., Yang, Y.D., Dhondt, S., James, N., Casimiro, I., Perry, P., Syed, A., Yang, H.B., Reemmer, J., Venison, E., Howells, C., Perez-Amador, M.A., Yun, J.G., Alonso, J., Beemster, G.T.S., Laplaze, L., Murphy, A., Bennett, M.J., Nielsen, E., and Swarup, R. (2012). AUX/LAX Genes Encode a Family of Auxin Influx Transporters That Perform Distinct Functions during Arabidopsis Development. Plant Cell **24,** 2874-2885.

Philippar, K., Ivashikina, N., Ache, P., Christian, M., Luthen, H., Palme, K., and Hedrich, R. (2004). Auxin activates KAT1 and KAT2, two K+-channel genes expressed in seedlings of Arabidopsis thaliana. Plant J **37,** 815-827.

Pimpl, P., Hanton, S.L., Taylor, J.P., Pinto-daSilva, L.L., and Denecke, J. (2003). The GTPase ARF1p controls the sequence-specific vacuolar sorting route to the lytic vacuole. Plant Cell **15,** 1242-1256.

Poulsen, L.R., Lopez-Marques, R.L., McDowell, S.C., Okkeri, J., Licht, D., Schulz, A., Pomorski, T., Harper, J.F., and Palmgren, M.G. (2008). The Arabidopsis P-4-ATPase ALA3 localizes to the Golgi and requires a beta-subunit to function in lipid translocation and secretory vesicle formation. Plant Cell **20,** 658-676.

Powell, R.D., and Morgan, P.W. (1970). Factors Involved in Opening of Hypocotyl Hook of Cotton and Beans. Plant Physiology **45,** 548-&.

Preuss, M.L., Schmitz, A.J., Thole, J.M., Bonner, H.K.S., Otegui, M.S., and Nielsen, E. (2006). A role for the RabA4b effector protein PI-4K beta 1 in polarized expansion of root hair cells in Arabidopsis thaliana. J Cell Biol **172,** 991-998.

Qin, G.J., Gu, H.Y., Zhao, Y.D., Ma, Z.Q., Shi, G.L., Yang, Y., Pichersky, E., Chen, H.D., Liu, M.H., Chen, Z.L., and Qu, L.J. (2005). An indole-3-acetic acid carboxyl methyltransferase regulates Arabidopsis leaf development. Plant Cell **17,** 2693-2704.

Rakusova, H., Fendrych, M., and Friml, J. (2015). Intracellular trafficking and PIN-mediated cell polarity during tropic responses in plants. Current Opinion in Plant Biology **23,** 116-123.

Rambourg, A., Clermont, Y., and Hermo, L. (1979). Three-dimensional architecture of the golgi apparatus in Sertoli cells of the rat. Am J Anat **154,** 455-476.

Ravikumar, R., Kalbfuss, N., Gendre, D., Steiner, A., Altmann, M., Altmann, S., Rybak, K., Edelmann, H., Stephan, F., Lampe, M., Facher, E., Wanner, G., Falter-Braun, P., Bhalerao, R.P., and Assaad, F.F. (2018). Independent yet overlapping pathways ensure the robustness and responsiveness of trans-Golgi network functions in Arabidopsis. Development **145**.

Rayle, D.L., and Cleland, R.E. (1992). The Acid Growth Theory of Auxin-Induced Cell Elongation Is Alive and Well. Plant Physiol **99,** 1271-1274.

Raz, V., and Ecker, J.R. (1999). Regulation of differential growth in the apical hook of Arabidopsis. Development **126,** 3661-3668.

Raz, V., and Koornneef, M. (2001). Cell division activity during apical hook development. Plant Physiology **125,** 219-226.

Reinhardt, D., Mandel, T., and Kuhlemeier, C. (2000). Auxin regulates the initiation and radial position of plant lateral organs. Plant Cell **12,** 507-518.

Renna, L., Stefano, G., Slabaugh, E., Wormsbaecher, C., Sulpizio, A., Zienkiewicz, K., and Brandizzi, F. (2018). TGNap1 is required for microtubule-dependent homeostasis of a subpopulation of the plant trans-Golgi network. Nat Commun **9**.

Richter, S., Kientz, M., Brumm, S., Nielsen, M.E., Park, M., Gavidia, R., Krause, C., Voss, U., Beckmann, H., Mayer, U., Stierhof, Y.D., and Jurgens, G. (2014). Delivery of endocytosed proteins to the cell-division plane requires change of pathway from recycling to secretion. Elife **3**.

Robinson, D., Ding, Y., and Jiang, L.W. (2016). Unconventional protein secretion in plants: a critical assessment. Protoplasma **253,** 31-43.

Roger, A.J., Munoz-Gomez, S.A., and Kamikawa, R. (2017). The Origin and Diversification of Mitochondria. Curr Biol **27,** R1177-R1192.
Sablowski, R. (2016). Coordination of plant cell growth and division: collective control or mutual agreement? Curr Opin Plant Biol **34,** 54-60.
Sack, F.D. (1991). Plant Gravity Sensing. Int Rev Cytol **127,** 193-252.
Saffer, A.M. (2018). Expanding roles for pectins in plant development. J Integr Plant Biol **60,** 910-923.
Sagan, L. (1967). On Origin of Mitosing Cells. J Theor Biol **14,** 225-&.
Santner, A., and Estelle, M. (2010). The ubiquitin-proteasome system regulates plant hormone signaling. Plant J **61,** 1029-1040.
Scheuring, D., Viotti, C., Kruger, F., Kunzl, F., Sturm, S., Bubeck, J., Hillmer, S., Frigerio, L., Robinson, D.G., Pimpl, P., and Schumacher, K. (2011). Multivesicular Bodies Mature from the Trans-Golgi Network/Early Endosome in Arabidopsis. Plant Cell **23,** 3463-3481.
Schoenaers, S., Balcerowicz, D., Breen, G., Hill, K., Zdanio, M., Mouille, G., Holman, T.J., Oh, J., Wilson, M.H., Nikonorova, N., Vu, L.D., De Smet, I., Swarup, R., De Vos, W.H., Pintelon, I., Adriaensen, D., Grierson, C., Bennett, M.J., and Vissenberg, K. (2018). The Auxin-Regulated CrRLK1L Kinase ERULUS Controls Cell Wall Composition during Root Hair Tip Growth. Curr Biol **28,** 722-+.
Schwark, A., and Schierle, J. (1992). Interaction of Ethylene and Auxin in the Regulation of Hook Growth .1. The Role of Auxin in Different Growing Regions of the Hypocotyl Hook of Phaseolus-Vulgaris. J Plant Physiol **140,** 562-570.
Scott, C.C., Vacca, F., and Gruenberg, J. (2014). Endosome maturation, transport and functions. Semin Cell Dev Biol **31,** 2-10.
Sebastian, T.T., Baldridge, R.D., Xu, P., and Graham, T.R. (2012). Phospholipid flippases: Building asymmetric membranes and transport vesicles. Bba-Mol Cell Biol L **1821,** 1068-1077.
Senechal, F., Wattier, C., Rusterucci, C., and Pelloux, J. (2014). Homogalacturonan-modifying enzymes: structure, expression, and roles in plants. J Exp Bot **65,** 5125-5160.
Sherrier, D.J., and Vandenbosch, K.A. (1994). Secretion of Cell-Wall Polysaccharides in Vicia Root Hairs. Plant J **5,** 185-195.
Silk, W.K., and Erickson, R.O. (1978). Kinematics of Hypocotyl Curvature. Am J Bot **65,** 310-319.
Simon, S., and Petrasek, J. (2011). Why plants need more than one type of auxin. Plant Sci **180,** 454-460.
Singh, M.K., and Jurgens, G. (2018). Specificity of plant membrane trafficking - ARFs, regulators and coat proteins. Semin Cell Dev Biol **80,** 85-93.
Singh, M.K., Kruger, F., Beckmann, H., Brumm, S., Vermeer, J.E.M., Munnik, T., Mayer, U., Stierhof, Y.D., Grefen, C., Schumacher, K., and Jurgens, G. (2014). Protein Delivery to Vacuole Requires SAND Protein-Dependent Rab GTPase Conversion for MVB-Vacuole Fusion. Current Biology **24,** 1383-1389.
Singh, M.K., Richter, S., Beckmann, H., Kientz, M., Stierhof, Y.D., Anders, N., Fassler, F., Nielsen, M., Knoll, C., Thomann, A., Franz-Wachtel, M., Macek, B., Skriver, K., Pimpl, P., and Jurgens, G. (2018). A single class of ARF GTPase activated by several pathway-specific ARF-GEFs regulates essential membrane traffic in Arabidopsis. Plos Genet **14**.
Smit, M.E., and Weijers, D. (2015). The role of auxin signaling in early embryo pattern formation. Current Opinion in Plant Biology **28,** 99-105.
Staehelin, L.A., and Moore, I. (1995). The Plant Golgi-Apparatus - Structure, Functional-Organization and Trafficking Mechanisms. Annu Rev Plant Phys **46,** 261-288.
Staehelin, L.A., and Kang, B.H. (2008). Nanoscale architecture of endoplasmic reticulum export sites and of Golgi membranes as determined by electron tomography. Plant Physiology **147,** 1454-1468.
Stefano, G., Renna, L., Chatre, L., Hanton, S.L., Moreau, P., Hawes, C., and Brandizzi, F. (2006). In tobacco leaf epidermal cells, the integrity of protein export from the endoplasmic reticulum and of ER export sites depends on active COPI machinery. Plant J **46,** 95-110.
Stein, I.S., Gottfried, A., Zimmermann, J., and Von Mollard, G.F. (2009). TVP23 interacts genetically with the yeast SNARE VTI1 and functions in retrograde transport from the early endosome to the late Golgi. Biochem J **419,** 229-236.
Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C.L., Paris, S., Galweiler, L., Palme, K., and Jurgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science **286,** 316-318.

Stepanova, A.N., Yun, J., Robles, L.M., Novak, O., He, W.R., Guo, H.W., Ljung, K., and Alonso, J.M. (2011). The Arabidopsis YUCCA1 Flavin Monooxygenase Functions in the Indole-3-Pyruvic Acid Branch of Auxin Biosynthesis. Plant Cell **23,** 3961-3973.
Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.Y., DoleZal, K., Schlereth, A., Jurgens, G., and Alonso, J.M. (2008). TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell **133,** 177-191.
Sterling, J.D., Quigley, H.F., Orellana, A., and Mohnen, D. (2001). The catalytic site of the pectin biosynthetic enzyme alpha-1,4-galacturonosyltransferase is located in the lumen of the Golgi. Plant Physiol **127,** 360-371.
Sterling, J.D., Atmodjo, M.A., Inwood, S.E., Kolli, V.S.K., Quigley, H.F., Hahn, M.G., and Mohnen, D. (2006). Functional identification of an Arabidopsis pectin biosynthetic homogalacturonan galacturonosyltransferase. P Natl Acad Sci USA **103,** 5236-5241.
Stierhof, Y.D., and El Kasmi, F. (2010). Strategies to improve the antigenicity, ultrastructure preservation and visibility of trafficking compartments in Arabidopsis tissue. Eur J Cell Biol **89,** 285-297.
Stierhof, Y.D., Viotti, C., Scheuring, D., Sturm, S., and Robinson, D.G. (2013). Sorting nexins 1 and 2a locate mainly to the TGN. Protoplasma **250,** 235-240.
Stowe-Evans, E.L., Harper, R.M., Motchoulski, A.V., and Liscum, E. (1998). NPH4, a conditional modulator of auxin-dependent differential growth responses in Arabidopsis. Plant Physiology **118,** 1265-1275.
Strasser, R. (2016). Plant protein glycosylation. Glycobiology **26,** 926-939.
Surma, M.A., Klose, C., and Simons, K. (2012). Lipid-dependent protein sorting at the trans-Golgi network. Bba-Mol Cell Biol L **1821,** 1059-1067.
Swarup, R., and Peret, B. (2012). AUX/LAX family of auxin influx carriers-an overview. Frontiers in Plant Science **3**.
Takahashi, K., Hayashi, K., and Kinoshita, T. (2012). Auxin activates the plasma membrane H+-ATPase by phosphorylation during hypocotyl elongation in Arabidopsis. Plant Physiol **159,** 632-641.
Takeuchi, M., Ueda, T., Yahara, N., and Nakano, A. (2002). Arf1 GTPase plays roles in the protein traffic between the endoplasmic reticulum and the Golgi apparatus in tobacco and Arabidopsis cultured cells. Plant J **31,** 499-515.
Tanaka, H., Kitakura, S., De Rycke, R., De Groodt, R., and Friml, J. (2009). Fluorescence Imalaina-Based Screen Identifies ARF GEF Component of Early Endosomal Trafficking. Curr Biol **19,** 391-397.
Tanaka, H., Nodzynski, T., Kitakura, S., Feraru, M.I., Sasabe, M., Ishikawa, T., Kleine-Vehn, J., Kakimoto, T., and Friml, J. (2014). BEX1/ARF1A1C is Required for BFA-Sensitive Recycling of PIN Auxin Transporters and Auxin-Mediated Development in Arabidopsis. Plant Cell Physiol **55,** 737-749.
Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M.K., Harper, R.M., Liscum, E., and Yamamoto, K.T. (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. Plant Cell **16,** 379-393.
Taylor-Teeples, M., Lanctot, A., and Nemhauser, J.L. (2016). As above, so below: Auxin's role in lateral organ development. Dev Biol **419,** 156-164.
Teh, O.K., and Moore, I. (2007). An ARF-GEF acting at the Golgi and in selective endocytosis in polarized plant cells. Nature **448,** 493-496.
Thomas, P.D., and Poznansky, M.J. (1989). Curvature and Composition-Dependent Lipid Asymmetry in Phosphatidylcholine Vesicles Containing Phosphatidylethanolamine and Gangliosides. Biochim Biophys Acta **978,** 85-90.
Tian, Q., and Reed, J.W. (1999). Control of auxin-regulated root development by the Arabidopsis thaliana SHY2/IAA3 gene. Development **126,** 711-721.
Torii, K.U. (2015). Stomatal differentiation: the beginning and the end. Current Opinion in Plant Biology **28,** 16-22.
Toyooka, K., Goto, Y., Asatsuma, S., Koizumi, M., Mitsui, T., and Matsuoka, K. (2009). A Mobile Secretory Vesicle Cluster Involved in Mass Transport from the Golgi to the Plant Cell Exterior. Plant Cell **21,** 1212-1229.
Trepat, X., Chen, Z., and Jacobson, K. (2012). Cell migration. Compr Physiol **2,** 2369-2392.

Uemura, T., Suda, Y., Ueda, T., and Nakano, A. (2014). Dynamic Behavior of the trans-Golgi Network in Root Tissues of Arabidopsis Revealed by Super-Resolution Live Imaging. Plant and Cell Physiology **55,** 694-703.

Uemura, T., Nakano, R.T., Takagi, J., Wang, Y.M., Kramer, K., Finkemeier, I., Nakagami, H., Tsuda, K., Ueda, T., Schulze-Lefert, P., and Nakano, A. (2019). A Golgi-Released Subpopulation of the Trans-Golgi Network Mediates Protein Secretion in Arabidopsis. Plant Physiology **179,** 519-532.

van Dop, M., Liao, C.Y., and Weijers, D. (2015). Control of oriented cell division in the Arabidopsis embryo. Current Opinion in Plant Biology **23,** 25-30.

Vandenbussche, F., Petrasek, J., Zadnikova, P., Hoyerova, K., Pesek, B., Raz, V., Swarup, R., Bennett, M., Zazimalova, E., Benkova, E., and Van Der Straeten, D. (2010). The auxin influx carriers AUX1 and LAX3 are involved in auxin-ethylene interactions during apical hook development in Arabidopsis thaliana seedlings. Development **137,** 597-606.

Vicre, M., Jauneau, A., Knox, J.P., and Driouich, A. (1998). Immunolocalization of beta-(1 -> 4) and beta-(1 -> 6)-D-galactan epitopes in the cell wall and Golgi stacks of developing flax root tissues. Protoplasma **203,** 26-34.

Viotti, C., Bubeck, J., Stierhof, Y.D., Krebs, M., Langhans, M., van den Berg, W., van Dongen, W., Richter, S., Geldner, N., Takano, J., Jurgens, G., de Vries, S.C., Robinson, D.G., and Schumacher, K. (2010). Endocytic and Secretory Traffic in Arabidopsis Merge in the Trans-Golgi Network/Early Endosome, an Independent and Highly Dynamic Organelle. Plant Cell **22,** 1344-1357.

Viotti, C., Kruger, F., Krebs, M., Neubert, C., Fink, F., Lupanga, U., Scheuring, D., Boutte, Y., Frescatada-Rosa, M., Wolfenstetter, S., Sauer, N., Hillmer, S., Grebe, M., and Schumacher, K. (2013). The Endoplasmic Reticulum Is the Main Membrane Source for Biogenesis of the Lytic Vacuole in Arabidopsis. Plant Cell **25,** 3434-3449.

Vogel, J.P., Woeste, K.E., Theologis, A., and Kieber, J.J. (1998a). Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of Arabidopsis confer cytokinin insensitivity and ethylene overproduction, respectively. P Natl Acad Sci USA **95,** 4766-4771.

Vogel, J.P., Schuerman, P., Woeste, K., Brandstatter, I., and Kieber, J.J. (1998b). Isolation and characterization of Arabidopsis mutants defective in the induction of ethylene biosynthesis by cytokinin. Genetics **149,** 417-427.

Voxeur, A., and Hofte, H. (2016). Cell wall integrity signaling in plants: "To grow or not to grow that's the question". Glycobiology **26,** 950-960.

Wakabayashi, K., Hoson, T., and Huber, D.J. (2003). Methyl de-esterification as a major factor regulating the extent of pectin depolymerization during fruit ripening: a comparison of the action of avocado (Persea americana) and tomato (Lycopersicon esculentum) polygalacturonases. J Plant Physiol **160,** 667-673.

Wang, A.L., Rao, V.R., Chen, J.J., Lussier, Y.A., Rehman, J., Huang, Y., Jager, R.D., and Grassi, M.A. (2014). Role of FAM18B in diabetic retinopathy. Mol Vis **20,** 1146-1159.

Wang, J., Sun, H.Q., Macia, E., Kirchhausen, T., Watson, H., Bonifacino, J.S., and Yin, H.L. (2007). PIP promotes the recruitment of the GGA adaptor proteins to the trans-Golgi TS recognition of the ubiquitin network and regulates their sorting signal. Mol Biol Cell **18,** 2646-2655.

Wattelet-Boyer, V., Brocard, L., Jonsson, K., Esnay, N., Joubes, J., Domergue, F., Mongrand, S., Raikhel, N., Bhalerao, R.P., Moreau, P., and Boutte, Y. (2016). Enrichment of hydroxylated C24-and C26-acyl-chain sphingolipids mediates PIN2 apical sorting at trans-Golgi network subdomains. Nat Commun **7**.

Weller, B., Zourelidou, M., Frank, L., Barbosa, I.C.R., Fastner, A., Richter, S., Jurgens, G., Hammes, U.Z., and Schwechheimer, C. (2017). Dynamic PIN-FORMED auxin efflux carrier phosphorylation at the plasma membrane controls auxin efflux-dependent growth. P Natl Acad Sci USA **114,** E887-E896.

Went, F.W. (1926). On Growth-Accelerating Substances in the Coleoptile of Avena sativa. Proceedings of the Section of Sciences, Koninklijke Akademie van Wetenschappen te Amsterdam **30,** 10-19.

Went, F.W., and Thimann, K.V. (1937). Phytohormones (The Macmillan Company).

Willige, B.C., and Chory, J. (2015). A current perspective on the role of AGCVIII kinases in PIN-mediated apical hook development. Frontiers in Plant Science **6**.

Willige, B.C., Ogiso-Tanaka, E., Zourelidou, M., and Schwechheimer, C. (2012). WAG2 represses apical hook opening downstream from gibberellin and PHYTOCHROME INTERACTING FACTOR 5. Development **139,** 4020-4028.

Willige, B.C., Ahlers, S., Zourelidou, M., Barbosa, I.C.R., Demarsy, E., Trevisan, M., Davis, P.A., Roelfsema, M.R.G., Hangarter, R., Fankhauser, C., and Schwechheimer, C. (2013). D6PK AGCVIII Kinases Are Required for Auxin Transport and Phototropic Hypocotyl Bending in Arabidopsis. Plant Cell **25,** 1674-1688.

Willingham, M.C., and Pastan, I. (1984). Endocytosis and exocytosis: current concepts of vesicle traffic in animal cells. Int Rev Cytol **92,** 51-92.

Wisniewska, J., Xu, J., Seifertova, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquie, D., Benkova, E., Scheres, B., and Friml, J. (2006). Polar PIN localization directs auxin flow in plants. Science **312,** 883-883.

Woeste, K.E., Ye, C., and Kieber, J.J. (1999). Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. Plant Physiology **119,** 521-529.

Wolf, S., Mouille, G., and Pelloux, J. (2009). Homogalacturonan methyl-esterification and plant development. Mol Plant **2,** 851-860.

Wolf, S., Mravec, J., Greiner, S., Mouille, G., and Hofte, H. (2012). Plant cell wall homeostasis is mediated by brassinosteroid feedback signaling. Curr Biol **22,** 1732-1737.

Wolf, S., van der Does, D., Ladwig, F., Sticht, C., Kolbeck, A., Schurholz, A.K., Augustin, S., Keinath, N., Rausch, T., Greiner, S., Schumacher, K., Harter, K., Zipfel, C., and Hofte, H. (2014). A receptor-like protein mediates the response to pectin modification by activating brassinosteroid signaling. Proc Natl Acad Sci U S A **111,** 15261-15266.

Wu, G.S., Cameron, J.N., Ljung, K., and Spalding, E.P. (2010). A role for ABCB19-mediated polar auxin transport in seedling photomorphogenesis mediated by cryptochrome 1 and phytochrome B. Plant J **62,** 179-191.

Xiao, C., Somerville, C., and Anderson, C.T. (2014). POLYGALACTURONASE INVOLVED IN EXPANSION1 Functions in Cell Elongation and Flower Development in Arabidopsis. Plant Cell **26,** 1018-1035.

Xu, J., and Scheres, B. (2005). Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. Plant Cell **17,** 525-536.

Xu, Y., Hortsman, H., Seet, L., Wong, S.H., and Hong, W. (2001). SNX3 regulates endosomal function through its PX-domain-mediated interaction with Ptdlns(3)P. Nat Cell Biol **3,** 658-666.

Yorimitsu, T., Sato, K., and Takeuchi, M. (2014). Molecular mechanisms of Sar/Arf GTPases in vesicular trafficking in yeast and plants. Front Plant Sci **5,** 411.

Young, R.E., McFarlane, H.E., Hahn, M.G., Western, T.L., Haughn, G.W., and Samuels, A.L. (2008). Analysis of the golgi apparatus in Arabidopsis seed coat cells during polarized secretion of pectin-rich mucilage. Plant Cell **20,** 1623-1638.

Zablackis, E., Huang, J., Muller, B., Darvill, A.G., and Albersheim, P. (1995). Characterization of the cell-wall polysaccharides of Arabidopsis thaliana leaves. Plant Physiol **107,** 1129-1138.

Zadnikova, P., Wabnik, K., Abuzeineh, A., Gallemi, M., Van Der Straeten, D., Smith, R.S., Inze, D., Friml, J., Prusinkiewicz, P., and Benkova, E. (2016). A Model of Differential Growth-Guided Apical Hook Formation in Plants. Plant Cell **28,** 2464-2477.

Zadnikova, P., Petrasek, J., Marhavy, P., Raz, V., Vandenbussche, F., Ding, Z., Schwarzerova, K., Morita, M.T., Tasaka, M., Hejatko, J., Van Der Straeten, D., Friml, J., and Benkova, E. (2010). Role of PIN-mediated auxin efflux in apical hook development of Arabidopsis thaliana. Development **137,** 607-617.

Zeeh, J.C., Zeghouf, M., Grauffel, C., Guibert, B., Martin, E., Dejaegere, A., and Cherfils, J. (2006). Dual specificity of the interfacial inhibitor brefeldin A for Arf proteins and Sec7 domains. J Biol Chem **281,** 11805-11814.

Zhang, G.F., and Staehelin, L.A. (1992). Functional Compartmentation of the Golgi-Apparatus of Plant-Cells - Immunocytochemical Analysis of High-Pressure Frozen-Substituted and Freeze-Substituted Sycamore Maple Suspension-Culture Cells. Plant Physiology **99,** 1070-1083.

Zhao, Y., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., and Chory, J. (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science **291,** 306-309.

Zheng, H.Q., Kunst, L., Hawes, C., and Moore, I. (2004). A GFP-based assay reveals a role for RHD3 in transport between the endoplasmic reticulum and Golgi apparatus. Plant J **37,** 398-414.

Zubieta, C., Ross, J.R., Koscheski, P., Yang, Y., Pichersky, E., and Noel, J.P. (2003). Structural basis for substrate recognition in the salicylic acid carboxyl methyltransferase family. Plant Cell **15,** 1704-1716.

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