Ethylene Regulates Differential Growth via BIG ARF-GEF-Dependent Post-Golgi Secretory Trafficking in Arabidopsis

Kristoffer Jonsson,a Yohann Boutté,b Rajesh Kumar Singh,a Delphine Gendre,a and Rishikesh P. Bhalerao,a,1

aUmeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University for Agricultural Sciences, SE-901 83 Umeå, Sweden
bCNRS-University of Bordeaux, UMR 5200 Membrane Biogenesis Laboratory, INRA Bordeaux Aquitaine, 33140 Villenave d’Ornon, France

INTRODUCTION

The apical hook develops immediately after seed germination, protecting the apical meristem as the etiolated seedling penetrates through soil. The curvature of the apical hook results from asymmetric cell elongation rates at opposite sides of the bending hypocotyl (Silk and Erickson, 1978; Raz and Koornneef, 2001), which are coordinated by the plant hormones ethylene and indole-3-acetic acid (IAA/auxin) (Li et al., 2004; Gallego-Bartolome et al., 2011). Ethylene promotes hook curvature largely by modulating auxin biosynthesis and distribution (Harper et al., 2000; Žádníková et al., 2010). Bending during hook development requires asymmetric distribution of auxin across the hypocotyl. The asymmetric auxin distribution with auxin response maxima localized on the concave side of the hook can be visualized using synthetic auxin response reporters such as DR5 (Vandenbussche et al., 2010; Zádníková et al., 2010). An increased auxin response on the concave side causes a reduced cell elongation rate relative to the convex side, resulting in hook formation. The establishment of auxin response maxima on the concave side of the apical hook relies on active, carrier-mediated polar auxin transport (PAT) (Vandenbussche et al., 2010; Žádníková et al., 2010). Members of both the AUX/LIKE-AUX1(LAX) influx carrier and PIN-FORMED (PIN) efflux carrier families play key roles in PAT-mediated hook development, underlined by the hook defects observed in aux/lax mutants, or by exposing seedlings to the carrier inhibitors N-(1-naphthyl)phtalamic acid or 1-naphtoxyacetic acid that disrupt PAT (Vandenbussche et al., 2010; Žádníková et al., 2010). The abundance and localization of PAT-mediating carriers is modulated by membrane trafficking processes. Whereas studies of the PIN family of auxin carriers have provided insights into how endocytosis facilitates their localization and abundance at the plasma membrane (PM) (Geldner et al., 2003; Dhonukshe et al., 2007), much less is known about the machinery involved in AUX/LAX protein trafficking, which also play an important role during hook development as suggested by the mutant phenotypes (Vandenbussche et al., 2010).

We have identified a key role for the evolutionarily conserved protein ECHIDNA (ECH), a plant homolog of the yeast Triacylglycerol lipase (TLG2/SYP4) interacting protein Tg12-Vesicle Protein 23 (TVP23), in mediating secretion of AUX1 influx carrier to the plasma membrane from the TGN during hook development and defects in BIG or ARF1 result in insensitivity to ethylene. Thus, our data indicate a division of labor within the ARF-GEF family in mediating differential growth with GNOM acting during the formation phase whereas BIGs act during the hook maintenance phase downstream of plant hormone ethylene.
the TGN, a post-Golgi compartment that serves as a hub for secretory and endocytic traffic. ARF-GEFs primarily catalyze nucleotide exchange for ARF GTPases, which mediate vesicle formation (Peyroche et al., 1996). Interestingly, the number of secretory vesicles at the TGN has been shown to be reduced in the ech mutant, implicating ECH in a hitherto unknown aspect of vesicle development (Boutté et al., 2013). The localization of ECH and BIGs at the TGN and the secretory defects observed for ech and big mutants prompted us to investigate whether BIGs like ECH were involved in hook development and whether they are involved in AUX1 trafficking in concert with ECH. Intriguingly, previous studies have hinted at a role for an ARF-GEF that is resistant to the action of the fungal toxin Brefeldin A (BFA) in AUX1 trafficking (Kleine-Vehn et al., 2006). BIG3, a member of the BIG family is resistant to BFA further supporting the investigation into the role of BIGs in AUX1 trafficking via TGN. Therefore, we initiated the characterization of BIGs and their target ARF1 in AUX1 trafficking.

Our data demonstrate that BIG4 colocalizes with ECH and ARF1 at the TGN and like ECH, BIG function is essential for ARF1 localization at the TGN. Disruption of ARF1 or BIG function phenocopies the AUX1 trafficking and hook developmental defects observed in ech. Moreover, big mutants, like ech, exhibit resistance to exaggeration of the apical hook by ethylene precursor ACC. Taken together, this work provides insight into the mechanism of ARF-GEF-mediated, ECH-dependent secretory trafficking at the TGN in hormonal control of differential growth.

RESULTS

The ARF-GEFs GNOM and BIG1–4 Act at Distinct Stages of Apical Hook Development

Following germination, wild-type seedlings progressively bend their hypocotyls to form an apical hook in the dark, a process that is complete 24 to 36 h postgermination (designated as the formation phase). The apical hook is maintained for ~48 h (maintenance phase), followed by a gradual opening over 72 to 96 h post-germination (opening phase), after which the hypocotyl becomes completely straight. We employed a genetic and pharmacological approach to investigate the role of ARF-GEFs in apical hook development, using the fungal toxin BFA, which inhibits the activity of ARF-GEFs (Peyroche et al., 1999). When wild-type seeds were germinated on media supplemented with 5 μM BFA, apical hook development was completely abolished (Figure 1A), indicating a role for ARF-GEFs in this process. The Arabidopsis thaliana genome harbors eight ARF-GEFs, divided into two subclasses, one comprising GNOM and GNL1-2 and the other BIG1-5, with BIG1-4 regulating processes distinct from BIG5 (Richter et al., 2014). Next, we took a genetic approach to obtain a better understanding of the contribution of distinct ARF-GEFs to hook development. We used Arabidopsis seedlings expressing a BFA-resistant variant of GNOM (GNOMR) (Geldner et al., 2003) and analyzed the effect of BFA on hook development in these seedlings (Figure 1A). In contrast to the wild type, the introduction GNOMR into the wild-type background completely restored hook formation, even in the presence of 5 μM BFA (Figure 1A). However, GNOMR seedlings were unable to maintain the apical hook in the presence of 5 μM BFA compared with seedlings grown without BFA (Figure 1A). We observed that GFP-tagged GNOM driven by its native promotor was expressed uniformly at equal levels during both hook formation and maintenance (Supplemental Figure 1). Thus, although expressed ubiquitously during distinct stages of hook development, GNOM acts primarily during the hook formation phase, whereas other ARF-GEFs may be essential during the subsequent stages of hook development.

ARF-GEFs BIG1–4 were recently shown to act redundantly and independently of GNOM during lateral root initiation (Richter et al., 2014). Therefore, we analyzed whether BIGs play a role in hook development. While big1–big4 single mutants, as well as various double and triple mutant combinations, displayed no discernible...
hook defects (Supplemental Figures 2A and 2B), big1 big2 big3 and big1/+ big2 big3 big4 mutants exhibited an apical hook development with a markedly shortened maintenance phase (Figure 1B). This indicates that BIG ARF-GEFs are not essential during the formation phase but act redundantly during the maintenance phase. A quadruple big mutant is not viable and could therefore not be analyzed (Richter et al., 2014). Of the BIG ARF-GEFs, only BIG3 is resistant to BFA (Richter et al., 2014). Thus, treating the big3 mutant with BFA allows simultaneous inhibition of BIG1-4 function as well as GNOM. While hook formation of wild-type plants remained unaffected in the presence of 0.5 μM BFA, big3 mutant treated with 0.5 μM BFA displayed severely perturbed apical hook development, with hook angle peaking at ~150° instead of 180° as in the wild type and with a completely abolished maintenance phase (Figure 1C). The hook defect in the BFA-treated big3 plants could be rescued with the introduction of an engineered, BFA-resistant variant of BIG4-YFP (ProUBQ10:BIG4-YFP) to the big3 background, but not with the introduction of a BFA-sensitive BIG4-YFP (ProUBQ10:BIG4-YFP) (Supplemental Figure 2C). These results further illustrate the functional redundancy among BIG1-4 family members during hook development. It has been previously shown that GNOM can take over the function of the BFA-resistant GN1L1 in the absence of GN1 (Richter et al., 2007). Therefore, we tested whether GNOM can similarly take over the function of BIGs in hook development. However, the introduction of GNOMR into big3 did not reverse the severe hook defect of BFA-treated big3 (unlike BFA-resistant BIG4) (Supplemental Figure 2D), indicating that GNOM cannot substitute for BIG1-4. Taken together, our results show that ARF-GEFs BIGs and GNOM are required at distinct stages of hook development.

**BIGs Act Redundantly in Ethylene-Mediated Hook Development**

Previous studies have demonstrated that the apical hook maintenance phase is regulated by ethylene (Raz and Koomneef, 2001), and exogenously added ethylene or ethylene precursor ACC induces exaggeration of the apical hook (Kang et al., 1987; Bleecker et al., 1988). Therefore, we investigated whether BIGs are involved in ethylene-mediated control of hook development. Hence, we examined whether the hook maintenance defect of BFA-treated big3 could be suppressed by ACC. While wild-type seedlings exhibited an exaggerated hook curvature in response to treatment with 10 μM ACC both in the presence and absence of 0.5 μM BFA (Figure 2A), reaching an average angle of 270°, the big3 mutants did not exaggerate their hook curvature in response to 10 μM ACC treatment when grown in the presence of 0.5 μM BFA (Figure 2B). This indicates that the BIG ARF-GEFs are required, and act independently of GNOM, during ethylene-mediated apical hook maintenance.

**BIGs Interact Genetically with ECH in Hook Development**

The hook defects in big mutants, e.g., attenuated maintenance and insensitivity of hook development to ethylene, are similar to those described in the ech mutant (Boutté et al., 2013). Furthermore, like ECH, BIG3 localizes to the TGN (Richter et al., 2014). Therefore, we examined whether BIG ARF-GEFs and ECH might act in a common pathway during hook development. We observed that hook development of the ech mutant displayed a strong hypersensitivity to BFA treatment, implying that ARF-GEF function may be compromised in the ech mutant (Figure 3A). Furthermore, an ech big2 big3 triple mutant exhibited enhanced defects in hook development when compared with ech and big2 big3 double mutants alone (Figure 3B), suggesting that ECH and BIG1-4 act synergistically in hook development.

**AUX1 Trafficking to the PM Requires BIG1-BIG4 Function**

We have previously demonstrated that the level of AUX1 at the PM is strongly reduced in the ech mutant (Boutté et al., 2013). Furthermore, the aux1 mutant displays insensitivity to ethylene during hook development (Vandenbussche et al., 2010), as do the ech and big mutants. This led us to investigate if AUX1 delivery to the PM requires BIG1-4 function. In contrast to mock-treated wild type and big3, or wild-type seedlings grown in the presence of 0.5 μM BFA, AUX1-YFP levels at the PM decreased to 43% of mock levels during the formation phase and 11% of mock levels during the maintenance phase in big3 mutant grown on medium supplemented with 0.5 μM BFA (Figure 3B). Compared with the strong reduction of AUX1-YFP plasma membrane levels during the maintenance phase in big3 seedlings grown on 0.5 μM BFA, the transcript levels of AUX1-YFP were only mildly reduced in big3 grown on 0.5 μM BFA during the maintenance phase (Supplemental Figure 3), indicating that the observed attenuation of PM localization of AUX1 in BFA-treated big3 is primarily posttranscriptional.

It has been suggested that AUX1 trafficking may rely on a BFA-resistant ARF-GEF (Kleine-Vehn et al., 2006). In agreement with this observation, no intracellular agglomerations of AUX1-YFP were observed in wild-type epidermal cells in the hook that were mock-treated, subjected to a 3-h treatment with 50 μM BFA, or alternatively pretreated with 50 μM cycloheximide (CHX) for 1 h prior to a 3-h treatment with 50 μM CHX together with 50 μM BFA (Figures 3A to 3C). While AUX1-YFP localization was unaffected in mock-treated big3 mutant (Figure 5D) like the wild type, big3 mutants treated with 50 μM BFA for 3 h displayed strong intracellular agglomerations of AUX1-YFP (Figure 5E). Importantly, this formation of AUX1-YFP agglomerations in big3 upon BFA treatment was blocked when the big3 seedlings were pretreated with CHX at 50 μM for 1 h prior to a 3-h treatment with 50 μM CHX + 50 μM BFA (Figure 5F). Additionally, the intracellular AUX1-YFP agglomerations in BFA-treated big3 seedlings strongly overlapped with the TGN-localized VHA-a1-RFP (Dettmer et al., 2006) (Pearson’s coefficient, 0.429 ± 0.083) (Figures 3G to 3L; Supplemental Figure 4A). By contrast, pretreatment with 50 μM CHX for 1 h followed by a 3-h treatment with 50 μM CHX + 50 μM BFA strongly agglomerated VHA-a1-RFP but not AUX1-YFP (Pearson’s coefficient 0.007 ± 0.055) (Supplemental Figures 4A and 4B). Importantly, the AUX1-YFP agglomeration in BFA-treated big3 was suppressed by expression of a BFA-resistant BIG4-RFP but not a BFA-sensitive BIG4-RFP (Supplemental Figures 5A to 5D) or expression of GNOMR (Supplemental Figures 5E and 5F). Thus, these results suggest that GNOM does not play a role in the trafficking of AUX1-YFP to the PM, in contrast to its role...
in PIN1 trafficking. Taken together, these results indicate that, like ECH, BIG1-4 mediate de novo AUX1 trafficking to the plasma membrane, via the TGN, independently of GNOM function.

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

BIG1-4 were recently shown to localize to the TGN in Arabidopsis roots (Richter et al., 2014). At least two domains have been identified in the TGN, one of which is labeled by VHA-a1, SYP61, and ECH, whereas the other of which only partially overlaps with VHA-a1 but is marked with RABA2a (Chow et al., 2008; Gendre et al., 2011; Uemura et al., 2014). Since ech and big1-4 mutants display very similar phenotypes, we investigated whether BIGs colocalize with ECH at the TGN. We observed a strong colocalization between ECH-YFP and BIG4-RFP (61%) as well as between ECH-YFP and the TGN-localized VHA-a1-RFP (64%) and BIG4-RFP and VHA-a1-GFP (65%) (Figures 6A to 6D), using a quantitative morphology-based method for assessing colocalization percentage (Boutté et al., 2006). This observation along with the phenotypes of the ECH and BIG ARF-GEF mutants strongly suggests that ECH and BIG ARF-GEFs operate at the same TGN domain. Therefore, we tested whether ECH is required for BIG ARF-GEF localization at the TGN. While BIG4-RFP exhibited an almost exclusively punctate labeling in wild-type seedlings (Figure 7A), BIG4-RFP was strongly mislocalized in ech mutants, exhibiting a diffuse rather than punctate pattern (Figure 7B). Similarly, we tested whether ECH localization required BIG ARF-GEF function to assess the localization interdependence of ECH and BIG ARF-GEFs. While ECH-YFP localized to punctate structures in mock-treated wild-type and big3, ECH-YFP agglomerated in the wild-type after 1 h of 50 μM BFA treatment (Figures 7C to 7E). By contrast, 1 h of 50 μM BFA treatment of big3 led to ECH-YFP exhibiting a diffuse pattern (Figure 7F) different from that observed in BFA-treated wild-type seedlings, indicating that ECH localization requires BIG ARF-GEF function and, hence, that the localization of ECH and BIG ARF-GEFs at the TGN is interdependent.

ECH has been shown to be important for the localization of VHA-a1 as well as other proteins such as YIPs and SYP61 at the TGN (Gendre et al., 2011, 2013). Disruption of the V-type proton ATPase function by Concanamycin A (ConcA) results in the mislocalization

Figure 2. BIG1–4 Are Required for Ethylene-Mediated Hook Maintenance.

(A) Wild-type seedlings respond to 10 μM ACC treatment with exaggerated hook curvature both in the presence or absence of 0.5 μM BFA.

(B) big3 mutant seedlings are unable to exaggerate in response to 10 μM ACC in the presence of 0.5 μM BFA. For each genotype, n = 16. Error bars represent ± of the mean.
of several TGN proteins, such as SYP61, that are also mislocalized in the ech mutant. This result prompted us to suggest that the mislocalization of TGN proteins in the ech mutant may be caused by the disruption of VHA-a1 function. Therefore, we tested whether the mislocalization of BIG ARF-GEFs in ech was due to disruption of VHA-a1 function by targeting VHA-a1 activity using ConcA. Our results show that a 2-h 5 mM ConcA treatment did not affect the localization of BIG3-YFP or BIG4R-YFP (Figures 7G to 7J). By contrast, the primary target of ConcA, VHA-a1-GFP, strongly agglomerated into large ConcA-induced bodies, indicating the effectiveness of the ConcA treatment (Figures 7K and 7L). Thus, ECH is required for the proper localization of BIG ARF-GEFs at the TGN independently of VHA-a1.

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

BIG3 has been previously shown to act as an ARF-GEF for the ARF1 GTPase in vitro (Nielsen et al., 2006). Since BIG4 colocalizes with ECH at the TGN, we investigated whether ARF1 also colocalizes with BIG ARF-GEFs at the TGN. We observed that ARF1-labeled structures colocalized with both BIG4 and VHA-a1 (40 and 47%, respectively) (Figures 8A to 8C), indicating that ARF1 and BIG4 localize to the same TGN domain. The mislocalization of BIG ARF-GEFs in the ech mutant led us to investigate whether the TGN localization of ARF1, the potential in vivo target of BIG3, could be compromised in the ech mutant. Whereas ARF1-GFP labeling in the wild type was largely restricted to punctate structures (Figure 9A), ARF1-GFP in the ech mutant exhibited a diffuse pattern with few punctate structures, indicating the mislocalization of ARF1 in the ech mutant (Figure 9F). To examine the role of BIGs in ARF1 localization, we visualized ARF1-GFP in the big3 mutant upon BFA treatment. While ARF1-GFP exhibited punctate labeling under mock conditions in both the wild type and the big3 mutant (Figures 9B and 9G), the punctate labeling of ARF1-GFP was lost after a 15-min treatment with 50 μM BFA in big3 (Figure 9H), before any discernable effect on its localization was observed in BFA-treated wild type (Figure 9C). By contrast, VHA-a1-GFP labeling was unaffected after a 15-min BFA treatment in both the wild type and big3 (Figures 9D, 9E, 9I, and 9J). The rapid mislocalization of ARF1 in big3 upon BFA treatment suggests that ARF1 localization may depend on TGN-localized BIG1-4 function. Introduction of a BFA-resistant BIG4-RFP but not a BFA-sensitive BIG4-RFP reversed the ARF1-GFP mislocalization in big3 upon a 30-min 50 μM BFA treatment, indicating that BIG ARF-GEF function like ECH is required for proper ARF1 localization at the TGN (Supplemental Figures 6A to 6D).

Figure 3. Loss of ECH Causes BFA Hypersensitivity, and ECH Interacts Genetically with BIG2 and BIG3.

(A) Hook development in ech is perturbed when exposed to 0.5 μM BFA, while wild-type seedlings remain unaffected. (B) ech big2 big3 triple mutant exhibits more severe apical hook development compared with ech and big2 big3 mutants. For each genotype, n = 16. Error bars represent ± of the mean.
ARF1 Members Are Essential for Ethylene-Mediated Apical Hook Development and AUX1 Trafficking

ARF1 localization at the TGN relies on BIGs and ECH. Therefore, we investigated whether ARF1 also plays a role in hook development, like BIG ARF-GEFs and ECH. However, the high redundancy among ARF1 subclass members (Xu and Scheres, 2005) makes it difficult to address the ARF1 function. Therefore, we generated transgenic plants expressing a dominant-negative GDP-locked mutant of the ARF1 member ARFA1c with low GTP affinity fused to CFP (ARFA1cT31N-CFP) (Dascher and Balch, 1994; Xu and Scheres, 2005), under control of a β-estradiol-inducible UBQ10 promoter to interfere with ARF1 function. In contrast to plants that expressed a wild-type variant of ARFA1c (ARFA1cWT-GFP) or untransformed wild-type controls (Figures 10A and 10B), induction of ARFA1cT31N-CFP mutant expression led to severely perturbed hook development. Upon induction of ARFA1cT31N-CFP, the hook formation peaked at 145° (Figure 10C) and the hook maintenance phase was completely abolished, closely resembling the ech and big mutant phenotypes. Furthermore, ACC treatment of the wild-type or ARFA1cWT-GFP seedlings resulted in an exaggerated hook angle of 270° (Figures 10A and 10B), while seedlings expressing ARFA1cT31N-CFP did not exaggerate hook curvature upon ACC treatment (Figure 10C). The transcript level of ARFA1cT31N-CFP upon β-estradiol induction was similar to that of ARFA1cWT-CFP (Supplemental Figure 7), indicating that the observed effect of ARFA1cT31N-CFP on hook development was not due to a higher expression of ARFA1cT31N-CFP relative to that of ARFA1cWT-CFP.

ARF1 colocalizes with BIGs at the TGN, and the disruption of ARF1 function, as in the ech or big mutants, results in failure to respond to ACC. Therefore, we examined whether ARF1 function is also required, like ECH and BIG1-4, for AUX1-YFP trafficking to the PM. AUX1-YFP fluorescence levels at the PM decreased to 25% of wild-type levels following ARFA1cT31N-CFP induction, whereas AUX1-YFP levels at the PM were unaffected by ARFA1cWT-GFP induction (Figures 11A to 11F; Supplemental Figure 8). In the wild type, as well as in seedlings expressing ARFA1cWT-GFP, AUX1-YFP was exclusively localized to the plasma membrane (Figures 11A, 11B, 11D, and 11E), with no intracellular signal detected. By contrast, a strong intracellular AUX1-YFP signal was observed in seedlings expressing ARFA1cT31N-CFP (Figure 11F). Thus, AUX1 delivery to the PM requires the function of the ARF1 subclass. These observations strongly suggest that ECH, BIG1-4, and ARF1 operate in such a way that the function of any of these ARF1 class members is required for AUX1 delivery to the PM.
a common pathway during hook development and AUX1 trafficking to the PM.

DISCUSSION

Plants exhibit remarkable plasticity during development and are able to modify their pattern of growth to adapt to the environment. They often rely on differential cell elongation to change their growth pattern, as exemplified in the apical hook development that follows seed germination. Here, we demonstrate that the GTPase ARF1 and its effectors ARF-GEFs of the BIG family play a role in apical hook development by mediating the secretory trafficking of the auxin influx carrier AUX1 from the TGN to the PM in concert with the previously described TGN-localized protein ECH.

Figure 6. BIG4 Colocalizes with ECH at the TGN.

Colocalization experiment shows that BIG4-RFP exhibits 61% overlap with ECH-YFP and 65% overlap with the TGN marker VHA-a1-GFP ([A], [C], and [D]), while ECH-YFP exhibits 64% overlap with VHA-a1-RFP ([B] and [D]). Five epidermal cells in each of ≥ 10 apical hooks at 48 h postgermination were analyzed for each treatment. Error bar represents SD of the mean. Bars = 10 μm.

Figure 7. BIG ARF-GEF and ECH Localization Is Interdependent, but BIG Localization Is Unaffected by Concanamycin A Treatment.

While BIG4-YFP exhibits punctate labeling in the wild type (A), in ech, BIG4-YFP labeling is diffuse (B). Under mock conditions, ECH-YFP exhibits punctate labeling in both the wild type and big3 (C) and (E). Upon 1 h of 50 μM BFA treatment, ECH-YFP agglomerates into large intracellular bodies in wild-type (D), while in big3, ECH-YFP exhibits a diffuse labeling upon 1 h of 50 μM BFA treatment (F). Under mock conditions, BIG3-YFP, BIG4-R-YFP, and VHA-a1-GFP exhibit punctate labeling ([G], [I], and [K]). Upon 2 h of 5 μM ConcA treatment, BIG3-YFP and BIG4-R-YFP remain punctate ([H] and [J]), while VHA-a1-GFP agglomerates (L). All experiments were performed in epidermal cells of the apical hook at 48 h postgermination. Bars = 20 μm.
Stage-Specific Role of ARF-GEFs in Apical Hook Development

The specific defects in secretory vesicle (SV) morphology and number previously observed in ech hinted at ECH being essential for proper secretory vesicle genesis at the TGN (Boutté et al., 2013). Other known players in vesicle formation are ARF-GTPases (D’Souza-Schorey and Chavrier, 2006) that cycle between GDP- and GTP-bound states and ARF-GEFs that play a key role in ARF GTPase function by facilitating the GDP-to-GTP exchange on ARF GTPases (Anders and Jürgens, 2008). Our results showed that hook development in the ech mutant is hypersensitive to treatment with the ARF-GEF-inhibitor BFA, suggesting that ARF-GEF function may be compromised in the ech mutant (Figure 3A). Our dissection of ARF-GEF involvement during hook development revealed that both the BFA-sensitive ARF-GEF GNOM and ARF-GEFs of the BIG family, BIG1-4, play a role in hook development. While the BFA-sensitive ARF-GEF GNOM is essential mainly during the formation phase of apical hook development (Figure 1A), the maintenance phase relies on the BIG ARF-GEF subclass members BIG1-4 (Figures 1B and 1C). It has previously been shown that ARF-GEFs can functionally replace each other. For example, GNOM can substitute for GNOM-LIKE1, and GNOM-LIKE2 can substitute for GNOM (Richter et al., 2007, 2011). However, this does not appear to be the case for GNOM and BIG1-4 in hook development, as the distinct ARF-GEFs have stage-specific roles, with BIG1-4 operating redundantly and independently of GNOM to mediate apical hook maintenance (Figures 1B and 1C; Supplemental Figures 2A to 2D). Moreover, since GNOM is expressed at all stages of hook development (Supplemental Figure 1), the stage-specific roles of GNOM and BIGs are not due to their differential stage-specific expression. Thus, the results presented here suggest that ARF-GEF regulation of hook development is separated temporally, with distinct pathways operating at different developmental phases. It remains to be seen whether the apical hook defects observed following the disruption of GNOM and BIG function are related.
to their distinct roles in trafficking, with GNOM participating in recycling (Geldner et al., 2003) and BIG1-4 involved in secretion rather than recycling (Richter et al., 2014).

BIG1-4 and ECH Act in Concert during Ethylene-Mediated Hook Development

The plant hormone ethylene is involved in hook maintenance, and ethylene-insensitive mutants, such as ein2, have severe defects in the maintenance phase (Guzmán and Ecker, 1990; Vandenbussche et al., 2010). Our data showed that BIG1-4 are required for ethylene-mediated hook maintenance (Figures 2A and 2B). Compared with the wild type, plants expressing ARFA1cT31N-CFP exhibit a shortened maintenance phase and insensitivity to 10 μM ACC treatment (C). For each genotypic and treatment, n ≥ 16. Error bars represent ±SE of the mean.

BIG1-4 Mediate AUX1 Trafficking to the PM at an ECH-Positive TGN Domain

Previous studies have revealed that AUX1 is required for ethylene-induced exaggeration of hook curvature and that in response to ethylene, AUX1 PM turnover increases preferentially on the concave side of the hook (Roman et al., 1995; Vandenbussche et al., 2010; Boutté et al., 2013). Delivery of de novo-synthesized AUX1 to the PM is strongly attenuated in the ech mutant, and hook development in the ech mutant is insensitive to ethylene (Boutté et al., 2013). In agreement with the genetic interaction between ECH and BIGs and the overall similarity of the big and ech mutant phenotypes, our data now revealed that BIG1-4 are essential for the delivery of newly synthesized AUX1 to the PM, independently of GNOM (Figures 4 and 5A to 5F; Supplemental Figures 5A to 5F). Furthermore, our colocalization study indicates that BIG3 and ECH reside on a similar TGN subdomain (Figures 6A to 6D). Thus, genetic and cell biological data suggest that both BIG1-4 and ECH are required at the TGN for AUX1 trafficking to the PM during hook development, highlighting the role of secretory trafficking in regulation of differential growth of the apical hook.

ECH and BIG1-4 Act in Concert Independently of VHA-a1 in AUX1 Trafficking from the TGN

TGN has a key role in secretion to the PM (Gendre et al., 2015). In recent years, the characterization of ECH and VHA-a1, a pathway in hook development where ethylene is an upstream regulator. In accordance with this hypothesis, we observed that an ech big2 big3 triple mutant displayed a strongly enhanced apical hook defect compared with the ech or big2 big3 double mutant (Figure 3B). Thus, BIG2 and BIG3 act together with ECH in a pathway that controls hook development downstream of ethylene.
component of the TGN-localized vacuolar H^+ ATPase, and other TGN-resident proteins have revealed certain details about the mechanisms underlying TGN function and protein secretion from the TGN to the PM (Dettmer et al., 2006; Gendre et al., 2011; Uemura et al., 2012; Asaoka et al., 2013). Disruption of VHA-a1 or ECH results in the mislocalization of TGN-resident proteins and alters TGN morphology (Dettmer et al., 2006; Viotti et al., 2010; Boutté et al., 2013). Disruption of TGN-localized vacuolar H^+ ATPase alters TGN pH levels, resulting in defective trafficking from the TGN (Luo et al., 2015). VHA-a1 has previously been connected to the secretion of BRI1 (Dettmer et al., 2006). However, despite the colocalization between ECH and VHA-a1 at the TGN as well as the mislocalization of VHA-a1 in the ech mutant, ECH-dependent AUX1 secretory trafficking in the apical hook and ECH-dependent pectin secretion in seed coat cells occur independently of VHA-a1 (Boutté et al., 2013; Gendre et al., 2013; McFarlane et al., 2013). Like ECH, BIGs (BIG3/4) localize to the TGN and colocalize with VHA-a1 (Richter et al., 2014) (Figures 6C and 6D) and ECH (Figures 6A and 6D). Thus, a crucial question to address was whether BIGs act together with VHA-a1 or independently of it in hook development. We observed that BIG localization was severely affected in the ech mutant, as BIG4-YFP exhibited a cytosol-like instead of a punctate pattern (Figures 7A and 7B). Similarly, ECH localization was perturbed by the disruption of BIG ARF-GEF function (Figures 7C to 7F), indicating the mutual dependence for TGN localization of ECH and BIGs. In contrast, when VHA-a1 function was inhibited with Conca, BIG ARF-GEF localization was unaffected (Figures 7G to 7L), indicating that the TGN localization of BIG ARF-GEFs analyzed here does not rely on VHA-a1 function despite the strong colocalization between them (Richter et al., 2014). Thus, while the secretion of AUX1 requires TGN function, it is mediated by BIG1-4 and ECH in a VHA-a1-independent manner during hook development. These observations reveal the complexity of TGN structure and function and indicate that even within a subdomain of the TGN, multiple distinct secretory pathways operate in a highly regulated manner.

**Figure 12. Model of AUX1 Secretory Trafficking at the TGN.**

**(A)** In the wild type, ECH and BIG ARF-GEFs localize to the TGN allowing ARF1 GTPases to be recruited, facilitating vesicle formation required for AUX1 delivery to the PM, a prerequisite for the ethylene response during hook development.

**(B)** In the absence of ECH, both BIG ARF-GEFs and ARF1 are lost from the TGN, leading to defects in AUX1 delivery to the PM and insensitivity to ethylene during hook development.

**(C)** When BIG ARF-GEF function is perturbed, ECH and ARF1 are mislocalized, which results in AUX1 delivery to the PM being hampered and insensitivity to ethylene during hook development.

**ARF1 GTPases Mediate Ethylene-Regulated Hook Development and Require ECH and BIG1-4 for Their Proper Localization**

ARF-GEFs regulate vesicle formation by activating GTPases of the ARF/Sar family (Peyroche et al., 1996; Anders and Jürgens, 2008). The ARF-GEF BIG3 was previously shown to catalyze GDP/GTP exchange specifically for members of the ARF1 subclass, localized mainly to the TGN and Golgi (Nielsen et al., 2006; Robinson et al., 2011). We observed that the disruption of ARF1 function by expressing a dominant-negative variant of ARFA1c (ARF1T31N) exhibited severely perturbed hook development (Figures 10A to 10C), with complete abolishment of the maintenance phase,
phenocopying ec and big1+/+ big2 big3 big4 or BFA-treated big3 mutant. Moreover, defects in hook development caused by the expression of the dominant negative ARF1C could not be suppressed by ethylene treatment as shown earlier for ec or big3 upon BFA treatment (Figure 10C). ARF1T31N, which has a low affinity for GTP, is thought to sequester endogenous ARF-GEFs by mimicking GDP-bound inactive ARF1, hampering the activation of endogenous ARF1, thereby mimicking the loss of ARF1 and ARF-GEF function (Dascher and Balch, 1994). Perturbing ARF1 function also greatly affected the delivery of AUX1 to the PM, as AUX1 was strongly retained in intracellular structures and highly reduced at the PM (Figures 11A to 11F; Supplemental Figure 8). This places the ARF1 into the same genetic pathway as ECH and BIG1-4 in hook development. Our data also showed that ARF1 colocalized strongly with BIG4 at the TGN (Figures 8A to 8C), and ARF1 labeling dramatically shifted from strictly punctate to highly diffuse when either BIG1-4 or ECH function was perturbed, suggesting that ARF1 is mislocalized and probably no longer associated with a membrane compartment in the absence of ECH or BIG ARF-GEFs (Figures 9A to 9C and 9F to 9H). These results suggest that ARF1 activation via ARF-GEF-mediated GDP/GTP exchange may have been compromised in the absence of ECH and BIG ARF-GEFs.

ARF1 has been associated with COPI-mediated pathways at the Golgi (Goldberg, 1999; Pimpl et al., 2003). ARF1 also localizes to the TGN in plants (Robinson et al., 2011) and has been demonstrated to have the capacity to mediate COPI-independent vesicle formation at the TGN in a cell-free system derived from PC12 cells (pheochromocytoma of the rat adrenal medulla) (Barr and Huttner, 1996). Interestingly, inhibiting BIG1-4 function does not affect COPI localization (Richter et al., 2014). Thus, BIG1-4 may regulate ARF1 function at the TGN in Arabidopsis. Similarly, defects in ec and bgs are most prominent at the TGN, and importantly BIGs are mislocalized from the TGN in ec (Figures 7A and 7B). Thus, ECH may also mediate ARF1-dependent processes preferentially at the TGN, potentially via a BIG ARF-GEF-dependent pathway.

We previously showed that the number of SVs at the TGN is reduced in the ec mutant, while the number of clathrin-coated vesicles (CCVs) remained unaffected (Boutil et al., 2013). Unlike CCVs, SVs may bear a thin coat or even lack a coat (Donohoe et al., 2007), forming via compositional modifications in membrane lipids that induce curvature of the membrane (Bard and Malhotra, 2006; Gendre et al., 2015). Notably, in vitro studies in rat pituitary GH3 cells demonstrated that Phospholipidase D (PLD) stimulates SV budding at the TGN (Chen et al., 1997). PLD mediates the conversion of the cylindrical phospholipid phosphatidylcholine to the more conical phosphatidic acid (Pappan et al., 1998), causing the membrane to arch. PLD activity is stimulated by ARF1, which provides a potential mechanistic framework for SV formation at the TGN (Chen et al., 1997). Analogous mechanisms for the formation of SVs from TGN have not been demonstrated as yet in plants. Nevertheless, our data showing reduced SVs in ec and bgs in cell biological and developmental phenotypes of ec and big mutants warrant the investigation of the role of ECH/BIGs/ARF1 and lipids in SV formation at the TGN in the future.

The TGN is a key hub for secretory and endocytic pathways. At least two distinct domains, defined by ECH/VHAa1/SYP61 and by RabA2a, appear to be involved in the trafficking of distinct TGN-PM and endocytic pathways (Dettmer et al., 2006; Chow et al., 2008; Gendre et al., 2011, 2013). Nevertheless, our knowledge of protein sorting and separation of diverse pathways from the TGN remains incomplete. We previously showed that ECH and VHA-a1, despite localizing to the same TGN domain, could have distinct functions, and ECH but not VHA-a1 is required for the trafficking of AUX1, a key component required for ethylene-mediated hook development (Boutil et al., 2013). Furthermore, ethylene application has been shown to increase the turnover of AUX1 on the concave side of the hook. Our results presented here reveal additional components of the AUX1 trafficking pathway essential to ethylene-mediated hook development (Figure 12). This pathway composed of BIG ARF-GEFs and their target ARF1 acts in AUX1 trafficking independently of VHA-a1 as described earlier for ECH (Boutil et al., 2013). As summarized in the model (Figure 12), when ECH is not present at the TGN, BIG localization at the TGN is affected and vice versa, which in turn results in the loss of ARF1 from the TGN and severe reduction of AUX1 levels at the PM and attenuation of the ethylene response in the hook (Figure 12). Both ARF1 and BIG ARF-GEFs have broad roles in trafficking relative to those uncovered so far for ECH. For example, ARF1 regulates additional processes independent of ECH, such as COPI-mediated retrograde trafficking from the Golgi (Stefano et al., 2006). Similarly, BIG1-4 operate in pathways distinct from ECH function, such as trafficking of cargo to the vacuole (Richter et al., 2014). Therefore, it is tempting to speculate that ECH might function as a component of the machinery that while requiring ARF1 and BIGs, could facilitate compartment and/or pathway specificity for trafficking via TGN. Unraveling the mechanisms behind trafficking pathway specificity may thus provide insight into the intricate complexity of endomembrane trafficking and how the multifaceted network of pathways controls myriad developmental processes spanning from embryogenesis to senescence.

**METHODS**

**Plant Material**

The Arabidopsis thaliana ecotype Columbia-0 (Col-0) and the following mutants were used in this study: ec (Gendre et al., 2011), big1, big2, big3, big4, big1 big3, big1 big4, big2 big3, big3 big4, big1 big2 big3, big1 big2 big4, big1 big3 big4, big2 big3 big4, and big1/+ big2 big3 big4 (Richter et al., 2014). The following transgenic lines were used: GNOMM696L-myc (GNOM®) (Geldner et al., 2003), ProAUX1:AUX1-YFP (Swarup et al., 2004), ProVHA-a1:VHA-a1-GFP, ProVHA-a1:VHA-a1-RFP (Dettmer et al., 2006), ProECH:ECH-YFP (Gendre et al., 2011), ProBIG3:BIG3-YFP, ProUBQ10:BIG4-YFP, ProUBQ10:BIG4-RFP, ProBIG3:BIG4-RFP, ProBIG3:BIG4-RFP (Richter et al., 2014), and ARF1-GFP (Xu and Scheres, 2005).

**Growth Conditions**

Plants were grown on plates supplied with 0.5x MS (2.2 g/L Murashige and Skoog nutrient mix [Duchefa]), 0.8% (w/v) plant agar (Duchefa), 0.5% (w/v) sucrose, and 2.5 mM MES (Sigma-Aldrich) buffered at pH 5.8 with KOH. For confocal microscopy analysis, seeds were stratified for 2 d at 4°C, given a 6-h white light treatment, and subsequently grown in darkness on vertically oriented agar plates at 21°C for the appropriate time (indicated for each experiment in the results section and/or figure legends). For time-lapse analysis of apical hook development, seedlings were grown vertically.
on plates in a dark room at 21°C illuminated only with an infrared LED light source (850 nm). Seedlings were photographed at 4-h intervals using a Canon D50 camera without an infrared filter.

**Inhibitor Treatments and Chemical Induction**

For time-lapse analysis of apical hook development, seedlings were grown on agar plates with the respective inhibitor/induction agent or equivalent amounts of solvent for mock treatments. For inhibitor pre-treatments of dark-grown seedlings, seedlings were incubated in liquid medium (0.5× MS) supplied with 0.5% [w/v] sucrose and 2.5 mM MES, pH adjusted to 5.8 with KOH) supplied with the indicated inhibitor or an equivalent amount of solvent during the time indicated. Stock solutions were 50 mM BFA, 5 mM ConcA, 100 mM ACC, and 50 mM CHX (Sigma-Aldrich), dissolved in DMSO. For induction of ARFA1c::GFP and ARFA1::GFP expression, seeds were germinated and grown on plates supplied with 5 mM [β-estradiol (Sigma-Aldrich)] or the equivalent amount of solvent from a 5 mM stock solution.

**Genotyping of big T-DNA Mutant Lines**

Genotyping was performed using a Phire Plant Direct PCR kit (Thermo Fisher Scientific), with the following primers. Primers used for big1 (SGK-458280) T-DNA mutant analysis are as follows: wild-type allele, 5'-CTAATCCGTCGCGGATTTT-3' and 5'-GGACTTGGCCTTCAATTTGGT-3'; T-DNA allele, 5'-CTAATCCGGCGGTATTGT-3' and 5'-ATATGAGCCTCATGATCTAGGC-3'. Primers used for big2 (SALK_033448) T-DNA mutant analysis are as follows: wild-type allele, 5'-GAAGGCGTCTGGGAAGAATC-3' and 5'-TCATCCACTTCCACCCAAG-3' and 5'-ATTGGCGGCTTGGGAA-3'. Primers used for big3 (SALK_046417) T-DNA mutant analysis are as follows: wild-type allele, 5'-TCAACTGGCCTGCAAGAAGA-3' and 5'-TCTGCTCTTGGGTGAA-3'; T-DNA allele, 5'-TCACTCCGCTGCAAGAAGA-3' and 5'-ATTGGCGGCTTGGGAA-3'. Primers used for big4 (SALK_086870) T-DNA mutant analysis are as follows: wild-type allele, 5'-TGCTGTCATTGCTGTTA-3' and 5'-TTGGTAAGGCTTGGCTGCT-3' and 5'-ATTGGCGGCTTGGGAA-3'.

For big1 heterozygosity analysis of the big1/+ big2 big3 big4 mutant, individual seedlings were genotyped after completion of the time-lapse imaging experiment.

**Confocal Laser-Scanning Microscopy and Quantitative Analyses of Fluorescence Intensity and Colocalization**

All fluorescence detection by confocal laser-scanning microscopy was performed using a Carl Zeiss LSM780 equipped with a 40x/1.2 W Corr M27. For examination of AUX1-YFP fluorescence intensities at the plasma membrane, the plasma membrane was acquired by manually outlining a 0.5-μm segmented line along the plasma membrane using ImageJ software. For each experiment, five epidermal cells from each of ≥10 seedlings were measured (n ≥ 50). For GNOM-GFP fluorescence intensity examination, the interior of individual cells was demarcated manually using polygonal selection tool in ImageJ, and fluorescence intensity was measured. For each time point, five epidermal cells from each of 10 seedlings were measured (n = 50). Colocalization of endomembrane compartments was performed using the simultaneous line-scanning mode, with a four-line average. Individual channels were segmented, subcellular objects were demarcated, and geometrical centers (centroids) of objects were calculated using the 3D objects counter plug-in in ImageJ. Colocalization of two objects was accepted when the distance between them was below the XY resolution (R) limit according to R = 0.61×NA. For each comparison, five epidermal cells from each of 10 seedlings were measured (n = 50). For AUX1-YFP and VHA-a1-RFP intracellular overlap analysis, the cell interior signal was separated from the plasma membrane signal by manual demarcation using the polygonal selection tool in ImageJ and selected as the region of interest. The ImageJ colocalization plug-in was used to obtain Pearson’s correlation coefficient for regions of interest of individual cells. For each experiment, five epidermal cells from each of 10 seedlings were analyzed (n = 50). For all quantitative analyses of fluorescence intensity, confocal images were acquired using identical acquisition parameters (laser power, photomultiplier, offset, zoom factor, and resolution) between the examined genotypes. All experiments were performed in the apical hook of dark-grown seedlings.

**Generation of Transgenic Plants**

ARF1::GFP and ARF1::CFP sequences were amplified from extracted genomic DNA from plants carrying these constructs (Xu and Scheres, 2005) using the following primers: CACCATGGGGTTGTCATTCGGAAAGTTG and TCTGCTCTTGGGTCGAAACT-3; T-DNA allele, 5'-CACCAACCAGA-3' and 5'-CTGGCGGAGGAAGAA-3'. Primers used for big1::GFP, ARF1 WT::GFP and ARF1 T31N::CFP into the pMDC7.b vector. All constructs were transformed into Col-0 wild-type plants. Plants were selected on agar plates supplied with hygromycin (25 µg/mL) (Duchefa). Three independent lines were analyzed.

**RNA Isolation and Quantitative Real-Time PCR Analysis**

Total RNA was extracted from 3-d-old dark-grown seedlings using a Spectrum Plant Total RNA isolation kit (Sigma-Aldrich) according to the manufacturer’s protocol. RNA (5 µg) was treated with RNase-free TURBO DNase (Life Technologies, Ambion), and 1 µg was then used for cDNA synthesis using an iScript cDNA synthesis kit (Bio-Rad). ELF1a was used as the reference gene for all experiments after screening and validation of a set of four different reference genes (UBQ, TIP, ELFa, and actin) using GeNorm software. CFP/GFP/YFP primers were designed from conserved sequences and the same primers were used for all experiments. Quantitative real-time PCR analyses were performed with a Roche LightCycler 480 II instrument, and relative expression values were calculated using the ∆Ct-method (∆Ct) to calculate relative expression values of genes of interest. Real-time PCR primers were as follows: CFP/GFP/YFP-Fwd, 5'-GACCAAGGCTTGCGAAGAAGAAACCTG-3' and 5'-TCATCCACTTCACCCAAGA-3' and 5'-ATTGGCGGCTTGGGAA-3'. Primers used for big1::GFP (SALK_096870) T-DNA mutant analysis are as follows: wild-type allele, 5'-TGCTGTCATTGCTGTTA-3' and 5'-TTGGTAAGGCTTGGCTGCT-3' and 5'-ATTGGCGGCTTGGGAA-3'.

For big1 heterozygosity analysis of the big1/+ big2 big3 big4 mutant, individual seedlings were genotyped after completion of the time-lapse imaging experiment.

**Statistical Analyses**

For statistical analyses, Microsoft Excel 2010 was used. For generation of box plot graphs, Minitab 16 was used. To evaluate statistical differences in plasma membrane intensities, Student’s t test was used. For all tests, *P < 0.05 and **P < 0.0001.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: ECH (At1g09330), BIG1 (At4g38200), BIG2 (At3g60860), BIG3 (At1g01980), BIG4 (At4g35380), GNOM (At1g13980), and ARFA1c (At2g47170).

**Supplemental Data**

- **Supplemental Figure 1.** GNOM is expressed ubiquitously throughout apical hook development.
- **Supplemental Figure 2.** BIG1-BIG4 act redundantly and independently of GNOM during apical hook development.
- **Supplemental Figure 3.** qRT-PCR analysis of AUX1-YFP expression.
Supplemental Figure 4. AUX1-YFP overlaps with TGN-marker VHA-a1-RFP in big3 upon BFA treatment.

Supplemental Figure 5. BFA-resistant BIG4 but not BFA-resistant GNOM reverses BFA-induced AUX1-YFP agglomerations in big3.

Supplemental Figure 6. BFA-resistant BIG4 reverses BFA-induced ARF1 mislocalization in big3.

Supplemental Figure 7. qRT-PCR analysis of inducible ARF1 expression.

Supplemental Figure 8. Quantification of AUX1-YFP PM fluorescence.

ACKNOWLEDGMENTS

We thank Gerd Jürgens for sharing materials and helpful discussions on the manuscript. We also thank Karin Schumacher, Malcolm J. Bennett, and Ben Scheres for sharing published materials. This work was supported by grants from the Knut and Alice Wallenberg Foundation, Vetenskapsrådet, Vinnova, and Berzelii. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

AUTHOR CONTRIBUTIONS

K.J. conceived, designed, drafted, and revised the article, and acquired, analyzed, and interpreted data. R.P.B. and Y.B. conceived, designed, drafted, and revised the article, and analyzed and interpreted data. D.G. analyzed and interpreted data and drafted the article. R.K.S. acquired, analyzed, and interpreted data and drafted the article. D.G. analyzed, and interpreted data. R.P.B. and Y.B. conceived, designed, and cell polarity in plants: studies on PIN proteins. J. Cell Sci. 20: 622–634.

REFERENCES


