Multiple roles for membrane-associated protein trafficking and signaling in gravitropism

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INTRODUCTION TO GRAVITROPISM

Gravitropism is a dynamic process that involves the perception of an organ’s abnormal orientation within the gravity field, a transduction of the corresponding information into a biochemical signal, the transmission of this signal to a site of response, and organ curvature. Proper curvature therefore requires the coordination of multiple cellular activities including signal transduction, phytohormone transport, and cell expansion. Published work discussed in this review, mostly on Arabidopsis, indicates that protein trafficking through the endomembrane system plays a critical role in all of these processes.

Gravitropism begins with signal perception. In Arabidopsis roots, the specialized cells that sense gravity, or statocytes, are located in the root tips within the columella region of the cap (Blancaflor et al., 1998; Tsugeki and Fedoroff, 1999; Kiss, 2000), in shoots, the endodermis contains the statocytes (Fujiaki et al., 2000; Kiss et al., 1989; Leitz et al., 2009). After amyloplast sedimentation, an auxin gradient is generated (part of the biochemical signal discussed above) and transmitted so that the auxin concentration on the lower side of the organ is higher than the concentration along its upper side (Ottenschlager et al., 2003). This typically promotes downward curvature of roots and upward curvature of shoots (Salisbury et al., 1988; Young et al., 1990).

The steps connecting amyloplast sedimentation and auxin redistribution in the signal transduction phase of gravitropism are still unclear, although several genes have been implicated in this phase. The molecular and functional analysis of some of these genes has suggested roles for endomembrane trafficking in this process. One possible model for signal perception involves the activation of stretch-activated mechanosensitive ion channels within membranes pressed upon by sedimenting amyloplasts (Leitz et al., 2009). Alternatively, in the ligand-receptor model, the activation of a transduction pathway occurs through productive interactions between sedimenting plastid-borne molecules and receptors associated with lower membranes (Braun, 2002). Lastly, in the hydrostatic pressure model, cellular machinery detects a pressure differential between the upper and lower sides of the statocytes caused by the weight of the entire protoplast on the cell wall (Staves, 1997). There is also substantial evidence for root gravity sensing outside of the columella cells that could involve an amyloplast-independent mechanism (Wolerton et al., 2002).

Researchers have proposed that several secondary messengers contribute to the signal transduction phase of gravitropism. For example, Ca2+ changes occur in response to gravitumulation, although studies have not found them in the columella cells (Pluthero and Trewavas, 2002; Toyota et al., 2008). Cytosolic pH changes, however, do occur in the columella cells upon gravitumulation, and changing the pH alters the gravitropic response (Scott and Allen, 1999; Monshausen et al., 2011). Inositol 1,4,5-triphosphate (InsP3) also appears to contribute to the formation of the auxin gradient possibly through a role in vesicle trafficking (Rivera et al., 1999; Wang et al., 2009).

In contrast to the signal perception phase of gravitropism, how a plant generates, maintains, and transmits the auxin gradient, as well as how this gradient dictates differential cell expansion, are better understood. The auxin efflux facilitators PIN-FORMED 3 (PIN3) and PIN7 show distinct relocalization to the lower side of the root cap columella cells in response to gravistemulation that initiates the differential flow of auxin toward the lower...
**ABP1** is required for auxin responses at the plasma membrane and Four SEDIMENTATION IN SHOOTS (Surpin et al., 2005). in independent role for endomembrane trafficking in gravitropism and disrupt the endomembrane system, these molecules is still underway, two of the compounds reduce hypocotyl gravitropic responses identified several small proteins that interact with auxin-responsive gene expression changes, and it has been pro- proposed to coordinate cell division and cell expansion (Shi and Yang, 2011). For more information on the overall gravitropic response, please see a recent review (Morita, 2010; Strohm et al., 2012).

**ENDOMEMBRANE SYSTEM COMPONENTS ARE IMPORTANT FOR GRAVITY PERCEPTION AND EARLY GRAVITY SIGNAL TRANSDUCTION**  

Endomembrane system components are required for normal shoot and root gravitropism in *Arabidopsis*. Endocytic pathways mediate the transport of proteins from the plasma membrane in order to control their recycling via the endosome or their degra- dation. Many proteins targeted to vacuoles are transported from the ER, to the Golgi, and then to the vacuole, although a Golgi-independent pathway also exists. Furthermore, some endocytosed plasma membrane proteins are also targeted to the vacuole. Pre- vacuolar compartments (PVCs), also called multivesicular bodies (MVBs), mediate Golgi or plasma membrane to vacuole transport. For more information, see a recent review on this process (Reyes et al., 2011). Genetic screens for shoot gravitropism mutants revealed a contribution of vesicular trafficking to vacuoles in grav- itropism. Similarly, a screen designed to find compounds that reduced hypocotyl gravitropic responses identified several small molecules that link gravitropism and endomembrane traffick- ing. Although characterization of the proteins that interact with these molecules is still underway, two of the compounds reduce gravitropic responses and disrupt the endomembrane system despite having no apparent effect on auxin, suggesting an auxin-independent role for endomembrane trafficking in gravitropism (Surpin et al., 2005).

**VACUULAR INTEGRITY IS ESSENTIAL FOR AMYLOPLAST SEGMENTATION IN SHOOTS**  

Four shoot gravitropism (sgr) mutants have been identified that share similar phenotypes and suggest a connection between vacuole integrity, amyloplast sedimentation, and shoot gravitropic responses. SGR3/VAM3 and SGR4/VTI11/ZIK are SNAREs, which are named for SNAP (soluble NSF attachment protein) receptors and are small proteins that mediate vesicle fusion. They are divided into vesicle-SNAREs (v-SNAREs), which are located on vesicle membranes, and target-SNAREs (t-SNAREs), which are located on target membranes. SGR3 is a t-SNARE (Sato et al., 1997), and SGR4 is a v-SNARE (Zheng et al., 1999).

SGR3/GRV2/KAM2 is a DnaJ domain-containing peripheral membrane protein that localizes to late endosomes (Silady et al., 2004, 2008). Lastly, SGR2 encodes a vacuole-localized protein homologous to the bovine testis phospholipid acid preferring phospholipase A1 (PA-PLA1; Kato et al., 2002).

sgr2, sgr3, sgr4, and sgr8 share reduced shoot gravitropic responses, abnormal amyloplast localization, and altered vacuole structures. sgr2, sgr3, sgr4, and sgr8 mutants all exhibit strongly reduced shoot gravitropic responses but normal or slightly enhanced phototropic and root gravitropic responses. sgr2 and sgr4 mutants also display very slow hypocotyl gravitropism (Fukaki et al., 1996b; Yamasuchi et al., 1997; Kato et al., 2002; Yano et al., 2003; Silady et al., 2004). All of these mutants show a generally intact tissue structure con- sist- ing of a single layer of epidermis, three to four layers of cortex, and one layer of endodermis, although the sgr2, sgr4, and sgr8 mutants show some pleiotropic phenotypes including altered cell size and shape (Kato et al., 2002; Yano et al., 2003, Silady et al., 2004). This suggests that these genes are likely to function directly in gravitropism and do not simply have missing or disorganized starches.

In wild-type plants, amyloplasts in shoot endodermal cells are found sedimented on the lower sides of the cells (Morita et al., 2002). They are wrapped in thin, tunnel-like cytoplasmic layers surrounded by vacuolar membranes that are transvacuolar lar strands, which pass through the vacuole and are connected to the peripheral cytoplasm. Amyloplasts can pass through these transvacuolar strands (Sato et al., 2005). However, in sgr2, sgr3, sgr4, and sgr8 mutants, the endodermal amyloplasts are found throughout both the upper and lower sides of the cells where they localize outside of the vacuole (instead of within the transvacuolar lar strands), often pressed against the cell periphery (Morita et al., 2002; Yano et al., 2003; Silady et al., 2004). At least sgr2 and sgr4 amyloplasts can be stained with potassium iodide, suggesting that they do accumulate starch, although a few amyloplasts appeared to contain slightly less starch than wild-type (Morita et al., 2002). Together, these data suggest that altered amyloplast localization, rather than reduced starch accumulation, results in the abnormal gravitropic responses of these mutants. sgr3, sgr5, sgr5, and sgr6 also show altered vacuolar phenotypes. sgr2 and sgr4 both have aberrant vacuolar components in the cytoplasm, although these compartments differ between mutants (Morita et al., 2002). sgr3 vacuolar membranes form irregular curves and do not properly surround the amyloplasts (Yano et al., 2003). sgr7 mutants have irregularly shaped vacuoles and aggre- gates of endosomes, which suggests that they might not properly fuse the tonoplast and vesicular membranes (Silady et al., 2008).

Golgi-to-vacuole targeting is critical for proper amyloplast localization in shoots. SGR2, SGR3, SGR4, and SGR8 are all expressed in all tissues exam- ined, and at least for SGR2, SGR1, and SGR4, expression in the endodermis is sufficient to rescue the gravitropic defects of the mutants (Zheng et al., 1999; Morita et al., 2002; Yano et al., 2003, Silady et al., 2004). This indicates that these proteins’ contribution to gravitropism occurs within the statocytes. In root cells, SGR4 colocalizes with ELV, a vacuolar cargo receptor located on the
ARG1 is required for the gravity-induced cytoplasmic alkalization of the columella cells. Both of these processes are required for the relocalization of PIN3 to the new lower peripheral membrane proteins that are necessary for full root gravitropism (Fukaki et al., 1997; Sedbrook et al., 1999; Guan et al., 2003; Stanga et al., 2009). Although the specific molecular function of ARG1 and ARL2 remains unclear, these data suggest that they play a role in the early gravity signal transduction steps that connect amyloplast sedimentation and auxin redistribution.

PHOSPHATIDYLINOSITOL SIGNALING MEDIATES VESICLE TRAFFICKING, AUXIN GRADIENT FORMATION, AND THE GRAVITROPIC RESPONSE

Phosphatidylinositol monophosphate 5-kinase (PIP5K) catalyzes the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2), a plasma membrane-localized phospholipid. PIP2 is then cleaved by phospholipase C (PLC) to produce the second messenger InsP3, which diffuses throughout the cell, and diacylglycerol (DAG), which stays in the membrane. Inositol polyphosphate 5-phosphatases (InsP5-pases) dephosphorylate InsP3 to stop its activity. In animals, InsP3 can trigger Ca2+ release from the ER and stimulate the cell plate. Additionally, inhibiting PLC also blocks the long-term InsP3 increase and reduces gravitropic bending (Perera et al., 2001). Some genes show InsP3-dependent changes in expression in response to gravitropic and/or phototropic stimuli, suggesting that this second messenger may play a key role in coordinating these two responses (Salinas-Mondragon et al., 2010).

Amyloplast inflorescence stems can perceive a change in orientation while at 4°C but cannot respond until after they are returned to room temperature (Fukaki et al., 1996a). InsP3 changes are similar in plants gravistimulated at 4°C and at room temperature, and plants expressing a constitutively active InsP5-5pase show decreased bending after gravistimulation at 4°C and a subsequent return to room temperature (Perera et al., 2001). These results support the hypothesis that phosphatidylinositol signaling functions early in gravity signal transduction.

Some Endomembrane System-Associated Proteins Mediate Early Gravity Signal Transduction Independently of Amyloplast Sedimentation

ALTERED RESPONSE TO GRAVITY 1 (ARG1/RHG) and its paralog ARG1-LIKE 2 (ARL2/GPS4) encode DnaJ-domain-containing peripheral membrane proteins that are necessary for full root and hypocotyl gravitropism (Fukaki et al., 1997; Sedbrook et al., 1999; Boonsirichai et al., 2003; Guan et al., 2003; Luessen et al., 2010). GFP-ARG1 fusions localize to components of the vesicle trafficking pathway including the ER, the Golgi, and vesicles near the plasma membrane, as well as the cell plate. Additionally, upon treatment with brefeldin A (BFA), which disrupts vesicle trafficking (Boomsma et al., 2003), ARG1 and ARL2 are required for the relocalization of PIN3 to the new lower sides of the columella cells upon gravistimulation, and at least ARG1 is required for the gravity-induced cytoplasmic alkalization of the columella cells. Both of these processes are important in generating an auxin gradient (Boonsirichai et al., 2003; Harrison and Masion, 2008). These genes are especially interesting because arg1 and arl2 mutants display normal phototropism, amyloplast starch accumulation, amyloplast sedimentation, responses to phytohormones, and responses to auxin transport inhibitors (Fukaki et al., 1997; Sedbrook et al., 1999; Guan et al., 2003; Stanga et al., 2009). Although the specific molecular function of ARG1 and ARL2 remains unclear, these data suggest that they play a role in the early gravity signal transduction steps that connect amyloplast sedimentation and auxin redistribution.
PLANTS CARRYING MUTATIONS IN GENES ASSOCIATED WITH PHOSPHATIDYLINOSITOL SIGNALING SHOW ALTERED GRAVITROPIC AND AUXIN-RELATED PHENOTYPES

PIN5K and InsP5-3-phosphatases are each encoded by 15 genes in Arabidopsis. pip5k2a seedlings have decreased PIP2 levels, and 5-ptase3 mutants are likely to have a decreased ability to dephosphorylate InsP3 (Wang et al., 2009; Mei et al., 2012). Therefore, it is not surprising that these mutants share many opposite phenotypes. pip5k2a seedlings respond slowly to gravity, while 5-ptase3 mutants show an enhanced response (Wang et al., 2009; Mei et al., 2012). In agreement with this finding, plants expressing a constitutively active InsP3 5-phosphatase do not exhibit the characteristic InsP3 increase in response to gravistimulation and show decreased gravitropic bending (Perera et al., 2006). Pip5k2 mutants are more sensitive to the polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) than are wild-type plants, which suggests impaired polar auxin transport in this mutant (Mei et al., 2012). In contrast, 5-ptase3 mutants show a reduced response to NPA, which indicates increased polar auxin transport (Wang et al., 2009). Plants carrying the constitutively active InsP3 5-phosphatase also show decreased basipetal auxin transport (Perera et al., 2006). Indeed, a greater percentage of 5-ptase3 mutants and a smaller percentage of pip5k2 mutants generate an asymmetric auxin gradient in roots in response to gravistimulation compared to wild-type seedlings, resulting in altered gravitropic phenotypes (Wang et al., 2009; Mei et al., 2012).

PHOSPHATIDYLINOSITOL SIGNALING AFFECTS VESICLE TRAFFICKING AND PIN PROTEIN TURNOVER

Phosphatidylinositol signaling is required for proper vesicle trafficking that leads to the establishment of an auxin gradient. PIN auxin efflux facilitators play important roles in controlling the direction and rate of auxin fluxes that allow for differential cell expansion upon gravistimulation (see The PIN Family of Auxin Efflux Facilitators). Normally PIN proteins cycle between the plasma membrane and endosomal compartments. This process is sensitive to BFA and requires clathrin-mediated endocytosis (Steinmann et al., 1999; Friml et al., 2002b; Geldner et al., 2003; Dhonukshe et al., 2007; Klein-Vehn et al., 2010). Compared to wild-type, 5-ptase3 mutants have an increased ability to internalize the endocytosis marker FM4-64, are less sensitive to BFA, and show faster resumption of PIN1 and PIN2 polar localization at the plasma membrane after BFA removal (Wang et al., 2009). In contrast, pip5k2 mutants show a decreased ability to internalize FM4-64, increased sensitivity to BFA, slower recovery after BFA removal, and decreased cycling of PIN2 and PIN3 (Mei et al., 2012). The phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin also results in altered PIN protein localization and decreased cycling of PIN2 and PIN3 (Mei et al., 2006). This causes auxin to accumulate in the lower sides of shoots and roots where it alters cell expansion rates to cause organ curvature (Salisbury et al., 1988; Bennett, 2003). Upon gravistimulation, the PIN3 and PIN7 auxin efflux carriers switch from a non-polar localization to a preferential distribution at the lower side of the plasma membrane (Friml et al., 2002b; Klein-Vehn et al., 2010). This causes auxin to accumulate in the lower sides of shoots and roots where it alters cell expansion rates to cause organ curvature (Salisbury et al., 1988; Young et al., 1990). Vesicle trafficking therefore plays a critical role in mediating this auxin gradient through its effects on the abundance, activity, and subcellular localization of auxin efflux and influx carriers. Membrane composition and differences in the sensitivity of certain cells to auxin over time also influence curvature kinetics (Willemsen et al., 2003; Bennitms and Scheres, 2008).

THE PIN FAMILY OF AUXIN EFFLUX FACILITATORS

There are eight PIN proteins in Arabidopsis, and at least five of them function directly or indirectly in gravitropism. This is achieved through their asymmetric localization at the plasma membrane, which can determine the direction of auxin flow (Wusiewska et al., 2006). These proteins often have overlapping functions; when one protein is non-functional, auxin-dependent ectopic expression of other PIN proteins can sometimes compensate for the loss (Biliou et al., 2005; Vieten et al., 2005).

PIN proteins are required for the generation and propagation of the gravity-induced auxin gradient

PIN1 localizes to the rootward sides of the cells that form the vasculature whereas PIN4 localizes to the rootward sides of the proximal meristem cells; the latter also shows non-polar localization in the columella cells (Friml et al., 2002a). These patterns suggest that PIN1 and PIN4 play indirect roles in gravitropism by contributing to auxin efflux through the vasculature to the columella cells (Galweiler et al., 1998; Geldner et al., 2001; Friml et al., 2002a). This is important for auxin to be transported to the root tip so that it can later be distributed laterally across the cap and up to the elongation zones upon gravistimulation.
FIGURE 1 | Cellular control of auxin carriers. (A) Phosphorylation by PINOID kinase and dephosphorylation by PP2A regulate PIN protein localization. (B) PIN proteins are removed from the plasma membrane through clathrin-mediated endocytosis into endocytic compartments. (C) PIN proteins may also be ubiquitylated and targeted to the vacuole via PVCs for degradation. (D) Alternatively, following endocytosis PIN proteins may be exocytosed in a selective, polar manner that requires the activity of an unidentified ARF GTPase. The activity of the GTPase is controlled by a GEF called GNOM, which removes the used GDP and allows fresh GTP to reload. (E) Treating plants with BFA inhibits GNOM, which likely inactivates the ARF GTPase. As a result, PIN proteins accumulate in intracellular aggregates termed BFA bodies. (F) Auxin can be actively transported across the plasma membrane. PIN proteins are gradient-powered auxin efflux carriers. Members of the ABC transporter family are ATP-driven and act as either auxin influx or efflux facilitators. AUX1 and its relative LAX are auxin influx carriers that use an existing ion gradient to allow auxin into cells.

While mutations in PIN4 cause root meristem disorganization that makes it difficult to analyze their gravitropic responses, pin3 mutants have normal roots with no gravitropic defects, suggesting that other PINs are able to compensate for the loss of this gene (Friml et al., 2002a). In contrast, PIN3 and PIN7 may function immediately upon gravistimulation to generate the initial auxin gradient across the cap. PIN3 is normally expressed in the upper S1 and S2 layers of the columella cells, while PIN7 localizes to the S2 and S3 tiers. However, PIN7 expands its expression into the S1 layer in pin3 mutants, suggesting its ability to compensate for the loss of PIN3 (Kleine-Vehn et al., 2010). In roots growing vertically, these proteins show a generally non-polar localization in the columella cells, but upon gravistimulation they are internalized and resorted into vesicles that direct them to the lower plasma membrane. This gravity-induced relocalization of the PIN3 and PIN7 proteins within the statocytes may be responsible for the development of a lateral auxin gradient across the cap, with accumulation on the new lower side of the root (Friml et al., 2003b; Kleine-Vehn et al., 2010). Consistent with this conclusion, the pin3 and pin7 mutants show gravitropism defects, and the pin3 pin7 double mutant shows stronger defects than either single mutant (Friml et al., 2002b; Kleine-Vehn et al., 2010).

PIN2/ERI1/AGR1/WAV6 localizes to the shootward sides of lateral root cap and epidermal cells where it plays a critical role in transporting auxin from the cap to the elongation zone both in vertically growing roots and upon gravistimulation. It also localizes to the rootward sides of the cortical cells in the meristem, where it may play a negative regulatory role that allows for optimal auxin fluxes in this region (Müller et al., 1998; Billos et al., 2005; Abas et al., 2006; Rahman et al., 2010). Here it may also contribute to an auxin reflux loop through the root epidermal and cortical cells in which the auxin maximum that forms on the lower side of the root is reinforced. pin2 mutants do not establish an auxin gradient upon gravistimulation and therefore exhibit gravitropic defects (Luschnig et al., 1998; Müller et al., 1998; Abas et al., 2006).

PIN protein regulation affects gravitropic responses

PIN proteins can be regulated at the levels of transcription, protein stability, subcellular localization, and transport activity (Petrásek
endocytosis and targeting to the vacuole are normally regulated by ubiquitylation (Figure 1C). However, in pin2 mutants in which six or more potential ubiquitylation sites are mutated, PIN2 is not internalized and targeted to the vacuole upon gravitostimulation. Therefore, PIN2 levels stay constant at the plasma membrane in these mutants, and these seedlings do not form a robust auxin gradient upon reorientation (Leitner et al., 2012). Short-term auxin treatment also interferes with intracellular PIN2 accumulation, but long-term treatment causes PIN2 internalization and degradation (Abas et al., 2006). This may reflect a feedback mechanism in which PIN2 is degraded after the auxin level reaches a threshold, preventing additional auxin transport and excessive root curvature. Ubiquitylation could control the rate of PIN2 degradation in this process.

BFA inhibits the targeting of PIN2 to the vacuole, which suggests the involvement of an ARF-GEF. However, plants expressing the BFA-resistant GNOM showed BFA-sensitive PIN2 vacuolar targeting, indicating that the ARF-GEF of interest is not GNOM. Like GNOM, SORTING NEXIN 1 (SNX1) localizes to endosomal compartments and is BFA-sensitive; however only SNX1 is sensitive to the PI3K inhibitor wortmannin (Jaillais et al., 2006; Kleine-Vehn et al., 2008). snx1 mutants resemble weak allele gnom mutants, and $snx1 gnom$ double mutants show enhanced abnormal phenotypes compared to the single mutants (Jaillais et al., 2006). This suggests that these genes function in different pathways but contribute to some of the same developmental processes. Upon wortmannin treatment, PIN2 and SNX1 colocalize in compartments, and PIN2 levels at the plasma membrane are reduced in snx1 mutants (Jaillais et al., 2006; Kleine-Vehn et al., 2008). Accordingly, long-term wortmannin treatment results in phenotypes reminiscent of altered auxin transport, including defective root and hypocotyl gravitropism (Jaillais et al., 2006). SNX1 may therefore contribute to a feedback mechanism involved in PIN2 retrieval for recycling through its ability to mediate PIN2 translocation from the PVC to the vacuole.

PIN protein localization also depends on its phosphorylation state, which is mediated in part by the serine-threonine kinase PINOID (PID) and type 2A protein phosphatase (PP2A), which act antagonistically (Michniewicz et al., 2007 Figure 1A). PP2A subunits are encoded by multiple genes including ROOTS CURL IN NPA 1 (RCNI1). Plants that overexpress PID, rcni1 mutants, and wild-type plants treated with the phosphatase inhibitor cantharidin all show increased shootward auxin transport, delayed auxin gradient formation upon gravitostimulation, and randomized root growth; these phenotypes are rescued by blocking polar auxin transport (Christensen et al., 2000; Benjamins et al., 2001; Rashotte et al., 2001). The elevated auxin transport in these plants probably leads to auxin depletion in the root meristem, which prevents auxin gradient formation (Benjamins et al., 2001; Rashotte et al., 2001). This increased auxin transport is attributed to a rootward
to shootward shift in the localization of some PIN proteins (Friml et al., 2004). PINOID and RCNI partially colocalize with PIN proteins and mediate the phosphorylation states of their central hydrophilic loops (Michniewicz et al., 2003). This means that they can affect PIN2-mediated auxin fluxes upon gravitumulation (Shin et al., 2005). More specifically, PP2A and a PINOID kinase family member are known to mediate the polar targeting of PIN2 in meristematic cortical cells, which is necessary for a full gravitropic response (Rahman et al., 2010). These experiments show that the phosphorylation status of PIN proteins affects their localizations and in turn their abilities to regulate gravitropism.

In addition to intracellular trafficking, protein degradation, and phosphorylation, a recent study suggests that small secretory peptides can also regulate PIN protein localization and affect gravitropism. GOLVEN (GVL) genes encode these peptides, and overexpression or knockdown of these genes generally results in reduced root and hypocotyl gravitropism. Treatment with some of these peptides, which act locally, also correlates with reduced auxin gradient formation upon reorientation and results in gravitropic defects. PIN2 mutants are resistant to GVL peptide treatment, and PIN2 levels increase in the membrane fractions of wild-type plants treated with GVL peptides (Whitford et al., 2012). Therefore, it is thought that the GVL peptides, along with auxin, mediate PIN2 trafficking in order to generate the auxin gradient necessary for root curvature.

PIN proteins may promote growth in the organ curvature phase of gravitropism

AUXIN can inhibit the internalization of many PIN proteins and prevent their constitutive cycling. This results in increased levels of PIN proteins at the plasma membrane, and so auxin stimulates its own efflux from cells. After gravitumulation, the inhibition of endocytosis corresponds with the formation of the auxin gradient (Faciorik et al., 2003). Therefore, the increased level of plasma membrane-associated PIN2 on the lower flank of gravitumulated roots may further enhance the auxin gradient.

AUXIN also triggers cell wall loosening that is necessary for cell elongation during root curvature. In Arabidopsis, PIN1 mediates local auxin accumulation, and its polar localization corresponds to the direction of mechanical stress in shoot apices (Hinsley et al., 2010). Work done in tomatoes shows that as tissue becomes more strained during growth, PIN1 shows an increase in overall abundance and a preferential localization at the plasma membrane. This contributes to auxin accumulation, which then promotes growth in a feed-forward loop. One possible mechanism for this is that local cell wall strain increases plasma membrane tension, which promotes exocytosis and blocks endocytosis. This could increase the amount of membrane-localized PIN1 relative to cytoplasmically-localized PIN1, although more complex models are also possible (Nakayama et al., 2012). It is possible that a similar process takes place upon gravitumulation, although this has not yet been addressed experimentally. For example, tissue strain during curvature could increase plasma membrane-localized PIN2 levels on the lower side of the root. This would increase the auxin concentration in this region and further inhibit curvature in a feed-forward manner.

THE ABC TRANSPORTER FAMILY OF AUXIN EFFLUX AND INFUX FACILITATORS

Members of the family of ATP-binding cassette (ABC) transporters couple ATP hydrolysis with the import and export of molecules such as xenobiotics, ions, sugars, lipids, peptides, and hormones including auxin across cell membranes. There are several lines of evidence that these proteins play critical roles in maintaining the auxin gradient that results in gravitropism.

ABC transporters regulate auxin fluxes

Multiple pieces of evidence support a role for several ABC-type transporters in auxin transport. First, the Arabidopsis PGP19/MDR1/ABCB19 protein and its closest relative ABCB1 directly act as auxin transporters when expressed in mammalian and yeast cells as well as in protoplast assays (Geisler et al., 2005; Yang and Murphy, 2009). Furthermore, abh19 single mutants and to a greater extent abh19 abh21 double mutants show decreased rootward auxin transport (Noh et al., 2001; Lewis et al., 2007). Similarly, plants carrying mutations in ABCB4/PGP4/MDR4, another ABC-type transporter with sequence similarity to ABCB1 and ABCB19, show decreased shootward auxin transport (Santulli et al., 2005; Terasaka et al., 2005; Lewis et al., 2007).

Interestingly, ABCB1 shows a distinct polar localization in different cell types at the upper edge of the distal elongation zone. In the endodermal cells its localization is always shootward, and in the cortical cells it is most often shootward (Geisler et al., 2005). A similar result was found for ABCB19 (Blakeslee et al., 2007). On the other hand, ABCB4 shows rootward localization in the epidermal cells at the upper edge of the distal elongation zone while displaying apolar localization in S3 columella and adjacent root cap cells (Terasaka et al., 2005). These distinct localization patterns may help generate differential levels of auxin accumulation in different cells.

A phenotypic analysis of these mutants is also compatible with a role for these proteins in auxin transport. Indeed, abh19 and abh21 abh21 mutants show epinastic, or downward-folding, cotyledons and first true leaves as do wild-type plants when treated with exogenous auxin (Noh et al., 2001). This is likely due to the improper accumulation of auxin in the cotyledons. These mutants also show increased sensitivity to 1-NAA, decreased sensitivity to NPA, and decreased auxin-responsive DR5::GUS expression (Geisler et al., 2005; Lin and Wang, 2005).

Together, these studies strongly suggest that these transporters help maintain proper auxin flow patterns, and additional work has shown that interactions between ABC transporters and other proteins play important roles in this process. Genetic interactions have been observed between ABCB1 and PINs, and coimmunoprecipitation and yeast two-hybrid experiments have shown that both ABCB1 and ABCB19 interact with PIN1 (Blakeslee et al., 2007). Additionally, abh19 and especially abh19 abh21 mutants show diffuse, punctate, and discontinuous PIN1 localization, which is likely to result in randomized directions of auxin efflux (Noh et al., 2003). Heterologous coexpression studies have also shown that the rate of auxin transport is increased when these proteins colocalize compared to when only one of them is present. In contrast, when PIN2 and either ABCB1, ABCB4, or ABCB19 are coexpressed in HeLa cells, IAA efflux decreases when compared
AGC kinases also mediate both PIN protein polarity and the auxin efflux activity of ABCB1 and ABCB19, suggesting that they regulate crosstalk between these auxin transporters (Christie et al., 2011; Henrichs et al., 2012). From these experiments, it appears that the ABC transporters and PIN proteins function separately but synergistically to provide both the specificity and the high rate of long-distance auxin transport.

Additionally, the immunophilin-like integral membrane protein required for brassinosteroid perception or signaling, TWISTED DWARF 1, interacts with both ABCB1 and ABCB19 (Geisler et al., 2003). twd1 mutants exhibit epinastic cotyledons and a strong reduction in polar auxin transport like abcb1 abcb19 double mutants, suggesting that ABCB1 and ABCB19 form a complex with TWD1 (Geisler et al., 2003). It is possible that TWD1 regulates the transport activity of ABCB1 and ABCB19 or that it mediates ABCB–PIN interactions.

**ABC transporters are required for normal gravitropic responses**

Several experiments show that ABC transporters function in the auxin transport phase of gravitropism. Interestingly, abcb19 hypocotyls respond to gravitropism twice as quickly as wild-type plants, and they also exhibit an enhanced phototropic response (Noh et al., 2003). Similarly, the abcb4 mutant shows a faster root gravitropic response than wild-type plants (Lewis et al., 2007). Experiments using the auxin-responsive DRS–GUS construct showed that these mutants form a more robust asymmetric auxin gradient across the root tip than wild-type plants (Lin and Wang, 2005; Lewis et al., 2007). The altered auxin efflux may therefore result in a steeper, although transient, auxin gradient upon gravistimulation. One possible explanation for this comes from studies showing that PIN2 mRNA levels decrease with distance from the root tip, while ABCB4 mRNA levels increase (Burnbaum et al., 2003). If this correlates with their contributions to auxin transport, the reduced shootward auxin transport as a result of the loss of ABCB19 or ABCB4 may cause auxin buildup in the elongation zone where it leads to an enhanced curvature response (Lewis et al., 2007). Surprisingly, despite the large reduction in rootward auxin transport, root gravitropic responses of abcb19 mutants are normal (Lewis et al., 2007). This could be due to compensation by other ABC transporters or PIN proteins.

A screen for compounds that reduce hypocotyl gravitropic responses identified a molecule called Gravacin that also causes decreased auxin sensitivity, decreased auxin transport, and endomembrane system defects (Surpin et al., 2005; Rojas-Pierce et al., 2007). Subsequent work showed that abcb19 and twd1, but not abcb4, are resistant to Gravacin (Rojas-Pierce et al., 2007). Gravacin targets ABCB19 and disrupts the ABCB19–PIN1 complexes, thereby interfering with their auxin transport activity (Rojas-Pierce et al., 2007). Using Gravacin to perturb ABCB19 but not PIN proteins may be useful in further characterizing the role of ABC transporters in auxin fluxes and gravitropism.

**The AUX and LAX Family of Auxin Import Carrier Proteins**

In addition to auxin efflux, auxin flow into cells also contributes to the auxin gradient. While auxin influx can occur by diffusion, the auxin influx carriers AUX1 and LAX can also actively import IAA (Marchant et al., 1999; Yang et al., 2006; Yang and Murphy, 2009; Pérét et al., 2012). Active auxin influx into particular cells might maintain proper auxin fluxes by countering auxin diffusion into other cells. aux1, but not lax, mutants are agravitropic, suggesting functional specialization within this gene family (Bennett et al., 1996; Pérét et al., 2012). Because aux2 mutants are defective in active auxin uptake, they are therefore resistant to exogenous IAA and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), but not 1-NAA, which can diffuse easily through membranes (Maher and Martindale, 1980; Bennett et al., 1996). Similarly, 1-NAA, but not 2,4-D, rescues the aux2 agravitropic root phenotype (Marchant et al., 1999). It is likely that 1-NAA is taken up by the root and redirected by an auxin efflux facilitator such as PIN2, which is expressed in the cortical and epidermal root tip cells like AUX1 (Müller et al., 1998; Marchant et al., 1999).

AUX1 functions in the signal transmission and curvature response phases, not the perception phase, of gravitropism. This is suggested by its expression in the regions of the root that respond to gravity (Marchant et al., 1999). Consistent with this result, AUX1 expression in only the lateral root cap and epidermal cells is sufficient to rescue the aux1 agravitropic phenotype (Swarup et al., 2005). This suggests that AUX1 contributes to gravitropism by facilitating shootward auxin transport from the root cap to the elongation zone (Swarup et al., 2005).

AUX1 also affects pH changes upon gravistimulation, suggesting a relationship between pH and auxin in gravitropism. Shortly after reorientation, wild-type roots show a decrease in pH on the upper side of the extracellular root surface and an increase on the lower side; this gradient occurs in the root cap as well as throughout the elongation zone (Monsma nouveau et al., 2011). It might contribute to cell wall loosening to allow for cell expansion or even to signal transmission itself: aux1 mutants, however, do not show this pH change. In fact, even when growing vertically, aux1 aux1 mutant extracellular root surfaces are uniformly acidic instead of showing dynamic pH fluctuations like wild-type roots. Both the pH gradient and the pH dynamics are rescued by introducing AUX1 into only the lateral root cap and epidermal cells (Monsma nouveau et al., 2011). It is possible that the pH gradient contributes to feedback mechanisms that regulate the gravity response by affecting AUX1-mediated auxin uptake.

**Conclusion**

Upon gravistimulation, amyloplasts sediment to the lower sides of the statocytes. In endodermal cells, SGR proteins play a key role in this process by maintaining vacuolar membrane integrity. The amyloplasts then trigger a signal transduction cascade that may involve proteins, calcium, and phosphatidylinositol signaling, which begins at the plasma membrane. Phosphatidylinositol signaling affects the cycling of auxin transporters, and changes in their localization at the plasma membrane cause auxin to accumulate in the lower side of the root and shoot. Here it affects cell elongation and causes the plant to realign itself with the gravity vector (Figure 2).

Therefore, through their roles in amyloplast sedimentation, phosphatidylinositol signaling, and auxin carrier localization,
membranes contribute in multiple ways to all phases of gravitropism. The evolution of these complex processes allows plants to adapt to changing environments and to integrate their responses to gravity with those to a wide variety of other stimuli including touch and moisture gradients. Future work in this area will continue to clarify how membrane-associated signaling and trafficking contribute to gravitropism and other areas of plant growth and development.

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CASPAR, T., and PICKARD, B. G. (1989). The steps of gravitropism are shown in the center core of the diagram. During plant reorientation, a plant is rotated relative to the gravity vector. This results in the sedimentation of dense amyloplasts within the statocytes. In roots the statocytes are the columnella cells, whereas in stems they are the endodermal cells. Each endodermal cell contains a large vacuole, and the amyloplasts must traverse it by tunneling through transvacuolar strands in order to reach the new lower side of the cell. This requires proper vacuole structure, which the SGR proteins mediate. Amyloplast sedimentation is then thought to activate signal transduction through second messengers, possibly calcium ions or proteins. Another second messenger is IP3, which is produced by cleavage of the phospholipid, PIP2. In a process that is not completely understood, the second messengers activate the relocalization of auxin transporters, such as PIN3 and PIN7 in the columnella cells. The new polarized distribution of these auxin efflux carriers changes the flow of auxin throughout the plant. This differential auxin transport affects cell elongation rates, thereby resulting in organ curvature as the plant grows.

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