

# Joining forces

## The interface of gravitropism and plastid protein import

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In flowering plants, gravity perception appears to involve the sedimentation of starch-filled plastids, called amyloplasts, within specialized cells (the statocytes) of shoots (endodermal cells) and roots (columella cells). Unfortunately, how the physical information derived from amyloplast sedimentation is converted into a biochemical signal that promotes organ gravitropic curvature remains largely unknown. Recent results suggest an involvement of the Translocon of the Outer Envelope of (Chloro) plastids (TOC) in early phases of gravity signal transduction within the statocytes. This review summarizes our current knowledge of the molecular mechanisms that govern gravity signal transduction in flowering plants and summarizes models that attempt to explain the contribution of TOC proteins in this important behavioral plant growth response to its mechanical environment.

### Introduction

Gravity provides a directional cue that enables plants to coordinate growth patterns. Using gravity as a guide, plants drive their roots into the soil and send their shoots skyward. Plants continue to coordinate their growth relative to the gravity vector not only throughout development, but also in response to their dynamic environments, which confront them with changes in light, humidity, oxygen, ions, nutrients, temperature and mechanical forces. To coordinate these various growth responses, a plant must convert the mechanical force of gravity into a chemical signal. This chemically-transduced signal will ultimately influence the growth pattern of the responding organ(s). While there are several reviews that discuss in great detail the extensive investigations into this process,<sup>1-3</sup> there are still many outstanding issues. In particular, it has long been a goal to understand the earliest mechanisms of gravity perception and signal transduction. Recently, we identified new genetic mutants that lend important insight into this enigmatic process. Additionally, these mutants could provide useful material for investigating protein import into plastids, especially amyloplasts.

To produce a gravitropic response, a plant must first be able to perceive gravity, then convert the mechanical force into a

chemical signal. This chemical signal will ultimately influence the growth pattern of the responding organ. In *Arabidopsis* shoots, gravity perception and response happen in overlapping sites. In *Arabidopsis* roots, there is a spatial separation between the primary site of gravity perception and early signal transduction, the root cap, and the responding site that develops a curvature, the distal elongation zone (DEZ). This separation makes *Arabidopsis* root gravitropism an ideal model to dissect the earlier phases from the later ones.<sup>1</sup>

Gravity sensing occurs primarily in the statocytes, which are the columella cells of the root and lie in the endodermal layer of the shoot. The statocytes are characterized by the presence of large, starch-filled amyloplasts. These plastids are denser than the surrounding cytoplasm, and tend to sediment to the bottom of statocytes. The movement and/or position of the amyloplasts contribute to the formation of a chemical signal, which is then transmitted basipetally to the DEZ, where it elicits a curvature response (Fig. 1A and B).

### Gravity Signal Transmission and Curvature Response

The phytohormone auxin is extremely important for the transmission of a gravitropic signal from the root cap to the DEZ. In a vertically-growing root, auxin is transmitted from its main site of synthesis in the shoot apex, through the vasculature to the root cap, where it is laterally distributed and then basipetally transmitted to the growing cells of the elongation zone.<sup>4</sup> According to the Cholodny-Went theory, a gravistimulated root will establish a lateral auxin gradient across the root cap, with the new bottom flank accumulating greater concentrations of auxin than the new top flank.<sup>5,6</sup> The auxin gradient in the root cap is then transmitted to the distal elongation zone, where it promotes differential cell elongation between the top and bottom sides, responsible for downward curvature (Fig. 1A and B).

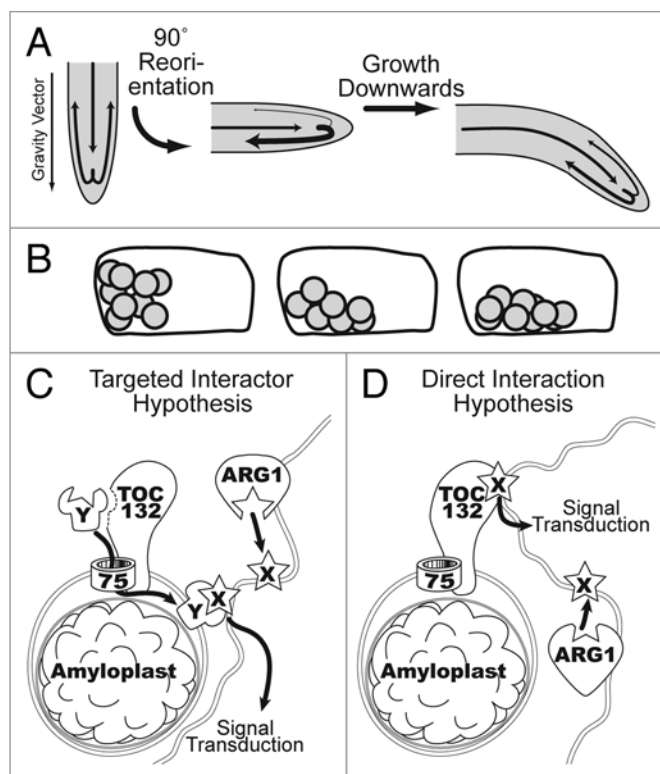
Abundant experimental and genetic evidence supports the Cholodny-Went theory. For instance, mutations in genes that affect auxin transport or response cause gravitropic defects (reviewed in ref. 7). Also, transgenic reporters of auxin levels in plants, such as the *GUS* or *GFP* genes under the control of the auxin-responsive artificial *DR5* promoter, are differentially activated in cells of the peripheral-cap and epidermis on the bottom side of gravistimulated roots.<sup>8-12</sup> Analysis of the auxin efflux facilitator, PIN3, and the corresponding mutant also lends support for the Cholodny-Went theory. *pin3* is an auxin transport mutant

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**Figure 1.** Schematic representation of the various phases of root gravitropism. (A) Fountain model of auxin transport in roots. The direction of auxin transport is represented by arrows whose widths reflect relative flow rates. In vertical roots auxin, which is mainly synthesized in young shoot tissues, is transported through the vasculature into the root tip where it is redistributed symmetrically toward more peripheral tissues of the lateral cap. There, auxin is transported basipetally toward the elongation zone where it inhibits cell elongation. Upon 90° reorientation within the gravity field, the lateral transport of auxin across the cap is re-directed downward, leading to a lateral gradient that, upon transmission to the elongation zone, promotes differential cellular elongation between upper and lower flanks, responsible for tip curvature. (B) The starch-statolith hypothesis proposes that the sedimentation of amyloplasts toward the new bottomside of gravistimulated root-cap columella cells triggers a transduction pathway that leads to cell polarization and lateral auxin transport toward to lower flank of the root (see A). (C and D) Models of TOC action in gravitropism. TOC132 may mediate the insertion of a gravity-signal transducer (Y) into the outer-envelope of amyloplasts. Upon amyloplast sedimentation within the statocytes, Y may interact with a plasma-membrane or ER-associated signal transducer (X), regulating its activity (C). Alternatively, TOC132 may interact directly with X upon gravistimulation thereby triggering signal transduction (D). In either case, ARG1 or ARL2 (not shown here) may modulate the location and/or activity of transducer X within the sensitive membrane. These figure panels were modified from figures published in Stanga et al.<sup>59</sup>

that affects gravitropism.<sup>13</sup> The PIN3 protein is expressed in statocytes where it localizes uniformly to the plasma membrane of vertically growing roots. In the root-cap statocytes, gravistimulation causes PIN3 to relocalize to the bottom side of the plasma membrane within about 2 minutes. This process is believed to promote the establishment of the lateral auxin gradient discussed above. It should however be cautioned that it is also possible that the gravity-induced PIN3 relocalization in the root statocytes results from a differential promotion of auxin efflux activity at

the bottom flank of the responding cells.<sup>14</sup> In fact, while *pin3* mutants are defective in both root and hypocotyl gravitropism, genetic analyses show that PIN3 is regulated by different mechanisms in the different organs.<sup>12</sup>

While auxin plays a crucial role in gravitropism, other phytohormones are also involved. Ethylene, brassinosteroids, abscisic acid, gibberellins, salicylic acid and jasmonic acid are all known to affect gravitropism.<sup>7</sup> However, their contribution is primarily due to their effects on auxin-related processes. Another phytohormone, cytokinin, is capable of inducing curvature when applied exogenously, although comprehensive genetic evidence for the involvement of cytokinin is currently lacking.<sup>15</sup>

### Perceiving Gravity

According to a long-standing idea, the starch-statolith hypothesis, it is the location and/or motion of the amyloplasts that provides the directional cue necessary for gravitropism.<sup>16</sup> Physical and genetic ablation experiments indicate the importance of statocytes to gravitropism.<sup>17,18</sup> While ablation of peripheral root cap cells did not alter root curvature, ablation of the innermost columella cells significantly altered root curvature without affecting growth rates. Of these inner columns, the second story columella cells (S2) contribute most to gravitropism.<sup>19</sup> Importantly, these S2 cells exhibit the largest amyloplast sedimentation velocities.

Artificial displacement of amyloplasts illustrates the importance of their movement to gravitropism. A natural property of the starch molecules that accumulate in these plastids is their diamagnetism, which induces them to move away from high-gradient magnetic fields (HGMF). When a HGMF is applied near the tip of a vertically oriented root, amyloplasts within the statocytes move away from the field. This lateral movement of the amyloplasts, away from the gravity vector, promotes a root-tip curvature in the direction of amyloplast displacement, as predicted by the starch statolith hypothesis.<sup>20</sup> Importantly, *phosphoglucosylmutase 1* mutants (*pgm1*), which contain starchless amyloplasts, did not curve when exposed to a lateral HGMF, suggesting that the curvature response was not due to other magnetic effects on the plant.

Studies on the developmental timing of the inception of gravisensitivity during germination and of starchless and starch-deficient mutants demonstrate the importance of sedimenting amyloplasts to gravitropism. For instance, in flax the onset of gravisensitivity was established to correlate with the appearance of mature, sedimentable amyloplasts during germination, 11 h before root emergence.<sup>21</sup> Similarly, *Arabidopsis* mutants with varying amounts of starch show varying levels of gravisensitivity that correlate with the ability of amyloplasts to sediment. Starch excess mutant (*sex1*) hypocotyls contain increased levels of starch and display enhanced sensitivity to gravistimulation.<sup>22</sup> On the other hand, mutants with intermediate levels of starch display little or moderately decreased sensitivity to gravistimulation. Similarly, *pgm1* mutant plants contain amyloplasts that lack starch and do not appear to settle to the bottom of statocytes upon gravistimulation. They show a severely attenuated gravity response.

The shoot statocytes of wild-type *Arabidopsis* plants contain large vacuoles that seem to impede amyloplast movement. Consequently, amyloplasts seem to sediment through transvacuolar strands in shoot statocytes upon gravistimulation.<sup>2</sup> This is quite distinct from what is seen in the columella cells of the root cap, which lack large vacuoles, thus allowing easy amyloplast sedimentation upon gravistimulation. Interestingly, several *shoot gravity response* (*sgr*) mutants of *Arabidopsis* were shown to be defective in genes that contribute to the biogenesis and/or function of their vacuoles, a phenotype that is accompanied by altered amyloplast sedimentation and gravitropic deficiency.<sup>22-27</sup>

While the starch-statolith hypothesis may be sufficient to explain most gravitropic sensing ability of flowering plant organs, it should be cautioned that several lines of evidence also suggest the existence of alternative mechanisms. One such line of evidence comes from analyses of plant gravitropism that involve a clever apparatus designed to keep a single region of a root at a constant angle from the gravity vector.<sup>28</sup> This apparatus, named the “ROTATO”, comprises a rotating stage controlled by a computer and microscope that monitor a selected region of the root and maintain it at a selected angle from the gravity vector. When the DEZ was maintained at a constant gravistimulating angle from vertical, the root continued to develop curvature long after the root cap had reached a vertical orientation, indicating that a secondary site of gravity sensing, distinct from the root cap, might exist in the DEZ. Because cells in this region of the root contain no sedimentable amyloplasts, it was suggested that gravity sensing in that region might involve a mechanism that detects the total weight of the protoplast on its cell wall. This hydrostatic-pressure model of gravity sensing was previously proposed to function in the large internodal cells of the alga *Chara*,<sup>29</sup> and some evidence also supports its involvement in gravity sensing by rice roots.<sup>30</sup> An alternative mechanism of gravity sensing in the root DEZ may help explain several unexpected observations on root gravitropism: (1) decapped maize roots display an actin turnover-dependent gravitropic response;<sup>31</sup> (2) a bidirectional movement of curvature can be detected by high-resolution time-lapse imaging approaches during the first 2–3 hours of gravistimulation, which includes an unexpected acropetal component;<sup>32</sup> and (3) the starchless *pgm1* mutants of *Arabidopsis* still show some response to gravistimulation despite displaying no evidence of amyloplast sedimentation.<sup>33,34</sup>

### Transducing Mechanical Forces into Chemical Signals

Although there is a bounty of experimental evidence supporting the starch-statolith hypothesis as well as more recent evidence suggesting the possibility of additional mechanisms, the means by which the first signal transduction events are triggered remain elusive. A few models have been proposed, involving mechanosensitive ion channels, receptor-ligand interactions, actin tensegrity and protoplast pressure.

Mechanosensitive ion channels are appealing candidates for gravity signal transduction because experimental evidence indicates the involvement of  $\text{Ca}^{2+}$  flux in gravitropism. Gravitropism

can be inhibited by chemically interfering with calcium channels, calmodulin,  $\text{Ca}^{2+}$  ATPases, or calcium itself.<sup>35-37</sup> Aequorin, a  $\text{Ca}^{2+}$  reporter, shows a biphasic cytosolic  $\text{Ca}^{2+}$  transient following gravistimulation of *Arabidopsis* seedlings.<sup>38</sup> This transient consists of an initial spike followed by a sustained secondary peak. The first peak seems to correlate with rotational stimulation of the seedlings whereas the latter appears associated with the signaling events that accompany gravity perception in shoots.<sup>39,40</sup> Unfortunately, the aequorin signal was so low that the experiment required observing large numbers of gravistimulated seedlings simultaneously, rendering efforts to identify subcellular patterns of  $\text{Ca}^{2+}$  difficult. Subsequent work showed that these signals derive from hypocotyls and petioles, but not roots, although the possibility that there are  $\text{Ca}^{2+}$  fluxes in roots, below the threshold of detection in this experimental system, could not be eliminated.<sup>39</sup>

Despite the evidence tying  $\text{Ca}^{2+}$  to gravitropism, it has not yet been shown that mechanosensitive ion channels are responsible for  $\text{Ca}^{2+}$  fluxes. Bioinformatic approaches have failed to identify orthologs to known eukaryotic mechanosensitive channels. However, ten *Arabidopsis* genes encoding orthologs to bacterial mechanosensitive channels (*MSL* genes) have been identified recently, and some members of this family may be able to contribute to gravitropism.<sup>41,42</sup> In particular, *MSL9* and *MSL10* are found in the plasma membrane of root cells. Future experiments may address a possible role for these channels in gravitropism or other forms of mechanotransduction. Additionally, an *Arabidopsis*  $\text{Ca}^{2+}$  channel recently implicated in mechanotransduction has been shown to rescue a stretch-activated  $\text{Ca}^{2+}$  channel in yeast.<sup>43</sup> This protein defines an additional class of potential mechanosensitive channels that warrant further study.

Further support for the role of  $\text{Ca}^{2+}$  in gravity signal transduction comes from experiments investigating the contribution of inositol-1,4,5-triphosphate (IP3) to that pathway. IP3 is involved in releasing  $\text{Ca}^{2+}$  from intracellular stores in both animals and plants.<sup>44</sup> Gravistimulation of oat and maize pulvini (graviresponsive organs at the base of monocot leaves) showed a biphasic IP3 response. Within the first few minutes after gravistimulation, IP3 levels fluctuated between upper and lower flanks of the pulvini. This response was followed by sustained increases of IP3 along the lower flank of the stimulated organ.<sup>45-47</sup> A similar response was observed in *Arabidopsis* inflorescence stems. Furthermore, hydrolysis of *Arabidopsis* IP3 by transgenic expression of human inositol polyphosphate 5-phosphatase coincided with a perturbation of inflorescence stem gravitropism.<sup>48</sup>

Within 1–2 minutes following gravistimulation, a pH spike can be observed in the cytoplasm of *Arabidopsis* columella cells, concomitant with an apoplastic acidification.<sup>49,50</sup> This is a transient flux that returns to the unstimulated state within 8–12 minutes. When caged protons are released in columella cells, the gravitropic response is delayed.<sup>49</sup> Chemicals that alter the pH balance of the apoplast also alter gravitropism: apoplastic alkalization delays gravitropism, apoplastic acidification enhances gravitropism.<sup>50</sup> The importance of starch to this process is emphasized by the observation that *pgm1* mutant roots show little to no pH spike following gravistimulation.<sup>49</sup>

The involvement of mechanosensitive ion channels fits well with the starch-statolith hypothesis whereby sedimenting amyloplasts would act as activators of the channels. The ability of the amyloplasts to sediment, which is correlated with starch content, would likewise be correlated with the ability to activate these mechanosensitive ion channels.<sup>51</sup> A similar, alternative mechanism could account for gravity perception. In this model, the weight of the protoplast produces tension differences between the top and bottom sides of a cell, which may differentially activate mechanosensitive ion channels.<sup>52</sup> As discussed above, this hydrostatic-pressure model derives from experimentation with the large Chara internodal cells, and as of yet has not been demonstrated to be functioning in Arabidopsis.

It has also been proposed that sedimenting amyloplasts might interact with the dynamic actin cytoskeleton network that is attached to the plasma membrane of the statocytes, thereby influencing membrane tension with potential impact on the gating of mechanosensitive ion channels embedded in those membranes. Interestingly, repeated gravistimulation of shorter duration than the time required for sedimenting amyloplasts to reach the bottom of the statocyte, but sufficient to alter interactions between amyloplasts and the dynamic cytoskeleton, is effective at inducing a gravitropic response. This observation supports a role for the actin cytoskeleton in transducing the mechanical information of amyloplasts movement to the peripheral membranes.<sup>53</sup> On the other hand, sedimenting plastids have also been proposed to locally disrupt the actin cytoskeleton in the statocytes, thereby altering tensions between the cytoskeleton and connected membranes with potential impact on membrane-associated mechanosensitive ion channels.<sup>54</sup> Unfortunately, these models of actin-mediated gravity signal transduction seem contradicted by the results of pharmacological experiments showing that disruption of the actin cytoskeleton increases, rather than inhibits, root<sup>55</sup> and shoot gravitropic curvature.<sup>56</sup> Actin is involved in many subcellular processes likely to function in gravitropism.<sup>57</sup> For instance, the actin cytoskeleton network has been implicated in amyloplast saltation, a process that constantly repositions amyloplasts within the statocytes, thereby potentially resetting the gravity sensing machinery.<sup>55</sup> It has also been proposed to mediate the subcellular localization of the PIN3 auxin efflux facilitator in the statocytes.<sup>13</sup> Therefore, precisely how it is involved in gravity signaling remains elusive.

There are also models that do not involve mechanosensitive ion channels. Again, studies of Chara outline a mechanism that could possibly have an Arabidopsis analog. It should be cautioned that while it may be possible to draw parallels between the gravitropic mechanisms of Chara and Arabidopsis, these organisms share a relatively distant common ancestor approximately 420–480 million years ago, plenty of time for many important features to diverge.<sup>58</sup> One such important difference is that statoliths in Chara are BaSO<sub>4</sub>-filled vacuoles, not amyloplasts. Despite these cytological differences, Chara is a useful system to distinguish between the possibilities of the protoplast-pressure model of gravity perception and a ligand-receptor model that postulates that ligands on the surface of sedimenting statoliths have to interact with receptors embedded in sensitive membranes

on the side of the cells in order to trigger gravity signal transduction. In a microgravity environment provided by parabolic flights, the weight of the protoplast would not be sufficient to cause the opening of mechanosensitive ion channels, as suggested by the protoplast-pressure model. Yet, Chara did exhibit gravitropic curvature under these conditions as long as the statoliths contacted a specialized surface of the plasma membrane.<sup>59</sup> These experiments suggest the possibility of a receptor-ligand interaction between two proteins, one displayed on the surface of the statolith, and the other on the sensitive region of the plasma membrane. We recently described two new mutants, *mar1* and *mar2* (discussed later), that suggest the exciting possibility that a similar mechanism might also exist in Arabidopsis.<sup>60</sup>

### ARG1: An Important Player in Gravity Signal Transduction

An important participant of early gravity signal transduction was identified in a screen designed to isolate Arabidopsis mutants with root gravitropic aberrations. *altered response to gravity1* (*arg1*) roots, when reoriented 90°, are slow to resume downward growth, though they retain the ability to respond to gravistimulation.<sup>61</sup> A similar phenotype is observed in *arg1* hypocotyls. This phenotype is not accompanied by any alterations in sensitivity to phytohormones or polar auxin transport inhibitors, growth rate or starch accumulation. Furthermore, phototropism is normal in *arg1*, demonstrating that the curvature defect is specific to gravitropism.

Transgenic rescue of the gravitropic defect with *ARG1* fused to promoters that drive expression in the statocytes suggest that ARG1 participates in early gravity signal transduction.<sup>9</sup> Because only the earliest phases occur within the root cap, rescuing the *arg1* defect by expressing *ARG1* with the *RCP1* promoter showed that ARG1 is indeed involved in the early stages of gravitropism. Likewise, expressing *ARG1* with the *SCR1* promoter, which drives expression within the endodermis, rescued the hypocotyl gravitropism defect of *arg1*.

Two paralogs of *ARG1* exist in Arabidopsis, named *ARG1-Like 1* and *ARG1-Like 2* (*ARL1* and *ARL2*). *arl2* mutants display similar phenotypes to *arg1*, while *arl1* does not show any defects. The *arl2 arg1* double mutant, the *arl1 arl2 arg1* triple mutant, and the *arg1* and *arl2* single mutants all display similar reorientation defects, indicating that *arl2* and *arg1* operate in the same genetic pathway.<sup>62</sup> However, *arg1* and *arl2* operate in a different genetic pathway than *pgm1*, because the *pgm1 arg1* and *pgm1 arl2* double mutants are more severely deficient in their gravitropic response than either single mutant. This distinction led to the identification of the *mar* mutations, which also enhance the gravitropic defect of *arg1*, and are discussed in the next section.

The ARG1 and ARL2 proteins are characterized primarily by two domains: a J-domain and a predicted coiled-coil region.<sup>61</sup> J-domains are widely conserved and constitute a broad family in Arabidopsis. Via interactions with heat shock cognate 70 (*hsc70*) chaperones, J-domain proteins modulate the folding, activity, targeting and abundance of different substrates.<sup>63</sup> Coiled-coil domains are involved in protein-protein interactions, and may

help to determine the protein substrates of ARG1 and ARL2.<sup>64</sup>

ARG1 is expressed ubiquitously in plants whereas ARL2 is expressed specifically in the statocytes.<sup>12,61</sup> Biochemical fractionation experiments showed association of ARG1 with a variety of different cellular membranes of the endomembrane system, along with the plasma membrane.<sup>9</sup> Subcellular localization using GFP- and myc-tagged ARG1 transgenes showed signals in discrete puncta throughout the cell, particularly in areas of high vesicle trafficking activity, such as the nascent phragmoplast. Localization to plastids was conspicuously absent. Similar studies demonstrated ARL2 association with the plasma membrane and some endomembranes of the root statocytes. Furthermore, when ectopically-expressed in other regions of the plant, ARL2-GFP was also found to associate with cellular regions undergoing intense vesicular trafficking activity, such as the phragmoplast.<sup>12</sup>

In addition to the evidence discussed already, two important observations support the conclusion that ARG1 and ARL2 participate in gravity signal transduction events. First, the cytoplasmic alkalization required for normal gravitropism is absent in *arg1-2* mutants.<sup>9</sup> This observation, coupled with the localization data discussed above, suggest that ARG1 may be modulating the localization and/or function of a proton pump. Secondly, the *arg1* and *arl2* mutants eliminate the contribution of the auxin efflux facilitator PIN3 to the gravitropic response by altering its accumulation at the bottom side of gravistimulated root statocytes.<sup>12</sup> As a consequence of this alteration, gravistimulated *arg1* and *arl2* roots are defective in their ability to establish a lateral auxin gradient, resulting in delayed gravitropic curvature. These data may reflect the role in the statocytes of cytoplasmic H<sup>+</sup> as a modulator of PIN3 localization and/or activity. Indeed, a similar relationship was established between a proton pyrophosphatase (AVP1) and a different auxin efflux facilitator, PIN1.<sup>65</sup> Interestingly, gravistimulated *arg1 pin3* and *arl2 pin3* hypocotyls have slower gravitropic bending than any single mutant.<sup>128</sup> However, during the first 6 hours after gravistimulation, the double mutants resemble the single mutants. Therefore, while ARG1 and ARL2 act in the same genetic pathway as PIN3 in the roots, they seem to act independently of PIN3 in the hypocotyls.

### Genetic Modifiers of *arg1*

We recently reported the identification of two new mutants, *modifiers of arg1 1* and *2* (*mar1*; *mar2*).<sup>60</sup> These genetic enhancers show little or no gravitropic defects on their own, yet roots and hypocotyls grow in random directions when in an *arg1* background. While the double mutants are agravitropic, their defect appears to affect the early stages of gravitropism only, as they respond normally to lateral light stimulation and exogenous application of phytohormones and auxin transport inhibitors. Importantly, these agravitropic double mutants have amyloplasts

**Table 1.** Characteristics of the main components of the TOC complex in Arabidopsis

Protein Family	Family Members	Locus	Mutants of Note	Comments	References
TOC75 Pores	TOC75-III	At3g46740	<i>toc75-III</i> ; <i>mar1</i>	null lethal; genetic interaction with <i>arg1</i>	Stanga et al 2009; Baldwin et al 2005
	TOC75-IV	At4g09080	<i>toc75-IV</i>	abnormal etioplasts	Baldwin et al. 2005
TOC159 Receptors	TOC159	At4g02510	<i>ppi2</i>	Pale leaves; abnormal chloroplast development	Bauer et al. 2000; Kubis et al. 2004; Ivanova et al. 2004
	TOC132	At2g16640	<i>mar2</i> ; <i>attoc132-1</i> ; <i>toc132-2</i>	Genetic interaction with <i>arg1</i> ; some functional redundancy with TOC120	Stanga et al 2009; Kubis et al. 2004; Ivanova et al. 2004
	TOC120	At3g16620	<i>attoc120-1</i> ; <i>toc120-2</i>	Defective root plastids in <i>toc120 toc132</i> mutant	Kubis et al. 2004; Ivanova et al. 2004
	TOC90	At5g20300	<i>toc90-1</i>	Role unknown	Kubis et al. 2004
TOC34 Receptors	TOC33	At1g02280	<i>ppi1</i>	Photosynthesis-related protein import	Jarvis et al. 1998; Kubis et al. 2003
	TOC34	At5g05000	<i>ppi3</i>	Abnormal root growth	Constan et al 2004

For each component, the names of the family members are provided, along with the loci encoding them, the mutations cited in this review and their phenotypes.

that appear normal in their overall morphology, starch content and sedimentation in response to gravity stimulation. Amazingly, though the *mar* mutants do not appear to have altered amyloplasts, the genes encode plastid-localized proteins, previously characterized as components of the Translocon of the Outer envelope of Chloroplasts (TOC), TOC75 and TOC132. Compared with other plastid types, chloroplasts are abundant and relatively simple to isolate. Therefore, much of the initial characterization of the TOC complex was done with isolated pea chloroplasts, and the complex earned its chloroplastic namesake.<sup>66-68</sup> It is important to note that additional plastid types require these translocons, and TOC-complex subunits are expressed in tissues devoid of chloroplasts.<sup>69</sup> To hypothesize how the TOC complex contributes to gravitropic signaling, we must first review some key observations in the broadly expanding field of plastidic protein import. Table 1 will help the reader through this section of our review. It summarizes the characteristics of the three main components of the TOC complex in Arabidopsis (TOC159, TOC75 and TOC34), the genes encoding them, the corresponding mutants and their phenotypes.

### The TOC Complex

Although plastids possess their own genetic machinery, most plastid proteins are encoded in the nuclear genome and translated in the cytosol.<sup>70</sup> In order for these plastidic proteins to function where they are needed, they must first translocate across two membranes, an outer envelope membrane and an inner envelope membrane. Chaperones maintain the proteins that are destined to be translocated, or preproteins, in an unfolded state and target them to the TOC complex.<sup>71</sup> The TOC complex is situated on the outer envelope of plastids. There, it interacts with the preproteins and translocates them across the outer envelope in a GTP-dependent process.<sup>72</sup> Once this step has happened, preproteins

are fed into an analogous protein complex of the inner envelope, the TIC complex, possibly by interacting with proteins in the inner membrane space.<sup>73,74</sup> The TIC complex completes the translocation, and preproteins adopt their final conformation or continue on to subplastidic destinations. As an important alternative to translocation, plastid-localized proteins may be targeted to the outer envelope via interactions with the TOC complex,<sup>75</sup> which could possibly reflect the mechanism of the TOC complex's involvement in gravitropism.

### TOC75: The Central Pore

The TOC complex comprises three primary subunits, named for their size (kDa) in pea: TOC159, TOC34 and TOC75. These are found with a stoichiometry of 1:4-5:4.<sup>76,77</sup> Each of the genes encoding these proteins is a member of a small gene family. TOC75 is the central pore through which all translocated preproteins pass. The central pore of TOC75 bears significant structural similarity to bacterial porin proteins. The key topological feature of these proteins is an array of short  $\beta$ -strands that form a  $\beta$ -barrel.<sup>78,79</sup> The Arabidopsis genome has two *TOC75*-like genes (in addition to a third pseudogene): *TOC75-III* and *TOC75-IV*.<sup>80</sup> A reverse genetic approach identified only subtle phenotypes associated with mutant forms of *TOC75-IV*. Structural abnormalities in etioplasts coupled with the *TOC75-IV* expression pattern suggest a role in dark-grown seedlings. In stark contrast to this subtle phenotype, an insertional allele of *TOC75-III* is lethal: embryos arrest at the 2-cell stage.<sup>80</sup> *mar1-1* is a hypomorphic allele of *TOC75-III*: while there is a mutation in a  $\beta$ -strand, *mar1-1* plants survive, though they are pale and stressed.<sup>60</sup> This partly-functional mutant may prove to be a valuable tool for dissecting the features of the TOC75 pores. Although the *mar1-1* mutants enhance the gravitropic phenotype of *arg1-2*, they also have pleiotropic defects even as single mutants, likely due to alterations in import of many critical proteins. In contrast, the *mar2-1* mutants appear very similar to wild type as single mutants, while producing the same agravitropic phenotype in the *arg1-2* background. *mar2-1* has a mutation in *TOC132*, one of the members of the *TOC159* gene family.

### TOC159: A 4-Member Family of GTPase Receptors

TOC159 interacts with preproteins during early stages of protein import, and is proposed to mediate preprotein recognition.<sup>67</sup> *TOC159* has three paralogs: *TOC132*, *TOC120* and *TOC90*.<sup>67,81,82</sup> These four proteins have highly conserved C-terminal membrane anchor domains (M-domains) and central GTP-binding domains (G-domain). Their N-terminal acidic domains (A-domains) vary considerably in length and are the major source of diversity amongst the four. Removal of the cytosolic domains, the A- and G-domains, from TOC159 nearly eliminates detectable binding of preproteins at the chloroplast surface.<sup>83</sup> However, preprotein translocation can still occur. A forward genetic screen for mutants defective in plastid protein import identified a mutant version of *TOC159* (*ppi2*;<sup>84</sup>). *ppi2* plants have an albino phenotype due to the arrested development of chloroplasts. Non-

photosynthetic plastids of the root, however, appear to develop normally. Furthermore, transcription of photosynthetic genes was repressed, and photosynthetic proteins were found not to accumulate. Consistent with these findings, expression analysis of the *TOC159* family members identified higher relative expression of *TOC159* in leaves compared to roots, while *TOC132* and *TOC120* are more abundant in roots than above-ground tissues.<sup>69,70,81</sup>

Analysis of RNA-null insertional alleles of these other paralogs supports a role for them in translocation of non-photosynthetic proteins.<sup>81</sup> No visible phenotypes were seen in *toc120* and *toc90* mutants. When two separate groups analyzed *toc132* mutants, one group detected no phenotype, while the other saw a very slight paleness in young plants, which developed into a reticulate pattern in older leaves.<sup>81,82</sup> Quantification of chlorophyll content detected a slight decrease. Ecotype differences may account for these subtle differences. Genetic analysis of these mutants revealed phenotypes not found in any single mutant.<sup>81</sup> *toc132 toc159* double mutants are lethal at an early stage of development, due to either a reduction in import of a broad range of proteins, or complete abrogation of a single import pathway that is only disrupted in either single mutant. Double mutant analysis also shows redundancy between *toc132* and *toc120*. The double mutants resemble the *ppi2* mutant, in that they are small and very pale. Unlike the *ppi2* mutant, *toc132 toc120* root plastids have structural abnormalities, notably a high proportion of large inclusion bodies. These experiments support the model that the TOC receptors derive functional specialization by interacting with different classes of preproteins. Further support comes from our interesting observation that while *TOC132* and *TOC120* share some functional redundancy, they retain functional specialization, as *arg1 toc120* mutants do not display the same random-growth phenotype we observe in *arg1 toc132* mutants.<sup>60</sup> It should however be cautioned here that the lower level of TOC120 expression relative to TOC132 in roots may be partially responsible for the latter result.

### TOC34: A 2-Member Family of GTPase Receptors

Like members of the TOC159 family, the third component of the TOC complex, TOC34, is also a membrane-anchored protein with a cytosolic GTPase-domain.<sup>85</sup> Unlike TOC159, the GTPase domain composes nearly the entirety of the cytosolic domain. TOC34 is also thought to have activity as a receptor, as it has been shown to interact directly with preproteins prior to translocation. The Arabidopsis *TOC34* gene family has two members: *TOC33* and *TOC34* (note that Arabidopsis *TOC33* is orthologous to pea *TOC34*;<sup>86</sup>). Like the TOC159 receptors, TOC33 and TOC34 have different affinities for different preproteins.<sup>87,88</sup> Additional *ppi* mutants may help to identify the different classes of preproteins transduced through TOC33 and TOC34. *ppi1* is a mutant form of the Arabidopsis *TOC33* gene, *ppi3* is a mutant form of the *TOC34* gene. Like *ppi2*, *ppi1* is specifically defective in expression, chloroplast import and accumulation of photosynthetic proteins.<sup>89</sup> On the other hand, *ppi3* mutants have shorter roots and aberrant root plastids, while their aerial tissues appear normal.<sup>90</sup> These phenotypes are consistent with the relative abundance of *TOC34* in the root and *TOC33* in leaves.<sup>69</sup> As such, *ppi3* is an interesting

candidate for possible involvement in root gravitropism; its contributions are being investigated currently.

### Targeting to the TOC Complex

The TOC complex is necessary for classical signal sequence-based targeting to the plastid.<sup>91</sup> Most nuclear-encoded plastid proteins possess a cleavable N-terminal transit peptide that is both necessary and sufficient for plastidic targeting.<sup>92</sup> TOC complex targeting sequences are diverse in both size and composition, which has so far precluded a thorough understanding of structural elements that determine targeting specificity. The diversity of transit peptide sequences may provide a means for the diverse types of TOC complexes to recognize subsets of preproteins.

Ample evidence supports the classical cleavable signal-sequence mechanism of plastidic targeting. However, recent evidence suggests that this is not the only process that can target proteins to the plastids. First, proteomic studies of isolated plastids have identified proteins lacking a cleavable signal sequence, suggesting an alternate mechanism of recognition or possibly even an alternative mechanism of translocation for some proteins.<sup>93</sup> Second, multiple targeting pathways for both intermembrane-space-localized and outer envelope proteins have been proposed that would not require a cleavable signal.<sup>94-96</sup> This raises an interesting possibility for the role of the TOC complex in gravitropic signaling: that the TOC complex mediates the insertion into the outer amyloplast envelope of a particular protein(s) that acts as a ligand (or receptor) that modulates gravity signal transduction.

### Model and Concluding Remarks

Other observations support the possibility of a ligand-receptor model of gravitropism: the wild-type-like sedimentation and saltation behaviors of the *mar2* mutant amyloplasts suggest not only that actin-related gravity signal transduction processes are unlikely to be affected in the *mar* mutants, but also that models requiring the mass of the plastids to trigger mechanosensitive ion channels are insufficient to explain the gravitropic defect of *mar2 arg1* mutants.<sup>60</sup>

As discussed above, the ARG1 and ARL2 proteins associate with both the plasma membrane and components of vesicle trafficking pathway, suggesting a role in mediating the targeting or activity of gravity signal transducers at the plasma membrane or at organelles of the secretory pathway.<sup>9</sup> Importantly, these J-domain proteins are conspicuously absent from plastids, suggesting that the genetic interactions between *arg1/arl2* and *toc132* do not represent a continuous physical interaction between the corresponding proteins. It is however possible that both ARG1/ARL2 and the TOC complex mediate the proper localization and/or activity of gravity signal transducers at distinct compartments within the cell that come together upon gravistimulation (plasma membrane or endoplasmic reticulum for ARG1, and plastid envelope for the TOC complex). In this context, the genetic interactions we observe between *arg1-2/arl2-3* and the *mar* mutations would reflect a decreased ability for these transducers to interact and promote gravity signal transduction when sedimenting plastids hit the peripheral ER or

plasma membrane of the mutant statocytes. Accordingly, removal of TOC132 would reduce the amount of plastid-associated transducer to a level that remains sufficient to trigger a normal gravitropic response in *ARG1 ARL2* plants, but is insufficient in the context of the attenuated pool of functional interacting ligand in *arg1-2* or *arl2-3* membranes. Similarly, removal of ARG1 or ARL2 may reduce the amount of peripheral membrane-associated signal transducer to a level that still permits a significant, though partially altered, gravitropic response in a wild-type TOC132 background, but becomes insufficient in a TOC132-deficient background.

In the context of the ligand-interaction model of gravity sensing, it is an important reminder that TOC75 also contributes to the proper targeting of plastid outer-envelope proteins, including members of the TOC159 family.<sup>75</sup> Therefore, the missense mutation in TOC75 associated with *mar1-1* may affect the proper targeting of the proposed plastid-associated transducer, which could potentially be TOC132 itself or another outer-envelope-associated protein.

While the ligand-receptor model of gravity sensing is compelling, it is also possible that the TOC complex affects gravitropism through an unidentified regulatory molecule. Indeed, plastids contribute to the manufacture and/or storage of important biological products.<sup>97</sup> However, the strong genetic interaction observed between *toc132* and *arg1-2* or *arl2-3* would require that the corresponding regulatory molecule be needed only in the absence of ARG1 or ARL2, a less likely scenario. Alternatively, internal plastid proteins may act in a signal transduction pathway without altering amyloplast structure or behavior. For example, a thylakoid-localized calcium-binding protein has recently been implicated in the transduction of stomatal-closing signals.<sup>98</sup> However, here again, such internal plastid protein would have to be needed for gravity signal transduction only in the absence of ARG1 or ARL2. Metabolic and/or proteomic profiling of *mar2-1 arg1-2* or *mar2-1 arl2-3* amyloplasts, along with the genetic identification of new participants of this genetic pathway, will be needed to further elucidate the molecular mechanisms that allow components of the TOC complex to contribute to gravity signal transduction.

To the best of our knowledge, the results discussed above constitute so far the strongest evidence consistent with a ligand-receptor model of gravity signal transduction in flowering plants. This model could be similar to the ligand-receptor model of gravity sensing that has been proposed to function in *Chara* rhizoids, based on careful evaluations of gravity sensing in the context of parabolic-flight microgravity environments.<sup>59</sup> Research is under way to test this exciting alternative scenario of gravity sensing and signal transduction.

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