A hormonal regulatory module that provides flexibility to tropic responses

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Abstract

Plants orient their growth depending on directional stimuli such as light and gravity, in a process known as tropic response. Tropisms result from asymmetrical accumulation of auxin across the responding organ relative to the direction of the stimulus, which causes differential growth rates on both sides of the organ. Here we show that gibberellins (GAs) attenuate the gravitropic reorientation of stimulated Arabidopsis hypocotyls. We show that the modulation occurs through induction of the expression of the negative regulator of auxin signaling *IAA19/MSG2*. The biological significance of this regulatory mechanism involving GAs and auxin seems to be the maintenance of a high degree of flexibility in tropic responses. This notion is further supported by observations that GA-deficient seedlings showed a much lower variance in the response to gravity compared to wild-type seedlings and that the attenuation of gravitropism by GAs resulted in an increased phototropic response. This suggests that the interplay between auxin and GAs may be particularly important for plant orientation under competing tropic stimuli.

Introduction

A hundred and thirty years ago, Darwin described how plants can sense their environment and orient themselves for optimal growth and development (Darwin, 1880). Among the signals that promote a tropic response in plants, gravity is unique in that it is constant and unidirectional. Besides, it generally induces the underground tissues to bend towards the signal, and the aerial parts against the stimulating vector. Like in other tropisms, when plants perceive a change in their position relative to the gravity vector, they respond by differential growth on either side of the affected organ (Esmon et al., 2005), and several hormones have been involved in the control of these responses. Among them, auxin is instrumental because it forms a lateral gradient in response to the stimulus and thus establishes the framework for differential growth (Rashotte et al., 2000; Esmon et al., 2006). The differential response to auxin on either side of an organ has been shown to depend on the correct functioning of polar auxin transport and activity of auxin efflux carriers (Friml et al., 2002), and also on the activity of specific Aux/IAA and AUXIN RESPONSE FACTOR (ARF) transcriptional regulators (Harper et al., 2000; Tatematsu et al., 2004). Moreover, brassinosteroids have been proposed to enhance tropic reorientation by facilitating polar auxin transport (Meudt, 1987; Li et al., 2005; Kim et al., 2007).

Gibberellins (GAs) are also known to promote cell expansion (Cowling and Harberd, 1999). The molecular mechanism of GA signaling proceeds through GA-induced degradation of repressor proteins of the DELLA family by the proteasome, thereby activating transcription of growth-promoting genes (Schwechheimer, 2008). Given that GAs regulate growth, sometimes as a subsidiary signal of auxin action (Frigerio et al., 2006), the obvious hypothesis is that GAs would mediate the promotion of differential growth during gravitropic reorientation. However, here we show molecular evidence for a different role of GAs on gravitropism through the attenuation of auxin responsiveness, that results in an increased ability to modulate growth under competing tropic signals.

RESULTS

Gibberellin deficiency enhances gravitropic reorientation

To test if GAs are necessary for the promotion of the differential cell expansion that underlies a tropic response, we examined the response of Arabidopsis etiolated hypocotyls to a gravitropic stimulus, under GA-limiting conditions. Surprisingly, paclobutrazol (PAC)-induced deficiency in GA biosynthesis not only did not impair gravitropic reorientation but, on the contrary, the hypocotyls of GA-deficient seedlings displayed an enhanced response to the gravitropic stimulus and a faster reorientation (Fig. 1A). This effect was observed at a low PAC concentration that did not inhibit seed germination, and was fully reverted by GA_3 application, demonstrating the specificity of the inhibitor. Moreover, the enhanced gravitropism was also evident in the *gai-1D* and *rga-∆17* mutants, which express dominant versions of the DELLA proteins GIBBERELLIN INSENSITIVE (GAI) and REPRESSOR OF GA1 (RGA) respectively, that constitutively block GA-induced growth (Peng et al., 1997; Dill et al., 2001), (Fig. 1B, Fig. S1). Since GA-deficiency also causes dwarfism, it is possible that the enhanced gravitropic response were due to an intrinsic capacity of smaller seedlings to display differential growth and bending. However, this is not the case, because transient induction of the dominant allele *gai-1D* increased the response to a gravitropic stimulus without affecting the size of the seedlings (Alabadí et al., 2008) (Fig. 2). Therefore, we conclude that GAs attenuate the gravitropic response in aerial tissues, and that this regulation is likely a direct consequence of DELLA activity.

Expression of *IAA19/MSG2* is repressed by DELLA proteins

DELLA proteins regulate gene expression in response to GAs (Zentella et al., 2007; de Lucas et al., 2008; Feng et al., 2008). Thus, to elucidate the molecular mechanism that underlies the regulation of gravitropism by GAs, we investigated by microarray analysis the transcriptional changes associated with transient expression of *gai-1D* in two-day-old dark-grown seedlings, i.e. under conditions

where it promotes gravitropism (Fig. 2). Transient expression of *gai-1D* led to altered expression of around 150 genes 4 hours after the inductive stimulus (Gallego-Bartolomé, Alabadí and Blázquez, manuscript in preparation). Remarkably, expression of *IAA19/MASSUGU2* (*IAA19/MSG2*), which encodes a member of the Aux/IAA family of proteins that negatively regulate auxin signaling (Tatematsu et al., 2004; Overvoorde et al., 2005), was steadily downregulated (Fig. 3A). The two closest paralogs of *IAA19/MSG2*, *IAA5* and *IAA6/SHORT HYPOCOTYL1* (*IAA6/SHY1*), were also repressed following *gai-1D* induction (Fig. 3A), and the expression of all these genes was consistently lower in dark-grown seedlings of the *gai-1D* and *rga-∆17* mutants, or in the presence of 1 µM PAC (Fig. S2).

To find out if the regulation of these genes by GAI was direct, we constructed a glucocorticoid-inducible version of *gai-1D* by fusing it to the rat glucocorticoid receptor domain (*GR*) (Lloyd et al., 1994; Aoyama and Chua, 1997), under the control of the *GAI* promoter. As expected, induction of gai-1D translocation into the nucleus by dexamethasone application caused repression of *IAA19/MSG2*, *IAA5* and *IAA6/SHY1*, and this repression was not blocked by cycloheximide (Fig. 3B).

The repression of *IAA19/MSG2* expression upon GA deficiency was also evident in etiolated seedlings harbouring a transcriptional fusion between the *IAA19/MSG2* promoter and the *GUS* reporter gene (Fig. 4). Higher expression levels were detected, as previously reported (Tatematsu et al., 2004), in the apical part of dark-grown seedlings, and the level of expression decreased upon PAC application in a dose-dependent manner.

IAA19/MSG2 mediates the regulation of gravitropism by gibberellins

The observation that *IAA19/MSG2* is a direct target for GAI transcriptional regulation provides a likely mechanism for the attenuation of gravitropism by GAs. The dominant *msg2-1* mutation that prevents IAA19/MSG2 destabilization by auxin has been shown to impair gravitropic responses (Tatematsu et al., 2004). Thus, to investigate the degree of involvement of IAA19/MSG2 in the repression of the gravitropic response by GAs, we asked whether DELLA accumulation would

alleviate the agravitropic phenotype of the seedlings that carry the hyperstable allele *msg2-1*. In agreement with our observation that PAC decreases the activity of the IAA19/MSG2 promoter (Fig. 4), growth of etiolated seedlings in the presence of 0.4 µM PAC prevented the accumulation in the nuclei of msg2-1:GFP protein expressed from the *IAA19/MSG2* promoter (Muto et al., 2007) (Fig. 5A). And, concurrently, this treatment restored an almost normal reorientation capacity to *msg2-1* mutant seedlings (Fig. 5B). We thus conclude that GAs modulate gravitropism at least partly through the transcriptional regulation of *IAA19/MSG2* expression.

Physiological relevance of the regulation of gravitropism by gibberellins

The results shown here indicate that the concentration of GAs –and hence the level of DELLA proteins– influences the gravitropic response of aerial tissues, but what is the physiological relevance of this regulation? Can changes in DELLA abundance within the physiological range still affect gravitropism? Indeed, seedlings of the quadruple DELLA KO mutant did suffer a delay in the gravitropic response when grown in the light (Fig. 6A), a situation that causes DELLA accumulation in the wild type (Achard et al., 2007), confirming that the ability to respond to gravistimulation directly depends on the relative concentration of DELLA proteins. In agreement with this, a quadruple DELLA KO mutant did not show any difference, compared to the wild type, in the speed or extent of reorientation after gravistimulation in darkness, when GAs are not in limiting concentrations (Fig. 6B).

However, a more critical consequence of the attenuation of auxin responsiveness by GAs is the increase in variance of the gravitropic response when large populations of Arabidopsis seedlings were examined right after germination. As shown in Fig. 7A, individual seedlings grown for 3 days in darkness displayed certain degree of inclination (between 5 and 10º) with respect to the gravity vector under normal GA concentrations, but this variation practically disappeared when GA biosynthesis or GA signaling were compromised (with PAC, or in the *gai-1D* mutant). The mechanism by which GAs cause this variance is very

likely equivalent to the one through which GAs control gravitropic reorientation (i.e., through IAA19/MSG2), given that *msg2-1* mutants showed a much larger variance compared to the wild type, which was consequently reduced by PAC (Fig. 7A).

To study whether the increase in variance of the gravitropic response caused by GAs could confer any adaptive advantage, we examined the behavior of seedling populations grown under two competing signals: a light source perpendicular to the gravity vector (i.e., photo- versus gravitropism). As shown in Fig. 7B, the increased gravitropic response caused by the presence of PAC, led to a reduction in phototropic orientation in wild-type seedlings. On the contrary, the aphototropic *phototropin1* (*phot1*) mutant, impaired in the main light receptor that regulates phototropism (Esmon et al., 2005), still responded to PAC with a severe increase in gravitropic response arguing against the direct regulation of phototropism by GAs (Tsuchida-Mayama et al., 2010). Competition between gravi- and phototropism has been proposed to be mediated by the phyA photoreceptor (Lariguet and Fankhauser, 2004; Whippo and Hangarter, 2004; Iino, 2006). Interestingly, the *phytochrome A* (*phyA*) mutant shows reduced response to PAC (Fig. 7B) suggesting a connection between GA action and the regulation of gravitropism by phyA. These observations highlight a specific role of GA-induced regulation of gravitropic response in a situation of competing environmental signals.

DISCUSSION

The work presented here reveals an unexpected role for GAs in the control of the response of plants to gravity, and highlights the physiological relevance of a novel interaction between DELLA proteins and the expression of *Aux/IAA* genes.

The main line of evidence that supports the relevance of IAA19/MSG2 in the control of gravitropism by GAs is the observation that the agravitropic phenotype caused by the dominant *msg2-1* allele was alleviated by inhibiting GA biosynthesis (Fig. 5B). This result at least indicates that transcriptional regulation of *IAA19/MSG2* by GA levels has a significant impact in the gravitropic response of etiolated seedlings. Our work also shows that the regulation of *IAA19/MSG2*

expression by DELLA proteins does not require protein synthesis (Fig. 3B). Since DELLA proteins do not bind DNA directly, it is highly likely that they regulate transcription of IAA9/MSG2 through the interaction with other transcription factors. Two such mechanisms have been proposed so far: the inhibition of DNA binding of members of the PHYTOCHROME-INTERACTING FACTOR (PIF) family of bHLH transcription factors (de Lucas et al., 2008; Feng et al., 2008), and the inhibitory interaction with JASMONATE-ZIM-DOMAIN (JAZ) proteins (Hou et al., 2010), which in turn regulate MYC2 activity in jasmonic acid signaling. There are no indications that JA signaling is involved in gravitropic responses, but it is reasonable to think that DELLA proteins interact with PIFs already present at high levels in dark-grown seedlings to directly regulate the expression of the target *Aux/IAA* genes. In agreement with this model, the expression of *IAA19* is strongly reduced in etiolated *pifq* mutants (Leivar et al., 2009), although there is no experimental evidence to date showing direct interaction between any PIF transcription factor and the *IAA19* promoter.

A critical issue in the control of the gravitropic response is the spatial localization of the machinery that perceives gravity and directs reorientation. Starch-loaded amyloplasts have been shown to be an integral part of the mechanism that allows gravity perception (Boonsirichai et al., 2003), and cells accumulating amyloplasts are located in the tip of the roots and in the endodermis of aerial tissues, such as the hypocotyl. Given that GAs affect gravitropic responses in hypocotyls, it is tempting to suggest that GA signaling interferes with the early events after gravity perception in the endodermis, but this hypothesis requires additional experimental evidence.

In any case, our observation that GAs regulate the gravitropic at an early stage does not diminish the role of the auxin gradient as the driving force in the orientation of the plant with respect to the gravity vector. Rather, GAs would act by fine tuning the formation of this gradient and modulating the responsiveness to this gradient in auxin responding cells. However, we cannot rule out that GAs also affect auxin relocalization, therefore establishing a reinforcing mechanism.

Interestingly, our results do not support an important role of GAs in the execution of cell expansion during gravitropic reorientation, but highlight a function of GAs in the generation of variance in the response. A higher degree of variance or noise in biological responses is a trait often selected by nature, and proposed to be at the core of the mechanisms that drive robust morphogenesis (Houchmandzadeh et al., 2002; Yucel and Small, 2006), and speciation (Braendle and Felix, 2008). In microorganisms, even adaptation of a population to the environment seems to be based, to some extent, on cell-to-cell variance within genetic circuits (Balaban et al., 2004; Sanchez and Kondev, 2008). Not many molecular mechanisms have been proposed that explain the generation of noise in cellular systems (Casal et al., 2004), and the attenuation of auxin response by GAs might represent one of such mechanisms to provide flexibility in situations under which plants face competing tropic signals. Such could be the case of plants that must optimize their access to light because of neighbors' proximity. The observation that DELLA protein concentration decreases in seedlings during the shade avoidance response (Djakovic-Petrovic et al., 2007) supports this scenario. In that case, part of the shade avoidance mechanism may involve the attenuation of gravitropism to allow bending against the gravity vector.

MATERIALS AND METHODS

Plants and growth conditions

Arabidopsis thaliana GA signaling dominant mutants *gai-1D* and *rga-Δ17*, and the quadruple loss-of-function *rga-24 gai-t6 rgl2-1 rgl1-1* (Cheng et al., 2004) are in the L*er* background, while the other lines used in this work, such as *msg2-1* (Tatematsu et al., 2004), *phot1* (Huala et al., 1997), *phyA* (Nagatani et al., 1993), *HS::gai-1D* (Alabadí et al., 2008), *pSCR::gai-1D:GR:YFP* (Úbeda-Tomás et al., 2008), *pMSG2::GUS* (Tatematsu et al., 2004), and *pMSG2::msg2-1:GFP* (Muto et al., 2007), are derived from Col-0 accession. For all experiments, seeds were surface sterilized and stratified for 4-7 days at 4ºC in darkness. Germination took place under continuous white fluorescent light (90–100 µmol $m^{-2}s^{-1}$) at 22°C.

Construction of transgenic lines

To obtain the transgenic line pGAI::gai-1D:GR, the *gai-1D* coding sequence was amplified from genomic DNA of the *gai-1D* mutant with primers: GAId-BamHI-F (GGA TCC ATG AAG AGA GAT CAT CAT CAT CA) and GAId-SacI-R (GAG CTC ATT GGT GGA GAG TTT CCA AGC CGA) and cloned into the pCR2.1 vector (Invitrogen), and the *Bam*HI-*Sac*I fragment was subcloned into pGreen0029-35S::GR (Hellens et al., 2000) to give rise to pG35::gai-1D:GR. The *GAI* promoter was PCR amplified from genomic DNA using oligos pGAI-KpnI-F (GGT ACC TGG GAC CAC AGT CTA AAT GGC GT) and pGAI-XbaI-R (TCT AGA GGT TGG TTT TTT TTC AGA GAT GGA), cloned into the pCR2.1 vector, and the *Kpn*I-*Xba*I fragment transferred into pG35::gai-1D:GR to construct pGAI::gai-1D:GR. Agrobacterium C58 pSOUP cells were transformed with pGAI::gai1-GR and *Arabidopsis* L*er* plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic seedlings with a 3:1 segregation ratio were selected based on their resistance to kanamycin.

Gene expression analysis

Total RNA extraction, cDNA synthesis and quantitative PCR, were carried out as described previously (Frigerio et al., 2006) using *EF1-α* expression for normalization. The primers used were: *IAA19* (CTC GGG CTT GAG ATA ACG GA and CCA CAT CTC TCC CCG GAA)*, IAA5* (AAC TAC GGC TAG GTC TTC CCG and AGA TGG ACT CAC CGG AGA CG) and *IAA6* (TGG CAA AGG AAG GTC TAG CAC and TGG AAG ACC CAA TCG AAG CT).

GUS staining was carried out as described previously (Frigerio et al., 2006).

Detailed description of the microarray experiment will be published somewhere else. Briefly, *HS::gai-1D* and Col-0 seedlings were grown for 3 days at 22ºC in darkness on half strength MS medium (Duchefa) with 0.8% w/v agar and 1% w/v sucrose, and then the plates were transferred to 37ºC for 30 min. Samples were collected before, and 1, 2, 4 h after the beginning of the heat treatment. Three independent biological replicates were used for the analysis. RNA amplification, labeling, and hybridization of microarray slides (70-mer oligonucleotide arrays that

represent the majority of the Arabidopsis genes: http://www.ag.arizona.edu/microarray) were carried out as previously described (Bueso et al., 2007). Hybridization was set up to isolate genes differentially expressed in *HS::gai-1D* compared to Col-0 at each time point.

Tropism tests

To determine the angle of seedling emergence, seedlings were grown for 3 days in darkness at 22ºC in a vertical orientation on plates containing half strength MS medium (Duchefa) with 0.8% w/v phytoagar and without sucrose, and supplemented with mock or 0.4 µM PAC. After 3 days the plates were photographed.

For reorientation experiments, the conditions were the same as above but after 3 days of growth the plates were reoriented 90º relative to the initial growth angle. Reorientation was recorded every hour under infrared light using CCD cameras coupled to Metamorph software as described by Schepens et al (Schepens et al., 2008).

To test blue light-induced phototropism, seedlings were grown for 3 days under 10 µmol $m^{-2}s^{-1}$ of continuous unilateral blue light at 22ºC in a vertical orientation on plates containing half strength MS medium (Duchefa) with 0.8% w/v phytoagar and without sucrose and supplemented with mock or 0.4 µM PAC. After 3 days, the plates were photographed.

In all cases, angles were measured using Image J software.

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Legends for figures

Fig. 1. Gravitropic reorientation of hypocotyls of GA deficient seedlings. Seedlings derived from L*er* and Col accessions were grown for 3 days in darkness, and the plates were turned 90º at time 0. Reorientation of the hypocotyls was monitored in darkness as explained in Materials and Methods. When used, paclobutrazol (PAC) concentration was 0.4 µM. Error bars represent SD (n>16 individual seedlings).

Fig. 2. Gravitropic reorientation after transient induction of *gai-1D*. Col and *HS::gai-1D* seedlings were grown for 3 days in darkness and subject to a 30-min heat shock at 37ºC before turning the plates 90º. Reorientation of the hypocotyls was monitored in darkness as explained in Materials and Methods. When used, paclobutrazol (PAC) concentration was 0.4 µM. Error bars represent SD (n>16 individual seedlings).

Fig. 3. Regulation of Aux/IAA gene expression by DELLA proteins. (**A**) Expression of *IAA5*, *IAA6* and *IAA19* determined by RT-qPCR in 3-day-old dark-grown *HS::gai-1D* seedlings after a 30-min heat shock at 37ºC. Values at each time-point are relative to the expression of each gene in seedlings not subject to heat shock. Error bars represent s.e.m. (n=3 biological replicates). (**B**) Expression of *IAA5*, *IAA6* and *IAA19* determined by RT-qPCR in 3-day-old dark-grown seedlings expressing a fusion between *gai-1D* and the glucocorticoid receptor (*GR*) under the control of the *GAI* promoter. 3-day-old dark-grown seedlings were transferred into flasks and incubated with soft shaking for 6 h with a mock solution or with 10 µM dexamethasone (DEX) to allow gai-1D:GR moving to the nucleus and regulate target genes. Alternatively, seedlings were also incubated with 10 µM cycloheximide (CHX) to prevent de novo protein synthesis during mock or DEX treatments. Values are relative to the expression of each gene in mock-treated seedlings. Error bars represent s.e.m. (n=3 biological replicates).

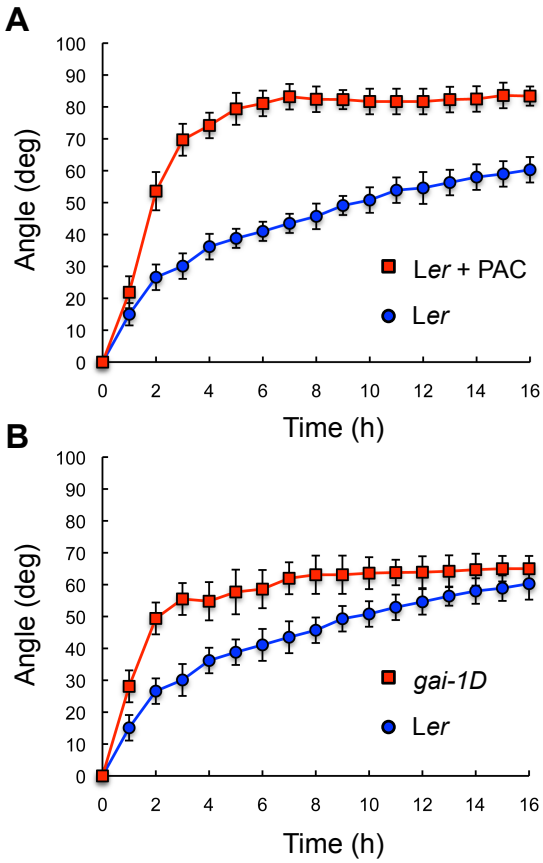
Fig. 4. Localization of MSG2/IAA19 expression in etiolated seedlings. GUS staining in 3-day-old dark-grown seedlings was done as indicated in Materials and Methods. Scale bar represents 1 mm.

Fig. 5. Involvement of IAA19/MSG2 in the regulation of gravitropism by GA. (**A**) Reduction of IAA19/Msg2-1 protein levels caused by impairement of GA biosynthesis. A *pMSG2::msg2-1:GFP* transgenic line was grown for 3 days in darkness with and without 0.4 µM PAC, and GFP fluorescence in hypocotyls was visualized under a confocal microscope. (**B**) Gravitropic reorientation of hypocotyls of *msg2-1* seedlings. Seedlings were grown for 3 days in darkness on control media or in media supplemented with 0.4 µM PAC, and the plates were turned 90º at time 0. Reorientation of the hypocotyls was monitored in darkness as explained in Materials and Methods. Error bars represent SD (n>16 individual seedlings).

Fig. 6. Gravitropic reorientation of hypocotyls of quadruple *della* (*gai rga rgl1 rgl2*) knockout mutants. Seedlings were grown for 3 days in darkness on MS, and the plates were turned 90º at time 0. In (A), seedlings were exposed to 150 µmol m^2 s^{-1} of white light during 8 h prior to reorientation, while in (B), dark-grown seedlings were used. All values between 2 and 16 hours in (A) are statistically different ($p<0.01$) between the *della* mutant and the parental control. PAC concentration was 0.4 µM. Error bars represent SD (n>46 individual seedlings).

Fig. 7. **Physiological relevance of the regulation of gravitropism by GAs.** (**A**) Distribution of hypocotyl orientation of 3-day-old seedlings in darkness. Seedlings were grown on vertical MS plates and the angle with respect to a horizontal line was recorded, with 90º being perfectly vertical. (**B**) Distribution of hypocotyl orientation of 3-day-old seedlings under continuous unilateral blue light (10 nmol $m^{-2}s^{-1}$), coming from the left as indicated by the arrow. The angle with respect to a horizontal line was recorded, with 90º being perfectly vertical. Boxes represent the middle quartiles around the median (bisecting line), while the whiskers represent the upper and lower quartiles. Blue and red colors depict growth in the absence

and presence of 0.4 μM PAC, respectively. The data represent the values of 65-130 seedlings.



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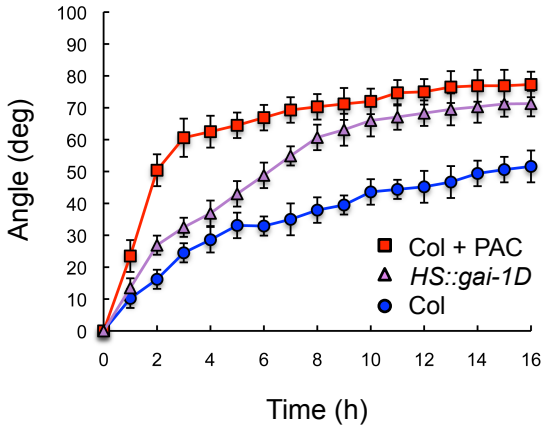

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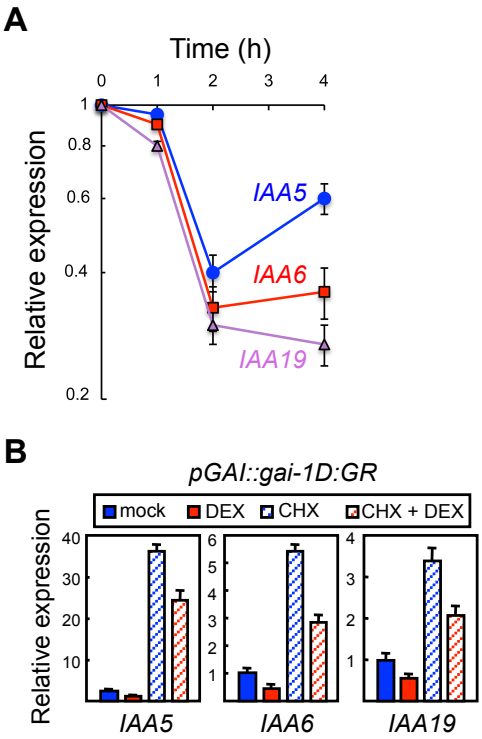

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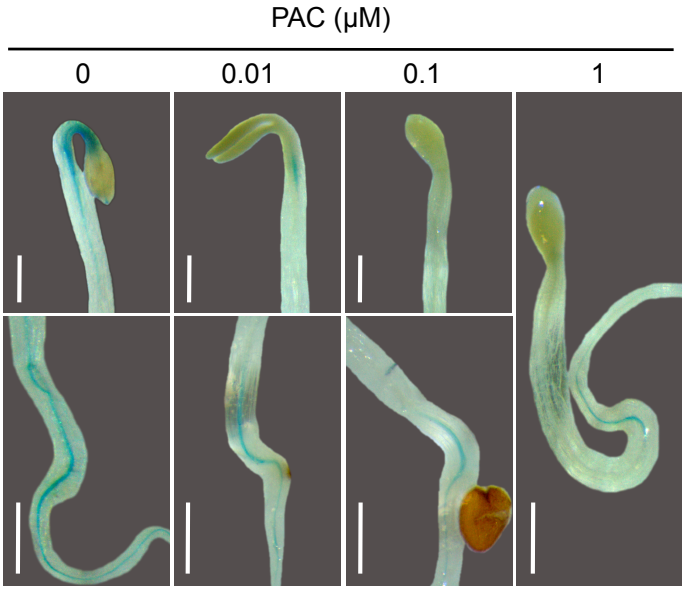

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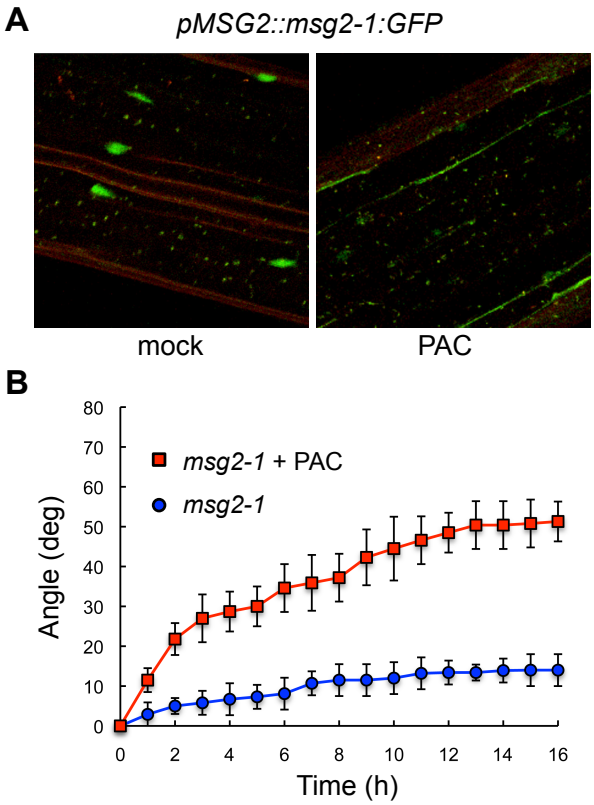

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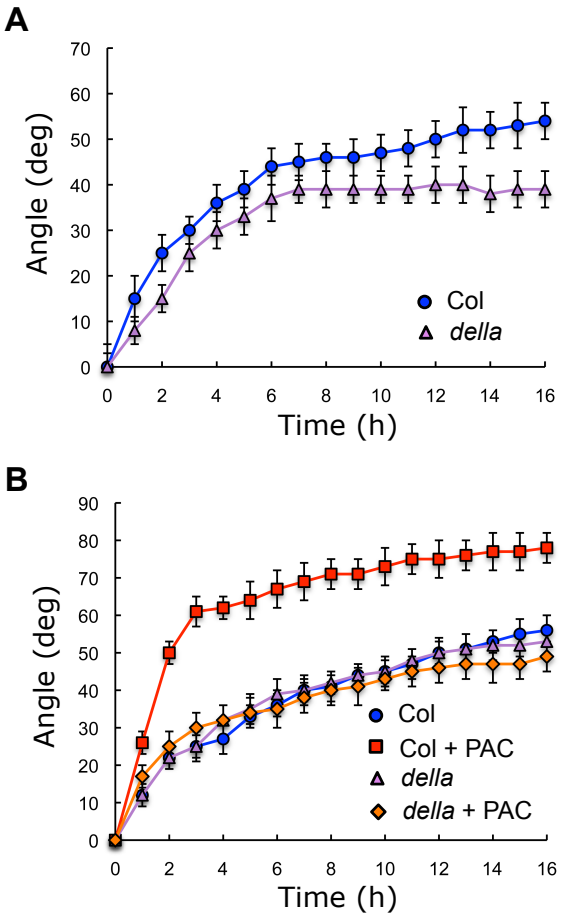

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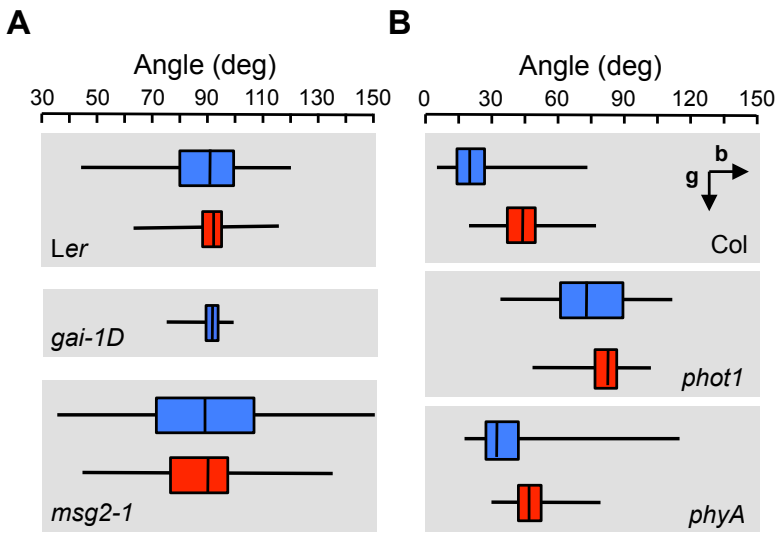

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