Hierarchy of hormone action controlling apical hook development in *Arabidopsis*.

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SUMMARY

The apical hook develops in the upper part of the hypocotyl when seeds buried in the soil germinate, and serves to protect cotyledons and the shoot apical meristem from possible damage caused by pushing through the soil. The curvature is formed through differential cell growth occurring at the two opposite sides of the hypocotyl, and it is established by a gradient of auxin activity and refined by the coordinated action of auxin and ethylene. Here we show that gibberellins (GAs) promote hook development through the transcriptional regulation of several genes of the ethylene and auxin pathways in *Arabidopsis*. The level of GA activity determines the speed of hook formation and the extent of the curvature during the formation phase independently of ethylene, likely by modulating auxin transport and response through *HLS1*, *PIN3*, and *PIN7*. Moreover, GAs cooperate with ethylene in preventing hook opening, in part through the induction of ethylene production mediated by *ACS5/ETO2* and *ACS8*.

INTRODUCTION

The acquisition of developmental innovations has accompanied the evolution of land plants (Langdale, 2008). A key innovation in seed plants is skotomorphogenesis (Wei *et al.*, 1994), an alternative to photomorphogenesis when seeds face germination in darkness, for example when they are buried in the soil. Importantly, skotomorphogenesis provides protection to emerging seedlings while pushing through the soil, especially to the shoot apical meristem (SAM) and cotyledons (Kami *et al.*, 2010). In dicotyledonous plants, these vital structures are protected by an apical hook in the hypocotyl that “pulls” them through the soil. Indeed, hookless mutants are not able to emerge when seeds germinate buried in the soil (Harpham et al., 1991).

The apical hook is mainly formed through differential elongation between the cells at opposite sides of the hypocotyl (Raz and Ecker, 1999). Hook development follows three phases: formation, maintenance, and opening (Raz and Ecker, 1999; Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010). The formation phase extends from the time when germination is completed until the hook curvature reaches ~180º and it usually takes ~24 h in *Arabidopsis thaliana*. Then, the curvature is actively maintained in parallel to extensive hypocotyl elongation. Hook maintenance can be interrupted by light, and then full opening is completed typically in 6 h (Liscum and Hangarter, 1993; Wu *et al.*, 2010). If seedlings are kept in the dark, the hook is maintained for 24 h, and opening is completed 70-90 h later (Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010).

The differential cell growth that underlies hook development is caused by an asymmetrical accumulation of auxin (Kuhn and Galston, 1992; Lehman *et al.*, 1996). Pharmacological treatments or mutations that affect either auxin accumulation (Boerjan *et al.*, 1995; Stepanova *et al.*, 2008; Zhao *et al.*, 2001), transport (Chaabouni *et al.*, 2009; Lehman *et al.*, 1996; Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010), or signalling (Li *et al.*, 2004; Nagpal *et al.*, 2000; Stowe-Evans *et al.*, 1998; Tatematsu *et al.*, 2004; Yang *et al.*, 2004; Žádníková *et al.*, 2010) influence apical hook development. Auxin accumulation marks the side with the lower growth rate in the apical hook (Kuhn and Galston, 1992; Raz and Ecker, 1999).

Besides auxin, other hormones participate in apical hook development. For example, exogenous treatment with ethylene induces the formation of exaggerated hooks whereas ethylene insensitive mutants are hookless (Guzman and Ecker, 1990). Similarly, gibberellins (GAs) are also required for correct hook development, given that a block in either GA synthesis or signalling results in a hookless phenotype (Achard *et al.*, 2003; Alabadí *et al.*, 2004; Vriezen *et al.*, 2004).

The concurrence of multiple hormones controlling a given output is a common theme in plant development (Alabadí and Blázquez, 2009; Alabadí *et al.*, 2009), although their precise mode of action is not always clear. For instance, in the case of hook development, ethylene influences the auxin pathway (Li *et al.*, 2004; Stepanova *et al.*, 2008; Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010), suggesting that ethylene requires auxin to control hook formation; but on the other hand, ethylene application is able to reverse the hook phenotype of the auxin mutant *nph4* (Harper *et al.*, 2000). Additionally, GAs act through ethylene in the control of hook development (Achard *et al.*, 2003; Vriezen *et al.*, 2004), but no molecular mechanism has been found yet.

To unveil the hierarchy of hormone action during hook development, we have investigated in detail the requirement for each hormone in a dynamic way from the time of hook formation to its opening phase, we have searched for gene targets downstream of GA action in the context of hook development, and we have tested the physiological relevance for this regulatory interactions in vivo.

RESULTS

Dynamics of GA-regulated apical hook development

To determine the phase of apical hook development in which GA activity is required, we performed a kinematic analysis of this process in L*er* wild type plants untreated and treated with the GA biosynthesis inhibitor paclobutrazol (PAC), as well as in *gai-1* and quintuple *della* mutants. *gai-1* encodes a dominant version of the DELLA protein GAI that constitutively inhibits GA signalling; the *della* mutant, which lacks all DELLA proteins of *Arabidopsis*, shows a fully activated GA pathway (Feng *et al.*, 2008; Peng *et al.*, 1997). Untreated wild type seedlings displayed the three phases of hook development (Figure 1a) (Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010). On the contrary, seedlings were not able to form the apical hook when treated with 0.2 μM

PAC; instead, they gradually entered into the opening phase (Figure 1a). *gai-1* mutants behaved similarly to PAC-treated seedlings although they started to form the hook, reaching a maximum angle of 121.4±9.5º 20 h after germination. Notably, *della* seedlings showed exaggerated apical hooks (the maximum angle was 241.8±7.9º) as a consequence of a faster kinetics of hook formation during the initial phase, whereas they behaved as the wild type during the other phases.

These results indicate that GA signalling is both necessary and limiting during the formation phase, and therefore the magnitude of hook curvature depends on this activity during the initial phase. In addition, GA activity is also necessary, yet not limiting, to delay hook opening.

GA control on hook development is dependent and independent upon ethylene activity

Exaggerated apical hooks also appear when ethylene activity is high (Guzman and Ecker, 1990). The exaggerated curvature in response to the ethylene precursor 1-aminocyclopropane-1-carboxylate acid (ACC) was due to a delay in the transition between formation and maintenance phases (Figure 1b) (Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010). Importantly, it was the level of GA activity, and not of ethylene, which set up the speed of hook formation (Figures 1b,c and S1). This suggests that both hormones act through different mechanisms during the initial phase, since ethylene is also necessary for hook formation (Vandenbussche *et al.*, 2010). To test if GA-mediated hook formation depends to some extent on ethylene activity, we analyzed hook development in the ethylene insensitive mutant *ein2-1* (Guzman and Ecker, 1990). *ein2-1* seedlings failed to complete hook formation (Vandenbussche *et al.*, 2010), whereas it was partially restored by GA-treatment (Figure 1c).

Analysis of mutants with low or null hormone activity suggested that both hormones are important to prevent hook opening (Figure 1a,c) (Vandenbussche *et al.*, 2010). The kinetics of hook opening was very similar in *della* and in wild type seedlings, and it remained unaltered when the latter were treated with a saturating amount of ACC (Figure 1b). Remarkably, the exaggerated hooks of *della* seedlings did not open after ACC-treatment (Figure 1b).

These results indicate that 1) GAs determine the rate of the hook formation and the extent of the curvature reached during this phase; 2) this role is partially independent of ethylene; 3) ethylene is necessary to complete this phase, although the response seems saturated; and 4) both hormones act jointly to prevent hook opening.

The expression of *ACS5*/*ETO2*, *ACS8*, and *HLS1* genes is regulated by the GA pathway

To elucidate the molecular mechanism by which GAs regulate hook development, we searched through microarray analysis for genes that could be relevant for this process among those rapidly regulated by gai-1 in 2-day-old *pHsp::gai-1* etiolated seedlings (Alabadí *et al.*, 2008) (Gallego-Bartolomé, Alabadí, Blázquez, unpublished). We found that the ethylene biosynthesis genes *ACC SYNTHASE8* (*ACS8*) and *ACS5/ETO2* (Vogel *et al.*, 1998; Yamagami *et al.*, 2003), and the ethylene-induced gene *HOOKLESS1* (*HLS1*) (Lehman *et al.*, 1996), were downregulated by gai-1. Analyses in *pHsp::gai-1*, *ProRGA:GFP-(rga-Δ17)*, and *Pro35S:gai-1* lines (Alabadí et al., 2008; Dill et al., 2001), and in *gai-t6 rga-24* double loss-of-function mutants (Dill and Sun, 2001; King *et al.*, 2001) confirmed their regulation by DELLAs (Figure 2a,b).

Their rapid response to gai-1 suggested that they might be direct targets. To confirm this, we examined their expression in *ProGAI:gai-1-GR* seedlings (Gallego-Bartolomé, Alabadí, Blázquez, submitted). As a control, we included the DELLA-induced gene *AtGA20ox2* gene in the analysis (Zentella *et al.*, 2007) (Figure 2b). Dexamethasone (DEX)-treatment repressed and induced *HLS1* and *AtGA20ox*, respectively, and this effect was not abolished by cycloheximide (CHX) indicating that regulation by gai-1 is independent of protein synthesis (Figure 2c). However, downregulation of *ACS5/ETO2* and *ACS8* by gai-1 requires the synthesis of a protein intermediate. The strong upregulation of *ACS8* by CHX could mask any effect of gai-1, and therefore we could not rule out the possibility of a direct effect of the DELLA protein. The transcription factor PIF5 promotes *ACS8* expression in etiolated seedlings (Khanna *et al.*, 2007). Since DELLAs regulate transcription by inhibiting several transcription factors of the PIF clade (Arnaud *et al.*, 2010; de Lucas *et al.*, 2008; Feng *et al.*, 2008), we tested whether this is the case for PIF5. GAI and PIF5 interacted *in vivo* in *Nicotiana benthamiana* leaves as shown by co-immunoprecipitation (Figure 2d) and bimolecular fluorescence complementation (BiFC) (Figure S2). Remarkably, chromatin immunoprecipitation (ChIP) showed that PIF5 binds *in vivo* to a G-box in the *ACS8* promoter in a GA-dependent manner in *Arabidopsis* (Figure 2e), suggesting DELLAs may repress *ACS8* expression by inhibiting PIF5.

ACS5/ETO2- and ACS8-mediated ethylene production contributes to hook development (Tsuchisaka *et al.*, 2009; Vogel *et al.*, 1998), and the activity of *HLS1* is central to mediate fully this effect (Lehman *et al.*, 1996; Roman *et al.*, 1995). Thus, our gene expression analysis suggests that GAs regulate hook development through the control of *HLS1* gene expression through direct regulation by DELLA proteins and via ethylene biosynthesis (Figure 2f).

GA-regulation of *ACS5/ETO2* and *ACS8* gene expression depends on the phase of hook development

To examine the temporal and spatial distribution of *ACS5/ETO2* and *ACS8* expression during hook development and their response to GAs, we used the *ProACS5:GUS* and *ProACS8:GUS* reporters (Tsuchisaka and Theologis, 2004). Their spatial and temporal expression patterns were similar (Figures 3a,b and S3). Staining was detected mainly in the hypocotyl vasculature, reaching the apical hook 36 h after germination. Both the timing and the extent of their response to GAs were somewhat different. The regulation of *ProACS5:GUS* expression upon GAs was evident 36 h after germination. Remarkably, GAs became limiting 36 h later, when GA-treatment resulted in augmented expression (Figure 3a). The dependence of *ProACS8:GUS* on GAs was also evident 36 h after germination (Figure 3b), although the response was already saturated. As expected, the PAC-effect on both reporter lines was reversed completely by simultaneous treatment with GAs (Figure S3). Hence, both the basal expression and the responsiveness to GAs of *ACS5/ETO2* and *ACS8* are subject to developmental regulation in the apical hook.

GAs support ethylene production in etiolated seedlings

Staining patterns of *ProACS5:GUS* and, to a lesser extent, of *ProACS8:GUS* in response to GAs support the idea that GAs promote ethylene biosynthesis in etiolated seedlings. To test it we measured ethylene production in etiolated L*er* wild type and *della* seedlings. The ability of wild type seedlings to produce ethylene decreased steadily during the first days after germination (Figure 3c). This trend was reversed in *della* seedlings, which produced more ethylene than the wild type after the second day. This timing is coincident with the dependence of *ACS8* and *ACS5/ETO2* expression upon GA activity (Figure 3a,b). Thus, the GA pathway may contribute to reach the minimum threshold level of ethylene needed to sustain a proper transition to hook maintenance and to delay hook opening in the wild type.

GAs regulate partly hook development by modulating PIF activity

The regulation of *ACS8* by the DELLA-PIF5 interaction (Figures 2d,e and S2), together with the fact that PIF1, PIF3, and PIF5 promote hook development (Khanna *et al.*, 2007; Kim *et al.*, 2011; Leivar *et al.*, 2008) suggests that PIFs could mediate the GA-regulation of this process. Indeed, *pif5* mutants showed a slight hypersensitivity in PAC-induced repression of *ACS8* and hook opening, whereas *Pro35S:PIF5-HA* seedlings were resistant (Figure S4a,b). In additional support of this hypothesis, *pif1 pif3 pif4 pif5* (*pif1/3/4/5*) seedlings (Leivar *et al.*, 2008; Shin *et al.*, 2009) did not form the apical hook and they immediately entered into the opening phase, whilst GA-treatment delayed hook opening for a few hours (Figure S4c). Analysis of the *pif3/4/5* mutant corroborated the significant role of PIF1 in this process, since these seedlings were able to delay the opening phase (Figure S4d). Remarkably, PIF1 was able to restore the GA-responsiveness during the formation phase. These results indicate that PIF activity is necessary at least for hook formation and that there is a temporal coincidence in the need of GA and PIF activities, suggesting a functional relationship in the control of this process.

HLS1 activity mediates GA effect on hook development

The partially ethylene-independent control of GAs on hook formation (Figure 1c) is consistent with a model by which GAs regulate *HLS1* directly (Figure 2f), and with GA activity being necessary to allow ethylene to exert its control on apical hooking (Achard *et al.*, 2003; Vriezen *et al.*, 2004). One-day-resolution analysis of hook development indicated that HLS1 is needed early after germination in the dark (Raz and Ecker, 1999). Our kinematic analysis confirmed previous results showing that *hls1-1* mutation prevented hook formation (Figure 4a). The dynamics of hook development was very similar in *hls1-1* mutants and in PAC-treated seedlings (Figures 1a and 4a), indicating that there is a temporal coincidence in the requirement of both activities during hook development. Besides, the hook phenotype of *hls1-1* seedlings was not affected by exogenous GA-treatment, whereas the wild type showed exaggerated hooks (Figure 4a).

To confirm that GAs regulate hook development through HLS1, we analyzed the effect that uncoupling *HLS1* expression from GA-regulation had on the GA-control of hook development. For that purpose, we prepared *Pro35S:YFP-HLS1* transgenic lines and analyzed their response to PAC. As hypothesized, Figure 4b shows that apical hooks of *Pro35S:YFP-HLS1* seedlings were partially resistant to PAC-induced opening. Furthermore, time-course analysis of *HLS1* expression showed that GA activity is needed to sustain its expression during hook development (Figure 4c). Nonetheless, *HLS1* transcript level was not increased in *della* mutants indicating that its regulation by GAs is already saturated.

GAs are needed to sustain differential auxin response during apical hook development

Asymmetrical auxin accumulation and response is essential for the differential cell growth underlying apical hook development (Lehman *et al.*, 1996; Li *et al.*, 2004; Vandenbussche *et al.*, 2010; Wu *et al.*, 2010; Žádníková *et al.*, 2010). Moreover, HLS1 is critical to establish the auxin response in the hook, since the asymmetric distribution of *ProDR5:GUS* staining in the apical hook is lost in *hls1* (Li *et al.*, 2004). Given the regulation of *HLS1* expression by GAs, we examined whether the *ProDR5:GUS* response was altered by GAs. By 18 h after germination, *ProDR5:GUS* staining was apparent at the concave side of the hook in control seedlings (Figure 5a,b) (Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010). Neither the intensity of the staining at the concave side nor the number of seedlings with differential staining was influenced by GA-treatment at this stage of development. Nevertheless, the percentage of seedlings with staining at the inner side of the hook was lower after treatment with 0.2 μM PAC. This result suggests that GAs are necessary to support differential auxin response during the formation phase. Stronger GA-dependence was observed during the maintenance and opening phases. At these two stages no *ProDR5:GUS* expression was detected at the upper zone of the hypocotyl of any PAC-treated seedling, where the apical hook should form, whereas GA-treatment enhanced the differential *ProDR5:GUS* staining at the concave side of the hook (Figure 5a,b). As expected, the PAC-effect was reversed completely by simultaneous treatment with GAs (Figure S3).

Remarkably, the *ProDR5:GUS* expression pattern is very similar in PAC-treated (Figure 5a) and in *hls1* seedlings (Li *et al.*, 2004). Despite the driving role proposed for HLS1 during apical hook development, its activity is not sufficient in the absence of polar auxin transport (Lehman *et al.*, 1996). In agreement, ACC-treatment does not revert the effects of the polar auxin transport inhibitor naphthylphthalamic acid (NPA) (Žádníková *et al.*, 2010). Similarly, 50 μM GA_3-treatment did not revert either the hookless phenotype or the altered *ProDR5:GUS* staining pattern caused by NPA-treatment (Figure 5c,d), which suppressed the exaggerated hooks of *della* seedlings (Figure 5d). The effects of GA- and ethylene-treatments on *ProDR5:GUS* during maintenance and opening phases are similar (Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010). Nevertheless, GAs might control auxin response independently of ethylene during the formation phase (Figure 1c). In fact, whereas indole-3-acetic acid (IAA)-treatment restores the apical hook to ethylene-insensitive mutants (Vandenbussche *et al.*, 2010), it was not able to restore it to PAC-treated seedlings and to *hls1-1* mutants (Figure 5e). In summary, these results draw new similarities between GAs and HLS1 activity, which suggests that they participate in the same pathway in the establishment and/or the interpretation of the auxin gradient during apical hook development.

GAs participate in maintaining *PIN3* and *PIN7* expression in the apical hook

Genetic analyses have implicated AUX1, LAX3, PIN1, PIN3, PIN4, and PIN7 in driving the auxin flux during apical hook development, and ethylene regulates the transcription of several of their genes (Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010). We asked whether GAs would also influence the expression of these genes. Expression of *PIN1*, *PIN4*, and *AUX1* was not altered by GAs during hook development (data not shown). Sustained expression of *PIN3* was dependent upon GAs during the maintenance and opening phases, whereas this dependence was evident earlier for *PIN7* (Figure S5a,b). These results are consistent with results of Figure 5e, and suggest that GAs might also promote hook development by maintaining proper expression of *PIN* genes needed to distribute the auxin flux from cotyledons (Žádníková *et al.*, 2010). To challenge this hypothesis, we investigated the response of *pin3 pin7* mutants to GAs. Double mutant seedlings were not able to complete hook formation and, importantly, they were resistant to GA-treatment (Figure 6). Interestingly, single mutants had contrasting behaviors: *pin3* mutants showed a milder defect on hook formation than *pin7*, whereas their response to GAs was quite affected; *pin7* seedlings responded to GAs similarly to the wild type despite having more disturbed hook formation than *pin3* (Figure S5c,d).

GA activity in the endodermis is required for apical hook development

Missexpression approaches have shown that the context outlined by the cell type may be determinant to define the output of hormone pathways (Jaillais and Chory, 2010). For instance, DELLA activity in the endodermis controls meristem size and overall growth in the root (Úbeda-Tomás *et al.*, 2009; Úbeda-Tomás *et al.*, 2008), whereas the epidermis is the key tissue for brassinosteroids to control shoot growth (Savaldi-Goldstein *et al.*, 2007). Thus, we examined whether GAs regulate hook development in a tissue-specific manner. We expressed gai-1 exclusively in the endodermis under the control of the *SCARECROW* promoter (*ProSCR:gai-YFP-GR*) (Úbeda-Tomás *et al.*, 2008), or in the epidermis under the control of the *MERISTEM LAYER1* promoter (*ProML1:GFP-gai-1*; Figure S6). Expression of *gai-1* in the endodermis but not in the epidermis impaired hook formation similar to the PAC-treatment or the *gai-1* mutation (Figure 7a). Since the *SCR* promoter is active in the hook endodermis starting 22 h after germination (Vandenbussche *et al.*, 2010), our results indicate that GA activity is necessary in the endodermis for the correct progression of hook development at least during the late formation phase, whereas it is dispensable in the epidermis. These results support further the functional relationship between GAs and PIFs sustaining hook development, since expression of *PIF1* only in the endodermis of the *pif1/3/4/5* mutant restores the hook (Kim *et al.*, 2011), indicating there is also a spatial coincidence in the requirement of both activities.

Next, to place the transcriptional network regulated by GAs in the context of the endodermis, we examined the activity of *ProDR5:GUS*, *ProPIN3:GUS*, and *ProPIN7:GUS* in F1 seedlings from crosses between the reporter lines and L*er* wild type, *ProML1:GFP-gai-1-11*, and *ProSCR:gai-YFP-GR* seedlings. Impairing GA signaling in the endodermis had the same effect on the expression of *ProDR5:GUS* and *ProPIN3:GUS* than PAC-treatment, whereas no effect was observed when GA signaling was blocked in the epidermis (Figure 7b). A tissue-independent effect was observed, however, when *ProPIN7:GUS* expression was examined. These results suggest that GAs control *PIN3* expression mainly from the endodermis and that confinement of its expression to the vascular bundle by PAC-treatment or *ProSCR:gai-YFP-GR* expression (see a magnification in Figure 7c), may impair to some extent the auxin flux towards outer tissues, in agreement with the disappearance of *ProDR5:GUS* from the concave side. In support of this, PIN3 is present in endodermis, cortex, and epidermis, whereas PIN7 and PIN4 are predominant in outer tissues (Žádníková *et al.*, 2010). The mild hook phenotype of *pin3* mutants indicate that other efflux carriers are involved, although less relevant for the GA-control on the hook. Moreover, GAs may impinge on other branches of the network, most likely *HLS1*, to regulate hook development from the endodermis.

DISCUSSION

The establishment of an apical hook is an intrinsic part of the skotomorphogenic developmental program and it depends on differential cell elongation on opposite sides of hypocotyls. The instructive molecular framework that guarantees this differential growth relies in the end on asymmetrical auxin response (Lehman *et al.*, 1996). Ethylene signalling represents one module of regulation that sustains this basic framework (Stepanova *et al.*, 2008; Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010), in a large part targeting *HLS1* transcription (Chaabouni *et al.*, 2009; Li *et al.*, 2004). Our results show that GAs impinge both on the ethylene pathway and on auxin distribution and response, and therefore it represents a new layer of regulation that ensures proper progression through all phases of hook development (Figure S7a).

GAs regulate hook formation independently of ethylene activity

Sustained asymmetric auxin activity is necessary during all phases for proper hook development (Chaabouni *et al.*, 2009; Lehman *et al.*, 1996; Wu *et al.*, 2010; Žádníková *et al.*, 2010). Ethylene plays its major role in a time-window that encompasses maintenance and opening phases and overlaps with a period of augmented sensitivity to the hormone (Raz and Ecker, 1999), whereas its role during the formation phase is minor (Figure S7b) (Knee *et al.*, 2000; Raz and Ecker, 1999; Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010). On the contrary, the GA pathway performs a prominent role during the initial phase, when the strength of its activity determines the speed of hook formation and the extent of hook curvature (Figure 1a). Importantly, this role of GAs is mostly independent of ethylene (Figure 1c). The high demand of GA activity for apical hooking is reminiscent of germination. The apical hook starts to form immediately after germination in darkness is completed. Germinating seeds require high levels of GAs to break dormancy (Cao *et al.*, 2005; Ogawa *et al.*, 2003; Penfield *et al.*, 2006). Our results suggest that this high GA activity might extend into the early stages of hook development to ensure a sustained GA response. Both processes may have similar mechanistic basis, the same GA response initiated in embryos during germination may continue later on in etiolated seedlings to promote apical hook development. In agreement, mutants with a hyperactive GA pathway show exaggerated growth of the embryo's axis (Cao *et al.*, 2005) and exaggerated hook curvature (Figure 1a). Moreover, GA biosynthesis and response take place mainly in the hypocotyl endodermis and cortex during germination (Ogawa *et al.*, 2003; Yamaguchi *et al.*, 2001). Remarkably, sustained GA activity specifically in hypocotyl endodermis is required for proper progression through hook formation (Figure 7).

GAs prevent hook opening in cooperation with the ethylene pathway

GAs are also required to prevent hook opening. This task is performed jointly with ethylene, and the transition to this phase is prevented only when the two hormones become not limiting (Figure 1b). This response suggests that this process might be controlled by a signalling element whose activity is regulated in cooperation by both pathways. For example, DELLA proteins could inactivate an ethylene-regulated transcription factor that negatively regulates opening, similar to their negative effect on PIFs (de Lucas *et al.*, 2008; Feng *et al.*, 2008). The apical hook, on the other hand, is not a vital structure when seedlings grow *in vitro*. The timing and kinetics of hook opening may respond solely to endogenous cues under these conditions. The identification of GAs and ethylene as elements imposing a brake to hook opening suggests that both pathways are targets of light signalling during de-etiolation. In fact, the GA pathway is downregulated by light (Achard *et al.*, 2007; Alabadí *et al.*, 2008; Reid *et al.*, 2002; Zhao *et al.*, 2007), which might help to turn off the hormonal network that prevents hook opening (see below). The activity of ethylene is high in etiolated seedlings (Zhong *et al.*, 2009), so it is reasonable to think that it is also reduced during de-etiolation. Indeed, light impinges negatively on ethylene signalling rather on ethylene levels to promote hook opening in *Arabidopsis* (Knee *et al.*, 2000). Besides, the expression of the ethylene- and GA-induced gene *HLS1* is repressed by light, which surely contributes to hook opening (Li *et al.*, 2004).

GAs regulate hook development by transcriptional regulation of auxin and ethylene pathways

How do GAs regulate progression through hook development? Our results indicate that GAs exert this regulation, or at least part of it, by transcriptional regulation of several elements of the signalling network that controls apical hooking. First, GAs impinge on the core of the mechanism by regulating expression of auxin transporter genes *PIN3* and *PIN7* (Figure S5c,d). Second, GAs influence the expression of two *ACS* genes involved in ethylene biosynthesis, *ACS5/ETO2* and *ACS8* (Figures 2a-c and 3a,b), as well as the expression of the ethylene-induced gene *HLS1* (Figures 2a-c and 4c), whose activity is necessary to control auxin responses in the hook (Lehman *et al.*, 1996; Li *et al.*, 2004). The kinetics of their transcriptional response suggests that DELLAs operate through different regulatory mechanisms depending on each case. Regulation of *PIN3* and *PIN7* seems an indirect consequence of DELLAs’ activity (data not shown). A similar case is found at the root meristem, where DELLAs downregulate *PIN* expression indirectly through ARR1 and SHY2 (Dello Ioio *et al.*, 2008; Moubayidin *et al.*, 2010). The downregulation of *HLS1* and *ACS8* is a direct consequence, whilst the fast regulation of *ACS5/ETO2* requires the synthesis of a protein intermediate (Figure 2c). Remarkably, DELLAs directly inhibit the activity of PIF5 to repress the expression of *ACS8* (Figure 2d,e), as previously seen with PIF3 and PIF4 for light-regulated genes (de Lucas *et al.*, 2008; Feng *et al.*, 2008). The expression of both *HLS1* and *ACS5/ETO2* is lower in *pif1/3/4/5* mutants than in the wild type (Leivar *et al.*, 2009; Shin *et al.*, 2009), suggesting that PIFs mediate their regulation by DELLAs as well. Nonetheless, the influence of PIFs may be indirect given that there are no G-boxes in the upstream promoter region of both genes.

Several pieces of evidence support the idea that regulation of *ACS* genes by GAs is relevant for ethylene production in etiolated seedlings. First, the *della* mutant produces more ethylene than the wild type (Figure 3c). Second, the timing for increased ethylene production in *della* mutants correlates with the increased expression of *ACS5/ETO2* upon GA-treatments (Figure 3a); the contribution of ACS8 activity to the extra ethylene in the *della* mutant may be lower. Third, this timing also coincides with the window of maximum ethylene sensitivity in the apical hook (Raz and Ecker, 1999). And fourth, ACS5/ETO2 and ACS8 contribute to ethylene-induced hook development (Tsuchisaka *et al.*, 2009; Vogel *et al.*, 1998).

The close connection of GAs with the auxin and ethylene pathways (Figure S7a) is manifested by the strong hook phenotype observed when the GA activity is compromised. Despite the role of the GA-mediated ethylene production may be minor, the regulation of *HLS1* and the auxin transporters surely have a deep contribution to hook development. For instance, the hookless phenotype caused by low GA levels is alleviated by overexpressing *HLS1* (Figure 4b). This idea is supported further by the staining patterns of *ProDR5:GUS* which are shared by PAC- or NPA-treatment (Figure 5a,c) and the *hls1* mutant (Li et al., 2004), and by the inability of IAA-treatment to restore the apical hook to PAC-treated and *hls1* seedlings (Figure 5e). We propose that GAs sustain differential auxin transport and response during the formation phase and that at least the latter might be mediated by HLS1 activity. This is based in three observations: first, there is a coincidence in the temporal requirement of HLS1 and GA activities during hook formation (Figures 1a and 4a). Second, *hls1* is epistatic over GA-application (Figure 4a). And third, *HLS1* expression is directly downregulated by DELLAs (Figure 2c). Notwithstanding, whereas GA activity is limiting to drive hook formation (Figure 1a), it is saturated to promote *HLS1* expression (Figure 4c). This suggests that there is another mechanism by which GAs regulate the formation phase besides transcriptional regulation of the *HLS1* gene.

EXPERIMENTAL PROCEDURES

Plant lines and growth conditions

Arabidopsis thaliana accessions L*er* and Col-0 were used as wild types. Mutants and transgenic lines used have been described: quintuple *della* (Feng *et al.*, 2008), *gai-1* (Peng *et al.*, 1997), *gai-t6 rga-24* (Dill and Sun, 2001; King *et al.*, 2001), *pRGA::GFP-(rga-Δ17)* (Dill *et al.*, 2001), *ProHsp:gai-1* and *Pro35S:gai-1* (Alabadí *et al.*, 2008), *ProSCR:gai-YFP-GR* (Úbeda-Tomás *et al.*, 2008), and *ProGAI:gai-1-GR* (Gallego-Bartolomé, Alabadí, Blázquez, submitted); *ein2-1* and *hls1-1* (Guzman and Ecker, 1990), and *pACS5::GUS* and *ProACS8:GUS* (Tsuchisaka and Theologis, 2004); *ProPIN7:GUS*, *pin7-1*, and *pin3-5* (Benkova *et al.*, 2003), *ProPIN3:GUS* (Friml *et al.*, 2002); *Pro35S:PIF5-HA* (Lorrain *et al.*, 2008), and *pif3/4/5* and *pif1/3/4/5* (Leivar *et al.*, 2008; Shin *et al.*, 2009). The *pin3-3 pin7^En* double mutant has been kindly provided by Dr Ykä Helariutta (Helsinki University).

Seeds were sterilized and stratified for 6 days in water at 4ºC. Germination took place under white fluorescent light (90–100 μmol m^{-2} s^{-1}) at 22°C for 6 h in a Percival growth chamber E-30B (http://www.percival-scientific.com). Seeds were plated in plates of half-strength MS medium with 0.8% (w/v) agar and 1% (w/v) sucrose supplemented with either 0.2 μM PAC, 50 μM GA_3, 10 μM ACC, 10 μM DEX, 0.1 μM IAA or 5 μM NPA and grown in darkness at 22ºC. For exogenous GA-treatment, seeds were stratified in 50 μM GA_3. For short-term treatments, seedlings were incubated in the dark in water supplemented with 10 μM CHX and/or 10 μM DEX. MS, PAC, GA_3, ACC, IAA and NPA were from Duchefa (http://www.duchefa.com). DEX and CHX were from Sigma (http://www.sigmaaldrich.com). Plates were placed vertically for kinematic analyses.

Real-time analysis of apical hook development

Real-time imaging of apical hook development and hook angle measurement were performed as described (Vandenbussche *et al.*, 2010; Žádníková *et al.*, 2010).

Analysis of reporter lines

β-glucuronidase (GUS) staining was performed as described (Žádníková *et al.*, 2010).

Construction of vectors and generation of transgenic lines The pENTR223 vector carrying the *HLS1* or ORF was obtained from the Arabidopsis Biological Resource Center (ABRC) and transferred into the *pEarleyGate104* vector (Earley *et al.*, 2006) by Gateway technology using the LR clonase (Invitrogen, http://www.invitrogen.com) to create *pEG::HLS1ox*.

The construction of *ProML1:GFP-gai-1*was as follows. The *gai-1* coding sequence was amplified from genomic DNA of the *gai-1* mutant with primers GAIdf (ATGAAGAGAGATCATCATCATCA) and GAIdr (ATTGGTGGAGAGTTTCCAAGCCGA) that included the attB1 and attB2 Gateway recombination sites (not shown), respectively. The PCR product was cloned into *pDONR221* (Invitrogen) by BP reaction, and then into the binary vector *pSBright:GFP* (Bensmihen *et al.*, 2005) by LR reaction to give rise to *pSBright:GFP-gai-1* construct. The *ML1* promoter was PCR-amplified using primers described (An *et al.*, 2004) and that included the *Hin*dIII recognition site. The PCR product was cloned into the *pCR2.1* vector and sequenced. After digestion with *Hin*dIII, the *ML1* promoter was cloned into the *Hin*dIII site of *pSBright:GFP-gai-1*, to create *ProML1:GFP-gai-1*.

Constructs were introduced in *Agrobacterium* strain C58 and used to transform *Arabidopsis* Col-0 wild type plants, *pEG:HLS1ox*, or L*er*, *ProML1:GFP-gai-1*. Transgenic seedlings in the T_1 and T_2 generations were selected on 50 μM glufosinate ammonium (Sigma). Transgenic lines with a 3:1 (resistant:sensitive) segregation ratio were selected, and 10 homozygous lines were identified in the T_3 generation. Data from two representative lines are shown.

Real-time quantitative RT-PCR

RNA extraction, cDNA synthesis, quantitative RT-PCR (qRT-PCR), analysis, and primer sequences for amplification of *AtGA20ox2* and *EF1-α* genes have been described (Frigerio *et al.*, 2006). qRT-PCR oligonucleotides for *ACS5/ETO2*, *ACS8*, and *HLS1* genes were: qRT-ACS5f (GCTGGTTCGACATCTGCGA), qRT-ACS5r (AGGCTCTGCAAGGCAAAACAT), qRT-ACS8f (GGTGCTACTCCGGCTAACGA), qRT-ACS8r

(TCCAGGATCAGCGAGACAAAA), qRT-HLS1f (CGATACCGTCCGTTTTCGAA), and qRT-HLS1r (GCCTTAGCCAAGTTATGCGC).

Ethylene measurements

Ethylene measurements were performed as described (Thain *et al.*, 2004), with the following modifications. 150-200 seeds were sterilized and sown in a 10 ml chromatography vial containing 5 ml of half-strength MS with 1% (w/v) sucrose and 0.8% (w/v) agar. The vial was kept 5 days at 4°C in darkness and subsequently exposed to white light for 6 h at 21°C to stimulate germination. Seedlings were grown in darkness (capped vials wrapped in aluminium foil). Every 24 h, the vials were flushed with hydrocarbon free air (Air Liquide, http://www.es.airliquide.com/) and ethylene in the headspace was detected with an ETD-300 photo-acoustic ethylene detector (Sensor Sense, http://www.sense.com.br).

Confocal microscopy

Images were taken using a Leica TCS SL confocal laser microscope (Leica Microsystems GmbH, http://www.leica-microsystems.com/) with excitation at 488 nm.

BIFC and co-IP assays

BIFC vectors *pMDC43-YFN* and *pMDC43-YFC* were provided by Dr Alejandro Ferrando (IBMCP). *pENTR* vectors carrying the coding sequence of *PIF5* and *GAI* were generated by the REGIA project (Paz-Ares and The Regia, 2002). PIF5 and GAI coding sequences were transferred into *pMDC43-YFC* and *pMDC43-YFN*, and into *pEarleyGate201* and *pEarleyGate104* (Earley *et al.*, 2006) for BIFC and co-IP, respectively, by Gateway using the LR clonase (Invitrogen). Each construct was introduced into *Agrobacterium* C58 cells, which were used subsequently to infiltrate leaves of *Nicotiana benthamiana*. BIFC analysis was performed as described (Scacchi *et al.*, 2009).

For co-IP, nuclear proteins were isolated from formaldehyde-fixed leaves. Immunoprecipitation was carried out with anti-HA antibody-coated paramagnetic beads (Miltenyi Biotec, http://www.miltenyibiotec.com/en/default.aspx) following manufacturer’s instructions. HA- and YFP-tagged proteins in the input and immunoprecipitated were detected by immunoblotting using anti-HA (Roche, https://www.roche-applied-science.com) and anti-GFP (Clontech, http://www.clontech.com/) antibodies.

ChIP and PCR amplification

Seedlings of *Arabidopsis* Col-0 and *Pro35S:PIF5-HA* transgenic line were grown at 22ºC for 3 days in darkness before fixation. ChIP assays were performed as described (Hornitschek *et al.*, 2009). qPCR oligonucleotides to amplify the region around the G-box were pACS8-F-1 (ATGGAAATTCACATCGTGCCTA) and pACS8-R-1 (GATGTCAGAGAAGAATGAGCACGT). The ORF region was amplified with the same oligonucleotides used to analyze *ACS8* gene expression by RT-qPCR.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. The GA activity determines the speed of hook formation.

Figure S2. GAI and PIF5 interact in plant cells.

Figure S3. GAs revert the PAC-effect on *ProACS5:GUS*, *ProACS8:GUS*, and *ProDR5:GUS*.

Figure S4. The activity of PIF transcription factors mediates the GA control on hook development.

Figure S5. PIN3, PIN7, and the regulation of hook development by GAs.

Figure S6. Specific expression of GFP-gai-1 in the epidermis.

Figure S7. Models explaining the pathway interactions and the timing of GA and ethylene action.

FIGURE LEGENDS

Figure 1. Regulation of apical hook development by GAs and ethylene.

(a) Kinematic analysis of hook development in L*er* wild type seedlings mock-treated and treated with 0.2 μM PAC, as well as in mock-treated *gai-1* and *della* seedlings.

(b,c) Kinematic analysis of hook development in L*er* wild type and *della* seedlings grown on control medium or with 10 μM ACC (b), as well as Col-0 wild type and *ein2-1* seedlings grown on control medium or with 50 μM gibberellic acid (GA_3) (c). Dotted vertical lines represent the transition between phases. All error bars represent s.e.m. (n>20).

Figure 2. GAs regulate the ethylene pathway in etiolated seedlings.

(a) Expression of *ACS5/ETO2*, *ACS8*, and *HLS1* in 2-d-old *pHsp::gai-1* seedlings subjected to a 30 min treatment at 37ºC; control seedlings were kept at 20ºC. Expression was determined by qRT-PCR and normalized to the respective control treatment.

(b) Thirty six-hour-old wild type L*er* and *gai-t6 rga-24* seedlings were grown on control medium or with 0.2 μM PAC. Expression was determined by qRT-PCR. PAC, fold change between PAC- and mock-treated wild type L*er* seedlings; *Pro35S:gai-1*, fold change between transgenic and wild type Col-0 seedlings; *rga-Δ17*, fold change between *ProRGA:GFP-(rga-Δ17)* and wild type L*er* seedlings; *gai-t6 rga-24* mock, fold change between *gai-t6 rga-24* and wild type L*er* seedlings; *gai-t6 rga-24* PAC, fold change between PAC-treated and mock-treated *gai-t6 rga-24* seedlings.

(c) Two-day-old *ProGAI:gai-1-GR* etiolated seedlings were incubated for 5 h in water or in water supplemented with either 10 μM DEX, 10 μM CHX, or both. (a-c) Expression was determined by qRT-PCR and normalized to the respective control treatment. Data represent mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results.

(d) co-IP showing the interaction between GAI and PIF5. YFP-GAI and HA-PIF5 were expressed either alone or together in leaves of *Nicotiana benthamiana*. Nuclear proteins were immunoprecipitated with anti-HA antibody-coated paramagnetic beads and detected by immunoblotting with either anti-HA or anti-GFP antibodies.

(e) qRT-PCR of a regulatory (G-box) or a control (ORF) sequence in the *ACS8* locus after ChIP with anti-HA. Analysis was performed in 36-hour-old Col-0 wild type and

Pro35S:PIF5-HA seedlings grown on control medium or with 0.2 μM PAC. Enrichment of the regulatory and control ORF sequences is shown after normalization to the input value. Data represent mean and standard deviation of three technical replicates from a representative experiment out of three biological replicates.
(f) Model: GAs control hook development by transcriptional regulation of *HLS1*, either directly or indirectly through regulation of ethylene biosynthesis.

Figure 3. Regulation of the ethylene pathway by GAs.
(a,b) Expression patterns of *ProACS5:GUS* (a) and *ProACS8:GUS* (b) during hook development in seedlings grown on control medium or with 0.2 μM PAC or 50 μM GA_3.
(c) GAs promote ethylene production in etiolated seedlings. The ability to produce ethylene per day was measured in wild type L*er* and quintuple *della* etiolated seedlings. Three independent sets of biological material were used for calculating mean values. Error bars represent s.e.m. The experiments were done twice with similar results.

Figure 4. HLS1 activity mediates the GA control on hook development.
(a) Kinematic analysis of hook development in Col-0 wild type and *hls1-1* seedlings grown on control medium or with 50 μM GA_3. Dotted vertical lines represent the transition between phases. Error bars represent s.e.m. (n>20).
(b) Hook angle of 1-day-old wild type Col-0 and *Pro35S:HLS1* seedlings grown on control medium or with 0.05 or 0.2 μM PAC. Error bars represent s.e.m. (n>20).
(c) qRT-PCR analysis of *HLS1* expression during hook development in wild type L*er* seedlings grown on control medium (M) or with 0.2 μM PAC, as well as in quintuple *della* seedlings. Thirty-six and 72 h data points were normalized to the expression value in the control wild type at the time point 18 h. Data represent mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results.

Figure 5. GAs regulate the differential auxin response in the apical hook.
(a,b) Expression pattern of *ProDR5:GUS* during hook development in seedlings grown on control media or with 0.2 μM PAC or 50 μM GA_3. Pictures of representative

seedlings are shown (a). The percentage of seedlings showing DR5 signal at the inner side of the hook is represented in (b). Data are mean of thee biological replicates, n>25 each. Error bars are s.d.
(c,d) Polar auxin transport mediates the GA regulation on hook development. Pictures of representative 1-day-old wild type Col-0 seedlings grown in control medium or with 50 μM GA_3, 5 μM NPA, or both (c). Hook angle of 1-day-old L*er* wild type and *della* seedlings grown in control medium or with 50 μM GA_3, 5 μM NPA, or both (d).
(e) Hook angle of 1-day-old Col-0 wild type and *hls1-1* seedlings grown in control medium or with 0.1 μM IAA, 0.2 μM PAC, or both. All error bars represent s.e.m. (n>20).

Figure 6. The contribution of PIN3 and PIN7 to GA-mediated hook development.
Kinematic analysis of hook development in Col-0 wild type and *pin3-3 pin7^En* double mutant seedlings grown on control medium or with 50 μM GA_3. Dotted vertical lines represent the transition between phases. Error bars represent s.e.m. (n>15).

Figure 7. GA activity in the endodermis controls hook development.
(a) Hook curvature was measured in 1-day-old L*er* wild type seedlings grown on control medium or in medium with 10 μM DEX or with 0.2 μM PAC; in *gai-1*, *ProML1:GFP-gai-1-4* and *ProML1:GFP-gai-1-11* (*ML1:gai*) seedlings grown on control medium, and in *ProSCR:gai-YFP-GR* (*SCR:gai-GR*) seedlings grown on control medium or with 10 μM DEX. All error bars represent s.e.m. (n>20).
(b,c) GUS staining of 1-day-old F1 etiolated seedlings from the crosses indicated in the main text, grown on control medium or in medium with 10 μM DEX or with 0.2 μM PAC (b). See a magnification of regions within orange squares in (c). Pictures of representative seedlings are shown.

Figure S1. The GA activity determines the speed of hook formation.

Kinematic analysis of hook development in L*er* wild type and *gai-1* seedlings grown on control medium or with 10 μM ACC. Dotted vertical lines represent the transition between phases. Error bars represent s.e.m. (n>20).

Figure S2. GAI and PIF5 interact in plant cells.

BiFC analysis in tobacco leaves between GAI and PIF5 fusions to N- and C-terminal fragments of YFP, respectively. Left, visible; right, YFP fluorescence.

Figure S3. GAs revert the PAC-effect on *ProACS5:GUS*, *ProACS8:GUS*, and *ProDR5:GUS*. Expression patterns of *ProDR5:GUS*, *ProACS5:GUS*, and *ProACS8:GUS* in seedlings grown on control medium, or on medium supplemented with 0.2 μM PAC or with 0.2 μM PAC plus 50 μM GA_3 for 36 h after germination.

Figure S4. The activity of PIF transcription factors mediate the GA control on hook development.

(a) qRT-PCR analysis of *ACS8* expression in 3-day-old wild type Col-0, *pif5*, and *Pro35S:PIF5-HA* seedlings grown on control medium (M) or with 0.2 μM PAC. All data were normalized to the expression value in the control wild type. Data represent mean and standard deviation of three technical replicates. Experiments were repeated twice with similar results.

(b) Hook angle of 3-day-old wild type Col-0, *pif5*, and *Pro35S:PIF5-HA* seedlings grown on control medium (M) or with 0.2 μM PAC. Error bars represent s.e.m. (n>20).

(c,d) Kinematic analysis of hook development in Col-0 wild type and *pif1/3/4/5* (c) and *pif3/4/5* (d) seedlings grown on control medium or with 50 μM GA_3. Dotted vertical lines represent the transition between phases. Error bars represent s.e.m. (n>20).

Figure S5. PIN3, PIN7, and the regulation of hook development by GAs.

(a,b) Expression patterns of *ProPIN3:GUS* (a) and *ProPIN7:GUS* (b) during hook development in seedlings grown on control medium or with 0.2 μM PAC or 50 μM GA_3.

(c,d) Kinematic analysis of hook development in Col-0 wild type and *pin3-5* (c) and *pin7-1* (d) seedlings grown on control medium or with 50 μM GA_3. Dotted vertical lines represent the transition between phases. Error bars represent s.e.m. (n>15).

Figure S6. Specific expression of GFP-gai-1 in the epidermis of etiolated seedlings.
Confocal image of a longitudinal section of the apical hook of a 1-day-old *ProML1:GFP-gai-1-11* seedling.

Figure S7. Models explaining the pathway interactions and the timing of GA and ethylene action.

(a,b) In etiolated seedlings proper activity of auxin is crucial for hook development (a). Its activity is sustained by GAs and ethylene at different levels, including auxin biosynthesis, transport, and response. Part of the GA control is exerted from the endodermis, for instance transcriptional regulation of *PIN3*. GAs and ethylene may exert this role independently or through common downstream signaling elements. Light act negatively on several branches of the hormonal network to promote hook opening. The contribution of the activity of the GA and ethylene pathways is different depending on the phase of hook development (b). GAs promote hook formation partly in a ethylene-independent manner, likely through HLS1, and the contribution of ethylene to this phase seems to be minor, whereas both pathways cooperate to prevent hook opening.

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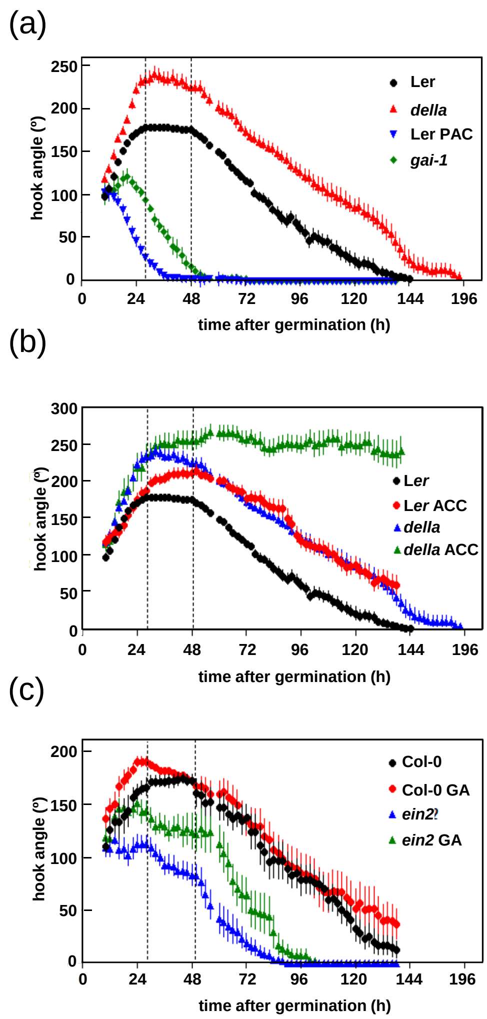

(a)
hook angle (º)
time after germination (h)
Ler
della
Ler PAC
gai-1
(b)
hook angle (º)
time after germination (h)
Ler
Ler ACC
della
della ACC
(c)
hook angle (º)
time after germination (h)
Col-0
Col-0 GA
ein2
ein2 GA

FIGURE 1

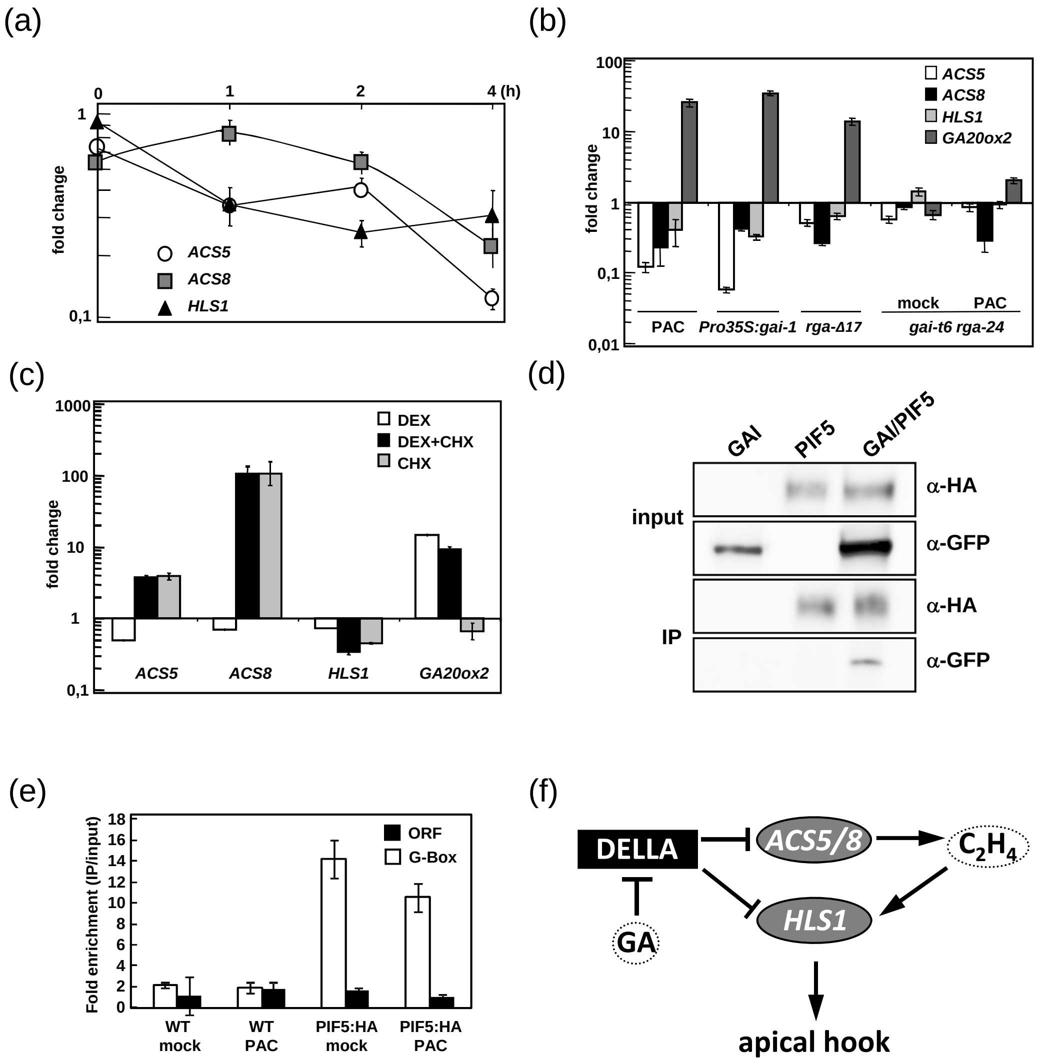
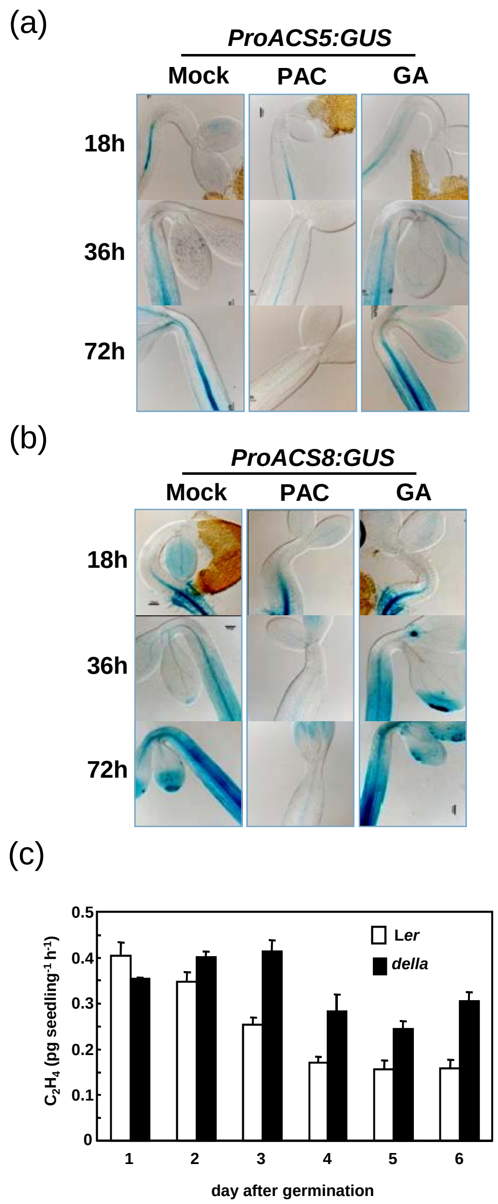

(a)
0
1
2
4 (h)
1
0,1
fold change
ACS5
ACS8
HLS1
(b)
100
10
1
0,1
0,01
fold change
ACS5
ACS8
HLS1
GA20ox2
PAC
Pro35S:gai-1
rga-Δ17
mock
PAC
gai-t6 rga-24
(c)
1000
100
10
1
0,1
fold change
DEX
DEX+CHX
CHX
ACS5
ACS8
HLS1
GA20ox2
(d)
GAI
PIF5
GAI/PIF5
input
IP
α-HA
α-GFP
α-HA
α-GFP
(e)
Fold enrichment (IP/input)
18
16
14
12
10
8
6
4
2
0
ORF
G-Box
WT mock
WT PAC
PIF5:HA mock
PIF5:HA PAC
(f)
DELLA
ACS5/8
C_2H_4
HLS1
GA
apical hook

FIGURE 2



(a)
ProACS5:GUS
Mock
PAC
GA
18h
36h
72h
(b)
ProACS8:GUS
Mock
PAC
GA
18h
36h
72h
(c)
C_2H_4 (pg seedling^{-1} h^{-1})
0.5
0.4
0.3
0.2
0.1
0
Ler
della
1
2
3
4
5
6
day after germination

FIGURE 3

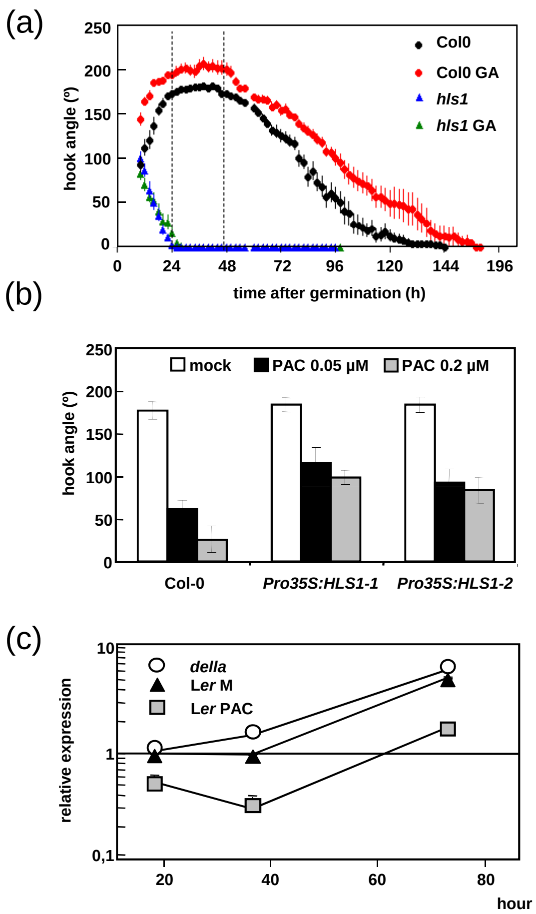

(a)
250
200
150
100
50
0
hook angle (º)
Col0
Col0 GA
hls1
hls1 GA
0
24
48
72
96
120
144
196
time after germination (h)
(b)
250
200
150
100
50
0
hook angle (º)
mock
PAC 0.05 µM
PAC 0.2 µM
Col-0
Pro35S:HLS1-1
Pro35S:HLS1-2
(c)
10
1
0,1
relative expression
della
Ler M
Ler PAC
20
40
60
80
hour

FIGURE 4

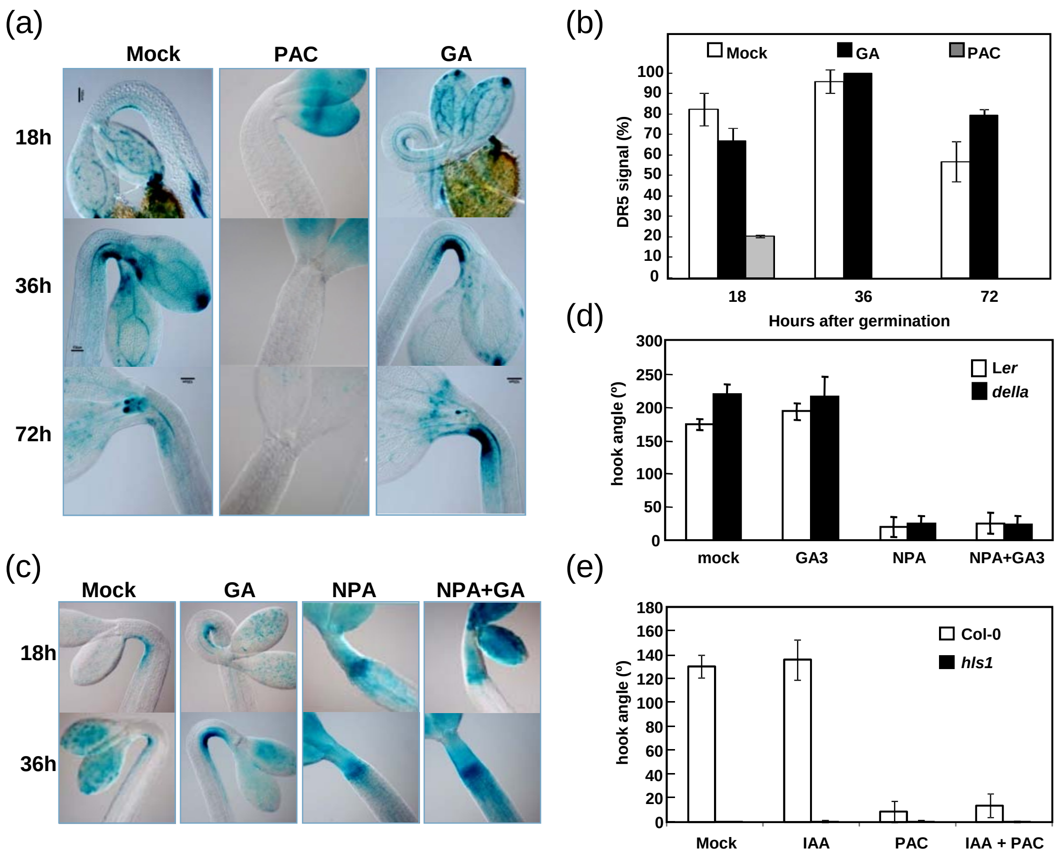

(a)
Mock
PAC
GA
18h
36h
72h
(b)
Mock
GA
PAC
DR5 signal (%)
18
36
72
Hours after germination
(c)
Mock
GA
NPA
NPA+GA
18h
36h
(d)
hook angle (º)
Ler
della
mock
GA3
NPA
NPA+GA3
(e)
hook angle (º)
Col-0
hls1
Mock
IAA
PAC
IAA + PAC

FIGURE 5

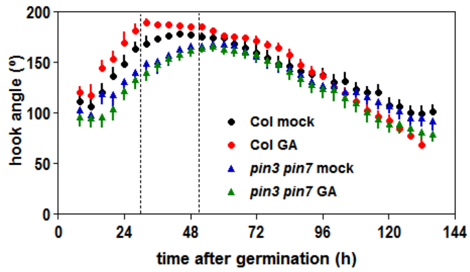

200
150
100
50
0
hook angle (°)
0
24
48
72
96
120
144
time after germination (h)
Col mock
Col GA
pin3 pin7 mock
pin3 pin7 GA

FIGURE 6

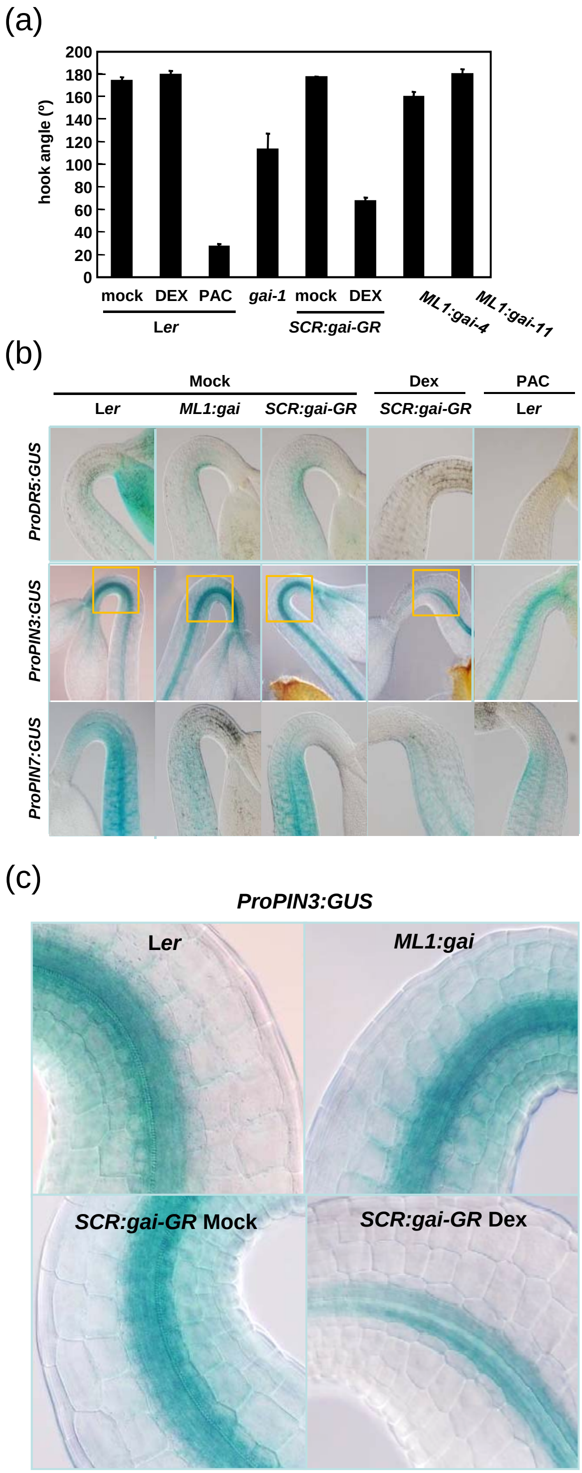

(a)
hook angle (°)
200
180
160
140
120
100
80
60
40
20
0
mock DEX PAC gai-1 mock DEX ML1:gai-4 ML1:gai-11
Ler
SCR:gai-GR
(b)
Mock
Dex
PAC
Ler
ML1:gai
SCR:gai-GR
SCR:gai-GR
Ler
ProDR5:GUS
ProPIN3:GUS
ProPIN7:GUS
(c)
ProPIN3:GUS
Ler
ML1:gai
SCR:gai-GR Mock
SCR:gai-GR Dex

FIGURE 7

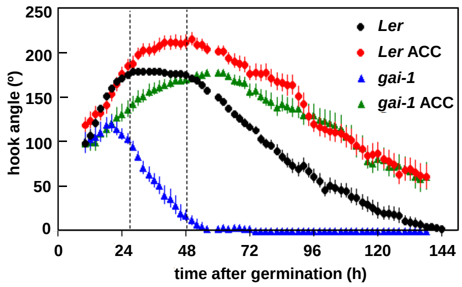

250
200
150
100
50
0
hook angle (°)
0
24
48
72
96
120
144
time after germination (h)
Ler
Ler ACC
gai-1
gai-1 ACC

FIGURE S1

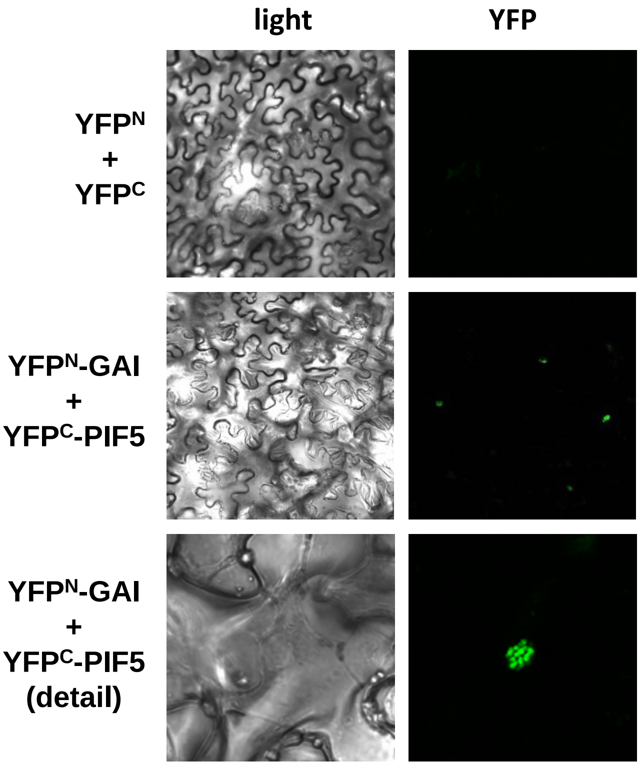

light
YFP
YFP^N + YFP^C
YFP^N-GAI + YFP^C-PIF5
YFP^N-GAI + YFP^C-PIF5 (detail)

FIGURE S2

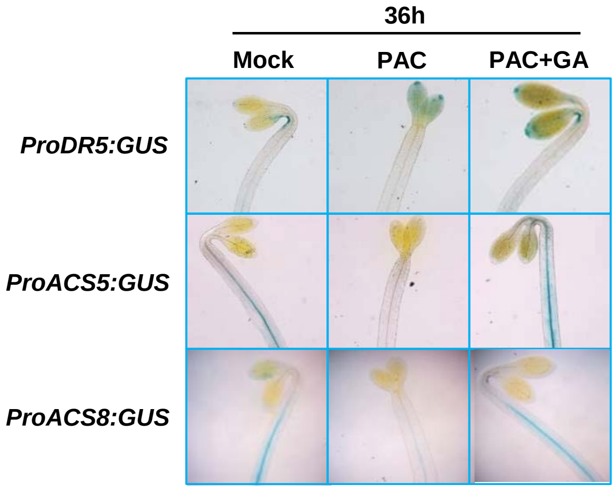


FIGURE S3

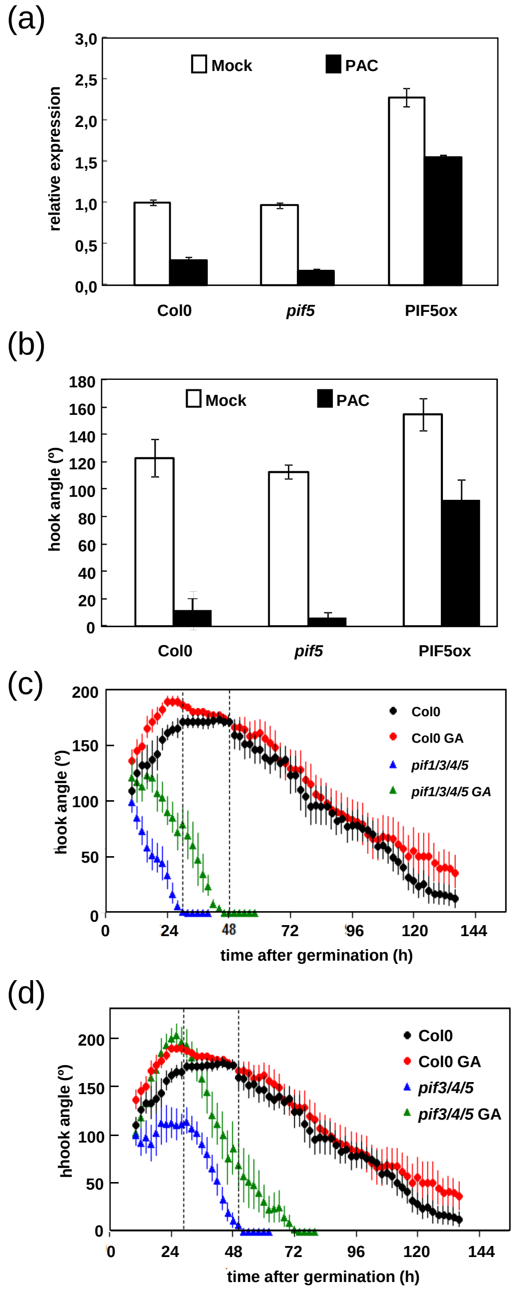
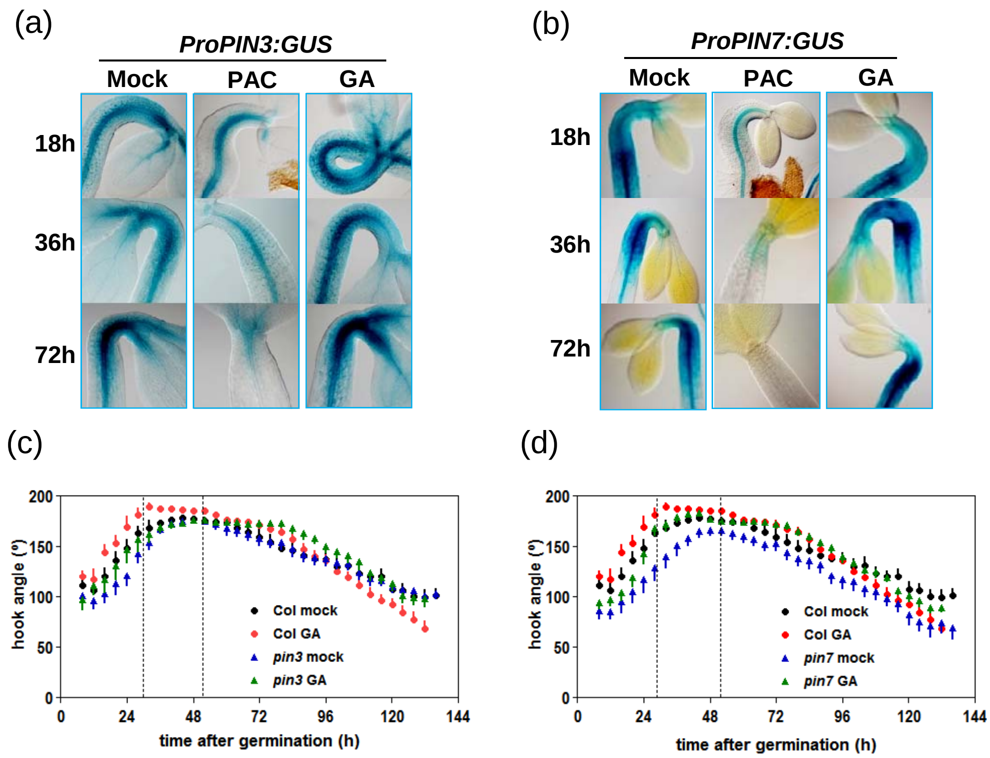

(a)
relative expression
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Mock
PAC
Col0
pif5
PIF5ox
(b)
hook angle (º)
180
160
140
120
100
80
60
40
20
0
Mock
PAC
Col0
pif5
PIF5ox
(c)
hook angle (º)
200
150
100
50
0
Col0
Col0 GA
pif1/3/4/5
pif1/3/4/5 GA
0
24
48
72
96
120
144
time after germination (h)
(d)
hhook angle (º)
200
150
100
50
0
Col0
Col0 GA
pif3/4/5
pif3/4/5 GA
0
24
48
72
96
120
144
time after germination (h)

FIGURE S4



(a)
ProPIN3:GUS
Mock
PAC
GA
18h
36h
72h
(b)
ProPIN7:GUS
Mock
PAC
GA
18h
36h
72h
(c)
hook angle (°)
time after germination (h)
Col mock
Col GA
pin3 mock
pin3 GA
(d)
hook angle (°)
time after germination (h)
Col mock
Col GA
pin7 mock
pin7 GA

FIGURE S5

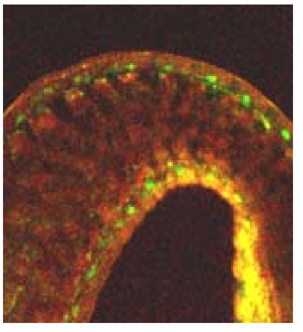
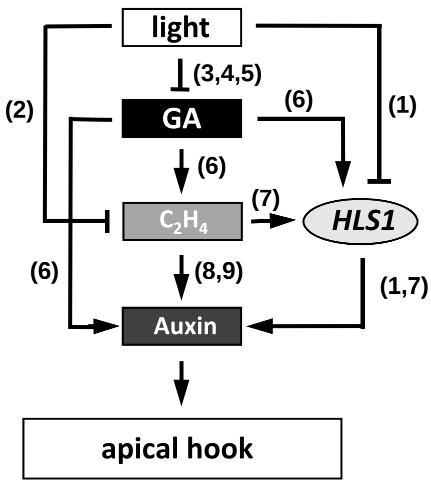


FIGURE S6

(a)


light
(3,4,5)
(2)
GA
(6)
(1)
(6)
C_2H_4
(7)
HLS1
(6)
(8,9)
(1,7)
Auxin
apical hook

(b)

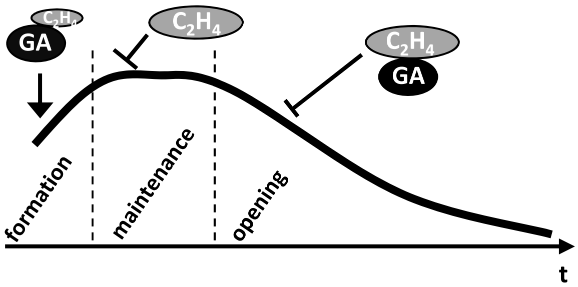

GA
C_2H_4
C_2H_4
C_2H_4
GA
formation
maintenance
opening
t

FIGURE S7