Gibberellins modulate light signaling pathways to prevent *Arabidopsis* seedling de-etiolation in darkness.

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Summary

In many plants, photomorphogenesis is the default developmental program after seed germination, and provides the key features that allow adaptation to light. This program is actively repressed if germination occurs in the absence of light, through a mechanism dependent on the E3 ubiquitin ligase activity encoded in *Arabidopsis* by COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1), which induces proteolytic degradation of transcription factors necessary for light-regulated development, such as HY5 (LONG HYPOCOTYL 5) and HYH (LONG HYPOCOTYL 5 HOMOLOG), and stabilization of transcription factors that promote skotomorphogenesis, such as PIF3 (PHYTOCHROME INTERACTING FACTOR 3). Seedlings deficient in gibberellin (GA) synthesis or signaling display a de-etiolated phenotype when grown in darkness, equivalent to the phenotype of *cop1* mutants, which indicates that the switch between photo- and skotomorphogenesis is also under hormonal control. Here we provide evidence for the existence of an interplay between GA and the COP1-mediated pathway, and identify HY5 and the PIF family as nodes of a regulatory network. This interaction occurs through distinct molecular mechanisms, based on the observation that GA signaling regulates HY5- but not PIF3- protein stability.

Introduction

Plant development is mostly post-embryonic. The basic axes of the plant body are established during embryo development, with a short root and the root apical meristem at one end, and with hypocotyl, cotyledons, and shoot apical meristem at the other. Growth and production of all new organs begins after germination. This, together with the sessile life style of plants, implies that they have multiple opportunities to modulate growth rate and development depending on the changing environmental conditions. Plants have developed complex systems to constantly monitor their surrounding environment. This information is integrated by endogenous cues such as hormones or the circadian clock to accordingly adjust their growth and development. This ability of plants is referred to as plasticity. The current hypothesis is that it is due to a complex web of interactions between signaling pathways coupling endogenous and environmental cues (Casal *et al.*, 2004).

The earliest example of plasticity in plant development occurs just after germination. Seedlings follow skotomorphogenic development if seeds germinate in the dark, whereas the alternative developmental program, photomorphogenesis, is triggered if seeds germinate in the light (Neff *et al.*, 1999). Light signaling initiated at the various photoreceptors conveys the inactivation of COP1, which acts as a global repressor of photomorphogenesis, this program being therefore the default pathway after germination (Huq, 2006; Wei *et al.*, 1994). Accordingly, dark-grown mutant seedlings defective in COP1 activity resemble wild-type seedlings grown in the light (Deng *et al.*, 1991). COP1 is an E3-ubiquitin ligase that after germination in darkness targets for degradation transcription factors that promote photomorphogenesis, whereas it allows accumulation of others that promote etiolated growth (Huq, 2006; Lorrain *et al*., 2006). The former group includes *LAF1* (*LONG AFTER FAR-RED LIGHT 1*), *HFR1* (*LONG HYPOCOTYL IN FAR-RED LIGHT 1*), *HYH*, and *HY5* (Ballesteros *et al.*, 2001; Duek and Fankhauser, 2003; Holm *et al.*, 2002; Oyama *et al.*, 1997). Mutant

seedlings deficient in any of these transcription factors are hyposensitive to light-induced de-etiolation, although this defect depends in some cases of the light quality. For example, *laf1* mutants do not respond properly to far-red light, while *hy5* mutants show defects under all light qualities tested (Ballesteros *et al.*, 2001; Koornneef *et al.*, 1980). The latter group includes PIF1, PIF3, and PIF4/SRL2, and mutant seedlings deficient in any of them are hypersensitive to light-induced de-etiolation. These activities also show preferences for different qualities of light; for instance, *srl2*/*pif4* and *pif3* mutants are hypersensitive to red light, whereas *pif1* mutants are hypersensitive to both red and far-red light (Huq and Quail, 2002; Kim *et al.*, 2003; Oh *et al.*, 2004; Shen *et al.*, 2005).

De-etiolation is also controlled by endogenous cues such as hormones. Different studies have shown that correct hormone homeostasis in etiolated seedlings is essential to properly control the transition between skotomorphogenesis and photomorphogenesis (Vandenbussche *et al.*, 2005). For instance, plants defective in either gibberellin (GA) or brassinosteroid metabolism or signaling are not able to fully repress photomorphogenesis after germination in darkness, and seedlings appear partially de-etiolated, *i.e.* they lose their apical hook, show open cotyledons, and expression of genes typically upregulated by light is elevated (Achard *et al.*, 2003; Alabadí *et al.*, 2004; Li *et al.*, 1996; Skezeres *et al.*, 1996; Vriezen *et al.*, 2004).

Is this developmental transition controlled independently by plant hormones and light? Or do they exert joint control on this process? We have addressed this question by studying whether the GA and light signaling pathways interact in the control of this developmental switch. We show that there exists interaction, and that GAs control this process by modulating the activity of the light signaling elements HY5 and PIFs, which therefore represent integration nodes for both pathways. These interactions, revealed in the context of photomorphogenic development, might also extend to other stages of plant development.

Results and discussion

GA repression of photomorphogenesis in darkness coincides with COP1 action

After germination in darkness, the activity of COP1 is critical during the first three days to establish the proper seedling developmental program –skotomorphogenesis *vs* photomorphogenesis (Hsieh *et al.*, 2000; Ma *et al.*, 2002). To establish whether GA and COP1 signaling exert joint control of the transition between these two alternative programs, we tested whether GA action was also restricted to the window of COP1 activity, or it would be continuously required during the whole period of etiolated growth. Interestingly, seedlings displayed a de-etiolated phenotype as long as GA biosynthesis had been prevented during only the first two days of growth after germination, as estimated by hypocotyl length, cotyledon opening, or *CAB2* expression (Figures 1a and 1b). This suggests that active GA biosynthesis during the first three days after germination in darkness is important in order to promote etiolated growth.

Nonetheless, to rule out the possibility that GAs accumulated during the first three days were enough for seedlings to undergo complete etiolation, we designed a second strategy in which GA signaling, instead of GA biosynthesis, was blocked. For that purpose, we prepared *Arabidopsis* transgenic lines expressing a dominant version of the negative GA signaling element GAI (GA INSENSITIVE) (Peng *et al.*, 1997), *gai-1*, under the control of a heat-shock inducible promoter (*Hsp*) (Matsuhara *et al.*, 2000). The *gai-1* mutation had been shown to confer partial de-etiolation in darkness, indicating that GAI participates in the GA signaling pathway controlling this response (Alabadí *et al.*, 2004). Three-day-old dark-grown *Hsp::gai-1* seedlings strongly and transiently expressed the *gai-1* mRNA in response to a 3 hr heat-shock treatment at 37ºC (Figure S1). Most notably, dark-grown *Hsp::gai-1* seedlings subject to a daily 3-hr heat-shock treatment starting 3 days after germination showed an etiolated

phenotype, whereas those that received the heat-shock starting 1 or 2 days after germination showed clear de-etiolation (Figures 1c and 1d). The reverse experiment supported the hypothesis of a temporal window for GA action, since a daily heat-shock treatment applied during the first 3 days after germination was enough to induce a strong de-etiolated phenotype, identical to control seedlings that received the heat-shock during 8 days, while seedlings that received the heat-shock only on the first and second days after germination were etiolated (Figure S2).

These results define a time limit of three days after germination during which GA activity determines, together with COP1, the nature of the developmental program that seedlings will follow. If this temporal coincidence truly reflects interaction between both pathways, then, according to the current model of COP1 repression of light signaling, one or more of the transcription factors regulated by COP1 would be expected to mediate the de-etiolation caused by reduced GA levels or signaling. They would represent integration nodes for the GA and light signaling pathways in the control of photomorphogenesis. Therefore, we surveyed the phenotype of *Arabidopsis* mutants defective in the activity of these transcription factors when GA synthesis was compromised in darkness.

The GA pathway targets HY5 activity to repress photomorphogenesis in darkness

Several genes are known to encode transcription factors required to establish photomorphogenesis: *LAF1*, *HFR1*, *HYH*, and *HY5* (Ballesteros *et al.*, 2001; Duek and Fankhauser, 2003; Holm *et al.*, 2002; Oyama *et al.*, 1997). Mutants defective in these genes do not de-etiolate properly in the light, and genetic analyses with some of these mutants have shown that loss-of-function alleles in these genes partially suppress the de-etiolated phenotype caused by *cop1* mutations in darkness (Kim *et al.*, 2002). Seedlings harboring loss-of-function mutations in *HFR1* and *LAF1* showed a de-etiolated phenotype in darkness in the presence of

1 µM paclobutrazol (PAC) which was not different to the phenotype of their corresponding wild-types (data not shown). However, the de-etiolation caused by PAC was partially suppressed in *hy5* mutants (Figures 2a, 2b, 3, and S3), although this ability was dependent on the genetic background. For example, *hy5-215* (Ang and Deng, 1994) and *hy5-ks50* (Oyama *et al.*, 1997) null alleles, in Columbia-0 (Col-0) and Wassilewskija (Ws) genetic backgrounds respectively, strongly suppressed the cotyledon opening phenotype caused by 1 µM PAC-treatment, contrasting with the weaker effect of *hy5-1* null mutant (Koornneef *et al.*, 1980), in Landsberg *erecta* (L*er*) genetic background. On the other side, only the *hy5-215* allele was able to partially suppress the hypocotyl growth arrest (Figure S3 and data not shown). The ability to suppress these phenotypes was also observed at lower doses of PAC, mainly in the Col-0 and Ws alleles (Figure S3 and data not shown). These differences depending on the genetic background are consistent with the strong component of natural genetic variation found in this GA response in *Arabidopsis* (D.A. and M.A.B., unpublished data). Therefore, HY5 activity is limiting for cotyledon opening and hypocotyl growth arrest in a physiological context with reduced GA levels.

Conversely, a transgenic line hypermorphic for HY5 activity, *HY5::S36A* (Hardtke *et al.*, 2000), as well as a transgenic line over-expressing the wild-type version of the protein from a constitutive promoter, *35S::HY5* (Ang *et al.*, 1998), were hypersensitive to a block in GA biosynthesis in darkness for the cotyledon opening trait (Figures 2a and 2b). The hypersensitivity was also observed at lower doses of PAC (data not shown). In both cases, the phenotype was opposite to that of the null *hy5* mutants. Interestingly, hyperactivity of the *HY5::S36A* transgene had been previously described only in the light, when COP1 is inactive (Hardtke *et al.*, 2000), whereas *35S::HY5* lines showed a wild-type phenotype both in the light and in the dark (Ang *et al.*, 1998). However, our results reveal their hyperactivity in

darkness in a GA-deficient physiological context, suggesting that the GA pathway may have a negative effect on HY5 levels or activity in etiolated seedlings.

We also studied these morphological traits in dark-grown, PAC-treated *hyh* mutant seedlings, which carry a null allele for the closest *HY5* homolog, *HYH* (in Ws background) (Holm *et al.*, 2002). This mutation did not affect hypocotyl growth (data not shown); however, contrary to *hy5*, loss of *HYH* function caused a hypersensitive response to PAC-treatment for cotyledon opening (Figure 3). This effect required the presence of HY5, as shown by epistasis analysis of *hy5 hyh* double mutants (Figure 3). This suggests that HYH may negatively regulate HY5 activity regarding cotyledon opening, at least in response to low GA levels. These two proteins interact in vivo (Holm *et al.*, 2002), and this result illustrates a specific effect of this interaction that may be relevant for the control of photomorphogenesis, and that seems to be intrinsically different of their redundant role as negative regulators of auxin signaling (Sibout et al., 2006).

Consistent with a broad involvement of HY5 in GA-mediated repression of photomorphogenesis, *hy5* mutants showed reduced expression of *CAB2* in response to several doses of PAC compared to the corresponding wild-type, whereas they were not affected in *RbcS* expression at any concentration of PAC tested (Figures 2c, 2d, S3 and data not shown). On the other side, *hyh* mutation did not affect the expression of any of the two markers, and seedlings of the *hy5-ks50 hyh* double mutant showed the same phenotype as the single *hy5-ks50* (Figure S3).

These results contrast with previous observations that etiolated *hy5* mutants did not show any defect in *CAB2* expression in response to a short red-light pulse (Anderson *et al.*, 1997), and with the dependency of the *RbcS* promoter activity upon HY5 in response to continuous light of different qualities (Osterlund *et al.*, 2000a). Our results suggest that distinct physiological conditions allow the identification of different limiting components of

the signaling network that controls the light-regulated switch between developmental programs.

The genetic evidence for the involvement of HY5 in the regulation of photomorphogenesis by GA points to the possibility that the GA pathway negatively regulates HY5 in darkness. A mechanism for this interaction is provided by the observation that HY5 protein accumulates in GA-deficient conditions in darkness (Figure 4b) without affecting *HY5* mRNA levels (Figure 4a). This accumulation was not apparent in seedlings over-expressing the potato ortholog of the positive GA signaling element *SLY1* (*SLEEPY 1*) (McGinnis *et al.*, 2003) (Figure 4b), which supports the participation of GA signaling in the regulation of HY5 protein levels. It is very likely that GA regulates HY5 stability through the modulation of COP1 activity, since neither exogenous GA nor PAC application affected HY5 levels in dark-grown seedlings of the weak allele *cop1-4* (Figures 4c and 4d). Moreover, COP1 protein levels were not significantly affected in response to altered GA levels in dark-grown seedlings (Figure 4e).

Our results suggest that HY5 acts as a target for the integration of multiple signaling pathways (including GA and light), a view which is in consonance with previous observations that HY5 also mediates the effect of exogenous cytokinins in blue-light induced accumulation of anthocyanin (Vandenbussche *et al.*, 2007).

The GA pathway enhances the activity of PIF transcription factors to promote etiolated growth

Opposite to HY5, which has a positive role on photomorphogenesis, other proteins such as PIF1, PIF3, and PIF4 (Huq and Quail, 2002; Kim *et al.*, 2003; Monte *et al.*, 2004; Shen *et al.*, 2005) have been proposed to also regulate skotomorphogenesis (Lorrain et al., 2006), based for instance on the phenotype of *pif1* mutants in darkness (Huq et al., 2004; Oh et al., 2004).

Besides, PIF1 and PIF3 accumulate in etiolated seedlings, and this accumulation has been shown to depend on COP1 at least for PIF3 (Bauer *et al.*, 2004; Park *et al.*, 2004; Shen *et al.*, 2005). Consistent with the hypothesis that GAs would regulate etiolated growth by interfering with light signaling elements, dark-grown seedlings of *pif1-1*, *pif1-2*, *pif3-1*, and *pif4*/*srl2* null alleles showed enhanced cotyledon opening and enhanced hypocotyl growth arrest in response to PAC treatment compared to the corresponding wild-type (Figures 5a-c and S4). The opposite phenotype for both traits was observed in a line over-expressing *PIF3* (Figures 5a-c and S4) (Kim *et al.*, 2003). Further support for the connection between GA and PIFs in the control of gene expression comes from the observation that genes regulated by PIF3 in darkness (Monte et al., 2004) are affected by GA in an equivalent way (Figure 5d). For instance, *ELIP-A* and *LhcB1.4*, two genes repressed by PIF3 in darkness were also repressed by GA, while two genes whose expression is induced by PIF3 in darkness (At1g55240 and At2g17500) were also upregulated by GA. As expected, *LHY*, a light-regulated gene whose expression is not dependent on PIF3, was not affected by PAC either.

These results are consistent with a model in which the promotion of growth by GAs would be mediated, to a large extent, by the positive regulation of the PIF proteins. In fact, this model is supported by the observation that the over-expression of *StSLY1* causes a PIF3-dependent resistance to de-etiolation in the absence of GA (Figure 6a and b). Since the negative GA signaling elements GAI and RGA (REPRESSOR OF *ga1-3*) are the main targets for SLY1 (Dill *et al.*, 2004; Fu *et al.*, 2004), and GAI and RGA mediate GA repression of photomorphogenesis in darkness (Alabadí *et al.*, 2004), it is reasonable to think that both proteins may participate in directly or indirectly regulating PIFs' activity. In fact, seedlings over-expressing *gai-1* (*35S::gai-1*) phenocopied *pif1-2* null mutant seedlings regarding chlorophyll accumulation in response to white light-induced de-etiolation (Figure 6c) (Huq *et al.*, 2004).

The mechanism by which the GA pathway would have a positive effect on PIFs' activity does not involve transcriptional regulation of these genes because the mRNA levels of *PIF1*, *PIF3*, and *PIF4* were similar in dark-grown, PAC-treated or untreated seedlings (Figure S5). To gauge the effect of GA activity on PIFs' protein levels, we used a transgenic *Arabidopsis* line over-expressing a myc-tagged version of PIF3 from the *35S* promoter (*PIF3-myc*) (Park *et al.*, 2004). Remarkably, PIF3-*myc* protein concentration was not reduced when GA synthesis was blocked. On the contrary, it seemed to be higher than in control seedlings (Figure 7a). Light induces PIF3 phosphorylation during seedling de-etiolation prior to degradation by the proteoseome (Al-Sady *et al.*, 2006), yet it seems that GA do not exert this control over PIF3 in dark-grown seedlings, since no slower migrating bands could be detected after running gels longer (data not shown). According to the genetic data shown above, this accumulated PIF3-*myc* protein should represent an inactive or less active version of the protein, or may be part of a higher order complex that inactivates the protein or reduces its activity. To examine this possibility, we examined the ability of PIF3 to induce the expression of two of its target genes (*ELIP-A* and *LhcB1.4*) in response to 1-hr red-light treatments (Figure 7b). In both cases, PAC impaired the observed PIF3-dependent rapid upregulation of these genes. As a control, *LHY* induction by light, which does not depend on PIF3, was not affected. In addition, at this stage of seedlings' life, the regulation of PIF3 by GA seems to be only relevant for etiolated growth, given that PAC-treatment did not affect the red-light induced degradation of the fusion protein (Figure 7a). It is important to remark that in other stages of development, such as during germination, regulation of the expression of DELLA genes by PIL5 (another member of the PIF family) is particularly relevant (Oh *et al.*, 2007), which indicates the high degree of interconnectivity between at least these two signaling pathways, and we cannot rule out that this phenomenon is also observed during de-etiolation.

All together, our results suggest that the GA pathway promotes etiolated growth by preventing the accumulation of an inactive form of the involved PIF proteins in dark-grown seedlings, a mechanism that is intrinsically different from the GA-dependent accumulation of HY5.

Light regulates the GA pathway during de-etiolation

How relevant is the observed modulation by GAs of the light signaling elements HY5 and PIFs in a natural context? The fate of etiolated seedlings in nature is to de-etiolate; thus, it is reasonable to think that if the mechanisms shown here to operate in darkness are physiologically relevant for the choice of the appropriate developmental program, the effect of GA regulation on light signaling should also be manifest upon the illumination of etiolated seedlings. Two pieces of evidence seem to support this view; first, light caused a dramatic and transient down-regulation of the expression of four of the genes encoding key enzymes in the GA biosynthetic pathway (*AtGA20ox1, 2* and *3*, and *AtGA3ox1*) (Figure 8a; see also Achard *et al.*, 2007). Simultaneously, expression of several genes encoding GA inactivating enzymes was increased, especially that of *AtGA2ox1*, whose transcript levels increased over two orders of magnitude in two hours (Figure 8a). Interestingly, transient downregulation of GA concentration upon illumination is not a species-specific regulation, since it is also observed in pea plants (Reid *et al*., 2002), in which GAs are the main hormones regulating photomorphogenesis (Alabadí *et al*. 2004). This transcriptional regulation presumably results in a depletion of active GAs in *Arabidopsis*, as shown in pea (Gil and García-Martínez, 2000; Folta *et al.*, 2003). Indeed this may be the case, since the hypocotyl growth arrest during blue light induced de-etiolation in *Arabidopsis* is dependent on GA levels (Folta *et al.*, 2003), and a GFP fusion of the DELLA protein RGA accumulates in elongating cells of hypocotyl 2 hours after transferring to light etiolated *Arabidopsis* transgenic seedlings (Achard *et al.*, 2007). Moreover, physiological relevance is not restricted to the control of cell expansion, as indicated by the observation that the kinetics of white light-induced expression of *CAB2* was delayed when seedlings were forced to undergo de-etiolation in the presence of exogenous

GA_3 (Figure 8b), or as shown above, the pace of chlorophyll accumulation in response to white light-induced de-etiolation is altered in *35S::gai-1* seedlings compared to the wild-type (Figure 6c).

A molecular model for the interaction between light and gibberellins for the control of photomorphogenesis

In summary, we propose that the accurate establishment of the most appropriate developmental program in an emerging seedling requires plastic interactions between light signaling and GAs that operate through at least two molecular mechanisms (Figure 8c): on one hand, the regulation of HY5 protein levels by COP1 and GAs, which determines the degree of activation or repression of photomorphogenesis; and, on the other hand, the regulation of protein concentration of the PIF transcription factors by COP1 with the additional level of regulation that represents the modification by GAs of PIFs' activity. A likely mechanism for this modification is provided by the physical interaction observed in vivo between DELLA and PIF proteins (Feng and Deng, unpublished; S. Prat, personal communication). In darkness, the high level of COP1 and GA signaling results in complete repression of photomorphogenesis caused by low levels of HY5; and skotomorphogenesis is allowed by the low concentration of DELLA proteins, which permits the activity of the PIF transcription factors. Upon illumination, the switch to photomorphogenic development is triggered by inactivation of COP1 signaling and transient accumulation of DELLA proteins, which result in instability and impairment of PIF activity, and also on accumulation of HY5. An implication of these findings is the identification of HY5 and the PIF proteins as two of the transcription factors that ultimately exert the regulation of gene expression in response to GA signaling. It is also important to remark that the interplay between light and GAs governs the whole plant life cycle, which implies that the interactions revealed in the context of photomorphogenic development very likely extend to other stages of plant development such as diurnal control of growth and shade avoidance (Djakovic-Petrovic *et al.*, 2007; Lorrain *et al.*, 2007).

Experimental Procedures

Plant strains and growth conditions

Arabidopsis thaliana accessions Col-0, L*er*, and WS were used as wild-type. Seeds were sown on sterile Whatman filter papers, placed in plates of 1/2 MS medium (Duchefa, Haarlem, The Netherlands), 0.8% (w/v) agar, 1% (w/v) sucrose, and stratified at 4°C for 6 days in darkness. Germination was induced by placing the plates for 8 hr under white fluorescent light (90-100 μmol s^{-1} m^{-2}) at 20ºC in a Percival E-30B. Next, plates were wrapped in several layers of aluminum foil and kept in darkness at 20ºC for the duration of the experiment. In experiments involving chemical treatments, filter papers harboring the seeds were transferred to control or treatment plates at the end of the 8 hr period of white light, then plates were wrapped in several layers of aluminum foil and kept in darkness at 20ºC for the duration of the experiment. Control plates for PAC treatments (1 μM; Duchefa, Haarlem, The Netherlands) contained 0.01% acetone (v/v, final concentration), whereas those involving GA_3 (10 μM; Duchefa, Haarlem, The Netherlands) contained 0.014% ethanol (v/v, final concentration).

De-etiolation was induced in white fluorescent light (90-100 μmol s^{-1} m^{-2}) or red-light (8-10 μmol s^{-1} m^{-2}). For red-light treatments, seedling plates were placed within a black box covered with an R (600-700 nm) filter (Carolina Biological Supply Co, Burlington, NC, USA). Manipulation of seedlings in darkness was performed under dim green safelight (560 nm, 15 nm half-band, <0.05 μmol s^{-1} m^{-2}) (Gil and García-Martínez, 2000).

Construction of vectors and generation of transgenic lines

To obtain the *Hsp::gai-1* and *35S::gai-1* constructs, the *gai-1* coding region was amplified by PCR from genomic DNA of the *gai-1* mutant with primers MB89 (5'-

GGGATCCGATGAAGAGAGATCATCATCA-3') and MB90 (5'-CCGGATCCGATGCATCTAATTGGTGGAGAGTTTC-3'), and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). Insert was excised either by *Nsi*I digestion and inserted into pCHF3 (Fankhauser *et al.*, 1999) cut with *Pst*I, to give rise the *35S::gai-1* construct, or by *Bam*HI and inserted into *Bam*HI-digested pTT101 (Matsuhara *et al.*, 2000), to give rise the *Hsp::gai-1* construct.

To obtain the *StSLY1ox* construct, a potato *SLY1* homolog (sharing 55% identity and 66% homology at the amino acid level with the *Arabidopsis* gene) was identified by searching the TIGR potato database (accession number TC112417). The coding region of this gene was amplified from a potato first strand cDNA pool using primers SLY5 (5´-ATGAAGCGGCAATTCGACGCCGGA-3') and SLY3 (5´-ACAGTAAAACCCAAACCTTAAGC-3'). The resulting PCR product was cloned into the pTZ57R/T vector (Fermentas, Burlington, Canada), excised from this plasmid by digestion with *Eco*RI/*Xba*I and then inserted into the pBinAR (Höfgen and Willmitzer, 1990) vector cut with these enzymes. All constructs were verified by sequencing.

Arabidopsis Col-0 plants were transformed with the various constructs by *Agrobacterium*-mediated DNA transfer (Clough and Bent, 1998). Transgenic seedlings in the T1 and T2 generations were selected by their resistance to the kanamycin antibiotic. Transgenic lines with segregation ratio 3:1 (resistant:sensitive) were selected, and several homozygous lines were identified in the T3 generation for each construct. Data from one representative line per construct are shown.

Protein extraction and western blots

Total proteins for analysis of HY5 and COP1 accumulation were extracted by homogenizing seedlings in 1 volume of cold extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1

mM EDTA, 1 mM DTT, 10% glycerol, 1 mM PMSF, and 1x complete protease inhibitor cocktail [Roche, Barcelona, Spain]). Extracts were centrifuged at 13.000 x g for 10 min at 4ºC. Protein concentration in supernatants was quantified by Bradford assay. Thirty μg of denatured total protein were separated in Novex® 4-20% Tris-Glycine gels (Invitrogen, Carlsbad, CA, USA) and transferred onto PVDF membrane (Bio-Rad, Barcelona, Spain).

Total proteins for analysis of PIF3-myc protein were extracted by homogenizing seedlings in 1 volume of cold extraction buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl pH 8.0, 8 M urea). Extracts were centrifuged at 13.000 x g for 10 min at 4ºC. Protein concentration in the supernatants was quantified using the *RC DC* Protein Assay (Bio-Rad, Barcelona, Spain). Forty μg of denatured total proteins were separated in Precise™ 8% Tris-HEPES-SDS gel (Pierce, Rockford, IL, USA) and transferred onto PVDF membrane (Bio-Rad, Barcelona, Spain).

Double mutant construction

Plants expressing the *StSLY1ox* transgene in the *pif3-1* mutant background were obtained by genetic crosses. Plants carrying the *StSLY1ox* transgene were selected among F2 seedlings based on their ability to grow better than the wild-type in the presence of PAC. Twenty plants with the widest rosette were selected, and the presence of the *StSLY1ox* transgene was verified by PCR using the above mentioned primers, SLY5 and SLY3, which did not amplify any PCR product from wild-type or *pif3-1* genomic DNA. Plants carrying the transgene were transferred to soil and genotyped for the *pif3-1* mutation, which is caused by a T-DNA insertion in the coding region, as described (Kim *et al.*, 2003). Three *pif3-1* homozygous plants were selected. Plants homozygous *pif3-1 StSLY1ox* were selected by phenotypic analysis in the F3 families. Seeds of each of the three F3 families were sown in 1/2 MS medium, 0.8% (w/v) agar, 1% (w/v) sucrose, containing 1 μM PAC and grown for 7 d in

darkness. The selection was based in the different hypocotyl phenotype of *pif3-1* and *StSLY1ox* plants when grown under this condition (see Figure 6A). After analyzing 50-60 F3 seedlings of each family, all seedlings from one of them were tall indicating that those seedlings were homozygous for the transgene.

"Real-time" quantitative RT-PCR

Total RNA extraction, cDNA synthesis, and quantitative PCR as well as primer sequences for amplification of GA metabolism and *EF1-α* genes has been described (Frigerio *et al.*, 2006). Primers for analyzing mRNA levels of *PIF1*, *PIF3*, *PIF4*, *ELIP-A*, *LhcB1.4*, *At1g55240*, *At2g17500*, and *LHY* by quantitative PCR are: PIF1f (5'-GTTGCTTTCGAAGGCGGTT-3') and PIF1r (5'-GCGCTAGGACTTACCTGCGT-3'), PIF3f (5'-CCACGGACCACAGTTCCAAG-3') and PIF3r (5'-ATCGCCACTGGTTGTTGTTG-3'), PIF4f (5'-GAGATTTAGTTCACCGGCGG-3') and PIF4r (5'-GGCACAGACGACGGTTGTT-3'), ELIPaf (5'-CGGTACAACAGCGATCTTGACA-3') and ELIPar (5'-CAACGCTTATGCCCTTGAAAA-3'), LhcB1.4f (5'-CGGCCTCCGAAGTATTTGG-3') and LhcB1.4r (5'-GGTGGGCTTGGAGGCTTT-3'), At1g55240f (5'-CCATCCCTTTGACGTCGATG-3') and At1g55240r (5'-CCGTGCTCGATCCAGGACTA-3'), At2g17500f (5'-CCAGCAATCTGCGATGAGG-3') and At2g17500r (5'-GGACCCTTTAATCAGCCGGA-3'), LHYf (5'-ACGAAACAGGTAAGTGGCGACA-3') and LHYr (5'-TGGGAACATCTTGAACCGCGTT-3').

To analyze expression of transgenic *gai-1* in the *Hsp::gai-1* seedlings, we used an oligonucleotide annealing in the 5'-UTR of the *HSP18.2* gene, which is included in the construct, as forward primer (5'-CCCGAAAAGCAACGAACAAT-3'), and an oligonucleotide annealing in the *gai-1* coding region as reverse primer (5'-

TCATTCATCATCATAGTCTTCTTATCTTGA-3'). Expression of *EF1-α* was used to normalize all expression data, as described in Frigerio *et al.* (2006).

Analysis of chlorophyll content.

Chlorophyll levels were measured as described by Neff and Chory (1998).

Supplementary Material

The following supplementary material is available with this article online:

Figure S1. Transgenic *gai-1* transcript levels transiently increase in response to a heat-shock treatment.

Figure S2. gai-1 activity during the first three days after germination is enough to induce de-etiolation.

Figure S3. Effects of the genetic background and the *hyh* mutation on HY5-dependent GA-phenotypes.

Figure S4. PAC dose-response assays for different *pif* mutants and transgenic lines.

Figure S5. GA signaling does not affect *PIF*'s expression level in dark-grown seedlings.

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Figure Legends

Figure 1. Temporal window of GA action in repressing photomorphogenesis in darkness.

(a, b) Wild-type Col-0 seedlings were grown in darkness in control media for 0 to 4 days before they were transferred to media containing 1 μM of the GA biosynthesis inhibitor paclobutrazol (PAC). Hypocotyl length and cotyledon opening (a), and *CAB2* transcripts levels (b) were determined in 10-day-old seedlings. Hypocotyl lengths and cotyledon opening angles were measured as previously described (Alabadí *et al.*, 2004); open and closed circles represent hypocotyl and angle between cotyledons, respectively. Error bars in (a) indicate standard error of the mean (n=15). In (b), total RNA was extracted and processed as previously described (Alabadí *et al.*, 2004). Blots were probed for *CAB2* and then re-probed for *18S rRNA* without previous stripping. *CAB2* signals were normalized to those of *18S rRNA* and signal level at time point zero was arbitrarily set to 1.

(c, d) Wild-type Col-0 and *Hsp::gai-1* dark-grown seedlings received a daily 3 hr heat-shock treatment at 37ºC for 0 to 8 days, starting at different days after germination. Hypocotyl length (c) and *CAB2* transcripts levels (d) were determined in 9-day-old seedlings. Error bars in (c) indicate standard error of the mean (n=15); open and closed circles represent *Hsp::gai-1* and wild-type seedlings, respectively. *CAB2* signals were normalized to those of *18S rRNA*; level at time point zero was arbitrarily set to 1.

Figure 2. HY5 activity mediates GA control of photomorphogenesis in darkness.

(a, b) Seven-day-old wild-type L*er* and *hy5-1* seedlings (left panel), and wild-type Ws, *35S::HY5* (*HY5ox*), and *HY5::S36A* (*hy5-S36A*) seedlings (right panel) were grown in darkness in control and in 1 μM PAC media. Two representative seedlings per genotype and per treatment are shown in (a). In (b), graphs show angle between cotyledons in PAC media, which were measured as previously described (Alabadí *et al.*, 2004). Error bars represent

standard error of the mean (n=15). Black and white bars represent wild-type and mutant lines, respectively. Cotyledon angle is 0 for all genotypes in control media. (deg) means degrees.

(c, d) *CAB2* (c) and *RbcS* (d) transcript levels in wild-type L*er* and *hy5-1* seven-day-old seedlings grown in the dark in control and in 1 μM PAC media. Each sample of total RNA was run and transferred to a membrane in duplicate, probed for *CAB2* or *RbcS*, and then re-probed for *18S rRNA* as described in Figure 1. Numbers below the panels indicate the *18S rRNA*-normalized intensity of the *CAB2* and *RbcS* signal relative to that of wild-type in control media, which was set arbitrarily to 1.

Figure 3. Interaction between HYH and HY5 in the regulation by GA of de-etiolation.

(a, b) Seven-day-old wild-type WS, *hy5-ks50*, *hyh*, and *hy5 hyh* seedlings were grown in darkness in control and in 1 μM PAC media. Two representative seedlings per genotype and per treatment are shown in (a). In (b), graph shows angle between cotyledons in PAC media. Error bars represent standard error of the mean (n=15). (deg) means degrees.

Figure 4. Gibberellins modulate HY5 protein levels.

(a) *HY5* transcript level in four-day-old wild-type Col-0 seedlings grown in the dark in control and 1 μM PAC media. Blot was re-probed for *18S rRNA* as a loading control.

(b) Four-day-old wild-type Col-0 and *StSLY1ox* seedlings (over-expressing the *Solanum tuberosum* ortholog of the *Arabidopsis SLY1* gene) were grown in darkness in control (-) or 1 μM PAC-media (+). Total proteins were extracted and HY5 accumulation was analyzed by Western-blot using anti-HY5 antibodies (Osterlund *et al.*, 2000b). CSN3 levels were used as loading control (Peng *et al.*, 2001). Total protein samples from four-day-old wild-type Col-0 light-grown seedlings (white fluorescent light, 90-100 μmol s^{-1} m^{-2}), and from four-day-old

hy5-215 dark-grown seedlings were used as controls for antibody specificity. Arrows indicate HY5 protein bands. Asterisk indicates a cross-reactive band also appearing in *hy5-215* extract.

(c) HY5 protein levels in four-day-old, dark-grown wild-type Col-0 and *cop1-4* mutant seedlings, which were grown in the presence (+) or in the absence (-) of 10 μM of GA_3. Protein levels were analyzed as described in (b).

(d) HY5 protein levels in four-day-old, dark-grown wild-type Col-0 and *cop1-4* mutant seedlings, which were grown in the presence (P) or in the absence (-) of 1 μM of PAC, or in media supplemented with 1 μM of PAC + 10 μM of GA_3 (P+G). Protein levels were analyzed as described in (b).

(e) Four-day-old wild-type Col-0 and *cop1-4* seedlings were grown in darkness in control (-), in 1 μM PAC-media (P), or in media supplemented with 1 μM of PAC + 10 μM of GA_3 (P+G). Total proteins were extracted and COP1 accumulation was analyzed by Western-blot using anti-COP1 antibodies (McNeils *et al*., 1994).

Figure 5. PIF genes mediate the promotion of skotomorphogenesis by gibberellins

(a, b, c) Seven-day-old wild-type Col-0, *pif1-1*, *pif1-2*, and *pif3* (left panel), wild-type Ws and *srl2* (middle panel), and wild-type L*er* and *PIF3ox* (right panel) seedlings were grown in darkness in control and in 1 μM PAC media. Two representative seedlings per genotype in PAC media are shown in (a). Hypocotyl lengths in both media (b) and angle between cotyledons in PAC media (c) were measured as previously described (Alabadí *et al.*, 2004); cotyledon angle is 0 for all genotypes in control media. In (b), closed triangle, circle, and square represent wild-type Col-0, WS, and L*er*, respectively. In (c), black and white bars represent wild-type and mutant lines, respectively. Error bars in (b) and (c) indicate standard error of the mean (n=15).

(d) Relative expression level of PIF3 target genes in 4-d-old seedlings grown in darkness, analyzed by "real-time" quantitative PCR (qRT-PCR). Error bars represent standard deviation (n=3).

Figure 6. Functional interaction between PIF proteins and GA signaling.

(a, b) Seven-day-old wild-type Col-0, *pif3-1*, *StSLY1ox*, and *pif3 StSLY1ox* seedlings were grown in darkness in control and in 1 μM PAC media. Hypocotyl length (a) and angle between cotyledons (b) in PAC media were measured as previously described (Alabadí *et al.*, 2004). Hypocotyl lengths (± standard error of the mean, n=15) in control media were 15.61±1.56 mm (wild-type Col-0), 17.13±0.77 mm (*pif3-1*), 15.78±0.78 mm (*StSLY1ox*), and 16.13±0.79 mm (*pif3 StSLY1ox*). Cotyledon angle is 0 for all genotypes in control media. Error bars in (a) and (b) indicate standard error of the mean (n=15).

(c) Four-day-old wild-type Col-0, *pif1-2*, and *35S::gai-1* seedlings were grown in darkness and transferred to white fluorescent light (90-100 μmol s^{-1} m^{-2}) for the indicated times. Chlorophyll from samples was extracted and quantified. Error bars represent the standard error of the mean (n=12).

Figure 7. Gibberellins regulate PIFs' activity in etiolated seedlings

(a) *PIF3-myc* seedlings were grown in darkness in control (con), 1 μM PAC (PAC), or 1 μM PAC + 10 μM GA_3 (PAC+GA_3) media for 4 days and transferred to red-light (10 μmol s^{-1} m^{-2}) for the indicated times, in minutes. Total proteins were extracted and PIF3-myc accumulation was analyzed by Western-blot using an anti-[c-myc]-peroxidase antibody (clone E910, Roche). DET3 levels were used as loading control (Duek *et al.*, 2004).

(b) Effect upon gene expression of a 1-hr red-light treatment of 4-d-old etiolated seedlings. Expression was analyzed by qRT-PCR, and fold induction was calculated relative to

expression of the corresponding genes before the treatment. Error bars represent standard deviation (n=3). Concentration of reagents is the same as above.

Figure 8. Light signaling regulates GA metabolism during de-etiolation.

(a) Three-day-old wild-type Col-0 seedlings were grown in the dark and transferred to white fluorescent light (90-100 μmol s^{-1} m^{-2}) for the indicated times, in hours (h). Transcript levels were determined by qRT-PCR. Maximum expression level for each gene was set to 1.

(b) Wild-type Col-0 seedlings were grown in the dark in control or in 10 μM GA_3 media for four days and then transferred to white fluorescent light (90-100 μmol s^{-1} m^{-2}) for the indicated times, in hours (h). Graph shows *CAB2* expression, which was analyzed as described in legend of Figure 1. Normalized *CAB2* signal at time point zero in the control sample was set to 1, and all other signals are relative to it. Open and closed circles represent control and GA_3-treated samples, respectively.

(c) Model illustrating interactions between light and GA pathways in the control of de-etiolation. After germination in the dark, COP1 is very active and promotes degradation of transcription factors inductors of photomorphogenesis, including HY5, and accumulation of active PIF proteins (circles), which support etiolated growth. Under this condition, high GA levels (yellow triangles) result in low DELLA accumulation (Vriezen *et al.*, 2004), thus largely preventing their negative effect on the activity of PIF proteins (squares), and their positive effect on HY5 accumulation. All these interactions lead to promotion of skotomorphogenesis. When GA levels are pharmacologically reduced with PAC, DELLA proteins stabilize (Vriezen *et al.*, 2004) and then partially inhibit PIF's activity and promote HY5 accumulation. This results in partial de-etiolation. During light-induced de-etiolation, GA levels decrease and DELLA proteins are stabilized (Achard *et al.*, 2007), subsequently

alleviating the negative effect of GA signaling on photomorphogenesis. Accumulating DELLAs may inhibit the activity of residual PIF proteins and enhance HY5 levels.

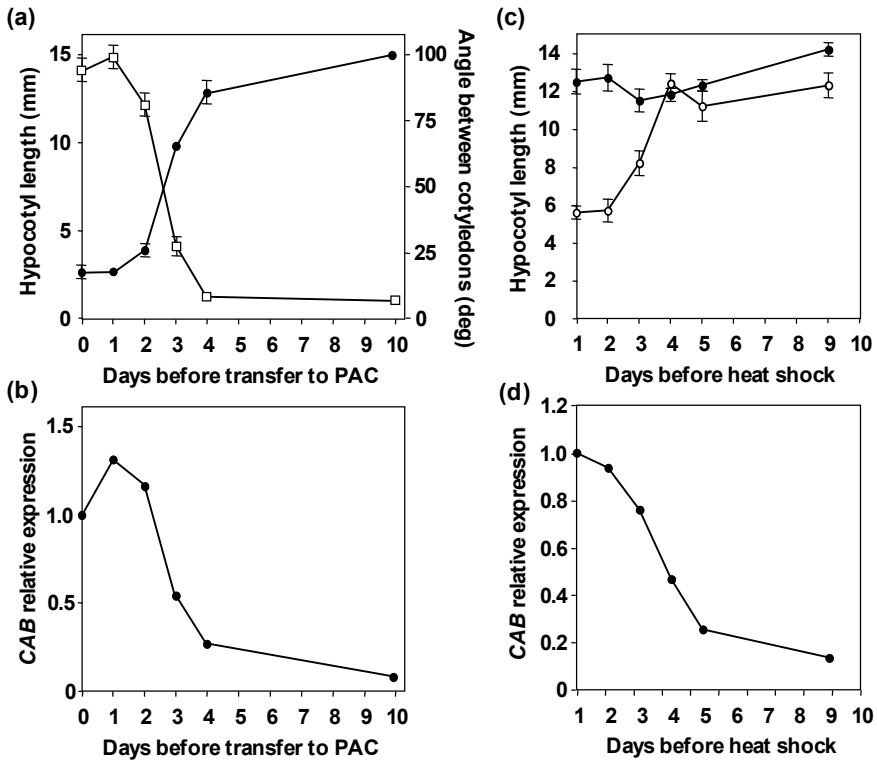

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Figure 1

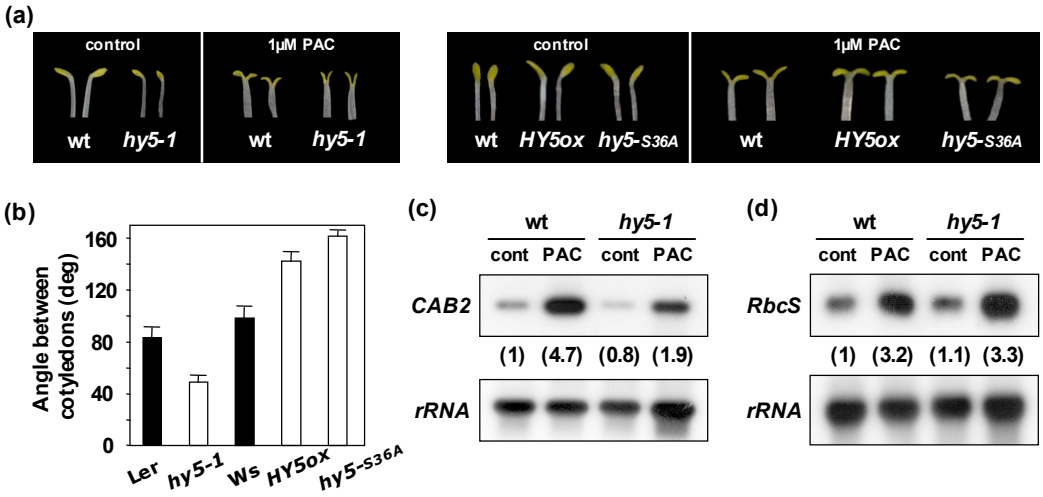

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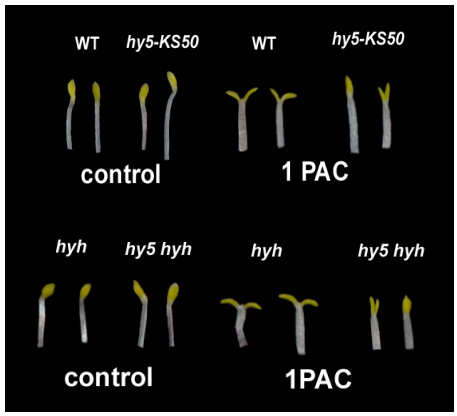
Figure 2

(a)



(b)

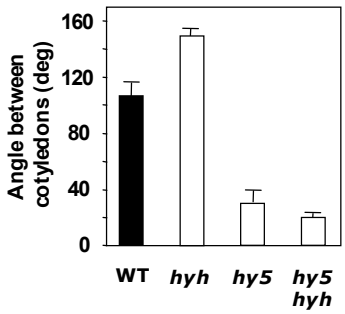


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Figure 3

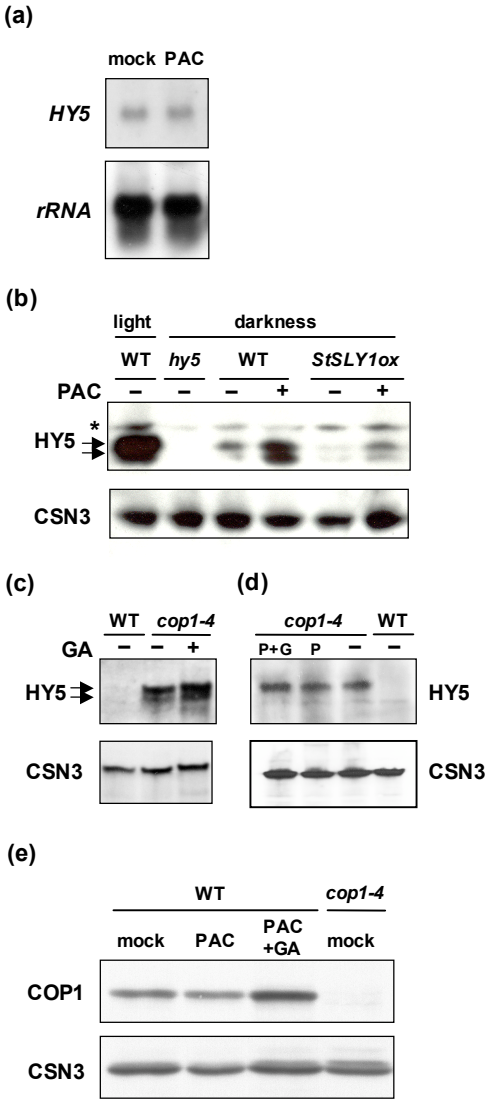

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Figure 4

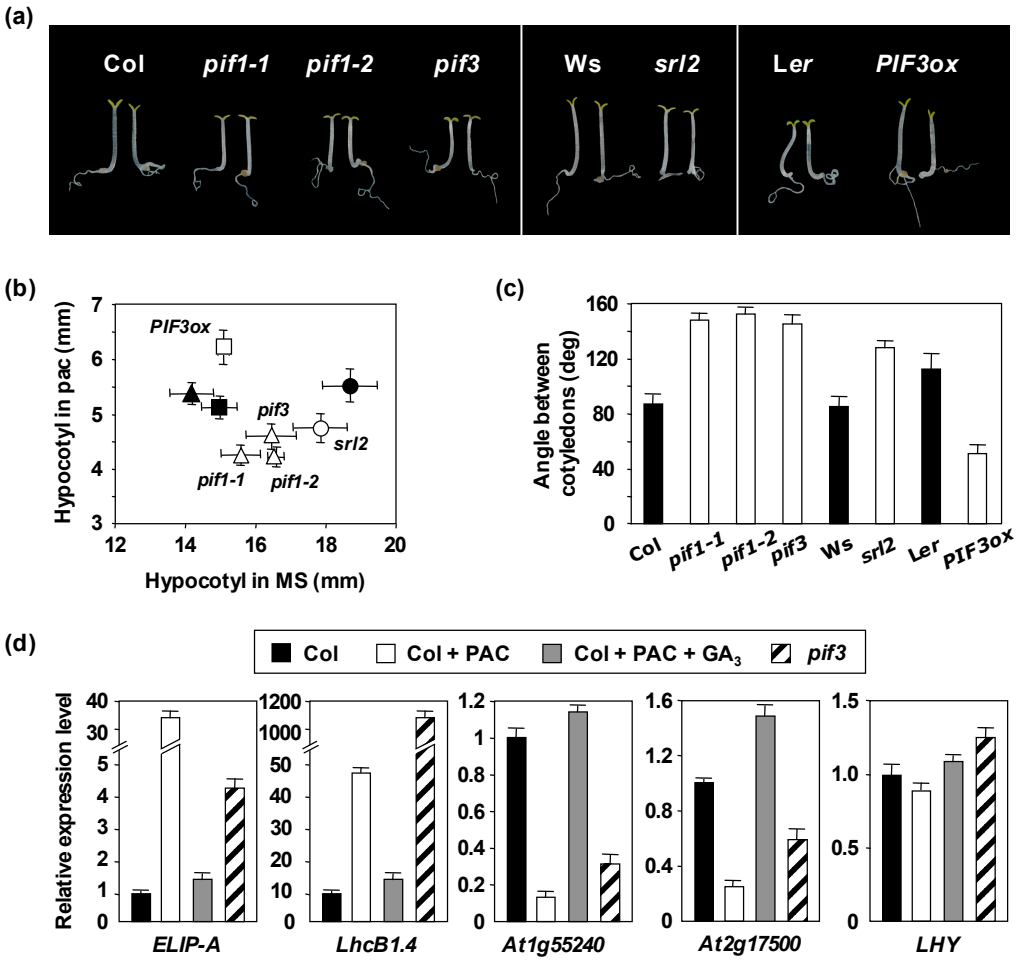

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Figure 5

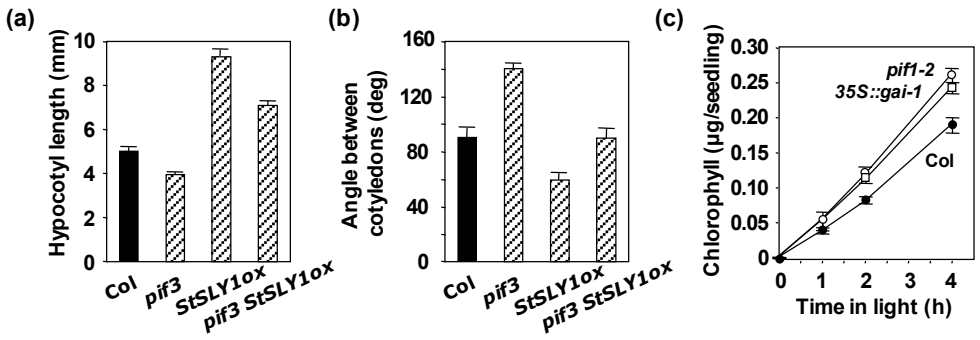

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Figure 6

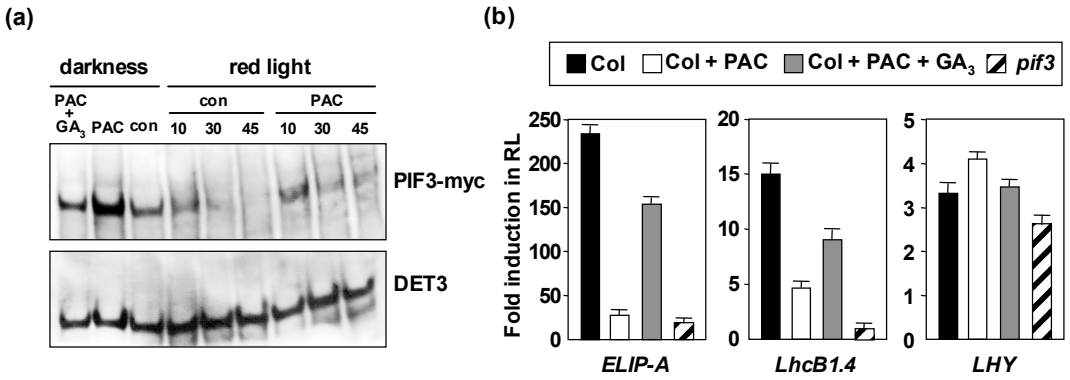

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Figure 7

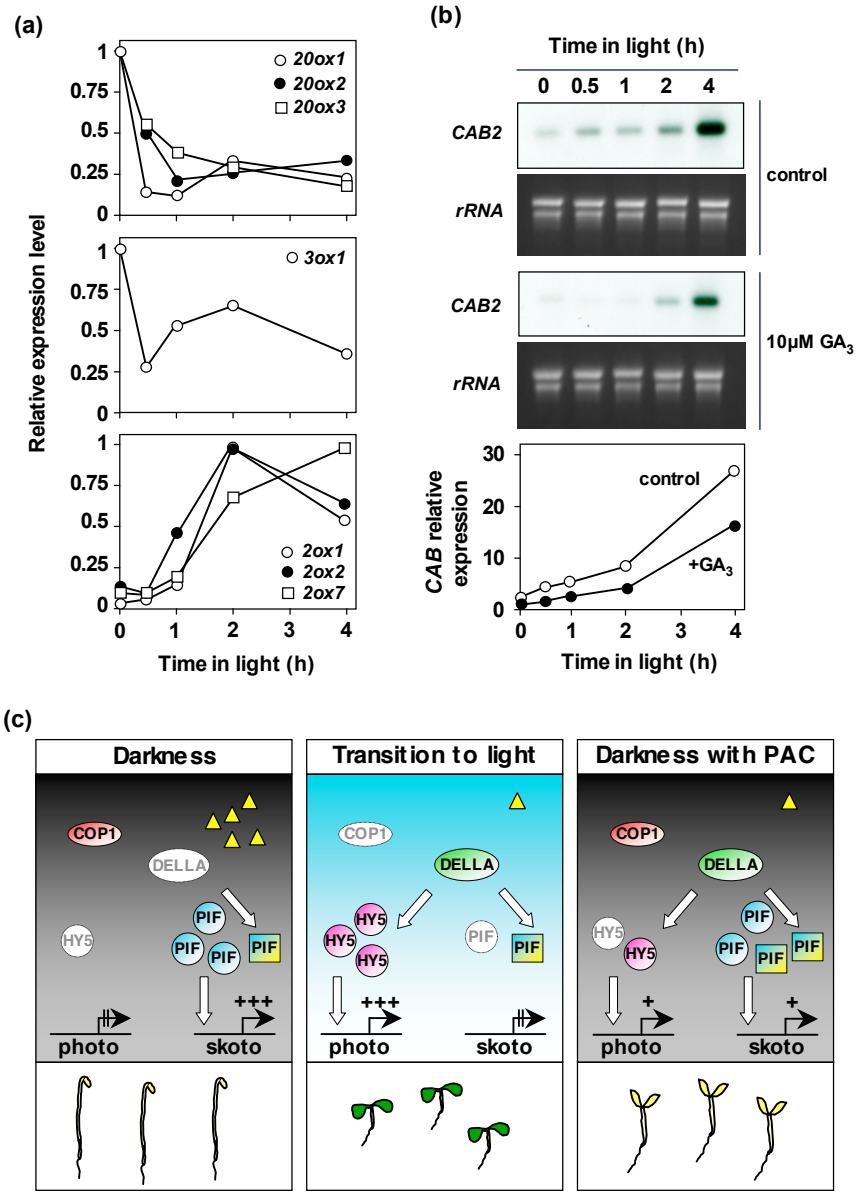

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Figure 8

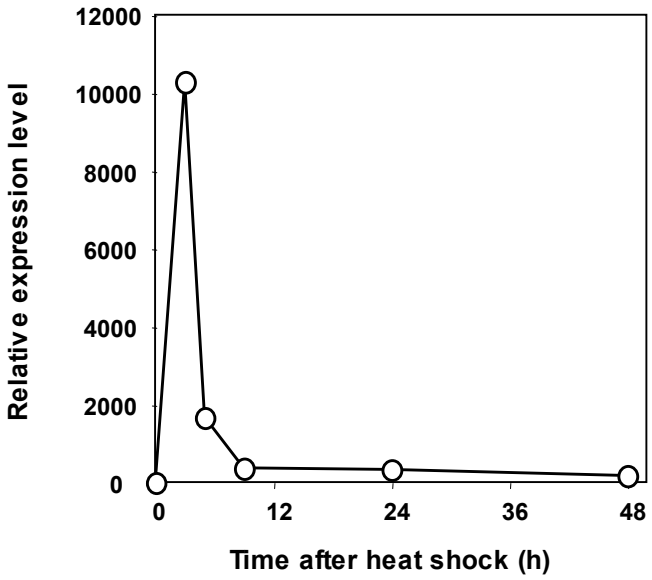

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Figure S1

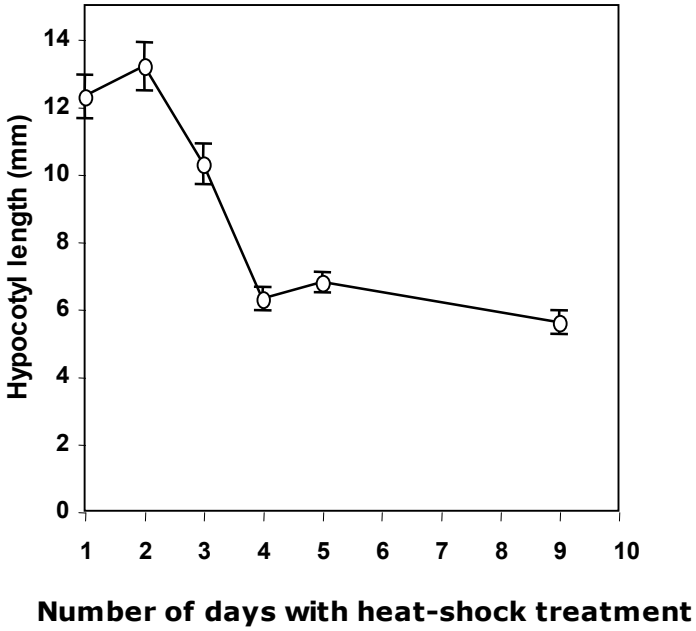

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Figure S2

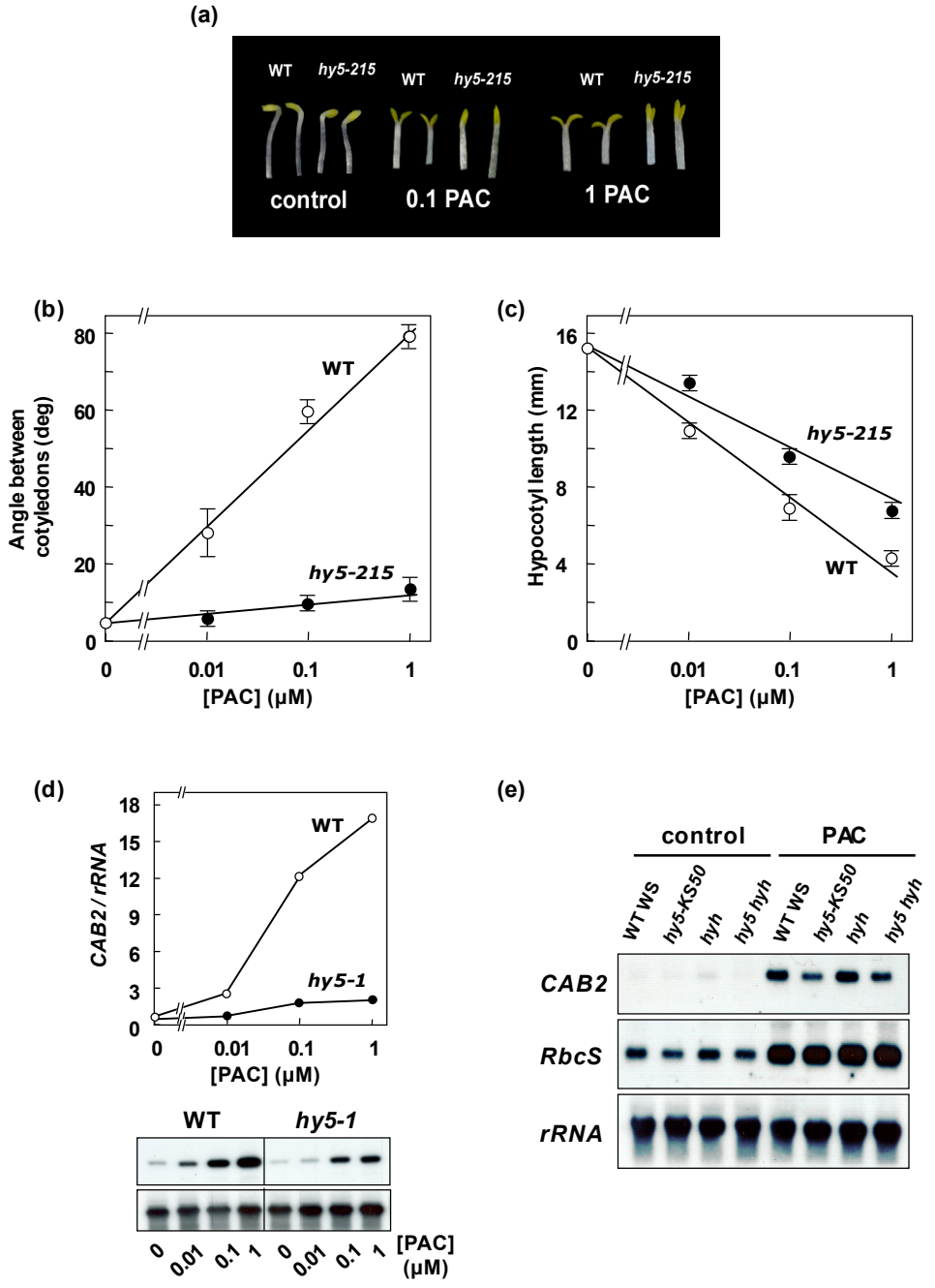

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Figure S3

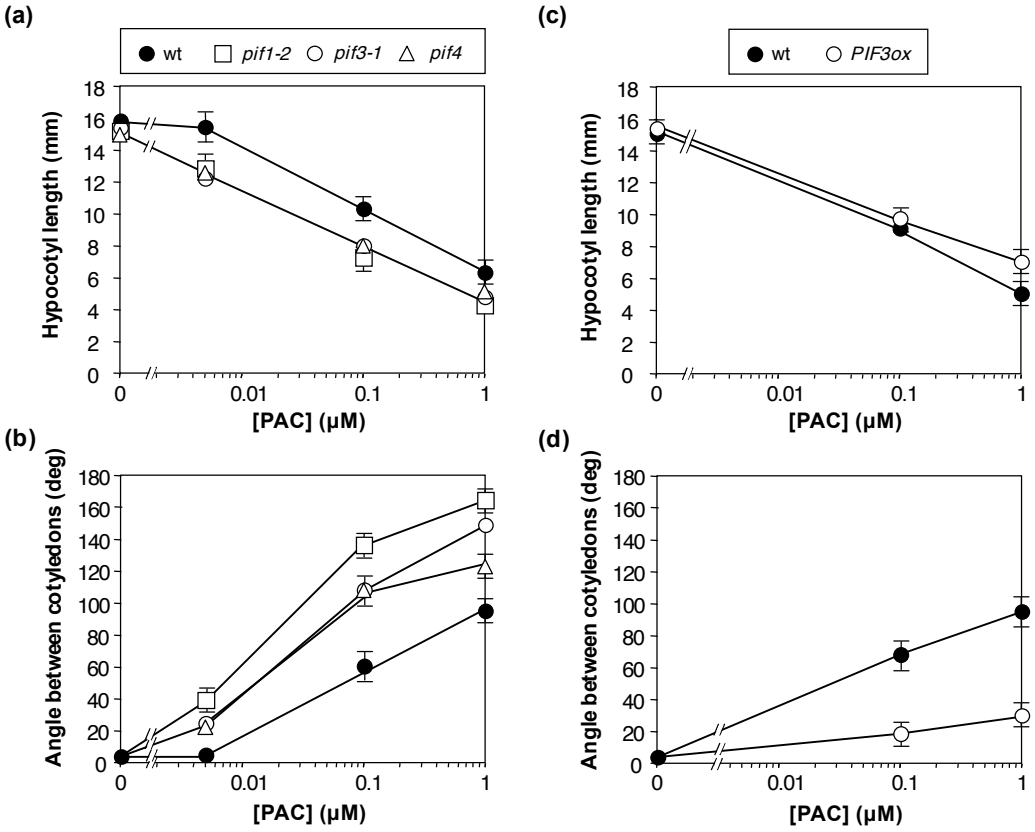

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Figure S4

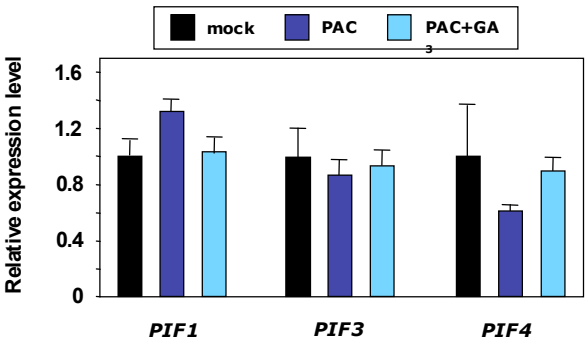

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Figure S5