Gibberellins Repress Photomorphogenesis in Darkness[1]

David Alabadí, Joan Gil, Miguel A. Blázquez, and José L. García-Martínez*

Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV), Valencia–46022, Spain

Plants undergo two different developmental programs depending on whether they are growing in darkness (skotomorphogenesis) or in the presence of light (photomorphogenesis). It has been proposed that the latter is the default pathway followed by many plants after germination and before the seedling emerges from soil. The transition between the two pathways is tightly regulated. The conserved COP1-based complex is central in the light-dependent repression of photomorphogenesis in darkness. Besides this control, hormones such as brassinosteroids (BRs), cytokinins, auxins, or ethylene also have been shown to regulate, to different extents, this developmental switch. In the present work, we show that the hormone gibberellin (GA) widely participates in this regulation. Studies from Arabidopsis show that both chemical and genetic reductions of endogenous GA levels partially derepress photomorphogenesis in darkness. This is based both on morphological phenotypes, such as hypocotyl elongation and hook and cotyledon opening, and on molecular phenotypes, such as misregulation of the light-controlled genes *CAB2* and *RbcS*. Genetic studies indicate that the GA signaling elements GAI and RGA participate in these responses. Our results also suggest that GA regulation of this response partially depends on BRs. This regulation seems to be conserved across species because lowering endogenous GA levels in pea (*Pisum sativum*) induces full de-etiolation in darkness, which is not reverted by BR application. Our results, therefore, attribute an important role for GAs in the establishment of etiolated growth and in repression of photomorphogenesis.

One of the most dramatic changes in plant growth and development occurs during the transition from life in the dark just after germination, to life in a light environment when the seedling emerges from soil. Development in darkness is referred to as skotomorphogenesis, whereas development in the light is referred to as photomorphogenesis. Skotomorphogenesis is characterized by an etiolated appearance of seedlings with a fast-growing hypocotyl or epicotyl, presence of an apical hook, and small and closed cotyledons or primary leaves. Moreover, these seedlings present etioplasts instead of chloroplasts, and the expression of genes that are normally light-regulated is repressed or kept at low, basal levels. When light triggers the photomorphogenic development, growth of hypocotyl or epicotyl is slowed down, cotyledons or primary leaves open and expand, etioplasts develop into chloroplasts, and the expression of light-controlled genes is up-regulated (Neff et al., 2000).

After germination, it is extremely important for plants to be able to maintain the skotomorphogenic development before reaching the light to preserve and protect both the shoot apical meristem and cotyledons or primary leaves. In many plants, photomorphogenesis is the default developmental pathway after germination (Wei et al., 1994). This is based on the fact that many lower plants lack an etiolated growth phase, and the new program, skotomorphogenesis, had to be invented partly to inhibit photomorphogenesis. Thus, plants have devoted several control mechanisms to ensure that it is prevented until the appropriate light condition is reached.

Mutants that resemble light-growing plants when they are growing in darkness have been identified in Arabidopsis. These mutants have helped to define several signaling pathways that repress seedling de-etiolation before emerging from soil. The main molecular device is based on the COP1, COP10, and DET1 proteins and the COP9 signalosome complex, which marks inducers of photomorphogenesis for degradation before the seedling reaches the light (Kim et al., 2002). The finding of orthologs of several of the corresponding genes in other species, such as pea (*Pisum sativum*) or tomato (*Lycopersicon esculentum*; Mustilli et al., 1999; Sullivan and Gray, 2000; Kim et al., 2002), suggests that this is a widely used and highly conserved mechanism.

Other mutants, such as the Arabidopsis *det2* or *cpd* or the tomato *extreme dwarf*, have helped to attribute a role for the hormones brassinosteroids (BRs) in the repression of photomorphogenesis and in establishing the etiolated developmental program in darkness (Chory et al., 1991; Li et al., 1996; Szekeres et al., 1996; Bishop et al., 1999). Those mutants are deficient in BR biosynthesis and show partial de-etiolation in darkness. This phenotype also appears in several *aux/iaa* mutants, which are resistant to auxin, also suggesting a role for this hormone in etiolation (Neff et al., 2000). A role for auxin in the repression of light-regulated

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* Corresponding author; e-mail jlgarcim@ibmcp.upv.es; fax 34–963877859.

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genes in etiolated seedlings is also revealed by the *doc1* mutant, which affects polar auxin transport, although no morphological de-etiolation in darkness is observed in this case (Gil et al., 2001). Moreover, treatments of Arabidopsis seedlings with cytokinins induce de-etiolation in darkness (Chory et al., 1994), although these effects are not observed in pea (Seyedi et al., 2001). All these results suggest that hormonal homeostasis in dark-grown seedlings plays an important role in maintaining the etiolated growing response until light is reached.

In this work, we have analyzed the contribution of GAs to the etiolated growth pattern and to repress photomorphogenesis in the dark. We have found that Arabidopsis plants with reduced GA levels show characteristics of light-grown plants when grown in darkness, including loss of the apical hook, inhibition of hypocotyl growth, cotyledon opening, and misregulation of light-controlled genes. Repression of photomorphogenesis by GAs seems to be a conserved mechanism because similar effects are observed in pea. In this species, the contribution of GAs to the repression process seems to be even more important, considering the complete leaf formation observed in dark-grown pea plants with low GA levels.

RESULTS

Paclobutrazol Treatment of Arabidopsis Seedlings Induces Partial Photomorphogenesis in Darkness

GAs mediate Arabidopsis hypocotyl growth in the dark (Cowling and Harberd, 1999); however, it is not known whether this effect represents repression of photomorphogenesis or light-independent control of cell expansion. To distinguish between these two possibilities, we examined other developmental aspects of seedling morphology in the dark: apical hook maintenance and cotyledon opening. As expected, 4-d-old WT seedlings grown in darkness showed an etiolated morphology with folded cotyledons and an apical hook (Fig. 1, A and B). However, when grown in the presence of 1 μM PAC, a GA biosynthesis inhibitor, they lost the apical hook. Furthermore, 6-d-old WT seedlings grown in the dark showed small and closed cotyledons (Fig. 1, A and C), whereas those grown in the presence of PAC showed partially opened cotyledons. WT morphology was completely restored in the presence of 10 μM GA_3. Unlike seedlings deficient in BR biosynthesis or treated with cytokinins (Chory et al., 1994; Nagata et al., 2000), PAC-treated seedlings grown in darkness for 42 d did not develop true leaves, although an enlargement of leaf primordia was visible (data not shown). The morphological changes induced in darkness when endogenous GA levels are reduced by PAC treatment, suggest that GAs are needed to complete the etiolated developmental program, at least at its early stages.

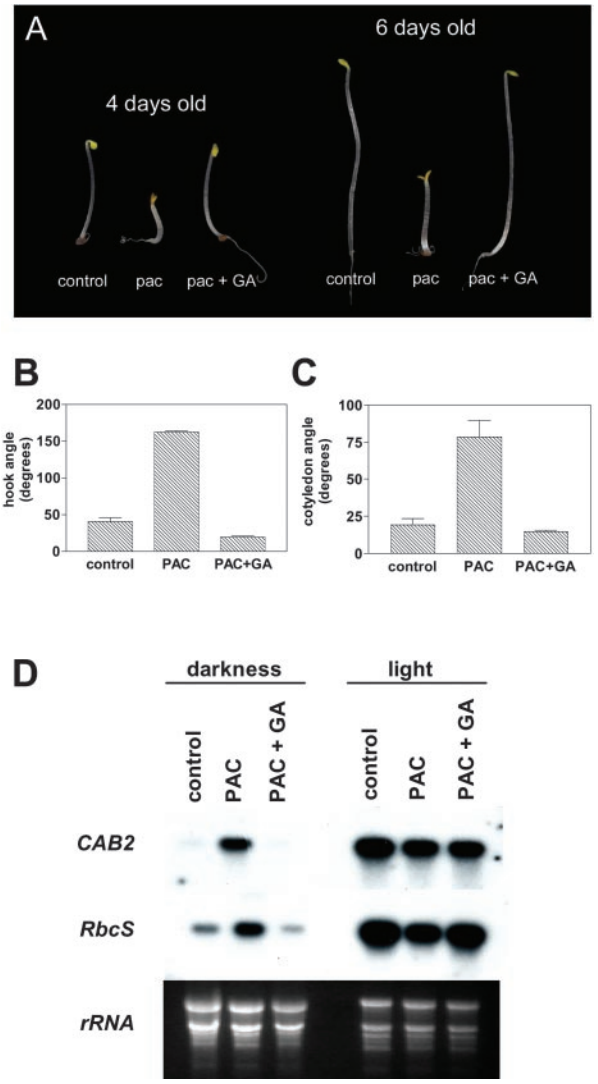

Figure 1. Effect of paclobutrazol (PAC) on photomorphogenesis of dark-grown Arabidopsis wild-type (WT) seedlings. A, Phenotypes of representative 4- and 6-d-old dark-grown seedlings for each treatment (see text). B, Hook angle of 4-d-old seedlings grown in darkness. Measurements are average of 10 to 15 seedlings ± SE per treatment. C, Cotyledon angle of 6-d-old seedlings grown in darkness. Measurements are average of 10 to 15 seedlings ± SE per treatment. D, Northern-blot analysis of *CAB2* and *RbcS* gene expression in Arabidopsis WT seedlings grown in darkness or in continuous white light for 10 d. Four micrograms of total RNA from light-grown seedlings, and 8 μg of total RNA from dark-grown seedlings was loaded per lane.

Misregulation of Light-Controlled Genes in PAC-Treated Arabidopsis Seedlings in Darkness

A common feature of seedlings undergoing constitutive photomorphogenesis in darkness is an increase in the expression level of light-regulated genes (Ma et al., 2003). In agreement with this, the expression of the light-regulated genes *CAB2* and *RbcS* in the dark was much higher in PAC-treated seedlings than in untreated controls (Fig. 1D). Addition of GA_3

to the growing media counteracted the effect of PAC and restored transcript levels to those of untreated controls. This misregulation was not observed in light-grown seedlings, suggesting that light can overcome the repression imposed by GAs.

Partial De-Etiolated Phenotype in GA-Deficient and GA-Signaling Arabidopsis Mutants

To confirm that the derepression of photomorphogenesis caused by PAC was indeed because of inhibition of GA biosynthesis, we examined the phenotype in the dark of the GA-deficient *ga1-3* mutant, which is a null allele of the gene encoding the enzyme that catalyzes the first step in the GA biosynthetic pathway (Sun et al., 1992). Figure 2A shows the loss of the apical hook and the extreme dwarf phenotype of *ga1-3* seedlings grown in darkness for 5 d compared with the WT. Although cotyledons of the *ga1-3* mutant remained folded after 8 d in darkness (Fig. 2A), this was likely because of a delay in the developmental timing caused by the deficiency of GAs because incubation for a few more days resulted in cotyledon opening in the mutant but not in the WT (data not shown). Furthermore, lack of GA biosynthesis in the *ga1-3* mutant caused derepression of the *CAB2* and *RbcS* genes in the dark (Fig. 2B), equivalent to the effect of PAC mentioned above. Reversion of this phenotype by exogenous GA_3 is consistent with the role of GAs in the repression of photomorphogenesis in the dark in Arabidopsis.

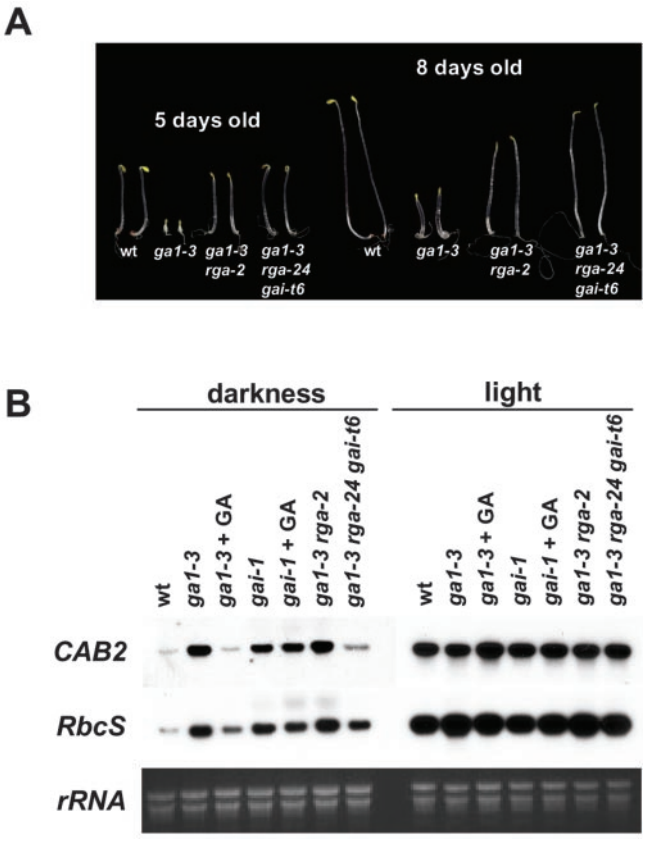

Figure 2. Effect of Arabidopsis GA biosynthesis and signaling mutants on photomorphogenesis in darkness. A, Phenotypes of representative 5- and 8-d-old dark-grown Arabidopsis GA biosynthesis and signaling mutants. B, Northern-blot analysis of *CAB2* and *RbcS* gene expression in Arabidopsis GA biosynthesis and signaling mutant seedlings grown in darkness or in continuous white light for 10 d. Four micrograms of total RNA from light-grown seedlings and 7 μg of total RNA from dark-grown seedlings were loaded per lane.

Several GA-signaling elements have been identified in Arabidopsis, with partially overlapping functions (Dill and Sun, 2001; King et al., 2001). For instance, loss-of-function mutations at the *GAI* and/or *RGA* loci are able to rescue the stem growth defects of *ga1-3* mutants but not defects in germination or flower development caused by reduced GA levels (Silverstone et al., 1997; Dill and Sun, 2001; King et al., 2001). To investigate whether GAI and RGA were involved in GA-dependent repression of photomorphogenesis in the dark, we studied if null mutations at these two loci were able to abolish the de-etiolated phenotype of *ga1-3* mutants. The short hypocotyl and the apical hook phenotypes of the *ga1-3* mutant were partially restored to the WT appearance by a mutation at the *RGA* locus (Fig. 2A) and completely restored by further elimination of GAI activity in the *ga1-3 rga-24 gai-t6* mutant. The role of GAI in controlling these processes was further supported by the darkness-induced phenotype of the semi dominant, GA-insensitive mutant *gai-1*, which also showed short hypocotyl and partial opening of the apical hook (Cowling and Harberd, 1999; data not shown). This indicates that both GAI and RGA are involved in the GA pathway controlling these morphological responses. The same seems to be true for the regulation of *CAB2* and *RbcS* mRNA levels. Transcript levels of both genes were elevated in the GA-insensitive *gai-1* allele compared with the WT, and these high levels were not restored to WT values by GA treatment. The expression of both genes was not as high as in the *ga1-3* mutant, suggesting that additional signaling elements are still mediating GA control of these genes. However, it is difficult to assess if RGA is the element involved in this signaling process because in the *ga1-3 rga-2* double mutant, both *CAB2* and *RbcS* mRNA levels were similar to those in the *ga1-3* mutant. This could be explained by redundancy with GAI because *CAB2* expression levels were restored to WT values in the triple mutant *ga1-3 rga-24 gai-t6*. Alternatively, additional GA-signaling elements might work in conjunction with GAI to regulate the expression of different sets of target genes. For instance, *RbcS* mRNA levels were still higher in the *ga1-3 rga-24 gai-t6* mutant than in WT, although they were lower than in *ga1-3* or *ga1-3 rga-2* plants.

BRs Restore the WT Molecular Phenotype of *CAB2* and *RbcS* in Dark-Grown Arabidopsis GA-Deficient Plants

The results shown above highlight overall similarities in the darkness-induced phenotypes between GA-deficient and -signaling mutants and those of BR-deficient mutants (Chory et al., 1991; Takahashi et al., 1995; Szekeres et al., 1996; Azpiroz et al., 1998). Besides, recent results indicate that BRs are able to partially rescue the short hypocotyl phenotype of *ga1-3* mutants in the dark (Steber and McCourt, 2001), raising the question of whether BRs would mediate GA repression of photomorphogenesis. Although 24-epibrassinolide (EBR) only partially reverted the PAC-induced morphological phenotypes (data not shown), EBR was nearly as effective as GA_3 to counteract *CAB2* and *RbcS* up-regulation in the absence of GAs (Fig. 3A). This observation suggests that BRs and GAs may act in parallel to regulate morphological aspects of growth in darkness, but that BRs mediate GA control of *CAB2* and *RbcS* expression. This hierarchy is supported by the inability of GAs to rescue the increased expression of both *CAB2* and *RbcS* in dark-grown seedlings of the *det2* mutant (Fig. 3B) or to revert the effect of treatment with the BR biosynthesis inhibitor brassinazole 220 (Sekimata et al., 2002; data not shown).

Effect of PAC on Development of Pea Seedlings in the Dark

Results obtained with Arabidopsis indicate that GAs participate in the repression of photomorphogenesis in darkness and that GA action requires BRs. We wondered whether GAs acted similarly in other plants. Pea plants represent an excellent experimental model, in which BRs do not participate in the repression of de-etiolation in the dark (Symons et al., 2002; Symons and Reid, 2003). Six-day-old, dark-grown pea seedlings had a typical etiolated phenotype, whereas in the presence of 10 μM PAC, they had short internodes and an open hook (Fig. 4A). After 2 weeks in darkness, PAC-grown seedlings also developed true leaves, with stipules and leaflets, although they were yellowish (data not shown). Afterward, leaf petioles kept on elongating, tendrils were formed eventually, and stipules and leaflets remained folded (Fig. 4B). Derepression by PAC of photomorphogenesis in the dark was completely reversed by simultaneous application of GA_3 (Fig. 4, A and B).

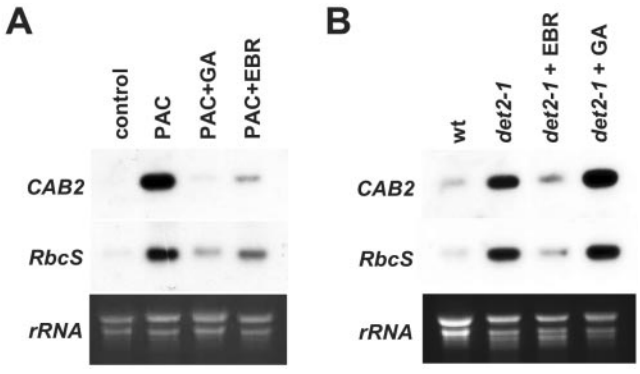

Figure 3. Interactions between GAs and BRs in the control of Arabidopsis photomorphogenesis in darkness. A, EBR treatment rescues the *CAB2* and *RbcS* WT expression level in 10-d-old WT Arabidopsis seedlings treated with PAC. *CAB2* and *RcbS* mRNA levels were analyzed by northern blot. Four micrograms of total RNA was loaded per lane. B, GA_3 does not rescue the molecular phenotype of *CAB2* and *RbcS* in *det2* mutants. WT and *det2-1* seedlings were grown for 10 d in darkness. *CAB2* and *RcbS* mRNA levels were analyzed by northern blot. Five micrograms of total RNA was loaded per lane.

Effect of *na* Mutation on Photomorphogenesis of Pea Seedlings in Darkness

Seedlings of the GA-deficient pea *na* mutant (Davidson et al., 2003) grown in the dark developed a photomorphogenic phenotype in contrast with the corresponding isogenic *Na* seedlings, which displayed a typical etiolated phenotype (Fig. 4, C and D). The phenotype of *na* seedlings was similar to that described for WT seedlings treated with PAC (Fig. 4, A and B). Application of GA_3 to *na* seedlings rescued the WT phenotype (Fig. 4C and D). It was necessary to apply a minimum of 1 μg of GA_3 per seedling to get full reversion of photomorphogenesis of *na* seedlings (Fig. 4E). Interestingly, the photomorphogenic repression effect of single applications of GA_3 to the seeds disappeared at later stages of seedling development (after about 24 d in darkness), probably as a result of dilution effect when the amount of GA_3 reaching the developing apex was not any more at a sufficient inhibitory concentration (data not shown). Application of brassinolide to *na* seedlings did not have any effect on photomorphogenesis (Fig. 4, C and D).

Effect of PAC and *na* Mutation on Transcript Levels of *RbcS* of Pea Seedlings Grown in the Dark

Transcript levels of *RbcS* were detected in the apical part but not in the stem of 6-d-old WT cv Alaska and *Na* seedlings grown in the dark (Fig. 5). *RbcS* transcripts were, however, more abundant in dark-grown seedlings of the WT cv Alaska in the presence of PAC and in *na* mutant plants (Fig. 5).

DISCUSSION

In this work, we present evidence that GAs are involved in the establishment of the seedling etiolated development in darkness and in the repression of photomorphogenesis under this condition, both in Arabidopsis and in pea. Although in Arabidopsis, GAs partially regulate de-etiolation and require BRs,

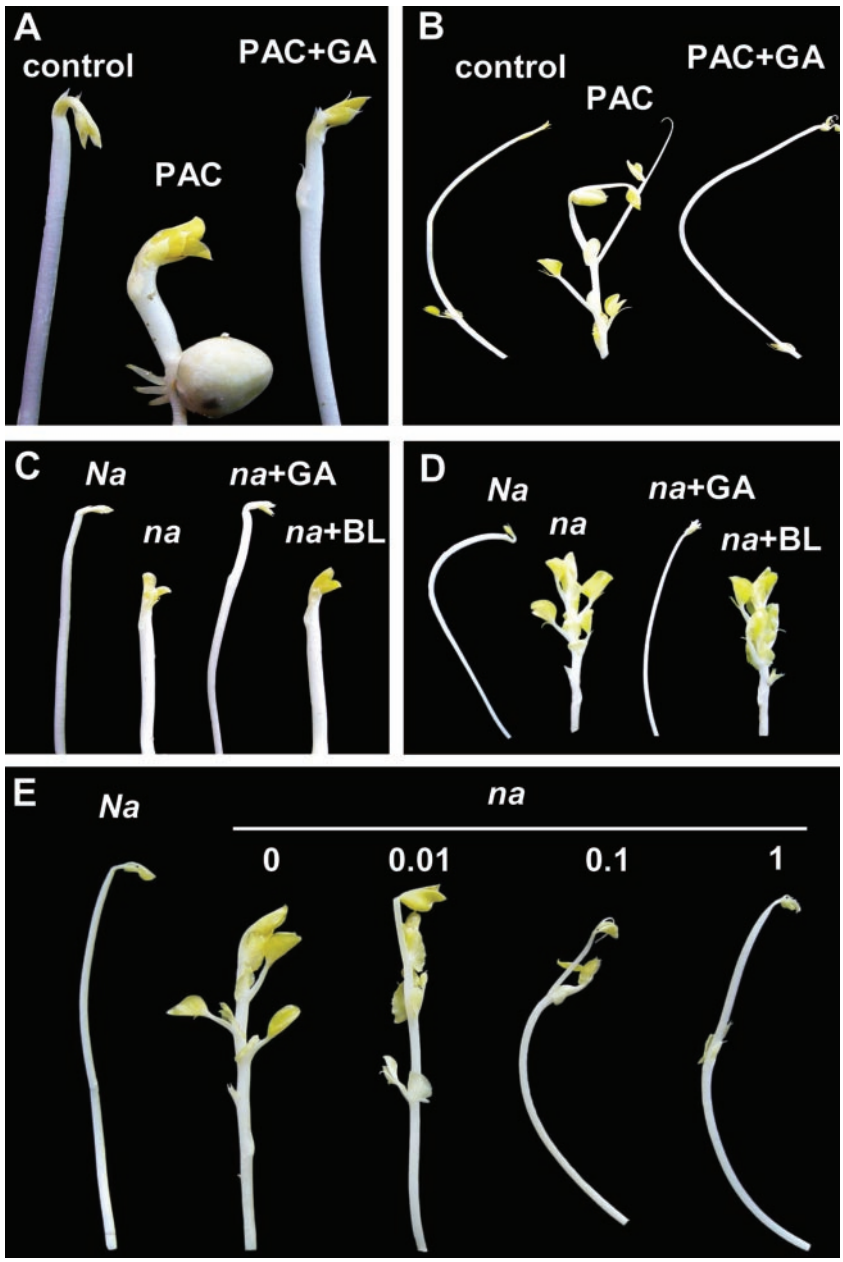

Figure 4. Effects of GA deficiency on photomorphogenesis of pea plants in darkness. A and B, Effect of PAC on photomorphogenesis of pea seedlings in the dark. Seeds of WT cv Alaska were watered with 10 μM PAC and cultured at 22°C in the dark for 6 (A) or 24 (B) d after seeding. GA_3 (1 μg) was applied to the seeds before planting, and to the seedlings after germination. C and D, Photomorphogenesis of *na* seedlings cultured in the dark for 6 (C) or 24 (D) d after seeding and effect of GA_3 (GA) and brassinolide (BL) application. E, GA_3 dose response on the reversal of photomorphogenesis of *na* pea seedlings cultured for 15 d in the dark. A single GA_3 dose was applied to dry seeds before germination. Numbers mean micrograms of GA_3 applied.

in pea, they are the main hormones in the regulation of skotomorphogenesis.

In Arabidopsis, the repression of the photomorphogenic developmental program and, hence, the induction of an etiolated growing response in darkness are mainly conducted through the concerted action of the COP9 signalosome with the COP1, COP10, and DET1 proteins, acting together as a repressor complex that is inactivated by light (Kim et al., 2002). This complex negatively modulates the expression of most of the light-controlled genes, leading to the etiolated phenotype observed in darkness (Ma et al., 2003). A similar global repressor mechanism might be active in pea, given the similarities between the dark-induced phenotypes of the *lip1* mutant, which affects the pea ortholog of COP1, with those of *cop1* mutants (Frances et al., 1992; Sullivan and Gray, 2000) and the almost ubiquitous presence of the COP9 signalosome in eukaryotes (Kim et al., 2002).

Although the COP machinery represents a mechanism to prevent photomorphogenesis in the absence of light, our results suggest that GAs could perform two different functions in seedling development: (a) the promotion of skotomorphogenic processes, such as hypocotyl elongation, and the formation and maintenance of the apical hook; and (b) the repression of photomorphogenesis in the dark, as represented by the regulation of light-induced genes.

It has long been known that GAs are necessary for cell expansion during hypocotyl growth, both in the light and in the dark (Cowling and Harberd, 1999;

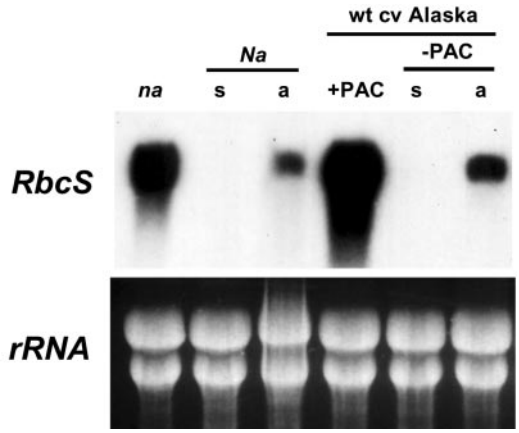

Figure 5. Effect of PAC and *na* mutation on *RbcS* transcript levels in pea. Twenty micrograms of total RNA was loaded per lane. s, Stem; a, apex.

Steber and McCourt, 2001; Figs. 1, 2, and 4). However, it is surprising that GAs participate in the establishment and maintenance of the apical hook, a process in which a prominent role has been attributed to other hormones, such as auxins and ethylene, both in Arabidopsis and in pea (Lehman et al., 1996; Peck et al., 1998; Raz and Ecker, 1999). Differential cell division and differential cell elongation contribute to the maintenance of this structure in Arabidopsis (Raz and Koornneef, 2001), so it is reasonable to think that GAs contribute to this process also through their regulation of cell expansion. Nevertheless, this may not be the only explanation: early in development, GAs have been recently found to activate the expression of the ethylene-induced gene *HLS1* (*HOOKLESS1*; Lehman et al., 1996; Ogawa et al., 2003), proposed to mediate differential auxin distribution in the apical part of the hypocotyl required to establish boundaries of growth and expansion in that region. This might also be the case in pea, in which two functional homologs of *HLS1* have been found (Du and Kende, 2001). Although we have shown that GAI and RGA mediate the GA-signaling branch controlling Arabidopsis apical hook formation (Fig. 2A), we still ignore the nature of the interactions between GAs and ethylene and auxin, but a molecular mechanism has been proposed in which GA-induced degradation of RGA is dependent on auxin in roots (Fu and Harberd, 2003).

The second mechanism by which GAs participate in seedling development in the dark is unexpected, and it involves active repression of light-regulated genes. Up-regulation of *CAB2* and *RbcS* in dark-grown seedlings with low GA levels resembles the misregulation of light-controlled genes shown by constitutive photomorphogenetic mutants in Arabidopsis and in pea. This suggests that in WT etiolated seedlings of both species, high GA signaling is needed to maintain properly repressed the expression of these and many other genes. That this function is different to the promotion of cell expansion involved in the processes mentioned above is supported by the observation that the *ga1-3 rga-2* mutant shows a rather normal etiolated phenotype in the dark but with high expression of *CAB2* and *RbcS* (Fig. 2). It has been proposed that the partial photomorphogenic phenotype of the Arabidopsis BR-deficient mutant *dwf4* may be caused by the close proximity of the shoot apex to the agar medium because of its dwarf growing habit (Azpiroz et al., 1998). However, we do not consider dwarfism to be the cause of the photomorphogenic phenotype of GA-deficient plants in the dark. First, results obtained with the Arabidopsis *dim* or the pea *lk* or *lkb* mutant plants, which show short hypocotyl and WT expression level of *CAB* or *RcbS* genes in darkness (Takahashi et al., 1995; Symons et al., 2002), clearly indicate that reduction in hypocotyl length does not necessarily affect the expression of these light-regulated genes (Szekeres et al., 1996). Second, we detected strong misregulation of *CAB2* or *RbcS* in *ga1-3 rga-2* mutant plants grown in darkness (Fig. 2B), despite that these plants showed hypocotyl length very close to that of WT (Fig. 2A).

Interaction between GAs and BRs in the control of physiological processes, such as hypocotyl elongation or seed germination, and in the control of gene expression in Arabidopsis has been reported (Bouquin et al., 2001; Steber and McCourt, 2001). Although both hormones seem to act oppositely in the control of the Arabidopsis *GASA1* gene, the general view is that both mediate positively in the control of common processes (Bouquin et al., 2001; Steber and McCourt, 2001), with BRs acting downstream of GAs or in a parallel pathway in the control of seed germination and hypocotyl elongation in the dark. Our results of gene expression analysis support this view because treatment with EBR abolishes up-regulation of light-regulated genes caused by lack of GAs, and GA treatment does not act in a reciprocal way in *det2* mutants (Fig. 3B). BRs, therefore, would mediate GA action on the expression of these genes in darkness, as suggested for other processes (Azpiroz et al., 1998; Ephritikhine et al., 1999; Bouquin et al., 2001). This is in contrast with the results in pea (Fig. 4, C and D), where BRs do not compensate for the lack of GAs.

What is the physiological relevance of GA regulation during the transition between skotomorphogenesis and photomorphogenesis? There is evidence that light signals could interact with GAs in the switch between etiolated and de-etiolated development. For instance, it is known that GAs are needed for hypocotyl growth of Arabidopsis and pea seedlings growing in darkness (Cowling and Harberd, 1999; Steber and McCourt, 2001; Figs. 1, 2, and 4) and that hypocotyls of Arabidopsis *phyB* mutants have an enhanced responsiveness to exogenously applied GAs (Reed et al., 1996) and altered expression of GA-biosynthetic genes (Yamaguchi et al., 1998). More-

over, a very fast decrease in the levels of the active GA_1, and also in the responsiveness to GAs, is observed in etiolated pea seedlings upon exposure to light (Ait-Ali et al., 1999; Gil and García-Martínez, 2000; O'Neill et al., 2000). The effect of light on GA_1 content occurs before any apparent morphological sign of de-etiolation, suggesting that light-induced de-etiolation in pea is mediated, at least in part, by a decrease of GA_1.

Comparison between pea and Arabidopsis suggests a common underlying pattern for the recruitment of hormones in the mechanism of repression of photomorphogenesis in darkness but also reveals different strategies. Although in Arabidopsis both the GA and BR pathways are active, only the former is active in pea because strong lines of evidence argue against a role for BRs in the repression of photomorphogenesis in this species (Symons et al., 2002; Symons and Reid, 2003). This suggests that different plant species have given dominant roles to different hormone signaling pathways to prevent de-etiolation in darkness. The functioning of the central and widely extended, COP1-based repressor mechanism might be buffered by additional layers of regulation, represented by different hormone signaling pathways. It is very likely that these additional regulatory mechanisms are under less selective pressure than the COP1-based mechanism, allowing different plant species to fix different hormones to perform this task and achieve a high level of plasticity.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis ecotypes Columbia-0 and Landsberg *erecta* were used as WT. *ga1-3*, *ga1-3 rga-2*, and *gai-1* seeds, all of them in Landsberg *erecta* background, were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). *ga1-3 rga-24 gai-t6* seeds, in the Landsberg *erecta* background, were kindly provided by Dr. Tai-ping Sun (Duke University, Raleigh, NC). *det2-1* seeds, in the Columbia-0 background, were kindly provided by Dr. Jianming Li (University of Michigan, Ann Arbor). To allow germination of seeds having the *ga1-3* mutation, which require GAs for germination (Koornneef and Van der Veen, 1980), they were incubated at 4°C for 3 d with a solution of 1 μM GA_3 (Fluka, Switzerland). Next, seeds were washed with several volumes of water and surface-sterilized with 20% (v/v) bleach for 15 min. Seeds were properly rinsed with sterile water and sown on 0.5× Murashige and Skoog agar plates containing 1% (w/v) Suc. Seeds of genotypes that do not require GA treatment for germination were incubated in water at 4°C for 3 d and surface sterilized and sown as described above. Germination was induced and synchronized by placing the plates under fluorescent white light (fluence rate of 40–60 μmol m^{-2} s^{-1}) at 20°C for 24 h. Seedlings growing in continuous light were kept under these conditions for a total of 10 d. For dark-growing seedlings, plates were wrapped in several layers of aluminum foil and kept at 20°C for different times (see text). Germination of *ga1-3*-containing seedlings shown in Figure 2A was further improved by removing the seed coat as previously described (Telfer et al., 1997).

Pea (*Pisum sativum*) seeds of WT V1 (selected from cv Alaska), and the pairs of lines WL1769 (genotype *NA*) and WL1766 (genotype *na-1*), kindly provided by Dr. James Reid (University of Tasmania, Australia) were used for the experiments. Seeds were germinated in vermiculite, irrigated with water, and grown at 22°C in the dark.

PAC (1 μM final concentration, Duchefa, Haarlem, The Netherlands), GA_3 (10 μM final concentration), and EBR (1 μM final concentration, Duchefa) were added to the Arabidopsis media after autoclaving. For pea treatments, PAC was applied to the vermiculite as 10 μM aqueous solution, and GA_3 was applied to dry seeds (1 μg per seed in 10 μL of ethanol solution) before seeding. GA_3 application was repeated 7 and 14 d after seeding to the hook. Brassinolide (10 ng, CIDtech Research Inc., Mississauga, Ontario, Canada) was applied to dry seeds and seedlings as described for GA_3. Seedling manipulations were carried out under dim-green safelight.

Analysis of Arabidopsis Hypocotyl Length and Cotyledon and Hook Opening

Arabidopsis seedlings in Figures 1A and 2A were placed on an acetate sheet and scanned at a resolution of 2,400 dots per inch. Cotyledon and hook opening were quantitated basically as described (Neff and Chory, 1998) using the Measure Angles tool of the ImageJ software (http://rsb.info.nih.gov/ij/). Hypocotyl length was also measured with the ImageJ software.

Northern-Blot Analysis

Arabidopsis total RNA was extracted from frozen, whole seedlings by using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Pea total RNA was extracted with TRIzol (Invitrogen/Life Technologies, Carlsbad, CA). RNA was run in 1% (w/v) agarose/formaldehyde gels and transferred onto Hybond N^+ filters (Amersham Biosciences, Little Chalfont, UK) following standard procedures. Filters were prehybridized and hybridized in 50% (v/v) formamide, 5× SSC, 0.5% (w/v) SDS, and 5× Denhart's at 42°C. Probes used corresponded to PCR fragments of the Arabidopsis *CAB2* and *RbcS-3A* genes and to the entire coding sequence of the pea *PsRbcS-3A* gene. *CAB2* fragment was amplified from Columbia-0 genomic DNA by using the oligonucleotides cab2F33 (5′-AAAGTTTCAATGGCCGCCTC-3′) and cab2R383 (5′-CCCACCTGCTGTGGATAACTT-3′) as forward and reverse primers, respectively. Arabidopsis *RbcS-3A* fragment was PCR amplified by using oligonucleotides SP6 and T7 as primers and EST 205G21T7 as template. Probes were ^{32}P labeled by standard procedures. Filters were washed twice for 10 min in 1× SSC/0.1% (w/v) SDS at 65°C and three times for 15 min in 0.1× SSC/0.1% (w/v) SDS at 65°C. Filters were exposed to Super RX films (Fuji Photo Film, Düsseldorf, Germany).

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