Nitric Oxide Regulates DELLA Content and *PIF* Expression to Promote Photomorphogenesis in Arabidopsis[1][W]

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The transition from etiolated to green seedlings involves a shift from hypocotyl growth-promoting conditions to growth restraint. These changes occur through a complex light-driven process involving multiple and tightly coordinated hormonal signaling pathways. Nitric oxide (NO) has been lately characterized as a regulator of plant development interacting with hormone signaling. Here, we show that Arabidopsis (*Arabidopsis thaliana*) NO-deficient mutant hypocotyls are longer than those from wild-type seedlings under red light but not under blue or far-red light. Accordingly, exogenous treatment with the NO donor sodium nitroprusside and mutant plants with increased endogenous NO levels resulted in reduced hypocotyl length. In addition to increased hypocotyl elongation, NO deficiency led to increased anthocyanin levels and reduced PHYB content under red light, all processes governed by phytochrome-interacting factors (PIFs). NO-deficient plants accordingly showed an enhanced expression of *PIF3*, *PIF1*, and *PIF4*. Moreover, exogenous NO increased the levels of the gibberellin (GA)-regulated DELLA proteins and shortened hypocotyls, likely through the negative regulation of the GA Insensitive Dwarf1 (GID1)-Sleepy1 (SLY1) module. Consequently, NO-deficient seedlings displayed up-regulation of *SLY1*, defective DELLA accumulation, and altered GA sensitivity, thus resulting in defective deetiolation under red light. Accumulation of NO in wild-type seedlings undergoing red light-triggered deetiolation and elevated levels of NO in the GA-deficient *ga1-3* mutant in darkness suggest a mutual NO-GA antagonism in controlling photomorphogenesis. PHYB-dependent NO production promotes photomorphogenesis by a GID1-GA-SLY1-mediated mechanism based on the coordinated repression of growth-promoting *PIF* genes and the increase in the content of DELLA proteins.

Light controls plant development through a complex mechanism that is dependent on the quality, intensity, timing, and duration of the incoming light. After embryonic development, germinated seeds can follow two main developmental programs, depending on whether early postgermination occurs in darkness or light. The most common situation in nature is that germinated seeds grow initially skotomorphogenically until cotyledons emerge from the ground. The deetiolation program starts with the loss of the apical hook and the opening of cotyledons to display maximal surface for photosynthesis (Alabadí and Blázquez, 2009). The dark-to-light transition is accompanied by an extensive transcriptional reprogramming that is mostly controlled by the function of two kinds of photoreceptors, phytochromes and cryptochromes, for red/far-red and UV/blue light, respectively (Jiao et al., 2007). The common understanding suggests that photomorphogenesis is the default program in plant development in such a way that skotomorphogenesis is the result of the efficient repression of the photomorphogenic program. Repression is based on the action of CONSTITUTIVE PHOTOMORPHOGENIC1 E3 ubiquitin ligase (Osterlund et al., 1999), which targets several photomorphogenesis-promoting transcription factors for degradation, including LAF1, HFR1, HY5, and HYH (Holm et al., 2002; Seo et al., 2003; Jang et al., 2005; Yang et al., 2005), but not others driving etiolated growth, such as the phytochrome-interacting factors PIF1, PIF3, and PIF4 (Castillon et al., 2007; Leivar et al., 2008b). To avoid skotomorphogenic growth in light, PIFs are rapidly phosphorylated and degraded by light-activated phytochromes (Shen et al., 2005, 2007; Al-Sady et al., 2006). The balance between the activities of both groups of transcription factors is thus determined by the transition from skotomorphogenesis to photomorphogenesis (Huq, 2006; Leivar et al., 2009; Shin et al., 2009). However, light is not the only factor controlling this developmental transition. Plant development is tightly subject to hormonal control, and this regulatory level represents an integration point between endogenous cues and stress-related environmental factors (Nemhauser, 2008; Alabadí and

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Blázquez, 2009; Wolters and Jürgens, 2009). Among major hormonal growth regulators, it is well known that GA signaling exerts an essential role in repressing photomorphogenesis under darkness (Alabadí et al., 2008; de Lucas et al., 2008; Feng et al., 2008). Repression is achieved through the GA-induced degradation of DELLA proteins, which are indeed negative modulators of PIF function (Achard et al., 2007; Alabadí et al., 2008; de Lucas et al., 2008; Feng et al., 2008), thus leading to hypocotyl elongation. The function of the GAs and its receptor GA Insensitive Dwarf1 (GID1) in DELLAs represents a regulatory module able to control plant growth under fluctuating environmental conditions (Harberd et al., 2009). This signaling module could allow growth arrest when plants face adverse environmental conditions, and it might function potentially as a link between stress-triggered responses and development (Achard et al., 2006, 2008; Navarro et al., 2008).

Many stress factors share the activation of rapid responses in the plant, characterized by the production of reactive oxygen and nitrogen species. Some of these molecules have been characterized as potential primary modulators because of their ubiquity and strong reactivity. Among them, nitric oxide (NO) has been extensively characterized as a regulator of stress-related responses (Hong et al., 2008; Neill et al., 2008) and developmental processes as well (He et al., 2004; Prado et al., 2004; Hu et al., 2005; López-Bucio et al., 2006). NO often exerts its regulatory activity in tight coordination with other regulators, including salicylic acid (Mur et al., 2008), jasmonic acid (Saito et al., 2009), abscisic acid (Saito et al., 2009), and ethylene (Ederli et al., 2006; Wang et al., 2009). It has also been reported that NO may function as a modulator of auxins in root development (Pagnussat et al., 2003; Guo et al., 2008; Lanteri et al., 2008). Conversely, NO production is activated by different hormones, including abscisic acid (Guo et al., 2003; Bright et al., 2006), cytokinins (Tun et al., 2008), auxins (Kolbert et al., 2008), jasmonic acid (Huang et al., 2004), and salicylic acid (Zottini et al., 2007; Hao et al., 2010). We have recently demonstrated that production of NO through two biosynthetic pathways involving nitrate reductase (NR/NIA) and nitric oxide-associated 1 (NOA1) activities is essential for the correct development of Arabidopsis (*Arabidopsis thaliana*), affecting a wide range of processes including seed production, germination, vegetative growth, and the control of stomatal closure (Lozano-Juste and León, 2010). NO's effect on seed germination is exerted in tight coordination with abscisic acid (Lozano-Juste and León, 2010), but the participation of other hormones such as GAs is also very likely. In fact, there is evidence about the interaction between GAs and NO in regulating germination (Beligni et al., 2002; Bethke et al., 2007). Interestingly, there are also some reports proposing that NO controls other aspects of development where GAs have an essential role, including hypocotyl elongation, the acquisition of photomorphogenic traits (Beligni and Lamattina, 2000; Tonón et al., 2010), and the growth and reorientation of pollen tubes (Prado et al., 2004). However, the underlying mechanism explaining how NO controls plant growth in connection with hormones remains unknown. Here, we show that NO counteracts GA signaling and thus promotes photomorphogenesis through a regulatory mechanism affecting DELLA protein accumulation positively and *PIF* expression negatively.

RESULTS

Increased Hypocotyl Elongation in Light-Grown NO-Deficient *nia1,2noa1-2* Mutant

Despite the delayed vegetative growth characteristic of *nia1,2noa1-2* seedlings (Lozano-Juste and León, 2010), we observed that their hypocotyls were unusually long, reaching a size that doubled that of wild-type seedlings 5 d after germination under white light (Fig. 1A). Enhanced hypocotyl elongation caused by NO deficiency was not evident under every growth condition; for instance, *nia1,2noa1-2* seedlings were as long as the wild type in darkness (Fig. 1A). Moreover, it was not the result of a negative effect of NO on the growth of the whole seedling, since the *nia1,2noa1-2* mutant displayed the opposite pattern in root elongation, with shorter roots than wild-type seedlings in light but no significant differences in darkness (Fig. 1B). The differential effect on hypocotyl elongation and root growth in light but not in darkness suggests that the *nia1,2noa1-2* mutant is fully able to repress photomorphogenesis in darkness, thus indicating that NO is not required for it. However, it displayed a defect in light-induced deetiolation, suggesting that NO is necessary for the repression of growth by light. To investigate whether NO would act in a specific branch of the light signaling pathway, we tested the ability of different light qualities to repress hypocotyl elongation in different NO-deficient mutant backgrounds. Figure 1C shows that blue and far-red light were able to repress hypocotyl elongation in all NO-deficient genotypes to the same extent observed in wild-type seedlings. However, under red light conditions, NO-deficient seedlings showed longer hypocotyls than wild-type seedlings, the effect being mild in the backgrounds with partial NO deficiency and additive in the triple mutant (Fig. 1C). Further characterization of the reduced inhibition of hypocotyl elongation of NO-deficient seedlings by red light showed that the differential effect was already significant by 4 d after germination. Up to 5 d after germination, when wild-type hypocotyl growth started to slow, the triple *nia1,2noa1-2* hypocotyls were still growing at the maximal rate (Supplemental Fig. S1A). The differential effect was also dependent on the fluence rate of red light, with significant differences in the inhibition of hypocotyl elongation of *noa1-2* and *nia1,2noa1-2* mutants at fluence rates as low as 1 μmol m^{-2} s^{-1} (Supplemental Fig. S1B). At 10 μmol m^{-2} s^{-1} red light, a 40%

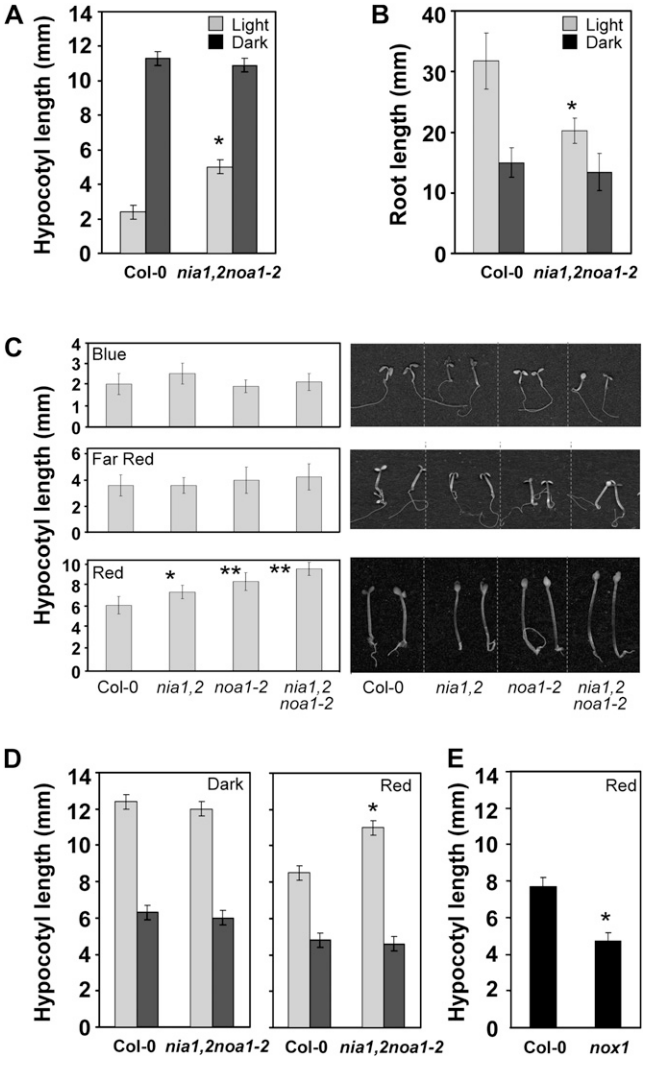

Figure 1. Hypocotyl and root elongation of NO-deficient plants under different light conditions. A, and B, Hypocotyl and root length, respectively, of the wild type and the *nia1,2noa1-2* mutant under white light (Light) or darkness (Dark). Hypocotyl and root length were measured in 5- and 10-d-old seedlings, respectively. C, Hypocotyl length was measured in 5-d-old seedlings grown under 16.5 μmol m^{-2} s^{-1} blue light, 5 μmol m^{-2} s^{-1} far-red light, and 20 μmol m^{-2} s^{-1} red light. D, Hypocotyl length in untreated (light gray bars) and NO-treated (250 μM SNP; dark gray bars) Col-0 and *nia1,2noa1-2* seedlings. E, Length of the hypocotyls of Col-0 and NO-overproducer *nox1* seedlings. Values are means ± SE of three independent experiments (at least 20 seedlings per experiment were measured). Asterisks represent statistically significant differential values at * $P < 0.005$ and ** $P < 0.001$ when comparing mutant versus the wild type under the same treatment conditions.

inhibition in wild-type hypocotyl length was observed, whereas only 25% was observed for *nia1nia2* and less than 5% for *noa1-2* and the triple mutant (Supplemental Fig. S1B). Importantly, differential hypocotyl elongation of *nia1,2noa1-2* versus accession Columbia (Col-0) seems to be due to reduced NO content, since both exogenous application of NO by the NO donor sodium nitroprusside (SNP) and the *nox1* mutant plant with increased NO content (He et al., 2004) resulted in shorter hypocotyls (Fig. 1, D and E), clearly showing that NO controls hypocotyl elongation.

PHYB Levels and Signaling Are Altered in a NO-Deficient Mutant

The reduced inhibition of hypocotyl elongation observed for NO-deficient seedlings only under red light conditions suggests that mutant seedlings are partially blind to red light, which may be caused by changes in the level or activity of the PHYB photoreceptor or by interference with PHYB signaling. If the *nia1,2noa1-2* mutant had altered PHYB levels or was impaired in PHYB function, it would be expected to phenocopy most if not every phenotype displayed by *phyB* mutant seedlings. Table I summarizes some of the traits characteristic of the *phyB* mutant phenotype and the correspondence in *nia1,2noa1-2* seedlings. Both mutants share overlapping traits, such as elongated hypocotyls under red light, pale green leaves with reduced chlorophyll content, low transpiration rates, accumulation of anthocyanins, and reduced branching. However, the elongated petioles, long root hairs, elongated stems, and early-flowering phenotype under a short-day photoperiod, which are characteristic of the *phyB* mutant, were not evident in *nia1,2noa1-2* mutant plants. In fact, the triple mutant was not impaired in PHYB production, since its level in the NO-deficient mutant did not differ significantly from that in wild-type seedlings in darkness (Fig. 2, A and B). However, a significantly reduced content of PHYB was detected in *nia1,2noa1-2* when compared with wild-type seedlings under red light (Fig. 2, A and B). Nevertheless, the *phyB* mutant was not altered in NO-triggered inhibition of hypocotyl elongation (Fig. 2C). All these observations suggest that NO-deficient plants might be altered in red light signaling but not in PHYB protein activity and that NO acts downstream of PHYB in this process.

PIFs Are Targets of the NO-Triggered Inhibition of Hypocotyl Growth

Since *nia1,2noa1-2* seedlings contained around 3-fold more anthocyanins than wild-type seedlings in light (Fig. 3C), and given that many of the genes encoding the enzymes of flavonol and anthocyanin biosynthesis are targets of the PIF3 transcription factor (Shin et al., 2007), NO might interfere with PHYB signaling through PIF3. As previously reported, PIF3, together with PIF4, negatively regulates PHYB content under red light conditions (Al-Sady et al., 2008; Leivar et al., 2008a), which is fully in agreement with the reduced levels of PHYB detected in *nia1,2noa1-2* seedlings only under red light (Fig. 2). Supporting this hypothesis, we have found that *nia1,2noa1-2* hypocotyls accumulate more *PIF3* transcript than wild-type seedlings under red light (Fig. 3A), and this increase was also detected for

Table I. *Comparative phenotypic analysis of phyB and nia1,2noa1-2 mutants*

Traits analyzed in the cited references were compared with those reported for the *nia1,2noa1-2* mutant (Lozano-Juste and León, 2010).

Trait	*phyB*	*nia1,2noa1-2*	Reference
Elongated hypocotyls	Yes	Yes	Reed et al. (1993)
Long petioles	Yes	Yes	Reed et al. (1993)
Pale leaves in light	Yes	Yes	Reed et al. (1993)
Elongated stems	Yes	No	Reed et al. (1993)
Reduced root elongation	Yes	Yes	Correll and Kiss (2005)
Long root hairs	Yes	No	Reed et al. (1993)
Early flowering	Yes	No	Reed et al. (1993); Guo et al. (1998)
Lower transpiration rate	Yes	Yes	Boccalandro et al. (2009)
Reduced branching	Yes	Yes	Reed et al. (1993)
Reduced seed germination	Yes	Yes	Shinomura et al. (1994)

other bHLH members of the *PIF* family such as *PIF1* and *PIF4* (Fig. 3A). Accordingly, the levels of these three *PIF* transcripts were significantly reduced upon treatment of wild-type seedlings with SNP (Fig. 3A). Furthermore, as expected for plants with enhanced PIF function (de Lucas et al., 2008; Feng et al., 2008), *nia1,2noa1-2* seedlings had an altered response to both GA_3 and the GA biosynthetic inhibitor paclobutrazol (PAC). Figure 3B shows that wild-type and *nia1,2noa1-2* hypocotyls treated with 200 nM PAC were 70% and 50% shorter, respectively, than those from untreated seedlings. At lower PAC concentrations, *nia1,2noa1-2* hypocotyls were always hyposensitive to PAC (Fig. 3C). Altered sensitivity to GAs was also observed in the *nia1,2noa1-2* mutant. Whereas *nia1,2noa1-2* hypocotyls reached their maximum length at GA_3 concentrations around 1 μM, wild-type hypocotyls increased elongation to at least 5 μM GA_3 (Fig. 3D). On the other hand, the hypocotyls of seedlings overexpressing *PIF3* were significantly more sensitive ($P < 0.05$) than wild-type seedlings to NO-mediated inhibition of hypocotyl elongation (Fig. 3F). By contrast, a quadruple *pif1pif3pif4pif5* mutant (*pifQ*) was almost completely insensitive to NO-triggered hypocotyl shortening (Fig. 3, E and F). All these data strongly suggest that there is an enhanced PIF function in NO-deficient mutants and also that PIFs are targets of the NO-triggered inhibition of hypocotyl growth.

NO Promotes DELLA Protein Accumulation

PIF activity is controlled by its association with DELLA proteins (de Lucas et al., 2008; Feng et al., 2008), whose levels are regulated by GAs through proteasome-mediated degradation (Dill et al., 2004: Achard et al., 2007; Harberd et al., 2009). The altered sensitivity of *nia1,2noa1-2* seedlings to PAC and GAs could also be explained by reduced levels of DELLAs in the NO-deficient seedlings. To test the possible effect of NO on DELLA accumulation, transgenic seedlings expressing a GFP-tagged version of the DELLA protein RGA under its endogenous promoter (*pRGA*::*GFP-RGA*) were treated with SNP. Figure 4A shows that in accordance with GA promoting DELLA degradation (Dill et al., 2004; Achard and Genschik, 2009), GFP-RGA fluorescence was absent both in roots and hypocotyls from GA-treated seedlings but, in turn, GFP-RGA-associated fluorescence, at nuclei, increased in NO-exposed seedlings (Fig. 4A). The increased amount of GFP-RGA protein was also detected in whole seedlings by western blot with anti-GFP antibodies (Fig. 4A). To check whether endogenous NO controls RGA levels, we generated *pRGA*::*GFP-RGA* transgenic plants in the *nia1,2noa1-2* background. Figure 4B shows GFP-RGA fluorescence in different backgrounds after transferring seedlings from darkness to light. Fluorescence was detected as soon as 2 h after the shift in hypocotyls of wild-type background, when almost no fluorescence was detected in hypocotyls of the NO-deficient background plants (Fig. 4B). By 4 h after the shift, GFP-RGA started to accumulate in the *nia1,2noa1-2* background, although its level was significantly lower than in the wild-type background, suggesting that NO is necessary for the timely light-dependent GFP-RGA accumulation. By contrast, hypocotyls of the *ga1-3* background showed enhanced GFP-RGA fluorescence even in darkness (Fig. 4B), due to the lack of GAs in this mutant (Achard et al., 2007). Moreover, we have further checked that this effect was not specific for RGA, and it is extended to all DELLAs. By using transgenic lines expressing tandem affinity purification (TAP)-tagged versions of the five DELLAs, we have analyzed the levels of DELLA proteins after treatment with the NO donor or GA_3. Western blots with anti-MYC antibody of whole seedling extracts showed increased protein levels of every DELLA in seedlings exposed to NO and decreased levels in GA-treated seedlings (Fig. 4C). Additionally, pretreatment with the NO donor and further application of GAs did not prevent GA-induced degradation of DELLAs (Fig. 4C), suggesting that NO is not modifying DELLA proteins, turning them resistant to proteolysis. We confirmed this idea by following TAP-RGA degradation upon exposure to increasing concentrations of GAs in NO-treated and untreated seedlings. Figure 4D shows that TAP-RGA was efficiently degraded in NO-treated seedlings to levels similar to those detected in untreated control seedlings. In fact, the degradation of

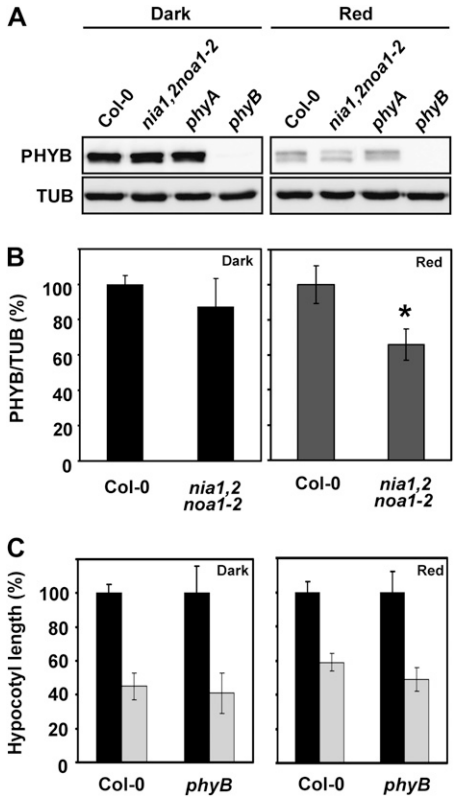

Figure 2. PHYB protein levels in wild-type Col-0, NO-deficient *nia1,2noa1-2*, *phyA*, and *phyB* mutants grown in darkness or red light. A, Western blots with anti-PHYB antibodies of protein samples from representative hypocotyls of the indicated genotypes grown for 3 d under darkness or 7 μmol m^{-2} s^{-1} red light. Western blots with anti-tubulin (TUB) are shown as loading controls. B, Quantification of PHYB levels normalized to TUB and expressed relative to the levels of Col-0. Values are means of three independent experiments ± SD. C, Relative hypocotyl length of untreated (black bars) and NO-treated (250 μM SNP; gray bars) Col-0 and *phyB* seedlings under dark and red light conditions. Values are means ± SE of three independent experiments (at least 20 seedlings per experiment were measured). Asterisks represent statistically significant differential values at $P < 0.05$ when comparing mutant versus the wild type under the same treatment conditions.

TAP-RGA by GA_3 seemed to be more efficient in NO-treated seedlings. Then, we explored whether active degradation of DELLAs in NO-treated seedlings is also mediated by the ATP-consuming proteasome by a previously reported cell-free assay system (Wang et al., 2009). Figure 4E shows no significant degradation of TAP-RGA proteins in the absence of exogenously added ATP in untreated seedlings, but degradation was observed in NO-treated seedlings, indicating that TAP-RGA in these extracts could be degraded by a proteasome-independent mechanism. However, both untreated and NO-treated TAP-RGAs were progressively degraded with time in ATP-treated extracts, the latter being more susceptible to degradation (Fig. 4F). Moreover, the proteasome inhibitor MG132 retarded TAP-RGA degradation in untreated controls but only to a limited extent in NO-treated samples (Fig. 4F), suggesting that in NO-treated samples, proteasome-independent hydrolysis also occurred. This alternative degradation mechanism could also explain the more efficient degradation of TAP-RGA in NO-treated seedlings upon GA_3 treatment (Fig. 4D) or in the absence of ATP (Fig. 4E).

We have also checked that increased tagged DELLA levels are not the result of an enhancing effect of NO on 35S-driven expression, as *35S*::*TAP-GFP* seedlings showed similar levels of GFP in NO-treated and control untreated seedlings (Supplemental Fig. S2). Moreover, *35S*::*TAP-GAIΔ17* and *35S*::*TAP-RGAΔ17* seedlings, expressing truncated versions of GAI and RGA resistant to GA-induced proteolysis, showed unaltered protein levels upon treatment (Fig. 4C). Increased DELLA levels upon NO treatment in *35S*::*TAP-DELLA* transgenic plants were then not likely due to transcriptional activation of *DELLA* genes, because the expression of these genes is under the control of the constitutive 35S promoter. Nevertheless, to check whether the enhanced accumulation of GFP-RGA in *pRGA*::*GFP-RGA* lines was due to the transcriptional control of *RGA* by NO, quantitative reverse transcription (RT)-PCR with RNA extracted from hypocotyls of wild-type seedlings exposed to the NO donor SNP was performed. We did not find significant changes in the transcript levels of *DELLA* genes in response to NO (Supplemental Fig. S3). Moreover, *DELLA* transcript levels were not altered in the NO-deficient *nia1,2noa1-2* mutant compared with wild-type seedlings (Supplemental Fig. S3). Only *RGL1* transcripts were significantly changed in the NO-deficient mutant when compared with Col-0, and it was not down-regulated, as expected for a putative role of NO as a gene activator (Supplemental Fig. S3).

Alternatively, the control of endogenous GA levels by NO might explain the accumulation of DELLAs in NO-treated seedlings. Regarding this, we have quantified the levels of GA biosynthesis and metabolism gene transcripts. Only the *GA20ox3* gene was down-regulated by NO treatment and up-regulated in the NO-deficient *nia1,2noa1-2* mutant (Supplemental Fig. S4). These data suggest that NO might reduce the biosynthesis of active GAs through specific effects on GA20ox3. However, this effect should be restricted to hypocotyl, because no other GA-related phenotype was observed in NO-deficient seedlings.

NO Induces DELLA Accumulation by Repressing *SLY1*

Wild-type hypocotyls grown in red light under increasing NO concentrations were progressively shortened. At 0.5 mM SNP, hypocotyl length was below 10% of those from untreated seedlings (Fig. 5A). Shortening of hypocotyls with increasing SNP concentrations correlated well with the accumulation of all DELLAs, with GAI being the less sensitive to NO (Fig. 5B). Additionally, Figure 5C shows that the inhibition of hypocotyl elongation exerted by NO under red light conditions

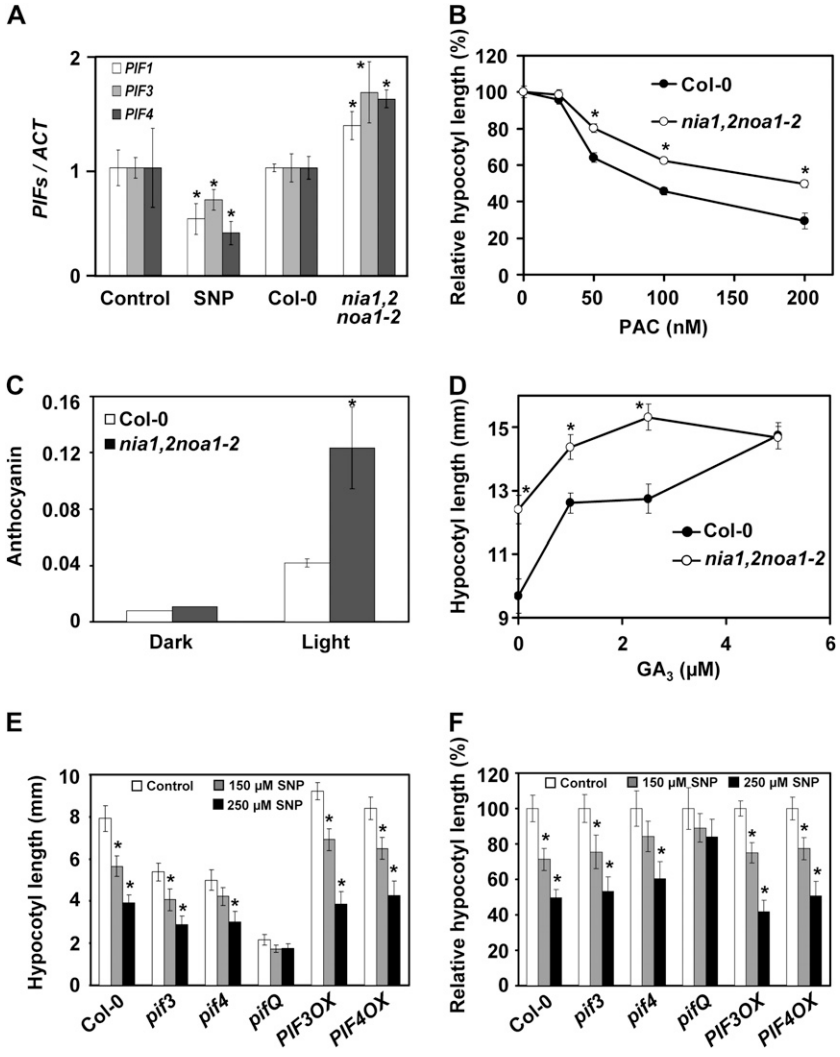

Figure 3. Functional connection between NO, GAs, and PIF proteins. A, *PIF1*, *PIF3*, and *PIF4* transcript levels in Col-0 or *nia1,2noa1-2* hypocotyls and on wild-type hypocotyls either treated with 1 mM SNP (NO donor) for 2 h or untreated as a control. B, Relative length of Col-0 and *nia1,2noa1-2* hypocotyls (mean ± SE) in seedlings exposed to the indicated concentrations of PAC. C, Anthocyanin levels in Col-0 and *nia1,2noa1-2* seedlings under dark or white light conditions expressed in arbitrary units of A_{530}. D, Length of Col-0 and *nia1,2noa1-2* hypocotyls (mean ± SE) treated with the indicated concentrations of GA_3. E and F, Total and relative length, respectively, of control untreated and SNP-treated hypocotyls in the indicated genotypes and concentrations. All the experiments were performed with seedlings grown under 20 μmol m^{-2} s^{-1} red light unless otherwise mentioned. Values are means of three biological replicates ± SE. For hypocotyl length, at least 20 seedlings per independent experiment were measured. Asterisks represent statistically significant differential values with at least $P < 0.05$ when comparing mutant versus the wild type for the same treatment conditions (A–D) or untreated versus treated samples in each genotype (E and F).

was partially dependent on DELLA function, as revealed by the slightly reduced response to NO detected in the *rgagai* double mutant and the more pronounced insensitivity detected in the quadruple mutant *4della*, lacking all DELLAs but RGL3 (Achard et al., 2006), and the *5della* global knockout mutant (Fig. 5C). In the concentration range from 0.1 to 0.5 mM SNP tested, the length of *4della* and *5della* hypocotyls was always 20% to 35% longer than wild-type hypocotyls (Fig. 5C). These data suggest that NO inhibits hypocotyl growth mostly through DELLA function. However, even at the lower NO donor concentration tested, around one-quarter of the effect was exerted via DELLA-independent mechanisms. These data point to DELLAs as the main but not only target for NO action in controlling growth, which is in agreement with the proposed regulation of *PIF1*, *PIF3*, and *PIF4* expression by NO indicated above.

DELLA levels are controlled by GAs through a mechanism dependent on GA perception by GID1, ubiquitination by the ubiquitin ligase Sleepy1 (SLY1), and further degradation by the proteasome. Whereas transcripts of genes coding for GID1s were not significantly affected by exogenous NO and only *GID1b* was down-regulated in *nia1,2noa1-2* seedlings (Fig. 6A), *SLY1* expression was significantly down-regulated by NO and up-regulated in the NO-deficient *nia1,2noa1-2* mutant (Fig. 6B). In agreement with an important role of SLY1 in NO-mediated inhibition of hypocotyl elongation, the loss- and gain-of-function mutants of SLY1, *sly1-10* and *sly1-D*, were less and more sensitive to NO, respectively, than wild-type seedlings (Fig. 6, D and F). Again, the insensitivity of *sly1-10* mutant to NO was not complete, indicating the existence of another pathway for NO regulation of hypocotyl elongation. Interestingly, although no transcriptional regulation of *GID1* genes by NO was observed (Fig. 6A), the double *gid1a,c* mutant hypocotyls were less sensitive to NO-triggered shortening (Fig. 6, C and E), thus suggesting that NO action requires the activity of both GID1a and GID1c receptors.

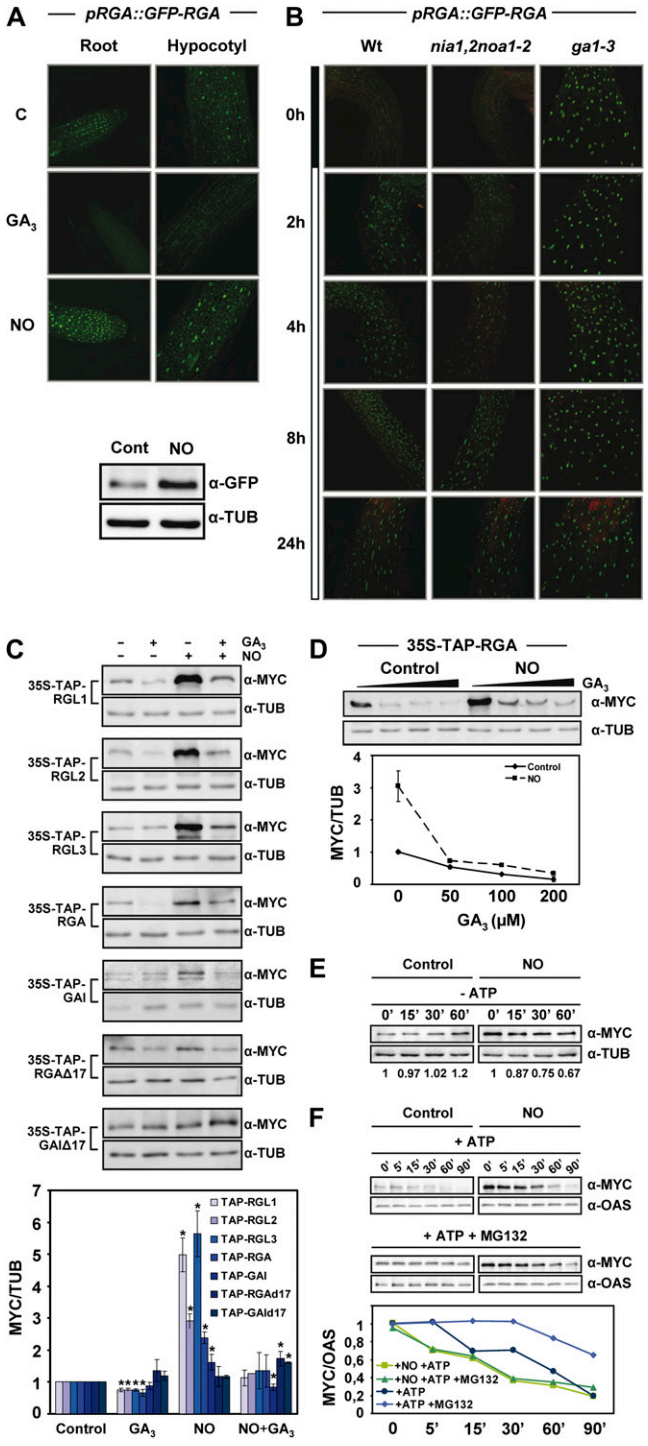

Figure 4. Effect of NO on DELLA protein accumulation. A, GFP-RGA in *pRGA::GFP-RGA* roots and hypocotyls, either untreated (control [C]) or treated for 2 h with 50 μM GA3 or 250 μM SNP as a source of NO, visualized by confocal microscopy. GFP-RGA levels and the loading control tubulin (TUB) were analyzed by western blot. B, GFP-RGA protein in hypocotyls of *pRGA::GFP-RGA* in wild-type, *nia1,2noa1-2*, and *ga1-3* backgrounds at different times after the shift from darkness to red light, as indicated in the bar at left. C, TAP-tagged versions of every DELLA protein were used to analyze the levels of each protein in seedlings treated (+) or not (−) with 50 μM GA_3 and/or 250 μM SNP (NO) for 2.5 h. TAP-DELLAs were detected with anti-MYC antibodies, and the levels of tubulin are shown as a loading control. The values normalized with tubulin and relative to control untreated samples were quantified, and the values shown in the bottom panel correspond to means of three independent experiments ± SD. Asterisks represent statistically significant differential values with at least $P < 0.05$ when comparing treated versus untreated controls in each genotype. D, GA-induced degradation of RGA in untreated (Control) and NO-treated (NO; as described in C) *35S-TAP-RGA* seedlings exposed to increasing GA_3 concentrations (0, 50, 100, and 200 μM). Values normalized to tubulin were quantified and are shown in the bottom panel as means of three independent experiments ± SE. E and F, Cell-free degradation assay of RGA in the absence (E) or presence (F) of ATP and the proteasome inhibitor MG132. Protein samples were incubated at room temperature for the indicated times and treatment conditions and detected with anti-MYC antibodies. Tubulin or *O*-acetyl-Ser(thiol)lyase 1 (OAS) was detected as a loading control (D–F). ATP and MG132 were used at 10 mM and 100 μM, respectively. The protein levels detected on western blots in E and F were quantified and normalized to TUB or OAS content.

NO Production Is Negatively Regulated by GAs

The proposed role of NO on photomorphogenesis would require physiological support to have a functional significance in the deetiolation process. We have tested whether the transition from darkness to light is accompanied by the endogenous production of NO in Arabidopsis. Endogenous NO content analysis in dark-grown hypocotyls shifted to red light has been performed by using 3-amino,4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) fluorescein that specifically detects NO. Figure 7 shows that NO-associated fluorescence was very low in dark-grown wild-type hypocotyls. However, by 1 h after the shift to red light, NO-associated fluorescence was raised along the hypocotyls (Fig. 7A). However, the increased NO-associated fluorescence upon transition to red light was not detected either in the *nia1,2noa1-2* or in the *phyB* mutant hypocotyls (Fig. 7A). Interestingly, the GA-deficient mutant *ga1-3* contained NO levels in darkness significantly higher than those detected in wild-type plants (Fig. 7A), suggesting that GAs exert a negative control on NO production in hypocotyls. In addition, no increase in NO was detected in the *ga1-3* mutant upon the shift to red light (Fig. 7A). As further support for the negative regulation exerted by GAs on NO production, both the wild type and the *ga1-3* mutant displayed reduced NO-associated fluorescence in GA_3-treated seedlings compared with untreated controls (Fig. 7B). By contrast, the GA-insensitive *gai-1D* mutant did not change its NO content upon treatment with exogenously supplied GAs (Fig. 7B).

DISCUSSION

Oxygen and nitrogen reactive species have important roles in plant development that are characteristic of developmental transitions (Gapper and Dolan, 2006; Tsukagoshi et al., 2010). These molecules have

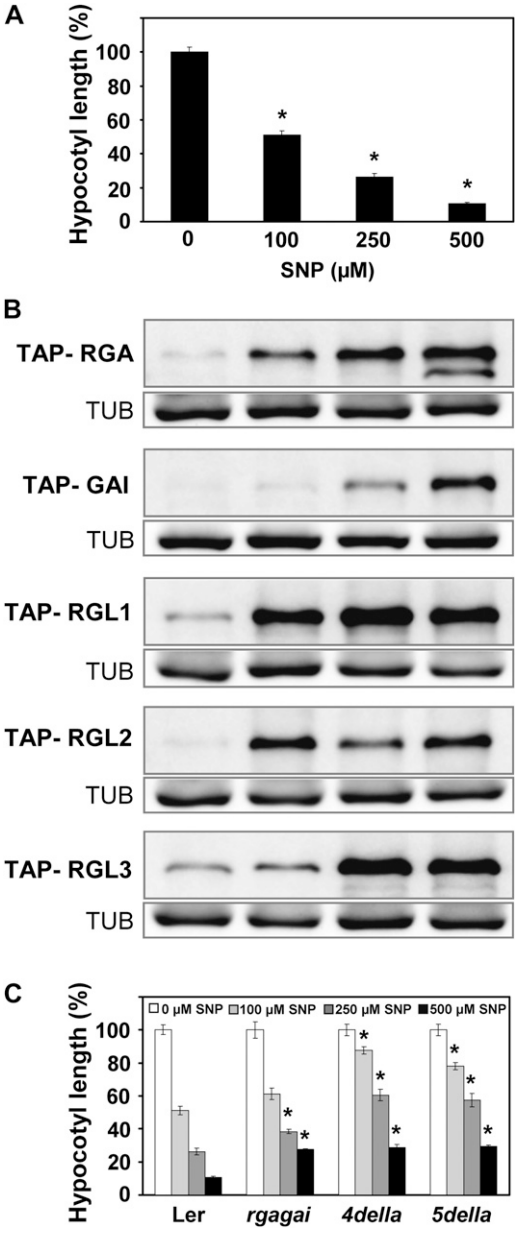

Figure 5. Effect of NO on hypocotyl elongation and DELLA content under red light. A and C, Hypocotyl length of wild-type L*er*, *rga-24gai-t6*, and quadruple (*4della*) and quintuple (*5della*) DELLA mutants was measured after growing seedlings in the indicated SNP concentrations for 3 d. Values of hypocotyl length are means ± SE of three independent experiments (at least 20 seedlings per experiment were measured). Asterisks represent statistically significant differential values with at least $P < 0.05$ when comparing hypocotyls of treated versus untreated wild-type seedlings (A) or mutant versus wild-type hypocotyls from different genotypes under the same treatment conditions (C). B, TAP-DELLA accumulation under the SNP concentrations shown in A was detected with anti-MYC antibodies, and the loading controls of tubulin (TUB) are included.

well-documented roles regulating the adaptation to changes in temperature (Allakhverdiev et al., 2008) or light (Li et al., 2009), conditions that regulate developmental processes like seed germination and seedling establishment (Bailly et al., 2008), hypocotyl elongation (Stavang et al., 2009), flowering (Ye et al., 2000), leaf senescence (Jing et al., 2008), and tuber formation (Agrawal et al., 2008). Among reactive species produced in plants in response to changes in their environment, NO has been the focus of increasing interest in recent years, since it participates in both defensive (Romero-Puertas et al., 2004; Wendehenne et al., 2004; Hong et al., 2008) and developmental (He et al., 2004; López-Bucio et al., 2006) processes. It has been reported that the addition of a NO donor to etiolated Arabidopsis seedlings promotes deetiolation

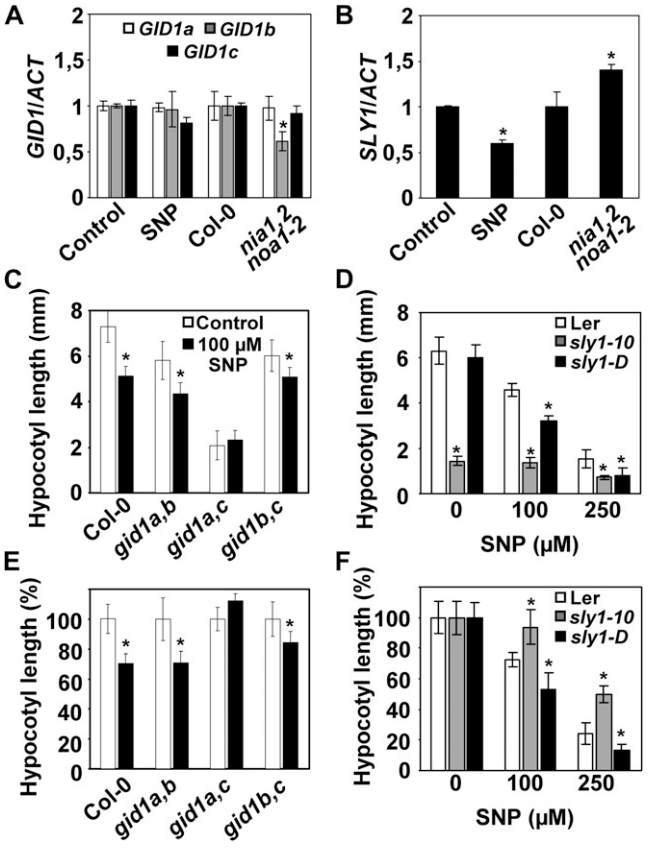

Figure 6. Functional connection between NO and the GA signaling components GID1s and SLY1. A and B, *GID1s* and *SLY1* transcript levels normalized to actin in hypocotyls of untreated (Control) and 1 mM SNP-treated wild-type seedlings as well as in the *nia1,2noa1-2* mutants and its wild-type background, Col-0. Values are means ± SE of three experiments. C and E, Total and relative hypocotyl length, respectively, of different combinations of double *gid1* mutants in untreated (white bars) and 100 μM SNP-treated (black bars) seedlings. D and F, Total and relative hypocotyl length of Col-0, the loss-of-function mutant *sly1-10*, and the gain-of-function mutant *sly1-D* at the indicated SNP concentrations. For C to F, values of hypocotyl length are means ± SE of three independent experiments (at least 20 seedlings per experiment were measured). Asterisks represent statistically significant differential values at $P < 0.05$ when comparing mutant versus wild-type hypocotyls under the same treatment conditions (A, B, D, and F) or treated versus untreated hypocotyls in each genotype (C and E).

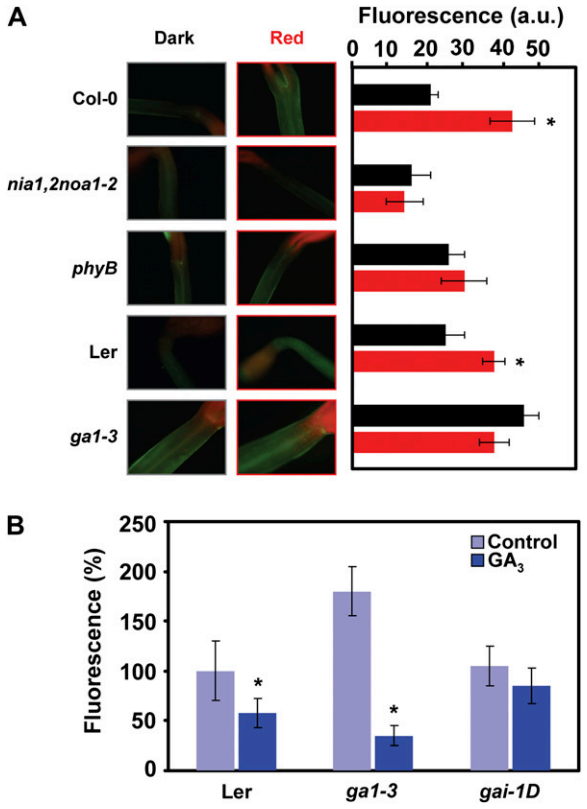

Figure 7. NO levels in the wild-type Col-0 and L*er* as well as in *nia1,2noa1-2*, *phyB*, *ga1-3*, and *gai-1D* mutant plants. A, Endogenous NO was visualized with the cell-permeable DAF-FM DA fluorescein in seedlings grown in darkness for 4 d and 1 h after the shift to red light (20 μmol m^{-2} s^{-1}). B, NO-related fluorescence in L*er*, *ga1-3*, and *gai-1D* in untreated (Control) and 50 μM GA_3-treated seedlings for 2.5 h. Values are shown relative to untreated wild-type levels. The right panel of A and B show the quantification of three independent experiments as mean values of fluorescence (arbitrary units [a.u.]) ± SE. Asterisks represent statistically significant differential values at $P < 0.05$ when comparing controls in darkness (A) or untreated (B).

and inhibits hypocotyl elongation (Beligni and Lamattina, 2000). In addition, a severe heat shock treatment (45°C) leads to a NO burst and the concomitant hypocotyl growth inhibition (Lee et al., 2008). Furthermore, the *hot 5-1* mutant, impaired in *S*-nitrosoglutathione reductase, which metabolizes the NO adduct *S*-nitrosoglutathione, was markedly susceptible to hot temperatures, accumulated increased concentrations of NO, and presented a deetiolated phenotype in darkness (Lee et al., 2008), the latter being dependent on the increased NO accumulation of the mutant (Lee et al., 2008). Accordingly, we have found that *nia1,2noa1-2* seedlings severely impaired in NO biosynthesis displayed elongated hypocotyls under red light conditions, in accordance with the previously reported data for a mutant impaired in the regulation of nitrate reductase gene expression (Lin and Cheng, 1997). Moreover, as presented in this study, a mutant that accumulates increased NO levels presented shorter hypocotyls than the wild type under the same conditions. All this evidence points to a positive role of NO in promoting photomorphogenesis.

However, nothing is known to date about the molecular mechanism by which NO is regulating photomorphogenesis. The observation that the triple mutant only showed elongated hypocotyls under red light but not under far-red or blue light conditions, and because PHYB is the main photoreceptor mediating deetiolation in red light (de Lucas et al., 2008), we first focused on PHYB as the target of NO. After comparing *phyB* mutant phenotypes with those of *nia1,2noa1-2* plants and, importantly, once we noticed that *nia1,2noa1-2* seedlings were able to produce wild-type levels of PHYB protein in darkness and also that the *phyB* mutant responds to NO like wild-type plants, it seemed to us that PHYB-dependent signaling and not its function was impaired in *nia1,2noa1-2* seedlings. This hypothesis agrees with previously reported data supporting a role for the NO second messenger cGMP in controlling the phytochrome signaling involved in some processes such as chloroplast development (Bowler et al., 1994). It is noteworthy to mention that by comparing the effect of NO on hypocotyl elongation under dark and red light conditions, we observed essentially similar responses (Supplemental Fig. S5). This suggests that NO in darkness can modulate the same signaling components than in red light. There are several essential downstream components of PHYB in red light signaling, but the interaction of PHYB with the PIF transcription factors may be one of the earliest. The fact that *nia1,2noa1-2* presented longer hypocotyls and contained more anthocyanins than wild-type seedlings pointed to an enhanced expression of *PIF* genes, and particularly of *PIF3*, in this mutant. In addition, the reduced accumulation of PHYB protein in the *nia1,2noa1-2* mutant under red light conditions fits well with an enhanced expression of *PIF3* in this mutant, given the previously described negative regulation of PHYB levels by PIF3 and PIF4 (Leivar et al., 2008a). Therefore, it is reasonable that the reduction in PHYB protein levels of the *nia1,2noa1-2* mutant in red light contributes significantly to the observed hypocotyl phenotype. We demonstrated that *PIF3* together with *PIF1* and *PIF4* were down-regulated by NO and up-regulated in NO-deficient seedlings. Interestingly, an interaction between HY5, HYH, and PIF4 regulating the light activation of *NR2/NIA2* expression in Arabidopsis has been described (Jonassen et al., 2009). These data suggest the existence of a regulatory loop between NR/NIA and PIFs with potential as a mechanism to integrate light- and NO-related factors controlling plant development.

Although it has been previously proposed that NO regulates hypocotyl elongation (Beligni and Lamattina, 2000; Tonón et al., 2010), no mechanism has been reported, to our knowledge, for explaining the control of hypocotyl growth by NO. We have identified PIFs as important regulators of hypocotyl elongation pheno-

types observed in NO-deficient seedlings. Furthermore, we found that the *pifQ* mutant is almost insensitive to the inhibition of hypocotyl elongation by NO. However, neither *pif3* nor *pif4* single mutants exhibited that phenotype, likely due to redundancy of PIF members in the NO control of hypocotyl length. Yet, the transactivating activity of PIFs is finely regulated through heterodimerization (Hornitschek et al., 2009) and/or interaction with other proteins, such as DELLAs, inhibiting their activity (de Lucas et al., 2008; Feng et al., 2008). The negative regulation of PIF function by NO (Fig. 3A) is accompanied by an increase in the accumulation of DELLA proteins (Fig. 4). It is well known that PIF3 and PIF4 physically interact with members of the DELLA family such as RGA, resulting in a reduction of the PIF3- and PIF4-triggered activation of their targets (de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolomé et al., 2010). After the dark-to-light transition, NO production leads to repressed *PIF* expression and enhanced DELLA accumulation, thus resulting in a very limited pool of transactivating PIFs. So, how is DELLA accumulation promoted by NO? First, we checked that NO-treated seedlings could degrade DELLA proteins in response to exogenously applied GAs (Fig. 4). Surprisingly, it seemed that NO-treated seedlings degraded DELLAs more efficiently in response to GAs than control-treated seedlings. This mechanism, far from being unusual, operates in other conditions where exaggerated DELLA accumulation is achieved due to the altered expression of important genes (Silverstone et al., 2007; Willige et al., 2007; Richter et al., 2010) or to inhibition of GA synthesis by PAC (Muangprom et al., 2005). After cell-free assays, we can propose that this is due to a proteasome-independent mechanism (Fig. 4). Then, we explored the possibility of NO regulating the transcription of genes coding for *DELLA*s. However, NO did not affect DELLA gene expression either in NO-deficient mutants or after NO treatment of wild-type plants (Supplemental Fig. S3). Eventually, NO might be controlling GA biosynthesis or catabolism. After an extensive analysis of the expression of *GA20oxidase* and *GA3oxidase* biosynthetic genes as well as *GA2oxidase* catabolic genes, only *GA20ox3* fulfills the criteria to be considered a potential target of NO in controlling GA production, as it was down-regulated upon exogenous NO treatment and up-regulated in the NO-deficient triple mutant (Supplemental Fig. S4). Nevertheless, we anticipate that changes in GA biosynthetic or catabolic gene expression in NO-treated or NO-deficient plants, if any, should be restricted to an organ, tissue, or limited number of cells, but certainly not to the whole seedling. *nia1,2noa1-2* plants did not show any general phenotype characteristic of GA overproducers; on the contrary, it had small shoots and produced seeds with low germination potential and increased dormancy (Lozano-Juste and León, 2010). An increase in GA levels specifically in the elongation zone of the hypocotyls might explain the phenotype of long hypocotyls of NO-deficient seedlings in red light, but this is technically difficult, and new methodologies or sensor tools should be developed in the future to accomplish this purpose.

Despite potential fine regulatory functions of NO on GA levels, NO certainly regulates GA signaling through the control of DELLA abundance and function. Our data support a role for NO inducing the accumulation of DELLAs (Fig. 4) despite finding no transcriptional induction of DELLA genes (Supplemental Fig. S3). Because DELLA protein levels are regulated through ubiquitin-proteasome-dependent degradation by the GID1-SCF^{SLY1} complex, we next focused our attention on this module as a possible target of NO action. First, *GID1a,b* and *GID1c* genes were not transcriptionally regulated by NO. However, the *gid1a,c* mutant, affected in the GID1 receptor isoforms, which are more involved in hypocotyl elongation (Griffiths et al., 2006; Stavang et al., 2009), showed a NO-resistant phenotype. Second, down-regulation of *SLY1* expression by NO and the up-regulation detected in the *nia1,2noa1-2* hypocotyls might explain the control of DELLA accumulation by NO. Moreover, the loss-of-function mutant *sly1-10* was partially insensitive in the inhibition of hypocotyl elongation by NO under red light (Fig. 6, D and F). In contrast to the short-hypocotyl phenotype of *sly1-10*, we have not found an altered hypocotyl length in *sly1-D* mutant seedlings under red light (Fig. 6, D and F), thus suggesting that the previously reported enhanced DELLA degradation by SLY1-D protein in roots (Fu et al., 2004) is not functional in the regulation of hypocotyl length under red light conditions. Additionally, *sly1-D* mutant seedlings responded unexpectedly stronger than wild-type seedlings to NO-triggered hypocotyl shortening (Fig. 6, D and F). It has been proposed that the SLY1-D protein interacts more efficiently than SLY1 with DELLAs and also that the interaction is increased by DELLA phosphorylation (Fu et al., 2004). In addition to the transcriptional regulation of the *SLY1* gene by NO described in this work, we cannot rule out the interference of NO in SLY1-DELLA interaction

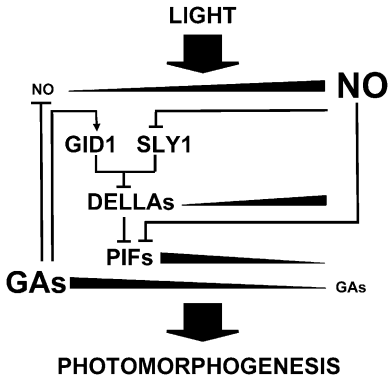

Figure 8. Scheme integrating NO and GA antagonist functions in the control of light-regulated photomorphogenesis through the balance between DELLAs and PIFs.

or even NO-mediated modifications of any of the components of the GA perception and signaling complex.

The fact that mutants in DELLA proteins, the GID1 receptor, or the SLY1 F-box behave as partially insensitive to NO strongly suggest that the GID1-DELLA-SLY1 complex is a target of NO in the control of hypocotyl length. Interestingly, the *sly1-10* mutant responded differentially to NO-triggered hypocotyl shortening under dark and red light conditions (Fig. 6, D and F; Supplemental Fig. S5E). The wild-type response to NO of the *sly1-10* mutant in darkness suggests that transcriptional control of *SLY1* by NO is mainly functional in light. This also points to PIFs as important targets of NO in darkness. Moreover, the *pifQ* mutant is almost completely insensitive to NO, thus suggesting that PIF proteins should have a pivotal role in the negative regulation exerted by NO on the basic GA signaling module. Furthermore, negative functional interaction between NO and GAs in controlling photomorphogenesis is somehow potentiated by the reciprocal negative effect of GAs on NO production, as demonstrated by the enhanced NO content, which can be reverted by GA_3 application, in the GA-deficient *ga1-3* mutant.

A model integrating the putative functions of NO, GAs, DELLA, and PIF proteins in controlling photomorphogenesis is depicted in Figure 8. The dark-to-light transition leads to increased levels of NO and decreases of GAs, which in turn would lead to a rapid increase in DELLA content as a result of less GID1-GA-DELLA-SLY1 functional interactions. The large pool of DELLA protein would associate with transcription factors, such as PIFs, whose transcription has been nevertheless repressed by NO. DELLAs acting as efficient scavengers of the free form of those transcription factors would lead to the arrest of hypocotyl growth, among other processes characteristics of photomorphogenesis. In the *nia1,2noa1-2* mutant, which is severely impaired in NO biosynthesis, the dark-to-light transition leads to a deficient production of NO and, therefore, to a loss of NO-mediated repression of *PIFs*. Simultaneously, ubiquitination by SLY1 and further degradation of DELLAs may be enhanced under low-NO conditions, thus leading to a decrease in DELLA content. As a result, the enlarged pool of free PIFs and/or their enhanced performance in *nia1,2noa1-2* seedlings would be responsible for a partially etiolated phenotype under light conditions.

MATERIALS AND METHODS

Plant Material and Treatments

Arabidopsis (*Arabidopsis thaliana*) accession Col-0 was the wild-type control of *nia1nia2*, *noa1-2*, *nia1,2noa1-2*, *gid1a,b*, *gid1a,c*, *gid1b,c*, *pif3-3*, *pif4-2*, and *pifQ* mutants. The single *ga1-3*, *gai-1D*, *sly1-10*, and *sly1-D*, double *rga-24gai-t6*, quadruple *4della*, and quintuple *5della* mutants and the transgenic plants *35S::TAP-RGA*, *35S::TAP-GAI*, *35S::TAP-RGL1*, *35S::TAP-RGL2*, *35S::TAP-RGL3*, *35S::TAP-RGAΔ17*, *35S::TAP-GAIΔ17*, and *pRGA::GFP-RGA* were used along with the Landsberg *erecta* (L*er*) wild-type background accession. Seeds were surface sterilized with 30% bleach and 0.01% Tween 20, washed extensively with milliQ sterile water, and sown in Murashige and Skoog medium supplemented with 0.8% agar and 1% Suc. After 3 d of stratification at 4°C, germination was synchronized by 3 h of illumination with white light and subsequent incubation in the dark for 22 h before transfer to the different growth conditions. White light-grown seedlings were grown at 19°C to 23°C under fluorescent white light (fluence rate of 70 μmol m^{-2} s^{-1}) with a 16-h-light/8-h-dark photoperiod. For different light quality treatments, seedlings were grown under continuous blue, far-red, or red light, provided by light-emitting diodes (Percival Science), at the indicated fluence rates (blue light, 16.5 μmol m^{-2} s^{-1}; far-red light, 5 μmol m^{-2} s^{-1}; red light, 20 μmol m^{-2} s^{-1}).

NO treatments were performed by photochemically mediated release of NO gas from a solution of SNP (Fluka) always contained in internal vessels separated from Murashige and Skoog medium, as reported previously (Bethke et al., 2006; Lozano-Juste and León, 2010). For gene expression or protein analysis, pulse treatments (2 h) of SNP were done. To test the SNP effect on hypocotyl growth, SNP treatment was performed upon seeds synchronized for germination and then maintained until the end point of the experiment. Freshly prepared GA_3 (Duchefa) and PAC (Duchefa) were added at the indicated concentrations.

Seedlings were manipulated in darkness under dim (below 0.05 μmol m^{-2} s^{-1}) green safelight when required.

For the generation of *pRGA::GFP-RGA* in the *nia1,2noa1-2* background, *pRGA::GFP-RGA* plants were crossed with *nia1,2noa1-2* and F2 and F3 progeny were PCR genotyped for *nia1*, *nia2*, and *noa1-2* mutations as reported previously (Lozano-Juste and León, 2010) and selected with kanamycin for the *pRGA::GFP-RGA* construct.

Hypocotyl Length Measurements

Seedlings were harvested from petri dishes, laid on acetate sheets, and scanned at 600 dots per inch. The resulting images were used for measuring hypocotyl length by using ImageJ software. Values of hypocotyl length are means ± SE of three independent experiments (at least 20 seedlings per experiment were measured).

Protein Extraction and Western Blot

Protein extraction and quantification were performed as reported previously (Lozano-Juste et al., 2011). Samples (15 μg) were separated on 8% polyacrylamide minigels (Bio-Rad; http://www.bio-rad.com) and transferred, when required, onto nitrocellulose membranes (GE Healthcare Spain; http://www.gehealthcare.com/eses/index.html).

GFP-tagged proteins were detected with a 1:8,000 dilution of a monoclonal anti-GFP antibody (Clontech), and TAP-tagged proteins were detected with a 1:10,000 dilution of anti-C-MYC tag-peroxidase conjugate antibody (Sigma). PHYB protein levels were detected with a 1:2,500 dilution of anti-PHYB monoclonal antibody from the laboratory of Akira Nagatani. Loading controls were performed with anti-tubulin antibodies (Sigma) or anti-OAS A1 antibodies from the laboratory of Cecilia Gotor. The secondary antibody was anti-mouse (1:10,000) or anti-rabbit (1:10,000) coupled to horseradish peroxidase, and further detection was performed with the ECL or the ECL Advance kit (GE Healthcare Spain; http://www.gehealthcare.com/eses/index.html). Images were captured with LAS3000 (Fuji) and quantified by using Image Gauge software (Fuji) where indicated.

Quantitative Real-Time PCR

Total RNA was isolated from hypocotyls of 5-d-old seedlings and further analyzed by quantitative RT-PCR techniques as described previously (Castillo and León, 2008). Transcript levels of *PIF1*, *PIF3*, *PIF4*, *RGA*, *GAI*, *RGL1*, *RGL2*, and *RGL3* genes coding for PIF and DELLA proteins, as well as GA biosynthetic and catabolic genes, were analyzed by quantitative RT-PCR using specific primers as reported previously (Frigerio et al., 2006; Alabadí et al., 2008). *GID1a*, *-1b*, *-1c*, and *SLY1* were analyzed with primers as follows: qGID1a-F, 5′-GTGACGGTTAGAGACCGCGA-3′; qGID1a-R, 5′-TCCCTCGGGTAAAAACGCTT-3′; qGID1b-F, 5′-TTACGGTCAAGGAACTCGGC-3′; qGID1b-R, 5′-TCGCCCTGACGGTTCTTTC-3′; qGID1c-F, 5′-CGGCTCAAATCTTCGATCTGG-3′; qGID1c-R, 5′-TTGGCATTTGCAGGGACTTTC-3′; qSLY1-F, 5′-GGGCAGAACCAGCTCAGATC-3′; qSLY1-R, 5′-TCTTCGGAAGCCACCAAGC-3′.

Anthocyanin Extraction and Quantification

Samples of 100 mg fresh weight of 5-d-old seedlings grown in darkness or white light were harvested, anthocyanin extracted, and quantified on acidic methanol buffer overnight at 4°C as reported previously (Francis, 1982). Anthocyanin content is expressed as A_{530} mg^{-1} fresh weight.

NO and GFP Detection by Fluorescence and Confocal Microscopy

The endogenous levels of NO in hypocotyls were determined by seedling staining with 15 μM DAF-FM DA for 1 h as described (Guo et al., 2003). NO-associated fluorescence was detected under UV illumination with a Nikon Eclipse fluorescence microscope using unchanged parameters for every measurement. Fluorescence intensity was quantified as reported previously (Lozano-Juste and León, 2010).

Fluorescence from *pRGA::GFP-RGA* was detected using a TCS SL confocal laser scanning microscope (Leica) with a 40× oil-immersion objective lens. For GFP and chloroplast autofluorescence, samples were excited with an argon laser at 488 nm. The fluorescence emission was collected between 497 and 537 nm for GFP (rendered in green) and between 579 and 647 nm band pass for chloroplast autofluorescence (rendered in red). TCS SL average projections of the Z-stack reconstructions (10 slices, 3 μm each) were taken and are presented in Figure 4. Ten seedlings were analyzed per experiment in three independent experiments.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Time and fluence rate dependence effect of red light on the elongation of wild-type and NO-deficient hypocotyls.

Supplemental Figure S2. Similar levels of GFP in NO-treated and control untreated *35S-TAP-GFP* seedlings.

Supplemental Figure S3. Relative transcript levels of genes coding for DELLA proteins in wild-type and NO-deficient hypocotyls.

Supplemental Figure S4. Biosynthetic pathway of gibberellins in Arabidopsis and levels of the biosynthetic and catabolic gene transcripts.

Supplemental Figure S5. Control experiments corresponding to Figures 1, 3, 5, and 6 performed in darkness.

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