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LETTERS

Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins

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Light and gibberellins (GAs) mediate many essential and partially overlapping plant developmental processes. DELLA proteins are GA-signalling repressors that block GA-induced development[1]. GA induces degradation of DELLA proteins via the ubiquitin/proteasome pathway[2], but light promotes accumulation of DELLA proteins by reducing GA levels[3]. It was proposed that DELLA proteins restrain plant growth largely through their effect on gene expression[4,5]. However, the precise mechanism of their function in coordinating GA signalling and gene expression remains unknown. Here we characterize a nuclear protein interaction cascade mediating transduction of GA signals to the activity regulation of a light-responsive transcription factor. In the absence of GA, nuclear-localized DELLA proteins accumulate to higher levels, interact with phytochrome-interacting factor 3 (PIF3, a bHLH-type transcription factor) and prevent PIF3 from binding to its target gene promoters and regulating gene expression, and therefore abrogate PIF3-mediated light control of hypocotyl elongation. In the presence of GA, GID1 proteins (GA receptors) elevate their direct interaction with DELLA proteins in the nucleus, trigger DELLA protein's ubiquitination and proteasome-mediated degradation, and thus release PIF3 from the negative effect of DELLA proteins.

Light and GA interact during *Arabidopsis thaliana* seedling development, regulating hypocotyl elongation, cotyledon opening and light-responsive gene expression; their pathways seem to converge at regulation of the abundance of DELLA proteins (GA pathway repressors)[3,6]. *Arabidopsis* has five DELLA proteins—RGA, GAI, RGL1, RGL2 and RGL3—defined by their unique DELLA domain and a conserved GRAS domain[4]. To analyse them *in vivo*, we raised antibodies against endogenous RGA and generated transgenic *Arabidopsis* expressing each of the five DELLA proteins with tandem affinity purification (TAP) tags (Supplementary Fig. 1). The response of DELLA protein levels to exogenously applied GA_3 (an active form of GA) or PAC (paclobutrazol, a GA biosynthesis inhibitor) was examined. We found that one-hour-long GA treatment eliminates the majority of DELLA proteins, and this GA effect can be largely prevented by 100 μM MG132 (a 26S proteasome-specific inhibitor). PAC, on the other hand, promotes over-accumulation of DELLA proteins (Fig. 1). These results show for the first time in *Arabidopsis* that all the DELLA proteins are under negative control by GA and the proteasome. Next, we generated lines expressing TAP-tagged RGAΔ17 and GAIΔ17, which lack a 17 amino acid motif within the DELLA domain that is required for GA-induced degradation[7,8]. As expected, TAP–RGAΔ17 and TAP–GAIΔ17 are completely resistant to GA and accumulate at higher levels than wild-type proteins, which cannot be further increased by PAC (Fig. 1, and

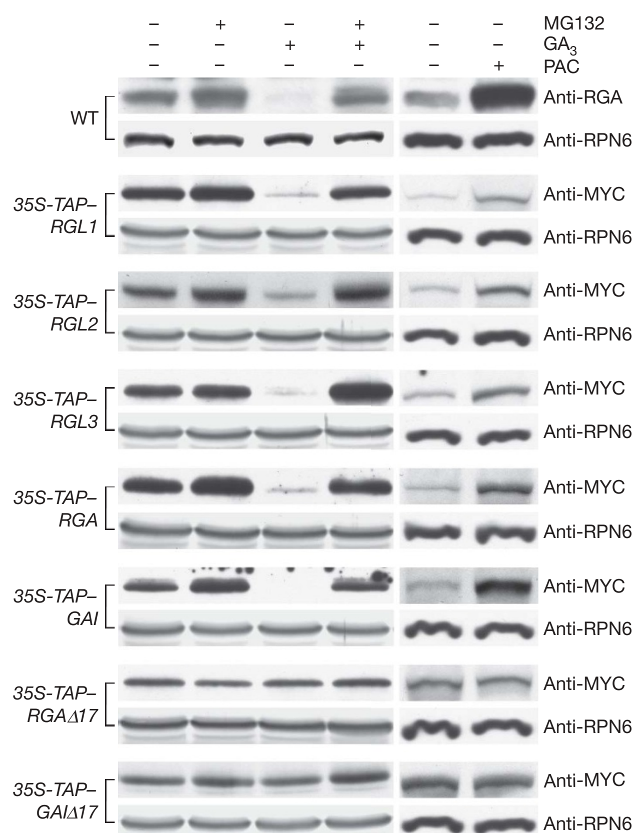

Figure 1 | Effect of GA_3, MG132 and PAC on DELLA protein abundance. Immunoblot analysis of RGA (by anti-RGA antibody) and TAP-DELLA proteins (by anti-MYC antibody) in various light-grown *Arabidopsis* seedlings (genotypes labelled to the left of each panel) treated with different combinations of GA_3, MG132 and PAC. Panels on the left (four lanes) and panels on the right (two lanes) are from two independent experiments using different protein gel systems. RPN6 immunoblotting (by anti-RPN6 antibody) is used as a loading control. WT, wild type.

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Supplementary Fig. 1b). *Arabidopsis* plants that overexpress these proteins show a dominant dwarf phenotype, reflecting enhanced DELLA activity (Supplementary Fig. 2), which also suggests that TAP–DELLA proteins retain normal DELLA function.

Inhibition of hypocotyl elongation, an important characteristic of photomorphogenesis, is shown to be repressed by GA in the dark and promoted by DELLA proteins in the light[3,6]. We further examined the possible mechanism of DELLA proteins in regulating photomorphogenesis. *Arabidopsis* seedlings have longer hypocotyls on GA-containing medium, whereas PAC dramatically inhibits the elongation of hypocotyls (Fig. 2a, b). Furthermore, the GA effect is more drastic in red light than in dark (Fig. 2b), consistent with the notion that the endogenous GA level is higher in dark-grown seedlings. In addition, *35S-TAP–RGAΔ17* and *35S-TAP-GAIΔ17* plants have much shorter hypocotyls than wild type, which cannot be rescued by GA. On the contrary, the hypocotyl of *rga-24 gai-t6* double mutants is longer than that of wild type, and is only partially inhibited by PAC. In a pentuple mutant (*della*) of all five *DELLA* genes, the hypocotyl length is comparable to that of GA-treated wild type, and PAC has no significant effect (Fig. 2a, b). Therefore, we reasoned that GA controls hypocotyl growth and affects photomorphogenesis status, mainly by regulating DELLA protein abundance.

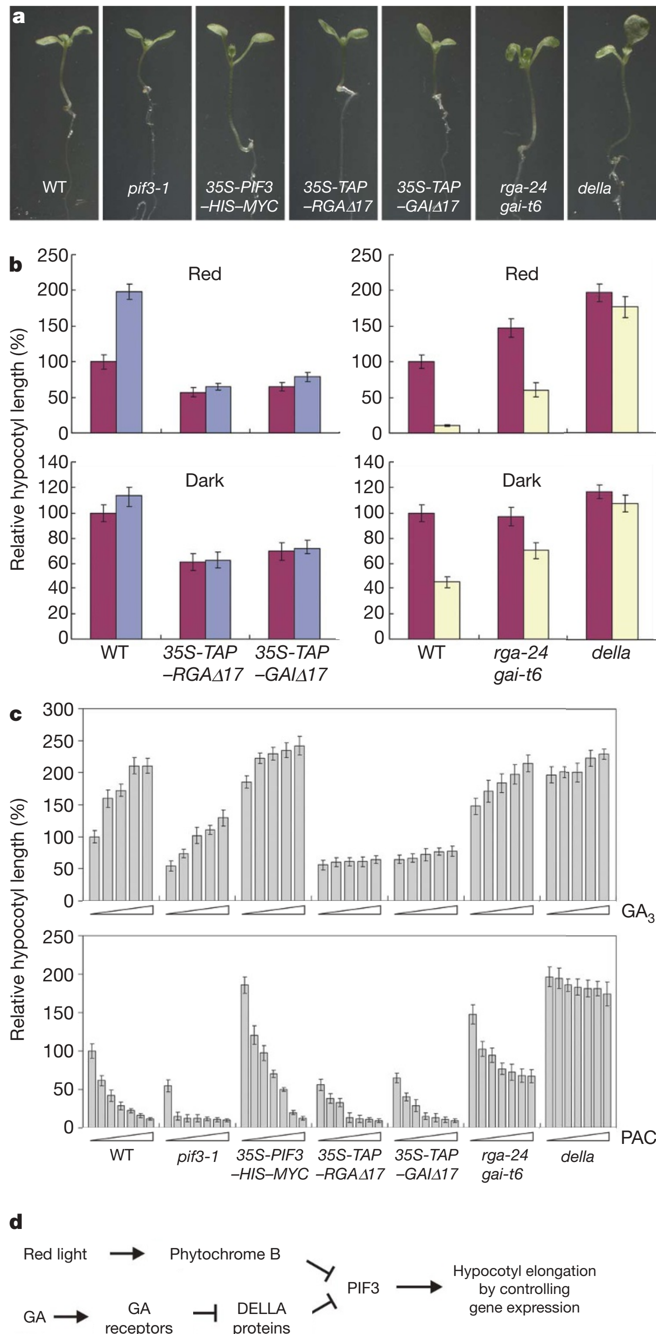

Figure 2 | DELLA proteins and PIF3 have opposite roles in regulating *Arabidopsis* hypocotyl elongation. **a**, Images of red-light-grown seedlings. **b**, Hypocotyl length measurement (mean ± s.d.) of untreated seedlings (red), or seedlings treated with 10 μM GA_3 (blue) or 1 μM PAC (yellow). **c**, Hypocotyl length measurement (mean ± s.d.) of red-light-grown seedlings treated with increasing amounts of GA_3 or PAC (see Methods). The concentrations of GA_3 used are 0, 0.5 μM, 1 μM, 2 μM and 5 μM (from left to right). The concentrations of PAC were 0, 0.01 μM, 0.02 μM, 0.05 μM, 0.1 μM, 0.2 μM and 0.5 μM (from left to right). In **b** and **c**, hypocotyl length of untreated wild-type seedlings is set to 100%. **d**, Simplified diagram depicting the genetic interaction of light and GA in the control of hypocotyl elongation by PIF3 and DELLA proteins. *della*, *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1*.

DELLA proteins are proposed to be transcription factors[4], and are required to localize to the nucleus for their function[9,10]. Genomic studies have revealed a number of GA-responsive genes that are regulated by *DELLA* genes[5]. However, using the chromatin immunoprecipitation (ChIP) technique in *35S-TAP–DELLA* lines, we were unable to observe specific binding of DELLA proteins to any of the 38 GA-responsive gene promoters tested (Supplementary Table 1). Thus, we hypothesize that DELLA proteins might regulate gene expression indirectly by controlling transcription factors. Because light and DELLA proteins both regulate hypocotyl growth, it seems possible that one, or more, of the well-known photomorphogenesis-related transcription factors might be a target of DELLA proteins. Among them, PIF3 is a good candidate, because it promotes hypocotyl elongation in red light[11]—the opposite of DELLA's function (Fig. 2a). Moreover, PIF3 has DNA-binding activity[12], interacts with the active form of phytochrome B (phyB)[13,14], and is negatively regulated by phytochrome through the ubiquitin/proteasome pathway[15–17], indicating it mediates signalling between light and gene expression. We observed that the *pif3-1* mutant has a short hypocotyl, and is partially resistant to GA and hypersensitive to PAC, mimicking *35S-TAP–RGAΔ17* and *35S-TAP–GAIΔ17* plants, whereas the PIF3 overexpression line shows a long hypocotyl and is hyposensitive to PAC, in a similar manner to GA-treated plants and *rga-24 gai-t6* and *della* mutants (Fig. 2c). These results imply that DELLA proteins may negatively regulate PIF3 in the control of hypocotyl elongation, representing a convergent point of light and GA pathways (Fig. 2d).

This regulation is probably mediated through physical interaction between PIF3 and DELLA proteins, as suggested by yeast two-hybrid and *in vitro* pull-down assays (Fig. 3a, d, and Supplementary Fig. 3). Moreover, bimolecular fluorescence complementation (BiFC) analysis detects direct RGA–PIF3 interaction in the nuclei of living plant cells (Fig. 3b). We further investigated this interaction using an immunoprecipitation approach. As shown in Fig. 3c, interaction between RGA and PIF3 is observed in dark-grown seedlings, in which PIF3 protein accumulates to reasonable abundance[15–17]. The interaction is also detectable in red light, when light-induced proteasomal degradation of PIF3 (refs 15–17) is blocked. The interaction is dependent on RGA abundance, such that PAC increases RGA–PIF3 interaction, whereas GA abolishes RGA accumulation and thus PIF3 is released. Importantly, under the condition that RGA–PIF3 interaction is enhanced, PIF3's effect on hypocotyl growth is largely impaired, and vice versa (Figs 2c, 3c), indicating that RGA-bound PIF3 has reduced activity. We tested whether DELLA proteins influence the previously reported interaction between phytochrome and PIF3 (refs 13, 14) by analysing the formation of nuclear speckles containing both phyB and PIF3 (ref. 15). Evidently, phyB–PIF3 interaction is essentially not affected by altering DELLA protein abundance (Supplementary Fig. 4). Therefore, DELLA protein binding most probably affects PIF3's transcription-regulation activity towards its target genes.

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This notion is supported by the observation that the RGA–PIF3 interaction *in vitro* is specifically inhibited by pre-incubating PIF3 with its cognate binding site, a G-box-containing DNA probe[12] (LHY; Fig. 3d, and Supplementary Fig. 3), which provides evidence that RGA–PIF3 and PIF3–DNA bindings are antagonistic. To test this *in vivo*, we selected five putative PIF3 target genes by analysing the published literature as well as taking into account results we obtained from a ChIP microarray analysis focused on PIF3, using a recently reported method[18]. By ChIP–PCR, we confirmed that these five promoters are bound by PIF3 as expected. In addition, we found that when DELLA protein level is increased by PAC, PIF3–promoter binding is severely reduced. On the other hand, removing DELLA proteins by GA treatment generally leads to enhanced occupancy of PIF3 on the promoters (Fig. 3e). We also noticed that, whereas GA and PAC do not significantly affect nuclear PIF3–MYC protein levels, they have slightly opposite effects on PIF3–MYC immunoprecipitation (Supplementary Fig. 5), which might be due to higher affinity of MYC antibody towards free PIF3–MYC than RGA-bound PIF3–MYC. Among the PIF3 target genes, At5g2120, At4g19030, At2g47890 and At1g34670 show light-responsive expression[19]. Interestingly, differential expression of At5g24120, At4g19030 and At2g47890 have also been reported in genomic studies focused on gene expression regulation by GA, *PIF3* or *DELLA* genes[5,20,21]. Subsequently, we used PCR with reverse transcription (RT–PCR) to check whether PIF3–promoter binding indeed affects gene expression. As shown in Fig. 3f, overexpressing PIF3 and reducing DELLA protein abundance (*della* mutant or GA treatment) have similar effect on the expression of two representative PIF3 target genes, whereas increasing DELLA protein abundance (overexpressing RGAΔ17 or PAC treatment) has the opposite effect. Overall, we demonstrate that DELLA proteins antagonize PIF3 function by protein–protein interaction and sequestration, which at least partly explains their effect on gene expression and the coordinated control of hypocotyl growth by light and GA.

Figure 3 | DELLA proteins bind PIF3 and inhibit PIF3 activity towards its target genes. **a**, β-galactosidase activities from yeast two-hybrid assays (mean ± s.d.). **b**, BiFC analysis of RGA and PIF3. The positions of nuclei are indicated by arrows. **c**, Co-immunoprecipitation of RGA with PIF3 in *35S-PIF3–His–MYC* seedlings. 'RGA' and 'Pre' indicate immunoprecipitation by anti-RGA antibody and pre-immune sera, respectively. **d**, Pull-down assays between His–PIF3 and MBP–RGA. The precipitated His–PIF3 was detected by anti-His antibody. MBP–RGA and MBP inputs were stained by Coomassie blue. **e**, ChIP–PCR analyses in dark-grown seedlings. **f**, Semi-quantitative RT–PCR analyses in dark-grown seedlings. Total, total protein extracts; IP, immunoprecipitation.

We next examined how the GA signal is relayed to affect DELLA protein abundance and thus DELLA–PIF3 interaction. Recently, GID1 proteins have been shown to act as nuclear GA receptors[22–25]. Through isolating and analysing *Arabidopsis gid1* mutants, we obtained results that are consistent with those reports[24,25], suggesting that GID1s are required for normal GA signalling and participate in light-induced development, possibly by inducing DELLA protein degradation (Supplementary Note 1 and Supplementary Fig. 6). We also confirmed the reported GA-dependent GID1–DELLA interaction[22–25], which requires the 17 amino acid motif within the DELLA domain, in yeast two-hybrid assays (Fig. 4a, b, and Supplementary Fig. 7). In addition, BiFC analysis of GID1c and RGA demonstrates their direct interaction in the nuclei of living plant cells (Fig. 4c). To test the effect of GA on GID1–DELLA interaction *in planta*, we used transgenic *Arabidopsis* expressing each of the three GID1 proteins with YFP or epitope tags for immunoprecipitation analyses. As shown in Fig. 4d, interaction of GID1a with each of the five DELLA proteins is detectable, and is greatly enhanced by GA. Furthermore, with MG132 treatment, immunoprecipitated DELLA proteins contain high-molecular-weight protein species, which react specifically with anti-ubiquitin antibody. Increasing amounts of these DELLA proteins that are predicted to be multi-ubiquitinated can be detected after GA treatment (Fig. 4d, e). On the other hand, GAIΔ17 shows no detectable interaction with GID1a and a nearly complete loss of multi-ubiquitination (Fig. 4d), suggesting that interaction with GID1s is essential for the multi-ubiquitination of DELLA proteins. We extended the interaction study to all three GID1 proteins, and the co-immunoprecipitation of each GID1 with endogenous RGA was detected (Fig. 4f–h). In these experiments, we lowered MG132 concentration (40 μM) and lengthened GA treatment time (two hours) to only partially inhibit the proteasome and allow a significant portion of RGA to be degraded. Nevertheless, GA-treated seedlings still have more GID1-bound RGA (Fig. 4f–h), implying that GID1–DELLA may be recognized by the ubiquitin/proteasome system as a heteromeric complex, and somehow DELLA proteins are preferentially ubiquitinated and degraded. This is supported by the

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observation that GID1a enhances RGA–SLY1 (E3 ubiquitin ligase subunit) interaction in the presence of GA[24]. Consistent with previous results in yeast[22], GID1b binds more RGA in untreated seedlings, and even with PAC treatment a small amount of GID1b-bound RGA can be detected (Fig. 4f-h). This implies a possible GA-independent pathway for GID1b to target DELLA proteins, which might be critical to keep DELLA proteins in check when the GA level is low.

The results reported here support a conclusion that GA signalling is initiated when GA molecules, the biosynthesis of which is induced by light[3], are sensed and bound by nuclear GID1 proteins. Then, GA-charged GID1s interact with DELLA proteins in the nucleus and target them for proteasomal degradation. When DELLA protein abundance is reduced, their interactive partners, for example, light-responsive and phytochrome-interacting transcription factors such as PIF3, are released from sequestration, and bind to their target promoters and regulate gene expression (Fig. 4i). PIF3 belongs to a transcription factor family defined by a conserved bHLH (basic-helix–loop–helix) domain, which has implicated function in DNA binding and dimerization[26]. In a similar way to PIF3, PIF4 (another phytochrome-interacting bHLH transcription factor) is also negatively regulated by DELLA proteins[27]. Moreover, DELLA proteins are shown to interact with the DNA-binding bHLH domain[27], consistent with our observation that RGA–PIF3 and PIF3–promoter interactions are mutually exclusive (Fig. 3). Two other bHLH proteins, PIL5 and SPT, are also involved in light and GA signalling, and have PIF3-like roles in hypocotyl growth[28,29], making them potential targets of DELLA proteins as well. Collectively, it is highly plausible that,

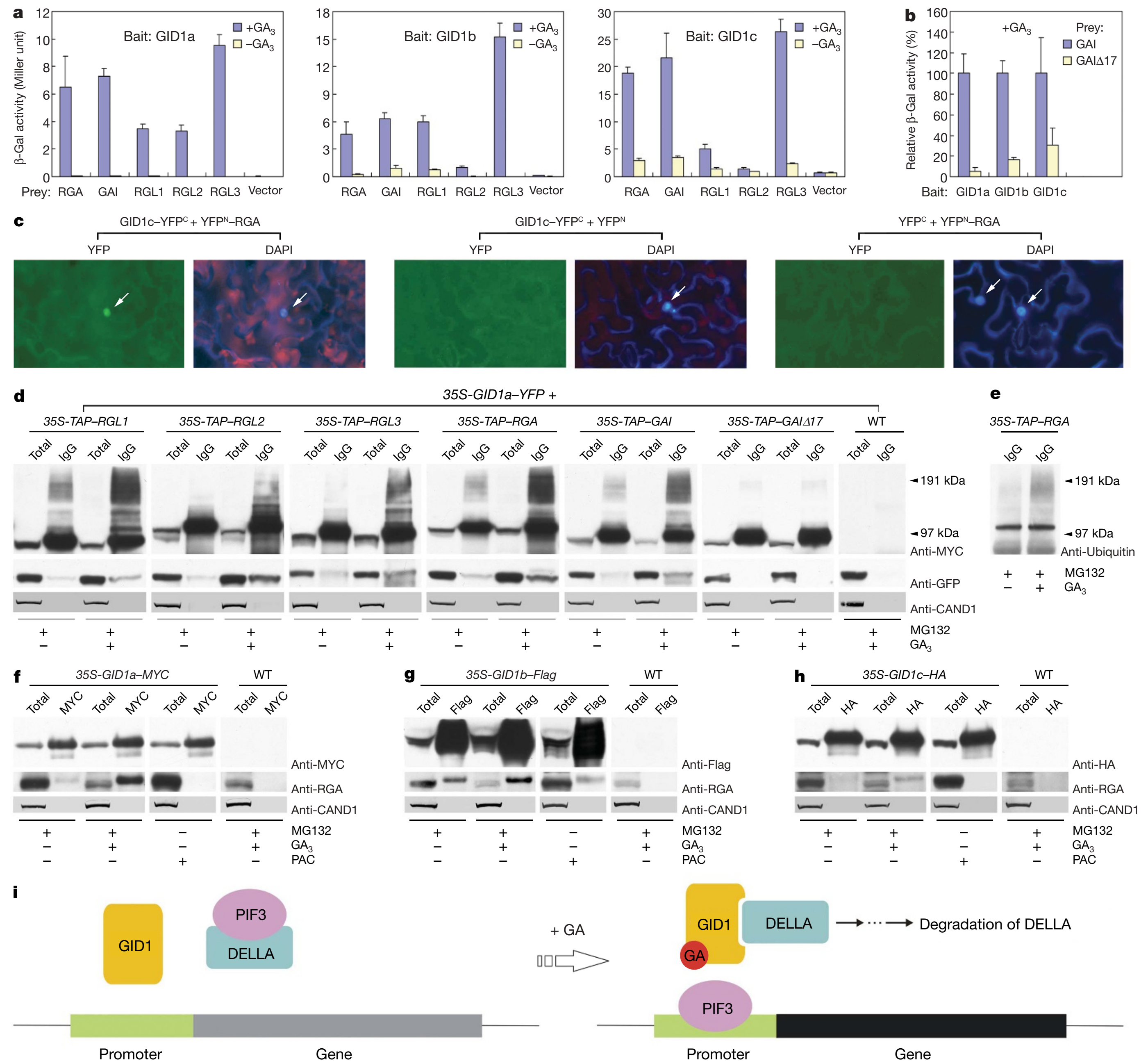

Figure 4 | GA-dependent interaction between GID1s and DELLA proteins. **a**, **b**, β-galactosidase activities from yeast two-hybrid assays (mean ± s.d.). In **b**, β-galactosidase activities from GAI–GID1 interactions are set to 100%. **c**, BiFC analysis of GID1c and RGA. The positions of nuclei are indicated by arrows. **d**, TAP-DELLA proteins interact with GID1a–YFP. **e**, Detection of multi-ubiquitinated TAP-RGA. In **d**, **e**, 'IgG' indicates immunoprecipitation by IgG-conjugated beads. **f–h**, RGA interacts with GID1a–MYC (**f**), GID1b–Flag (**g**), and GID1c–HA(haemagglutinin) (**h**). 'MYC', 'Flag' and 'HA' indicate immunoprecipitation by respective antibodies. **i**, A working model of the nuclear protein interaction cascade in GA signalling.

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through modulating multiple phytochrome-interacting transcription factors, DELLA proteins play a key part in integrating the regulatory effect of light and GA on gene expression and plant development.

METHODS SUMMARY

The procedures for *Arabidopsis* plant growth, yeast two-hybrid analyses, protein and chromatin immunoprecipitations, and subcellular localization studies are described previously[15,18,30]. CAND1 is used as a negative control in protein immunoprecipitation experiments. Histone H1 is used as an internal control in RT–PCR. Primers used in ChIP–PCR and RT–PCR are listed in Supplementary Tables 1 and 2. MG132 treatment is carried out by vacuum infiltration. GA and PAC treatments are carried out by either applying GA_3 to the seedlings or supplementing plant growth medium with GA_3 or PAC. BiFC experiments are carried out between transiently expressed *Arabidopsis* proteins in tobacco leaves. *In vitro* pull-down assays are performed using recombinant proteins purified from bacteria, in the presence of either a canonical G-box containing DNA probe (LHY) or a mutant G-box-containing DNA probe (G-mut).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions X.W.D. conceived the project, and S.F. and X.W.D. together designed the experiments. L.-M.F. designed some of the experiments. S.F. and G.G. performed chromatin immunoprecipitation. C.M. and G.G. analysed the *gid1* mutants and performed RT–PCR. Y.W. made the anti-RGA antibody. Y.W., L.C., F.W. and L.Y. performed the yeast two-hybrid analyses. J.Z. and F.W. performed *in vitro* pull-down assays. C.M. and J.M.I.-P. performed the BiFC experiments. S.K. and E.S. performed phyB and PIF3 subcellular localization studies. X.F. provided the *rga-24 gai-t6* double and *della* pentuple mutants. S.F. performed all other experiments. S.F. and X.W.D. wrote the manuscript.

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METHODS

Plant materials and growth conditions. The wild-type *Arabidopsis* ecotypes used in this study are Landsberg *erecta* and Columbia-0. The T-DNA insertion alleles, *gid1a-2* and *gid1c-1*, were verified by PCR-based genotyping. They turn out to be identical to the alleles published in a recent report[24]. The point mutation alleles, *gid1b-2* and *gid1b-3*, were isolated from the *Arabidopsis* TILLING collection (http://tilling.fhcrc.org/). To grow *gid1a-2 gid1b-3 gid1c-1* triple mutant, embryos are mechanically removed from seed coat and allowed to germinate on MS medium plates. For hypocotyl elongation, ChIP–PCR, RT–PCR and subcellular localization experiments, *Arabidopsis* seeds are sown on filter papers in MS medium plates and placed under continuous white light. After 24 h, filter papers with seeds are transferred onto new MS plates, or MS plates containing various concentrations of GA_3 or PAC, and then placed in the dark or red light for six days.

Generation of transgenic *Arabidopsis* lines. DNA fragments containing full-length open reading frames of five *DELLA* genes (*RGA*, *GAI*, *RGL1*, *RGL2* and *RGL3*) and two deletion mutants (*RGAΔ17* and *GAIΔ17*) are cloned via Gateway reactions into the binary N-terminal TAP-tag vector[31]. DNA fragments containing full-length open reading frames of *GID1* genes (*GID1a*, *GID1b*, and *GID1c*) are cloned into YFP-, Flag-, MYC- and HA-tag vectors, respectively. Subsequently, the fragments that contain the gene-coding sequence plus the tag are subcloned into the binary pJIM19 vector[18]. The binary constructs are introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation.

GA, MG132 and PAC treatments. For protein immunoprecipitation and immunoblotting experiments, 10-day-old seedlings are vacuum-infiltrated with 40 μM or 100 μM MG132 (dissolved in DMSO) or DMSO alone for ten minutes and kept immersed in the same solution for two hours. Then GA_3 is added in the solution to a final concentration of 100 μM, and after one- or two-hour incubation, plant tissues are harvested. For PAC treatment, *Arabidopsis* seeds are sown on MS medium, and after 24 h they are transferred to MS medium containing 1 μM PAC. Ten-day-old seedlings are harvested.

Yeast two-hybrid and immunoprecipitation assays. Yeast two-hybrid analyses and protein immunoprecipitation using antibody- and IgG-conjugated beads are performed as described previously[30]. A complementary DNA fragment corresponding to amino acids 1 to 201 of RGA is cloned into *Escherichia coli* expression vector pET32a, and purified recombinant protein is used to immunize rabbits for generating polyclonal antibodies. The anti-CAND1 and anti-CSN5 antibodies are described previously[30,32]. For immunoprecipitation with anti-RGA antibodies, 10 μl affinity-purified anti-RGA antibodies are incubated with protein extracts for four hours. Then, 50 μl protein A agarose beads are used to precipitate the immune complex. CAND1 is used as a negative control in protein immunoprecipitation experiments. Chromatin immunoprecipitations (ChIP) are performed as described previously[18]. For each ChIP, 40 μl anti-MYC antibody-conjugated beads are used.

Bimolecular fluorescence complementation analysis. Full-length open reading frames of RGA, PIF3 and GID1c are cloned via Gateway reactions into the binary pBiFC vectors containing either amino- or carboxy-terminal yellow fluorescence protein (YFP) fragments (YFP^N and YFP^C)[27]. Each of the three proteins is fused with both YFP fragment vectors, as either an N-terminal or C-terminal fusion, thus resulting in four constructs per protein. For example, we have four fusion configurations for RGA, RGA–YFP^C, RGA–YFP^N, YFP^C–RGA, and YFP^N–RGA. To test the interactions between RGA and PIF3, and between GID1c and RGA, we examined all eight possible pair-wise combinations via an adapted transient BiFC assay. Leaves of 2–4-week-old tobacco (*Nicotiana benthamiana*) plants are infiltrated with *Agrobacterium* (GV3101) strains containing individual BiFC construct pairs, as well as a binary plasmid expressing the p19 protein of tomato bushy stunt virus to suppress gene silencing[27]. Epidermal cell layers are examined under the microscope for fluorescence using the green fluorescent (GFP) filter 3–4 days after infiltration. For RGA and PIF3, we detected positive nuclear BiFC interaction signals in four combinations (YFP^N–RGA with YFP^C–PIF3, YFP^N–RGA with PIF3–YFP^C, RGA–YFP^N with YFP^C–PIF3 and RGA–YFP^N with PIF3–YFP^C). For GID1c and RGA, we detected positive nuclear BiFC interaction signals in six of the eight pair-wise combinations, except two (YFP^C–GID1c with YFP^N–RGA and YFP^C–GID1c with RGA–YFP^N).

***In vitro* pull-down assays.** A DNA fragment encoding full-length RGA protein is cloned into pMal-C2X vector, obtaining a fusion with the maltose-binding protein (MBP). MBP–RGA and MBP proteins are purified from bacteria by amylose resin beads. His–PIF3 protein is purified from bacteria containing pPIF3–RSETb plasmid by Ni-NTA agarose. The canonical G-box containing LHY probe and G-box mutant (G-mut) probe are described previously[12]. Before pull down, His–PIF3 (1 μg) is pre-incubated with 10 pM, 20 pM and 40 pM LHY probe or G-mut probe for 20 min. Then, MBP–RGA (1 μg) or MBP (0.3 μg) together with amylose resin beads is added and the mixture is incubated for 30 min. After washing five times with buffer supplemented with DNA probes (the same concentrations as used in the pre-incubation), the precipitated PIF3 is analysed by immunoblotting with anti-His antibody.

Subcellular localization study of phyB–YFP and PIF3–CFP(cyan fluorescent protein) fusion proteins. Hypocotyl cells of dark-grown seedlings and dark-grown seedlings exposed to 2 min, 1 h or 18 h of red-light irradiation are analysed under microscope. Epifluorescent, light and confocal laser scanning microscopy are performed as previously described[15].

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