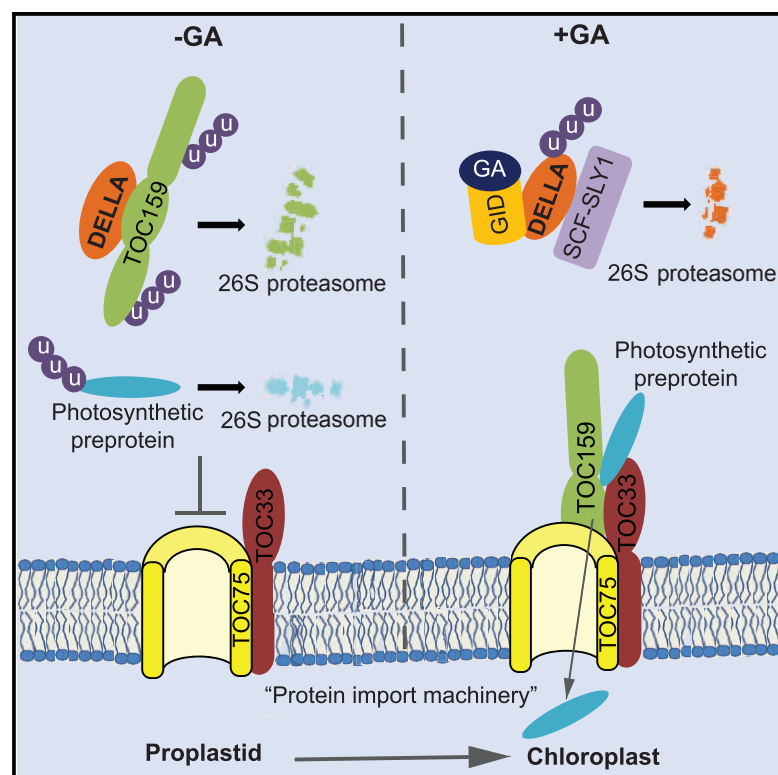


Current Biology

Chloroplast Biogenesis Controlled by DELLA-TOC159 Interaction in Early Plant Development

Graphical Abstract



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In Brief

Chloroplast biogenesis is critical for survival of germinating seeds. Shanmugabalaji et al. show that gibberellic acid (GA) controls chloroplast biogenesis. When GA is low, the chloroplast import receptor **TOC159** interacts with **DELLA** and together with its cargo proteins via the UPS. A framework for proplastid-to-chloroplast transition is provided.

Highlights

- Plant hormone gibberellic acid (GA) controls the proplastid to chloroplast transition
- Low gibberellic acid (GA) enables direct interaction of **DELLA** with **TOC159**
- **DELLA** promotes the degradation of **TOC159** by UPS- blocking chloroplast biogenesis
- Un-imported chloroplast proteins degraded by UPS under low GA



Chloroplast Biogenesis Controlled by DELLA-TOC159 Interaction in Early Plant Development

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SUMMARY

Chloroplast biogenesis, visible as greening, is the key to photoautotrophic growth in plants. At the organelle level, it requires the development of non-photosynthetic, color-less proplastids to photosynthetically active, green chloroplasts at early stages of plant development, i.e., in germinating seeds. This depends on the import of thousands of different preproteins into the developing organelle by the chloroplast protein import machinery [1]. The preprotein import receptor TOC159 is essential in the process, its mutation blocking chloroplast biogenesis and resulting in albino plants [2]. The molecular mechanisms controlling the onset of chloroplast biogenesis during germination are largely unknown. Germination depends on the plant hormone gibberellic acid (GA) and is repressed by DELLA when GA concentrations are low [3, 4]. Here, we show that DELLA negatively regulates TOC159 protein abundance under low GA. The direct DELLA-TOC159 interaction promotes TOC159 degradation by the ubiquitin/proteasome system (UPS). Moreover, the accumulation of photosynthesis-associated proteins destined for the chloroplast is downregulated post-transcriptionally. Analysis of a model import substrate indicates that it is targeted for removal by the UPS prior to import. Thus, under low GA, the UPS represses chloroplast biogenesis by a dual mechanism comprising the DELLA-dependent destruction of the import receptor TOC159, as well as that of its protein cargo. In conclusion, our data provide a molecular framework for the GA hormonal control of proplastid to chloroplast transition during early plant development.

RESULTS AND DISCUSSION

Chloroplasts are the photosynthetic organelles of plants and members of the larger plastid family. Chloroplast biogenesis as the transition from proplastids (undifferentiated plastids in plant

embryos) to chloroplasts in very young plants has rarely been investigated so far and promises new insight into the controlling mechanisms. The assembly of the photosynthetic apparatus during chloroplast biogenesis depends on the import of thousands of different preproteins by the chloroplast protein import machinery. The core of the preprotein import machinery at the outer chloroplast membrane consists of the TOC75 protein-conducting channel and the import receptors TOC33 and TOC159 [5–7]. In seeds, DELLA factors cooperate to stall seedling establishment by promoting abscisic acid (ABA) synthesis and accumulation upon seed imbibition. *Arabidopsis* DELLAs are encoded by five genes: *RGL1*; *RGL2*; *RGL3*; *RGA*; and *GAI* [3]. Gibberellic acid (GA)-dependent downregulation of DELLA abundance is central in chlorophyll and carotenoid biosynthesis during the differentiation of functional chloroplasts and also regulates chloroplast division and grana stacking in mesophyll cells [8, 9].

TOC159 and RGL2 Protein Levels Are Inversely Regulated during Seed Germination

RGL2 plays a predominant role in repressing seedling establishment because *RGL2* mRNA expression is positively regulated by ABA, thus introducing a positive feedback loop sustaining high RGL2 accumulation [10–12]. On Murashige and Skoog (MS) medium, favorable to germination, RGL2 protein was detected until 24 hr after imbibition, after which increasing GA concentrations lead to its downregulation, consistent with previous results [13]. TOC159 protein levels began to increase strongly after RGL2 disappeared 36–48 hr after imbibition (Figure 1A). Under unfavorable germination conditions, such as canopy light or high temperatures, GA synthesis is repressed, which promotes DELLA accumulation. To mimic those conditions, seeds were placed on MS medium containing paclobutrazol (PAC), an inhibitor of GA synthesis. As expected, PAC-treated seeds accumulated high RGL2 protein levels, which remained high while TOC159 levels were reduced, consistent with the absence of greening (Figure 1B). We also compared the effects of PAC on wild-type (WT) to those on the *della* single mutant *rgl2* and the triple mutant *rgl2/gai/rga*, in which seedling germination and chloroplast biogenesis proceeds despite reduced concentrations of GA (Figure S1A). In PAC-treated WT seeds, TOC159 levels were strongly diminished when compared to untreated seeds, and in *rgl2* and *rgl2/gai/rga* mutants, no PAC effects could be observed (Figures S1B and S1C). Like TOC159, TOC33 was diminished in



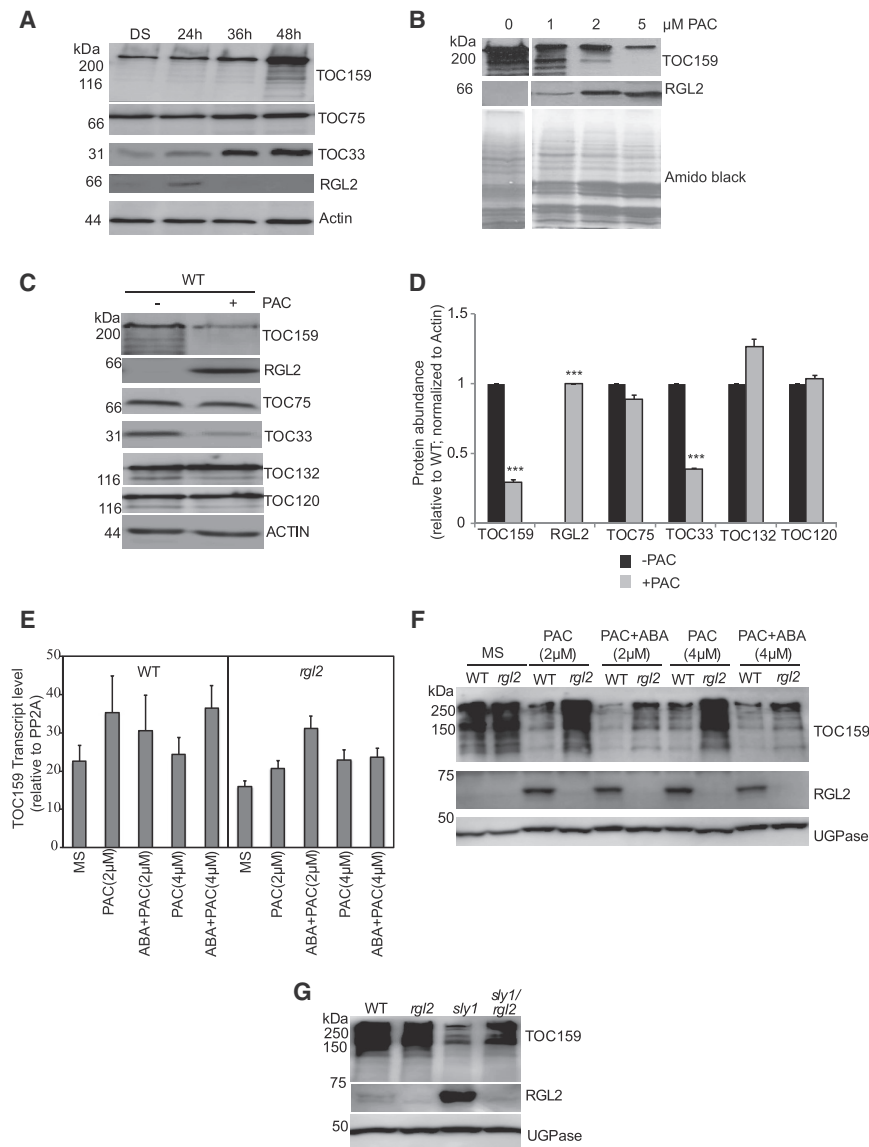


Figure 1. RGL2 Negatively Regulates TOC159 Protein under Low GA

(A) Western blot analysis of WT dry seeds (DSs) and seedlings germinated for 24, 36, and 48 hr, using antibodies to TOC159, TOC75, TOC33, and RGL2. Actin was used as a loading control.

(B) Total protein extracts from 66-hr-old WT seedlings grown in the presence or absence of PAC (1, 2, and 5 μ M) were analyzed by immunoblotting using antibodies to TOC159 and RGL2. Amido Black staining of the membrane shows equal loading.

(C and D) Total protein extracts of 66-hr-old WT seedlings grown in the presence or absence of PAC (5 μ M) were analyzed by immunoblotting using antibodies to TOC159, RGL2, TOC132, TOC120, TOC75, and TOC33 (C), and specific bands were quantified and normalized with respect to actin. Data are relative to WT (D). Error bars indicate $\pm$ SEM ($n = 4$). Student's t test; *** $p < 0.005$.

(E) TOC159 transcript levels measured from WT and *rgl2* seedlings grown in the absence or presence of PAC (2 or 4 μ M), ABA (5 μ M), and PAC+ABA. Data represent means $\pm$ SD of TOC159 mRNA level relative to PP2A ($n = 4$).

(F) Western blot analysis of WT and *rgl2* seedlings grown in the absence or presence of PAC (2 or 4 μ M), ABA (5 μ M), and PAC+ABA, using antibodies to TOC159, RGL2, and UGPase (loading control).

(G) Total protein extracts of 24-hr-old WT, *rgl2*, *sly1*, and *sly1/rgl2* seedlings, analyzed by immunoblotting using antibodies to TOC159, RGL2, and UGPase.

See also Figure S1.

WT in the presence of PAC (Figures 1C and 1D). Interestingly, PAC treatment had no effect in WT on the levels of TOC132 and TOC120, two homologs of TOC159 that are not specifically required for chloroplast biogenesis (Figures 1C and 1D) [14].

TOC159 Protein Level Is Downregulated Posttranscriptionally under Low GA

We next explored how DELLA factors could repress TOC159 accumulation when GA synthesis is inhibited. Under low-GA conditions, RGL2, together with other DELLA factors, promotes endogenous ABA accumulation [10, 11, 15]. Thus, in PAC-treated WT seeds, the diminished TOC159 protein levels (Figures 1B, 1C, and 1F) could potentially be attributed to endogenous ABA repressing TOC159 mRNA accumulation or negatively regulating TOC159 accumulation posttranscriptionally. We tested these hypotheses in turn. PAC-treated WT and *rgl2* seeds had similar TOC159 mRNA expression, showing that low TOC159 accumulation in PAC-treated WT seeds relative to

rgl2 mutant seeds cannot be attributed to low TOC159 mRNA levels (Figure 1E) [16]. Mutant seeds lacking one or several DELLA factors do not accumulate endogenous ABA upon imbibition in presence of PAC. In order to evaluate whether endogenous ABA negatively regulates TOC159 accumulation posttranscriptionally, we added ABA together with PAC in the germination plates in order to compensate for low endogenous ABA levels in mutant seeds lacking DELLA factors. Thus, we incubated PAC-treated *rgl2*, *rgl2/gai/rga*, and *rgl1/rgl2/rgl3/rga/gai* (*della*-null mutant) seeds with ABA, which blocked their germination and greening as for WT seeds (Figure S1A). Strikingly, TOC159 levels remained low in PAC- plus ABA-treated WT seeds relative to *rgl2*, *rgl2/gai/rga*, and *rgl1/rgl2/rgl3/rga/gai* (Figures 1F and S1B–S1D).

We also analyzed TOC159 protein levels in the *sly1-2* mutant, which accumulates high RGL2 protein levels upon imbibition [10]. The *sly1* mutant cannot germinate or green unless the tegument is removed [17]. *sly1-2* seeds had visibly lower TOC159 levels compared to WT and *rgl2-13*. The *sly1-2* phenotype was rescued in the *sly1-2/rgl2-13* double mutant, which had near-WT levels of TOC159 (Figure 1G). Collectively, these results therefore are not consistent with the notion that high endogenous ABA levels triggered by DELLA factors under low-GA

conditions are responsible for repressing TOC159 accumulation in seeds. Rather, they strongly suggest that DELLA factors could directly repress TOC159 accumulation posttranscriptionally in a manner unrelated to their role in stimulating endogenous ABA levels.

TOC159 and DELLA Proteins Interact Directly

Up to here, our understanding of the role of RGL2 and other DELLAs in proplastid to chloroplast transformation during seed germination is very limited. To explore it, we carried out a yeast two-hybrid screen using RGL2 as the bait, and TOC159 itself was identified as an interaction partner of DELLAs. The TOC159 protein is composed of three domains: the N-terminal acidic domain (A-domain), central GTPase domain (G-domain), and C-terminal membrane domain (M-domain) (Figure S2) [2]. All DELLA factors physically interacted with the TOC159 G-domain in the two-hybrid assay (Figure 2A). The G-domain of the receptor TOC33 failed to interact with DELLA, indicating specificity of the DELLA–TOC159 G-domain interaction (Figures 2A and S2).

Co-immunoprecipitation of GFP-TOC159GM and MYC-RGL2 from extracts of co-infiltrated *Nicotiana benthamiana* leaves confirmed the interaction *in planta* (Figure 2B). In vegetative plants, GFP-TOC159GM localizes at the outer envelope of the chloroplast membrane, whereas RFP-RGL2 is localized in the nucleus. However, when RFP-RGL2 and GFP-TOC159GM were expressed together, fluorescence co-localized in the nucleus (Figure 2C). Fluorescence also appeared in the nucleus by bimolecular fluorescence complementation (BiFC) upon co-expression of YFC-TOC159GM and YFN-RGL2 in *N. benthamiana* leaves (Figure 2D). NTAP-TOC159GM isolated from transgenic seedlings grown in the presence of PAC was associated with RGL2 as well as TOC75 (Figure 3A). In a reverse experiment, TOC159 associated with RGL2-HA purified from estradiol-inducible *RGL2-HA* seedlings grown in the presence of PAC (Figure 3B). This corroborates the notion that RGL2 interacts with TOC159 *in vivo*. Moreover, recombinant RGL2 inhibited the *in vitro* outer membrane insertion of synthetic TOC159 in isolated chloroplasts, suggesting that it also functions to sequester TOC159 (Figure S3). Consistent with this notion, DELLAs have been shown to sequester interaction partners other than TFs in the nucleus, such as the prefoldin complex [18].

DELLA Promotes TOC159 Degradation by the Ubiquitin/Proteasome System

Besides their role in interfering with transcription factor (TF) activity [19–22], it has been demonstrated that DELLA mediates phytochrome interacting factor (PIF) degradation by the ubiquitin/proteasome system (UPS) [23]. TOC159 is a known target of ubiquitination and proteasome degradation [24, 25]. Therefore, DELLAs may promote TOC159 degradation via the UPS to repress chloroplast biogenesis under low-GA conditions. To evaluate this hypothesis, we first addressed whether TOC159 regulation by the UPS could be detected. PAC-treated WT seedlings incubated with MG132 (a proteasome inhibitor) had increased TOC159 levels, implicating degradation by the UPS (Figures 3C and 3D). Ubiquitination of TOC159 was confirmed after immunoprecipitating GFP-TOC159GM and anti-ubiquitin western blotting in the presence of PAC (Figure 3E).

Ubiquitin-Proteasome-Mediated Degradation of TOC159 Is SP1 Independent under Low GA

Chloroplast outer membrane ubiquitin E3 ligase SP1 has been implicated in the remodeling of the TOC complex by the UPS during plastid developmental transitions as well as stress, and TOC159, -75, and -33 are known targets of SP1 [24, 26]. To address the role, if any, of SP1 in DELLA-mediated TOC159 degradation, we analyzed PAC-treated *sp1* mutant seedlings. In the presence of PAC, TOC33, -75, and -159 were decreased in WT, but not in *rgl2* or *rgl2/rga/gai*. Surprisingly, only TOC33 and -75, but not TOC159, were increased in the presence of PAC in *sp1* when compared with WT (Figures 3F and 3G). This provides evidence for a role of SP1 in the PAC-induced degradation of TOC33 and TOC75 but suggests that a currently unknown ubiquitin ligase is specifically involved in DELLA-dependent degradation of TOC159 by the UPS.

Levels of Photosynthesis-Associated Preproteins Are Regulated at the Posttranscriptional Level under Low GA

We provide evidence that DELLA interacts with TOC159 under low-GA conditions, which leads to degradation of TOC159 by UPS and diminishes the levels of the chloroplast protein import receptor. This in itself would be expected to impede chloroplast biogenesis. However, it also raises the question of the fate of preproteins destined for the chloroplast during early seed germination. Very high concentrations of ABA or PAC (20 μ M) repress the expression of photosynthesis-associated genes in the embryo [27]. However, the expression of most photosynthesis-associated genes did not change significantly in WT in the presence of moderate PAC concentrations (5 μ M) when compared with *rgl2* in early seed germination [16]. We then investigated the accumulation of photosynthesis-associated as well as several non-photosynthetic proteins (FBN1A, FBN4, and MDH) in the presence or absence of low PAC concentrations (1 μ M). In the absence of PAC, WT seedlings accumulated higher levels of photosynthesis-associated proteins, but not of the non-photosynthetic proteins (Figures 4A, 4B, S4A, and S4B). Moreover, *rgl2* accumulated higher levels of photosynthesis-associated proteins than WT at the early stages of seed germination (Figures S4C and S4D). These results suggest regulation of pre-protein abundance at the posttranscriptional level.

An Un-imported Model Preprotein Is Degraded by the UPS under Low GA

It has been shown that un-imported chloroplast-destined preproteins accumulating in the cytosol are targeted for the UPS by the C terminus of Hsc70-interacting protein (CHIP) ubiquitin E3-ligase [28]. To address un-imported preprotein fate under low-GA conditions, we used RbcS(TP)-GFP (the transit peptide of the small subunit of rubisco preprotein fused to GFP) expressed in WT as a model. Seedlings treated with low PAC concentrations were import deficient as judged by the absence of imported GFP protein (Figure 4C). Furthermore, higher molecular mass, un-imported RbcS(TP)-GFP preprotein was detected by western blotting (Figure 4C) together with weak GFP fluorescence at the chloroplast periphery (Figure 4D). In the absence of PAC, imported RbcS(TP)-GFP was detected by western blotting (Figure 4C) and resulted in strong GFP fluorescence inside chloroplasts

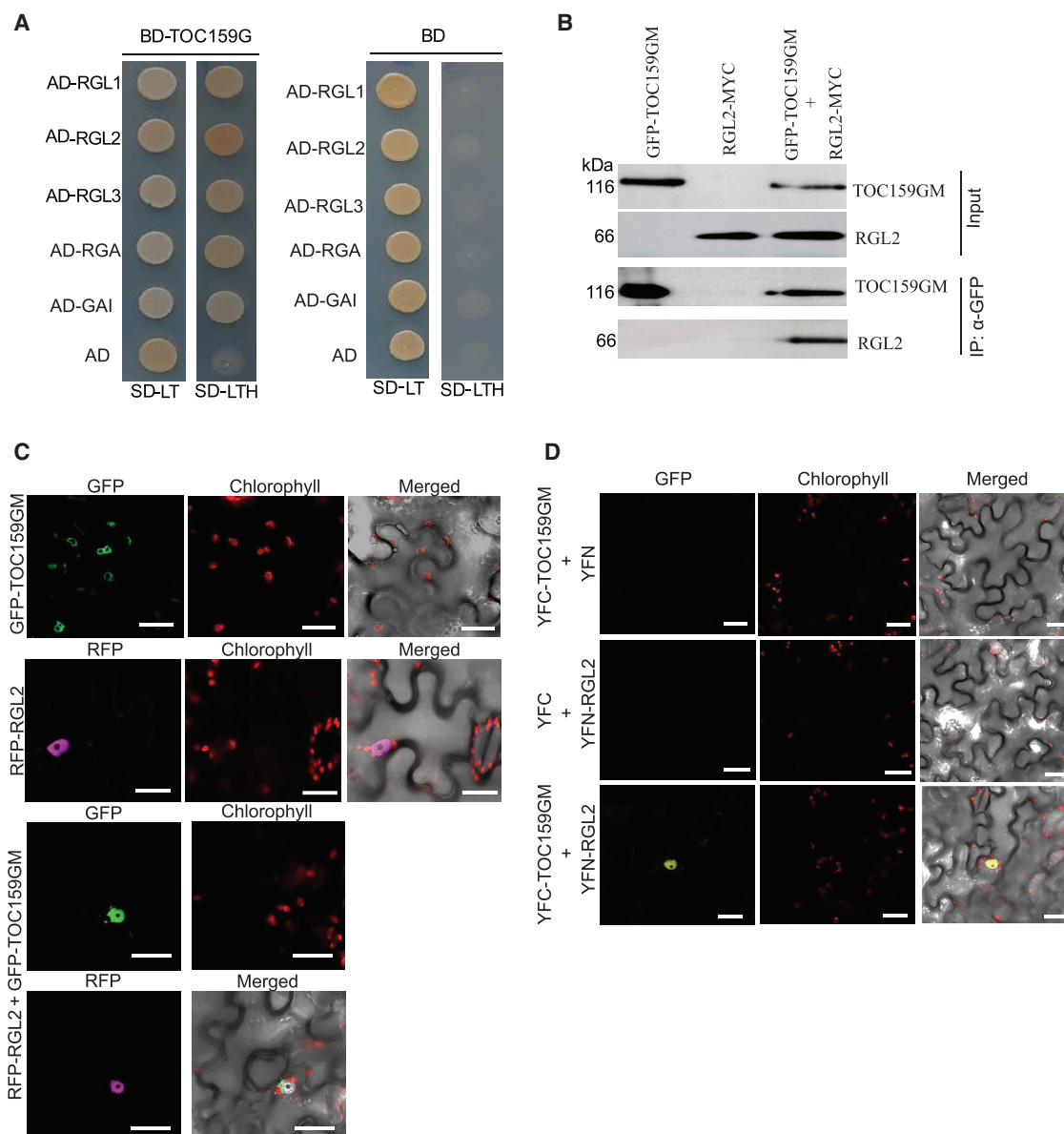


Figure 2. DELLA Specifically Interacts with TOC159 G-Domain

(A) Yeast two-hybrid interaction between the TOC159 G-domain and DELLA proteins on –Leu, –Trp and –Leu, –Trp, –His medium. AD, activation domain; BD, binding domain; empty vector was used as a control.

(B) Co-immunoprecipitation from *N. benthamiana* leaves expressing GFP-TOC159GM, RGL2-MYC, or both. Total protein extracts (input) were incubated with anti-GFP beads, and the recovered proteins (IP) were analyzed by western blotting using anti-GFP and MYC antibodies to detect TOC159GM and RGL2, respectively.

(C) Confocal microscopy images showing localization of GFP-TOC159GM and RFP-RGL2 in transiently transformed *N. benthamiana* leaves and co-localization confocal microscopy images of GFP-TOC159GM and RFP-RGL2 in co-transformed *N. benthamiana* leaves. The scale bars represent 20 μ m.

(D) BiFC confocal microscopy images showing TOC159GM and RGL2 interaction in *N. benthamiana* leaves co-expressing TOC159GM and RGL2 fused to the C- (YFC) and N-terminal (YFN) portions of YFP, respectively. No fluorescence was observed with the corresponding empty vectors (upper panel). The scale bars represent 20 μ m.

See also Figure S2.

(Figure 4D). These results show that low GA inhibits import of the model chloroplast preprotein and negatively regulates its concentration. Immunoprecipitation of un-imported RbcS(TP)-GFP under low GA followed by western blotting revealed higher molecular mass bands. Anti-ubiquitin western blotting indicated that these are due to ubiquitination of RbcS(TP)-GFP (Figures 4E and 4F).

Conclusions

Our studies provide a molecular frame work for the GA hormonal control of chloroplast biogenesis during seed germination. Environmental stress decreases the concentrations of GA during seed imbibition, and when GA levels are low the DELLA (RGL2) accumulates [10–12]. Under such conditions, seed germination

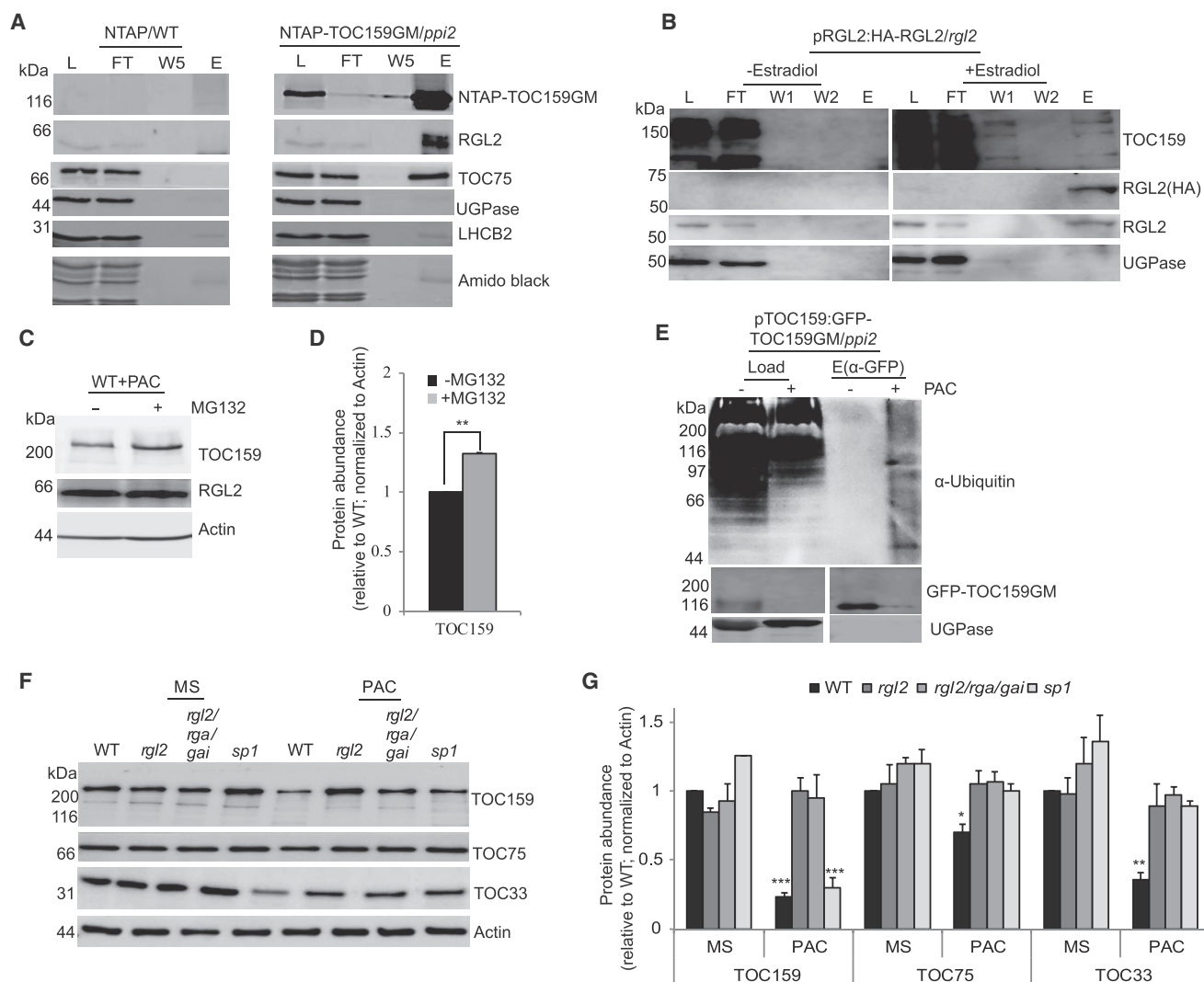


Figure 3. TOC159 Interacts with RGL2 and Is Degraded via the UPS under Low GA

(A and B) Immunoprecipitated NTAP-TOC159GM protein complex was analyzed by western blotting using immunoglobulin G (IgG) (to detect NTAP-TOC159GM), antibodies to RGL2, TOC75, UGPase, and LHCB2; NTAP/WT seedlings were used as a negative control (E, eluate; FT, flow-through; L, load; W5, last wash; A), and immunoprecipitated RGL2-HA protein complex was analyzed by western blotting using antibodies to HA (to detect HA-RGL2), RGL2, TOC159, and UGPase (B).

(C and D) Total protein extracts of 3-day-old WT seedlings grown on PAC treated with or without MG132, analyzed by immunoblotting using antibodies to TOC159, RGL2, and actin as a loading control (C), and specific bands were quantified and normalized with respect to actin. Data are relative to WT (D). Error bars indicate $\pm$ SEM ($n = 4$). Student's t test; ** $p < 0.01$.

(E) Immunoprecipitated GFP-TOC159GM protein complex was analyzed by western blotting using antibodies to GFP (to detect GFP-TOC159GM), ubiquitin, and UGPase.

(F and G) Total protein extracts of 66-hr-old seedlings grown on MS medium in the presence or absence of PAC were analyzed by immunoblotting using antibodies to TOC159, TOC75, and TOC33 proteins. Actin was used as a loading control (F), and specific bands were quantified and normalized to WT grown without PAC (G). Error bars indicate $\pm$ SEM ($n = 3$). Student's t test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

See also Figure S3.

as well as chloroplast biogenesis are attenuated. Under low GA, a posttranslational mechanism contributes to the delay of chloroplast biogenesis by limiting the chloroplast protein import pathway at the level of the TOC complex. Direct interaction with DELLA promotes the degradation of the TOC159 import receptor by the UPS prior to its assembly in the TOC complex. In the yeast two-hybrid screen, TOC159 interacted with all five

DELLAs. This suggests that DELLA-promoted TOC159 degradation as a means to control protein import into the chloroplast occurs at additional stages of development or under different types of stress in vegetative plants. As the import receptor TOC159 is essential for chloroplast biogenesis (the *ppi2* loss-of-function mutant has an albino phenotype), it is a very suitable target for interference with chloroplast biogenesis [2]. The chloroplast

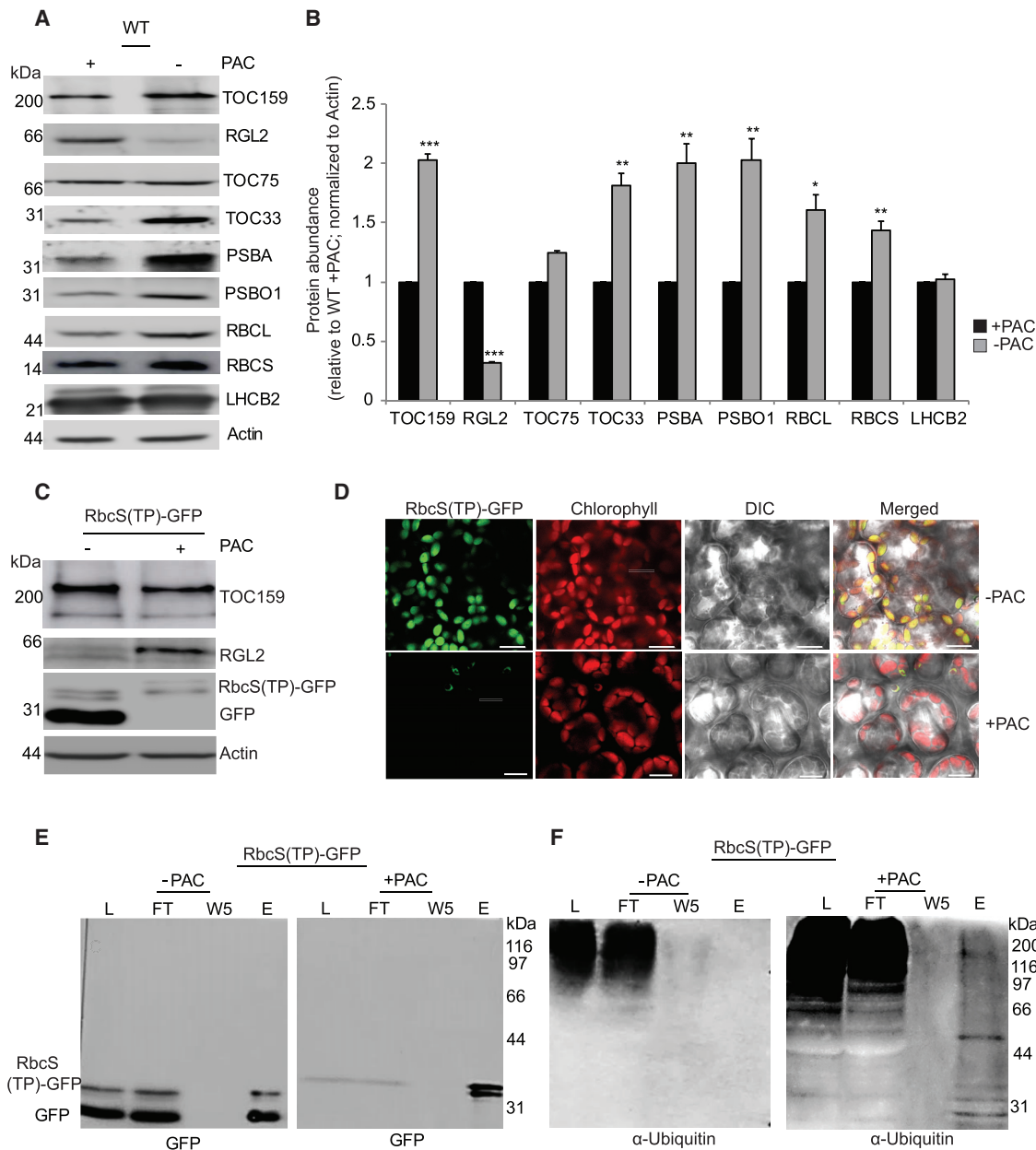


Figure 4. Low GA Concentration Reduces Accumulation of Photosynthesis-Associated Proteins and Promotes UPS-Mediated Degradation of the Un-imported Model Preprotein RbcS(TP)-GFP

(A and B) Total protein extracts of 48-hr-old WT seedlings grown in the presence or absence of PAC (1 μ M), analyzed by immunoblotting using antibodies to TOC159, RGL2, TOC75, TOC33, and several photosynthesis-associated proteins (A). Specific bands were quantified and normalized with respect to actin. Data are relative to WT + PAC (B). Error bars indicate $\pm$ SEM (n = 4). Student's t test; *p < 0.05; **p < 0.01; ***p < 0.005.

(C) Total protein extracts of 5-day-old RbcS(TP)-GFP-expressing seedlings grown in the presence or absence of PAC (1 μ M), analyzed by immunoblotting using antibodies to TOC159, RGL2, GFP, and actin (loading control).

(D) Confocal microscopy images of cotyledons of 5-day-old RbcS(TP)-GFP-expressing seedlings grown in the presence or absence of PAC (1 μ M). The scale bars represent 20 μ m.

(E and F) Immunoprecipitation of RbcS(TP)-GFP from 5-day-old seedlings treated with and without PAC. Total protein extracts (L) were incubated with anti-GFP beads, and immunoprecipitated proteins were analyzed by western blotting using antibodies to GFP (to detect RbcS(TP)-GFP products; E) and ubiquitin (F). Different but identically loaded blots were used for western analysis (F).

See also Figure S4.

outer membrane ubiquitin E3-ligase SP1 [24] does not appear to be required for the DELLA-dependent degradation, presumably because the affected TOC159 never integrates into the outer membrane. Moreover, co-localization of TOC159 and RGL2 in the nucleus suggests that a cytoplasmic or nuclear E3-ligase is implicated. In contrast to TOC159, the reduced levels of TOC33 under low GA could be attributed to SP1 activity. SP1-independent degradation of TOC159 hints at the activity of an unknown E3-ligase. In the *ppi2* mutant lacking TOC159, many nuclear genes encoding photosynthesis-associated proteins are strongly downregulated [2]. Under the low-GA/PAC conditions used in this study, TOC159 was diminished but the photosynthesis-associated genes were still expressed as in the absence of PAC. Still, the corresponding proteins were accumulating at reduced levels. Using RbcS(TP)-GFP as an *in vivo* model protein for chloroplast import, we provided evidence that ubiquitination of a preprotein in the cytosol and degradation by the UPS occurs when import is blocked. In view of the low-GA-induced import defect, this mechanism may be responsible for the removal of preproteins from the cytosol. Potentially, the cytosolic ubiquitin E3-ligase CHIP is involved in this scenario, as it is known to target un-imported preproteins [28]. When conditions become favorable for seed germination, GA accumulates to higher levels. The GA-Gibberellin insensitive dwarf1 (GID) complex binds to DELLA, leading to its ubiquitination by the F-box protein SLY1 and the SCF E3 ligase followed by degradation via the UPS [29], which in turn stabilizes TOC159. The comparatively low levels of TOC159 in the *sly1* mutant that accumulates RGL2 to high levels lend support to this scenario. Upon destruction of DELLA, TOC159 is immediately available for assembly into the TOC complex, which allows full import of photosynthesis-associated proteins into the chloroplast. Such a mechanism can assure a rapid switch from heterotrophic to photoautotrophic growth (visible as seedling greening) as soon as conditions allow. This in turn may improve the seedling's chances for survival in a challenging and stressful environment.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at <https://doi.org/10.1016/j.cub.2018.06.006>.

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AUTHOR CONTRIBUTIONS

F.K., L.L.-M., and V.S. designed experimentation. V.S. and M.R. performed yeast two-hybrid screening. H.C. performed RT-PCR. L.L.-M. and H.C. analyzed gene expression. H.C. analyzed TOC159 protein in *rgl2*, *sly1*, and *sly1/rgl2* lines. V.S. and G.G. performed *in vivo* localization and BiFC experiments. V.S. and S.A. engineered GFP-TOC159GM lines and performed quantitative western blot. V.S. and H.C. did *in vivo* pull-down assays. V.S. carried out all other experiments. V.S. and F.K. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TOC159	[2]	N/A
TOC75	[30]	N/A
TOC33	[31]	N/A
TOC132	[24]	N/A
TOC120	[32]	N/A
RGL2	[10]	N/A
FBN1A	[33]	N/A
MDH	[34]	N/A
PSBA	Agrisera	AS01016S; RRID: AB_1832037
PSBO	Agrisera	AS142824; RRID: AB_1031788
LHCB2	Agrisera	AS01003; RRID: AB1832080
RBCS	Agrisera	AS07259; RRID: AB_1031806
RBCL	Agrisera	AS03037; RRID: AB_2175406
IgG	MP Biomedicals	55944; RRID: AB_2334717
anti-GFP	Sigma	G6795; RRID: AB_563117
anti-actin	Sigma	A0480; RRID: AB_476670
anti-MYC	Cell signaling	2276S; RRID: AB_331783
anti-UGPase	Agrisera	AS05 086; RRID: AB_1031827
anti-Ubiquitin	Cell signaling	3936; RRID: AB_331292
anti-mouse IgG conjugated with HRP	Sigma	A5278; RRID: AB_258232
anti-rabbit IgG conjugated with HRP	Millipore	AP132P; RRID: AB_90264
Bacterial and Virus Strains		
<i>E. coli</i> strain DH5a	Invitrogen	18265017
<i>E. coli</i> strain BL21 DE3	NEB	C2527H
<i>Agrobacterium tumefaciens</i> C58	[35]	N/A
Yeast-Y187	Clontech	630457
Yeast-Y2H GOLD	Clontech	630498
Chemicals, Peptides, and Recombinant Proteins		
Murashige and Skoog Basal Medium	Duchefa Biochemie	M022
BASTA	Sigma	45520
Paclobutrazol	Sigma	43900
Abscisic acid	Sigma	A1049
Yeast nitrogen base without aminoacid	BD Difco	DF0919073
Complete CSM-Trp	Formedium	DCS0141
Complete CSM-Leu	Formedium	DCS0091
Complete CSM-Trp/-Leu	Formedium	DCS0561
Complete CSM-Trp/-Leu/-His	Formedium	DCS0971
Complete CSM-Trp/-Leu/-His/-Ade	Formedium	DCS1221
3-Amino-1,2,4-triazole	Sigma	A8056
MG132	AbMole	M1902
Protease inhibitors	Sigma	P9599
μMACS GFP-tagged beads	Miltenyi Biotec	130091125
Human IgG	MP Biomedicals	855908
Triton X-100	Roche	10789704001

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
RQ1 RNase-Free DNase	Promega	M6101
Improm II reverse transcriptase	Promega	A3800
oligo(dT)15 primer	Promega	C1101
Power SYBR Green PCR master mix	Applied Biosystems	4367659
BP clonase	Invitrogen	11789020
LR clonase	Invitrogen	11791020
Matchmaker Gold Yeast Two-Hybrid System	Clontech	630489
TNT Quick-coupled Translation System	Promega	L1170
ECL Plus western blotting substrate	Pierce	32132
Experimental Models: Organisms/Strains		
<i>ppi2</i>	[2]	N/A
<i>rgl2-13</i>	[36]	N/A
<i>sp1-3</i>	[24]	N/A
<i>sly1-2</i>	[17]	N/A
<i>sly1-2/rgl2-13</i>	This paper	N/A
<i>rgl2/rga/gai</i>	[37]	N/A
<i>rgl1/rgl2/rgl3/rga/gai</i>	[37]	N/A
NTAP-TOC159GM/ <i>ppi2</i>	[38]	N/A
NTAP/WT	[38]	N/A
pTOC159:GFP-TOC159GM/ <i>ppi2</i>	This paper	N/A
35S:RbcS(TP)-GFP/WT	This paper	N/A
Oligonucleotides		
See Table S1	This paper	N/A
Recombinant DNA		
pGBKT7	Clontech	630443
pGADT7	Clontech	630442
pGBKT7-ΔRGL2	This paper	N/A
pGBKT7-TOC159G	This paper	N/A
pGBKT7-TOC159M	This paper	N/A
pGBKT7-TOC33G	This paper	N/A
pGADT7-TOC159G	This paper	N/A
pGADT7-TOC33G	This paper	N/A
pGADT7-RGL1	This paper	N/A
pGADT7-RGL2	This paper	N/A
pGADT7-RGL3	This paper	N/A
pGADT7-RGA	This paper	N/A
pGADT7-GAI	This paper	N/A
pGBKT7-T	Clontech	630489
pGADT7-p53	Clontech	630489
pGADT7-Lam	Clontech	630489
pENTR221	Invitrogen	12536017
pB7WGF2	http://www.psb.ugent.be/gateway/	N/A
pYFN43	[39]	N/A
pYFC43	[39]	N/A
pB7WGR2	http://www.psb.ugent.be/gateway/	N/A
pET28a-RGL2-HIS	[10]	N/A
pB7WGF2-pTOC159:GFP-TOC159GM	This paper	N/A
pYFN43-TOC159GM	This paper	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pYFC43-RGL2	This paper	N/A
pB7WGF2-35S:GFP-TOC159GM	This paper	N/A
pB7WGR2-RGL2	This paper	N/A
pEarleyGate 203-RGL2-MYC	This paper	N/A
Software and Algorithms		
ImageQuant TL	GE Healthcare	N/A
Quantity One 4.6 software	Bio-rad	N/A
LAS software	Leica	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Felix Kessler (felix.kessler@unine.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant materials and growth conditions

Wild-type and mutant plants were in the Columbia-0 (Col-0), Wassilewskija (Ws), and Landsberg *erecta* (Ler) ecotype. The *sp1-3* and *rgl2-13* mutants used in this study were in the Col-0 ecotype and previously described [24, 36]. The *sly1-2 rgl2-13* double mutant was generated by crossing homozygous *rgl2-13* and *sly1-2* plants followed by genotyping. For genotyping the *sly1-2* allele, PCR was performed using dcaps primers followed by *DraI* digestion. The *della* multiple mutant (N16298) are in the Ler background and were described previously [21]. The different combinations of *della* mutants used (*rgl2-SK54 rga-28 gai-t6*) and the pentuple *della* mutant are in the Col-0 background as previously described [37]. The *ppi2* mutant, NTAP/WT, and NTAP-TOC159GM/*ppi2* lines used in this study were in the Ws ecotype and previously described [38]. Plants were grown either on soil under long-day conditions (16 h light (120 $\mu\text{mol m}^{-2} \text{s}^{-1}$), 8 h dark, 21°C, 70% relative humidity) or on Murashige and Skoog (MS) medium under short-day conditions (8 h light (120 $\mu\text{mol m}^{-2} \text{s}^{-1}$), 16 h dark, 21°C, 70% relative humidity).

Seedling treatment

Seeds were surface-sterilized and placed on MS medium containing the desired concentrations of paclobutrazol (PAC) (5 μM , 2 μM , 1 μM), abscisic acid (ABA) (5 μM), or both. Plates were then placed under standard growth conditions for 66 hours, unless indicated otherwise. For *in vivo* co-immunoprecipitation (CoIP) experiments and proteasome inhibitor experiments, the seedlings were further treated with MG132 (100 μM) in liquid MS medium by vacuum infiltration for 20 min. The MG132 vacuum-infiltrated seedlings were immersed in liquid MS medium for the next 12 hours. The corresponding volumes of solvents for PAC, ABA, and MG132 (ethanol, DMSO) were used in control experiments.

Plant transformation and transgenic lines

The coding sequence for TOC159GM was amplified from the pET21d-TOC159GM construct [38] with TOC159GM -F(GATE)/TOC159GM -R(GATE) primers and ligated into the pENTR221 vector using BP clonase (Invitrogen) and inserted into the pB7WGF2 binary vector (<http://www.psb.ugent.be/gateway/>) using the LR clonase (Invitrogen), resulting in a vector coding for the GFP-TOC159GM fusion under the control of the 35S promoter. The TOC159 promoter was PCR amplified from genomic DNA using pTOC159-F(*SpeI*)/pTOC159-R (*HindIII*) primers, digested with *SpeI*/*HindIII*, and used to replace the cauliflower mosaic virus 35S promoter in the GFP-TOC159GM construct. The binary construct pTOC159:GFP-TOC159GM was introduced into *Agrobacterium tumefaciens* C58 and stably transformed into heterozygous *ppi2* plants using the floral dip method [40]. Transformants were selected on phosphinotricine-containing medium and complemented homozygous lines were selected and named pTOC159:GFP-TOC159GM/*ppi2*.

METHOD DETAILS

Yeast two-hybrid assays

The DELLA protein N-terminal part results in auto-activation in the yeast two-hybrid assay [20]. To avoid auto-activation, the sequence encoding a RGL2 fragment between amino acids 113 and 547 was amplified by PCR using primers RGL2-F(*NdeI*)/RGL2-R(*NcoI*), digested with *NdeI*/*NcoI*, and ligated into the pGBKT7 (Clontech) vector to generate BD- Δ RGL2. The cDNA library obtained from 36-hour-old 5 μM PAC-, 3 μM ABA-treated *Arabidopsis* seedlings fused to the GAL4-AD in pGADT7 (Clontech) vector transformed into yeast strain Y187, and BD- Δ RGL2 transformed into yeast strain Y2H GOLD, was used as the bait vector for yeast

two-hybrid screening. The independent transformants were selected on SD –Leu –Trp –Ade –His plates with 5 mM 3-aminotriazol (3-AT). The cDNA fragments corresponding to the TOC159 GTPase domain (TOC159G), TOC159 membrane domain (TOC159M), and TOC33 GTPase domain (TOC33G) were ligated into the pGADT7 (GAL4-AD) and pGBKT7 (BD fusion) vectors. The full-length cDNA sequences of RGL1, RGL2, RGL3, RGA, and GAI in pET28a were digested with *NotI* and further treated with Klenow enzyme to fill in the cohesive end and digested with *NdeI*. The blunt-ended and *NdeI*-digested fragments were ligated into the *SmaI*/*NdeI* sites of the pGADT7 vector. Yeast cells carrying the bait vector were then co-transformed with the prey plasmids containing the full-length DELLA fragments and/or the various TOC159 and TOC33 coding sequences. The empty bait vector (BD) was used as a negative control. BD-T, AD-p53, and AD-Lam were used as the positive and negative controls.

Bimolecular fluorescence complementation (BiFC) assays

Full-length RGL2 was PCR amplified from pET28a-RGL2 [10] using primers RGL2-F(GATE)/RGL2-R1(GATE), ligated into the pENTR221 vector by BP clonase (Invitrogen), and inserted into the binary vectors pYFN43 and pYFC43, containing the N- and C-terminal halves of GFP, respectively. TOC159GM from pENTR221 (described above) was inserted by LR-reaction (Invitrogen) into the binary pYFN43 and pYFC43 vectors [39]. The C-F(GATE)/C-R(GATE) primers were used to remove the *ccdB* cassette from the pENTR221 vector using BP clonase (Invitrogen) and inserted into the binary vectors pYFN43 and pYFC43 without any coding sequence. The vectors encoding the YFN and YFC fusions with or without TOC159GM/RGL2 constructs were transformed into *A. tumefaciens* C58Ci strain. All different combinations of constructs were co-infiltrated into leaves of 2–3-week-old *Nicotiana benthamiana* and the p19 protein of tomato bushy stunt virus [41] was used to suppress gene silencing in *N. benthamiana* leaves. Infiltrated leaves were analyzed after 2 days using a Leica TCS SL confocal microscope. Images were captured and analyzed using LCS lite software (Leica).

Transient expression assays

pB7WGF2 binary vector coding for the GFP-TOC159GM fusion under the control of the 35S promoter (described above). The coding sequence of RGL2 was PCR amplified from pET28a-RGL2 using primers RGL2 –F(GATE)/RGL2 –R(GATE), ligated into the pENTR221 vector using BP clonase (Invitrogen), and inserted into the pB7WGR2 binary vector (<http://www.psb.ugent.be/gateway/>) using LR clonase (Invitrogen) to recombine RFP-RGL2 fusion under the control of the 35S promoter. Both constructs were transformed into *A. tumefaciens* strain C58Ci. Single constructs (GFP-TOC159GM, RFP-RGL2) and the combination of both constructs were co-infiltrated into leaves of 2–3-week-old *N. benthamiana*; the p19 protein of tomato bushy stunt virus was used to suppress gene silencing in *N. benthamiana* leaves. Infiltrated leaves were analyzed after 2 days under a Leica TCS SL confocal microscope. Images were captured and analyzed using LCS lite software (Leica).

Confocal laser scanning microscopy

Seeds of the *Arabidopsis thaliana* chloroplast protein import reporter line 35S:RbcS(TP)-GFP/WT were surface-sterilized and plated on MS medium containing 1 μ M PAC under short-day conditions for 5 days. The fluorescence was directly observed under a Leica TCS SL confocal microscope. Images were captured and analyzed using LCS lite software (Leica).

RNA extraction and gene expression analysis

Total RNA was extracted from seeds using the phenol:chloroform method as described [42]. Total RNAs were treated with RQ1 RNase-Free DNase (Promega) and reverse-transcribed using Imprim II reverse transcriptase (Promega) and oligo(dT)15 primer (Promega) according to the manufacturer's recommendations. Quantitative RT-PCR was performed by using QuantStudio Real Time PCR (ThermoFisher Scientific) and Power SYBR Green PCR master mix (Applied Biosystems). Relative transcript level was calculated by using the comparative Δ Ct method and normalized to the **PP2A** (*At1g69960*) gene transcript levels.

Protein extraction and immunoblotting

Samples of identical fresh weight from each plant were frozen in liquid nitrogen and proteins were extracted using AP extraction buffer (100 mM Tris pH 8, 2% β -mercaptoethanol, 4% SDS, 20% glycerol) followed by acetone precipitation. SDS-PAGE and immunoblotting were performed as described [35, 42]. After protein transfer, the nitrocellulose membranes were stained with amido black. To probe the blots, primary antibodies recognizing TOC159 [2], TOC75 [30], TOC33 [31], TOC132 [24], TOC120 [32], FBN1A [33], MDH [34] and RGL2 [10] were used. To detect photosynthesis-associated proteins, antibodies recognizing RBCS, RBCL, PSBA, PSBO, and LHCB2 were purchased from Agrisera, Sweden. Additional antibodies recognizing affinity tags or non-photosynthetic housekeeping proteins were IgG (Cell Signaling Technology), anti-Ubiquitin (Cell Signaling Technology), anti-MYC (Cell Signaling Technology), anti-GFP (Sigma), anti-actin (Sigma), and anti-UGPase (Agrisera). Secondary antibodies were anti-rabbit IgG conjugated with horseradish peroxidase (Millipore), or anti-mouse IgG conjugated with horseradish peroxidase (Sigma). Chemiluminescence was detected using ECL Plus Western Blotting Detection Reagents (Pierce) and developed using a GE Amersham Imager 600. Band intensities were quantified using ImageQuant TL (GE Healthcare) software.

In planta ColP from transient expression system in *N. benthamiana*

Full-length RGL2 was PCR amplified from pET28a-RGL2 using the primers RGL2-F(GATE)/RGL2-R2(GATE), inserted into the pENTR221 vector by BP clonase (Invitrogen), and further recombined into the pEarleyGate 203 binary vector [43] with LR clonase

to obtain a RGL2-MYC fusion construct. The GFP-TOC159GM (described above) and RGL2-MYC fusion constructs were transformed into *A. tumefaciens* strain C58Ci and co-infiltrated into 2–3-week-old *N. benthamiana*; the p19 protein of tomato bushy stunt virus was used to suppress gene silencing in *N. benthamiana* leaves. Total proteins were extracted in lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.2% v/v protease inhibitors (Sigma P9599), 20 μ M MG132, and 10% glycerol. Anti-GFP antibodies conjugated to microbeads (μ MACS GFP-tagged beads; Miltenyi Biotec) were used to isolate the immunoprotein complexes. The eluates were resolved by SDS-PAGE, and GFP-TOC159GM and RGL2-MYC were detected using anti-GFP and anti-MYC antibodies, respectively.

In vivo CoIP

36-hour-old NTAP/WT and NTAP-TOC159GM/*ppi2*, PAC (5 μ M)-treated transgenic seedlings were transferred to MG132-containing medium for 12 hours. Total proteins were extracted in “lysis buffer (as above)” and IgG-Sepharose resin was used to isolate protein complexes. Eluates were analyzed by western blotting using anti-RGL2, IgG, anti-TOC75, anti-LHCB2 and anti UGPase antibodies for detection of RGL2, NTAP-TOC159GM, TOC75, LHCB2, and UGPase protein. 36-hour-old pTOC159:GFP-TOC159GM/*ppi2*, PAC (5 μ M) treated transgenic seedlings were transferred to MG132-containing medium for 12 hour. One-week-old pTOC159:GFP-TOC159GM/*ppi2* transgenic seedlings transferred to MG132-containing medium for 12 hours were used as a control. Total proteins were extracted in “lysis buffer (as above)” and anti-GFP antibody conjugated to microbeads (μ MACS GFP-tagged beads; Miltenyi Biotec) was used to isolate protein complexes. Ubiquitinated TOC159GM protein complexes were detected by western blotting using anti-GFP, anti-Ubiquitin, and anti-UGPase antibodies for detection of GFP-TOC159GM, Ubiquitinated protein complexes, and UGPase protein. 35S:RbcS(TP)-GFP/WT seeds were grown in the presence or absence of PAC (1 μ M) for 5 days and tested for ubiquitination as described above. Total proteins were extracted in “lysis buffer (as above),” and anti-GFP antibodies conjugated to microbeads (μ MACS GFP-tagged beads; Miltenyi Biotec) were used to isolate immunoprotein complexes. Eluates were analyzed by western blotting and probed with anti-GFP and anti-Ubiquitin antibodies for detection of RbcS(TP)-GFP, GFP, and Ubiquitinated protein complexes. 72-hour-old estradiol inducible pRGL2:HA-RGL2, PAC (10 μ M)-treated transgenic seedlings with or without estradiol (50 μ M). Total proteins were extracted in lysis buffer containing 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.1% Triton X-100, 1mM DTT, 0.1% protease inhibitors (Sigma P9599), 5 μ M MG132, and 10% glycerol, and HA-beads (Anti-HA affinity matrix; Roche) were used to isolate protein complexes. Eluates were analyzed by western blotting and probed with anti-HA, anti-RGL2, anti-TOC159, and anti-UGPase antibodies for detection of HA-RGL2, RGL2, TOC159, and UGPase protein.

Expression and purification of recombinant proteins

RGL2-His tag was overexpressed in *Escherichia coli* strain BL21(DE3) transformed with expression vector pET28a-RGL2-HIS. Expression was induced using 0.4mM isopropyl- β -D-thiogalactopyranoside at 25°C for 12 h. Bacterial pellets were lysed by sonication in 50 mM Tris-HCl pH 8, 150 mM NaCl, and 5 mM imidazol followed by centrifugation for 30 min at 14,000 rpm (Sorvall, SS-34). The supernatant was filtered through a 0.45 μ M nitrocellulose filter and RGL2-His₆ protein was purified from the supernatant fraction by nickel-nitrilotriacetic acid agarose affinity chromatography. Eluates were dialyzed against phosphate buffered saline (PBS). 10% glycerol was added to the purified RGL2-His₆ protein and stored at –80°C.

Chloroplast TOC159 targeting and protein import assay

Isolation of pea chloroplasts and *in vitro* protein import assays were performed as described [44, 45]. Isolated chloroplasts were incubated with *in vitro*-synthesized [³⁵S] methionine-labeled TOC159 (TNT_T7 Quick-coupled Transcription/Translation System; Promega) in the presence or absence of recombinant RGL2 protein, using bovine serum albumin as a negative control for recombinant RGL2 protein. As a negative control of TOC159 targeting, *in vitro* translated [³⁵S]-methionine labeled preprotein of the small subunit of Rubisco (p-RBCS) was incubated with isolated chloroplasts in the presence or absence of recombinant RGL2 protein, using BSA as a control. Selected samples were treated with the thermolysin protease to remove non-imported [³⁵S]-methionine labeled proteins. 25 μ g of re-isolated chloroplasts were separated by SDS-PAGE. Gels were stained with Coomassie Blue, dried, and analyzed using a Phosphorimager (Molecular Imager FX; Bio-Rad).

QUANTIFICATION AND STATISTICAL ANALYSIS

For protein quantification, immunoblot band intensities were quantified using ImageQuant TL (GE Healthcare) software and the data are shown as mean $\pm$ SEM. Sample size, n, for each experiment is given in the figure legends. For transcript quantification, the data are shown as mean $\pm$ SEM. Sample size, n, for each experiment is given in the figure legends. Statistical analysis was carried out by Student's t test, with p values higher than 0.05 being considered non-significant (n.s.) while p values lower than 0.05 being considered significant for the analyzed data and indicated as: *, p < 0.05; **, p < 0.01; ***, p < 0.005. For [³⁵S]-methionine-labeled protein quantification, radioactive bands were quantified using ImageQuant TL (GE Healthcare) software and the data are shown as mean of two independent biological samples having given apparently similar results.

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Supplemental Information

Chloroplast Biogenesis Controlled by DELLA-TOC159

Interaction in Early Plant Development

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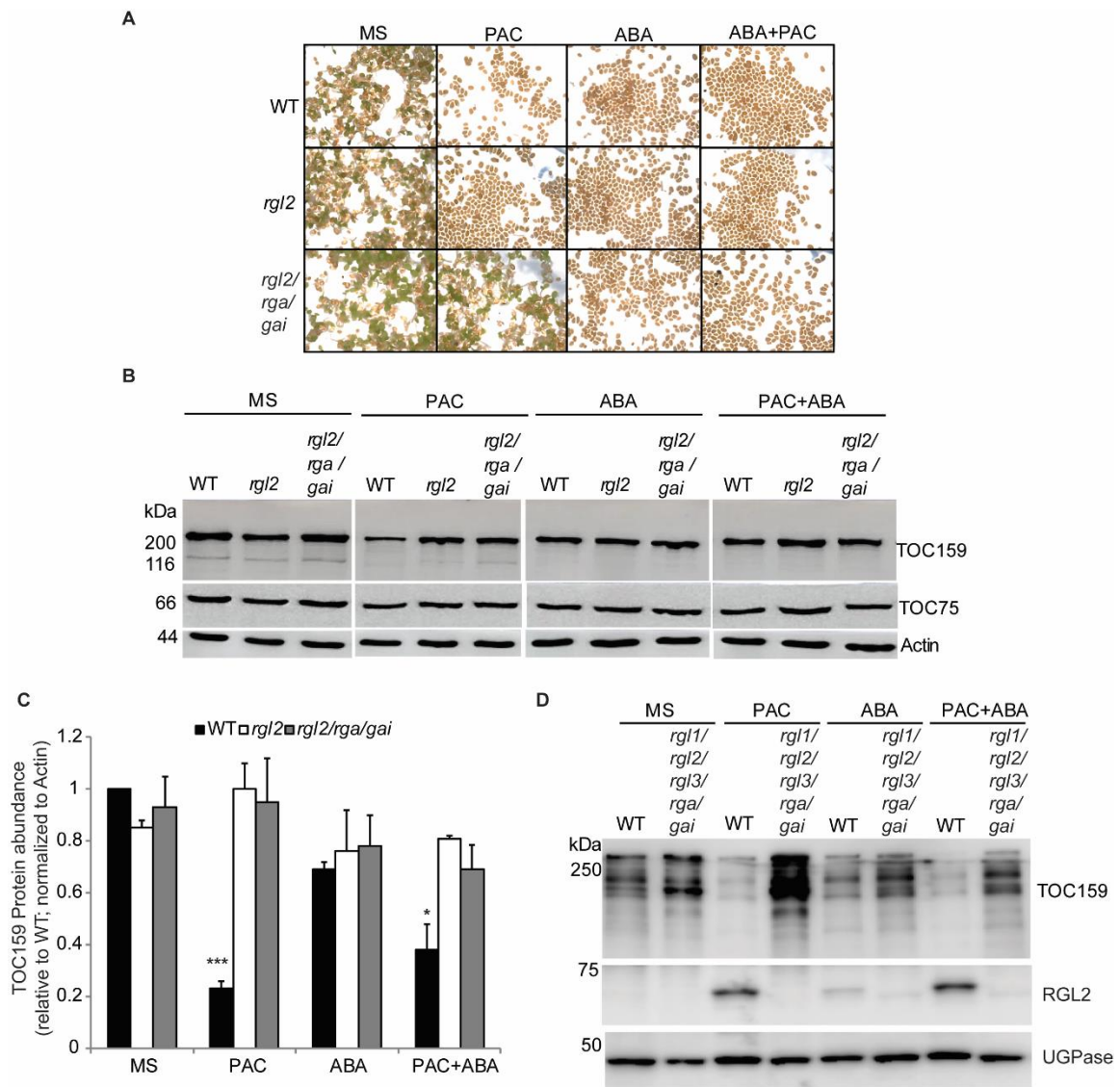


Figure S1. TOC159 protein level is not regulated by ABA, related to Figure 1

(A-C) WT, *rgl2*, and *rgl2/rga/gai* seedlings grown on MS medium with or without PAC (5 μ M), ABA (5 μ M), and ABA+PAC, documented at 66 hours (A) and total protein extracts were analyzed by immunoblotting using antibodies to TOC159, TOC75, actin (B) and quantification of specific bands were normalized with respect to actin. Data are relative to WT (C) Error bars indicate $\pm$ SEM (n=3). (Student's t-test; * p <0.05; *** p <0.005). For quantification, the same data were used in Fig. 3F for TOC159 protein quantification.

(D) Western blot analysis of WT and *rgl1/rgl2/rgl3/rga/gai* (*della* null mutant) seedlings grown in the absence or presence of PAC (5 μ M), ABA (5 μ M), and PAC+ABA, using antibodies to TOC159, RGL2, and UGPase (loading control).

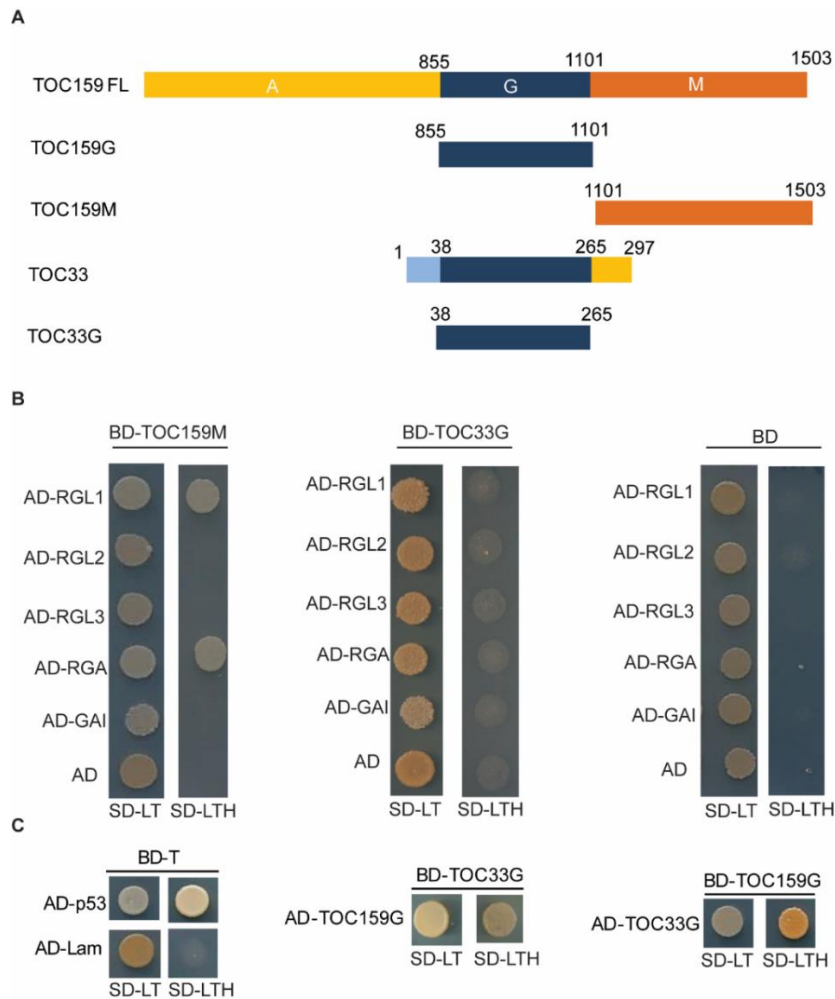


Figure S2. DELLAs interact with the G-domain of TOC159 but not with that of TOC33, related to Figure 2

(A) Schematic representation of full-length TOC159 composed of N-terminal acidic domain (A-domain), GTPase domain (G-domain; TOC159G), and C-terminal membrane domain (M-domain; TOC159M), and full-length TOC33 with GTPase domain (TOC33G).

(B) Yeast two-hybrid analysis of BD-TOC159M and BD-TOC33G interactions with AD-RGL1, AD-RGL2, AD-RGL3, AD-RGA, and AD-GAI vector on –Leu, –Trp (SD-LT) and –Leu, –Trp, –His (SD-LTH) media. Empty bait vector was used as a negative control.

(C) Yeast two-hybrid analyses of (left panel) BD-T interaction with AD-p53 and AD-Lam vector on SD-LT and SD-LTH media, as a control for functional yeast two-hybrid assays; and of interactions of (center panel) BD-TOC33G with AD-TOC159G and (right panel) vice versa on SD-LT and SD-LTH media, as a positive control for the BD-TOC33G interaction.

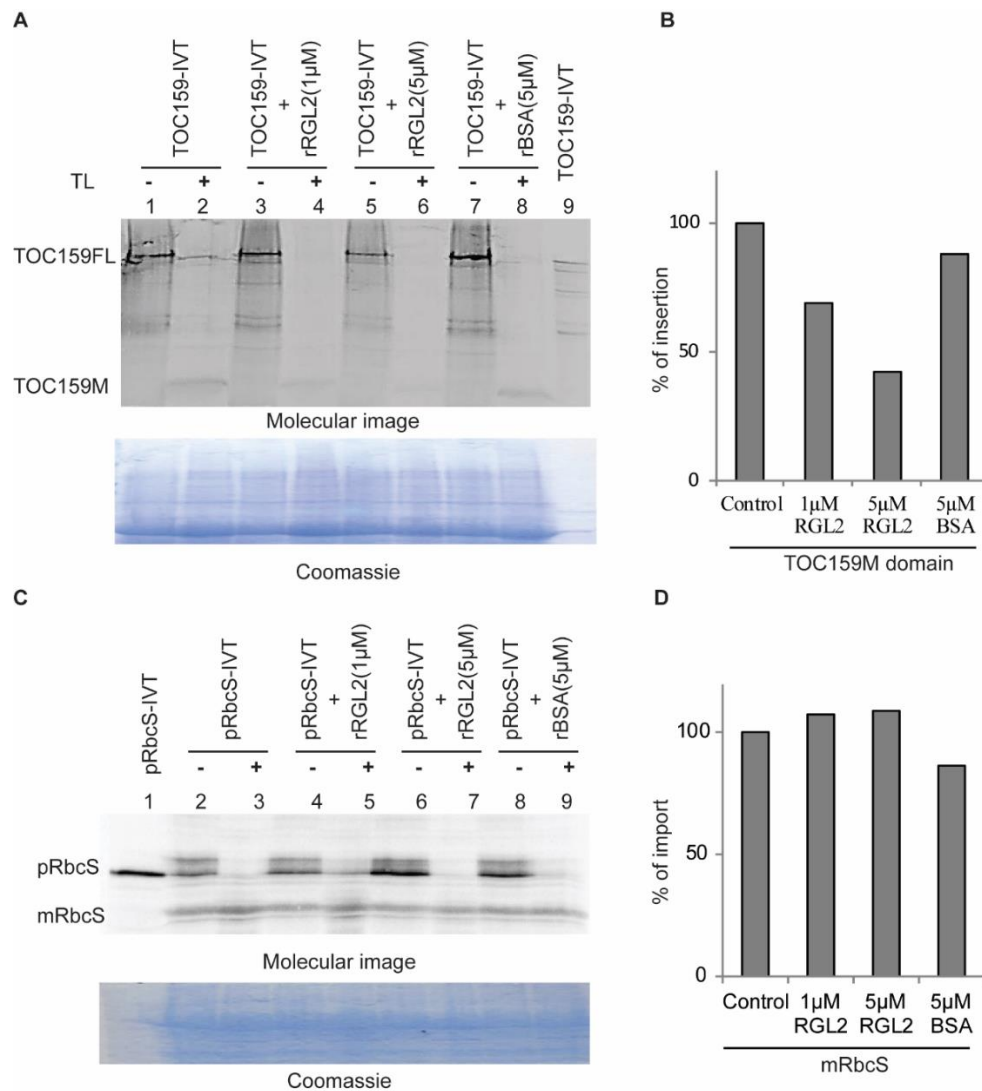


Figure S3. Recombinant RGL2 (rRGL2) binds to TOC159 and interferes with its insertion into the chloroplast membrane, related to Figure 3

(A-B) *In vitro*-synthesized, [35 S]methionine-labeled TOC159 (TOC159FL) was incubated with isolated pea chloroplasts in the presence or absence of recombinant RGL2 or BSA. Chloroplasts were reisolated and incubated with thermolysin (TL). Samples were analyzed by SDS-PAGE and Coomassie Blue staining followed by PhosphorImager visualization and quantification. A section of the Coomassie Blue-stained gel is shown as a loading control. Quantitative analysis of data from replicate experiments using ImageQuant TL software (GE Healthcare). The TL-resistant fragment (TOC159M) of *in vitro*-synthesized [35 S]-methionine labeled TOC159 incubated with chloroplasts in the absence of rRGL2 or BSA (lane 2 in A) was set to 100% (B).

(C-D) *In vitro*-translated, [35 S]-methionine labeled pRbcS was incubated with isolated pea chloroplasts in the presence of recombinant RGL2 or BSA. Chloroplasts were reisolated and incubated with TL. Samples were analyzed by SDS-PAGE and Coomassie Blue staining followed by PhosphorImager visualization and quantification. A section of the Coomassie Blue-stained gel is shown as a loading control. Quantitative analysis of data from replicate experiments using ImageQuant TL software (GE Healthcare). Imported, TL-resistant [35 S]-methionine labeled mRbcS in the absence of rRGL2 OR BSA (lane 3 in C) was set to 100% (D).

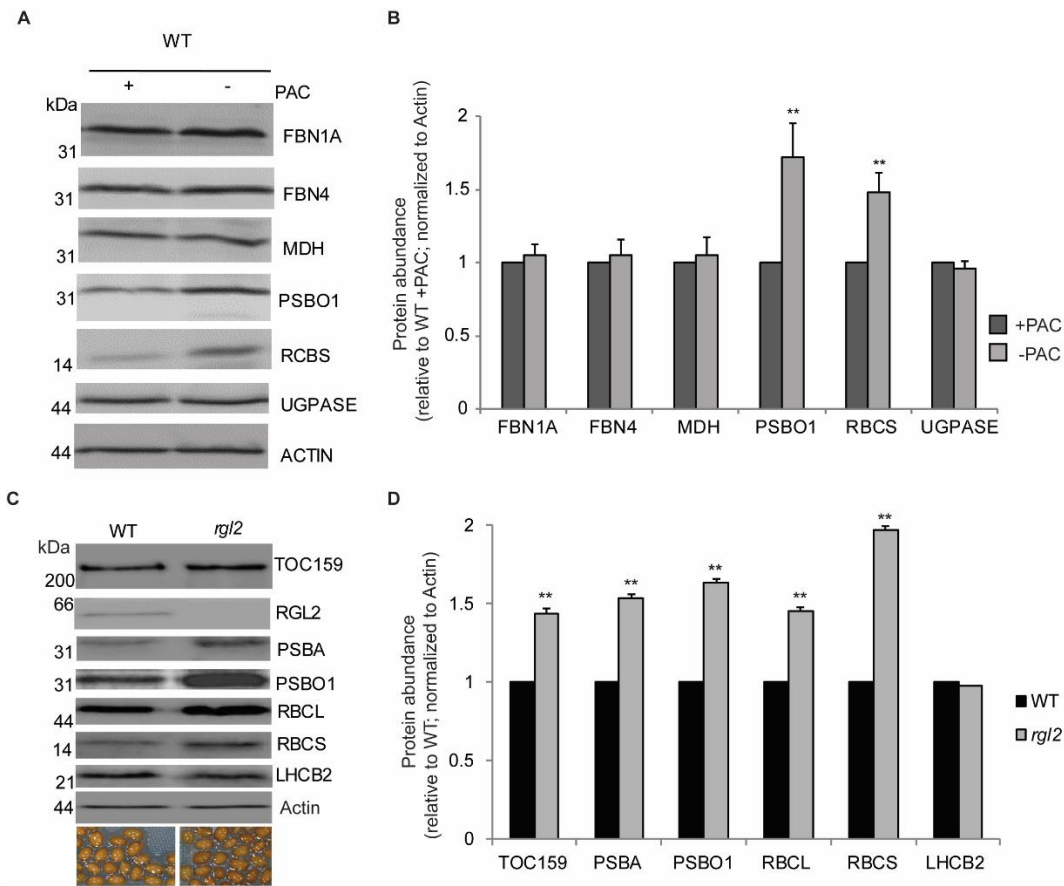


Figure S4. Low GA does not reduce accumulation of non-photosynthetic proteins and precocious accumulation of photosynthesis-associated proteins in *rgl2*, related to Figure 4

(A and B) Total protein extracts of 48-hour-old WT seedlings grown in the presence or absence of PAC (1 μ M), analyzed by immunoblotting using antibodies to chloroplast non-photosynthetic proteins fibrillin 1A (FBN1A), fibrillin 4 (FBN4), malate dehydrogenase (MDH), cytosolic UGPase and photosynthesis-associated proteins PSBO1, RBCS (A) Specific bands were quantified and normalized with respect to actin. Data are relative to WT +PAC (B). Error bars indicate $\pm$ SEM (n=4). (Student's t-test; *p<0.05; **p<0.01; ***p<0.005).

(C-D) Total protein extracts of 28-hour-old WT and *rgl2* seedlings were analyzed by immunoblotting using antibodies to TOC159, RGL2, and several photosynthesis-associated proteins and specific bands were quantified and normalized with respect to actin. Data are relative to WT. Error bars indicate $\pm$ SEM (n=3). (Student's t-test; **p<0.01)

Primer name	Sequence (5'→ 3')
RGL2-F(<i>Nde</i> I)	GCTTCTCATATGCTTGACACGACC
RGL2-R(<i>Nco</i> I)	GCAAGCTTCGGTACTCAGGCGAG
TOC159G-F(<i>Eco</i> RI)	CGTTCCGAATTCATGTCCAATTCTAATGTCACAATGGC
TOC159G-R(<i>Bam</i> HI)	GGAG AGGCGGGATTCTTATCGGAAACCAAATAC
TOC159GM-F(GATE)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCCAATTCTAATGTCACAATGGC
TOC159GM-R(GATE)	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGTACATGTCGTACTTGTCGTTTCGTCGC
pTOC159-F(<i>Spe</i> I)	CACTGAAAGCTTAAACCTGATTTTAC
pTOC159-R (<i>Hind</i> III)	CGACCACCACTAGTATCCTTCGGCTTTGC
RGL2-F(GATE)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGAGAGGATACGGAGAAACATGG
RGL2-R1(GATE)	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GGC GAG TTT CCA CGC CGA GG
RGL2-R2(GATE)	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ATA GGC GAG TTT CCA CGC CGA GG
C-F(GATE)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTAG TCCAAATGTTCTTCTCAAACG
C-R(GATE)	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATGTTTTTATTTTGTACAC
TOC159G-F(<i>Eco</i> RI)	CGT TCCGAATTCATGTCCAATTCTAATGTCACAATGGC
TOC159G-R(<i>Bam</i> HI)	GGAGAGGCGGAATTCTTATCGGAAACCAAATAC
TOC159 INT	GAATAGGGTTTTAATCGGAAG
TOC159 A3R	TGCCACATCAACATGCACTG
LB5	GATGCAATCGATATCAGCCAATTTTAGAC
PP2A RT F	GGACCGGAGCCAACCTAGGA
PP2A RT R	GCTATCCGAACCTTCTGCCTCATT
TOC159 RT F	CACAGTCTTGCTCTAGCTAG
TOC159 RT R	CTCCTCTGACCACATATGCC
rga-28F	ACCACGGGACTTCTTCTTCAT
rga-28R	TGGACTAAACGAACACCGTTC
rgl1-Sk62F	TGAAGAGAGAGCACAACCACC
rgl1-Sk62R	AGCTCGAAGACCGAGTTAACC
rgl3-3F	GTTACATACACATGTTCTCGTTCTCGA
rgl3-3R	ATTGCCTCTTCTAGTAATGCCTTCAATGAC
gai-t6F	AGCATCAAGATCAGCTAAAG
gai-t6R	CTAGATCCGACATTGAAGGA
gai-t6	TCGGTACGGGATTTTCGCAT
rgl2-13F	ATGGCTGAAGTAGCACAGAAGCTTGAACA
rgl2-13R	GGCAATCAGCTGTTGCCCGTCTCACTGGTG
sly1-2F	ACTCTCTTTACCTCTGGCCTCTTT
sly1-2R	ACTTACCAATCAACAAACTCC

Table S1. Primers used in this study. Related to STAR Methods.