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DELLA Proteins Physically Interact with CONSTANS to Regulate Flowering under Long Days in *Arabidopsis*

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Abstract

Proper timing of flowering is essential for reproduction of plants. Although it is well known that both light and gibberellin (GA) signaling play critical roles in promoting flowering in *Arabidopsis thaliana*, whether and how they are integrated to regulate flowering remain largely unknown. Here we show through biochemical studies that DELLA proteins physically interact with CONSTANS (CO). Furthermore, the interaction of CO with NF-YB2 is inhibited by the DELLA protein, RGA. Our findings suggest that regulation of flowering by GA signaling in leaves under long days is mediated, at least in part, through repression of DELLA proteins on CO, providing a molecular link between DELLA proteins, key components in GA signaling pathway, and CO, a critical flowering activator in photoperiod

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signaling pathway.

1. Introduction

Light is a pivotal environmental factor that acts to profoundly regulate multiple aspects of plant growth and development, such as seed germination, photomorphogenesis, and floral transition [1-4]. Sense of daily duration of light is critical for many plants to switch from vegetative to reproductive development. In *Arabidopsis thaliana*, a B-box zinc finger transcription factor expressing in leaves, called CONSTANS (CO), is a key component to accelerate flowering under long days (LDs) [5]. CO exerts function mainly by binding to the promoter and activating the expression of *FLOWERING LOCUST* (*FT*) directly, whose encoding protein is known to be a candidate of florigen [6]. The *FT* gene transcribes in leaves, and the FT protein acts as a mobile signal transferring from leaves to shoot apical meristem (SAM) through the phloem system to promote flowering [7]. The expression of *CO* is regulated by circadian clock. Under LDs, *CO* expression declines in the morning, rises in the afternoon, and peaks at dusk. It is known that CO protein accumulates at afternoon, and undergoes degradation in the dark via the 26S proteasome pathway mediated by the COP1-SPA complex [2,8], critical negative regulators of light signaling. Under light, photoactivated blue light receptors cryptochromes (CRY) act to inhibit the function of COP1-SPA complexes through direct interactions, resulting in stabilization of CO protein [9-11].

Gibberellin (GA) is diterpene plant hormone that is also involved in regulating a variety of plant developmental processes, including seed germination, hypocotyl /stem elongation, and floral initiation [12]. It is known that GA promotes the interactions of its receptors, GID1, with DELLA proteins, RGA, GAI, RGL1, RGL2, and RGL3, leading to subsequent ubiquitination and degradation of these proteins mediated by E3 ubiquitin-ligase $SCF^{SLY1/GID2/SNZ}$ and 26S proteasome, respectively [13-15]. Therefore, DELLA proteins act as key repressors of GA signaling [16]. Genetic studies suggest that GA signaling promote flowering under both SDs and LDs [17-19]. Biochemical and molecular studies indicate that under SDs and LDs, DELLA proteins interfere with the transcriptional activity of SPLs through their direct interactions and repress the expression of the MADS box genes in SAM.

Moreover, DELLA proteins also interact with SPLs to reduce miR172 levels to promote the abundance of its targets encoding the AP2-like transcription factors and inhibit the expression of *FT* in leaves under LDs [17,19]. The DELLA domain is known to be critical for GA promotion of the degradation of DELLA proteins, and deletion of the N-terminal 17 amino acids in this domain results in stabilization of DELLA proteins in response to GA [20]. Moreover, transgenic plants expressing RGA lacking these 17 amino acids within its DELLA domain (RGAd17) display a severely late flowering phenotype under LDs [19], indicating a strong role for the stabilized version of DELLA protein, RGAd17, in inhibiting flowering.

It has been suggested that the Nuclear Factor Y (NF-Y) complex is involved in both light and GA signaling to promote flowering [21-23]. It consists of NF-YA, NF-YB, and NF-YC subunits, which interact with each other, and form heterotrimeric transcriptional complex and regulate gene expression by binding to the CCAAT box [24]. In *Arabidopsis thaliana*, both RGA and CO interact with NF-YA2, NF-YB2/B3, and NF-YC3/C9, respectively [23]. Genetic and biochemical studies demonstrate that CO regulation of flowering is dependent on NF-Y complex. Moreover, it's reported that CO interacts with NF-Y complex and binds to the promoter of *FT* and *SUPPRESSOROF OVEREXPRESSION OF CO1* (*SOC1*), suggesting that CO/NF-Y complex regulates the transcription of downstream genes involved in the regulation of flowering. By contrast, RGA prevents NF-Y from binding to the promoter of *SOC1* [22].

It is known that regulation of hypocotyl elongation by GA signaling is mediated through repression of the transcriptional activity of Phytochrome-Interacting Factors 3 and 4 (PIF3 and PIF4), pivotal negative regulators of photomorphogenesis [25], through direct interactions of DELLA proteins with PIF3 and PIF4 [26,27]. Although much is known about how light or GA signaling regulates flowering independently, whether and how they actually interact to regulate this process is largely unknown. Here, we show by biochemical studies that DELLA proteins physically interact with CO. Furthermore, RGA acts to inhibit the interaction of CO with NF-YB2, likely through the interactions of RGA with CO and NF-YB2. Our results suggest that light and GA signaling may be integrated to regulate flowering under LDs, at least in part, through direct DELLA-CO interaction in leaves. This interaction, likely as well as the interaction of DELLA proteins with the NF-Y complex, may prohibit the association of CO with NF-Y complex, leading to repression of the transcription of *FT* in vasculature of

leaves and delay of flowering under LDs.

2. Materials and methods

2.1 Yeast two hybrid assay

Yeast two hybrid assay was performed according to the user manual for Yeastmaker Yeast Transformation System (Clontech Laboratories, Inc.). Combinations of AD and BD vectors were cotransformed into AH109 cells via the PEG/LiAc transformation procedure. Three days after transformation, yeast cells were spread on either SD-Trp/Leu/His (SD-T-L-H) plates supplemented with 25μM 3-Amino-1,2,4-triazole (3-AT) or SD-Trp/Leu/His/Ade (SD-T-L-H-A) plates for interaction test.

2.2 Pull-down assay

Fragments encoding the full length CO, RGA, GAI, and NF-YB2 were cloned into pGEX-4T-1 and pCOLD-TF vector, respectively. GST, GST-CO, His-TF, His-RGA, His-GAI, and His-NF-YB2 proteins were expressed in *E. coli* (Rosetta). His-TF, His-RGA, His-GAI, and His-NF-YB2 were purified according to the manufacturer's protocol (QIAGEN). His-TF (histidine-tagged Trigger Factor) is expressed and purified from *E.coli* harboring the empty pCOLD-TF vector, which served as negative control. For the pull-down assay to detect the interaction of DELLA proteins with CO, bait proteins GST-CO was firstly incubated with 20μL Glutathione beads (MagneGST Glutathione particles, Promega) in TBST buffer (50 mM Tris-HCl, pH 7.5, 150mM NaCl, 0.2%Triton-x-100 and 10% glycerol) at 4°C for 1 h, and washed three times with the same buffer. The beads were then re-suspended by 1 ml TBST buffer and prey proteins His-RGA, His-GAI and His-TF were added to the solution. The mixture was incubated for another 1 h at 4°C and washed with TBST buffer for three times. For the pull-down assay to detect the effects of RGA on the interaction of CO with NF-YB2, bait proteins (GST and GST-CO) were firstly incubated with 20μL Glutathione beads in TBST buffer at 4°C for 1 h, and washed three times with the same buffer. The beads were then re-suspended by 1 ml TBST buffer and His-NF-YB2 proteins were added to the solution. The mixture was incubated for another 1 h at 4°C and washed with TBST buffer for three times. The beads were then re-suspended again by 1 ml TBST buffer and mixed with

His-RGA or His-TF for 1 h and washed with TBST buffer for three times. Proteins were eluted into SDS loading buffer and analyzed by Western blot. Prey proteins were detected by anti-His antibody (NewEast) and bait proteins were visualized by Coomassie Brilliant Blue staining.

2.3 Protein Cellular Co-localization Study

For protein co-localization in tobacco, fragments encoding YFP, CO-YFP, CFP, RGA-CFP, CFP-GAI, RGL1-CFPand RGAd17-CFP were cloned into pHB vector [28] to generate the indicated constructs, respectively. A mixture of *Agrobacterium* harboring CFP-fusion and YFP-fusion constructs plus p19-expressing *Agrobacterium* strain with a ratio of 1:1:1 was introduced into tobacco leaves by infiltration. After 2 days, leaves were collected for confocal microscopic examination (Leica TCS SP5II confocal laser scanning microscope).

2.4 Co-immunoprecipitation Assays

Construction of *35S::Myc-CO* described previously [29]. Fragments comprising *35S::RGA-Flag*, *35S::GAI-Flag*, *35S::RGAd17-Flag* and *35S::cLUC-Flag* were cloned into pCambia1300. Flag-tagged proteins were coexpressed with Myc-CO in tobacco leave leaves. After 2 days, samples from tobacco were homogenized in lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.2% Trition-X-100, 1mM Pefabloc, cocktail, 50 μM MG132). After centrifugation, supernatant was incubated with 20 μL anti-Flag magnetic beads (Sigma) for 30 min at 4°C. The immunoprecipitates were washed 3 times with lysis buffer and eluted with 0.5 μg/μL 3×flag peptide (Sigma) in 50 μL lysis buffer. The elutes were subjected to Western blot analysis with anti-Flag (Sigma) and anti-Myc (Millipore) antibodies, respectively.

2.5 GA and PAC treatments

Seeds were sown on solid Murashige and Skoog (MS) medium and incubated at 4°C for 3 d. Before treatments, seedlings were grown at 20°C under continuous fluorescent white light for 7 d. GA_3 and PAC dissolved in ethanol and diluted using MS was added to different concentrations, or an equivalent amount of ethanol diluted in MS was added as a mock treatment. Seedlings were then incubated for 2 h in solution under light and collected for Western blot analysis with anti-Myc and anti Actin (Abmart) antibodies.

3.Results

3.1 The DELLA protein GAI interacts with CO in yeast cells and pulls down CO in vitro

CO and DELLA proteins are pivotal components in light and GA signaling pathways, which act to promote and repress flowering time under LDs in the leaf vasculature, respectively [2,8,19]. To explore whether DELLA proteins might directly regulate CO activity, we firstly examined the possible physical interaction of a DELLA protein, GAI, with CO through yeast two-hybrid system. To do this, we prepared a bait construct expressing theGAL4 DNA binding domain (BD) fused to full-length GAI protein, and a prey construct expressing the GAL4 activation domain (AD) fused to full-length CO protein (Figure 1A). The yeast cells coexpressing either AD and BD-GAI fusion protein or AD-CO fusion protein and BD or AD-CO and BD-GAI fusion proteins grew well on the nonselective medium (Figure 1 B, left panel). However, when grown on the selective medium, the yeast cells coexpressing either AD and BD-GAI or AD-CO and BD hardly grew, whereas those coexpressing AD-CO and BD-GAI were able to grow (Figure 1B, right panel). These results indicate that GAI directly interacts with CO in yeast cells. It has been shown that the CCT (CONSTANS, CONSTANS-LIKE, TOC1) domain of CO is required for its interaction with COP1 or NF-Y complex to regulate flowering [2,8,21]. To determine whether the CCT domain of CO might also be required for its interaction with GAI, we prepared two constructs expressing CO fragments with (COΔ1-305) or without (COΔ306-374) this domain (Figure 1A), and performed yeast two-hybrid assay. We found that COΔ1-305 interacted with GAI, whereas COΔ306-374 did not (Figure 1B and 1C), indicating that the CCT domain may mediate the interaction of CO with GAI.

Next, we confirmed GAI-CO interaction by performing in vitro pull-down assay with GST-tagged CO and His-tagged GAI fusion protein expressed and purified form *E.coli*. The results demonstrate that GAI is strongly pulled down by CO. We also expressed and purified another DELLA protein, RGA, from *E.coli*, and performed the pull-down assay together with GAI. We found that CO also pulls down RGA (Figure 1D). Taken together, these results suggest that DELLA proteins may interact with CO directly.

3.2 DELLA proteins interact with CO in plant cells

With the demonstration that DELLA proteins interact with CO in yeast cells and in vitro, we asked whether these proteins might also physically interact in plant cells. Because CO is a nuclear protein and is found in nuclear bodies (NBs) [2,21], we postulated that if DELLA proteins indeed interact with CO in vivo, they would be localized together with CO. To test this postulation, we firstly prepared constructs expressing three DELLA proteins, RGA, GAI and RGL1, tagged with cyan fluorescent protein (CFP) (RGA-CFP, CFP-GAI, and RGL1-CFP), and a construct expressing CO tagged with yellow fluorescent protein (YFP) (CO-YFP), respectively. The recombinant proteins encoded by these constructs were transiently expressed individually or together in tobacco leaves. When co-expressed with YFP, RGA-CFP, CFP-GAI and RGL1-CFP were evenly diffused in the nucleus, respectively (Figure 2A, rows 2-4). However, when coexpressed with CO-YFP, all these DELLA proteins were colocalized to the same NBs of CO-YFP (Figure 2A, rows 6-8), indicating possible direct interactions of DELLA proteins with CO in plant cells. Next, we performed co-immunoprecipitation (co-IP) assay with tobacco leaves coexpressing Flag-tagged RGA or GAI and Myc-tagged CO proteins, and found that Myc-CO was pulled down by IP of RGA-Flag and GAI-Flag, respectively (Figure 2B). Since Myc-CO was not pulled down by IP of the Flag-tagged control protein, firefly luciferase C terminus (cLUC) (Figure 2B), these results indicate specific interactions of DELLA proteins with CO in planta.

To determine whether DELLA domain is required for the interactions of DELLA proteins with CO, we performed protein cellular colocalization assay by transiently coexpressing CO-YFP and RGAd17 tagged with CFP (RGAd17-CFP) in tobacco leaves, and found that they are localized to the same NBs (Figure 2A, row 9). To further confirm RGAd17-CO interaction, we performed co-IP assay with samples transiently coexpressing RGAd17-Flag and Myc-CO fusion proteins in tobacco leaves. As shown in Figure 2C, Myc-CO is pulled down by IP of RGAd17-Flag, but not by IP of cLUC-Flag, indicating that RGAd17 interacts with CO in vivo. Taken together, these results demonstrate that DELLA proteins interact with CO in plant cells.

3.3 The abundance of CO protein is not regulated by GA signaling

It is shown that DELLA proteins and CO act to repress and activate *FT* transcription, respectively [6,17,18]. To explore whether GA signaling might regulate CO protein abundance, we examined CO protein levels in transgenic seedlings overexpressing Myc-CO treated with different concentrations of GA or its biosynthesis inhibitor, paclobutrazol (PAC), by Western blotting. The results demonstrate that neither GA nor PAC treatments affect CO abundance (Figure 3A). Since it is well established that GA treatment triggers rapid degradation of DELLA proteins, while PAC treatment enhances the accumulation of DELLA proteins, this result indicates that the fluctuation of DELLA proteins exerts no effects on CO protein levels. In other words, GA signaling does not likely regulate CO protein abundance.

3.4 The interaction of CO with NF–YB2 is repressed by RGA in vitro

It has been shown recently that CO interacts with NF-YB2 and bind to the promoters of *FT* and *SOC1* to activate their expression, respectively. Moreover, NF-YB2 interacts with both CO and DELLA [22,23]. Genetic studies suggest that NF-YB subunits play a critical role in mediating CO promotion of flowering [22]. Since it is now demonstrated that DELLA proteins interact with CO, we asked whether they might interrupt the interaction of CO with the NF-Y complex. To test this possibility, we expressed and purified a NF-Y complex subunit, NF-YB2, tagged by histidine (His-NF-YB2), from *E. coli* and performed pull-down assays with His-NF-YB2, His-RGA, and GST-CO fusion proteins. As shown in Figure 3B, NF-YB2 is pulled down by GST-CO, which is consistent with the previous study [23]. Interestingly, the interaction of GST-CO with His-NF-YB2 was progressively reduced as the amount of His-RGA increased, whereas in the absence of His-RGA but in the presence of a control protein, His-TF, GST-CO strongly pulled down His-NF-YB2, indicating that RGA represses the interaction of CO with NF-YB2 in a dose-dependent manner.

4. Discussion

It has long been known that the GA biosynthesis deficient mutants display a severely late flowering phenotype under SDs [30,31], indicating that GA plays a critical role in the regulation of flowering time under SDs. Previous studies have shown that GA promotes flowering

through activating positively-acting genes including *SOC1* and *LEAFY* (*LFY*) in SAM under SDs [32,33]. The following demonstrations suggest that GA is also involved in the regulation of flowering time under LDs. (1) GA biosynthesis deficient mutants *ga1*, the GA receptor mutant *gid1a-c* and the transgenic plants expressing RGAd17 display a late flowering phenotype under LDs [19,30,34]. (2) Transgenic plants overexpressing GA2ox7, an enzyme catabolizing the active GAs, show a late flowering phenotype under LDs, while the loss-of-function mutant of the four DELLA proteins, *gai-t6 rga-t2 rgl1-1 rgl2-1*, show an early flowering phenotype under LDs [18,23]. (3) DELLA proteins act to inhibit the transcription of *FT* and *TSF* in leaves under LDs [17,18]. However, whether DELLA proteins directly regulate CO through physical interaction is not known. In the present study, we have revealed the physical interaction of DELLA proteins with CO through combined approaches of yeast two-hybrid, pull-down, protein cellular colocalization, and co-IP assays. In an attempt to evaluate the significance of the interactions of DELLA proteins with CO, we analyzed CO protein levels upon exogenous GA or PAC application, and found that the CO protein abundance is not affected by GA or PAC. Given that GA and PAC treatments promote and inhibit the degradation of DELLA proteins, respectively, our results indicate that the interactions of DELLA proteins with CO do not affect CO protein stability. In conjunction with the previous demonstration that GA signaling is not involved in the regulation of *CO* transcription [18], our findings suggest that the regulation of *FT* transcription by GA signaling in leaves is not mediated through its regulation of CO protein abundance.

What might be the consequence of the interactions of DELLA proteins with CO? In an attempt to address this question, we examined the effects of RGA on the interaction of CO with NF-YB2 through in vitro pull-down assay, and found that RGA represses the interaction of CO with NF-YB2 in a dose-dependent manner. This result suggests that abundant DELLA proteins may be needed to efficiently inhibit the interaction of CO with NF-YB2. It is shown that the *pSUC2::DELLAd17* (*GAId17*, *RGAd17*, *RGL1d17*, *RGL2d17*, *RGL3d17*) lines flower late under LDs, but earlier than the *co* mutant [2,8,17]. We speculate that the less pronounced late flowering phenotype of *SUC2::DELLAd17* lines compared to *co* mutant may result from the following. (1) The levels of DELLAd17 proteins expressed in these lines may not be high enough to inhibit the activity of all the CO molecules. (2) Even when the levels of

DELLAd17 proteins are high enough, they may not be able to completely inhibit the interaction of CO with NF-YB2 in vivo, since other components other than DELLA proteins may be involved in this process. In view of our and others' previous demonstrations that the interaction of COP1 with its E3 ubiquitin ligase enhancer, SPA1, is inhibited by both CRY1-COP1 and CRY1-SPA1 interactions [9,10], we speculate that the interactions of RGA with both CO and NF-YB2 may be involved in RGA suppression of the interaction of CO with NF-YB2. Given that the CCT domain of CO mediates its interactions with NF-YB2 [21] and GAI (this study), it is reasonable to speculate that at least the interactions of DELLA proteins with CO may prohibit the association of NF-YB2 with CO. Based on previous studies and the present work, we propose a model to present how light and GA signaling is integrated to regulate flowering time under LDs (Figure 4). During the dark phase of LDs, CRY is inactive, and COP1 is fully functional and interacts with CO to promote its ubiquitination and degradation [2,8,35,36]; During the light phase of LDs, photo-activated CRY inhibits the repression effects of COP1 on CO through CRY-COP interaction, leading to the accumulation of CO protein. When GA level is low, DELLA proteins accumulate at high levels and are able to efficiently inhibit CO activity, leading to late flowering; whereas when GA level is high, DELLA proteins predominantly undergo degradation and CO is capable of fulfilling its transcriptional activation activity on *FT*, thus accelerating flowering.

It has been proposed that GA promotion of flowering in leaves under LDs is not dependent on CO, since *CO* transcription is not regulated by GA [19]. Moreover, genetic studies indicate that the *della co* quintuple mutant flowers slightly later than the *della* quadruple mutant under LDs, but significantly earlier than the *co* single mutant [23], which appears to be in conflict with the notion that CO acts downstream of DELLA proteins (Figure 4). However, these seemingly discrepancies or complication can be reconciled by our findings. On one hand, although GA signaling does not regulate either *CO* transcription or CO protein accumulation, it can regulate CO activity through the interaction of DELLA proteins with CO; On the other hand, it is important to note that DELLA proteins appear to play a more important role in SAM than in leaves in the regulation of flowering under LDs. If DELLA proteins did not regulate flowering in SAM under LDs, the *della co* quintuple mutant would flower much later than the *della* quadruple mutant, likely similar to the *co* mutant.

Therefore, the results obtained from our biochemical and previous genetic studies suggest that regulation of flowering in leaves under LDs by GA signaling proceeds, at least in part, through direct interactions of DELLA proteins with CO (Figure 4), which is reminiscent of the established molecular link between DELLA proteins and PIF3/4 that is used to integrate GA and light signaling to regulate photomorphogenic development [26,27].

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Figure legends:

Fig. 1. GAI physically interacts with CO in yeast cells and binds to CO in vitro.

(A) Yeast two-hybrid constructs comprising fragments of GAI and CO fused to the GAL4 DNA binding domain (BD) or GAL4 activation domain (AD), respectively.

(B-C) Yeast two-hybrid assays showing that the interaction of CO with GAI is mediated through its CCT domain. Transformed yeast cells were grown on nonselective (SD-T-L) or selective media (SD-T-L-H) supplemented with 25 mM 3-AT, which acts to repress the autonomous transcriptional activity of BD-GAI.

(D) GST pull-down assay showing the interactions of CO with RGA and GAI. The recombinant GST-CO pulls down His-RGA and His GAI, but not the His-TF control protein. CBB denotes Coomassie Brilliant Blue staining.

All these experiments were independently repeated for three times.

Fig. 2. DELLA proteins interact with CO in plant cells.

(A) Colocalization study in tobacco cells showing DELLA proteins, RGA, GAI and RGL1, colocalize to the same nuclear bodies (NBs) of CO in tobacco cells. Scale bar, 3μm. These analyses were performed in three biological replicates.

(B-C) Co-IP assay showing interactions of RGA and GAI with CO. Flag-tagged RGA, GAI and RGAd17 proteins or the control cLUC were co-expressed with Myc-tagged CO in tobacco leaves, respectively. The immunoprecipitates were detected by anti-Flag and anti-Myc antibodies. These analyses were performed in two biological replicates.

Fig. 3. RGA represses the interaction of CO with NF-YB2 in vitro.

(A) CO abundance is not affected by GA or PAC treatments. The *35S::Myc-CO* and wild type (Col) seedlings were grown under continuous write light condition for 7 d. Total proteins extracted from seedlings treated with different concentrations of GA_3, PAC or ethanol mock for 2 h were subjected to immunoblot analysis with anti-Myc antibody. This analysis was performed in two biological replicates. (B) GST pull-down assay showing repression of the association of CO with NF-YB2 by RGA. His-TF (His-tagged Trigger Factor) served as negative control. This analysis was performed in three biological replicates.

Fig. 4. A model describing crosstalk between light and GA signaling to regulate flowering. Under LDs, light activated photoreceptors, CRY1 and CRY2, stabilize CO by inhibiting the activity of COP1/SPA complex through direct CRY-COP1 and CRY-SPA interactions. The transcriptional activation activity of CO on *FT* is repressed by the interactions of DELLA proteins with CO, and likely also with NF-Y. Arrows and T bars denote positive and negative regulation, respectively.