

DELLA-GAF1 Complex Is a Main Component in Gibberellin Feedback Regulation of GA20 Oxidase ²¹

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Gibberellins (GAs) are phytohormones that regulate many aspects of plant growth and development, including germination, elongation, flowering, and floral development. Negative feedback regulation contributes to homeostasis of the GA level. DELLAs are negative regulators of GA signaling and are rapidly degraded in the presence of GAs. DELLAs regulate many target genes, including *AtGA20ox2* in *Arabidopsis* (*Arabidopsis thaliana*), encoding the GA-biosynthetic enzyme GA 20-oxidase. As DELLAs do not have an apparent DNA-binding motif, transcription factors that act in association with DELLA are necessary for regulating the target genes. Previous studies have identified GAI-ASSOCIATED FACTOR1 (GAF1) as such a DELLA interactor, with which DELLAs act as coactivators, and *AtGA20ox2* was identified as a target gene of the DELLA-GAF1 complex. In this study, electrophoretic mobility shift and chromatin immunoprecipitation assays showed that four GAF1-binding sites exist in the *AtGA20ox2* promoter. Using transgenic plants, we further evaluated the contribution of the DELLA-GAF1 complex to GA feedback regulation. Mutations in four GAF1-binding sites abolished the negative feedback of *AtGA20ox2* in transgenic plants. Our results showed that GAF1-binding sites are necessary for GA feedback regulation of *AtGA20ox2*, suggesting that the DELLA-GAF1 complex is a main component of the GA feedback regulation of *AtGA20ox2*.

GAs are tetracyclic diterpenoid phytohormones that regulate many aspects of plant growth and development, including seed germination, leaf expansion, stem elongation, root growth, flower induction, and anther development (Richards et al., 2001). GA biosynthetic enzymes have been characterized using genetic, molecular, and biochemical approaches (Yamaguchi, 2008). In *Arabidopsis* (*Arabidopsis thaliana*), six biosynthetic enzymes have been identified. Endogenous levels of GAs are fine-tuned by feedback control of GA biosynthetic enzymes at several steps (Yamaguchi, 2008; Fukazawa et al., 2011; Hedden and Thomas, 2012). The final steps in GA biosynthesis are catalyzed by GA 20-oxidase and GA 3-oxidase, the expression of which is controlled by endogenous GA levels. *AtGA20ox1*, *AtGA20ox2*, *AtGA20ox3*, and *AtGA3ox1* are highly up-regulated in GA-deficient mutants, whereas they are down-regulated after the application of GAs

(Chiang et al., 1995; Phillips et al., 1995; Rieu et al., 2008). GA-deactivation genes also respond to endogenous GA levels. For example, *GA2ox1* and *GA2ox2* are up-regulated by GA treatment (Thomas et al., 1999). GA feedback regulation has been shown to depend on GA signaling components, including the GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), DELLA proteins, an F-box adaptor subunit of SCF E3 ubiquitin ligase, and SPINDLY (Sasaki et al., 2003; Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006; Sun, 2011; Hauvermale et al., 2012). *Arabidopsis* contains five DELLAs, GIBBERELLIN INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, which act as negative regulators of GA signaling (Sun and Gubler, 2004). Binding of GA to GID1 enhances the interaction between GID1 and DELLAs, resulting in the rapid degradation of DELLAs via the ubiquitin-proteasome pathway (Sun, 2011). Under GA-deficient conditions, DELLA proteins accumulate in the nucleus and regulate the expression of target genes. Zentella et al. (2007) have identified 14 early GA-responsive genes, including *AtGA20ox2* and *AtGA3ox1*, as targets of DELLAs, which activate the expression of these genes.

Because DELLAs do not have an apparent DNA-binding motif, it was hypothesized that DNA-binding proteins are involved in the direct regulation of these genes along with DELLAs. Indeed, several transcription factors have been identified as DELLA-interacting proteins. For example, DELLAs regulate hypocotyl elongation by interacting with PHYTOCHROME INTERACTING FACTOR (PIF) and BRASSINAZOLE RESISTANT1

¹ This study was supported in part by grants from the Japan Society for the Promotion of Science (JSPS) to J.F. (26440148) and Y.T. (15H04392) and by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan to J.F. (24116525) and Y.T. (24118004).

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J.F. and Y.T. designed the research; J.F., M.M., S.W., C.M., and T.I. performed experiments; J.F. and Y.T. wrote the article.

www.plantphysiol.org/cgi/doi/10.1104/pp.17.00282

(BZR1), which promote hypocotyl elongation (de Lucas et al., 2008; Feng et al., 2008; Bai et al., 2012). DELLAs inhibit the activities of these transcriptional activators by titrating their DNA-binding activity. GAs trigger the degradation of DELLAs, which release PIFs to activate target genes, including β -EXPANSIN and PACLOBUTRAZOL RESISTANCE, thus promoting hypocotyl elongation. The mechanism of GA-dependent transactivation has been explained by the degradation of DELLAs that titrated transcriptional activators.

However, multiple GA-regulated genes, including *AtGA20ox2* and *AtGA3ox1*, are repressed by GAs. The mechanism of GA feedback regulation is difficult to explain based on the titration of PIF and BZR1 by DELLAs. Moreover, a chromatin immunoprecipitation (ChIP) assay indicated that DELLAs associate with the promoters of several genes (Zentella et al., 2007), while in the titration model, the DELLA complex could not bind to the promoter of target genes. Thus, DELLAs may have another function in the regulation of these genes. DELLAs activate gene expression through transcription factors, including type B ARABIDOPSIS RESPONSE REGULATORS1 (Marín-de la Rosa et al., 2015), SQUAMOSA PROMOTER BINDING PROTEIN-LIKE9 (Yamaguchi et al., 2014), ABSCISIC ACID INSENSITIVE3 (ABI3), and ABI5 (Lim et al., 2013). These findings indicated novel functions for

DELLAs. Recently, we identified a DELLA-binding transcription factor, designated GAI-ASSOCIATED FACTOR1 (GAF1), and revealed a new role of DELLAs as transcriptional coactivators (Fukazawa et al., 2014). GAF1 is a transcription factor with zinc finger motifs that shows similarity to maize (*Zea mays*) INDETERMINATE1 (ID1; Colasanti et al., 1998). The Arabidopsis genome contains 16 ID1-related proteins (IDD, for ID1 domain protein). The maize ID1 and Arabidopsis IDD family proteins bind to the consensus sequence TTTTGTCG (Kozaki et al., 2004). GAF1 also interacts with the co-repressor TOPLESS-RELATED (TPR). DELLAs and TPR act as coactivators and a corepressor of GAF1, respectively. By removing DELLA, GAs convert the GAF1 complex from transcriptional activator to repressor. This model can explain the GA feedback regulation. In fact, we have identified GA feedback-regulated genes, including *GA20ox2*, *GA3ox1*, and *GID1b*, as GAF1 target genes. Gel-shift analysis and ChIP assay showed that GAF1 binds directly to the *GA20ox2* and *GID1b* promoters (Fukazawa et al., 2014). These data indicated that the DELLA-GAF1 complex is involved in GA feedback regulation of *AtGA20ox2*. Various other transcription factors involved in GA feedback regulation, such as YABBY1, AT-HOOK PROTEIN OF GA FEEDBACK1 (AGF1),

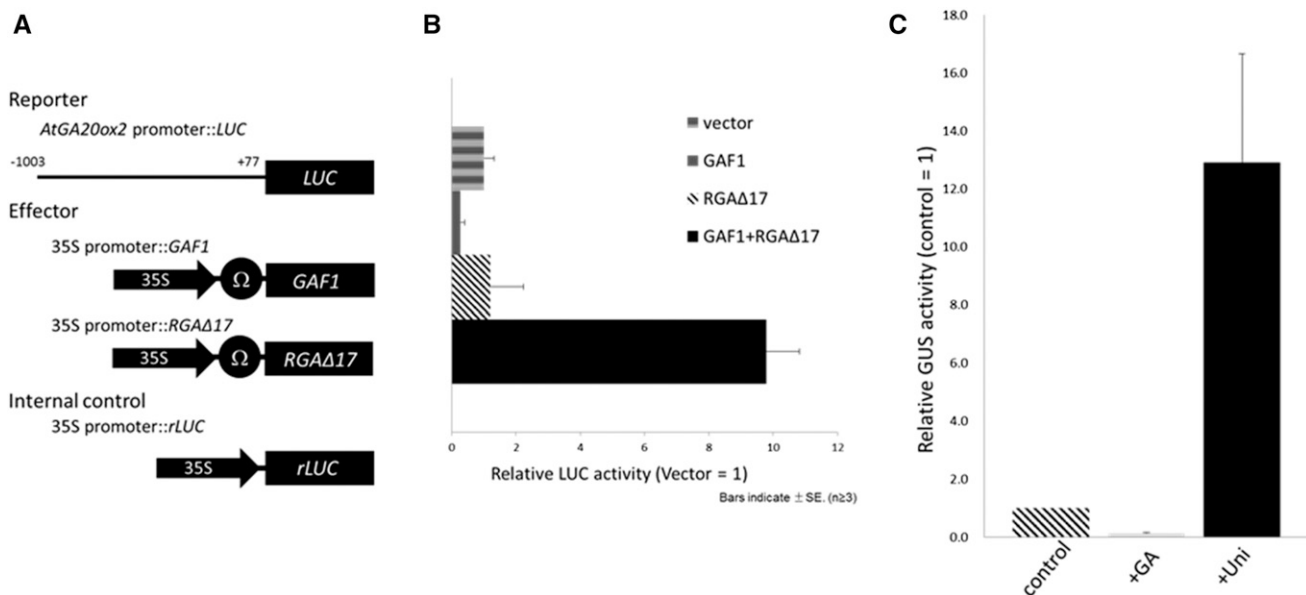


Figure 1. A 1,003-bp region of the *AtGA20ox2* promoter is regulated by the DELLA-GAF1 complex and responds to GA. **A**, Schematic representation of the reporter and effector. A 1,003-bp fragment of the *AtGA20ox2* promoter was fused to the *LUC* gene. The effector plasmid expressed the full-length GAF1 or RGA Δ 17 under the control of the CaMV 35S promoter with a viral translation enhancer (Ω). **B**, Transactivation assay of GAF1 and RGA Δ 17. The effector, reporter, and internal control constructs were cotransformed into Arabidopsis leaves. The transfected leaves were incubated for 20 h, and then, LUC and rLUC activities were measured. The results are shown as LUC/rLUC activity. Error bars indicate \pm SE ($n = 3$). **C**, Fluorometric GUS assay for GA negative feedback in transgenic Arabidopsis plants carrying the 1,003-bp *AtGA20ox2* promoter::GUS construct. Gray and black bars represent the GUS activities of plants treated for 1 week with GA₃ and uniconazole (Uni), respectively. The striped bar represents the GUS activity of untreated control plants, which was arbitrarily set to 1. The mean activities of five independent transgenic lines for each construct are shown. Error bars indicate \pm SE.

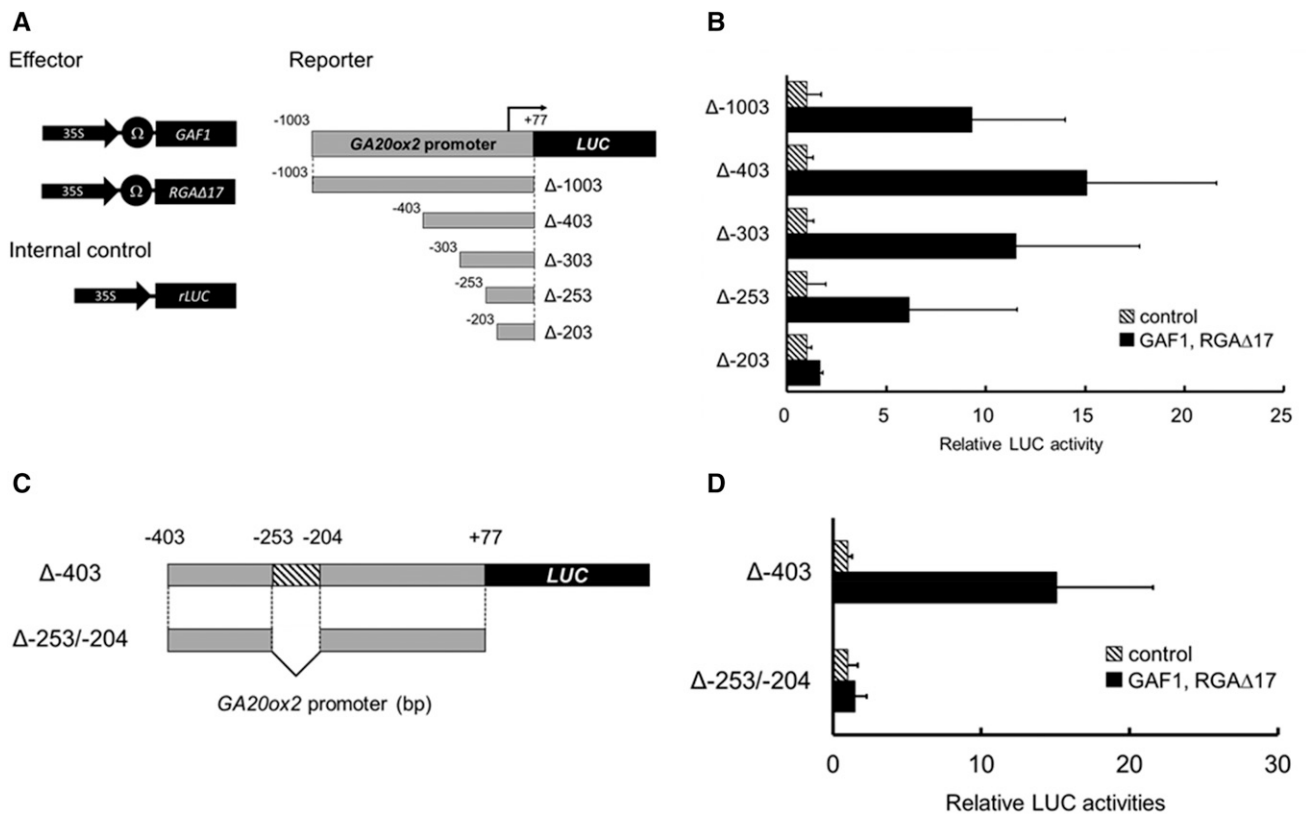


Figure 2. Identification of the DELLA-GAF1-regulated region of the *AtGA20ox2* promoter using a transactivation assay of *GAF1* and *RGAA17*. A and C, Reporter, effector, and internal control constructs used in the assay are shown in A. The constructs were cotransformed into Arabidopsis leaves. B and D, The transfected leaves were incubated for 20 h, and then, LUC and rLUC activities were measured. The results are shown as LUC/rLUC activity. Error bars indicate SD ($n = 3$).

REPRESSION OF SHOOT GROWTH (RSG), and *YABBY4*, have been reported (Dai et al., 2007; Matsushita et al., 2007; Fukazawa et al., 2010; Yang et al., 2016).

In this study, we aimed to investigate how important the contribution of the DELLA-GAF1 complex to GA feedback regulation of *AtGA20ox2* is compared with that of other factors. We identified multiple *GAF1*-binding sites in the promoter of *AtGA20ox2* and evaluated the effect of mutation of each of these *GAF1*-binding sites on GA feedback regulation.

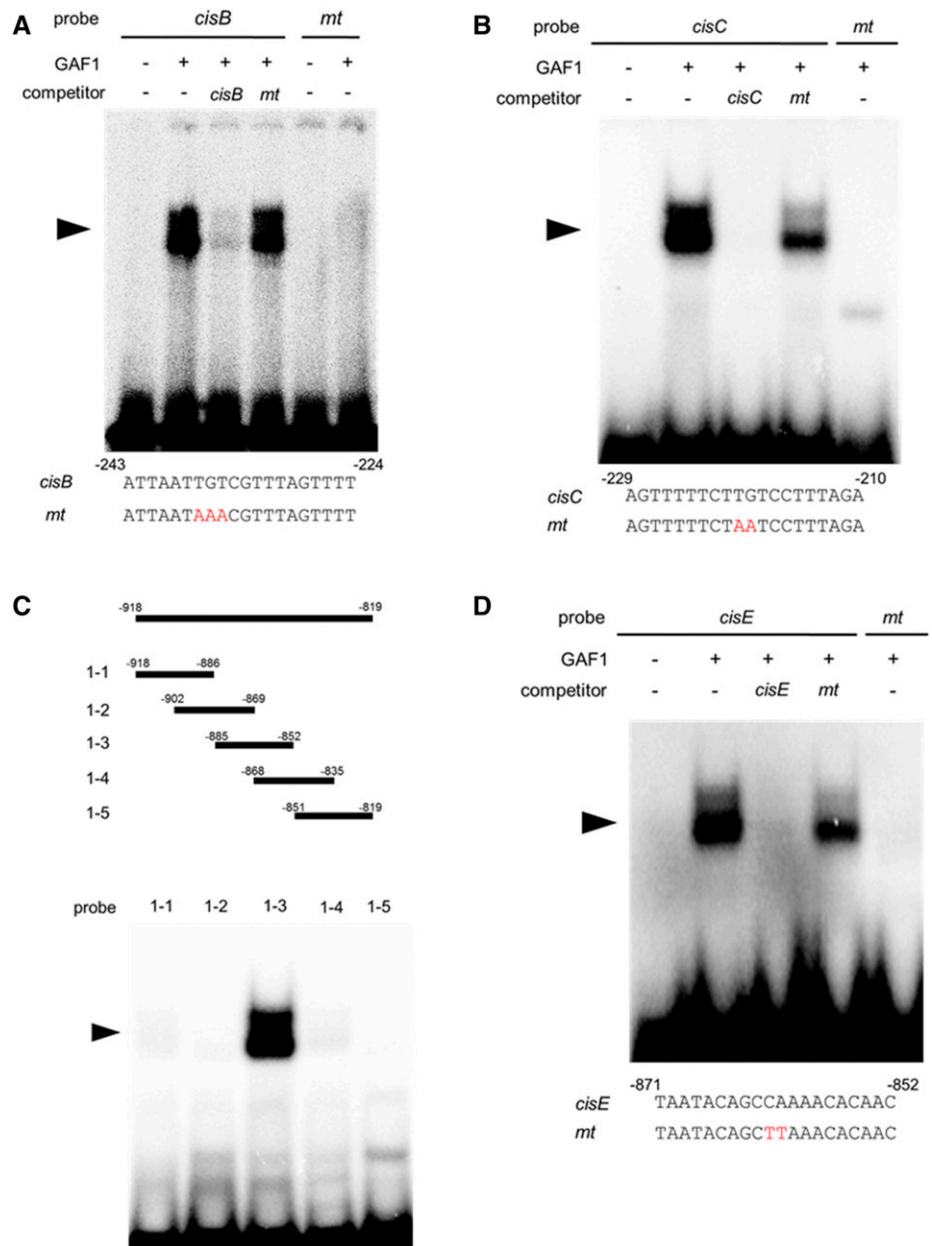
RESULTS

The *AtGA20ox2* Promoter Is Activated by the DELLA-GAF1 Complex and Is Responsible for GA Feedback Regulation

The Arabidopsis genome carries five *GA20ox* genes, three of which (*AtGA20ox1*, *AtGA20ox2*, and *AtGA20ox3*) function partially redundantly at most developmental stages, including floral transition, while *AtGA20ox4* and *AtGA20ox5* have very minor roles (Rieu et al., 2008; Plackett et al., 2012). *AtGA20ox1*, *AtGA20ox2*, and *AtGA20ox3* are under negative feedback regulation by GA, especially, the expression of *AtGA20ox2* is most responsive to endogenous GA levels, while

AtGA20ox4 and *AtGA20ox5* expression is not affected by GA levels. Previously, we found that DELLA-GAF1 is involved in the feedback regulation of *AtGA20ox2* (Fukazawa et al., 2014). To determine whether DELLA-GAF1 directly regulates the transcription of *AtGA20ox2*, we carried out a transactivation assay using Arabidopsis leaves. Effector plasmids expressing *GAF1* and/or DELLA under the control of the cauliflower mosaic virus (CaMV) 35S promoter were cotransformed with a reporter plasmid containing a *LUC* reporter gene driven by the *AtGA20ox2* promoter (−1,003 to +77, where +1 indicates the transcription start site; Fig. 1A). Base-position numbering of the *AtGA20ox2* promoter was changed from ATG as +1 in our previous study (Fukazawa et al., 2014) to the transcription start site as +1 in this study. DELLA-GAF1 activated the *AtGA20ox2* promoter-LUC fusion (Fig. 1B), indicating that the *AtGA20ox2* promoter is indeed regulated by the DELLA-GAF1 complex. In this transactivation assay, we used *rga-Δ17* as the constitutively active DELLA protein. *rga-Δ17* is a mutant protein with a 17-amino acid deletion within the DELLA domain of RGA, rendering it resistant to degradation by GAs (Dill et al., 2001). To investigate whether this promoter region of *AtGA20ox2* is sufficient for GA feedback regulation, we generated transgenic plants carrying the promoter fused with a *GUS* reporter

Figure 3. Identification of GAF1-binding regions in the *AtGA20ox2* promoter in vitro. A and B, Gel retardation assays using recombinant GAF1 protein. A, Oligonucleotides containing *cisB* (–243 to –224, wild type; lanes 1–4) or *mtcisB* (*mt*; lanes 5 and 6) were used as probes. B, Oligonucleotides containing *cisC* (–229 to –210, wild type; lanes 1–4) or *mtcisC* (*mt*; lane 5) were used as probes. Red letters indicate mutated bases. Wild type and *mt* indicate competition with a 200-fold excess of unlabeled wild-type and mutated probe, respectively. The specific GAF1-DNA complexes are indicated by arrowheads. +, Addition to the reaction mixtures; –, omission from the reaction mixtures. C, Gel retardation assay using recombinant GAF1 protein. Thirty-base-pair regions of the *AtGA20ox2* promoter were used as probes. D, Oligonucleotides containing *cisE* (–871 to –852, wild type; lanes 1–4) or *mtcisE* (*mt*; lane 5) were used as probes. Red letters indicate mutated bases. Wild type and *mt* indicate competition with a 200-fold excess of unlabeled wild-type and mutated probe, respectively. The specific GAF1-DNA complexes are indicated by arrowheads. +, Addition to the reaction mixtures; –, omission from the reaction mixtures.



gene and examined the effect of applying GA₃ or uniconazole, an inhibitor of GA biosynthesis. Uniconazole-treated transgenic plants exhibited higher GUS activity than did untreated transgenic plants (Fig. 1C). In contrast, GA₃ repressed GUS activity. These results indicated that the *AtGA20ox2* promoter is regulated by the DELLA-GAF1 complex in a transient assay and GA levels in plants.

Identification of the cis-Acting Elements of the *AtGA20ox2* Promoter for the DELLA-GAF1 Complex

To define the cis-acting elements in the *AtGA20ox2* promoter for the DELLA-GAF1 complex, we generated a series of 5' deletions of the *AtGA20ox2* promoter fused with a *LUC* reporter gene (Δ–403, Δ–303, Δ–253, or Δ–203)

and we examined whether DELLA-GAF1 activated these promoters in a transactivation assay using Arabidopsis leaves. The effector plasmids expressing GAF1 and DELLA under the control of the CaMV 35S promoter were cobombarded with a reporter plasmid (Fig. 2A). The DELLA-GAF1 complex activated Δ–1003, Δ–403, Δ–303, and Δ–253 but not Δ–203 (Fig. 2B), and an internal deletion (Δ–253/–204) in the promoter Δ–403 disrupted the activation by DELLA-GAF1 (Fig. 2, C and D). These data suggested that at least a cis-acting element for the DELLA-GAF1 complex is located between –253 and –204. Previously, we identified two GAF1-binding sites, –968 to –949 (*cisA*) and +6 to +26 (*cisD*), in the *AtGA20ox2* promoter by ChIP assay (Fukazawa et al., 2014). Although Δ–203 and internal deletion (Δ–253/–204) promoters include *cisD*,

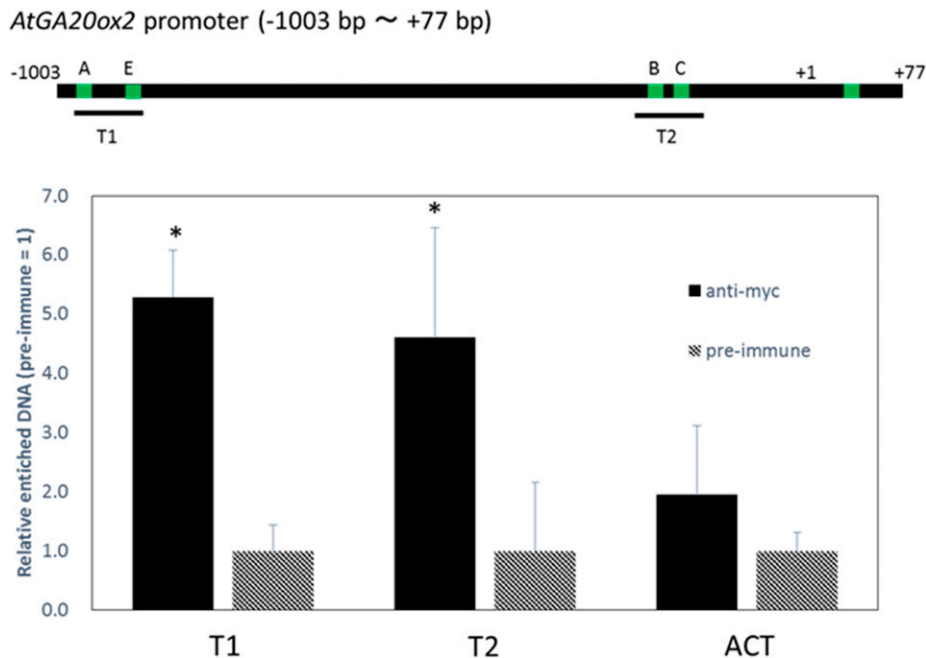


Figure 4. GAF1 binds to multiple regions of the *AtGA20ox2* promoter in vivo. ChIP assays were performed with anti-GST or anti-GFP in *RGAPro:RGA-GFP* transgenic plants. Green bars indicate GAF1-binding sites of the *AtGA20ox2* promoter. The coprecipitated level of each DNA fragment was quantified by real-time PCR and normalized to the input DNA. The relative coprecipitated level of each DNA fragment using anti-GST compared with a DNA fragment using anti-GFP are shown in the graph. ACT, ACTIN. Error bars indicate the SD of three biological replicates ($n = 3$). *, $P < 0.05$ versus preimmune DNA fragment enrichment by Student's *t* test.

DELLA-GAF1 could not activate these promoters. Thus, *cisD* seemed not to play an important role in the transcriptional activation of *AtGA20ox2* by DELLA-GAF1. Therefore, among the four GAF1-binding sites found in the *AtGA20ox2* promoter, the *cisD* region was excluded from further analysis. Taken together, these results suggest that one or more functional cis-elements for the DELLA-GAF1 complex exist in the *AtGA20ox2* promoter between -253 and -204.

Identification of GAF1-Binding Sites in the *AtGA20ox2* Promoter

GAF1 belongs to the IDD transcription factor family and binds to *ID1-cis*, a consensus motif TTTTGTCC (Fukazawa et al., 2014). Two sequences similar to *ID1-cis*, AATTGTCC and TCTTGTCC, were found in the region -253 to -204 of the *AtGA20ox2* promoter (+1, transcription start site). To investigate whether GAF1 binds to these sequences, a gel-shift assay was performed. Recombinant GAF1 protein specifically bound to AATTGTCC and TCTTGTCC sequences, named *cisB* and *cisC*, respectively (Fig. 3, A and B). The newly identified GAF1-binding sequences had substitutions at 2 bp of the 8-bp *ID1-cis* sequence, suggesting that a yet unidentified GAF1-binding sequence might exist in the *AtGA20ox2* promoter. Therefore, we searched for novel GAF1-binding sites in the region -1,003 to -403 using a comprehensive gel-shift assay. In this analysis, we examined which 100-bp DNA fragments between -1,003 and -403 inhibit the binding of GAF1 to *ID1-cis*. Complex formation of the *ID1-cis* probe with GAF1 was inhibited by the DNA fragment from -918 to -819 (Supplemental Fig. S1), indicating the presence of an unidentified GAF1-binding site in this region of the *AtGA20ox2* promoter. Further gel-shift assays revealed a novel GAF1-binding

site, located from -885 to -852 (Fig. 3C). And we identified a new GAF1-binding site, named *cisE* (Fig. 3D).

To investigate the binding of GAF1 to the *AtGA20ox2* promoter in vivo, we carried out ChIP analysis using transgenic plants expressing myc-tagged GAF1 under the control of the CaMV 35S promoter. The results showed that GAF1 binds to two regions, T1 (*cisA* and *cisE*) and T2 (*cisB* and *cisC*; Fig. 4). The ChIP assay did not allow distinguishing between *cisA* and *cisE* and between *cisB* and *cisC* because the binding sites are too close; however, these results indicated that GAF1 binds directly to at least two regions in the *AtGA20ox2* promoter in vivo.

Mutations in GAF1-Binding Sites of the *AtGA20ox2* Promoter Disrupt Transcriptional Activation by DELLA-GAF1

To determine whether the GAF1-binding sites identified in vitro are functional in vivo in the activation of the *AtGA20ox2* promoter by the DELLA-GAF1 complex, we constructed a series of mutant promoter-LUC fusions, in which mutations were introduced to eliminate the GAF1-binding sequences (Fig. 5, A and B). Internal deletion analysis showed that the region -253 to -204 of the *AtGA20ox2* promoter, including *cisB* and *cisC*, is important for promoter activation by DELLA-GAF1 (Fig. 2C). We identified *cisE* as a new GAF1-binding site in the *AtGA20ox2* promoter. We investigated whether the four GAF1-binding sites (i.e. *cisA*, *cisB*, *cisC*, and *cisE*) in the *AtGA20ox2* promoter are involved in transcriptional activation by DELLA-GAF1. Promoters with a single cis-element mutation (*mtE*) or two combinations of triple cis-element mutations (*mtABC* or *mtBCE*) of the four GAF1-binding sites were still activated by DELLA-GAF1, although the transactivation activity was lower than that of the wild-type promoter (Fig. 5C). Mutations in all four GAF1-binding sites (*mtABCE*)

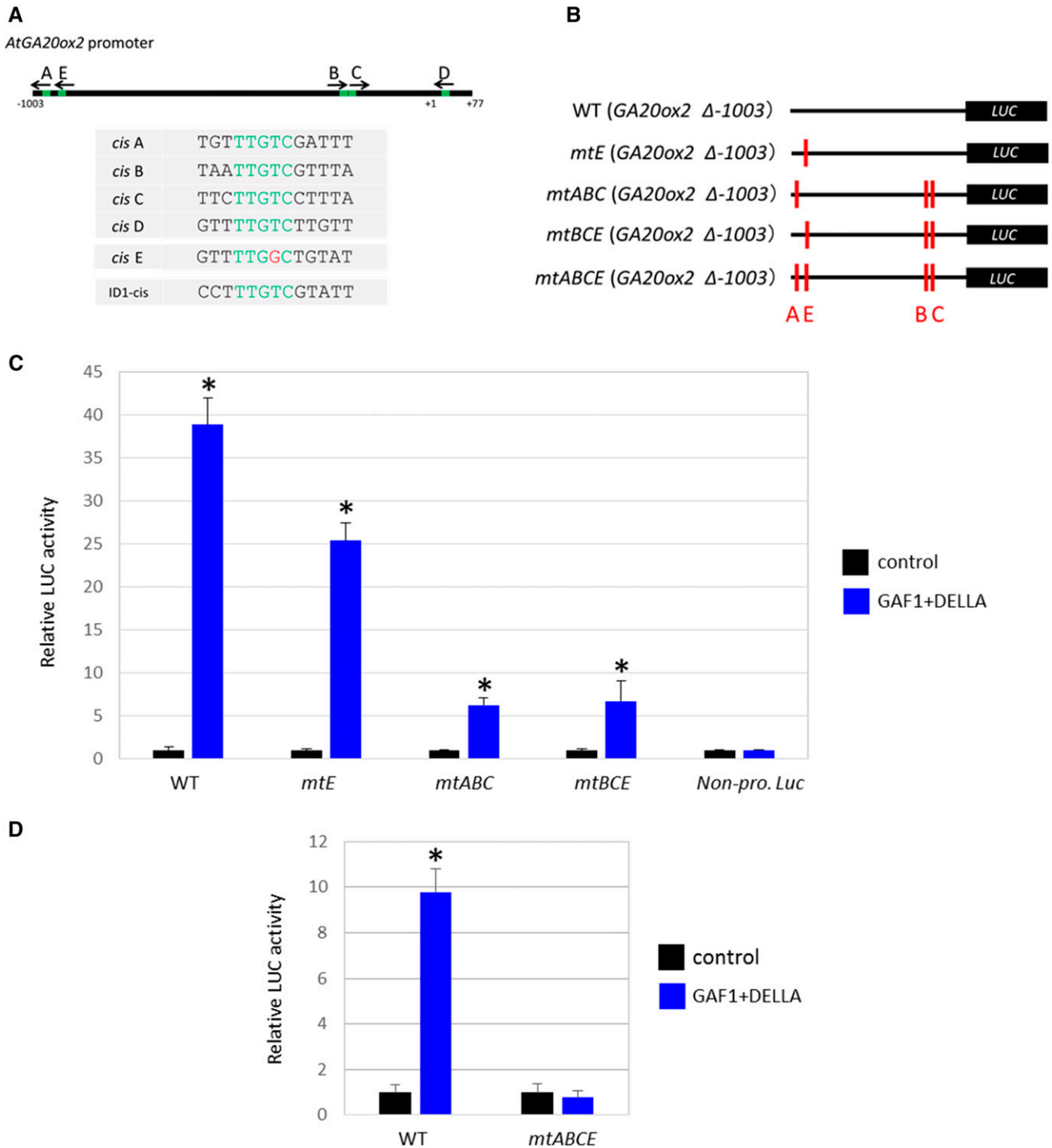


Figure 5. Mutation of four GAF1-binding sites completely abolishes DELLA-GAF1 activation of the *GA20ox2* promoter. *A*, Comparison between ID1-*cis* and GAF1-binding sites in the *AtGA20ox2* promoter. Green letters show conserved sequence among these GAF1-binding sites. Arrows indicate the direction of each GAF1-binding site. *B*, Transactivation assays of GAF1 and RGA. Reporter constructs of each *AtGA20ox2* promoter mutant fused with LUC used in the assay are shown. Red bars indicate mutations in GAF1-binding sites of the *AtGA20ox2* promoter. *C* and *D*, The constructs were cobombarded into Arabidopsis leaves. The transfected leaves were incubated for 20 h, after which the LUC and rLUC activities were measured. The results are shown as LUC/rLUC activity. WT, Wild type. Error bars indicate SD ($n = 6$). *, $P < 0.05$ versus control by Student's *t* test.

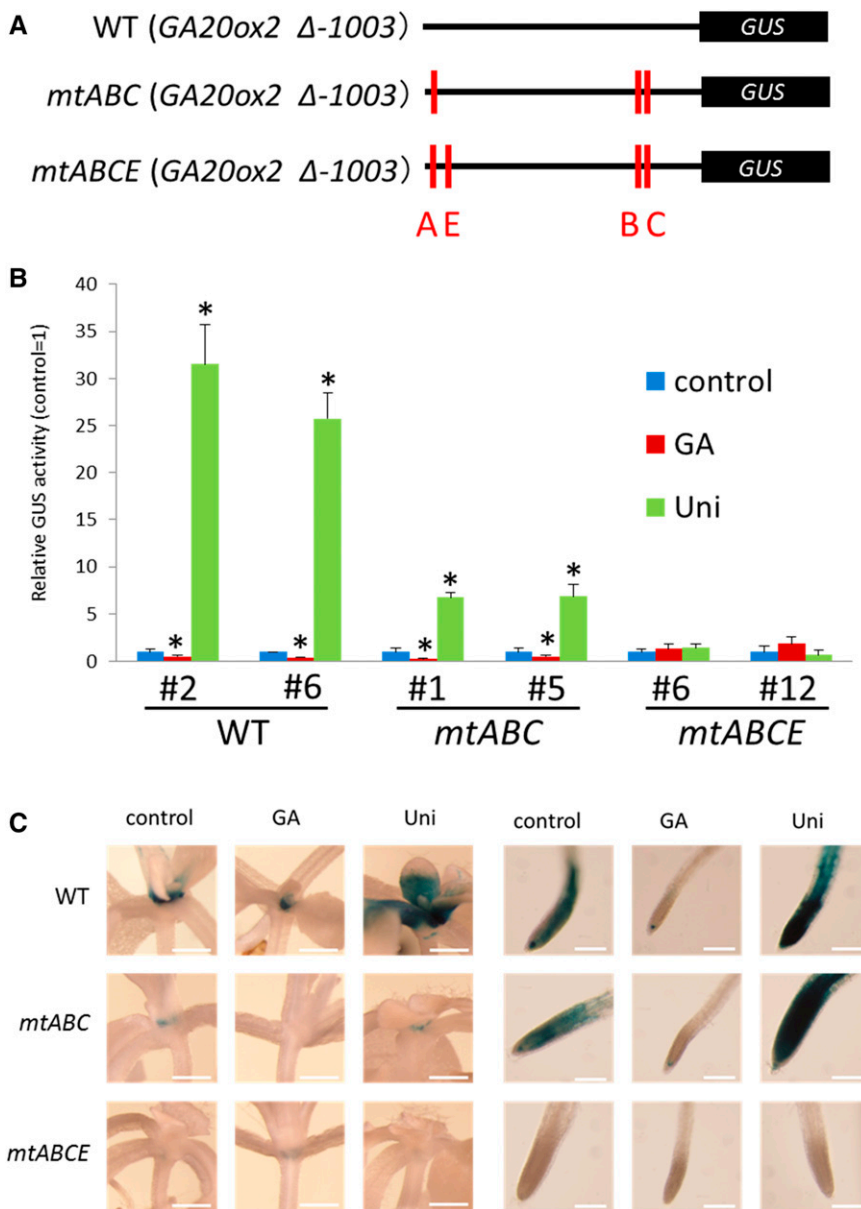


Figure 6. Mutation of four GAF1-binding sites completely abolishes the GA responsibility of the *AtGA20ox2* promoter. A, Reporter constructs of each *AtGA20ox2* promoter mutant fused with GUS used in the assay. Red bars indicate mutations in GAF1-binding sites of the *AtGA20ox2* promoter. B, Fluorometric GUS assay for GA negative feedback in transgenic Arabidopsis plants carrying 1,003 bp of *AtGA20ox2* promoter::GUS, *mtABCAtGA20ox2* promoter::GUS (including three mutations), or *mtABCEAtGA20ox2* promoter::GUS (including four mutations). The red and green bars represent the GUS activities of plants treated for 1 week with GA₃ and uniconazole (Uni), respectively. The blue bars represent the GUS activity of untreated control plants, which was arbitrarily set to 1 (control = 1). The mean activities of eight independent transgenic lines for each construct are shown. Error bars indicate s.d. *, *P* < 0.05 versus control by Student's *t* test. C, Comparison of GUS staining patterns in transgenic plants carrying the wild-type (WT), *mtAtGA20ox2* promoter::GUS (*mtABC*), and *mtAtGA20ox2* promoter::GUS (*mtABCE*) constructs with those of the mutant versions. Meristematic regions of shoot and root tip in seedlings were observed at 12 d. Bars = 0.05 mm (shoot) or 0.2 mm (root tip).

completely abolished transcriptional activation by DELLA-GAF1 (Fig. 5D). This loss-of-function experiment showed that at least one GAF1-binding site is necessary for DELLA-GAF1-mediated activation of *AtGA20ox2*.

GAF1-Binding Sites Are Necessary for GA Feedback Regulation of *AtGA20ox2*

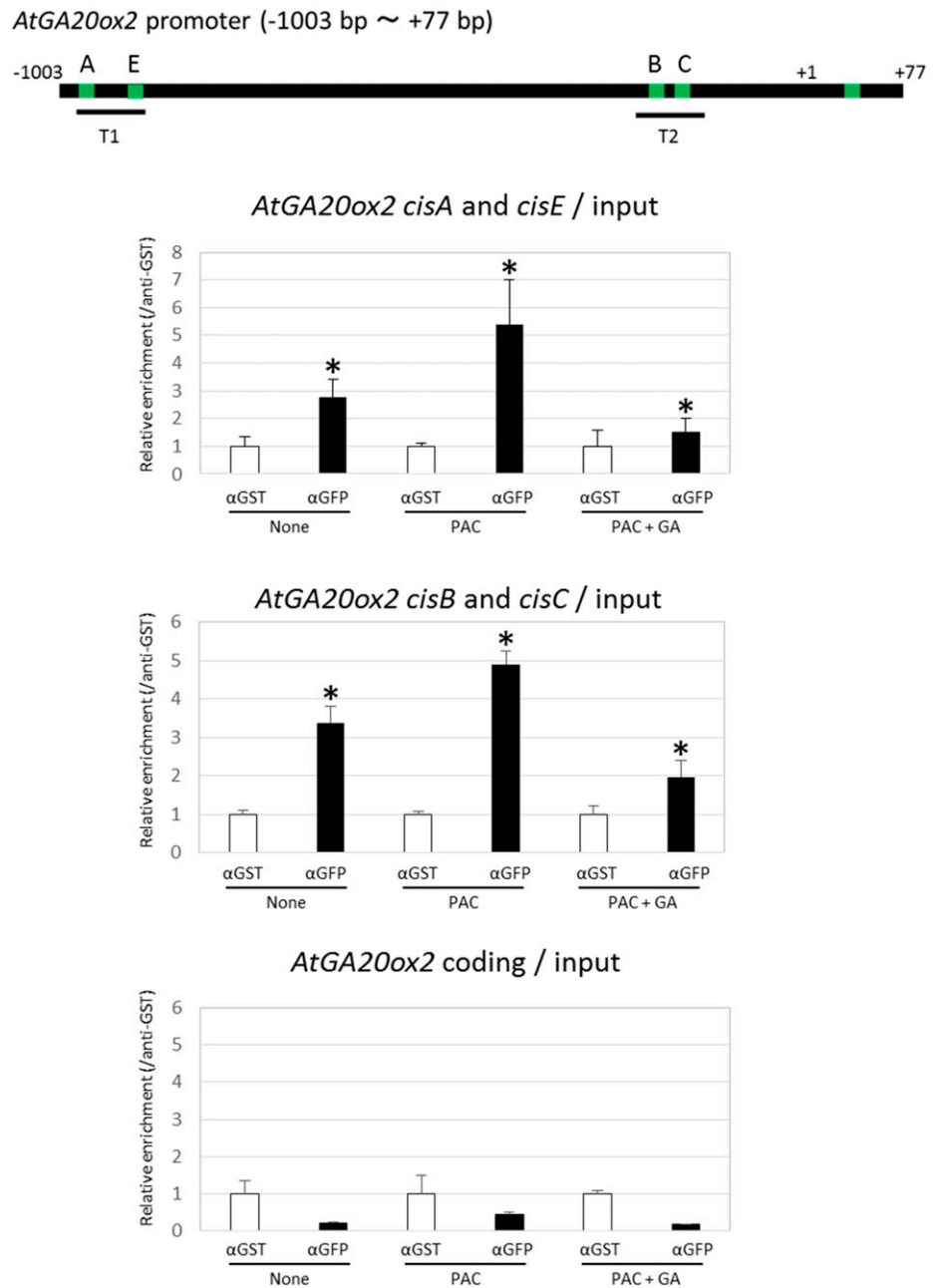
To determine whether the four GAF1-binding sites are involved in the GA negative feedback regulation of *AtGA20ox2*, we generated transgenic plants carrying mutant promoter-GUS constructs, in which mutations were introduced to eliminate the GAF1-binding sites (Fig. 6A), and GUS activity of each transgenic plant was confirmed by fluorometric GUS enzyme analysis. As expected, these mutations affected the GA negative feedback regulation

of *AtGA20ox2*. Two combinations of triple cis-element mutations (*mtABC* or *mtBCE*) among four functional GAF1-binding sites in the *AtGA20ox2* promoter decreased its response to GA (Fig. 6B). Mutation of all four GAF1-binding sites (*mtABCE*) completely inhibited GA feedback regulation of the promoter (Fig. 6B). Thus, at least one GAF1-binding site in the *AtGA20ox2* promoter is necessary for GA negative feedback, suggesting that the DELLA-GAF1 complex is a main component in GA feedback regulation of *AtGA20ox2*.

GAF1-Binding Sites Are Involved in Tissue-Specific Expression in Root Tip and Shoot Apices

To evaluate the contribution of the GAF1-binding sites of the *AtGA20ox2* promoter to the tissue-specific expression

Figure 7. DELLAs bind to the GAF1-binding region of the *AtGA20ox2* promoter in a GA-dependent manner. The DELLA complex binds to the *AtGA20ox2* promoter in vivo. ChIP assays were performed with anti-GST or anti-GFP using transgenic plants carrying with *RGAPro*: RGA-GFP that were treated with paclobutrazol (PAC) or PAC + GA₃ or left untreated for 1 week. The coprecipitated level of each DNA fragment T1, including *AtGA20ox2*, *cisA*, and *cisE*, T2, including *AtGA20ox2*, *cisB*, and *cisC*, or *AtGA20ox2* coding, was quantified by real-time PCR and normalized to the input DNA. The relative coprecipitated levels of each DNA fragment using anti-GST were set to 1, and relative enrichments of each DNA fragment using anti-GFP versus a DNA fragment using anti-GST are shown in the graphs. Error bars indicate the SD of three biological replicates ($n = 3$). *, $P < 0.05$ versus control by Student's *t* test.



of *AtGA20ox2*, we compared GUS expression patterns in transgenic plants carrying *AtGA20ox2* promoter-GUS and mutant *AtGA20ox2* promoter-GUS constructs. Histochemical analysis indicated that *AtGA20ox2* is expressed mainly in the root tip and shoot apices (Fig. 6C), consistent with the expression pattern of *GAF1* promoter:GUS (Fukazawa et al., 2014). These observations indicated that *GAF1* could regulate the expression of *AtGA20ox2* in root tip and shoot apices. The expression of mutant *AtGA20ox2* promoter-GUS (*mtABC*) and mutant *AtGA20ox2* promoter-GUS (*mtABCE*) in shoot apices and in root tip decreased and abolished the response of *AtGA20ox2* to GAs, respectively. The responses to GAs and uniconazole in root apices of

mutant *AtGA20ox2* promoter-GUS (*mtABC*) transgenic plants were higher than those in shoot apices (Fig. 6C). Together, these results indicate that binding of *GAF1* to the *AtGA20ox2* promoter is necessary for GA feedback regulation of *AtGA20ox2* and its tissue-specific expression in the root tip and shoot apices.

GA-Induced DELLA Degradation Decreases the Binding of DELLA to *GAF1*-Binding Sites of the *AtGA20ox2* Promoter

In GA feedback regulation, endogenous GA levels affect the amount of DELLA protein, which is reflected in the expression level of *AtGA20ox2* (Phillips et al., 1995;

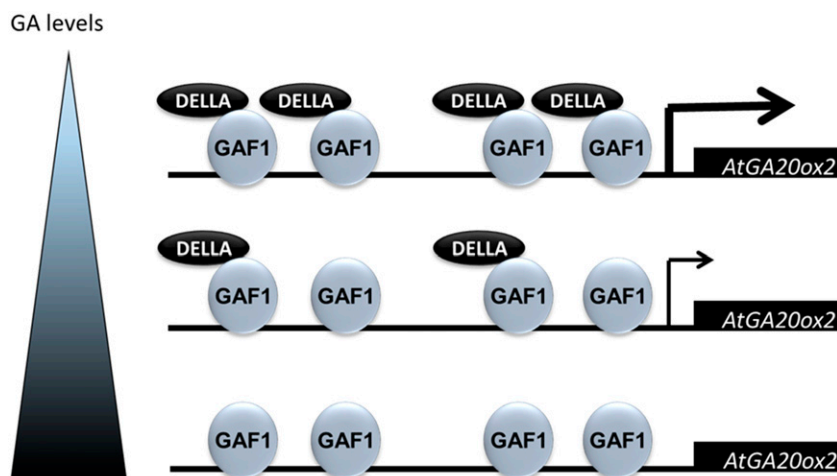


Figure 8. Proposed model of GA feedback regulation by the DELLA-GAF1 complex in a GA-dependent manner. In GA feedback regulation, endogenous GA levels affect the amount of DELLA protein, which is reflected in the expression level of *AtGA20ox2*. A decrease in GAs promotes DELLA accumulation, resulting in increased DELLA binding to GAF1-binding sites on the *AtGA20ox2* promoter and the activation of *AtGA20ox2* expression, whereas exogenous GA treatment suppresses the accumulation of DELLA, thus decreasing the amount of DELLA on the *AtGA20ox2* promoter and *AtGA20ox2* expression. DELLAs associate with GAF1 on the *AtGA20ox2* promoter and might control the feedback regulation of *AtGA20ox2*.

Middleton et al., 2012). We hypothesized that a decrease in GAs would promote DELLA accumulation, after which DELLA would bind to the GAF1-binding sites of the *AtGA20ox2* promoter in vivo, whereas exogenous GA treatment would promote the degradation of DELLAs, resulting in decreased availability of DELLA for binding to GAF1 sites on the *AtGA20ox2* promoter. To confirm this, we performed a ChIP assay using transgenic plants carrying *RGApro:RGA-GFP*. A ChIP assay using anti-GFP antibody showed that that RGA-GFP binds to two regions in the *AtGA20ox2* promoter, T1 (*cisA* and *cisE*) and T2 (*cisB* and *cisC*). In contrast, RGA-GFP did not bind to the coding region of *AtGA20ox2*. Moreover, the binding of RGA-GFP to the *AtGA20ox2* promoter was increased by treatment with paclobutrazol, an inhibitor of GA biosynthesis, and decreased by GAs (Fig. 7).

DISCUSSION

The DELLA-GAF1 Complex Is Involved in GA Feedback Regulation of *AtGA20ox2*

Previously, we generated transgenic plants expressing a mutant form of GAF1 that cannot bind to DELLAs (Δ PAM) under the control of the CaMV 35S promoter in the *ga1-3* background, a GA-deficient mutant. The up-regulation of *AtGA20ox2* in *ga1-3* was repressed by overexpression of Δ PAM, indicating that the DELLA-GAF1 complex is involved in GA feedback regulation (Fukazawa et al., 2014). In this study, we identified four GAF1-binding sites in the *AtGA20ox2* promoter, and these GAF1-binding sites were found to be necessary for GA feedback regulation of *AtGA20ox2*. In addition, we showed that the stabilization and localization of GAF1-GFP protein does not change with or without GA and that Δ PAM could bind to DNA (Fukazawa et al., 2014). These results indicated that DELLA is not required for binding of GAF1 to DNA, but DELLA is required for the activation of target genes, such as *AtGA20ox2*. Based on these data and ChIP analysis, we provide a model in which a decrease in GAs promotes the accumulation of DELLAs, upon

which DELLA binds to GAF1-binding sites of the *AtGA20ox2* promoter in vivo, whereas exogenous GA treatment promotes the degradation of DELLAs, reducing the amount of DELLA available to bind to the *AtGA20ox2* promoter. Because DELLA proteins do not have an apparent DNA-binding motif, they associate with GAF1 to bind to the *AtGA20ox2* promoter and might control the feedback regulation of *AtGA20ox2* (Fig. 8).

GAF1-Binding Sequences in the Promoter of GA Feedback-Regulated Genes

GAF1 belongs to the IDD family of transcription factors, which bind to the consensus sequences TTTTGTTCG and TTTGTCGTATT (Kozaki et al., 2004). Four out of five GAF1-binding sites identified in the *AtGA20ox2* promoter, *cisA*, *cisB*, *cisC*, and *cisD*, include the TTGTC sequence. GAF1 also binds to *cisE*, but this site apparently does not have a TTGTC sequence; however, it does have a similar sequence, TTGGC. The maize ID1 protein also binds to the TTTTGGCGTTAT sequence, which does not include a complete consensus sequence (Kozaki et al., 2004). Comparison of these binding sequences suggests that TTTTG(G/T)CNNTA might be a more appropriate consensus sequence for GAF1 binding. *AtGA3ox1* and *GID1b* also are activated by the DELLA-GAF1 complex (Fukazawa et al., 2014). TTGTC or TTGGC was found in the *AtGA3ox1* and *GID1b* promoter, suggesting that the DELLA-GAF1 complex also is involved in the GA feedback regulation of these genes.

GAF1-Binding Sequences in the *AtGA20ox2* Promoter Are Necessary for Tissue-Specific Expression of *AtGA20ox2* in the Shoot Apices and Root Tip

The expression of *AtGA20ox1* and *AtGA20ox2* in seedlings is strongly down-regulated by GA (Phillips et al., 1995). In contrast, the expression of these genes is up-regulated in the *ga1-3* GA-deficient mutant. The

DELLA proteins GAI and RGA are major repressors of GA-promoted vegetative growth (Dill and Sun, 2001; King et al., 2001). mRNAs of RGA and GAI are detected in all tissues throughout plant development (Silverstone et al., 1998; Lee et al., 2002; Tyler et al., 2004). Rieu et al. (2008) investigated the effect of the *gai-t6* and *rga-24* loss-of-function mutations on the expression of *GA20ox* in the *gai-3* background. The expression of *AtGA20ox1* and *AtGA20ox2* was decreased significantly in *gai-t6 rga-24 gai-3*, suggesting that both proteins are involved in GA feedback regulation of *AtGA20ox1* and *AtGA20ox2*; however, the expression patterns of GAI and RGA are different from those of *AtGA20ox1* and *AtGA20ox2* in seedlings. GAI and RGA are expressed ubiquitously, whereas the expression of *AtGA20ox1* and *AtGA20ox2* is restricted to the meristematic tissue (Plackett et al., 2012). Because the expression pattern of GAF1 seemed similar to that of *AtGA20ox1* and *AtGA20ox2* (Fukazawa et al., 2014), GAF1 may be a determinant of the tissue-specific expression of *AtGA20ox1* and *AtGA20ox2* in GA-deficient conditions. *AtGA20ox1* and *AtGA20ox2* are the most highly expressed *GA20ox* genes during vegetative and early reproductive development, and both genes act redundantly to promote hypocotyl and internode elongation as well as flowering time. Because the mutants *ga20ox1 ga20ox2* and *gaf1 gaf2* exhibit short-hypocotyl and late-flowering phenotypes (Rieu et al., 2008; Plackett et al., 2012; Fukazawa et al., 2014), DELLA-GAF1 might be involved in the expression of *AtGA20ox2* in the hypocotyl and shoot apex.

The DELLA-GAF1 Complex Is a Major Component of GA Feedback Regulation

The levels of bioactive GAs in plants are maintained by feedback regulation. In *Arabidopsis*, *AtGA20ox1*, *AtGA20ox2*, *AtGA20ox3*, and *AtGA3ox1* are down-regulated by exogenous GAs. Feedback regulation of these genes did not occur in the *penta della* mutant (Livne et al., 2015), indicating that DELLA proteins are necessary for GA feedback regulation. Various other transcription factors involved in GA feedback regulation have been reported (Yamaguchi, 2008; Fukazawa et al., 2011). For example, RSG is involved in the GA feedback regulation of *AtGA20ox1* (Fukazawa et al., 2000, 2010). The 14-3-3 proteins and NtCDPK1 (for Ca²⁺-dependent kinase1) control the localization of RSG in a GA-dependent manner (Igarashi et al., 2001; Ishida et al., 2004, 2008; Ito et al., 2010, 2014). AGF1 was identified as a regulator of feedback regulation of *AtGA3ox1* in *Arabidopsis* (Matsushita et al., 2007). These transcription factors are involved in regulating the expression of *GA20ox* and *GA3ox*. They might act synergistically with the DELLA-GAF1 complex in GA feedback regulation. However, the relationship between these transcription factors and the DELLA-GAF1 complex is not clear at present.

Recently, it was reported that brassinosteroids (BRs) also regulate GA biosynthesis. The expression of *AtGA20ox1* and *AtGA3ox1* genes is lower in *cpd*, a BR-deficient mutant, and *bri1-1*, a BR-insensitive mutant, than in the wild

type (Unterholzner et al., 2015). In addition, treatment with BR strongly increased *AtGA20ox1* expression in *cpd*. BES/BZR1, a transcription factor regulating BR signaling, binds directly to *AtGA20ox1* and *AtGA3ox1* promoters. Although the GA levels are decreased in *cpd* and *bri1-1* (Unterholzner et al., 2015), GA feedback regulation does not occur in these mutants, suggesting that BR signaling can affect GA feedback regulation. However, GA application reduced *AtGA20ox1* expression in *bri1-1*, suggesting that the feedback repression of GA production does not rely on BR signaling. Moreover, *AtGA20ox2* is not a target of BZR1 (Unterholzner et al., 2015). In GA-deficient conditions, accumulated DELLA interacts with BZR1 and inhibits its DNA-binding ability (Bai et al., 2012), thus inhibiting BZR1-dependent activation of its target genes, including GA biosynthetic genes. These data indicate that the regulation of GA biosynthetic genes by BZR1 is not involved in GA feedback regulation.

In this study, we showed that the DELLA-GAF1 complex is a main component of the GA feedback regulation of *AtGA20ox2*. Further investigation of how the DELLA-GAF1 complex regulates other target genes will help to reveal the molecular mechanisms of the fine-regulation of GA negative feedback and provide insights into the interaction between the control of endogenous levels of GAs and GA signaling.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All transgenic lines in this study were derived from *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) wild type. To generate transgenic plants overexpressing MYC-tagged GAF1, a 4×MYC tag was amplified and cloned into the *NotI* site of pBJJ4-GAF1. To generate mutant plants, *AtGA20ox2* promoter and mutated *AtGA20ox2* promoters were cloned into the *Sall*-*Bgl*III site of the binary vector pBI101. The primers are listed in Supplemental Table S1. *Agrobacterium tumefaciens*-mediated *Arabidopsis* transformation was carried out by the floral dip method (Clough and Bent, 1998). The primer sets for cloning are listed in Supplemental Table S1. Plants were grown in a controlled growth chamber at 22°C under white light illumination (16 h of light/8 h of dark).

Application of GA₃ to *AtGA20ox2* or *mtAtGA20ox2* Promoter:GUS Transgenic Plants

Seven-day-old seedlings of transgenic plants grown on one-half-strength Murashige and Skoog (1/2 MS) agar medium were transferred to 1/2 MS agar plates containing uniconazole or GA₃ and grown for an additional 1 week. To investigate GA sensitivity, plants were treated or not with 1 mg L⁻¹ uniconazole, 1 μM paclobutrazol, or 10 mg L⁻¹ GA₃.

Transactivation Assay

GA20ox2 promoters with a deletion from positions -1,003 to +77 (+1, transcription start site) were cloned into the *Sall*-*Bgl*III site of the p-less GUS vector, which is a pUC18-based plasmid that contains the *GUS* gene cassette of pBI101 (Takahashi et al., 1995). All primers used for transient assay analysis are shown in Supplemental Table S1. *GAF1* and *RGAA17*, an internal deletion mutant of *RGA*, were cloned into the *NotI*-*XhoI* site of the pJ4 vector, which carries the CaMV 35S promoter with a viral translation enhancer, the Ω sequence (Fukazawa et al., 2000), to be used as effectors. Transactivation assays were performed as described previously (Ohta et al., 2001). Reporter plasmid (1.2 μg) and 1.6 μg of each effector plasmid were used for each bombardment. For the normalization of reporter gene

activity, 0.4 μg of reference plasmid was cobombarded. Bombarded leaves were incubated in darkness for 20 h, after which luciferase activity was quantified. The data are presented as averages of three independent biological replicates.

Gel Retardation Assay

Gel retardation assays were performed following the procedure described previously (Fukazawa et al., 2000, 2010). GAF1 was cloned into the *NotI-XhoI* site of the pET30b vector (Novagen). Recombinant protein 6xHis-GAF1 was expressed and affinity purified from *Escherichia coli* BL21 (DE3) pLysE using Ni²⁺ resin (Novagen). The nucleotide sequences of the double-stranded oligonucleotides used for the gel mobility shift assays are described in Supplemental Table S1. The oligonucleotides were annealed and then labeled using [α -³²P]dCTP and the Klenow fragment of DNA polymerase I. Binding mixtures contained 50 fmol of labeled probe, 1 μg of purified recombinant GAF1 or 1 μg of control extract of *E. coli*, and 2 μg of poly(dI/dC). DNA competitor was used at 100-fold excess molar concentration. The binding buffer consisted of 20 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 50 mM KCl, 1 mM EDTA, 10% (v/v) glycerol, and 2 μM ZnCl₂. Reactions were incubated at 4°C for 30 min and loaded onto 4% (w/v) polyacrylamide gels containing 6.7 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 3.3 mM sodium acetate.

Fluorometric GUS Enzyme Analysis

Transgenic plants were homogenized in the extraction buffer described by Jefferson et al. (1987). After removal of the cell debris by centrifugation at 15,000g for 10 min at 15°C, GUS activities were determined using 1 mM 4-methylumbelliferyl glucuronide as a substrate at 37°C. Before the feedback assay experiment, we confirmed GUS activity in each transgenic plant.

Histochemical Staining

Kanamycin-resistant transgenic plants were histochemically stained to detect GUS activity by immersing seedlings in a staining solution (100 mM sodium phosphate buffer, pH 7, with 50 mM NaCl, 1 mM potassium ferricyanide, 0.1% [v/v] Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide) overnight at 37°C. After staining, the samples were immersed in a fixing solution (5% [v/v] formaldehyde, 5% [v/v] acetic acid, and 20% [v/v] ethanol), followed by dechlorophyllation in 70% (v/v) ethanol.

ChIP Assay

The ChIP experiment was performed following the procedure described previously (Fukazawa et al., 2010) with some modifications. In brief, 2-week-old 4xmyc-GAF1 transgenic or Col-0 plants were cross-linked in 1% (v/v) formaldehyde by vacuum filtration for 10 min and incubated at 4°C for 1 h. Aliquots of each protein sample were immunoprecipitated with anti-GST (Santa Cruz Biotechnology), anti-myc (MBL International), and anti-GAI (generated in house) antibodies for 12 h at 4°C. Chromatin-antibody complexes were precipitated with salmon sperm DNA/protein G agarose beads at 4°C for 2 h. Primers used for ChIP analysis are listed in Supplemental Table S1. The coprecipitated level of each DNA fragment was quantified by real-time PCR using specific primer sets and normalized to input DNA. The coprecipitated levels of preimmune serum or anti-GST antibody (immunoprecipitated DNA/input DNA) were set to 1. The results are shown as relative DNA enrichment. Error bars indicate SD ($n = 3$).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *GAF1* (At3g50700), *RGA* (At2g01570), *AtGA20ox2* (At5g51810), and *ACTIN2* (At3g18780).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Identification of the GAF1-binding region in the *AtGA20ox2* promoter.

Supplemental Table S1. Primers used in this work.

ACKNOWLEDGMENTS

We thank Dr. Belay T. Ayele (University of Manitoba) for helpful comments on the article.

Received March 2, 2017; accepted September 14, 2017; published September 15, 2017.

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