Large-Scale Identification of Gibberellin-Related Transcription Factors Defines Group VII ETHYLENE RESPONSE FACTORS as Functional DELLA Partners[1][C][W]

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DELLA proteins are the master negative regulators in gibberellin (GA) signaling acting in the nucleus as transcriptional regulators. The current view of DELLA action indicates that their activity relies on the physical interaction with transcription factors (TFs). Therefore, the identification of TFs through which DELLAs regulate GA responses is key to understanding these responses from a mechanistic point of view. Here, we have determined the TF interactome of the Arabidopsis (*Arabidopsis thaliana*) DELLA protein GIBBERELLIN INSENSITIVE and screened a collection of conditional TF overexpressors in search of those that alter GA sensitivity. As a result, we have found RELATED TO APETALA2.3, an ethylene-induced TF belonging to the group VII ETHYLENE RESPONSE FACTOR of the APETALA2/ethylene responsive element binding protein superfamily, as a DELLA interactor with physiological relevance in the context of apical hook development. The combination of transactivation assays and chromatin immunoprecipitation indicates that the interaction with GIBBERELLIN INSENSITIVE impairs the activity of RELATED TO APETALA2.3 on the target promoters. This mechanism represents a unique node in the cross regulation between the GA and ethylene signaling pathways controlling differential growth during apical hook development.

The extraordinary plasticity that characterizes plant development is thought to rely on a complex network of interacting signaling pathways (Casal et al., 2004). Hormones play important roles in this network, acting in many instances as second messengers that connect environmental cues to modulate plant growth and development (Lau and Deng, 2010; Rymen and Sugimoto, 2012). In addition, extensive cross regulation between hormonal signaling pathways provides an additional level of regulation to this network (Depuydt and Hardtke, 2011).

The GA metabolic pathway is responsive to environmental factors both biotic and abiotic (Achard et al., 2006, 2007a, 2007b; Zhao et al., 2007; Alabadí et al., 2008; Navarro et al., 2008; Stavang et al., 2009). Moreover, the GA pathway is also modulated by endogenous factors, such as the circadian clock (Arana et al., 2011), as well as other hormones, including auxins, ethylene, or cytokinins (Jasinski et al., 2005; Frigerio et al., 2006; Achard et al., 2007a). This places GAs as central players mediating the integration of environmental cues with growth and development. The five-member DELLA protein family is the master negative regulator in the GA signaling cascade (Davière and Achard, 2013). Complete removal of DELLA activity in the *dellaKO* combination mutant results in constitutive activation of all GA responses (Ikeda et al., 2001; Cheng et al., 2004; Feng et al., 2008; Jasinski et al., 2008). DELLAs are transcriptional regulators with stabilities that are negatively regulated by GAs in such a way that they accumulate in the

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nucleus when GA levels drop but are degraded by the 26S proteosome when GA levels rise. Our current view of DELLA action indicates that these proteins exert their pervasive control on plant growth and development through regulating the activity of diverse transcription factors (TFs; Schwechheimer, 2011) and other regulatory proteins, including chromatin remodelers, like Switch/Sucrose Non-Fermenting Chromatin Remodeling Complex subunit 3C and PICKLE (Sarnowska et al., 2013; Zhang et al., 2014), by physical interaction. Therefore, understanding GA-mediated control of plant development would require identification of its downstream transcriptional mediators.

Here, we have determined a TF interactome of the Arabidopsis (*Arabidopsis thaliana*) DELLA protein GIBBERELLIN INSENSITIVE (GAI) by yeast (*Saccharomyces cerevisiae*) two-hybrid assays (Y2Hs). Our results show that DELLAs can interact with many structurally diverse TFs, suggesting that they act as central signaling hubs in the plant connecting different signaling cascades. The

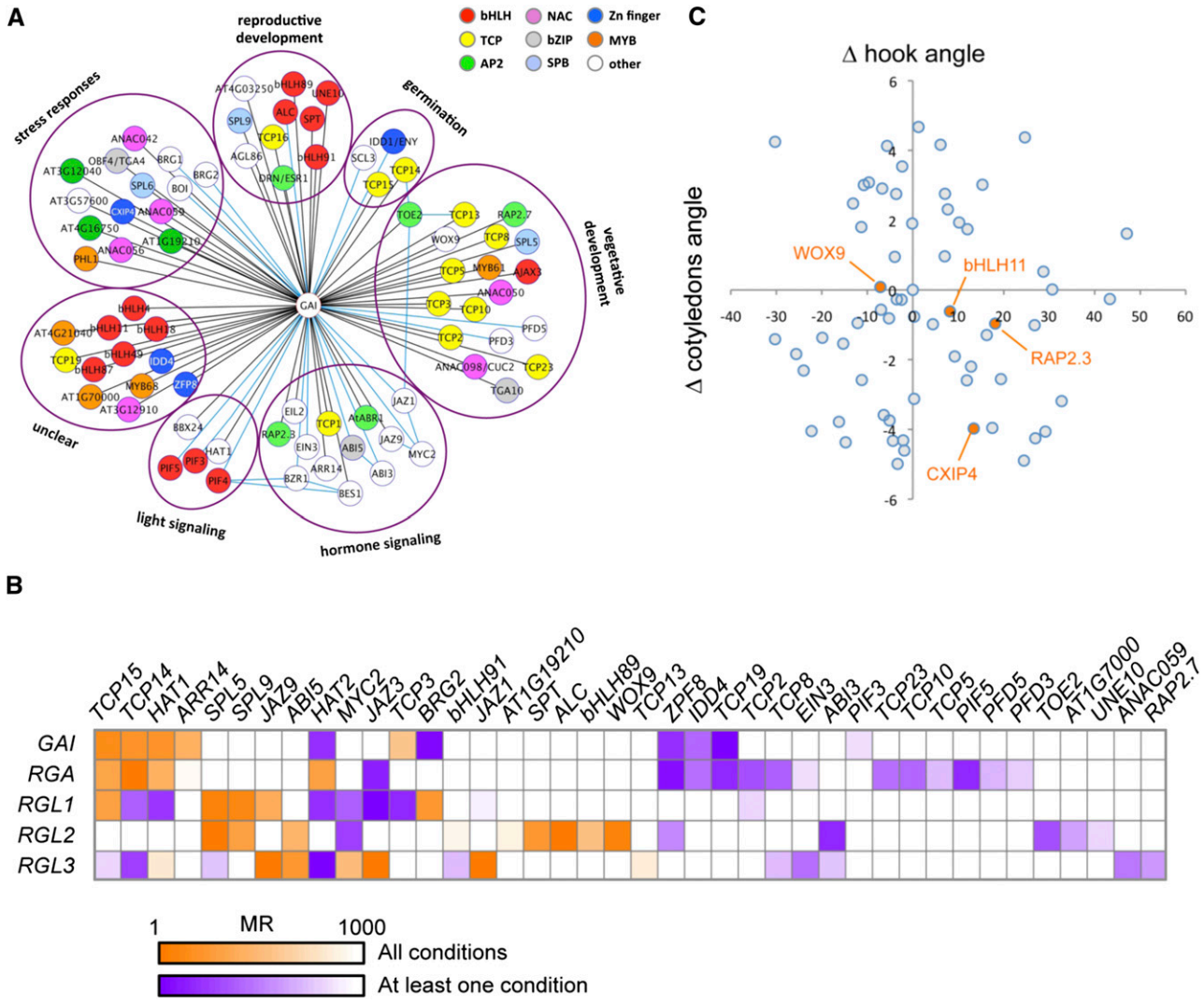

Figure 1. Identification of TFs involved in GA signaling. A, Visualization of the GAI interactome. Interactors are grouped depending on the biological process in which they participate based on the literature or in the Arabidopsis Information Resource annotation. Black edges indicate interactions identified in this work; light blue edges indicate interactions reported primarily in the literature. Colors of nodes group the TFs by family. B, Coexpression analysis of *DELLA* genes and the interacting TFs. The confidence of the coexpression is indicated by the mutual rank (MR) index according to ATTED-II (lower values indicate higher confidence). C, Identification of TFs with overexpression that affects GA-dependent hook formation but not GA-dependent folding of the cotyledons in dark-grown seedlings. Each dot represents a transgenic line overexpressing a single TF. The graph shows the variation (in degrees) in hook angle (*x* axis) and the angle between cotyledons (*y* axis) with respect to the behavior of a wild-type control line all growing in the presence of PAC (see "Materials and Methods"). Positive hook angle values and negative cotyledon angle values indicate that the transgenic line is more resistant to the effects of PAC.

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interactome confirms known interactions but also identifies many novel TF binding partners, which, therefore, represent promising targets for additional research into plant GA signaling. As a follow-up of this approach and as a way of validating the use of this network as a key resource for the identification of unique DELLA functions, we have further investigated the physiological relevance of the interaction with a particular TF, RELATED TO APETALA2.3 (RAP2.3), that belongs to the group VII ETHYLENE RESPONSE FACTORS (ERFs) of the APETALA2 (AP2)/ethylene responsive element binding protein (EREBP) superfamily. Functional evaluation of the GAI-RAP2.3 interaction has provided new insights, to our knowledge, into the mechanism of cross regulation between the GA and ethylene pathways, particularly in the control of apical hook development.

RESULTS AND DISCUSSION

The DELLA Protein GAI Interacts with Multiple TFs

To determine a TF interactome of the DELLA protein GAI, we screened by Y2H an arrayed library containing approximately 1,200 TFs from Arabidopsis (Castrillo et al., 2011) using the GRAS (for GAI, REPRESSOR OF *ga1-3*, SCARECROW) domain of GAI as bait (M5GAI). After testing all pairwise interactions between GAI and the TFs in the library, we identified 66 interactions that corresponded to 57 unique TFs (Supplemental Table S1), including 2 known interactors, PHYTOCHROME INTERACTING FACTOR3 (PIF3) and PIF4 (de Lucas et al., 2008; Feng et al., 2008). Figure 1A shows the GAI interactome visualized with Cytoscape (Shannon et al., 2003). The interacting TFs belong to 15 of 39 families represented in the library (Supplemental Table S2). The overall diversity of interactors found in this study is in line with the variety reported in the literature (Locascio et al., 2013b) and suggests that there is not a clear, strong bias for any particular TF family. However, 20% of the interactors belong to the TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTOR (TCP) family (12 of 23 interactors present in the library). TCPs share certain structural similarities with basic helix-loop-helixes (bHLHs) that resides in their DNA binding domain (Aggarwal et al., 2010), and at least in the case of the bHLH PIF4, a region including this domain acts as interacting surface with DELLA proteins (de Lucas et al., 2008), suggesting that this structure might perform the same role as well for the TCPs.

The number of unique interacting partners is likely an underestimation given that (1) the library that we screened contains only 75% of the more than 1,500 partners encoded in the Arabidopsis genome (Riechmann et al., 2000), and (2) DELLAs also perform cellular roles by interacting with proteins that are not TFs, such as PREFOLDIN3 and PREFOLDIN5 (Locascio et al., 2013a). Moreover, in support of the functional connection between DELLAs and the partners identified in the Y2H screening, a coexpression analysis performed with ATTED-II (Obayashi et al., 2014) rendered statistically significant coexpression for 68% of the interactors with at least one of five *DELLA* genes (Fig. 1B and Supplemental Table S3).

The structural variety of the GAI-interacting TFs (Supplemental Table S2; Locascio et al., 2013b) reflects the diversity of processes in which DELLAs are involved (Fig. 1A and Supplemental Table S1), which provides a molecular framework to the notion that DELLA proteins act as true signaling hubs, controlling many aspects of plant development and survival (Claeys et al., 2014). Remarkably, the fact that the GA metabolism and hence DELLA levels are very sensitive to changes in the environmental conditions (Sun, 2010) places DELLAs as potential signaling hubs connecting many aspects of plant physiology with environmental changes.

Identified DELLA Interactors Affect GA Responses

In a parallel effort to identify TFs involved in GA signaling, we sought to find those with overexpression that would alter the sensitivity to endogenous GAs. For this, we took advantage of the TRANSPLANTA (TPT) collection of Arabidopsis lines for conditional TF overexpression under the regulation of a β-estradiol-inducible promoter (Coego et al., 2014). It has been reported that GAs promote skotomorphogenesis, such that etiolated seedlings growing under GA-limiting conditions display photomorphogenic traits, such as short hypocotyls, an open apical hook, and unfolded cotyledons (Alabadí et al., 2004, 2008; Achard et al., 2007b; Gallego-Bartolomé et al., 2011). We screened 641 TPT lines representing 276 TFs growing in darkness under two different conditions: 10 nM GA biosynthesis inhibitor paclobutrazol (PAC) with 5 μM β-estradiol for 3 d to score their apical hook angle and 1 μM PAC with 5 μM β-estradiol for 7 d to score the angle between cotyledons. Most TPT lines displayed a behavior equivalent to the control line, but we found several TFs with overexpression that either enhanced or reduced the phenotype caused by GA deficiency in one of the screenings (Fig. 1C and Supplemental Table S4).

When we focused our attention on the TFs that interfered only with the control of the apical hook by GAs, we found that overexpression of RAP2.3, CATION EXCHANGER1 INTERACTING PROTEIN4, and bHLH11 caused enhanced sensitivity to GAs, whereas overexpression of WUSCHEL-RELATED HOMEOBOX9 caused the opposite effect (Fig. 1C). RAP2.3 belongs to the group VII ERFs of the AP2/EREBP superfamily (Nakano et al., 2006). Given that (1) no member of this family has been previously reported as a DELLA interactor (Locascio et al., 2013b) and (2) it participates in the ethylene signaling cascade (Büttner and Singh, 1997), which is known to control apical hook formation (Guzmán and Ecker, 1990), we decided to investigate the functional connection between GAI and RAP2.3.

GAI Interacts with RAP2.3

We reconfirmed the interaction between M5GAI and RAP2.3 by Y2H (Fig. 2, A and B). Moreover, the ability

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of RAP2.3 to interact with DELLAs is not restricted to GAI, because it also interacted with the equivalent truncated version of RGA (RGA52; Supplemental Fig. S1). It was previously shown that RGA interacts with PIF4 through a region encompassing its DNA binding domain, impairing PIF4 DNA binding (de Lucas et al., 2008). Other TFs also interact with DELLAs through

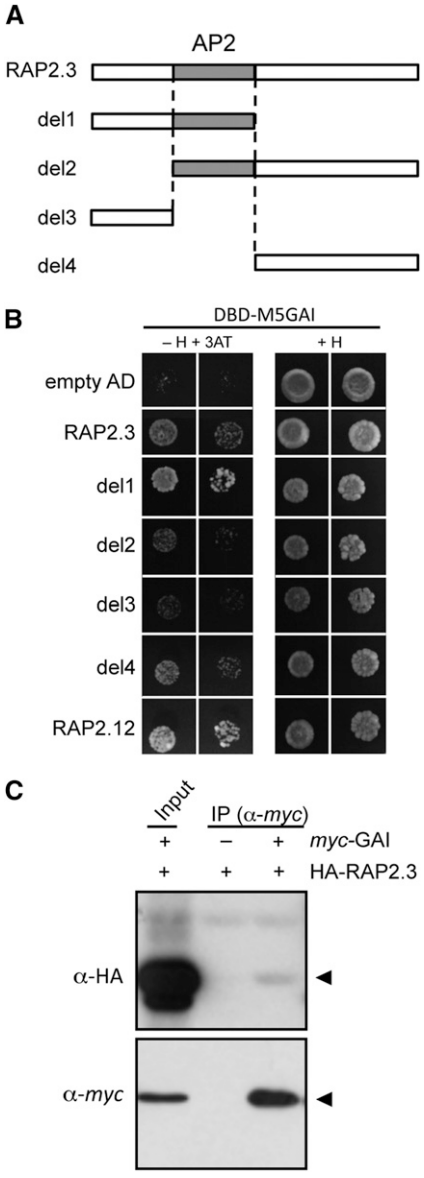

Figure 2. GAI interacts physically with RAP2.3. A, Scheme that shows the different deletions of RAP2.3 used for the Y2Hs. AP2 denotes the DNA binding domain. B, Y2Hs showing the interactions between M5GAI and full-length RAP2.3, four truncated versions, and RAP2.12. Two serial dilutions per yeast clone are shown. AD, Gal4 activation domain; DBD, Gal4 DNA binding domain; +H, control plate including His in the medium; 3AT, 3-amino-1,2,4-triazole. C, Co-IP performed in Arabidopsis protoplasts showing the interaction between *myc*-GAI and HA-RAP2.3. Arrowheads indicate HA-RAP2.3 and *myc*-GAI in top and bottom, respectively.

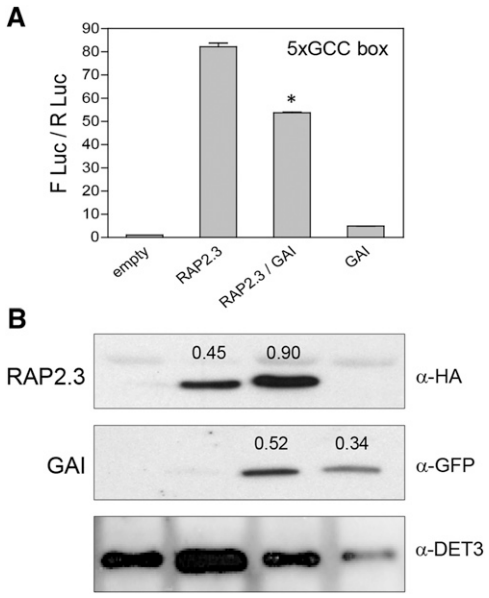

Figure 3. GAI inhibits RAP2.3. A, Cells of *A. tumefaciens* harboring the reporter construct (5× GCC box) were infiltrated in leaves of *N. benthamiana* alone (empty) or together with cells harboring effector constructs (RAP2.3, RAP2.3/GAI, or GAI). Firefly LUC activity was normalized to *Renilla* LUC. Values were normalized with respect to the ratio obtained for the reporter construct alone. Three biological repeats were performed, and error bars represent SEM. *, LUC activity in leaves expressing RAP2.3 and RAP2.3/GAI is significantly different ($P < 0.01$). B, Western-blot analysis of the effector protein levels in one of three biological replicates. Numbers in blots indicate the relative protein levels after normalization to DE-ETIOLATED3 (DET3) that served as a loading control.

specific parts of the protein (for instance, BRASSINAZOL RESISTANT1 [BZR1;Gallego-Bartolomé et al., 2012] or MYC2 [Hong et al., 2012]). We prepared four deleted versions of RAP2.3 (Fig. 2A) and tested their ability to interact with M5GAI to determine where GAI bound within RAP2.3. Strikingly, only del1, which contains the highly conserved amino terminus and the AP2 DNA binding domain, was able to interact strongly with M5GAI (Fig. 2B). These results suggest that both the N-terminal part and the AP2 domain are needed to support the interaction. We speculated that the ability of the RAP2.3 to bind DNA might be affected on interaction with GAI, because del1 includes the AP2 domain. Interestingly, both M5GAI and RGA52 also interacted with RAP2.12 (Fig. 2B and Supplemental Fig. S1), a close relative of RAP2.3 (Nakano et al., 2006), suggesting that this ability might extend to all other members of the group VII ERFs.

Next, we investigated if the interaction also occurs in planta by performing coimmunoprecipitation (co-IP) assays in Arabidopsis protoplasts cotransfected with *myc*-GAI and hemagglutinin (HA)-RAP2.3. The fusion of the HA tag at the N terminus of RAP2.3 will prevent degradation through the N-end rule pathway (Gibbs et al., 2014). As shown in Figure 2C, HA-RAP2.3 was efficiently pulled down from extracts

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of protoplasts by anti-*myc* antibodies only in the presence of *myc*-GAI, indicating that both proteins are able to interact in plants as well.

GAI Counteracts RAP2.3-Mediated Transcriptional Activation

As mentioned above, the ability of several TFs to bind their DNA targets is inhibited on interaction with DELLAs. For instance, this is the case for several PIFs (de Lucas et al., 2008; Feng et al., 2008; Cheminant et al., 2011; Gallego-Bartolomé et al., 2011) and BZR1 (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). In other cases, the interaction with the TF occurs in the vicinity of the promoters of certain genes to promote their expression (Lim et al., 2013; Park et al., 2013). RAP2.3 binds in vitro and in vivo to the GCC box (Büttner and Singh, 1997; Yang et al., 2009; Gibbs et al., 2014), and it is able to activate transcription when transiently overexpressed in Arabidopsis leaves (Yang et al., 2009). To test the effect that the interaction with GAI may have on the transcriptional activation ability of RAP2.3, we performed transactivation assays in leaves of *Nicotiana benthamiana*. As reported, we placed the *LUCIFERASE* (*LUC*) gene under the control of a synthetic promoter containing five copies of a 29-nucleotide fragment from the promoter of the ethylene-induced gene *HOOKLESS1* (*HLS1*), which contains a single GCC box (Fujimoto et al., 2000). A similar reporter construct has been used to show the ability of several RAP2.3-related TFs to activate or repress transcription (Fujimoto et al., 2000; Song et al., 2005). As shown in Figure 3A, LUC activity strongly increased when HA-RAP2.3 was expressed together with the reporter construct, which is in agreement with RAP2.3 being a transcriptional activator (Yang et al., 2009). Importantly, when yellow fluorescent protein (YFP)-GAI was co-expressed together with HA-RAP2.3 in the same leaves, LUC activity was significantly reduced, despite the fact that HA-RAP2.3 accumulated to a slightly higher level (Fig. 3B). These results suggest that GAI prevents either the DNA binding ability of RAP2.3 or its capacity to activate transcription.

DELLAs Prevent the Binding of RAP2.3 to the Promoter of Its Target Genes in Vivo

To identify genes potentially regulated as a consequence of DELLA-RAP2.3 interaction, we performed a meta-analysis between sets of genes regulated by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Goda et al., 2008) and those misregulated in the *dellaKO* mutant (Arana et al., 2011). We selected four genes that were up-regulated by ACC and down-regulated by DELLAs: At5g44120, At4g31940, At2g41260, and At4g19690 (Fig. 4A). The effect of a conditional overaccumulation of RAP2.3 on the expression of these genes was assayed in dark-grown seedlings that

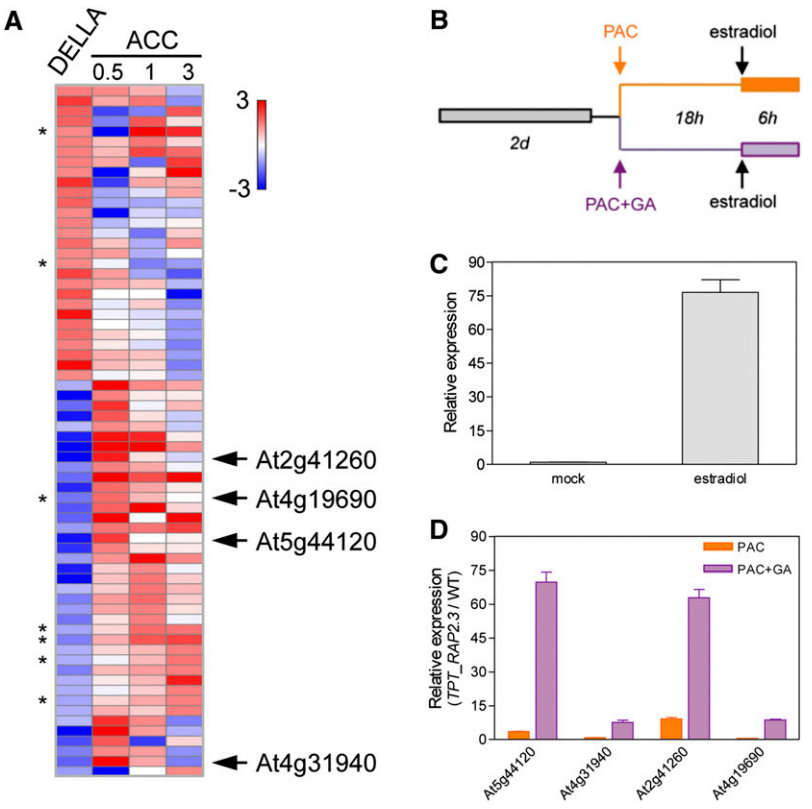

Figure 4. RAP2.3 activity is modulated by DELLA levels in Arabidopsis. A, Heat map illustrating the results of a meta-analysis that identifies genes putatively coregulated by DELLAs and the ethylene pathway. Red and blue denote induced and repressed genes, respectively. Column 1 shows the expression of the wild type with respect to a *della*KO mutant. Bar = log2 fold change. Numbers on top of the heat map indicate hours of ACC treatment. Selected genes for additional analyses are marked by arrows and their respective AGI code. *, Genes that are direct targets of EIN3 (Chang et al., 2013). B, Scheme depicting the experimental setup to assay the activity of RAP2.3 in a context with high (PAC) and low (PAC + GA) DELLA levels. C, Expression of *RAP2.3* in the *TPT_RAP2.3* line after β-estradiol treatment. D, Expression of four selected genes using the experimental design depicted in B and measured by qRT-PCR. Expression levels of each gene were normalized against *EF1α*. Three biological repeats were performed; error bars are SEM.

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accumulated high or low DELLA levels because of a treatment with 10 μM PAC or 10 μM PAC plus 1 μM GA_4, respectively (Fig. 4B shows the experimental design). To induce *RAP2.3*, we used a transgenic line from the TPT collection expressing its open reading frame (ORF) under the control of a β-estradiol-inducible promoter (*TPT_RAP2.3*; Fig. 4C; Coego et al., 2014). The expression of the four target genes was higher when RAP2.3 accumulated in a context deprived of DELLA proteins (PAC + GA; Fig. 4D). This suggests that RAP2.3 does, indeed, promote expression of these genes and that interaction with DELLA perturbs its activity. These results corroborate those observed in transactivation assays in *N. benthamiana* (Fig. 3).

To determine whether GAI affects the ability of RAP2.3 to bind target promoters or activate their transcription, we studied the binding of RAP2.3 to a variety of promoters in vivo by chromatin immunoprecipitation (ChIP). None of four target genes used for expression analysis in Figure 4 contain the canonical GCC box within the 1,500 nucleotides immediately upstream of the ATG. Therefore, to identify putative binding sites for RAP2.3, we used the motif discovery tool MotifLab (Klepper and Drabløs, 2013). We scanned the promoters using binding matrices obtained from in vitro DNA binding assays with TFs of the ERF family, including RAP2.3 (Franco-Zorrilla et al., 2014). We identified putative target sequences in each promoter that we called generically GCC-like boxes (Fig. 5A). For ChIP assays, we used a transgenic line overexpressing an HA-tagged mutant version of the RAP2.3 protein (*35S::MA-RAP2.3-HA*). This mutant version of RAP2.3 is constitutively stable; it is resistant to N-end rule-mediated degradation, because the Cys at position 2 is replaced by the stabilizing residue Ala (Gibbs et al., 2011, 2014). We did not detect in vivo binding of the RAP2.3 to the region containing the GCC-like box in the promoter of At2g41260 and At4g19690 in etiolated seedlings (Supplemental Fig. S2A). However, the regions containing GCC-like boxes in the promoters of At4g31940 and At5g44120 were efficiently coimmunoprecipitated with MA-RAP2.3-HA (Fig. 5B). Importantly, the enrichment of these regions after co-IP was significantly higher when seedlings were grown in conditions that favor DELLA degradation (PAC + GA) than in seedlings that accumulate DELLAs (PAC). Thus, these results collectively suggest that DELLAs inactivate RAP2.3 by blocking its DNA binding activity in what seems like a common mechanism for DELLA-mediated modulation of TF activity (Davière and Achard, 2013).

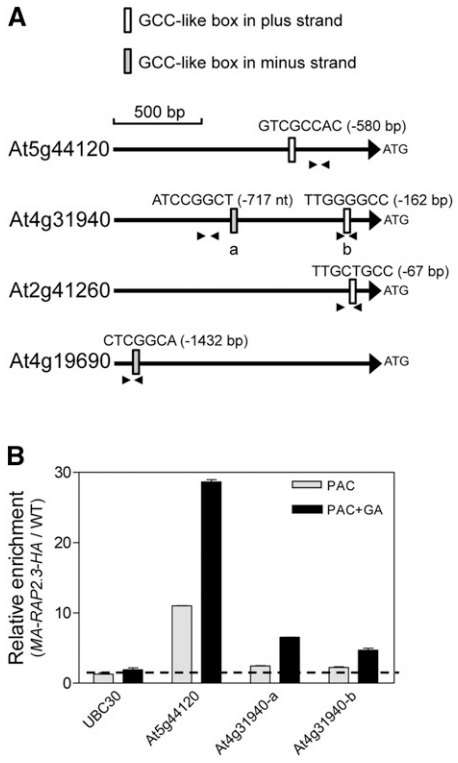

Figure 5. DELLAs inhibit the binding of RAP2.3 to target promoters in vivo. A, Localization of the GCC-like boxes in the −1,500-bp upstream region (from the ATG) of the selected genes. The sequence of the putative cis elements identified by MotifLab is shown as it appears in the plus strand along with the distance to the ATG. Arrowheads indicate the position of the sequences used to design the primers for ChIP analysis. B, ChIP of MA-RAP2.3-HA followed by quantitative PCR of selected target genes in 4-d-old seedlings grown in darkness in 0.5 μM PAC with or without 1 μM GA_4. *UBIQUITIN-CONJUGATING ENZYME30* (*UBC30*) is a control gene with a promoter that is not bound by MA-RAP2.3-HA. Values represent the fold enrichment of RAP2.3-bound DNA in immunoprecipitated samples relative to the control gene *HSF* (At4g17740) and then to the total input DNA. Data are means and SDs of two technical replicates from a representative experiment of two experiments.

The DELLA-RAP2.3 Interaction Mediates Apical Hook Opening

The development of the apical hook typical of etiolated seedlings is regulated by GAs and ethylene (Abbas et al., 2013). In particular, both pathways jointly prevent premature apical hook opening in darkness (Gallego-Bartolomé et al., 2011). Recently, it has been shown that DELLAs counteract the effect of ethylene during apical hook development by inhibiting the activity of ETHYLENE INSENSITIVE3 (EIN3) through physical interaction, providing a mechanism for the coregulation of this process by GAs and ethylene (An et al., 2012). It was previously shown that *RAP2.3* expression is induced by ethylene (Büttner and Singh, 1997). Significantly, EIN3 binds in vivo to the *RAP2.3* promoter (Chang et al., 2013) and activates its expression in etiolated seedlings (Fig. 6A), suggesting that RAP2.3 could also participate in the mechanism regulating apical hook development by GAs and ethylene. To unambiguously show the involvement of RAP2.3 in this process, we investigated the hook phenotype in a loss-of-function transferred DNA (T-DNA) insertional mutant (WiscDsLox247E11). We named this mutant *rap2.3-2* after the *rap2.3-1* allele previously described (Ogawa et al., 2007). A combined treatment of

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wild-type etiolated seedlings with 12.5 μM ACC plus 1 μM GA_4 provoked the formation of hooks with an exaggerated curvature (Fig. 6B; Gallego-Bartolomé et al., 2011; An et al., 2012). Remarkably, *rap2.3* mutant seedlings were partially resistant to treatment with both hormones, and the hook angle was significantly reduced in the mutant compared with the wild type, having a similar angle to that observed when ACC alone is supplied (Fig. 6B). Hooks of mutant and wild-type seedlings were equally responsive to separate treatments with 12.5 μM ACC and 1 μM GA_4, suggesting that, under these conditions, RAP2.3 activity is not limiting, most likely because of genetic redundancy with the other members of the group VII ERFs. To address this issue, we investigated if other members of group VII ERFs, RAP2.2 and RAP2.12, were also involved in this physiological response. *rap2.2* mutant seedlings showed a wild-type response for all treatments, whereas *rap2.12* behaved like *rap2.3*, indicating that it also mediates the control of hook development by GAs and ethylene (Fig. 6B). To fully determine the involvement of group VII ERFs, we prepared a quintuple mutant defective in all five genes that we named *erfVII* (*rap2.2 rap2.3 rap2.12 hypoxia-responsive erf1* [*hre1*] *hre2*). This mutant showed a more marked defect in the response to ACC + GA and also revealed a defect in the response to GAs (Fig. 6B). On the contrary, its response to ACC was like the wild type. These results suggest that RAP2.3 and RAP2.12 are the most important group VII ERFs controlling hook development by the joint action of GAs and ethylene. The fact that *erfVII* seedlings show a full response to ACC, whereas their response to GAs is diminished suggests that the activity of these TFs is more relevant for GAs to control hook development than for ethylene.

To understand this process in more detail, we looked at the effect of PAC on apical hook angle in the presence or absence of overaccumulated RAP2.3. In

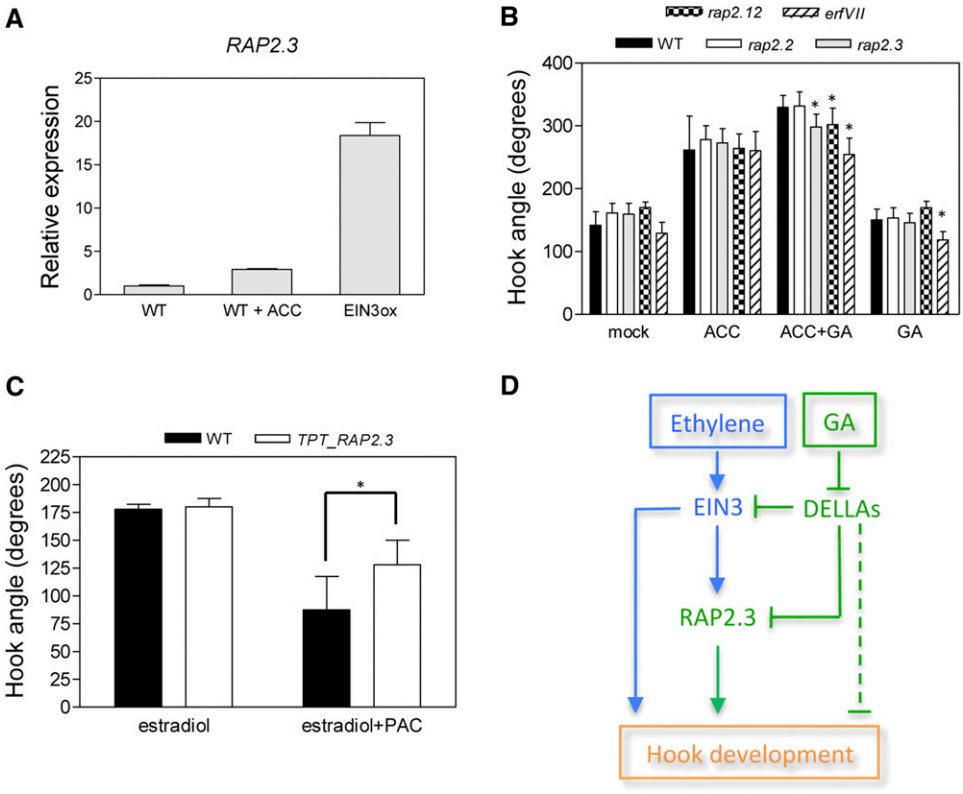

Figure 6. RAP2.3 regulates hook development downstream of GA and ethylene pathways. A, Expression of *RAP2.3* in 4-d-old dark-grown seedlings measured by qRT-PCR. Expression levels were normalized against *EF1α*. Three biological repeats were performed; error bars are SEM. B, Three-day-old wild-type Col-0, *rap2.2*, *rap2.3*, *rap2.12*, and *erfVII* seedlings were grown in darkness in control media (mock) and media supplemented with 12.5 μM ACC, 12.5 μM ACC plus 1 μM GA_4, or 1 μM GA_4. Graph shows the apical hook angle. *, Hook angles of wild-type and mutant seedlings are significantly different in the ACC + GA and GA treatments ($P < 0.05$). Error bars represent SEM ($n = 15$). C, Graph showing the apical hook angle of 3-d-old wild-type and *TPT_RAP2.3* seedlings grown in darkness in the presence of 5 μM β-estradiol or 5 μM β-estradiol plus 0.02 μM PAC. *, Hook angles of wild-type and *TPT_RAP2.3* seedlings are significantly different in the estradiol + PAC treatment ($P < 0.01$). Error bars represent SEM ($n = 15$). D, Model depicting the cross regulation between the GA and ethylene pathways in the control of hook development. Arrows and bars indicate positive and negative effects, respectively. The dashed line defines ethylene-independent control of hook development by DELLAs. WT, Wild type. [See online article for color version of this figure.]

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wild-type seedlings, 0.02 μM PAC induced hook opening (Fig. 6C). However, in *TPT_RAP2.3* seedlings treated with β-estradiol, this PAC-induced opening was perturbed (Fig. 6C), further indicating an important GA-associated role for RAP2.3 during this developmental process. Taken together, these results suggest that DELLAs might also regulate the apical hook development by inactivating RAP2.3 in addition to EIN3 (An et al., 2012).

CONCLUSION

The DELLA-TF interactome presented here has identified a wide range of unique protein-protein interactions, and therefore can be used as a tool for investigating new molecular mechanisms to explain (1) how GAs regulate physiological processes and (2) the interaction between GAs and other hormone or signaling pathways. Our functional studies add a unique molecular insight into the mechanism that controls apical hook development by GAs and ethylene (Fig. 6D). RAP2.3 represents an EIN3-dependent branch of ethylene signaling, formerly exemplified by ERF1 (Solano et al., 1998), which participates in the transcriptional cascade triggered by the hormone in the control of this developmental process. RAP2.3 regulates sets of genes that are not directly regulated by EIN3, which is the case of three of four genes shown in Figure 4 (Chang et al., 2013), expanding this way the effect on gene expression triggered by ethylene. Interestingly, *HLS1*, a major target of EIN3 in the regulation of hook development, is not an in vivo target of RAP2.3, because (1) its expression was not altered after *RAP2.3* induction or in the *rap2.3* mutant (Supplemental Fig. S2, B and C) and (2) MA-RAP2.3-HA was not able to bind to the GCC box in its promoter (Supplemental Fig. S2A), despite the fact that it was able to regulate transcription from a fragment of this promoter containing the cis element (Fig. 3A).

The interaction of DELLAs with EIN3 and RAP2.3 indicates that GAs regulate the ethylene-triggered transcriptional cascade at various levels and that they could be relevant at several stages of hook development (Fig. 6D). For instance, low levels of DELLAs during skotomorphogenesis ensure the proper activity of both EIN3 and RAP2.3 and perhaps, RAP2.12 as well to keep the hook closed. An additional scenario where the negative regulation of both TFs by DELLAs could be relevant is to promote hook opening during deetiolation. DELLAs and EIN3 levels increase and decrease, respectively, during this transition (Achard et al., 2007b; Zhong et al., 2009), although the accumulation of DELLAs is faster. Therefore, inactivating both EIN3 and RAP2.3 by physical interaction would be an efficient and rapid way to sequester these proteins and counteract the ethylene-dependent mechanism that maintains a closed hook.

Group VII ERFs are degraded through the N-end rule pathway in response to the simultaneous presence of nitric oxide (NO) and oxygen (Gibbs et al., 2011, 2014; Licausi et al., 2011). The role of group VII ERFs as NO and oxygen sensors and the reported NO-dependent accumulation of DELLA proteins in response to light (Lozano-Juste and León, 2010), therefore, suggest that the interaction with DELLAs might be relevant in other physiological contexts as well.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) accession Columbia-0 (Col-0) was used as the wild type; *35S::MA-RAP2.3-HA*, *35S::EIN3*, and *TPT_RAP2.3* lines have been described (Chao et al., 1997; Coego et al., 2014; Gibbs et al., 2014). Homozygous *rap2.3-2* seedlings (T-DNA insertion line WiscDsLox247E11) were identified in a segregating F2 population by PCR-based genotyping using the gene-specific oligonucleotides 5′-CCATCCCACCAACCAAGTTAACGTGA-3′ and 5′-GCAGATCTGGGAAGTTGAGCTTGGC-3′ and an oligonucleotide annealing at the left border of the T-DNA as primers. Seeds of the insertion line were obtained from the Arabidopsis Biological Resource Center. T-DNA insertion mutants *hre1*, *hre2*, *rap2.2-1*, *rap2.3-1*, and *rap2.12-1* were described previously (Gibbs et al., 2014). Homozygous quintuple insertion mutant *erfVII* (*rap2.2-1 rap2.3-1 rap2.12-1 hre1 hre2*) was generated by crossing lower group VII ERF combination mutants and identified by PCR using T-DNA and gene-specific primers sets as described (Gibbs et al., 2014). For all experiments, seeds were surface sterilized and sown in sterile filter papers in plates containing one-half-strength Murashige and Skoog medium (Duchefa) with 0.8% (w/v) agar and 1% (w/v) Suc and were kept for 4 to 7 d at 4°C in darkness. Germination was induced under continuous white fluorescent light (90–100 $\mu mol\ m^{-2}\ s^{-1}$) at 22°C for 8 to 12 h.

After germination was induced, seeds of the different genotypes were transferred to treatment plates that were kept in darkness at 22°C. For gene expression analyses, 4-d-old seedlings were used. For continuous treatments, wild-type, *EIN3ox*, and *TPT_RAP2.3* seedlings were grown in media supplemented with mock solution, 12.5 μM ACC (Sigma), or 5 μM β-estradiol (Sigma). For short-term treatments, 2-d-old wild-type and *TPT_RAP2.3* seedlings were soaked for 18 h in a solution containing 10 μM PAC (Duchefa) or 10 μM PAC plus 1 μM GA_4 (Duchefa) and then treated with 5 μM β-estradiol for 6 additional h. Seedlings were handled under green safe light.

For apical hook angle experiments, 3-d-old wild-type and *rap2.3-2* seedlings were grown in darkness in media containing mock solution, 12.5 μM ACC, 12.5 μM ACC plus 1 μM GA_4, or 1 μM GA_4. Wild-type and *TPT_RAP2.3* seedlings were grown in media containing 5 μM β-estradiol or 0.02 μM PAC plus 5 μM β-estradiol for 3 d. The screening of the *TPT* lines for hook angle phenotypes was performed in 3-d-old seedlings growing in 0.01 μM PAC plus 5 μM β-estradiol. The screening of the TPT lines for cotyledon opening phenotypes was performed in 7-d-old seedlings growing in 1 μM PAC plus 5 μM β-estradiol. Seedlings were scanned and hook or cotyledon angles were measured using the Image J software.

Y2Hs

The coding sequence of a GAI truncated version (M5GAI) was cloned into *pDEST32* (Invitrogen) to create a fusion with the Gal4 DNA binding domain (Gallego-Bartolomé et al., 2012), and it was used to transform the *Saccharomyces cerevisiae* haploid yeast strain pJ694α. The screening of the TF library was performed as previously described (Castrillo et al., 2011). Positive clones were selected in medium containing 1 mM 3-amino-1,2,4-triazole (Sigma).

The *RGA52* truncated version cloned into *pDEST32* has been described (Gallego-Bartolomé et al., 2012). Full-length and truncated versions of *RAP2.3* were obtained by PCR using primers listed in Supplemental Table S5 and cloned into *pCR8/GW/TOPO* (Invitrogen). The *pENTR* vector containing the ORF of *RAP2.12* was obtained from the Arabidopsis Biological Resource Center. Full-length ORFs and truncated versions were transferred into *pDEST22* (Invitrogen) to create Gal4 activation domain fusions. Assays were preformed in the yeast strain AH109 (Clontech). Yeasts were tested for interactions in synthetic dextrose/-Leu/-Trp/-His medium plus 5 mM 3-amino-1,2,4-triazole.

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Co-IP

Arabidopsis cell suspension derived from wild-type Col-0 roots was used for protoplast isolation (Mathur and Koncz, 1998). Transfections of protoplasts were performed as described (Fülöp et al., 2005), with 3 μg each of *myc-GAI* and *HA-RAP2.3* expression constructs. Transfected protoplasts were cultured for 16 h at room temperature and then lysed in extraction buffer (25 mM Tris-HCl, pH 7.8, 5 mM EGTA, 10 mM $MgCl_2$, 75 mM NaCl, 10% [v/v] glycerol, 0.2% [v/v] Tween 20, 2 mM dithiothreitol, and 1% [v/v] plant protease inhibitor cocktail; Sigma). In co-IP assays, proteins were incubated in a total volume of 100 μL of extraction buffer containing 150 mM NaCl, 0.2 mg mL^{-1} bovine serum albumin, and 1.5 μg of anti-*c-myc* antibody (clone 9E10; Covance). Immunocomplexes were captured on Protein G-Sepharose beads (GE Healthcare), washed three times in 500 μL of washing buffer (1× Tris-buffered saline, 5% [v/v] glycerol, and 0.1% [v/v] Igepal CA-630), and eluted by boiling in 25 μL of 1.5× Laemmli sample buffer. Proteins were then resolved by SDS-PAGE and blotted to a polyvinylidene difluoride membrane (Millipore). The presence of HA-RAP2.3 and *myc*-GAI proteins was detected by monoclonal anti-HA-peroxidase conjugate antibody (clone 3F10; Roche) and anti-myc antibody (clone 9E10; Roche) with ECL Reagent (GE Healthcare).

Reporter Construct and Transcriptional Assays

A synthetic regulatory cassette consisting of five copies of a 29-nucleotide fragment from the *HLS1* promoter that contains one GCC box (AGCCGCCATTTATGAGTTAACGCAGACAT) upstream of the minimal *35S* promoter and the Ω translational enhancer was synthesized by GeneScript and cloned into the *Pst*I and *Nco*I sites of the *pGreenII 0800-LUC* vector (Hellens et al., 2005). The *RAP2.3* coding sequence was cloned into the *pEarleyGate-201* vector (Earley et al., 2006) to create an HA-RAP2.3 fusion to be used as an effector. The effector construct that expresses *YFP-GAI* has been described (Gallego-Bartolomé et al., 2012).

Leaves of 4-week-old *Nicotiana benthamiana* were infiltrated with *Agrobacterium tumefaciens C58* cells carrying the constructs. The ratio of cells with reporter and effector constructs was 1:4. Firefly and the control Renilla LUC activities were assayed from leaf extracts with the Dual-Glo Luciferase Assay System (Promega) and quantified with a GloMax 96 Microplate Luminometer (Promega). The proper expression of effector proteins was tested by western-blot analysis. HA-RAP2.3 and YFP-GAI fusions were detected with anti-HA (3F10; Roche) and anti-GFP (ab290; Abcam) antibodies.

Gene Expression

For gene expression analysis, total RNA was extracted with the E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek) according to the manufacturer's instructions. Complementary DNA synthesis was performed with the SuperScript II First-Strand Synthesis System (Invitrogen). Quantitative reverse transcription (qRT)-PCR was performed as described using the *EUKARYOTIC TRANSLATION ELONGATION FACTOR1-α* (*EF1-α*) gene for normalization (Frigerio et al., 2006). Primers used for PCR are listed in Supplemental Table S5.

ChIP

35S::MA-RAP2.3-HA seedlings were grown for 4 d at 22°C in one-half-strength Murashige and Skoog medium plates containing 0.5 μM PAC with and without 1 μM GA_4. Wild-type Col-0 seedlings were used as the control. ChIP was performed as described previously (Saleh et al., 2008) using Dynabeads Protein A (Invitrogen) and an anti-HA antibody (ab9110; Abcam). Relative enrichment was calculated by normalizing the amount of target DNA first to the internal control gene *HEAT-SHOCK FACTOR* (At4g17740) and then to the corresponding amount in the input. Primers used to amplify the target regions are listed in Supplemental Table S5.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: GAI (At1g14920), RGA (At2g01570), RAP2.2 (At3g14230), RAP2.3 (At3g16770), RAP2.12 (At1g53910), HRE1 (At1g72360), and HRE2 (At2g47520).

Supplemental Data

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

Supplemental Figure S1. RGA interacts physically with RAP2.3 and RAP2.12.

Supplemental Figure S2. HLS1 gene is not a transcriptional target of RAP2.3.

Supplemental Table S1. List of GAI interacting partners found in this study.

Supplemental Table S2. List of the TF families including the TFs identified in this study.

Supplemental Table S3. Coexpression analysis for the interactors and the DELLA genes.

Supplemental Table S4. Values for hook and cotyledon angles in the TPT screening.

Supplemental Table S5. List of oligonucleotides used in this study.

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