Floral homeotic genes are targets of gibberellin signaling in flower development

Hao Yu*, Toshiro Ito*, Yuanxiang Zhao*, Jinrong Peng†, Prakash Kumar‡, and Elliot M. Meyerowitz*§

*Division of Biology 156–29, California Institute of Technology, Pasadena, CA 91125; †Department of Biological Sciences, Faculty of Science, National University of Singapore, 10 Science Drive 4, Singapore 117543; and §Institute of Molecular and Cell Biology, 30 Medical Drive, National University of Singapore, Singapore 117609

Contributed by Elliot M. Meyerowitz, April 3, 2004

Gibberellins (GAs) are a class of plant hormones involved in the regulation of flower development in Arabidopsis. The GA-deficient ga1-3 mutant shows retarded growth of all floral organs, especially abortive stamen development that results in complete male sterility. Until now, it has not been clear how GA regulates the late-stage development of floral organs after the establishment of their identities within floral meristems. Various combinations of null mutations of DELLA proteins can gradually rescue floral defects in ga1-3. In particular, the synergistic effect of rga-t2 and rgl2-1 can substantially restore flower development in ga1-3. We find that the transcript levels of floral homeotic genes APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG) are immediately up-regulated in young flowers of ga1-3 upon GA treatment. Using a steroid-inducible activation of RGA, we further demonstrated that these floral homeotic genes are transcriptionally repressed by RGA activity in young flowers whereas the expression of LEAFY (LFY) and APETALA1 (AP1) is not substantially affected. In addition, we observed the partial rescue of floral defects in ga1-3 by overexpression of AG. Our results indicate that GA promotes the expression of floral homeotic genes by antagonizing the effects of DELLA proteins, thereby allowing continued flower development.

Materials and Methods

Plant Materials. All Arabidopsis mutants used in this study are in the Landsberg erecta (Ler) background unless stated otherwise. They were grown at 22°C in continuous light. To break dormancy, all seeds with ga1-3 background were imbibed in 100 μM GA at 4°C for 7 days, and then rinsed thoroughly with water before sowing. Mutant lines ga1-3, rgl1-1, rgl2-1, gai-16, rga-t2, ga1-3 rgl1-1, ga1-3 rgl2-1, ga1-3 gai-16, ga1-3 rga-t2, and ga1-3 gai-16 rga-t2 have been described (22). The other mutant lines in this study were created by cross-pollination between the above

Abbreviations: GA, Gibberellin; GR, glucocorticoid receptor; LFY, LEAFY; AG, AGAMOUS; PI, PISTILLATA; AP1, APETALA1; AP2, APETALA2; AP3, APETALA3.

1To whom correspondence should be addressed. E-mail: meyerow@its.caltech.edu.

© 2004 by The National Academy of Sciences of the USA

www.pnas.org/cgi/doi/10.1073/pnas.0402377101

PNAS | May 18, 2004 | vol. 101 | no. 20 | 7827–7832
relevant mutants, and their genotypes were verified as reported (21, 22).

To create gal-1-3 rga-t2 35S::RGA-GR, gal-1-3 rga-t2 was treated weekly with 100 μM GA and transformed with the binary vector harboring the 35S::RGA-GR cassette. Transgenic plants containing 35S::RGA-GR were screened by Basta selection and further tested for phenotypic effects by dexamethasone treatment. We isolated one transgenic line, which contains only one transgene insertion and shows the phenotype closely resembling gal-1-3 after dexamethasone treatment, to cross with gal-1-3 rga-t2 to generate gal-1-3 35S::AG-GR.

To create 35S::AG-GR, Arabidopsis ecotype Landsberg erecta (Ler) was transformed with the binary vector harboring the 35S::AG-GR cassette (T.I. and E.M.M., unpublished results). We selected a transgenic line, which contains only one transgene insertion and shows the phenotype closely resembling 35S::AG after dexamethasone treatment, to cross with gal-1-3 to generate gal-1-3 35S::AG-GR.

Dexamethasone treatment and sample collection were as described (30).

**Plasmid Constructs.** We constructed a derivative pGreen0229TI vector by cloning the cauliflower mosaic virus 35S coat protein gene (35S) promoter with tandem enhancers and transcriptional terminator into the KpnI and XhoI sites of pGreen0229 (31). The hormone-binding domain of the rat glucocorticoid receptor (GR) was amplified from pRl-ΔGR (32) by the primers GR1 (5′-TCCCCGGGGGATCTGAAGCTCGAA-3′) and GR2 (5′-GCTCTAGAGCTCAGTCATTTTGATGA-3′). The amplified GR fragment was cut with BamHI and XbaI and cloned into the corresponding sites of the pGreen0229TI:GR to generate pGreen0229TI:GR. The entire RGA cDNA was amplified by RT-PCR with the primers RGA-G1 (5′-AAGGATCCACCTGAGTCCGACGTC-3′) and RGA-G2 (5′-CTCTAGACTCTAATCATGCCTAG-3′), and the amplified fragment was digested by PstI and BamHI and subsequently cloned into the corresponding sites of pGreen0229TI:GR to create a 35S::RGA-GR cassette.

**Analysis of Gene Expression.** To investigate gene expression in young flowers, we selected inflorescence apices containing floral buds younger than stage 10. Total RNA was extracted by RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed by using the ThermoScript RT-PCR system (Invitrogen). Under our RT-PCR conditions, we performed 22–25 cycles of amplification to make sure that quantification for all genes examined was within a linear range. The amplified PCR products were detected as described (33). RT-PCR was repeated three times by using samples collected separately.

Primers designed for RT-PCR were as follows: AP1-P1 (5′-GGATCCAGATGCCACGTCGCGG-3′) and AP1-P2 (5′-GCGGCTCTAGAGCTCAGTCATTTTGATGA-3′) for APETALA1 (API); AP2-P1 (5′-AGAGGATCCACCTGAGTCCGACGTC-3′) and AP2-P2 (5′-CTCTAGACTCTAATCATGCCTAG-3′) for APETALA2 (API2); AP3-P1 (5′-AGCTGCGTCGTCTTGAG-3′) and AP3-P2 (5′-GCTCTAGAGCTCAGTCATTTTGATGA-3′) for PI; AP1-P1 (5′-GGATCCAGATGCCACGTCGCGG-3′) and AG-P2 (5′-GCTCAGGAACTTGAAGGCCAAGC-3′) and AG-P2 (5′-GCTCAGGAACTTGAAGGCCAAGC-3′) and AG-P2 (5′-GCTCAGGAACTTGAAGGCCAAGC-3′) and AG-P2 (5′-GCTCAGGAACTTGAAGGCCAAGC-3′) and AG-P2 (5′-GCTCAGGAACTTGAAGGCCAAGC-3′) for ATCCGTAGAAGATCCACGCCAGCAT-3′) for AG; LFP-P1 (5′-GGATCCACCTGAGTCCGACGTC-3′) and LFP-P2 (5′-GGATCCACCTGAGTCCGACGTC-3′) for ATCCGTAGAAGATCCACGCCAGCAT-3′) for LFP; and TUB2-P1 (5′-CTCACTCAGGAGCTTCTCC-3′) and TUB2-P2 (5′-CTCACTCAGGAGCTTCTCC-3′) for β-tubulin (TUB2). Some other primers were according to the following references: WUSCHEL (WUS) (34), SUPERMAN (SUP) (35), and SEPALLATA 3 (SEP3) (36).

**In Situ Hybridization.** Nonradioactive in situ hybridization was performed according to a published protocol (37). Synthesis of antisense probes has been described (38). Sections of both WT and gal-1-3 plants were placed on the same slide, which was hybridized and detected under the same conditions. The comparable panels for different probes in in situ figures were recorded from the same slide.

**Results and Discussion.** DELLAs in Arabidopsis include GAI, RGA, RGL1, RGL2, and RGL3, which contain a conserved DELLAL domain at their N termini (18, 25). The stability of these proteins is thought to be reduced in the presence of GA (25–28). It has been suggested that DELLAS proteins play repressive roles in various aspects of plant growth and development (18–24). Flowers in GA-deficient mutants gal-1-3 possess undeveloped floral organs...
in all four whorls. In particular, stamen development, including filament elongation and pollen maturation, is abortive. Recently reported work (29) and our observations (Fig. 6, which is published as supporting information on the PNAS web site) show that various combinations of null mutations of DELLA proteins gai-t6, rga-t2, rgl1-1, and rgl2-1 (22) can rescue floral phenotypes of ga1-3 to different degrees (Table 1). These results suggest that RGA and RGL2 play major functions in repressing the continued growth of floral organs, and that the sequence of the importance of DELLA proteins involved in flower development is RGA, RGL2, RGL1, and GAI.

**GA Promotes Flower Development Partly by Up-Regulating Floral Homeotic Genes.** To identify the downstream genes regulated by GA signaling, we examined the expression of a set of genes involved in floral patterning (12) upon GA treatment, which included the floral meristem identity gene LFY, floral homeotic genes AP1, AP2, AP3, PI, and AG, and floral organ identity and growth regulators WUS, SUP, and SEP3. Our results showed that the expression of B and C function genes AP3, PI, and AG was up-regulated in inflorescence apices of ga1-3, ~2 h after GA treatment (Fig. 1A) whereas expression of the other genes was not substantially changed under the conditions tested (data not shown). Although the selected inflorescence apices for RT-PCR contained floral buds from stages 1 to 10, RNA of old floral buds after stage 5 would be expected to constitute the greatest part of the RNA isolated from the batch of floral buds (T.L. and E.M.M., unpublished data). Thus, up-regulation of these floral homeotic genes after GA treatment mostly reflected a change in their transcript levels in older floral buds after stage 5, after the time when the floral meristems had already established floral organ identity (39). Because GA treatment is sufficient to restore the normal growth of floral organs in ga1-3 flowers, we suggest that the promotion of floral homeotic gene expression by GA signaling may be important for the continued development of floral buds to late-stage flowers, which have already established floral organ identity.

To further investigate the potential involvement of GA signals in the promotion of floral homeotic gene expression, we performed in situ hybridization with relevant probes, to ga1-3 and WT inflorescences. The B function gene AP3 and C function gene AG were expressed at lower levels in ga1-3 than in WT plants (Figs. 2 and 3) although their expression domains were not changed. Such reduction of expression levels was observed through the whole process of flower development in ga1-3 but was particularly evident by stage 8 (Figs. 2 B and E and 3 B and F). On the contrary, the expression of LFY, AP2 (data not shown), and AP1 (Fig. 7, which is published as supporting information on the PNAS web site) was not noticeably changed in ga1-3, which is consistent with the RT-PCR result (Fig. 1A). As compared with their expression in ga1-3, expression of AP3 and AG was also higher in ga1-3 rgl2-1 rga-t2 (data not shown), which showed significant rescue of floral defects of ga1-3, indicating that GA may up-regulate the expression of target genes in flower development by overcoming the effects of DELLA proteins, especially RGA and RGL2.

Our data showed that GA can specifically and continuously promote the expression of B and C function genes during flower development, but not the LFY, AP1, and AP2 genes. The significance of this finding lies in two aspects. First, compared with the promotion of LFY expression by GA in the control of flowering time (40), LFY expression in emerging floral meristems is independent of GA signaling. Flower phenotypes in ga1-3 suggest that, without GA, the normal expression of LFY in young floral meristems is sufficient to promote the transcript levels of floral meristic genes to establish normal floral organ identity, but not enough to secure the continued development of floral organs. Although we cannot exclude the possibility that GA signaling may coordinate with LFY in early development of floral meristems, our results suggest that GA can promote expression of floral homeotic genes independently of LFY activity in late-stage flowers, where LFY expression is absent. Second, it has been reported that GA signals can greatly promote petal development in ap1-1 and ap2-1 (41), indicating the possible presence of an A function-independent pathway in floral organogenesis that can be induced by GA signal transduction. This finding may be explained by the observation that GA can up-regulate B and C function genes, but not A function genes.

**Inducible Activation of RGA.** Among the identified DELLA proteins, RGA plays a more prominent role than GAI, RGL1, and RGL2 in mediating GA signaling during flower development (29). RGA contains a putative nuclear localization signal, and an RGA fusion with green fluorescent protein is localized in the nucleus of onion epidermal cells, indicating that RGA functions...
in the nucleus, perhaps as a transcriptional regulator (42). To further elucidate the mechanistic links between GA signaling and the activity of downstream genes in flower development, we created a steroid-inducible version of RGA in transgenic plants containing the RGA protein fused to the hormone-binding domain of a rat GR under the control of a 35S promoter. Posttranslational activation of RGA can be achieved in plants transgenic for this construct by dexamethasone treatment, which releases the fusion protein bound in the cytoplasm by means of the rat protein domain to the nucleus (43).

The loss-of-function rga mutation can partially rescue a wide range of phenotypic defects in ga1-3 plants, such as stem elongation, flowering time, and flower development (ref. 42 and Fig. 6C). To closely examine the effects of RGA activity, we transformed ga1-3 rga-t2 double mutants with 35S::RGA-GR. The rationale is that, if the RGA-GR protein is biologically functional, activation of RGA by dexamethasone should revert the rescued phenotypes of ga1-3 rga-t2 to those of ga1-3. We subsequently isolated one ga1-3 rga-t2 35S::RGA-GR transgenic line, which showed phenotypic reversion from ga1-3 rga-t2 to ga1-3 after weekly treatment with dexamethasone (Fig. 4A and B). This result indicates that the RGA-GR fusion protein has similar biological functions as WT RGA and allows control of RGA activity in a glucocorticoid-dependent manner.

Published work (29) and our genetic analysis indicate that RGA is a key regulator of GA signaling involved in the control of continued development of floral organs, as reflected in the major phenotypic difference between ga1-3 rgl2-1 rga-t2 and ga1-3 rgl2-1. The former genotype showed significant rescue of floral defects as compared with the latter (Table 1). As RGL2 is the second most important DELLA protein after RGA in the control of flower development; lack of RGL2 activity in ga1-3 would potentially remove a majority of the redundant repressive effects with RGA. Thus, in the ga1-3 rgl2-1 rga-t2 background, the steroid-inducible activation of RGA could reveal major genes responding to RGA activity. We crossed ga1-3 rga-t2

Fig. 2. In situ localization of AP3 expression in WT plants (A–C) and ga1-3 mutants (D–F). (A and D) An inflorescence apex with stage-2 and stage-4 flowers. (B and E) A stage-8 flower. (C and F) A stage-10 flower. (Bars = 100 μm.)

Fig. 3. In situ localization of AG expression in WT plants (A–D) and ga1-3 mutants (E–H). (A and E) An inflorescence apex with a stage-4 flower. (B and F) A stage-6 flower. (C and G) A stage-9 flower. (D and H) A stage-12 flower. (Bars = 100 μm.)
expression of these genes (data not shown). These results imply that RGA may control the expression of these floral homeotic genes in an indirect way. Also, whereas RGA may specifically regulate the B and C function genes, it does not seem to regulate AP1 and LFY because their expression did not respond either to GA treatment or to RGA activity (Fig. 1 and data not shown).

To further confirm that floral homeotic genes act downstream of GA signaling in later stages of flower development, we generated ga1-3 35S::AG-GR, where a biologically active AG-GR fusion protein can be induced by dexamethasone (T.I. and E.M.M., unpublished results). If down-regulation of AG expression is partially responsible for ga1-3 floral phenotypes, provision of additional AG activity by dexamethasone should at least restore some phenotypic defects. This suggestion was confirmed by the following observations. Dexamethasone treatment of ga1-3 35S::AG-GR provided functional AG activity, causing the pheno- typic rescue of ga1-3 flowers with elongated stamens and pistils (Fig. 5B). At a later stage, stamen development was at least partially rescued (Fig. 5D), which eventually resulted in partial fertility (Fig. 5F). However, mock-treated plants still

**Floral Homeotic Genes Function Downstream of GA Signaling.** Using the established steroid-inducible activation of RGA, we further studied whether the expression of floral homeotic genes is repressed by RGA activity. Dexamethasone treatment of inflorescence apices of ga1-3 rgl2-1 rga-t2 35S::RGA-GR for <6 h caused very little change in AP3, PI, and AG RNA levels, but 8 h of treatment resulted in a 2-fold reduction of transcript levels of these three genes (Fig. 1B), which was consistent with the up-regulation of the expression of these floral homeotic genes by GA treatment. Thus, B and C function genes are transcriptionally repressed by RGA, which is the major mediator of GA signaling involved in flower development.

However, our results demonstrated that the expression of AP3, PI, and AG did not respond to RGA activity within 4 h of treatment by dexamethasone (Fig. 1B). Furthermore, a combined treatment of dexamethasone and cycloheximide, an inhibitor of translation, for 6 h did not reveal any alteration of
developed as gpd1-3, with retarded growth of all floral organs and infertility (Fig. 5A, C, and E). These observations suggest that the promotion of AG is necessary for continued development of reproductive organs, and that AG is a target of GA signaling in flower development.

Taken together, the work presented here suggests that GA promotes normal development of floral organs partly by up-regulating the expression of floral homeotic genes AP3, PI, and AG. GA achieves this effect by suppressing the function of two DELLA proteins, RGA and RGL2. It has been shown recently that GA regulates cell elongation in filament development and cellular differentiation in anthers leading from microspores to mature pollen grains (29). Our results indicate that GA may perform these functions by regulating the late functions of floral homeotic genes. Indeed, continuous AG activity seems to be necessary for promoting the growth of WT stamens with sporogenous cells, elongated filaments, and dehiscent 4-located anthers (T.I. and E.M.M., unpublished results). Thus, GA signaling in flower development is possibly coordinated by a regulatory network involving DELLA proteins and floral homeotic genes.

The absence of typical DNA-binding domains in DELLA proteins indicates that these transcriptional regulators may form complexes with other transcription factors to control the expression of downstream genes (18, 22, 23, 42). Because DELLA proteins function in a wide range of plant developmental programs, their involvement in flower development may be mediated by additional flower-specific regulators. It will be interesting to clarify whether floral homeotic genes are simply the downstream targets of DELLA proteins, or, alternatively, whether they may also interact with DELLA proteins as region-specific cofactors.

We thank Dr. Nick Harberd and Dr. Tai-Ping Sun for helpful comments and critical reading of the manuscript. This work was funded by National Institutes of Health Grant GM45697 (to E.M.M.). H.Y. was supported in part by an overseas postdoctoral fellowship from the National University of Singapore.