Global Identification of DELLA Target Genes during Arabidopsis Flower Development

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Gibberellin (GA) plays important roles in regulating many aspects of plant development. GA derepresses its signaling pathway by promoting the degradation of DELLA proteins, a family of nuclear growth repressors. Although the floral organ identity is established in flowers of the GA-deficient mutant ga1-3, the growth of all floral organs is severely retarded. In particular, abortive anther development in ga1-3 results in male sterility. Genetic analysis has revealed that various combinations of null mutants of DELLA proteins could gradually rescue floral organ defects in ga1-3 and that RGA is the most important DELLA protein involved in floral organ development. To elucidate the early molecular events controlled by RGA during flower development, we performed whole-genome microarray analysis to identify genes in response to the steroid-inducible activation of RGA in ga1-3 rgl2 rga 35S:RGA-GR. Although DELLA proteins were suggested as transcriptional repressors, similar numbers of genes were down-regulated or up-regulated by RGA during floral organ development. More than one-third of RGA down-regulated genes were specifically or predominantly expressed in stamens. A significant number of RGA-regulated genes are involved in phytohormone signaling or stress response. Further expression analysis through activation of RGA by steroid induction combined with cycloheximide identified eight genes as immediate targets of RGA. In situ hybridization and transgenic studies further showed that the expression pattern and function of several selected genes were consistent with the predictions from microarray analysis. These results suggest that DELLA regulation of floral organ development is modulated by multiple phytohormones and stress signaling pathways.

GAs are a class of tetracyclic diterpenoid phytohormones that control many aspects of plant development throughout the plant life cycle (Richards et al., 2001; Sun and Gubler, 2004; Fleet and Sun, 2005). The “release of restraint” model has proposed that the “ground state” of the GA signaling is repressive and that GAs regulate plant growth and development by suppressing a group of DELLA proteins (Dill and Sun, 2001; Silverstone et al., 2001; Harberd, 2003). A total of five DELLA proteins belonging to the plant-specific GRAS family of putative transcriptional regulators have been identified in the Arabidopsis (Arabidopsis thaliana) genome (Bolle, 2004). The highly conserved N-terminal DELLA domain of these proteins, which is absent in other GRAS members, is required for GA-mediated protein degradation by a ubiquitin/26S proteasome-dependent proteolysis process (Silverstone et al., 1998; Dill et al., 2001, 2004; Richards et al., 2001; Sasaki et al., 2003; Fu et al., 2004).

The GA-induced DELLA proteolysis is mediated by the recently identified GA receptors. GA-INSENSITIVE DWARF1 (GIDI), which encodes a soluble protein with similarity to hormone-sensitive lipases, was first identified as a GA receptor in rice (Oryza sativa; Ueguchi-Tanaka et al., 2005). Subsequently, its homologs GID1a, GID1b, and GID1c were identified and characterized as the major GA receptors in Arabidopsis with functional redundancy and specificity (Griffiths et al., 2006; Nakajima et al., 2006; Iuchi et al., 2007). Further studies have shown that the GID1 receptors interact with DELLA proteins through their DELLA domains in a GA-dependent manner, which possibly leads to a conformational change of DELLA proteins and their subsequent degradation via the ubiquitin-proteasome pathway (Griffiths et al., 2006; Nakajima et al., 2006; Willige et al., 2007). The F-box proteins GID2 in rice and SLEEPY1 in Arabidopsis have been identified as components of SCF E3 ubiquitin ligase complexes that target the GA-GID1-DELLA complex for proteasome-mediated degradation (McGinnis et al., 2003; Sasaki et al., 2003; Griffiths et al., 2006). Recent studies have demonstrated that environmental signals, such as salt and light, and other phytohormones, such as ethylene, auxin, and abscisic acid (ABA), regulate plant growth by affecting the GA-induced destabilization of DELLA proteins (Achard...
et al., 2003, 2006; Fu and Harberd, 2003). Auxin promotes root growth by enhancing the destabilization of DELLA proteins, whereas stress-induced ethylene and ABA signaling suppress plant growth by delaying the progress of the GA-induced degradation of DELLA proteins. Therefore, DELLA proteins can integrate responses from different phytohormones and signals of adverse conditions, permitting appropriate modulation of plant growth in response to changes in the natural environment (Achard et al., 2006).

GAs are known to play various roles in plant reproductive development, and different species respond differently to GAs (Pharis and King, 1985). In Arabidopsis, the GA-deficient mutants, such as ga1-3, exhibited retarded growth of all four whorls of floral organs, especially petals and stamens (Wilson et al., 1992; Goto and Pharis, 1999). In ga1-3, abnormal anther development characteristic of arrested microsporogenesis prior to pollen mitosis prevents the formation of mature pollen, resulting in complete male sterility (Cheng et al., 2004). All of these GA-deficient phenotypes could be rescued by application of exogenous GA (Koornneef and van der Veen, 1980), suggesting that GAs play an important role in regulating floral organ development. Genetic crossing studies have demonstrated that various combinations of null mutants of DELLA proteins can gradually rescue floral organ defects of ga1-3 to different degrees (Cheng et al., 2004; Yu et al., 2004b). In particular, the synergistic effect of rga-t2 and rgl2-1 can substantially rescue the floral phenotype of ga1-3 with normal petals, pistils, and much developed stamens. These observations suggest that GA-dependent floral organ development is largely mediated through DELLA proteins.

To date, several microarray analyses have been carried out to identify genes affected by DELLA proteins or GA in Arabidopsis at different developmental stages, including seed germination and seedling and floral development (Ogawa et al., 2003; Cao et al., 2006; Nemhauser et al., 2006; Zentella et al., 2007). Among these studies, Cao et al. (2006) compared transcriptomes in developing flowers of ga1-3, ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1, and wild-type plants. That study revealed that GA could regulate downstream genes during flower development in either a DELLA-dependent or a DELLA-independent manner. However, as the differentially expressed genes in that study were identified to some extent as a result of secondary or long-term effects of metabolism and growth, such as floral morphological changes in loss-of-function mutants, the early molecular events controlled by DELLA proteins during floral organ development remain unclear.

In this study, we aimed to identify early targets of DELLA proteins involved in flower development. As RGA plays a more prominent role than other DELLA proteins in mediating GA’s effect on flower development, we focused on the identification of RGA target genes using established ga1-3 rgl2-1 rga-t2 35S:RGA-GR transgenic plants expressing a steroid-inducible version of RGA, which has been shown to retain the same biological function as RGA (Cheng et al., 2004; Yu et al., 2004b). Posttranslational activation of RGA in these plants could be achieved by treating inflorescence apices with the steroid hormone dexamethasone. Since degradation of the induced RGA protein was prevented in the background of ga1-3 rgl2-1 rga-t2, this inducible system allowed us to identify transcriptomes immediately responding to RGA activity prior to morphological changes of treated flowers, thus permitting the investigation of an immediate effect of RGA on floral organ development.

Our microarray results revealed an almost similar number of genes that were down-regulated or up-regulated by RGA at the inflorescence apex after 4 h of induction. Over 38% of RGA down-regulated genes were expressed in stamen and more than one-third of down-regulated genes were specifically or predominantly expressed in stamen, suggesting that regulation of stamen development is a major molecular event controlled by DELLA proteins during flower development. We also found that a significant number of RGA-regulated genes were involved in phytohormone signaling or the stress response. This observation indicates that the regulation of floral organ development by DELLA proteins is modulated by multiple phytohormones or environmental signals.

RESULTS

Microarray Analysis of RGA-Regulated Genes in Flower Development

Previous genetic analyses have shown that RGA plays a more prominent role than other Arabidopsis DELLA proteins in repressing floral organ development (Cheng et al., 2004; Yu et al., 2004b). In order to identify transcriptomes immediately responding to RGA activity during flower development, we performed microarray analysis of an established steroid-inducible version of RGA (RGA-GR) in ga1-3 rgl2-1 rga-t2 (Yu et al., 2004b). Mock-treated ga1-3 rgl2-1 rga-t2 35S:RGA-GR plants showed the same floral phenotypes as ga1-3 rgl2-1 rga-t2, in which rgl2-1 rga-t2 could significantly rescue the floral defects in ga1-3 (Fig. 1A). On the contrary, dexamethasone-treated flowers of ga1-3 rgl2-1 rga-t2 35S:RGA-GR displayed the same retarded growth of stamens, petals, and carpels as flowers of ga1-3 rgl2-1 (Fig. 1B; Yu et al., 2004b). In addition, dexamethasone treatment was able to produce other phenotypes related to RGA repression, such as impaired leaf expansion and stem elongation (Fig. 1C). These observations demonstrate that the RGA-GR fusion protein is biologically functional and that posttranslational activation of RGA-GR by dexamethasone is sufficient to regulate RGA downstream genes, thus resulting in the repression of plant development, including flower development.

Our previous study showed that the induction of RGA activity in ga1-3 rgl2-1 rga-t2 35S:RGA-GR in-
nescence apices for more than 6 h altered the expression of several floral homeotic genes, which, however, did not occur in the presence of cycloheximide, an inhibitor of protein translation (Yu et al., 2004b). This indicates that RGA induction for more than 6 h imposes some indirect effects on downstream genes during flower development. In addition, as the growth of floral organs and microsporogenesis are initially arrested at around floral stage 10 in ga1-3 (Smyth et al., 1990; Cheng et al., 2004), DELLLA proteins should affect the transcriptomes in flowers earlier than stage 10. Thus, to identify early genes in response to RGA activity, we collected inflorescence apices of ga1-3 rgl2-1 rga-t2 35S:RGA-GR containing floral buds younger than stage 10, which were mock treated or treated with dexamethasone for 4 h. We first compared the transcriptomes in ga1-3 rgl2-1 rga-t2 35S:RGA-GR activated by dexamethasone relative to mock treatment, which revealed the genes responding to dexamethasone and its induced RGA activity (Fig. 1D, Dex vs Mock). Second, we compared the transcriptomes in dexamethasone-treated ga1-3 rgl2-1 rga-t2 35S:RGA-GR relative to those in dexamethasone-treated non-transgenic ga1-3 rgl2-1 rga-t2, which revealed the genes responding to the induced RGA activity and 35S:RGA-GR transgene locus effect (Fig. 1D, Dex vs NT Dex). Only genes showing consistently altered expression in these two comparisons were chosen as RGA-regulated genes. Three sets of biologically independent replicates were collected for microarray analysis. Genes up- or down-regulated by RGA were defined independently as those with a statistically significant change in three treatment/control pairs ($P < 0.05$). We applied a 1.5-fold cutoff for the genes with $P < 0.05$. According to these criteria, 413 RGA up-regulated and 393 RGA down-regulated genes were identified in three biological replicates (Supplemental Tables S1 and S2).

To validate the genes identified in our microarray analysis, we randomly examined the expression of some genes using independent sets of RNA samples prepared from dexamethasone- and mock-treated inflorescence apices of ga1-3 rgl2-1 rga-t2 35S:RGA-GR by semiquantitative reverse transcription (RT)-PCR (Fig. 2). For these selected genes, the relative changes of gene expression in dexamethasone- and mock-treated samples were consistent with those revealed by microarray, although the absolute values for mRNA abundance were not always consistent in both analyses. These RT-PCR results confirmed that the microarray data obtained were reproducible and that the RGA-inducible system coupled with microarray analysis could identify genes whose expression was affected by RGA activity within a short time span.

### Functional Assessment of RGA-Regulated Transcriptome

All of the identified 806 genes were subjected to functional assessment by the NetAffx Gene Ontology Mining Tool (https://www.affymetrix.com/analysis/netaffx/go_analysis_netaffx.affx; Table I). Among the 393 RGA down-regulated genes, 366 genes each had an assigned molecular function based on amino acid homology, and the other 27 genes were unknown. The largest group of down-regulated genes (a total of 171 genes) encoded metabolic enzymes, of which a large number of genes were involved in the remodeling of cell wall structure, such as those...
encoding cell wall proteins (Pro-rich cell wall proteins, extensins, Hyp-rich glycoproteins [HRGPs], Gly-rich proteins [GRPs], expansins), glycoproteins (arabinoxylan, xyloglucan transferase, polygalacturonase), and pectin-related enzymes (Showalter, 1993; Catoire et al., 1998; Micheli, 2001). Compared with the percentage of metabolic genes (around 27%) in the Arabidopsis genome (The Arabidopsis Information Resource; www.arabidopsis.org), the metabolic genes accounted for 44% of total genes down-regulated by RGA during flower development. On the contrary, ratios of the genes encoding transcription factors and proteins with protein- or nucleic acid-binding activity, which are possibly involved in gene regulatory or signal transduction pathways, were lower than those of the whole genome (Table I). The significant overrepresentation of metabolic genes demonstrated a predominantly repressive effect of RGA on cellular metabolism rather than other regulatory pathways during flower development, implying that RGA may directly repress metabolic processes relevant to floral organ development.

Among the 413 RGA up-regulated genes, 380 genes each had an assigned molecular function (Table I). Similar to the RGA down-regulated genes, metabolic genes were overrepresented, accounting for about 35% of total RGA up-regulated genes. One unique feature for RGA up-regulated genes was the overrepresentation of genes encoding transcription factors (11%) and proteins with protein-binding (17%) or nucleic acid-binding (15%) activity, whereas their ratios in the whole genome were only 6%, 7%, and 7%, respectively. These genes included those encoding transcription factors of the zinc finger, MYB, bHLH, WRKY, and bZIP families and those encoding proteins related to the ubiquitination pathway, such as F-box, SKP, SPOP, and RING zinc finger proteins. These results imply that RGA might be able to activate regulatory genes involved in the repression of floral organs during flower development. This is consistent with a recent study showing that DELLA proteins promote the expression of downstream negative components, including putative transcription factors and ubiquitin E2/E3 enzymes, in seedling development (Zentella et al., 2007).

Figure 2. RT-PCR confirmation of RGA-regulated genes from microarray analysis. Expression analysis was performed with total RNA from inflorescence apices of mock-treated (M) or dexamethasone-treated (D) ga1-3 rgl2-1 rga-t2 35S:RGA-GR plants. This analysis was repeated on three independent samples, and a representative gel photo is presented. The b-tubulin gene (TUB2) was amplified as a control. Primer pairs for individual genes are listed in Supplemental Table S10. A, Expression of RGA down-regulated genes in response to RGA activation. B, Expression of RGA up-regulated genes in response to RGA activation.

A previous microarray experiment compared transcriptomes in unopened floral buds of ga1-3, ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1, and wild-type plants (Cao et al., 2006). This study uncovered genes that are differentially expressed in ga1-3, which exhibits retarded growth of floral organs, and ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 and wild-type plants, which have normal floral organs. A comparative analysis revealed that only 47 RGA down-regulated genes (12.0%) or nine RGA up-regulated genes (2.2%) identified in our microarray analysis overlapped with the DELLA down-regulated or up-regulated genes in floral buds identified by Cao et al. (2006; Supplemental Table S3). On the contrary, 120 genes (106 GA up-regulated and 14 GA down-regulated) previously identified as DELLA-independent or partially dependent genes in floral buds were found to be immediately regulated by RGA in our study (Supplemental Table S3). Thus, a majority of genes immediately regulated by RGA during floral organ development, as revealed in our study, are not differentially expressed in loss-of-function mutants. These observations indicate that the method used in this study is more sensitive to uncover DELLA immediate target genes.

In another recent microarray analysis, Zentella et al. (2007) uncovered DELLA-regulated genes in shoots of 8-d-old seedlings by inducing the expression of a dominant GA-insensitive mutant protein, rga-D17. When we compared this list with our list of RGA-regulated genes, only 29 genes (3.6%) overlapped (Supplemental Table S4), demonstrating that DELLA proteins essentially regulate distinct sets of genes in the control of seedling and flower development.

We found that three genes showed consistent responses in the above experiments and our analysis.
**Table I. Gene ontology of RGA-regulated genes during flower development**

RGA-regulated genes were analyzed by the NetAffx Gene Ontology Mining Tool (https://www.affymetrix.com/analysis/netaffx/go_analysis_netaffx4.affx). RGA Up and RGA Down represent RGA up-regulated genes and down-regulated genes, respectively.

| Molecular Function                        | Total | RGA Up | RGA Down |
|------------------------------------------|-------|--------|----------|
| **Catalytic activity**                   |       |        |          |
| Total                                     | 316   | 145    | 171      |
| Hydrolase                                 | 110   | 43     | 67       |
| Transferase                               | 99    | 46     | 53       |
| Oxidoreductase                            | 78    | 29     | 49       |
| Lyase                                     | 19    | 11     | 8        |
| Ligase                                    | 18    | 18     | 0        |
| Isomerase                                 | 5     | 2      | 3        |
| Helicase                                  | 3     | 1      | 2        |
| **Binding activity**                      |       |        |          |
| Total                                     | 333   | 190    | 143      |
| Ion binding                               | 124   | 58     | 66       |
| Protein binding                           | 94    | 69     | 25       |
| Nucleic acid binding                      | 86    | 43     | 43       |
| Nucleotide binding                        | 69    | 48     | 21       |
| Lipid binding                             | 21    | 1      | 20       |
| Tetrapyrrole binding                      | 18    | 2      | 16       |
| Oxygen binding                            | 15    | 2      | 13       |
| Carbohydrate binding                      | 12    | 5      | 7        |
| Co-factor binding                         | 10    | 7      | 3        |
| Vitamin binding                           | 3     | 3      | 0        |
| Drug binding                              | 1     | 0      | 1        |
| Steroid binding                           | 1     | 1      | 1        |
| Hormone binding                           | 1     | 1      | 0        |
| Amine binding                             | 1     | 1      | 0        |
| Selenium binding                          | 1     | 1      | 0        |
| Pattern binding                           | 1     | 1      | 0        |
| **Transporter activity**                  |       |        |          |
| Total                                     | 44    | 17     | 27       |
| **Transcription factor/regulator activity**|       |        |          |
| Total                                     | 58    | 45     | 13       |
| Zinc finger                               | 10    | 9      | 1        |
| MYB                                       | 5     | 3      | 2        |
| WRKY                                      | 3     | 2      | 1        |
| Scarecrow                                 | 3     | 3      | 0        |
| bHLH                                      | 2     | 0      | 2        |
| AP2 domain                                | 2     | 2      | 0        |
| MADS                                      | 1     | 0      | 1        |
| Others                                    | 32    | 26     | 6        |
| **Enzyme regulator activity**             |       |        |          |
| Total                                     | 17    | 4      | 13       |
| **Signal transducer activity**            |       |        |          |
| Total                                     | 16    | 7      | 9        |
| **Structural molecule activity**          |       |        |          |
| Total                                     | 7     | 2      | 5        |
| **Nutrient reservoir activity**           |       |        |          |
| Total                                     | 6     | 1      | 5        |
| **Motor activity**                        |       |        |          |
| Total                                     | 5     | 1      | 4        |
| **Translation regulator activity**        |       |        |          |
| Total                                     | 4     | 3      | 1        |
| **Function not assigned**                 |       |        |          |
| Total                                     | 3     | 2      | 1        |

| **Total no. of RGA-regulated genes**      | 806   | 413    | 393      |

At1g75900, encoding an extracellular lipase (EXL3), was down-regulated by RGA, while At3g63010 and At2g39570, encoding AtGID1b and an ACT domain-containing protein, respectively, were up-regulated by RGA (Supplemental Tables S1–S4). Therefore, it is possible that DELLA proteins constitutively regulate these genes in the whole process of plant development. In particular, AtGID1b, a GA receptor gene in Arabidopsis, has been identified as a direct target of RGA in seedling development (Zentella et al., 2007). This result, together with our observation of the immediate regulation of AtGID1b by RGA, indicates that modulating GA homeostasis is required for both seedling and flower development.

**Expression of RGA-Regulated Genes in Different Floral Organs**

We further investigated the spatial expression of RGA-regulated genes in various floral organs by analyzing our data with the Genevestigator online meta-analysis tool (Zimmermann et al., 2004), which includes a database collection of more than 3,000 array results in Arabidopsis. The largest group of RGA-
regulated genes was highly expressed in stamens and pollen, which occupied 19.4% or 38.2% of total up-regulated or down-regulated genes, respectively (Table II; Supplemental Tables S5 and S6). The second largest group of genes was highly expressed in petals, which occupied 9.9% of RGA-regulated genes (Table II; Supplemental Tables S5 and S6). On the contrary, the percentages of genes enriched in other floral organs, such as carpels, sepals, and pedicels, were relatively low. These results indicate that while RGA affects genes expressed in all floral organs, it mainly regulates the development of stamens and petals. This is well consistent with the previous molecular genetic studies showing that RGA mainly hinders the growth of petals and stamens and the formation of mature pollen during flower development (Cheng et al., 2004; Yu et al., 2004b).

To understand the spatial specificity of expression of RGA-regulated genes, we compared our data with the microarray data set containing genes specifically or predominantly expressed in one type of floral organ (Wellmer et al., 2004; Fig. 3; Supplemental Table S7). Interestingly, 135 of 393 RGA down-regulated genes (34.4%) were specifically or predominantly expressed in stamen, whereas only 19 of 413 RGA up-regulated genes (4.6%) exhibited specific expression in stamen. A low percentage of or even no RGA-regulated genes were specifically expressed in other floral organs. These results strongly suggest that DELLA proteins regulate stamen or pollen development through a group of stamen-specific genes.

We found that RGA down-regulated genes included some previously published stamen-specific genes. A group of oleosin genes encoding GRPs (GRP14, -16, -17, -19, and -20) are expressed in tapetum and pollen coat (Alves-Ferreira et al., 1997; Mayfield et al., 2001; Kim et al., 2002). Genes encoding several family II EXLs (EXL4, -5, and -6) are expressed in pollen coat (Mayfield et al., 2001; Wellmer et al., 2004). A few known stamen-specific gene, AT1g27 (ASK2), is highly expressed in tapetum (Rubinelli et al., 1998). AT2g43230, encoding a putative protein kinase, and At1g61160, encoding the SHAGGY-like kinase ASK2, are predominantly expressed in pollen (Tichtinsky et al., 1998; Wellmer et al., 2004). A few known stamen-specific genes were identified as RGA up-regulated genes. For example, the Arabidopsis STamen Development

| Genes Carpel Petal Sepal Stamen and Pollen Pedicel |
|------------------|---------|---------|---------|-----------------|-------|
| RGA down (393)   | 23 (5.9%) | 39 (9.9%) | 14 (3.6%) | 150 (38.2%) | 21 (5.3%) |
| RGA up (413)     | 5 (1.2%)  | 41 (9.9%) | 30 (7.3%) | 80 (19.4%)  | 23 (5.6%) |

This analysis was performed by Genevestigator (https://www.genevestigator.ethz.ch). The number and ratio of RGA-regulated genes, which are highly expressed in a specific floral organ, are indicated. In this Genevestigator analysis (Supplemental Tables S5 and S6), the expression of a gene at different tissues or organs of whole plants was normalized, with the value of the highest signal intensity set at 100% and the absence of signal set at 0%. We define the high expression of a gene in a specific floral organ as when its expression level reaches at least 75% of the highest signal intensity in the whole plants. The parameters chosen in this analysis were ATH1 22k array (Selected array type), Hierarchical Clustering, Euclidean (Distance), Average Linkage (Linkage), Plant Organs (Meta Analysis), and Linear (Scale Type). RGA up and RGA down represent RGA up-regulated genes and down-regulated genes, respectively.
regulated genes overlapped with JA-responsive genes (Fig. 4; Supplemental Table S8). In total, 134 (16.7%) RGA-regulated genes identified in this study were likely JA-responsive genes involved in stamen development. For example, genes encoding THIONIN2.1 and JASMONIC ACID CARBOXYL METHYLTRANSFERASE, which were induced by JA (Xie et al., 1998; Seo et al., 2001), were immediately down-regulated by RGA in our microarray analysis.

Glucosinolates (GSLs), a group of bioactive secondary metabolites in the Brassicaceae family derived from amino acids, function in plant defense against herbivores and microorganisms (Mikkelsen et al., 2003). Methyl jasmonate treatment up-regulates the levels of indole GSLs and some of the aliphatic GSLs, such as 8-methylthiooctyl GSL and 8-methylsulfinyloctyl GSL. MYB28 (At5g61420, also called PRODUCTION OF METHIONINE-DERIVED GLUCOSINOLATE1), which is a positive regulator for the production of aliphatic GSLs at basal levels in Arabidopsis (Hirai et al., 2007), was down-regulated by both JA and RGA (Supplemental Table S8). In addition, two cytochrome P450 family genes encoding CYP79B2 and CYP83A1, which are involved in the production of indole and aliphatic GSLs (Hull et al., 2000; Bak and Feyereisen, 2001), were also down-regulated by RGA. Identification of these genes involved in GSL production suggests that the interaction between the JA pathway and DELLA proteins may control amino acid metabolism during stamen development.

**RGA-Regulated Genes Are Related to Other Hormones or the Stress Response**

ABA plays an antagonistic role to GA in cereal aleurone and vegetative tissues and interacts with the GA pathway by either acting downstream of DELLA proteins (Gomez-Cadenas et al., 2001; Gubler et al., 2002; Zentella et al., 2002, 2007) or stabilizing DELLA proteins (Achard et al., 2006). Several genes in response to ABA treatment were identified as RGA-regulated genes in our microarray analysis. They included At5g53820 and At5g08350, encoding ABA-responsive proteins, and the genes encoding LATE EMBRYOGENESIS ABUNDANT proteins (At4g13560, At1g52680, At2g46140, and At3g38760; Supplemental Tables S1 and S2). In addition, three putative WRKY transcription factors were also identified in our study. WRKY genes are known to be involved in biotic and abiotic stress responses, senescence and trichome development, and responses of hormones such as ABA (Eulgem et al., 2000). Thus, RGA-mediated regulation of flower development may involve ABA-dependent or related genes.

Ethylene is known to modulate Arabidopsis reproductive growth, such as flower senescence and fruit maturation (Kieber, 1997). These developmental processes could be modulated by DELLA proteins because RGA up-regulated genes identified in this study...
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included several ethylene-related genes (Supplemental Table S1) such as At1g22300, encoding a putative ethylene-responsive calmodulin-binding protein (SR1), and At1g04310, encoding the ethylene receptor ETHYLENE RESPONSE SENSOR2 (ERS2; Hua et al., 1998).

RGA up-regulated genes also included KNAT2 (At1g70510), which is a member of the class I KNOTTED-like homebox (KNOX) transcription factors (Reiser et al., 2000). It has been shown that Arabidopsis KNOX proteins, including KNAT2, activate cytokinin biosynthesis (Jasinski et al., 2005; Yanai et al., 2005), whereas KNOX proteins in tobacco (Nicotiana tabacum), tomato (Solanum lycopersicum), and Arabidopsis could suppress GA biosynthesis (Sakamoto et al., 2001; Hay et al., 2002). Thus, KNOX function seems to be mediated by both cytokinin and GA pathways, and regulation of KNAT2 by RGA may provide an essential link between these two pathways during flower development.

As low concentrations of auxin promote the GA-mediated destabilization of DELLA proteins (Achard et al., 2006), we also compared our microarray data with the auxin-responsive genes in wild-type inflorescence apices (Nagpal et al., 2005). Only two unknown genes, At4g23870 and At1g29510, which were auxin induced and RGA regulated, were identified.

Since DELLA proteins integrate responses to various hormonal and environmental signals of adverse conditions (Achard et al., 2006), we further investigated the relationship between RGA-regulated genes and stress-response pathways by comparing our microarray data with the transcriptionomes controlled by the Snf1-related protein kinase KIN10, which is the central integrator of transcription networks in plant stress and energy signaling in Arabidopsis (Baena-Gonzalez et al., 2007). Interestingly, 118 (28.6%) RGA up-regulated genes overlapped with the genes induced by KIN10, while only eight (2.0%) RGA down-regulated genes were repressed by KIN10. There were few genes that were either RGA down-regulated and KIN10 induced or RGA up-regulated and KIN10 repressed (Supplemental Table S9). As a significant number of RGA up-regulated genes are induced by KIN10, it is likely that RGA and KIN10 act in concert to promote the expression of downstream genes involved in flower development in response to various stress conditions.

Expression Analysis of Immediate Targets of RGA

To identify promising candidates that could mediate RGA’s effect on flower development, we selected genes that either show significant expression changes in our microarray analysis or encode potentially important regulatory proteins for further expression analysis in response to RGA activity. We first examined the time course expression of these genes in the inflorescence apices of ga1-3 rgl2-1 rga-t2 35S:RGA-GR-containing floral buds younger than stage 10 that underwent either dexamethasone or mock treatment for 0, 2, 4, and 8 h. Second, we applied a combined treatment of dexamethasone and cycloheximide, an inhibitor of protein synthesis, at the 4-h time point to identify potential immediate targets of RGA. To examine whether these genes are regulated by GA, their expression was also analyzed in mock- and GA-treated ga1-3 plants.

Quantitative real-time PCR analyses revealed eight genes that were potentially immediate targets of RGA, because the combined treatment of dexamethasone and cycloheximide resulted in a similar increase or decrease in expression of these genes as observed with dexamethasone treatment alone (Fig. 5). Since RGA modulates the expression of these genes independently of protein synthesis, they might be immediate targets of transcriptional regulation by RGA. Although there are some fluctuations in their expression levels, four genes (At3g28830, At3g16750, At3g62950, and At5g49450) showed consistent down-regulated or up-regulated expression in response to the induced RGA activity at different time points compared with their expression at 0 h. Three other genes (At3g62230, At5g61420, and At2g17840) were either down-regulated or up-regulated after 4 h of RGA induction. The expression of At5g23290 was up-regulated by RGA within 4 h of induction but down-regulated afterward.

As expected, the expression of six genes under GA treatment was opposite to that induced by RGA activity under dexamethasone treatment, suggesting that these genes are regulated by DELLA proteins in response to GA. On the contrary, two genes, At3g62230 and At3g62950, exhibited a similar expression trend by induced RGA activity and GA treatment. As GA also affects gene expression in a DELLA-independent mode (Cao et al., 2006), the eventual effect of GA on RGA-regulated genes might reflect the outcome of both the RGA-dependent and -independent pathways.

To study the spatial expression of these genes in floral organs, we performed in situ hybridization on mock- and dexamethasone-treated ga1-3 rgl2-1 rga-t2 35S:RGA-GR flowers. We found that three of eight genes demonstrated altered expression specifically in anthers in response to RGA activity (Fig. 6). At15g49450 (AtbZIP1), a member of the group S1 Arabidopsis bZIP transcription factor family, was immediately up-regulated by RGA (Fig. 5). A previous study has shown that group S1 proteins, including AtbZIP1, can form heterodimers with group C bZIP proteins to directly regulate the Arabidopsis Pro dehydrogenase gene (ProDH; At3g30775; Satoh et al., 2004; Weltmeier et al., 2006). Both AtbZIP1 and ProDH were found as RGA up-regulated and KIN10-induced genes in this study, indicating that AtbZIP1 is probably relevant to RGA regulation of floral organ development in response to stress. In situ localization of AtbZIP1 showed that its expression was weak in all floral organs but immediately induced by RGA in anthers at floral stage 10 (Fig. 6, A–C; Smyth et al., 1990).
mental Table S7) and immediately down-regulated by RGA. In situ hybridization confirmed that At3g62230 was localized in anthers of wild-type and mock-treated ga1-3 rgl2-1 rga-t2 35S:RGA-GR flowers at stage 10 (Fig. 6, D and E). Its expression was specifically restricted in sperm cells of pollen after mitotic division at floral stage 12 (Fig. 6, G and H). Upon dexamethasone treatment, At3g62230 transcripts were rapidly repressed by RGA activity (Fig. 6, F and I). Another gene, MYB28 (At5g61420), which regulates the production of aliphatic GSLs (Hirai et al., 2007), was detected in anthers at floral stage 10 and down-regulated by RGA upon dexamethasone treatment (Fig. 6, J–L). These results suggest that these genes might mediate RGA function in anther or male gametophyte development.

**Functional Characterization of Novel RGA-Regulated Genes**

To understand the function of immediate target genes of RGA, we further characterized two RGA
up-regulated genes, *AtbZIP1* and *At3g62950*, by generating overexpression transgenic plants. Our assumption is that if RGA acts as a growth repressor in flower development, overexpression of its immediately up-regulated genes would more or less compromise floral organ development similar to that exhibited by excessive activity of RGA in *ga1-3*. Over-expression of *AtbZIP1* did not exhibit abnormal phenotypes in floral shape and organization (Fig. 7, A and B). However, anther dehiscence of 35S: *AtbZIP1* was delayed with the release of a considerably reduced number of pollen grains (Fig. 7F) compared with those of the wild type (Fig. 7E) at floral stage 14 (Smyth et al., 1990). Alexander staining of these anthers at floral stage 13 revealed that most of the pollen in 35S: *AtbZIP1* were aberrant without cytoplasmic contents (Fig. 7, I and J). As a result, the siliques of 35S: *AtbZIP1* were short and stunted with some unfertilized ovules (Fig. 7, K and L). These observations suggest that an excessive amount of *AtbZIP1* expression is detrimental to pollen development.

*At3g62950*, encoding a glutaredoxin-like protein, was an immediate target up-regulated by RGA (Fig. 5; Supplemental Table S1). *At5g61420*, encoding MYB28, was expressed strongly in sepals but weakly in other floral organs (Supplemental Table S5). In addition to retarded vegetative growth (data not shown), overexpression of *At3g62950* also impaired floral organ development of
early flowers in the primary inflorescence, resulting in undeveloped petals and stamens and male sterility because of nondehiscent anthers (Fig. 7, C and G). These phenotypes resembled those of ga1-3 (Fig. 7, D and H), implying that alteration of At3g62950 expression may be responsible for floral defects in ga1-3. At least 31 glutaredoxin genes that possess a typical glutathione-reducible CXXC or CXXS active site have been identified in the Arabidopsis genome (Rouhier et al., 2004). ROXY1, a member of the glutaredoxin family, is required for petal development in Arabidopsis (Xing et al., 2005). At3g62950 shares 49% amino acid identity with ROXY1. The overexpression phenotype of At3g62950 suggests that it may play a more extensive role than ROXY1 in flower development.

DISCUSSION

Identification of RGA-Regulated Genes in Flower Development

The DELLA proteins are conserved repressors of GA signaling that play regulatory roles in a wide range of processes during plant growth, including seed germination, root growth, leaf expansion, stem elongation, and the development of reproductive organs (Dill and Sun, 2001; Silverstone et al., 2001; Harberd, 2003; Fleet and Sun, 2005). Several recent studies have shed light on the functional mechanism by which DELLA proteins regulate downstream molecular events in Arabidopsis vegetative development (Zentella et al., 2007; de Lucas et al., 2008; Feng et al., 2008). DELLA proteins play two important roles in Arabidopsis seedling development (Zentella et al., 2007). They maintain GA homeostasis by directly up-regulating the expression of GA biosynthetic and GA receptor genes, and they promote the expression of other negative regulators involved in the transcriptional regulation or proteolysis of downstream GA response components. The interaction between DELLA proteins and phytochrome-interacting factors (PIFs), a group of bHLH-type transcription factors, prevents PIFs from binding to their target gene promoters, thus affecting the expression of genes involved in PIF-mediated light control of plant development such as hypocotyl elongation (de Lucas et al., 2008; Feng et al., 2008).

In this study, we have identified early target genes of DELLA proteins involved in flower development by whole-genome microarray analysis of genes in

Figure 7. Phenotypes of overexpression of AthZIP1 (At5g49450) and At3g62950. A to D, Comparison of inflorescence apices of the wild type (A), 35S:AtbZIP1 (B), 35S:At3g62950 (C), and ga1-3 (D). E and F, Comparison of stage 14 flowers of the wild type (E) and 35S:AtbZIP1 (F). G and H, Comparison of the fully developed early flowers in the primary inflorescence of 35S:At3g62950 (G) and ga1-3 (H). Further development of these flowers is arrested. I and J, Alexander’s staining of pollen viability in stage 13 flowers of the wild type (I) and 35S:AtbZIP1 (J). Arrows in J indicate abnormal pollen without cytoplasmic content. Bars = 50 μm. K and L, Comparison of siliques (K) and developing seeds (L) in wild-type and 35S:AtbZIP1 plants. Arrows in L indicate unfertilized ovules.
response to the steroid-inducible activation of RGA in ga1-3 rgl2-1 rga-t2 35S:RGA-GR inflorescence apices. The microarray results have been confirmed by semi-quantitative RT-PCR analysis of randomly selected genes. Further studies by quantitative real-time PCR coupled with treatment by a protein synthesis inhibitor have revealed eight RGA immediate target genes. Three of them are differentially expressed in anthers in response to RGA, while two of them, when overexpressed in transgenic plants, inhibit floral organ development like DELLA proteins. These results suggest that our RGA-inducible system coupled with microarray analysis is competent at discovering RGA target genes in floral organ development.

**RGA Regulates Cell Expansion during Flower Development**

The flowers of the GA-deficient mutant ga1-3 typically exhibit retarded elongation of petals, stamens, and pistils and arrested anther development. Loss-of-function mutants of DELLA proteins can rescue almost all of these phenotypes (Cheng et al., 2004; Yu et al., 2004b). Microscopic analysis has revealed that the arrest of stamen filament growth in ga1-3 is due to the defective elongation of filament epidermal cells (Cheng et al., 2004), indicating that GA may mainly affect cell expansion rather than cell division in floral organs. Thus, it is possible that DELLA proteins affect the expression of genes involved in cell expansion, a process that is largely dependent on cell wall modification in plants. We have identified a group of RGA-regulated genes that encode proteins involved in the remodeling and modification of cell wall structure, such as Pro-rich cell wall proteins, HRGPs, GRPs, and expansins (Showalter, 1993; Catoire et al., 1998; Micheli, 2001). The expression of these genes is distributed widely in floral organs, including stamens, pollen, carpels, petals, and sepals (Supplemental Tables S5 and S6), indicating that RGA regulation of cell expansion is a general mechanism for various floral organs. Since the majority of genes encoding proteins involved in cell wall structure are RGA down-regulated, RGA may regulate cell expansion mainly in a repressive manner. Furthermore, metabolic genes are significantly overrepresented in both RGA down-regulated and up-regulated genes. This suggests that RGA could closely regulate a large number of metabolic genes in various aspects of flower development, including cell expansion in floral organs, which also requires the acceleration of basic metabolic processes.

**RGA Predominantly Regulates Stamen and Pollen Development**

Although RGA-regulated genes are found in various floral organs, over 38% of RGA down-regulated genes are highly expressed in stamens (Table II), and over one-third of these down-regulated genes are specifically or predominantly expressed in stamens (Supplemental Table S7). These results suggest that DELLA proteins negatively regulate stamen development through a group of stamen-specific genes.

Arabinogalactan proteins (AGPs) are HRGPs that are massively glycosylated and particularly abundant in cell walls, plasma membranes, and extracellular secretions (Showalter, 2001). Previous studies have shown that AGPs are expressed in pollen or tapetum in several plant species and may be related to signal transduction between the tapetum and immature pollen (Southworth and Kwiakowski, 1996; Scutt and Gilmartin, 1998). Seven genes, encoding AGP1, -6, -11, -15, -23, -24, and -40, have been identified as RGA down-regulated genes in our study (Supplemental Table S2), and six of these genes are specifically or predominantly expressed in stamens (Supplemental Table S7). Although the function of AGPs in stamen development remains unclear, specific enrichment of a group of RGA down-regulated AGPs in stamens implies that GA may promote AGPs in stamen development. As AGPs anchored in the plasma membrane positively mediate GA-induced α-amylase production in barley (Hordeum vulgare) aleurome cells (Suzuki et al., 2002), it would be interesting to investigate whether a similar process that results in starch mobilization occurs in stamen development.

In ga1-3, microsporogenesis is arrested at around floral stage 10 prior to pollen mitosis, which is mainly due to the repressive function of RGA and RGL2 (Cheng et al., 2004). We have found that at least one-third of RGA-regulated genes are expressed in the male gametophyte at various stages of microsporogenesis (Supplemental Fig. S1). These include the homologs of several reported genes involved in pollen development. **MALE STERILITY2** (MS2) encodes a fatty acyl reductase acting in the formation of pollen wall substances (Aarts et al., 1997), and **MS5** encodes a protein that has a putative ribonuclease P activity and probably affects male meiosis (Glover et al., 1998). Two genes, At4g33790 and At3g51280, encoding MS2- and MS5-like proteins, respectively, are identified as RGA down-regulated genes. In addition to pollen development, GA is also required for normal pollen tube growth in the sexual reproduction of plants (Singh et al., 2002; Swain et al., 2004). Cellulose, hemicellulose, and pectin are three major carbohydrates making up the primary cell wall in most plant cells, while the wall of the pollen tube tip is only composed of a single layer of pectin. Thus, pectin methylesterases (PMEs) likely play a central role in pollen tube growth (Tian et al., 2006). Our RGA down-regulated list includes a group of genes encoding putative PMEs, such as VANGUARD1 (VGD1; At2g47040) and VGDH1 (At2g47030), both of which are represented by the same probe set identifier in the Affymetrix array, and VGDH2 (At3g62170). VGD1 is expressed specifically in pollen grains and pollen tubes (Jiang et al., 2005). The null vgd1 mutants are normal in female fertility and are able to undergo pollen germination. However, the penetration rate of vgd1 pollen tubes is reduced sig-
significantly, resulting in smaller and shorter siliques with fewer seeds compared with wild-type plants. Two other PME genes, AtPPME1 (At1g69940) and At5g07410, represented by the same probe set identifier by RGA. AtPPME1 plays a role in determining the shape of the pollen tube and the rate of its elongation (Tian et al., 2006). Identification of a group of RGA down-regulated PME genes in unpollinated floral buds indicates that GA is required to relieve RGA repression of these genes during pollen development so that the pollen tube can elongate normally after pollination.

Taken together, these results suggest that RGA predominately represses stamen development during flower development and is involved in all phases of microsporogenesis and pollen tube growth.

Interaction of RGA and Phytohormones during Flower Development

JA regulates at least three developmental pathways in stamen maturation: filament elongation, anther dehiscence, and pollen viability (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001). It was shown recently that the floral homeotic gene AGAMOUS regulates late stamen maturation by directly controlling a JA biosynthetic gene, DEFECTIVE IN ANther DEHISCENCE1 (Ito et al., 2007). Over 16% of RGA-regulated genes in our microarray list are JA-responsive genes involved in stamen development (Mandaokar et al., 2006), suggesting that the cross talk between DELLA proteins and JA could regulate stamen development. As metabolic pathways related to cell wall rearrangement are highly active in developing stamens, both RGA and JA regulate the accumulation of transcripts putatively involved in the remodeling and modification of cell walls, which include a group of genes encoding GRPs and EXLs (Showalter, 1993; Mayfield et al., 2001; Mandaokar et al., 2006). In addition, RGA and JA may interact to affect amino acid metabolism during stamen development. MYB28, which is down-regulated by both RGA and JA, is a master transcription factor regulating the pathway from Met to aliphatic GSLs and is essential for the biosynthesis of aliphatic GSLs at a basal level in Arabidopsis (Hirai et al., 2007). Its expression in flowers is down-regulated after 4 h of RGA induction, which occurs even in the presence of the protein synthesis inhibitor (Fig. 5), indicating that it is an immediate target of transcriptional regulation by RGA. In the flower at stage 10, MYB28 expression is specifically localized in anthers and immediately down-regulated by induction of RGA activity (Fig. 6, J–L). Thus, RGA could closely regulate MYB28 to affect amino acid metabolism during stamen development. As JA treatment has been shown to regulate the levels of aliphatic GSLs and MYB28 is JA responsive (Mikkelsen et al., 2003; Mandaokar et al., 2006), it is possible that MYB28 is a central regulator that mediates the interaction between DELLA proteins and the JA pathway.

Ethylene has been proposed to act similarly to or possibly in concurrence with jasmonic acid as a signaling molecule controlling anther dehiscence in tobacco, because ethylene insensitivity causes the delayed degeneration of the stomium cells and dehydration (Rieu et al., 2003). Anther dehiscence involves the processes of thickening of secondary cell walls, consecutive degeneration of various anther tissues, changes in carbohydrate metabolism, and movement of water out of the anther (Keijzer, 1987; Clément and Audran, 1995; Bots et al., 2005). GA deficiency or overdose produces nondehiscent anthers in various plant species (Colombo and Favret, 1996; Murray et al., 2003; Zhang et al., 2007), indicating that a fine-tuned GA signaling is important for anther dehiscence. RGA-regulated genes include some genes involved in water or osmotic stress, such as aquaporin-like genes, dehydrins, and Pro oxidase genes. In addition, IRREGULAR XYLEM13 (IRX13; At5g03170), which probably functions in the differentiation of tracheary elements (Mitsuda et al., 2005), has been identified as an RGA down-regulated gene. IRX13, encoding a fasciclin-like AGP, is up-regulated by a transcription factor, NAC SECONDARY WALL THICKENING PROMOTING FACTOR1, that controls secondary wall thickening required for anther dehiscence (Mitsuda et al., 2005). These results suggest that RGA might be involved in the regulation of water movement and cell wall thickening, thus affecting anther dehiscence. Since RGA up-regulated genes include those encoding the regulators in the ethylene signaling pathway, such as ERS2 and SR1, ethylene control of anther dehiscence would be more or less modulated by RGA. On the other hand, ethylene inhibits root growth and flowering in Arabidopsis partly by enhancing the accumulation of DELLA proteins (Achard et al., 2003, 2006, 2007). Thus, anther dehiscence could also be mediated through the effect of ethylene on the stabilization of DELLA proteins. Further analysis will be required to elucidate how the interaction between DELLA proteins and ethylene signaling affects genes involved in anther dehiscence.

DELLA proteins have been proposed to play a key integrative role in the phytohormone signal response network (Achard et al., 2003, 2006; Fu and Harberd, 2003). In agreement with this, our results suggest that the function of DELLA proteins in floral organ development is mediated by other phytohormones in addition to GA. In particular, DELLA proteins likely at least interact with JA and ethylene signaling pathways to regulate stamen development.

RGA Regulates Flower Development by Activating Stress-Related Genes

It has been suggested that GA-promoted destabilization of DELLA proteins integrates plant responses to environmental signals under adverse conditions and
that the growth restraint conferred by DELLa proteins promotes survival, probably via the redirection of resources (Achard et al., 2006). It is unknown how this mechanism would affect the function of DELLa proteins in the control of flower development. Two Snf1-related protein kinases, KIN10 and KIN11, have been identified as central regulators that coordinate stress, sugar, and developmental signals to globally regulate plant metabolism, energy balance, growth, and survival in Arabidopsis (Baena-Gonzalez et al., 2007). KIN10 targets a remarkably broad set of genes that orchestrate transcription networks, promote catabolism, and suppress anabolism, which is necessary for plants to restrain their growth and eventually survive under stress. Our comparative analysis has revealed that over 28% of RGA up-regulated genes are induced by KIN10. This result suggests that DELLa proteins and KIN10 may partly mediate similar plant responses to adversity. As alteration of KIN10/11 and DELLa expression affects flower development in Arabidopsis (Cheng et al., 2004; Yu et al., 2004b; Baena-Gonzalez et al., 2007), they may act synergistically to promote the expression of a similar set of downstream genes involved in flower development as part of the plant response to stress.

Among the RGA up-regulated and KIN10-induced genes, AtbZIP1 (At5g49450) and At5g22920 are immediate targets of RGA, because their expression is up-regulated by RGA in the presence of the protein synthesis inhibitor (Fig. 5). AtbZIP1 expression is weak in wild-type floral organs but immediately induced by RGA in anthers at floral stage 10 (Fig. 6, A–C). A previous study has shown that AtbZIP1 forms a heterodimer with group C bZIP proteins to activate the expression of ProDH (At3g30775), which is induced in response to hypoosmotic conditions occurring during rehydration after stress recovery (Satoh et al., 2004; Weltmeier et al., 2006). Interestingly, both AtbZIP1 and ProDH are RGA up-regulated and KIN10-induced genes. The observations of specific expression of AtbZIP1 in anthers and delayed anther dehiscence in 35S:AtbZIP1 indicate that the activity of AtbZIP1 and ProDH regulated by RGA and KIN10 may be relevant to water movement during anther development, in which anthers first take up water for growth but dehydrate before dehiscence (Bots et al., 2005).

As key repressors of GA-responsive growth, DELLa proteins accumulate in the nucleus and are rapidly degraded in response to GA (Silverstone et al., 2001; Fu et al., 2002). A key question regarding the function of DELLa proteins, which do not have a DNA-binding domain, is how they regulate downstream genes, thus mediating the growth restraint on plants. Recent studies of the interaction between RGA and bHLH-type transcription factor PIFs have shed light on this question, with the first piece of evidence showing that RGA interacts with key transcription factors to control the expression of a group of relevant genes (de Lucas et al., 2008; Feng et al., 2008). When we compared our microarray data with those known PIF-regulated genes (data not shown), very few genes were found to overlap, suggesting that DELLa function may depend on various transcription factors to regulate diverse developmental programs. This is supported by another study showing that RGA is consistently associated with the promoters of eight DELLa-responsive genes (Zentella et al., 2007). Since these genes do not contain consensus sequences in RGA-associated regions, various transcription factors are likely involved in mediating RGA function.

Our microarray analysis has identified almost a similar number of genes that are up-regulated or down-regulated by RGA within a short time span. At least four genes have been found to be immediately up-regulated by RGA even in the presence of the protein synthesis inhibitor (Fig. 5), suggesting that RGA could intrinsically activate them independently of protein synthesis. As the change of expression of RGA-regulated genes could be mediated by RGA-interacting transcription factors, the exact function of DELLa proteins in the transcription regulatory complex needs to be further investigated.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type and transgenic Arabidopsis (Arabidopsis thaliana) plants of the same Landsberg erecta ecotype were grown at 22°C under long days (16 h of light/8 h of dark). Generation of gat-3 rgl2-1 rga-12 35S:RGA-GR transgenic plants was as described previously (Yu et al., 2004b). Seeds with gat-3 background were imibed in 100 μM GA3 at 4°C for 7 d to break dormancy and rinsed thoroughly with water before sowing.

Tissue Collection for Microarray Analysis

Inflorescence apices of gat-1-3 rga-12 rga-2-1 rga-12 35S:RGA-GR transgenic plants were mock treated (0.03% ethanol and 0.015% Silwet L-77) or treated with 10 μM dexamethasone and 0.015% Silwet L-77. As a control, inflorescence apices of gat-1-3 rga-12 rga-12 nontransformed plants were also treated under the same conditions. After 4 h, the treated inflorescence apices containing floral buds younger than stage 10 were collected and immediately frozen in liquid nitrogen. Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen), and its quality was assessed by gel electrophoresis. To ensure that dexamethasone treatment of these plants was effective, some dexamethasone- and mock-treated plants were continuously cultivated to observe the phenotypic change of floral organs.

Microarray Experiments and Data Analysis

Three sets of biologically independent replicates were used for each treatment. Total RNA (5 μg) extracted from each set of samples was used for cDNA synthesis followed by copy RNA (cRNA) labeling and fragmentation according to the manufacturer’s instructions using the One-Cycle Target Labeling and Control Reagents Kit (Affymetrix). Fragmented cRNAs were hybridized on the Affymetrix Arabidopsis ATH11 high-density gene array for 16 h followed by washing and staining using GeneChip Fluidics Station 450. GeneChip Scanner was used to scan the probe arrays, and signals were obtained by GeneChip Operating Software (GCOS). The GCOS-generated data files (CHP file) were imported into GeneSpring GX 7.3.1 (Agilent) for further analysis. All samples were normalized per chip to the 50th percentile and per gene to median signals. Cross-gene error model was used in the data interpretation. Genes were filtered by Affymetrix flags to appear as “present” or marginal in at least 6 out of 9 profiles. This reduced 22,810 total genes to 13,622 genes. Up- or down-regulated genes were defined independently as those with a statistically significant change in three treatment/control pairs.
(P < 0.05). We first compared the transcriptomes in ga1-3 rgl2-1 rga-t2 35S:RGA-GR activated by dexamethasone treatment relative to mock treatment and then compared the transcriptomes in dexamethasone-treated ga1-3 rgl2-1 rga-t2 35S:RGA-GR relative to those in dexamethasone-treated non-transgenic ga1-3 rgl2-1 rga-t2. Only genes that consistently showed altered expression over 1.5-fold in these two comparisons in three biological replicates were chosen as RGA-regulated genes. For meta-analysis with other data sets, Microsoft Excel was used to manage and filter the microarray data. Spatial analysis of gene expression in various floral organs was performed online by Genevestigator (Zimmermann et al., 2004; https://www.genevestigator.ethz.ch/at/).

Quantitative Real-Time PCR and Semiquantitative RT-PCR

For the time course experiments, inflorescence apices of ga1-3 rgl2-1 rga-t2 35S:RGA-GR plants containing floral buds younger than stage 10 were collected at 0, 2, 4, and 8 h after a single mock or dexamethasone treatment. To examine the effect of cycloheximide (10 μM) and GA4 (100 μM) on gene expression, inflorescence apices were collected 4 h after a single treatment. Total RNAs were isolated using RNeasy RNA isolation kit (Qiagen) and reverse transcribed by the ThermoScript RT-PCR system (Invitrogen). Quantitative real-time PCR assays were performed in triplicate on a 7900HT Fast Real-Time PCR system (Applied Biosystems) using TUBULIN2 (TUB2) as an internal control as described previously (Liu et al., 2007). The efficiency of each pair of primers was determined based on its standard curve obtained from a series of 10-fold diluted template DNAs. The difference between the cycle threshold (Ct) of target genes and the Ct of control primers (Ct(target) - Ct(control)) was used to obtain the normalized expression of target genes. Each experiment was repeated three times using samples collected separately. Semiquantitative RT-PCR was performed as described previously (Yu et al., 2004b). Primers used for gene expression analysis are listed in Supplemental Table S10.

Plasmid Construction and Plant Transformation

To generate overexpression constructs of RGA-regulated genes, the coding regions of the selected genes were amplified from inflorescence cDNAs using primers with adaptors containing appropriate restriction sites (Supplemental Table S10). The resulting PCR products were digested with restriction enzymes and cloned into the corresponding sites of pGreen-35S (Yu et al., 2004a). The overexpression constructs were transformed into wild-type Landsberg erecta plants via Agrobacterium tumefaciens-mediated transformation, and the transformants were screened by Basta selection.

In Situ Hybridization

Nonradioactive in situ hybridization experiments were performed as described previously (Yu et al., 2004a). Primers used for the generation of digoxigenin-labeled antisense cRNA probes are listed in Supplemental Table S10.

Raw and processed microarray data are available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the series identifier GSE10019.

Supplemental Data

The following materials are available in the online version of this article.
Supplemental Figure S1. Expression of RGA-regulated genes at different pollen developmental stages.
Supplemental Table S1. List of genes up-regulated by RGA during flower development.
Supplemental Table S2. List of genes down-regulated by RGA during flower development.
Supplemental Table S3. Comparison of RGA-regulated genes revealed in this study with DELLA-dependent or -independent genes in floral buds.
Supplemental Table S4. Comparison of RGA-regulated genes revealed in this study with DELLA-responsive genes in young seedlings.

Supplemental Table S5. Spatial expression of RGA up-regulated genes in various floral organs analyzed by Genevestigator (https://www.genevestigator.ethz.ch).
Supplemental Table S6. Spatial expression of RGA down-regulated genes in various floral organs analyzed by Genevestigator (https://www.genevestigator.ethz.ch).
Supplemental Table S7. List of RGA-regulated genes that are specifically or predominantly expressed in various floral organs.
Supplemental Table S8. Comparison of RGA-regulated genes revealed in this study with JA-responsive genes in stamen development.
Supplemental Table S9. Comparison of RGA-regulated genes revealed in this study with KIN10-regulated genes.
Supplemental Table S10. List of primers used in this study.

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