Molecular Mechanism of Gibberellin Signaling in Plants

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■ **Abstract**

The hormone gibberellin (GA) plays an important role in modulating
diverse processes throughout plant development. In recent years, significant progress
has been made in the identification of upstream GA signaling components and *trans*-
and *cis*-acting factors that regulate downstream GA-responsive genes in higher plants.
GA appears to derepress its signaling pathway by inducing proteolysis of GA sig-
naling repressors (the DELLA proteins). Recent evidence indicates that the DELLA
proteins are targeted for degradation by an E3 ubiquitin ligase SCF complex through
the ubiquitin-26S proteasome pathway.

Key Words GA-responsive genes, hormone signal transduction, SCF complex,
DELLA proteins, GARC

INTRODUCTION

Bioactive gibberellins (GAs) are plant hormones that promote stem and leaf growth. In some species, GAs also induce seed germination and modulate flowering time and the development of flowers, fruits, and seeds. The GA perception and signal transduction pathway converts the GA signal into alterations in gene expression and plant morphology. Although the GA receptor has not been identified, significant progress has been made recently in identifying upstream GA signaling components and *trans-* and *cis-*acting factors that regulate downstream GA-responsive genes in higher plants. Accumulating evidence indicates that there is a close interaction between GA metabolism and GA response pathways to maintain GA homeostasis in plants. There are several recent reviews on GA metabolism and/or GA signaling pathways (49, 50, 73, 84, 97, 117, 135). This review highlights the newly discovered molecular mechanism of GA-induced proteolysis of GA signaling repressors, and the recent microarray and biochemical studies that have identified new GA-responsive genes and factors that regulate transcription of these genes.

GA SIGNALING COMPONENTS IDENTIFIED BY GA-RESPONSE MUTANTS

GA-Response Mutants

To identify GA signaling components, mutants with altered stem heights in a variety of species have been isolated (reviewed in 102, 117). Mutants with constitutively active GA responses have a slender and paler-green phenotype, which mimics wild-type plants that are overdosed with GA. Mutants that are impaired in the GA signaling resemble the GA biosynthesis mutants, i.e., dark-green dwarf with compact leaves, and some with reduced fertility. But their phenotype cannot be rescued by GA treatment. Several additional mutant screens have also been employed to isolate new GA-response mutants. These screens include (*a*) mutations that confer altered sensitivity to GA biosynthesis inhibitors (61), (*b*) suppressor mutations that rescue the dwarf phenotype in a GA-deficient background (112) or a reduced GA-response background (133), (*c*) mutations that alter expression of a GA-responsive promoter-reporter fusion gene (77, 92), and (*d*) suppressors

of an ABA-insensitive mutant *abi1* (115). These genetic approaches have allowed researchers to clone a number of genes that encode positive and negative regulators of GA signaling.

Positive Regulators of GA Signaling

Characterization of loss-of-function (recessive), GA-unresponsive dwarf mutants has identified several positive regulators of GA signaling. The *dwarf1* (*d1*) (79) and *GA-insensitive dwarf2* (*gid2*) (106) mutants in rice and the *sleepy1* (*sly1*) (115) mutant in *Arabidopsis* have a semidwarf phenotype, but they cannot be rescued by GA treatment. Pharmacological studies in cereal aleurones suggest that the heterotrimeric G protein plays a role in GA signaling (62). This hypothesis is supported by the finding that *D1* encodes a putative α-subunit of the heterotrimeric G protein (2, 36). However, an alternative GA signaling pathway must exist because the *d1* null mutant is not as dwarf as a severe GA biosynthetic mutant, even though *D1* seems to be a single gene in the rice genome (127). In *Arabidopsis*, $G\alpha$ is also encoded by a single gene, *GPA1*. The null *gpa1* alleles are less responsive to GA in seed germination (128). However, unlike the rice *d1* mutant, the *gpa1* mutants have a normal final plant height (128). Therefore, the heterotrimeric G proteins may function differently in various species.

GID2 and *SLY1* encode homologous F-box proteins, which are likely to be a subunit of the SCF complex, a class of the ubiquitin E3 ligases (76, 106). In addition to the F-box protein, the SCF complex contains SKP, Cullin, and a RING-domain protein Rbx (23, 53). Binding of the F-box proteins to SKP1 in the SCF complexes is via the conserved N-terminal 40–60–amino acid F-box domain. The C-terminal protein-protein interaction domain of the F-box protein is responsible for recruiting specific target proteins to the SCF E3 complex for ubiquitination and subsequent degradation by the 26S proteasome. Recently, SCF-mediated proteolysis has been shown to regulate many developmental processes in plants, including floral development, circadian rhythm, light-receptor signaling, senescence, and hormone signaling (reviewed in 15, 47, 53, 129). GID2 and SLY1 appear to modulate the GA responses by controlling the stability of a class of negative regulators SLR/RGA of GA signaling (see next section for details).

PICKLE (*PKL*), another putative positive regulator of GA signaling in *Arabidopsis*, encodes a putative CHD3 chromatin-remodeling factor (82). The semidwarf phenotype of the *pkl* mutants resembles other GA-response mutants (81). Interestingly, *pkl* has a unique embryonic root phenotype that is not present in other GA-biosynthesis or -response mutants (81). The root phenotype has a low penetrance in the *pkl* mutants. But treatment with a GA-biosynthesis inhibitor [paclobutrazol (PAC) or uniconazole (UNI)] increases the penetrance of this phenotype, whereas applications of GA have an opposite effect. These results suggest that PKL may mediate GA-induced root differentiation during germination. Recently, several embryonic identity genes have been shown to be upregulated in the *pkl* seedlings, suggesting that PKL normally represses expression of these genes

during seed germination (98). However, expression of these genes is not affected by UNI treatment. Future characterization of genes whose expression is affected by both GA and the *pkl* mutation will help to elucidate the relationship between PKL and GA signaling.

Photoperiod-Responsive 1 (*PHOR1*) in potato was isolated as a gene whose transcript level increased in leaves under short-day conditions (1). Inhibition of *PHOR1* expression in transgenic potato using an antisense *PHOR1* construct results in a semidwarf phenotype and a reduced GA response in elongating internodes. Overexpression of PHOR1 confers a longer internode phenotype and an elevated GA response, further supporting the role of PHOR1 as an activator of GA signaling. PHOR1 contains seven armadillo repeats, which are present in armadillo and β-catenin proteins, components of Wnt signaling in *Drosophila* and vertebrates (1). In tobacco BY2 cells, GA stimulates nuclear localization of transiently expressed PHOR1-GFP from the cytoplasm. These results suggest that PHOR1 may activate transcription of GA-induced genes.

Negative Regulators of GA Signaling

Several negative regulators of GA signaling have been isolated by characterization of the recessive (loss-of-function) slender mutants and the dominant (gain-of-function) GA-unresponsive dwarf mutants. One of the slender mutants, *spindly* (*spy*) in *Arabidopsis*, was first identified as a mutant seed that germinated in the presence of PAC (61). Additional *spy* alleles have been isolated as suppressors of the GA-deficient mutant *ga1-3* and a GA-unresponsive dwarf *gai-1* (112, 133). The null *ga1-3* mutant is a nongerminating, male-sterile dwarf because *GA1* encodes an enzyme for the first committed step in GA biosynthesis. Recessive *spy* alleles partially rescue all the phenotypes in *ga1-3*, indicating that SPY inhibits an early step in GA signaling. Transient expression of the SPY barley homolog (HvSPY) in aleurone protoplasts inhibits GA-induced α-amylase gene expression (99), further supporting the role of SPY in GA response. The SPY sequence is highly similar to that of the Ser/Thr *O*-linked N-acetylglucosamine (*O*-GlcNAc) transferases (OGTs) in animals (60, 101). OGTs contain the tetratricopeptide repeats (TPRs) near their N termini and a C-terminal catalytic domain. Animal OGTs modify target proteins by attaching GlcNAc moiety to Ser/Thr residues, which may interfere or compete with kinases for phosphorylation sites (22). *O*-GlcNAc modification is a dynamic protein modification, which is implicated in regulating many signaling pathways (131). Purified recombinant SPY protein has OGT activity in vitro (124). Some *spy* phenotypes (e.g., abnormal phylotaxy of flowers) are not present in GA-treated wild-type *Arabidopsis*, indicating that SPY also regulates additional cellular pathways (120). A second OGT gene [*SECRET AGENT* (*SEC*)] in *Arabidopsis* was isolated recently (48). Although the *sec* mutant has no obvious phenotype, the *spy sec* double mutant is synthetic lethal in gamete and seed development. Like animal OGTs, the active SPY probably functions as a homotrimer, formed by protein-protein interaction via the TPRs. Overexpression

of the SPY TPRs in transgenic *Arabidopsis* and petunia confers a *spy*-like pheno-type, suggesting that elevated TPRs alone may block the SPY function by forming inactive heterodimers with SPY and/or by interacting with the target proteins of SPY (58, 126). Because SPY is detected in both cytoplasm and nucleus in plant cells, similar to the localization of animal OGTs, the target proteins of SPY could be present in both cellular compartments (121).

Another negative regulator of GA signaling, SHORT INTERNODES (SHI) in *Arabidopsis*, has been identified by the dwarf phenotype of the dominant *shi* mutant that overexpressed the *SHI* gene (31). SHI contains a zinc-finger motif, suggesting its potential role in transcriptional regulation or ubiquitin-mediated proteolysis. Transient expression of SHI in barley aleurone cells inhibits GA induction of α-amylase expression, further supporting its role in GA signaling (32). However, the loss-of-function *shi* alleles show no obvious phenotype, probably owing to functional redundancy of several homologous genes in *Arabidopsis* (32).

The DELLA proteins, a class of GA signaling repressors, are highly conserved in *Arabidopsis* (RGA, GAI, RGL1, RGL2, RGL3) and several crop plants, including maize (d8), wheat (Rht), rice (SLR1), barley (SLN1), and grape (VvGAI) (8, 84). The DELLA proteins belong to a subfamily of the plant-specific GRAS protein family (91). The *Arabidopsis* genome contains over 30 GRAS family members, all of which have a conserved C-terminal region (123). Their N termini are more divergent, however, and probably specify their roles in different cellular pathways. A conserved and unique domain (named DELLA, after a conserved amino acid motif) is present near the N terminus of the DELLA proteins (85, 110). The other GRAS proteins with known functions are also regulators of diverse developmental pathways, including radial patterning (26, 52), axillary meristem formation (71, 107), shoot meristem maintenance (116), and phytochrome A signaling (7). The DELLA proteins are likely to function as transcriptional regulators (85, 110). They contain (*a*) polymeric Ser/Thr motifs (poly S/T), which could be targets of phosphorylation or glycosylation; (*b*) Leu heptad repeats (LHR), which may mediate protein-protein interactions; (*c*) putative nuclear localization signals (NLS); and (*d*) a putative SH2 phosphotyrosine binding domain (87) (Figure 1). Several DELLA proteins direct the GFP fusion protein into plant cell nuclei (reviewed in 84), and the GFP-RGA and SLR1-GFP fusion proteins can function to rescue the phenotype of the *rga/ga1-3* or the *slr1* mutant, respectively (57, 111). Although the DELLA proteins do not have a clearly identified DNA-binding domain, they may act as coactivators or corepressors by interacting with other transcription factors that bind directly to the DNA sequence of GA-regulated genes.

RGA and GAI in *Arabidopsis* were the first two DELLA proteins to be identified. The loss-of-function *rga* alleles partially suppress most of the phenotype of the GA-deficient mutant *ga1-3*, except seed germination and floral development (112). The gain-of-function (semidominant) *gai-1* mutant has a GA-unresponsive dwarf phenotype (66). Subsequently, the loss-of-function *gai-t6* allele has been shown to confer resistance to PAC in vegetative growth (85). These genetic studies indicate that RGA and GAI are negative regulators of GA response. Cloning of

GAI and *RGA* reveals that their encoded proteins share 82% identity in their amino acid sequences (85, 110). RGA and GAI have partially redundant functions in maintaining the repressive state of the GA signaling pathway, but RGA plays a more predominant role than GAI. The *rga* and *gai* null alleles interact synergistically in processes partially rescued by *rga*, although the *gai* null allele alone has little effect (29, 64). Removing both RGA and GAI function allows for a complete derepression of many aspects of GA signaling, including rosette leaf expansion, flowering time, and stem elongation. Three additional DELLA proteins, RGL1, RGL2, and RGL3 (for RGA-like), are present in *Arabidopsis* (29, 86). *Arabidopsis* transgenic lines expressing a dominant *35S::rgl1* transgene (containing the DELLA motif deletions) exhibit a GA-unresponsive dwarf phenotype (132). In addition to modulating vegetative growth, RGL1 may also affect seed germination because a transgenic line in which *RGL1* expression is silenced is resistant to PAC during germination (132). However, in a separate study, Lee et al. (69) isolated one *rgl1* and three *rgl2* Ds insertion mutants. Only the *rgl2* alleles, not the *rgl1* allele, confer PAC resistance during seed germination (69). The discrepancy between these two studies can be explained by two possibilities. First, the PAC-resistant phenotype in germinating seeds of the silenced *rgl1* line could be due to cosuppression of the *RGL2/RGL3* genes because expression of these genes was not examined in the mutant seed. Alternatively, the *rgl1* allele with a Ds insertion (69) may be a weak allele because the Ds insertion is located 68 bp upstream of the ATG translational start codon. However, the unpublished RNA blot data indicate that this insertion eliminates *RGL1* gene expression (69). Taken together, the role of RGL1 in seed germination needs to be re-examined. Also, the role of RGL3 in GA signaling and the specific repressors that are responsible for modulating flower development have not been determined.

Studies of RGA/GAI orthologs in several crops indicate that the function of these DELLA proteins in repressing GA signaling is highly conserved between dicots and monocots. Interestingly, unlike *Arabidopsis*, only one RGA/GAI functional ortholog is present in rice [SLR1 (55)] and another in barley [SLN1 (18)]. Consequently, GA-independent stem growth is achieved by removing only SLR1 or SLN1, respectively, in these species. It is not clear why *Arabidopsis* contains five DELLA proteins. Perhaps multiple functionally overlapping genes are required for fine-tuning GA-regulated development in dicots. Isolation and characterization of DELLA proteins in additional species will allow us to determine whether the differences in the DELLA-protein copy numbers correlate with monocot versus dicot division.

Additional Components of GA Response Pathway

Several putative GA-response *Arabidopsis* mutants have been isolated using GA-responsive promoter-reporter fusion genes as markers. One screen used a mutagenized transgenic line that carried the dual reporters, i.e., GA-inducible promoter *GASA1-GUS* and *GASA1-luciferase* (*LUC*) fusion genes (92). Another screen

employed *GA20ox1* promoter–*LUC* fusion gene (77). Expression of the *GA20ox1* promoter is feedback inhibited by GA (89). Some of these mutants show altered sensitivity to GA and to an inhibitor of GA biosynthesis, and/or have altered expression of GA feedback-regulated genes *GA20ox1* or *GA3ox1*. But the overall phenotypes of these mutants do not completely resemble the GA-unresponsive dwarf or slender mutants (described in previous sections). Three *LUC-superexpressor* mutants (*leu1*, *fpa1-3*, and *fpa1-4*), isolated from the *GA20ox1-LUC* mutant screen, have been characterized in more detail (77). Although *leu1* is a semidwarf, it has light-green leaves and produces rounder seeds. The mRNA levels of *GA20ox1* and *GA3ox1* are elevated in *leu1*, supporting the conclusion that the semidwarf phenotype is the result of impaired GA response. Surprisingly, GA treatment of *leu1* further increases *GA20ox1* and *GA3ox1* expression. *leu1* is allelic to *fragile fiber2*, and *LEU1* encodes a katanin p60 subunit, a microtubule-severing AAA-ATPase (AtKSS) (9). Mutations in this gene result in random orientations of the cortical microtubules (CMT) in elongating cells and cause defective cell elongation (11). Additionally, cellulose microfibril deposition is abnormal in *fra2/leu1* (9, 12). AtKSS is likely to play a role in GA-induced cell elongation because GA promotes transverse orientation of CMT in elongating cells (109). Consistent with this hypothesis, elongation defects of stems and leaves of the *leu1* mutant are not rescued by GA, whereas flowering time of *leu1* is similar to wild type, and *LEU1* mRNA accumulation is induced by GA (9). The *fpa1-3* and *fpa1-4* mutants exhibit a more elongated seedling phenotype, and seed germination of *fpa1-4* is less sensitive to GA-biosynthesis inhibitor, indicating that these mutants may have increased GA responses. However, they showed a delayed flowering phenotype and are allelic to previously isolated late-flowering mutants *fpa1*. FPA1 is a positive regulator in the autonomous (photoperiod-independent) pathway that controls flowering time. GA promotes flowering, in parallel to the autonomous pathway, which also interacts with the GA pathway to accelerate flowering (95). The role of FPA in GA response and floral induction requires further investigation.

Derepression of GA Signaling by GA-Induced Degradation of the DELLA Proteins

The uniqueness of the DELLA domain hints that this region may specify the role of the DELLA subfamily of GRAS proteins in GA response. Recent studies of the dwarf mutants containing the mutations in the DELLA domain illustrate that this domain is important for the inactivation of the DELLA proteins by the GA signal. The initial evidence came from the discovery that the gain-of-function *gai-1* allele contains an in-frame deletion in the *GAI* gene, which results in the loss of 17 amino acids spanning the DELLA motif (85). Peng et al. (85) hypothesized that deletions in the gai-1 protein make it a constitutive repressor of GA response. Similar internal deletions or N-terminal truncations in other DELLA proteins in different species also results in a GA-unresponsive dwarf phenotype (reviewed in 84). The most notable example is the semidwarf wheat cultivars that greatly

facilitated an increased grain yield during the Green Revolution in the 1960s and 1970s. All these wheat cultivars contain deletions in the DELLA domain of an *Rht* (for reduced height) gene (87). Studies of *sln1* in barley and *Vvgai* in grape also showed that single amino acid substitutions around the DELLA motif confer a GA-unresponsive dwarf phenotype (8, 18).

Studies of the effect of GA on *RGA* gene expression first revealed the molecular mechanism by which GA inactivates the DELLA-protein function. Immunoblot analysis and confocal microscopy demonstrate that the levels of both the RGA and GFP-RGA proteins are reduced rapidly by GA treatment (111), although GA only causes a slight increase in the amount of the *RGA* mRNA in *Arabidopsis* seedlings (110). Similar results have also been obtained from studies of SLR1 in rice (57) and SLN1 in barley (43). Nevertheless, the GA signal may regulate other DELLA proteins via different mechanisms. For example, GFP protein fusions with RGL1 or GAI remain stable after GA treatment (30, 132). However, these results need to be verified by analyzing the endogenous RGL1 and GAI proteins.

The DELLA Domain and C Terminus of the DELLA Proteins are Essential for Their Degradation

As described above, deletions within the DELLA domain of the DELLA proteins result in a GA-unresponsive dwarf phenotype. The sequences missing in *gai-1* are identical between GAI and RGA, and an identical mutation (*rga-Δ17*) in the *RGA* gene (with or without the *GFP* fusion) conferred a GA-insensitive severe dwarf phenotype in the transgenic *Arabidopsis* (28). The finding of GA-dependent rapid reduction of RGA protein accumulation prompted the question, Does GA treatment affect the rga-Δ17 protein accumulation? Immunoblot analysis and confocal microscopy showed that the levels of rga-Δ17 and GFP-(rga-Δ17) proteins were unaffected by GA treatment (28). Thus, GA-inducible RGA protein disappearance depends on the presence of the DELLA motif. These results also support the conclusion that GA-induced protein degradation, rather than reduced translational rate, leads to the rapid disappearance of the RGA protein. Mutations in the DELLA motif in SLR1 and SLN1 also made the mutant proteins resistant to GA-induced degradation (43, 57). Therefore, the DELLA motif is essential for DELLA protein degradation in response to the GA signal, and deletion of this motif confers a GA-unresponsive dwarfing phenotype through resistance of the mutant protein to GA-induced proteolysis.

Additional functional motifs in SLR1 have been identified by overexpressing slr1 mutant proteins with various internal in-frame deletions or a C-terminal truncation in transgenic rice (57). In this study (57), each *slr* mutant gene was fused to the *GFP* gene and expressed under the control of the constitutive *Act1* promoter. A second highly conserved motif (called VHYNP) is present in the DELLA domain (Figure 1). Deletion of the VHYNP motif or sequences between the DELLA and VHYNP motifs also block GA-dependent protein degradation and confer a GA-unresponsive dwarf phenotype. Expression of poly S/T/V-deleted slr1

confers a severe dwarf phenotype, but the mutant protein did not accumulate to a higher level than wild-type SLR1 and remains GA responsive. Thus, the poly S/T/V region may play a regulatory role in SLR1 activity. Overexpression of the LHR1-deleted slr1 does not cause any obvious phenotype, even though the mutant protein is resistant to GA-induced degradation. It has been proposed that LHR is needed for SLR1 dimerization, which may be required for SLR1 activity and for GA-dependent degradation. The C-terminal truncated slr1 (deletion starts from the VHIID region) is unresponsive to GA, indicating that the DELLA domain alone is insufficient for GA-induced degradation. Similar results have been obtained from studies of SLN1 in barley (43) and RGA in *Arabidopsis* (A. Dill & T-p. Sun, unpublished results). Interestingly, overexpression of this C-terminal truncated slr1 protein confers a slender phenotype, similar to that of the loss-of-function *slr1* mutant. The dominant-negative effect of this truncated protein suggests that it interferes with the wild-type SLR1, probably by forming nonfunctional heterodimers. Additional site-directed and deletion mutagenesis is needed to further define other functional motifs in the DELLA proteins.

Degradation of RGA and SLR1 via the Ubiquitin-Proteasome Pathway, Targeted by an E3 Ligase SCF Complex

Genetic studies suggest that SLY1 in *Arabidopsis* and GID2 in rice are positive regulators of GA signaling (106, 115). Because both SLY1 and GID2 proteins contain an F-box domain, their predicted function (as part of an SCF complex) is to modulate the stability of GA-response component(s). Recent results indicate that RGA and SLR1 are likely the direct targets of SLY1 and GID2, respectively. The *sly1* and *gid2* dwarf mutants accumulate much higher levels of the RGA and SLR1 proteins, respectively, in comparison to that in wild type (76, 106). A null *rga* allele partially suppresses the dwarf phenotype of the *sly1* mutant, indicating that the elevated RGA level contributed to the dwarfness of *sly1* and that RGA is downstream of SLY1 in the GA signaling pathway (76). In a yeast two-hybrid assay, GID2 interacts with OsSkp2 (one of the Skp homologs in rice), supporting the conclusion that GID2 is part of an SCF complex (106). These results imply that proteolysis of RGA and SLR1 is targeted by SCF^{SLY1} and SCF^{GID2}, respectively, through the ubiquitin-26S proteasome pathway. By immunoprecipitation and immunoblotting, polyubiquitinated SLR1 is detected in protein extracted from wild-type plants pretreated with a proteasome inhibitor MG132 (106). In addition, GA treatment increases the accumulation of ubiquitinated SLR1 under this condition. Recent studies in barley also show that GA-mediated degradation of the SLN1 protein and induction of α-amylase expression are inhibited by proteasome inhibitors (34).

Posttranslational modifications of target proteins, most commonly phosphorylation, are often required for recognition by the SCF E3 ligases (65). In vivo labeling experiments and phosphatase treatment demonstrate that the SLR1 protein is present in two (phosphorylated and unphosphorylated) forms in the *gid2*

mutant. The slower mobility form is the phosphorylated SLR1 because it corresponds to the ^{32}P-labeled form and it disappeared after phosphatase treatment. In wild type, only the unphosphorylated SLR1 is detected by immunoblotting (106). GA treatment causes an increase in the phosphorylated SLR1 in *gid2*, suggesting that SLR1 phosphorylation is induced by GA and that GID2 may preferentially target the phosphorylated SLR1 for degradation. A model that summarizes the recent findings on GA-induced proteolysis of the DELLA proteins is shown in Figure 2*a*. However, more than one phosphorylated form of the DELLA protein may be present and may have different functional properties. In *Arabidopsis* and barley, gain-of-function mutations in the DELLA motif (rga-Δ17 and sln1d) also result in accumulation of a slower mobility form (form II), in addition to the regular form (form I) (28, 43). Furthermore, the GA-insensitive dwarf mutant *gse1* shows an increased accumulation of form II of SLN1. The *GSE1* gene has not been cloned, but epistasis analysis showed that *GSE1* is upstream of *SLN1* (19). Thus, form II may be the active form and the change in mobility may be due to posttranslational modifications (43). If form II is phosphorylated, these results suggest that not all phosphorylated SLN1 or RGA proteins can be targeted for GA-induced proteolysis. Future biochemical studies are needed to determine the nature of form II proteins and their role in GA signaling.

MODEL OF GA SIGNALING PATHWAY IN *ARABIDOPSIS*

A revised model of GA signaling pathway in *Arabidopsis* is shown in Figure 2*b*. The DELLA proteins (RGA, GAI, RGL1, RGL2, and perhaps RGL3) are putative transcriptional regulators that directly or indirectly inhibit GA-activated genes. SPY is also a negative regulator of the GA response pathway. OGT target sites are often rich in Ser/Thr and located near Pro, Val, or acidic residues (74). SPY may inhibit the GA signaling pathway by activating RGA and GAI because the poly S/T region of the RGA/GAI proteins contains putative OGT modification sites (85, 110). In animal systems, GlcNAc modification could facilitate nuclear localization, increase protein stability, and/or alter protein activity (114, 131). The GA signal derepresses its signaling pathway by promoting RGA protein degradation through the SCF[SLY1]-proteasome pathway. This model also proposes that GAI activity is inhibited by SLY, but the mechanism involved is not clear because the GFP-GAI fusion protein is unresponsive to GA.

A quantitative response to the amount of GA signal is incorporated into this model. The concentrations of bioactive GAs determine the degree of activation of the GA response pathway by controlling the extent of RGA/GAI/RGLs inactivation through SLY1. RGA and GAI are the major repressors that modulate GA-induced leaf and stem growth, and also transition from vegetative-to-reproductive phase (flowering time). RGL1 and RGL2 play a role in regulating seed germination. It is likely that RGL3 also plays a role in seed germination and/or flower development. Although not shown in Figure 2, RGA and GAI may also play a minor role in

flower development (29). RGL1 to RGL3 activities may also be modulated by SPY and SLY1 because *spy* and *sly1* mutations affect all GA-regulated developmental processes.

Alternative models also exist to explain the results of previous genetic studies. For example, SPY may act downstream from RGA/GAI/RGLs. Thus, biochemical studies are needed to demonstrate that RGA/GAI/RGLs are the direct substrates of SPY. Additional epistasis analysis between *sly1* and *gai*/*rgl*s will help to determine whether GAI and RGLs are regulated by SLY1. Future biochemical and genetic studies are needed to incorporate the heterotrimeric G protein, SHI, and PKL into this *Arabidopsis* model.

CEREAL ALEURONE CELLS: A MODEL SYSTEM FOR STUDYING GA SIGNAL TRANSDUCTION AND GENE EXPRESSION

The function of cereal aleurone layers in germinated cereal grains is to synthesize and secrete hydrolytic enzymes to degrade the starchy endosperm reserves (59, 84). Following imbibition, GA_1 is released from germinated embryos into the endosperm, inducing the synthesis of hydrolytic enzymes in aleurone cells. The action of GA in aleurone cells can be blocked by ABA. The isolated aleurone offers many advantages over other GA-regulated responses: a single cell type, lack of a hormone source other than embryo, ready ability to test the function of GA signaling genes in aleurone tissue by transient expression experiments, and GA-responsive protoplasts can be isolated.

Profiling of gene expression in barley aleurone cells has shown that nearly 200 genes are transiently upregulated after 12-h treatment with GA_3 (P. Bethke, A. Fath, Y. Hwang, T. Zhu & R. Jones, personal communication). Although many of these genes encode hydrolytic enzymes such as α-amylase, proteases, ribonu-cleases, and β-glucanases, other genes encoding enzymes involved in general metabolism, transcription, transport of metabolites, and cell death are also upreg-ulated by GA. The response of α-amylase genes (α-*Amy1* and α-*Amy2* subclasses) to GA has been extensively studied over the past few decades and is by far the best-characterized GA response in aleurone cells (59, 73).

Over the past decade, cereal aleurone has been used intensively to analyze GA signal transduction and gene expression. Genetic, molecular, and biochemical approaches have identified a number of upstream regulators of α-amylase gene expression; many of which are described in the previous section. D1 (127), GID2 (106), cGMP (88), and arabinogalactan proteins (119) act as positive regulators of GA signaling in aleurone cells, whereas the DELLA proteins SLR1/SLN1/RHT (18, 55), SPY (99), HRT (93), and KGM (134) act as negative regulators.

Time-course studies show that SLN1 protein in barley aleurone cells is targeted for proteasome-mediated degradation within 10 min of GA application (Figure 3) (34, 43). Little is known about the direct target(s) of SLN1 repression in aleurone
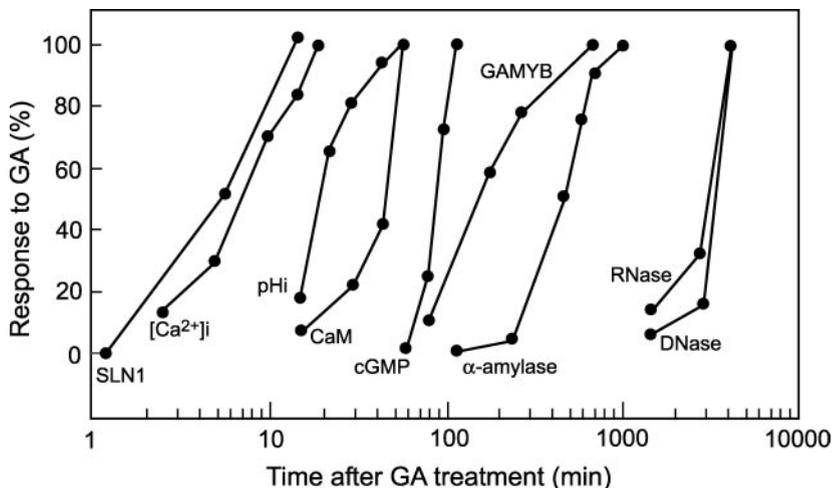
Figure 3   Time course of GA-induced responses of barley and wheat aleurone tissue. The graph has been adapted from Reference 5 and includes data from several studies (10, 13, 20, 43, 45, 51, 88, 108). For SLN1, the percentage response to GA represents decreasing amounts of SLN1 protein in response to GA-induced degradation.

cells except that cGMP may play an intermediary role between SLN1 and early-response genes (42, 88). There is a lag of approximately 1 h between GA-induced SLN1 degradation and increased expression of early-response genes such as the transcription factor GAMYB (Figure 3). Expression of hydrolase genes, such as *α-Amy* genes, starts soon after the expression of early-response genes and is followed by cell death 36–48 h after GA treatment.

In parallel to the molecular genetic studies that have revealed the roles of several upstream GA signaling components, considerable progress has been made in our understanding of molecular mechanisms of downstream transcriptional regulation of GA-responsive gene expression in aleurone cells. Recent developments in our understanding of *cis*-acting elements and transcription factors involved in GA-regulated gene expression in aleurone cells are reviewed in the next two sections.

cis-Acting Elements in Hydrolase Genes

The development of transient expression assays in aleurone cells has led to rapid progress in our understanding of transcriptional regulation of hydrolase gene expression, in particular α-amylase genes, by GA signaling pathways. Various GA-response complexes containing a GA-response element (GARE) have been identified in the promoters of hydrolase genes (Figure 4a). Functional analysis of *α-Amy1* gene promoter identified a 21-bp fragment of a barley *Amy-1* promoter that conferred GA responsiveness to a minimal 35S promoter (113). Mutation of a highly conserved TAACAAA sequence within the 21-bp sequence and similar elements in

other hydrolase promoters abolished GA responsiveness of the promoters in transient expression analyses, thus confirming the role of the TAACAAA-like boxes as a GARE (16, 44, 100, 118, 125). There appears to be flexibility in the orientation of the GARE within hydrolase promoters. Two cysteine protease promoters from barley and rice (16, 118) have the GARE in the opposite direction to those found in *α-Amy* promoters. Redundancy of the GARE in hydrolase promoters appears to be uncommon, considering that multiple functional TAACAAA-like boxes are found only in the rice REP-1 promoter (118).

Other components of the GA-response complexes (GARC) of hydrolase gene promoters include the pyrimidine box (C/TCTTTT), the TATCCAC box, the CAACTC box, and the Box1/O2S-like element. A high level of GA-dependent expression of barley *α-Amy1* promoter requires the presence of a GARE together with a pyrimidine and TATCCAT box, whereas the barley *α-Amy2* promoter requires an additional *cis*-acting element, the Box1/O2S-like element (44, 68). In the rice and barley cysteine proteinase promoters, the GARE acts in concert with a pyrimidine box and CAACTC elements, which are putative MYB binding sites (16, 118). It is likely that differences in the GARCs in various hydrolase gene promoters underlie differences in quantitative and/or qualitative aspects of their GA response in aleurone cells. It has been known for some time that *α-Amy1* and *α-Amy2* genes are regulated differentially in germinated barley grains, with *α-Amy1* expressed before *α-Amy2* genes (17).

Until now the focus has been on identifying *cis*-acting elements in promoters of hydrolase genes. It is expected that future studies will also examine the promoters of early- and late-response genes such as GAMYB (42, 45) and ribonuclease (10). In addition, GA-responsive genes identified by microarray analysis can be organized into self-organizing maps to group genes with similar expression characteristics. By identifying *cis*-acting elements that are common to promoters of similarly expressed genes, researchers will be able to build a picture of the modular organization of various GARCs in GA-responsive promoters.

Transcription Factors that Bind to GARC Regulatory Elements

The importance of the TAACAAA box in hydrolase gene promoters has focused attention on identifying cognate transcription factors that act as regulators of hydrolase gene expression. HvGAMYB, a GA-regulated MYB transcription factor, binds specifically to the TAACAAA-like boxes in hydrolase gene and transactivates both *α-Amy* and other GA-responsive hydrolase gene promoters in the absence of GA (Figure 4b) (16, 45, 46). Mutation of the TAACAAA-like boxes blocks both HvGAMYB binding and transactivation of hydrolase gene promoters, indicating that its ability to activate hydrolase gene promoters is dependent on the presence of a GARE (16, 45). Transient RNAi experiments have demonstrated that HvGAMYB is necessary for GA activation of an *α-Amy2* gene promoter in barley aleurone cells (136). GA-induced increases in *α-Amy2* gene promoter activity are blocked in aleurone cells expressing *HvGAMYB* RNAi.
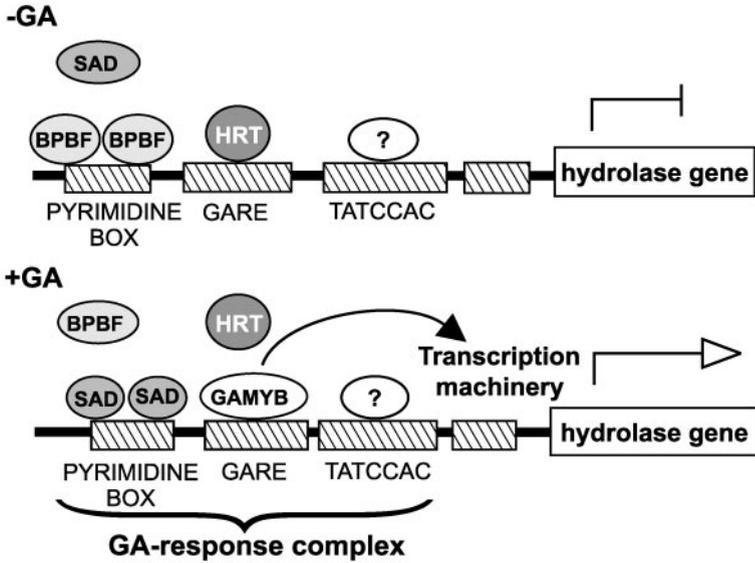
a.

GARE	Gene
A**TAACAAA**CTC	α-*Amy1/6-4* (−144 to −134)
G**TAACAGA**GTC	α-*Amy2/32b* (−120 to −110)
C**TAACGTA**GCA	*REP-1* (−148 to 138)
G**TAACAGA**AAG	*REP-1* (−80 to −90)
G**TAACAGA**ATG	*EPB-1* (−141 to 152)
TAACRDA	Consensus GARE

b.

c.
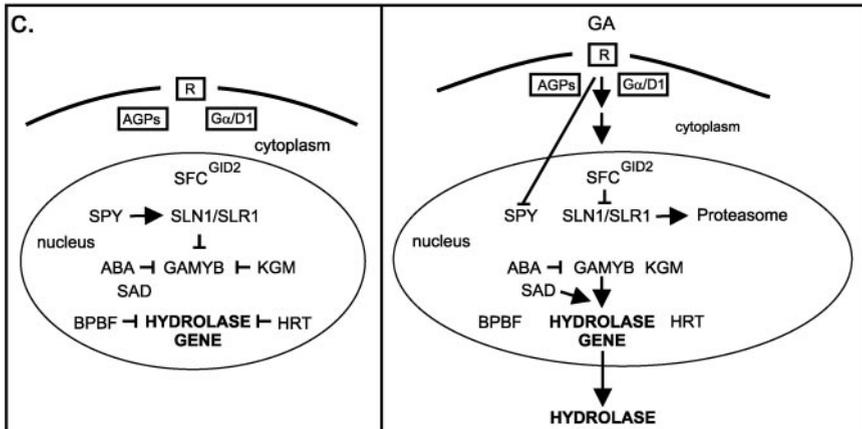
HvGAMYB mRNA and protein content in aleurone cells increases rapidly in response to GA treatment (1–2 h) preceding the rise in *α-Amy* gene expression (Figure 3). Studies so far indicate that the increase in HvGAMYB expression in GA-treated aleurone cells is due in part to an increase in *HvGAMYB* transcription (42, 43). The transcription rate of the *HvGAMYB* gene is twice as high in nuclei isolated from GA-treated aleurone layers than in nuclei from control layers. A similar level of response to GA has been reported for a *HvGAMYB* promoter:*GUS* gene expressed transiently in aleurone cells (42).

There is also growing evidence for posttranscriptional regulation of GAMYB expression and function in aleurone cells through a number of different mechanisms. The observation that the increases in GAMYB protein are much higher than those detected for GAMYB transcript in GA-treated aleurone layers indicates that GAMYB translation and/or stability may be a major target for GA signaling (43, 134). On the basis of recent evidence, it is tempting to speculate that microRNA (miRNA) may be involved in the regulation of translation of GAMYB transcripts. *Arabidopsis* and rice miRNA sequences containing complementary sequences to GAMYB-like genes have been reported, but their function remains to be demonstrated (96). Phosphorylation of GAMYB has been proposed as a potential mechanism for regulation of GAMYB function in aleurone cells. KGM, a Ser-Thr kinase related to Mak-like kinases, has been identified as a HvGAMYB-binding partner in a yeast two-hybrid screen (134). Transient expression of KGM blocks HvGAMYB transactivation of a barley *α-Amy1* promoter, indicating that KGM acts as a repressor of GAMYB. The repressor activity of KGM is dependent on a conserved Tyr residue in the activation loop of KGM. Although a number of potential KGM phosphorylation sites have been identified in HvGAMYB, it is not yet clear that KGM acts on HvGAMYB via a kinase-dependent or kinase-independent

←

Figure 4 GAREs and molecular mechanisms of GA signaling in cereal aleurone cells. (*a*) Sequences of GAREs identified in GA-responsive promoters of the *α*-amylase genes (44, 100, 113) and cysteine protease genes (16, 118). A consensus sequence derived from the compilation of the GAREs is also shown (R = A or G; D = A, G, T). (*b*) A generalized *α*-amylase GA-response complex (GARC) with SAD/BPBF and GAMYB/HRT binding sites. The DNA binding with one finger (DOF) proteins, SAD and BPBF, bind to the pyrimidine box, whereas GAMYB and HRT proteins bind to the GARE. (*c*) Model of GA signaling in cereal aleurone cells. (*Left*) In the absence of applied GA, SLN1/SLR1, KGM, BPBF, and HRT repress hydrolase gene expression in cereal aleurone cells. (*Right*) Following the addition of GA to aleurone cells, GA signaling is initiated at a plasma membrane–bound receptor (R) that is closely associated with a G protein (Gα/D1) and arabinogalactan proteins (AGP). GA signaling depresses SLN1/SLR1 repressor activity in the nucleus via activation of the SCF[GID2] complex. SPY repression is also inactivated by GA signaling. Following the degradation of SLN1/SLR1, hydrolase gene expression is activated by GAMYB and SAD.

mechanism. RNA analysis reveals that KGM is present in both GA-treated and non-GA-treated aleurone cells. How and when KGM activity is regulated in aleurone cells remains to be determined, but it is probably involved in blocking HvGAMYB activation of hydrolase genes in the absence of GA.

A second transcription factor that binds to the region containing the GARE in a barley α-Amy1 promoter has been identified. HRT, a nuclear-localized zinc-finger protein expressed in aleurone cells, binds specifically to a 21-bp promoter fragment that contains a GARE and can repress GA-induced expression of α-Amy1 and α-Amy2 promoters in transient expression experiments (93). Although it is clear that HRT functions as a repressor of α-Amy genes in aleurone cells, further work is required to determine the HRT binding site within the 21-bp promoter fragment and how GA regulates its activity.

There is evidence that different DOF transcription factors bind to pyrimidine boxes in hydrolase GARCs and have competing functions in regulating hydrolase gene expression in aleurone cells (56, 78, 130). BPBF, a barley DOF transcription factor that binds to the pyrimidine boxes of cathepsin B-like protease and α-Amy2 gene promoters, represses GA-induced expression of Al21 gene promoter (78). In contrast, another barley DOF, SAD, functions as a transcriptional activator of the Al21 promoter (56). Both these DOFs interact with HvGAMYB, indicating that different DOF-interacting partners may regulate HvGAMYB function (27, 56). Future studies will reveal how this combinatorial control regulates hydrolase gene expression in aleurone cells. One model may be that SAD-GAMYB interaction activates hydrolase gene expression and BPBF-GAMYB interaction switches off hydrolase gene expression prior to programmed cell death (78). Box2/O2S element, a component of α-Amy2 GARC, is the binding site for members of the WRKY gene family, but little is known about their function and regulation (104).

The demonstration of protein-protein interaction between two GARC binding factors is a start in identifying subunits that make up the transcriptional complexes that bind to GA-responsive hydrolase gene promoters. Proteomic approaches such as yeast two-hybrid will be useful in identifying other subunits of the transcriptional complexes. The function of newly identified subunits can be rapidly analyzed in transient expression analyses using overexpression and RNAi approaches. Future work is also needed to identify how these multicomponent transcriptional complexes are recruited to the GARCs. It is expected that future studies will also examine the role of chromatin remodeling in regulating the function of these transcriptional complexes.

In summary, a model of GA signaling in aleurone cells is shown in Figure 4c. The many striking similarities between this model and the model of GA signaling in *Arabidopsis* (Figure 2b) indicate that the molecular mechanisms regulating the derepression of DELLA proteins is conserved between cereals and *Arabidopsis*. Further genetic and biochemical studies are needed to identify these conserved early-signaling steps, and the aleurone layer will continue to be a very useful system to analyze these molecular interactions in the cytoplasm and nucleus.

GA SIGNALING AND GENE EXPRESSION
IN OTHER CELL TYPES

GA-regulated genes have been identified in a number of tissues and their respective promoters analyzed to identify putative GAREs (6, 67, 70, 90). TAACAAA-like sequences have been identified in promoters of rice expansin genes. Analysis of the promoter of OsEXP4, a GA-regulated expansin expressed in elongating internodes of deepwater rice, revealed the presence of a TAACAAA-like box and two other *α-Amy* GARC *cis*-acting elements (pyrimidine box and Box1/O2S) (70). Further work is required to test the function of these putative GARC elements in the OsEXP4 promoter. Other GA-regulated genes that contain TAACAAA-like boxes in their promoters include the Gerbera *GEG* gene and tomato invertase genes (67, 90).

DNA microarray chips have provided a powerful tool for identifying GA-regulated genes in various plant tissues. In a microarray study of GA-regulated gene expression in germinating *Arabidopsis* seeds, 138 GA-upregulated genes and 120 GA-downregulated genes have been detected in stratified and imbibed *ga1-3* seeds treated with GA_4 for 12 h (83). In germinating wild-type seeds, the expression of many of these GA-responsive genes that were identified in *ga1-3* seeds paralleled increases in endogenous GA_4 content. Early-GA-regulated genes encode a number of transcription factors that are likely to act as a transcriptional cascade, activating the expression of downstream genes involved in cell elongation and other germination processes. Genes encoding transcription factors that are rapidly induced by GA_4 in *Arabidopsis* seeds include *AtMYB34/ATR1*, *DOF*, and *ATHB-16*. The role of the DOF and ATHB-16 are unknown, but AtMYB34/ATR1 is a positive regulator of the *ASA1* gene, which encodes a component of an early Trp biosynthetic enzyme (3). It is of interest to note that 20% of promoters of GA-upregulated genes contain TAACAAA-like sequences, indicating that many promoters contain a different GARE from that found in cereal hydrolase genes (83). Some of the GA-response genes identified in GA-treated imbibed *ga1-3* seeds show altered expression prior to changes in endogenous GA_4 content in germinating wild-type seeds. This result indicates that these genes may also be regulated by other factors in wild-type seeds.

The detection of putative GAREs similar to those found in cereal hydrolase genes raises the question whether GAMYB has a role in other GA-regulated processes outside the aleurone layer. Studies so far indicate that GAMYB is expressed in a number of other GA-responsive tissues, including the shoot apex, anthers, and subcrown internode (21, 40, 41, 80). The gene targets in these tissues have not yet been identified, but in *Arabidopsis* the GA-responsive *LEAFY* gene promoter has been proposed as a target for *Arabidopsis* GAMYB-like genes in the shoot apex (6). Increases in the transcript of *AtMYB33*, a *GAMYB*-like gene in *Arabidopsis*, have been detected in the shoot apex prior to *LEAFY* expression (41). Similarly, *LtGAMYB* expression is induced in the shoot apex in response to GA and long-day treatment (40). In anthers, HvGAMYB is expressed nuclei of the epidermis, endothecium, middle layer, and tapetum during the early stages of

anther development (80). Expression persists in the two outer layers until dehiscence. The identification of *GAMYB* knockout mutants in rice and *Arabidopsis* will assist in determining the role of *GAMYB* genes in GA-regulated processes outside the aleurone layer (M. Matsuoka, personal communication; T. Millar & F. Gubler, personal communication).

FEEDBACK REGULATION OF GA METABOLISM BY THE ACTIVITY IN THE GA RESPONSE PATHWAY

The activity of the GA response pathway is tightly linked to the activities in GA biosynthesis and catabolism by a feedback mechanism (reviewed in 50, 84). GA 20-oxidases (20ox) and GA 3-oxidases (3ox) are enzymes that catalyze the final steps in the synthesis of bioactive GAs, whereas GA 2-oxidases (2ox) are responsible for GA deactivation. In the constitutive GA-response mutants, such as rice *slr1*, barley *sln1*, pea *la cry^s*, and *Arabidopsis rga/gai-t6*, the bioactive GAs and/or the transcript levels of the *GA20ox* and/or *GA3ox* genes are present at lower levels than in wild type (24, 29, 55, 75). In contrast, GA-unresponsive dwarf mutants (e.g., rice *d1*, *gid2*, maize *D8*, and *Arabidopsis gai-1*, *shi*, *sly1*) accumulate higher amounts of bioactive GAs and *GA20ox* and/or *GA3ox* mRNAs (31, 35, 76, 85, 106, 122, 127). Although *GA2ox* expression has not been examined in the GA-response mutants, mRNA levels of some *GA2ox* genes were reduced in GA-deficient conditions and elevated by GA treatment. These results indicated that GA homeostasis is achieved by a close interaction between the GA metabolism and GA signaling pathways. However, none of the components that mediate the feedback regulation have been identified. Future molecular genetics and biochemical studies will be necessary to elucidate the molecular mechanism involved.

INTERACTIONS BETWEEN GA AND OTHER HORMONE SIGNALING PATHWAYS

In recent years, there has been a rapid increase in our knowledge of how growth and development is regulated by molecular interactions between hormone response pathways (37). This has been facilitated by the increasing availability of hormone response mutants, together with the increasing generation of data by microarray analyses and use of reporter genes.

In cereal aleurone cells, activation of Ca^{2+}/calmodulin signaling pathways by GA plays an important role in the synthesis and secretion of hydrolases. As shown in Figure 3, the rapid decline of SLN1 protein parallels transient increases in cytosolic calcium and precedes other GA-induced changes in signaling molecules such as calmodulin and intracellular pH. Evidence so far indicates that GA-induced changes in Ca^{2+}/calmodulin signaling targets membrane-based components in the ER and vacuole; however, these changes do not affect hydrolase gene expression

(5). An ER Ca^{2+}-ATPase, which supplies Ca^{2+} for the synthesis of Ca^{2+}-containing metalloenzymes such as α-amylase, is a target for Ca^{2+}/calmodulin signaling (14, 39). Another membrane target is a slow vacuolar channel that releases Ca^{2+} from the protein storage vacuole (4).

ABA blocks a number of GA responses in plants, including hydrolase expression in aleurone cells, flowering, leaf growth, and germination. In aleurone cells, it is clear that ABA inhibition of hydrolase gene expression does not act through the repressor of GA signaling, SLN1. The demonstration that (*a*) α-*Amy* gene expression in the *sln1* loss-of-function mutant is still responsive to ABA and that (*b*) GA-induced SLN1 degradation is not blocked by ABA indicates that ABA acts downstream of SLN1 in aleurone cells (43, 136). Because ABA only partly blocks GA-induced increases in *HvGAMYB* transcript and protein (43; F. Gubler, unpublished data), it appears likely that ABA acts at multiple sites to block α-*Amy* gene expression. Transient overexpression of an ABA-upregulated protein kinase PKABA1 represses GA-regulated expression of GAMYB and α-*Amy* genes (42, 136). However, recent experiments with *PKABA1* RNAi have failed to demonstrate that PKABA1 is necessary for ABA repression of GA signaling in aleurone cells. Further work is needed to determine whether ABA signaling pathways consist of two pathways, a PKABA1-dependent and a PKABA1-independent pathway. Anoxia also blocks α-*Amy* gene expression in rice, and results indicate that it blocks GA signaling downstream of SLR1 (72). Evidence is emerging that GA signaling may also regulate ABA metabolism and signaling. The shoots (but not the roots) of the rice *slr1* mutant contain a level of ABA elevated in comparison to that in wild type (54). However, the barley *sln1* mutant has a level of ABA similar to that in wild-type plants (18). In *Arabidopsis*, the *spy1-3* mutant is less sensitive to ABA in seed germination (115). It appears likely that derepression of GA signaling overrides the ABA inhibition of germination. However, whether this effect is direct or indirect is unclear.

Recent evidence indicates that GA-dependent degradation of RGA in *Arabidopsis* roots is regulated by shoot-apex-derived auxin (33). GA promotion of root growth in *Arabidopsis* is inhibited by RGA and GAI. Depriving the *Arabidopsis* roots of shoot-derived auxin reduces the ability of GA to promote degradation of GFP-RGA (and presumably endogenous RGA), thereby restricting root growth. Furthermore, the kinetics of GA-induced GFP-RGA degradation is delayed in an auxin signaling mutant *axr1-12*, which provides further support that auxin signaling interacts with GA signaling components in roots. However, recent studies showed that *axr1* also affects other signaling pathways because AXR1 is required for regulating SCF complex function by conjugation (also called neddylation) of the RUB (related-to-ubiquitin) protein to the cullin subunit of the SCF complexes (25). Therefore, the effect of *axr1* on GFP-RGA stability could also be the result of an impaired SCF^{SLY1}. In contrast to the above study, a strong GA-induced internode elongation (although not maximum) was observed in decapitated peas where endogenous auxin is very low (103), suggesting that the effect of auxin on GA responses may be species and/or organ dependent. Recent studies of

mutations in the *Arabidopsis BIG* gene (encoding a calossin-like protein) suggest that this gene not only plays a role in normal auxin efflux, but also affects GA, cytokinin, and light responses (38, 63). Interactions between auxin signaling and GA promotion of stomata formation in hypocotyls in *Arabidopsis* have also been described, but where the auxin signal interacts with the GA response pathway is not yet clear (105). Microarray data from germinating *Arabidopsis* seeds suggest that GA may promote both auxin synthesis and transport, together with ethylene synthesis and response (83). It is also clear that GA signaling is interconnected closely with a number of other signaling pathways, but molecular mechanisms of these interactions remain poorly understood (63, 94).

CONCLUSIONS

Recent molecular genetics and biochemical approaches have significantly furthered our understanding of GA signaling in plants. The newly available whole-genome expression profiling and proteomic technology will be additional powerful tools to facilitate the identification of the GA receptor(s), new GA signaling components, and tissue-specific GA-regulated genes.

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