Involvement of 14-3-3 Signaling Protein Binding in the Functional Regulation of the Transcriptional Activator

REPRESSION OF SHOOT GROWTH by Gibberellins

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REPRESSION OF SHOOT GROWTH (RSG) is a tobacco (Nicotiana tabacum) transcriptional activator with a basic Leu zipper domain that regulates endogenous amounts of gibberellins (GAs) by the control of a GA biosynthetic enzyme. The 14-3-3 signaling proteins have been suggested to suppress RSG by sequestering it in the cytoplasm. Here, we show that RSG phosphorylation on Ser-114 is important for 14-3-3 binding. We found that GA levels regulate the intracellular localization of RSG. RSG translocated into the nucleus in response to a reduction in GA levels. GA treatment could reverse this nuclear accumulation. The GA-induced disappearance of RSG–green fluorescent protein from the nucleus did not depend on protein degradation. By contrast, the mutant RSG (S114A) that could not bind to 14-3-3 continued to be localized predominantly in the nucleus after GA application. Analysis of the mRNA levels of GA biosynthetic genes showed that the feedback regulation of the GA 20-oxidase gene was inhibited in transgenic plants expressing a dominant negative form of RSG. Our results suggest that RSG is negatively modulated by GAs by 14-3-3 binding and might be involved in GA homeostasis.

INTRODUCTION

Gibberellins (GAs), which are tetracyclic diterpenoid growth factors, are essential regulators of many aspects of plant development, including seed germination, stem elongation, flower induction, and anther development. The GA biosynthetic pathway is well established, and genes encoding the enzymes for all of the steps of the major pathway have been isolated from Arabidopsis thaliana (for reviews, see Hedden and Phillips, 2000; Olszewski et al., 2002). Both endogenous developmental programs and environmental stimuli affect the expression of these enzymes. Therefore, elucidating the transcriptional regulation of GA biosynthetic enzymes is crucial to identify the molecular mechanisms involved in plant development and to understand how these mechanisms help plants adapt to changes in their environment. A factor that affects the transcription of GA biosynthetic enzymes is GA itself. In GA-deficient Arabidopsis mutants (e.g., ga1-3), the expression of GA4 encoding GA 3-oxidase (formerly GA 3β-hydroxylase) and GA5 encoding GA 20-oxidase is higher than in the wild type, and this increased expression can be reduced by GA application (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995). The mechanisms of negative feedback seem to contribute to a homeostasis of GA levels. Although the GA feedback regulation has been shown to depend on GA signaling components, including DELLA regulators, SPINDLY, and PHOTOPERIOD-RESPONSIVE 1 (for a review, see Olszewski et al., 2002), its molecular mechanisms are still largely unknown.

RSG (for REPRESSION OF SHOOT GROWTH) is a tobacco (Nicotiana tabacum) transcriptional activator with a basic Leu zipper domain that is involved in the regulation of endogenous amounts of GAs (Fukazawa et al., 2000). The dominant negative form of RSG repressed the expression of the ent-kaurene oxidase gene of the GA biosynthetic pathway in transformed tobacco plants. This downregulation reduced endogenous amounts of GAs and severely inhibited the process of cell elongation of stems, resulting in a dwarf phenotype. Thus, RSG regulates the morphology of plants by controlling endogenous amounts of GAs.

Recently, genomics studies using microarrays have revealed changes in gene expression profiles in response to developmental programs and environmental stimuli. To know how these alterations in gene expression pattern are induced, the functional regulation of the transcription factors must be investigated. Phosphorylation, in particular, plays critical roles in the binding of transcription factors to DNA and to other regulatory proteins and the proteolysis of the transcription factors in other eukaryotes (for a review, see Brivanlou and Darnell, 2002). However, the understanding of posttranslational regulations of transcription factors is still limited in plants. We identified the 14-3-3 signaling proteins as RSG binding partners (Igarashi et al., 2001).
The 14-3-3 proteins form a highly conserved family of homodimeric and heterodimeric proteins (for reviews, see van Hemert et al., 2001; Tzivion and Avruch, 2002). Although a single conserved cellular function of 14-3-3 proteins is not apparent, an important common characteristic is their ability to bind to other proteins; >70 such examples exist. Through these binding reactions, the 14-3-3 proteins appear to act as molecular scaffolds or chaperones. The biological roles of 14-3-3 complexes have been demonstrated in signal transduction, subcellular targeting, and cell cycle control. The 14-3-3 proteins can also act as allosteric cofactors modulating the catalytic activity of their binding partners. In plants, 14-3-3 binding proteins include a variety of key metabolic enzymes, such as nitrate reductase, sucrose-phosphate synthase, and plasma membrane H^+ -ATPase (for reviews, see Sehnke et al., 2002; Roberts, 2003). The 14-3-3 proteins bind to phosphorylated motifs containing phosphoserine residues of RSxpSXP and RXY/FXpSXP (pS indicates a critical phosphoserine) in their target proteins (Yaffe et al., 1997). A sequence surrounding Ser-114 of RSG that is closely related to the conventional 14-3-3 binding motif was essential for 14-3-3 binding. However, it remains to be determined whether the phosphorylation of Ser-114 of RSG is involved in the regulation of RSG binding to 14-3-3.

Transport of proteins across the nuclear envelope is highly selective and can be temporally regulated. For example, some transcription factors are maintained in an inactive state in the cytoplasm until a signal is received that promotes their translocation into the nucleus, either downstream of a particular signal transduction pathway or at a specific point in the cell cycle (for a review, see Kaffman and O’Shea, 1999). The S114A mutant of RSG that could not bind to 14-3-3 proteins was localized predominantly in the nucleus, whereas wild-type RSG was distributed throughout the cell (Igarashi et al., 2001). Thus, a function of 14-3-3 proteins may be to bind RSG and thereby sequester RSG in the cytoplasm so that it is unable to regulate its target genes in the nucleus. When RSG dissociates from 14-3-3 proteins in response to developmental programs and environmental stimuli, it accumulates in the nucleus. There, it may activate its target genes, including a GA biosynthetic gene. To understand the role of RSG in plant development, stimuli that affect the intracellular localization of RSG through 14-3-3 binding should be identified. In this study, we found that GA levels regulate the intracellular localization of RSG. Our studies also suggest a possible involvement of RSG in the feedback regulation of GA biosynthesis.

RESULTS

14-3-3 Binding Requires RSG Phosphorylation on Ser-114

Phosphorylation is one of the most frequent and important posttranslational modifications of proteins. We examined whether phosphorylation is required for the interaction of RSG with 14-3-3. Our previous result that RSG prepared by in vitro translation binds to 14-3-3 (Igarashi et al., 2001) suggested that RSG is phosphorylated by an endogenous kinase present within the rabbit reticulocyte lysate. To test this, we used a broad-spectrum Ser/Thr protein kinase inhibitor, K252a. The presence of K252a during the in vitro translation did not reduce the total amount of radiolabeled RSG protein produced; however, K252a resulted in a marked inhibition of RSG binding to 14-3-3 (Figure 1A). Thus, phosphorylation by Ser/Thr kinase(s) within the reticulocyte lysate is required for RSG to interact with 14-3-3. We previously identified a sequence in RSG closely related to the conventional 14-3-3 binding motif and demonstrated that Ser-114 in the motif is essential for 14-3-3 binding. Furthermore, the Lys-52 residue in the amphipathic groove of 14-3-3 involved in phosphoserine recognition was necessary for RSG binding (Igarashi et al., 2001). Taken together, these results suggest that the RSG binding to 14-3-3 is regulated by the phosphorylation of Ser-114 in RSG.

To determine whether the phosphorylation of Ser-114 is important for the interaction, we performed competition experiments using synthetic peptides consisting of amino acids 108 to 122 of RSG that were either phosphorylated on Ser-114 or Ser-112 or unphosphorylated (Figure 1B). Recombinant maltose binding protein (MBP)–14-3-3 was absorbed to amylose resin. The immobilized 14-3-3 fusion protein was incubated with in vitro–translated, 35S-Met–labeled RSG followed by affinity chromatography. The interaction between RSG and 14-3-3 proteins was disrupted by incubation with excess peptide containing phosphorylated Ser-114 but not with unphosphorylated peptide (Figure 1C). Furthermore, the peptide with phosphorylated Ser-112 as well as the unphosphorylated one showed little, if any, inhibition of the interaction between RSG and 14-3-3 proteins (Figure 1C). This confirms that phosphorylation of Ser-114 of RSG is critical for 14-3-3 binding.

Recently, we have found a protein kinase that phosphorylates Ser-114 of RSG, using an in-gel kinase assay of tobacco cell extracts with affinity-purified glutathione S-transferase (GST)–RSG fusion proteins as substrates and those of S114A mutant version of RSG as negative controls. The identified protein kinase RSGK (for RSG kinase) phosphorylates RSG but not the S114A mutant (our unpublished results). Recombinant RSG that was expressed in and purified from Escherichia coli could not bind to 14-3-3 proteins because bacteria lack protein kinase activity that phosphorylates Ser-114 of RSG. To test whether phosphorylation of bacterially expressed RSG by RSGK might promote binding of RSG to 14-3-3 proteins, in vitro kinase assays using GST-RSG fusion proteins as substrates were performed, and products were subjected to GST pull-down assays. The 14-3-3 binding domain of RSG (amino acids 69 to 140) was phosphorylated in vitro by RSGK, resulting in binding by 14-3-3 (Figures 1D and 1E). The S114A mutation of RSG eliminated both phosphorylation by RSGK and the ability to interact with 14-3-3 proteins (Figures 1D and 1E). To confirm that Ser-114 is the site of RSGK-catalyzed phosphorylation in vitro, we used antibodies that specifically recognize phospho-Ser-114 of RSG. Immunoblot analysis clearly showed that RSGK phosphorylates RSG at Ser-114 in vitro (Figure 1F). These results indicate that the phosphorylation of Ser-114 is required for 14-3-3 binding to RSG.

Inhibition of Phosphorylation Promotes Nuclear Localization of RSG

Phosphorylation-dependent binding of RSG to 14-3-3 proteins suggests that intracellular localization of RSG is regulated by
Figure 1. Phosphorylation of RSG is Required for the Binding to 14-3-3.

(A) Inhibition of RSG binding to 14-3-3 by K252a. 35S-Met–labeled full-length RSG was synthesized by in vitro translation in the presence of various concentrations of the protein kinase inhibitor K252a (0, 1, and 10 μM). Ten percent of the in vitro–translated protein was saved as an input control. Full-length MBP–14-3-3 or MBP–14-3-3 (K52E) fusion protein was conjugated to amylose resin and incubated with 35S-Met–labeled RSG. K52E, a mutant version of 14-3-3 that cannot interact with RSG, was used as a negative control. The 14-3-3–associated proteins were resolved by SDS-PAGE. After electrophoresis, proteins were visualized by fluorography and Coomassie blue staining. The amount of bound 35S-Met–labeled RSG was normalized against each input and is shown in the bar graph as arbitrary units, as the bind of RSG to MBP–14-3-3 without K252a was 1.0.

(B) Amino acid sequences of synthetic peptides (pep).

(C) Competition experiments using synthetic peptides. The full-length MBP–14-3-3 fusion protein was conjugated to amylose resin and incubated with 35S-Met–labeled full-length RSG in the presence of unphosphorylated or Ser-114–phosphorylated peptide or Ser-112–phosphorylated peptide at concentrations of 0.1, 0.5, and 1 mM. A control reaction (Ctrl) contains no synthetic peptide. The 14-3-3–associated proteins were resolved by SDS-PAGE. After electrophoresis, proteins were visualized by fluorography and Coomassie blue staining. The amount of bound 35S-Met–labeled RSG was normalized against the amount of Coomassie-stained MBP–14-3-3 in each sample and is shown as arbitrary units in the bar graph, as the bind of RSG to MBP–14-3-3 without a synthetic peptide was 1.0.

(D) Phosphorylation of RSG by RSGK in vitro. The 14-3-3 binding domain of RSG (amino acids 69 to 140) or that of the S114A mutant version of RSG was expressed as a GST fusion protein. The GST fusion proteins were phosphorylated with recombinant RSGK in the presence of [γ-32P]ATP. Aliquots of each reaction were subjected to SDS-PAGE. After electrophoresis, the phosphoproteins were detected by a phosphor imager, and total GST fusion proteins were visualized by Coomassie Brilliant Blue (CBB) staining.

(E) Phosphorylation of RSG promotes binding to 14-3-3. Bacterially expressed GST–RSG (amino acids 69 to 140) or the S114A mutant version was either left unphosphorylated (−) or phosphorylated (+) with recombinant RSGK using unlabeled ATP. The GST fusion proteins were immobilized on GSH-Sepharose beads and incubated with full-length MBP–14-3-3 protein. RSG or the S114A mutant version–associated proteins were resolved by SDS-PAGE. After electrophoresis, proteins were visualized by Coomassie blue staining.

(F) Phosphorylation site of RSG. Bacterially expressed GST–RSG (amino acids 69 to 140) was either left unphosphorylated (−) or phosphorylated (+) with recombinant RSGK using unlabeled ATP and was then subjected to immunoblot analysis with antibody specific for phospho-Ser-114 of RSG. Total GST fusion proteins were visualized by Coomassie Brilliant Blue (CBB) staining.
phosphorylation. To confirm this, we examined the effect of K252a on the intracellular localization of RSG using transgenic tobacco plants in which the fusion gene of RSG and green fluorescent protein (GFP) was expressed under the control of the 35S promoter of the Cauliflower mosaic virus. The treatment of epidermal cells of leaves with K252a led to a significant redistribution of RSG-GFP within 30 min from mainly cytoplasmic (Figure 2A) to almost exclusively nuclear localization (Figure 2B). Therefore, the cytoplasmic sequestration of RSG is phosphorylation dependent. This result also shows that the continual phosphorylation of RSG is required for cytoplasmic sequestration of RSG.

**GAs Regulate Intracellular Localization of RSG**

RSG shuttles continuously between the nucleus and the cytoplasm. The cytoplasmic localization of RSG is the result of a steady state situation in which RSG enters the nucleus and is exported more rapidly back to the cytoplasm (Igarashi et al., 2001). Exclusion of RSG from the nucleus via active nuclear export keeps RSG in an inactive state by preventing its access to target genes of the nucleus in unstimulated cells. Nuclear accumulation of RSG might be induced by internal and external stimuli through the dephosphorylation of Ser-114 in RSG. We then explored the stimuli that affect the intracellular localization of RSG. GA biosynthesis is modified by GA itself in a type of feedback regulation (for a review, see Olszewski et al., 2002). An interesting supposition is that endogenous amounts of GAs regulate the intracellular localization of RSG. To test this possibility, we examined the effect of applying an inhibitor for GA biosynthesis, uniconazole P, to transgenic tobacco plants in which RSG-GFP was expressed. Uniconazole P led to the redistribution of RSG-GFP from a mainly cytoplasmic to an almost exclusively nuclear localization (Figure 3A, b and c), with the alteration of plant morphology to dwarf (Figure 3A, a). This nuclear accumulation of RSG-GFP was reversed to a mainly cytoplasmic localization with the application of GA3 to the transgenic plants via soil (Figure 3A, d). Intracellular localization of GFP, a control, was not affected by uniconazole P or GA3 treatment (data not shown). To investigate the kinetics of cytoplasmic migration of RSG-GFP in response to GAs, transgenic plants expressing RSG-GFP were sprayed with a solution of GA3. Translocation of RSG-GFP from the nucleus to the cytoplasm was observed within 2 h after GA3 treatment (Figure 3B, a to c). These results showed that the intracellular localization of RSG is regulated by the endogenous amounts of GAs. The plant hormones brassinolide and 2,4-D also promote cell elongation; however, applying either of these did not alter the intracellular localization of RSG-GFP from the cytoplasm to the nucleus (data not shown).

**The S114A Mutation Inhibited Cytoplasmic Migration by GAs**

The S114A mutant of RSG that cannot bind 14-3-3 proteins localized exclusively in the nucleus (Igarashi et al., 2001). To investigate whether this mutation affected the migration of RSG from the nucleus to the cytoplasm in response to exogenous GAs, we applied GA3 to the uniconazole P–treated transgenic plants expressing the S114A-GFP mutant. In contrast with RSG-GFP, S114A-GFP continued to be localized predominantly in the nucleus after treatment with GA3 (Figure 3A, e to g). Exogenous GA3 treatment did not affect the nuclear localization of S114A-GFP in the uniconazole P–untreated transgenic tobacco plants either (data not shown). These results suggested that the regulation of the intracellular localization of RSG by GAs depends on the binding of 14-3-3 proteins to RSG.

**GAs Did Not Promote RSG Degradation**

RGA (for REPRESSOR OF GA1-3) of Arabidopsis and SLR1 (for SLENDER RICE1) of rice (Oryza sativa) are negative regulators of GA signaling that contain a DELLA domain (for a review, see Olszewski et al., 2002). GA treatments caused the disappearance of the RGA and SLR1 protein from the nuclei of plant cells.

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**Figure 2.** Effect of K252a on the Intracellular Localization of RSG.

Epidermal cells of leaves of transgenic tobacco plants expressing wild-type RSG-GFP were observed by confocal laser scanning microscopy. Fluorescence signals of GFP are displayed.  
(A) Intracellular localization of RSG-GFP in epidermal cells of leaves before treatment with K252a.  
(B) Intracellular localization of RSG-GFP in epidermal cells of leaves treated with 5 μM K252a for 30 min. Bar = 50 μm.
Figure 3. GAs Regulate the Intracellular Localization of RSG.

(A) Confocal microscopic images of GFP fluorescence in leaves of transgenic tobacco plants expressing RSG-GFP. (a) The effect of uniconazole P on the growth of tobacco. The control untreated tobacco plant is at left, the uniconazole P–treated tobacco plant is in the center, and the GA-treated tobacco plant after treatment with uniconazole P is at right. (b) to (d) Confocal microscopic images of GFP fluorescence in leaves of transgenic tobacco plants expressing RSG-GFP. To inhibit GA biosynthesis, the transgenic plants were treated with uniconazole P (c) and then with GA3 for 3 d (d). The control indicates normal growth conditions without any treatment (b). (e) to (g) Confocal microscopic images of GFP fluorescence in leaves of transgenic tobacco plants expressing S114A-GFP. The transgenic plants were treated with uniconazole P (f) and then treated with GA3 for 3 d (g). The control indicates normal growth conditions without any treatment (e). Uni, plants treated with uniconazole P for 7 d; Uni → GA, plants treated with uniconazole P for 7 d and then with GA3 for 3 d. Bar = 100 μm.

(B) Effect of GA3 on the intracellular localization of RSG-GFP in epidermal cells of leaves. For the GA treatment, the transgenic plants were treated with uniconozol P (a) and then were sprayed with 100 μM GA3 and observed after 2 h (b) and 6 h (c). GFP fluorescence was visualized by fluorescence microscopy. Note that GFP fluorescence was visible in the cytoplasm near the plasma membrane 2 h after GA3 treatment (b). Bar = 100 μm.
through protein degradation (Silverstone et al., 2001; Itoh et al., 2002). Recent studies have shown that SLEEPY1 of Arabidopsis and GA-INSENSITIVE DWARF 2 of rice are putative F-box subunits of an Skp-1-Cullin-F-box protein E3 ubiquitin ligase that mediates the GA-induced degradation of DELLA regulators (McGinnis et al., 2003; Sasaki et al., 2003). We then examined RSG-GFP protein levels by immunoblot analysis after GA3 treatment. Figure 4A shows that RSG-GFP protein levels were not reduced by GA3 treatment. The S114A mutation of RSG that diminished the interaction with 14-3-3 did not affect the protein levels of the GFP fusion protein (S114A-GFP) after GA3 treatment (Figure 4B). These results suggested that the disappearance of RSG-GFP from the nucleus did not depend on protein degradation mechanisms. Subsequently, we confirmed the results with the GFP fusion protein by analyzing the behavior of the native RSG protein. Consistent with the data presented for the RSG-GFP fusion protein, no clear change of the RSG protein level was detected in the GA3-treated tobacco plants (Figure 4C).

**Dominant Negative RSG Inhibits the Feedback Regulation of the GA 20-Oxidase Gene**

It has been shown that GA biosynthesis is affected by the activity of the GA response pathway through a feedback mechanism (Hedden and Phillips, 2000). Our results that the intracellular localization of RSG is regulated by GA levels lead us to speculate (Hedden and Phillips, 2000). Our results that the intracellular localization of RSG is regulated by GA levels lead us to speculate that RSG plays a role in the feedback regulation of GA biosynthetic genes. We examined whether the ent-kaurene oxidase gene, a target of RSG (Fukazawa et al., 2000), was regulated by endogenous amounts of GAs. However, the mRNA of the gene was not accumulated by the application of uniconazole P, an inhibitor for GA biosynthesis (Figure 5A), indicating that the tobacco ent-kaurene oxidase gene was not under the control of GA-negative feedback. Similar results have been reported in Arabidopsis (Helliwell et al., 1998).

One possibility that remained to be examined was that RSG might be directly or indirectly involved in the transcriptional regulation mechanism. Subsequently, we confirmed the results with the GA biosynthetic genes. RT-PCR was performed, and the products were amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of the NKO panel indicate the relative levels of NKO transcript after standardization using arcA as a loading control. The value of SR1 (− Un) was arbitrarily set to 1.0. Uni, uniconazole P.

(B) Effect of the dominant negative form of RSG on the expression of GA biosynthetic genes. RT-PCR was performed, and the products were detected by DNA gel blot hybridization using tobacco cDNAs encoding GA biosynthetic enzymes. Tobacco arcA (Ishida et al., 1993) was amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of the panels indicate the relative levels of mRNAs of GA biosynthetic genes after standardization using arcA as a loading control. The value of SR1 was arbitrarily set to 1.0. NtCPS, tobacco arcA (Ishida et al., 1993) was amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of panels indicate the relative levels of mRNAs of GA biosynthetic genes after standardization using arcA as a loading control. The value of SR1 was arbitrarily set to 1.0. Uni, uniconazole P.

(C) Feedback regulation of the GA 20-oxidase gene was inhibited by the dominant negative form of RSG. Transgenic tobacco plants expressing a dominant negative form of RSG and control SR1 plants were treated (+) or untreated (−) with uniconazole P for 1 week as indicated. The mRNA levels of GA 20-oxidase and GA 3-oxidase were examined by RT-PCR. These tobacco genes, Ntc12, encoding GA 20-oxidase, and Nty, encoding GA 3-oxidase, are known to be under GA feedback regulation (Kusaba et al., 1998; Ueguchi-Tanaka et al., 1998; Itoh et al., 1999). After PCR reactions, the products were detected by DNA gel blot hybridization. Tobacco arcA was amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of the NtCPS panel indicate the relative levels of NtCPS transcript after standardization using arcA as a loading control. The value of SR1 (− Un) was arbitrarily set to 1.0. Uni, uniconazole P.

Figure 5. Effect of the Dominant Negative Form of RSG on the Feedback Regulation of GA Biosynthetic Genes.

(A) Effect of uniconazole P on the expression of tobacco ent-kaurene oxidase (NKO) mRNA. SR1 tobacco plants were treated (+) or untreated (−) with uniconazole P for 1 week as indicated. The mRNA levels of NtKO were examined by RT-PCR. After amplification, the products were detected by DNA gel blot hybridization. Tobacco arcA (Ishida et al., 1993) was amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of the NtKO panel indicate the relative levels of NtKO transcript after standardization using arcA as a loading control. The value of SR1 (− Un) was arbitrarily set to 1.0. Uni, uniconazole P.

(B) Effect of the dominant negative form of RSG on the expression of GA biosynthetic genes. RT-PCR was performed, and the products were detected by DNA gel blot hybridization using tobacco cDNAs encoding GA biosynthetic enzymes. Tobacco arcA (Ishida et al., 1993) was amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of panels indicate the relative levels of mRNAs of GA biosynthetic genes after standardization using arcA as a loading control. The value of SR1 was arbitrarily set to 1.0. NtCPS, tobacco ent-kaurene oxidase synthase; NtKS, tobacco ent-kaurene synthase; NtKO, tobacco ent-kaurene oxidase; NtKO, tobacco arcA (Ishida et al., 1993) was amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of the panels indicate the relative levels of mRNAs of GA biosynthetic genes after standardization using arcA as a loading control. The value of SR1 was arbitrarily set to 1.0. Uni, uniconazole P.

(C) Feedback regulation of the GA 20-oxidase gene was inhibited by the dominant negative form of RSG. Transgenic tobacco plants expressing a dominant negative form of RSG and control SR1 plants were treated (+) or untreated (−) with uniconazole P for 1 week as indicated. The mRNA levels of GA 20-oxidase and GA 3-oxidase were examined by RT-PCR. These tobacco genes, Ntc12, encoding GA 20-oxidase, and Nty, encoding GA 3-oxidase, are known to be under GA feedback regulation (Kusaba et al., 1998; Ueguchi-Tanaka et al., 1998; Itoh et al., 1999). After PCR reactions, the products were detected by DNA gel blot hybridization. Tobacco arcA was amplified in the same reaction and used as an internal control of RT-PCR. The values at the bottom of the NtCPS panel indicate the relative levels of NtCPS transcript after standardization using arcA as a loading control. The value of SR1 (− Un) was arbitrarily set to 1.0. Uni, uniconazole P.
regulation of GA biosynthetic genes other than the ent-kaurene oxidase gene when endogenous GA levels were decreased. To test this possibility, we investigated the expression levels of the genes encoding the enzymes for all steps of the major GA biosynthetic pathway in transgenic plants in which the dominant negative form of RSG (Fukazawa et al., 2000) was expressed to repress the endogenous RSG function. For this experiment, we isolated the tobacco cDNAs encoding putative ent-copalyl diphosphate synthase, ent-kaurene synthase, and ent-kaurenoic acid oxidase with degenerate primers. The amino acid sequences of the predicted products of the tobacco cDNAs were 71, 66, and 69% identical and 95, 93, and 94% similar to those of the Arabidopsis genes, respectively (Sun and Kamiya, 1994; Yamaguchi et al., 1998; Helliwell et al., 2001). Analyses by RT-PCR and DNA gel blot hybridization showed that the endogenous amounts of GAs by repressing the expression of the ent-kaurene oxidase gene in transgenic plants (Fukazawa et al., 2000). The mRNA of the ent-copalyl diphosphate synthase gene was slightly increased in the transgenic plants. However, the significance of this observation is not clear at present. A series of genes encoding the enzymes involved in the GA biosynthetic pathway has been cloned from a variety of species. The genes of GA 20-oxidase and GA 3-oxidase are regulated by feedback mechanisms in several plants, including Arabidopsis and tobacco (Hedden and Phillips, 2000). If RSG is involved in GA-negative feedback regulation, the functional inhibition of RSG caused the suppression of the feedback regulation of either or both of these two genes. As shown in Figure 5B, the GA 3-oxidase gene was upregulated in a GA-negative feedback manner in the GA-deficient transgenic tobacco plants; however, the GA 20-oxidase gene was not upregulated in the same transgenic plants. The increased expression of GA 3-oxidase gene in the transgenic plants was reduced by GA3 treatment (our unpublished results). Furthermore, the accumulation of the GA 20-oxidase mRNA was not induced, even by the treatment with uniconazole P, in the transgenic tobacco plants in which the function of RSG was repressed, whereas it was clearly induced by the treatment with uniconazole P in the control SR1 tobacco plants (Figure 5C). These results suggest that RSG may directly or indirectly play a role in the feedback regulation of the GA 20-oxidase gene.

DISCUSSION

Appropriate intracellular localization plays a central role in regulating the normal function of proteins and the physiology of the cell. Proteins that sense internal and external stimuli can transmit information to the nucleus either by interaction with other signaling molecules or by their own movement from the cytoplasm to the nucleus. A growing number of transcription factors function by a regulated nuclear–cytoplasmic translocation mechanism to control specific gene expression (Kaffman and O’Shea, 1999).

The 14–3–3 proteins bind, in a phosphorylation-dependent fashion, to several important cytoplasmic and nuclear proteins, including Cdc25 and Raf-1, and function, at least in part, to sequester proteins in particular states or subcellular compartments (for a review, see Tzivion and Avruch, 2002). Masking of the motif that affects the intracellular localization of the ligand by 14–3–3 proteins could be a mechanism for the determination of the cytoplasmic or nuclear localization of nucleocytoplasmic shuttling proteins. It appears that the ligand, rather than 14–3–3, dictates the resulting subcellular location. Brunet et al. (2002) proposed that 14–3–3 functions as a type of molecular chauffeur and that the destination of the 14–3–3–bound complex is determined by instructions contained within the sequence and structure of the bound cargo rather than through any intrinsic properties of 14–3–3.

In addition to the regulation of intracellular localization, 14–3–3 binding can alter the ability of the target protein to interact with other partners. The Bcl-XL protein has a strong antiapoptotic effect in mammalian cells. This protein is inactivated by binding to the bcl-xL/bcl-2-associated death promoter homolog (BAD) protein, resulting in cell death. Cell survival stimuli promote 14–3–3 binding to BAD, which interferes with the ability of BAD to bind and inactivate Bcl-xL, thus inhibiting apoptosis (Datta et al., 2000). Furthermore, because each 14–3–3 dimer can bind up to two distinct ligand molecules (Muslin et al., 1996; Yaffe et al., 1997), 14–3–3 proteins could work as molecular scaffolds that allow the interaction between signaling proteins that do not associate directly with each other. Our results suggested that the regulation of the intracellular localization of RSG by GAs depends on 14–3–3 binding. Dissociation of RSG from 14–3–3 in response to a decrease in GA levels could promote both the nuclear accumulation of RSG and the formation of a new complex with RSG in the nucleus.

Endogenous GA levels are subject to negative feedback regulation through GA signaling (Hedden and Phillips, 2000). Studies of this homeostatic regulation may provide a link between two important areas of phytohormone research: signaling and control of endogenous amounts of hormones. The dominant negative form of RSG repressed the upregulation of the GA 20-oxidase gene in GA-deficient plants, suggesting that RSG...
may play a role in the regulation of GA feedback. In response to a decrease in GA levels, RSG could promote the transcriptional activation of the GA 20-oxidase gene either directly or indirectly. We previously found that a target of RSG is the ent-kaurene oxidase gene in the GA biosynthetic pathway (Fukazawa et al., 2000); however, the expression of the gene is not controlled in a negative feedback manner by bioactive GAs. A possible explanation for this observation is that RSG changes binding partners to regulate the GA 20-oxidase gene instead of the ent-kaurene oxidase gene when endogenous GA levels are decreased. The ability of basic Leu zipper transcription factors to form homodimers or heterodimers expands the repertoire of target genes that they can regulate (Motohashi et al., 1997). Decreases in GA levels of plants could induce de novo synthesis or modification of an RSG binding partner(s) and/or modification of RSG, resulting in the alteration of the target gene of RSG through the formation of a new complex. Additionally, RSG could bind a transcription factor that is involved in feedback regulation and help its nuclear translocation in response to a decrease in GA levels. In this context, we observed that the ent-kaurene oxidase mRNA was increased (1.9-fold) in the transgenic plants expressing S114A-GFP, whereas the levels of the GA 20-oxidase mRNA in the same transgenic plants were comparable to those of control SR1 plants (our unpublished results). This might suggest that the upregulation of the GA 20-oxidase gene requires cell signaling events in addition to the nuclear accumulation of RSG in response to a decrease in GA levels.

Recent studies showed that distinct gene expression programs can be differentially regulated by a single transcription factor. For instance, the p53 tumor suppressor protein of mammals is a transcription factor whose most important function in suppressing tumor formation is likely the induction of apoptosis. In response to severe DNA damage, p53 switches the targets from genes involved in cell cycle arrest to those involved in cell death. The phosphorylation status of p53 protein and the binding protein regulate the change of p53 target genes (Oda et al., 2000; Samuels-Lev et al., 2001). Another remarkable example is the yeast transcription factor Met4, which activates the genes involved in the biosynthesis of the sulfur-containing amino acids Met and Cys. However, excess Met in the rich medium inhibited the binding of Met4 to the promoters of these genes and recruited it to those of genes required for the production of S-adenosylmethionine. It was found that the ubiquitination of Met4 not only regulates the stability but also controls the selective activation of one set of Met4-responsive genes and not another (Kuras et al., 2002). These results indicate that both internal and external stimuli can control the differential recruitment of a single transcription factor to distinct promoters, thereby diversifying transcriptional activator specificity, and suggest that cells have evolved elaborate mechanisms to coordinately control gene expression and, at the same time, discriminate between different pathways by subtle mechanisms we have only begun to appreciate.

Another finding is that the feedback regulation of the GA 3-oxidase gene was not affected in the transgenic tobacco plants in which the function of RSG was repressed, although that of GA 20-oxidase gene was impaired in the same plants (Figure 5). This result may suggest that the feedback regulation of the GA 20-oxidase gene and the GA 3-oxidase gene is based on different mechanisms, at least in part.

We propose that the GA signal causes the disappearance of RSG from the nucleus through 14-3-3 binding (Figure 6). Because RSG binding to 14-3-3 requires the phosphorylation of Ser-114 in RSG, the phosphorylation state of Ser-114 of RSG might be under the regulation of GA signaling. Reversible protein phosphorylation on Ser and Thr residues is essential for the regulation of numerous cellular functions and signal transduction pathways. Control of this process is achieved by the modulation of the activities of the protein kinases and phosphatases, which catalyze the opposing phosphorylation and dephosphorylation reactions, respectively.

DELLA proteins are nuclear repressors of plant GA responses. GA signaling promotes the derepression of GA responses via the ubiquitin proteasome–mediated degradation of DELLA repressors. Most Skp1-Cullin-F-box protein ubiquitin ligase requires modification of the target protein for interaction between the target and F-box proteins, and phosphorylation is one of the most common types of modification of target proteins (Wu et al., 2003). Indeed, phosphorylation was implicated in the regulation of the 26S proteasome-mediated proteolysis of the barley (Hordeum vulgare) DELLA protein SLENDER1 (Fu et al., 2002) and rice SLR1 (Sasaki et al., 2003) in response to GAs.

We found that Ser/Thr kinase activity is required for the apparent cytoplasmic localization of wild-type RSG. The kinases might inhibit the nuclear import or promote the nuclear exclusion of wild-type RSG through enhancing the association of RSG with 14-3-3 proteins downstream of GA signaling. An attractive possibility is that identical kinases or kinases in the same signaling cascade are involved in the phosphorylation of both RSG and DELLA regulators to quench their activities in response to GAs. Known mammalian protein kinases that have been reported to phosphorylate their substrates on residues generating 14-3-3 binding sites include protein kinase A (Guthridge et al., 2000; Duckworth et al., 2002), protein kinase B/Akt (Brunet et al., 1999), checkpoint kinase Chk1 (Peng et al., 1997; Sanchez et al., 1997), C-TAK1 (for Cdc twenty-five C-associated protein kinase) (Muller et al., 2001), and calcium/calmodulin-dependent protein kinase (McKinsey et al., 2000). Functional homologs of these proteins in plants could be candidates for kinases that phosphorylate Ser-114 of RSG in response to GAs.

METHODS

Plant Growth Conditions and Chemical Treatments

The effects of chemicals were examined on plants grown in soil individually in 1-liter beakers. Transgenic tobacco (Nicotiana tabacum cv Petite Havana SR1) plants (4 weeks after germination) received 250 mL of 34 μM uniconazole P (Wako, Osaka, Japan), 100 mL of 20 μM GA3, 100 mL of 4.5 μM 2,4-D, or 100 mL of 1 μM brassinolide. Control plants received 250 or 100 mL of water. Plants were grown under continuous light at 28°C.

In Vitro RSG-14-3-3 Binding Assay

L-[35S]Met-labeled RSG was prepared in vitro using rabbit reticulocyte lysate as described previously (Igarashi et al., 2001). Where indicated, the
Ser/Thr kinase inhibitor K252a was included in the in vitro translation reaction. Portions of 35S-RSG protein were diluted with a NET-gel buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40) and a protease inhibitor cocktail (Pierce, Rockford, IL) and incubated with MBP-14-3-3 or MBP-14-3-3 (K52E) fusion protein (Igarashi et al., 2001) coupled to amylase resin (New England Biolabs, Beverly, MA) for 2 h at 4°C. Each agarose resin was washed twice with a NET-gel buffer and once with 50 mM Tris-HCl, pH 7.6, and 0.1% Nonidet P-40. The bead-bound proteins were then analyzed by SDS-PAGE and visualized by fluorography or Coomassie Brilliant Blue staining. Quantification of the signal was performed using a Fuji Film imaging system (BAS-1800II and LAS-1000 plus; Tokyo).

For the peptide competition assay, phosphorylated (108-NHFRSLp-SVDAFFDDG-122 and 108-NHFRRpLSVDAFFDDG-122) and unphosphorylated peptides consisting of amino acids 108 to 122 of RSG were prepared in PBS (11.9 mM sodium phosphate, pH 7.4, 2.7 mM KCl, and 136.9 mM NaCl). Peptides were synthesized by standard Fmoc [N-9-fluorenyl]methoxycarbonyl] chemistry. MBP-14-3-3 fusion proteins were incubated with synthetic peptides at the indicated concentrations (0, 0.1, 0.5, or 1.0 mM) for 1 h at 4°C followed by incubation with 35S-RSG protein. The beads were extensively washed with the buffer and subjected to SDS-PAGE as noted above.

**Kinase Assay and GST Pull-Down Assay**

The 14-3-3 binding domain of RSG (amino acids 69 to 140) or that of S114A mutant was cloned into pGEX-SX-2 (Amersham Bioscience, Piscataway, NJ). For experiments to phosphorylate RSG in vitro, GST-RSG and MBP-RSGK were affinity purified with GSH-Sepharose beads (Amersham Bioscience) or amylase resin. Kinase assays were performed using 0.1 μg of MBP-RSGK as the kinase and 20 μg of GST-RSG or GST-S114A mutant as the substrate in 100 μL of reaction mixture containing 10 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 0.5 mM CaCl₂, 0.1% Triton X-100, 0.1% β-mercaptoethanol, and 1 mM ATP (cold assay) or 100 μM ATP supplemented with (γ-32P)ATP (10 to 20 μCi/reaction) (hot assay) at 30°C for 30 min. For GST pull-down assays, phosphorylated GST-RSG or GST-S114A was incubated with GSH-Sepharose beads and affinity-purified MBP-14-3-3 for 1 h at 4°C in binding buffer consisting of 100 mM Mops-NaOH, pH 6.5, 10 mM MgCl₂, 0.1% Triton X-100, and 1% β-mercaptoethanol. Each bead was washed extensively with the binding buffer followed by elution with a binding buffer containing 10 mM GSH. Eluted proteins were resolved by SDS-PAGE. After electrophoresis, the phosphoproteins were detected by the phosphor imager BAS-2000 (Fuji Film), and total proteins were resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

**Generation of Anti-pSer-114-RSG Antibody**

The phospho-Ser-114-specific antisera was raised against a chemically synthesized phosphopeptide NPRPLNHFRSLpSVDA. The antisera was further affinity purified through a phosphopeptide-conjugated Sepharose CL-4B column. To deplete antibodies that recognize unphosphorylated peptide, the affinity-purified anti-phospho-Ser-114-RSG antibody was then passed through a column conjugated with unphosphorylated peptide NPRPLNHFRSLVD. The purified antibody reacts strongly with the phosphopeptide but not with the unphosphorylated peptide.

**Microscopy**

Leaf sections of plants were mounted in MS (Wako, Tokyo, Japan) supplemented with 0.3 M sucrose on glass slides with the abaxial side of the leaf up. Where indicated, leaf sections were incubated with 5 μM K252a for 15 min before observation.

Fluorescence signals were investigated by a confocal laser scanning microscope (TCS SP, Leica Microsystems, Heidelberg, Germany) equipped with an Ar/Kr laser. Specimens were excited with the 488-nm line of the argon ion laser, and emission lights were dispersed and recorded in a window from 550 to 570 nm for detection of GFP fluorescence and from 600 to 670 nm for chlorophyll autofluorescence. After successive scanning along with x axis for the appropriate interval, the three-dimensional images of GFP fluorescence were constructed from each optical section.

Fluorescence signals were also investigated by an epifluorescence microscope (Nikon eclipse E600; Nikon, Tokyo) equipped with a CCD camera (Nikon DXM1200). Images were exported as TIFF format files and further processed with bit map–based image editing software (COREL PHOT-PAINT; COREL, Dublin).

**Protein Analysis**

Tobacco leaves were homogenized in liquid nitrogen by grinding with a pestle and mortar. Cell lysates were extracted in 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 50 mM Naf, 1 mM NaVO₄, 20% glycerol, and 1% Nonidet P-40 containing a protease inhibitor cocktail (Pierce) and a phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Proteins in extracts were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and then reacted with anti-RSG antisera followed by horseradish peroxidase–conjugated second antibody. Chemiluminescence was detected and quantified with an imaging system (LAS-1000 plus; Fuji Film). Total protein was visualized by Coomassie Brilliant Blue staining.

**Isolation of Tobacco cDNA Homologs of GA Biosynthetic Enzymes**

To isolate the cDNA clones encoding tobacco ent-copalyl diphasphate synthase (NtCPS), tobacco ent-copalyl diphasphate synthase (NtPS), and tobacco ent-kaurenoic acid oxidase (NtkAO), we performed RT-PCR using total RNA isolated from young tobacco leaves and the degenerate primers 5’-GCNTAYGAYACNGCNGTGGTNGC-3’ and 5’-AANGC-CATNGCNTRCRTC-3’ for NtCPS, 5’-AARYTNCGNCTAYTGGY-TAYTTY-3’ and 5’-CCAYTCNGCYTNCNKARCAT-3’ for NtkAO, and 5’-ATGYTAYTNAAYGCGNGNCAYA-3’ and 5’-ACYTACCCACCGTTNGGDA-3’ for NtkAO. Samples were heated to 94°C for 5 min and then subjected to 30 cycles of 94°C for 30 s, 53°C for 60 s, and 74°C for 60 s. DNA fragments were isolated after gel electrophoresis and used for secondary PCR reactions. The resulting products were cloned into pUC18 and sequenced.

**RT-PCR**

For RT-PCR studies, total RNA from stems was converted into cDNA using SuperScript II (Invitrogen, Carlsbad, CA) and oligo(dT)₁₂₋₁₈ (Amersham Bioscience). PCR was performed with cDNA derived from 0.5 μg of total RNA (or 10 ng of genomic DNA as a control) with Extaq (Takara, Shiga, Japan). The primer sequences were 5’-GGC-CAATTTCTGTTGACGTAATCAATA-CATTCC-3’ and 5’-CACTTTGATAGAAAATGTATCTAAG-3’ for NtGA2ox₁, and 5’-CATNTYAYRACTGNAYCNCCYTTN-GDAD-3’ for NtGA3ox. Samples were heated to 94°C for 5 min and then subjected to 30 cycles of 94°C for 30 s, 53°C for 60 s, and 74°C for 60 s. DNA fragments were isolated after gel electrophoresis and used for secondary PCR reactions. The resulting products were cloned into pUC18 and sequenced.
20-oxidase and the mode of expression (Kusaba et al., 1998; Ueguchi-Tanaka et al., 1998). The PCR reaction was run for 18, 21, and 24 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 90 s to ensure that amplifications were within the linear range. The PCR products were size separated on a 1% (w/v) agarose gel, blotted onto Biodyne B (Pall, East Hills, NY), and hybridized with [32P]-labeled gene-specific DNA probes. After overnight hybridization in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS at 65°C, blots were washed twice in 2× SSC containing 0.1% SDS at 65°C before autoradiography.

Sequence data of the tobacco cDNAs from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AB170034 (NCPS), AB170035 (NkKS), and AB170036 (NkKAO).

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