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Involvement of RSG and 14-3-3 Proteins in the Transcriptional Regulation of a GA Biosynthetic Gene

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Abstract

Our study focused on the functional analysis of RSG, a tobacco transcriptional activator with a bZIP domain. Expression of a dominant-negative form of RSG severely inhibited the process of cell elongation in stems and reduced the endogenous amounts of GA_1, which is the major active gibberellin (GA) in tobacco in transgenic plants. To investigate the function of RSG in the regulation of GA amounts, we identified a target gene of RSG. This gene encodes *ent*-kaurene oxidase in the GA biosynthetic pathway. These results indicate that RSG regulates plant morphology through the transcriptional control of a GA biosynthetic enzyme. We identified the 14-3-3 signaling proteins as RSG-binding partners. The 14-3-3 proteins, which constitute a highly conserved isoform of homo- and heterodimeric molecules, associate with a number of signaling molecules to regulate growth, apoptosis and cell cycle progression. The mutant version of RSG that could not bind to 14-3-3 proteins exhibited a higher transcriptional activity than wild-type RSG. Consistent with this observation, the mutant RSG that could not bind to 14-3-3 proteins was predominantly localized in the nucleus, whereas wild-type RSG was distributed throughout the cell. 14-3-3 proteins negatively modulate RSG, which is involved in the regulation of endogenous amounts of GAs by controlling its intracellular localization.

Key words: 14-3-3; bZIP protein; Cell elongation; Gibberellins; Nuclear export; RSG; Target gene

Introduction

Development of multicellular organisms is a complex process during which cells proliferate, differentiate and elongate in response to inductive cues to form the tissues that will eventually comprise a body. During that time, various genes are turned on

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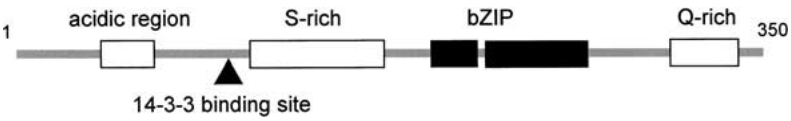

Figure 1. Schematic domain structure of RSG. S-rich, serine-rich region; Q-rich, glutamine-rich region.

and off in different developing cells, tissues and organs. This process is regulated by a variety of developmental programs and environmental stimuli which ultimately lead to the nucleus. There the transduced signals exert regulatory control over transcription factors that bind to specific *cis*-regulatory elements which then activate or repress expression of specific sets of genes. Indeed, genetic studies have revealed that either loss-of-function or gain-of-function mutations in proteins that are involved in transcriptional regulation severely impact cell growth, differentiation, and elongation. Gibberellins (GAs) are essential regulators of many aspects of plant development, including stem elongation, seed germination, and flowering. Both endogenous developmental programs and environmental stimuli affect the endogenous amounts of GAs by controlling the expression of GA biosynthetic enzymes. Therefore, elucidating the transcriptional regulation of GA biosynthetic enzymes is crucial for identifying the molecular mechanisms involved in plant development and to understand how these mechanisms help plants adapt to changes in their environment. In this review, we focused on a bZIP transcription factor, RSG, that is involved in the control of endogenous amounts of GAs and its functional regulation by 14-3-3 proteins.

A bZIP Transcriptional Activator RSG

RSG (for repression of shoot growth) is a DNA-binding protein with a basic leucine zipper (bZIP) domain that is isolated from tobacco (Fukazawa and others 2000). An acidic region, preceded by a phenylalanine cluster near the N terminus (Uesugi and others 1997), a serine-rich region, and a glutamine-rich region in the C-terminal region may serve as transcriptional activation domains (Figure 1). The *Arabidopsis* genome sequence contains 75 distinct members of the bZIP family, of which approximately 50 are not described in the literature. Using common domains, the AtbZIP family can be subdivided into 10 groups (Jakoby and others 2002). Thirteen RSG-related genes are found in the *Arabidopsis* genome (Figure 2) and 12 of them are classified into group I. Unlike other bZIP proteins, which have a conserved arginine residue at position −10 relative to the first leucine residue in the leucine-zipper region, members of group I share a lysine residue at this position. This arginine residue at position −10 is important for the DNA binding specificity of bZIP proteins. Substitution for the arginine residue by lysine at position −10 changes the optimal binding site of the yeast bZIP protein GCN4 (Suckow and others 1994) from the palindromic ATF/CRE (for activating transcription factor 1/cAMP response element) site (GACGTC) to the pseudopalindromic AP-1 (for activator protein 1) site (TGACTCA).

The bZIP proteins generally function as either homodimers or heterodimers. Dimerization specificity depends on the amino acid sequences of the two zipper regions. Thus, each bZIP protein in the cell can form dimers with only a small set of other bZIP proteins. Actually, RSG selectively forms a dimer with itself and its related protein but not with other classes of plant bZIP proteins, including GBF1, HY5 TGA1a, and TAF-1 (Fukazawa and others 2000).

Functional Redundancy of Regulatory Genes

A standard approach in plant development research is the analysis of morphological mutants. However, when a regulatory gene belongs to a multigene family and has functions that are the same as those of other members of the family, loss-of-function mutations and expression of antisense RNA or siRNA (for small inhibitory RNA) might not result in a phenotype. A clear example of this is provided by studies of the ethylene receptors of *Arabidopsis*. Single loss-of-function mutation in *ETHYLENE RESPONSE1* (*ETR1*)-related genes does not exhibit defects in ethylene response (Hua and Meyerowitz 1998). Mice that bear loss-of-function mutations in a wide variety of important genes, including those encoding c-Src (Soriano and others 1991), the nerve growth factor receptor (Lee and others 1992), and MyoD (Rudnicki and others 1992), are viable and have no obvious change in phenotype. Functional redundancy may represent a widespread feature in some regulatory networks controlling complex developmental processes in multicellular organisms. GA-deficient plant mutants display characteristic phenotypes that include dark green leaves and stunted growth attributable to the inhibition of stem

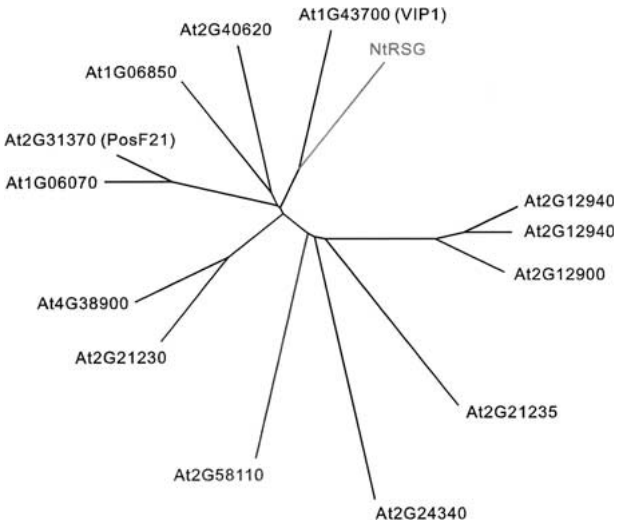

Figure 2. Classification of RSG-related bZIP proteins of *Arabidopsis*. Dendrogram generated using CLUSTALW (http://clustalw.genome.ad.ip) based on the predicted amino acid sequences of 13 RSG-related genes of *Arabidopsis* with tobacco RSG. At2G58110 that has a lysine residue at position −10 relative to the first leucine residue in the leucine-zipper region is shown in blue. Tobacco RSG is shown in red.

elongation, especially during the cell elongation phase. Most of the mutants had defects in the genes that encode GA biosynthetic enzymes and these mutants helped in the isolation of GA biosynthesis genes (Olszewski and others 2002); however, transcriptional or posttranslational regulators of GA biosynthetic enzymes had not been identified from molecular genetic studies. It was suggested that these genes are either functionally redundant or essential for embryogenesis.

Arabidopsis has about four times as many bZIP genes as yeast, worm and human. Genetic and molecular studies of a few of these *Arabidopsis thaliana* bZIP (AtbZIP) factors show that they regulate diverse biological processes such as pathogen defense, light and stresses signalling, seed maturation and flower development. A tobacco bZIP transcription factor RSG belongs to a small gene family (Fukazawa and others 2000). An *Arabidopsis* mutant in which one of RSG-related genes was interrupted by a T-DNA insertion did not show an obvious change in phenotype (Babiychuk and others 1997). Furthermore, transgenic tobacco plants in which the antisense construct of RSG was expressed did not show remarkable morphological alteration. Thus, to investigate the role of RSG in plant development, other strategies, along with studying insertion mutants, would be necessary. We tried to repress the function of RSG by using a dominant negative form of RSG.

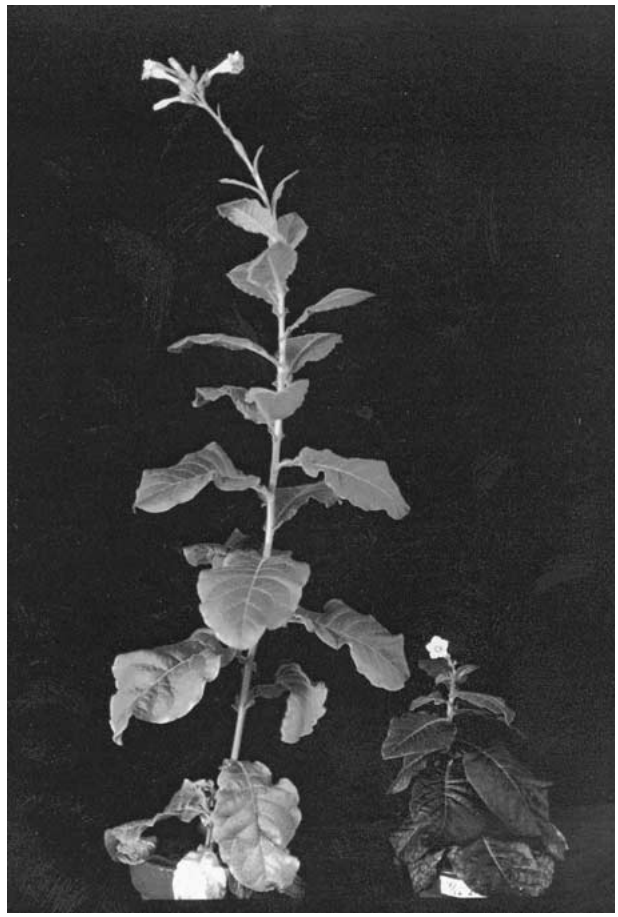

Figure 3. Phenotypes of transgenic tobacco plants expressing the dominant negative form of RSG. Comparison of SR1 tobacco (left) and the transgenic tobacco expressing the dominant negative form of RSG (right).

Dominant Negative Strategy

Removal of either the activation domain or the DNA binding domain, whether occurring naturally or constructed *in vitro*, can produce a dysfunctional transcription factor. Such mutant proteins can inhibit the function of wild-type factors in a dominant-negative fashion. For example, a dominant-negative form of PG13 (the gene product of *g13*), a member of the TGA1a bZIP family, suppresses the activity of wild-type TGA1a/PG13 in transgenic tobacco plants (Rieping and others 1994). These findings suggest that the mutant version of RSG without activation domains could repress the activity of full-length RSG and its related bZIP proteins. We confirmed that the transcriptional activity of full-length RSG was selectively repressed by the

bZIP domain of RSG in yeasts (Fukazawa and others 2000).

To repress endogenous RSG, we generated transgenic tobacco plants in which the dominant negative form of RSG was expressed under the control of the 35S promoter of the cauliflower mosaic virus. This expression severely inhibited the process of cell elongation of stems, resulting in a dwarf phenotype (Figure 3). The leaves of the transformed plants were smaller than those of control SR1 tobacco plants, had a wrinkled surface, and were slightly dark green. Whereas the growth of both root and callus from the transgenic tobacco plants was comparable to that of the control SR1 tobacco plants in tissue culture, shoot growth was severely inhibited. This result shows that the dominant-negative form of RSG did not inhibit the overall growth of tobacco but specifically inhibited the growth of the shoot.

Dominant Negative Form of RSG Reduced GA Amounts

GA-deficient *Arabidopsis* mutants display characteristic phenotypes, including dark green leaves and a dwarf growth habit attributable to reduced stem elongation (Hedden and Phillips 2000; Olszewski and others 2002). Because the dwarf phenotypes of transformed tobacco plants, in which the dominant-negative form of RSG was expressed, seemed similar to those of GA-deficient mutants, we examined the effect of applying GAs to the transformed tobacco plants. The GAs restored internode length as well as the size and surface of transformed tobacco plant leaves to the same as those of control SR1 tobacco plants. GA perception and signal transduction pathways appeared to be normal in the transgenic tobacco plants because GAs restored stem elongation and overdosing produced thin, elongated plants. These observations suggested that the dwarf phenotypes of the transgenic tobacco plants were due to the decrease in the endogenous amounts of GAs. In fact, the endogenous amounts of GA_1, the major active GA of tobaccos, in the transgenic plants was only 15% of that in the control SR1 plants (Fukazawa and others 2000). The dominant-negative form of RSG inhibited cell elongation through decreasing the endogenous amounts of GAs.

Target Genes of RSG

One problem with the strategy of using dominant-negative mutations is the potential for influencing other, unrelated regulatory proteins. Although RSG does not interact with those known plant bZIP proteins that we have tested, we cannot completely rule out the possibility that the morphological changes of transformed plants could reflect the cross-inhibition of other transcription factors. Identifying the target gene of RSG would provide the most direct evidence that RSG regulates the endogenous amounts of GAs. We found that GA_{53} was decreased in the transgenic tobacco plants in which the dominant negative form of RSG was expressed (Kamiya and Takahashi unpublished results). This suggested that the lesion in the transgenic tobaccos could involve the enzymes upstream of GA 20-oxidase in the GA biosynthesis. The GA biosynthetic pathway has been well characterized by using biochemical techniques as well as studying mutants defective in biosynthesis (Hedden and Phillips 2000; Olszewski and others 2002). We isolated tobacco cDNA homologs of all the known genes encoding enzymes involved in GA biosynthesis and examined their expression in the transgenic plants in which the dominant negative form of RSG was expressed. The *ent*-kaurene oxidase mRNA was evidently decreased in the transgenic plants (Fukazawa and others 2000), whereas mRNAs for other GA biosynthetic enzymes were not reduced (Fukazawa and Takahashi unpublished results). The *ent*-kaurene oxidase protein catalyzes *ent*-kaurene to *ent*-kaurenoic acid (Helliwell and others 1999) and is upstream of GA 20-oxidase in the GA biosynthetic pathway. These results indicated that the decrease in the GA amounts in the transgenic tobacco plants was due to the repression of the *ent*-kaurene oxidase gene expression. Tobacco *ent*-kaurene oxidase promoter-β-*glucuronidase* (*GUS*) was activated by RSG in the transient assay system with tobacco mesophyll protoplasts. Furthermore, recombinant RSG directly bound to the tobacco *ent*-kaurene oxidase promoter in gel retardation experiments (Fukazawa and Takahashi unpublished results). Thus, a target of RSG is the *ent*-kaurene oxidase gene in GA biosynthetic pathway and its regulation might be direct.

GA biosynthesis reactions are compartmentalized within the plant cell (Olszewski and others 2002). The *ent*-kaurene oxidase is a cytochrome P450 enzyme that is thought to associate with the endoplasmic reticulum (ER); however, it is not clear how *ent*-kaurene is transported from the plastid to the ER membrane. Because the N-terminal portion of the *ent*-kaurene oxidase directs green fluorescent protein (GFP) to the outer envelope membrane of the plastid, Helliwell and others (2001) proposed that the *ent*-kaurene oxidase provides a crucial link between the plastid- and ER-located steps of the GA

biosynthetic pathway. The expression pattern of *Arabidopsis ent*-kaurene oxidase gene (*GA3*) is consistent with the sites of GA action. The mRNA level is low in matured leaves and high in the elongating stems and further increased in the inflorescence (Helliwell and others 1998). RSG may regulate the gene expression of the *ent*-kaurene oxidase that links the physically separated early and the later steps of GA biosynthesis in response to developmental programs and environmental stimuli. A transcription factor usually regulates the expression of several target genes. The possibility that under certain conditions RSG potentially regulates the genes for other proteins involved in GA biosynthesis in addition to the *ent*-kaurene oxidase gene should not be overlooked.

14-3-3 Protein is an RSG Binding Partner

To understand how transcription factors control organisms, their target genes and the functional regulation mechanism must be identified. However, information regarding target genes and posttranslational regulations of transcription factors is still limited in plants. We found that RSG regulates the morphology of plants by controlling the *ent*-kaurene oxidase gene in GA biosynthetic pathway. Although GA biosynthesis is restricted to specific regions, including actively growing and elongating tissues (Smith 1992), RSG is expressed ubiquitously in plant organs. This apparent inconsistency suggests an involvement of posttranscriptional and/or posttranslational modifications of the transcription factor. One possible mechanism for the functional regulation of RSG is the interaction of RSG with accessory proteins, including other transcriptional activators, repressors, general transcription factors, coactivators, or chaperones.

To identify proteins that interact specifically with RSG, we performed a yeast two-hybrid screen using RSG as bait. One group of isolated clones encoded 14-3-3 proteins. The 14-3-3 proteins, which constitute a highly conserved isoform of homodimeric and heterodimeric molecules, associate with a number of different signaling proteins, including Raf-1, Bad, Cdc25, and telomerase (van Hemert and others 2001; Tzivion and Avruch 2002). On the basis of their interaction with various ligands, 14-3-3 proteins have been proposed to be important in controlling intracellular signaling pathways (Aitken 1996). In addition, 14-3-3 proteins work as molecular chaperones or regulate the intracellular localization of their binding partners (Kumagai and Dunphy 1999; Yang and others 1999; Seimiya and others 2000). In plants, 14-3-3 proteins include the regulator for the H^+-ATPase in the plasma membrane (Korthout and de Boer 1994; Marra and others 1994; Oecking and others 1994) and a protein that specifically inhibits nitrate reductase activity from spinach cells (Bachmann and others 1996; Moorhead and others 1996). The plant 14-3-3 proteins also are found as part of a transcriptional DNA binding complex, and have been reported to associate with G-box DNA binding complexes and a TATA box-binding protein (Lu and others 1992; Schultz and others 1998).

There are at least seven distinct genes for 14-3-3 in mammalian cells, giving rise to nine isotypes (α, β, γ, ϵ, δ, η, σ, τ, and ξ, with α and δ being phosphorylated forms of β and ξ, respectively) (Aitken and others 1995). The *Arabidopsis* 14-3-3 family consists of 13 members (ω, ψ, χ, ϕ, υ, ρ, π, o, ν, μ, λ, κ, ϵ) (Sehnke and others 2002). Expression of π has yet to be confirmed experimentally (Rosenquist and others 2001). Two additional truncated 14-3-3-like genes were also found in the *Arabidopsis* genome (Rosenquist and others 2001). 14-3-3s are currently designated by Greek letters, with the mammalian isoform names generally chosen from the beginning of the alphabet and the *Arabidopsis* isoforms chosen from the end of the alphabet. Comparison of the *Arabidopsis* 14-3-3 family isoforms reveals that the isoforms all share a conserved core region, with the N and C termini being divergent.

14-3-3 Protein Negatively Regulates RSG

The three-dimensional crystal structures of 14-3-3 consists of a dimer with a bundle of nine antiparallel α-helices in each monomer (Liu and others 1995; Xiao and others 1995). Many ligand proteins containing the conserved phosphorylated motifs bind into a large groove formed by amphiphilic helices of 14-3-3 (Muslin and others 1996; Yaffe and others 1997). The interaction of RSG with 14-3-3 proteins in plant cells was confirmed by immunoprecipitation experiments (Igarashi and others 2001). It has been demonstrated that 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 (Muslin and others 1996; Yaffe and others 1997). A sequence closely related to the conventional 14-3-3 binding motif was found surrounding Ser-114 of RSG. We demonstrated that Ser-114 is a critical serine for binding to 14-3-3 with a yeast two-hybrid assay. Of note, similar 14-3-3 binding motifs are found in RSG-related bZIP proteins of *Arabidopsis* (Table 1). The mutant version of RSG (S114A) that

Table 1. Putative 14-3-3 Binding Motif found in RSG-related bZIP Proteins of *Arabidopsis*

Protein	Predicted binding sites
NtRSG	RSL*S*VD[116]
At1g43700 (VIP1)	RSF*S*VD[37], RSN*S*MD[73]
At2g21230	RSV*S*MD[302], RSM*S*QP[81]
At4g38900	RSV*S*VD[323], RSM*S*QP[103]
At1g06850	RSR*S*DD[42]
At2g31370 (PosF21)	HSQ*S*MD[164]
At1g06070	HSQ*S*MD[172]
At2g40620	HSL*S*VD[122]
At2g12940	RSV*S*MD[90]
At2G13150	RSL*S*VD[91]
At2G21235	RSN*S*AK[184]
At2G24340	HSM*T*SQ[117]
At2G12900	RSM*T*QP[19]
At1G58110	RSS*S*DS[100]
Consensus	RSX*S*X[P/−]

Critical serine or threonine residues on 14-3-3 binding are underlined.

could not bind to 14-3-3 proteins exhibited a higher transcriptional activity than did wild-type RSG in the transient assay system with tobacco cultured cells, indicating that tobacco 14-3-3 negatively regulates RSG (Igarashi and others 2001).

14-3-3 Directs the Intracellular Localization of RSG

Signal transduction pathways are complex networks of biochemical reactions that ultimately culminate in specific patterns of nuclear gene expression mediated by transcription factors. Intensive studies have revealed a variety of posttranslational regulation mechanisms of transcription factors, including the interaction with coactivators and general transcription factors. Another possible mechanism, in which the ability of transcription factors is not necessarily affected, is the regulation of intracellular localization (Kaffman and O'Shea 1999). One example of a protein that has been shown to bind to 14-3-3 proteins through the conserved motif containing a phosphoserine residue is Cdc25. Cdc25 is a phosphatase that dephosphorylates and activates the cyclin-dependent kinase Cdc2, leading to entry into mitosis. The binding of 14-3-3 proteins has little or no effect on the phosphatase activity of *Xenopus* Cdc25; however, 14-3-3 proteins sequester Cdc25 in the cytoplasm, regulating its subcellular localization to prevent access of Cdc25 to the Cdc2-cyclin B substrates (Peng and others 1997; Kumagai and Dunphy, 1999; Yang and others 1999). This finding prompted us to examine the possibility that 14-3-3 proteins regulate RSG by controlling its intracellular localization. Consistent with the result that 14-3-3 negatively regulates RSG, the mutant RSG (S114A) 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 and others 2001). 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. Thus, 14-3-3 proteins negatively modulate RSG through the regulation of intracellular localization (Figure 4).

The binding of 14-3-3 proteins might inhibit the nuclear import and/or promote the nuclear exclusion of wild-type RSG. Masking of the nuclear localization sequence and/or exposure of the nuclear export sequence (NES) of the complex could be a consequence of 14-3-3 binding to RSG. Brunet and others (2002) proposed that 14-3-3 functions as a type of ''molecular chauffeur'' where 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. Alternatively, 14-3-3 binding could result in a conformational alteration of RSG that changes the interaction with nuclear import and/or export machinery. In *Xenopus*, binding of 14-3-3 proteins to Cdc25 markedly reduces the nuclear import rate of Cdc25, allowing nuclear export mediated by CRM1 to predominate (Yang and others 1999). Similarly, a variety of mammalian proteins involved in transcriptional control are regulated through 14-3-3 binding, including FKHRL1, histone deacetylase, TAZ, and MITR (van Hemert and others 2001; Tzivion and Avruch 2002). Plants do not appear to have homologs of Cdc25 (Arabidopsis Genome Initiative 2000). However, in our study, we found that 14-3-3 binding to a plant bZIP transcription factor is responsible for its cytoplasmic localization. Thus, the nuclear-cytoplasmic partitioning of regulatory factors by 14-3-3 proteins appears to be an evolutionarily ancient mechanism.

Several studies have shown that 14-3-3 proteins also associate with plant transcription factors, including the G-box binding complex (Lu and others 1992), VP1, bZIP protein EmBP1 (Schultz and others 1998), general transcription factor TBP, and TFIIB (Pan and others 1999). Because 14-3-3 proteins apparently are assembled into protein-DNA complexes with these proteins, a role of 14-3-3 could be to provide the link of activator-activator and activator-general transcription factors but not

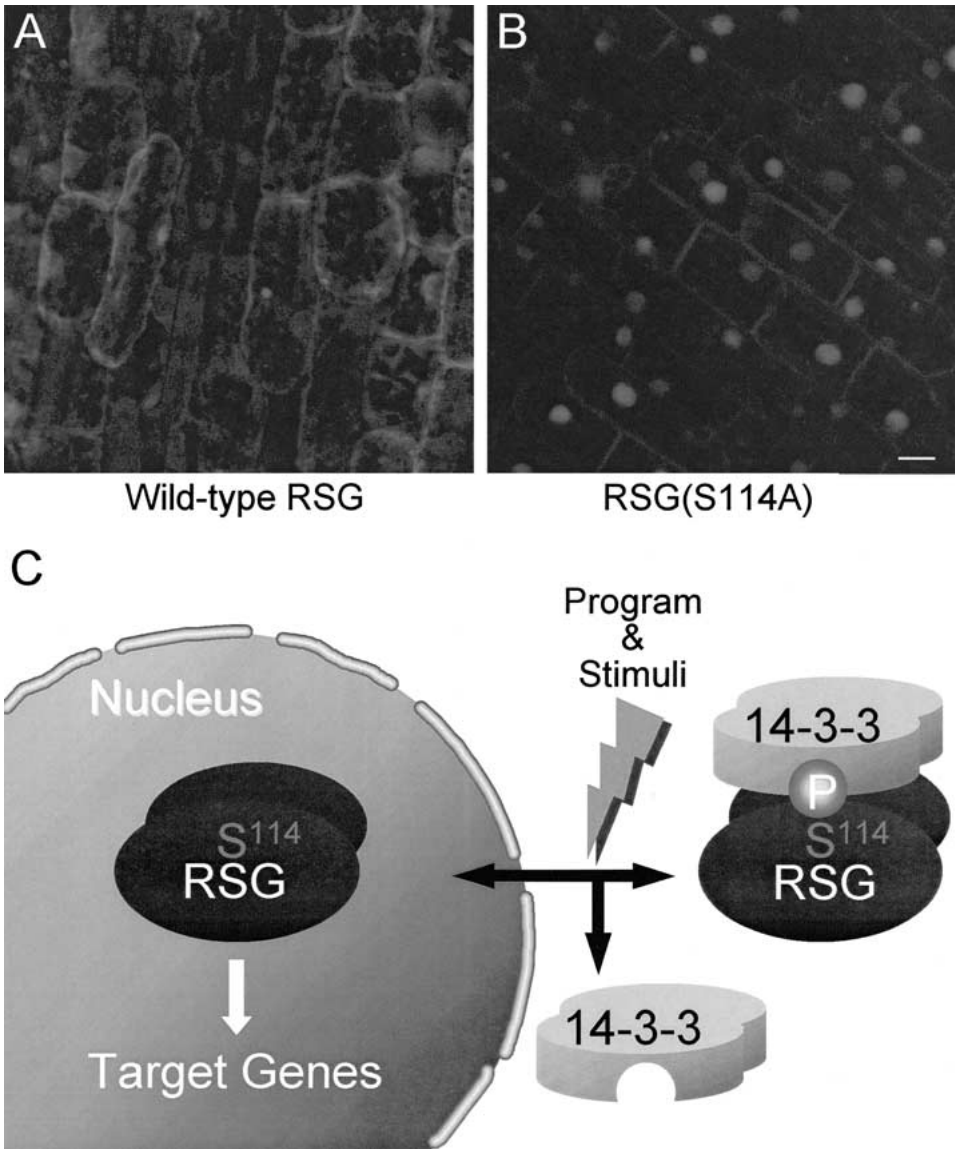

Figure 4. 14-3-3 proteins regulate the function of RSG by controlling its intracellular localization. Effects of the S114A mutation on the intracellular localization of RSG. Intracellular localization of GFP fusion proteins in transgenic tobacco plants was analyzed by confocal laser scanning microscopy. (**A**) Epidermal cells of stems of transgenic tobacco plants expressing wild-type RSG-GFP. Fluorescence signals of GFP (green) are displayed. (**B**) Epidermal cells of stems of transgenic tobacco plants expressing S114A-GFP. Fluorescence signals of GFP are displayed. Bar in (**B**) = 20 μm for (**A**) and (**B**). (**C**) A model for functional regulation of RSG by 14-3-3. RSG protein continually shuttles back and forth between the nucleus and the cytoplasm. Binding of 14-3-3 proteins to the phosphorylated serine 114 residue of the RSG protein inhibits nuclear import and/or enhances nuclear export to shift the equilibrium of RSG to a predominantly cytoplasmic localization. Inhibition of 14-3-3 binding leads to the rapid redistribution of RSG to the nucleus, where it activates transcription of the target genes including a GA biosynthetic gene.

the sequestration of the ligands in the cytoplasm, as in the cases of RSG and Cdc25.

RSG Shuttles

The apparent cytoplasmic localization of RSG in plant cells suggests that RSG could be tethered to a cytoplasmic structure by a static mechanism that prevents its release and subsequent movement to the nucleus. Alternatively, the cytoplasmic localization of RSG may reflect the balance of a dynamic nuclear import/export process in which RSG is mobile and can enter the nucleus but is exported rapidly to the cytoplasm. To distinguish between these hypotheses,

we examined the effect of leptomycin B (LMB), a potent inhibitor that blocks nuclear export (Kudo and others 1999). This drug inhibits CRM1/exportin 1, a receptor that mediates the nuclear export of proteins containing a NES (Fornerod and others 1997; Fukuda and others 1997; Ossareh-Nazari and others 1997). Treatment with LMB led to a significant redistribution of RSG within 45 min from a mainly cytoplasmic to an almost exclusively nuclear localization (Igarashi and others 2001). The result indicated that RSG is exported actively to the cytoplasm and that its intracellular localization is regulated by a dynamic mechanism rather than a static retention mechanism. The apparent 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. Because mRNA for RSG is expressed in various organs (Fukazawa and others 2000), posttranscriptional and/or posttranslational modifications of RSG should be necessary for the appropriate spatial and temporal regulation of GA amounts in plants. Exclusion of RSG from the nucleus via active nuclear export suggests that the nuclear localization of RSG may be allowed only in a limited number of cells and in a restricted time frame during plant development. The intracellular compartmentalization of RSG could play a role in the strictly controlled GA biosynthesis of plants. Both internal and external stimuli might affect the endogenous amounts of GAs through controlling nucleo-cytoplasmic shuttling of RSG. The search for stimuli that affect the intracellular localization of RSG is under way.

VIP1 (for VirE2-interacting protein 1) is a RSG-related bZIP protein in *Arabidopsis* (Figure 2). This protein is identified as a binding protein of *Agrobacterium* VirE2 that coats the transported T-DNA molecule and is thought to play a role in nuclear import of VirE2 (Tzfira and others 2001). Nuclear import of VirE2 could depend on the nucleo-cytoplasmic shuttling of VIP.

Regulation of 14-3-3 Binding

A protein kinase inhibitor, K252a resulted in a marked inhibition of RSG binding to 14-3-3 and of sequestration of RSG in the cytoplasm (Ishida and Takahashi unpublished results). Furthermore, our recent competition experiments using synthetic peptides showed that the phosphorylation of Ser-114 of RSG is important for 14-3-3 binding to RSG (Ishida and Takahashi unpublished results). Thus, intracellular localization of RSG is regulated by 14-3-3 binding through phosphorylation status of Ser-114. Reversible protein phosphorylation on serine and threonine 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. When RSG dissociates from 14-3-3 proteins in response to developmental programs and environmental stimuli, through the dephosphorylation of Ser-114, it accumulates in the nucleus. There it may activate its target genes, including a GA biosynthetic gene. Identification of the kinases and phosphatases that modify the phosphorylation status of Ser-114 of RSG will improve our understanding of the molecular mechanisms regulating endogenous amounts of GAs.

However, phosphorylation of target proteins might not be the only factor controlling the binding of the 14-3-3. Binding of 14-3-3s to phosphorylated nitrate reductase requires millimolar concentrations of a divalent cation such as Mg^{2+}. Athwal and Huber (2002) found that micromolar concentrations of the polyamines, $spermidine^{4+}$ and $spermine^{3+}$, can substitute for divalent cations in modulating 14-3-3 action. The *ACL5* gene of *Arabidopsis* encodes spermine synthase, one of the polyamine biosynthetic enzymes (Hanzawa and others 2000). Recessive mutations in the *ACL5* gene result in a severe reduction in the length of stem internodes. Although the molecular mechanism is unknown, this result suggested that polyamines play a role in the regulation of plant development. The polyamine content of plant cells changes developmentally and in response to stresses (Kumar 1997). It might be possible that polyamines may express at least part of their biological action by binding to 14-3-3s, thereby promoting their interactions with various target proteins, including RSG.

14-3-3 family proteins can dimerize via their N-terminal domains, as we demonstrated in a two-hybrid assay (Igarashi and others 2001). Each 14-3-3 dimer can bind up to two distinct ligand molecules (Muslin and others 1996; Yaffe and others 1997). Thus, 14-3-3 proteins are thought to sometimes work as molecular scaffolds that allow interaction between signaling proteins that do not associate directly with each other (Braselmann and McCormick 1995). Because 14-3-3 binding proteins include various signaling factors, such as kinases and phosphatases (Aitken 1996), there could be other mechanisms in which RSG functions are regulated by another 14-3-3 binding protein via a 14-3-3 dimer as an adaptor. Investigation of how the interaction between RSG and 14-3-3 is controlled by both internal and external signals will help

reveal the molecular mechanisms for the fine regulation of the endogenous amounts of GAs that control many aspects of plant development.

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