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RESEARCH ARTICLE

Arabidopsis Homologs of a c-Jun Coactivator Are Present Both in Monomeric Form and in the COP9 Complex, and Their Abundance Is Differentially Affected by the Pleiotropic *cop*/*det*/*fus* Mutations

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The CONSTITUTIVE PHOTOMORPHOGENIC9 (COP9) complex is a nuclear localized, multisubunit protein complex essential for repression of light-mediated development in Arabidopsis. Mutations that abolish the complex result in constitutive photomorphogenic development in darkness and pleiotropic developmental defects in both light and darkness. Here, we report the identification of two apparently redundant genes, *AJH1* and *AJH2*, that encode a subunit of the COP9 complex. Both AJH1 and AJH2 share high amino acid sequence identity (62 and 63%, respectively) with JAB1, a specific mammalian coactivator of AP-1 transcription. The proteins encoded by these two genes are present in both complex and monomeric forms, whereas complex formation is in part mediated by the direct interaction with FUSCA6. In addition, the stability of the monomeric AJH proteins requires functional *COP1* and *DEETIOLATED1* loci. Together with the fact that the previously known subunit FUSCA6 is an Arabidopsis homolog of human GPS1, a negative regulator of AP-1 transcription, our data suggest that the COP9 complex may contain both negative and positive regulators of transcription. Therefore, the COP9 complex may achieve its pleiotropic effects on Arabidopsis development by modulating activities of transcription factors in response to environmental stimuli.

INTRODUCTION

Light signals play a critical role in controlling the development of higher plants. Under light conditions, young seedlings display characteristic photomorphogenic development. This developmental fate is exemplified by seedlings displaying open and expanded cotyledons, inhibition of hypocotyl elongation, cell differentiation, and high-level expression of light-inducible genes (Deng, 1994). In contrast, dark-grown seedlings undergo skotomorphogenesis. This developmental fate results in seedlings displaying an apical hook on closed and unexpanded cotyledons, elongation of hypocotyl cells, and low-level expression of light-inducible genes.

Various genetic mutant screens have been designed to identify components in the light-mediated signal transduction pathway in Arabidopsis (Pepper et al., 1994; von Arnim and Deng, 1996). One group of mutant screens has identified positive regulatory components of the light signal transduction cascade (von Arnim and Deng, 1996; Oyama et al., 1997). Those include the photoreceptors and the transcription factor *LONG HYPOCOTYL5* (*HY5*). A second set of mutant screens has identified intermediate negative regulators of light-signaling components known collectively as the *cop*/*det*/*fus* (for *constitutive photomorphogenic*/*deetiolated*/*fusca*) mutants (Miséra et al., 1994; Kwok et al., 1996). Mutations in eleven pleiotropic *COP*/*DET*/*FUS* loci (e.g., *COP1*, *DET1*, *COP8*, *COP9*, *COP10*, *COP16*, *FUS6*/*COP11*, *FUS4*, *FUS5*, *FUS11*, and *FUS12*) have been identified. Their wild-type gene products seem to play roles in repressing light-regulated development and are essential for overall development in Arabidopsis (Deng et al., 1991; Wei and Deng, 1992; Castle

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and Meinke, 1994; Miséra et al., 1994; Pepper et al., 1994; Wei et al., 1994). All of the pleiotropic *cop*/*det*/*fus* mutants display very similar, if not identical, pleiotropic mutant phenotypes, that is, light-grown phenotypes in the absence of light. These phenotypes include alterations in both general cell morphology and gene expression (Deng et al., 1991; Wei and Deng, 1992; Castle and Meinke, 1994; Miséra et al., 1994; Pepper et al., 1994; Wei et al., 1994; Kwok et al., 1996; Oyama et al., 1997).

The Arabidopsis *COP9* gene was identified initially by mutations that resulted in phenotypes seen in the pleiotropic *cop*/*det*/*fus* mutants (Wei et al., 1992). Cloning and molecular studies of *COP9* indicate that it encodes a protein exclusively present in a high molecular weight, nuclear-localized protein complex known as the COP9 complex (Wei et al., 1994b). Biochemical purification of the COP9 complex revealed that it is multisubunit in nature, with a total molecular mass of ~550 kD (Chamovitz et al., 1996). Interestingly, initial studies of two mutants in the pleiotropic *cop*/*det*/*fus* collection, *fus6* (also called *cop11*) and *cop8*, indicate that the COP9 complex is unstable in these mutants (Wei et al., 1994b). This result suggests that FUS6 and COP8 may define integral subunits or important factors necessary for either the formation and/or stability of the COP9 complex. In the case of FUS6, it has been demonstrated recently that it is clearly a subunit of the COP9 complex (Chamovitz et al., 1996; Staub et al., 1996).

To date, FUS6 and COP9 remain the only two fully characterized components of the Arabidopsis COP9 complex. Most recently, a mammalian counterpart of the COP9 complex has been purified and characterized (Seeger et al., 1998; Wei and Deng, 1998). It has been shown that both mammalian and plant COP9 complexes consist of eight highly homologous subunits (Wei et al., 1998). To better understand the molecular function(s) of the COP9 complex, we decided to further characterize the new subunits of the COP9 complex in Arabidopsis and to determine which of the other pleiotropic *COP*/*DET*/*FUS* loci may encode the remaining components of the COP9 complex. Here, we characterize a new subunit of the COP9 complex. In addition, we provide an overall assessment of how many of the pleiotropic *COP*/*DET*/*FUS* loci may encode other components of the COP9 complex or how they may affect the activity of the complex. Based on these findings, the potential role of the COP9 complex in the repression of photomorphogenesis is discussed.

RESULTS

Identification and Molecular Characterization of AJH1 and AJH2, the Arabidopsis JAB1 Homologs

To further characterize the subunit composition of the COP9 complex, we subjected putative subunits copurifying with the COP9 complex to peptide sequencing. One peptide sequence of 14 amino acids, SSLDSHLLDLLWNK, was obtained from a 42-kD copurifying protein (designated as the p42 band) (see Figure 1C of Chamovitz et al., 1996). This peptide sequence is 100% identical to a putative coding region in a single Arabidopsis expressed sequence tag (EST) clone (GenBank accession number R65432). Using this EST clone as a probe, we obtained its full-length cDNA clone by screening a 7-day-old etiolated Arabidopsis seedling library (data not shown). Sequencing analysis indicated that this cDNA (designated as p42A) contains an open reading frame of 357 amino acids with a predicted molecular mass of 40 kD (Figure 1). In the process, a second cDNA, designated p42B, was also identified. This cDNA encodes a protein of 358 amino acids, sharing 85% amino acid identity and the same molecular mass with the p42A-encoded protein (Figure 1). The two cDNAs are also very similar at the nucleotide level, sharing 86% identity. Genomic DNA gel blot analyses at various stringency levels confirmed that there are no other homologs of p42A and p42B in the Arabidopsis genome (data not shown).

The proteins encoded by p42A and p42B are highly homologous to JAB1 (for c-Jun activation domain binding protein 1), a recently identified specific coactivator of AP-1 transcription in humans (Figure 1; Claret et al., 1996). Therefore, we designated the two genes defined by the p42A and p42B cDNA clones as *AJH1* and *AJH2*, respectively, for Ara-

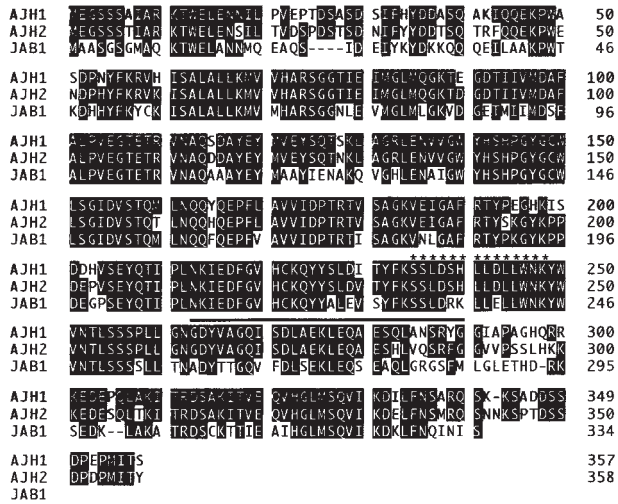

Figure 1. Protein Sequence Comparison of AJH1, AJH2, and JAB1.

Black regions indicate amino acids identical among the three proteins. Broken lines represent gaps introduced to optimize alignment. Asterisks above amino acids 235 to 248 of AJH1 and AJH2 represent the peptides sequenced from the p42 protein band from COP9 complex purification (see Figure 1C in Chamovitz et al., 1996). The line over amino acids 263 to 290 of AJH1 marks the approximate predicted coiled-coil region of AJH1, AJH2, and JAB1. GenBank accession numbers for the nucleotide sequences of *AJH1* and *AJH2* are AF087413 and AF087412, respectively.

bidopsis JAB1 homologs 1 and 2. Throughout their entire lengths, AJH1 and AJH2 are, respectively, 62 and 63% identical at the amino acid level to human JAB1. In fact, the internal 225–amino acid region of AJH1 and AJH2 (from amino acids 61 to 285) shares even greater amino acid identity (76 and 77%, respectively) with the corresponding JAB1 internal region (from amino acids 57 to 281). At the N terminus, both AJH1 and AJH2 share 40% amino acid identity with JAB1, whereas at the C terminus, AJH1 and AJH2 share 39 and 37% amino acid identity with JAB1, respectively. In addition, AJH1, AJH2, and JAB1 all exhibit lower amino acid identity (29, 28, and 31%, respectively) with *Schizosaccharomyces pombe pad1*$^+$, an essential fission yeast gene whose gene product is a positive regulator of Pap1-dependent transcription (Toda et al., 1991; Shimanuki et al., 1995). Interestingly, Pap1 was identified as an AP-1–like leucine zipper transcription factor, whereas Pad1 has been recently shown to be a component of the proteasome in *S. pombe* (Spataro et al., 1997). The observed similarity of the AJH proteins to Pad1 is consistent with the observation that many of the mammalian COP9 complex components also are related to distinct subunits of the regulatory complex of the 26S proteasome (Wei et al., 1998). Based on this striking amino acid conservation between plant and human proteins, we conclude that we have cloned the JAB1 homologs from Arabidopsis.

Besides a putative coiled-coil structure (from amino acids 264 to 290 in AJH1, amino acids 266 to 288 in AJH2, and amino acids 265 to 282 in JAB1; Figure 1), recent database search analyses indicate that *JAB1*, *AJH1*, *AJH2*, and *pad1*$^+$ are all members of the *MOV34* gene family, whose products are characterized by a highly conserved region at the N-terminal part of their proteins (Asano et al., 1997). This N-terminal region, which comprises amino acids 53 to 143 of JAB1 and amino acids 57 to 147 of AJH1 and AJH2, has been found in a subset of components of the proteasome and the human eukaryotic translation initiation factor 3 complex. The precise molecular function of this large region of similarity among different proteins is unclear, but it may be involved in large multiprotein complex assembly.

Both *AJH1* and *AJH2* Are Located on Chromosome 1 and Do Not Overlap with Any Known *COP*/*DET*/*FUS* Loci

Previous near-saturating genetic screens have identified 11 *COP*/*DET*/*FUS* loci (Miséra et al., 1994; Kwok et al., 1996). To determine whether *AJH1* and *AJH2* correspond to any of these loci, we determined the genetic map position for both genes. Both genes mapped to chromosome 1 (at 52 and 149 centimorgans, respectively) in the Arabidopsis genome (data not shown). *AJH1* is located on yeast articial chromosome (YAC) clone CIC5F4, whereas *AJH2* is located on YAC CIC7E8. The fact that neither of these YAC clones overlaps with any of the known *COP*/*DET*/*FUS* loci (S.F. Kwok and X.-W. Deng, unpublished data) is consistent with our notion that *AJH1* and *AJH2* are functionally redundant and thus that the corresponding single-gene mutations would not have been recovered by the typical *cop*/*det*/*fus* mutant screen.

The AJH Proteins, COP9, and FUS6 Coimmunoprecipitate from Total Soluble Arabidopsis Protein Extracts

As a first step toward confirming that the AJH proteins are indeed part of the COP9 complex, rabbit antibodies were raised against the recombinant AJH1 protein. The specific antibodies subsequently were purified (see Methods) and shown to recognize both recombinant AJH1 and AJH2 proteins (data not shown). Immunoprecipitation with the purified polyclonal antibodies raised against the recombinant AJH1 protein confirmed specific coimmunoprecipitation of COP9 and FUS6 with the single protein band that is reactive to the AJH antibodies (Figure 2). This single band, recognized by the specific AJH antibodies, most likely represents a combination of both AJH1 and AJH2 (see below). In addition, FUS6-specific antibodies were able to coimmunoprecipitate FUS6, COP9, and the specific protein band recognized by the AJH antibodies (Figure 2). These results show that the protein(s) that is recognized by the AJH antibodies is stably

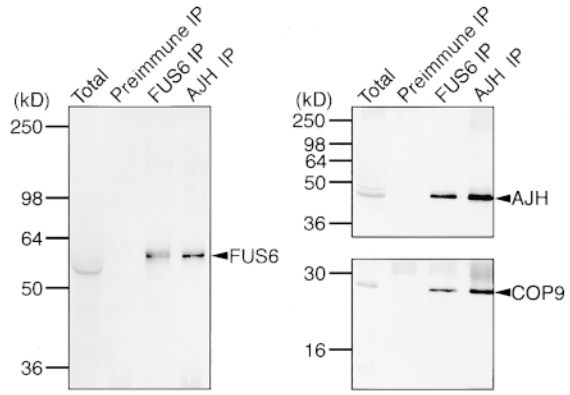

Figure 2. Coimmunoprecipitation of the Arabidopsis JAB1 Homologs with COP9 and FUS6.

Immunoprecipitation extracts using AJH preimmune antibodies (Preimmune IP), FUS6-specific antibodies (FUS6 IP), and AJH-specific antibodies (AJH IP) are indicated. Preimmune immunoprecipitation represents preimmune serum from the same rabbit before injection with the GST–AJH1 antigen (see Methods). Total indicates seedling extracts before immunoprecipitation. At left is the immunoblot from immunoprecipitation extracts probed with affinity-purified FUS6 antibodies. At top right is the immunoblot of the same set of immunoprecipitation extracts probed with the purified AJH antibodies. The immunoblot of the same set of immunoprecipitation extracts probed with affinity-purified COP9 antibodies is shown at bottom right. The numbers at left indicate the positions of molecular mass markers in kilodaltons.

associated with COP9 and FUS6 in the same multiprotein complex.

Both AJH1 and AJH2 Exist as Monomeric and Complexed Forms

To test whether the AJH protein(s) is exclusively present in the COP9 complex, we conducted gel filtration analyses of total Arabidopsis protein extracts. In wild-type seedling extracts, AJH antibodies recognized a protein band that cofractionated in high molecular mass fractions (eluting from fractions 11 to 13 at ~500 kD) identical to those for COP9 (Figure 3). However, at the same time, the AJH antibodies recognized a band that was also present in the fractions corresponding to the AJH monomeric form (eluting from fractions 19 to 20) (Figure 3). Further examination of the fractionation profiles of wild-type seedlings grown under different light regimes failed to reveal dramatic differences in the molar distribution of the complex versus monomeric form of the protein(s) recognized by the AJH antibodies (Figure 3).

To examine whether the specific protein band(s) recognized by the AJH antibodies contains only one or both AJH proteins, which are predicted to have the same molecular weight and cannot be separated on SDS-PAGE, we took advantage of the distinct isoelectric points of the two AJH proteins. Based on their theoretical isoelectric point differences (5.05 for AJH1 and 5.11 for AJH2), we performed two-dimensional gel analyses to resolve AJH1 and AJH2. As shown in Figure 4, both complex and monomeric fractions from wild-type seedlings have two detectable spots on immunoblots of two-dimensional gels. The spots correspond to the size and theoretical isoelectric points for AJH1 and AJH2, respectively. Judging from the relative intensity of the two spots, the ratios of the proteins in the complex and monomeric fractions are quite similar for AJH1 and AJH2. This result suggests that AJH1 and AJH2 are similarly present in both complex and monomeric forms, further supporting the notion that AJH1 and AJH2 are functionally redundant.

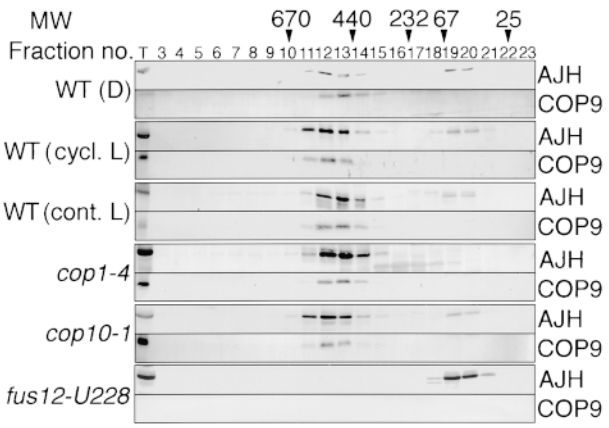

Figure 3. Representative Immunoblots of Gel Filtration Analyses of 8-Day-Old Wild-Type and Various *cop*/*det*/*fus* Seedling Extracts.

All fractions were examined for the presence of AJH and COP9 proteins. Numbers above the arrowheads indicate the approximate molecular masses (MW; given in kilodaltons). T represents total soluble protein extracts before gel filtration. Numbers 3 to 23 are fraction numbers. WT (D), WT (cycl. L), WT (cont. L), *cop1-4*, *cop10-1*, and *fus12-U228* refer to the seedling genotypes and growth conditions used for the gel filtration immunoblot analyses. The Arabidopsis JAB1 homologs (AJH) and COP9 bands are marked at right and were detected by their respective specific antibodies. Identical extracts were used for each pair of AJH and COP9 immunoblots. D, dark-grown seedlings; cycl. L, photoperiod light-grown seedlings; cont. L, seedlings grown under continuous light; WT, wild type.

The AJH Monomers and the COP9 Complex Are Differentially Affected by the Pleiotropic *cop*/*det*/*fus* Mutations

To determine the effect, if any, that the pleiotropic *cop*/*det*/*fus* mutations have on the stability and/or formation of the complex and monomeric forms of the AJH proteins, we investigated the distribution of AJH proteins by gel filtration analyses (Figure 3 and Table 1). In all *cop*/*det*/*fus* mutants examined, the presence of the COP9 complex strictly correlates with the ability of AJH proteins to incorporate into the complex form. Based on this analysis, the pleiotropic *cop*/*det*/*fus* mutants can be categorized into three classes based on their effects on the complex and/or monomeric forms of the AJH proteins (Table 1). The first class of mutants (class I) includes the *det1* and *cop1* mutants. For all of the *COP1* and *DET1* alleles examined, the AJH proteins were undetectable as monomers, whereas the complex form was present. This absence of the AJH proteins in the monomeric form may be attributed to a possible role of COP1 and DET1 in regulating the stability of AJH monomers.

The second class of mutants (class II), which is represented by *cop10*, has no detectable effects on the ability of the AJH proteins to form a complex or monomer and appears identical to the wild type. Thus, COP10 defines a subclass of its own, whose role in repressing photomorphogenesis does not involve structural alteration of the COP9 complex or AJH monomers. For example, COP10 may act downstream of COP9, and the COP9 complex may function to activate *COP10* expression or activity. Alternatively, COP10 may function to modulate the activity of the COP9 complex in ways not involving global structural changes to the complex.

The third class (class III) includes the *cop8*, *fus8*, *cop9*, *fus6*/*cop11*, *fus12*, *fus11*, *fus4*, and *fus5* mutants. All of these mutants lack the COP9 complex and thus the complex form of the AJH proteins, whereas there was no effect on the accumulation of monomeric AJH proteins in these mutants. Two of these genes, *COP9* and *FUS6*, previously have been demonstrated to encode subunits of the COP9 complex (Staub et al., 1996; Wei et al., 1994b). Therefore, it

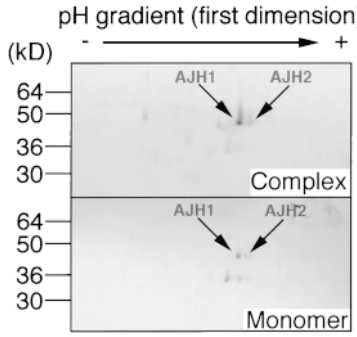

Figure 4. Two-Dimensional Gel Analyses of AJH1 and AJH2 Isoforms.

At top is an immunoblot of AJH proteins from complex fractions 11 to 14 (Figure 3) that were combined and then separated by two-dimensional gel analysis. At bottom is an immunoblot of AJH monomer fractions 19 to 20 (Figure 3) that were combined and separated by two-dimensional gel analysis. Protein extracts for the two-dimensional gel analyses are from 8-day-old, cycling light–grown (16 hr of light and 8 hr of dark), wild-type seedlings, indicated as WT (cycl L) in Figure 3. The arrows indicate expected spots for AJH1 and AJH2 proteins. The protein A–purified antibodies recognize both AJH1 and AJH2. The numbers at left indicate the positions of molecular mass markers in kilodaltons. The orientation of electrodes (− and +) in the isoelectric focusing gel is indicated by the arrow.

is possible that most of the class III mutants define genes that encode subunits of the COP9 complex. Mutations in these genes would disrupt the complex stability and therefore its function. It is also possible that some of the class III mutants may define genes whose wild-type functions are to positively regulate the expression of some of the COP9 complex subunits. Alternatively, the gene products defined by the class III loci may be specifically involved in the multiprotein assembly of the COP9 complex but are not part of the mature complex. In addition, the presence of AJH monomers in the class III mutants clearly indicates that the monomeric form of the AJH proteins is not sufficient to repress photomorphogenesis in darkness, because mutants containing only the monomer all exhibited *cop*-like phenotypes. However, whether the monomeric form of the AJH proteins has cellular functions distinct from that of the COP9 complex is unclear at present. Nevertheless, these results strongly support our claim that the AJH proteins are an integral subunit of the COP9 complex and that many of the pleiotropic *COP*/*DET*/*FUS* genes may encode the other subunits of the COP9 complex.

The AJH Monomers and the COP9 Complex Localize to Distinct Cellular Compartments

To gain further insight into the possible function of the complex and monomeric forms of the AJH proteins, we examined their subcellular localization. Immunofluorescence labeling of wild-type root protoplasts indicates that total AJH proteins are present in both the nucleus and the cytoplasm, regardless of the light conditions (Figures 5A to 5H). Because previous studies suggested that the COP9 complex is nuclear localized (Chamovitz et al., 1996), the observed cytoplasmic staining of AJH proteins may be largely due to the AJH monomeric forms. To confirm this observation, we examined immunostaining of AJH proteins in various mutants in which the AJH proteins only accumulated as a complex form (class I) or monomeric form (class III). The *cop1-4* mutant, a class I representative, did not accumulate any immunochemically detectable AJH monomer, and AJH proteins were predominantly localized in the nucleus (Figures 5I to 5L). However, *fus12-U228*, a class III mutant, accumulated only monomeric AJH proteins, and the AJH proteins did not appear to localize predominantly in the nucleus (Figures 5M to 5P). Rather, the AJH proteins appeared to have a more diffuse cytoplasmic staining pattern in the *fus12-U228* mutants. Therefore, the monomeric AJH proteins are mainly cytoplasmic, whereas the COP9 complex is

Table 1. Effects of the *cop*/*det*/*fus* Mutations on the Accumulation of the AJH Monomer and the COP9 Complex

Genotype[a]	AJH Complex[b]	AJH Monomer[b]	COP9 Complex[b]
WT (D)	+	+	+
WT (cycl. L)	+	+	+
WT (cont. L)	+	+	+
Class I			
cop1-1	+	−	+
cop1-4	+	−	+
cop1-5	+	−	+
cop1-6	+	−	+
det1-1	+	−	+
det1-8	+	−	+
Class II			
cop10-1	+	+	+
Class III			
cop8-1	−	+	−
fus8-S253	−	+	−
cop9-1	−	+	−
fus7-S100	−	+	−
fus6-1	−	+	−
fus6-T379	−	+	−
fus12-U228	−	+	−
fus12-R380	−	+	−
fus11-U203	−	+	−
fus4-414	−	+	−
fus5-S234	−	+	−
fus5-T379	−	+	−

[a] The wild-type samples WT (D), WT (cycl. L), and WT (cont. L) are wild-type seedlings grown under continuous darkness, cycling white light, or continuous white light conditions, respectively.

[b] (+) is given when either the complex or monomeric form is present; (−) represents undetectable complex and monomer on immunoblots.

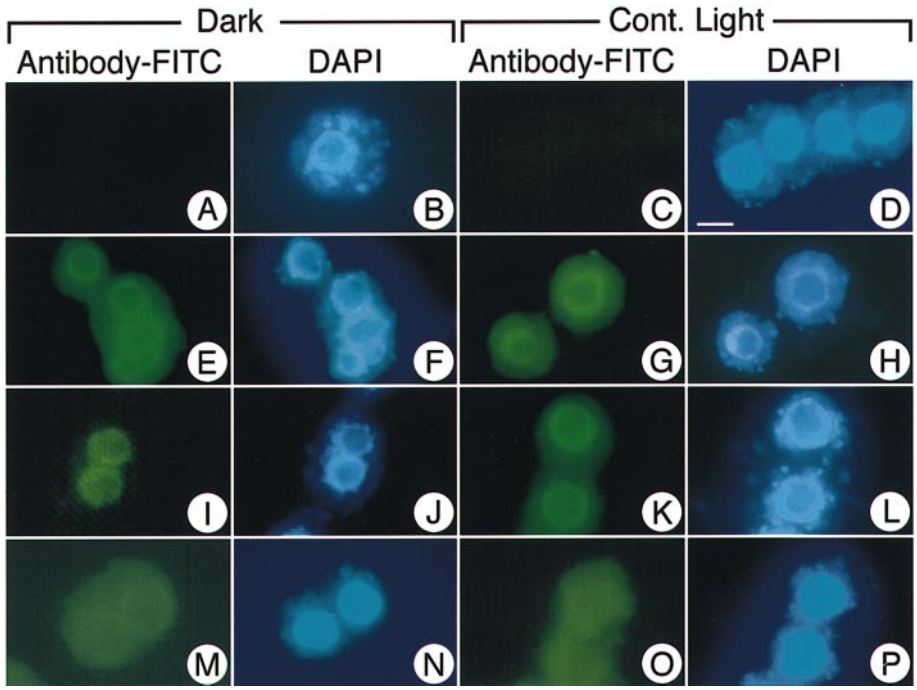

Figure 5. AJH Subcellular Localization in Isolated Arabidopsis Root Protoplasts.

(A) to **(H)** Protoplasts from wild-type roots.
(I) to **(L)** Protoplasts from *cop1-4* (class I).
(M) to **(P)** Protoplasts from *fus12-U228* (class III).
(A) and **(C)** show protoplasts stained with protein A–purified AJH1 preimmune serum; **(E)**, **(G)**, **(I)**, **(K)**, **(M)**, and **(O)** show protoplasts stained with protein A–purified anti-AJH1 antibodies. **(B)**, **(D)**, **(F)**, **(H)**, **(J)**, **(L)**, **(N)**, and **(P)** show 4′,6-diamidino-2-phenylindole (DAPI) staining of protoplasts identical to those shown in **(A)**, **(C)**, **(E)**, **(G)**, **(I)**, **(K)**, **(M)**, and **(O)**, respectively. **(A)**, **(B)**, **(E)**, **(F)**, **(I)**, **(J)**, **(M)**, and **(N)** are from dark-grown seedling root protoplasts. **(C)**, **(D)**, **(G)**, **(H)**, **(K)**, **(L)**, **(O)**, and **(P)** are from continuous light–grown (Cont. Light) seedling root protoplasts. FITC, fluorescein isothiocyanate. Bar in **(D)** = 15 μm for **(A)** to **(P)**.

predominantly nuclear localized. Because the AJH1 and AJH2 protein sequences do not contain canonical nuclear localization signals, the cytoplasmic localization of the AJH monomers is consistent with our previous finding that the COP9 complex may require some form of preassembly before translocating into the nucleus (Chamovitz et al., 1996).

The AJH Proteins and COP9 Have Similar Expression Patterns

We used immunoblot analysis to examine the expression pattern of the AJH proteins in Arabidopsis tissue types. As shown in Figure 6A, AJH proteins were ubiquitously expressed in various Arabidopsis tissues, with high expression in floral and root tissues and much lower expression in seedlings and siliques. This expression pattern is identical to that of COP9 (Figure 6A). This ubiquitous pattern of expression suggests that the COP9 complex may play a more global role in mediating developmental responses than simply during early seedling development. In addition, the expression of the AJH proteins did not seem to be light modulated because they are constitutively expressed regardless of light conditions (Figure 6B).

Reduced Levels of AJH Proteins in Arabidopsis Seedlings Result in Partial Constitutive Photomorphogenic Development in Darkness

To further confirm a specific role for the AJH proteins in light-regulated seedling development, we generated transgenic plants that express sense and antisense AJH1 full-length cDNAs driven by the strong cauliflower mosaic virus 35S RNA promoter (see Methods). Among those transgenic lines, two lines from sense transgenes and 13 lines from antisense transgenes exhibited an observable photomorphogenic phenotype in darkness. The results from representative lines are presented in Figure 7. When grown in complete darkness, the selected transgenic Arabidopsis seedlings expressing either the AJH1 sense or antisense construct resulted in seedlings displaying a partial constitu-

tive photomorphogenic phenotype. This partial *cop*-like phenotype is typified by dark-grown seedlings displaying open and enlarged cotyledons, no apical hook, and also a modest but significant decrease in hypocotyl length when compared with wild-type seedlings (Figure 7). Interestingly, several AJH1 lines that were transformed with the AJH1 sense construct also displayed a *cop*-like phenotype in darkness. This is most likely due to a cosuppression effect in these sense transgenic lines because cosuppression is commonly observed in transgenic plants. Indeed, protein blot analysis indicated that both sense and antisense lines that exhibited phenotypes had at least a fivefold reduction in AJH proteins (Figure 7, bottom). The fact that none of the sense and antisense transgenic lines displayed a drastic phenotype, as seen in the *cop9* or *fus6* mutants, may be due to residual AJH2 protein remaining in the cells (below our detection limit). This was anticipated because antisense or cosuppression by the *AJH1* transgene may not work efficiently on *AJH2* gene expression because of their sequence divergence. Nonetheless, the specific photomorphogenic phenotype associated with reduced AJH protein levels clearly indicates a specific role for AJH proteins in light-mediated Arabidopsis seedling development.

AJH1 Interacts Directly with FUS6 in the Yeast Two-Hybrid Assay

To investigate the subunit interactions within the COP9 complex, we used a yeast two-hybrid assay to determine whether AJH1 can interact directly with COP9 or FUS6, the two currently known subunits. Figure 8 indicates that AJH1

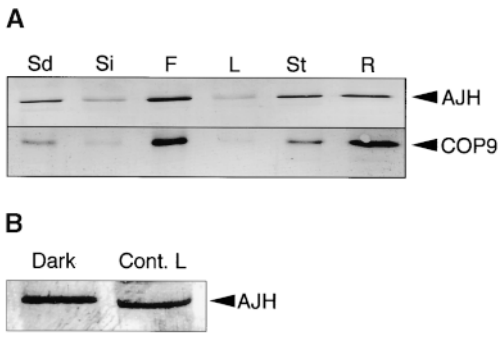

Figure 6. Expression Patterns of Arabidopsis JAB1 Homologs and COP9.

(A) AJH proteins and COP9 have similar expression patterns in various tissues. Sd, Si, F, L, St, and R indicate seedlings, siliques, flowers, leaves, stems, and roots, respectively, from wild-type plants (see Methods for details). At top is an immunoblot using protein A–purified anti-AJH1 antibodies. At bottom is an immunoblot using anti-COP9 antibodies.

(B) AJH protein levels from 8-day-old wild-type seedlings grown under darkness or continuous light (Cont. L) conditions.

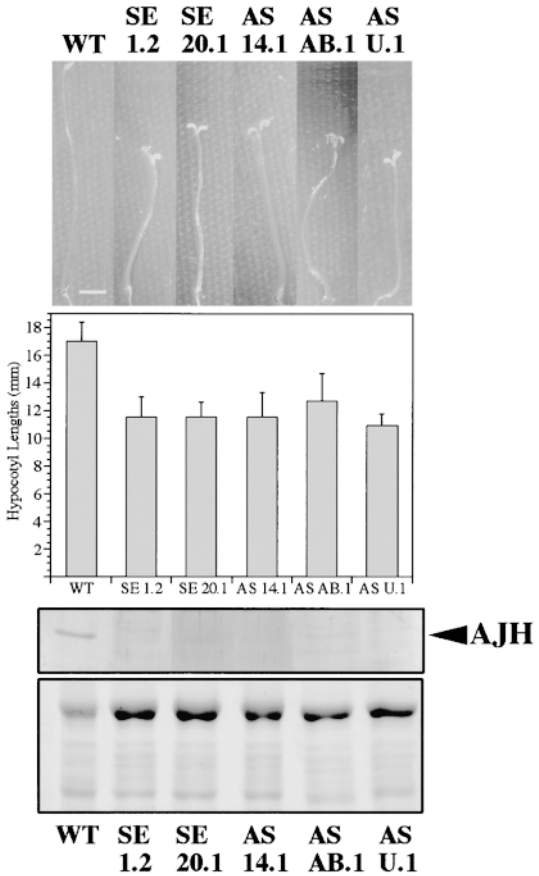

Figure 7. Reduction of AJH Protein Levels in Transgenic Seedlings Results in Partial Constitutive Photomorphogenesis in Darkness.

A morphological (top) and hypocotyl (middle) length comparison of wild-type and transgenic seedlings transformed with either an AJH1 sense or antisense construct is shown. AJH protein levels from these seedlings are shown in the gel second from the bottom. At bottom, an identical protein gel was stained with Coomassie Brilliant Blue R 250; it is shown below the AJH1 blot to confirm the equal loading of each extract. All transgenic seedlings were from the T_3 generation and were grown in complete darkness for 10 days at 22°C on GM solid media containing 1% sucrose. All seedlings at the top were photographed at the same magnification. Hypocotyl lengths are estimated by averaging at least 12 seedlings (*y* axis, in millimeters), and the error bars represent the standard deviation from the mean. For protein gel blots of AJH proteins, 10 μg of total soluble protein was loaded on each lane and subjected to immunoblotting with anti-AJH1 antibodies. The seedlings examined are wild-type Columbia seedlings (WT), two representative AJH1 sense (SE) transgenic lines (lines 1.2 and 20.1), and three representative AJH1 antisense (AS) transgenic lines (lines 14.1, AB.1, and U.1).

can interact directly with FUS6 but not COP9 in the yeast two-hybrid system. The overall structure of FUS6 is necessary for its interaction with AJH1 because each tested truncated form of FUS6 (i.e., with deletions of the leucine-rich region and the C-terminal coiled-coil motif [amino acids 139 to 441], the C-terminal coiled-coil motif [amino acids 358 to 441], or the N-terminal coiled-coil motif [amino acids 1 to 137]) was unable to interact with AJH1 (Figure 8). On the other hand, the interactive domain of AJH1 with FUS6 appears to be within the N-terminal two-thirds of the protein. The putative coiled-coil motif of AJH1 is not essential for its interaction with FUS6 because deletion of most of this region still results in reduced yet significant interactions with FUS6.

DISCUSSION

Implications for a Role of the COP9 Complex in Modulating Transcription

We demonstrate here that a new subunit of the Arabidopsis COP9 complex is encoded by a small gene family composed of *AJH1* and *AJH2.* These genes are most likely to encode the Arabidopsis homologs of human JAB1, a known coactivator of AP-1–dependent transcription (Claret et al., 1996). JAB1 has been shown to interact directly with c-Jun and JunD, but not v-Jun or JunB, stabilizing their interaction with their cognate AP-1 binding sites and specifically activating AP-1–dependent transcription in a c-Jun– and JunD-dependent manner (Claret et al., 1996). The recent observation that the purified human COP9 complex contains a kinase activity toward the c-Jun protein in vitro (Seeger et al., 1998) further implies a biochemical mode for this regulatory interaction between the COP9 complex and c-Jun in animal cells. From the high amino acid similarity between both of the Arabidopsis AJH proteins and human JAB1, it is possible that the AJH proteins may function like JAB1 in modulating transcription factor activity during repression of photomorphogenesis.

Interestingly, the mammalian FUS6 homolog, GPS1, was recently identified as a molecule involved in the suppression of Gα-activating mutations in the yeast *Saccharomyces cerevisiae*, and a chimeric GPS1 and FUS6 construct was shown to be active in the suppression of the yeast pheromone pathway (Spain et al., 1996). This result suggests that the mammalian GPS1 and the Arabidopsis FUS6 proteins

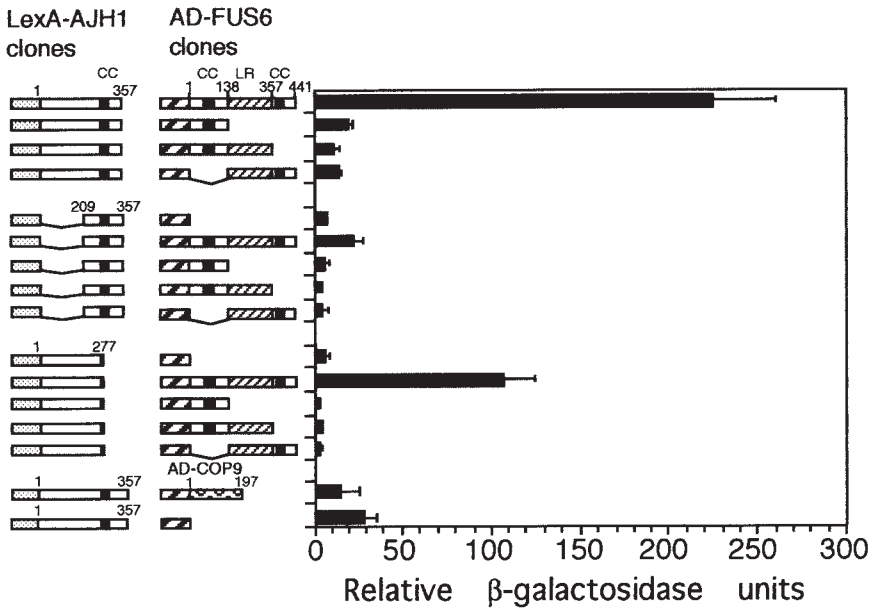

Figure 8. AJH1 Interacts with FUS6 but Not COP9 in the Yeast Two-Hybrid Assay.

The indicated portions of AJH1 were fused to the LexA DNA binding domain, the indicated portions of FUS6 or full-length COP9 were fused to the activation domain (AD), and they were tested in pairwise fashion, along with a reporter construct (pSH18-34), by using the yeast two-hybrid system (see Methods). The FUS6 activation domain fusion (AD) constructs containing the N-terminal coiled-coil (CC) domain of FUS6 are from amino acids 1 to 138, the AD construct containing the N-terminal coiled-coil and the leucine-rich (LR) patch is from amino acids 1 to 357 of FUS6, and the AD construct containing the deleted N-terminal coiled-coil is from amino acids 138 to 441 of FUS6. The indicated values are averages of relative β-galactosidase activities determined by a solution assay with four to six independent clones; error bars represent standard deviations from the mean.

may have conserved functions. In addition, overexpression of GPS1 in vitro in human cell lines was also shown to modulate the JNK/MAPK (for Jun N-terminal kinase/mitogen-activated protein kinase) signaling pathway by specifically interfering with JNK activity and thus regulating the phosphorylation of c-Jun and the activity of AP-1–dependent transcription (Kallunki et al., 1996; Spain et al., 1996). Thus, the functional conservation between GPS1 and FUS6 suggests that the COP9 complex may function in regulating a trimeric G protein and kinase-containing pathway(s) during light-mediated development in Arabidopsis. This is not unexpected because trimeric G proteins and protein kinases have been implicated in light signal transduction in higher plants (Romero and Lam, 1993; Bowler et al., 1994; McNellis et al., 1994b).

It is important to note that the mammalian AJH homolog JAB1 appears to have a function opposite that of GPS1 in modulating c-Jun and AP-1–dependent transcription. JAB1 appears to play a role in specifically coactivating c-Jun–dependent transcription and stabilizing c-Jun binding to DNA. Therefore, it appears that the COP9 complex may contain distinct subunits that are capable of either negatively or positively regulating transcription factors. It is possible that by fine-tuning modular activities between coactivators and repressors in the COP9 complex, plants may achieve optimal regulation in mediating various developmental processes in response to environmental stimuli ranging from light to hormones. However, our results indicate that plant AJH proteins exist in both a complex and a monomeric form. This difference in the distribution of the AJH proteins clearly adds another level of modularity to the system. It is possible that the monomeric form of the AJH proteins may function during other stages of development.

The COP9 Complex and Its Relationship to COP1 Action

COP1 has been shown to be an autonomous repressor of photomorphogenesis, and this function is attributed to COP1's ability to localize predominantly to the nucleus in darkness and to the cytoplasm in the light (von Arnim and Deng, 1994, 1996). This nuclear-cytoplasmic partitioning is compromised in all of the mutants that define the pleiotropic *COP*/*DET*/*FUS* loci (Chamovitz et al., 1996; von Arnim et al., 1997). Interestingly, we have also shown here that many of the pleiotropic *COP*/*DET*/*FUS* gene products may be integral subunits of the COP9 complex. Although the molecular mechanisms are not clear at present, it is possible that the direct or indirect interactions between COP1 and the COP9 complex may be essential for COP1's stability or retention in the nucleus, and light may specifically modulate these interactions. However, it remains to be determined how this aspect of the function of the COP9 complex relates to its ability to regulate the activity of transcription factors. Because it has been demonstrated recently that within the nucleus, COP1 also acts to interact directly with and regulate transcription factors (Ang et al., 1998; Yamamoto et al., 1998), it will also be of interest to determine whether the COP9 complex and COP1 regulate distinct, partially overlapping transcription factors or the same set of downstream target transcription factors.

Most of the Pleiotropic *COP*/*DET*/*FUS* Genes Are Required for Building the COP9 Complex

Our gel filtration analyses of all the pleiotropic *cop*/*det*/*fus* mutants indicate that at least eight of 11 mutations clearly affect the ability of COP9 and AJH proteins to accumulate in the complexed form. These include mutations in *COP8*, *COP9*, *FUS6*, *FUS4*, *FUS5*, *FUS8*, *FUS11*, and *FUS12.* This result provides convincing evidence that some of these pleiotropic *COP*/*DET*/*FUS* gene products may actually be subunits of the COP9 complex. In fact, both COP9 and FUS6 have already been shown to be subunits of the COP9 complex (Staub et al., 1996). However, the recent characterization of both plant and mammalian COP9 complexes indicates that the core complexes consist of eight distinct subunits (Wei et al., 1998). Because nine pleiotropic *COP*/*DET*/*FUS* loci are required for the accumulation of the COP9 complex in Arabidopsis, it is likely that a minority of this group of the pleiotropic *COP*/*DET*/*FUS* gene products may not be subunits of the COP9 complex. Rather, they may affect the assembly, formation, and/or stability of the COP9 complex, even though they are not part of the mature complex.

METHODS

Plant Material and Growth Conditions

Plant germination and growth conditions were described previously (Wei and Deng, 1992). Unless stated otherwise, seeds were first vernalized and then grown under a cycling long-day photoperiod (16 hr of light at 48 μmol m^{-2} sec^{-1} and 8 hr of dark) for 8 days at 22°C. The growth condition for continuous light experiments was 24 hr of light at 75 μmol m^{-2} sec^{-1}. We used *cop1-1*, *cop1-4*, *cop1-6*, and *det1-1* mutant alleles from *Arabidopsis thaliana* ecotype Columbia (McNellis et al., 1994a; Pepper et al., 1994). The *cop1-5*, *cop9-1*, and *cop10-1* mutations were in the Arabidopsis ecotype Wassilewskija; *fus4-414* was in the Dijon background (McNellis et al., 1994a; Wei et al., 1994a; Kwok et al., 1996). All other mutant seeds were from the Landsberg *erecta* ecotype (Miséra et al., 1994; Pepper et al., 1994; Kwok et al., 1996).

Protein Extraction and Gel Filtration

Total soluble protein extracts were obtained by homogenizing cycling, light–grown (16 hr of light and 8 hr of dark), 8-day-old, wild-type seedling tissue in a 1.5-mL microcentrifuge tube by use of a pellet pestle motor (Kontes Scientific, Vineland, NJ) in a buffer containing 25 mM Tris, pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 10

mM β-mercaptoethanol, and 1 mM fresh phenylmethylsulfonyl fluoride. Total soluble protein extracts were filtered through a 0.2-μm syringe filter, and ~200 μg of total soluble protein extract was fractionated through a Superose 6 HR 10/30 gel filtration column (Pharmacia Biotechnology, Piscataway, NJ) with a buffer containing 1.5 mM NaH_2PO_4, 8 mM Na_2HPO_4, 150 mM NaCl, and 2 mM $MgCl_2$. All gel filtrations were performed at 4°C. Fractions (0.5 mL) were collected and concentrated with Strataclean resin (Stratagene, La Jolla, CA) and resuspended in 2 × SDS sample buffer (Staub et al., 1996). Equal volumes of each fraction were loaded onto an SDS–polyacrylamide gel for analyses and immunoblotting.

Protein extracts for tissue-specific immunoblots were taken from 21-day-old, continuous light–grown, wild-type plants, except for seedlings that were from 8-day-old, cycling light–grown (16 hr of light and 8 hr of dark) plants. Root extracts were isolated from 2-week-old plants. Total soluble extracts were resuspended in SDS-PAGE sample buffer, and equal amounts of total protein (~10 μg) were loaded for each tissue sample on SDS-PAGE. The gels were subjected to standard immunoblotting procedures, as described previously (Staub et al., 1996). All protein concentrations for both tissue-specific immunoblotting and gel filtration immunoblotting were determined by use of a protein assay reagent kit (Bio-Rad).

Two-Dimensional Gel Analysis

Total wild-type soluble protein extracts were fractionated in a Superdex 200 gel filtration column (Pharmacia Biotechnology), and fractions were collected. Complex fractions (fractions 11 to 13) and monomer fractions (fractions 19 and 20) for the AJH proteins from gel filtration were combined, respectively, and concentrated on a 10-kD cut-off Microsep microconcentrator (Pall Filtron Inc., Northborough, MA). Approximately 50 μg of total soluble protein was loaded onto a first-dimension tube gel (model SE200; Hoeffer, San Francisco, CA) with a pH gradient ranging from 3 to 10 by using premixed ampholines (Pharmacia Biotechnology). Tube gels were resolved according to the manufacturer's recommended conditions and in the second dimension by using standard SDS-PAGE analyses followed by standard immunoblot procedures (Staub et al., 1996).

Antibody Production, Immunoprecipitation, and Immunoblot Analyses

The full-length cDNA clone for AJH1 was cloned in-frame in the pGEX vector (Pharmacia Biotechnology). The resulting glutathione *S*-transferase (GST)–AJH1 fusion protein was overproduced in *Escherichia coli* and purified as insoluble inclusion bodies from an acrylamide gel and then eluted by an electroelutor (Bio-Rad). The electroeluted GST–AJH1 protein was used to immunize rabbits to raise polyclonal antibodies. AJH1 antibodies were purified using a protein A column (Pharmacia Biotechnology) according to the manufacturer's recommendations. Coimmunoprecipitation experiments used methods identical to those described previously (Staub et al., 1996). FUS6 and COP9 antibodies were described previously (Wei et al., 1994b; Staub et al., 1996).

Arabidopsis Root Protoplast Immunofluorescence

Protoplast isolation was conducted using methods similar to those used by Staub et al. (1996), with the following modifications to the protocol. (1) Six-day-old wild-type, 8-day-old *cop1-4*, and 9-day-old *fus12-U228* seedlings were used to isolate root protoplasts. (2) Protein A–purified antibodies raised against AJH1 were used as the primary antibody (at a 1:500 dilution). (3) The secondary antibody was a goat anti–rabbit antibody conjugated with fluorescein (used at a 1:200 dilution) (Sigma). 4′,6-Diamidino-2-phenylindole was used at 5 μg/mL to determine nuclear staining.

Production of AJH1 Sense and Antisense Transgenic Lines

A full-length AJH1 cDNA was cloned either in the sense or antisense orientation downstream of the 35S promoter in the pROK2 transformation vector (Baulcombe et al., 1986). Constructs were transformed into wild-type Columbia ecotype plants via vacuum infiltration, and ~20 independent transgenic lines for each construct were established. Dark-grown seedling phenotype screening of the transgenic lines revealed two sense lines and 13 antisense lines exhibiting an observable partial photomorphogenic phenotype. Those lines were established, and representative lines were selected for the further detailed analysis presented in this study (lines SE 1.2 and 20.1 for the sense expression construct and lines AS 14.1, AB.1, and U.1 for the antisense construct). All transgenic seeds used in this study were from the T_3 generation and were grown in darkness for 10 days on GM media with 1% sucrose (Wei and Deng, 1992).

Yeast Two-Hybrid Assay

All LexA fusion constructs were cloned as translational fusions to the LexA DNA binding domain of vector pEG202 (Guarente, 1983). All activation domain fusions were cloned as in-frame fusions to the acidic domain of vector pJG4-5 (Guarente, 1983). Pairwise combinations of LexA clones and activation domain clones were cotransformed with a β-galactosidase reporter construct (pSH18-34) to *Saccharomyces cerevisiae* EGY48, as previously described (Chen et al., 1992). Six to eight independent transformants were inoculated with 2 mL of SC media lacking histidine, uracil, and tryptophan supplemented with 2% galactose and 1% raffinose (McNellis et al., 1996). Cultures (0.2 mL) used for liquid β-galactosidase activity assays were described previously (Ausubel et al., 1994). Relative β-galactosidase activities were calculated according to previously described methods (McNellis et al., 1996). The remaining yeast cultures were lysed in SDS sample buffer, and protein expression for each respective construct was confirmed by immunoblotting using anti-LexA antibodies, protein A–purified AJH antibodies, affinity-purified FUS6 antibodies, or affinity-purified COP9 antibodies.

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