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Arabidopsis *FUSCA5* Encodes a Novel Phosphoprotein That Is a Component of the COP9 Complex

Baruch Karniol, Przemyslaw Malec,[1] and Daniel A. Chamovitz[2]

Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel

The COP9 complex is a regulator essential for repression of light-mediated development in Arabidopsis. Using partial amino acid sequence data generated from purified COP9 complexes, we cloned the Arabidopsis cDNA encoding the 27-kD subunit of the COP9 complex and showed that it is encoded by the previously identified *FUSCA5* (*FUS5*) locus. *fus5* mutants exhibit constitutive photomorphogenic phenotypes similar to those of *cop9* and *fus6.* Point mutations in *FUS5* that led to a loss of FUS5 protein were detected in four *fus5* allelic strains. FUS5 contains the PCI/PINT and mitogen-activated protein kinase kinase activation loop motifs and is highly conserved with the mammalian COP9 complex subunit 7 and the *Aspergillus nidulans* AcoB proteins. FUS5 is present in both complex and monomeric forms. In the COP9 complex, FUS5 may interact directly with FUS6 and COP9. Mutations in FUS6 and COP9 result in a shift in the electrophoretic mobility of FUS5. This shift can be mimicked by in vitro phosphorylation of FUS5 by plant extracts. These findings further support the hypothesis that the COP9 complex is a central and common regulator that may interact with multiple signaling pathways.

INTRODUCTION

Light is the major environmental signal regulating plant development. Light signals perceived by the photoreceptors are transduced to the nucleus to regulate gene expression and development (reviewed in Fankhauser and Chory, 1997; Mustilli and Bowler, 1997). Genetic screens in Arabidopsis have identified 10 negative regulators of light signaling, known collectively as the *cop*/*det*/*fus* (for *constitutive photomorphogenic*/*deetiolated*/*fusca*) mutants (Kwok et al., 1996). Mutations at any of these loci cause dark-grown mutant seedlings to mimic light-grown wild-type seedlings at the levels of both gross morphology and gene expression. The pleiotropic nature of the mutants together with various genetic data imply that their gene products act at the nexus between multiple signal inputs of upstream photoreceptors and a variety of downstream regulatory cascades controlling specific aspects of cellular differentiation. However, these loci have roles beyond mediating the switch in plants from dark-grown to light-grown development because severe mutations at each of these loci lead to plant death after the seedling stage. This indicates that the wild-type gene products play an essential role during normal vegetative development, although this role remains poorly defined (Castle and Meinke, 1994; Miséra et al., 1994; Mayer et al., 1996). Furthermore, the COP9 complex is also conserved in animal systems (Chamovitz and Deng, 1995; Seeger et al., 1998; Wei et al., 1998).

Because several of the pleiotropic *cop*/*det*/*fus* mutants are phenotypically similar, it has been proposed that at least several of these loci encode proteins that function in proximity to each other in the same signaling pathway to control the primary switch between dark-grown and light-grown growth patterns. Direct evidence for this hypothesis was provided by showing that the COP9 protein is a component of a multisubunit protein complex that also contains FUS6 as a subunit (Chamovitz et al., 1996). The COP9 complex is localized to the nucleus in plants and binds heparin (Chamovitz et al., 1996); however, a biochemical function for this complex is not known.

Indirect evidence for the function of the COP9 complex has been provided in animal systems in which proteins highly similar to COP9 complex components have been identified (Chamovitz and Deng, 1995; Spain et al., 1996). Ectopic overexpression of the human FUS6 ortholog, GPS1, inhibits mitogen-activated protein kinase (MAPK) pathways in both yeast and mammalian cells (Spain et al., 1996). The recently reported mammalian COP9 complex contains JAB1, a c-JUN–activating binding protein (Seeger et al., 1998; Wei et al., 1998). The human COP9 complex (also termed the signalosome) appears to phosphorylate c- and D-Jun in vitro (Seeger et al., 1998), suggesting that the COP9 complex may be directly involved in MAPK pathways.

Recent reports have highlighted the similarities among the COP9 complex, eIF3 complex, and the 19S regulatory complex of the proteasome (Glickman et al., 1998; Wei et al., 1998).

[1] Current address: Jan Zurzycki Institute of Molecular Biology, Jagiellonian University, PL-31-120 Krakow, Poland.

[2] To whom correspondence should be addressed. E-mail chamd@post.tau.ac.il; fax 972-3-6409380.

All three complexes are multisubunit and similar in size, and subunits of all three complexes share a similar motif, termed the PCI (for proteasome–COP9 complex initiation; Hofmann and Bucher, 1998) or PINT (for proteasome–Int6–Nip1–Trip15; Aravind and Ponting, 1998) domain. Although the biological significance of the PCI/PINT domain remains to be determined, the domain, together with the other similarities mentioned above, suggests that these complexes share an overall architecture and that there may be some form of interaction between the complexes.

As part of a broad effort to elucidate the structure and biochemical activity of the COP9 complex, we decided to begin by first characterizing its subunit composition. Here, we report the cloning of the gene that encodes the p27 subunit of the complex and show that it corresponds to one of the previously identified *cop*/*det*/*fus* loci. Our results indicate that phosphorylation of p27 may play a role in COP9 complex function.

RESULTS

Cloning and Genetic Structure of p27

The first step toward defining the biochemical activity and the subunit composition of the COP9 complex was to analyze the complex by biochemically purifying it from cauliflower (Chamovitz et al., 1996) and sequencing the peptides from the copurified proteins (Karniol et al., 1998). The amino acid sequence was determined for four peptides from the 27-kD protein that copurifies with the COP9 complex (Karniol et al., 1998). All of these peptides are encoded by a 1000-bp region on Arabidopsis chromosome 1. A 400-bp fragment of this region was amplified by polymerase chain reaction (PCR), and the PCR product was used as a probe to screen a cDNA library. Five positive plaques were obtained, and the longest cDNA insert (~1 kb) present in one of the clones was sequenced completely on both strands. This cDNA clone contains an in-frame stop codon upstream of the first ATG codon of a 225–amino acid open reading frame. The predicted gene product has calculated molecular mass of 27 kD and a pI of 5.23. Comparison of the cDNA with the genomic sequence revealed that the p27 gene contains nine exons and eight introns (Figure 1). Portions of this cDNA have not been identified in the Arabidopsis expressed sequence tag database.

The chromosomal region containing p27 is located at the top of Arabidopsis chromosome 1, flanked by the simple sequence length polymorphism marker nga59 and CER1 (Figure 2a). The *FUS5* locus is found at the top of chromosome 1, 4.5 centimorgans below nga59 on the genetic map (Kwok et al., 1996). Although there are often discrepancies between genetic and physical maps, these data suggest that *FUS5* may encode p27. As shown in Figure 2B, *fus5* mutants show structural and developmental phenotypes that are characteristic of the pleiotropic *cop*/*det*/*fus* mutants (Miséra et al., 1994; Kwok et al., 1996). Dark-grown *fus5* mu-

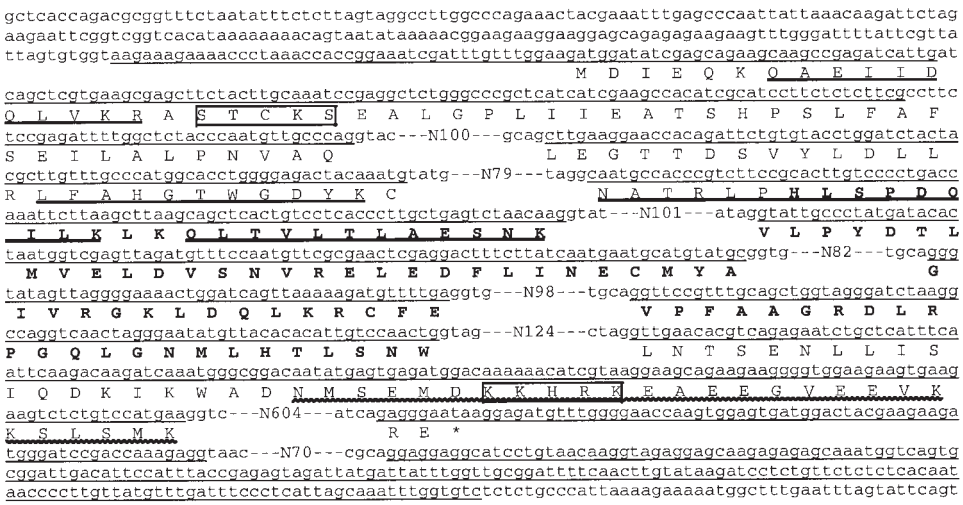

Figure 1. Genomic, cDNA, and Deduced Protein Sequence of the Gene Encoding p27.

The genomic sequence from nucleotides 40,190 to 37,659 in bacterial artificial chromosome clone T7123 (GenBank accession number U89959) is shown. The cDNA sequence is underlined. Introns are designated by their first and last four nucleotides separated by a dashed spacer designating the length of the intervening sequence. The underlined amino acid sequences correspond to the four peptides sequenced from cauliflower (Karniol et al., 1998). Residues shown in boldface comprise the PCI domain. The wavy line designates the coiled-coil domain. The boxed STCKS sequence is the putative MAPK activation motif, and the boxed KKHRK is a putative nuclear localization sequence. Asterisk denotes the stop codon.

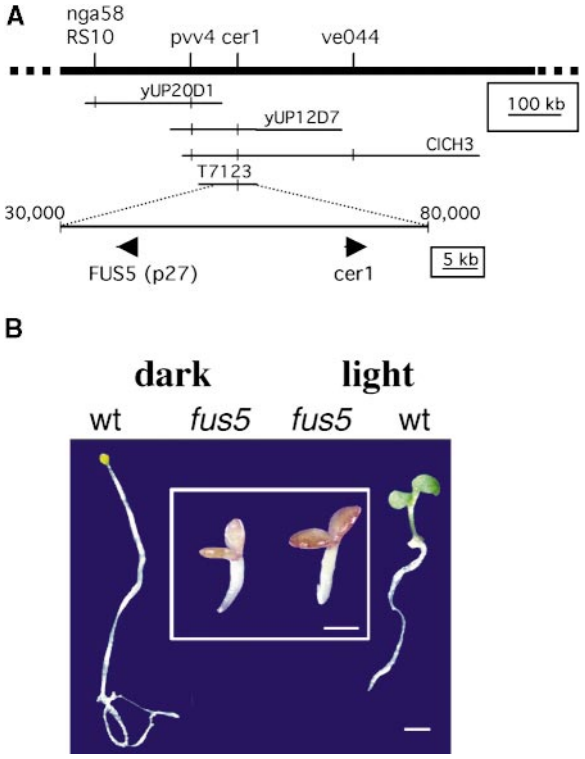

Figure 2. Genetic and Phenotypic Description of *FUS5*.

(A) Genomic map of the top of Arabidopsis chromosome 1. yUP20D1, yUP12D7, and ClCH3 are anchored yeast artificial chromosome clones, and T7123 is the bacterial artificial chromosome clone that has been sequenced as part of the Arabidopsis Genome Initiative (accession number U89959). The arrowheads designate the direction of the *FUS5* and *cer1* genes. This map has been modified from the one provided through the Arabidopsis Genome Center (University of Pennsylvania, Philadelphia; http://cbil.humgen.upenn.edu/~atgc/ATGCUP.html).

(B) *fus5* mutants display light-grown phenotypes in light and darkness. Wild-type (wt) and *fus5* seedlings were grown in complete darkness or in light for 5 days. *fus5-1* is shown as a representative of *fus5-1*, *fus5-2*, *fus5-3*, and *fus5-4*, which are phenotypically indistinguishable. Bar = 1 mm.

tants exhibit short hypocotyls and open, expanded cotyledons similar to light-grown *fus5* and wild-type seedlings and accumulate high levels of anthocyanin. In addition, *fus5* mutants develop chloroplasts in the dark, express high levels of light-inducible genes, although grown in darkness, and die after the seedling stage in light and darkness (Kwok et al., 1996).

p27 Is Absent in *fus5* Seedlings

As a first step toward determining whether p27 is encoded by the *FUS5* locus, we analyzed the accumulation of p27 protein in *fus5* seedlings. The full-length p27 cDNA was expressed in *Escherichia coli*, purified, and used to immunize rabbits for the production of polyclonal antibodies. p27-specific antibodies were subsequently affinity purified (see Methods).

Immunoblot analysis using affinity-purified antibodies to probe protein extracts from wild-type seedlings and several *fus5*, *cop9*, *fus6*, and *cop1* mutants is shown in Figure 3A. Two specific proteins with apparent molecular masses of 30 and 33 kD were detected in the wild-type and *cop1-6* seedlings but were absent from all *fus5* seedlings. In the *cop9* and *fus6* mutants, which lack the COP9 complex (Wei et al.,1994; Chamovitz and Deng, 1997), both the 33- and 30-kD proteins were absent, and a 30.5-kD protein was detected. To examine the accumulation of COP9 in the same *fus5* mutants, we probed a similar protein blot with affinity-purified anti-COP9 antibodies. All *fus5* mutants contained no detectable COP9 (data not shown). This result is consistent with our earlier finding that *fus6* mutants, which lack the COP9 complex, also lack any detectable level of COP9 (Wei et al., 1994). These results suggest that *FUS5* encodes a specific member of the COP9 complex and that mutations in this locus result in a loss of the COP9 complex.

fus5 Mutants Contain Mutations in the Gene Encoding p27

Whereas the results shown in Figure 3A suggest that p27 is encoded by *FUS5*, the lack of p27 protein in the *fus5* mutants could be a pleiotropic effect of a mutation at another locus. To determine whether p27 is encoded by the *FUS5* locus, we amplified the p27 gene from four independent *fus5* alleles and compared its sequence to that of the wild type from the same ecotype. All *fus5* mutant alleles were generated by ethyl methanesulfonate mutagenesis (Miséra et al., 1994). Different single base pair transitions were identified in the gene encoding p27 of each *fus5* mutant (S234, S239, U264, and T339b, renamed as given in Figure 3B). These transitions resulted in a severe change in the p27 protein. The p27 gene in *fus5-1* has a G-to-A transition that changes the Trp-74 TGG codon in the second exon to a TGA stop codon. Mutations found in the p27 gene in *fus5-2, fus5-3*, and *fus5-4* all affect putative exon–intron junctions. The independently generated *fus5-2* and *fus5-4* alleles both had a G-to-A transition in the consensus 3′ splice junction of the third intron, changing it from AG:G to AA:G. A guanine at this position is universally conserved in all introns and is essential for completion of the splicing process (Brown et al., 1996). Similar splicing mutations have been detected in a number of Arabidopsis genes, including *PHYA*, *GA1*, *AGAMOUS*, *DET1*, and *COP1* (Brown et al., 1996). The *fus5-2* mutation changes the splicing junction AG:C to GG:C at the 3′ end of the first intron, which also leads to an aberrant p27 protein. A similar AG-to-GG mutation was found in the *cop1-6* gene, leading to a truncated transcript (McNellis et al., 1994). This molecular analysis, which correlates with the

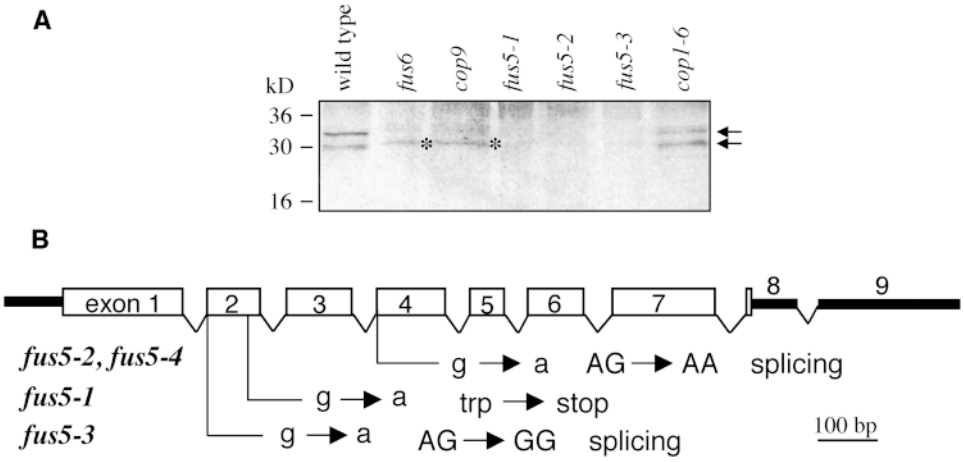

Figure 3. Analysis of *fus5* Mutants.

(A) FUS5 accumulation in wild-type and mutant strains. Ten micrograms of total soluble protein was separated by SDS-PAGE on a 12.5% acrylamide gel and reacted with affinity-purified antibodies raised against FUS5. The arrows indicate the 30-kD (top) and 33-kD (bottom) proteins. The mobility-shifted protein detected in *cop9* and *fus6* is indicated by asterisks.

(B) Summary of point mutations in the *FUS5* gene in the *fus5* mutants. Coding regions are shown as boxes, and noncoding cDNA is shown as a thick line. The placement of introns is indicated as given in Figure 1. The mutation for each mutant is shown as is the corresponding functional change. Alleles have been renamed as follows: *fus5-1*, *fus5*-S234; *fus5-2*, *fus5*-U264; *fus5-3*, *fus5*-T339; and *fus5-4*, *fus5*-S239.

lack of p27 protein in the *fus5* mutants, strongly indicates that p27 is encoded by the *FUS5* locus.

FUS5 Is Present in Both High Molecular Mass and Monomeric Forms

In both *cop9* and *fus6* mutants, neither the COP9 nor FUS6 protein has been detected; however, in the wild type, COP9 and FUS6 are found only in the COP9 complex and not in a monomeric form (Staub et al., 1996; Chamovitz and Deng, 1997). Therefore, the functional form of COP9 and FUS6 reside only within the COP9 complex (Wei et al., 1994a). The absence of the 30- and 33-kD proteins in *cop9* and *fus6* (Figure 3) is consistent with the prediction that FUS5 is a member of the COP9 complex, together with COP9 and FUS6. However, the detection of a 30.5-kD protein in the *cop9* mutants suggests that FUS5 may also exist in a form independent of the COP9 complex. To substantiate this prediction, we used gel filtration chromatography to fractionate native proteins from extracts of wild-type seedlings.

The elution profile of FUS5 from gel filtration chromatography and the relative position of molecular mass standards are shown in Figure 4. This elution profile indicates that FUS5 is found in different forms: FUS5 elutes from the gel filtration column in high molecular mass fractions (Figure 4, fractions 12 to 14) corresponding to the reported gel filtration elution profile of COP9 and FUS6 in the COP9 complex (Wei et al., 1994; Chamovitz et al., 1996; Staub et al., 1996; Kwok et al., 1998), but it is also found in fractions corresponding to the FUS5 monomer (Figure 4, fractions 19 and 20).

FUS5 Interacts with Other Components of the COP9 Complex

To further substantiate the finding that FUS5 is a subunit of the COP9 complex and to initiate a study of the structure of the COP9 complex, we examined the possibility that FUS5 interacts directly with one or more subunits of the complex in a heterologous system. To this end, we have used the yeast two-hybrid assay (Gyuris et al., 1993). As shown in Figure 5, FUS5, FUS6, and COP9 were fused to either the LexA DNA binding domain or the yeast transcription activation domain. FUS5 alone did not interact with the activation domain, and the COP9–AD or FUS6–AD fusion proteins did not interact with the LexA domain. However, the LexA–FUS5 fusion protein clearly interacted with COP9 and FUS6.

p27 Sequence Features

Computer searches using the predicted amino acid sequence of FUS5 revealed that this polypeptide is highly similar to the AcoB protein (20% identity and 29% similar-

ity) from *Aspergillus nidulans* (GenBank accession number U1801265) and the recently reported S7a and S7b (27 and 31% identity and 40 and 43% similarity, respectively) subunits of the mammalian COP9 complex (GenBank accession numbers AF071316 and AF071317). FUS5 contains a PCI/PINT domain found in many of the subunits of the proteasome, the COP9 complex, and the eIF3 complex, which is followed by a coiled-coil structure. FUS5 contains a putative nuclear localization signal between residues 202 and 206—KKHRK (Hicks and Raikhel, 1995). This motif is similar to the simian virus 40 nuclear localization signal and consists of a four-residue pattern composed of three basic amino acids and a histidine or proline. FUS5 also contains several consensus phosphorylation sites and a canonical MAPK kinase activation loop motif (Ser-X-X-X-Ser, where X is any amino acid) between amino acids 19 and 23 (Figure 1).

Two iterations of PSI-BLAST (Altschul et al., 1997) revealed that FUS5 and its close orthologs (AcoB, S7a, and S7b) are significantly similar to mouse COP9 complex subunit 2. This result is surprising because subunit 2, similar to mouse COP9 complex subunits 1, 3, 4, 5, and 6, is similar to subunits of the lid of the 19S regulatory complex of the proteasome (Glickman et al., 1998; Wei et al., 1998). No similarity was found between the mouse homologs of COP9 and FUS5 (subunits 8 and 7, respectively) and subunits of the proteasome regulatory lid. Although both FUS5 and mouse COP9 complex subunit 2 contain PCI/PINT domains, the similarity extends to other regions of the protein, suggesting that FUS5 and subunit 2 may have a common ancestor.

FUS5 Is a Phosphoprotein

Several reports have suggested that phosphorylation may be involved in COP9 complex activity (Spain et al., 1996; Seeger et al., 1998). The primary sequence of FUS5 contains several putative phosphorylation sites, suggesting that it may be a phosphoprotein. To determine if FUS5 is a phosphoprotein, we checked for the ability of recombinant FUS5 to be phosphorylated in vitro by plant extracts. Recombinant FUS5 was incubated with γ-^{32}P-ATP in the presence or absence of wild-type Arabidopsis protein extracts and separated by SDS-PAGE (see Methods). To ensure that any phosphoprotein detected is FUS5 and not autophosphorylation of a protein contained in the plant extract, after the kinase reaction, we immunoprecipitated FUS5 from the reaction mixture by using anti-FUS5 antibodies (Figure 6A, lanes 3 and 6). As shown in Figure 6A, recombinant FUS5 has no autophosphorylation activity (lane 7) but is phosphorylated in the presence of plant extract (lanes 2, 3, 5, and 6). No qualitative difference in FUS5 phosphorylation was detected for dark-grown or light-grown extracts. No qualitative difference in background autophosphorylation activity was detected for dark-grown or light-grown extracts (Figure 6A, lanes 1 and 4). Similar in vitro phosphorylation reactions with other recombinant substrates available in our laboratory, such as p105 (Karniol et al., 1998), did not result in phosphorylated products (data not shown).

To determine the effect of phosphorylation on the electrophoretic mobility of FUS5, we incubated purified FUS5 with plant extracts in either the presence or absence of ATP. Protein gel blot analysis after SDS-PAGE showed that electrophoretic mobility of FUS5 is influenced by phosphorylation such that it appears $\sim$2 kD larger than the nonphosphorylated protein (Figure 6B). This shift in electrophoretic mobility is specific for active phosphorylation, because the mobility shift is dependent on the presence of Arabidopsis extracts, and stopping the reaction at time 0 by boiling for 5 min inhibits phosphorylation and thus results in no change in electrophoretic mobility. Furthermore, whereas the efficiency of in vitro phosphorylation reactions are variable (Coligan et al., 1995) and often well below 50% of the exogenous substrate is phosphorylated, as seen in Figure 6B, lane 3, 81% of FUS5 is found in the upper band after the phosphorylation reaction, suggesting a high specificity of the FUS5 kinase. Mass spectroscopic analysis of the unphosphorylated and phosphorylated proteins showed that the phosphorylated FUS5 is 352 D larger than the unphosphorylated protein, indicating that four residues on FUS5 are phosphorylated by the plant extract.

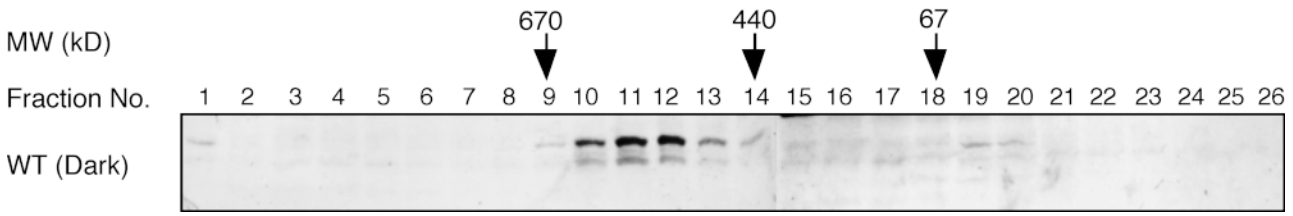

Figure 4. Gel Filtration Profile of FUS5.

Total soluble Arabidopsis protein was separated by Superose 6 gel filtration chromatography, and the resulting fractions were analyzed by protein gel blotting with anti-FUS5 antibodies. Fraction numbers (0.5 mL per fraction starting from the void volume) are labeled at top. The elution peaks for thyroglobulin (670 kD), apoferritin (440 kD), and BSA (67 kD) are designated above the fractions. MW, molecular weight; WT, wild type.

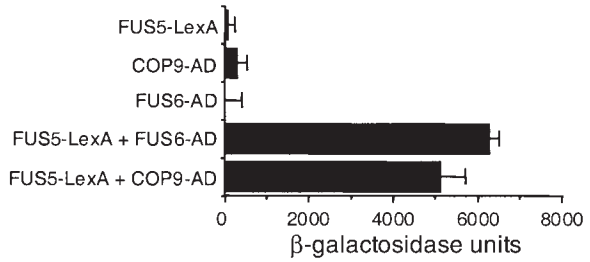

Figure 5. FUS5 Interacts with Both COP9 and FUS6 in the Yeast Two-Hybrid Assay.

The relative *lacZ* reporter gene activity in yeast cells for the different combinations of plasmids is shown. lacZ activity in the negative controls (i.e., FUS5–LexA, COP9–AD, and FUS6–AD) represents the background levels in yeast cells. Five individual transformants were used to measure relative lacZ activity for each pairwise combination. Error bars represent standard deviation.

DISCUSSION

We report the cloning of a novel component of the Arabidopsis COP9 complex. Previous genetic studies suggested that some of the *COP*/*DET*/*FUS* loci might encode components of the complex. Here, we have shown that the p27 component of the COP9 complex is encoded by the *FUS5* locus.

Several lines of evidence indicate that p27 is a component of the COP9 complex and that its isolation is not simply a result of nonspecific copurification. First, p27 copurifies with the COP9 complex in an equimolar ratio with COP9 and FUS6 (Chamovitz et al., 1996). Second, p27 is found in a large protein complex of identical size to the COP9 complex. Third, p27 interacts in yeast with known components of the COP9 complex. This interaction in yeast appears to be specific because (1) p27, COP9, and FUS6 by themselves do not activate the yeast assay and (2) COP9 and FUS6 do not interact with each other and share little if any amino acid similarity. Interestingly, the p42 subunit of the complex, Arabidopsis JAB1 homolog 1 (AJH1), was recently shown also to interact with FUS6 (Kwok et al., 1998).

The strongest proof for the identification of p27 as a component of the COP9 complex is provided through genetics. Mutations in at least three loci, *COP9, FUS6*, and *COP8*, lead to a loss of the complex and cause identical *cop*/*det*/*fus*–conferred phenotypes (Wei et al., 1994; Chamovitz and Deng, 1997). The gene encoding p27 maps to the *FUS5* locus, and mutations in *FUS5* result in phenotypes identical to those provoked by mutations in *COP9*, *FUS6*, and *COP8*, suggesting that p27 is encoded by *FUS5.* This hypothesis is strongly supported by our finding that four independent alleles of *fus5* contain point mutations in the gene encoding p27, which lead to the loss of the p27 protein in these mutants. Taken together, these findings lead us to conclude that p27 is encoded by *FUS5* and that FUS5 is a component of the COP9 complex. Thus, we expect that other subunits will also be encoded by other *COP*/*DET*/*FUS* loci.

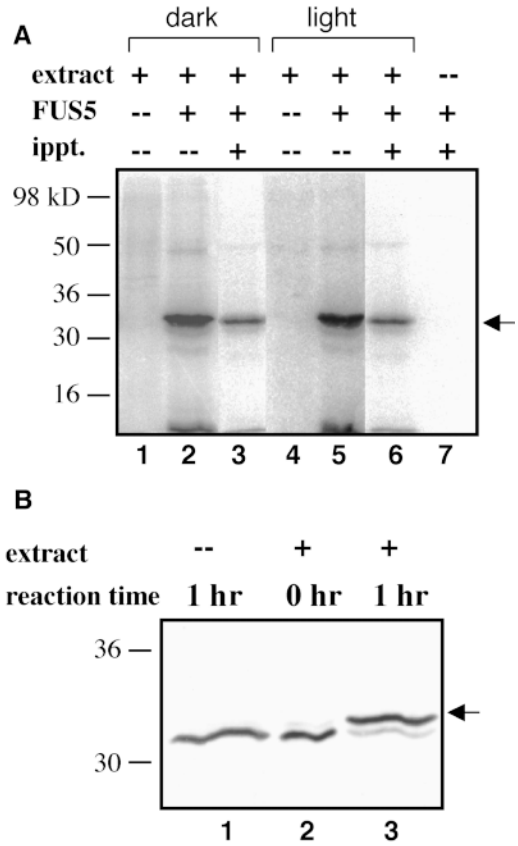

Figure 6. FUS5 Is a Phosphoprotein.

(A) FUS5 undergoes in vitro phosphorylation. One microgram of FUS5 was incubated with γ-^{32}P-ATP in the presence (+) of buffer (lane 7) or protein extracts from dark-grown (lanes 2 and 3) and light-grown (lanes 5 and 6) Arabidopsis seedlings. FUS5 was immunoprecipitated (ippt.) after incubation (lanes 3 and 6). Autophosphorylation activity in protein extracts from dark-grown (lane 1) and light-grown (lane 4) Arabidopsis seedlings in the absence (--) of exogenous FUS5 is shown. The arrow designates FUS5.

(B) Phosphorylation of FUS5 retards electrophoretic mobility. The FUS5 protein was incubated with kinase buffer and ATP in the presence (+; lanes 2 and 3) or absence (--; lane 1) of Arabidopsis protein extracts, separated by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-FUS5 antibodies. The reaction in lane 2 was stopped at time 0 by boiling for 5 min. The reactions in lanes 1 and 3 were stopped by boiling after 1 hr of incubation at room temperature. The arrow designates phosphorylated FUS5. The relative amounts of the upper and lower bands in lane 3 were determined using National Institutes of Health Image, version 1.57.

The two proteins with different electrophoretic mobilities that were immunodetected by using the anti-FUS5 antibody in both the wild type and *cop1-6* are products of the same gene. If these two proteins were the products of two different genes, we would expect that only one of them would be missing in the *fus5* mutant strains, but this is not the case. Both proteins are absent in all *fus5* mutant strains, although both are present in strains known to have an intact COP9 complex, such as wild type and *cop1-6.* In our gel filtration analysis, the FUS5 doublet was also detected in fractions corresponding to the COP9 complex. However, we do not know whether these two proteins represent different functional forms of FUS5, or whether they are simply an artifact of SDS-PAGE. It should be noted that both FUS6 and COP9 sometimes appear as a doublet after SDS-PAGE (Chamovitz et al., 1996; Chamovitz and Deng, 1998).

The mobility shift of FUS5 between that of the wild type and the *cop9* and *fus6* mutants is most likely physiologically relevant. Although we cannot be certain whether this mobility shift is a downshift of the 33-kD protein or an upshift of the 30-kD protein, this change in electrophoretic mobility was detected only in mutants that lacked the COP9 complex and not in the *cop1-6* mutant that retained an intact COP9 complex. We suggest that in the absence of the complex, FUS5 goes through different post-translational modifications affecting its mobility during SDS-PAGE.

Although we still do not know the basis of this mobility shift, one possibility is that differential phosphorylation affects the mobility of FUS5. FUS5 is phosphorylated in vitro, and this phosphorylation decreases the mobility of the recombinant protein by $\sim$2 kD, suggesting that differential phosphorylation may contribute to the mobility shift detected in vivo. Furthermore, preliminary evidence indicates that two distinct kinases act on FUS5 (P. Malec and D.A. Chamovitz, unpublished data). As discussed below, COP9 complex components have been implicated in regulating or participating in several kinase pathways; thus, the phosphorylation of FUS5 may be significant.

Previously, it was reported that the COP9 complex may exist in different forms. Seeger et al. (1998) reported that the human COP9 complex (referred to as the signalosome) is found as a 450-kD species and as two larger species of up to 1000 kD. Wei et al. (1994) reported a light-dependent shift in the size of the COP9 complex. The gel filtration analysis of FUS5 further supports the idea that COP9 complex components can function in different forms. FUS5 is found both as a monomer and as part of the 500-kD COP9 complex. Similar results were also found for AJH1 (Kwok et al., 1998).

FUS5 is conserved between the plant and animal kingdoms both at the level of primary sequence and as a member of the COP9 complex. The FUS5 ortholog in mammals was recently shown to be a component of the COP9 complex (Seeger et al., 1998; Wei et al., 1998). However, no obvious orthologs for FUS5 were detected in the *Saccharomyces cerevisiae* genome. Neither have *S. cerevisiae* orthologs been detected for FUS6 or COP9 (Chamovitz and Deng, 1997). Based on this and other data, we originally hypothesized that the COP9 complex has an essential role in regulating cellular differentiation in multicellular organisms (Chamovitz and Deng, 1997).

The finding of gene products highly similar to FUS5 in *A. nidulans* furthers the development of this hypothesis. Asexual sporulation in *A. nidulans* involves the formation of conidiophores, which are specialized multicellular reproductive structures (Adams et al., 1998). Conidiation in *A. nidulans* is an inductive developmental process involving both intrinsic and external signals. Mutations in *AcoB* result in an asporogenous phenotype that is insensitive to inducing signals (Lewis and Champe, 1995). One of these inductive signals is light. Light control of conidiation in *A. nidulans* is reminiscent of phytochrome-mediated responses found in plants, because it is induced by red light and suppressed by far-red light (Mooney and Yager, 1990). Also, similar to phytochrome-mediated signaling (Bowler and Chua, 1994), a heterotrimeric G protein modulates the induction of conidiation. The role of a putative FUS5 ortholog in the regulation of a G protein–mediated developmental pathway in *A. nidulans* is similar to the role of GPS1 in modulating the MAPK pathway in mammalian cells (Spain et al., 1996) and perhaps to the role of the human COP9 complex in interacting with the MAPK pathway (Seeger et al., 1998). Thus, AcoB appears to function in developmental regulation similar to the COP9 complex in higher organisms. However, we do not know whether *A. nidulans* contains a COP9 complex or proteins similar to other components of the complex. Whereas *A. nidulans* may not be considered a multicellular organism, in contrast to *S. cerevisiae*, it does develop multicellular structures. We therefore modify our original hypothesis to state that the COP9 complex has an essential role in regulating multicellular differentiation.

METHODS

Plant Materials and Growth Conditions

The *fusca5* (*fus5*) allelic mutants S234, S239, U264, and T339b are in the *Arabidopsis thaliana* Landsberg *erecta* background (Miséra et al., 1994). The *constitutive photomorphogenic cop9-1* and *fus6-1* mutants are in the Wassilewskija background (Wei et al., 1994a). Wild-type plants are in the Arabidopsis Columbia or Landsberg *erecta* ecotype. Plant germination and growth conditions in darkness and white light were as described previously (McNellis et al., 1994). Light- and dark-cycle conditions were 16 hr of white light at 75 μmol m^{-2} sec^{-1} and 8 hr of darkness.

Isolation of the Arabidopsis p27 cDNA Clone

Internal peptide sequences of the cauliflower COP9 complex p27 subunit were described in Karniol et al. (1998). All four peptides are putatively encoded by the same region on chromosome 1. This

region has been sequenced as part of the Arabidopsis Genome Initiative (Arabidopsis bacterial artificial chromosome clone T7123). This region was amplified by polymerase chain reaction (PCR), and the 0.4-kb PCR product was used as a probe to clone the corresponding cDNA. The probe was labeled by using the digoxigenin labeling method (Boehringer Mannheim). To obtain the full-length cDNA sequence, an Arabidopsis CD4-7 cDNA library was screened (Newman et al., 1994). For cDNA screening, 5×10^5 phages displayed on duplicate sets of filters (positively charged nylon membranes; Boehringer Mannheim) were prehybridized in 5 × SSC (850 mM NaCl, 85 mM trisodium citrate·$2H_2O$, pH 7.0), 0.02% SDS, 0.01% *N*-lauroylsarcosine, and 1% blocking reagent for 2 hr at 68°C. Hybridization was performed in the same buffer containing the 0.4-kb probe for 16 hr at 68°C. Filters were washed to a final stringency of 0.1% SSC and 0.1% SDS at 68°C. Five putative positive plaques were picked and rescreened until purified. The sizes of the inserts were determined by PCR by using SP6 and T7 as primers. One phage containing the longest insert (~1.1 kb) was selected. The amplified DNA from the longest clone was cloned into a pGMT vector (Promega) and cleaved with SalI and NotI, and the resulting 1.1-kb fragment was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA), followed by sequencing of both strands (ABI 377; Perkin-Elmer). Sequence comparisons were made using the facilities at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Sequence alignments were made using the Clustal X program (Thompson et al., 1997).

Sequencing and Analysis of Wild-Type and Mutant Genes

For sequencing the gene encoding p27 in the *fus5* mutant alleles, we made synthetic oligonucleotide primers (18 to 19 bp ⩾ 50% GC) to enable the sequencing of the exons from the mutants and the wild type of the same ecotype. Genomic DNA was prepared from the wild-type and mutant seedlings (Dellaporta et al., 1983), and four sets of gene-specific primers were used to amplify fragments from the *FUS5* gene by using PCR. Conditions for PCR were as follows: 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, using Expand High Fidelity (Boehringer Mannheim) in a PTC-100 thermal cycler (MJ Research, Watertown, MA). The PCR products were sequenced using the same specific primers. Any alteration observed in the DNA sequence of the mutant alleles in comparison to the wild type was reconfirmed by sequencing at least two additional independent PCR products from independent DNA preparations.

Antibody Production and Affinity Purification

The coding region of *FUS5* minus the first three codons was cloned into a pGEX-4t-1 vector (Pharmacia). The plasmid construct was verified by sequencing. The resulting glutathione *S*-transferase–FUS5 fusion protein was overproduced in *Escherichia coli* and purified over glutathione–agarose beads (Pharmacia), according to the manufacturer's instructions. The fusion protein was digested with thrombin, leaving proline, serine, and glycine at the N terminus of FUS5, and the FUS5 protein was used for production of rabbit polyclonal antibodies and also for immobilization to an *N*-hydroxysuccinimide HiTrap column (Pharmacia). Antibodies bound to the FUS5 protein were eluted with a low-pH buffer (2 M glycine and 1 mM EGTA, pH 2.5) for 30 min. The resulting affinity-purified anti-FUS5 antibodies were neutralized by the addition of 1 M Tris-HCl, pH 8.8.

Protein Extraction and Immunoblot Analysis

Seedlings were homogenized in a buffer containing 10% glycerol, 10 mM NaCl, 10 mM $MgCl_2$, and 5 mM EDTA with freshly added proteinase inhibitors, including 0.5 mM phenylmethylsulfonyl fluoride and 5 mg/mL leupeptin. The protein concentrations were determined by using the Bradford assay (Darbre, 1986). Equal amounts of soluble protein were electrophoresed on 12.5 or 15% SDS–polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with affinity-purified rabbit polyclonal antibodies against the FUS5 protein. Bound antibodies were detected with alkaline phosphatase–coupled secondary antibodies, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Gel Filtration Chromatography

Total homogenates were prepared as described above. The total homogenate was microcentrifuged for 20 min at maximum speed, and the supernatant was filtered through a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI). Approximately 100 μg of total soluble protein was fractionated through a Superose 6 HR column (Pharmacia), with PBS plus 10 mM $MgCl_2$ and 10% glycerol, at a flow rate of 0.3 mL min^{-1}. All fractionations were performed at 4°C. Fractions of 0.5 mL each were collected and concentrated by Strata Clean Resin Beads (Stratagene). The protein standards were as follows: thyroglobulin (669 kD), apoferritin (443 kD), catalase (232 kD), aldolase (158 kD), and BSA (66 kD).

Yeast Two-Hybrid Assay

The coding region of the *FUS5* cDNA, minus the first three codons, was cloned into the EcoRI-NotI site of pEG202 to make an in-frame fusion with LexA. The generated plasmid was designated pEG-FUS5. pJG-COP9, pJG-FUS6 (Kwok et al., 1998), pEG-FUS5, and a reporter plasmid (pSH18-34) were transformed into yeast strain EGY48. The selection for transformants and the assay for their β-galactosidase activities were essentially as described previously (McNellis et al., 1996). Relative β-galactosidase activities were calculated according to Ausubel et al. (1995).

In Vitro Phosphorylation of the P27 Protein

Preparation of Arabidopsis Extract

Arabidopsis seedlings (Columbia ecotype) were grown for 5 days on Murashige and Skoog medium (Sigma) with 1% agar, in darkness, at 22°C. To obtain extracts from light-treated tissue, seedlings were exposed for 4 hr to white fluorescent light (500 μmol m^{-2}) before extraction. All subsequent manipulations were done under a green safelight, at 4°C. The plant tissue was frozen in liquid nitrogen, ground to a fine powder, and homogenized in modified TEF buffer (Lu et al., 1994) containing 20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 25 mM NaF, 0.1 mM orthovanadate, and 0.1 mM phenylmethylsulfonyl fluoride (1 mL of buffer per 1 g of seedlings). The extract was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged (20,000*g* for 30 sec). The resulting supernatant was used as a kinase source in phosphorylation of recombinant p27 protein.

In Vitro Phosphorylation and Immunoprecipitation

The reaction mixture contained 5 μL of 2 × reaction buffer (50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 mM NaF), 1 to 1.5 μL of seedling extract (1 μg of total proteins), 1 μg of purified recombinant p27, and 2 mCi of γ-^{32}P-ATP (New England Nuclear, Boston, MA) in a final volume of 10 μL. Samples were incubated at room temperature in darkness for 1 hr and then diluted to 300 μL by using ice-cold immunoprecipitation buffer containing 10 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, and 0.1% Tween 20. One microliter of affinity-purified anti-p27 antibodies was subsequently added, and samples were mixed by gentle shaking in darkness at 4°C for 5 hr. Ten microliters of protein A beads (Sigma) was added, and samples were mixed for 1 hr. Protein A beads were collected by centrifugation and washed five times with 1 mL of PBS containing RIPA detergents (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.5% SDS). The beads were boiled for 5 min in 2 × SDS sample buffer; proteins were resolved on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was dried and exposed to an autoradiography screen (Fuji Photo Film Co., Kanagawa, Japan). The screen was read and analyzed using a BAS PhosphorImager (Fuji). Nonradioactive phosphorylation of p27 was as given above, but with ATP added rather than γ-^{32}P-ATP. For mass spectroscopy of p27, the proteins were resolved by HPLC on a 2.1 × 30-mm C-8 column (model Aquapore RP-300; Applied Biosystems, Foster City, CA) and eluted with a linear gradient of 15 to 65% acetonitrile in 0.05 TFA. p27 was analyzed by electrospray ion trap mass spectrometry (LCQ; Finnigan, San Jose, CA) in the positive ion mode. Molecular mass was estimated with the deconvolution algorithm.

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NOTE ADDED IN PROOF

To reconcile differences in terminology, the COP9 complex is now referred to as the COP9 signalosome (**Wei, N., and Deng, X.-W.** [1999]. Making sense of the COP9 signalosome. Trends Genet. **15,** 98–103).