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Arabidopsis *cop8* and *fus4* Mutations Define the Same Gene That Encodes Subunit 4 of the COP9 Signalosome

Giovanna Serino,[a] Tomohiko Tsuge,[a,b] Shing Kwok,[a] Minami Matsui,[b] Ning Wei,[a] and Xing-Wang Deng[a,1]

[a] Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut 06520-8104
[b] Laboratory for Photoperception and Signal Transduction, Frontier Research Program, Institute of Physical and Chemical Research (RIKEN), Saitama 351-01, Japan

The pleiotropic *constitutive photomorphogenic*/*deetiolated*/*fusca* (*cop*/*det*/*fus*) mutants of Arabidopsis exhibit features of light-grown seedlings when grown in the dark. Cloning and biochemical analysis of COP9 have revealed that it is a component of a multiprotein complex, the COP9 signalosome (previously known as the COP9 complex). Here, we compare the immunoaffinity and the biochemical purification of the COP9 signalosome from cauliflower and confirm its eight-subunit composition. Molecular cloning of subunit 4 of the complex revealed that it is a proteasome–COP9 complex–eIF3 domain protein encoded by a gene that maps to chromosome 5, near the chromosomal location of the *cop8* and *fus4* mutations. Genetic complementation tests showed that the *cop8* and *fus4* mutations define the same locus, now designated as *COP8*. Molecular analysis of the subunit 4–encoding gene in both *cop8* and *fus4* mutants identified specific molecular lesions, and overexpression of the subunit 4 cDNA in a *cop8* mutant background resulted in complete rescue of the mutant phenotype. Thus, we conclude that *COP8* encodes subunit 4 of the COP9 signalosome. Examination of possible molecular interactions by using the yeast two-hybrid assay indicated that COP8 is capable of strong self-association as well as interaction with COP9, FUS6/COP11, FUS5, and Arabidopsis JAB1 homolog 1, the latter four proteins being previously defined subunits of the Arabidopsis COP9 signalosome. A comparative sequence analysis indicated that COP8 is highly conserved among multicellular eukaryotes and is also similar to a subunit of the 19S regulatory particle of the 26S proteasome.

INTRODUCTION

Plant development is highly plastic and often optimized according to the prevailing environmental conditions, of which light is one of the most important (Kendrick and Kronenberg, 1994). In response to light, Arabidopsis seedlings undergo photomorphogenic development, whereas dark-grown seedlings display etiolation or skotomorphogenic development (von Arnim and Deng, 1996). Analyses using genetic screening designed to identify mutants that display a complete light-grown phenotype in darkness have defined a total of 11 *CONSTITUTIVE PHOTOMORPHOGENIC*/*DEETIOLATED*/*FUSCA* (*COP*/*DET*/*FUS*) loci (Chory et al., 1989; Deng et al., 1991; Miséra et al., 1994; Kwok et al., 1996). Dark-grown mutant seedlings of this group display a pleiotropic phenotype, including open and expanded cotyledons, a short hypocotyl, chloroplast development, stomatal maturation, expression of light-regulated genes, and anthocyanin accumulation. In addition, mutants carrying strong mutations in this group of genes are lethal after the seedling stage, suggesting an essential role of the affected genes for normal plant development. The recessive nature of all of the *cop*/*det*/*fus* mutations characterized to date indicates that the proteins encoded by those loci are negative regulators of photomorphogenic development in the dark, whereas light releases their repressive activity.

At least three genes from this group, *COP9*, *FUS6*/*COP11*, and *FUS5*, encode subunits of a nuclear enriched protein complex, the COP9 signalosome (also known as the COP9 complex; Chamovitz et al., 1996; Staub et al., 1996; Karniol et al., 1999; Wei and Deng, 1999). Mutations in *COP9*, *FUS5*, or *FUS6*/*COP11* result in no detectable amounts of the COP9 signalosome (Kwok et al., 1998; Karniol et al., 1999; Wei and Deng, 1999), suggesting that the stability of the complex requires the coordinated accumulation of all subunits. Because the COP9 signalosome does not accumulate in the mutants of five additional *COP*/*DET*/*FUS* loci, these genes may either encode the other subunits of the complex or be absolutely required for its stability or formation.

The COP9 signalosome is highly conserved among multicellular eukaryotic organisms. Molecular characterization of

[1] To whom correspondence should be addressed at the Department of Biology, Osborn Memorial Laboratories, OML 301, Yale University, P.O. Box 20-8104, 165 Prospect St., New Haven, CT 06520-8104. E-mail xingwang.deng@yale.edu; fax 203-432-5726.

mammalian COP9 signalosome components has revealed that several of them may play a role in cell signaling (Seeger et al., 1998; Wei et al., 1998; Wei and Deng, 1999). For example, human subunit 5, counterpart of Arabidopsis JAB1 homolog 1 (AJH1) and AJH2, has been defined as JAB1, a c-Jun coactivator (Claret et al., 1996), and has also been shown to regulate the abundance of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Tomoda et al., 1999). Human subunit 1, or Gps1, which is the counterpart of Arabidopsis FUS6/COP11, can suppress the lethality of the Gα gene deletion in yeast when overexpressed and repress c-Jun N-terminal kinase activity in mammalian cells (Spain et al., 1996). Human subunit 6 is also called hVip, a protein able to interact with the human immunodeficiency virus protein Vpr and to modify its nuclear localization (Mahalingam et al., 1998). Lastly, subunit 2 corresponds to Alien/Trip15, a protein isolated based on its ligand-dependent interaction with the thyroid hormone receptor and retinoic acid X in a yeast two-hybrid assay (Lee et al., 1995; Dressel et al., 1999). On the other hand, all eight COP9 signalosome subunits have overall similarities to the eight subunits of the lid subcomplex of the 19S regulatory particle of the 26S proteasome (Glickman et al., 1998a). Each pair of corresponding subunits from the COP9 signalosome and the 26S proteasome exhibits sequence similarity over the entire lengths of the proteins (Wei et al., 1998). This similarity not only suggests a common evolutionary origin but also may indicate a role for the COP9 signalosome in regulating protein degradation. Indeed, a direct physical interaction between subunits of the COP9 signalosome and the proteasome has been suggested recently (Kwok et al., 1999).

The preliminary coimmunoprecipitation analysis of the cauliflower COP9 signalosome also indicated an eight-subunit composition in plants (Wei et al., 1998). To date, half of these subunits remain uncharacterized. As a first step toward understanding the biochemical nature of the cellular function of the COP9 signalosome, it is necessary to molecularly characterize all subunit components. A comparison of the purified cauliflower COP9 signalosome by immunoaffinity procedures and an improved biochemical procedure further confirmed the eight-subunit composition of the COP9 signalosome. Peptide sequence analyses of these copurified proteins led to the identification of four uncharacterized subunits. Here, we report the molecular characterization of one new subunit, subunit 4, and the identification of its corresponding *COP*/*FUS* locus.

RESULTS

Biochemical and Affinity Purification of the Plant COP9 Signalosome

In our previous biochemical purification of the COP9 signalosome (Chamovitz et al., 1996), a total of 15 major protein bands copurified with the cauliflower COP9 protein. Characterization of those protein bands led to the identification of three subunits, FUS6/COP11 (Chamovitz et al., 1996; Staub et al., 1996), AJH1/AJH2 (Kwok et al., 1998), and FUS5 (Karniol et al., 1999), in addition to COP9 itself. However, some of the copurified proteins were not integral components of the complex (Karniol et al., 1998). Recent characterization of the mammalian COP9 signalosome indicated that it contains eight distinct subunits (Seeger et al., 1998; Wei and Deng, 1998; Wei et al., 1998). Indeed, our initial coimmunoprecipitation analysis of the cauliflower COP9 signalosome with antibodies raised against two distinct subunits also suggested an eight-subunit composition of the cauliflower COP9 signalosome (Wei et al., 1998).

To further characterize the molecular identity of the remaining subunits of the plant COP9 signalosome, we performed large-scale preparative affinity purification of the cauliflower COP9 signalosome using antibodies raised against the FUS6/COP11 subunit. As shown in Figure 1 (left), a set of seven major protein bands can be copurified according to the Coomassie Brilliant Blue R-250 staining pattern. The major protein bands were labeled p22, p27, p36, p40, p45, p47, and p54, according to their estimated molecular mass in kilodaltons. To verify this subunit composition profile, we had improved the previously developed biochemical procedure (see Methods; Wei et al., 1998) and were able to purify the same set of protein bands in the COP9 signalosome fractions (Figure 1, right). In addition, two protein bands of 50 and 52 kD were also present. Those proteins were shown to be FUS6/COP11 (Chamovitz et al., 1996; data not shown). Interestingly, those two FUS6/COP11 bands were depleted from the affinity-purified COP9 preparation, possibly because of their tight binding to the immobilized FUS6/COP11 antibodies, which might have rendered them resistant to elution. The protein gel blot analysis clearly indicates that p22 is COP9 (data not shown). Therefore, the remaining six major copurified protein bands, together with two representative minor protein bands (p32* and p37*), were subjected to protein sequence analysis (Wei et al., 1998). An updated summary of these data is presented in Table 1.

Clearly, each of the major copurified proteins corresponded to a distinct subunit of the mammalian COP9 signalosome. Furthermore, two proteins, p40 and p27, matched the cloned Arabidopsis AJH1/AJH2 (Kwok et al., 1998) and FUS5 (Karniol et al., 1999) proteins, respectively, whereas the two minor protein bands (Figure 1) represented partial degradation products of p40 (AJH1/AJH2) or p36, respectively. Based on their similarity to the mammalian subunits, the eight subunits are designated as subunits 1 to 8 (S1 to S8; Figure 1; Wei et al., 1998). Moreover, the peptide sequences of p40 (S5) suggested that cauliflower also has two genes that may be similar to *AJH1* and *AJH2* (Kwok et al., 1998) for the same subunit (Table 1). In Arabidopsis, S1, S7, and S8 correspond to FUS6/COP11, FUS5, and COP9, respectively. The remaining four subunits, S2, S3, S4, and

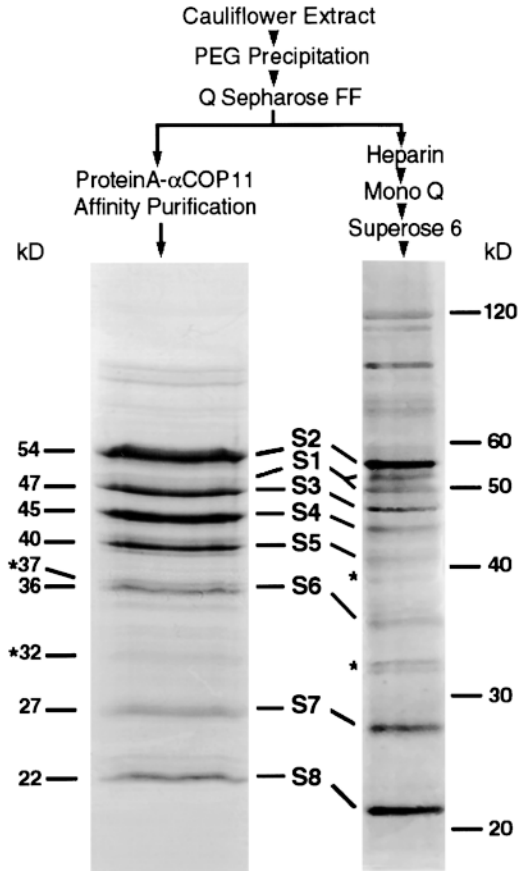

Figure 1. Purification Procedure and Subunit Composition of the Cauliflower COP9 Signalosome.

The immunoaffinity-purified complex is shown at the left, and the biochemically purified complex is shown at the right. The major steps of purification are shown in both cases. Protein bands for the affinity-purified COP9 signalosome (left) were stained with Coomassie blue; the protein profile of the biochemically purified COP9 signalosome preparation (right) was silver-stained. Asterisks denote minor protein bands that were later shown to be partial degradation products: p37 is a degradation product of p40, and p32 is a degradation product of p36. Band assignment to each subunit (S1 to S8) is based on peptide sequencing or on protein gel blot analysis (see text or Table 1 for details). Note that the affinity-purified COP9 signalosome preparation is depleted of FUS6/COP11 (S1) subunits, possibly because of their tight binding to the affinity column and resistance to our elution scheme.

S6, are plant COP9 signalosome subunits that have not been molecularly characterized.

Cloning and Sequence Analysis of the Subunit 4 Gene

To characterize the subunit 4 gene (now termed *AtS4*), we used three distinct peptide sequences obtained from the cauliflower S4 band to search the Arabidopsis database (http://genome-www.stanford.edu/Arabidopsis/). This search identified a partial expressed sequence tag (EST) clone that was missing the 5′ portion of the gene (35B4T7; GenBank accession number T04273). The EST clone was obtained and completely sequenced. This partial cDNA sequence was then used to search the Arabidopsis genomic database, and a recently sequenced genomic P1 clone covering the *AtS4* gene was found. From the known human S4 sequence and the *AtS4* genomic sequence, we were able to design specific primers to obtain a cDNA clone covering the full-length open reading frame by using reverse transcription–polymerase chain reaction (RT-PCR) amplification. The coding region of the cDNA is 1194 bp long and encodes a protein with an isoelectric point of 4.76 and a calculated molecular mass of 44.9 kD, which is very close to its apparent molecular mass of 47 kD in cauliflower (Figure 2A). The predicted AtS4 sequence matches well with the peptide sequences from cauliflower (Figure 2B). As shown in Figure 2A, the AtS4 protein is closely related to its mouse counterpart COPS4 (49% identity and 68% similarity) and has homologs in other multicellular organisms, such as *Cionia intestinalis* (Ascidiaceae family) and *Drosophila melanogaster* (46 and 48% identity, respectively). As shown in Figure 2A, the similarities among the subunit 4 homologs extend to the entire sequence, except for the N and C termini of the proteins. Interestingly, like most other COP9 signalosome subunits, no homolog of AtS4 can be detected in the genome of the yeast *Saccharomyces cerevisiae*.

Analysis of the amino acid sequence of AtS4 reveals a leucine-rich region in the N-terminal region (amino acids 23 to 66) and a proteasome–COP9 complex–eIF3 (PCI) domain encompassing amino acids 190 to 362 (Figure 3). The PCI domain is a common protein motif found in components of the lid subcomplex of the proteasome, the COP9 signalosome, and the eIF3 complex (Hofmann and Bucher, 1998). Within the PCI domain, a putative helix-loop-helix domain is centered around amino acids 294 and 302. Direct comparison of the genomic and cDNA sequences revealed that the *AtS4* genomic sequence contains 13 exons and 12 introns (Figure 3).

The *cop8* and *fus4* Mutations Are Allelic and Map to the Same Chromosomal Region as *AtS4*

Mutations in eight pleiotropic *COP*/*DET*/*FUS* loci result in the apparent absence of the COP9 signalosome in Arabidopsis (Kwok et al., 1998; Wei and Deng, 1999). Among them, three loci, *COP9*, *FUS6*/*COP11*, and *FUS5*, have been demonstrated to encode subunits of Arabidopsis COP9 signalosome (Wei et al., 1994a; Staub et al., 1996; Karniol et al., 1999). Therefore, one of the remaining *COP*/*DET*/*FUS* genes might encode AtS4. The P1 clone containing *AtS4* is very close to the *DFR* (dihydroflavonol 4-reductase) marker, located at position 102 centimorgans on chromosome 5 of

Table 1. Summary of the Peptide Sequences from the Subunits of the Affinity-Purified Cauliflower COP9 Signalosome

Molecular Mass (kD)	Subunit	Peptide Sequence	Related to		% Identity	
			Arabidopsis	Mammals	vs. Arabidopsis Sequences	vs. Mammalian Subunits
p54	S2	KSAVTRNYSEK	NA[a]	COPS2 (TRIP15)	100	100
	S2	KWNTQLK	NA	COPS2 (TRIP15)	83	50
	S2	KNERLWFK	NA	COPS2 (TRIP15)	100	85
p47	S4	KAYLPDK	COP8	COPS4	85	33
	S4	KSTVLDRAMIEHNNLLSASK	COP8	COPS4	100	83
	S4	KRKFLDAALRYYSISQIEK	COP8	COPS4	88	44
p45	S3	KDGMVRFLEDPEQYK	NA	COPS3	100[b]	60
	S3	KSIQGLSASPGDLSALHG	NA	COPS3	85[b]	25
	S3	KEAEMHVLQMIQDGQIHALINQK	NA	COPS3	78[b]	65
p40	S5	KDILFNSARQSDK	AJH1	COPS5 (JAB1)	92	38
	S5	KNILTVEQPDSSSSDGIFYYDEAS	AJH2	COPS5 (JAB1)	65	8
	S5	KQYYSLDITYFK	AJH1	COPS5 (JAB1)	100	66
	S5	KRVQISALAL	AJH2	COPS5 (JAB1)	90	70
	S5	KITVEQVHGLMSQVIKDILFNSA	AJH1	COPS5 (JAB1)	100	69
p37[c]	S5	KISDDHVSEYQTIPLNK	AJH1	COPS5 (JAB1)	100	78
	S5	KQYYSLDITYFK	AJH1	COPS5 (JAB1)	100	66
p36	S6	KLHPLVMLNISD	NA	COPS6 (hVIP[d])	NA	83
	S6	KGRTVEIFNSFELLLD	NA	COPS6 (hVIP)	NA	56
	S6	KMLNSRIRVLHQYLGSMQK	NA	COPS6 (hVIP)	NA	31
p32[c]	S6	KMLNSRIRVLHQYLAA	NA	COPS6 (hVIP)	NA	43
	S6	KALMDINESPVYVLLNPTINHAQK	NA	COPS6 (hVIP)	NA	39
p27	S7	KEAEEGVEELK	FUS5	COPS7	90	30[e]
	S7	KWADNMSEIDK	FUS5	COPS7	90	18[e]

[a] NA, not available.
[b] Identity to a *Lycopersicon chilense* cDNA clone (LCU19099, GenBank accession number U19099).
[c] Degradation products.
[d] Human Vpr interacting protein (Mahalingam et al., 1998).
[e] Identity to mouse COP9 complex S7a (GenBank accession number AAC33904).

the Arabidopsis genome. By using simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers (see Methods), a systematic mapping analysis of available *cop*/*det*/*fus* mutations revealed that both the *cop8-1* and the *fus4-414* mutations reside close to the genomic location for AtS4 (Figure 4).

The *cop8-1* mutation (Wei et al., 1994b) was detected in line 2817, which was identified from the first Feldmann T-DNA–tagged collection (Feldmann, 1991). This same line was also independently identified as carrying the *emb134* mutation (Castle and Meinke, 1994). Moreover, *emb134* was reported to be allelic to the previously known *fus8* mutations and thus was assigned another name, *fus8-1* (Castle and Meinke, 1994; Miséra et al., 1994). However, our mapping data revealed that a previously used standard *fus8* allele, *fus8-S253* (Miséra et al., 1994), was located on chromosome 4. This is not the same chromosome on which the *cop8-1* (*emb134*) mutation resides (data not shown). Therefore, the initial assignment of this *cop8-1* (*emb134*) mutation to the *FUS8* locus appears to be incorrect.

The fact that both *cop8-1* and *fus4-414* mutants accumulate undetectable or diminished amounts of the COP9 signalosome (Kwok et al., 1998) and are located in the same region of chromosome 5 prompted us to analyze whether the two mutations are allelic to each other. We performed genetic crosses between *cop8-1* and *fus4-414* mutants. Because the homozygous mutant plants are lethal for both mutations, the plants heterozygous for each mutation were used in the cross. Among the F_1 progeny, 27 wild-type and 10 *fus* mutant seedlings were recovered. This segregation of wild type to *fus* is close to the 3:1 ratio expected from the allelic mutations. Therefore, we conclude that *cop8-1* and *fus4-414* are allelic and define the same locus, which we designated as *COP8*.

Close examination of the *cop8-1* and *fus4-414* mutants revealed some subtle differences in their phenotypes (Figure 5). The cotyledons in the mature *cop8-1* seeds are purple, whereas the seed color of *fus4-414* mutant seeds is normal and similar to that of the wild type (Figure 5C). Despite this difference in seed color, both mutants exhibit similar seed-

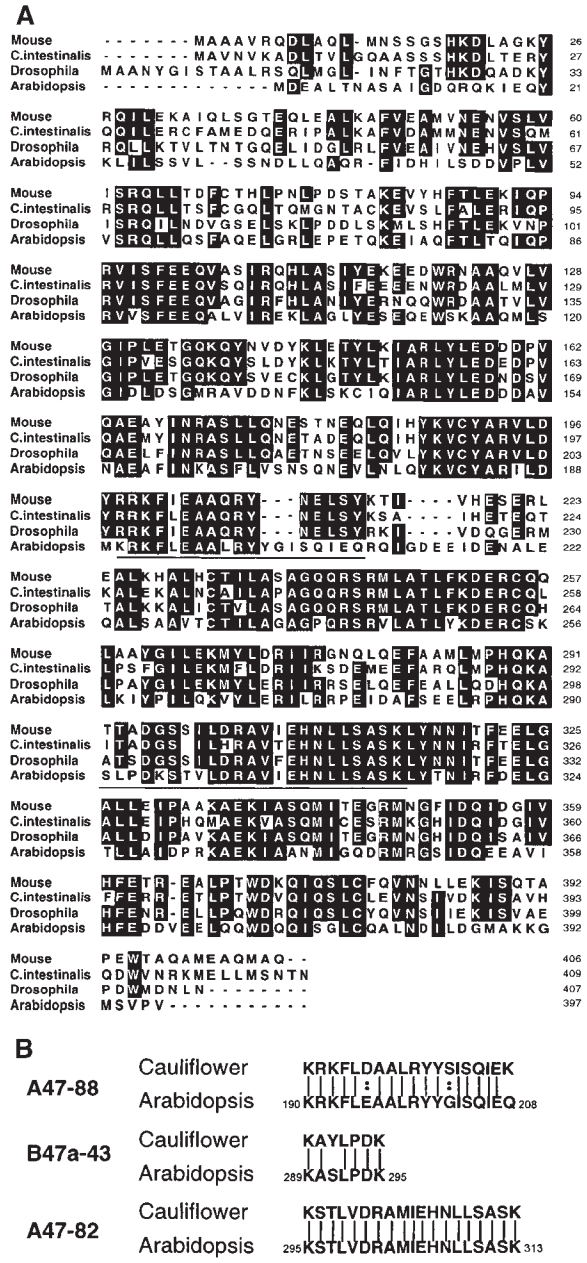

Figure 2. Comparison of the COP8 Sequence with Its Homologs from Other Organisms.

(A) Protein sequences of COP8 and its homologs from mouse, *C. intestinalis*, and Drosophila. Boxed residues indicate amino acid identity. The underlined sequences correspond to the locations of three peptides sequenced from cauliflower. Numbers at right indicate the positions of the last amino acid residues. Note that the second and third peptide sequences (B47a-43 and A47-82) are adjacent to each other. The GenBank accession numbers for the three homologs are AAD28607 (Drosophila), Z83760 (*C. intestinalis* COS41.8), and AAC33901 (mouse). The Arabidopsis AtS4 GenBank accession number is AF176089.

(B) Comparison of peptide sequences from cauliflower COP8 with the corresponding Arabidopsis COP8 protein sequences. Vertical bars indicate identity; colons indicate similarity. The numbers for the Arabidopsis peptides indicate the first and last amino acid positions.

ling phenotypes and are lethal after the seedling stage (Figures 5A and 5B). Interestingly, the F_1 seeds that are transheterozygous for both *fus4-414* and *cop8-1* mutations exhibit the purple color and resemble seeds of the *cop8-1* parent (Figure 5C). Therefore, as far as the seed color phenotype is concerned, the *cop8-1* allele is dominant over the *fus4-414* allele.

Both *cop8* and *fus4* Mutations Contain Molecular Lesions in the *AtS4* Gene

To determine whether the *COP8* gene encodes AtS4, we sought to identify mutations in the *AtS4* sequence in *cop8-1* and *fus4-414* mutants. Specific primers were designed to amplify by PCR both the cDNA and genomic sequences of the *AtS4* gene from the homozygous mutants and their corresponding wild-type strains. Sequence analysis of those PCR products revealed a 12-bp deletion in the *AtS4* gene of the *cop8-1* mutant (Figure 3). This deletion removes the splice site between exon III and intron III. Sequence analysis of the corresponding cDNA region revealed that this mutation leaves the unspliced intron III in the mature mRNA, which in turn leads to premature termination of the open reading frame. The resulting mutant COP8-1 protein has only the N-terminal 125 amino acids plus another nine amino acids derived from the intron III sequence before the open reading frame terminates (Figure 3). The *cop8-1* mutant was initially isolated from a T-DNA insertional mutagenized line, but the mutation did not cosegregate with kanamycin resistance conferred by the T-DNA (Wei et al., 1994b). One could reasonably speculate that the deletion found resulted from insertion followed by excision of the T-DNA.

Sequencing of the *fus4-414* allele identified a single base pair substitution at the last nucleotide position of intron VII. Sequence analysis of the mutant cDNAs indicated that this mutation leads to the skipping of exon VIII in the mature mRNA (Figure 3). The predicted mutant version of the protein will be missing the 15 amino acids (positions 251 to 265) encoded by exon VIII. The lethal phenotype of the mutation indicated that the missing sequence within the PCI domain is critically important for the function of AtS4. Nonetheless, the molecular lesions found in those two mutations are consistent with the fact that the *fus4-414* allele is a weaker mutation than *cop8-1*. The presence of specific mutations in both *cop8-1* and *fus4-414* mutant alleles indicated that the *COP8* gene encodes AtS4 of the COP9 signalosome.

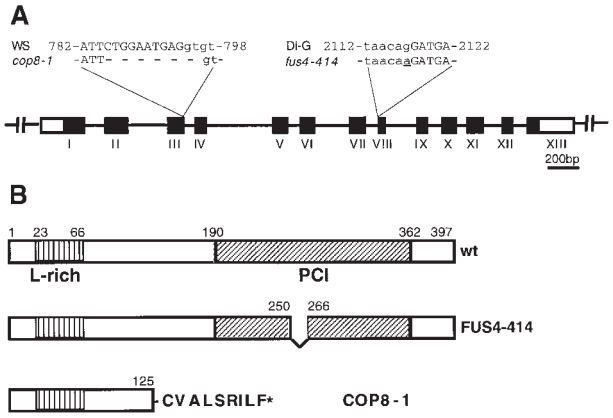

Figure 3. Schematic Representation of the *COP8* Gene and Lesions in the *cop8-1* and *fus4-414* Mutants.

(A) The genomic structure of the *COP8* locus and the molecular nature of the mutations found in the *cop8-1* and *fus4-414* alleles are provided at the top. Introns are shown as lines; the boxes and the roman numerals indicate exons, with the 5′ and 3′ untranslated regions shown by white boxes and the protein-coding region denoted by black boxes. This sequence is contained within the sequence of the P1 clone MBD2 (GenBank accession number AB008264). The two inserts show the mutated sequences found in *fus4-414* and *cop8-1* when compared with the respective wild types, Wassilewskija (WS), and Dijon-G (Di-G). The numbers indicate the sequence position within the wild-type genomic clone, with the first nucleotide of the starting amino acid codon as position 1.

(B) Diagram of wild-type (wt) and mutant versions of COP8 proteins. The predicted nine amino acids and the stop codon (asterisk) in the cop8-1 mutant protein are indicated. The striped and cross-hatched boxes indicate the locations of the leucine-rich region and the PCI domain, respectively. The numbers indicate positions of the amino acids.

Overexpressed *AtS4* Can Rescue the Phenotype of the *cop8* Mutant

To further confirm that *COP8* encodes AtS4, we introduced the full-length *AtS4* cDNA under the control of the cauliflower mosaic virus 35S promoter into the *cop8-1* mutant. Because of the lethality of the homozygous *cop8-1* mutation, the *AtS4* cDNA construct was transformed into plants heterozygous for *cop8-1*. The stably transformed plants heterozygous for both *cop8-1* and the transgene were selected and examined. In the absence of phenotype rescue, a 3:1 ratio of wild-type to mutant seedlings would be expected from the progeny of a selfed plant heterozygous for the *cop8-1* mutation and the transgene. If the *AtS4* transgene can rescue the mutant phenotype, then the ratio of wild-type to mutant plants in the same progeny should be 15:1 rather than 3:1, provided that the transgene is at a single locus unlinked to *COP8*. An even higher wild type–to–mutant ratio is expected if the transgene is present at multiple loci. Table 2 summarizes the segregation analyses of two such transformed lines. Both transgenic lines most likely contain two loci of the transgene, according to the segregation ratio of the T-DNA marker for gentamycin resistance. The greatly reduced number of mutants in the progeny (approximately two in 100) compared with the expected one-quarter homozygous *cop8-1* mutants indicated that the transgene was capable of rescuing the mutant phenotype. This further confirms that *COP8* encodes AtS4. From now on, we refer to AtS4 as COP8.

The Mutated COP8 Protein in the *fus4-414* Allele May Exhibit Reduced Ability to Assemble into the COP9 Signalosome

To reveal whether the mutated COP8 proteins in the *cop8-1* and *fus4-414* mutants accumulate and are capable of complex assembly, we raised polyclonal antibodies against COP8 (see Methods). As shown in Figure 6A, protein gel blot analysis revealed that the anti-COP8 antibody detects a 48-kD protein in the wild type, whereas it detects a smaller 46-kD protein of reduced amount in the *fus4-414* mutant and no protein in the *cop8-1* mutant. The reduced protein size observed in *fus4-414* mutants is consistent with the predicted 15–amino acid deletion in the COP8 protein (Figure 3). In contrast, the preimmune serum detected no protein in either wild-type or mutant extracts (Figure 6B). This result clearly suggests that the anti-COP8 antibodies are specific. Furthermore, it reveals that the mutated COP8 protein accumulates in the *fus4-414* mutants but at a reduced amount.

To reveal the effect of the *cop8-1* and *fus4-414* mutations on COP9 signalosome accumulation, we first analyzed the accumulation patterns of two representative subunits, FUS6/COP11 and AJH1/AJH2, in those mutant backgrounds. As shown in Figures 6C and 6D, the accumulation of FUS6/COP11 was markedly diminished in the *fus4-414* mutants and completely abolished in the *cop8-1* mutants, whereas AJH protein accumulation was not affected in either mutant. Because AJH proteins are expected to be stable in the monomeric form (Kwok et al., 1998), the *cop8* and *fus4* muta-

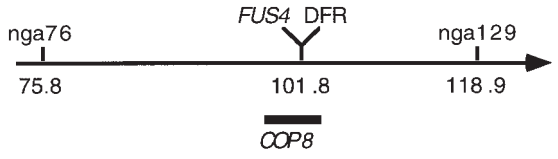

Figure 4. *cop8-1* and *fus4-414* Map to the Same Region on Chromosome 5.

Shown is the map position determined for *cop8-1* and *fus4-414* within the region of chromosome 5 near the marker DFR. The molecular markers used for generating the map are shown above the line, and their positions (in centimorgans) are indicated below. The arrow indicates the direction of the bottom telomere of chromosome 5.

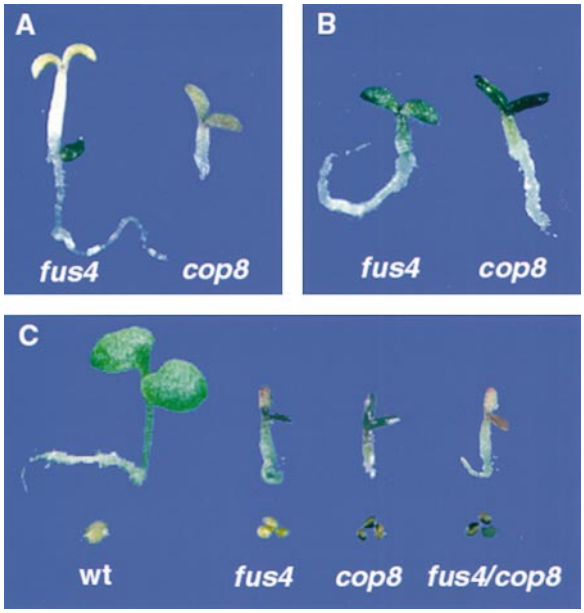

Figure 5. Seedling and Seed Phenotypes of *cop8-1* and *fus4-414* and Their Transheterozygote.

(A) Dark-grown 8-day-old *cop8-1* and *fus4-414* seedlings.
(B) Light-grown 8-day-old *cop8-1* and *fus4-414* seedlings.
(C) A comparison of 5-day-old light-grown seedlings and the respective seeds of (left to right) the wild type, *fus4-414*, *cop8-1*, and *fus4/cop8* transheterozygote.

tions were not expected to affect AJH accumulation. However, because FUS6/COP11 is stable only in the COP9 signalosome, the absence of COP8 in *cop8-1* mutant is expected to abolish the COP9 signalosome and thus FUS6/COP11. Therefore, the diminished accumulation of FUS6/COP11 in the *fus4-414* mutant implies that the COP8 mutant protein cannot effectively promote assembly of the COP9 signalosome.

To confirm this notion, we subjected the COP8 protein to gel filtration analysis and compared the results with those obtained with the AJH proteins. As shown in Figure 6E, both COP8 and AJH proteins also accumulated in forms (fractions 17 and 18, ~100 kD) that are smaller than the COP9 signalosome. Whereas the majority of wild-type COP8 was present in the COP9 signalosome (peak around fraction 12, 500 kD), the mutated COP8 protein is predominantly present in the fractions outside of the COP9 signalosome (fractions 17 and 18). This suggests that the 15–amino acid deletion of the COP8 protein in the *fus4-414* mutant background markedly compromised its capability to form the COP9 signalosome. The fact that this 15–amino acid deletion is located within the PCI domain implies a role of the COP8 PCI domain in its ability to assemble into the COP9 signalosome. We noted that trace amounts of the mutated COP8 were present in the COP9 signalosome in the *fus4-414* mutant (Figure 6E; see fractions 12 and 13), which is consistent with the trace amounts of FUS6/COP11 also detected in this mutant (Figure 6C).

AtS4 Is Capable of Direct Interaction with Four Other Subunits of the COP9 Signalosome

To characterize the overall architecture of the COP9 signalosome, we decided to examine the interactions of COP8 with other known components of the complex, using a yeast two-hybrid assay (Ausubel et al., 1995). It has already been shown that FUS6/COP11 interacts with itself, AJH1, and FUS5 (Kwok et al., 1998, 1999; Karniol et al., 1999), and FUS5 also interacts with COP9 (Karniol et al., 1999). As shown in Figure 7A, COP8 in the LexA configuration is able to interact specifically with FUS6/COP11, AJH1, FUS5, and COP9. On the other hand, COP8 in the activation domain configuration interacts with AJH1, FUS5, and COP9 but fails to interact with FUS6/COP11 (Figure 7B). It is not clear why the interaction between COP8 and FUS6/COP11 can be demonstrated in only one of the two configurations. Perhaps the COP8 and FUS6/COP11 interaction requires COP8 in a stable dimeric configuration, which might be feasible only

Table 2. Phenotype Complementation of the *cop8-1* Mutation by 35S–*AtS4*

		Gentamycin		Ratio	Phenotype[b]		Ratio
Line[a]	Total	Res.[c]	Sens.[d]	Res.:Sens.	Wild Type	*cop8*	Wild Type:Mutant
46	112	104	8	12.3:1	98	2	49:1
52	204	188	16	9.0:1	200	4	50:1

[a] Seedlings of the T_2 progeny were scored for T-DNA marker gentamycin resistance in 100 μg/mL gentamycin plates.
[b] The seedling phenotype was scored on regular growth medium plates (Wei and Deng, 1992). All seedlings were grown in continuous light for 10 days.
[c] Res., resistant.
[d] Sens., sensitive.

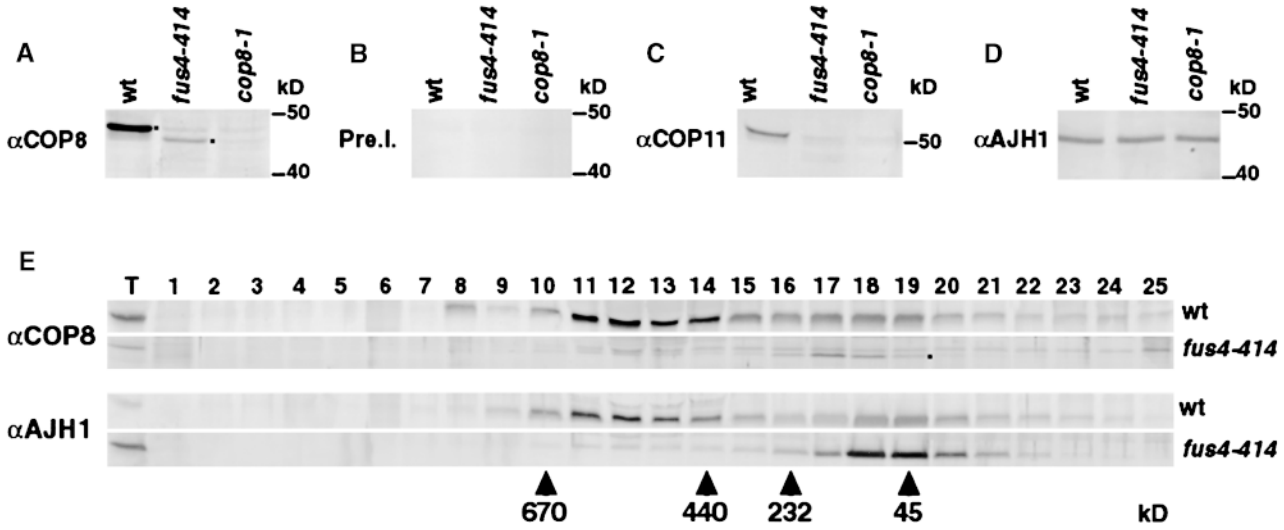

Figure 6. Effect of *cop8* and *fus4* Mutations on Accumulation of the COP9 Signalosome and Its Representative Subunits COP8, FUS6/COP11, and AJH1/AJH2.

Accumulation in the wild-type (wt), *fus4-414*, and *cop8-1* mutants is shown in **(A)** to **(D)**. Fifteen micrograms of total soluble protein extract from 8-day-old light-grown wild-type, *fus4-414*, and *cop8-1* seedlings was subjected to protein gel blot analysis.

(A) Protein gel blot probed with anti-COP8 antibodies (αCOP8). The dots indicate the wild-type protein and its mutated form in *fus4-414*.

(B) Protein gel blot probed with preimmune serum (Pre.I.) corresponding to the anti-COP8 antibodies.

(C) Protein gel blot probed with anti-COP11 (αCOP11) antibodies.

(D) Protein gel blot probed with anti-AJH1 (αAJH1) antibodies.

(E) The COP9 signalosome accumulates in reduced quantities in *fus4* mutants. Total soluble protein extracts from 8-day-old light-grown wild-type and *fus4-414* seedlings were separated by gel filtration and subjected to immunoblot analysis by using anti-COP8 antibodies (αCOP8; top) (the dot indicates the FUS4-414 protein in the *fus4-414* mutants) and anti-AJH1 antibodies (αAJH1; bottom). Lane T indicates the total extract used in the experiment, and lanes 1 to 25 indicate the fractions in the gel-filtration elution. Numbers below the arrowheads indicate the peak position of the molecular mass markers in kilodaltons.

Note that a weak background signal at the wild-type COP8 position is visible in both *cop8-1* and *fus4-414* mutants in **(A)**; it becomes more evident in the gel-filtration analysis of the *fus4-414* mutant proteins shown in **(E)** because this immunoblot was developed for a long time. Numbers at right in **(A)** to **(D)** indicate molecular mass markers.

when COP8 is fused to LexA. All of the protein constructs examined express the predicted protein in yeast at similar amounts, as determined by protein gel blot analysis (data not shown); therefore, the observed differences in reporter expression should indicate the relative strength of their interactions. Also, notably, COP8 strongly interacts with itself, suggesting that it is possible that two copies of COP8 could be present in the COP9 signalosome. This would be consistent with the fact that the S4 band has the strongest Coomassie blue staining in the affinity-purified COP9 signalosome (Figure 1).

Relationship of the AtS4 Homologs and Their Proteasome Paralogs in Eukaryotic Organisms

The COP9 signalosome has been hypothesized to share a common evolutionary origin with the lid subcomplex of the 19S regulatory particle of the 26S proteasome (Wei and Deng, 1999). This hypothesis is based on the observation that the eight mammalian COP9 signalosome subunits exhibit one-to-one similarity to the eight subunits of the lid subcomplex (Glickman et al., 1998a; Wei and Deng, 1999). Seeking further insights about this evolutionary relationship, we conducted a comparative analysis of the COP8 homologs and their paralogs in the 26S proteasome among representative eukaryotic organisms. For the proteasome paralogs, we selected proteins from representative taxonomic groups, including yeast Rpn5 (Glickman et al., 1998b) and human p55 (Saito et al., 1997), and their homologs in Caenorhabditis and Drosophila. Those sequences were then compared with the COP8 homologs from similar groups of organisms. The relationship of all of those sequences is depicted in Figure 8. This analysis clearly defines two distinct families of proteins: the COP9 signalosome COP8 family and the proteasome Rpn5 family. This grouping correlates with the functionality of the proteins, regardless of the organisms, and supports the notion that the two groups of

proteins diverged from the very beginning during the evolution of eukaryotes.

DISCUSSION

The Subunit Composition of the Plant COP9 Signalosome

Comparison of immunoaffinity and classic biochemical purification of the cauliflower COP9 signalosome has further confirmed our early conclusion that the COP9 signalosome contains eight core subunits (Wei et al., 1998; Wei and Deng, 1999). Our data reported here reaffirm our early coimmunoprecipitation analyses using antibodies raised against COP11 and AJH1 (Wei et al., 1998). Together, three main arguments support the conclusion that the eight major copurified protein bands represent the subunit composition of the plant COP9 signalosome. First, the same eight protein bands copurified with the COP9 signalosome in multiple independent purification procedures. Any protein other than the integral components of the COP9 signalosome would be extremely unlikely to be copurified by all purification procedures. Second, the composition of the eight subunits is consistent with that of the mammalian COP9 signalosome defined recently (Wei et al., 1998). Third, molecular identities of the eight proteins match exactly with the eight subunits of the mammalian COP9 signalosome. The peptide sequences and the immunoblot analyses allowed us to identify four previously reported subunits of the plant COP9 signalosome: S1 as FUS6/COP11, S5 as AJH 1/AJH2, S7 as FUS5, and S8 as COP9. Aside from the molecular characterization of S4 as COP8 in this report, the remaining three subunits are in the process of being molecularly characterized.

Compared with our initial biochemical purification of the cauliflower COP9 signalosome (Chamovitz et al., 1996), several copurified proteins evidently were eliminated with our new procedures. For example, in our previously purified fractions, protein sequence analysis revealed not only seven true subunits of the COP9 signalosome subunits but also two eIF3 complex subunits and two subunits of the 26S proteasome (Karniol et al., 1998; data not shown). Although possible direct protein–protein interactions between the COP9 signalosome and both eIF3 and proteasome complexes have been implied (Karniol et al., 1998; Kwok et al., 1999), coimmunoprecipitation studies have suggested that those three complexes are not stably associated in plant cell extracts. Our improved purification procedures can eliminate both eIF3 and proteasome complexes from the COP9 signalosome and thus should facilitate future characterization of the biochemical activity of the COP9 signalosome. However, it is interesting that two distinct protein motifs, the PCI domain and the Mpr1p–Pad1p–N terminus domain, are exclusively present among subunits of the COP9 signalosome, the eIF3 complex, and the proteasome (Hofmann and Bucher, 1998; Wei and Deng, 1999). Therefore, whether the cofractionation of these related proteins in our previous biochemical purification is of any functional relevance remains to be defined.

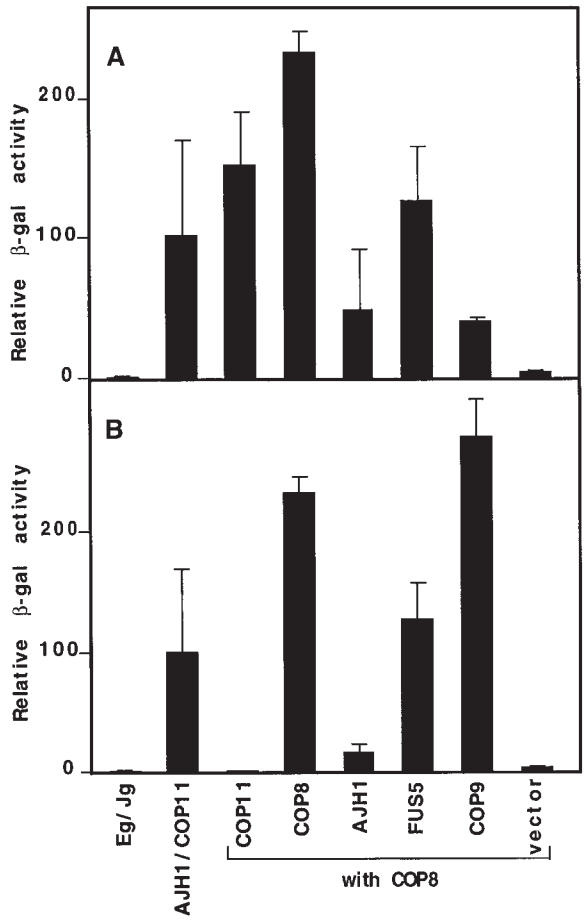

Figure 7. Interaction of COP8 with Other Subunits of the COP9 Signalosome.

Full-length COP8 was fused either to the LexA DNA binding domain or to the activation domain and assayed for its interaction, in a pairwise fashion, with other COP9 signalosome subunits. The relative LacZ activity shown is the average of at least six independent transformants.

(A) LexA–COP8 fusion versus activation domain fusions of COP11, COP8, AJH1, FUS5, COP9, or activation domain vector control (vector).

(B) Activation domain–COP8 fusion versus LexA fusion constructs of COP11, COP8, AJH1, FUS5, COP9, or LexA alone (vector).

The first bars in **(A)** and **(B)** denote the negative controls with the two empty vectors (Eg, LexA empty vector; Jg, activation domain empty vector). The interaction of AJH1 and COP11 (second bars) was used as a positive control. Error bars represent standard deviation from the mean. β-gal, β-galactosidase.

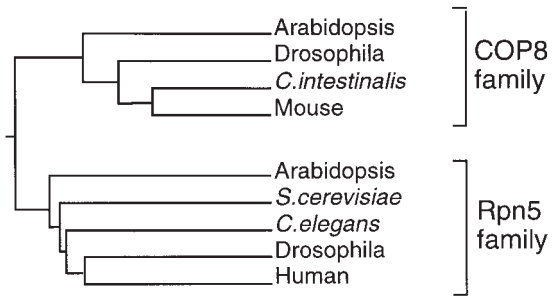

Figure 8. Phylogenetic Relationships of the COP8 Family and Their Corresponding Proteasome Paralogs.

The evolutionary tree was obtained by the Jotun Hein algorithm using the Megalign program (gap penalty, 11; gap length, 3) (DNAstar, Madison, WI). The accession numbers for the COP8 homologs are given in the legend to Figure 2. To date, we have not found any homolog of COP8 in the *Caenorhabditis elegans* genome. GenBank accession numbers for the proteasome paralogs are NP002807 (human p55), Z74195 (yeast Rpn5), U40029 (*C. elegans* protein F10G7.8), LD27261 (Drosophila), and AB016893 (Arabidopsis). The COP8 family proteins are described in the legend to Figure 2.

Subunit 4 of the COP9 Signalosome Is Encoded by *COP8*, Which Is Defined by Mutations in Both *cop8* and *fus4*

The genetic complementation test clearly suggested that the *cop8-1* and *fus4-414* mutations are allelic. Because they both mapped to the same location in the genome, they probably define the same genetic locus. Furthermore, the fact that both *cop8-1* and *fus4-414* mutations have molecular lesions in the gene encoding AtS4 of the COP9 signalosome confirms that those two mutations define the same genetic locus, which we named *COP8*. Together with the ability of the overexpressed *AtS4* cDNA to rescue the phenotype of the *cop8-1* mutant, these data confirm that *COP8* encodes AtS4 of the COP9 signalosome. With this new finding, four of the 11 pleiotropic *COP*/*DET*/*FUS* genes—*FUS6*/*COP11*, *COP9*, *FUS5*, and *COP8*—are shown to encode subunits of the Arabidopsis COP9 signalosome.

Similar to its homologs in mice and humans, the COP8 C-terminal region contains the PCI domain, a motif also found in five other components of the COP9 complex (Wei et al., 1998) and in several components of both the eIF3 complex and the 26S proteasome (Hofmann and Bucher, 1998). The function of this domain is not known; it could be a site for protein–protein interaction within the same complex or could play a role in macromolecular assembly. Our observation that the 15–amino acid deletion in the COP8 PCI domain markedly compromised its ability to assemble into the COP9 signalosome (Figure 6E) would support this notion. The N-terminal half contains a leucine-rich region that is common to many protein-interactive motifs. As a subunit of the COP9 signalosome, COP8 seems to be capable of interacting directly with all four of the characterized subunits of the Arabidopsis COP9 signalosome. This suggests a central location for COP8 among the COP9 signalosome subunits. In addition, COP8 is also capable of interacting with itself, similar to the FUS6/COP11 subunit (Kwok et al., 1999). Indeed, both COP8 and COP11 may be present in more than one copy within the complex. However, our subunit interaction is based only on a yeast two-hybrid assay (Figure 5). This assay has its limitations: it is assaying only the interaction of two subunits in isolation and may not necessarily reflect the interactions in plant cells. Further investigation is required to obtain a complete picture of the subunit interaction and configuration within the COP9 signalosome.

The COP9 Signalosome and the 26S Proteasome: Evolutionary Implications

Sequence analysis has revealed sequences related to that of COP8 in representative groups of multicellular organisms from *C. intestinalis* and *Drosophila* to humans. The high degree of sequence identity (46 to 49%) among these proteins suggests an essential and conserved function during evolution. Interestingly, no homolog was observed among the sequenced genomes of any unicellular organism, suggesting that the COP9 complex might have evolved specifically as a result of the increasing complexity necessary for the regulation of multicellular development.

Database searches also indicate that COP8 shares sequence similarities with the Rpn5 family of proteins. Rpn5, one of the eight proteins identified as subunits of the 26S proteasome lid subcomplex from yeast, has homologs in all eukaryotic organisms, including plants and animals. The lid subcomplex is part of the 19S regulatory particle of the 26S proteasome. The similarity of COP8 to this group of proteins ranges from 20 to 25% in sequence identity and 50 to 55% in sequence similarity. A relationship comparison (Figure 8) suggested that the two families of proteins have possibly evolved from a common precursor during the early stages of eukaryotic cell evolution. Later on, COP8 (thus, the COP9 signalosome) found its role in multicellular organisms and maintained it to the present day. In present-day single-cell eukaryotes, such as *S. cerevisiae*, the counterpart for COP8 or the COP9 signalosome might be lost, possibly because of its nonessential role.

METHODS

Plant Material and Growth Conditions

Plant germination and growth conditions of *Arabidopsis thaliana* were as previously described (Wei and Deng, 1992). All seedlings were vernalized for a week at 4°C before transfer to growth cham-

bers. Unless stated otherwise, the growth chamber was used under a cycling long-day photoperiod (16 hr of light at 48 mmol m^{-2} sec^{-1} and 8 hr of dark) at 22°C. Wild-type plants were in the Columbia, Wassilewskija, or Dijon-G backgrounds, and *fus4-414* was in the Dijon-G background (Miséra et al., 1994). *cop8-1* was in the Wassilewskija background (Wei et al., 1994b). For the allelism test, crosses between *fus4-414* and *cop8-1* were performed by using heterozygous *fusca fus4-414* and *constitutive photomorphogenic cop8-1* mutants. The plants heterozygous for those mutations were identified by the purple seeds or seedling phenotypes. The F_1 generation from the cross was used for analysis.

Large-Scale Immunoaffinity Purification of the COP9 Signalosome

Cauliflower heads purchased in a local market were used as the starting material for purification. All procedures were performed at 4°C. Seven hundred grams of fresh cauliflower tissue was homogenized in a blender with the same volume of extraction buffer (containing 50 mM Bis-Tris propane, pH 6.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 200 mM NaCl, 0.01% Nonidet P-40, and 5 mM β-mercaptoethanol). The extract was clarified by passage through four layers of cheesecloth and centrifugation at 13,000*g* for 30 min. A 50% polyethylene glycol (PEG) stock solution in buffer A (0.5 M Bis-Tris propane, pH 6.4, and 10% glycerol) was added to the supernatant to a final PEG concentration of 8%. After mixing for 20 min, the sample was centrifuged at 10,000*g* for 15 min. The supernatant was applied to a 250-mL Q Sepharose Fast Flow (Pharmacia Biotechnology, Uppsala, Sweden) ion-exchange column equilibrated with 0.2 M NaCl in buffer A. The fractions containing the COP9 complex (assayed by immunoblotting with anti-COP9– and anti-COP11–specific antibodies) were eluted at 0.2 to 0.44 M NaCl. The salt concentration of the sample was adjusted to 0.3 M NaCl, and the pH was adjusted to 7.0. The sample was loaded onto an affinity column made by coupling 6 mg of affinity-purified anti-COP11 antibodies (Staub et al., 1996) to a 8-mL column with protein A–Sepharose beads (Sigma) equilibrated with buffer E (20 mM Bis-Tris propane, pH 7.0, and 10% glycerol). The column was washed with 0.5 M NaCl and 0.1% Tween 20 in buffer E, and the complex was eluted with 0.1 M glycine, pH 2.7. The complex-containing fractions were concentrated with Microsep 10 (Filtron, Northborough, MA), resuspended in 2 × sample buffer, loaded on a 12% SDS–polyacrylamide gel, and visualized by Coomassie Brilliant Blue R 250 (Sigma) staining. The bands corresponding to the different COP9 signalosome subunits were excised from the gel and subjected to protein digestion and peptide sequencing, as previously described (Wei et al., 1998).

Biochemical Purification of the Plant COP9 Signalosome

For biochemical purification, 70 g of cauliflower tissue was subjected to protein extraction, PEG precipitation, and ion-exchange chromatography, as described for affinity purification above. The salt concentration of the complex-containing fraction from the ion-exchange column was adjusted to 0.1 M NaCl, and the sample was loaded onto a 5-mL heparin affinity column (Pharmacia Biotechnology) equilibrated with 0.1 M NaCl in buffer B (Bis-Tris propane, pH 7.0, and 10% glycerol). The complex was eluted at 0.6 mM NaCl with buffer B. The sample was then diluted three times with buffer C (Bis-Tris propane, pH 8.0, and 10% glycerol) and applied to a 1-mL Mono-Q column (Pharmacia Biotechnology) equilibrated with 0.2 M NaCl. The complex was eluted at 0.2 to 0.44 M NaCl gradient in buffer C. The complex-containing fraction was concentrated to 200 μL with Microsep 10 (Filtron) and applied onto a Superose 6 HR 10 gel-filtration column (Pharmacia Biotechnology). The complex was eluted at a flow rate of 0.25 mL/min in buffer D (20 mM Tris, pH 7.0, 150 mM NaCl, and 2 mM $MgCl_2$). Fractions (0.5 mL) were collected and concentrated with Strataclean resin (Stratagene, La Jolla, CA), resuspended in 2 × sample buffer, loaded on SDS–polyacrylamide gels, and visualized by silver staining (Silver Stain Plus; Bio-Rad).

RNA and DNA Extraction

RNA extraction was performed by using Trizol reagent (Life Technologies, Rockville, MD). DNA extraction was performed according to Edwards et al. (1991).

Isolation of the *AtS4* cDNA and Sequence Analysis of AtS4 in the *cop8* and *fus4* Mutants

The three peptide sequences (A47-88, B47a-43, and A47-82; see Table 1) were used to search the Arabidopsis database: a partial expressed sequence tag (EST) clone (35B4T7; GenBank accession number T04273; obtained from the Arabidopsis Biological Resource Center, Columbus, OH) was retrieved. The EST sequence and the three peptides were also found to correspond to a completely sequenced P1 clone (MBD2) on chromosome 5 (GenBank accession number AB008264) as a part of the Arabidopsis Genome Sequencing Project. The complete full-length clone was obtained by using reverse transcription–polymerase chain reaction (RT-PCR) (Advantage RT-for-PCR kit; Clontech, Palo Alto, CA) of RNA extracted from 1-week-old Columbia seedlings by using primers specific for the 5′ and 3′ ends of the gene. The PCR conditions were as follows: 35 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C. Ultma Taq (Perkin-Elmer, Foster City, CA) was used. The PCR product was cloned in the vector pCR-Script SK+ (Stratagene), and the sequence was obtained by direct sequencing. The first methionine residue was determined by the following observation—a stop codon and a clear TATA box were found 42 and 60 bp, respectively, upstream of the ATG codon—and by comparison with COP subunit 4 (S4) sequence from its animal counterpart.

To determine the mutation of the *AtS4* gene in the *cop8* and *fus4* mutants, 1 μg of total RNA from 1-week-old seedlings homozygous for either *fus4-414* or *cop8-1* and their respective wild-type ecotypes was used for RT-PCR (see above). Mutations were further confirmed by PCR and sequencing of the corresponding genomic region. The PCR conditions were the same as mentioned above. For RT-PCR, the PCR products were run on agarose gel, isolated by using a QIAEXII gel extraction kit (Qiagen Inc., Chatsworth, CA), and then directly sequenced. For the genomic DNA, at least four independent PCR products for each of the mutant alleles and their corresponding ecotypes were cloned into the vector pCR-Script SK+ and sequenced to confirm the mutation. The sequence data were analyzed by using the LaserGene software (DNAstar Inc., Madison, WI).

cop8 and *fus4* Mapping

F_2 mapping populations were generated by crossing heterozygous *cop8-1* (Wassilewskija ecotype) and *fus4-414* (Dijon-G) to both

Columbia and Landsberg *erecta* wild-type ecotypes. PCR-based mapping using simple sequence length polymorphisms (SSLP) and cleaved amplified polymorphic sequences (CAPS) was performed under previously published conditions (Bell and Ecker [1994] for SSLP; Konieczny and Ausubel [1993] for CAPS). The only modifications were as follows. The protocol for DNA preparation was identical to that of Edwards et al. (1991). DNA was isolated from individual single F_2 mutant seedlings and used for PCR-based mapping; an average of 27 seedlings (54 chromosomes) were examined to determine linkage to the SSLP or CAPS marker. Linkage was estimated by the numbers of recombinant chromosomes.

Complementation of the *cop8* Mutation by the *AtS4* cDNA

The 35S-AtS4 overexpression construct obtained by cloning a full-length AtS4 cDNA in the binary vector pPZPY122 (Yamamoto et al., 1998) was transformed into *cop8-1* heterozygous plants via vacuum infiltration, and ~40 independent lines were generated. The T_1 gentamycin-resistant plants were selfed, and the T_2 generation of two representative lines was used for the analysis in this study.

Antibody Production, Protein Extraction, Gel Filtration, and Immunoblot Analysis

The full-length cDNA clone for COP8 (AtS4) was cloned in the vector pRSETC (Invitrogen, Carlsbad, CA). The resulting histidine-tagged fusion protein was overexpressed in *Escherichia coli*, purified from inclusion bodies, and used to produce rabbit polyclonal antibodies, as reported previously (Kwok et al., 1998). Other antibodies used were anti-COP11 (Staub et al., 1996) and anti-AJH1 (Kwok et al., 1998).

Total soluble protein extracts from *fus4-414*-/-, *cop8-1*-/-, and wild-type Columbia seedlings were obtained and used for all immunoblot analyses, as previously described (Staub et al., 1996). For gel filtration, the total soluble protein extract was fractionated through a Superose 6 HR 10/30 gel-filtration column (Pharmacia Biotechnology) and analyzed by immunoblotting, as previously described (Kwok et al., 1998).

Yeast Two-Hybrid Assay

The full-length cDNA clone for COP8 was cloned into the pEG202 and pJG4-5 vectors (Gyuris et al., 1993; Ausubel et al., 1995). The two new plasmids were designated pEG-COP8 and pJG-COP8. The other plasmids used in the assay were pEG-AJH1, pJG-AJH1, pEG-COP9, pJG-COP9, pEG-COP11, pJG-COP11 (Kwok et al., 1998), pEG-FUS5, and pJG-FUS5 (Karniol et al., 1999). All of the LexA fusion constructs were transformed with the reporter pSH18-34 (Ausubel et al., 1995) in yeast strain EGY48. The activation domain fusion constructs were transformed in yeast strain L40 (Invitrogen). The transformants were selected and mated (Bendixen et al., 1994). Six to 12 of the mated colonies were inoculated with 2 mL of liquid media lacking histidine, uracil, and tryptophan and supplemented with 2% galactose and 1% raffinose (Kwok et al., 1998), after which they were assayed for β-galactosidase activity, as previously described (Kwok et al., 1998).

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