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Evidence for FUS6 as a Component of the Nuclear-Localized COP9 Complex in Arabidopsis

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The pleiotropic *CONSTITUTIVE PHOTOMORPHOGENIC* (*COP*), *DEETIOLATED* (*DET*), and *FUSCA* (*FUS*) loci are essential regulatory genes involved in the light control of seedling developmental patterns in Arabidopsis. Although *COP1*, *DET1*, *COP9*, and *FUS6* (also called *COP11*) have been cloned, their biochemical activities and interactions remain elusive. We have recently suggested that multiple pleiotropic *COP*, *DET*, and *FUS* genes may encode subunits of a large regulatory complex. In this study, we generated specific antibodies against Arabidopsis FUS6 and show that accumulation of both COP9 and FUS6 is coordinated in the pleiotropic *cop*, *det*, and *fus* mutant backgrounds and in wild-type plants throughout development. Both COP9 and FUS6 cofractionated into identical high molecular mass fractions in an analytical gel filtration assay, and neither was found in its monomeric form. Moreover, antibodies raised against either COP9 or FUS6 selectively coimmunoprecipitated both proteins. We have also developed an Arabidopsis protoplast immunolocalization assay and demonstrated that the COP9 complex is localized in the nucleus and that its nuclear localization is not affected by light conditions or tissue types. The integrated genetic and biochemical results strongly support the conclusion that both COP9 and FUS6 are components of the nuclear-localized COP9 complex. Therefore, we have provided the strongest evidence for the conclusion that at least some of the pleiotropic *COP*, *DET*, and *FUS* loci act in the same signaling pathway.

INTRODUCTION

Light is one of the most important environmental stimuli regulating plant growth and development. Perhaps the most dramatic example of this is the light control of seedling development in dicotyledonous plants such as Arabidopsis. Light signals turn on the photomorphogenic pathway of development; the seedlings are characterized by short hypocotyls and open and enlarged cotyledons. In contrast, dark-grown seedlings develop according to the skotomorphogenic pathway. These seedlings develop elongated hypocotyls with apical hooks and small cotyledons before developmental arrest (Kendrick and Kronenberg, 1994). Mutants with altered light responses in their seedling developmental program have been identified and used to dissect the light signaling network (Chory, 1993; Deng, 1994; Quail, 1994; McNellis and Deng, 1995). Arabidopsis mutants with reduced sensitivity to light exhibit a characteristic long hypocotyl phenotype in the light. These include mutants lacking phytochromes and a blue light receptor (Ahmad and Cashmore, 1993; Quail et al., 1995) as well as the long hypocotyl mutants *hy5* (Koornneef et al., 1980) and *fhy1* and *fhy3* (Whitelam et al., 1993) that define signaling components downstream of the photoreceptors.

Arabidopsis mutants exhibiting light-grown seedling characteristics when grown in the dark have been identified in multiple *deetiolated* (*det*) and *constitutive photomorphogenic* (*cop*) loci (Chory, 1993; Deng, 1994; McNellis and Deng, 1995; Kwok et al., 1996). Of these, *det1*, *cop1*, and *cop8* to *cop15* display the most pleiotropic phenotypes. When grown in the dark, they all have short hypocotyls, expanded cotyledons, high levels of light-dependent gene expression, and high levels of chlorophyll production in roots. They are also all epistatic to mutations in both phytochromes A and B and the HY4 blue light receptor (Chory, 1993; Ang and Deng, 1994; Deng, 1994; Wei et al., 1994a; Kwok et al., 1996). The recessive nature of these mutations and their pleiotropic phenotypes indicate that the normal function of these loci is to suppress photomorphogenic development in the absence of light signals. Interestingly, all of these mutants have been independently identified as *fusca* (*fus*) mutants for their dark purple cotyledons, resulting from accumulation of high levels of anthocyanins during embryogenesis and early seedling development (Castle and Meinke, 1994; Miséra et al., 1994; Kwok et al., 1996). Severe mutants from all 10 pleiotropic *cop*, *det*, and *fus* loci are lethal after the seedling stage, indicating that these loci are essential for plant development under normal light conditions (Kwok et al., 1996).

Four of the *DET*, *COP*, and *FUS* genes have been cloned. Although *COP9* (Wei et al., 1994b), *FUS6* (Castle and Meinke,

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1994; also known as *COP11*, Wei et al., 1994a), and *DET1* (Pepper et al., 1994) encode completely novel proteins, COP1 has a novel combination of a Zn finger, a coiled-coil region, and WD-40 repeats characteristic of the β subunit of trimeric G proteins (Deng et al., 1992; McNellis et al., 1994a). Overexpression of *COP1* in transgenic Arabidopsis seedlings led to partial suppression of photomorphogenesis, as characterized by significant hypocotyl elongation under continuous far-red and blue light conditions as well as by photoperiodic white light (McNellis et al., 1994b). This result provides direct evidence supporting the hypothesis that COP1 represses photomorphogenesis in darkness, whereas light signals abrogate such repression and turn on the photomorphogenic pathway. Studies using β-glucuronidase reporter fusion proteins have suggested that both COP1 and DET1 act in the nucleus in suppressing photomorphogenic development (Pepper et al., 1994; von Arnim and Deng, 1994). Moreover, light modulation of COP1 activity involves a light-dependent nucleocytoplasmic partitioning of COP1 in hypocotyl cells of seedlings (von Arnim and Deng, 1994).

Our previous studies have established that COP9, a 23-kD protein, exists exclusively as a large (≥560 kD) protein complex (Wei et al., 1994b). In addition, we reported that the steady state level of COP9 was significantly reduced in *cop8* and *fus6* mutants but not in *cop1*, *det1*, *cop10*, or *hy5* mutants. Based on this observation, we hypothesized that COP8 and FUS6 are important for either the formation or the stability of the COP9 complex (Wei et al., 1994b). Recently, it has been shown that FUS6 (COP11) and COP9 can be copurified from cauliflower head extracts (Chamovitz et al., 1996), strongly implying that COP9 and FUS6 are components of the same multisubunit protein complex.

We report here extensive immunological evidence that FUS6 is physically associated with COP9 in vivo and thus is a structural component of the COP9 complex. Furthermore, we demonstrate that the COP9 complex is localized in the nucleus, and its nuclear localization is not affected by light condition or tissue type. These results show definitively that at least some of the pleiotropic genes at the *COP*, *DET*, and *FUS* loci act together in the nucleus in the same signal transduction pathway to repress photomorphogenic plant development.

RESULTS

Arabidopsis FUS6 Has an Apparent Molecular Mass of 51 kD and Its Accumulation Is Not Light Regulated

To test whether FUS6 is part of the COP9 complex, antibodies against FUS6 were generated. The *FUS6* gene (Castle and Meinke, 1994) was amplified by polymerase chain reaction from wild-type plant cDNA, and a full-length recombinant FUS6 protein was expressed in *Escherichia coli*. The purified protein was used to immunize rabbits for the production of polyclonal antibodies. The FUS6-specific antibodies were subsequently affinity purified (see Methods).

Immunoblot analysis using affinity-purified FUS6 antibodies to probe protein extracts from wild-type plants and several *fus6* mutants is shown in Figure 1A. A specific band at the expected size for FUS6 (~51 kD; Castle and Meinke, 1994) accumulates in wild-type seedlings but is absent from two different T-DNA insertion mutants (*fus6-2* and *fus6-1*) predicted to disrupt the *FUS6* locus (Castle et al., 1993; Castle and Meinke, 1994) and an ethyl methanesulfonate–induced allele (*fus6-T379*; Miséra et al., 1994). The absence of the 51-kD band in known *fus6* mutants confirmed its identity as FUS6. The large subunit of ribulose-1,5-bisphosphate carboxylase, which can be seen as a faint shadow above FUS6 in Figure 1A, accumu-

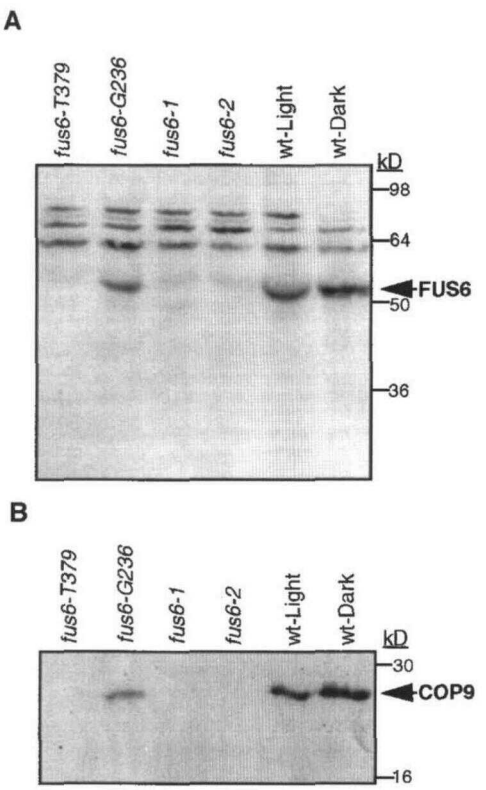

Figure 1. FUS6 (COP11) and COP9 Accumulation in Wild-Type Plants and Four *fus6* Mutants.

(A) Immunoblot analysis of FUS6.
(B) Immunoblot analysis of COP9.
Six-day-old light- and dark-grown wild-type (wt) seedlings and 9-day-old light-grown *fus6* seedlings were used for the analysis. Each lane contains equal amounts of total protein extracts (10 μg per lane). The mobility of the FUS6-specific band (arrowhead) is affected by the accumulation of the large subunit of ribulose-1,5-bisphosphate carboxylase, which can be seen as a faint background band, particularly in light-grown plants. The anti-FUS6 antibodies used also cross-reacted with three (light-grown samples) or two (dark-grown sample) bands of high molecular mass in all protein extracts. Note that COP9 (arrowhead) accumulates only in protein samples containing FUS6. The numbers at right indicate molecular mass markers in kilodaltons.

lates to very high levels in light-grown tissues and alters the mobility of FUS6 slightly. Our anti-FUS6 antibodies also reacted to three additional bands of higher molecular masses (~63 to ~80 kD) in all extracts from light-grown wild-type plants and *fus6* mutants, whereas the sample from dark-grown wild-type plants contained only the two smaller bands (Figure 1A). Because these three immunoreacting bands are present in equivalent amounts in all *fus6* mutants tested, we conclude that they are cross-reacting signals and are not derived from the *FUS6* gene.

The protein gel blot analysis (Figure 1A) clearly shows that FUS6 is equally abundant in both light- and dark-grown tissues, despite reportedly lower transcript levels in the dark (Castle and Meinke, 1994). This lack of light regulation of its steady state protein level is very similar to that of Arabidopsis COP9 (Figure 1B; Wei et al., 1994b).

The FUS6 Protein Is Required for Normal Accumulation of COP9

Among the four *fus6* mutant alleles examined (Figure 1A), the *fus6-G236* mutant accumulates the apparently full-length 51-kD FUS6 protein, whereas none was detected in the other three alleles. Because *fus6-G236* is an ethyl methanesulfonate–induced lethal allele (Miséra et al., 1994), it presumably carries a missense mutation that abolishes protein function.

To examine the COP9 accumulation in the same *fus6* mutants, we probed an identical immunoblot by using affinity-purified COP9 antibodies (Figure 1B). Like FUS6, the abundance of COP9 (23 kD) in wild-type seedlings was not affected by light conditions, as was previously observed (Wei et al., 1994b). However, COP9 accumulated only in protein extracts in which FUS6 was present and was absent from mutants that do not accumulate FUS6. Most strikingly, COP9 accumulated to wild-type levels in the *fus6-G236* mutant, which accumulates full-length (nonfunctional) FUS6. This result indicates a structural requirement of FUS6 for stability of the COP9 complex that is separable from its function in light signaling, and this is consistent with either a direct interaction of COP9 and FUS6 or their presence in the same protein complex.

Coordinate Accumulation of COP9 and FUS6 in Different *cop* and *det* Mutants

If COP9 and FUS6 are members of the same multiprotein complex, their accumulation will most likely be affected equally by mutations in the other pleiotropic *cop* and *det* loci. Therefore, we examined protein extracts from both dark- and light-grown wild-type and several available mutant strains. Figures 2A and 2B show the immunoblot analysis for FUS6 and COP9, respectively. Compared with wild-type extracts, FUS6 levels were unchanged in the *cop1-4*, *det1-1*, and *cop10-1* mutants, regardless of light treatment. In contrast, FUS6 was absent from the *cop8-S253* and *cop9-1* mutants under both light conditions. As is evident from the immunoblot (Figure 2B),

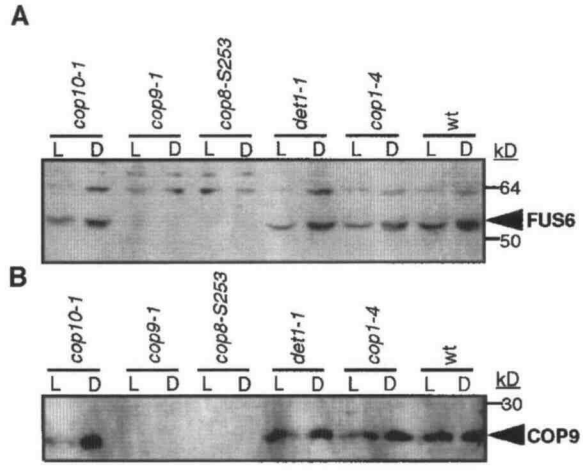

Figure 2. COP9 and FUS6 Accumulation Is Coordinated in the Different *cop* and *det* Mutants.

(A) Immunoblot analysis of FUS6.
(B) Immunoblot analysis of COP9.
Six-day-old wild-type (wt) seedlings and 9-day-old *cop* and *det* seedlings were used for the analysis. Each lane contains equal amounts of total protein extracts (10 μg per lane). Note that some of the light-grown samples are slightly underloaded, as determined by the intensity of the ~63-kD cross-reacting band. The positions of COP9 and FUS6 are indicated (arrowheads). The numbers at right indicate molecular mass markers in kilodaltons. L, light grown; D, dark grown.

the COP9 accumulation is similar to that of FUS6 in all cases, being absent in both the *cop8* and *cop9* mutants. These results indicate that mutations at all three loci, COP8, COP9, and FUS6, affect the stability of the COP9 complex equally. This also confirmed our previous suggestion that COP8 is required for formation or stability of the COP9 complex (Wei et al., 1994b).

FUS6 Has the Same Organ Expression Pattern as COP9

Both *cop9* and *fus6* mutants show the same range of phenotypes, including constitutive photomorphogenesis, suppression of root plastid development, and lethality after the seedling stage, suggesting that their gene products are required throughout the development of the plant. Indeed, COP9 was found to be ubiquitous, although at different levels, in all organs of Arabidopsis plants (Wei et al., 1994b). To test whether FUS6 is expressed in a similar fashion, the accumulation of COP9 and FUS6 in different organs of both seedlings and adult plants was examined. As shown in Figure 3A, immunoblot analysis of protein extracts from a variety of wild-type plant organs indicates that FUS6 accumulated in all organs tested, confirming its general role throughout plant development. A low level of FUS6 was observed in adult leaves, stems, and siliques; higher levels were found in young flowers and whole seedlings, with the highest amounts found in rapidly dividing young

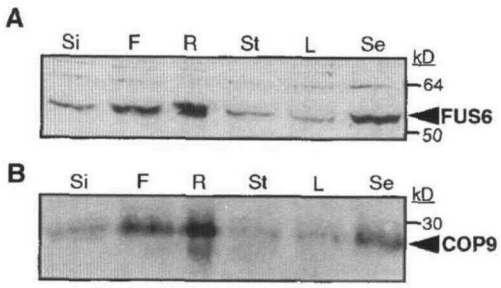

Figure 3. COP9 and FUS6 Have the Same Organ Expression Pattern.

(A) Immunoblot analysis of FUS6.
(B) Immunoblot analysis of COP9.
Shown is an immunoblot analysis of total protein extracts (10 μg per lane) from 6-day-old wild-type seedlings (Se), adult plant leaves (L), stems (St), roots (R), flowers (F), and green siliques (Si) from 3-week-old agar medium–grown young seedlings. The positions of COP9 and FUS6 are indicated (arrowheads). The numbers at right indicate molecular mass markers in kilodaltons.

root tissues. This expression pattern of FUS6 is exactly identical to that of COP9 (Figure 3B). These results suggest that both FUS6 and COP9 are present at a higher level per total protein basis in actively dividing meristematic tissues. Most importantly, both COP9 and FUS6 accumulated to the same relative amounts in all tissues examined, which is consistent with their being in the same protein complex.

COP9 and FUS6 Cofractionate in Identical High Molecular Mass Protein Fractions

It has been shown previously that COP9 exists only in a high molecular mass protein complex, with no monomer form of the protein being detected. Therefore, the functional form of COP9 resides only within the COP9 complex (Wei et al., 1994b). The previous genetic evidence and coordinated accumulation of COP9 and FUS6 shown above are consistent with the prediction that they may be members of the same protein complex. To substantiate this prediction, we used gel filtration chromatography to fractionate native protein complexes from extracts of wild-type light-grown seedlings. The elution fractions from the Superdex 200 HR column with the relative position of molecular mass standards are shown in Figure 4. As was previously observed, COP9 elutes from the column exclusively in high molecular mass fractions (Figure 4B, fractions 4 to 7) between the 669- and the 443-kD size standards. The size estimate for the complex of ~560 kD (Wei et al., 1994b) was confirmed by Coomassie Brilliant Blue R 250 staining of an identical protein gel, which showed that ribulose-1,5-bisphosphate carboxylase (~550 kD) peaks also in fractions 5 and 6 (data not shown). Consistent with our previous report (Wei et al., 1994b), no monomer 23-kD COP9 was observed (Figure 4D). As predicted, FUS6 also eluted from the gel filtration column in the identical high molecular mass fractions as COP9 (Figure 4A, fractions 4 to 7). Importantly, no monomeric (51-kD) FUS6 was observed (Figure 4C). In addition, FUS6 and COP9 cofractionated solely in the identical high molecular mass fractions when total soluble protein extracts of dark-grown seedlings were used for the analysis (data not shown). These results indicate that like COP9, FUS6 also exists only within the COP9 complex in both light and dark conditions.

COP9 and FUS6 Coimmunoprecipitate from Total Soluble Protein Extracts

If COP9 and FUS6 are physically associated within the same multiprotein complex, it should be feasible to coimmunoprecipi-

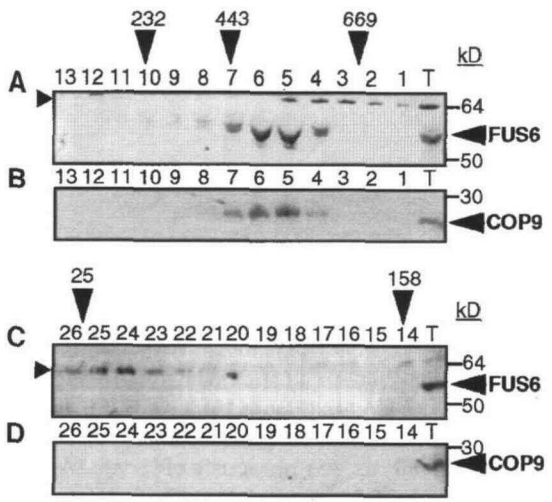

Figure 4. COP9 and FUS6 Cofractionate in Identical High Molecular Mass Fractions.

(A) FUS6 immunoblot analysis of high molecular mass gel filtration fractions (larger than ~160 kD).
(B) COP9 immunoblot analysis of high molecular mass gel filtration fractions (larger than ~160 kD).
(C) FUS6 immunoblot analysis of lower molecular mass gel filtration fractions (smaller than ~160 kD).
(D) COP9 immunoblot analysis of lower molecular mass gel filtration fractions (smaller than ~160 kD).
Protein extracts from the upper parts (hypocotyls and cotyledons) of light-grown wild-type seedlings were analyzed by gel filtration chromatography, and the elution fractions (0.5 mL each, labeled 1 to 26) were separated by SDS-PAGE and blotted onto a membrane. Because of the limitations of the protein gel, the 26 fractions were analyzed in two separate gels. The identical blots were probed with antibodies raised against FUS6 (**[A]** and **[C]**) or COP9 (**[B]** and **[D]**). The positions of COP9 and FUS6 are marked by horizontal arrowheads at right. Lanes T contain total extract before fractionation. The elution peaks of size standards (vertical arrowheads) are shown. Note the absence of monomeric COP9 (23 kD) or FUS6 (51 kD) in the immunoblots. The black triangles at left indicate a cross-reactive protein of ~63 kD observed from FUS6 antibodies. The numbers at right indicate molecular mass markers in kilodaltons.

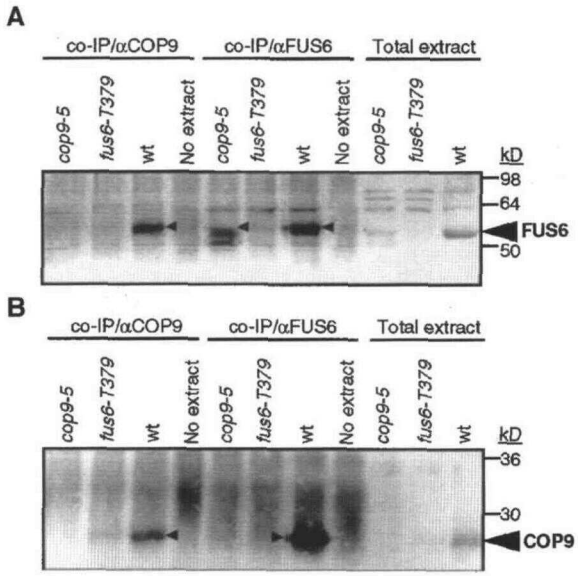

Figure 5. Coimmunoprecipitation of COP9 and FUS6.

(A) FUS6 immunoblot analysis of anti-FUS6 and anti-COP9 immunoprecipitates and total protein extracts.

(B) COP9 immunoblot analysis of anti-FUS6 and anti-COP9 immunoprecipitates and total protein extracts.

Either anti-COP9 or anti-FUS6 antibodies conjugated to protein A–agarose beads (co-IP/αCOP9 or co-IP/αFUS6) were used for immunoprecipitations from total soluble protein extracts (100 μg each) of light-grown wild-type (wt), *fus6-T379*, and *cop9-5* mutants. Antibody-conjugated protein A beads were incubated without protein extract (No extract) to show the position of any cross-reacting immunoglobulins. The immunoprecipitates were run on a 12% SDS–polyacrylamide gel, which was then cut in half; the upper part was probed with anti-FUS6 antibodies and the lower part with anti-COP9 antibodies. Total extracts before immunoprecipitation (10 μg each) were included to show the position of COP9 and FUS6. Note the altered mobility of FUS6 due to the presence of the large subunit of ribulose-1,5-bisphosphate carboxylase in the total wild-type extract before immunoprecipitation, whereas in the immunoprecipitated samples, the ribulose-1,5-bisphosphate carboxylase is completely depleted. Small arrowheads indicate full-length 51-kD FUS6 and COP9 in the immunoprecipitates. The positions of COP9 and FUS6 are indicated at right (large arrowheads). The numbers at right indicate molecular mass markers in kilodaltons.

tate them both using either of the specific antibodies. To test this association, we first covalently coupled the affinity-purified antibodies to protein A–agarose beads, mixed them with protein extracts from light-grown wild-type seedlings or mutants, and washed away the unbound cellular proteins. The affinity-bound proteins were then eluted and examined by immunoblot analysis, as shown in Figures 5A and 5B, together with the total protein extracts as controls. The immunoblot clearly shows that both COP9 and FUS6 were precipitated from the wild-type extract by using either anti-COP9– or anti-FUS6–conjugated protein A–agarose beads. As expected (see Figure 1), when the extract from the severe *fus6-T379* mutant was used, no FUS6 or COP9 protein was precipitated by either of the antibodies. Likewise, when anti-COP9–conjugated protein A–agarose beads were used for immunoprecipitation from the extract of the lethal *cop9-5* mutant, which has a splicing defect in the *COP9* gene (N. Wei and X.-W. Deng, unpublished data), no COP9 protein was observed. However, precipitation from the *cop9-5* sample by using the anti-FUS6–conjugated protein A–agarose beads identified at least three FUS6-immunoreactive bands. These protein species were of smaller molecular mass than the 51-kD FUS6 protein and presumably represent intermediates in FUS6 degradation, suggesting that a mutation preventing accumulation of any one member may destabilize the complex and target the other members for degradation. These results confirm the physical association of COP9 and FUS6 and definitively demonstrate that both are subunits of the same protein complex.

Nuclear Localization of the COP9 Complex Is Not Affected by Tissue Type and Light Condition

To understand the cellular function of the COP9 complex, its subcellular localization and its possible response to light and development signals were examined. Because in wild-type seedlings all detectable COP9 and FUS6 are present within the COP9 complex (Wei et al, 1994b), we expected that the subcellular localization pattern of either COP9 or FUS6 would represent that of the COP9 complex. To localize the COP9 protein within the cell, we developed an indirect immunofluorescence staining procedure using Arabidopsis protoplasts that were isolated from different organs of seedlings grown in different light conditions. This procedure was refined based on the localization patterns of known proteins in wild-type plants (see Methods for details). These include tubulin, a cytoskeletal protein, and the Sm antigens of the RNA splicing machinery, a well-defined nuclear marker.

The microtubule staining patterns in protoplasts from light- and dark-grown seedlings appeared as a cytoplasmic network of continuous fine fibers, indicating that the cytoskeletal structure of the protoplasts was well preserved in our procedure (data not shown). The Y12 antibody raised against the Sm antigen consistently gave an exclusively nuclear staining pattern, as evidenced by 4′,6-diamidino-2-phenylindole (DAPI) staining of the nucleus of the same protoplast (data not shown). We have also used antibodies raised against human RNA polymerase II in Arabidopsis protoplasts, and the expected nuclear staining was obtained (data not shown). Because this procedure resulted in correct localization patterns with both cytoplasmic and nuclear antigens, we then proceeded to localize COP9 and FUS6 with our affinity-purified antibodies.

Protoplasts were isolated from cotyledon, hypocotyl, and root tissues of light- and dark-grown wild-type seedlings and

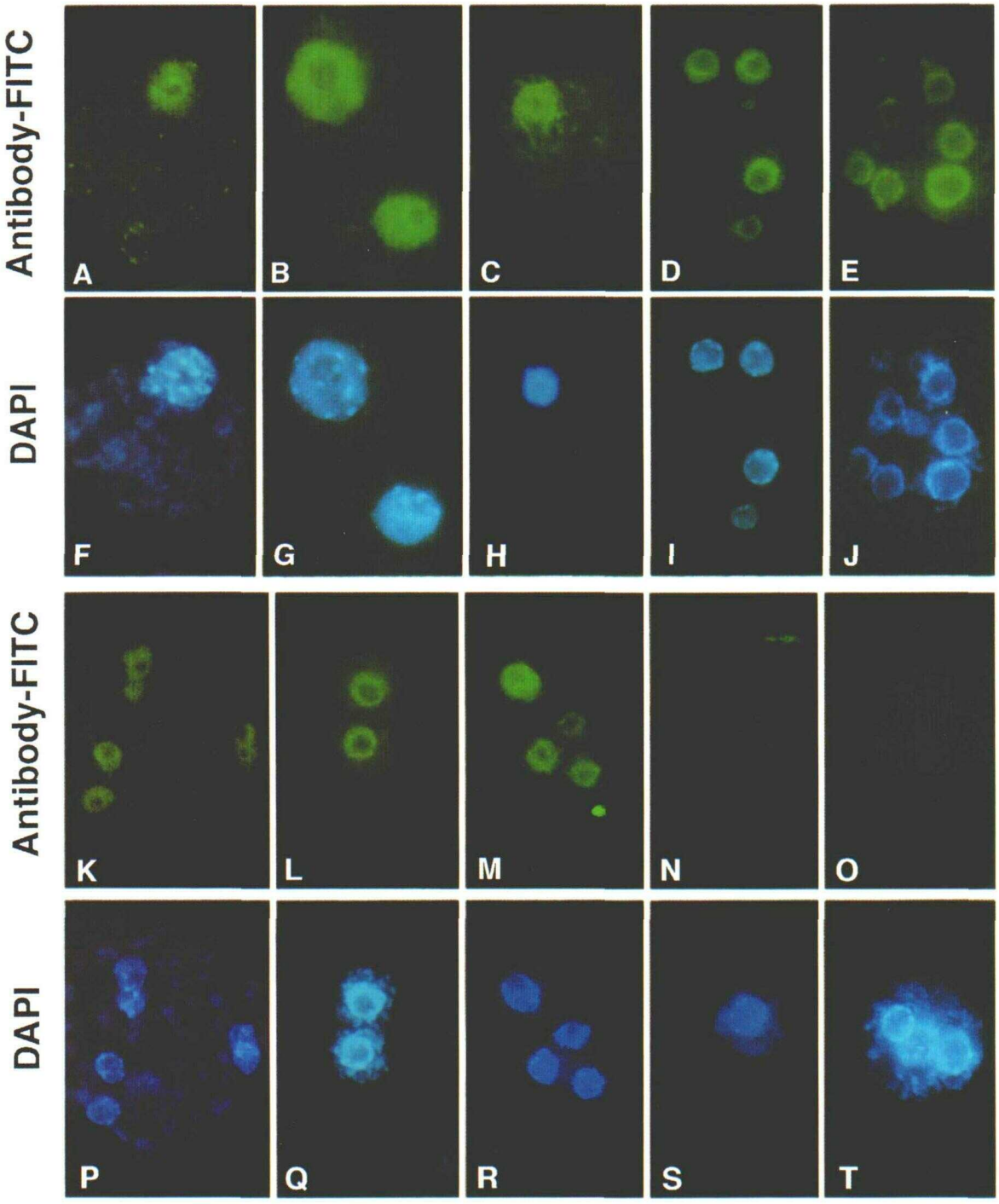

Figure 6. COP9 and FUS6 Subcellular Localization in Isolated Arabidopsis Protoplasts.

(A) and **(F)** Protoplasts from light-grown seedling cotyledons stained with anti-COP9 antibodies.
(B) and **(G)** Protoplasts from light-grown seedling hypocotyls stained with anti-COP9 antibodies.
(C) and **(H)** Protoplasts from dark-grown seedling hypocotyls stained with anti-COP9 antibodies.
(D) and **(I)** Protoplasts from light-grown seedling roots stained with anti-COP9 antibodies.
(E) and **(J)** Protoplasts from dark-grown seedling roots stained with anti-COP9 antibodies.
(K) and **(P)** Protoplasts from light-grown seedling cotyledons stained with anti-FUS6 antibodies.

immunodecorated with anti-COP9 antibodies. As is evident from the representative samples shown in Figures 6A to 6J, COP9 was found in the nuclei of cells derived from every organ tested, regardless of the light or dark growth conditions. Similar to the Sm antigen (data not shown), the COP9 staining signal seemed to localize to the nucleoplasm and was excluded from the central nucleolar area, which was lightly stained with DAPI. The nucleolus appeared especially large in young root cells; hence, a ringlike localization pattern was observed for COP9 (Figures 6D and 6E), as was the case with some other nuclear proteins, including the Sm antigen (data not shown). These results indicate that the COP9, and therefore the COP9 complex, is nuclear localized in all organs of Arabidopsis seedlings, and that the COP9 complex localization does not seem to be affected by light signals.

To further confirm the localization pattern of the COP9 complex, we also examined the subcellular localization of FUS6 in several tissue types. However, the intensity of the cross-reactive bands observed by using our newly generated antibody precluded its use for immunofluorescence studies. Therefore, we developed a more stringent affinity purification procedure (see Methods) that dramatically decreased the signal from the cross-reactive bands. As shown in Figure 7, with the more stringently purified antibodies, FUS6 is the only prominent band observed in wild-type seedling extracts, and the ~63-kD cross-reacting band is only barely visible in extracts from both wild-type and *fus6-2* (a null allele) seedlings.

We used this highly specific FUS6 antibody preparation for immunofluorescent localization in protoplasts derived from light- and dark-grown cotyledons and hypocotyls and light-grown root tissues. Similar to that of COP9, FUS6 localized to the nucleus regardless of light conditions in all cell types examined, and representatives are shown in Figures 6K to 6R. Similar to that of COP9, the most intense FUS6 signal was observed in the nucleoplasm, and it seemed to be somewhat excluded from the central nucleolar region. A ringlike localization pattern for FUS6 was commonly observed in root cells and in dark-grown tissues. Neither COP9 nor FUS6 antibodies showed any specific staining pattern when *fus6-2* seedlings were used for the immunofluorescence assay (Figures 6N, 6O, 6S, and 6T). These results conclusively suggest that both COP9 and FUS6, and therefore the entire COP9 complex, are nuclear localized in all tissue types of Arabidopsis under both light or dark conditions.

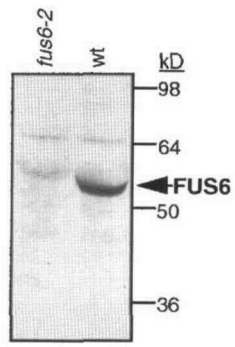

Figure 7. High-Stringency Affinity-Purified Anti-FUS6 Antibodies Detect Only the FUS6 Protein.

Total protein extracts (10 μg per lane) from 6-day-old light-grown wild-type (wt) seedlings and 9-day-old light-grown *fus6-2* seedlings were used for the analysis. Note that FUS6 protein is the major band in the protein immunoblot and that the ~63-kD protein species is only barely detectable. Abundant accumulation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase results in a faint background band in extracts from both the wild type and the mutant above the position of the FUS6 protein. The numbers at right indicate molecular mass markers in kilodaltons.

DISCUSSION

In this article, we have presented substantial evidence to confirm the previous genetic hypothesis that at least some of the 10 pleiotropic *COP*, *DET*, and *FUS* loci act in the same regulatory pathway and most likely in the same regulatory nuclear complex to suppress photomorphogenic development in darkness (Wei et al., 1994b; McNellis and Deng, 1995). Specifically, we have raised polyclonal antibodies against Arabidopsis FUS6 and used it to test several predictions based on the premise that COP9 and FUS6 are components of the same regulatory complex. First, the accumulation of COP9 and FUS6 is coordinated throughout plant development and within all mutants examined. Most strikingly, the *fus6-G236* mutant, which still accumulates the apparently full-length (but nonfunctional) 51-kD FUS6 protein, also accumulates COP9. Second, an analytical size fractionation assay of total protein extracts from Arabidopsis seedlings showed that FUS6 exists only in large

Figure 6. (continued).

(L) and **(Q)** Protoplasts from dark-grown seedling hypocotyls stained with anti-FUS6 antibodies.
(M) and **(R)** Protoplasts from light-grown seedling roots stained with anti-FUS6 antibodies.
(N) and **(S)** Protoplasts from light-grown *fus6-2* mutant seedlings stained with anti-COP9 antibodies.
(O) and **(T)** Protoplasts from light-grown *fus6-2* mutant seedlings stained with anti-FUS6 antibodies.
Protoplasts shown in **(A)** to **(E)** and **(N)** were stained with affinity-purified anti-COP9 antibodies; their corresponding DAPI labelings are shown in **(F)** to **(J)** and **(S)**, respectively, to indicate the positions and patterns of the nuclei. Protoplasts shown in **(K)** to **(M)** and **(O)** were stained with affinity-purified anti-FUS6 antibodies; their corresponding DAPI labelings are shown in **(P)** to **(R)** and **(T)**, respectively. The magnification used for **(A)** to **(J)** is up to twice that of **(K)** to **(T)**. Protoplasts from dark-grown cotyledons, which might be unavoidably contaminated with those from residual hypocotyl tissue, also showed a nuclear staining pattern for COP9 and FUS6 (data not shown).

molecular mass fractions that are identical to those of COP9. Finally, antibodies raised against either COP9 or FUS6 selectively coimmunoprecipitate both proteins from total soluble protein extracts of wild-type seedlings, confirming a physical association of the two proteins.

Together with the previous genetic data (Wei et al., 1994a, 1994b) and recent copurification of COP9 and FUS6 from cauliflower (Chamovitz et al., 1996), our data definitively show that FUS6 is a structural member of the COP9 complex. The stability of the entire complex apparently requires the coordinate accumulation of all of the subunits. Analogous situations have been reported in many other systems, for example, in the accumulation of the photosystem I and II complexes (reviewed in Rochaix and Erickson, 1988). Because the genetic data suggest that COP8 is also required for the stability of the COP9 complex, we predict that this factor is also part of the complex. However, the final confirmation of this prediction awaits the molecular cloning of the *COP8* gene.

The cytological data shown here strongly suggest that the COP9 complex, which includes FUS6 and probably COP8, acts within the nucleus. This conclusion is consistent with recent studies of COP9 in cauliflower, in which protoplast immunostaining was used, and of a β-glucuronidase–COP9 fusion in transgenic Arabidopsis seedlings (Chamovitz et al., 1996). Cellular localization studies with DET1 (Pepper et al., 1994) and COP1 (von Arnim and Deng, 1994) have also shown that they act within the nucleus. Together, these results suggest that all four cloned pleiotropic *COP*, *DET*, and *FUS* genes encode nuclear regulators. This raises an interesting question regarding how COP1, DET1, the COP9 complex, and the other uncloned genes interact. Our genetic evidence suggests that mutations in *COP1*, *DET1*, and *COP10* have no effect on COP9 or FUS6 accumulation, suggesting that their gene products are not involved in the formation or the stability of the COP9 complex. However, it remains to be seen whether these factors interact in a more transient fashion or indirectly and whether light plays a role in those potential interactions.

It is reasonable to assume that suppression of photomorphogenic development involves global regulation of gene expression. The fact that all four cloned pleiotropic *COP*, *DET*, and *FUS* genes define nuclear regulators is consistent with the notion that their gene products may act directly in regulating gene expression, perhaps as transcription factors themselves or by regulating the activity of transcription factors. This notion is also supported by the observed similarity of COP1 to the Drosophila TFIID subunit $dTAF_{II}80$ (Dynlacht et al., 1993) and the observation that light inactivation of COP1 is concomitant with its exclusion from the nucleus (von Arnim and Deng, 1994). Finally, it should be mentioned that both COP9 and FUS6, the only two characterized components from the COP9 complex, are highly related to human genes, although the function of their human counterparts is not known (Chamovitz and Deng, 1995). Nevertheless, this observation suggests that the COP9 complex may be an evolutionarily conserved nuclear regulator in all higher eukaryotes. Therefore, further understanding of the composition and biochemical function of the Arabidopsis COP9 complex not only may provide insights into the mechanism of light signaling in plants, but also may help us to understand the cellular function of their human counterparts.

METHODS

Plant Materials and Growth Conditions

The *cop9-1*, *cop10-1*, *fus6-1*, and *fus6-2* (also known as *cop11-2* and *cop11-1*, respectively) mutants are in the *Arabidopsis thaliana* ecotype Wassilewskija background (Wei et al., 1994a), *cop1-4* and *det1-1* are in the Arabidopsis ecotype Columbia background (McNellis et al., 1994a; Pepper et al., 1994), and the *cop8-S253*, *fus6-G236*, and *fus6-T379* alleles are in the Landsberg *erecta* background (Miséra et al., 1994). Wild-type plants are in the Arabidopsis Columbia or Wassilewskija ecotype. Plant germination and growth conditions in darkness and white light were as described previously (Wei and Deng, 1992; McNellis et al., 1994a). Unless mentioned otherwise, growth conditions for light-grown plants were 16 hr of white light at 75 μmol m^{-2} sec^{-1} and 8 hr of darkness.

Protein Extraction and Gel Filtration Chromatography

Tissues were homogenized in a buffer containing 10 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 10 mM β-mercaptoethanol, and 25 mM Tris-Cl, pH 7.5, with freshly added proteinase inhibitor phenylmethylsulfonyl fluoride at 1 mM. Homogenates were centrifuged for 5 min at 4°C to remove debris. Protein concentrations in the homogenates were determined by using the Bio-Rad protein assay reagent kit. Equal concentrations of protein were used for all immunoblot analyses of total protein extracts.

For gel filtration chromatography, the homogenate was microcentrifuged for 10 min, 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 Superdex 200 HR column (Pharmacia, Uppsala, Sweden), with PBS plus 10 mM $MgCl_2$, at a flow rate of 0.5 mL/min. All fractionations were performed at 4°C. Fractions were collected and concentrated by centrifugal ultrafiltration with Micros 10 concentrators (Filtron, Northborough, MA). An equal volume of individual fractions was used for immunoblot analysis. The protein standards for size estimation of the gel filtration complex(es) were thyroglobulin (669 kD), apoferritin (443 kD), catalase (232 kD), aldolase (158 kD), and chymotrypsinogen (25 kD).

Antibody Production, Immunoprecipitation, and Immunoblot Analysis

The full-length open reading frame of the wild-type *FUS6* (*COP11*) cDNA was amplified by polymerase chain reaction, using specific primers based on the published gene sequence (Castle and Meinke, 1994), and cloned into a pGEX vector (Pharmacia) and the pMAL2 vector (New England Biolabs, Beverly, MA). Plasmid constructs were verified by sequencing. The resulting glutathione *S*-transferase (GST)–FUS6 and maltose binding protein (MBP)–FUS6 fusion proteins were overproduced in *Escherichia coli* and purified by using glutathione–agarose

beads (Sigma) or amylose resin (New England Biolabs), respectively, according to the manufacturers' suggestions. GST–FUS6 protein was used for production of rabbit polyclonal antibodies. Similar to the previous procedure (Wei et al., 1994b), anti-FUS6 antibodies were subsequently affinity purified by using the MBP–FUS6 fusion protein immobilized to an *N*-hydroxysuccinimide Hi-Trap column (Pharmacia). This Hi-Trap column uses an agarose support containing activated *N*-hydroxysuccinimide ester groups, which react with ligands containing amino groups to give stable amide bonds. The affinity-purified FUS6-specific antibodies have no cross-reactivity with purified GST protein itself as determined by immunoblot analysis (data not shown). This antibody preparation was used for the experiments shown in Figures 1 to 5. For highly monospecific anti-FUS6 antibodies used in immunofluorescence studies (Figures 6 and 7), elution of antibodies from the MBP–FUS6 affinity column was preceded by extensive washing in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, and 0.5% deoxycholate) containing 0.5% SDS.

For immunoprecipitations, affinity-purified antibodies were first coupled to protein A–agarose beads (Sigma) by standard procedures (Harlow and Lane, 1988). Briefly, antibodies and protein A beads (~1 mg antibodies/mL wet beads) were mixed for 1 hr at room temperature; the beads were then collected by centrifugation and washed three times in 0.2 M sodium borate, pH 9.0. The beads were resuspended in 500 μL of 0.2 M sodium borate, pH 9.0, with 20 mM dimethylpimelimidate. After 30 min of shaking at room temperature, the reaction was stopped by incubating in 0.2 M ethanolamine, pH 8.0, for 2 hr. The antibody-coupled protein A beads were washed three times and stored in 250 μL of PBS solution. A 15-μL aliquot of these beads was mixed with 100 μg of total soluble protein in 300 μL of PBS containing 0.1% Tween 20 (PBS-T) with gentle shaking at 4°C for 6 hr. The beads were collected by microcentrifugation and washed six times with 1 mL of PBS-T. The affinity-bound proteins were eluted from the beads by boiling for 5 min in 2 × SDS sample buffer before loading onto a 12% SDS–polyacrylamide gel.

Total soluble and gel filtration protein samples were mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and then loaded onto a 10% (for FUS6) or 12% (for COP9) SDS–polyacrylamide gel. The protein gels were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and probed with affinity-purified polyclonal antibodies raised against COP9 (Wei et al., 1994b) or FUS6 at 1:500 dilution. Detection was based on horseradish peroxidase–conjugated goat anti–rabbit antibodies ($F[ab']_2$ fragment) (Pierce, Rockford, IL).

Arabidopsis Protoplast Immunofluorescence Staining

The procedure for protoplast preparation was modified from those published in Jones et al. (1990) and Matsui et al. (1995). First, different organs (cotyledon, hypocotyl, and root) of the seedlings were separated and cut into pieces by using razor blades. The plant tissues were added to protoplast digestion solution containing 1% cellulase R10, 0.25% macerozyme R10 (Yakult Pharmaceutical Inc., Tokyo), 10 mM Mes, pH 5.7, 0.4 M mannitol, 0.1% BSA, 30 mM $CaCl_2$, and 5 mM β-mercaptoethanol. The digestion continued for 2 to 3 hr at room temperature with shaking at 60 rpm. The digestion mixture was filtered through a 149-μm nylon filter. The resulting protoplasts from the filtrate were pelleted by spinning at 65*g* in a clinical centrifuge for 6 min. The supernatant was removed, and the protoplasts were washed by resuspending them in the wash buffer (0.4 M mannitol, 4 mM Mes, and 20 mM KCl, pH 5.7), followed by another spin under the same conditions. The protoplasts were laid onto polylysine precoated eight-well slides (Carlson Scientific Inc., Peotone, IL) and were allowed to bind to the slides for 1 to 2 hr at room temperature.

The protoplasts were fixed with 2% formaldehyde (2% paraformaldehyde was used in some cases) for 10 min and then permeabalized with 0.5% Nonidet P-40 for 5 to 10 min. The cells were fixed again by soaking in 1:1 (v/v) cold methanol–acetone at −20°C for 15 min, with one change of the solution. This step also removes chlorophylls, which otherwise give intense autofluorescence. The slides containing fixed protoplasts were air dried.

All immunoreactions were performed at room temperature. A well with no primary antibody was always included in each eight-well slide to control for the background generated from secondary antibodies or autofluorescence background from improper fixation and protoplast preparation. First, the protoplasts were rehydrated in PBS for at least 30 min. This was followed by blocking in 2% BSA in PHEM (60 mM Pipes, 25 mM Hepes, pH 6.9, 10 mM EGTA, 2 mM Mg_2Cl) for 1 hr. The cells were incubated with an appropriate dilution of primary antibodies for 1 to 2 hr. Unbound antibodies were removed by washing for 5 min each in PBS, PBS-T, and again in PBS twice. The secondary antibodies, which are conjugated with either fluorescein (Sigma) or Texas-Red (Molecular Probes Inc., Eugene, OR), were incubated with the samples for 1 hr and then subjected to the wash series as described above. The samples were mounted in SlowFade antifade reagent (Molecular Probes Inc.) containing 1 mg/mL of 4′,6-diamidino-2-phenylindole and viewed through a Leica light microscope (Leica, Deerfield, IL).

The primary antibodies used are as follows: affinity-purified rabbit polyclonal antibodies raised against COP9 (Wei et al., 1994b) at 1:25; monospecific affinity-purified FUS6 antibody at 1:300; monoclonal anti-α-tubulin antibody from mouse ascites fluid (clone DM 1A; Sigma) diluted at 1:400; and Y12, a mouse monoclonal antibody against human small nuclear ribonucleoprotein Sm, at 1:1000.

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