

# Characterization of Two Subunits of *Arabidopsis* 19S Proteasome Regulatory Complex and its Possible Interaction with the COP9 Complex

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The nuclear localized, multi-subunit COP9 complex (or COP9 signalosome) is a key developmental modulator involved in repression of photomorphogenesis. In an effort to further define the molecular actions of the COP9 complex, a yeast two hybrid interactive screen was undertaken to identify proteins that could directly interact with one subunit of this complex, namely FUS6/COP11. This screen identified one specific interactive protein, AtS9, that is likely the *Arabidopsis* non-ATPase S9 (subunit 9) of the 19S regulatory complex from the 26S proteasome. AtS9 specifically interacts with FUS6/COP11 *via* the C-terminal domain of FUS6/COP11, which is distinct from the N-terminal domain necessary for FUS6/COP11 to interact with itself. As anticipated, AtS9 is not a member of the COP9 complex, but tightly associates with an ATPase subunit of the *Arabidopsis* 19S proteasome regulatory complex, AtS6A. Since all three proteins, FUS6/COP11, AtS9, and AtS6A, are present as complexed forms *in vivo*, the observed interaction implies that the COP9 complex may directly interact with the 19S regulatory complex of the 26S proteasome or other potential AtS9-containing complex. This notion is consistent with the parallel tissue-specific expression patterns and the similar, predominantly nuclear localization of both the COP9 complex and the AtS9 protein.

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**Keywords:** *Arabidopsis*; COP9; protein degradation; proteasome; photomorphogenesis

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## Introduction

*Arabidopsis* seedlings can follow two contrasting developmental strategies: photomorphogenesis (de-etiolation) in the light or skotomorphogenesis (etiolation) in darkness (von Arnim & Deng, 1996). One component involved in this light mediated

developmental switch is the COP9 complex, whose deficiency results in constitutive photomorphogenic development in darkness and lethality post-seedling development (Wei *et al.*, 1994a; Wei & Deng, 1996). These results suggest that the COP9 complex is not only important for light regulated development, but it is also essential for adult viability. Initial characterization of the COP9 complex indicates that it is a large and multi-subunit protein complex predominantly localized in the nucleus (Chamovitz *et al.*, 1996; Staub *et al.*, 1996; Kwok *et al.*, 1998). Interestingly, the COP9 complex has also been shown to be present in other multicellular organisms, including mammals (Seeger *et al.*, 1998; Wei & Deng, 1998). Complete characterization of the purified COP9 complexes from plants and mammals indicates that the complexes are highly conserved, and that they contain eight distinct yet conserved subunits (Seeger *et al.*, 1998; Wei *et al.*, 1998). Although four individual subunits have been implicated respectively in AP-1-mediated transcription activation, thyroid hormone regulation, and cell cycle progression, the specific

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Abbreviations used: eIF3, eukaryotic translation initiation factor 3; AtS9, *Arabidopsis* 19S proteasome regulatory complex subunit 9; HTH, helix-turn-helix; ORF, open reading frame; EST, expressed sequence tag; AAA, ATPases associated with diverse activation; DAPI, 4'-6-diamidino-2-phenylindole; BAC, bacterial artificial chromosome; YAC, yeast artificial chromosome; HIV, human immunodeficiency virus; pfu, plaque forming units.

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molecular and cellular function(s) of the COP9 complex remains to be elucidated (Wei *et al.*, 1998).

On the other hand, the molecular characteristics of the COP9 complex subunits provide information regarding the evolutionary origin of the COP9 complex. A careful sequence analysis indicates that the COP9 complex is closely related to the 19S proteasome regulatory complex (Wei *et al.*, 1998). First, the eight subunits of the COP9 complex exhibit significant similarity to eight distinct non-ATPase subunits of the 19S regulatory complex of the 26S proteasome, and thus can be classified as their respective paralogues. Second, all eight subunits of the COP9 complex contain one of the two newly defined protein motifs, the PCI or MPN domain, that is present among subunits of the proteasome regulatory complex, the COP9 complex, and the eIF3 complex (eukaryotic translation initiation factor 3; Hoffmann & Bucher, 1998; Wei *et al.*, 1998). Since the single cell yeast *Saccharomyces cerevisiae* genome does not contain any homologues of the COP9 complex subunits (Wei *et al.*, 1998), it is reasonable to speculate that the action of the COP9 complex is likely to be specific to the functions of multicellular eukaryotes. Nevertheless, given the homologies to subunits of the 19S regulatory complex of the 26S proteasome, the COP9 complex may play some cellular role related to the proteasome and protein degradation.

Regulated degradation of many cytoplasmic and nuclear proteins is primarily through the function of the 26S proteasome. The proteolytic core of the 26S proteasome, the 20S proteasome, is regulated by a number of endogenous protein complexes. Among the best studied regulators of the 20S proteasome are the 11S and 19S regulatory complexes (Tanaka & Tsurumi, 1997). The combination of the 19S regulatory complex and 20S proteasome core forms the 26S proteasome, the most common form in eukaryotic cells. In all eukaryotic cells, the 19S regulatory complex is made up of two main classes of subunits: the non-ATPases and the ATPases. The non-ATPase of the 19S complex constitute about 11 or so subunits. However, there are six ATPases in the 19S complex, most of which are encoded by a unique multigene family of homologous polypeptides conserved during evolution (Tanaka & Tsurumi, 1997). It has been recently demonstrated (Glickman *et al.*, 1998b) that at least in yeast, the 19S regulatory complex consists of two subcomplexes: the base subcomplex which contains all ATPase subunits and three non-ATPase subunits, and the led subcomplex which contains eight non-ATPase subunits. Interestingly, the led subcomplex included all the eight non-ATPase subunits of the 19S regulatory complex of proteasome which share similarity with the COP9 complex subunits. Therefore, it is reasonable to assume that the led subcomplex and the COP9 complex share common evolutionary origin.

To gather molecular evidence for a specific biochemical function of the COP9 complex, we have

initiated several screens for non-subunit interactive partners of the COP9 complex by using representative COP9 subunits as probes. Here, we report an *Arabidopsis* component identified as interactive factor with the FUS6/COP11 subunit. Since this factor is a component of the 19S regulatory complex of the 26S proteasome, it provides important implications about the biochemical activity of the COP9 complex.

## Results

### Identification of a FUS6-interactive factor by the yeast two hybrid interaction trap screen

To identify specific factors interacting with FUS6/COP11, a yeast two-hybrid interactive screen was undertaken to identify proteins that may interact with LexA-FUS6 as a bait. A six day-old light-grown *Arabidopsis* seedling cDNA expression library was used for the interactive clone screening. Five independent interactive clones, all derived from the same gene, were obtained after screening  $2 \times 10^6$  clones. The largest cDNA insert of the five was sequenced completely, and found to contain a 915 bp DNA fragment that encoded a potential open reading frame (ORF) of 267 amino acid residues. Database searches using the obtained cDNA sequence revealed four *Arabidopsis* EST clones (39A10T7, F2D12T7, 97O8T7, and G9H3T7) representing the same gene (see Materials for GenBank accession numbers). All four EST clones were obtained, and the complete nucleotide sequence of the two longest EST clones (39A10T7 and G9H3T7) was determined (GenBank accession number AF083890). As shown in Figure 1, this analysis revealed a complete ORF of 421 amino acid residues, encoding a predicted 48.1 kDa polypeptide.

### The FUS6/COP11-interactive protein is likely a subunit of the 19S proteasome regulatory complex

Insight into a possible cellular role of the FUS6-interactive protein was gained by the high degree of protein sequence homology (50% identity and 72% similarity) with a recently reported human gene identified as a subunit (S9) of the 19S regulatory complex from the human 26S proteasome (HuS9, Figure 1(a); Hoffman & Rechsteiner, 1997). Further database searches using the predicted full-length protein sequence of the FUS6/COP11 interactive protein identified likely homologues in all represented taxonomic groups, including protozoa (*Toxoplasma gondii*), fungi (*S. cerevisiae* and *Schizosaccharomyces pombe*), nematodes (*Caenorhabditis elegans* and *Schistosoma mansoni*), insects (*Drosophila*), and mammals (mouse, pig, and humans). As shown in Figure 1(a), these proteins share 50-70% identity with each other over their entire length. Considering the conservative amino acid substitutions, their simi-



terminus of the AtS9 and FUS6 proteins (Figure 1(b)). Both proteins have a high overall leucine/isoleucine content (at ~25%) toward the C-terminal half (Figure 1(b)). This leucine/isoleucine-rich region, together with the C-terminal coil motif, corresponds to the PCI domain (Hoffmann & Bucher, 1998). As shown in Figure 1(c), the C-terminal region of the AtS9 and FUS6 proteins also contains region reminiscent of a helix-turn-helix (HTH) motif, which is found in numerous transcriptional activators that bind DNA (Mushegian & Koonin, 1996) and conserved in the AtS9 homologues (Figure 1(c)).

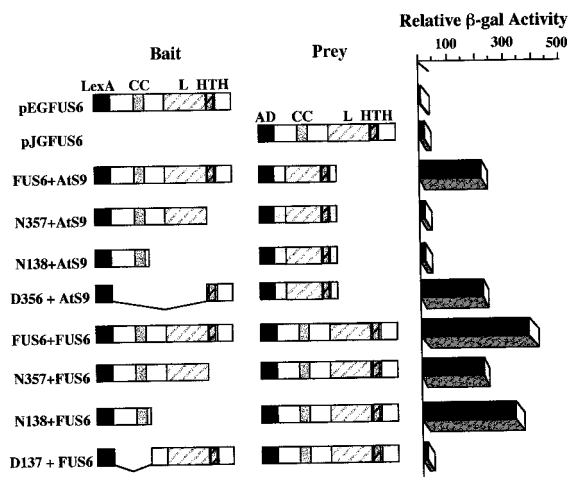
### FUS6 interacts with AtS9 and itself through distinct domains

The specificity of the interaction between FUS6 and AtS9 and their responsible domains were further analysed using the yeast two-hybrid interaction assay. As shown in Figure 2, the FUS6-AtS9 interaction is ~200-fold above negative controls. Removal of the C-terminal 85 amino acid residues of FUS6 (N357) abolishes its interaction with AtS9. Likewise, the FUS6 deletion construct containing only the N-terminal 138 amino acid residues of FUS6 (N138) also shows no interaction with AtS9. In contrast, the D356 construct, which contains

only the C-terminal 85 amino acid residues of FUS6, interacts with AtS9 to a similar degree as the full-length FUS6. These results indicate that the AtS9 interactive site resides on the FUS6 C terminus. Interestingly, FUS6 was also found to interact with itself (~400-fold above negative controls). In contrast with the FUS6-AtS9 interaction, the FUS6-FUS6 interaction is not significantly reduced in the FUS6 N138 and N357 deletions. However, removal of the N-terminal 137 amino acid residues of FUS6 (D137) completely abolishes this self-interaction. Therefore, the FUS6 self-interactive site is located in its own N terminus. Taken together, these results suggest that although FUS6 interacts with itself *via* its N-terminal 137 amino acid residues, this interaction is at a different site than that with AtS9 and does not interfere with the FUS6-AtS9 interaction.

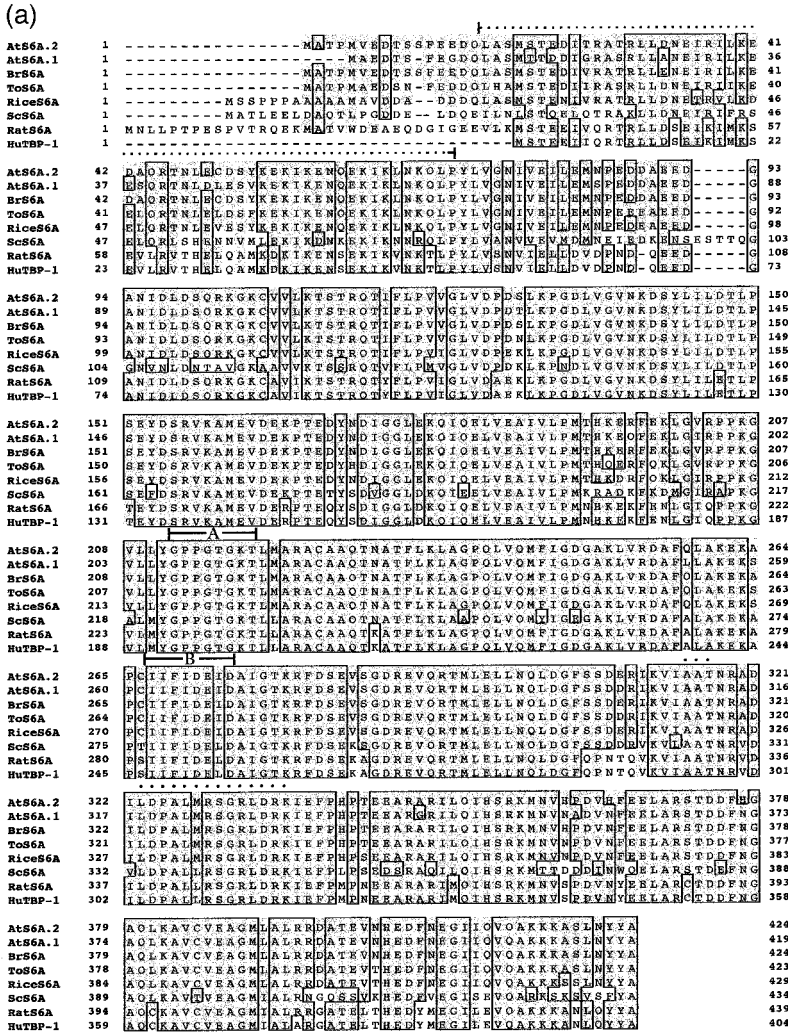
### The identification of an *Arabidopsis* gene homologous to a human ATPase subunit of the 19S proteasome regulatory complex

To further confirm that AtS9 is a true subunit of the 19S regulatory complex for the *Arabidopsis* 26S proteasome, we tested whether AtS9 is physically associated with other expected subunits of the *Arabidopsis* 19S regulatory complex. To this end, we used the highly conserved ATPase domain of the ATPase subunits of the 19S regulatory complex to search for available *Arabidopsis* ESTs encoding these subunits. We identified a 1.3 kb EST, 89E7T7, that contains a partial ORF for a predicted ATPase subunit of the 19S regulatory complex. We subsequently screened an *Arabidopsis* seven day-old light-grown seedling cDNA expression library, and obtained a full-length cDNA clone (see Methods). Conceptual translation of the cDNA clone (GenBank accession number AF081573) indicates it has an ORF of 424 amino acid residues, encoding a protein of about 47 kDa. This predicted protein is highly similar (94% identity and 99% similarity) to another *Arabidopsis* sequence deposited in GenBank (accession number AC000106). Both *Arabidopsis* genes are highly homologous to the subunit 6A of the 19S regulatory complex of the 26S proteasome from other organisms representing a wide range of taxonomic groups from yeast to human (Glickman *et al.*, 1998a; Richmond *et al.*, 1997). Thus, we designated our gene as AtS6A.2 and the other known *Arabidopsis* gene as AtS6A.1. A representative amino acid lineup of six homologous proteins with the two *Arabidopsis* proteins is shown in Figure 3(a). All homologues of AtS6A vary in length ranging from 404 to 439 amino acid residues (length differences mostly at the N termini). These AtS6A homologues show amino acid identity with AtS6A.1, ranging from 68% (*S. cerevisiae*) to 99% (*Brassica rapa*) throughout the entire length of their proteins.



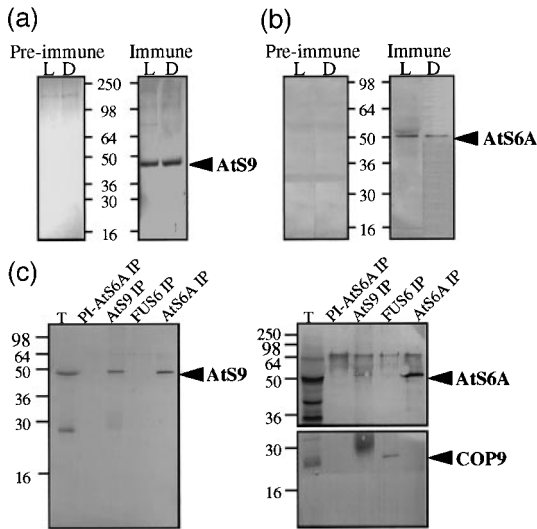
**Figure 2.** FUS6 interacts with AtS9 and itself through distinct domains in the yeast two-hybrid assay. The control constructs pEGFUS6, pJGFUS6 and Bait + Prey constructs are listed on the left. Bait constructs are fused to the LexA DNA-binding domain. Prey constructs are fused to the synthetic yeast transcription activation domain (AD). For interaction assays between FUS6 mutant proteins and AtS9, the longest partial AtS9 clone originally isolated from the two-hybrid screen was used. Protein structural features are the coiled-coil domain (CC, gray shaded box), leucine/isoleucine-rich domain (L, gray striped rectangle) and the helix-turn-helix domain (HTH, striped box). The relative  $\beta$ -galactosidase ( $\beta$ -gal) reporter activity in yeast cells were determined from at least two independent experiments using ten individual transformants each. Standard error was less than 10% in all cases.

**Figure 3.** Sequence comparison and structure features of AtS6A and its homologues. (a) Best-fit alignment of AtS6A and its homologues. *AtS6A.2* and *AtS6A.1* (accession numbers AF081573 and AC000106) refer to the two genes encoding for subunit 6A (also called 6') of the 19S regulatory complex from *Arabidopsis thaliana*. BrS6A, ToS6A, RiceS6A, ScS6A, RatS6A and HuTBP-1 refer to the S6A (or S6') subunit genes from *Brassica rapa*, tomato, rice, *S. cerevisiae*, rat and human, respectively. The GenBank accession numbers for these six genes are: D88663, X74426, D17788, X73569, D83522, and M34079, respectively. HuTBP-1 refers to the human Tat Binding Protein 1 which has been shown to be the human S6A/S6' subunit of the 19S regulatory complex (Ohana *et al.*, 1993). The three distinctive structural motifs of AtS6A (and other AAA ATPases) are shown. The coiled-coil motif is represented by broken lines, the ATPase A and B box motifs are indicated above their respective sequences by A and B and the RNA/DNA helicase motif is indicated by dots over their amino acid residues. Identical amino acid residues are boxed and similar amino acid residues are shaded. (b) Structural features of the AAA-family of proteasomal ATPases. The AAA ATPase family of genes contain three major motifs as described in the text. The coiled-coil motif is indicated by a gray shaded box, the ATPase motifs (A and B) are indicated by stippled boxes and the helicase motifs are indicated by diagonal striped boxes. The consensus amino acid sequence is indicated for each motif. X refers to any amino acid residues, while numbers between motifs indicated conserved amino acid spacing between each individual motif. The diagram is not drawn to scale.



S6A/S6' is one of the subunits of the 19S regulatory complex that associates with the 20S proteasome core in an ATP-dependent manner to form the active 26S complexes, providing substrate specificity and ATP dependence to the enzyme (Seeger *et al.*, 1997). This class of ATPases is collectively known as AAA-family of ATPases (AAA for ATPases associated with diverse activities; Nacken, 1997; Rivkin *et al.*, 1997). All members of this family of ATPase have three distinctive motifs (Figure 3(b)). At the N-terminal end, they have a coiled-coil domain. In

some cases, this coiled-coil domain is reminiscent of a leucine zipper, with valine, methionine, and isoleucine substituting for leucine at times (as in Rat S6A/S6' in Figure 3(b)). The middle portion of the polypeptide has a highly conserved ATPase motif, which is divided into an A box, important for ATP binding, and a B box, important for ATP hydrolysis. The C-terminal end of the proteins contain a putative RNA/DNA helicase motif. Additionally, the ATPase and the helicase motifs also maintain conserved spacing between themselves (Figure 3(b)).



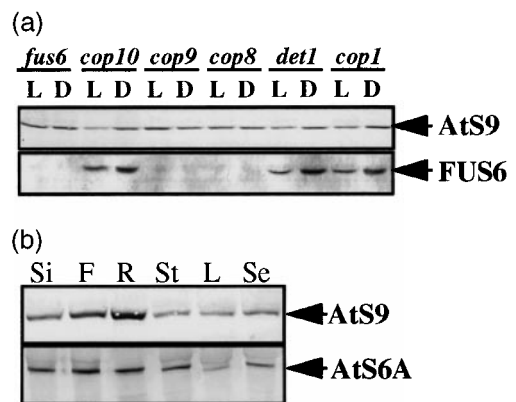
**Figure 4.** Characterization of the antibodies raised against AtS9 and AtS6A and their co-immunoprecipitation analysis. (a) and (b) Represent identical protein gels (10  $\mu$ g/lane) containing extracts from wild-type light (L) or dark-grown (D) seedlings probed with either pre-immune or immune rabbit antiserum. Note the presence of a single protein species of 48 kDa only in the blot probed with immune AtS9 antiserum (a) and a single protein species of about 51 kDa only in the blot probed with immune AtS6A antiserum (b). (c) Co-immunoprecipitation of AtS6A and AtS9. Left, Western blot of various immunoprecipitation (IP) extracts using protein-A purified antiserum and then probed with protein-A purified AtS9 antibodies. Right top, the same IP extracts as left Western blot, but probed with protein-A purified AtS6A antibodies. Right bottom, Western blot of the same set of IP extracts probed with protein-A purified COP9 antibodies. T represents total soluble protein extract before immunoprecipitation, and PI-AtS6A IP refers to the IP extracts with AtS6A pre-immune serum.

### AtS9 and AtS6A co-immunoprecipitate each other but are not members of the COP9 complex

To determine if AtS9 and AtS6A are members of the same complex, specific polyclonal antibodies were generated and purified (see Methods). Figure 4(a) and (b) show immunoblot analyses of wild-type protein extracts probed with either anti-AtS9 or anti-AtS6A antibodies. Anti-AtS9 antibodies reacted with a single  $\sim$ 48 kDa protein species (Figure 4(a)), in agreement with the predicted molecular mass of the full-length AtS9 cDNA. Anti-AtS6A.2 antibodies reacted with a single  $\sim$ 51 kDa protein species (Figure 4(b)), migrating slightly slower than the expected. Since *AtS6A.1* and *AtS6A.2* are redundant genes encoding the same subunit (S6A/S6') of the 19S regulatory complex, the  $\sim$ 51 kDa protein band that is recognized by the antibodies raised against recombinant *AtS6A.2*-encoded protein probably represents a mixture of both protein species

encoded by the *AtS6A.1* and *AtS6A.2* genes. Both AtS9 and AtS6A protein expression do not seem to be light modulated, since their expression levels are similar between continuous light or continuous dark conditions (Figure 4(a) and (b)). In addition, no protein bands are detected when AtS9 and AtS6A pre-immune rabbit antiserum are used in identical immunoblots. Therefore, we conclude that the observed 48 kDa and the 51 kDa protein species are the *Arabidopsis* AtS9 and AtS6A, respectively.

To examine whether AtS9 and AtS6A can stably associate with each other, co-immunoprecipitation experiments were carried out with the purified antibodies. As shown in Figure 4(c), AtS9 antibodies coupled to protein-A beads immunoprecipitate itself and AtS6A (left, Figure 4(c)). Similarly, AtS6A antibodies coupled to protein-A beads also immunoprecipitate AtS9 and itself (top right, Figure 4(c)). In contrast, both AtS6A pre-immune antibodies or FUS6 antibodies coupled to protein-A beads are unable to immunoprecipitate AtS9 or AtS6A. However, FUS6 antibodies can clearly immunoprecipitate COP9 as reported (Figure 4(c), bottom right; Staub *et al.*, 1996). Taken together, these results strongly suggest that AtS6A and AtS9 can stably associate with each other *in vivo*. However, AtS6A and AtS9 are not tightly associated with the COP9 complex. Our size fractionation analysis (S.F.K., J.M.S. & X.-W.D., unpublished data) clearly indicated a co-fractionation of AtS6A and AtS9 in large molecular mass fractions consistent with the conclusion that they are components of the proteasome.



**Figure 5.** AtS9 and AtS6A accumulation patterns in different photomorphogenic mutants and organ types. (a) Identical protein gel blots are shown that contain extracts (10  $\mu$ g/lane) from continuous light-grown (L) or dark-grown (D) *fus6-1*, *cop10-1*, *cop9-1*, *cop8-1*, *det1-1* and *cop1-4* mutant seedlings. The top panel is a protein gel blot probed with AtS9 antiserum. The bottom panel is a protein gel blot probed with FUS6 immune antiserum. (b) Protein gel blots containing extracts (10  $\mu$ g/lane) from wild-type siliques (Si), or whole tissues from flowers (F), roots (R), stems (St), leaves (L) and whole seedlings (Se) were probed with either AtS9 or AtS6A antibodies.

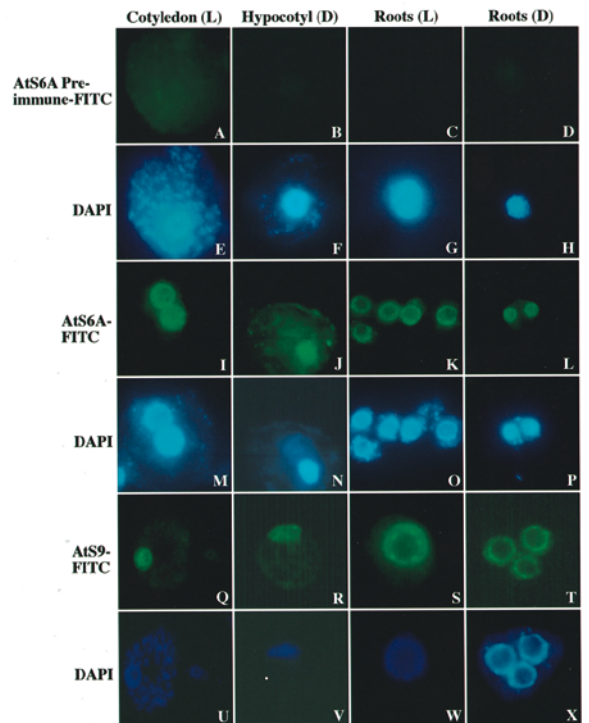
To further confirm that AtS9 is not part of the COP9 complex, we examined the accumulation of AtS9 in several of the available pleiotropic *cop/det/fus* mutants. This is based on the co-ordinated protein accumulation pattern of FUS6 and COP9 subunits of the COP9 complex, because mutations in any one member prevents the accumulation of these two proteins in the cell (Wei *et al.*, 1994a,b; Kwok *et al.*, 1998). As shown in Figure 5(a), AtS9 accumulates to similar levels in all of the pleiotropic *cop/det/fus* mutants, regardless of the light conditions. Most importantly, AtS9 levels are normal in the *fus6*, *cop9*, and *cop8* mutants, all of which are defective in the COP9 complex as indicated by the absence of FUS6 protein accumulation in these three mutants. These results further confirm that AtS9 is not a member of the COP9 complex, but rather is part of the 19S regulatory complex from *Arabidopsis*.

### Both AtS9 and AtS6A are expressed in a similar organ specific patterns as that of the COP9 complex

To obtain support for a possible functional interaction between the COP9 complex and 19S regulatory complex of the proteasome, we examined the organ-specific expression patterns of both AtS9 and AtS6A, and compared that to the tissue expression profile of the COP9 complex reported (Staub *et al.*, 1996; Kwok *et al.*, 1998). Figure 5(b) shows that the AtS9 and AtS6A proteins accumulate in all organs tested, with the highest abundance in root and floral tissues. This ubiquitous expression patterns in various *Arabidopsis* organs suggest AtS9 and AtS6A are required in all organs and many developmental stages. Importantly, although AtS9 and AtS6A are not members of the COP9 complex, their expression patterns are similar to FUS6 and other components of the COP9 complex (Chamovitz *et al.*, 1996; Staub *et al.*, 1996). This similarity in the tissue expression pattern between AtS9 and the COP9 complex would support a physiological role of the observed AtS9-FUS6 interaction.

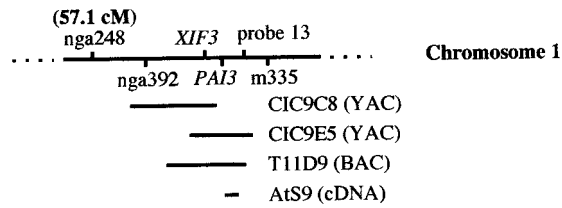
### AtS9 and AtS6A are predominantly localized to the nucleus

We have shown that the COP9 complex is largely nuclear localized, regardless of light conditions or tissue types (Staub *et al.*, 1996; Chamovitz *et al.*, 1996). To further substantiate the feasibility of the predicted interaction between the COP9 complex and the 19S regulatory complex of the proteasome *in planta*, the cellular localization of AtS9 and AtS6A was examined using an *Arabidopsis* protoplast immunolocalization assay (Matsui *et al.*, 1995). A representative set of cell staining patterns is shown in Figure 6. Indirect immunofluorescence staining of wild-type cells using the purified anti-AtS6A antibodies (Figure 6A to P) indicates that AtS6A has a staining pattern pre-



**Figure 6.** Immunofluorescence of AtS6A and AtS9 in *Arabidopsis* protoplasts. A to D, Protoplasts probed with purified AtS6A pre-immune antiserum and detected with FITC secondary antibody. E to H, Corresponds to protoplasts from A to D stained with DAPI. I to L, Protoplast probed with purified AtS6A antiserum and detected with FITC secondary antibody. M to P, DAPI staining of corresponding protoplasts from I to L, respectively. Q to T, Protoplasts probed with purified AtS9 antiserum and detected with FITC secondary antibody. U to X, DAPI staining of corresponding protoplasts from Q to T, respectively. All protoplasts were made from tissues of wild-type seven day-old dark or light-grown seedlings as indicated.

minantly on the nucleus that overlaps with nuclear DNA staining using 4'-6-diamidino-2-phenylindole (DAPI), but also has some diffuse cytoplasmic staining. This pattern of staining is observed in all tissue types examined, including light-grown cotyledons (Figure 6I) and roots (Figure 6K) and dark-grown hypocotyls (Figure 6J) and roots (Figure 6L). This AtS6A-specific staining is consistent with predominantly nuclear staining patterns of its human counterpart, also named TBP-1 (Ohana *et al.*, 1993). As expected, immunofluorescence of protoplasts using anti-AtS9 antibodies indicated a similar predominantly nuclear localization (overlapping with the DAPI staining) with some diffuse cytoplasmic staining (Figure 6Q to 6X). Again, this nuclear staining is observed in all cell types examined, including light-grown cotyledons (Figure 6Q) and roots (Figure 6S) and dark-grown hypocotyl cells (Figure 6R) and roots (Figure 6T). Together, this result suggests that the *Arabidopsis* 19S regulatory complex of proteasome is largely nuclear localized



**Figure 7.** Diagram of the region of chromosome 1 containing the *AtS9* gene. Molecular markers near position 57.1 (cM) are shown above the line. Relative positions of YAC and BAC clones and the *AtS9* cDNA are shown below the line representing chromosome 1.

and similar to the cellular localization pattern of the COP9 complex.

### ***AtS9* is a single copy gene that maps to chromosome 1**

Low stringency Southern blot analyses indicate that *AtS9* is a single-copy gene in *Arabidopsis* (data not shown). In order to determine if *AtS9* may represent a previously characterized *Arabidopsis* gene, the chromosome map position of *AtS9* was determined as shown in Figure 7. The full-length *AtS9* cDNA was used to probe a BAC (bacterial artificial chromosome) library containing the *Arabidopsis* genome (available from the *Arabidopsis* Biological Resource Center, ABRC). A positive BAC (T11D9) was identified and shown to hybridize with two YAC (yeast artificial chromosome) clones, CIC9C8 and CIC9E5, that mapped to a region on the top of chromosome 1 (position 57 cM). These two YAC clones were obtained and confirmed by hybridization with an *AtS9* cDNA probe. Multiple restriction enzyme digestions revealed that the *AtS9* genomic DNA resides on a 2 kb *DraI* fragment within CIC9E5 (Figure 7). However, none of the known light-signal transduction genes have been reported to reside in this chromosomal location. Therefore, *AtS9* most likely represents a previously uncharacterized locus.

## **Discussion**

### **Implications of the observed interaction between the 19S proteasome regulatory complex and the COP9 complex**

Our results here indicate that we have identified two components, *AtS9* and *AtS6A*, of the *Arabidopsis* 19S regulatory complex and that *AtS9* can interact with *FUS6*. Since all three proteins, *FUS6*, *AtS9*, and *AtS6A*, are present as complexed forms *in vivo* (Staub *et al.*, 1996; data not shown), the observed *FUS6* and *AtS9* interaction suggests that the COP9 complex may directly interact with the 19S regulatory complex of the 26S proteasome. Since *AtS9* and *AtS6A* are not stably associated with the COP9 complex, it implies that the 19S regulatory complex may possibly associate and

function with the COP9 complex in a transient manner. This is not unlikely, given the strong and specific *AtS9-FUS6* interaction, the overlapping cellular localization pattern of *AtS9*, *AtS6A*, and the COP9 complex, and the parallel organ accumulation profiles between *AtS9* and *AtS6A* with the COP9 complex subunits.

Since the COP9 complex contains putative repressors (*FUS6*) and activators (AJH) of transcription (Spain *et al.*, 1996; Claret *et al.*, 1996), it can be postulated that the COP9 complex may function in modulating the activity of transcription factors. This effect on transcription factors and the possible role of the COP9 complex in modulating steroid hormone receptors and the cell cycle could all be potentially integrated by a unified function of the COP9 complex in modulating degradation of specific regulatory proteins in diverse pathways. One putative mechanism is through selective phosphorylation/dephosphorylation of specific regulatory proteins by the COP9 complex, and then designating them for proteolysis by the proteasome. This would be consistent with the observed kinase activity of the human COP9 complex, also known as the JAB1-containing signalosome, which has been shown to phosphorylate I $\kappa$ B and c-Jun *in vitro* (Seeger *et al.*, 1998). Furthermore, multiple lines of evidence indicate that the degradation of c-Jun requires either an ubiquitin-dependent or an ubiquitin-independent proteasome degradation pathway (Jariel-Encontre *et al.*, 1997). The hypothesis that c-Jun may be a possible target for degradation *via* the COP9 complex is not unlikely, since one subunit of the JAB1-containing signalosome is JAB1, a specific c-Jun interacting protein that functions to co-activate AP-1-dependent transcription (Claret *et al.*, 1996). Clearly, further experiments will be needed to confirm or disprove this possibility.

### ***AtS9* and *AtS6A* may have functions beyond cellular protein degradation**

Although both *AtS9* and *AtS6A* are the non-ATPase and ATPase subunits, respectively, of the 19S regulatory complex of the 26S proteasome, these proteins may have functions in addition to protein degradation. For instance, *AtS9*, *FUS6*, and the *AtS9* homologues all display a characteristic HTH domain at the C-terminal end of their proteins. This domain has been found in many DNA-binding transcriptional factors, and suggests *AtS9* may be a DNA binding protein. Another example is subunit 6A of the 19S regulatory complex. The human S6A (or S6'), also named TBP-1, was originally identified as a protein that interacted with the viral protein Tat of type 1 human immunodeficiency virus (HIV; Ohana *et al.*, 1993). Although, it is unable to bind DNA itself, TBP-1 has been shown to be a strong transcriptional activator when brought into close proximity of specific promoters (Ohana *et al.*, 1993). It is possible that like TBP-1, *AtS6A* may also function as a putative

transcriptional activator. When tightly associated with AtS9, which has a potential DNA-binding motif, they may function as specific transcriptional regulator in addition to their role in modulating the proteasome. This notion would be consistent with the observation that AtS9 and AtS6A are abundantly present in the nucleus. Also, the fact that mutations in many subunits of the 19S complex result in cell cycle arrest and chromosome instability in *S. cerevisiae* (Tanaka & Tsurumi, 1997) could be explained by an alternative function of these proteins in addition to their role in the degradation of cell cycle regulators.

### The implication of FUS6 self interaction

Using the yeast two-hybrid assay, we also show that FUS6 is capable of interacting with itself *via* its N-terminal coiled-coil domain. The self interaction of FUS6 is particularly interesting, since it may imply that there are two FUS6 molecules within each COP9 complex and that they contact each other. Previous gel filtration studies with FUS6 showed that FUS6 is present only in the COP9 complex, and there is no dimer outside of the complex (Staub *et al.*, 1996). This observation is not unlikely, since the purification of both the mammalian and plant COP9 complexes indicate each complex consists of eight distinct subunits with the accumulative molecular mass of the subunits adding up to only 330 kDa, which is much smaller than the 500-550 kDa size predicted by gel filtration studies. This discrepancy may be due to a combination of two possibilities: unusual shape of the complex or multiple copies of a subset of the subunits in the complex (Wei *et al.*, 1998). Therefore, multiple copies of FUS6 in each functional COP9 complex would be a likely possibility. Nevertheless, the sequence motif responsible for this self-interaction is distinct from that responsible for interacting with AtS9.

## Methods

### Yeast two-hybrid interaction screen

The full-length *Arabidopsis* FUS6 cDNA was fused in frame to the LexA-DNA-binding domain in plasmid pEG202 (Guarente, 1993) to yield plasmid pEGFUS6. Yeast strain EGY48 was transformed with pEGFUS6 and the resulting line, EGY48 (pEGFUS6), was established for re-transformation with cDNA library-containing plasmids. The cDNA library was constructed using *Arabidopsis* six day-old light-grown seedlings, and the library was cloned into plasmid pJG4-5 *via* EcoRI and XhoI (Guarente, 1993). The pJG library plasmids were transformed into the EGY48 (pEGFUS6) parent line and leucine prototrophs selected. In a screen of  $2 \times 10^6$  yeast colonies harbouring cDNA library plasmids, 16 independent putative FUS6-interactive clones were identified. The insert in each of the 16 clones was characterized by restriction enzyme digestion and found to represent four different cDNA classes. The AtS9 were represented five times and gives the most reproducible and strongest interaction with LexA-FUS6. Therefore, we only selected

AtS9 for further characterization. Quantitative  $\beta$ -gal actinidase activity measurements for the interaction strength were carried according to published procedure (McNellis *et al.*, 1996; Ausebel *et al.*, 1994).

### cDNA cloning

Using the highly conserved ATPase domain of the ATPase subunits of the 19S regulatory complex, we searched GenBank for available *Arabidopsis* ESTs for these subunits. We identified a 1.3 kb EST, 89E7T7 (GenBank accession number T20899), that contains a partial ORF for the 6A (or 6') ATPase subunit of the 19S regulatory complex. To identify a full-length cDNA clone, a non-size selected  $\lambda$ ZAP II cDNA expression library constructed using light-grown seven day-old Landsberg *erecta* seedlings was screened. The  $5 \times 10^5$  plaque forming units (pfu) were screened using a [ $\alpha$ - $^{32}$ P]dCTP random primed *Sall*/*Hind*III 450 bp N-terminal fragment from EST 89E7T7 (1.3 kb) according to manufacturer's conditions (Boehringer Mannheim). Four independent clones were identified and subjected to excision of the pBluescript cDNA insert *via* manufacturer's conditions (Stratagene, La Jolla, CA). All four excision clones were checked for insert size by digestion of excised plasmid with *Eco*RI and *Not*I or *Sall* and *Not*I, and the longest clone, AtS6A.2 z1-1b2, was analysed and used for all further experiments. Complete sequencing of cDNA clone AtS6A.2 z1-1b2 indicated that it was a full-length clone for AtS6A.2 with a putative methionine, stop codon, and a poly(A) tail in the 3' untranslated region. Conceptual translation of AtS6A.2 z1-1b2 indicated that it encoded a protein of 424 amino acid residues.

To identify the EST clones for AtS9, the sequence of the partial cDNA AtS9 cloned obtained from the yeast two hybrid screening (using the LexA-FUS6 bait) was used to search GenBank for available *Arabidopsis* ESTs. EST clones 39A10T7, F2D12T7, 97O8T7, and G9H3T7 (GenBank accession numbers T04552, AA042373, T22161, and N95863, respectively) were obtained from ABRC. All clones were sequenced completely on both strands. EST clones 39A10T7 and G9H3T7 together constituted a full-length cDNA for AtS9.

### Plant materials and growth conditions

The constitutive photomorphogenic *cop8-1*, *cop9-1*, *cop10-1*, *fus6-1* mutants are in the *Arabidopsis thaliana* ecotype Wassilewskija background (Wei *et al.*, 1994a) while *cop1-4* and *det1-1* are in *Arabidopsis* ecotype Columbia background (McNellis *et al.*, 1994; Wei *et al.*, 1994b). Unless stated otherwise, the wild-type plants used are all in the *Arabidopsis* Columbia ecotype. Plant germination and growth conditions in darkness and white-light were as described (Wei & Deng, 1992; McNellis *et al.*, 1994). Unless specified otherwise, light-grown plants were under 16 hours of white-light at  $75 \mu\text{mol m}^{-2} \text{s}^{-1}$  and eight hours of darkness.

### Antibody production, immunoprecipitation, and immunoblot analysis

For AtS9 antiserum production, the 915 bp *Eco*RI fragment from the pJG4-5 vector containing the original partial AtS9 cDNA isolate was inserted into the *Eco*RI site of plasmid pET15b. The resulting pETAAtS9 plasmid contained the C-terminal  $\sim 25$  kDa ORF of AtS9 fused in frame to a T7-Tag peptide, and was expressed from the

inducible *Escherichia coli* tac promoter. The orientation of the fusion gene was verified by sequencing and subsequently by expression of the fusion protein. The resulting AtS9 protein was overproduced in *E. coli* and accumulated in inclusion bodies. Inclusion bodies were solubilized by sonication in SDS-PAGE loading buffer and subsequent boiling for ten minutes. Total protein from inclusion bodies were separated on a SDS/12% polyacrylamide gel, and the AtS9 polypeptide band was excised from the gel and electroeluted using a Bio-Rad electroelutor (Bio-Rad). After dialysis against PBS, the purified AtS9 protein (at 250 µg per 500 µl per injection) was used to immunize rabbits for the production of polyclonal antiserum. Anti-AtS9 antibodies were subsequently purified using protein-A agarose beads immobilized to a Hi-Trap column (Pharmacia).

For AtS6A antibody production, a protocol similar to the AtS9 antibody production was used. The only exception was that the 89E7T7 EST was cloned into pGEX 5X-3 as a translational fusion to GST via *Sall*-*NotI* restriction sites (Pharmacia). The fusion protein was also overproduced in *E. coli* and subjected to the same purification scheme as AtS9 to isolate protein for injection into rabbits. AtS6A antibodies were purified using the same method as that for AtS9 antibody purification.

Co-immunoprecipitation experiments and protein gel blotting were performed as described (Staub *et al.*, 1996). All antibodies used were protein-A purified. Protein-A purified polyclonal antibodies raised against COP9 (Wei *et al.*, 1994b) were used at 1:100, while protein-A purified AtS9, AtS6A, AJH (Kwok *et al.*, 1998), or FUS6 (Staub *et al.*, 1996) were used at 1:1000. Detection was based on horseradish peroxidase-conjugated goat anti-rabbit antibodies (F[ab']<sub>2</sub> fragment) used at 1:5000 (Pierce Scientific).

#### Arabidopsis protoplast immunofluorescence staining

The procedure for protoplast preparation and immunofluorescence was similar to those described (Matsui *et al.*, 1995; Staub *et al.*, 1996). All protoplasts were mounted on eight-well slides with SlowFade anti-fade reagent (Molecular Probes Inc.) containing 1 mg/ml of DAPI and viewed through a Leica light microscope. Protein-A purified rabbit polyclonal antiserum raised against AtS9 and AtS6A were used at a dilution of 1:1000 and 1:500, respectively. Pre-immune AtS6A antiserum was used at 1:500 dilution. Secondary antibody used was a goat anti-rabbit antibody conjugated to fluorescein (FITC; Sigma) and was used at a dilution of 1:400.

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#### References

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994). *Current Protocols in Molecular Biology*, vol. 27, pp. 13.6.2-13.6.4, John Wiley and Sons, New York.
- Chamovitz, D. A., Wei, N., Osterlund, M. T., von Arnim, A. G., Staub, J. M., Matsui, M. & Deng, X.-W. (1996). The COP9 complex, a novel multi-subunit nuclear regulator involved in light control of a plant developmental switch. *Cell*, **86**, 115-121.
- Claret, F.-X., Hibi, M., Dhu, S., Toda, T. & Karin, M. (1996). A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. *Nature*, **383**, 453-457.
- Glickman, M. H., Rubin, D. M., Fried, V. A. & Finley, D. (1998a). The regulatory particle of the *Saccharomyces cerevisiae* proteasome. *Mol. Cell. Biol.* **18**, 3149-3162.
- Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A. & Finley, D. (1998b). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalsome and eIF3. *Cell*, **94**, 615-623.
- Guarente, L. (1983). Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.* **101**, 181-191.
- Hoffman, L. & Rechsteiner, M. (1997). Molecular cloning and expression of subunit 9 of the 26S proteasome. *FEBS Letters*, **404**, 179-184.
- Hoffmann, K. & Bucher, P. (1998). PCI and MPN-domains: scaffold for multi-protein complexes or proteasome regulators? *Trends Biochem.* In the press.
- Jariel-Encontre, I., Salvat, C., Steff, A.-M., Pariat, M., Acquaviva, C., Furstoss, O. & Piechaczyk, M. (1997). Complex mechanisms for *c-fos* and *c-jun* degradation. *Mol. Biol. Rep.* **24**, 51-56.
- Kwok, S. F., Solano, R., Tsuge, T., Chamovitz, D. A., Matsui, M., Ecker, J. R. & Deng, X.-W. (1998). *Arabidopsis* homologs of a c-jun coactivator are present both in monomeric form and in the COP9 complex and their abundance is differentially affected by the pleiotropic *cop/det/fus* mutations. *Plant Cell*, **10**, 1779-1790.
- Lupas, A., Van Dyke, M. & Stock, J. (1991). Predicting coiled coils from protein sequences. *Science*, **252**, 1162-1164.
- Matsui, M., Stoop, C. D., von Arnim, A. G., Wei, N. & Deng, X.-W. (1995). *Arabidopsis* COP1 protein specifically interacts *in vitro* with a cytoskeleton-associated protein, CIP1. *Proc. Natl Acad. Sci. USA*, **92**, 4239-4243.
- McNellis, T. W., von Arnim, A. G., Araki, T., Komeda, Y., Miséra, S. & Deng, X.-W. (1994). Genetic and molecular analysis of an allelic series of *cop1* mutants suggests functional roles for the multiple protein domains. *Plant Cell*, **6**, 487-500.
- McNellis, T. W., Torii, K. U. & Deng, X.-W. (1996). Expression of an N-terminal fragment of COP1 confers a dominant-negative effect on light-regulated seedling development in *Arabidopsis*. *Plant Cell*, **8**, 1491-1503.
- Mushegian, A. R. & Koonin, E. V. (1996). Sequence analysis of eukaryotic developmental proteins: ancient and novel domains. *Genetics*, **144**, 817-828.
- Nacken, W. (1997). Members of the AAA-gene family are involved in early embryogenesis of vertebrates. *Biochim. Biophys. Acta*, **1354**, 1-6.

- Ohana, B., Moore, P. A., Ruben, S. M., Southgate, C. D., Green, M. R. & Rosen, C. A. (1993). The type 1 human immunodeficiency virus Tat binding protein is a transcriptional activator belonging to an additional family of evolutionary conserved genes. *Proc. Natl Acad. Sci. USA*, **90**, 138-142.
- Richmond, C., Gorbea, C. & Rechsteiner, M. (1997). Specific interactions between ATPase subunits of the 26S protease. *J. Biol. Chem.* **272**, 13403-13411.
- Rivkin, E., Culligan, E. B., Tres, L. L. & Kierszenbaum, A. L. (1997). A protein associated with the manchette during rat spermiogenesis is encoded by a gene of the TBP-1-like subfamily with highly conserved ATPase and protease domains. *Mol. Reprod. Dev.* **47**, 77-89.
- Seeger, M., Ferrell, K. & Dubiel, W. (1997). The 26S proteasome: a dynamic structure. *Mol. Biol. Rep.* **24**, 83-88.
- Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., *et al.* (1998). A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. *FASEB J.* **12**, 469-478.
- Spain, B. H., Bowdish, K. S., Pacal, A. R., Staub, S. F., Koo, D., Chang, C. Y., Xie, W. & Colicelli, J. (1996). Two human cDNAs, including a homolog of *Arabidopsis* FUS (COP11), suppress G-protein- and mitogen-activated protein kinase-mediated signal transduction in yeast and mammals. *Mol. Cell. Biol.* **16**, 6698-6706.
- Staub, J. M., Wei, N. & Deng, X.-W. (1996). Evidence for FUS6 as a component of the nuclear-localized COP9 complex in *Arabidopsis*. *Plant Cell*, **8**, 2047-2056.
- Tanaka, K. & Tsurumi, C. (1997). The 26S proteasome: subunits and functions. *Mol. Biol. Rep.* **24**, 3-11.
- von Arnim, A. & Deng, X.-W. (1996). A role for transcriptional repression during light control of plant development. *BioEssays*, **18**, 905-910.
- Wei, N. & Deng, X.-W. (1992). COP9: a new genetic locus involved in light-regulated development and gene expression in *Arabidopsis*. *Plant Cell*, **4**, 1507-1518.
- Wei, N. & Deng, X.-W. (1996). The role of the COP/DET/FUS genes in light control of *Arabidopsis* seedling development. *Plant Physiol.* **112**, 871-878.
- Wei, N. & Deng, X.-W. (1998). Characterization and purification of the mammalian COP9 complex, a conserved nuclear regulator initially identified as a repressor of photomorphogenesis in higher plants. *Photochem. Photobiol.* **68**, 237-241.
- Wei, N., Kwok, S. F., von Arnim, A. G. & Deng, X.-W. (1994a). *Arabidopsis* COP8, COP10, and COP11 genes are involved in repression of photomorphogenic development in darkness. *Plant Cell*, **6**, 629-643.
- Wei, N., Chamovitz, D. A. & Deng, X.-W. (1994b). *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. *Cell*, **78**, 117-124.
- Wei, N., Tomohiko, T., Serino, G., Dohmae, N., Takio, K., Matsui, M. & Deng, X.-W. (1998). The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. *Curr. Biol.* **8**, 919-922.

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