Characterization of the Last Subunit of the Arabidopsis COP9 Signalosome: Implications for the Overall Structure and Origin of the Complex

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The COP9 signalosome (CSN) is an evolutionarily conserved protein complex that resembles the lid subcomplex of proteasomes. Through its ability to regulate specific proteasome-mediated protein degradation events, CSN controls multiple aspects of development. Here, we report the cloning and characterization of AtCSN2, the last uncharacterized CSN subunit from Arabidopsis. We show that the AtCSN2 gene corresponds to the previously identified FUS12 locus and that AtCSN2 copurifies with CSN, confirming that AtCSN2 is an integral component of CSN. AtCSN2 is not only able to interact with the SCFCop subunit AtCUL1, which is partially responsible for the regulatory interaction between CSN and SCFCop, but also interacts with AtCUL3, suggesting that CSN is able to regulate the activity of other cullin-based E3 ligases through conserved interactions. Phylogenetic analysis indicated that the duplication and subsequent divergence events that led to the genes that encode CSN and lid subunits occurred before the divergence of unicellular and multicellular eukaryotic organisms and that the CSN subunits were more conserved than the lid subunits during evolution. Comparative analyses of the subunit interaction of CSN revealed a set of conserved subunit contacts and resulted in a model of CSN subunit topology, some aspects of which were substantiated by in vivo cross-link tests.

INTRODUCTION

The COP9 signalosome (CSN) is an evolutionarily conserved protein complex that was identified initially in Arabidopsis as a repressor of light-regulated development (Wei and Deng, 1992; Wei et al., 1994; Chamovitz et al., 1996). Genetic screening aimed at identifying Arabidopsis mutants that display an altered developmental response to light led to the identification of the class of mutants known as pleiotropic cop/det/fus mutants (Hardtke and Deng, 2000). These mutants are characterized by a constitutive photomorphogenic phenotype, regardless of the light condition used, and in the light accumulate anthocyanin, which is responsible for their purple color (fusca) (Wei and Deng, 1996). Furthermore, the severe cop/det/fus mutations lead to growth arrest and lethality after the seedling stage, indicating that their corresponding genes control, in addition to photomorphogenesis, other essential developmental programs. At present, 10 distinct pleiotropic COP DET/FUS loci have been identified and characterized, 5 of which have been shown to encode CSN subunits (Wei et al., 1994; Staub et al., 1996; Karniol et al., 1999; Serino et al., 1999; Peng et al., 2001b). CSN contains a total of eight subunits, is expressed ubiquitously in plant tissues, and is remarkably conserved throughout evolution, indicating that it plays a fundamental role in higher eukaryotes (Wei and Deng, 1999). This conclusion is supported by reports that CSN loss-of-function mutations lead to cell cycle arrest in fission yeast and to postembryonic growth arrest in Drosophila (Freilich et al., 1999; Mundt et al., 1999).

An increasing amount of evidence suggests that CSN is involved in the regulation of the ubiquitin-proteasome system, one of the major cellular pathways responsible for protein degradation (Schwechheimer and Deng, 2001). A key step on this pathway is the attachment of a polyubiquitin chain to the substrate that will be degraded. This occurs through an enzymatic cascade that includes three separate enzymatic activities designated E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (Hershko and Ciechanover, 1998). The E3 class of enzymes is very diverse and is responsible for both selecting the appropriate substrate and promoting the formation of the ubiquitin chain. Once the substrate is ubiquitylated, it is recognized and degraded by the proteasome. The proteasome is composed of a 20S catalytic particle and of a 19S regulatory particle. The 19S regulatory particle consists of two subcomplexes, the lid and the base (Glickman et al., 1998). The lid is necessary for the recognition of the ubiquitin chain and the subsequent deubiquitylation of the substrate.
(Verma et al., 2001), which is unfolded by the base and injected into the 20S catalytic particle for proteolysis (Glickman, 2000).

Remarkably, CSN exhibits a striking homology, in both complex composition and subunit sequence, with the lid of the proteasome (Wei et al., 1998). In addition, CSN is necessary for the regulation of E3 activity. For example, CSN interacts with the plant ubiquitin ligase SCFTIR1 and is necessary for the cleavage of the ubiquitin-like protein RUB1/NEDD8 from the CUL1 subunit of SCF ubiquitin ligases in human and plants (Lyapina et al., 2001; Schwechheimer et al., 2001, 2002; Cope et al., 2002). The RUB1/NEDD8 modification enhances SCF activity, probably through the recruitment of its cognate E2 (Kawakami et al., 2001). The CUL1 subunit of SCF belongs to the cullin family, which contains six members in human (Kipreos et al., 1996). Only hCUL1, hCUL2, and hCUL5 have been shown to be part of functional ubiquitin ligase complexes (Lyapina et al., 1998; Stebbins et al., 1999; Querido et al., 2001). However, all six cullins have been reported to interact with the same ring finger protein, RBX1, and to have the potential of being modified by RUB1/NEDD8, suggesting that all of them could form ubiquitin ligase complexes (Hori et al., 1999; Ohta et al., 1999). Interestingly, in human, all six cullins can copurify with CSN (Lyapina et al., 2001). By removing RUB1/NEDD8 from the cullin, CSN could trigger the termination of the polyubiquitination process and the release of the substrate from a wide array of ubiquitin ligases. This finding is in agreement with the recent report that CSN mutants accumulate higher amounts of polyubiquitylated proteins (Peng et al., 2001a).

Together, these results suggest that CSN acts as a central regulator of the protein degradation process, ensuring that a specific substrate will be modified by ubiquitin and properly degraded by the proteasome.

To reveal the molecular mechanism of CSN action, a thorough analysis of its composition and structure is needed. Here, we report the cloning and characterization of subunit 2 (AtCSN2) of the COP9 signalosome. We show that AtCSN2 is encoded by FUS12, one of the Arabidopsis COP/DET/FUS loci. Furthermore, we report that AtCSN2 can make direct contact with distinct members of the cullin family from Arabidopsis. We propose that this is a conserved interaction module through which the COP9 signalosome can regulate multiple cullin-based E3 ligases. This study concluded the characterization of the Arabidopsis CSN subunits. It allowed us to investigate the phylogenetic relationships between CSN and the structurally related lid of the proteasome and to perform a comprehensive interaction analysis among all subunits.

RESULTS

Cloning and Molecular Characterization of the AtCSN2 Gene

To clone the gene that encodes subunit 2 of the Arabidopsis CSN, we used the CSN2 peptide sequences obtained from the purified cauliflower CSN (Serino et al., 1999) to search the Arabidopsis database (http://www.Arabidopsis.org). A partial EST clone (H4H12T7) was retrieved. Using specific primers, we performed a 5′ rapid amplification of cDNA ends analysis to obtain the 5′ portion of the gene (see Methods). Meanwhile, we were able to retrieve a full-length genomic clone encompassing the complete open reading frame for AtCSN2 from the Arabidopsis database. According to the sequence of the genomic clone and our 5′ rapid amplification of cDNA ends analysis, we amplified by reverse transcription PCR the full-length cDNA for AtCSN2.

The full coding region of the cDNA for AtCSN2 is 1317 bp long and encodes a protein of 439 amino acids with a predicted molecular mass of 51 kD. This molecular mass is slightly smaller than our predicted size for the cauliflower CSN2, which runs in SDS-PAGE with an apparent molecular mass of 54 kD (Serino et al., 1999). Two possible explanations for this discrepancy can be hypothesized: as a result of its conformation, the cauliflower CSN2 protein could run abnormally in SDS-PAGE. Alternatively, the cauliflower CSN2 protein could be modified post-translationally and thus have a molecular mass higher than predicted. Indeed, potential phosphorylation and glycosylation sites are found within the AtCSN2 amino acid sequence.

The AtCSN2 protein is closely related to its homologs from human (hCSN2; 60% identity) and Schizosaccharomyces pombe (SpCSN2; 46% identity). In fact, CSN2 and CSN5 are the two most conserved subunits of the complex (Figure 1A). Interestingly, like all other CSNs with the exception of CSN5, we found no AtCSN2 homolog in the genome of Saccharomyces cerevisiae. As shown in Figure 1A, the identity among CSN2 subunits is particularly high in the C-terminal half of the protein, except for the last few amino acids. This conserved region includes the PCI domain (Proteasome-COP9 complex-eIF3), which encompasses amino acids 244 to 409. The PCI domain is a protein motif found in the other five CSN subunits and in components of the lid and the eIF3 complex (Hofmann and Bucher, 1998). Arabidopsis and human CSN2 also contain a sequence that has homology with a TPR region (amino acids 42 to 96), which could be a site of protein–protein interaction (Blatch and Lasalle, 1999). Four peptides sequenced from the cauliflower CSN2 display almost exact matches with AtCSN2 (Figure 1B).

The AtCSN2 Gene Maps to the FUS12 Locus, Whose Mutants Contain Molecular Lesions in the Gene

Mutations in seven pleiotropic COP/DET/FUS loci result in the apparent absence of CSN in Arabidopsis (Kwok et al., 1998; Wei and Deng, 1999). Five of those loci (COP9, FUS6/COP11, FUS5, COP8/FUS4, and FUS11) have been demonstrated to encode subunits of Arabidopsis CSN (Wei et al., 1994; Staub et al., 1996; Karniol et al., 1999; Serino et al., 1999; Peng et al., 2001b). Only two COP/DET/FUS genes (COP16 and FUS12) remain to be cloned, and these do not accumulate detectable CSN (Kwok et al., 1998). Therefore, it is possible that one of the two could encode AtCSN2. Indeed, the genomic clone that contains AtCSN2 is very close to the FUS12 locus, which has been mapped to position 61.2 centimorgan on chromosome II of the Arabidopsis genome (Kwok et al., 1996).

To determine whether the FUS12 gene encodes AtCSN2, we sought to identify mutations in the AtCSN2 sequence in two available alleles of fus12: fus12-R380 and fus12-U228 (Miséra et al., 1994; Kwok et al., 1998). The cDNA and genomic se-
quences of the AtCSN2 gene were amplified by PCR from the homozygous mutants and their corresponding wild-type ecotype (see Methods). Sequence analysis of the PCR products revealed a point mutation (transition from G to A) in the AtCSN2 gene of the fus12-U228 mutant (Figure 2A). This mutation removes the splice site between exon II and intron III, leaving the unspliced intron III in the mature mRNA and leading to premature termination of the open reading frame. The resulting mutant FUS12-U228 protein has only the first N-terminal 35 amino acids followed by a premature stop codon (Figure 2B). Similarly, sequencing of the fus12-R380 allele identified a single base pair substitution at the last nucleotide position of intron IV, introducing a premature stop. The predicted mutant version of the protein is only 135 amino acids long (FUS12-R380; Figure 2B).

Both fus12 alleles used for this analysis are lethal after the seedling stage and do not accumulate CSN (Kwok et al., 1998), indicating that the PCI domain is critical for the stability of AtCSN2 and of the whole complex.

Expression of AtCSN2 Rescues the Phenotype of the fus12 Mutant

To further confirm that AtCSN2 is encoded by the FUS12 gene, a genomic fragment containing the complete AtCSN2 open reading frame together with its own promoter was introduced in the fus12-R380 background to test functional complementation (GCSN2; Figure 2A). Because the homozygous fus12-R380 mutant is lethal, the transgene was transformed into heterozygous plants. The stably transformed plants heterozygous for both fus12-R380 and the transgene were selected, and their progeny were 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 fus12-R380 mutation and the transgene. If the AtCSN2 transgene rescues the mutant phenotype, the ratio of wild-type to mutant plants in the progeny should be 15:1 rather than 3:1, provided that the transgene is at a single locus unlinked to FUS12. Table 1 summarizes the segregation analyses of the T2 generation for six selected lines with a single T-DNA insertion per genome (3:1 ratio of resistant to sensitive). The wild-type to mutant ratios obtained are very similar to the 15:1 expected in the case of genetic complementation. Figure 2C shows that the phenotype of the fus12 mutant was rescued completely by the introduction of the AtCSN2 wild-type genomic clone. This result confirms that FUS12 encodes AtCSN2.

AtCSN2 Is an Integral Component of the COP9 Signalosome

Although CSN can accumulate stably only if all eight subunits are present, some of its single subunits can be stable as monomers, as has been demonstrated for CSN5 and CSN7 (Karniol et al., 1998; Kwok et al., 1998). CSN1, CSN3, CSN4, CSN6, and CSN8 appear to be stable only within the complex (Wei et al., 1994; Staub et al., 1996; Serino et al., 1999; Peng et al., 2001a, 2001b). To ascertain whether AtCSN2 cofractionates with the CSN and whether it exists in any other form, we raised polyclonal antibodies against the full-length AtCSN2 protein and performed gel-filtration analysis of the total protein extracts from light-grown seedlings (see Methods). The elution profiles of AtCSN2 and other representative CSN subunits are shown in Figure 3A. Like most CSN subunits, AtCSN4 is present only in the complex form, whereas AtCSN5 exists in both monomeric
Figure 2. The FUS12 Locus from Arabidopsis Corresponds to the Gene That Encodes AtCSN2.

(A) Genomic structure of the AtCSN2 locus and molecular nature of the mutations found in the fus12-U228 and fus12-R380 alleles. Introns are shown as lines; boxes and Roman numerals indicate exons. The 5’ and 3’ untranslated regions are shown by gray boxes, and the protein-encoding region is denoted by black boxes. A genomic fragment containing the complete genomic sequence of AtCSN2 together with its own promoter is indicated at top (GCSN2) and was used for the genetic complementation experiment. The two inserts are the mutated sequences found in fus12-R380 and fus12-U228. Numbers indicate base pairs.

(B) Wild-type and mutant versions of AtCSN2 proteins. The light gray boxes indicate the TPR homology region; the dark gray box indicates the location of the PCI domain. Numbers indicate amino acid positions.

(C) Seedling phenotype of Landsberg wild type (Ler-0), fus12-R380, and fus12-R380/AtCSN2, a representative line obtained by transforming the fus12-R380 mutant with GCSN2, indicating complementation of the mutant phenotype. All seedlings were grown in the light for 5 days.

and complex forms. AtCSN2 cofractionates with AtCSN4 and with the complexed form of AtCSN5, indicating that AtCSN2 is a component of CSN and that it is stable only when associated with the complex.

To further substantiate that AtCSN2 is part of CSN, we immunoprecipitated AtCSN2 from crude cell extracts and examined the immunoprecipitated protein by immunoblot analysis for the presence of other CSN subunits. As shown in Figure 3B, the AtCSN2 antibody, but not the preimmune serum, was able to immunoprecipitate AtCSN4 and AtCSN5 and, in a reciprocal experiment, was immunoprecipitated by AtCSN4 antibodies (data not shown). In addition, an AtCUL1 antibody immunoprecipitated the SCF<sup>TRP</sup> subunit ASK1 and AtCSN2 (Figure 3C). These results indicate that AtCSN2 is associated physically with CSN in vivo and that it is present only in the complex form.

AtCSN2 represents the last subunit of Arabidopsis CSN to be characterized, so this study concludes the genetic and molecular characterization of all eight AtCSN subunits. Table 2 contains a summary of all eight AtCSN subunits and their identities to their respective human counterparts.

**AtCSN2 Interacts Directly with Arabidopsis Cullin3**

As mentioned above, it has been shown that CSN can copurify with all six human cullins and that hCSN2 interacts directly with hCUL1 (Lyapina et al., 2001). Because AtCSN2 has been demonstrated to interact with AtCUL1 in the two-hybrid system (Schwechheimer et al., 2001), we hypothesized that, like human CSN, Arabidopsis CSN might be able to bind distinct cullin-containing complexes through a direct cullin-AtCSN2 interaction.

To test this hypothesis, we searched the Arabidopsis genome database (http://www.Arabidopsis.org) for Arabidopsis homologs of the human cullins. A recent sequence similarity search revealed that the Arabidopsis genome contains 11 cullin-related genes (Shen et al., 2002). In our analysis, we selected the six clones that displayed the closest homology with the six human cullins, according to a Basic Local Alignment Search Tool (BLAST) search using the human cullin sequences as queries. Figure 4A illustrates the results of our phylogenetic comparison between human and Arabidopsis cullins. The phylogenetic tree divides into three major branches. The first branch contains the human cullins hCUL1, hCUL2, and hCUL5, which do not seem to have any close homolog in Arabidopsis. The second branch contains only three Arabidopsis cullins: the previously characterized AtCUL1 (Gray et al., 1999) and two close homologs. The third branch is divided into two subgroups: the first contains the human cullins hCUL4A and hCUL4B and their Arabidopsis homolog, and the second contains hCUL3 and two highly homologous Arabidopsis proteins. We decided to focus our studies on the Arabidopsis CUL3 homolog At1g26830 (renamed AtCUL3) and went on to test its possible interaction with AtCSN2. We chose AtCUL3 because of its sequence divergence from AtCUL1 and its close homology with hCUL3, which has been shown to coinmunoprecipitate with the human CSN (Lyapina et al., 2001). We took advantage of a LexA-based yeast two-hybrid assay to assess possible interactions between AtCUL3 and AtCSN2 (see Methods). As a positive control, we used AtCUL1, which has been reported to interact with AtCSN2. These results are shown in Figure 4B. Remarkably, AtCUL3 interacted with AtCSN2 more strongly than AtCUL1. Therefore, CSN binds, and possibly regulates, different cullin-based E3 ligases in a conserved manner through direct contacts between AtCSN2 and cullins.

**Phylogenetic Analysis of CSN and Lid Subunits**

CSN exhibits a striking similarity, in terms of subunit composition and overall structure, to the lid subcomplex of the protea-
some (Glickman et al., 1998). Furthermore, each CSN subunit is similar to a specific lid subunit. Therefore, it has been suggested that the two complexes might have evolved from a common ancestor. The availability of complete sets of mammalian and plant CSN subunits prompted us to investigate the evolutionary relationships of the lid and the CSN. Therefore, we conducted a phylogenetic analysis of all eight CSN subunits and their lid paralogs by comparing their amino acid sequences from representative organisms. The sequences used for the analysis are indicated in the supplemental data online, and the results obtained from this analysis are shown in Figure 5. The phylogenetic trees for each CSN subunit and its corresponding lid subunit (RPN) always consist of two clades. It is important to note that, for every tree, the subunits from one of the two protein complexes, although from different organisms, including yeast, are grouped in the same clade. Therefore, the duplication and subsequent divergence events that led to the genes that encode CSN and lid subunits occurred before the divergence of unicellular and multicellular eukaryotic organisms. In our analysis, every tree reveals the same branching pattern and thus reflects the evolutionary history of the organisms selected. For instance, the yeast sequences (budding yeast and/or fission yeast), which are the most diverged, always lie at the base of the clade, whereas the sequences from budding yeast and fission yeast are clustered together as a basal group in the RPN3, RPN7, RPN8, and RPN12 trees, whereas only budding yeast sequences are found at the base of the branch in the RPN3, RPN6, and RPN11 trees. In RPN5 and RPN9, Arabidopsis is grouped together at the top of the clade. The sequences from Caenorhabditis elegans, however, are not positioned stably in all of the trees, probably because their amino acid substitution rates for each subunit differ from those of other organisms.

We also noticed that the branching patterns among the eight RPN subunits of the lid differ slightly. For example, the budding and fission yeast are clustered together as a basal group in the RPN7, RPN8, and RPN12 trees, whereas only budding yeast sequences are found at the base of the branch in the RPN3, RPN6, and RPN11 trees. In RPN5 and RPN9, Arabidopsis is clustered together with budding and fission yeast. These differences might be attributable to a slightly uneven evolutionary rate among the sequences of individual subunits, possibly reflecting their different functions. One important point from our sequence comparison is that the CSN subunits are more conserved than the lid subunits, which implies that CSN subunits either originated later or were exposed to greater selective pressure than the lid subunits. More sequence information from other organisms, especially from the basal unicellular organisms, is needed to reveal the complete evolutionary history of CSN and lid subunits.

**Table 1. Phenotype Complementation of the fus12 Mutation by Genomic AtCSN2**

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Resistant</th>
<th>Sensitive</th>
<th>Resistant:Sensitive Ratio</th>
<th>Wild Type</th>
<th>fusca</th>
<th>Wild-Type:fusca Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2.1</td>
<td>90</td>
<td>30</td>
<td>3.0:1</td>
<td>90</td>
<td>7</td>
<td>14.8:1.1</td>
</tr>
<tr>
<td>T2.3</td>
<td>80</td>
<td>28</td>
<td>2.9:1</td>
<td>110</td>
<td>9</td>
<td>14.7:1.2</td>
</tr>
<tr>
<td>T2.4</td>
<td>94</td>
<td>32</td>
<td>2.9:1</td>
<td>150</td>
<td>11</td>
<td>14.9:1.0</td>
</tr>
<tr>
<td>T2.6</td>
<td>85</td>
<td>27</td>
<td>3.0:8</td>
<td>90</td>
<td>8</td>
<td>14.6:1.3</td>
</tr>
<tr>
<td>T2.10</td>
<td>105</td>
<td>36</td>
<td>2.9:1</td>
<td>115</td>
<td>9</td>
<td>14.8:1.1</td>
</tr>
<tr>
<td>T2.11</td>
<td>94</td>
<td>31</td>
<td>3.0:1</td>
<td>120</td>
<td>10</td>
<td>14.7:1.2</td>
</tr>
</tbody>
</table>

The segregation ratios were calculated on T2 seedlings. Resistant and sensitive ratios were calculated according to the ability of the seedlings to survive on gentamycin-containing Murashige and Skoog (1962) medium (see Methods). The mutant (fusca) and wild-type phenotypes were scored at 10 days after germination on Murashige and Skoog (1962) medium without antibiotics (see Methods).

**Pair-Wise Yeast Two-Hybrid Interaction Analysis of the Eight COP9 Signalosome Subunits from Arabidopsis and Mammals**

A key issue lacking from our current understanding of CSN is its full complement of subunit–subunit contacts and the three-dimensional topology of the complex. In an effort to map CSN topology, several attempts have been made that used electron microscopy, the yeast two-hybrid assay, and in vitro binding assays (Kapelari et al., 2000; Fu et al., 2001). Previous yeast two-hybrid studies have been used successfully to reveal interactions among several known CSN subunits. For instance, we have shown that AtCSN4, AtCSN5, and AtCSN6 are capable of interactions with other specific CSN subunits, whereas an extensive interaction study among selected CSN subunits has revealed the existence of a central core within the complex composed of CSN4, CSN5, CSN6, and CSN7 (Kwok et al., 1998; Serino et al., 1999; Fu et al., 2001; Peng et al., 2001a). With the cloning of the last subunit, we performed a comprehensive pair-wise interaction screen of all eight CSN subunits from Arabidopsis (Figure 6A). In our assay, all eight subunits were fused to both the LexA DNA binding domain (DB) and the activation domain (AD). The constructs then were transformed into yeast strains suitable for the interaction assay (see Methods).

Subunit interactions were quantified using a lacZ reporter gene. Positive interactions in the plate assay (Figure 6A) were tested again with a liquid β-galactosidase activity assay (Figure 6B) (see Methods). All of the protein constructs examined, with the exception of DB-AtCSN6, expressed the predicted fusion proteins in yeast at similar levels, as determined by immunoblot analysis (data not shown). DB-AtCSN6 was found to be lethal to yeast; the only colonies recovered from the DB-AtCSN6 transformation plate were white in all assays and did not express any LexA fusion protein. Therefore, with the exception of DB-AtCSN6, the observed differences in reporter expression should indicate the relative strength of the interaction. In summary, 23 interacting pairs were identified from this comprehensive assay: CSN1–CSN1, CSN1–CSN2, CSN1–CSN3, CSN1–CSN4, CSN1–CSN5, CSN1–CSN7, CSN1–CSN8, CSN2–CSN3, CSN2–CSN4, CSN2–CSN5, CSN2–CSN6, CSN3–CSN4, CSN3–CSN5, CSN3–CSN6, CSN3–CSN7, CSN3–CSN8, CSN4–CSN5, CSN4–CSN6, CSN4–CSN7, CSN4–CSN8, CSN5–CSN6, CSN5–CSN7, CSN5–CSN8, CSN6–CSN7, CSN6–CSN8, and CSN7–CSN8.
CSN2–CSN4, CSN3–CSN3, CSN3–CSN6, CSN3–CSN7, CSN3–CSN8, CSN4–CSN4, CSN4–CSN5, CSN4–CSN6, CSN4–CSN7, CSN4–CSN8, CSN5–CSN6, CSN6–CSN7, CSN7–CSN8, and CSN8–CSN8. Based on these data, we confirmed all previously reported results (Serino et al., 1999; Fu et al., 2001; Peng et al., 2001a) and detected six new interactions. For instance, we were able to test the binding ability of AtCSN2 for the first time and found that it interacts very strongly with AtCSN1 and, less intensely but very reproducibly, with AtCSN4. Although the AtCSN1–AtCSN2 interaction seems to be very well conserved, because it has been found in fission yeast and human as well (Mundt et al., 1999; Tsuge et al., 2001), the interaction between AtCSN4 and AtCSN2 is novel and has not been found for the corresponding subunits of any other organism tested. Remarkably, some of the interactions were demonstrated in only one of the two possible configurations. This find-

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**Table 2. Summary of the Eight AtCSN Subunits and Their Corresponding Genes**

<table>
<thead>
<tr>
<th>Subunit Number</th>
<th>Designated Name</th>
<th>Gene/Locus Name</th>
<th>Identity to Human (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AtCSN1</td>
<td>FUS6/COP11</td>
<td>44.7</td>
<td>Staub et al. (1996)</td>
</tr>
<tr>
<td>2</td>
<td>AtCSN2</td>
<td>FUS12/COP12</td>
<td>61.1</td>
<td>This work</td>
</tr>
<tr>
<td>3</td>
<td>AtCSN3</td>
<td>FUS11/COP13</td>
<td>42.0</td>
<td>Peng et al. (2001b)</td>
</tr>
<tr>
<td>4</td>
<td>AtCSN4</td>
<td>COP8/FUS4</td>
<td>49.5</td>
<td>Serino et al. (1999)</td>
</tr>
<tr>
<td>5</td>
<td>AtCSN5</td>
<td>AJH1 and AJH2</td>
<td>62.0</td>
<td>Kwok et al. (1998)</td>
</tr>
<tr>
<td>6</td>
<td>AtCSN6</td>
<td>AtCSN6a and AtCSN6b</td>
<td>39.8</td>
<td>Peng et al. (2001a)</td>
</tr>
<tr>
<td>7</td>
<td>AtCSN7</td>
<td>FUS5/COP15</td>
<td>34.4</td>
<td>Karniol et al. (1999)</td>
</tr>
<tr>
<td>8</td>
<td>AtCSN8</td>
<td>COP9/FUS7/FUS8</td>
<td>32.3</td>
<td>Wei et al. (1994)</td>
</tr>
</tbody>
</table>
ing could be attributable to misfolding of the proteins when fused to either the DB or the AD moiety, resulting in fusion proteins that cannot function in the yeast interaction assay.

To examine the evolutionary conservation of CSN interactions, we performed a similar experiment with the mammalian CSN subunits and tested all of their possible pair-wise interactions (see Methods). The average results from the liquid β-galactosidase activity assays for all combinations are shown in Figure 6C. Because the DB-CSN3 fusion protein showed transactivation activity by itself, the interaction of CSN3 with other subunits was tested only in the AD-CSN3 configuration. For the other seven subunits, the pair-wise interactions were examined in both vector configurations. All of the proteins were expressed at comparable levels in yeast, as judged by immunoblot analysis of the total yeast protein extracts (data not shown). The results from this comprehensive yeast two-hybrid assay confirmed 11 pair-wise interactions and 2 self-interactions already found for the Arabidopsis CSN subunits and revealed 2 new self-interactions. In summary, the subunit-subunit interactions among mammalian CSN subunits are as follows: CSN1–CSN2, CSN1–CSN3, CSN1–CSN4, CSN2–CSN4, CSN2–

CSN6, CSN3–CSN4, CSN3–CSN6, CSN3–CSN8, CSN4–CSN6, CSN4–CSN7, CSN4–CSN8, and CSN5–CSN6. In addition, self-interactions were found for four subunits: CSN2, CSN4, CSN6, and CSN8, suggesting that they could form dimers or oligomers within the complex or mediate potential CSN–CSN interactions. It is worth noting that, although the CSN2 self-interaction was not detected for AtCSN2, it was clearly detected in mammals. In conclusion, even though we found fewer positive interacting pairs with the mammalian subunits, the results from

Figure 4. AtCSN2 Interacts with Different Members of the Cullin Family.
(A) Phylogenetic relationship of the six human cullins and their putative Arabidopsis homologs. The tree was generated using the CLUSTAL algorithm in the Megalign program (DNASTAR).
(B) AtCSN2 interacts with AtCUL1 and AtCUL3. The interactions between AD-AtCSN2 and AtCUL1 or AtCUL3 fused to the DB are shown by black bars. The background controls (AtCUL1 or AtCUL3 interacting with AD alone) are indicated with gray bars. Numbers on the y-axis are average values of at least six independent transformants. Error bars represent SD.

Figure 5. Evolutionary Relationships of CSN and Lid Subunits (RPN) from Selected Organisms.
Numbers above the branches indicate support values. The GenBank accession numbers for the proteins listed are given in the supplemental data online. at, Arabidopsis thaliana; ce, Caenorhabditis elegans; dm, Drosophila melanogaster; hs, Homo sapiens; sc, Saccharomyces cerevisiae; sp, Schizosaccharomyces pombe.

A

CSN1+RPN7

B

CSN2+RPN6

C

CSN3+RPN3

D

CSN4+RPN5

E

CSN5+RPN11

F

CSN6+RPN8

G

CSN7+RPN9

H

CSN8+RPN12

hsCSN1

dmCSN1

atCSN1

ceCSN1

spCSN1

hsRPN7

dmRPN7

atRPN7

ceRPN7

spRPN7

scRPN7

hsCSN1

dmCSN1

atCSN1

ceCSN1

spCSN1

hsRPN6

dmRPN6

atRPN6

ceRPN6

spRPN6

scRPN6

hsCSN4

dmCSN4

atCSN4

ceCSN4

spCSN4

hsRPN5

dmRPN5

atRPN5

ceRPN5

spRPN5

scRPN5

hsCSN6

dmCSN6

atCSN6

ceCSN6

spCSN6

hsRPN8

dmRPN8

atRPN8

ceRPN8

spRPN8

scRPN8

hsCSN8

dmCSN8

atCSN8

ceCSN8

spCSN8

hsRPN12

dmRPN12

atRPN12

ceRPN12

spRPN12

scRPN12
the two yeast two-hybrid matrices complement and largely overlap each other.

Interacting Pairs among CSN Subunits Can Be Identified by Correlation Analysis of Their Evolutionary Rates

The data obtained for the interactions of mammalian and Arabidopsis subunits demonstrate that the topological architecture of CSN likely remained conserved throughout evolution. This finding suggests that it would be feasible to compare the interacting pairs among different organisms to ascertain their conservation and coevolution. Proteins that interact are known to coevolve, because molecular interactions require precise structures that impose constraints on the evolutionary rate (Fraser et al., 2002). If two proteins are correlated in their functions, the changes of amino acids in one protein will cause changes in the other. Therefore, to substantiate the CSN subunit–subunit interactions, we measured the correlation of the evolutionary rates of the genes that encode CSN subunits. A similar approach has been used to identify interacting proteins in other systems (He and Wu, 1993). To this end, we compared the ratio of nonsynonymous and synonymous substitution rates ($dN/dS$) of the CSN subunits from selected organisms (see Methods). $dN$ and $dS$ represent the number of nonsynonymous and syn-
Identity of subunit 2 of the COP9 signalosome (AtCSN2) as FUS12, thus bringing to six the number of CSN subunits identified by their corresponding COP/DET/FUS loci. Definitive evidence confirmed that AtCSN2 is a subunit of the COP9 signalosome. Thus, the cloning of AtCSN2 concludes the molecular characterization of the eight AtCSN subunits and made possible an overall topology and interaction analysis of the entire complex.

DISCUSSION

Here, we report the molecular identity of subunit 2 of the COP9 signalosome (AtCSN2) as FUS12, thus bringing to six the number of CSN subunits identified by their corresponding COP/DET/FUS loci. Definitive evidence confirmed that AtCSN2 is a subunit of the COP9 signalosome. Thus, the cloning of AtCSN2 concludes the molecular characterization of the eight AtCSN subunits and made possible an overall topology and interaction analysis of the entire complex.

An Updated Structural Topology Map for CSN

Our comprehensive subunit interaction study also provides further insight into the molecular architecture of the COP9
with and regulates the ubiquitin ligase SCF TIR1. This interaction was demonstrated previously that in Arabidopsis, CSN interacts with and regulates the ubiquitin ligase SCF TIR1. This interaction is direct, because specific SCF subunits interact with specific CSN subunits. Among those subunits, AtCSN2 has been shown to interact with AtCUL1. We report here that AtCSN2 also can interact with AtCUL3, which is another protein belonging to the cullin family that is distantly related to AtCUL1 (Figure 6). It is possible that part of the reason why CSN2 is one of the two most conserved subunits during evolution is their need to cooperate, through direct physical contacts, with the cullins.

Our new findings are in agreement with the current model, in which the COP9 signalosome regulates several classes of cullin-containing ubiquitin ligases and possibly other classes of E3s as well, probably through contacts between specific CSN subunits and selected members of the protein degradation machinery (Schwechheimer et al., 2001, 2002). This notion is supported by the recent cloning of COP10, a ubiquitin-conjugating enzyme variant that binds to COP1, to a putative E3 ligase, and to specific CSN subunits that are different from those that contact SCF complexes (Figure 8) (Suzuki et al., 2002). Remarkably, mutations in COP10, COP1, and CSN yield a similar seedling phenotype (Kwok et al., 1996). However, although cop1 and cop10 weak alleles are viable and can survive to the adult stage, all of the CSN mutant alleles found to date are lethal. This would be expected if CSN functions as a general regulator of many E3 ligases, which control a variety of developmental processes (Figure 8).

**METHODS**

**Plant Material and Growth Conditions**

Plant germination and growth conditions for Arabidopsis thaliana were as described previously (Wei and Deng, 1992). All seedlings were vernalized for 1 week at 4°C before transfer to growth chambers. Unless stated otherwise, the growth chamber was used under a cycling long-day photoperiod (16 h of light at 48 μmol m⁻² s⁻¹ and 8 h of dark) at 22°C. Wild-type plants were in the Columbia or Landsberg background. fus12-R380 and fus12-U228 were in the Landsberg background (Miséra et al., 1994).
Isolation of the AtCSN2 cDNA and Sequence Analysis of AtCSN2 in the fus12-R380 and fus12-U228 Alleles

Four peptide sequences from three different HPLC fractions (A54-29, A54-73, and A54-50; Figure 1B) (Serino et al., 1999) were used to search the Arabidopsis database (http://www.Arabidopsis.org). A partial EST clone (H4H1277; obtained from the ABRC [Columbus, OH]) was retrieved. The EST sequence and the four peptides also were found to correspond to a completely sequenced BAC clone (T20P8) on chromosome II as part of the Arabidopsis genome-sequencing project.

RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA). The 5′ end of the gene was determined using the 5′ rapid amplification of cDNA ends kit (Invitrogen) according to the manufacturer’s instructions. The complete full-length cDNA clone was obtained by reverse transcription PCR (Advantage RT-for-PCR kit; Clontech, Palo Alto, CA) of RNA extracted from 1-week-old Columbia seedlings using 21-bp primers specific for the 5′ and 3′ ends of the gene, starting from the ATG and the stop codon, respectively. 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. Pfu polymerase (Invitrogen) was used. The PCR product was cloned with the TOPO-TA cloning kit in the vector pCR-II (Invitrogen), and the sequence was verified. The PCR product was cloned with the TOPO-TA cloning kit in the vector pCR-II (Invitrogen), and the sequence was confirmed by direct sequencing. The first Met residue was determined by comparison with the CSN2 amino acid sequence from its animal counterpart (Figure 1A) and by the observation that a stop codon and a clear TATA box were found 42 and 60 bp, respectively, upstream of the ATG codon.

To determine the mutation of the AtCSN2 gene in the fus12 alleles, 1 μg of total RNA from 1-week-old seedlings homologous for each allele and their respective wild-type ecotypes was used for reverse transcription PCR (see above). Mutations were confirmed further by PCR and sequencing of the corresponding genomic region. The PCR conditions were the same as those described above. The PCR products were isolated using the QIAEXII gel extraction kit (Qiagen, Chatsworth, CA) and then sequenced directly. For the genomic DNA, at least four independent PCR products for each of the mutant alleles and their corresponding ecotypes were cloned in vector pCRII with the TOPO-TA cloning kit (Stratagene) and sequenced to confirm the mutation. The sequence data were analyzed using LaserGene software (DNASTAR, Madison, WI).

Complementation of the fus12 Mutation by the AtCSN2 cDNA

The BAC clone T20P8, obtained from the ABRC, was digested with the enzyme XbaI to generate a fragment of 6762 bp that contains the complement TATA box were found 42 and 60 bp, respectively, upstream of the ATG codon.

The 5′ end of the gene was determined using the 5′ rapid amplification of cDNA ends kit (Invitrogen) according to the manufacturer’s instructions. The complete full-length cDNA clone was obtained by reverse transcription PCR (Advantage RT-for-PCR kit; Clontech, Palo Alto, CA) of RNA extracted from 1-week-old Columbia seedlings using 21-bp primers specific for the 5′ and 3′ ends of the gene, starting from the ATG and the stop codon, respectively. 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. Pfu polymerase (Invitrogen) was used. The PCR product was cloned with the TOPO-TA cloning kit in the vector pCR-II (Invitrogen), and the sequence was confirmed by direct sequencing. The first Met residue was determined by comparison with the CSN2 amino acid sequence from its animal counterpart (Figure 1A) and by the observation that a stop codon and a clear TATA box were found 42 and 60 bp, respectively, upstream of the ATG codon.

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

The full-length cDNA clone for AtCSN2 was cloned in the vector pGEX4T1 (Amersham Pharmacia Biotech). The resulting glutathione S-transferase–AtCSN2 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-CSN4 (Serino et al., 1999), anti-AtCSN5 (Kwok et al., 1998), anti-TBP (Schwechheimer et al., 2001), anti-AtCUL1, and anti-ASK1 (Gray et al., 1999).

Total soluble protein extracts from fus12-R380, fus12-U228, and wild-type Columbia and Landsberg seedlings were obtained and used for all immunoblot analyses, as described previously (Staub et al., 1996). For gel filtration, the total soluble protein extract was fractionated through a Superose 6 HR 10/30 gel-filtration column (Amersham Pharmacia Biotech) and examined by immunoblot analysis, as described previously (Kwok et al., 1998).

Yeast Two-Hybrid Assay

The full-length cDNA clone for AtCSN2 was cloned as a translational fusion to the LexA DNA binding domain (in pEG202) or to the transcription activation domain (in pJG4-5) (Golemis et al., 1994). The two new plasmids were designated pEG-AtCSN2 and pJG-AtCSN2. For the Arabidopsis CSN two-hybrid pair-wise interaction assay, the other plasmids used in the assay were pEG-AtCSN1 and pJG-AtCSN1 (formerly FUS6/ COP11; Kwok et al., 1998); pEG-AtCSN3 and pJG-AtCSN3 (Itoh et al., 2001b); pEG-CSN4 and pJG-CSN4 (formerly COP8; Serino et al., 1999); pEG-AtCSN5 and pJG-AtCSN5 (formerly AUH1; Kwok et al., 1998); pEG-AtCSN6 and pJG-AtCSN6 (Peng et al., 2001a); pEG-AtCSN7 and pJG-AtCSN7 (formerly FUS5; Karniol et al., 1999); and pEG-AtCSN8 and pJG-AtCSN8 (formerly COP9; Kwok et al., 1998). For the CSN two-hybrid assay with mammalian subunits, all constructs have been reported previously (Lyapina et al., 2001; Tsuge et al., 2001). For the cullin interaction assay, the full-length cDNA for AtCUL3 was cloned in the pE vector (pEG-AtCUL3). The other clones used were pEG-AtCUL1 (Schwechheimer et al., 2001) and pJG-AtCSN2 (this study). All of the LexA fusion constructs were transformed into the yeast strain EGY48. The activation domain fusion constructs were transformed into the yeast strain L40 (Invitrogen), which contains a β-galactosidase reporter gene. The transformants were selected and mated (Bendixen et al., 1994), and the resulting diploids were placed in medium selective for the diploids and containing X-Gal as a substrate for detection of the interaction. Selected pair-wise interactions were analyzed further by liquid assay, as described previously (Serino et al., 1999).

Phylogenetic and Correlation Analyses

The CSN sequences were obtained by a Basic Local Alignment Search Tool (BLAST) search of all databases at the National Center for Biotechnology Information World Wide Web site (http://www.ncbi.nlm.nih.gov/). A complete list of the sequences used, together with their GenBank accession numbers, is provided in the supplemental data online. All phylogenetic analyses were performed using maximum likelihood with PAUP (Phylogenetic Analysis Using Parsimony and other methods) version 4 (Rogers and Swofford, 1998).

For ratio of nonsynonymous versus synonymous substitution rate (dN/dS) correlation studies, the confirmed amino acid sequences from different organisms of the same CSN subunit were aligned using CLUSTALX (Thompson et al., 1997). The DNA sequences of the genes that code for the subunits then were aligned manually according to their deduced amino acid sequences. The YN00 program in PAML version 3.0 (http://abacusgene.ucl.ac.uk/software/paml.html) was used to calculate the dN/dS between pairs of gene sequences of the same subunit from different organisms. The correlation between pairs of dN/dS was determined using Excel (Microsoft, Redmond, WA) to evaluate the subunit-to-subunit relationship. The statistical significance of the correlation analysis was measured with the Pearson coefficient.

In Vivo Cross-Linking

The top layer tissues of the cauliflower (Brassica oleracea botrytis) inflorescences (~1 mm) were incubated with 10 volumes of 1% formaldehyde in PBSM buffer (1.76 mM KH2PO4, 10 mM Na2HPO4, 136 mM NaCl, 2.6 mM KCl, 5 mM MgCl2, 10% glycerol) under vacuum for 30 min.
The reaction was stopped by adding Gly to a final concentration of 0.125 M for 5 min. The tissue was washed three times with protein extraction buffer before protein extraction (Peng et al., 2001a). Protein extracts were subjected to SDS-PAGE and immunoblot analysis with antibodies to selected CSN subunits. For immunoprecipitation, the formaldehyde-treated protein extracts were subjected to denaturing treatment with 8 M urea. The urea was removed by gradient buffer exchange, and the soluble proteins were used for immunoprecipitation studies.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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NP199433 (At5g46210), NP171797 (At1g02980), NP175007 (At1g43140), NP_003469 (hCUL5), NP174005 (At1g26830), NP176266 (At1g69670), NP_003581 (hCUL3), AF077188 (hCUL4A), NP_003579 (hCUL4B), Accession numbers for the human proteins and Arabidopsis cullins shown in Figure 4 are as follows: NP.003583 (hCUL1), AAD23581 (hCUL2), NP.003581 (hCUL3), AF077188 (hCUL4A), NP.003579 (hCUL4B), NP.003484 (hCUL5), NP174005 (At1g26830), NP176266 (At1g69670), NP199433 (At5g46210), NP171797 (At1g02980), NP175007 (At1g43140), and AAK76704 (AICUL1).

REFERENCES


