Arabidopsis CAND1, an Unmodified CUL1-Interacting Protein, Is Involved in Multiple Developmental Pathways Controlled by Ubiquitin/Proteasome-Mediated Protein Degradation

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Ubiquitin/proteasome-mediated protein degradation controls various developmental pathways in eukaryotes. Cullin-containing complexes are both versatile and abundant groups of RING family ubiquitin E3 ligases, whose activities are subject to control by RUB/Nedd8 (for related to ubiquitin/neural precursor cell-expressed developmentally downregulated 8) modification of their cullin subunits. Here, we report the identification of an Arabidopsis thaliana counterpart of human CAND1 (cullin-associated and neddylation-dissociated) and demonstrate that it can preferentially interact with unmodified CUL1. The Arabidopsis cand1-1 null mutant displays distinct phenotypes, including late flowering, aerial rosettes, floral organ defects, low fertility, dwarfism, loss of apical dominance, and altered responses to multiple plant hormones. Molecular analyses show that many of these defects are because of compromised activity of CUL1-containing ubiquitin E3 ligases, indicating that CAND1 is required for their optimal activity. Furthermore, the cand1-1 mutant displays a partial constitutive photomorphogenic phenotype and has defects in HY5 degradation in the absence of light, a process mediated by a different RING family E3, COP1. Thus, our data provides genetic support for a critical role of CAND1 in regulating various ubiquitin E3 ligases and their targeted cellular and developmental pathways.

INTRODUCTION

The ubiquitin/proteasome system is a universal selective proteolysis system in eukaryotes, in which target proteins are ubiquitinated and subsequently degraded by the 26S proteasome. Protein ubiquitination requires the coordinated action of a series of three distinct enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (Hershko and Ciechanover, 1998). Ubiquitin E3 ligases are capable of recruiting substrates and catalyzing the transfer of ubiquitin moieties from the E2 to the substrates. Thus, they are largely responsible for the substrate specificity of the ubiquitin/proteasome system (Vierstra, 2003).

Cullin-containing complexes, which belong to the RING superfamily, are probably the most abundant group of ubiquitin E3 ligases. Cullin proteins can be clustered phylogenetically into five clades (Risseeuw et al., 2003). CUL1 is the best characterized cullin, which forms the SKP1/CUL1/ROC1/F-box protein (SCF) complex. SCF complexes can recruit ubiquitin-conjugated E2s through the RING finger protein ROC1 (also known as RBX1 or HRT1) and different substrates through divergent F-box proteins (Deshaies, 1999; N. Zheng et al., 2002). Some other cullin family members have also been shown to form SCF-like ubiquitin E3 ligase complexes in mammalian cells. CUL2 (or CUL5) forms a complex with ROC1, elongin B, elongin C, and BC-box proteins (Kamura et al., 1998, 2001; Kaelin, 2002). CUL3 forms a complex with the ROC1 and BTB proteins (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003b; Xu et al., 2003). CUL4A forms complexes with ROC1, DDB1, and DDB2, or CSA, or DET1/COP1 (Groisman et al., 2003; Wertz et al., 2004).

In Arabidopsis thaliana, although multiple cullins are present, only CUL1-containing SCF-type ubiquitin E3 ligases have been characterized at the biochemical and functional levels. On the other hand, Arabidopsis COP1, a repressor of photomorphogenesis in darkness, has been studied in detail. COP1 is a RING finger protein (von Arnim and Deng, 1993) and exhibits in vitro ubiquitin E3 ligase activity toward photomorphogenesis-promoting transcription factors on its own (Saijo et al., 2003; Seo et al., 2003), hence representing a different type of RING family E3. Recently, its human counterpart has been suggested to be part of a ubiquitin E3 ligase complex (Zwart et al., 2004). The ubiquitin-like protein RUB (for related to ubiquitin; Arabidopsis and yeast), also known as Nedd8 (for neural precursor cell-expressed developmentally downregulated 8; human),
CAND1's developmental functions will be essential.

each of the mammalian studies was performed in vitro or in
ubiquitin E3 ligases (J. Zheng et al., 2002). However, because
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vitro cullin-containing ubiquitin E3 ligase activities are inhibited
to antagonize RUB/Nedd8 modification, it is not surprising that in
2003; Oshikawa et al., 2003). Because CAND1 binding appears
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lines inhibits CUL1/SKP1 binding, and either RUB/Nedd8 deconjugation
are required to regulate assembly and activity of cullin-containing ubiquitin E3 ligases (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003). However, various genetic studies suggest that CN3 is necessary for the optimal activity of multiple cullin-containing ubiquitin E3 ligases (Schwechheimer et al., 2001; Cope et al., 2002; Feng et al., 2003; Groisman et al., 2003; Liu et al., 2003; Pintard et al., 2003a; Wang et al., 2003). Therefore, it is hypothesized that dynamic cycles of RUB/Nedd8 deconjugation and deconjugation are required to regulate assembly and activity of cullin-containing ubiquitin E3 ligases (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wolf et al., 2003). However, the mechanism for this regulation and the key players remain to be identified.

Recently, human CAND1 (cullin-associated and neddylation-dissociated), previously known as TIP120A (TATA binding protein interacting protein; Yogosawa et al., 1996), has been identified as a potential key player in the assembly of cullin-containing ubiquitin E3 ligases and has been shown to selectively bind unmodified cullins. Overexpressing CAND1 in cultured cell lines inhibits CUL1/SKP1 binding, and either RUB/Nedd8 modification or SKP1 binding dissociates CAND1 from CUL1 (Liu et al., 2002; J. Zheng et al., 2002; Hwang et al., 2003; Min et al., 2003; Oshikawa et al., 2003). Because CAND1 binding appears to antagonize RUB/Nedd8 modification, it is not surprising that in vitro cullin-containing ubiquitin E3 ligase activities are inhibited by CAND1 (Liu et al., 2002; J. Zheng et al., 2002; Min et al., 2003). Still, like CN3, CAND1 knockdown in cultured mammalian cells causes accumulation of SCF substrates, implying that CAND1 might be important for the optimal activity of cullin-containing ubiquitin E3 ligases (J. Zheng et al., 2002). However, because each of the mammalian studies was performed in vitro or in cultured cells, a validation by genetic analysis and revelation of CAND1's developmental functions will be essential.

In this article, we report a molecular genetic analysis of the Arabidopsis CAND1 gene. We demonstrate that Arabidopsis CAND1 is preferentially associated with unmodified CUL1 in vivo. More importantly, we provide critical genetic evidence that CAND1 acts positively to regulate multiple ubiquitin E3 ligases and their associated developmental processes in plants.

RESULTS

Identification of the Arabidopsis CAND1 Gene

A homology search using human CAND1 sequence (Liu et al., 2002) identified a CAND1 homolog, At2g02560, in the Arabidopsis genome. The presence of multiple EST clones as well as a full-length cDNA (RAFL09-95-108) in the RIKEN collection supports the expression of At2g02560. The full-length open reading frame (ORF) of Arabidopsis CAND1 was cloned by RT-PCR using RNA isolated from wild-type Arabidopsis seedlings, and sequence analysis confirmed its 100% identity to the reported full-length cDNA.

The Arabidopsis CAND1 gene has 28 exons and encodes a protein of 1219 amino acids (accession number AY099857). The sequence identity of Arabidopsis CAND1 with other eukaryotic CAND1s is significant: 43% identity for mammals, 35% for Drosophila melanogaster, 33% for Caenorhabditis elegans, and 22% for fission yeast (Schizosaccharomyces pombe). A phylogenetic tree based on CAND1 protein sequence homologies shows that CAND1 is evolutionarily conserved (Figure 1A). In each of those organisms, the RUB/Nedd8 conjugation pathway is essential (Osaka et al., 2000; Tateishi et al., 2001; Kurz et al., 2002; Ou et al., 2002; Dharmasiri et al., 2003). Interestingly, CAND1 seems to be missing in budding yeast (Saccharomyces cerevisiae), where RUB/Nedd8 conjugation is not essential (Lammer et al., 1998; Liakopoulos et al., 1998). This is consistent with the notion that CAND1 may be functionally connected with RUB/Nedd8 modification.

Identification of cand1 T-DNA Insertional Mutants

To investigate the developmental function of Arabidopsis CAND1, we took advantage of the superior genetics available in Arabidopsis. We searched the available Arabidopsis T-DNAinsertional mutagenesis collections and obtained three independent lines (Figure 1B; Alonso et al., 2003; Rosso et al., 2003; see Methods). All three T-DNA insertion mutations studied behave recessively (data not shown). Polyclonal antibodies raised against Arabidopsis CAND1 can detect a single band migrating around 120 kD in wild-type Arabidopsis protein extracts but not in any of the three cand1 homozygous T-DNA insertion alleles (Figure 1C). Thus, all three mutants likely represent true null or severe loss-of-function mutations for CAND1. Interestingly, the CUL1 protein level, in either the RUB-modified form or unmodified form, is not affected by any of the three cand1 mutations (Figure 1C).

Initial Phenotype Analysis of cand1 Mutants

All three cand1 alleles produce similar phenotypes (Figure 1D). Basically, cand1 affects multiple aspects of the plant phenotype throughout development. Germination and seedling stage growth of cand1 mutants under normal conditions appear to be the same.
as wild-type Arabidopsis (data not shown). The phenotypes of cand1 mutants become more obvious later during development. As shown in Figure 1D, the rosette leaves of cand1 mutants are much smaller than those of wild-type plants and have a wavy morphology. The cand1 mutants flower later than wild-type plants, with an increased number of rosette leaves (Figures 1D and 4A), indicating that the vegetative to reproductive growth transition of the primary shoot apical meristem is affected. The growth of axillary meristems is also abnormal, where a large number of smaller leaves are produced (Figure 1D). Furthermore, the mutants have very low fertility, with less than one seed produced on average per silique (data not shown). Possibly
because of their poor fertility, mutant plants continue to make new flowers while wild-type plants of the same age start to senesce (Figure 1D).

Other notable phenotypes of cand1 mutants include dwarfism and loss of apical dominance. When examined closely, dwarfism largely results from reduced stem elongation (Figure 1D; data not shown), which is analogous to the gibberellin pathway mutants (Harberd et al., 1998). The cand1 mutants have a strong increase in the number of secondary inflorescences (Figure 1D), an indication that they have lost apical dominance, which is also observed in auxin response mutants (Lincoln et al., 1990; Estelle, 1992). In later sections, we will provide evidence that both gibberellin and auxin pathways are affected in the cand1-1 mutant.

35S Promoter–Driven FLAG-CAND1 Partially Complements the cand1-1 Mutation

To further confirm that the phenotypes observed are indeed attributable to cand1 mutation, we constructed a chimeric gene using the constitutive 35S promoter of Cauliflower mosaic virus to drive expression of a CAND1 cDNA, with three copies of flag tags at the N terminus of CAND1 protein. This construct was introduced into a cand1-1 mutant background to test functional complementation. Because the homozygous cand1-1 mutant produces very few seeds and is severely dwarfed, we first stably transformed the transgene into Arabidopsis plants heterozygous for cand1-1 mutation. In T1 generation, we selected plants resistant to antibiotic markers for both the transgene and the T-DNA and further confirmed their genotypes through PCR-based genotyping and segregation in their selfed progenies (data not shown).

We examined expression levels of the FLAG-CAND1 fusion protein from the transgene as well as the endogenous CAND1 protein using total flower protein extracts. As shown in Figure 2A, although it’s driven by a strong constitutive promoter, the level of FLAG-CAND1 protein is lower than endogenous CAND1 (lines 3-12 and 5-2). The FLAG–CAND1 expression is not enhanced in the cand1-1 mutant background either (Figure 2A, lines 5-4 and 6-7). As a likely result of this low expression level of the FLAG–CAND1, cand1-1 mutant phenotypes are only partially rescued by the transgene. Nevertheless, all of the key defects of cand1 mutants discussed in the previous section, including late flowering time, low fertility, reduced plant size, loss of apical dominance, and other features, are partially rescued to various extents (Figure 2B, lines 5-4 and 6-7).

Furthermore, we identified several cosuppression lines, in which endogenous CAND1 protein abundance is downregulated by the presence of the transgene, and no FLAG–CAND1 fusion protein is expressed (Figure 2A, lines 5-22 and 6-2). Remarkably, like the partially rescued lines, the cosuppression lines also display intermediate phenotypes compared with wild-type and cand1 mutants (Figure 2B). Thus, this observation supports the notion that FLAG–CAND1 is fully active and that the partial rescue in lines 5-4 and 6-7 is because of the low levels of the FLAG–CAND1 protein. Taken together, our data corroborates the linkage between the phenotypes and the cand1 mutations. These
results also indicate that the level of CAND1 is critically important, suggesting that CAND1 may act as a rate-limiting factor in some cullin-containing ubiquitin E3 ligase function.

CAND1 Preferentially Interacts with Unmodified CUL1 in Vivo

Because FLAG-CAND1 is functional, it was used in an initial attempt to detect in vivo association of CAND1 and CUL1 by immunoprecipitation. When we pulled down FLAG-CAND1 using anti-FLAG antibody in extracts from the transgenic seedlings, we were able to detect the presence of unmodified CUL1 in the precipitate (Figure 3A). This result suggests that, like in mammals, CAND1 interacts with unmodified CUL1 in Arabidopsis. Because of the low ratio of RUB-modified CUL1 to unmodified CUL1 in the transgenic seedlings, the low sensitivity of this coimmunoprecipitation does not allow a conclusion to be drawn as to whether the RUB-modified CUL1 was excluded from precipitation or whether it was simply below the detection threshold (Figure 3A).

Next, to determine the specificity of the CAND1/unmodified CUL1 interaction, we took advantage of wild-type Arabidopsis plants that express the CUL1 protein with a modified tandem affinity purification (TAP) tag fused at either the N or C terminus. Whereas nearly half of the TAP-CUL1 (N-terminal fusion) is modified by RUB, all of the CUL1-TAP (C-terminal fusion) exists in the unmodified form (Figure 3B). Because RUB modification takes place at the CUL1 C terminus, a big C-terminal fusion such as TAP (34 kD) presumably blocks RUB conjugation. On the other hand, an N-terminal TAP fusion has no spatial conflict with RUB conjugation and for some unknown reason slows down RUB deconjugation. The different properties of these two fusion proteins provide an ideal system for testing their differential associations with CAND1 in vivo. Indeed, CAND1 can be readily detected in the IgG precipitate of CUL1-TAP but not in TAP-CUL1 (Figure 3B). Additionally, consistent with observations in human cells (Liu et al., 2002; J. Zheng et al., 2002), CAND1 is not coimmunoprecipitated with ASK1-TAP (C-terminal fusion) in vivo (Figure 3C). This observation demonstrates that CAND1 does not interact with the C-terminal TAP tag itself, and the association between CAND1 and CUL1-TAP is specific.

To further characterize the interaction between Arabidopsis CAND1 and CUL1, we tested their interaction in a yeast two-hybrid assay. Wild-type CUL1 interacts with CAND1 in yeast, as shown by an eightfold increase in β-galactosidase (β-gal) activity over the vector control (Figure 3D). When the RUB modification site on CUL1 (del Pozo and Estelle, 1999) is mutated from Lys to Arg (K682R), the β-gal activity is further increased by 10-fold (Figure 3D). It is shown in Figure 3E that Arabidopsis CUL1 is indeed modified by RUB in yeast, whereas the mutant CUL1 protein is not. Moreover, it appears that the amount of the mutated CUL1(K682R) and the total amount of the modified and unmodified wild-type CUL1 are of similar levels (Figure 3E). Thus,

Figure 3. CAND1 Selectively Interacts with Unmodified CUL1 in Vivo.

(A) Unmodified CUL1 was precipitated together with FLAG-CAND1. Seedling protein extracts prepared from wild-type Arabidopsis and 35S:FLAG-CAND1 transgenic Arabidopsis (line 6-7) were incubated with anti-FLAG antibody conjugated agarose (α-FLAG). The precipitates and the total extracts were subjected to immunoblot analysis with antibodies against FLAG, CUL1, and TATA binding protein (TBP).

(B) and (C) CAND1 associates with CUL1-TAP but not with TAP-CUL1 or ASK1-TAP in vivo. Total flower protein extracts prepared from wild-type Arabidopsis, 35S:CUL1-TAP, 35S:TAP-CUL1, and 35S:ASK1-TAP transgenic Arabidopsis were incubated with IgG-coupled sepharose. The precipitates and the total extracts were subjected to immunoblot analysis with antibodies against CAND1, TATA binding protein (TBP), and CUL1 (B) or MYC (C).

In (A) to (C), arrowheads indicate protein positions, and T indicates total protein extract. Anti-TBP (TATA binding protein) antibody is used as a pull-down control.

(D) CAND1 interacts with RUB modification site mutated CUL1 more strongly than with wild-type CUL1 in yeast two-hybrid assays. β-gal activity resulted from CUL1(K682R) and CAND1 interaction is set to 100%. Error bars represent standard deviation (n = 4).

(E) Arabidopsis CUL1 is modified by RUB in yeast, which can be abolished by a point mutation at its RUB modification site. Yeast strains used in two-hybrid assays (labeled at the top) were subjected to immunoblot analysis with antibodies against CUL1 and CAND1. Arrowheads indicate protein positions. The asterisk marks a nonspecific band cross-reacting with anti-CUL1 antibodies, which is also used as a loading control of the total protein amount in each lane.
the residual interaction observed between wild-type CUL1 and CAND1 could be because of the presence of a fraction of the wild-type CUL1 in the unmodified form in yeast. It is also interesting to note that in this yeast two-hybrid assay, the N terminus of CUL1 is fused to LexA (25 kD), which argues against the possibility that N-terminal TAP tag might prevent TAP-CUL1 and CAND1 from interacting with each other in vivo. Collectively, our studies show that CAND1 preferentially interacts with unmodified CUL1.

Loss of CAND1 Results in an Aberrant Vegetative-to-Reproductive Transition and Flower Development

As described earlier, cand1 mutants flower later than wild-type plants, with an increased number of rosette leaves before bolting (Figures 1D and 4A). Even after they begin to flower, many more cauline leaves are still produced on the shoots, as compared with wild-type plants (Figure 1D). Furthermore, aerial rosettes are found in normal axillary branch positions (Figures 4B to 4E). The formation of aerial rosettes is attributable to the failure of axillary branch internodes to elongate immediately as they normally do in Columbia-0. This phenotype is observed in the late-flowering Arabidopsis ecotype Sy-0 (from Isle of Skye, UK), reflecting a defect in the vegetative/reproductive growth switch in the axillary meristem (Poduska et al., 2003).

The cand1 mutant flowers have all the right organs, although they are much smaller (Figure 4F). We found that the mutant flower is poorly pollinated (data not shown). As a result, the siliques of the mutant are very short and contain hardly any seeds (Figure 4G).

Arabidopsis UFO is required for the expression of B-class floral organ identity genes that control petal and stamen development and encodes an F-box protein that incorporates into an SCF-type ubiquitin E3 ligase (Samach et al., 1999; Wang et al., 2003). We have previously reported a 35S:UFO-MYC overexpression line (UM), in which B-functions become ectopic and greatly enhanced, causing enlarged petal size, increased number of stamens, and filamentous carpels (Wang et al., 2003; Figure 4I). We introduced the UFO-MYC transgene into the cand1-1 mutant background by genetic crossing. The cand1-1 mutants expressing UFO-MYC were selected by protein gel blot analysis (Figure 4H), and their flower phenotypes were compared with UM and cand1-1. Essentially, the flowers of UM/cand1-1 are identical to those of cand1-1 mutant and drastically different from those of UM (Figure 4I). Consistent with the phenotype observation, the UFO-MYC protein level is greatly downregulated in UM/cand1-1 flowers (Figure 4H). Evidently, SCF-UFO activity is suppressed in the cand1-1 mutant, implying a positive regulatory role for cand1-1 mutant. The extracts were then subjected to immunoblot analysis with anti-CAND1, anti-MYC, and anti-RPN6 antibodies. Arrowheads indicate protein positions. The asterisks mark two cross-reacting bands. RPN6 is used as a loading control.

Figure 4. Abnormal Development of Shoot Apex and Flower in the cand1-1 Mutant.

(A) Flowering time of wild-type Arabidopsis and cand1-1 mutant, as indicated by average number of rosette leaves at the time of bolting. The identities of the plants are labeled at the bottom. Error bars represent standard deviation (n = 6).
(B) to (E) Aerial rosettes are formed in the axils of the cand1-1 mutant. Samples were taken from different parts of the same plant, including the bottom (B), the middle (C), and the tip of the axillary branch (D) and (E).
(F) Comparison of wild-type and cand1-1 flowers. The flowers were dissected to reveal the floral organs. Pictures were taken under the same magnification.
(G) Comparison of wild-type and cand1-1 siliques.
(H) UFO-MYC protein level is greatly reduced in the cand1-1 mutant background. Protein samples were extracted from flowers of wild-type Arabidopsis, 35S:UFO-MYC transgenic Arabidopsis (UM), cand1-1 mutant expressing 35S:FLAG-CAND1 transgene (UM/cand1-1), and
CAND1 in maintaining SCF\textsuperscript{UFO} ubiquitin E3 ligase activity during floral organ development.

The cand1-1 Mutant Has Reduced Responsiveness to Jasmonate and Auxin

Another well-studied F-box protein in Arabidopsis is COI1, the central regulator of the jasmonate pathway (Xie et al., 1998; Xu et al., 2002). The cand1-1 mutant shows a dosage-dependent resistance to the root growth inhibition effect caused by jasmonates, especially at low concentrations of jasmonates (2 and 5 \textmu M), where cand1-1 has an \textasciitilde 50\% increase in resistance compared with the wild type (Figure 5A). The cand1-1 coi-1 double mutant has no enhanced resistance to jasmonates compared with the coi-1 single mutant (data not shown). This supports the idea that SCFCOI1 controls most of the jasmonate-triggered responses (Feng et al., 2003) and that CAND1 acts to optimize its activity. However, unlike UFO-MYC, the COI1 protein level is not changed in the cand1-1 mutant (data not shown). Thus, further evidence is required to substantiate that the enhanced resistance to jasmonates observed in cand1-1 is because of the reduction in SCFCOI1 activity.

The loss of apical dominance in the cand1 mutants is reminiscent of some auxin pathway mutants. Indeed, we were able to demonstrate that root growth inhibition by auxin in cand1-1 mutant is moderately relieved, which is comparable to axr1-3, a weak axr1 mutant (Figure 5B). In an independent study, Chuang et al. (2004) identified a point mutation for Arabidopsis CAND1, which also causes an auxin-resistant phenotype and has synergistic effects with the tir1-1 mutation. Like UFO and COI1, TIR1 also encodes an F-box protein, and SCFTIR1 is required for auxin responses (Gray et al., 1999). Together, these data provide evidence that optimal SCFTIR1 activity requires CAND1 function.

CAND1 Is Required for Proper Gibberellin Signaling

As mentioned earlier, the dwarf phenotype of cand1 mutants might be caused by defects in gibberellin pathways. To test this hypothesis, we examined whether loss of CAND1 impairs gibberellin (GA) signaling pathways that also involve a specific SCF-type ubiquitin E3 ligase. SCFSLY1 is suggested to be responsible for targeting the GA pathway repressor of ga1-3 (RGA) for degradation in Arabidopsis (McGinnis et al., 2003). Indeed, whereas RGA protein in wild-type Arabidopsis is barely visible in protein gel blot analysis, sly1-10, a mutant of the F-box protein in SCFSLY1, can accumulate a high level of RGA (McGinnis et al., 2003; Figure 6A). Noticeably, the RGA protein level is also elevated in the cand1-1 mutant (Figure 6A), which likely contributes to the mutant dwarf phenotype.

RGA protein is destabilized rapidly upon addition of GA (Silverstone et al., 2001; McGinnis et al., 2003; Figure 6B, lanes 1 and 2). However, in the cand1-1 mutant, the GA-induced RGA protein reduction is partially compromised (Figure 6B, lanes 3 and 4). Therefore, it is conceivable that CAND1 is a positive regulator of SCFSLY1 and that in cand1 mutants, SCFSLY1 cannot achieve optimal activity, which results in a reduction of GA signaling.

It is interesting to note that GA responses regulate floral pathway integrators (Simpson and Dean, 2002), which could provide a possible explanation for the late flowering phenotype of cand1 mutants. In addition, GA is also essential for petal and stamen development in Arabidopsis (Harberd et al., 1998), indicating another potential cause of the cand1 mutant flower phenotypes.

CAND1 Represses Photomorphogenesis by Promoting HY5 Degradation in Darkness

As stated earlier, loss of CAND1 does not affect germination and seedling stage growth under normal conditions. However, when cand1-1 seedlings are grown in darkness, they exhibit mild constitutive photomorphogenic phenotypes, with short hypocotyls and opened cotyledons (Figure 7A). It has been shown that the extent of photomorphogenic development is directly correlated with the abundance of HY5, a photomorphogenesis-promoting transcription factor (Osterlund et al., 2000).
Consistent with this, we found that dark-grown cand1-1 mutant seedlings, whose degree of photomorphogenesis is in between light-grown and dark-grown wild-type seedlings, also accumulate an intermediate level of HY5 protein (Figure 7C, lanes 1 to 3). HY5 is degraded by the 26S proteasome upon transfer from light to dark (Osterlund et al., 2000; Figure 7D, lanes 1 to 3). Evidently, HY5 protein is more stable in the cand1-1 mutant (Figure 7D, lanes 4 to 6).

The targeted degradation of HY5 in darkness requires COP1, a ubiquitin E3 ligase for HY5 (Saijo et al., 2003), and COP10, an E2 ubiquitin-conjugating enzyme variant (Suzuki et al., 2002). We crossed the cand1-1 mutant with cop1-6 and cop10-4, both of which are nonlethal alleles, to obtain genetic evidence for the functional interactions of CAND1 with COP1 and COP10. Remarkably, the dark-grown double mutants cop1-6 cand1-1 and cop10-4 cand1-1 have shorter hypocotyls than their parental single mutants (Figure 7B) and accumulate higher levels of HY5 as well (Figure 7C, lanes 4 to 7). These data suggest that CAND1 has synergistic effects with COP1 and COP10 in promoting HY5 degradation and in repressing photomorphogenesis in darkness. Considering that human COP1 has most recently been indicated to be an integral part of a CUL4A-containing ubiquitin E3 ligase (Wertz et al., 2004), it will be interesting to find out if this function of CAND1 is dependent on Arabidopsis CUL4.

**DISCUSSION**

In this study, we characterized Arabidopsis CAND1, a crucial player in the regulation of cullin-containing ubiquitin E3 ligases. We demonstrated that CAND1 has the ability to selectively interact with unmodified CUL1 in vivo. The Arabidopsis cand1-1 null mutant exhibits serious defects in various aspects of development, and we showed that many of these defects are caused by compromised activities of CUL1-containing SCF-type ubiquitin E3 ligases. Therefore, CAND1 functions as a positive regulator of HY5 expression.

**Figure 6.** Proper GA Signaling Requires CAND1. 
(A) The GA pathway negative regulator RGA accumulates in the cand1-1 mutant. Flower protein extracts from wild-type Arabidopsis, cand1-1, rga-24 (negative control), and sly1-10 mutants were subjected to immunoblot analysis with anti-RGA and anti-RPN6 antibodies. (B) The cand1-1 mutant fails to rapidly destabilize RGA upon GA treatment. Eight-day-old wild-type and cand1-1 seedlings were treated with GA3 for 2 h. Subsequently, protein extracts were prepared from treated (+) and untreated (−) seedlings and subjected to immunoblot analysis with anti-RGA and anti-RPN6 antibodies. The arrowheads in (A) and (B) indicate protein positions. RPN6 is used as a loading control.

**Figure 7.** CAND1 Functions in Photomorphogenesis by Regulating HY5 Degradation. 
(A) and (B) Loss of CAND1 leads to constitutive photomorphogenesis in dark and enhances phenotypes of weak cop alleles. Different Arabidopsis lines (labeled at the top) were grown in complete darkness for the indicated number of days. All pictures were taken under the same magnification. (C) The cand1-1 mutation causes hyperaccumulation of HY5 in dark-grown seedlings. Seedling protein extracts from 4-d-old light-grown wild-type Arabidopsis and cand1-1 mutant were prepared and blotted with anti-HY5 and anti-RPN6 antibodies. (D) HY5 is degraded less efficiently in the cand1-1 mutant than in wild-type Arabidopsis. Four-day-old light-grown seedlings of wild-type Arabidopsis and cand1-1 mutant were transferred to complete darkness. Samples were collected at different time points starting from the transfer (indicated at the top) and blotted with anti-HY5 and anti-RPN6 antibodies. The arrowheads and asterisk in (C) and (D) indicate protein positions and a cross-reacting band, respectively. HY5 has two forms: unphosphorylated (bottom band) and phosphorylated (top band). RPN6 is used as a loading control.
regulator of cullin-containing E3s. In addition, we found that CAND1 also works together with COP1 and COP10 to promote HY5 degradation, which sheds new light on the control of photomorphogenesis.

Physical Interaction between CAND1 and CUL1

Here, we provide three lines of direct in vivo evidence supporting that Arabidopsis CAND1 is an unmodified CUL1-interacting protein, similar to its human counterpart (Liu et al., 2002; J. Zheng et al., 2002; Hwang et al., 2003; Min et al., 2003; Oshikawa et al., 2003). First, only the unmodified form of CUL1 is detected in the immunoprecipitate of FLAG-CAND1 (Figure 3A). Second, CAND1 can be pulled down together with the unmodified CUL1-TAP; contrarily, TAP-CUL1, which is hyper-RUB modified, does not pull down any CAND1 (Figure 3B). Third, when the RUB modification site on Arabidopsis CUL1 is mutated and its RUB modification in yeast is prevented (Figure 3E), the interaction between Arabidopsis CUL1 and CAND1 in yeast increases by 10-fold (Figure 3D). It is interesting to note that in yeast approximately half of the wild-type CUL1 actually exists in the unmodified form, yet its ability to interact with CAND1 decreases ~10-fold compared with CUL1(K682R) mutant protein, despite the fact that the two CUL1 proteins have almost equal abundance (Figures 3D and 3E). In Arabidopsis, TAP-CUL1 has a high rate of RUB modification in comparison with CUL1-TAP (Figure 3B). If this causes a similar scale of decrease in the interaction between the unmodified form of TAP-CUL1 and CAND1, the interaction might fall below the detection limit of our IgG pull-down experiment (Figure 3B).

The observation mentioned above can be taken as an indication that the interaction between CUL1 and CAND1 may be labile and dynamic in vivo. What is consistently observed in both Arabidopsis and yeast is that if CUL1 is not able to undergo RUB modification or if its capacity for RUB modification is reduced, its interaction with CAND1 is strong. On the contrary, when CUL1 is hyper-RUB modified or with a high capacity for RUB modification, the interaction between CUL1 and CAND1 decreases dramatically, to an extent much below expectation based on the abundance of the remaining CUL1 in the unmodified form (Figure 3). Taken together, it suggests that the binding of CAND1 to unmodified CUL1 is quickly turned over by RUB modification, and only when RUB modification cannot take place, CAND1 binding to CUL1 will be sustained.

In vivo, when a target protein somehow signals a need for degradation, the substrate/SKP1/F-box protein subcomplex promotes RUB/Nedd8 modification of CUL1, which leads to CAND1 dissociation from CUL1. Reciprocally, when RUB/Nedd8 is cleaved from modified CUL1 by CSN, CAND1 binds unmodified CUL1 with high affinity, displacing the SKP1/F-box protein. Therefore, through CAND1, the RUB/Nedd8 conjugation and deconjugation cycle can be directly linked to the assembly/disassembly of cullin-containing ubiquitin E3 ligases. This model implies that although CAND1 binding is an important step in the RUB/Nedd8 conjugation and deconjugation cycles, it may not affect the RUB/Nedd8 modification status of cullins, which is supported by our data (Figure 1C).

Regulation of SCF-Type Ubiquitin E3 Ligases by CAND1

The unique binding characteristic of CAND1 turns out to be the key to its biological function. It prevents the formation of functional cullin-containing ubiquitin E3 ligases by sequestering ROC1/cullin. Consistently, various in vitro assays show that CAND1 acts as a negative regulator of SCF ubiquitin E3 ligase activities (Liu et al., 2002; J. Zheng et al., 2002; Min et al., 2003). However, CAND1 binding of unmodified cullins should be considered as an intermediate step in the cullin-containing complex dynamic assembly/disassembly cycles; hence, CAND1 is an integral component in the overall ubiquitin/proteasome-mediated degradation of protein substrates (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wolf et al., 2003). This would suggest a positive role for CAND1 in ubiquitin E3 ligase–mediated developmental processes.

We revealed the physiological and developmental roles of CAND1 in Arabidopsis through examination of T-DNA insertional mutants. Multiple SCF-type ubiquitin E3 ligases are likely to be compromised in the cand1-1 mutant, including SCFUFO, SCFTRI1, SCFCOI1, and SCFSLY1. SCFUFO promotes the expression of AP3 and PI, the B-class floral organ identity genes (Samach et al., 1999). Flower phenotypes of UFO overexpression are overridden by cand1-1 mutation, which suggests that SCFUFO requires CAND1 for its optimal activity (Figure 4). SCFCOI1 and SCFTR1 control jasmonate and auxin pathways, respectively (Gray et al., 1999; Xu et al., 2002). Loss of CAND1 seems to affect their activities, because the cand1-1 mutant has reduced responses to both jasmonate and auxin (Figure 5). Reduction of SCFTR1 activity is also indicated by the loss of apical dominance in cand1 mutants (Figure 1D). SCFSLY1 targets the GA pathway repressor RGA for degradation, stimulating the GA response (McCinnis et al., 2003). The cand1-1 mutant accumulates a higher level of RGA protein than the wild type (Figure 6A) and fails to rapidly degrade RGA upon GA treatment (Figure 6B). Adult cand1 mutant plants are dwarfed, with severely reduced stem elongation (Figure 1D). This evidence suggests that CAND1 is necessary for normal SCFSLY1 function and proper GA response. Collectively, our genetic studies have undoubtedly established CAND1 as a positive regulator of CUL1-containing SCF-type ubiquitin E3 ligases.

It is clear that RUB/Nedd8 conjugation and deconjugation cycles are important for many CUL1-containing SCF-type ubiquitin E3 ligases because loss or reduction of RUB/Nedd8 modification, RUB/Nedd8 cleavage, or absence of CAND1 will impair their activities. Why is dynamic assembly/disassembly important for the activity of cullin-containing E3? One theory is that F-box proteins assembled in the SCF complexes are subjected to the E3's self-ubiquitination activity (Zhou and Howley, 1998; Wirbelauer et al., 2000; Li et al., 2004). By limiting their incorporation into the complexes, they can be stabilized and work more efficiently when needed (J. Zheng et al., 2002; Cope and Deshaies, 2003; Wei and Deng, 2003). In the cand1-1 mutant, we observed a drastic decrease of UFO–MYC overexpression level (Figure 4H), but the endogenous COI1 level is not changed (data not shown). Obviously, there is no simple answer to this question.
The nonlethal nature and relative absence of a phenotypic defect during embryonic and seedling development of the null cand1 mutant suggested that CAND1 is not required for all SCF-type ubiquitin E3 ligases because the cut1 null mutant itself is embryonic lethal (Shen et al., 2002). Also, considering the fact that defects in RUB conjugation (Dharmasiri et al., 2003) or deconjugation (Serino and Deng, 2003; Wei and Deng, 2003) cause severe seedling growth retardation and adult lethality, CAND1 may not be an absolutely essential component in mediating RUB/Nedd8 conjugation and deconjugation cycles of all cullin-containing ubiquitin E3 ligases.

Control of Flowering and Photomorphogenesis by CAND1

We successfully related several cand1 mutant phenotypes to the activities of known CUL1-containing SCF-type ubiquitin E3 ligases. There are other abnormalities of cand1 mutants that cannot be readily explained in this way, for example, the late flowering phenotype and aerial rosettes (Figures 1D and 4A to 4E). Reduction of SCF-type ubiquitin E3 ligase activities in cand1 mutants may play a part in this because GA pathway mutants, as well as mutants for the two F-box proteins ZTL and FKF, which are involved in circadian clock control, are late flowering. Both GA and circadian clock pathways act upstream of floral pathway integrators that regulate flowering time (Nelson et al., 2000; Somers et al., 2000; Simpson and Dean, 2002). However, mutations in these pathways alone do not cause formation of aerial rosettes. Another example is the flower phenotype (Figures 4F and 4G), which apparently cannot be attributed to the inactivity of any single cullin-containing ubiquitin E3 ligase known in Arabidopsis. A quick explanation is that a combination of defects in multiple ubiquitin E3 ligase activities eventually causes cand1 phenotypes, keeping in mind that there are at least 694 putative F-box proteins in the Arabidopsis genome for CUL1-containing SCF-type ubiquitin E3 ligases (Gagne et al., 2002) and that there are many other cullins yet to be examined. In addition, we cannot eliminate a role for cullin-independent CAND1 function in flower timing and flower development.

Photomorphogenesis is repressed in darkness through the proteasomal degradation of photomorphogenesis-promoting transcription factors, such as HY5 (Osterlund et al., 2000). In this pathway, the E3 is COP1, a different RING family ubiquitin E3 ligase (Saijo et al., 2003; Seo et al., 2003). COP10, a ubiquitin-conjugating enzyme variant, is also necessary for HY5 degradation (Suzuki et al., 2002). Surprisingly, CAND1 has a role in promoting HY5 degradation, as shown by the high level of HY5 in the dark-grown constitutive photomorphogenic cand1-1 mutant (Figures 7A and 7C), and in the failure of the cand1-1 mutant to rapidly destabilize HY5 upon transfer from light to dark (Figure 7D). Furthermore, CAND1 has synergistic genetic interactions with both COP1 and COP10 (Figures 7B and 7C), supporting its direct involvement in repressing photomorphogenesis. Interestingly, human COP1 has recently been shown to assemble into a CUL4A-containing ubiquitin E3 ligase for c-Jun (Wertz et al., 2004), and Arabidopsis COP1 does form complexes in vivo (Saijo et al., 2003). Therefore, it is measurable to speculate that CAND1’s regulation of photomorphogenesis is realized by modulating anorthologous ubiquitin E3 ligase in Arabidopsis that contains COP1 and CUL4 and targets photomorphogenesis-promoting transcription factors. Alternatively, it is possible that another cullin-containing ubiquitin E3 ligase may be indirectly involved in regulating COP1 E3 activity.

METHODS

Plant Materials, Growth Conditions, and Hormone Treatments

The axr1-3, axr1-12 (Lincoln et al., 1990), cop1-1 (Xie et al., 1998), rga-24 (Silverstone et al., 1998), sly1-10 (McGinnis et al., 2003), cop1-6 (McNellis et al., 1994), and cop10-4 (Suzuki et al., 2002) mutants and the 35S:UM (Wang et al., 2003) transgenic plants were described previously. The wild-type Arabidopsis thaliana plants used in this study were of the Columbia-0 ecotype.

To grow Arabidopsis seedlings, seeds were surface sterilized, put on MS plates (Gibco, Cleveland, OH) containing 1% sucrose, and cold treated at 4°C for 3 to 5 d before being placed in a standard, continuous white light growth chamber or in complete darkness at 22°C. To obtain adult plants, 7- to 9-d-old light-grown seedlings were transferred to soil and grown in a standard long-day (16 h light/ 8 h dark) growth room.

For root growth inhibition assays, Arabidopsis seedlings were first grown on normal MS medium for 4 d and then transferred to MS medium containing different concentrations of methyl jasmonate (Bedoukian, Danbury, CT) or 2,4-D (Sigma, St. Louis, MO). Root length was measured 4 d after the transfer. GA treatment experiments were performed as previously described (Silverstone et al., 2001).

Cloning of Arabidopsis CAND1 and Isolation of cand1 T-DNA Insertional Mutants

Full-length ORF of CAND1 was amplified by RT-PCR from wild-type Arabidopsis seedlings with forward primer (5’-CGCGGATCCGATGGCGAGAATTACAGTTTC-3’) and reverse primer (5’-ATGGAGATCCTGCGATCCGCTTACTCACTATTCCGGATTGC-3’). The BamHI/Hind fragment of the PCR product was cloned into pEG202 (Origene, Rockville, MD) and then sequenced. This construct is named as pEG-CAND1, which served as the PCR template for subsequent cloning of CAND1 into other vectors.

Based on a database search, we found one T-DNA insertion line for CAND1 in the GABI-Kat collection (Line ID 13H10; Rosso et al., 2003). Plants homozygous for the T-DNA insertion were identified by PCR-based genotyping. This insertional mutant is named cand1-1. The mutation cosegregates with the T-DNA insertion through a backcross to wild-type plants. Two additional T-DNA insertional mutants, cand1-2 and cand1-3, were found in the Salk collection (from Salk_094797 and Salk_110967, respectively; Alonso et al., 2003). The mutant plants were identified within segregating populations by phenotype resemblance to the cand1-1 mutant and confirmed by PCR-based genotyping. When not specified, we use cand1 to indicate all three mutants throughout the text.

Generation of Transgenic Arabidopsis Plants

A KpnI/SalI fragment containing full-length CAND1 ORF was inserted into pFP3PZY122 (Feng et al., 2003). Then, an XbaI/SalI fragment with the inserted DNA was subcloned into pJJM19(BAR), a plant binary vector that has basta-resistance marker and the 35S promoter of Cauliflower mosaic virus.

Arabidopsis plants heterozygous for cand1-1 mutation were used in the transformation of 35S:FLAG-CAND1 transgene. Transgenic plants were selected with sulfadiazine (100 µg/ml; Sigma) and gulosinate-ammonium (20 µg/ml; Riedel-de Haën, Seelze, Germany). The cand1-1 mutants carrying 35S:FLAG-CAND1 transgene were identified by PCR-based genotyping.
Full-length ORFs of Arabidopsis CUL1 and ASK1 were cloned via Gateway reactions (Invitrogen, Carlsbad, CA) into plant binary vector with either N-terminal or C-terminal modified TAP tag fusion (Saigo et al., 2003). The transgenes were individually introduced into wild-type Arabidopsis. Transgenic plants were selected with gentamicin (200 μg/mL; Sigma).

Immunoblot Assays and Antibodies

Arabidopsis tissues were homogenized in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor (Roche, Indianapolis, IN). The extracts were centrifuged twice at 4°C for 10 min each, and the protein concentration in the supernatant was determined by Bradford assay (Bio-Rad, Hercules, CA). Protein samples were boiled in sample buffer, run on SDS-PAGE gels (8, 12, or 17.5%), and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were probed with different primary antibodies. A BamHI/Motl fragment containing the first 530 nucleotides of CAND1 ORF was cloned into pET-28a (Novagen, Madison, WI). This construct encodes a fusion protein with 6X His tags and CAND1’s N-terminal 210 residues. The fusion protein was expressed in Escherichia coli and purified with nickel-nitrilotriacetic acid agarose beads (Qiagen, Valencia, CA). Polyclonal antibodies were raised by immunizing rabbits using purified fusion protein as antigen.

Other primary antibodies used in this study include anti-CUL1 (Wang et al., 2002), anti-RGA (Silverstone et al., 2001), anti-HYS (Osterlund et al., 2000), anti-CO1 (Xu et al., 2002), anti-RPN6 (Kwok et al., 1999), anti-TBP (Schwechheimer et al., 2001), anti-MYC (Saigo et al., 2003), and anti-FLAG (Sigma).

Yeast Two-Hybrid and in Vivo Pull Down Analyses

Procedures for yeast two-hybrid assays and bait construct for CUL1 have been described previously (Schwechheimer et al., 2001; Wang et al., 2002). To create a CUL1(K682R) fragment, the adenine position at 2045 of CUL1 ORF was mutated by PCR method to guanosine. A Sal fragment containing full-length CAND1 ORF was cloned into pJG4/5 (Origene) to make the CAND1 prey construct. For protein expression level detection, yeast cells were boiled directly in sample buffer and subjected to immunoblot analysis.

The FLAG antibody pull-down and IgG pull-down experiments were performed as previously described (Feng et al., 2003; Saigo et al., 2003) with minor modifications. The recipe of lysis/binding/washing buffer was 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 10 mM NaF, 2 mM Na3VO4, 25 mM β-glycerophosphosphate, 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor (Roche). The acid elution and concentration steps were omitted.

Sequence data for the protein sequences used in the phylogenetic analysis have been deposited with the EMBL/GenBank data libraries under accession numbers XP_125901 (mouse), NP_060918 (human), NP_609389 (Drosophila), NP_178360 (Arabidopsis), NP_507244 (C. elegans), and NP_593268 (S. pombe). The Arabidopsis CAND1 gene has been deposited under the accession number AY099887.

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Arabidopsis CAND1, an Unmodified CUL1-Interacting Protein, Is Involved in Multiple Developmental Pathways Controlled by Ubiquitin/Proteasome-Mediated Protein Degradation
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