The Arabidopsis COP9 SIGNALOSOME INTERACTING F-BOX KELCH 1 Protein Forms an SCF Ubiquitin Ligase and Regulates Hypocotyl Elongation


ABSTRACT The regulation of protein turnover by the ubiquitin proteasome system (UPS) is a major posttranslational mechanism in eukaryotes. One of the key components of the UPS, the COP9 signalosome (CSN), regulates ‘cullin–ring’ E3 ubiquitin ligases. In plants, CSN participates in diverse cellular and developmental processes, ranging from light signaling to cell cycle control. In this work, we isolated a new plant-specific CSN-interacting F-box protein, which we denominated CFK1 (COP9 INTERACTING F-BOX KELCH 1). We show that, in Arabidopsis thaliana, CFK1 is a component of a functional ubiquitin ligase complex. We also show that CFK1 stability is regulated by CSN and by proteasome-dependent proteolysis, and that light induces accumulation of the CFK1 transcript in the hypocotyl. Analysis of CFK1 knockdown, mutant, and overexpressing seedlings indicates that CFK1 promotes hypocotyl elongation by increasing cell size. Reduction of CSN levels enhances the short hypocotyl phenotype of CFK1-depleted seedlings, while complete loss of CSN activity suppresses the long-hypocotyl phenotype of CFK1-overexpressing seedlings. We propose that CFK1 (and its regulation by CSN) is a novel component of the cellular mechanisms controlling hypocotyl elongation.

Key words: Arabidopsis thaliana; ubiquitin; COP9 signalosome; proteasome; F-box protein; hypocotyl elongation; seedling development.

INTRODUCTION

In plants, the regulation of protein turnover by the ubiquitin proteasome system (UPS) is a key posttranslational mechanism underlying diverse cellular processes. One of the central components of the UPS, the COP9 signalosome (CSN), is conserved from yeast to animals and has been shown to regulate many aspects of plant development (Wei et al., 2008).

CSN consists of eight subunits and is required to ensure the activity of cullin–RING ubiquitin ligases (CRLs) in vivo (Chamovitz et al., 1996; Serino et al., 1999; Lyapina et al., 2001; Schwechheimer et al., 2001). CRLs represent an extensive class of multi-subunit E3 ubiquitin ligases, each consisting of a core module containing a member of the cullin family and the RING domain protein RBX1, which recruits E2 ubiquitin-conjugating enzymes to the ligases (Hua and Vierstra, 2011). The cullin–RBX1 core binds to one of several hundreds of possible adaptor proteins, each of which appears to target a distinct array of substrates for ubiquitination

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Received 23 January 2013; accepted 24 February 2013
and proteasomal degradation (Hua and Vierstra, 2011). CRL complexes containing the CUL1 member of the cullin family are named SCF (SKP1, Cullin, and F-box protein; Feldman et al., 1997; Skowyra et al., 1997). In SCF complexes, the substrate adaptors are the F-box proteins (FBPs), which are tethered to the CUL1–RBX1 core through the linker protein SKP1 via their F-box domain (Bai et al., 1996).

CRLs are regulated by the conjugation and removal of the ubiquitin-related small protein RUB1/NEDD8 on their cullin subunit (Hua and Vierstra, 2011). RUB1/NEDD8 conjugation (neddylation) induces a conformational change that favors CRL activity (Duda et al., 2008; Saha and Deshaies, 2008; Yamoah et al., 2008). The opposite reaction, named deneddylation, promotes CRL inactivation and is catalyzed by CSN, via its subunit S (Lyapina et al., 2001; Schwechheimer et al., 2001; Zhou et al., 2001; Cope et al., 2002; Wee et al., 2005). CSN inactivates CRLs also in a non-catalytic fashion, suggesting that CSN regulates CRL activity by multiple mechanisms (Fischer et al., 2011; Emberley et al., 2012; Enchev et al., 2012; Zhou et al., 2012). CSN-mediated CRL inactivation prevents the autocatalytic degradation of CRL substrate adaptors in vivo, thus sustaining CRL activity (Wee et al., 2005; Cope and Deshaies, 2006; Denti et al., 2006). Indeed, several FBPs were found to be more unstable in csn mutants of Schizosaccharomyces pombe (Zhou et al., 2003; Schmidt et al., 2009), Neurospora crassa (He et al., 2005), and in human CSN knockdown cells (Cope and Deshaies, 2006; Denti et al., 2006).

In Arabidopsis, CSN has been shown to interact directly with the conserved cullin and RING subunits of CRLs and has therefore the potential of regulating virtually all CRLs (Lyapina et al., 2001; Schwechheimer et al., 2001; Serino et al., 2003; Hua and Vierstra, 2011; Enchev et al., 2012). Indeed, several CRL-mediated pathways have already been shown to be dependent on CSN in Arabidopsis. CSN regulates seedling de-etiolation in part through a CUL4–RBX1–CDD complex, and controls gibberellin, auxin, and jasmonic acid response, as well as flower development, through the regulation of distinct SCF complexes (Schwechheimer et al., 2001; Feng et al., 2003; Wang et al., 2003; Dohmann et al., 2008; Chen et al., 2010; Dohmann et al., 2010). CSN has been also shown to regulate plant immunity through a CRL3 ubiquitin ligase (Spoel et al., 2009). However, many growth and developmental defects of the csn mutants can not be explained based only on the CRLs identified so far, suggesting that additional CSN downstream effectors still await identification. We have used a biochemical approach to identify novel substrates of CSN function. In this paper, we report the isolation and functional characterization of a new F-box protein co-purifying with CSN, which we denominated CFK1 (COP9 SIGNALOSOME INTERACTING F-BOX KELCH 1). We show that, in Arabidopsis, CFK1 is a component of an SCF ubiquitin ligase and that it is a target of CSN activity. In addition, we show that transcription of the CFK1 gene is strongly up-regulated by light specifically in the hypocotyl, and that CFK1 is involved in the regulation of hypocotyl elongation through cell expansion.

**RESULTS**

The CSN Co-Purifies with the CFK Proteins

To identify proteins interacting with CSN, we utilized a large-scale preparative affinity purification of the CSN from cauliflower (Serino et al., 1999). In addition to the bands corresponding to the eight CSN subunits, several other bands that were present in the purified sample in sub-stoichiometric amounts were subjected to protein sequencing analysis, allowing the identification of eight novel CSN-interacting proteins (Supplemental Table 1). In this paper, we describe the characterization of one of these proteins, which we denominated CFK (COP9-INTERACTING F-BOX KELCH).

To identify CFK in Arabidopsis, we used three distinct peptide sequences obtained from the cauliflower band to search the Arabidopsis genome database (www.Arabidopsis.org). This analysis indicated a 100% match of the three peptides to two homologous proteins, At5g42350 and At5g42360, which were denominated CFK1 and 2, respectively. The two proteins had a predicted molecular weight of 64 kDa and were 98% identical in terms of amino acid sequence (Figure 1A). The CFK proteins do not have close homologs in animals, while CFK orthologs with the same domain organization are present also in plants as distant as poplar (Populus trichocarpa) and rice (Oryza sativa) (Supplemental Figure 1A). Clear CFK1 homologs were not found in Selaginella moellendorfﬁ, nor in the moss Physcomitrella patens, suggesting that these proteins appeared later in the evolution of vascular plants. Arabidopsis genome analysis revealed that the CFK1 and 2 genes are adjacent to each other on the lower arm of chromosome V, and are oriented in opposite directions (Supplemental Figure 1B).

Sequence analysis and genome annotations indicated that the newly identified proteins contain an identical F-box domain in their N-terminus and three kelch repeats downstream of the F-box (Figure 1A). Kelch repeats, consisting of repeated sequence motifs with hallmark residues spaced at regular intervals, have been implied in protein–protein interactions (Hudson and Cooley, 2008). In CFK1, the F-box domain spans from amino acid residue 135–175 (Figure 1A), while the three putative kelch repeats span over residues 184–231, 232–282, and 355–402, respectively (Figure 1B).

Both CFK proteins are predicted to be nuclear by the SubCellular Proteomic Database (SUBA) (Heazlewood et al., 2007), and a putative bipartite nuclear localization sequence can be found between residues 25 and 41 in CFK1, and between residues 24 and 40 in CFK2 (Figure 1A). To determine the subcellular localization of CFK1 in vivo, we generated plants expressing a CFK1–YFP fusion protein under the control of the CFK1 endogenous promoter (PCFK1:CFK1–YFP).
Confocal microscopy analysis shown that, in hypocotyl cells from light-grown seedlings, CFK1–YFP was predominantly nuclear localized in hypocotyl epidermis cells (Figure 1C).

**CFK1 Is a Component of an SCF Ubiquitin Ligase**

The presence of an F-box domain in the CFK proteins suggested that they might be part of an SCF ubiquitin ligase complex. Given the high identity between the two proteins, we focused our further analysis on CFK1 and sought to assess a possible direct interaction with ASK1 (Arabidopsis homolog of SKP1, Saccharomyces cerevisiae Suppresser of Kinetochoore 1), a conserved SCF subunit which has been shown to interact directly with many FBPs (Gagne et al., 2002). We carried out a yeast two-hybrid assay with full-length CFK1 fused to the LexA DNA Binding Domain (bait) and a prey consisting of full-length ASK1 fused to the LexA Activation Domain (Figure 1D). The results obtained indicated that CFK1 interacts very strongly with ASK1, but not with CUL1, another SCF component. This interaction represents a true molecular partnership, since a bait consisting of only the C-terminal region of CFK1 (CFK1ΔF-box) fused to the LexA DNA Binding Domain was not able to interact with ASK1. Thus, the interaction between CFK1 and ASK1 requires the F-box in the former protein.

To confirm that CFK1 is part of an SCF ubiquitin ligase complex in planta, we produced Arabidopsis plants harboring a construct encoding three copies of the HA epitope, translationally fused to full-length CFK1 and under the control of the constitutive CaMV-35S promoter (P35S:HA–CFK1) (Supplemental Figure 2A–2C). Total protein extracts from wild-type (Col-0) and P35S:HA–CFK1 plants were immunoprecipitated with antibodies against HA, followed by immunoblotting with antibodies against the conserved SCF subunit CUL1 and against the CSN subunit CSN6. HA–CFK1 was able to co-immunoprecipitate with CSN6, thus reconfirming an
interaction between CFK1 and the CSN (Figure 1E, lane 4). In addition, HA–CFK1 was able to co-immunoprecipitate with both neddylated and deneddylated forms of CUL1 (Figure 1E, lane 4). Thus, HA–CFK1 is present in SCF complexes regardless of their neddylation status. It should be noted, however, that the amount of deneddylated CUL1 which co-immunoprecipitates with HA–CFK1 might be enhanced by the CSN activity still present in the extract after cell lysis (Bennett et al., 2010).

To further validate the association of CFK1 with an SCF complex, we examined the co-fractionation of HA–CFK1 with CUL1 using gel-filtration chromatography (Figure 1F). Consistently with the result that CFK1 and CUL1 physically interacted in the co-immunoprecipitation experiments, HA–CFK1 and CUL1 had partially overlapping fractionation profiles and co-fractionated in fractions corresponding to about 250 kDa. The broader elution profile of CUL1 could be due to the presence of CUL1 in other multiprotein complexes with other FBPs. Interestingly, HA–CFK1 could be detected only in the complex form, indicating that HA–CFK1 is not stable as a monomer. These results indicate that HA–CFK1 is specifically associated with CUL1 in vivo; they also confirm that an SCF[HA–CFK1] complex is correctly assembled in vivo.

**CSN Protects CFK1 from 26S Proteasome-Mediated Degradation**

Some FBPs are intrinsically unstable and their half-life can be regulated by a proteasome-dependent mechanism (Zhou and Howley, 1998; Galan and Peter, 1999). To test whether CFK1 was itself a substrate of the UPS, we tested whether it was ubiquitinated in vivo. Total protein extracts prepared from wild-type (Col-0) and from P35S:HA–CFK1 seedlings were subjected to immunoprecipitation, followed by immunodetection. A significant increase in the production of higher-molecular-mass species was observed when HA–CFK1 was immunoprecipitated from P35S:HA–CFK1 seedlings treated with the proteasome inhibitor MG132 (Lee and Goldberg, 1998) (Figure 2, top panel). Subsequent immunoblot analysis using antibodies against ubiquitin (anti-Ub) indicated that the higher-molecular-mass species corresponded to ubiquitinated HA–CFK1 (Figure 2, bottom panel). This result indicates that CFK1 is ubiquitinated in vivo.

To establish whether CFK1 is a substrate of proteasome activity, 6-day-old P35S:HA–CFK1 seedlings were treated with cycloheximide (CHX) to inhibit protein synthesis, MG132 to inhibit proteasome activity, or with a combination of the two for 4 h, and the total protein extracts were subjected to immunodetection. None of the inhibitors used led to a significant change in CFK1 mRNA levels (Supplemental Figure 3A). However, as shown in Figure 3A, addition of MG132 was indeed effective in stabilizing HA–CFK1, especially in the absence of CHX, while the protease inhibitor PMSF was ineffective in this respect. This result confirmed that CFK1 is a substrate of the proteasome.

The stability of a subset of FBPs has been shown to be regulated by CSN in Saccharomyces pombe, N. crassa, Caenorhabditis elegans, and mammals (He et al., 2005; Wee et al., 2005; Cope and Deshaies, 2006; Luke-Glaser et al., 2007). Our finding that CFK1 co-purifies with CSN prompted us to investigate whether stability of the former is regulated by the latter. To this goal, we crossed the P35S:HA–CFK1 transgenic line (in the Col-0 ecotype) to the csn4-1 mutant (cop8-1, in the Ws ecotype; Serino et al., 1999), which lacks CSN and accumulates deneddylated CUL1 (Gusmaroli et al., 2007). Because homozygous csn4-1 mutants are seedling-lethal, the P35S:HA–CFK1 construct was first introduced into a heterozygous csn4-1 background; homozygous csn4-1 P35S:HA–CFK1 plants were then identified in the selfed F2 population.

We first determined HA–CFK1 half-life in the presence or absence of CSN, by assessing HA–CFK1 protein levels in 6-day-old P35S:HA–CFK1 (both in the Col-0 and in the Col-0/Ws background) or P35S:HA–CFK1 csn4-1 seedlings. No detectable difference in protein abundance was observed in the steady-state levels of HA–CFK1 protein from these seedlings (Figure 3B). We therefore treated P35S:HA–CFK1

![Figure 2. CFK1 Is Ubiquitinated In Vivo.](image-url)

Immunoblot analysis with α-HA (top) and α-ubiquitin (α-Ub, bottom) of protein extracts from 6-day-old wild-type (Col-0) and P35S:HA–CFK1 seedlings incubated for 4 h in 50 μM MG132 and immunoprecipitated with α-HA resin. MWs are indicated in kiloDalton (kDa). Asterisks: aspecific bands. A minor aspecific binding of the α-HA resin to ubiquitinated proteins was observed (bottom panel).
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(both in the Col-0 and in the Col-0/Ws background) and csn4-1 P35S:HA–CFK1 seedlings with CHX to inhibit protein synthesis, and the total protein extracts were subjected to time-course RT–PCR and immunodetection. CFK1 mRNA was expressed at comparable levels in wild-type seedlings at all time points tested, while it was expressed at slightly higher levels in csn4-1 mutants (Supplemental Figure 3B). In contrast, in either Col-0 or Col-0/Ws wild-type seedlings, HA–CFK1 protein levels were reduced after 4 h of incubation with CHX and were undetectable after 8 h. Remarkably, HA–CFK1 levels were clearly reduced after only 2 h of incubation with CHX in csn4-1 mutants, indicating that the absence of CSN increased the instability of HA–CFK1 (Figure 3C). Taken together, these results provide convincing evidence that HA–CFK1 stability is regulated at the posttranslational level by CSN and the UPS.

Light Promotes CFK1 Transcript Abundance in the Hypocotyl

A quantitative real-time RT–PCR (qRT–PCR) analysis indicated that expression of the CFK1 gene was slightly higher (about 1.5-fold) in whole Arabidopsis seedlings grown in the light than in the dark (Figure 4A). To confirm this result, we analyzed CFK1–YFP protein localization in dark and in light-grown seedlings. As shown in Figure 4B (left panel), in dark-grown, 3-day-old PCFK1:CFK1–YFP seedlings, YFP fluorescence was mainly localized in the unexpanded cotyledons and in the root. In light-grown seedlings, expression of

Figure 3. HA–CFK1 Is Regulated by the Proteasome and by the CSN.
(A) Immunoblot (top) and relative densitometric analysis (bottom) of the effects of DMSO, cycloheximide (CHX), MG132, and PMSF on HA–CFK1 protein accumulation. Protein extracts were prepared from 6-day-old P35S:HA–CFK1 seedlings mock treated with DMSO or treated with 50 μM MG132, 100 μM CHX, a combination of the two, or 4 mM PMSF for 4 h. Equal protein loading was confirmed with α-Rpt5.
(B) Immunoblot (top) and relative densitometric analysis (bottom) of the effects of the csn4-1 mutation on the steady-state levels of HA–CFK1. Protein extracts were prepared from 6-day-old Col-0, Col-0/Ws (wild-type sibling from the cross), or csn4-1 seedlings harboring P35S:HA–CFK1. Bars represent the mean ± SEM.
(C) Immunoblot (left) and relative densitometric analysis (right) of the effects of CHX on HA–CFK1 levels in the wild-type and in csn4-1 mutants. 6-day-old Col-0, Col-0/Ws, or csn4-1 seedlings harboring P35S:HA–CFK1 were incubated with CHX as indicated. Equal protein loading was confirmed with α-Rpt5. Bars are means ±SEM.
PCFK1:CFK1–YFP remained almost unchanged in the cotyledons and in the root, while it was increased in the hypocotyl (Figure 4B, right panel). To further investigate the relationship between light and CFK1 accumulation in the hypocotyl, we determined the kinetics of change in CFK1–YFP fluorescence following transfer of 6-day-old seedlings from dark to light. We found that hypocotyls from dark-grown seedlings began to exhibit increased CFK1–YFP fluorescence within 1 h of onset of light exposure, with subsequent increase in CFK1–YFP levels proportional to the duration of light exposure (Figure 4C). We further confirmed that the observed increase in CFK1–YFP fluorescence was genuinely associated with an increase in CFK1–YFP protein level via immunodetection of CFK1–YFP in hypocotyl extracts (using antibodies against GFP; Figure 4D, top panel). This experiment revealed a progressive increase in the level of immunologically detectable CFK1–YFP that reached a plateau after around 3–4 h (Figure 4D, bottom panel).

Further measurements of CFK1 transcript abundance by qRT–PCR of hypocotyls of 6-day-old Col-0 seedlings grown in the dark and moved for 4 h in the light revealed that light induces CFK1 transcription of approximately 2.5-fold (Figure 4E). We concluded that light promotes CFK1 transcript accumulation in the hypocotyl.

**Light Does Not Regulate CFK1 Stability**

Because we have shown earlier that CFK1 protein stability is regulated by the UPS, we next asked whether light, which affects CFK1 transcription, also affects CFK1 protein stability. To this goal, we used P35S:HA–CFK1 seedlings, in which HA–CFK1 is expressed from the constitutive 35S promoter, and determined HA–CFK1 protein levels in hypocotyls following transfer of 6-day-old P35S:HA–CFK1 seedlings from dark to light. As shown in Figure 5A, similar HA–CFK1 protein levels were observed in the hypocotyls of seedlings in both conditions, suggesting that light exposure did not have any detectable effect on HA–CFK1 hypocotyl accumulation. Furthermore, blocking the proteasome activity with MG132 resulted in approximately the same relative level of HA–CFK1 stabilization in both dark- and light-grown P35S:HA–CFK1 seedlings (Figure 5B). CHX-chase experiments also demonstrated a similar half-life for HA–CFK1 in light-grown and dark-grown
CFK1 Controls Hypocotyl Elongation

Taken together, these data indicate that light does not affect CFK1 protein stability and that it regulates CFK1 mainly at the transcriptional level.

Overexpression and Down-Regulation of CFK Genes Affect in Opposite Ways Hypocotyl Elongation

The dependence of CFK1 transcript accumulation upon light specifically and only in the hypocotyl suggested a possible involvement of CFK1 in light-controlled hypocotyl growth. Indeed, P35S:HA–CFK1 seedlings showed an approximately 30% increase in hypocotyl length when compared to the wild-type under continuous white light (Wc), as shown in Figure 6A. In Arabidopsis, both cryptochromes, which respond to blue/UV-A light, and phytochromes, which sense red/far-red light, are necessary for normal hypocotyl elongation. Consequently, we set to assess the light-quality dependence of this long-hypocotyl phenotype by growing seedlings for 6 d in the dark or under continuous red (Rc), far-red (FRc), or blue (Bc) light. P35S:HA–CFK1 seedlings showed long hypocotyls under all light qualities, as well as in the dark (Figure 6A and 6B), suggesting that CFK1 promotes hypocotyl elongation, independently of light quality.

To further substantiate the involvement of CFK1 in regulating hypocotyl growth, we retrieved a T-DNA insertional mutant of CFK1 (cfk1-1), in which the T-DNA was inserted 664 nucleotides after the ATG start codon, leading to a complete absence of the full-length transcript (Supplemental Figure 5A–5C). When compared to their wild-type siblings (Col 1b), cfk1-1 mutant seedlings displayed moderately but significantly shorter hypocotyls only under Rc light (Figure 6A and 6B).

Because the high identity between the CFK1 and CFK2 genes raised the possibility of a functional redundancy between the two genes, we generated plants where both genes were knocked down simultaneously by RNA interference. These CFKRNAi plants harbor a hairpin containing inverted repeats of 295 nucleotides identical to both CFK1 and CFK2 under the control of a β-estradiol-inducible promoter (Supplemental Figure 5D). Among the T3 transgenic seedlings obtained, two representative lines, showing a significant reduction in CFK1 and CFK2 expression when treated with β-estradiol (Supplemental Figure 5E), were selected for further experiments.

Interestingly, the modest hypocotyl phenotype of the cfk1-1 mutant was enhanced in CFKRNAi plants: when grown on inductive media, CFKRNAi seedlings showed a reduction in hypocotyl length under Rc and FRc light and, to a lower extent, under Bc light (Figure 6A and 6B). This inhibition of hypocotyl elongation was dependent on the concentration of the inducer and was not observed in pER8–GFP control seedlings (Supplemental Figures 5F and 6A). In contrast, no alteration in hypocotyl length was observed in CFKRNAi seedlings grown in the dark in the presence of the inducer (Figure 6A and 6B). These results corroborate the notion that CFK1 acts as a positive regulator of hypocotyl elongation, and suggest a putative functional redundancy between CFK1 and CFK2. Because CFK2 mRNA levels in cfk1-1 mutant did not significantly differ from their corresponding wild-type controls (Supplemental Figure 5C), a cross-regulatory effect between the two homologous genes can be ruled out, suggesting that CFK1 and CFK2 cooperate for the promotion of the hypocotyl
CFK1 Regulates Hypocotyl Elongation

In Arabidopsis, most, if not all, cells of the hypocotyl are formed in the embryo. Consequently, after germination, hypocotyl growth is mainly the result of longitudinal cell expansion (Gendreau et al., 1997). To assess whether the hypocotyl phenotype observed in seedlings with altered CFK1 expression was due to a difference in cell size or in cell number, we counted the cells in the outer cortex layer of the hypocyls of 6-day-old P35S:HA–CFK1, cfk1-1, CFKRNAi, and their respective control seedlings. As shown in Figure 6D, all lines had very similar cell numbers. The absence of a significant difference in cell number indicates that the long-hypocotyl phenotype of P35S:HA–CFK1 seedlings and the short hypocotyl phenotype of cfk1-1 and CFKRNAi seedlings are due to an altered cell longitudinal expansion, rather than cell division.

The results described so far support a role for CFK1 in increasing hypocotyl cell size and prompted us to ask whether CFK1 was also required for cell expansion in another organ. Because Arabidopsis cotyledons are known to grow post-embryonically mainly by cell expansion after an early phase of cell divisions (Tsukaya et al., 1994), we asked whether CFK1 might also mediate cotyledon expansion. We measured the area of the cotyledons from 6-day-old P35S:HA–CFK1, cfk1-1, β-estradiol-induced and -uninduced CFKRNAi plants, and from their respective wild-type controls grown under Rc light, where the hypocotyl phenotype is particularly evident. As shown in Figure 6E, a statistically significant increase in cotyledon size was observed in P35S:HA–CFK1 seedlings, while a slight decrease in cotyledon elongation, though they may not share completely overlapping functions.

Further comparison of P35S:HA–CFK1 and CFKRNAi seedlings grown on inductive and non-inductive medium under increasing fluence rates of different light qualities and in the dark showed that P35S:HA–CFK1 seedlings were taller than the wild-type, with a more evident fluence response mainly under low fluences of Rc light. In addition, CFKRNAi seedlings, but not pER8–GFP controls (Supplemental Figure 6B), had significantly shorter hypocyls than the wild-type under low fluences of Rc and FRc and, to a lower extent, under low fluences of Bc light (Figure 6C). At higher fluence rates, when light intensity nearly approaches saturation for hypocotyl growth inhibition, the differences between CFKRNAi hypocotyls and their corresponding controls became smaller. These results indicate that light might modulate CFK1-mediated promotion of hypocotyl elongation.

CFK1 Regulates Cell Size

Figure 6. CFK1 Regulates Hypocotyl Elongation by Regulating Cell Size. (A) Hypocotyls of 6-day-old representative cfk1-1, CFKRNAi, and P35S:HA–CFK1 and control seedlings. Seedlings were grown in continuous white (Wc), red (Rc), far-red (FRc), blue (Bc) light, or in dark (D). For cfk1-1, control was a wild-type sibling obtained among the progeny from a cfk1-1 heterozygous line (Col 1b). CFKRNAi seedlings were grown in the presence of DMSO (negative control) or 10 μM β-estradiol (β-EST). For P35S:HA–CFK1, Col-0 was used as a control. Light intensities: W (70 μmol m−2 s−1), R (15 μmol m−2 s−1), FR (0.06 μmol m−2 s−1), B (3.2 μmol m−2 s−1). Scale bars = 1 mm. (B) Quantification of hypocotyl lengths. Data are means ± SEM (n > 30, three replicates per genotype). (C) Fluence rate responses of CFKRNAi and P35S:HA–CFK1 seedlings. CFKRNAi, P35S:HA–CFK1 seedlings and their controls were grown in various fluence rates of Rc, FRc, and Bc. CFKRNAi seedlings were grown in the presence of DMSO or 10 μM β-estradiol (β-EST). Measurements are expressed as a fold-change (FC) compared to their controls. Error bars represent the variation in SEM of this FC response (n > 30, three replicates/fluence/genotype). (D) Hypocotyl outer cortex cells cell numbers of 6-day-old CFK lines and their respective control seedlings grown in Rc. SD, standard deviation (n > 30 per genotype). (E) Measurement and representative images of cotyledon areas from 6-day-old cfk1-1, CFKRNAi, P35S:HA–CFK1, and their control seedlings grown in Rc (15 μmol m−2 s−1). Data are means ± SEM (n > 30, three replicates/genotype).
size was observed in induced CFKRNAi seedlings. No notice-
able change in cotyledon size was observed for the cfk1-1
mutant compared to its wild-type control or for pERB–GFP
control seedlings (Figure 6E and Supplemental Figure 6C). We
concluded that CFK1 is a positive regulator of hypocotyl and
cotyledon cell expansion in Arabidopsis seedlings.

The Phenotype of a CSN Partial Loss-of-Function
Mutant Can Be Enhanced by Reduced CFK1 Levels

Light-grown CSN mutants have dramatically short hypocotyls
(Kwok et al., 1996). The physical interaction between CFK1
and CSN suggests that CSN-mediated hypocotyl inhibition
might also require CFK1 function. The requirement of CSN
and CFK1 for proper hypocotyl growth was further assessed
in a genetic interaction study, by crossing the CFKRNAi line
to a weak CSN mutant allele (csn5a-2) (Gusmaroli et al., 2004;
Dohmann et al., 2005; Gusmaroli et al., 2007). When we intro-
duced the CFKRNAi construct into the csn5a-2 homozygous
mutant background, the hypocotyl length of the resulting
csn5a-2 CFKRNAi seedlings was slightly enhanced compared
to the parents, when grown in Rc (Figure 7A). This sug-
gests an additive effect between csn5a-2 and the CFKRNAi
transgene. On the other hand, CFK1 overexpression could not
compensate for the severe phenotype of a CSN knock-
out mutant (csn4-1), as judged by the phenotypical analysis
of P35S:HA–CFK1 csn4-1 seedlings (Figure 7B).

DISCUSSION

In this work, we present the characterization of CFK1, a new
plant-specific F-box protein co-purifying with CSN. We show
that, in Arabidopsis, CFK1 is a component of an SCF complex
and is a target of CSN activity; that transcription of the CFK1
gene is strongly light-dependent specifically in the hypocotyl;
and that CFK1 is a positive regulator of hypocotyl elongation.

CFK1 contains an F-box motif in its N-terminus, and our
work demonstrates that this domain of CFK1 is functional,
since it is responsible of the interaction of CFK1 with the ASK1
subunit of SCF complexes. We also provide evidence that
CFK1 interacts in vivo and co-fractionates with CUL1, indicat-
ing that CFK1 forms a functional SCF<sub>CFK1</sub> complex in vivo.

Many FBPs from different organisms have been shown to
be intrinsically unstable, their half-life being regulated by a
proteasome-dependent mechanism (Zhou and Howley, 1998;
Galan and Peter, 1999; Mathias et al., 1999; Kao et al., 2000; Rouillon et al., 2000). Our results demonstrate that CFK1 falls
into this category; CFK1 intrinsic instability might be neces-
sary to allow a rapid switching of SCF complexes specific-
ity, thereby enabling cells or organisms to adapt quickly to
changing physiological conditions and cell cycle progression
(Galan and Peter, 1999).

A recent report indicates that the abundance of
CORONATINE INSENSITIVE1 (COI1), another F-box protein
from Arabidopsis, is strictly maintained at an appropriate and
stable level, and that dissociated COI1 is degraded through
the 26S proteasome pathway (Yan et al., 2013). Similarly, the
inclusion in an SCF complex might play an essential role in
regulating the stability of CFK1. Indeed, similarly to COI1
(Feng et al., 2003), we could not detect the presence of a
CFK1 monomer in size fractionation experiments, suggesting
that CFK1 is stable only within the SCF complex.

Because we found CFK1 in a screen for novel CSN interac-
tors, CSN might also be required to regulate the function of
SCF<sub>CFK1</sub>. It has been suggested that CSN promotes CRL activity
in vivo by counteracting the autocatalytic breakdown of CRL
adaptors such as FBPs (Zhou et al., 2003; He et al., 2005; Wee
et al., 2005). Consequently, in csn mutants, CRL substrates
are stabilized, while CRL substrate adaptors are destabilized.
Consistently with this model, we show here that CSN pro-
 motes CFK1 stability. Our results indicate that the same pro-
 cess—CFK1 degradation by the proteasome—is taking place
both in wild-type and in csn mutants, but that it is accelera-
ted in the absence of the CSN. In addition, we show that
a reduction in CSN levels enhances the hypocotyl phenotype
of CFKRNAi seedlings. This additive effect could be explained
by the fact that the possible residual CFK1 and CFK2 activity
present in the CFKRNAi lines might be further lowered by a
reduction in CSN levels. On the other hand, the finding that
a complete loss of CSN activity suppresses the CFK1 overexpres-
 sor phenotype suggests that CSN is required for CFK1 func-
tion. Indeed, in the absence of CSN, CFK1 half-life is reduced,
and its function might be compromised.

Our work provides strong evidence that the CFK1 tran-
script level is tightly controlled by light in an organ-specific
fashion: CFK1 is expressed at much higher levels specifically
in the hypocotyl of light-grown seedlings as compared to
dark-grown ones, while expression in other organs (root,
cotyledons) is similar in light or dark. CFK1 half-life, however,
remained the same regardless of the light conditions used,
indicating that CFK1 stability is independent of light. Hence,
light regulates CFK1 expression but not CFK1 protein stability.

We also show that CFK1 promotes hypocotyl elonga-
tion by regulating cell expansion. Indeed, the fact that
cotyledon size—another developmental process controlled
mainly by cell expansion in seedlings—is oppositely regu-
lated in P35S:HA–CFK1 and CFKRNAi seedlings reinforces
the hypothesis that CFK1 is involved in the control of cell
expansion. Our results also suggest that CFK1-mediated pro-
motion of hypocotyl elongation requires light, which
indeed we have shown to be necessary for CFK1 transcrip-
tional activation.

CFK1 and CFK2 might play similar, but not identical, func-
tions: the two proteins share a 98% amino acid identity, and
RNAi-mediated silencing of both CFK1 and CFK2 leads to a
more severe phenotype than the loss of just CFK1. However,
the weakness of the cfk1 hypocotyl phenotype is not due to a
compensation of the missing CFK1 transcript by the CFK2 tran-
script, since this latter in the cfk1-1 mutant is not higher than
in the wild-type. Further biochemical, molecular and genetic studies will be critical to uncover the function of CFK2.

The incorporation of CFK1 into an SCF<sup>CFK1</sup> complex, together with the phenotype of CFK1 reduction-of-function and gain-of-function transgenic lines, suggests that CFK1 might direct the ubiquitination and subsequent degradation of one or more negative regulators of cell size. Since kelch repeats are known to form a β-propeller typically involved in protein–protein interactions (Adams et al., 2000), the predicted kelch repeats at the C-terminus of CFK presumably provide the necessary sites to bind specific substrates. The future isolation of SCF<sup>CFK1</sup> substrates will help in understanding the molecular mechanisms of CFK protein(s) function, and their regulation by light and by the CSN.

**METHODS**

**Plant Material and Growth Conditions**

Wild-type, CFKRNAi, PCFK1:CFK1–YFP, and P35S:HA–CFK1 plants were of the Columbia (Col-0) ecotype. The csn4-1 (cop8-1) mutant is of the Wassilewskija (Ws) ecotype (Serino et al., 1999). The cfk1-1 mutant, corresponding to the WiscDsLox506C02 line (Woody et al., 2007), is in the Col-0 ecotype, and the respective T-DNA insertion is located 664 bp downstream of the ATG. The csn4-1 P35S:HA–CFK1 transgenic line was obtained by crossing the csn4-1 heterozygous mutant with P35S:HA–CFK1 plants.

Unless otherwise noted, Arabidopsis thaliana seedlings were surface-sterilized and grown on solid MS medium with Gamborg’s vitamins (Duchefa; www.duchefa.com) at 22°C with a light intensity of 130 μmol m<sup>-2</sup> s<sup>-1</sup>. For fluence curve and phenotypical experiments, seed plates were exposed to white light for 8 h to stimulate germination, and then transferred to the appropriate light conditions. For experiments involving β-estradiol, cycloheximide (CHX), MG132, and solvent mock experiments, seedlings were grown in the conditions indicated in the text.

**Transgenic Plants**

To generate PCFK1:CFK1–YFP transgenic plants, a fragment of 3022 bp, encompassing the full-length gene, and 1333 bp upstream of the ATG of CFK1 was amplified from genomic DNA with the primers promCFK1–YFPFWD and promCFK1–YFPREV. The YFP gene was amplified from the pM999YFP vector by using the primers YFPFWD and YFPREV, and cloned downstream of the PCFK1:CFK1 transgenic line was obtained by crossing the csn4-1 heterozygous mutant with P35S:HA–CFK1 plants.

To generate P35S:HA–CFK1 transgenic plants, a sequence encoding three HA epitopes was amplified from the Alligator2 vector (www.isv.cnrs-gif.fr/jg/alligator/) with the primers KpnFWD and EcoRIREV. The amplified fragment was cloned in the pENTR1A vector (Invitrogen; www.invitrogen.com) to generate the pENTR–3xHA plasmid. Full-length CFK1 was amplified from cDNA by using the CFKEcoRI and CFKXhoI primers, and cloned in the pENTR–3xHA plasmid to generate the pENTR–HA–CFK1 clone. The HA–CFK1 fragment was
recombined into the Gateway vector pH2GW7 containing the CaMV 35S promoter (Karimi et al., 2002).

To generate CFKRNAi plants, a region corresponding to 295 bp of CFK1 and CFK2, located (for CFK1) 1096–1391 nucleotides downstream of the ATG and with no homology to other sequences in the Arabidopsis genome (CFK2), was amplified with CFKFX and CFKRK (sense), and CFKB and CFKFC (antisense). The two amplified fragments were cloned in the vector pHannibal (Wesley et al., 2001), and the resulting hairpin RNA construct was cloned in the β-estradiol-inducible XVE pER8 vector (Zuo et al., 2000).

All constructs were introduced into the Agrobacterium tumefaciens strain GV3101 and transformed into Arabidopsis Col-0 plants (Clough and Bent, 1998). Approximately 40 independent lines were generated per construct. The T1 hygromycin-resistant plants were selfed, and the T2 and T3 generations were used for the analysis. Primers are listed in Supplemental Table 2.

Purification of COP9 Signalosome Interacting Proteins and Mass Spectrometry Analysis

COP9 signalosome immuno-affinity purification and mass spectrometry analysis were performed according to Serino et al. (1999) using anti-CSN5 antibodies. The affinity purified complex was subject to SDS–PAGE and several bands present in low sub-stochiometric amounts compared to the eight CSN subunits were excised and subjected to protein sequencing.

Sequence Retrieval, Alignments, and Domain Analysis

Amino acid sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and visualized with the BOXSHADE server (www.ch.embnet.org/software/BOX_form.html). Protein domains were identified by using the SMART database (http://smart.embl.de/) (Letunic et al., 2009). β strands were identified by comparison of results obtained from the PsiPred (http://bioinf.cs.ucl.ac.uk/psipred/) and the Jpred (www.compbio.dundee.ac.uk/www-jpred/) servers.

Fluorescence Microscopy

For DAPI staining and YFP fluorescence of hypocotyl cells, hypocotyls were mounted in DAPI solution (DAPI 5 μg ml⁻¹; PIPES 50 mM, EGTA 5 mM, NP-40 0.1%, and DMSO 10%) and observation on a Leica TCS SPE confocal microscope. YFP fluorescence images of whole seedlings were taken with an Axioskop 2 plus microscope (Zeiss; www.zeiss.com/) equipped with a ProgRes C10 camera (Jenoptik; www.jenoptik.com).

Protein Extraction, Immunoprecipitation, Gel-Filtration, and Immunoblot Analyses

For all experiments with Arabidopsis plant extracts, proteins were extracted in IP buffer as described in Serino et al. (2003). HA–CFK1 was detected with monoclonal antibodies to HA (Sigma). CUL1, CSN6, TBP, and Rpt5 (Regulatory Particle 5a or At6A) were detected with specific antibodies (Gray et al., 1999; Kwok et al., 1999; Peng et al., 2001; Schwechheimer et al., 2001; Gusmaroli et al., 2007).

For gel filtration, the total soluble protein extract was fractionated through a Superose 6 HR 10/30 gel-filtration column (GE Healthcare; www.gelifesciences.com) followed by immunodetection (Kwok et al., 1998).

For co-immunoprecipitation analyses, 500–1000 μg of total proteins were incubated with 20 μl of monoclonal anti-HA immobilized onto Sepharose fast flow beads (Sigma; www.sigmaaldrich.com) for 4 h at 4°C. The beads were washed three times with the IP buffer and subjected to immunodetection.

For in vivo ubiquitination analyses, light-grown 6-day-old 35S:HA–CFK1 seedlings were incubated with 50 μM MG132 for 4 h. Crude extracts were then prepared according to Lee et al. (2009) and subjected to immunoprecipitation, followed by immunodetection.

For MG132, PMSF, and CHX analysis, seedlings were ground in IP buffer and equal amounts of proteins were subjected to immunoblot analysis.

For densitometric analysis, mean intensities were background subtracted and normalized to the loading control using the ImageJ software v.1.43 (http://rsb.info.nih.gov/ij). Error bars were derived by error propagation calculation. For all HA–CFK1 and YFP densitometric analyses, the levels of HA–CFK1 or YFP from three independent experiments were quantified by densitometry analysis (bottom panel), with the relative HA–CFK1 or YFP at the start of the experiment set at 1.

Yeast Two-Hybrid Assay

Full-length CFK1 and a portion of the CFK1 gene depleted of the F-box domain (obtained by PCR amplified with the CFK1FW3 and CFK1REV3 primers) were cloned in the pEG202 vector and used as a bait. The pJG–ASK1 and the pJG–CUL1 vectors have been described by Schwechheimer et al. (2001). The bait constructs were transformed into yeast strain EGY48, while prey constructs were transformed into yeast strain L40. The plate and liquid assays were performed as described by Golemis and Khazak (1997).

qRT–PCR

At least three independent RNA extractions, reverse transcriptions, and qPCR analyses were carried out for each experiment. Total RNA was extracted from whole seedlings according to Vittorioso et al. (1998). 1 μg of RNA was retrotranscribed with the SuperScript III Reverse Transcriptase kit (Invitrogen). Samples were amplified with the Power SYBR Green PCR Master Mix (Applied Biosystems; www.appliedbiosystems.com/) on a Lightcycler apparatus (Bio-Rad; www.bio-rad.com/). Primers CFKFWRT and CFKREV were used for CFK1; primers CFKFWRT and CFKREV were used for CFK2; primers CFKFW and CFKREV were used to analyze simultaneously both CFK transcripts. Target genes were analyzed using the lcyicer software (Bio-Rad) and normalized to the endogenous ACTIN2 control.
Samples were considered statistically significant for a Student's t-test value of \( p < 0.05 \). For \( CFK1 \) experiments, the \( CFK1 \) expression level of dark-grown wild-type seedlings was used as reference and set at 1. Error bars were derived by error propagation calculation. All primers are listed in Supplemental Table 2.

### Hypocotyl Length, Hypocotyl Cell Number, and Cotyledon Area Measurements

Hypocotyl length and cotyledon area measurements were taken from seedlings flattened on their agar plates. Images were taken using a digital camera and analyzed with the ImageJ software. Hypocotyl outer cortex cell number was determined by counting cells from the base to the top of the hypocotyl of at least 30 seedlings previously incubated in chloral hydrate (3:8 ratio of water and chloral hydrate) for 24 h at room temperature, using an Axioskop 2 plus microscope (Zeiss; [www.zeiss.com](http://www.zeiss.com)).

### Accession Numbers

Sequence data used in this study can be found in the GenBank database under the following accession numbers: \( CFK1 \) (NP_199050), \( CFK2 \) (NP_199051), OsCFK1 (NP_001063890); PtcFK1 (XM_002300600.1); PtcFK2 (XM_002307765.1).

### SUPPLEMENTARY DATA

Supplementary Data are available at [Molecular Plant Online](http://www.molplant.org).

### FUNDING

S.I. was supported by an EMBO short-term fellowship and B.L. by a Sapienza Università di Roma fellowship. This work was supported by research grants from Ministero dell’Istruzione, Università e Ricerca, Progetti di Ricerca di Interesse Nazionale, and from Sapienza Università di Roma to P.C.; from Ministero degli Esteri (Italy–Japan 269/0127680 to G.S. and T.T. and Italy–China 260/140270 to G.S.); from Consiglio Nazionale delle Ricerche (to G.S. and G.M.); and from the Japanese Society for the Promotion of Science (to G.S. and T.T.).

### ACKNOWLEDGMENTS

We thank the European Arabidopsis Stock Centre NASC for providing the WiscDsLox506C02 seeds. We also thank Nam Hai-Chua for providing the pER8 and the pER8–GFP vector, the Beijing Genomics Institute, and Jun Wang and Jian Wang for protein sequence analysis, Qi Xie for the gift of the anti-ubiquitin antibody. No conflict of interest declared.

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