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The role of CFK proteins in Arabidopsis development

Dottorando
Anna Franciosini

Docente guida
Prof. Giovanna Serino

Tutore   Coordinatore
Prof. Giuseppe Macino   Prof. Irene Bozzoni

Anna Franciosini

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GLOSSARY

Arabidopsis thaliana: small flowering plant that is widely used as a model organism in plant biology. Arabidopsis is a member of the Brassicaceae family.

COP9 signalosome (or CSN): a multi-subunit protein complex that is structurally related to the lid of the 26S proteasome. One subunit removes the ubiquitin fold protein NEDD8 from the cullin subunit in CRL E3s.

Cotyledons: the leaves of a seedling formed during embryonic development

CRL: cullin–RING ligase, a superfamily of ubiquitin ligases. It typically consists of a cullin family member as a molecular scaffold and of a small RING protein, RBX1 which forms the core complex. An active CRL is formed when the cullin–RBX1 core is assembled with a substrate binding module that recruits substrates.

F-box protein: substrate receptor protein of CUL1-based CRLs; binds to SKP1-type adaptor proteins.

Hypocotyl: the part of the stem of a young seedling that is situated underneath the cotyledon and above the root.

Proteasome: highly conserved protein degradation machine that consists of the 20S proteasome and 19S regulatory particles.

SCF: SKP1–cullin–F-box complex. It is a type of CRL ubiquitin ligase that contains a SKP1 adaptor protein, CUL1–RBX1 and an F-box-containing substrate receptor, which confers substrate specificity.

Seedling: young plant developing out of a plant embryo from a seed.

Ubiquitin: small protein present in all eukaryotic cells that participates in a variety of cellular functions including protein degradation.

UPS: ubiquitin-proteasome system; protein ubiquitination by the concerted action of an E1 ubiquitin activating enzyme, an E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase, and the subsequent proteolytic degradation of the polyubiquitinated protein by the proteasome.

SUMMARY

The regulation of protein turnover by the ubiquitin proteasome system (UPS) is a major post-translational 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 an effort to identify novel plant processes controlled by the CSN, in our laboratory we isolated two novel plant-specific proteins, CFK1 and CFK2, that interact with the CSN. We have shown that in *Arabidopsis thaliana* CFK1 is a component of a functional ubiquitin ligase complex, named SCF^{CFK1}.

During my PhD, I have demonstrated 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.
With my PhD work, I propose that CFK1 (and its regulation by CSN) is a novel component of the cellular mechanisms controlling hypocotyl elongation in Arabidopsis.

INTRODUCTION

CSN: Structure and Function

- CSN Architecture

The COP9 Signalosome (or CSN) is a multi-protein complex conserved in all eukaryotic organisms and initially isolated in plants as a repressor of light-dependent development (Wei and Deng 1992).

The CSN is composed of eight core subunits, denominated from CSN1 to CSN8 (Table 1). This composition is conserved among all eukaryotic organisms, with the exceptions of some unicellular fungi and of *C.elegans*, which possess smaller versions of the complex (Wei et al. 2008) (Table 1). Genes coding for CSN subunits 1-4 and 7-8 were found in the initial genetics screens, while CSN5 and CSN6 were identified as subunits after the biochemical purification of the complex. The reason why CSN5 and CSN6 were not isolated in the genetic screens is likely due to the fact that they are both encoded by two genes in *Arabidopsis* (Stratmann and Gusmaroli 2012).

Each CSN subunit contains one of two conserved domains: subunits CSN1-4 and CSN7-8 contain a PCI (proteasome, COP9 Signalosome and initiation factor 3) domain, while CSN5 and CSN-6 have a MPN domain (Mpr1 and Pad1 N-terminal) (Table 1) (Pick et al. 2009; Enchev et al. 2010).

The PCI domain displays a bipartite fold consisting of a N-terminal helical bundle and a C-terminal winged helix, which are connected through a central helix (Dessau et al. 2008). This domain mediates protein-protein interaction within multi-protein complexes and is therefore essential for the maintenance of the structural integrity of the complexes. The MPN domain contains a β-sheet motif with nine β-strands, surrounded by three α-helices (Sanches et al. 2007; Zhang et al. 2012).

DOMAINS	S.cerevisiae	S.pombe	C.elegans	Drosophila	Human	A.thaliana
PCI	Csn11	Csn1	CSN1	CSN1	CSN1	CSN1/FUS6
PCI	Csn10	Csn2	CSN2	CSN2	CSN2	CSN2/FUS12
PCI	-	Csn3	CSN3	CSN3	CSN3	CSN3/FUS11
PCI	Rpn5*	Csn4	CSN4	CSN4	CSN4	CSN4/COP8
MPN+	Csn5	Csn5	CSN5	CSN5	CSN5	CSN5
MPN-	Csi1	-	CSN6	CSN6	CSN6	CSN6
PCI	Csn9	Csn7	CIF-1	CSN7	CSN7	CSN7/FUS5
PCI	-	-	-	CSN8	CSN8	CSN8/COP9

* also a proteasome subunit (Serino and Pick 2013)

Table 1 – CSN Subunit Composition in Different Organisms (Franciosini et al., 2013).

Structural and biochemical studies have indicated that the MPN domain is present in two distinct versions. The first one, known as MPN+/JAMM (for Jab1/MPN/Mov34), is found in the CSN5 subunit, and harbors a metalloproease motif that is responsible for the catalytic activity of the complex (Sanches et al. 2007). This activity is essential for the removal of an ubiquitin-like peptide, NEDD8 (neural precursor cell expressed, developmentally down-regulated 8) (called RUB1 in plants), from the cullin subunit of the cullin-RING type of ubiquitin ligases (CRLs) (Figure 1) (Cope et al. 2002; Maytal-Kivity et al. 2002). The second version, which has recently been re-named MPN-, is located in CSN6, lacks the metal coordinating residues and is therefore biochemically

inactive, but likely plays a structural or regulatory function (Nezames and Deng 2012; Zhang et al. 2012; Serino and Pick 2013).
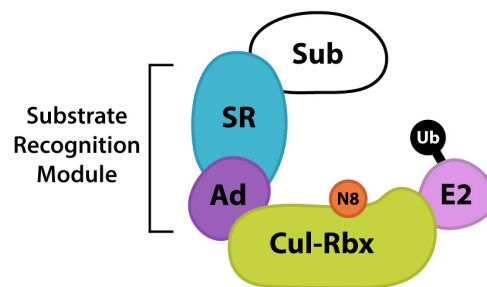

Figure 1. Model of a CRL Ubiquitin Ligase. The CRLs are composed by an enzymatic core that contains a cullin scaffold subunit and Rbx1 (Cul-Rbx), that interacts with the E2 Ub-conjugating enzyme. Specific substrates are recruited to the core by a substrate recognition module consisting of an adaptor protein (Ad) and a substrate receptor (SR). In the CRL active form, cullin subunit is modified by the attachment of the NEDD8 (N8) peptide (Franciosini et. al, 2013).

Because pure crystals of the entire complex have not been obtained to date, a tridimensional analysis of CSN architecture based on X rays crystallography is not available. However, several other approaches have been used to circumvent this problem and to allow a preliminary characterization of CSN structure. Mass spectrometry analysis and electron microscopy studies have contributed to elucidate the topology and the structure of the CSN (Sharon et al. 2009; Enchev et al. 2012). These data have shown that the human CSN is composed by two symmetrical modules, CSN1/2/3/8 and CSN4/5/6/7, connected by the interaction between CSN1 and CSN6 (Sharon et al. 2009) (Figure 2). High resolution electron-microscopy has provided the latest structure model depicting the refined subunit organization of the complex (Enchev et al. 2012).
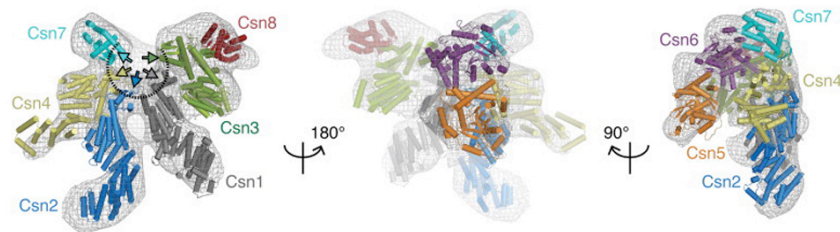
Figure 2 - Model of the CSN Structure. Left, PCI cluster side view. A dotted arc and color-coded arrows indicate the approximately coplanar positions of the winged-helix domains. MPN subunits are omitted for clarity. Center, opposite side, characterized by a protrusion formed by the two MPN domain subunits, Csn5 and Csn6. Right, view showing the edge of the coplanar PCI cluster. The protrusion formed by the Csn5 and Csn6 MPN subunits is left of the PCI cluster (Enchev et al. 2012).

- CSN Regulates CRLs Ubiquitin Ligases

A major breakthrough which led to the discovery of the biochemical activity of the CSN came from the laboratories of Raymond Deshaies and Xing-Wang Deng. By using human and *Arabidopsis*, these laboratories found a physical direct and functional interaction between CSN and CRLs, one of largest class of E3 ubiquitin ligases.

As shown in Figure 1, all CRLs enzymes are composed of a backbone cullin subunit (CUL1, CUL3 and CUL4 in *Arabidopsis*) (Shen et al. 2002; Gingerich et al. 2005) that interacts via its C-terminus with a RING-box protein 1 (RBX1) subunit, which functions in turn as a platform for the E2 (Ubiquitin-conjugating enzyme) charged with ubiquitin (Kleiger et al. 2009). At its N-terminus, the cullin subunit binds specific substrate recognition modules that recognize and deliver appropriate substrates for ubiquitylation. Different sub-classes of CRLs exist, depending on the different assembly based on the type of cullin subunit, and each cullin interacts with a different class of substrate recognition

modules (Hua and Vierstra 2011). In CUL1-based CRLs, the substrate recognition module is composed of a F-box protein, that is responsible for the interaction with the substrate, and which is anchored to CUL1 through the SKP1 (S-phase kinase-associated protein 1) adaptor subunit, called ASK1 (Arabidopsis SKP1-like1) in *Arabidopsis*. CUL1-containing CRL complexes are named SCFs (Skp1/Cul1/F-box) (Hua and Vierstra 2011). CUL3-based ubiquitin ligases contain a BTB/POZ (broad complex/tramtrack/bric-a-brac and Pox virus and Zinc finger) subunit that that serves as the substrate recognition module. CUL4-based CRLs contain a substrate recognition module composed of a DWD (DDB1-binding/WD-40 domain) protein, which interacts with the substrate, and of a DDB1 (DNA damage binding protein 1) adaptor that connects DWD proteins to and CUL4 (Hua and Vierstra 2011).

The *Arabidopsis* genome, unlike the genome of other organisms, encodes a remarkable large number of genes related to protein degradation. In *Arabidopsis* there are more than 700 F-box proteins, 85 DWD and 80 BTB/POZ substrate receptors; thus, an incredible number of CRLs can be assembled. This suggests that, in this sessile organism, protein degradation has a crucial role in cellular and developmental processes (Vierstra 2009). Because the CSN has been shown to interact with all three types of CRLs (Lyapina et al. 2001; Gusmaroli et al. 2007; Hotton and Callis 2008) in *Arabidopsis*, this complex might be required to ensure the proper life span of hundreds, if not thousands, specific proteins. This might help explaining the highly pleiotropic phenotype of *csn* mutants, because lack of CSN would cause malfunction of multiple CRLs (Table 2).

Plant Processes	CRL	Substrate	References
Photomorphogenesis	COP1, CDD-CUL4	phyA, phyB, HYS, HYH, LAF1, HFR1	Chen et al., 2006
Flower Development	SCF^{UFO}	Unknown	Wang et al., 2003
Auxin Perception	SCF^{TIR1}	AUX/IAA Proteins	Schwechheimer et al., 2001
Jasmonate Response and Plant Defense	SCF^{COI1}	JAZ Proteins	Feng et al., 2003; Hind et al., 2011
Gibberellin Signaling	SCF^{SLY}	DELLA Proteins	Dohmann et al., 2010
R-Mediated Resistance	SGT1-associated SCF	Unknown	Liu et al., 2002
SA-Mediated Defense	CUL3-based Ubiquitin Ligases	NPR1	Spoel et al., 2009

Table 2 – Representative Plant Processes Controlled by the CSN (Franciosini et al., 2013).

- The CSN and CAND1-Regulated CRL Cycle

CRLs function is regulated by the covalent attachment (neddylation) or removal (de-neddylation) of the NEDD8 peptide on their cullin subunit. NEDD8 is conjugated to CRLs by a three-steps cascade reaction similar to ubiquitination cascade. NEDD8 binding promotes substrate ubiquitination, because it leads to a conformational change on the CRL that allows the transfer of the ubiquitin peptide from the E2 enzyme to the substrate (Figure 3). CSN removes NEDD8 from the CRLs, through its catalytic CSN5 subunit leading to CRL inactivation (Lyapina et al. 2001; Cope et al. 2002).

Evidence from plants and other organism show that CRL neddylation and deneddylation are required for the proper functioning of the CRLs (Wei and Deng 2003). Understanding the precise mechanism of how the cycle of CRL neddylation and deneddylation is still a focus of active research. In the current model, the substrate itself plays a role in the regulation of the CRL cycle. In presence of a substrate, the substrate receptor subunit can inhibit de-neddylation of its cullin partner, and prevent CSN activity in order to ensure substrate degradation (Stratmann and Gusmaroli 2012) (Figure 3). Thus, substrate availability promotes accumulation of active, NEDD8-conjugated CRL complexes. After substrate ubiquitination and degradation, the CRL can recruit the CSN and become de-neddylated, or alternatively, can undergo auto-ubiquitination of its own substrate receptor subunit, followed by release of the cullin-RBX1 core (Figure 3). CSN has high affinity for its reaction products, and might remain bound to the cullin-Rbx1 core. When substrates are again available, CSN is displaced, CRLs become neddylated and come back in their activated form (Emberley et al. 2012).

CSN regulation of CRLs works in concert with another regulation module, centered around the protein CAND1 (Cullin-associated and neddylation dissociated 1). CAND1 binds the unmodified cullin-RBX core, preventing it from the association with the other subunits of the CRL complex. This results in an inactive CRL, and the active state is restored only when the levels of its respective substrate recognition module increase. The substrate recognition module then displaces CAND1, allowing cullin neddylation and substrate ubiquitination. CAND1 might play the role of a substrate receptor exchange factor, and stimulate CRL activity, thus allowing the exchange of different substrate recognition modules on the same cullin-Rbx1 scaffold (Bennett et al. 2010; Pierce et al. 2013) (Figure 3). In other words, after substrate degradation and CSN-mediated deneddylation, the cullin-Rbx1 core can follow two different routes: (1) it can interact with another substrate, promote CSN displacement, and become neddylated, or (2) can bind

CAND1, enter in an "exchange regime" and as a result associates with a newly available substrate recognition module. This triggers the dissociation of CAND1. A neddylation event completes the cycle, and CRL is now back in its active form again (Bennett et al. 2010; Pierce et al. 2013).

CSN regulates the CRL cycle also in a non-catalytic fashion. Results obtained for SCF and CUL4-based CRL suggest that CSN occludes two CRL functional sites: the Cul1-Rbx1 C-terminal domain and the substrate receptor, thus maintaining CRL assembly. This indicates that this CSN-mediated inhibition by steric hindrance could be conserved among all CRLs. Thus, CSN works as a CRL inhibitor by deneddylation and protein interactions, with a result that it promotes sustained functions of CRLs by maintaining its stability by facilitating a rapid and efficient substrate turnover in vivo (Fischer et al. 2011; Enchev et al. 2012).
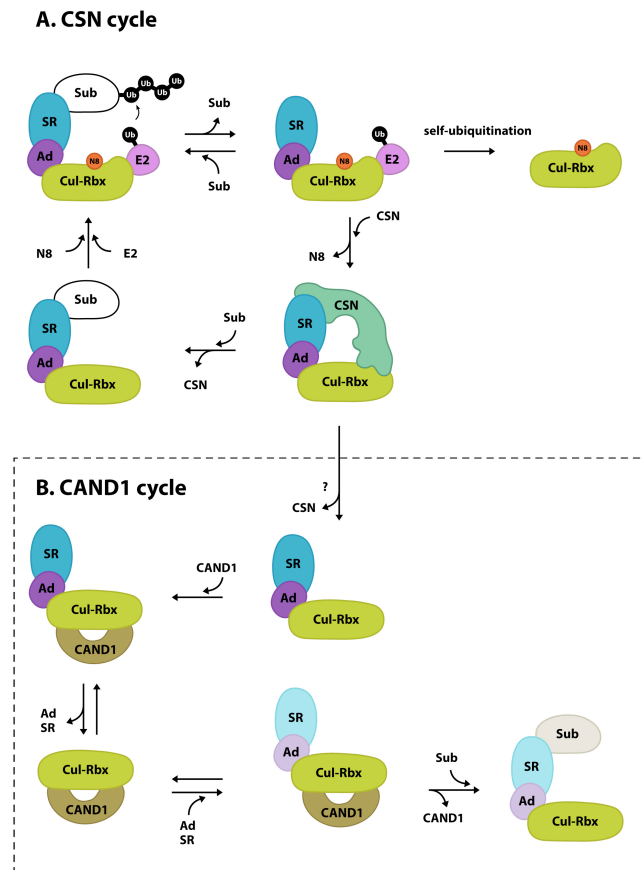
Figure 3 - Proposed Regulatory CRLs Cycles Involving CSN and CAND1.
A completely assembled CRL binds a E2 Ub-conjugating enzyme and poly-ubiquitinates a substrate (upper left). Upon consumption of the substrate the CRL complex could either be subjected to auto-ubiquitination and degradation of the substrate receptor (SR), or recruit the CSN and be deneddylated. The reappearance of the substrate leads to displacement of the CSN and to the reformation of neddylated active CRL complex, completing the CSN cycle. In addition, following the dissociation of the CSN that occurs in an unknown manner, CRL can enter in the CAND1 cycle. The protein CAND1 binds the cullin-Rbx1 scaffold (Cul-Rbx), promoting the dissociation of the adaptor (Ad) and the substrate receptor from Cul-Rbx. The availability of a second Ad-SR module leads to the dissociation of CAND1 and to the formation of a new CRL complex (Franciosini et al., 2013).

CSN Roles in Plant Development

Arabidopsis csn mutants have a pleiotropic phenotype, which includes a deregulated activation of light induced development, as well as defect in other multiple cellular pathways, which culminates in growth arrest prior to the transition to the adult stage. This simple observation suggests a crucial role of the CSN in controlling many aspects of plant life (Stratmann and Gusmaroli 2012).

In the last 20 years, research carried out in *Arabidopsis* has actively contributed to identify the plant developmental pathways regulated by the CSN, and its involvement in a plethora of plant processes well beyond photomorphogenesis has emerged. Some of these processes are described below and summarized in Table 2.

- CSN in Photomorphogenesis and Other Light-Regulated Processes

The CSN was initially discovered during genetic screens for constitutive photomorphogenic development in darkness, which led to the identification of 9 non-allelic *cop/det/fus* mutants (Wei and Deng 1992; Wei and Deng 2003). While dark-grown wild type Arabidopsis seedlings undergo skotomorphogenesis, a developmental program leading to elongated hypocotyls and closed, unexpanded cotyledons, the *cop/det/fus* mutants display a constitutive photomorphogenic phenotype, characterized by the deregulated activation of light-induced development (photomorphogenesis), even when grown in the absence of light. These mutants display short hypocotyls and open cotyledons, along with the deregulated expression of light-inducible genes and multifaceted defects in several cellular and developmental pathways that ultimately result in lethality at the seedling stage. Of the nine *COP/DET/FUS* loci identified in the initial genetic screens, six loci correspond to CSN subunits, and the remaining

three, *COP1*, *COP10* and *DET1*, are not part of the CSN (Sullivan et al. 2003; Yi and Deng 2005). Extensive biochemical and genetic studies have now defined that these proteins are part of two additional complexes: the $\underline{C}$OP1–$\underline{S}$UPPRESSOR OF $\underline{P}$HYA-105 (SPA) complex and the $\underline{C}$OP10–$\underline{D}$ET1–$\underline{D}$DB1 (CDD) complex (Yanagawa et al. 2004; Zhu et al. 2008; Lau et al. 2011). These two complexes cooperate together to induce ubiquitination and proteasome-mediated degradation of positive regulators of light responses (Lau and Deng 2012).

COP1 is itself an ubiquitin ligase and interacts with several photomorphogenesis-promoting factors, such as the phytochromes A and B, and the transcription factors HY5 ($\underline{E}$LONGATED $\underline{H}$YPOCOTYL FACTOR 5), HYH ($\underline{HY}$5-$\underline{H}$OMOLG), LAF1 ($\underline{L}$ONG $\underline{A}$FTER $\underline{F}$AR-RED FACTOR1) and HFR1 (LONG $\underline{H}$YPOCOTYL IN $\underline{F}$AR-$\underline{R}$ED) (Jang et al. 2010; Lau and Deng 2012). However, COP1 requires the entire COP1-SPA and the CDD complexes to promote the ubiquitination and degradation of photomorphogenesis-promoting factors in darkness. In the dark, these complexes function therefore as master repressors of photomorphogenesis, by triggering the ubiquitination and degradation of transcriptional factors that activate specific light responses. On the contrary, in the presence of light, activated photoreceptors repress COP1 function and allow the accumulation of the photomorphogenesis-promoting transcription factors, resulting in photomorphogenic development (Lau and Deng 2012). This differential mechanism of COP1 function relies also on its cellular localization. In fact, upon light exposure, when the photomorphogenesis takes place, COP1 is shuttled from the nucleus to the cytoplasm, where it is not able to promote the degradation of HY5 and the other transcription factors (Osterlund et al. 1999; Lau and Deng 2012).

The similar photomorphogenic phenotype between *csn* mutants and *cop1* suggests that they might work together to regulate photomorphogenesis. Indeed, CSN is necessary for COP1 nuclear translocation and CDD complex stabilization (Wang et al. 2009).

In addition COP1 interacts directly with CSN1 subunit and both COP1 and the CSN interact with CUL4 (Chen et al. 2006; Lau and Deng 2012).

- CSN in Flower Development

CSN regulates the SCF complex SCF^{UFO}, which contains the F-box protein UFO (UNUSUAL FLORAL ORGANS) and which is required for the proper regulation of floral meristem identity and for the floral organ development (Wang et al. 2003). The molecular mechanism is not fully elucidated, but probably CSN works together with SCF^{UFO} to promote the ubiquitination of negative regulators of the expression of *APETALA3* (*AP3*), an homeotic gene required to specify petal and stamen identities. Accordingly, weak *csn* mutants show defects in floral development, and *AP3* decreased expression, indicating that CSN, by mediating SCF^{UFO} activity, regulates flower development (Stratmann and Gusmaroli 2012).

CSN in Plant Hormone Signaling

Some F-box proteins can function both as hormone receptors and substrate recognition subunits. The involvement of the CSN in hormonal pathways was first revealed by the interaction with SCF^{TIR1} in auxin signaling (Schwechheimer et al. 2001). Since then, its involvement in jasmonic acid and gibberellin signaling through regulation of the corresponding SCF activity have been determined (Feng et al. 2003; Dohmann et al. 2010) (Table 2). SCF ubiquitin ligases are therefore a central component of hormone perception and signaling, and since their activity is under the control of the CSN, the CSN itself occupies a very crucial role in plant hormone response.

- Auxin Signaling

Auxin (indole-3-acetic acid or IAA) regulates many developmental plant processes, including embryogenesis, root and stem elongation, apical dominance, phototropism and gravitropism, and lateral root initiation, by inducing a rapid change in auxin-responsive gene expression .

The key player in auxin signaling is SCF^{TIR1}, that promotes auxin-dependent degradation of negative regulators of auxin response. The F-box protein TIR1 belongs to a small family of related F-box proteins, that includes five additional members called Auxin Signaling F-box, or AFBs (AFB1-5). These F-box proteins function as the auxin receptor, directly interacting with auxin (Mockaitis and Estelle 2008; Yu et al. 2013). Auxin binding increases the affinity of SCF^{TIR1} for its substrates, the Aux/IAA repressor proteins, leading to their ubiquitination and degradation. This allows the release of the Auxin Response Factors (ARFs), a large family of transcription factors which binds promoters of auxin responsive genes, and stimulates ARF-dependent transcription. Thus, auxin, by promoting the degradation of Aux/IAA proteins through SCF^{TIR1}, causes the transcription activation of auxin responsive genes (Santner and Estelle 2010).

CSN physically interacts with SCF^{TIR1}, regulates its activity, and is required for the stabilization of the F-box protein TIR1 (Schwechheimer et al. 2001). This is in agreement with the model that proposes CSN to be necessary to prevent the auto-ubiquitination of CRL substrate recognition subunits. Plants with reduced level of CSN show reduced degradation of Aux/IAA and decreased auxin response, underling the relevance of this complex in regulating auxin signaling (Santner and Estelle 2010).

- Jasmonic Acid Signaling

Jasmonic acid (JA) is an important signaling molecule that mediates plant responses to biotic and abiotic stress, as well as other aspects of plant development such as growth and fertility (Gfeller et al. 2010).

Similarly to the auxin sensor SCF^{TIR1}, the SCF^{COI1} ubiquitin ligase, which contains the F-box protein COI1 (CORONATINE-INSENSITIVE1) functions as the JA receptor. After its interaction with JA, COI1 is able to bind the JAZ (jasmonate ZIM-domain) family of transcriptional regulators, and SCF^{COI1} promotes their ubiquitination and degradation, *via* the proteasome pathway (Yan et al. 2013; Zhou et al. 2013). In absence of the hormone, JAZ proteins actively repress a sub-family of MYB transcription factors, which bind the *cis*-acting elements of JA-response genes. In presence of JA, JAZ proteins are degraded, allowing the expression of JA-induced genes (Vierstra 2009; Wager and Browse 2012).

Similarly to SCF^{TIR1}, CSN associates physically with SCF^{COI1} (Feng et al. 2003), regulates its function, and is thus essential for proper JA signaling. In fact, plants with reduced level of CSN are not able to respond properly to JA, and an adequate cellular level of CSN is required for proper JA responses (Stratmann and Gusmaroli 2012).

- Gibberellin Signaling

Gibberellins (GAs) regulate diverse growth and developmental processes such as seed germination, stem elongation, leaf expansion and flower development.

GAs responses are negative regulated by a class of proteins, named DELLA proteins, from their conserved DELLA motif, composed by five members. The five DELLA proteins have both redundant and specialized functions, finally resulting in the repression of GAs

signaling. GAs perception is mediated by their receptor GID1 (Giberellin-Insensitive Dwarf1) (Wang and Deng 2011). In presence of GAs, the GID1 receptor binds the DELLA proteins, recruits the ubiquitin ligase $SCF^{SLEEPY1}$ and promotes the ubiquitination of DELLAs, thus inducing their degradation by the proteasome. SCF^{SLY1}-mediated degradation releases DELLA-dependent block, and thus promotes the activation of GA positive regulators, such as the bHLH transcription factors PIFs (phytochrome-interacting factor) (Dill et al. 2004; Ariizumi et al. 2011; Wang and Deng 2011).

SCF^{SLY1} fails to efficiently degrade RGA (REPRESSOR OF *ga1-3*), a member of DELLA proteins family in *csn* mutants. Furhermore, *csn* hypomorphic mutants show developmental defects, including in germination and hypocotyl elongation, which might be ascribed to an inefficient activity of SCF^{SLY1} and to the resulting accumulation of DELLA proteins. Thus, the repression of GA signaling could be the cause of some of growth defects displayed by *csn* mutants (Dohmann et al. 2010).

CSN in plant pathogen response

- R-Mediated Resistance

Pathogens, when they get in contact with their plant host, use effectors proteins that disrupt immunity response and promote successful infection. Plant cells have evolved sophisticated signaling pathways to recognize and respond to pathogen-delivered effectors. One of these pathways relies on disease resistant proteins (R), which recognize pathogen effectors and induce the so-called R protein-mediated response. This leads to hypersensitive localized cell death response (HR) at the infection site, rapid oxidative burst, and activation of various defense responsive genes (Craig et al. 2009).

The first report of an involvement of the CSN in pathogen response came from studies in *Nicotiana tabacuum*. Two proteins, RAR1 (REQUIRED FOR Mla12 RESISTANCE 1) and SGT1 (SUPPRESSOR OF G2 ALLELE OF SKP1), have been defined as a converging point between ubiquitination and R genes-mediated resistance. In fact, RAR1 and SGT1 associate with an SCF-type ubiquitin ligase and with the CSN, and both associations are required for *R* gene-mediated responses to induce resistance against a variety of pathogens (Liu et al. 2002; Craig et al. 2009). This SGT1-associated SCF ligase and CSN might therefore work in concert to target negative regulators of the defense response, which have yet to be identified. Such regulators could act as repressors of selected defense genes (Craig et al. 2009).

- JA-Mediated Defense

CSN regulates also the response to pathogenic insects and fungi, through its already described interaction with the SCF^{COI} ligase, which mediates JA responses. In fact, JA is not only involved in the regulation of plant development, but also in plant defense (Gfeller et al. 2010). JA accumulates in response to wound in the injury sites, where it is perceived by SCF^{COI1} and promotes JAZ proteins degradation, thus resulting in the expression of a group of JA-responsive genes which encode several plant defense proteins involved in resistance to herbivores and necrotrophic fungal pathogens (Hind et al. 2011; Stratmann and Gusmaroli 2012).

- Salicylate-Mediated Defenses

After pathogen infection, plants may respond by increasing the production of salicylic acid (SA) (Craig et al. 2009). This increase in SA levels causes in turn the SA-dependent upregulation of pathogenesis-related (*PR*) genes.

A key component of such response is NPR1 (nonexpressor of pathogenesis related genes), a transcriptional positive regulator of *PR* genes. In standard conditions, NPR1 is found mainly in an oligomeric form and sequestered in the cytoplasm. Upon pathogen infection, SA accumulates and promotes NPR1 reduction to a monomeric state, resulting in translocation of NPR1 monomer to the nucleus. Within the nucleus, NPR1 co-activates transcription of *PR* and other response genes. After the activation of gene expression, NPR1 is recruited by a CUL3-based ubiquitin ligase, ubiquitinated and degraded. This degradation -which also requires a proper functioning CSN- is essential to limit the transcriptional activation of *PR* genes, avoiding a constitutive defense response in the absence of infection (Spoel et al. 2009). This mechanism of action is conserved well beyond *Arabidopsis*, since CSN-silenced tomato plants also display upregulation of *PR* genes (Stratmann and Gusmaroli 2012).

CSN plays therefore a double role in plant defense against pathogens. On one hand, it positively regulates JA- and wound-dependent gene expression, and on the other hand it functions negatively on the SA-dependent *PR* gene expression (Stratmann and Gusmaroli 2012).

The F-Box Protein CFK1

- The CSN Co-Purifies with CFK1, a New F-box Protein of *Arabidopsis thaliana*

As described in the previous paragraphs, in Arabidopsis the CSN is a key regulator of several cellular and developmental pathways. 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. In order to identify novel CSN-mediated plant processes, in the laboratory of Giovanna Serino in which I carried

out my PhD project, a biochemical approach was used to identify novel proteins that interact with the CSN in cauliflower. This analysis led to the identification of a new protein, that was named CFK (COP9 SIGNALOSOME INTERACTING F-BOX KELCH 1), that direct interacts with the CSN. In Arabidopsis CFK is encoded by two genes, *CFK1* and *CFK2* (At5g42350 and At5g42360), and the corresponding proteins share the 98% of identity in terms of amino acid sequence (Figure 4).

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 4A). 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 to 175 (Figure 4A), while the three putative kelch repeats span over residues 184-231, 232-282, and 355-402 respectively (Figure 4B).

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*) (Figure 5A). Clear CFK1 homologs were not found in *Selaginella moellendorffi*, nor in the moss *Physcomytrella 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 (Figure 5B).
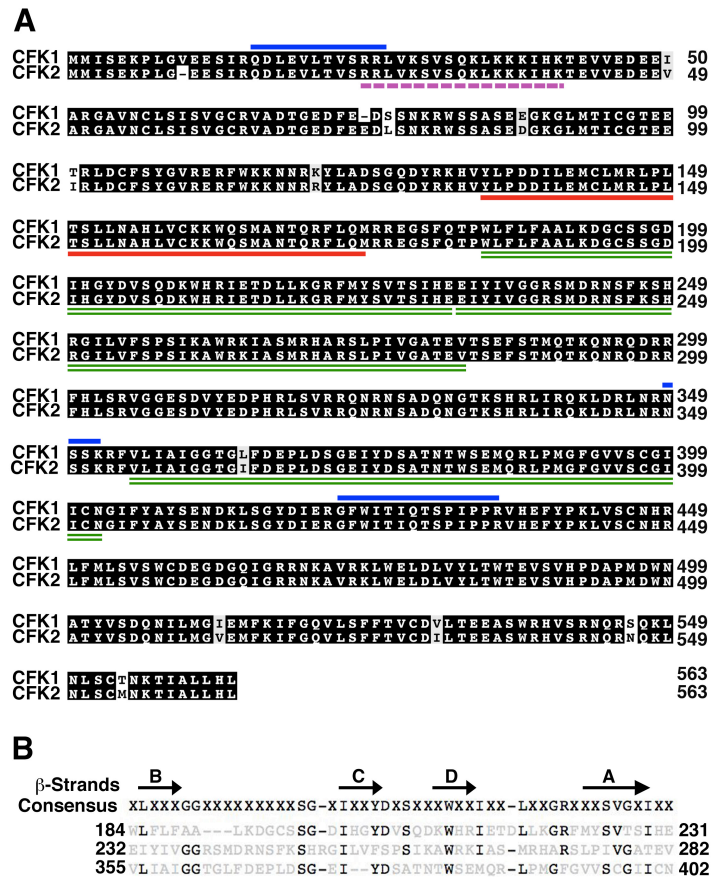
A

```
CFK1 MMISEKPLGVEESIRQDLEVLTVSRRLVKSVSQKLKKKIHKTEVVEDEEI 50
CFK2 MMISEKPLG-EESIRQDLEVLTVSRRLVKSVSQKLKKKIHKTEVVEDEEV 49

CFK1 ARGAVNCLSISVGCRVADTGEDFE-DSSNKRWSSASEEGKGLMTICGTEE 99
CFK2 ARGAVNCLSISVGCRVADTGEDFEEDLSNKRWSSASEDGKGLMTICGTEE 99

CFK1 TRLDCFSYGVRERFWKKNNRKYLADSGQDYRKHVYLPDDILEMCLMRLPL 149
CFK2 IRLDCFSYGVRERFWKKNNRRYLADSGQDYRKHVYLPDDILEMCLMRLPL 149

CFK1 TSLLNAHLVCKKWQSMANTQRFLQMRREGSFQTPWLFLFAALKDGCSSGD 199
CFK2 TSLLNAHLVCKKWQSMANTQRFLQMRREGSFQTPWLFLFAALKDGCSSGD 199

CFK1 IHGYDVSQDKWHRIETDLLKGRFMYSVTSIHEEIYIVGGRSMDRNSFKSH 249
CFK2 IHGYDVSQDKWHRIETDLLKGRFMYSVTSIHEEIYIVGGRSMDRNSFKSH 249

CFK1 RGILVFSPSIKAWRKIASMRHARSLPIVGATEVTSEFSTMQTKQNRQDRR 299
CFK2 RGILVFSPSIKAWRKIASMRHARSLPIVGATEVTSEFSTMQTKQNRQDRR 299

CFK1 FHLSRVGGESDVYEDPHRLSVRRQNRNSADQNGTKSHRLIRQKLDRLNRN 349
CFK2 FHLSRVGGESDVYEDPHRLSVRRQNRNSADQNGTKSHRLIRQKLDRLNRN 349

CFK1 SSKRFVLIAIGGTGLFDEPLDSGEIYDSATNTWSEMQRLPMGFGVVSCGI 399
CFK2 SSKRFVLIAIGGTGIFDEPLDSGEIYDSATNTWSEMQRLPMGFGVVSCGI 399

CFK1 ICNGIFYAYSENDKLSGYDIERGFWITIQTSPIPPRVHEFYPKLVSCNHR 449
CFK2 ICNGIFYAYSENDKLSGYDIERGFWITIQTSPIPPRVHEFYPKLVSCNHR 449

CFK1 LFMLSVSWCDEGDGQIGRRNKAVRKLWELDLVYLTWTEVSVHPDAPMDWN 499
CFK2 LFMLSVSWCDEGDGQIGRRNKAVRKLWELDLVYLTWTEVSVHPDAPMDWN 499

CFK1 ATYVSDQNILMGIEMFKIFGQVLSFFTVCDVLTEEASWRHVSRNQRSQKL 549
CFK2 ATYVSDQNILMGVEMFKIFGQVLSFFTVCDILTEEASWRHVSRNQRNQKL 549

CFK1 NLSCTNKTIALLHL 563
CFK2 NLSCMNKTIALLHL 563
```

B

```
β-Strands          B →              C →    D →          A →
Consensus  XLXXXGGXXXXXXXXXXSG-XIXXYDXSXXXWXXIXX-LXXGRXXXSVGXIXX
      184  WLFLFAA---LKDGCSSG-DIHGYDVSQDKWHRIETDLLKGRFMYSVTSIHE 231
      232  EIYIVGGRSMDRNSFKSHRGILVFSPSIKAWRKIAS-MRHARSLPIVGATEV 282
      355  VLIAIGGTGLFDEPLDSG-EI--YDSATNTWSEMQR-LPMGFGVVSCGIICN 402
```

Figure 4 – The F-Box Proteins CFK1 and CFK2. (A) Amino acid sequence comparison of CFK1 and CFK2 proteins from *Arabidopsis*. Blue lines indicate the position of the three sequenced peptides. The potential nuclear localization sequence, the F-box motif and the three kelch repeats are defined by the dashed purple line, the red line, and the green double lines (green), respectively. Right side numbers indicate amino acid position. Dashes denote gaps. The black or grey shading denotes identity or similarities between the two proteins, respectively. **(B)** Alignment of the three putative kelch repeats from CFK1. Top row: consensus sequence. The amino acid position of each repeat is showed at the left and right of the sequence. Conserved residues are highlighted in black. Arrows mark the putative four β-strands, named from A to D (Franciosini et al. 2013).
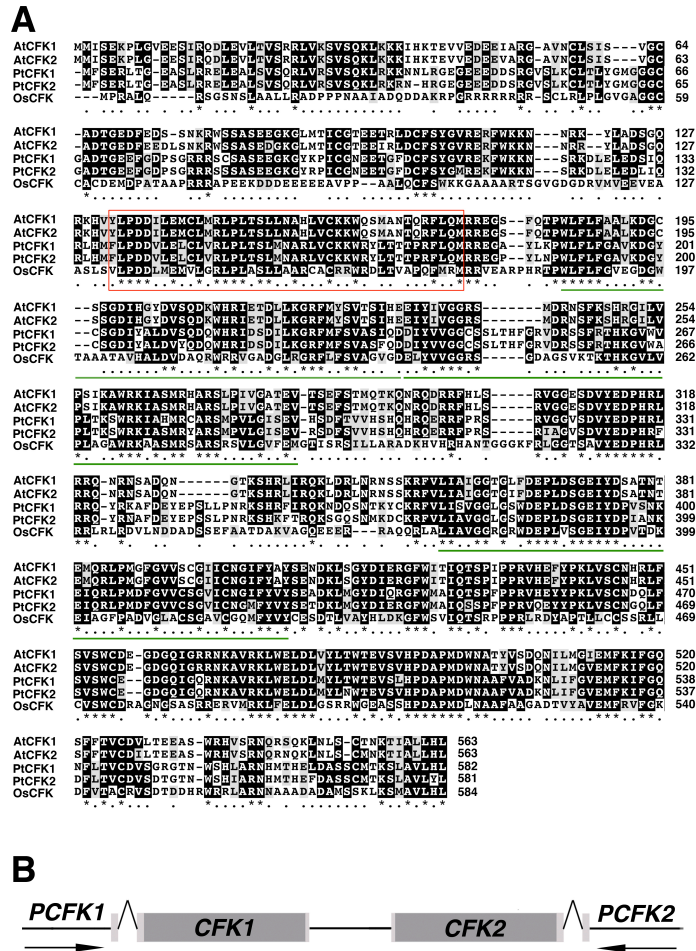
A

B

Figure 5 - CFK Proteins Are Conserved Among Vascular Plants and Are Encoded by Two Genes in *Arabidopsis thaliana*. (A) Aminoacid sequence alignment of CFK proteins from different plant species. Dashes denote gaps. The residue length of each protein is showed at the end of the sequence. The locations of the F-box domain and of the three kelch repeats are indicated by the red square and by the green lines, respectively. The black or grey shading denote amino acid identity or similarities among the proteins. Asterisks denote conserved residues in at least 50% of the sequences. At, *Arabidopsis thaliana*, Os, *Oryza sativa*, Pt, *Populus trichocarpa*. Amino acid sequences were aligned

using ClustalW2 (http://www.ebi.ac.uk/Tools/es/cgi-bin/clustalw2) and visualized with the BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html). **(B)** Genomic organization of *CFK1* (At5g42350) and *CFK2* (At5g42360). Solid boxes indicate translated sequences (black) and transcribed but not translated regions (grey). Horizontal black lines represent intergenic sequences while exponential symbol denotes introns. Arrows on bottom indicate the 5'->3' directions of the two genes. *PCFK1* and *PCFK2* indicate the promoters of the two genes (Franciosini et al. 2013).

- 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, in the laboratory where I carried out my PhD project, they focused further analysis on CFK1 and sought to assess a possible direct interaction with ASK1 (<u>A</u>rabidopsis homolog of SKP1, *Saccharomyces cerevisiae* <u>S</u>uppressor of <u>K</u>inetochore 1), a conserved SCF subunit which has been shown to interact directly with many F-box proteins (Gagne et al. 2002). Through an yeast two-hybrid assay, a very strong interaction between ASK1 and the F-box protein CFK1 was demonstrated.

To confirm that CFK1 is part of an SCF ubiquitin ligase complex *in planta*, 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*) were produced (Figure 6A-C).
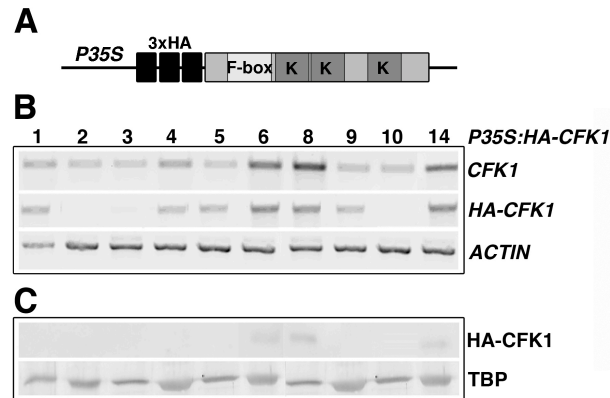
Figure 6 - Characterization of the Arabidopsis *P35S:HA-CFK1* Overexpression Lines. (A) Graphic representation of the construct used to transform *Arabidopsis thaliana* plants. *P35S*, CaMV 35S promoter. The sequence encoding the three HA repetitions are represented by black boxes; the sequences encoding the F-box domain and the three kelch repeats (K) are represented by a light grey and a dark grey box, respectively. **(B)** RT-PCR analysis of the levels of *CFK1* and of *HA-CFK1* mRNAs in ten independent transgenic lines (lines 1-6, 8-10 and 14). The *ACTIN2* cDNA was used as positive control for the RT-PCR reactions. Lines 6, 8 and 14 were used in the subsequent analyses. **(C)** Immunoblot analysis of the same lines shown above with antibodies to HA. Equal protein loading was confirmed by probing with antibodies to TBP (TATA Binding Protein).

A immunoprecipitation experiment, using *P35S:HA-CFK1* transgenic line, has shown that HA-CFK1 interacts with the CSN subunit CSN6, thus re-confirming an interaction between CFK1 and the CSN. In addition, CFK1 co-immunoprecipitated with the conserved SCF subunit CUL1, suggesting that CFK1 is present in SCF complexes. A gel filtration chromatography further validated the association of CFK1 with an SCF complex, indicating that HA-CFK1 is specifically associated with CUL1 *in vivo* and also confirming that an SCF[HA-CFK1] complex is correctly assembled *in vivo* (Franciosini et al. 2013) (Fig 7).
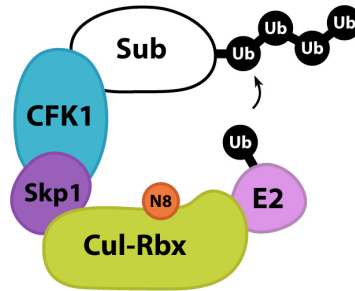
Figure 7 - The SCF^{CFK1} Ubiquitin Ligase. The F-box CFK1 is a component of an SCF ubiquitin ligase *in vivo*. In Arabidopsis, CFK1 interacts with the adaptor protein Skp1, and recruits a specific substrate (Adapted from Franciosini et al., 2013).

AIMS

As described in the Introduction, the CSN is a multi-protein complex, conserved across the eukaryotes, and required for the proper function of a large class ubiquitin ligases, the CRLs. Since the discovery of the CSN, a wealth of research has increased the understanding of the CSN involvement in the control of a plethora of a plant cellular processes including hormone signaling and development (Schwechheimer et al. 2001; Feng et al. 2003; Wang et al. 2003; Dohmann et al. 2010), cell cycle progression (Dohmann et al. 2008), photomorphogenesis (Chen et al. 2006), and stress responses (Liu et al. 2002; Hind et al. 2011). However, growth and developmental defects displayed by *csn* mutants suggest that, in Arabidopsis, the CSN may be involved in other pathways, apart from the processes mentioned above. Thus, due to the importance of this complex in plant and all other eukaryotes, in the last years the main research topic of laboratory in which I carried out my PhD project has been to elucidate the function of CSN in Arabidopsis, attempting to find novel plant processes controlled by the CSN. With this purpose, in our laboratory a new plant F-box protein, named CFK1, that direct interacts with the CSN was identified. Although the first studies have shown that CFK1 forms an active ubiquitin ligase complex, called SCF^{CFK1}, *in planta,* contributing to define its molecular function, the physiological role of CFK1 remained elusive.

The main goal of my PhD project has been to understand the function of the novel plant F-box protein, CFK1, in Arabidopsis development.

To set out to investigate the biological function of CFK1, in the first part of my PhD project I addressed general questions on the regulation of CFK1 in plant at post-transcriptional and translational level. In the recent years, it has been demonstrated that the stability of a subset of F-box protein is dependent on the CSN in *Schizosaccharomyces pombe* (Zhou et al. 2003; Schmidt et al.

2009), in *Neurospora crassa* (He et al. 2005), and in CSN knockdown human cells (Cope and Deshaies 2006; Denti et al. 2006). Arabidopsis genome encodes for more than 700 F-box proteins (Hua and Vierstra 2011), however the stability of these proteins has not been linked to the CSN so far. Accordingly to the evidences from other organism, I found that the plant specific F-box protein CFK1 is a target of the CSN, and that it is a substrate of the proteasome. In addition, given that in plant the CSN is involved in the light signaling response, I also investigated a putative relationship between light and CFK1, establishing that the light strongly promotes *CFK1* mRNA accumulation in the hypocotyl.

To shed light on the physiological function of CFK1 in Arabidopsis, during the second part of my PhD I analyzed the hypocotyl elongation, in response to light, of seedlings that overexpress or down-regulate *CFK1*, showing that CFK1 is a novel positive component of the molecular mechanism that controls hypocotyl elongation. In addition, further analysis have suggested that CFK1 affects hypocotyl elongation through increasing cell size, and that CFK1 function is dependent on the activity of the CSN.

RESULTS

CSN Protects CFK1 from 26S Proteasome-Mediated Degradation

Some F-box proteins 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 ubiquitin proteasome system, 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 8, top panel). Subsequent immunoblot analysis using antibodies against ubiquitin (anti-Ub) indicated that the higher molecular mass species corresponded to ubiquitinated HA-CFK1 (Figure 8, 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 four hours, and the total protein extracts were subjected to immunodetection. None of the inhibitors used led to a significant change in *CFK1* mRNA levels (Figure 10A). However, as shown in Figure 9A, addition of MG132 was indeed effective in stabilizing HA-CFK1, especially in absence of CHX, while the protease inhibitor PMSF was ineffective in this respect. This result confirmed that CFK1 is a substrate of the proteasome.
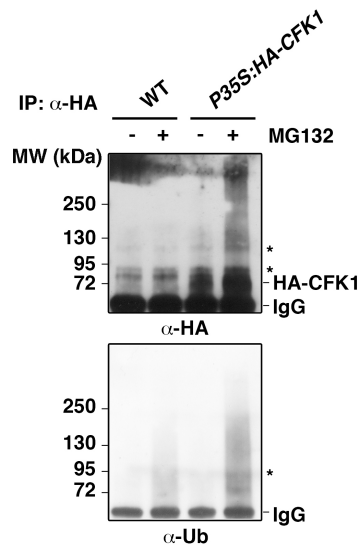
Figure 8 – CFK1 Is Ubiquitinated *In Vivo*. Immunoblot analysis with α-HA (top) and α−ubiquitin (α-Ub, bottom) of protein extracts from 6-day-old wild-type (Col-0) and P35S:HA-CFK1 seedlings incubated for 4 hours in 50 μM MG132 and immunoprecipitated with α-HA resin. MWs are indicated in kiloDalton (kDa). Asterisks: aspecific bands. A minor aspecific binding of the α-HA resin to ubiquitinated proteins was observed (bottom panel) (Franciosini et al. 2013).

The stability of a subset of F-box proteins has been shown to be regulated by CSN in *Saccharomyces pombe*, *Neurospora crassa*, *Caenorhabdtis 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 neddylated CUL1 (Gusmaroli et al. 2007). Because homozygous *csn4-1* mutants are seedling-lethal, the *P35S:HA-CFK1* construct was first introduced into an 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 9B). We therefore treated *P35S:HA-CFK1* (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 immunodetction. *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 (Figure 10B). In contrast, in either Col-0 or Col-0/Ws wild-type seedlings, HA-CFK1 protein levels were reduced after 4 hours incubation with CHX and were undetectable after 8 hours. Remarkably, HA-CFK1 levels were clearly reduced after only 2 hours incubation with CHX in *csn4-1* mutants, indicating that the absence of CSN increased the instability of HA-CFK1 (Figure 9C). Taken together, these results provide convincing evidence that HA-CFK1 stability is regulated at the post-translational level by CSN and the UPS.
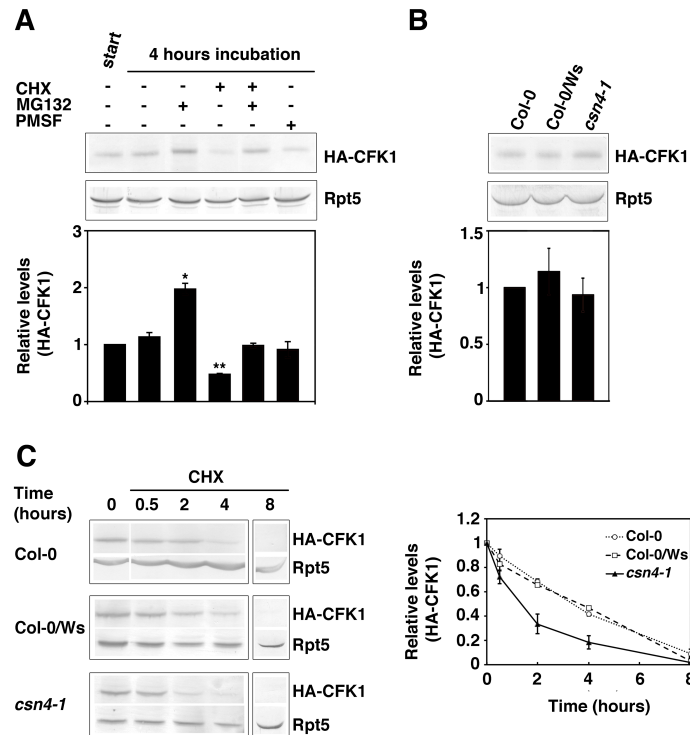
Figure 9 - HA-CFK1 Is Regulated by the Proteasome and by the CSN. **(A)** Immunoblot (top) and relative densitometric analysis (bottom) of the effects of DMSO, cycloheximide (CHX), MG132 and PMSF on HA-CFK1 protein accumulation. Protein extracts were prepared from 6-day-old *P35S:HA-CFK1* seedlings mock treated with DMSO or treated with 50 mM MG132, 100 mM CHX, a combination of the two, or 4 mM PMSF for 4 hours. Equal protein loading was confirmed with a-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 a-Rpt5. Bars are means ± SEM (Franciosini et al. 2013).
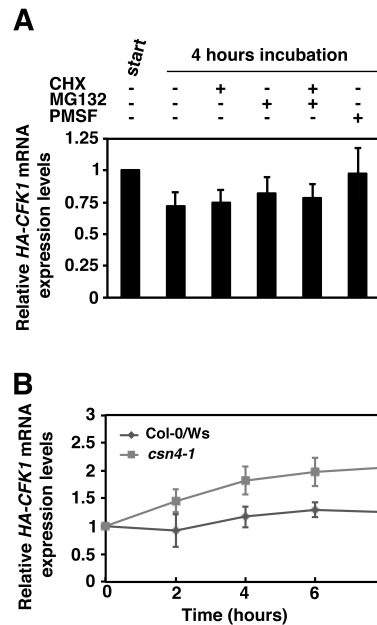
Figure 10 - *HA-CFK1* **mRNA Levels Are not Altered in** *P35S:HA-CFK1* **Seedlings Treated with Cycloheximide (CHX) and MG132, but Are Higher in Absence of CSN. (A)** RT-PCR analysis of *HA-CFK1* mRNA steady state levels in 6-day old *P35S:HA-CFK1* seedlings mock treated with DMSO or treated with 100 μM CHX, 50 μM MG132, a combination of the two, or 4 mM PMSF for four hours. Data were normalized to *ACTIN2*. There were no statistically significant effects according to a two-tailed paired t-test. **(B)** 6-day-old wild-type (Col-0/Ws) or *csn4-1* seedlings harboring the *P35S:HA-CFK1* transgene were incubated with CHX for the times indicated in the Figure and subjected to RT-PCR analysis. CFK1 primers amplified both endogenous *CFK1* and the *HA-CFK1* transgene. Data were normalized to *ACTIN2*. There were no statistically significant effects according to a two-tailed paired t-test.

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 11A). To confirm this result, we analyzed CFK1-YFP protein localization in dark and in light grown seedlings. As shown in Figure 11B (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 *PCFK1:CFK1-YFP* remained almost unchanged in the cotyledons and in the root, while it was increased in the hypocotyl (Figure 11B, 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 1h of onset of light exposure, with subsequent increase in CFK1-YFP levels proportional to the duration of light exposure (Figure 11C). 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 11D, 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 11D, 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 hours in the light revealed that light induces *CFK1* transcription of approximately 2.5-fold (Figure 11E). We concluded that light promotes *CFK1* transcript accumulation in the hypocotyl.
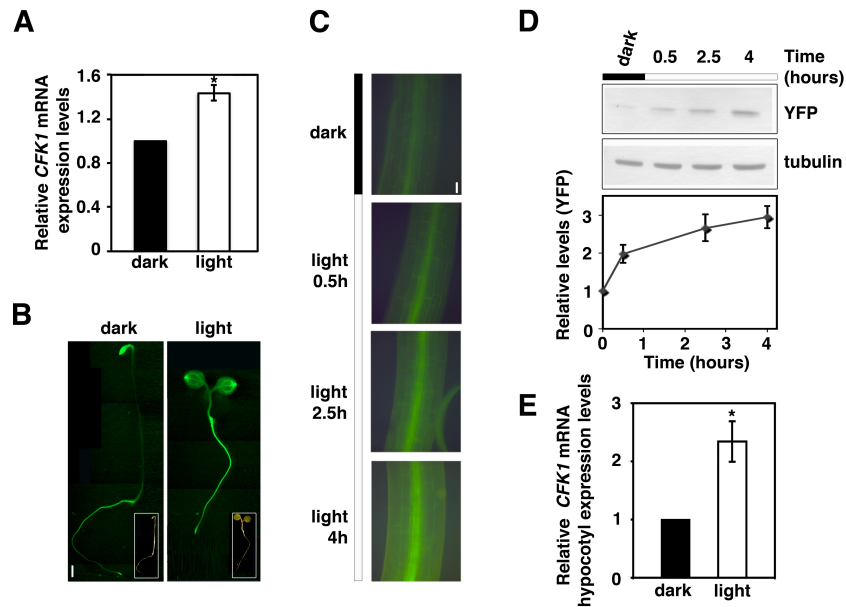
Figure 11 - Light Promotes *CFK1* Transcript Accumulation. (A) qRT-PCR profiles of *CFK1* expression in 6-day-old Col-0 seedlings grown under dark and light conditions. *CFK1* transcript levels were normalized to that of *ACTIN2*. Dark was used as reference and set to 1. Data are means ± SEM (P < 0.05, *) of three independent experiments. **(B)** YFP fluorescence of *PCFK1:CFK1-YFP* seedlings grown in dark (left) and light (right) for three days. Scale bar, 1mm. The inserts show the same two seedlings under bright field illumination. **(C)** YFP fluorescence in elongating cells of hypocotyls of *PCFK1:CFK1-YFP* seedlings grown in the dark for 5 days and then transferred to light, for the times indicated. Note that CFK1 nuclear staining is less obvious, due to the use of a conventional epifluorescence microscope for this figure. Scale bar, 50 mm. **(D)** Immunoblot (top) and relative densitometry analysis (bottom) of YFP levels in hypocotyls of *PCFK1:CFK1-YFP* seedlings grown in the dark for 5 days, transferred to white light for the times indicated and subjected to immunodetection with a-GFP. Tubulin serves as a loading control. Bars represent the mean ± SEM. **(E)** qRT-PCR profiles of *CFK1* expression in hypocotyls from 6-day-old Col-0 seedlings grown in the dark for 5 days (dark) and then transferred to white light for four hours (light). The levels of *CFK1* expression were normalized to that of *ACTIN2*. Dark was used as reference with its expression levels set at 1. Data are means ± SEM (P < 0.05, *) from three independent experiments.

Light Does Not Regulate CFK1 Stability

Because we have shown earlier that CFK1 protein stability is regulated by the ubiquitin proteasome system, 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 12A, 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 12B). CHX-chase experiments also demonstrated a similar half-life for HA-CFK1 in light-grown and dark-grown seedlings (Figure 13). Taken together, these data indicate that light does not affect CFK1 protein stability and that it regulates *CFK1* mainly at the transcriptional level.
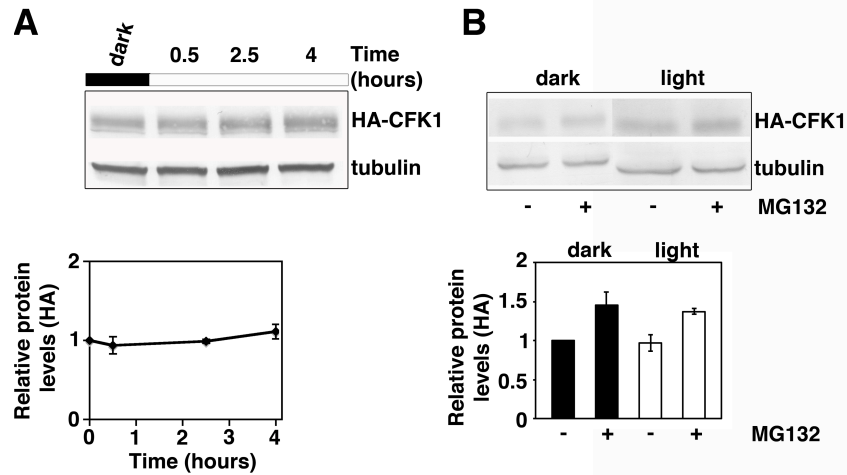
Figure 12 - Light Does Not Affect CFK1 Stability. (A) Immunoblot (left) and relative densitometry analysis (right) of HA-CFK1 levels in hypocotyls of *P35S:HA-CFK1* seedlings grown in the dark for 5 days and then transferred to white light for the times indicated. Tubulin serves as a loading control. Bars: mean ± SEM (might be too small to be distinguished). **(B)** Immunoblot (left) and relative densitometry analysis (right) of HA-CFK1 levels in whole 6-day-old light- and dark-grown *P35S:HA-CFK1* seedlings, mock treated with DMSO or treated with 50 mM MG132 for two hours. Tubulin serves as a loading control. Dark was used as reference with its expression levels set to 1. Bars are means ± SEM.
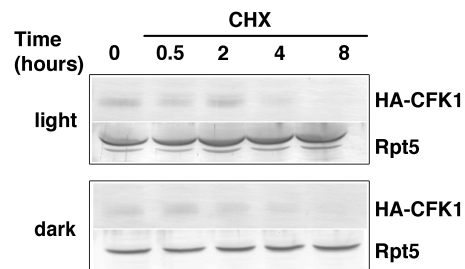
Figure 13 - CFK1 Stability is Similar in Dark and Light-Grown Seedlings.
6-day-old Col-0 seedlings harboring the *P35S:HA-CFK1* transgene were grown
in light or dark and incubated with CHX for the times indicated in the Figure,
followed by immunodetection with antibodies to HA. Equal protein loading was
confirmed by probing with anti-Rpt5 antibody.

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 14A. 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 days 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 14A-B), 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, leading to a complete absence of the full-length transcript (Figure 15A-C). When compared to their wild-type siblings (Col 1b), *cfk1-1* mutant seedlings displayed moderately but significantly shorter hypocotyls only under Rc light (Figure 14A-B).

Because the high identity between the *CFK1* and *CFK2* genes raised the possibility of a functional redundance 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 (Figure 15D). Among the T3 transgenic seedlings obtained, two representative lines, showing a significant reduction in *CFK1* and *CFK2* expression when treated with β-estradiol (Figure 15E), 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 14A-B). This inhibition of hypocotyl elongation was dependent on the concentration of the inducer and was not observed in *pER8-GFP* control seedlings (Figures 15F and 16A). In contrast, no alteration in hypocotyl length was observed in *CFKRNAi* seedlings grown in the dark in the presence of the inducer (Figure 6A-B). 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 (Figure 15C), 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 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 (Figure 16B), had significantly shorter hypocotyls than the wild type under low fluences of Rc and FRc and, to a lower extent, under low fluences of Bc light (Figure 16C). 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.
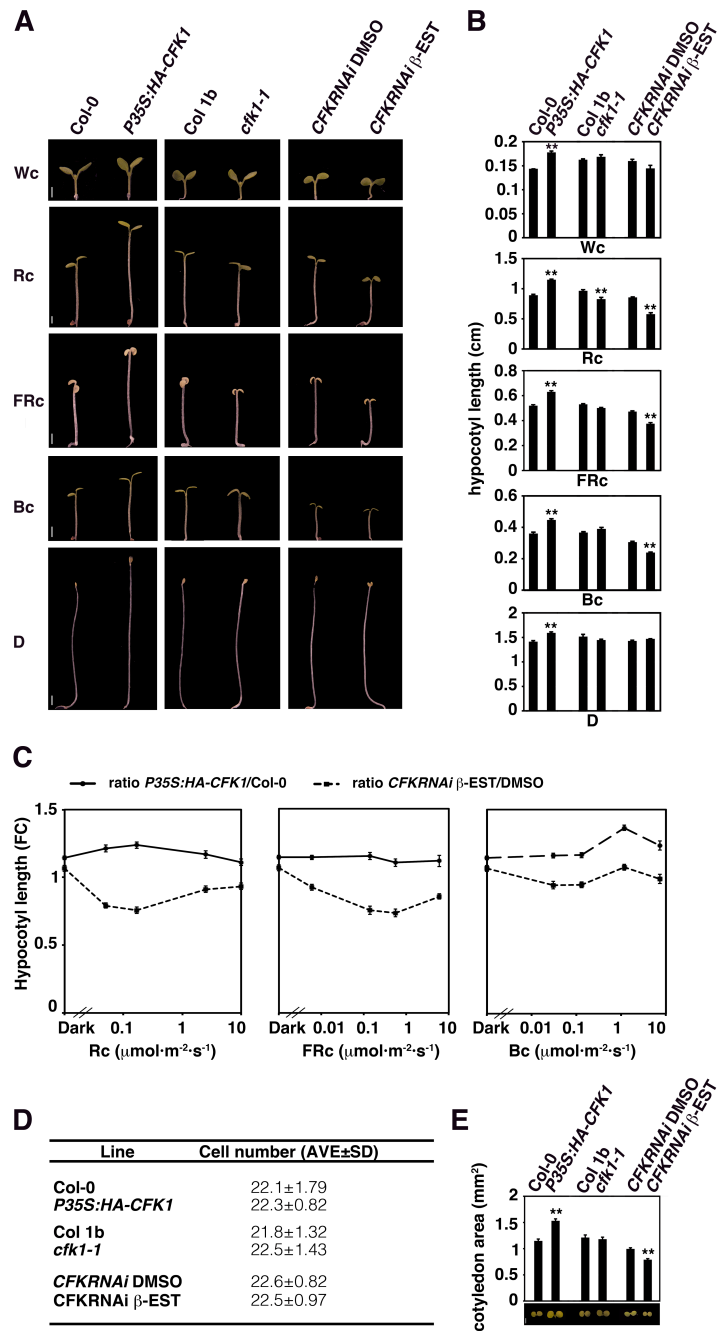
A

B

C

D

Line	Cell number (AVE±SD)
Col-0	22.1±1.79
P35S:HA-CFK1	22.3±0.82
Col 1b	21.8±1.32
cfk1-1	22.5±1.43
CFKRNAi DMSO	22.6±0.82
CFKRNAi β-EST	22.5±0.97

E

Figure 14 - 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 presence of DMSO (negative control) or 10 mM b-estradiol (b-EST). For *P35S:HA-CFK1*, Col-0 was used as a control. Light intensities: W (70 μmol$\cdot$m$^{-2}\cdot$s^{-1}), R (15 μmol$\cdot$m$^{-2}\cdot$s^{-1}), FR (0.06 μmol$\cdot$m$^{-2}\cdot$s^{-1}), B (3.2 μmol$\cdot$m$^{-2}\cdot$s^{-1}). Scale bars:1 mm. **(B)** Quantification of hypocotyl lengths. Data are mean $\pm$ SEM ($n > 30$, three replicates per genotype. **, P<0.01. **(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 mM b-estradiol (b-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$\cdot$m$^{-2}\cdot$s^{-1}). Data are means $\pm$ SEM ($n > 30$, three replicates/genotype).
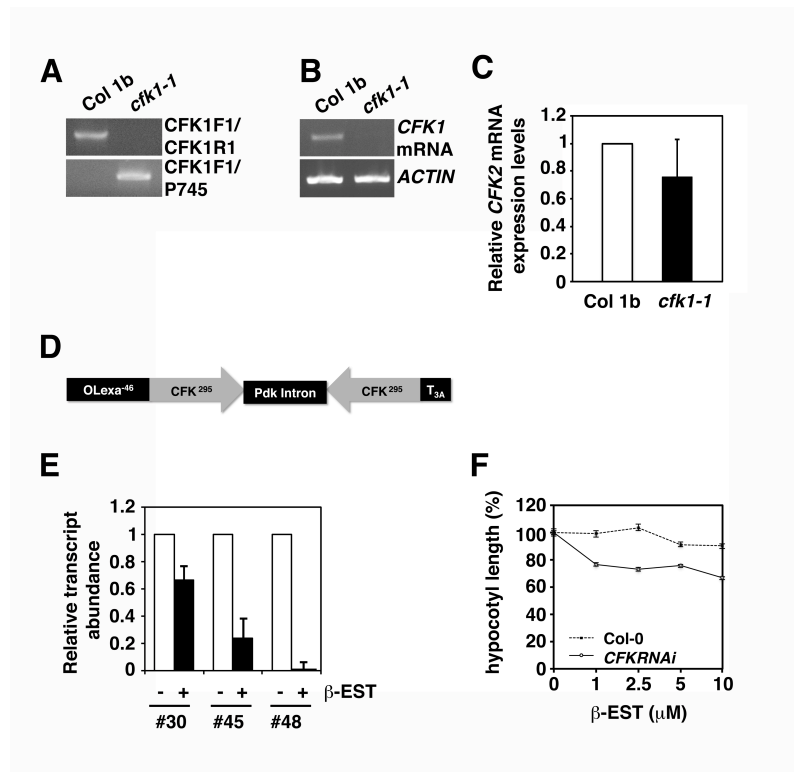
Figure 15 - Characterization of the Arabidopsis *cfk1-1* Mutant and of the *CFKRNAi* Transgenic Lines. (A) PCR-based genotype analysis of Arabidopsis *cfk1-1* homozygous mutant and wild-type plants. Primers CFK1F1 and CFK1R3 were used to specifically amplify the *CFK1* wild-type (Col 1b); primers CFK1F1 and P745 were used to specifically amplify the *CFK1::T-DNA* insertion allele. **(B)** Detection of *CFK1* specific transcript in 6-day-old *cfk1-1* homozygous mutant and its wild-type sibling (Col 1b) by RT-PCR. Primers CFK1F1 and CFK1R1 were used to specifically amplify the *CFK1* gene. Primers to *ACTIN2* were used as control of the RT-PCR reaction. **(C)** qRT-PCR analysis of *CFK2* expression in *cfk1-1* and their corresponding wild-type (Col 1b) seedlings. The levels of *CFK2* mRNAs were normalized to that of *ACTIN2*. Col 1b was used as a reference with its expression levels set at 1. Error bars represent SD. The experiment was performed in triplicate and data from one representative experiment is shown. **(D)** Schematic representation of the *CFKRNAi* construct. For simplicity, only the region between the operator and the terminator of the XVE vector is shown. OLexA indicates the eight copies of the LexA operator sequence; 46, the 46 35S minimal promoter; CFK[295], the 295

conserved nucleotides of *CFK1* and *CFK2*; Pdk intron, the pyruvate dehydrogenase kinase intron derived form the pHannibal vector; T3A, rbcsS3A poly(A) adenylation sequence. Figure modified from Zuo et al., 2000. **(E)** qRT-PCR analysis for *CFK1* and *CFK2* transcripts in β-estradiol-induced *CFKRNAi* seedlings shows an evident decrease of *CFK1* and *–2* transcript levels. Three independent 5-day-old *CFKRNAi* transgenic lines (#30, #45, #48) were incubated in a liquid medium containing DMSO, or 10 μmolar β-estradiol for 24 hours. The overall level of *CFK1* and *-2* transcripts was simultaneously assayed using primers designed to detect both transcripts. DMSO was used as reference for expression levels and set to 1. Error bars represent SEM ($P < 0.05$) from three replicates. Lines 45 and 48 were used in the subsequent analyses. **(F)** Dose-Response Curve of *CFKRNAi* Transgenic Seedlings to β-estradiol. *CFKRNAi* seedlings were germinated and cultured in the presence of increasing concentrations of β-estradiol as indicated in the Figure for six days and their hypocotyls were measured. The results are reported as percent inhibition, calculated from the equation hypocotyl length of β-estradiol-treated plants/hypocotyl length of DMSO-treated plants. Data are presented as mean ± SEM (n > 30, three replicates).
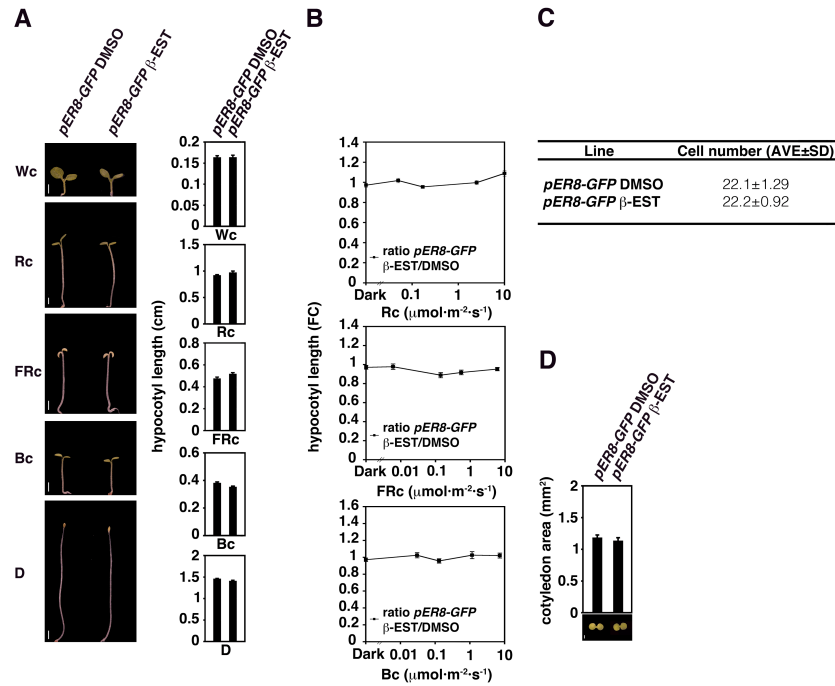
Figure 16 - Phenotypic Characterization of the pER8-GFP Transgenic Line.
(A) Hypocotyl phenotypes (left) and quantification of hypocotyl lengths (right) of 6-day-old *pER8-GFP* seedlings grown in presence of DMSO (negative control) or in presence of 10 mM β-estradiol (β-EST). Seedlings were grown for 8 h in darkness, then transferred to continuous white (Wc) red (Rc), far-red (FRc), blue (Bc), or kept in dark (D). Light intensities were as follows: W (70 μmol·m-2·s-1), R (15 μmol·m-2·s-1), B (3.2 μmol·m-2·s-1) FR (0.06 μmol·m-2·s-1). Scale bars correspond to 1 mm. Data represent the mean and SEM of at least 30 seedlings. **(B)** Fluence rate responses of *pER8-GFP* seedlings. Seedlings were grown for 8 h in darkness, then transferred to various photon fluence rates of continuous red (Rc, top panel), far-red (FRc, middle panel), and blue (Bc, bottom panel), in presence of DMSO (negative control) or 10 μM β-estradiol (β-EST). Measurements are expressed as a fold change (FC) with respect to their controls (β-EST vs DMSO). Error bars represent the variation in SEM of this FC response (n > 30, three replicates per fluence per genotype). **(C)** Hypocotyl outer cortex cells cell numbers of 6-day-old *pER8GFP* lines grown with or without the inducer in Rc light. SD, standard deviation (n > 30 per genotype). **(D)** Cotyledon area (top) and representative photographs (bottom) from 6-day-old

seedlings grown in continuous red light, in presence of DMSO (negative control) or 10 μM β-estradiol (β-EST) in continuous red light (32 μmol·m-2·s-1) (top panel). Data are presented as mean ± SEM (n > 30, three replicates per genotype).

CFK1 Regulates Cell Size

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 hypocotyls of 6-day-old *P35S:HA-CFK1, cfk1-1, CFKRNAi* and their respective control seedlings. As shown in Figure 14D, 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 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 size was observed in induced *CFKRNAi* seedlings. No noticeable change in cotyledon size was observed for the *cfk1-1* mutant compared to its wild-type control, or for *pER8-*

GFP control seedlings (Figures 14E and 16C). 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-Funtion 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 introduced 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 17A). This suggests 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 17B).

A

B
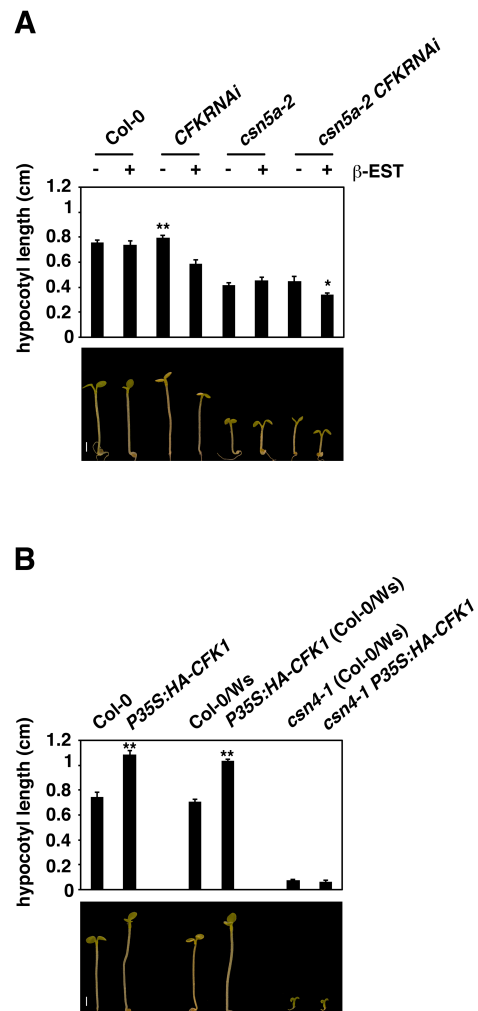
Figure 17 - Genetic Relationships Between *CFK* Transgenic Lines and *csn* Mutants. (A) Measurement and representative images of hypocotyl length of 6-day-old Col-0, *CFKRNAi, csn5a-2* and *csn5a-2 CFKRNAi* seedlings. Seedlings were grown in Rc, in presence of DMSO or 10 mM b-estradiol (b-EST). **(B)** Measurement (top) and representative photographs (bottom) of hypocotyl length of 6-day-old Col-0, *P35S:HA-CFK1,* Col-0/Ws*, P35S:HA-CFK1* (Col-0/Ws), *csn4-1* (Col-0/Ws) and *csn4-1 P35S:HA-CFK1* seedlings. Seedlings were grown in Rc.

DISCUSSION

For this PhD thesis I have focused on the characterization of CFK1, a new plant specific F-box protein co-purifying with CSN. I show that in *Arabidopsis* CFK1, that is a component of an SCF complex, 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.

Many F-box proteins 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). The results obtained in this thesis demonstrate that CFK1 falls in this category; CFK1 intrinsic instability might be necessary to allow a rapid switching of SCF complexes specificity, 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.

Because we found CFK1 in a screen for novel CSN interactors, CSN might also be required to regulate the function of SCF^{CFK1}. It has been suggested that CSN promotes CRL activity *in vivo* by counteracting the autocatalytic breakdown of CRL adaptors such as F-box proteins (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. Consistent with this model, the data presented here show that CSN promotes CFK1 stability. My results indicate that the same process - CFK1 degradation by the proteasome - is taking place both in wild-type and in *csn* mutants, but that it is accelerated in the

absence of the CSN. In addition, 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 overexpressor phenotype suggests that CSN is required for CFK1 function. Indeed, in the absence of CSN, CFK1 half-life is reduced, and its function might be compromised.

This work provides strong evidence that the *CFK1* transcript 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.

I also show that CFK1 promotes hypocotyl elongation by regulating cell expansion. Indeed, the fact that cotyledon size - another developmental process controlled mainly by cell expansion in seedlings - is oppositely regulated in *P35S:HA-CFK1* and *CFKRNAi* seedlings reinforces the hypothesis that CFK1 is involved in the control of cell expansion. The results presented here also suggest that CFK1-mediated promotion of hypocotyl elongation require light, which indeed we have shown to be necessary for CFK1 transcriptional activation.

CFK1 and CFK2 might play similar, but not identical, functions: 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* transcript, 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^{CFK1} 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^{CFK1} substrates will help understanding the molecular mechanisms of CFK protein(s) function, and their regulation by light and by the CSN.

MATHERIALS AND 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; http://www.duchefa.com/) at 22°C with a light intensity of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For fluence curve and phenotipical experiments, seeds plates were exposed to white light for 8 hours to stimulate germination, and then transferred to the appropriate light conditions. For experiments involving b-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 fragment in the pGreen 0179 vector (Hellens et al. 2000).

To generate *P35S:HA-CFK1* transgenic plants, a sequence encoding three HA epitopes was amplified from the Alligator2 vector (http://www.isv.cnrs-gif.fr/jg/alligator/) with the primers KpnFWD and EcoRIREV. The amplified fragment was cloned in the pENTR1A vector (Invitrogen; http://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 (CFK[295]), was amplified with CFKFX and CFKRK (sense), and CFKFB 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 b-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 Table 3.

Fluorescence Microscopy

YFP fluorescence images of whole seedlings were taken with an Axioskop 2 plus microscope (Zeiss; http://www.zeiss.com/) equipped with a ProgRes C10 camera (Jenoptik; http://www.jenoptik.com).

Protein Extraction, Immunoprecipation, 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, YFP and tubulin were detected with monoclonal antibodies to HA, YFP and tubulin (Sigma). Rpt5 (Regulatory Particle 5a or At6A), was detected with specific antibodies (Peng et al. 2001).

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. The antibody to ubiquitin was provided by Qi Xie.

For MG132, PMSF and CHX analysis, seedlings were ground in IP buffer and equal amount 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.

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)(Vittorioso et al., 1998). 1μg of RNA was retrotrascribed with the SuperScript III Reverse Transcriptase kit (Invitrogen). Samples were amplified with the Power SYBR Green PCR Master Mix (Applied Biosystems;

http://www.appliedbiosystems.com/) on a Lightcycler apparatus (Bio-Rad; http://www.bio-rad.com/). Primers CFK1FWRT and CFK1REVRT were used for *CFK1*; primers CFK2FWRT and CFK2REVRT were used for *CFK2;* primers CFKFFW and CFKRREV were used to analyze simultaneously both *CFK* transcripts. Target genes were analyzed using the Icycler software (Bio-Rad) and normalized to the endogenous *ACTIN2* control. Samples were considered statistically significant for 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 Table 3.

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; 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).

PRIMER NAME	SEQUENCE
CFKEco	5'-GGATTCATGATGATTTCTGAGAAA-3'
CFKFB	5'-GGATCCGATGAGCCACTGGATTCAGG-3'
CFKFC	5'-ATCGATGCCCATCTCCTTCATCGCAC-3'
CFKFX	5'-CTCGAGGATGAGCCACTGGATTCAGG-3'
CFKFFW	5'-AGGAGGCTTGTGAAAAGTGT-3'
CFKRK	5'-GGTACCGCCCATCTCCTTCATCGCAC-3'
CFKRREV	5'-TCAGCAACTCTGCAACCAAC-3'
CFKXhoI	5'-CTCGAGTCAGAGATGAAGCAATGC-3'
CFK1FWD	5'-GAATTCCAAACACCGTGGTTGTTT-3'
CFK1FWD3	5'-GAATTCCAAACACCGTGGTTGTTT-3'
CFK1FWRT	5'-AACGAAAAGGGAAAGCGAAATTG-3'
CFK1F1	5'-ATTGGGAGTGGAGGAGTC-3'
CFK1REV	5'-GAGCTCAGTCTCTACTTCGTTACG-3'
CFK1REVRT	5'-CAAACTTCACGCCAAGAGAAGC-3'
CFK1REV3	5'-CTCGAGTCAGAGATGAAGCAATGC-3'
CFK1R1	5'-CTCTTATTACTTGAATCC-3'
EcoRIREV	5'-GAATTCTGGATCTCCTGCATAGTC-3'
KpnFWD	5'-GGTACCATGGCATACCCATACGAC-3'
promCFK1-YFPFWD	5'-CTGCAGAATAGATATTGAAATAGTGC-3'
promCFK1-YFPREV	5'-TCTAGAGAGATGAAGCAATGCAATGG-3'
YFPFWD	5'-GATATCGATGGTGAGCAAGGG-3'
YFPREV	5'-GCGGCCGCTTACTTGTACAGCTCG-3'
CFK2FWRT	5'-GGTCTCCGAGGTCTCAATTATT-3'
CFK2REVRT	5'-AAGACCATCATTTTCCACAGAT-3'

Table 3 – Primers used in this work.

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Anna Franciosini