Arabidopsis CIPK26 interacts with KEG, components of the ABA signalling network and is degraded by the ubiquitin–proteasome system

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Abstract

The RING-type E3 ligase, Keep on Going (KEG), is required for early seedling establishment in Arabidopsis thaliana. Post-germination, KEG negatively regulates abscisic acid (ABA) signalling by targeting Abscisic Acid Insensitive 5 (ABI5) for ubiquitination and subsequent degradation. Previous reports suggest that the role of KEG during early seedling development is not limited to regulation of ABI5 abundance. Using a yeast two-hybrid screen, this study identified Calcineurin B-like Interacting Protein Kinase (CIPK) 26 as a KEG-interacting protein. In vitro pull-down and in planta bimolecular fluorescence complementation assays confirmed the interactions between CIPK26 and KEG. In planta experiments demonstrated that CIPK26 was ubiquitinated and degraded via the 26S proteasome. It was also found that turnover of CIPK26 was increased when KEG protein levels were elevated, suggesting that the RING-type E3 ligase is involved in targeting CIPK26 for degradation. CIPK26 was found to interact with the ABA signalling components ABI1, ABI2, and ABI5. In addition, CIPK26 was capable of phosphorylating ABI5 in vitro. Consistent with a role in ABA signalling, overexpression of CIPK26 increased the sensitivity of germinating seeds to the inhibitory effects of ABA. The data presented in this report suggest that KEG mediates the proteasomal degradation of CIPK26 and that CIPK26 is part of the ABA signalling network.

Key words: ABA, ABI5, CIPK, E3 ligase, KEG, 26S proteasome, ubiquitin.

Introduction

Ubiquitination is an important post-translational modification that regulates protein function in eukaryotic cells. The covalent attachment of ubiquitin to selected targets has a range of consequences including changes in protein activity, localization, and stability (Vierstra, 2009). The outcome of protein ubiquitination is determined by the number of ubiquitin molecules and by the topology of the polyubiquitin chain that is attached to the substrate. For example, the attachment of a polyubiquitin chain generated using lysine-48 linkages generally serves as a signal for degradation of the modified protein by the 26S proteasome, a multi-subunit protease (Vierstra, 2009). By controlling the abundance of regulatory proteins such as transcription factors, the ubiquitin–proteasome system enables organisms to control their cellular milieu to transition through developmental stages and respond to changes in the environment.

The covalent attachment of ubiquitin to targets involves the sequential action of three enzymes. First, E1 (ubiquitin-activating enzyme) activates ubiquitin in an ATP-dependent reaction. E2 (ubiquitin-conjugating enzyme) then accepts the activated ubiquitin, forming a thioester linked E2-ubiquitin (E2-Ub) intermediate. The substrate-recruiting E3 (ubiquitin ligase) facilitates the transfer of ubiquitin from the E2-Ub intermediate onto the target.

Abbreviations: ABA, abscisic acid; BD, binding domain; BiFC, bimolecular fluorescence complementation; CHX, cycloheximide; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; MG132, Z-Leu-Leu-Leu-CHO; PSI, N-[(phenylmethoxy)carbonyl]-l-isoleucyl-L-α-glutamyl-tert-butyl ester-N-[(1S)-1-formyl-3-methylbutyl]-L-alaninamide; YFP, yellow fluorescent protein.

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Ubiquitin ligases are categorized based on the type of domain used to recruit the E2-Ub intermediate. The largest category of E3s uses the Really Interesting New Gene (RING) domain to bind to E2-Ub. The RING domain consists of an octet of zinc-coordinating cysteine and histidine residues that form a cross-brace structure that is essential for ubiquitin transfer (Lorick et al., 1999). The RING domain is found in both monomeric and multi-subunit E3 ligases. Monomeric RING-type E3s contain both the E2 and substrate-binding domains within a single polypeptide, whereas multi-subunit or CULLIN (CUL)-based E3 ligases use different subunits for E2 and substrate-binding functions. Monomeric RING-type E3 ligases can be further subcategorized based on the presence of predicted substrate-binding domains (Stone et al., 2005). Keep on Going (KEG) is a monomeric RING-type E3 ligase that is part of the seven-member Ankyrin repeat domain-containing subgroup (Stone et al., 2005, 2006). KEG is a unique member of this subgroup because, in addition to the RING domain and Ankyrin repeats, the protein also contains a kinase domain and a series of HERC2-like repeats. The HERC2-like repeats are involved in protein interactions and may mediate the dimerization of KEG (Gu and Innes, 2011). There are no other proteins in the predicted Arabidopsis proteome with a domain organization similar to KEG. However, homologues of KEG are found in other plant genomes including Oryza sativa, Medicago truncatula and Populus trichocarpa (Stone et al., 2006).

KEG negatively regulates abscisic acid (ABA) signalling by targeting the ABA-responsive bZIP transcription factor Abscisic Acid Insensitive 5 (ABI5) for degradation by the 26S proteasome (Stone et al., 2006; Liu and Stone, 2010). ABA functions during adaptive responses to environmental stress and also plays a pivotal role as a growth regulator during normal development (Himmelbach et al., 2003). After germination, seedlings sense their nutritional and environmental conditions and modify their growth to ensure survival. At this developmental checkpoint, ABA promotes the accumulation and activation of ABI5 to suspend seedling growth until conditions improve (Lopez-Molina et al., 2001, 2003). Multiple lines of evidence support the requirement for KEG in maintaining low levels of ABI5 in the absence of ABA. KEG mutants accumulate extremely high levels of ABI5, keg-1 seedlings display a severe post-germinative growth-arrest phenotype that can be partially rescued by loss of ABI5. KEG interacts with and ubiquitinates ABI5 in vitro, and a functional KEG E3 ligase is required to rescue the keg-1 growth-arrest phenotype and return ABI5 levels to those of the wild type (Stone et al., 2006; Liu and Stone, 2010).

ABA promotes KEG self-ubiquitination and degradation by the 26S proteasome (Liu and Stone, 2010). In combination with other factors, this reduction in KEG levels allows the accumulation of ABI5 and the subsequent ABA-mediated changes in gene expression required for growth arrest. Transgenic Arabidopsis seedlings that overexpress ABI5 do not undergo post-germinative growth arrest, which suggests that the accumulation of ABI5 only partially accounts for the severity of the keg phenotype. keg-1 abi5-I seedlings have normal and expanded cotyledons but fail to develop beyond the seedling stage (Stone et al., 2006). Therefore, KEG has a greater influence on plant development than the regulation of ABI5 abundance. In order to understand the essential role KEG plays during development, we used a yeast two-hybrid screen to identify KEG-interacting proteins. Calcineurin B-like Interacting Protein Kinase (CIPK) 26 was isolated as a KEG-interacting protein.

In Arabidopsis, there are 26 members of the CIPK family (Weinl and Kudla, 2009) which are also referred to as succrose non-fermenting-1 (SNF1)-related protein kinase 3 (SnRK3) (Hrabak et al., 2003). The CIPK family is defined by the presence of an N-terminal Ser/Thr kinase domain with homology to succrose non-fermenting-1 kinase (SNF) from yeast and AMP activated protein kinase (AMPK) from animals, a NAF domain and a protein phosphatase interaction (PPI) domain (Ohta et al., 2003; Weinl and Kudla, 2009) (Fig. 1A). The NAF domain acts as an autoinhibitory module and binding of Ca$^{2+}$ sensing calcineurin B-like (CBL) proteins to the NAF domain is thought to relieve inhibition (Guo et al., 2001; Gong et al., 2002a). In addition to relieving autoinhibition, CBL proteins also direct the cellular localization of CIPK proteins (Batistic et al., 2010). Genetic loss-of-function and gain-of-function analyses have revealed that CIPKs can also modulate ABA responses (Gong et al., 2002b; Kim et al., 2003; Pandey et al., 2008).

Here we demonstrate that CIPK26 interacts with KEG, which targets the kinase for ubiquitin-dependent proteasomal degradation. Overexpression of CIPK26 renders transgenic plants hypersensitive to ABA. Consistent with this observation, CIPK26 interacts with ABI1 and ABI2, two negative regulators of ABA signalling. In addition, CIPK26 interacts with ABI5 and is capable of phosphorylating ABI5 in vitro. These results suggest that CIPK26 plays a role during ABA signal transduction and that KEG targets CIPK26 for degradation.

**Materials and methods**

**Plant materials**

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was grown as described by Liu and Stone (2010). Nicotiana benthamiana (tobacco) plants were grown under a photoperiod of 8 h of light and 16 h of dark at 23 °C.

The previously described keg-1 (Salk_049542) obtained from the Callis laboratory (University of California, Davis, CA, USA) was originally from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003; Stone et al., 2006). cipk26-1 (Salk_005859) and cipk26-2 (Salk_074944) were obtained from ABRC. Seedlings were genotyped using PCR. For reverse transcription (RT)-PCR, RNA was isolated from 8-d-old seedlings using TRIzol reagent (Sigma-Aldrich) according to the manufacturer’s instructions. All primers used in this study are listed in Supplementary Table S1 at JXB online.

**Cloning**

Unless specified, all cloning was done using Gateway cloning technology (Invitrogen). CIPK26 cDNA and segments were generated by RT-PCR (see above). A Phusion site-directed mutagenesis kit (Finnzymes) was used to introduce mutations into CIPK26 cDNA to generate CIPK26N<sup>TV</sup> and CIPK26N<sup>KR</sup> (Gong et al., 2002a,b). The cloning of full-length ABI5 and wild-type and RING mutant...
versions of KEG cDNAs were as described previously (Liu and Stone, 2010). ABI1 and ABI2 cDNAs were obtained from ABRC.

A 17-β-estradiol-inducible KEG expression vector was generated from the activator/responder vector pLB12 (Brand et al., 2006) and pEarleyGate 201 (Earley et al., 2006). In brief, the 35S promoter was introduced in pLB12 upstream of the chimaeric transcription factor XVE. Using the XhoI restriction sites, pLB12 was digested and then ligated with pEarlyGate201 lacking the 35S promoter. KEG cDNA was then introduced into the resulting vector to create 35S:XVE/OlexA:HA-KEG referred to in this report as OlexA:HA-KEG. In this vector, there is constitutive expression of XVE, and addition of 17-β-estradiol stimulates expression of KEG from the OlexA promoter. All nucleotide sequences were confirmed by DNA sequencing (Génome Québec Innovation Centre, McGill University, Montreal, Quebec, Canada).

Yeast two-hybrid screen
The yeast two-hybrid Gateway destination vectors (pNLexAattR and pJZ4attR) and reporter (pJK103) were gifts from the Finley laboratory (Wayne State University, Detroit, MI, USA) and the screen protocol was as described by Serebriiskii et al. (2001). A cDNA library (prey) made from Arabidopsis siliques, flower buds, and germinating seeds (Norclone Biotech Laboratories) was a gift from the Gazzarrini laboratory (University of Toronto, Ontario, Canada). Using the RING, kinase, and Ankyrin portion of KEG as bait, approximately 350 yeast colonies were selected for by growth on medium lacking leucine. Positive clones were then selected for by streaking yeast colonies onto medium containing 5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside (BioShop Canada). Purified plasmids were then sequenced (Génome Québec). One isolated clone represented CIPK26 and interaction was verified by a β-galactosidase activity liquid assay (Clontech Yeast Protocols Handbook).

Plant transformation
To generate 35S:GFP-CIPK26 transgenic plants, the full-length CIPK26 cDNA was introduced into the pMDC43 Gateway plant transformation vector (Curtis and Grossniklaus, 2003). The resulting plasmid was then transformed into Agrobacterium tumefaciens strain GV3101. The floral dip method (Clough and Bent, 1998) was used to introduce the transgene into Col-0. T1 transgenic plants were selected for by antibiotic resistance, and expression of the transgene was confirmed by Western blot (see below).

To generate 35S:GFP-CIPK26 OlexA:HA-KEG double-transgenic plants, 35S:GFP-CIPK26 (line 1) was transformed with OlexA:HA-KEG as described above. T1 transgenic plants were selected for by antibiotic resistance, and expression of the transgene was confirmed by Western blot (see below).

Plant protein extraction and Western blot analysis
Protein extracts from 6-d-old 35S:GFP-CIPK26 seedlings were subject to Western blot analysis with green fluorescent protein (GFP) antibodies. To assess the expression of HA-KEG, 4-d-old 35S:GFP-CIPK26 OlexA:HA-KEG seedlings were grown in liquid medium and treated overnight with ethanol (control, solvent) or with 20 µM 17-β-estradiol (Sigma). Protein extracts were subjected to Western blot analysis with haemagglutinin (HA) antibodies. For MG132 treatments, 6-d-old 35S:GFP-CIPK26...
seedlings were treated for 16 h with or without the proteasome inhibitors 50 μM Z-Leu-Leu-Leu-CHO (MG132), and 50 μM N-[(phenylmethoxy)carbonyl]-i-isoleucyl-l-α-glutamyl-tert-butyl ester-N-[[(S)-1-formyl-3-methylbutyl]-l-alaninamide (PSI; Boston Biochem). GFP-trap beads (20 μl; Chromotek) were used according to the manufacturer’s instructions to immuno-precipitate GFP-CIPK26 from protein extracts (approximately 300 μl at 5 μg μl⁻¹) prepared from 3SS:GFP-CIPK26 seedlings treated for 16 h with or without 50 μM MG132 and 50 μM PSI. Isolated proteins were then subjected to Western blot analysis with GFP or ubiquitin antibodies as described previously (Liu and Stone, 2010).

Degradation assays

For cell-free degradation assays, CIPK26N was introduced via Gateway cloning into the modified pDEST257 destination vector (Liu and Stone, 2010). Recombinant proteins were expressed in Escherichia coli strain Rosetta (DE3) and purified using nickel-charged resin (Bio-Rad) according to the manufacturer’s protocols. Cell-free degradation assays were carried out as described by Wang et al. (2009). To examine degradation of GFP-CIPK26 in planta, an assay described by Joo et al. (2008) was used.

To demonstrate the effect that KEG has on GFP-CIPK26 protein abundance in planta, 4-d-old 3SS:GFP-CIPK26 OlexA:HA-KEG seedlings were grown for 24 h in liquid medium and then treated overnight with ethanol (solvent, control) or with 20 μM 17-β-estradiol. Seedlings were then treated with 1 mM cycloheximide (CHX), and tissue was collected at the indicated time points and protein extracted for Western blot analysis as described above.

Localization and bimolecular fluorescence complementation (BiFC) assay

Agrobacterium transformed with the appropriate binary plasmids was grown and prepared for transient expression as outlined previously (Liu et al., 2012). For localization of CIPK26, CIPK26 cDNA was introduced into the Gateway-compatible pEarleyGate101 plant transformation vector (Earley et al., 2006). Gateway-compatible BiFC vectors pEarleygate201-YN and pEarleygate202-YC were a gift from Dr Yuhai Cui’s laboratory (Agriculture and Agri-Food London, ON, Canada; Lu et al., 2010). CIPK26, KEG, ABI1, ABI2, and ABI5 cDNAs were recombined into the appropriate pEarleygate201-YN and pEarleygate202-YC vectors. Leaves were assayed for fluorescence 48 h after infiltration for both subcellular localization and the BiFC assay.

Glutathione S-transferase (GST) pull-down assay

CIPK26N, CIPK26C, segments of KEG (RK, A, and H; see Results), ABI1 and ABI2 cDNAs were introduced into modified pDEST257 or pDEST565 (Liu and Stone, 2010). Pull-down assays were carried out as described previously (Schechtman et al., 2003).

ABA inhibition of germination assay

Seeds were collected from plants of each genotype grown under the same conditions and harvested at the same time. Seeds were stratified at 4 °C in the dark for 3 d and then placed at 22 °C with continuous light for 36 h. Germination was defined as radicle emergence through the seed coat. Assays were repeated three times with three replicates per trial and at least 30 seedlings per replicate.

Phosphorylation assays

Various combinations of wild-type and mutated forms of nickel-purified His-Flag-CIPK26N and His-Flag-AB15 were incubated at 30 °C for 30 min in 30 μl kinase assay buffer (20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, and 10 μCi [γ-32P]ATP). Addition of SDS-loading buffer and boiling for 5 min stopped the reaction and samples were separated by 7.5% SDS-PAGE. The gel was dried with a gel dryer and phosphorylated protein was detected by autoradiography.

The in-gel kinase activity assay was preformed as described previously (Liu et al., 2008) with the following modifications: protein extract prepared from various genotypes treated with or without 50 μM ABA for 3 h was separated on an SDS-polyacrylamide gel embedded with 0.1 mg ml⁻¹ of His-purified His-Flag-ABI5 protein in the separating gel as a substrate.

Fluorescent microscopy

Fluorescent images were acquired using a Zeiss LSM 510 META inverted confocal laser scanning microscope (Carl Zeiss MicroImaging) equipped with a 25× oil-immersion objective. Yellow fluorescent protein (YFP) fluorescence was detected at an excitation wavelength of 514 nm, and emissions were collected between 530 and 600 nm. For GFP fluorescence, an excitation wavelength of 488 nm was used and emissions were collected between 475 and 525 nm. To show the outline of each epidermal cell, a single slice of the fluorescent and bright-field images were overlaid.

Results

KEG interacts with CIPK26

Yeast two-hybrid screens were performed to identify proteins that interact with KEG. Full-length KEG (RKAH) and portions of KEG (RING, kinase and Ankyrin region (RKA), Ankyrin repeats only (A), and Ankyrin and HEC2-like repeats (AH)) fused to the LexA DNA-binding domain (BD) were used as bait to screen an Arabidopsis cDNA library generated from mRNA isolated from flowers, siliques, and germinating seeds (Fig. 1A). To prevent ubiquitination and degradation of interacting proteins, the RING domain of KEG was made non-functional by changing two essential zinc-coordinating cysteine residues to alanine (R^AA) (Stone et al., 2006). A cDNA clone containing the partial open reading frame of AT5G21326 was found to restore growth on selection medium and repeatedly induced higher β-galactosidase activity when co-expressed with BD-R^AA-KA relative to controls (Fig. 1A, B). The isolated cDNA encoded for a member of the CIPK/SnRK3 family named CIPK26.

GST pull-down and BiFC assays were used to confirm the interaction between KEG and CIPK26. Segments of KEG fused to GST (GST-RK, GST-A and GST-H) were used in pull-down assays with 6×His- and Flag-tagged portions of CIPK26 (His-Flag-CIPK26N and His-Flag-CIPK26C) (Fig. 1A). Full-length CIPK26 is insoluble when expressed in E. coli cells, which prohibited purification of sufficient protein for analysis. Under stringent isolation conditions, GST-RK was able to pull down His-Flag-CIPK26N, which contains the kinase domain of CIPK26 (Fig. 1C; top left panel), while GST-RK was not able to pull down His-Flag-CIPK26C (Fig. 1C; top right panel).

The CIPK/SnRK3 proteins constitute a subfamily of the SnRks, which also include SnRK1 and SnRK2 kinases. A protein sequence similarity tree was generated to establish the phylogenetic relationship between CIPK26 and other CIPK/SnRK3 family members (Supplementary Fig. S1 online). CIPK26 was most closely related to CIPK3, CIPK9,
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and CIPK23. Using microarray expression data available via Genevestigator©, CIPK26 was found to be widely expressed with its highest expression in seedlings (Supplementary Fig. S2 at JXB online). Similar to other CIPK family members (Batistic et al., 2010), CIPK26 was localized to the nucleus and cytoplasm (Fig. 2A, B). YFP-tagged CIPK26 (CIPK26-YFP) was observed in the nucleus and cytoplasm of Nicotiana benthamiana (tobacco) epidermal cells transiently transformed via Agrobacterium tumefaciens-mediated transformation (Fig. 2A). A similar subcellular localization pattern was observed in stably transformed Arabidopsis seedlings overexpressing GFP-tagged CIPK26 (GFP-CIPK26) (Fig. 2B).

For BiFC analysis, tobacco leaf epidermal cells were transiently co-transformed with 35S:CIPK26-YFPN and 35S:KEG-YFPC to express CIPK26 fused to the amino-terminal portion of YFP (YFPN) and KEG fused to the carboxy-terminal portion of YFP (YFPC). A strong fluorescence signal was observed in cells co-expressing CIPK26-YFPN and KEG-YFPC (Fig. 2C), which indicated reconstitution of the YFP protein as a result of interaction between CIPK26 and KEG. The fluorescent signal was observed throughout the cytoplasm, as well as in punctuate structures. These punctuate structures may represent the trans-Golgi/early endosome vesicle localization of KEG, as described by Gu and Innes (2011). Infiltration with 35S:CIPK26-YFPN or 35S:KEG-YFPC alone did not result in a fluorescent signal (data not shown).

**CIPK26 is ubiquitinated and degraded by the 26S proteasome**

The observation that KEG, a RING-type E3 ligase, interacts with CIPK26 suggests that CIPK26 protein levels may be regulated via the ubiquitin-proteasome system. A cell-free degradation assay was first used to determine whether CIPK26 is turned over and if degradation is proteasome dependent. His-Flag-CIPK26N was incubated with extracts prepared from wild-type seedlings supplemented with or without MG132, a specific inhibitor of the 26S proteasome (Fig. 3). In the absence of MG132, the levels of His-Flag-CIPK26N gradually decreased over time (Fig. 3, top panel). The degradation of His-Flag-CIPK26N was markedly slower in the presence of MG132, indicating that turnover of CIPK26 involved proteolysis by the 26S proteasome (Fig. 3, bottom panel).

To facilitate further analysis of CIPK26, two independent 35S:GFP-CIPK26 transgenic lines were generated. Western blotting with GFP antibodies was used to verify the expression of the transgene. The GFP antibodies detected two proteins, one of approximately 80 kDa, the expected size of GFP-CIPK26, and a larger migrating form (Fig. 4A, upper panels). These proteins were not detected in extracts prepared from wild-type and control (transformed with empty vector) plants (Fig. 4A, upper panels). To confirm that the detected proteins corresponded to GFP-CIPK26, GFP-trap beads were used in immunoprecipitation assays with extracts prepared from wild-type and 35S:GFP-CIPK26 (line 1) transgenic seedlings. Western blotting with GFP antibodies showed that the GFP-trap beads pulled down GFP-CIPK26 of increasing molecular weight (Fig. 4A, lower panel). These results suggested that CIPK26 is modified in planta and this modification(s) resulted in a range of migrating species. To

![Fig. 2. CIPK26 interacts with KEG in planta.](image)
determine whether the high-molecular-weight forms of CIPK26 represented phosphorylated proteins, GFP-CIPK26 isolated using GFP-trap beads was simultaneously subjected to Western blot analysis with GFP antibodies (Supplementary Fig. S3A, left panel, at JXB online) and phosphothreonine (P-Thr) antibodies (Supplementary Fig. S3A, right panel) that detected phosphorylated proteins. Comparison of the two blots showed that the P-Thr antibodies detected proteins corresponding to some of the higher-molecular-weight forms of GFP-CIPK26.

A modified cell-free degradation assay was used to monitor the turnover of full-length CIPK26 in protein extracts prepared from 35S:GFP-CIPK26 (lines 1 and 2) seedlings pre-treated with or without MG132. ATP, which is required for 26S proteasome activity, was added to plant extracts (zero time point) and the levels of GFP-CIPK26 were then determined at the indicated time points. The levels of GFP-CIPK26 gradually decreased over time for both transgenic plant lines (Fig. 4B, top panel; Supplemental Fig. 3B). The decrease in GFP-CIPK26 levels was significantly slower in protein extracts prepared from 35S:GFP-CIPK26 (line 1) seedlings pre-treated with MG132 (Fig. 4B, bottom panel). In addition, treatment of 35S:GFP-CIPK26 (line 1) seedlings with MG132 and another 26S proteasome inhibitor, PSI, repeatedly resulted in an increase in GFP-CIPK26 levels compared with levels in untreated seedlings (Fig. 4C).

To determine whether CIPK26 is ubiquitinated in planta, GFP-trap beads were used to isolate GFP-CIPK26 from protein extracts prepared from 35S:GFP-CIPK26 (line 1) seedlings with or without proteasome inhibitors. Ubiquitin antibodies detected a high-molecular-weight smear of GFP-CIPK26, indicative of ubiquitinated proteins, in extracts isolated from MG132- and PSI-treated seedlings (Fig. 4D).

In 35S:GFP-CIPK26 transgenic plants, the 35S promoter continuously drives expression of GFP-CIPK26 and therefore GFP-CIPK26 protein is continuously replenished. If CIPK26 is indeed being degraded by the 26S proteasome, then inhibition of protein synthesis via treatment with CHX should result in a decrease in GFP-CIPK26, and treatment with MG132 should prevent this reduction in protein levels. As predicted, the levels of GFP-CIPK26 in transgenic seedlings treated overnight with CHX alone were significantly lower than those of mock (DMSO)-treated seedlings (Fig. 4E, compare lanes 1 and 2). The reduction in GFP-CIPK26 protein was not observed in seedlings treated with MG132 prior to and during incubation with CHX (Fig. 4E, lane 3). GFP-CIPK26 protein levels were also significantly reduced when MG132 was removed just prior to incubation of seedlings with CHX (Fig. 4E, lane 4). Together, the above results demonstrated that CIPK26 is ubiquitinated and turned over by the 26S proteasome.

**KEG contributes to the turnover of CIPK26**

Double-transgenic *Arabidopsis* plants constitutively expressing GFP-CIPK26 (35S:GFP-CIPK26) and HA-KEG (OlexA:HA-KEG) under the control of an estradiol-inducible promoter were used to observe the stability of GFP-CIPK26 during a CHX chase experiment. Treatment of double-transgenic seedlings overnight with estradiol resulted in accumulation of HA-KEG (Fig. 5, top panel). CHX was added to the medium after estradiol treatment and the abundance of GFP-CIPK26 protein was monitored at various time points. Over time, the level of GFP-CIPK26 was rapidly reduced in estradiol-treated seedlings compared with that in untreated seedlings that did not express HA-KEG (Fig. 5, middle panel). As a control for the assay, estradiol treatment did not affect the stability of GFP-CIPK26 in single-transgenic 35S:GFP-CIPK26 seedlings (Fig. 5, left panel). Taken together, these results suggested that KEG contributes to the turnover of CIPK26.

**CIPK26 interacts with components of the core ABA signalling network, ABI1 and ABI2, and overexpression alters ABA sensitivity**

Some members of the CIPK family have been found to alter ABA sensitivity, implicating them in the regulation of ABA signal transduction (Gong et al., 2002b; Kim et al., 2003; Pandey et al., 2008). In addition, clade A type 2C protein phosphatases (PP2Cs) can selectively interact with various CIPKs (Ohta et al., 2003; Lan et al., 2011; Lee et al., 2007). Abscisic Acid Insensitive 1 (ABI1) and ABI2 are PP2Cs that negatively regulate ABA signal transduction through dephosphorylation of SnRK2 kinases (Ma et al., 2009; Park et al., 2009). GST pull-down assays were first used to determine whether CIPK26 could also interact with ABI1 and ABI2. GST-tagged ABI1 (GST-ABI1) and GST-ABI2 were both able to interact with and pull down His-Flag-CIPK26C, the portion of CIPK26 that contains the PPI domain (Fig. 6A). The interaction between CIPK26 and ABI1 or ABI2 was verified in planta using BiFC assays. Co-expression of ABI1-YFPN with CIPK26-YFPc (Fig. 6B) in tobacco leaf epidermal cells resulted in a fluorescent signal in the nucleus and cytoplasm, further suggesting an interaction between ABI1 and CIPK26. Similarly,
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Fig. 4. CIPK26 is ubiquitinated and turned over by the 26S proteasome in planta. (A) Upper panel: protein expression levels of GFP-CIPK26 in two independently generated 35S:GFP-CIPK26 transgenic plant lines. Middle panel: ponceau S staining shows protein loading. Bottom panel: immunoprecipitation (IP) of GFP-CIPK26 from protein extracts prepared from Col-0 and 35S:GFP-CIPK26 (line 1) seedlings using GFP-trap beads. Multiple migrating higher-molecular-weight forms of GFP-CIPK26 were detected in extracts from 35S:GFP-CIPK26 (line 1). (B) Cell-free degradation assay using GFP-CIPK26. Protein extracts were prepared from 6-d-old 35S:GFP-CIPK26 (line 1) seedlings treated with (+) or without (–) 50 µM MG132. (C) CIPK26 protein accumulates in the presence of MG132. WB analysis showing the levels of GFP-CIPK26 in 4-d-old 35S:GFP-CIPK26 (line 1) seedlings treated with (+) or without (–) 50 µM MG132 and PSI. (D) CHX treatment reduces the accumulation of CIPK26. Four-d-old 35S:GFP-CIPK26 (line 1) seedlings were treated with DMSO (lane 1), 1 mM CHX (lane 2), 50 µM MG132 and 1 mM CHX (lane 3) or 50 µM MG132 prior to addition of 1 mM CHX (lane 4). All blots are representative of at least two trials. Ponceau S staining shows the protein loading.
The above results suggested that CIPK26 mediates ABA signalling. To further explore this hypothesis, we investigated the possibility that ABI5 might be a target of CIPK26 kinase activity. CIPK26 was found to interact with ABI5 in yeast two-hybrid (Fig. 7A) and BiFC (Fig. 7B) assays. For BiFC, co-expression of 

\[ 35S:GFP-CIPK26-YFP \] 

resulted in a strong fluorescent signal in the nucleus and a weaker fluorescent signal in the cytoplasm of some cells (Fig. 7B). Agrobacterium-mediated transformation of each construct alone did not produce fluorescent signals (data not shown). Kinase assays were then used to determine whether CIPK26 could phosphorylate ABI5. His-Flag-CIPK26N and a constitutively active His-Flag-CIPK26N\^{TD} were both able to phosphorylate His-Flag-ABI5 \textit{in vitro} (Fig. 7C). An inactive version of the kinase, His-Flag-CIPK26N\^{KR}, was unable to phosphorylate His-Flag-ABI5 (Fig. 7C).

The severe growth-arrest phenotype observed for \textit{keg} mutants may be partially explained by the findings that CIPK26 is capable of phosphorylating ABI5 and that KEG regulates the turnover of both CIPK26 and ABI5. In the \textit{keg} mutant background, accumulation of CIPK26 may lead to the phosphorylation of the accumulated transcription factor, resulting in growth arrest. If this is indeed the case, then the phosphorylation status of ABI5 in \textit{keg} seedlings should differ from that of the wild type. An in-gel kinase assay utilizing His-Flag-ABI5 as the cast substrate and protein extracts prepared from untreated \textit{keg-1}, untreated wild-type, and ABA-treated wild-type seedlings was carried out to assess the phosphorylation of ABI5. The pattern of ABI5 phosphorylation observed in \textit{keg-1} was more similar to that of ABA-treated than that of untreated wild-type seedlings (Fig. 7D). Of note was the observation that a kinase of the same molecular weight as CIPK26, approximately 50 kDa, phosphorylated ABI5 in \textit{keg} as well as in ABA-treated wild-type seedlings, but not in untreated wild-type seedlings.

**Discussion**

Previous work has shown that KEG, a RING-type E3 ligase, negatively regulates ABA signal transduction by targeting...
Proteasomal degradation of CIPK26

However, the importance of this E3 ligase is not limited to regulation of ABI5 abundance. In this study, we identified CIPK26 as a KEG-interacting protein. We found that CIPK26 is unstable and is degraded by the 26S proteasome under normal growth conditions. In addition, we provided evidence that KEG mediates the proteasomal degradation of CIPK26. CIPK26 was found to interact with ABI1, ABI2, and ABI5, components of the ABA signalling network. In addition, CIPK26 was able to phosphorylate ABI5 in vitro. In accordance with acting as a positive regulator of ABA signalling, overexpression of CIPK26 increased the sensitivity of germinating seeds to the inhibitory effects of the hormone.

The interaction between KEG and CIPK26 may have various outcomes. For example, CIPK26 may phosphorylate KEG, altering E3 ligase activity. Alternatively, KEG may ubiquitinate CIPK26, targeting the kinase for degradation. In this report, we have provided evidence for the latter. Using Arabidopsis double-transgenic plants, we demonstrated that upregulation of KEG expression increased CIPK26 degradation, suggesting that KEG promotes the turnover of the kinase. Many kinases are unstable and subject to ubiquitin-dependent proteolysis. The distantly related mammalian AMPK is subject to ubiquitin-mediated degradation by the E3 ligase cell death-inducing DFF45-like effector α (Cidea) to regulate energy consumption (Qi et al., 2008). In Arabidopsis, the abundance of SnRK1.2/AKIN11 is reduced through the 26S proteasome in response to phosphate starvation (Fragoso et al., 2009). SnRK1.1/AKIN10 is targeted for degradation by the Pleiotropic Response Locus 1 (PRL1), a subunit of a CUL-based E3 ligase (Lee et al., 2008). There is also evidence to suggest that ABA controls the abundance of SnRK1 from wheat (Coello et al., 2012). Another example of hormone-related kinase degradation is Brassinosteroid-insensitive 2 (BIN2), which is targeted for proteasomal degradation by an unknown E3 ligase in the presence of brassinosteroid (Peng et al., 2008).

Ubiquitin-mediated degradation of CIPK26 contributes an additional layer of complexity to CIPK regulation. CIPK proteins have an autoinhibitory NAF domain, and

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Fig. 6. CIPK26 interacts with ABI1 and ABI2 and has altered ABA sensitivity. (A) GST pull-down assays using bead-bound GST, GST-ABI1 or GST-ABI2 and bacterial cell lysates containing His-Flag-CIPK26C. GST-ABI1 and GST-ABI2 were able to pull down His-Flag-CIPK26C (upper panel) as detected by Western blotting (WB) with Flag antibodies. Coomassie Brilliant Blue (CBB) staining is used to visualize the amount of GST and GST fusion proteins used in each assay (bottom panels). Blots are representative of two independent trials. (B, C) BIFC analysis in N. benthamiana leaf epidermal cells co-expressing ABI1, or ABI2 and CIPK26 fused to the amino (YFP<sup>N</sup>) or carboxyl (YFP<sup>C</sup>) portions of YFP. Right panels show transmitted light images merged with fluorescence images from a single optical section. An enlargement of the nucleus is shown in the inset. (E) Percentage germination of Col-0, control, 35S:GFP-CIPK26 line 1, and 35S:GFP-CIPK26 line 2 seeds in the presence (0.25 and 0.5 µM) or absence of ABA. Germination was scored as radical emergence after 36h. 35S:GFP-CIPK26 line 1, and 35S:GFP-CIPK26 line 2 were significantly different from the wild type at both ABA concentrations (*, P < 0.05). Statistical analysis was performed using Student’s t-test. The graph represents three independent trials with n ≥30 seeds per trial. Results are shown as means ± standard error.
Ca\textsuperscript{2+}-sensing CBL proteins are thought to bind to this NAF domain to relieve inhibition and activate the kinase (Weinl and Kudla, 2009). This model has been demonstrated with CBL1- or CBL9-mediated activation of CIPK23 (Xu et al., 2006). Alternatively, CIPK proteins can also be activated by phosphorylation within the activation loop (Guo et al., 2001). The identities of upstream kinases that phosphorylate CIPKs within the activation loop are unknown, and the relationship between CBL-mediated activation versus activation loop phosphorylation is unclear. The interaction between clade A PP2Cs such as ABI1/ABI2 and CIPKs creates a phosphorylation/dephosphorylation module (Ohta et al., 2003; Lee...
In *Arabidopsis*, there are nine clade A PP2Cs that selectively interact with various CIPKs (Lee et al., 2007; Lan et al., 2011). For example, CIPK23 specifically interacts with AKT1-interacting PP2C1 (AIP1) but not with ABI1 or ABI2 (Lee et al., 2007). Using regulation of AKT1 as a model, Lan et al. (2011) suggested that clade A PP2Cs counteract the activities of CIPK similar to how ABI1/ABI2 inactivate SnRK2s during early ABA receptor-binding events. The interaction between CIPK26 and ABI1/ABI2 may represent a kinase-phosphatase pair that regulates ABA signal transduction. However, further research is required to determine the exact mechanism underlying CIPK26 activation.

The finding that CIPK26 can phosphorylate ABI5 in vitro suggests that the kinase may contribute to the ABA response through ABI5 phosphoregulation. ABI5 protein activity is regulated through three ABA-dependent mechanisms: increased transcription, inhibition of proteasomal degradation, and changes in phosphorylation status (Lopez-Molina et al., 2001, 2002). ABI5 is highly unstable and ABA induces molecular events that prohibit its degradation (Lopez-Molina et al., 2001; Liu and Stone, 2010). However, simple protein accumulation is not sufficient to produce an ABA response because transgenic plants over-expressing ABI5 do not display an ABA-related phenotype in the absence of the hormone (Brocard et al., 2002; Lopez-Molina et al., 2003). The accumulated ABI5 must be activated in order to bring about the changes in gene expression required for growth arrest. ABI5 has multiple phosphorylation sites including four within its conserved regions, C1-C4, and is probably phosphorylated by multiple kinases in planta including SnRKs and calcium-dependent protein kinases (Furihata et al., 2006; Fujii et al., 2007; Piskurewicz et al., 2008; Nakashima et al., 2009; Sirichandra et al., 2010). Interestingly, a CIPK from rice, OsCK1, can phosphorylate the ABRE-binding factor, OREB1, within the C4 domain (Hong et al., 2011). Phosphorylation within these conserved regions can transactivate members of the ABI5 bZIP family (Furihata et al., 2006; Fujii et al., 2007; Hong et al., 2011). The contribution of each kinase to the stability and activation of ABI5 bZIP family members remains to be defined experimentally.

Regulation of ABI5 activity via CIPK26-dependent phosphorylation may aid in explaining the severity of the keg mutant phenotype. Unlike other ABI5 overexpressing plants, KEG mutants accumulate a high-molecular-weight and possibly phosphorylated form of ABI5 (Brocard et al., 2002; Lopez-Molina et al., 2003; Stone et al., 2006). Accumulation of CIPK26 in keg may interfere with CIPK26 regulation, resulting in phosphorylation of targets such as ABI5. CIPK26-mediated phosphorylation of ABI5 in keg may somehow mimic ABA signalling, leading to the growth arrest of keg seedlings in the absence of the hormone. As CIPK26 and KEG are found on the same chromosome, it is not possible to generate keg cipk26 double mutants to determine whether loss of CIPK26 could rescue the KEG growth-arrest phenotype. The results presented here demonstrate that CIPK26 is degraded through the 26S proteasome and that KEG is involved in mediating this degradation. Our results strongly suggest that KEG may target CIPK26 for degradation to further negatively regulate ABA signalling.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Sequence similarity tree of CIPK26 and related family members including SnRK2s and SnRK1s.

**Supplementary Fig. S2.** Tissue expression profile of CIPK26. Mean values were collected from Genevestigator® on an Agronomics whole genome tiling array. Results are shown as means ± standard error.

**Supplementary Fig. S3.** (A) Phosphorylation of CIPK26 in *CIPK26*-overexpressing seedlings. Protein extracts prepared from 6-d-old 35S:*GFP-CIPK26* (line 1) seedlings were incubated with GFP-trap beads. GFP-trap-isolated proteins were split equally between Western blot (WB) analysis using GFP (left panel) and P-Thr (right panel) antibodies. (B) Cell-free degradation assay using 35S:*GFP-CIPK26* (line 2). Protein extracts were prepared from 6-d-old 35S:*GFP-CIPK26* (line 2) seedlings.

**Supplementary Fig. S4.** *cipk26* T-DNA lines do not show altered sensitivity to the effects of ABA on germination. (A) Schematic of *CIPK26* depicting the positions of the T-DNA insertions. Arrows indicate the positions of primers used in RT-PCR. (B) RT-PCR analysis of 8-d-old Col-0, *cipk26*-1, and *cipk26*-2 seedlings showing expression of *CIPK26* and *ubiquitin10* (control). (C) Percentage germination of Col-0, *cipk26*-1, and *cipk26*-2 seeds in the presence of ABA. Sterilized seeds were cold stratified for 3 d on MS medium containing different concentrations of ABA (0, 1, and 3 μM). Germination was scored as radical emergence after 36 h. The Col-0, *cipk26*-1, and *cipk26*-2 lines were not significantly different from each other at 0, 1, and 3 μM ABA (P >0.05). Statistical analysis was performed using Student’s *t*-test (*n* ≥30 seeds per trial). The assay was done in triplicate. Results are shown as means ± standard error.

**Supplementary Table S1.** List of the primers used in this study.

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