Investigation of two Arabidopsis RING-type E3 ligases, KEG and XBAT32.

by

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With love, I dedicated this work to my family.

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ABSTRACT

E3 ubiquitin ligases are a family of proteins that facilitate the covalent attachment of ubiquitin onto target proteins. Protein activity, function, and stability are influenced by ubiquitination and consequently ubiquitination has a major impact on cellular functions and organism physiology. In plant biology, E3 ligases mediate normal growth and development but also facilitate adaptive responses to environmental stress through hormone dependent and independent pathways. Under specific environmental and developmental conditions, E3 ligases mediate the degradation of signalling proteins, transcription factors, biosynthetic enzymes and effector proteins. By using the model plant *Arabidopsis thaliana* to investigate E3 ligases and their targets, we can better understand how plants control their cellular milieu under normal growth conditions and during stress.

Precisely controlled production of the hormone ethylene impacts the growth and development of plants. Knockout lines of the Arabidopsis RING-type E3 ligase, *XBAT32*, overproduce ethylene which alters lateral root production. In this thesis I present a model whereby XBAT32 regulates ethylene biosynthesis through the ubiquitin-mediated degradation of ethylene biosynthesis proteins. In this work, I show that the stability of ethylene biosynthesis proteins, 1-aminocyclopropane-1-carboxylate synthase (ACS)4 and ACS7, are influenced by XBAT32. I discuss a model wherein XBAT32 ubiquitinates ACS4 and ACS7 to regulate the production of ethylene.

Keep on Going (KEG) is a large multidomain E3 ligase that is essential for growth of Arabidopsis seedlings as *KEG* knockout lines arrest growth after germination. KEG is a regulator of abscisic acid (ABA) signalling and acts by targeting an ABA-responsive transcription factor, ABSCISIC ACID INSENSITIVE 5 (ABI5), for degradation. However, genetic studies suggest that KEG has other ubiquitination targets. In this work, I identified Calcineurin B-like Interacting Protein Kinase (CIPK) 26 as a KEG-interacting protein. Here I provide evidence that CIPK26 is a target of KEG's E3 ligase activity and that CIPK26 acts as part of an ABA signalling cascade. I also provide evidence that the kinase activity of CIPK26 influences its own stability and the stability of KEG. In this thesis, I provide a model wherein KEG and CIPK26 share reciprocal regulation to mediate ABA signalling.

LIST OF ABBREVIATIONS USED

ABA	Abscisic acid
ABI1	Abscisic acid insensitive 1
ABI2	Abscisic acid insensitive 2
ABI5	Abscisic acid insensitive 5
ABRC	Arabidopsis Biological Resource Center
ABRE	Abscisic acid response *element*
ACC	1-aminocyclopropane-1-carboxylate
ACS	1-aminocyclopropane-1-carboxylate (ACC) synthase
AD	Activation domain
AdoMet	*S*-adenosylmethionine
AIP1	AKT1-interacting PP2C1
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate activated protein kinase
ATP	Adenosine triphosphate
AVG	aminoethoxyvinylglycine
BD	DNA Binding domain
BiFC	Bimolecular Fluorescence complementation
BIN	Brassinosteroid-insensitive 2
BTB	Broad complex/tramtrack/bric-a-brac
bZIP	Basic leucine zipper
CBL	Calcineurin B-like proteins
CDPK	Calcium dependent protein kinase
CHX	Cycloheximide
CIPK	CBL-Interacting protein Kinase
CIPK26	CBL-interacting protein kinase 26
Col-0	Columbia - 0
CUL	Cullin
DMSO	Dimethylsulfoxide
EBF	EIN3-binding F box protein
EIL	Ethylene insensitive like
ein	*ethylene insensitive*

ETO1	Ethylene over-producer
etr	*ethylene receptor*
GFP	Green Fluorescent protein
GST	Glutathione S-transferase
HA	Hemagglutinin A
HECT	Homology to E6-Associated Carboxyl-Terminus
HERC	HECT and RCC1-like
His	Histidine
IgG	Immunoglobulin G
K	Lysine
KEG	Keep On Going
Lys	Lysine
MAPK	Mitogen activated protein kinase
MS	Murashige and Skoog
PCR	Polymerase chain reaction
PP2C	Protein phosphatase 2C
PPI	Protein phosphatase interactions
PRL	Pleiotropic Response Locus 1
P-Thr	Phospho threonine
R	Arginine
RING	Really interesting new gene
RT-PCR	Reverse transcription-polymerase chain reaction
RUB	Related to ubiquitin
SNF	Sucrose non-fermenting
SnRK	SNF-related kinases
TOE	Target of ETO1/EOL1/2
Ub	Ubiquitin
UBA	Ubiquitin-activating enzyme
UBC	Ubiquitin-conjugating enzyme
UPS	Ubiquitin Proteasome System
XBAT32	XB3 ortholog 2 in *Arabidopsis thaliana*
YFP	Yellow Fluorescent protein

ACKNOWLEDGEMENTS

CHAPTER 1:
INTRODUCTION

Complex plant signalling networks enable plants to complete their life cycles and respond to changes in their environment. Plants synthesize small molecule hormones, which act through signalling cascades to coordinate environmental cues into their genetic framework. These hormones account for many aspects of plant physiology and growth. An overall goal of this thesis is to investigate how specific proteins contribute to plant growth and response to the environment. By uncovering molecular mechanisms that account for fundamental plant processes we can address agronomic problems and solutions.

In this thesis I use the model plant *Arabidopsis thaliana* (Arabidopsis) to investigate the role of protein E3 ubiquitin ligases. The super family of E3 ligases is defined by the ability to mediate the attachment of ubiquitin onto specific target proteins (Vierstra, 2009). E3 ligases work in conjunction with an E1 activating enzyme (UBA) that first activates ubiquitin using ATP. The E2 conjugating enzyme (UBC) then binds ubiquitin creating an E2-ubiquitin intermediate. Specific targets are bound by the E3 ligase and the E2 and E3 coordinate the transfer of ubiquitin onto the target (Figure 1). E3 ligases can be divided into three groups, RING, U-box and HECT, based on the domain they use to interact with the E2 (Smalle and Vierstra, 2004). RING-type E3 ligases can be monomeric or cullin (CUL) based multi-subunit E3s with separate E2 binding proteins and substrate binding proteins. In general, a target protein may undergo several rounds of ubiquitination creating a poly-ubiquitin chain. There are multiple consequences of protein ubiquitination including change in cellular localization, and change in activity. However, to date the most commonly described outcome of ubiquitination is degradation of the target protein through the 26S proteasome (Vierstra, 2009). The Arabidopsis genome contains more than 1,300 predicated E3 ligases, which can categorized into subfamilies based on domain composition (Stone *et al*., 2005). To study E3 ligases, I have focused on two monomeric RING-type E3 ligases named Keep On Going (KEG) and XB3 ortholog 2 in Arabidopsis (XBAT32).

My primary research focus is on KEG, which plays an essential role during post-germinative growth. *KEG* knockout lines exhibit a severe growth arrest phenotype during
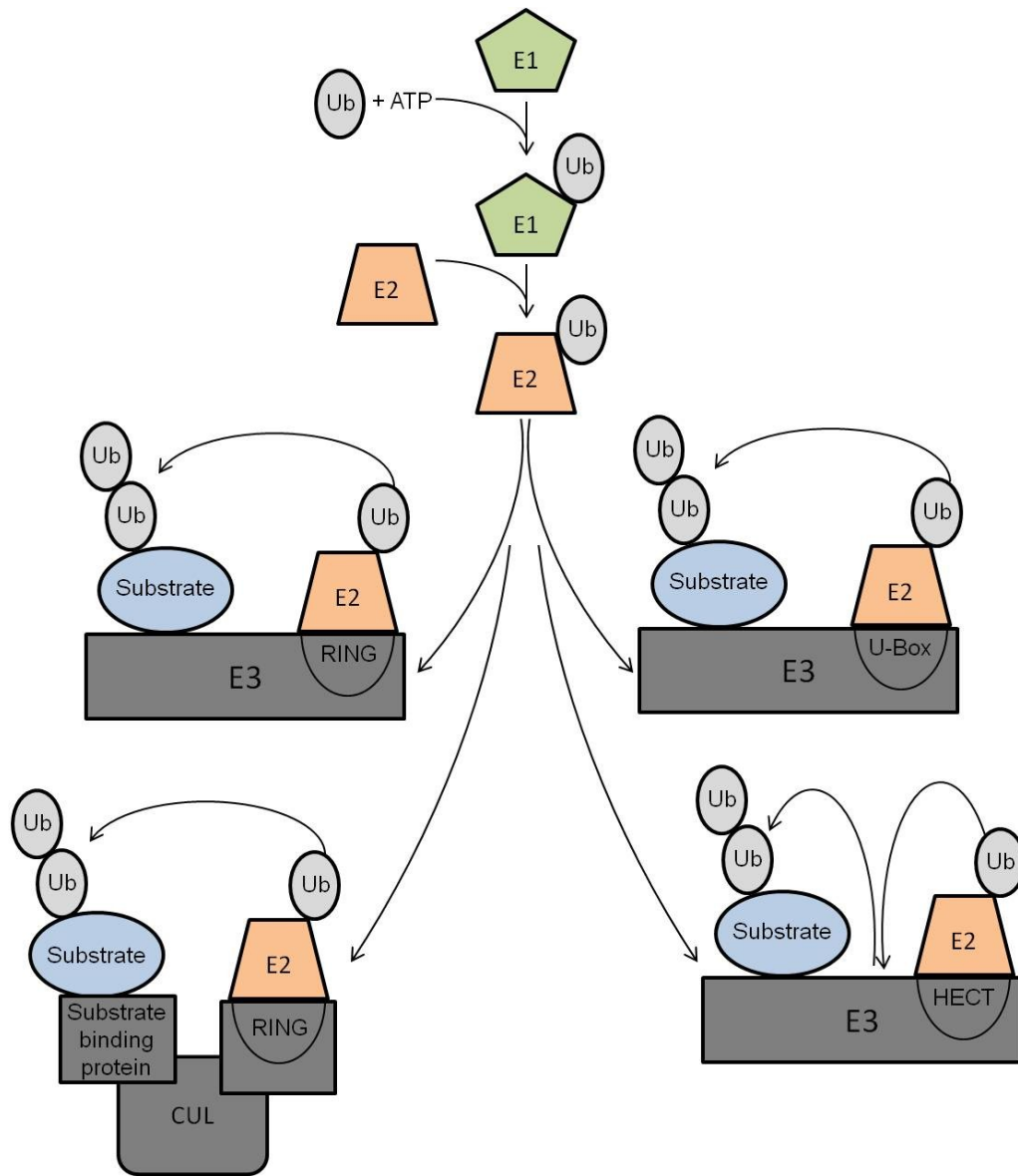
Figure 1. The ubiquitination pathway and types of E3 ligases. Ubiquitin is activated with ATP by the E1 and then transferred to the E2 forming an E2-ubiquitin intermediate. Specific substrates are bound by the E3 ligase and the E2 and E3 coordinate the transfer of ubiquitin onto the substrate. E3s are categorized based on the presence of a RING, HECT or U-box E2 binding domain. The RING-type E3s are subdivided into monomeric or multi-subunit E3 ligases.

the seedling stage (Stone *et al.*, 2006). KEG is part of the protein interaction network, which mediates abscisic acid signal transduction. The production of abscisic acid is increased during certain developmental processes and during unfavorable growth conditions. ABA induces signalling cascades that mediate changes in physiology through adjustment in gene expression or through immediate effector proteins (Weiner *et al.*, 2010). In the absence of ABA, KEG ubiquitinates an ABA-responsive transcription factor, Abscisic Acid Insensitive-5 (ABI5). This transcription factor is then subsequently degraded by the 26S proteasome preventing expression of ABA-responsive genes (Stone *et al.*, 2006; Liu and Stone, 2010). However, accumulation of ABA induces molecular signalling events that lead to a change in KEG substrate specificity. Rather than ubiquitinating Abscisic Acid Insensitive-5, KEG self-ubiquitinates and is degraded through the 26S proteasome (Liu and Stone, 2010). This self-regulation mechanism displayed by KEG allows ABI5 protein to accumulate, become activated, and induce ABA-responsive gene expression (Figure 2). The molecular signalling events that regulate KEG stability are unknown and regulation of monomeric E3 ligase activity is a relatively unexplored area of plant biology. Genetic studies suggest that ABI5 is not likely to be the only substrate of KEG's E3 ligase activity. This leaves two questions surrounding KEG; first, what are proteins that interact with KEG to regulate KEG activity and second, what are additional ubiquitination targets of KEG.

The overall goal of my PhD work was to identify KEG interacting proteins and characterize the function of their interaction. Using a yeast two hybrid screen, I identified CBL-Interaction Protein Kinase 26 (CIPK26) as a KEG interacting protein. Chapters 3 and 4 explore this interaction and suggest that under normal growth conditions CIPK26 is a KEG ubiquitination target (Chapter 3). In Chapter 4, I explore evidence which suggests that when CIPK26 is active, CIPK26 phosphorylates KEG to regulate KEG stability.

Another part of my thesis investigates the ubiquitination targets of the E3 ligase XBAT32. Previous work on this E3 ligase suggests that XBAT32 contributes to regulation of ethylene production. An *XBAT32* knockout line overproduces ethylene and displays an ethylene-related lack of lateral root phenotype (Prasad *et al.*, 2010). Using a yeast two hybrid screen I identified two ethylene biosynthesis proteins (ACS4 and ACS7)
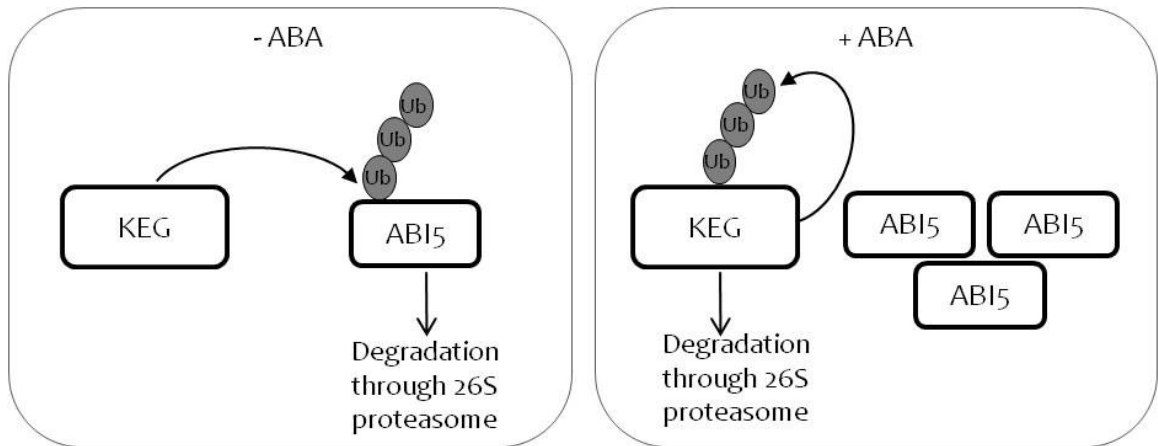
Figure 2. Model of KEG activity in the presence and absence of ABA. In the absence of ABA KEG inhibits ABI5 by ubiquitination. In the presence of ABA KEG self-ubiquitinates and ABI5 accumulates.

as XBAT32 interacting proteins. Chapter 2 explores this interaction and provides evidence that ACS4 and ACS7 are ubiquitination targets of XBAT32.

This PhD dissertation is in a publications based format. Chapters 2 and 3 are published in peer-reviewed journals. Chapter 4 is an unpublished manuscript. Each chapter contains its own abstract, introduction, methods, results and discussion section. All references have been placed together in the reference section. Two review articles can be found in the appendices and they provide a review of the subject matter. Each manuscript contains a statement about author contributions. Copyright permission letters can be found in Appendix C.

CHAPTER 2:

The Arabidopsis RING-type E3 ligase XBAT32 mediates the proteasomal degradation of the ethylene biosynthetic enzyme, 1-aminocyclopropane-1-carboxylate synthase 7.

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Contributions:

WJL: Participated in design of all assays, performed all assays except for assays displayed in Figure 4B and Figure 9. Wrote the manuscript.

JKB: Performed assay seen in Figure 4B and Supplemental Figure 9B.

SLS: Participated in design of all assays, prepared Figure 3 and Figure 9A and revised the manuscript.

Summary

E3 ubiquitin ligases select specific proteins for ubiquitin conjugation, and the modified proteins are commonly degraded through the 26S proteasome. XBAT32 is a RING-type E3 ligase involved in maintaining appropriate levels of ethylene. Previous work has suggested that XBAT32 modulates ethylene production by ubiquitinating two ethylene biosynthesis enzymes, ACS4 (type-II isoform) and ACS7 (type-III isoform). In Arabidopsis, conserved sequences within the C-terminal tail of type-I and -II 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) isoforms influence ubiquitin-dependent proteolysis. ACS7, the sole Arabidopsis type-III ACS, contains a truncated C-terminal tail that lacks all known regulatory sequences, which suggests that this isoform may not be subject to ubiquitin-mediated proteasomal degradation. Here we demonstrate *in planta* that ACS7 is turned over in a 26S proteasome-dependent manner and that degradation of ACS7 requires the E3 ligase XBAT32. Furthermore, the ethylene-related phenotypes that result from overexpression of *ACS7* in wild-type plants are greatly exaggerated in *xbat32-1*, suggesting that XBAT32 is required to attenuate the effect of overexpression of *ACS7*. This observation is consistent with a role for XBAT32 in the ubiquitin-mediated degradation of ACS7. The dark-grown phenotype of *xbat32-1*

seedlings overexpressing *ACS7* can be effectively rescued by aminoethoxyvinylglycine, an inhibitor of ACS activity. The degradation rate of ACS4 is also significantly slower in the absence of XBAT32, further implicating XBAT32 in the ubiquitin-mediated degradation of ACS4. Altogether, these results demonstrate that XBAT32 targets ethylene biosynthetic enzymes for proteasomal degradation to maintain appropriate levels of hormone production.

Introduction

The attachment of one or more ubiquitin molecules to an internal lysine of a substrate is a regulatory mechanism utilized by all eukaryotes. Ubiquitin attachment serves a wide range of functions from targeting proteins for degradation via the 26S proteasome to the activation of signalling proteins (Sun and Chen, 2004; Vierstra, 2009). The outcome of ubiquitination can be signified by the lysine residue used for ubiquitin chain assembly (polyubiquitination), as well as by the number of ubiquitin molecules attached to the target protein. Lysine-48 linked polyubiquitin chains have been well documented to signal degradation through the 26S proteasome, while functional modifications generally involve lysine-63 linked chains, monoubiquitination (attachment of a single ubiquitin molecule) or multi-monoubiquitination (attachment of multiple ubiquitin molecules). The attachment of ubiquitin to a target requires the sequential action of three enzymes: E1 (ubiquitin-activating enzyme, UBA), E2 (ubiquitin-conjugating enzyme, UBC) and E3 (ubiquitin ligase). The conjugation cascade begins with the ATP-dependent activation of ubiquitin by E1. The activated ubiquitin is then transferred to E2 forming an E2-ubiquitin (E2-Ub) intermediate. The ubiquitin ligase interacts with both the E2-Ub and the target protein, and coordinates the transfer of ubiquitin from E2-Ub onto the target. As the substrate recruiting component, E3 determines target specificity and is considered the major component of the pathway. Ubiquitination plays an integral role in almost all aspects of plant growth and development. The importance of the pathway to plant biology is reflected in its function as a regulator of the synthesis, perception and signal transduction of all the major hormones including auxin, gibberellic acid, ethylene and abscisic acid (Santner and Estelle, 2010).

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A significant proportion of the Arabidopsis genome (over 6%) is dedicated to the ubiquitin proteasome system (UPS). The majority of these genes (approximately 1300) encode for the substrate-recruiting ubiquitin ligases (Stone *et al.*, 2005; Vierstra, 2009). E3 ligases can be categorized into three main groups depending on the type of domain used to interact with the E2-Ub intermediate: the Really Interesting New Gene (RING), Homology to E6-Associated Carboxyl-Terminus (HECT) or the U-box domains. The majority of Arabidopsis E3 ligases contain a RING domain, which uses eight conserved cysteine and/or histidine residues to assemble two zinc ions into an interweaved structure that is essential for E2 binding and ubiquitin ligase activity (Freemont, 1993; Lorick *et al.,* 1999). RING-type E3s can function as monomeric E3s, which contain the substrate-binding and E2-binding function within the same protein, or as multi-subunit E3 ligases, which have these interaction domains on separate components of the complex. The Arabidopsis monomeric RING-type E3 family is quite large, with 479 members identified to date (Kosarev *et al.*, 2002; Stone *et al.*, 2005). The RING-type E3 family can be further divided into 30 subfamilies based on the presence of potential substrate-binding domains such as BRCT, ankyrin repeats and the vWA domain (Stone *et al.*, 2005).

XBAT32 (for XB3 ortholog 2 in *Arabidopsis thaliana*) is a member of the XBAT RING-type E3 subfamily and contains a series of ankyrin repeats that facilitates protein–protein interaction, followed by a RING domain (Bork, 1993; Nodzon *et al.*, 2004; Stone *et al.*, 2005). Loss of *XBAT32* results in a number of ethylene-related phenotypes. Most notably, *xbat32-1* produces few to no lateral roots without any observable effects on primary root growth (Nodzon *et al.*, 2004; Prasad *et al.*, 2010). A significant overproduction of ethylene contributes to the reduced lateral root phenotype of *xbat32-1* seedlings (Prasad *et al.*, 2010). Similar to *xbat32-1*, ethylene-overproducing mutants, such as *eto1*, and constitutive ethylene-signalling mutants, such as *ctr1*, also display a reduced lateral root phenotype (Negi *et al.*, 2008). Conversely, ethylene-insensitive mutants, such as *etr1-3* and *ein2-5*, have increased numbers of lateral roots (Negi *et al.*, 2008). Initiation of root branching requires the localized accumulation of auxin, which activates a quiescent pericycle cell that undergoes a series of coordinated divisions to produce a lateral root primordium. The newly formed primordia expand and emerge out

of the primary root to produce a lateral root (De Smet *et al.,* 2007; Dubrovsky *et al.,* 2008). Proper auxin synthesis, transport and signalling are essential for lateral root development (Péret *et al.,* 2009). The inhibitory effect of increasing ethylene levels on lateral root formation is due to the hormone's ability to alter auxin transport, which prohibits the localized accumulation of auxin (Negi *et al.,* 2008; Lewis *et al.,* 2011).

Ethylene biosynthesis is tightly controlled, and production is kept at relatively low levels during most stages of plant development (Wang *et al.,* 2002; Chae and Kieber, 2005). Ethylene production can be dramatically increased under certain environmental and developmental conditions such as pathogen attack and fruit ripening. The rate-limiting step and key point of regulation during ethylene biosynthesis is the conversion of *S*-adenosylmethionine (AdoMet) to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) by a family of enzymes called ACC synthases (ACSs) (Yang and Hoffman, 1984; Wang *et al.,* 2002). The eight members of the Arabidopsis ACS family all contain a highly conserved catalytic domain and a variable C-terminal tail (Yamagami *et al.,* 2003). The ACS enzymes can be classified into three types (I, II or III) based on the presence of phosphorylation sites found in the C-terminal tail. Type-I ACS isoforms (ACS2 and ACS6 in Arabidopsis) contain the longest C-terminal tail with a calcium-dependent protein kinase (CDPK) phosphorylation site followed by three mitogen-activated protein kinase (MAPK) phosphorylation sites (Tatsuki and Mori, 2001; Liu and Zhang, 2004; Sebastià *et al.,* 2004). Type-II ACS isoforms (ACS4, -5, -8, -9 and -11 in Arabidopsis) contain a shorter C-terminal tail with a single CDPK phosphorylation site. Type-III ACS (ACS7 in Arabidopsis) isoforms contain the shortest C-terminal tail with no identifiable phosphorylation sites (Argueso *et al.,* 2007). The sequences in the C-terminal tails of type-I and -II ACSs influence the stability of the isoforms.

Unphosphorylated type-I ACSs are rapidly degraded by the 26S proteasome, while phosphorylation by stress-responsive MAPKs, MPK6 and MPK3, drastically increases ACS2 and ACS6 protein levels (Liu and Zhang, 2004; Joo *et al.,* 2008; Han *et al.,* 2010). The CDPK phosphorylation of tomato type-II ACS, LeACS2, also appears to prohibit turnover (Sebastià *et al.,* 2004; Kamiyoshihara *et al.,* 2010). The shorter C-terminal tail and lack of phosphorylation sites in type-III ACSs raises the question of how the stability of these isoforms is controlled and if they are also turned over by the UPS.

The E3 ubiquitin ligase responsible for targeting Arabidopsis type-I ACSs for proteasomal degradation has not yet been identified. In Arabidopsis, type-II ACSs are targeted for ubiquitination by broad complex/tramtrack/bric-a-brac (BTB) ETO1 and ETO1-like 1/2 (EOL1/2) proteins which function as the substrate-recruiting component of Cullin3-based E3 ligase complexes (CUL3-BTB) (Wang *et al.*, 2004; Yoshida *et al.*, 2005; Christians *et al.*, 2009). The fact that XBAT32 interacts with ACS4 suggests that the RING-type E3 ligase may modulate the stability of this type-II ACS (Prasad *et al.*, 2010). Mutations within the C-terminal tails of ACS5 and ACS9 prevent recognition of the enzymes by ETO1 and EOL1/2 leading to elevated protein levels and overproduction of ethylene (Vogel *et al.*, 1998; Chae *et al.*, 2003; Yoshida *et al.*, 2006; Christians *et al.*, 2009). The ETO1/EOL1/2 recognition sequence, referred to as TOE, also includes the CDPK phosphorylation site. However, it is not known if CDPK phosphorylation influences the stability of the enzymes.

We have previously provided evidence which suggests that the abundance of ACS7 may be influenced by the UPS (Prasad *et al.*, 2010). ACS7 was found to interact with XBAT32 in yeast two-hybrid assays and XBAT32 can catalyze the attachment of ubiquitin to ACS7 during *in vitro* ubiquitination assays (Prasad *et al.*, 2010). Here we demonstrate that despite the truncated C-terminal tail, ACS7 is turned over by the UPS and XBAT32 is required for the degradation of ACS7 *in planta*. In addition, the ethylene overproduction phenotype that is observed in wild-type plants overexpressing *ACS7* is exaggerated in the *xbat32-1* background, suggesting that XBAT32 contributes to attenuating the effects of overexpression of *ACS7,* which is consistent with XBAT32 targeting ACS7 for degradation. XBAT32 was also shown to interact with and ubiquitinate ACS4 *in vitro* (Prasad *et al.*, 2010). Using a cell-free degradation assay we also demonstrate that XBAT32 is involved in the turnover of ACS4.

Results

ACS7 is degraded through the 26S proteasome

With the exception of ACS7, the C-terminal extensions of Arabidopsis ACS isoforms have been suggested or demonstrated to modulate protein stability (Argueso *et al.*, 2007). Possible points of regulation include MAPK and/or CDPK phosphorylation sites and E3 ligase target sequences (Figure 3). The truncated C-terminal tail is not

unique to ACS7, the sole Arabidopsis type-III isoform. A number of ACSs found in other plant species also lack the regulatory sequences (Figure 3). Even though ACS7, a type-III ACS protein, can interact with and be ubiquitinated by XBAT32 *in vitro*, the apparent lack of regulatory sequences found in type-III ACS proteins raises the question of whether these ACS proteins are subject to ubiquitin-dependent degradation *in planta*. To determine if ACS7 is degraded by the UPS, we utilized a cell-free degradation assay to monitor the turnover of the enzyme in the presence and absence of MG132, a specific inhibitor of the 26S proteasome. Histidine (His) and Flag tagged recombinant ACS7 (His-Flag-ACS7) protein was incubated with plant extracts prepared from wild-type seedlings in the presence and absence of MG132. The His-Flag-ACS7 gradually disappeared when incubated with protein extracts from wild-type seedlings, and this degradation was blocked by MG132 (Figure 4A). This demonstrates that ACS7 is turned over by the 26S proteasome.

The covalent attachment of a polyubiquitin chain to a lysine residue on a target protein is a prerequisite for degradation by the 26S proteasome. ACS7 contains a single lysine residue (lys435) in its C-terminal tail (Figure 3). Therefore we investigated the possibility that lys435 was a site of ubiquitin attachment. Cell-free degradation assays were used to compare the stability of His-Flag-ACS7 to that of His-Flag-ACS7(K435R) which contains a lysine 435(K435) to arginine (R) mutation. If lys435 is used for ubiquitination, then loss of the lysine should retard degradation of ACS7. Unexpectedly, His-Flag-ACS7(K435R) was degraded much faster than His-Flag-ACS7 (Figure 4B). The degradation of His-Flag-ACS7(K435R) was blocked by MG132, suggesting that the increased turnover of the mutant ACS7 was carried out by the 26S proteasome (Figure 4B). These results suggest that lys435 is not utilized by the ubiquitination pathway. However, the fact that the mutation altered the rate of degradation suggests that the C-terminal tail lysine somehow influences the stability of ACS7.

ACS7 degradation is dependent on the RING E3 ligase XBAT32

Previous studies identified XBAT32 as a potential E3 ligase that targets ACS7 for degradation by the 26S proteasome (Prasad *et al.*, 2010). To determine if XBAT32

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                                                      CDPK            MAPK
        ACS6  (409)  EPGWFRVCFANMDHKTMETALERIRVFTSQLEEETKPMA----ATTMMAKKKKK--CWQSNLRLSFSDTRRFDDGFFSPHSPVPPSPLVRAQT
  Type-I LeASC2 (407) EPGWFRVCFANMDDGTVDIALARIRRFVGVEKSGDK---------SSSMEKKQQ--WKKNNLRLSFSKRMYDESVLSPLSSPIPPSPLVR---
        ACS2  (409)  EPGWFRICFANMDDDTLHVALGRIQDFVSKNKNKIVEKASENDQVIQNKSAKKLK-WTQTNLRLSFRRL-YEDGL-SSPGIMSPHSPLLRA--
        ACS11 (396)  EPGWFRVCFANMSDQTMEVAMDRVKGFVDNNNG------GKQKRTMWDTRRRS--LINKWVSKLSSVTCESER-------------------
  Type-II ACS4 (402) EPGWFRVCFANMIDETLKLALKRLKMLVDDENSSRRC-QKSKSERLNGSRKKTMSNVSNWVFRLSFHDREAEER------------------
        ACS9  (401)  EPGWFRVCFANMSEDTLDLAMKRLKEYVESTDSRRVI-SKSSHDRIKSLRKKT---VSNWVFRVSWTDRVPDER------------------
        ACS5  (401)  EPGWFRVCFANMSEDTLDLALKRLKTFVESTDCGRMI-SRSSHERLKSLRKKT---VSNWVFRVSWTDRVPDER------------------
        LeACS3 (401) EPGWFRVCFANMSEDTLDLAMRRIKDFVESTAPNATN-HQNQQQSNANSKKKS---FSKNVFRLSFNDRQRER------------------
        ACS8  (401)  EPGWFRVCFANLSEETLKVALDRLKRFVDGPSP-TRR-SQSEHQRLKNLRKMK---VSNWVFRLSFHDREPEER-----------------
        LeACS4 (411) EVGWFRVCFANIDDQTMEIALARIRMFMDAYNNVNKNGVMKNKHNGRGTTYDLTPQMGSTMKMLLA-----------------------
        ACS7  (414)  EVGWFRVCFANMSENTLEIAIKRIHEFMDRRRRF---------------------------------------------------------
  Type-III CsACS2 (408) EPGWFRVCFANMSEKTLHVALDRIRRFMERMKKENEAN-----------------------------------------------------
        CmACS-7(408) EPGWFRVCFANMSEKTLHVALDRIRRFMERMKKENEAN-----------------------------------------------------
        PsACS3 (406)  EPGWFRVCFANMSEQTLEVALKRIHNFMERRERSWNLFSSL--------------------------------------------------
        PcASC2a(412) EPGWFRVCFANMSEQTLGIALTRIHNFMEKRERAC-------------------------------------------------------
        PtACS5 (405)  EPGWFRVCFANMSEPTLAVALKRIHDFMDQRKTEAIASPPQQ------------------------------------------------
        PtACS6 (405)  EPGWFRVCFANMSEETLEVALKRIHNFMEQRKTASS------------------------------------------------------
        PgACS1 (415)  EPGWFRVCFANMSEHTLQVSLQRIQGFMDSKQN---------------------------------------------------------
        PmACS2 (421)  EPGWFRFCFANMDDQTVQESLNRIHLFVESKTPNQRGMAG--------------------------------------------------
        PgACS4 (493)  EPGWFRICFSTLSCQDMTVALQRIREFSKHCKRSS-------------------------------------------------------
        PmACS4 (496)  EPGWFRICFGTLSSQDLTVALQRIQEFSKRCKRSP-------------------------------------------------------
                BOX 7
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Figure 3. Alignment of the variable C-terminal tail of type-I, -II and -III 1-aminocyclopropane-1-carboxylate synthase (ACS) enzymes from *Arabidopsis thaliana*. Alignment includes ACS enzymes from *Solanum lycopersicum* (Le; tomato), *Cucumis sativus* (Cs; cucumber), *Prunus salicina* (Ps; plum), *Pyrus communis* (Pc; Pear), *Picea glauca* (Pg; white spruce), *Pseudotsuga menziesii* (Pm; Douglas-fir), *Cucumis melo* (Cm; melon) and *Populus trichocarpa* (Pt; poplar). A portion of the conserved catalytic domain (BOX 7) is included in the alignment as a reference. Amino acids involved in regulating the stability of the different types of ACS isoforms are highlighted. Mitogen-activated protein kinase (MAPK) phosphorylation sites found in type-I ACSs are in bold. The calcium-dependent protein kinase (CDPK) phosphorylation site found in type-I and -II ACS proteins are in bold and underlined. The ETO1 interaction domain (target of ETO1 sequence or TOE) identified in tomato type-II ACS, LeACS3, is italicized. The two clusters of basic amino acids that negatively impact the stability of ACS6 are underlined. The ACS7 lysine-435 mutated in this study is in bold and underlined.
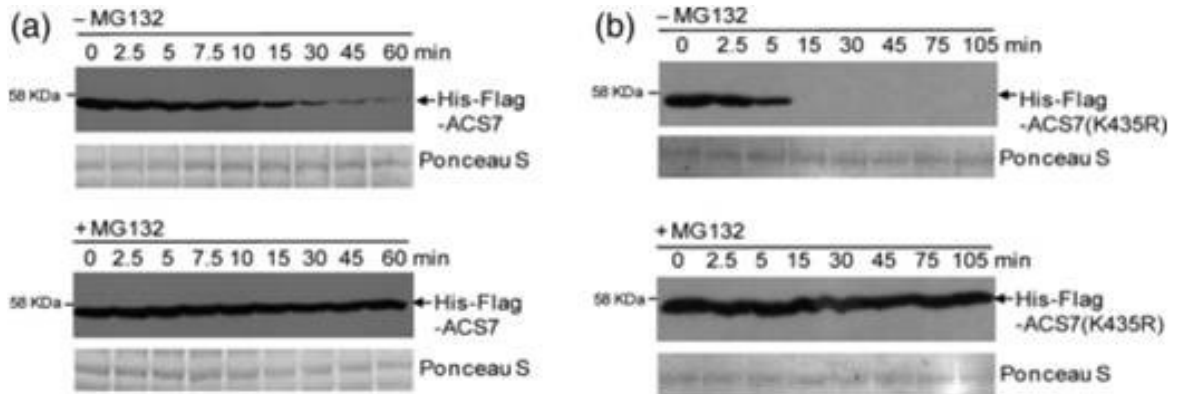
Figure 4. Proteasome dependent degradation of ACS7, a type-III 1-aminocyclopropane-1-carboxylate synthase (ACS) isoform. (a) Stability of ACS7 in cell-free degradation assays. His-Flag-ACS7 protein was incubated with plant extracts from 8-day-old wild-type seedlings with (+) or without (−) 40 μm MG132. (b) Stability of the ACS7 mutant ASC7(K435R) in cell-free degradation assays. Histidine (His)-Flag-ACS7(K435R) protein was incubated with plant extracts from 8-day-old wild-type seedlings with (+) or without (−) 40 μm MG132. The level of His-Flag-ASC7 remaining at the indicated time points was determined by western blot analysis using His antibodies. Ponceau S staining confirms equal protein loading. min, minutes.

influences the abundance of ACS7 *in planta*, we compared the stability of ACS7 in transgenic wild-type (*35S:HA-ACS7*/Col-0) and *xbat32-1* (*35S:HA-ACS7/xbat32-1*) plants overexpressing hemagglutinin (HA) tagged ACS7 using an modified assay from Joo *et al.* (2008). *35S:HA-ACS7*/Col-0 and *35S:HA-ACS7/xbat32-1* seedlings (corresponding to L2 and L1, respectively, from Figure 7A) were first treated with MG132. Following removal of the proteasome inhibitor, seedlings were incubated with the protein synthesis inhibitor cycloheximide (CHX) and the level of HA-ACS7 was determined at the indicated times (Figure 5A). Consistent with the results obtained from the cell-free degradation assays, the levels of HA-ACS7 in wild-type seedling gradually decreased over time (Figure 5A). In contrast, the level of HA-ACS7 protein remained stable in *xbat32-1* seedlings (Figure 5A). The lack of degradation of HA-ACS7 observed in *xbat32-1* seedlings demonstrates a requirement for XBAT32 in the proteasome-mediated turnover of ACS7. Furthermore, treatment of *35S:HA-ACS7*/Col-0 seedlings (corresponding to L2 from Figure 7A) with MG132 repeatedly caused an increase in HA-ACS7 levels (Figure 5B). The accumulation of HA-ACS7 is not observed in MG132-treated *35S:HA-ACS7/xbat32-1* seedlings (corresponding to L1 from Figure 7A), suggesting a lack of degradation by the UPS in the *xbat32-1* background (Figure 5B).

XBAT32 is involved in the proteasomal degradation of ACS4

ACS4 belongs to the type-II class of ACS proteins. Turnover of the type-II class of ACS proteins via ETO1/EOL1/2-mediated ubiquitination has been previously demonstrated (Wang *et al.*, 2004; Christians *et al.*, 2009). However, interaction of ACS4 with XBAT32 suggests that ACS4 may be regulated by multiple E3 ligases and that the CUL4-BTB E3 ligase is not solely responsible for degradation of the enzyme. If XBAT32 contributes to the ubiquitin-mediated degradation of ACS4, then the half-life of ACS4 should be prolonged in the *xbat32-1* seedlings. To compare the half-life of ACS4 in *xbat32-1* and wild-type seedlings we utilized a cell-free degradation assay. Recombinant His and Flag tagged ACS4 (His-Flag-ACS4) was incubated with plant extracts prepared from either *xbat32-1* or wild-type seedlings. We repeatedly observed a slower rate of degradation for His-Flag-ACS4 in *xbat32-1* extracts compared with that of wild-type (Figure 6A). The turnover of His-Flag-ACS4 in both *xbat32-1* and wild-type
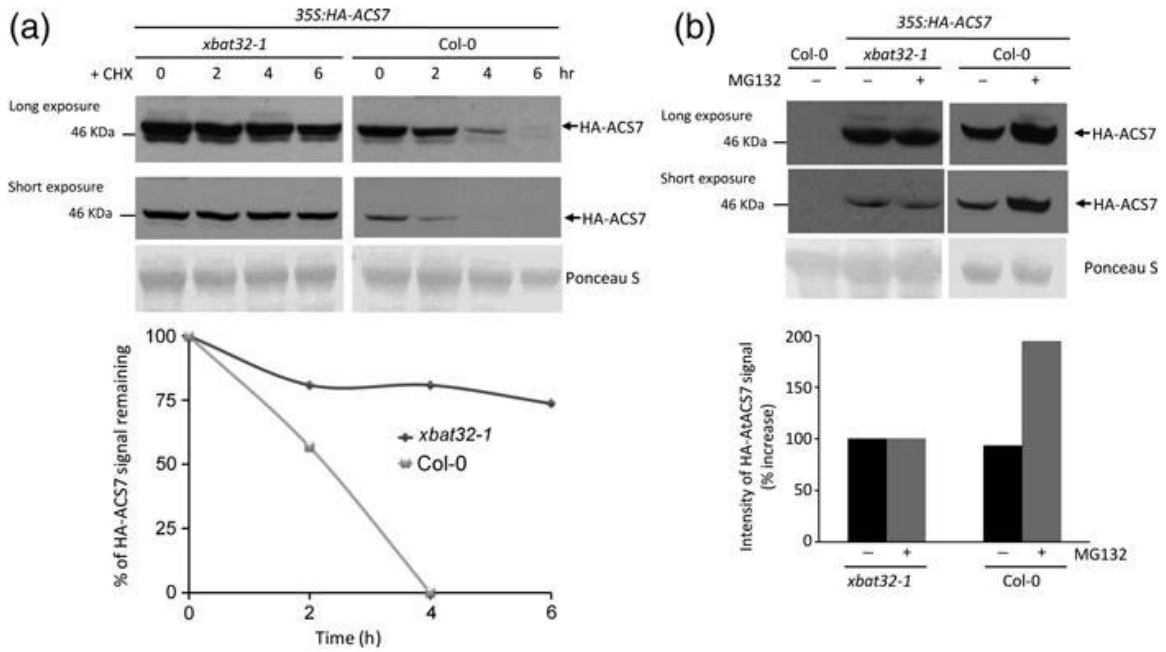
Figure 5. Proteasomal degradation of ACS7 is dependent on the presence of XBAT32. (a) Twelve-day-old 35S:HA-ACS7/Col-0 and 35S:HA-ACS7/xbat32-1 seedlings were pre-treated with 30 μm MG132 for 16 h. Following removal of MG132, 1 mm cycloheximide (CHX) was added and the levels of hemagglutinin (HA)-ACS7 were determined by western blot analysis with HA antibodies. Ponceau S staining confirms equal protein loading. Bottom graph displays HA-ACS7 signal intensity based on the short exposure (b). 12-day-old Col-0, 35S:HA-ACS7/xbat32-1 and 35S:HA-ACS7/Col-0 seedlings were treated with (+) or without (−) 30 μm MG132 for 16 h. The levels of HA-ACS7 were determined by western blot analysis with HA-antibodies. Ponceau S staining confirms equal protein loading for each treatment. The graph illustrates changes in HA-ACS7 signal intensity following treatment with MG132 from three separate trials. Signal intensities from each blot was determined using ImageJ software and shown as a percentage (%) of untreated (– MG132, control).
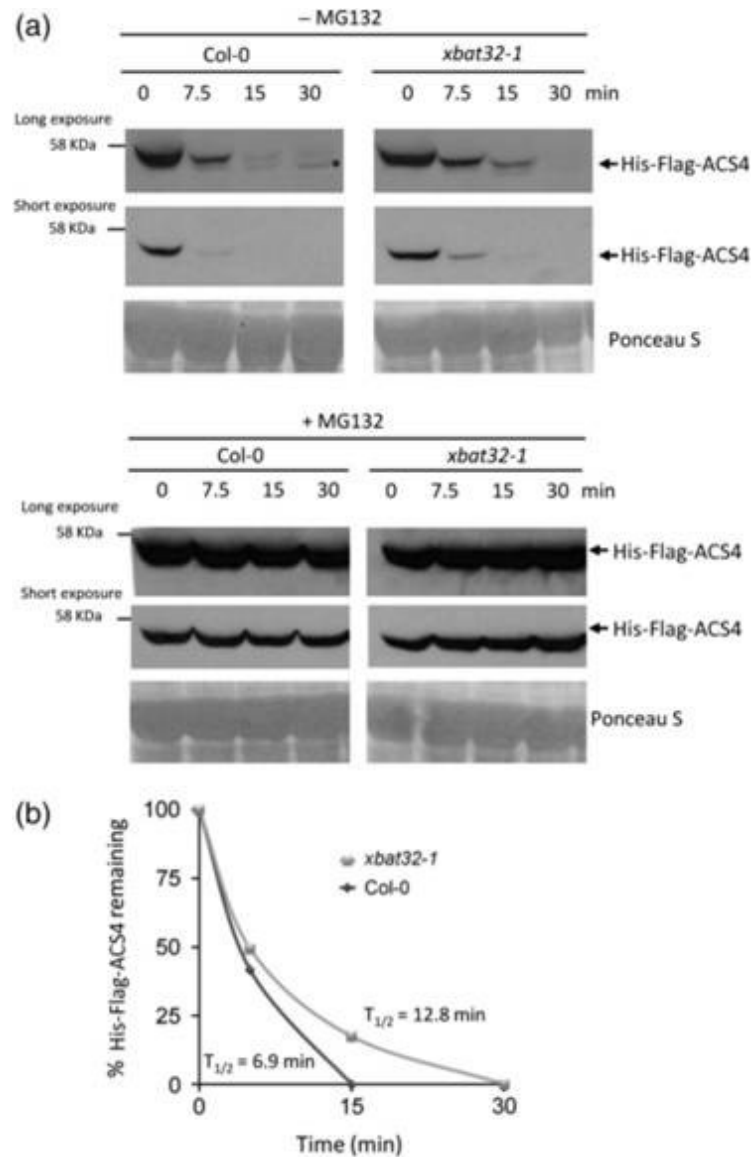
Figure 6. XBAT32 contributes to the turnover of ACS4. (a) Cell-free degradation assays showing the turnover of ACS4. His-Flag-ACS4 protein was incubated with plant extracts from 8-day-old Col-0 and *xbat32-1* seedlings with (+) or without (−) 40 μm MG132. The levels of histidine (His)-Flag-ACS4 remaining at the indicated time points were determined by western blot analysis with His antibodies. Ponceau S staining confirms equal protein loading. '*' Indicates a cross-reactive band. (b) Half-life ($T_{1/2}$) of His-Flag-ACS4 in the *xbat32-1* background compared with wild-type Col-0 based on signal quantification from short exposure. min, minutes.

plant extracts was blocked by MG132 (Figure 6A). The half-life of His-Flag-ACS4 was markedly longer in assays using extracts from *xbat32-1* seedlings (12.8 min) compared with wild-type (6.9 min) (Figure 6B). These results suggest that XBAT32 contributes to the proteasomal-dependent turnover of ACS4. The C-terminal tail of ACS4 contains a number of lysine residues, four of which (Lys424, -427, -452 and -453) are found in all or almost all type-II ACSs (Figures 3 and Figure 9 in the Supporting Information). To determine if these lysine residues are involved in ACS4 degradation we investigated the effects of loss of all four lysine residues on ACS4 stability. A cell-free degradation assay was used to compare the turnover of His-Flag-ACS4 and His-Flag-ACS4(RR-RR) with Lys424, -427, -452 and -453 mutated to arginine (R). His-Flag-ACS4 and His-Flag-ACS4(RR-RR) recombinant proteins were incubated with extracts prepared from wild-type seedlings. Unexpectedly, the reduction in His-Flag-ACS4(RR-RR) levels occurred at a much faster rate than His-Flag-ACS4 (Figure 9B in Supporting Information). These results are similar to those obtained for His-Flag-ACS7(K435R), where substituting a lysine residue for arginine in the C-terminal tail increased the proteasomal-dependent turnover of the protein (Figure 4B). These results suggest that these lysine residues in the ACS4 C-terminal tail are not utilized for ubiquitination but instead seem to be involved in stabilizing the protein.

The overexpression phenotypes of ACS7 are exaggerated by the loss of XBAT32

Results obtained from the degradation assays strongly suggest that increased stability of ACS4 and ACS7 contributes to the *xbat32-1* phenotype. To further investigate this possibility we determined the effect of overexpression of *ACS7* on *xbat32-1* development. Since considerably less is known about the regulation of ACS7 stability and ACS4 is known to be degraded by multiple E3 ligases, we limited further analysis to understanding the relationship between XBAT32 and ACS7. Transgenic wild-type (*35S:HA-ACS7*/Col-0) and *xbat32-1* (*35S:HA-ACS7*/*xbat32-1*) plants overexpressing *HA-ACS7* were phenotypically analysed. Figure 7(A) shows the expression levels of HA-ACS7 in two independent (L1 and L2) wild-type (*35S:HA-ACS7*/Col-0) and *xbat32-1* (*35S:HA-ACS7*/*xbat32-1*) transgenic lines. As described previously, 4–5-week-old *xbat32-1* displays reduced plant height compared with wild-type (Nodzon *et al.*, 2004). Interestingly, plants from both *35S:HA-ACS7*/Col-0 transgenic lines (L1 and L2)
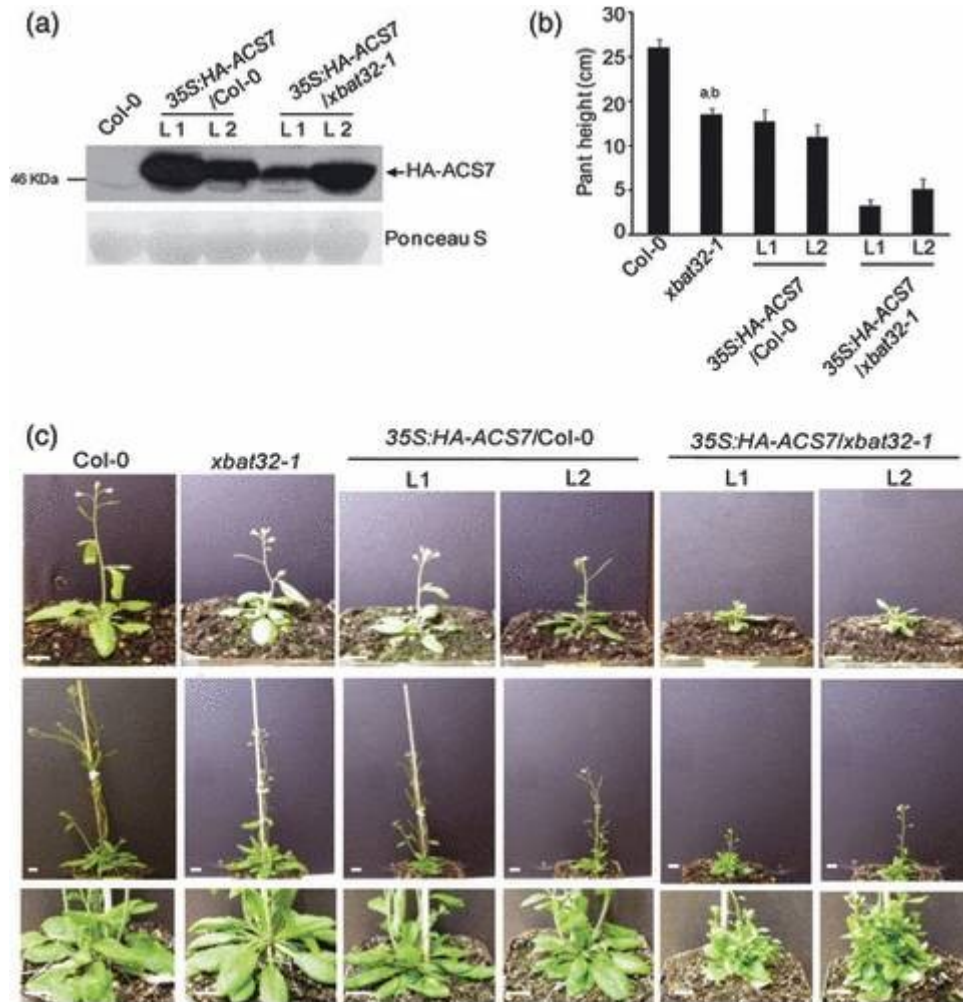
Figure 7. The *xbat32-1* background exaggerates the *ACS7* overexpression phenotype in adult plants.
(a) Expression levels of hemagglutinin (HA) ACS7 in *35S:HA ACS7*/Col-0 and *35S:HA ACS7*/*xbat32-1*
in independently generated transgenic plant lines (L1, line 1; L2, line 2). Protein extracts were prepared
from 8 day old seedlings and HA-ACS7 was detected by western blot analysis using HA antibodies.
Ponceau S staining shows equal protein loading. (b) Average height of 5 week old Col-0, *xbat32-1*,
35S:HA ACS7/Col-0 and *35S:HA ACS7/xbat32-1* plants. *xbat32-1* is not significantly different from
35S:HA ACS7/Col-0 (L1 and L2) plants (a; $P > 0.05$). *xbat32-1* is significantly different from *35S:HA
ACS7/xbat32-1* (L1 and L2) plants (b; $P < 0.05$). Statistical analysis was performed using Student's t test.
$n \geq 10$ plants, error bars represent ± standard error. (c) Representative of Col-0, *xbat32-1,*
35S:HA-ACS7/Col-0 and *35S:HA ACS7/xbat32-1* plants at 4 weeks (top panels) and 5 weeks (middle
panels). The bottom panels show the rosette phenotype of 7 week old *35S:HA ACS7*/Col-0 (L1 and L2)
and *35S:HA ACS7/xbat32-1* (L1 and L2) compared with Col-0 and *xbat32-1*. Scale bar = 1 cm.

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were shorter than wild-type, with plant height comparable to that of *xbat32-1* plants (Figure 7B,C). This suggests that levels of ACS7 in the overexpressing transgenic wild-type plants, *35S:HA-ACS7*/Col-0, mirrored that of the levels of endogenous ACS7 protein in *xbat32-1* plants. Plants from both *35S:HA-ACS7*/*xbat32-1* lines (L1 and L2) were severely delayed in growth compared with *xbat32-1* and *35S:HA-ACS7*/Col-0. At 4 weeks bolting was observed for very few *35S:HA-ACS7*/*xbat32-1* plants (<25%), whereas most *35S:HA-ACS7*/Col-0 (>70%), and all *xbat32-1* (100%) and wild-type Col-0 (100%) had produced a bolt (Figure 7C top panels). At 5 weeks the number of bolted *35S:HA-ACS7*/*xbat32-1* plants had increased (>60%) and the average height of the bolted plants was half that of *xbat32-1* and *35S:HA-ACS7*/Col-0 plants (Figure 7B,C middle panel). At 7 weeks the rosettes of *xbat32-1* and *35S:HA-ACS7*/Col-0 plants were similar to wild-type with the leaves of *35S:HA-ACS7*/Col-0 appearing only slightly smaller (Figure 7C bottom panels). This is in contrast to the *35S:HA-ACS7*/*xbat32-1* plants, which displayed a compact rosette with an increased number of small leaves, a dwarfed stature, and reduced apical dominance (Figure 7C bottom panels). The phenotypes observed between *35S:HA-ACS7*/*xbat32-1* and *35S:HA-ACS7*/Col-0 plants are significantly different and demonstrate that overexpression of *HA-ACS7* in the *xbat32-1* background has a greater effect on plant development than overexpression in wild-type background. This observation is consistent with XBAT32 mediating the degradation of ACS7. The exaggerated phenotype of the *35S:HA-ACS7*/*xbat32-1* lines may be because these lines display the effects of both stabilized endogenous ACS7 and transgenic HA-ACS7. In addition, unlike in the *xbat32-1* background, the HA-ACS7 protein expressed in wild-type is still being targeted for degradation.

The phenotypes observed for *35S:HA-ACS7*/*xbat32* plants, including short stature and a compact rosette, is reminiscent of the ethylene overproducer *eto1* and ethylene signalling mutant *ctr1*. To further investigate the ethylene phenotype, growth of *35S:HA-ACS7*/*xbat32-1* seedlings was examined in the light and dark. A well-documented effect of ethylene on light-grown seedlings is the inhibition of primary root growth and increased root hair production (Masucci and Schiefelbein, 1994; Tanimoto *et al.*, 1995). The primary root length of *35S:HA-ACS7*/Col-0 and *35S:HA-ACS7*/*xbat32-1*
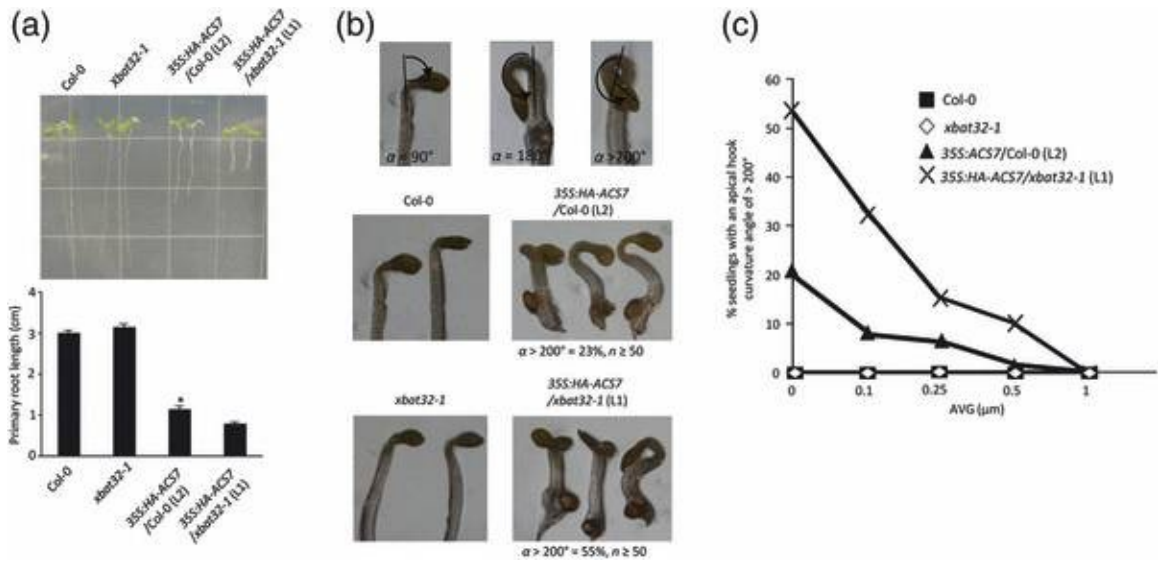
Figure 8. The effect of ACS7 overexpression is enhanced in *xbat32-1* seedlings compared with wild-type. (a) Light-grown 9-day-old Col-0, *xbat32-1*, *35S:HA-ACS7*/Col-0 (L2) and *35S:HA-ACS7*/*xbat32-1*(L2) seedlings. The graph shows the average primary root length of 9-day-old seedlings. *35S:HA-ACS7*/Col-0 (L2) is significantly different from *35S:HA-ACS7*/*xbat32-1*(L2) (*$P < 0.05$). Statistical analysis was performed using Student's *t*-test. The error bar represents ± standard error. (b) The top panel displays a schematic of how the angle of apical hook curvature was determined. The bottom panels display dark-grown 3-day-old Col-0, *xbat32-1*, *35S:HA-ACS7*/Col-0 (L2) and *35S:HA-ACS7*/*xbat32 -1* (L2) seedlings. The percentage (%) of seedlings with an apical hook curvature angle of >200° is shown ($n \geq 50$). (c) Dark-grown 3-day-old Col-0, *xbat32-1*, *35S:HA-ACS7*/Col-0 (L2) and *35S:HA-ACS7*/*xbat32 -1* (L1) seedlings on growth media supplemented with increasing concentrations of aminoethoxyvinylglycine (AVG). The graph shows the percentage (%) of seedlings with an apical hook curvature of >200° ($n \geq 30$/genotype/treatment).

was greatly reduced compared to wild-type and *xbat32-1* (Figure 8A). However, consistent with the requirement for XBAT32-mediated turnover of ACS7, the primary root length of *35S:HA-ACS7*/*xbat32-1* was significantly shorter than*35S:HA-ACS7*/Col-0 (Figure 8A). In addition, overexpression of *ACS7* in the *xbat32-1* background resulted in a drastic increase in root hair production compared with all other genotypes (Figure 8A). Both *35S:HA-ACS7*/Col-0 and *35S:HA-ACS7*/*xbat32-1* exhibited the classic triple response observed in ethylene-treated dark-grown wild-type seedlings, including shorter and thicker hypocotyls, shorter roots and exaggerated apical hooks (Figure 8B middle and bottom panels). Both *35S:HA-ACS7*/Col-0 and *35S:HA-ACS7*/*xbat32-1* displayed a very similar reduced hypocotyl length after 3 and 4 days compared with Col-0 and *xbat32-1* (data not shown). However, one major difference in the triple response phenotype between these two transgenic lines is that the number of *35S:HA-ACS7*/*xbat32-1* seedlings with an exaggerated angle of apical hook curvature, as defined by Lehman *et al.,* (1996) to be >200°, was higher than that of *35S:HA-ACS7*/Col-0 (Figure 8B). A schematic displaying how the angle of hook curvature was adapted from Vandenbussche *et al.,* (2010) and Lehman *et al.,* (1996) and is displayed in the top panel of Figure 8(B). Treatment with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, blocked formation of the exaggerated apical hooks for both *35S:HA-ACS7*/Col-0 and *35S:HA-ACS7*/*xbat32-1* seedlings (Figure 8C). However, a greater concentration of AVG was required to eliminate the formation of exaggerated apical hooks by *35S:HA-ACS7*/*xbat32-1* seedlings compared with *35S:HA-ACS7*/Col-0 (Figure 8C). This result further suggests greater ethylene production through increased stability of ACS7 in *xbat32-1* seedlings compared with wild-type.

Discussion

Previous studies implicated the RING-type E3 ligase XBAT32 in the proteolytic control of two ethylene biosynthetic enzymes, ACS4 and ACS7 (Prasad *et al.*, 2010). Here we provide evidence which supports a role for XBAT32-mediated proteasomal degradation of ACS4 and ACS7 by showing that XBAT32 contributes to the ethylene-related phenotypes and the stability of the enzymes. Using transgenic plants we have demonstrated that ACS7 is gradually degraded by the 26S proteasome in wild-type seedlings while ACS7 levels remain consistent in *xbat32-1* seedlings. Furthermore,

inhibition of proteasome activity induces accumulation of ACS7 protein in wild-type seedlings but not in *xbat32-1* seedlings. These results demonstrate that despite the lack of known regulatory sequences within the C-terminal tail of ACS7, turnover of the enzyme occurs via the UPS. Although an E3 ligase has been previously identified for ACS4, we show that XBAT32 also contributes to the turnover of ACS4. Using a cell-free degradation assay we demonstrate that ACS4 has a longer half-life in the absence of XBAT32 compared with a control.

In our previous study we demonstrated that the reduced lateral root phenotype displayed by *xbat32-1* is caused by an increase in ethylene levels (Prasad *et al.*, 2010). The overproduction of ethylene is most likely due to the lack of XBAT32-mediated ubiquitination and the resulting accumulation of ACS proteins. Evidence for a functional relationship between XBAT32 and ACS7 comes from the analysis of transgenic wild-type and *xbat32-1* plants that overexpress ACS7. Overexpression of *ACS7* in wild-type seedlings produced a number of phenotypes that can be attributed to elevated ethylene production, including inhibition of primary root elongation and increased root hair production (Kieber *et al.*, 1993; Masucci and Schiefelbein ,1994; Tanimoto *et al.*, 1995; Le *et al.*, 2001; Christians *et al.*, 2009). These phenotypes were exaggerated in the *xbat32-1* overexpressing *ACS7* with greater inhibition of primary root length and increased production of root hairs. Similarly, stabilization of ACS6 through phosphomimic mutations also inhibited primary root elongation and increased root hair formation (Liu and Zhang, 2004; Joo *et al.*, 2008). These results support the notion that ACS7 is stabilized in the *xbat32-1* seedlings and that this increased stability contributes to the ethylene-related phenotypes.

A common phenotype of ethylene-overproducing mutants, such as *eto1*, is reduced plant height (Christians *et al.*, 2009). Similarly, *xbat32-1* overproduces ethylene and plants are shorter than wild-type (Nodzon *et al.*, 2004; Prasad *et al.*, 2010). Interestingly, overexpression of *ACS7* in wild-type plants reduced plant height to a level that is very similar to *xbat32-1* suggesting that overexpression of *ACS7* in wild-type mimics the *xbat32-1* phenotype. A further reduction in plant height is observed following overexpression of *ACS7* in *xbat32-1*. The difference in the effect of the *35S:HA-ACS7* transgene between Col-0 and *xbat32-1* may be attributed to the accumulation of

endogenous ACS7. Since XBAT32 modulates the abundance of ACS7, the *xbat32-1* plants would have higher levels of endogenous ACS7 than wild-type. Therefore, expression of the *35S:HA-ACS7* transgene would be expected to have a greater additive effect in the *xbat32-1* background compared with wild-type. This would account for the phenotype displayed by the *35S:HA-ACS7*/Col-0 (L1) line which has the highest expression of the transgene but still did not produce an exaggerated phenotype as seen with *35S:HA-ACS7/xbat32-1* lines.

In addition, ACS7 protein (endogenous or transgenic) is still being turned over in wild-type background. Therefore, despite the variation in protein levels the enzyme is more unstable in wild-type plants compared with *xbat32-1*. The results suggest that the difference in phenotype in the transgenic plants is not only linked to the expression of the transgene but to the increased stability of the enzyme in the *xbat32-1* background.

The results points to a model in which XBAT32 mediates the proteasomal degradation of ACS7. A bushy phenotype, a compact rosette with an increase number of small leaves, dwarfed stature and reduced apical dominance, seen only in the *xbat32-1* plants overexpressing *ACS7,* is reminiscent of other mutants that overproduce ethylene. For example, the ethylene-overproducing *related to ubiquitin 1* (*rub1*) *rub2* double mutant displayed a similar bushy and compact rosette phenotype (Bostick *et al.*, 2004). The lack of a bushy phenotype seen in wild-type plants overexpressing *ACS7* suggests that the functional XBAT32 is able to decrease the stability of endogenous ACS7 and transgenic HA-ACS7 throughout development, bringing ethylene levels closer to that of wild-type. In the *xbat32-1* background, ACS7 protein is stable, producing an exaggerated ethylene-related phenotype. However, we cannot rule out the possibility that *xbat32-1* displays an ethylene-overproduction phenotype though a mechanism independent of ACS proteins, and that overexpression of *ACS7* in *xbat32-1* merely results in an additive effect that does not correlate XBAT32 with mediating the abundance of ACS7.

Overexpression of a ubiquitination target in a plant that is missing the corresponding E3 ligase has been previously documented to result in an exaggerated phenotype. Overexpression of Ethylene Insensitive3-like1 (EIL1), a target of the F-box protein EIN3-binding F box protein 1 and 2 (EBF1/2), in an *ein3 ebf1 ebf2* background but not wild-type resulted in plants with stunted sepals and petals and a protruding

gynoecium (Binder *et al.*, 2007; An *et al.*, 2010). While *ein3 ebf1 ebf2* do display numerous ethylene response phenotypes, these plants do not display this floral phenotype and neither does overexpression of *EIL1* in wild-type plants (An *et al.*, 2010). The accentuated phenotype was suggested to be the result of constitutively activated ethylene signalling due to EIL1 accumulation. Consequently, we propose that the exaggerated ethylene-related phenotypes observed for *xbat32-1* plants overexpressing *ACS7* is a result of increased stability and over-accumulation of endogenous ACS7 and transgenic HA-ACS7.

Induction of ethylene synthesis is controlled through increased transcription and stabilization of ACS proteins. Several lines of evidence demonstrate that regulation of ACS protein stability occurs via the variable C-terminal extensions. The C-terminal tail of type-I ACS6 is sufficient to confer instability on otherwise stable reporter proteins (Joo *et al.*, 2008). ACS6 is more stable following phosphorylation at multiple sites by a stress-activated MAPK, MPK6, and degradation of ACS6 is inhibited by phosphomimic mutations (Liu and Zhang, 2004; Joo *et al.*, 2008). A CDPK phosphorylation site is also found along with the MAPK sites in all type-I ACSs. However, the role of the CDPK phosphorylation in regulating the stability of type-I ACSs is not clear. Most type-II ACSs contain a TOE sequence within the C-terminal tail that mediates interaction with ETO1/EOL1/2 E3 ligase and is required for degradation. *eto2* and *eto3* are dominant negative mutations in the TOE sequence of ACS5 and ACS9, respectively, that render the enzymes stable (Vogel *et al.*, 1998; Chae *et al.*, 2003). A CDPK phosphorylation site is found within the TOE sequence. However, loss of the CDPK phosphorylation site does not seem to influence the ability of ACS5 to bind to ETO1/EOL1, at least in yeast (Christians *et al.*, 2009). ACS7, a type-III isoform, has a truncated C-terminal tail and lacks both the MAPK and CDPK phosphorylation sites. A search of other plant genomes identified a number of ACSs that also contain truncated C-terminal extensions. The lack of regulatory motifs in the type-III ACSs places into question the ability of the UPS to influence the stability of these enzymes. As shown in this report, ACS7 protein is targeted by the UPS. Whether or not phosphorylation may be required for regulating ACS7 stability remains to be determined. There is some evidence to suggest that ACS7 may be phosphorylated, albeit within the catalytic region. ACS7, along with ACS6 and

ACS8, were found to interact with a 14-3-3 protein which usually requires a phosphorylated recognition motif for target interaction (Chang *et al.*, 2009). The lack of known regulatory sequences in ACS7's C-terminal tail suggest that environmental or development signals that influence the stability of ACS7 may be communicated through its corresponding E3 ligase, XBAT32. Expression of *XBAT32* is inhibited by ethylene (Stepanova *et al.*, 2007). Thus a feed-forward regulatory mechanism may allow ACS7 protein to accumulate through decreased expression of *XBAT32*.

Loss of lysine residues within the C-terminal tail of ACS4 and ACS7 unexpectedly increased the proteasomal-mediated degradation of these proteins, suggesting that these lysines are not target sites for ubiquitin conjugation. Replacement of lysine with arginine is a common directed mutation because both are basic amino acids and are similar in structure. The basic amino acids within the C-terminal tail of ACS6 were found to influence the stability of the enzyme (Joo *et al.*, 2008). Substituting the lysine residues for acidic asparagines stabilized ACS6 without the need for phosphorylation, suggesting that the presence of basic amino acids somehow destabilized the protein (Joo *et al.*, 2008). Conversely, substituting the lysine residues for neutral alanines was found to destabilize ACS6 (Joo *et al.*, 2008). In the case of ACS4 and ACS7, substitution of lysine for the more strongly basic arginine may have a greater negative impact on the stability of the enzymes. Collectively, it seems that charges within the C-terminal tail may influence the ability of the UPS to efficiently recognize and target the ACSs for degradation.

The lack of regulatory sequences within the C-terminal tail of ACS7 has also raised questions as to whether ACS7 can rapidly respond to abiotic or biotic stress. ACS7 is an important contributor to ethylene production, as two recent studies have documented a reduced production of ethylene in *acs7-1* seedlings (Tsuchisaka *et al.*, 2009; Dong *et al.*, 2011). Dong *et al.* (2011) also demonstrated that *ACS7* mutants are actually more tolerant of salt stress compared with wild-type. This phenotype is consistent with the observation that *xbat32-1* plants are hypersensitive to salt stress (Prasad and Stone, 2010). This suggests that XBAT32 regulation of ACS7 protein levels is important for tolerance of salt stress.

ACS4 is a type-II ACS and contains the TOE motif required for interaction with the BTB E3 ligases ETO1 and EOL1. XBAT32 would then be an additional E3 ligase that contributes to the turnover of ACS4. It is not uncommon for multiple E3 ligases to ubiquitinate the same target. In mammalian systems many E3s promote the ubiquitination of the DNA damage checkpoint transcription factor p53 (Benkirane *et al.*, 2010). In Arabidopsis, both RING-type E3 ligase KEG and DWA1/2 contribute to ubiquitin-mediated degradation of ABSCISIC ACID INSENSITIVE 5 (ABI5) (Stone *et al.*, 2006; Lee *et al.*, 2010; Liu and Stone, 2010). Multiple E3 ligases that target the same substrate probably do so under different environmental conditions or at different stages of development. While the expression pattern varies between *ETO1* and *EOL1/2*, all three have a broad expression pattern (Christians *et al.*, 2009). In contrast, the expression of *XBAT32* is restricted to the roots, leaves and flowers and is not found in stem or siliques (Nodzon *et al.*, 2004). ETO1, EOL1/2 and XBAT32 may then function to turn over ACS4 under specific developmental conditions or in specific tissues. Collectively, our results demonstrate that ACS7, a type-III ACS, is subject to degradation by the UPS and that XBAT32 modulates the abundance of both ACS4 and ACS7.

Experimental Procedures

Plant materials and growth conditions

Seeds were surface-sterilized with 50% (v/v) bleach and 0.1% Triton X-100. Sterilized seeds were plated on half-strength Murashige and Skoog (MS) medium containing 0.8% agar and 1% sucrose. Seeds were stratified at 4°C for 2–3 days and then germinated under continuous light at 22°C. Seven-day-old seedlings were transferred from MS to soil and grown under photoperiodic cycles of 16 h light and 8 h dark at 22°C in a growth chamber.

Identification of T-DNA insertional plants

An *xbat32-1* (Salk_015002) T-DNA insertional line that harbors a T-DNA insert in At5g57740 was obtained from the Arabidopsis Biological Resource Center (ABRC; Alonso *et al.*, 2003). *xbat32-1* has been previously described by Nodzon *et al.* (2004) and Prasad *et al.* (2010).

Plant transformation

ACS7 cDNA was obtained from the ABRC and was introduced via Gateway (Invitrogen, http://www.invitrogen.com/) into the pEarleyGate 201 plant transformation vector (Earley *et al.*, 2006). 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 wild-type Colombia-0 (Col-0) and *xbat32-1* plants generating *35S:HA-ACS7*/Col-0, and *35S:HA-ACS7*/*xbat32-1*, respectively. T_1 transgenic plants were selected by growing seedlings on MS supplemented with 50 μM DL-phosphinothricin (Sigma-Aldrich, http://www.sigmaaldrich.com/). Herbicide-resistant plants were transferred to soil and presence of the transgene was confirmed by polymerase chain reaction (PCR). Homozygous transgenic plants were selected from the T_3 generation based on resistance to DL-phosphinothricin and used for all assays.

Sample preparation and immunoblot analysis

To determine the expression levels of HA-ACS7 in *35S:HA-ACS7*/Col-0 lines (L1 and L2) and *35S:HA-ACS7*/*xbat32-1* lines (L1 and L2) protein was extracted from 8-day-old seedlings. To compare the degradation of ACS7 in wild-type versus *xbat32-1* *in planta* degradation assays were modified from Joo *et al.* (2008). Twelve-day-old *35S:HA-ACS7*/Col-0 and *35S:HA-ACS7*/*xbat32-1* seedlings grown in liquid MS media were treated for 16 h with the proteasome inhibitor MG132 (30 μM) (Boston Biochem, http://www.bostonbiochem.com/). MG132 was removed by washing seedlings with liquid MS before incubation with liquid MS medium containing cycloheximide (1 mM) (Bioshop, http://www.bioshopcanada.com/). Time zero was collected immediately after the addition of cycloheximide and tissue was snap frozen in liquid nitrogen. Other samples were collected at the indicated time points. To assess the effect of MG132 on protein abundance, 12-day-old *35S:HA-ACS7*/Col-0 and *35S:HA-ACS7*/*xbat32-1* seedlings grown in liquid MS media were treated for 16 h with or without MG132 (30 μM). Dimethyl sulfoxide (DMSO; the solvent for MG132) was used as a control. Following treatment, samples were collected and snap frozen in liquid nitrogen.

Total protein was extracted from samples using 20 mM (TRIS)-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5% glycerol, and protease inhibitor cocktail tablets (Roche Diagnostics, http://www.roche.com/diagnostics/). The concentration of protein extract

was determined using the Bradford reagent (Sigma-Aldrich). Samples were separated on 7.5% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. Blots were blocked with 5% non-fat milk powder in TBST (50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) at room temperature (25 degrees) before membranes were incubated with mouse anti-HA (Sigma-Aldrich) at 1:5000 dilution for 1 h. After three 10-min washes with TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG at 1:5000 dilution for 1 h (Sigma-Aldrich). Washed membranes were visualized using an enhanced Lumi-Light Western Blotting Substrate kit (Thermo Scientific,http://www.thermoscientific.com/) following the manufacturer's instructions. Ponceau S staining was used to confirm equal protein loading.

Cell-free degradation assays

ACS4 and ACS7 cDNAs obtained from ABRC and were introduced via Gateway into the modified pDEST527 (a FLAG tag was inserted into the vector to create a Flag-His tag) destination vector to obtain Flag-His tagged recombinant proteins. pDEST527 was obtained from Addgene (plasmid 11518 donated by Dominic Esposito, National Cancer Institute). Lysine-435 of ACS7 and Lys424, -427, -452, and -453 of ACS4 was changed to arginine to generate ACS7(K435R) and ACS4(RR-RR) using a Phusion site-directed mutagenesis kit (New England Biolabs, http://www.neb.ca) and the following primers:

ACS7(K435): 5′-GTTCGAGGAGGAGGACGATGTCAA-3′ and
5′-CGTTTAGTCTTTCGCTTTTACTCTTTTGGC-3′;
ACS4 (K424, K427): 5′-ATTGCGTTGAGGAGAATACATGAGTT-3′ and
5′-CTCCAAAGTGTTCTCACTCATATTAGCAAAAC-3′;
ACS4(K452, K453): 5′-TTGCTTTAAGGAGATTGAGGATGTTGGT-3′ and
5′-GCTTTAATGTCTCATCAATCATGTTCGC-3′.

Recombinant proteins were expressed in *Escherichia coli* strain Rosetta (DE3) and purified using nickel-charged resin (Bio-Rad,http://www.bio-rad.com/) according to the manufacturer's protocols.

Plant extract was obtained from 8-day-old Col-0 or *xbat32-1* seedlings using the extraction buffer [25 mM TRIS-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl$_2$, 4 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM DTT, and 10 mM ATP] described by Wang

et al. (2009). Seedlings were snap frozen in liquid nitrogen and then ground in extraction buffer. Cell debris was removed by two 10-min centrifugation at 20 000 *g* at 4°C. The supernatant was collected and protein concentration was determined by the Bradford assay. Total protein concentration was adjusted using extraction buffer. For proteasome inhibitor treatments, 40 μM MG132 was added to protein extracts for a 20-min pre-treatment period. Approximately 150 ng of recombinant His-Flag-ACS4 or His-Flag-ACS7 protein was incubated in 200 μl of plant extracts (containing 1 mg total protein) at 30°C. Samples were taken at indicated intervals and abundances of His-Flag-ACS4 or His-Flag-ACS7 were determined by immunoblots using anti-His antibodies (Sigma-Aldrich) followed by horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich). Quantification of band intensity was done using ImageJ software (Abramoff *et al.*, 2004). In brief, images were converted to binary and then the analyse particle function was used to outline each band and measure its size.

Growth assays

For the primary root growth assay *35S:HA-ACS7*/Col-0, *35S:HA-ACS7*/*xbat32-1*, *xbat32-1* and Col-0 seeds were surface-sterilized, plated on half-strength MS and stratified for 3 days at 4°C. Seeds were then germinated and grown vertically for 9 days in continuous light at 22°C. Root length was quantified using ImageJ software. For dark-grown assays, surface-sterilized seeds were plated on half-strength MS medium with or without various concentrations of AVG (Sigma-Aldrich) and then stratified for 3 days in the dark at 4°C. Seeds were then transferred to the dark for 3 days at 22°C. The angle of hook curvature was measured using the ImageJ software angle tool. All assays were carried out at least twice, with three replicates per trial.

Supplementary Material
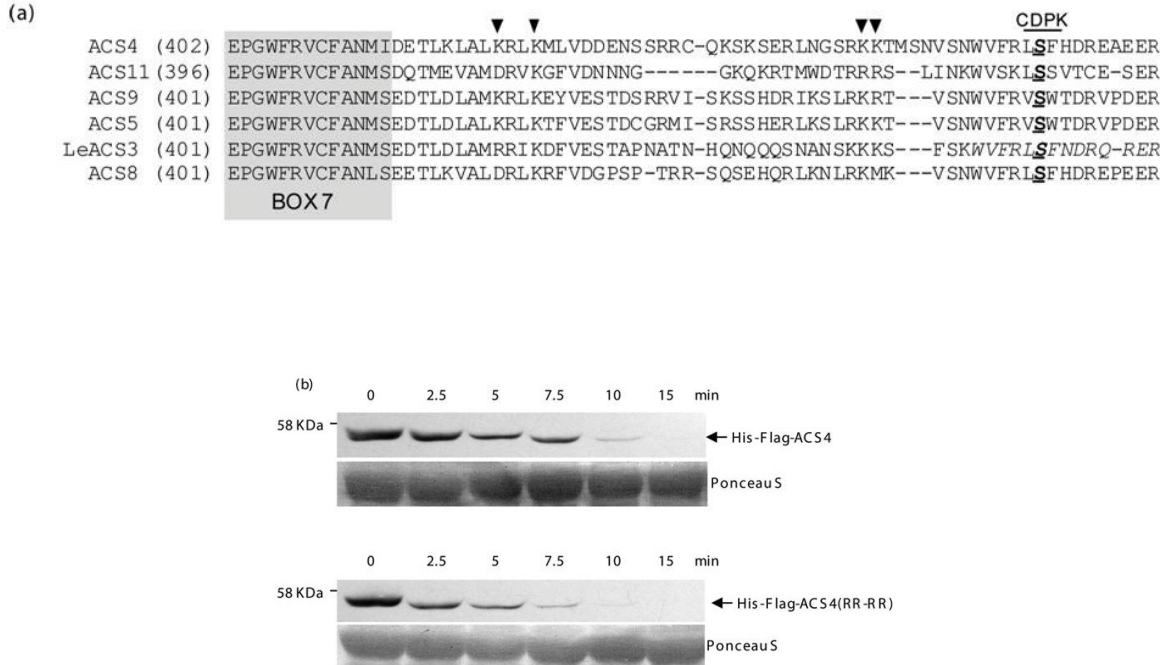
Figure 9. Basic amino acids in ACS4 C-terminal extension influence proteolysis of the enzyme. (a). Alignment of the C-terminal region of *Arabidopsis thaliana* and *Solanum lycopersicum* (Le; tomato) type-II ACS enzymes. A portion of the conserved catalytic domain (BOX 7) is included in the alignment as a reference. The calcium-dependent protein kinase (CDPK) phosphorylation site is in bold and underlined. The ETO1 interaction domain (TOE sequence) of tomato type-II ACS, LeACS3, is italicized. Arrows indicate ACS4 lysines (K) residues (424, 427, 452, and 453) that were mutated to arginine (R) to generate ACS4(RR-RR). (b). Cell-free degradation assay. His-Flag-ACS4 and His-Flag-ACS4(RR-RR) proteins were incubated with plant extracts from 8-day-old wild-type seedlings. The level of His-Flag-ACS4 (top panel) and His-Flag-ACS4(RR-RR) (bottom panel) remaining at the indicated time points were determined by western blot

Acknowledgements

CHAPTER 3:

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

Contributions:
WJL: Participated in experimental design and performed assays shown in Figure 10,12,13, 14 and 15. Wrote the manuscript and participated in revisions.
H.L: Participated in experimental design and performed assays seen in Figure 11 and 16.
A.S: Contributed to yeast two hybrid screen.
A. M-H. Contributed to data in Figure 15D.
SLS. Participated in design experimental design and participated in revisions.

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.

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.

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 tricocarpa* (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 abi5-1* 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 sucrose 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 sucrose 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) (Figure 10A). 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.*, 2002*a*). 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.*, 2002*b*; 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 8h of light and 16h 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 CIPK26N cDNA to generate CIPK26N^{TD} and CIPK26N^{KR} (Gong *et al.*, 2002*a*,*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 chimeric transcription factor XVE. Using the *Xho*I 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 16h with or without the proteasome inhibitors 50 μM Z-Leu-Leu-Leu-CHO (MG132), and 50 μM *N*-[(phenylmethoxy)carbonyl]-L-isoleucyl-L-α-glutamyl-tert-butyl ester-*N*-[(1S)-1-formyl-3-methylbutyl]-L-alaninamide (PSI; Boston Biochem). GFP-trap beads (20 μl; Chromotek) were used according to the manufacturer's instructions to immunoprecipitate GFP-CIPK26 from protein extracts (approximately 300 μl at 5 μg μl^{-1}) prepared from *35S:GFP-CIPK26* seedlings treated for 16h 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 pDEST527 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 *35S:GFP-CIPK26 OlexA:HA-KEG* seedlings were grown for 24h in liquid medium and then treated overnight with ethanol (solvent, control) or with 20 µM 17-β-estradiol. Seedlings were then treated with 1mM 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 pEarleyagte201-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 pEarlygate201-YN and pEarlygate202-YC vectors. Leaves were assayed for fluorescence 48h 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 pDEST527 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 days and then placed at 22 °C with continuous light for 36h. 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-ABI5 were incubated at 30 °C for 30min in 30 μl kinase assay buffer (20mM Tris/HCl, pH 7.5, 50mM NaCl, 1mM dithiothreitol, 0.1% Triton X-100, and 10 μCi [γ-^{33}P]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 3h was separated on an SDS-polyacrylamide gel embedded with 0.1mg 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 514nm, and emissions were collected between 530 and 600nm. For GFP fluorescence, an excitation wavelength of 488nm was used and emissions were collected between 475 and 525nm. 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 HERC2-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 (Figure 10A). 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 (Figure 10A, 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) (Figure 10A). 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 (Figure 10C; top left panel), while GST-RK was not able to pull down His-Flag-CIPK26C (Figure 10C; 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 (Figure 17 Supplementary Figures). CIPK26 was most closely related to CIPK3, CIPK9, and CIPK23. Using microarray expression data available via Genevestigator©, *CIPK26* was found to be widely expressed with its highest expression in seedlings (Figure 18 Supplementary Figures). Similar to other CIPK family members (Batistic *et al.*, 2010), CIPK26 was localized to the nucleus and cytoplasm (Figure 11A, 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 (Figure 11A). A similar subcellular localization pattern was observed in stably transformed *Arabidopsis* seedlings overexpressing GFP-tagged CIPK26 (GFP-CIPK26) (Figure 11B).
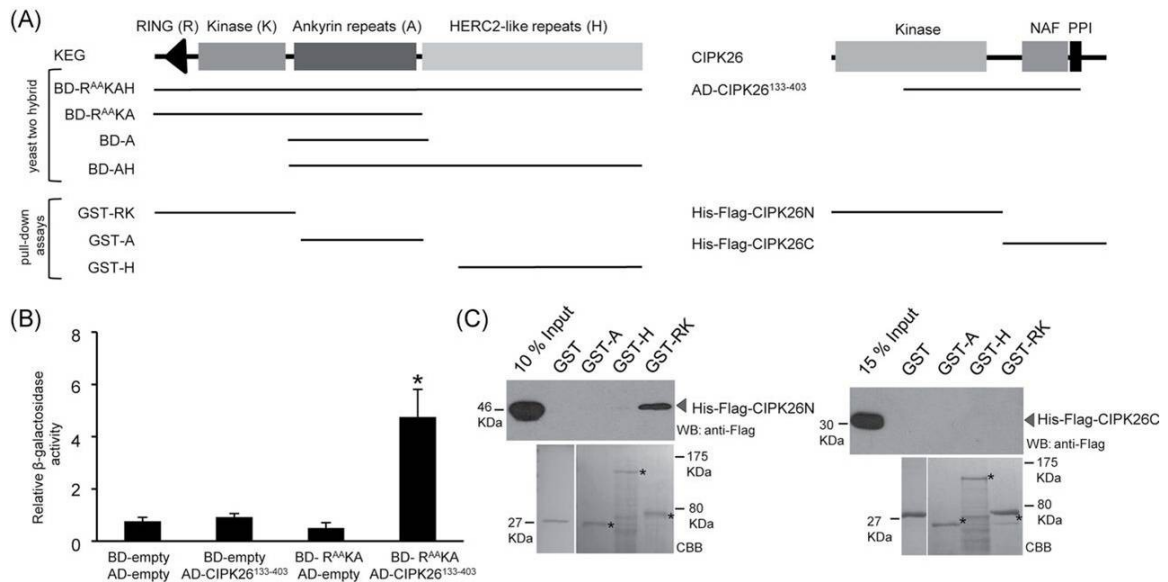
Figure 10. CIPK26 interacts with KEG *in vitro*. (A) Schematic representation of KEG and CIPK26 deletion constructs used in yeast two-hybrid screens and GST pull-down assays. A schematic of the partial segment of CIPK26 identified via the yeast two-hybrid screen is also indicated. (B) Interaction between BD-R^{AA}KA and AD-CIPK26$^{133–403}$ indicated by β-galactosidase activity of soluble extracts prepared from yeast colonies. β-Galactosidase assays were done in triplicate. Results are shown as means ±standard error. β-Galactosidase activity from yeast transformed with BD-R^{AA}KA and AD-CIPK26$^{133–403}$ constructs was significantly different from all other combinations (*, $P < 0.05$). Statistical analysis was performed using Student's *t*-test. (C) GST pull-down assays using bead-bound GST, GST-RK, GST-A or GST-H and bacterial cell lysates containing His-Flag-CIPK26N or His-Flag-CIPK26C. GST-RK was able to pull down His-Flag-CIPK26N (upper left panel) and not His-Flag-CIPK26C (upper right panel) as detected by Western blot (WB) analysis with Flag antibodies. Coomassie Brilliant Blue (CBB) staining was used to visualize GST and the GST fusion proteins used in each assay (bottom panels; * indicates the relevant band). The blots shown are representative of two independent trials.

(A) 35S:CIPK26-YFP (Tobacco)
Fluorescence   Merged
100 µm   100 µm

(B) 35S:GFP-CIPK26 (Arabidopsis)
Fluorescence   Merged
20 µm   20 µm

(C) 35S:CIPK26 -YFP^N / 35S:KEG-YFP^C
Fluorescence   Merged
100 µm   100 µm
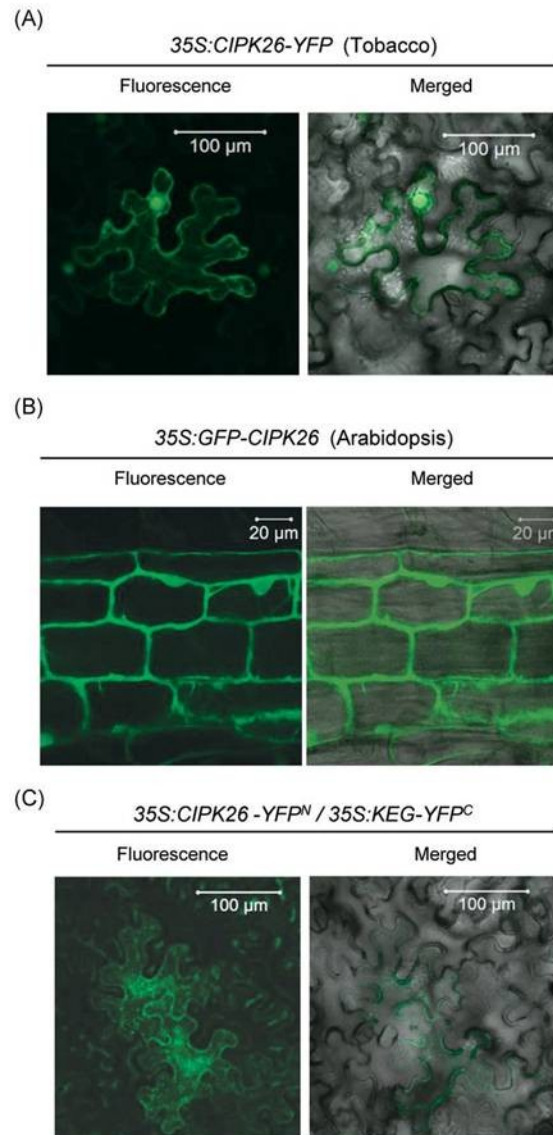
Figure 11. CIPK26 interacts with KEG *in planta*. (A) Localization of CIPK26–YFP in transiently transformed *N. benthamiana* epidermal cells. (B) Localization of CIPK26 in 4-d-old *35S:GFP-CIPK26* transgenic *Arabidopsis* seedlings. (C) BiFC analysis in *N. benthamiana* leaf epidermal cells expressing full-length CIPK26 and KEG fused to the amino (YFP^N) or carboxyl (YFP^C) portions of YFP, respectively. Fluorescence indicates interaction between CIPK26-YFP^N and KEG-YFP^C. Left panels: fluorescent image from a single optical section; right panels: transmitted light images merged with fluorescence image from a single optical section.

For BiFC analysis, tobacco leaf epidermal cells were transiently co-transformed with *35S:CIPK26-YFP N* and *35S:KEG-YFP C* to express CIPK26 fused to the amino-terminal portion of YFP (YFP^N) and KEG fused to the carboxyl-terminal portion of YFP (YFP^C). A strong fluorescence signal was observed in cells co-expressing CIPK26-YFP^N and KEG-YFP^C (Figure 11C), 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-YFP N* or *35S:KEG-YFP C* 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 (Figure 12). In the absence of MG132, the levels of His-Flag-CIPK26N gradually decreased over time (Figure 12, 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 (Figure 12, 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 80kDa, the expected size of GFP-CIPK26, and a larger migrating form (Figure 13A, upper panels). These proteins were not detected in extracts prepared from wild-type and control (transformed with empty vector) plants (Figure 13A, 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
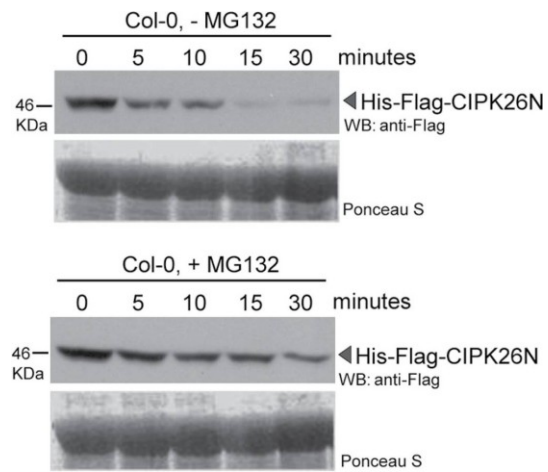
Figure 12. CIPK26 is turned over by the 26S proteasome *in vitro*. Cell-free degradation assay using recombinant His-Flag-CIPK26N. His-Flag-CIPK26N was incubated with protein extracts from 8-d-old Col-0 seedlings with (+) or without (−) 50 μM MG132.
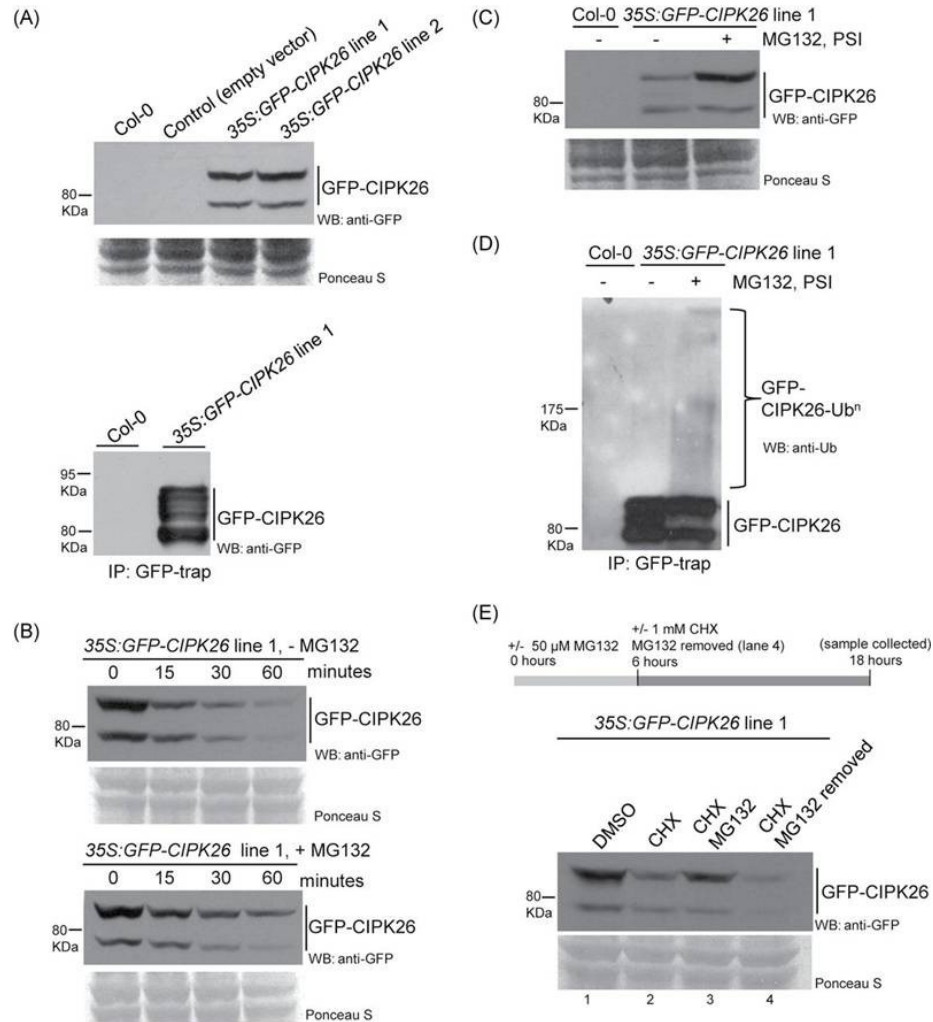
Figure 13. CIPK26 is ubiquitinated and turned over by the 26S proteasome *in planta*. (A) Upper panel: protein expression levels of GFP-CIPK26 in two independently generated *35S:GFP-CIPK26* transgenic plant lines. Middle panel: ponceau S staining shows protein loading. Bottom panel: immunoprecipitation (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) CIPK26 is ubiquitinated *in planta*. GFP-trap immunoprecipitated (IP) GFP-CIPK26 from 4-d-old *35S:GFP-CIPK26* (line 1) seedlings treated with (+) or without (–) 50 μM MG132 and PSI were subject to WB analysis with ubiquitin (Ub) antibodies. High-molecular-weight smearing observed with ubiquitin antibodies after proteasome inhibitor treatment indicated ubiquitinated forms of GFP-CIPK26. (E) CHX treatment reduces the accumulation of CIPK26. Four-d-old *35S:GFP-CIPK26* (line 1) seedlings were treated with DMSO (lane 1), 1mM CHX (lane 2), 50 μM MG132 and 1mM CHX (lane 3) or 50 μM MG132 prior to addition of 1mM CHX (lane 4). All blots are representative of at least two trials. Ponceau S staining shows the protein loading.

molecular weight (Figure 13A, lower panel). These results suggested that CIPK26 is modified *in planta* and this modification(s) resulted in a range of migrating species. To 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 (Figure 19 Supplementary Figures left panel) and phosphothreonine (P-Thr) antibodies (Figure 19 Supplementary Figures 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 (Figure 13B, top panel; Figure 19 Supplemental Figures). The decrease in GFP-CIPK26 levels was significantly slower in protein extracts prepared from *35S:GFP-CIPK26* (line 1) seedlings pre-treated with MG132 (Figure 13B, 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 (Figure 13C).

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 treated 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 (Figure 13D).

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 (Figure 13E, 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 (Figure 13E, lane 3). GFP-CIPK26 protein levels were also significantly reduced when MG132 was removed just prior to incubation of seedlings with CHX (Figure 13E, 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 (Figure 14, 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 (Figure 14, 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 14, 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.*, 2002*b*; 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
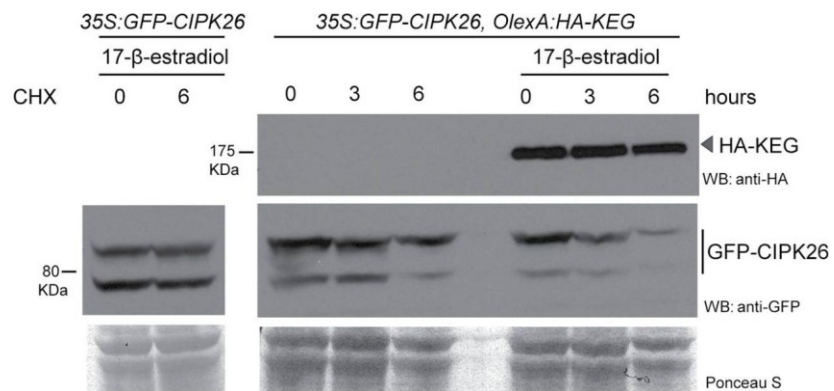
Figure 14. KEG is involved in the turnover of CIPK26. Four-d-old *35S:GFP-CIPK26 OlexA:HA-KEG* double-transgenic seedlings were treated overnight with or without estradiol. CHX was then added to the growth medium and the protein abundance of HA-KEG (top panel) and GFP-CIPK26 (middle panel) were assessed at the indicated time points. As a control, *35S:GFP-CIPK26* lacking the KEG transgene was subjected to estradiol treatment followed by CHX treatment. Ponceau S shows the protein loading. All blots are representative of a least two trials.

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 (Figure 15A). The interaction between CIPK26 and ABI1 or ABI2 was verified *in planta* using BiFC assays. Co-expression of ABI1-YFP^N with CIPK26-YFP^C (Figure 15B) in tobacco leaf epidermal cells resulted in a fluorescent signal in the nucleus and cytoplasm, further suggesting an interaction between ABI1 and CIPK26. Similarly, co-expression of ABI2-YFP^C with CIPK26-YFP^N (Figure 15C) in tobacco leaf epidermal cells resulted in a fluorescent signal in the nucleus and cytoplasm. *Agrobacterium*-mediated transformation of each construct alone did not produce a fluorescent signal (data not shown). Together, these results provided strong evidence for CIPK26 interaction with the ABA-related phosphatases, ABI1 and ABI2.

The interaction between CIPK26 and ABI1/ABI2 suggested a role for the kinase in ABA signalling. To determine whether CIPK26 is involved ABA signalling, we analysed the ABA response of two independent T-DNA insertional mutant lines, *cipk26-1* (Salk_005859) and *cipk26-2* (Salk_074944), which were confirmed to lack expression of the *CIPK26* transcript (Figure 20 Supplementary Figures). No obvious phenotypic differences were observed between *cipk26-1* or *cipk26-2* and wild-type plants grown under standard growth conditions (data not shown). The germination rate of *cipk26-1* and *cipk26-2* seeds in the presence of ABA was compared with that of the wild type. The germination rate of both *cipk26-1* and *cipk26-2* in the presence and absence of ABA was similar to that of the wild type (Figure 20C Supplementary Figures). This finding was not unexpected because *CIPK26* is part of a large gene family and may function redundantly with other closely related family members. To overcome the issue of redundancy, we analysed the response of *35S:GFP-CIPK26* (lines 1 and 2) to ABA-induced inhibition of germination (Figure 15D). Germination of *35S:GFP-CIPK26* (lines 1 and 2) seeds were significantly reduced in the presence of 0.25 and 0.5 μM ABA compared with wild-type and control plants (Figure 15D). These results suggested that the kinase functions as a positive regulator of plant responses to ABA.
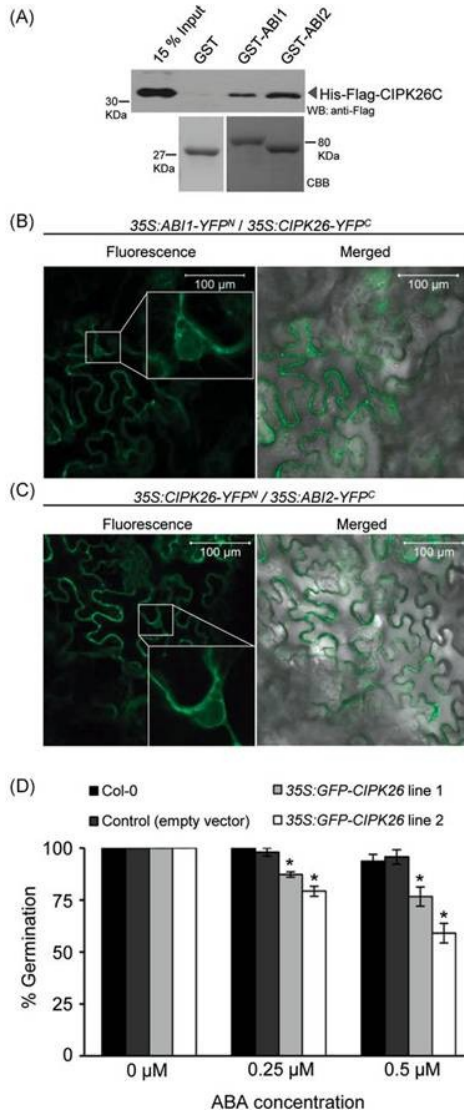
Figure 15. CIPK26 interacts with ABI1 and ABI2 and alters ABA sensitivity. (A) GST pull-down assays using bead-bound GST, GST-ABI1 or GST-ABI2 and bacterial cell lysates containing His-Flag-CIPK26C. GST-ABI1 and GST-ABI2 were able to pull down His-Flag-CIPK26C (upper panel) as detected by Western blotting (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^N) or carboxyl (YFP^C) 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 $n \geq 30$ seeds per trial. Results are shown as means ± standard error.

CIPK26 is a functional kinase that interacts with and phosphorylates ABI5

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 (Figure 16A) and BiFC (Figure 16B) assays. For BiFC, co-expression of *35S:CIPK26-YFP N* and *35S:ABI5-YFP C* resulted in a strong fluorescent signal in the nucleus and a weaker fluorescent signal in the cytoplasm of some cells (Figure 16B). *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 *in vitro* (Figure 16C). An inactive version of the kinase, His-Flag-CIPK26N^{KR}, was unable to phosphorylate His-Flag-ABI5 (Figure 16C).

The severe growth-arrest phenotype observed for *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 *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 *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 *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 *keg-1* was more similar to that of ABA-treated than that of untreated wild-type seedlings (Figure 16D). Of note was the observation that a kinase of the same molecular weight as CIPK26, approximately 50kDa, phosphorylated ABI5 in *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 ABI5 for ubiquitin-mediated degradation (Stone *et al.*, 2006; Liu and Stone, 2010). However, the importance of this E3 ligase is not limited
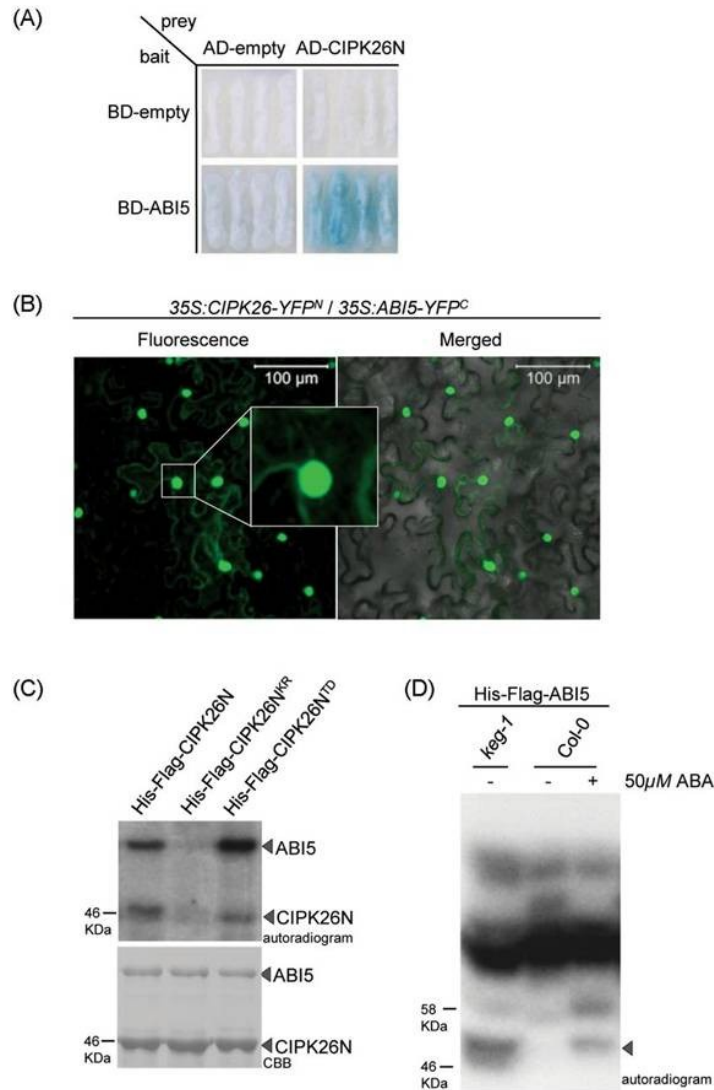
Figure 16. CIPK26 interacts with ABI5 and phosphorylates ABI5 *in vitro*. (A) ABI5 interacts with CIPK26N in yeast two-hybrid screens. ABI5 was fused to a LexA DNA-binding domain (BD) and tested for interaction with CIPK26N fused to the activation domain (AD). (B) BiFC analysis in *N. benthamiana* leaf epidermal cells co-expressing CIPK26 and ABI5 fused to the amino (YFP^N) or carboxyl (YFP^C) portions of YFP, respectively. Right panel: transmitted light images merged with fluorescence images from a single optical section. An enlargement of the nucleus is shown in the inset. (C) *In vitro* phosphorylation assays using His-Flag-ABI5 and His-Flag-CIPK26N, inactive His-Flag-CIPK26N^{KR}, or constitutively active His-CIPK26N^{TD}. Top panel: autoradiogram showing phosphorylation of ABI5 by CIPK26N and CIPK26N^{TD}. Bottom panel: Coomassie Brilliant Blue (CBB) staining showing the amounts of purified protein used in the assay. (D) In-gel kinase assays using His-Flag-ABI5 as the cast substrate. Protein extracts were prepared from 8-d-old *keg-1*, ABA (50 µM)-treated and untreated Col-0 seedlings. The autoradiogram shows phosphorylation of ABI5 by various kinases in each protein extract. The arrowhead indicates phosphorylation of ABI5 by an approximately 50kDa kinase in *keg-1* and ABA-treated Col-0 seedlings.

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 Ca^{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 *et al.*, 2007). 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 overexpressing *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 m*ay 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 demonstrated 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.
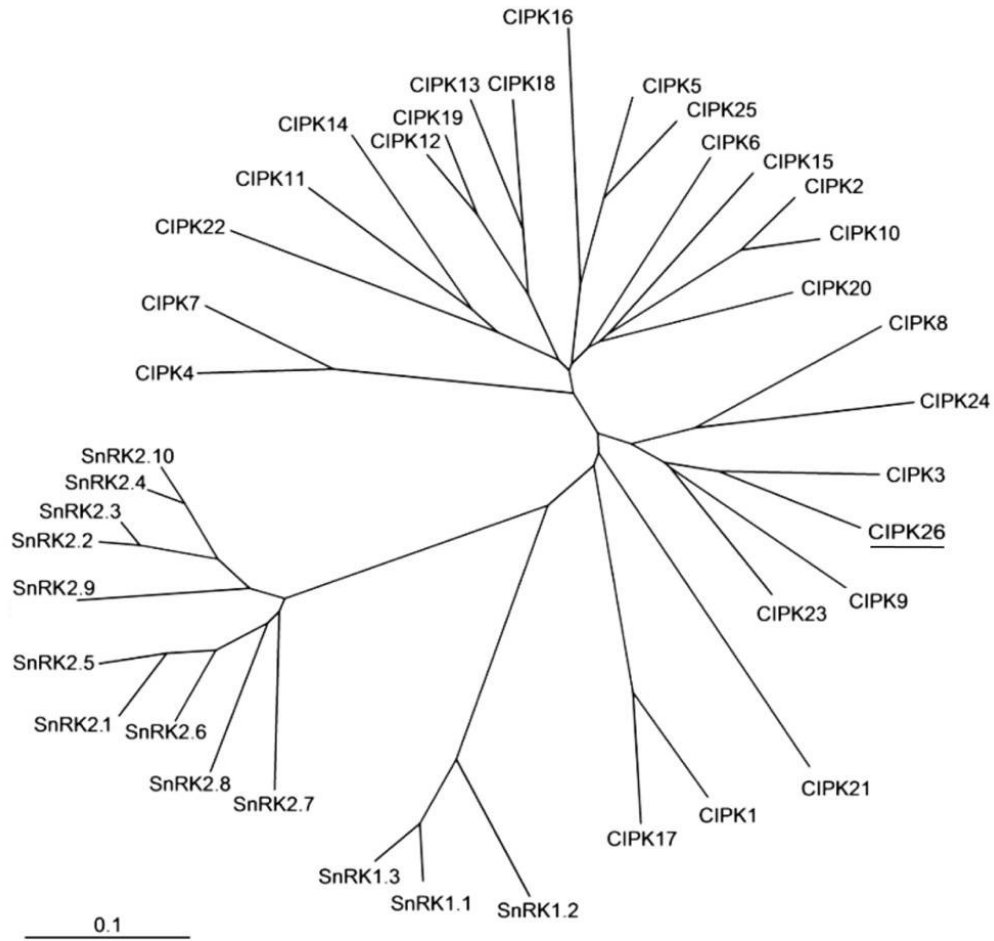
Figure 17. Sequence similarity tree of CIPK26 and related family members including SnRK2s and SnRK1s.
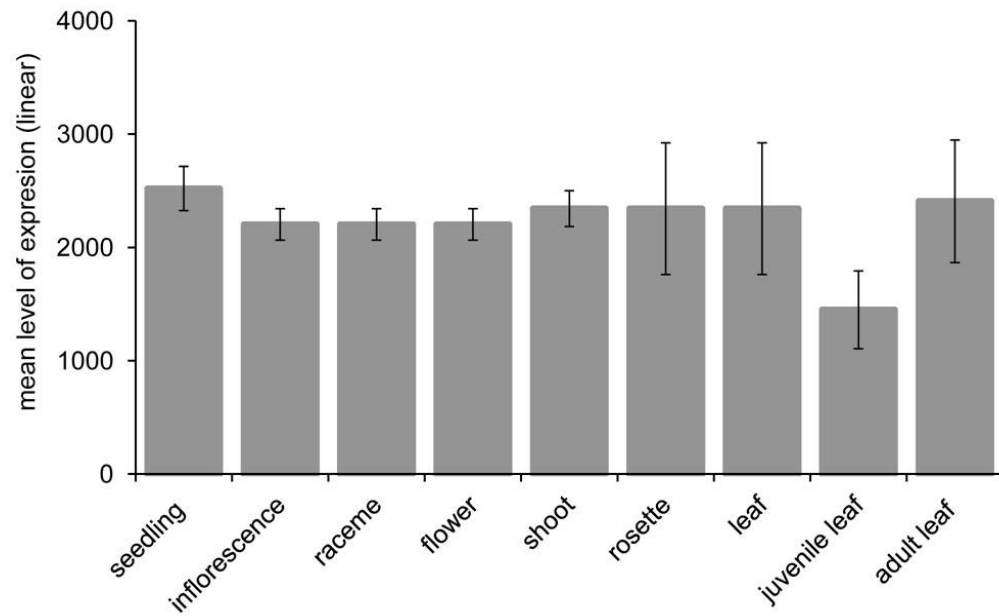
Figure 18. Tissue expression profile of *CIPK26*. Mean values were collected from Genevestigator© on an Agronomics whole genome tiling array. Results are shown as means ±standard error.
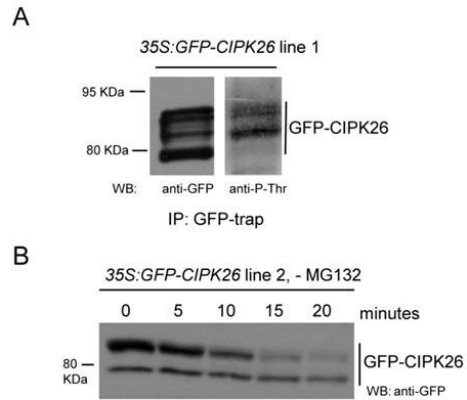
Figure 19. Phosphorylation of CIPK26 and cell-free degradation assay. (A) Phosphorylation of CIPK26 in *CIPK26*-overexpressing seedlings. Protein extracts prepared from 6-d-old*35S:GFP-CIPK26* (line 1) seedlings were incubated with GFP-trap beads. GFP-trap-isolated proteins were split equally between 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.
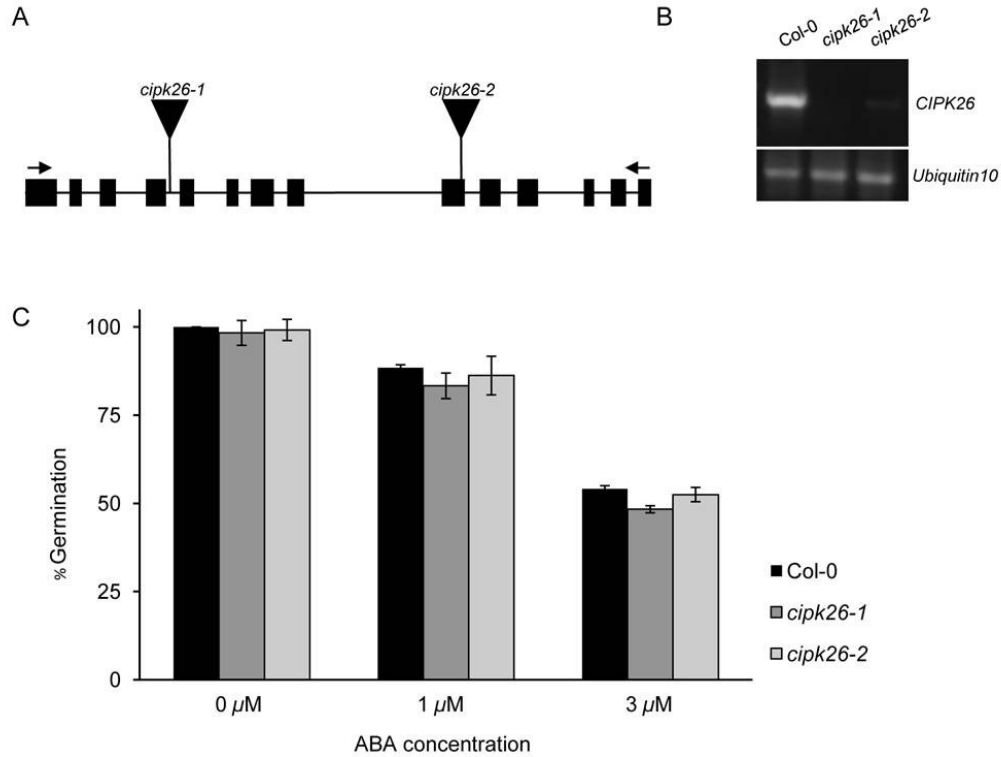
Figure 20. Analysis of *CIPK26* knockout lines. *cipk26* T-DNA lines do not show altered sensitivity to the effects of ABA on germination. (A) Schematic of *CIPK26* depicting the positions of the T-DNA insertions. Arrows indicate the positions of primers used in RT-PCR. (B) RT-PCR analysis of 8-d-old 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 36h. 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 ($n \geq 30$ seeds per trial). The assay was done in triplicate. Results are shown as means ± standard error.

Supplementary Table

Name	Primers*
Full length *CIPK26*	**F:** *GGGGACAAGTTTGTACAAAAAAGCAGGCTT*CATGAATCGG CCAAAGGTTCAGCGTC **R:***GGGGACCACTTTGTACAAGAAAGCTGGGT*CTTTGCTTAGACCAGAGCTCTCCCA
CIPK26N *(1bp–870bp)*	**F:***GGGGACAAGTTTGTACAAAAAAGCAGGCTT*CATGAATCGGCCAAAG GTTCAGCGTC **R:***GGGGACCACTTTGTACAAGAAAGCTGGGT*CAGCGTCCACATCATCCAAGTTGGC
CIPK26C *(805bp–317bp)*	**F:***GGGGACAAGTTTGTACAAAAAAGCAGGCTT*CAAGAAAAATTACAAGCCAGCTGTTTTCG **R:***GGGGACCACTTTGTACAAGAAAGCTGGGT*CTTTGCTTAGACCAGAGCTCTCGCCA
CIPK26N[TD] (T172)	**F:**TGGTCTTCTTCACGATGCATGCGGAACT **R:**RTCACCCCTGACTTGTCGGGACAACG
CIPK26N[KR] (K42)	**F:**ACGCGTGGCTCTCCGTATACTTGATAAGGA **R:**TCTCCTGTCTCAGTATTAACAGCACACCGA
cipk26-1	**F:**AGCATAAGATGGCTGAACAGG **R:**CAGGAGCAGCATAGTTTGGAG
cipk26-2	**F:**ACCGTAACGAAGTGAGCAATG **R:**TTCTTATTCTGGGTTTTGGGG
UBQ10	**F:** TTACATGAAACTAAACATTGAACTTC **R:**TCAATTCTCTCTACCGTGATCAAG
ABI1	**F:***GGGGACAAGTTTGTACAAA AAAGCAGGCTT*CATGGAGGAAG TATCTCCGGCGAT **R:***GGGGACCACTTTGTACAAGAAAGCTGGGT*CGTTCAAGGGTTTGCTCTTGAGTTTCC
ABI2	**F:***GGGGACAAGTTTGTACAAA AAAGCAGGCTT*CATGGACGAAG TTTCTCCTGCAG **R:***GGGGACCACTTTGTACAAGAAAGCTGGGT*CATTCAAGGATTTGCTCTTGAATTT
35S promoter	**F:***GGGGACAAGTTTGTACAAAAAAGCAGGCTA*TCTCAGAATTCCAATCCCAC **R:***GGGGACCACTTTGTACAAGAAAGCTGGGT*CATCCCTTACGTCAGTGGAG
pEarlyGate201	**F:**ACACGCTCGAGTATAAGAGC **R:**ATTCGGCGTTAATTCAGTAC

* italicized nucleotides indicate AttB sequences for Gateway cloning

Table 1. List of the primers used in this study.

Acknowledgements

CHAPTER 4:

The kinase activity of CIPK26 influences its own stability and that of its E3 ligase, KEG.

Wendy J Lyzenga, Hongxia Liu and Sophia L. Stone.

Contributions:

WJL: Participated in experimental design and performed all assays except for Figure 23 which was done in collaboration with HL and except for preparation of sample for mass spectrometry analysis. Wrote the manuscript and participated in revisions.

HL: Collaborated with WJL on the assay displayed in Figure 23 and prepared sample for mass spectrometry analysis.

SLS: Participated in experimental design. Searched phosphorylation sites from Protein Phosphorylation Database. Revised the manuscript.

Abstract

Ubiquitination is a common and essential post-translational modification found in eukaryotic cells. E3 ubiquitin ligases facilitate the covalent attachment of ubiquitin onto select target proteins. In plant biology, E3 ligases mediate normal growth and development and facilitate hormone signalling. Keep on Going (KEG) is a large multidomain E3 ligase that is essential for growth of Arabidopsis seedlings. *KEG* mutants arrest growth after germination highlighting the importance of this E3 ligase during early seedling establishment. Previous work has shown that KEG functions to negatively regulate abscisic acid (ABA) signalling by targeting ABA-responsive transcription factors ABSCISIC ACID INSENSITIVE 5 (ABI5), ABRE Binding Factors 1 (ABF1), and ABF3 for degradation. However, in the presence of ABA, KEG is subject to proteasomal degradation as a form of negative regulation. Here we show that a constitutively active version of CIPK26 is more stable than wild type and a kinase dead version. We show that CIPK26 can phosphorylate KEG *in vitro* and, using a modified cell-free degradation assay, we provide evidence that CIPK26 promotes the degradation of KEG. In this report we suggest a model wherein active CIPK26 phosphorylates KEG to induce proteasomal degradation of the E3 thereby allowing active CIPK26 to accumulate along with other KEG targets and promote ABA responses.

Introduction

The ubiquitin proteasome system (UPS) is responsible for the specific degradation of cellular proteins and regulates a wide range of cellular processes. Three enzymes, the E1 (ubiquitin-activating enzyme, UBA), E2 (ubiquitin-conjugating enzyme, UBC) and E3 (ubiquitin ligase), facilitate ubiquitination in a stepwise fashion. The E1 first activates ubiquitin with ATP and then transfers the activated ubiquitin onto the E2. The E3 ligase confers specificity to the process by directly selecting targets for ubiquitination and binding to the E2. The E2 and E3 coordinate transfer of ubiquitin onto the target. The diverse and large families of E3 ligases across species highlight the importance of this UPS component to eukaryotic biology (Weissman *et al*., 2011). Almost all aspects of plant growth and development are regulated through the UPS in some manner. Proteolytic degradation plays an indispensible role in the regulation of the signal transduction of plant hormones including abscisic acid (Santner and Estelle, 2010)

Keep on Going (KEG) is a RING-type E3 ligase that plays an essential role during plant development. *KEG* knockout lines germinate but fail to develop into adult plants (Stone *et al*., 2006). KEG is a large protein consisting of a RING domain, kinase domain, Ankyrin repeats and HERC2-like repeats. KEG's role during plant development appears to be multifaceted as evidence suggests that KEG regulates intracellular protein trafficking via the endomembrane system and plays a substantial role in the regulation of ABA signalling (Chen *et al*., 2013, Liu and Stone, 2013, Gu and Innes, 2013; Stone *et al*., 2006). The production of ABA is increased during many phases of plant development such as seed maturation and transition from seed to seedling stage. ABA is also produced in response to abiotic stresses such as drought (Himmelbach *et al*., 2003; Weiner *et al*., 2010). ABA acts through a multilayered signalling cascade that induces expression of genes containing ABRE elements in their promoters. In the absence of ABA, KEG negatively regulates the abundance of members of the bZIP subfamily of transcription factors, ABI5, ABF1 and ABF3 (Liu and Stone, 2013; Chen *et al*., 2013; Stone *et al*., 2006). However, in the presence of ABA, KEG inhibits its own activity through self-ubiquitination and subsequent degradation (Liu and Stone, 2010). Loss of either *ABI5*, *ABF1* or *ABF3* in the *keg* background is able to partially rescue the severe growth arrest phenotype of *keg* confirming that mis-regulation of ABA signalling contributes to the *keg*

phenotype (Chen *et al*., 2013; Stone *et al*., 2006). However, lack of a complete rescue suggests that KEG has other targets and additional roles. Previously, we performed a yeast two hybrid screen to identify other KEG interacting proteins and identified CBL-Interacting Protein kinase 26 (CIPK26) (Lyzenga *et al*., 2013).

The CIPK family of kinases are also known as SNF1-related kinase 3s (SnRK3)s and along with SnRK2s and SnRK3s fall under the Calcium dependent protein kinase (CDPK)-SnRK super family of kinases. SnRK2s and SnRK3s are unique to the plant kingdom but their kinase domain shares homology to sucrose non-fermenting-1 kinase (SNF) from yeast and AMP activated protein kinase (AMPK) from animals (Kolukisaoglu *et al*., 2004; Hrabak *et al*., 2003). CIPK/SnRK3s regulate abiotic stress response and facilitate ion homeostasis through regulation of various ion transporters (Yu *et al*., 2014). There is also evidence that CIPK family members play various roles in ABA signalling (Yu *et al*., 2014; Lyzenga *et al.,* 2013).

CIPKs/SnRK3s (referred to as CIPKs from here on) appear to be regulated and activated through a variety of mechanisms. CIPKs contain a self-inhibitory NAF domain and binding of Ca^{2+} sensing CBL proteins to the NAF relieves inhibition. Recent reports also show that phosphorylation of the activation loop by unknown upstream kinases and phosphorylation of CBL proteins by their interacting CIPKs also contribute to full activation of CIPKs (Chaves-Sanjuan *et al*., 2014; Hashimoto *et al*., 2012; Ohta *et al*., 2003). Adding to the complexity, we have also shown that a CIPK (CIPK26) is unstable and regulated through ubiquitin-mediated degradation (Lyzenga *et al*., 2013).

There are many interconnections between phosphorylation and ubiquitination. Phosphorylation of ubiquitination targets can create phosphodegrons which promote recognition by the UPS or prevent ubiquitination (Hunter, 2007). Phosphorylation can also influence the activity of E3 ligases by allosteric inhibition or activation (Hunter, 2007). Many E3s are themselves subject to proteasomal degradation and phosphorylation can promote/inhibit their own degradation (Weissman *et al*., 2011). The negative regulatory proteasomal degradation of KEG is controlled through phosphorylation. In the presence of ABA a phosphorylation event promotes KEG ubiquitination and subsequent degradation (Liu and Stone, 2010). While previous work has demonstrated that CIPK26 is degraded through the 26S proteasome and that KEG contributes to this degradation

(Lyzenga *et al.*, 2013), conditions that influence the stability of CIPK26 have not been investigated. In addition, the upstream signalling mechanism that induce ABA-mediated KEG ubiquitination has not been identified. In this report we demonstrate that the kinase activity of CIPK26 influences its stability; a constitutively active version is stable while a kinase dead version is unstable. We provide evidence that the kinase activity of CIPK26 promotes KEG degradation through the 26S proteasome and suggest a model where an activated CIPK26 is stable because it phosphorylates KEG to promote KEG ubiquitination and degradation.

Materials and Methods

Cloning, Mutagenesis and generation of transgenic plants

Gateway cloning was used to generate all constructs unless indicated (Invitrogen). Generation *CIPK26*, *CIPK26^{TD}* and *CIPK26^{KR}* cDNA are described in Lyzenga *et al.*, (2013). Full length *CIPK26, CIPK26^{TD}* and *CIPK26^{KR}* cDNA were introduced into the pEarleyGate101 gateway plant transformation vector (Earley *et al.*, 2006) or pMDC43 vector (Curtis and Grossniklaus, 2003) creating C-terminal yellow fluorescence protein (YFP) and hemagglutinin (HA) tag (CIPK26-YFP-HA) or N-terminal green fluorescent protein (GFP-CIPK26) respectively. Both vectors use the cauliflower mosaic virus ^{35}S promoter. The resulting plasmids were then transformed into *Agrobacterium tumefaciens* strain GV3101. Transgenic plants were generating in *Arabidopsis thaliana* ecotype Colombia-0 (Col-0) using the floral dip method (Clough and Bent, 1998) and T1 transgenic plants were selected for by growth on MS medium supplemented with basta (Sigma-Aldrich). Herbicide resistant plants were transferred to soil and presence of the transgene was confirmed by PCR and western blot.

To assess the effect of MG132 on protein accumulation four-day-old *35S:CIPK26-YFP-HA, 35S:CIPK26^{TD}-YFP-HA, 35S:CIPK26^{KR}-YFP-HA* transgenic seedling were transferred to liquid MS and treated with 30μM MG132 or DMSO for 16 hours before tissue was snap frozen in liquid nitrogen. 40μg of protein extract from each treatment was subjected to western blot analysis using HA antibodies to detect CIPK26-YFP-HA. Assay was repeated and band intensity as quantified using ImageJ software (Abramoff *et al.*, 2004).

Transient Protein Expression in Nicotiana benthamiana

Nicotiana benthamiana (tobacco) plants were grown and Agrobacterium strain GV3101 was transformed and prepared for transient expression as previously described (Lyzenga *et al.*, 2013). Equal volumes of appropriately transformed Agrobacterium cultures were used for infiltrating tobacco leaves. When comparing protein abundance between two infiltrations, one leaf was injected on either side of the mid-vein and used for comparison. Thirty-two hours after infiltration, tobacco leaves were injected with 50 μM MG132 or DMSO, injected area was excised and was incubated in 50 μM MG132 or DMSO for 16 hours before tissue was snap frozen in liquid nitrogen. Tissue was ground protein extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, and protease inhibitor cocktail tablets (Roche Diagnostics)). 40 μg of extracted protein was used in western blot analysis with GFP antibodies to detect expression of GFP-CIPK26.

Cell-free Assays

The cell-free degradation assays were preformed as described in Lyzenga *et al.* (2013). In brief, tissue from four-day-old *35S:CIPK26-YFP-HA, 35S:CIPK26^{TD}-YFP-HA, 35S:CIPK26^{KR}-YFP-HA* transgenic seedlings were ground in protein extraction buffer extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5% glycerol, and protease inhibitor cocktail tablets (Roche Diagnostics) and at time zero, 10 mM $MgCl_2$ and 10 mM ATP was added to 200 μL of plant extracts (containing 1 mg total protein) and incubated at 30°C. Equal volume of sample was taken at each indicated interval and the reaction was stopped by the addition of SDS-loading buffer and snap frozen in liquid nitrogen. The abundance of CIPK-YFP-HA was determined by western blotting using HA antibodies.

The cell-free degradation assay in Figure 25 was preformed as described above with modifications. Tissue from six-day-old *35S:HA-KEG* seedlings was ground in protein extraction buffer. At time zero 10 mM $MgCl_2$, 10 mM ATP and approximately 100ng of nickel-affinity His-Flag-CIPK26N^{TD} or His-Flag-CIPK26N^{KR} recombinant proteins was added to 200 μL of plant extracts (containing 1 mg total protein) and incubated at 30°C. As a control 50 μM MG132 was added to protein extraction buffer

before time zero and the addition of ATP, MgCl$_2$ and His-Flag-CIPK26N^{TD}. As a second control, recombinant protein buffer only was added along with ATP and MgCl$_2$ at time zero. Equal volume of sample was taken at each indicated interval and the reaction was stopped by the addition of SDS-loading buffer and snap frozen in liquid nitrogen. The abundance of HA-KEG was determined by western blotting using HA antibodies.

Kinase assay

Recombinant Flag-His-tagged CIPK26 and Flag-His-tagged KEG were purified using Nickel affinity gel (Sigma) and were used for *in vitro* kinase assays. The 5' region (1bp to 870bp) of *CIPK26, CIPK26^{KR}, CIPK26^{TD}* cDNA that encodes for the amino terminal kinase domain (CIPK26N, CIPK26N^{KR}, CIPK26N^{TD}) were introduced into modified pDEST527 gateway destination vector to obtain Flag-His-tagged recombinant proteins (Liu and Stone, 2010). The RING and Kinase segment contains amino acids 1-467; primers used to generate the RING and Kinase domains of KEG (RK) can be found in Liu and Stone, (2010). Vectors were transformed into *Escherichia coli* strain Rosetta (DE3) and protein expression induced with 1uM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma) and purified with Nickel affinity gel (Sigma) according to manufacturer's instructions. Combinations of wild type and mutated forms of His-Flag-CIPK26N and His-Flag-RK 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 DTT, 0.1% Triton X-100, and 10 μCi of [γ-^{33}P]ATP). The reaction was stopped by the addition of SDS-loading buffer and boiling for 5 minutes. Samples were separated on a 7.5% SDS-PAGE gel and the gel was dried with a gel dryer and phosphorylated protein was detected by autoradiography.

Identification of KEG phosphorylation sites.

Experimentally determined KEG phosphorylation sites from untreated samples were gathered from Plant Protein Phosphorylation Database (Gao *et al*., 2009). To determine KEG phosphorylation sites from ABA-treated samples we immunoprecipitated HA-KEG using affinity HA-beads, according to manufactures protocol (Sigma), from 7-day old *35S:HA-KEG* seedlings that have been treated with 30 μM MG132 for 2 hours

followed by 16 hours with 50 μM ABA. The purified protein was separated with SDS-PAGE and stained with coomassie brilliant blue (Sigma). The HA-KEG band was excised from the gel and sent for liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis at the Sheldon Biotechnology Centre McGill University.

Results

The kinase activity of CIPK26 influences its stability

Previous work has demonstrated that CIPK26 is degraded through the 26S proteasome (Lyzenga *et al*., 2013). To investigate factors that influence that stability of CIPK26, we generated kinase dead (GFP-CIPK26^{KR}) and constitutively active (GFP-CIPK26^{TD}) versions of CIPK26 and compared the turnover of the kinase mutants to that of CIPK26 (GFP-CIPK26). Initially, we examined the accumulation of GFP-CIPK26, GFP-CIPK26^{KR}, and GFP-CIPK26^{TD} in the presence and absence of MG132 using transient expression in tobacco leaf epidermal cells. MG132 blocks the 26S proteasome and therefore causes accumulation of unstable protein. By comparing the relative amounts of protein from an untreated and MG132 treated samples we can assess relative protein stability. We observed an increase in the abundance of GFP-CIPK26 in the presence of MG132 compared to untreated samples (Figure 21A, compare lanes 1 and 2). This MG132-induced accumulation of GFP-CIPK26 is consistent with previous work showing that CIPK26 is unstable (Lyzenga *et al*, 2013). The two migrating forms corresponding to GFP-CIPK26 were also previously observed and are discussed in Lyzenga *et al*. (2013). Interestingly, we could barely detect GFP-CIPK26^{KR} in the absence of MG132 (Figure 21A, lane 3). We observed a large increase in the abundance of GFP-CIPK26^{KR} when samples were treated with MG132 (Figure 21A, compare lanes 3 and 4). Only a slight increase was observed for GFP-CIPK26^{TD} when the samples were treated with MG132 (Figure 21A, compare lanes 5 and 6). These results suggest that GFP-CIPK26^{KR} is more rapidly degraded through the 26S proteasome than GFP-CIPK26 and GFP-CIPK26^{TD}. The results also suggest that GFP-CIPK^{TD} is relatively stable compared to GFP-CIPK26 and GFP-CIPK26^{KR}.

To investigate if this trend would also occur *in planta* we generated stable transgenic Arabidopsis plants overexpressing *CIPK26-YFP-HA*, *CIPK26^{KR}-YFP-HA*, or
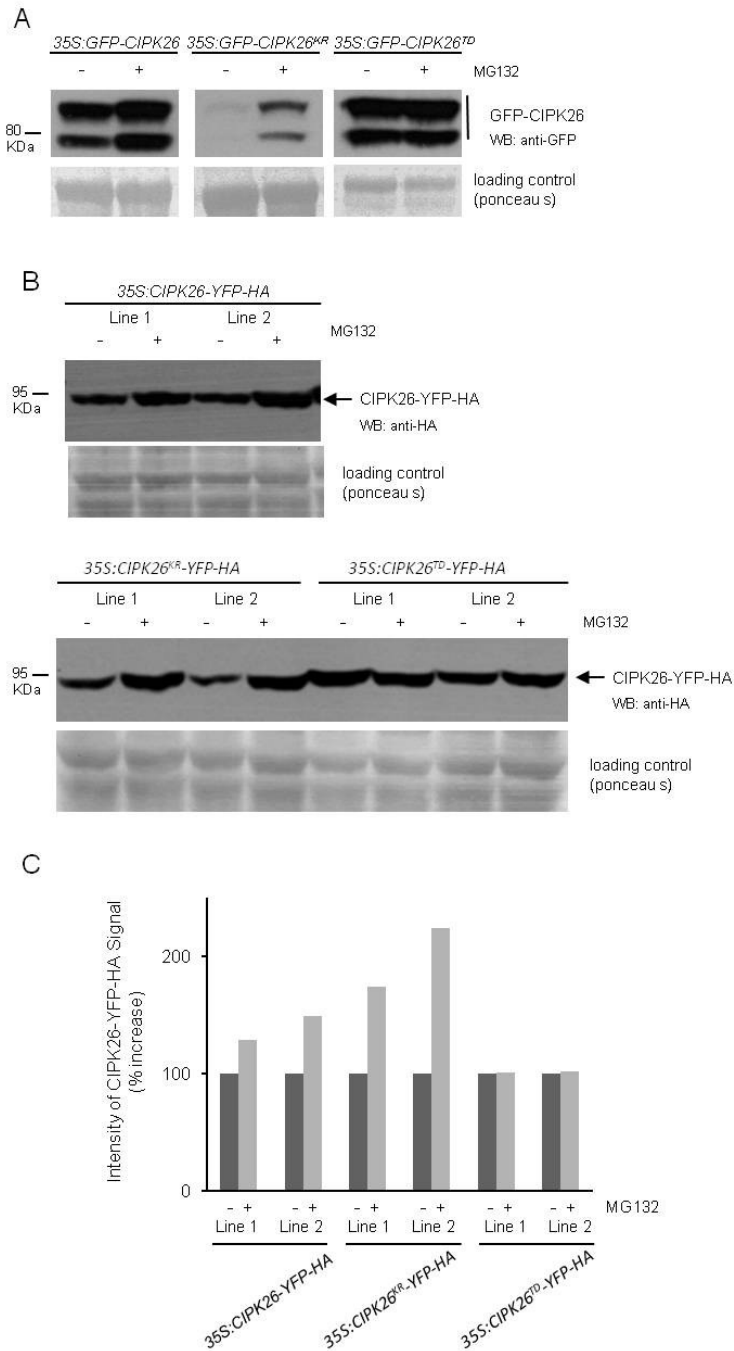
Figure 21. The kinase activity of CIPK26 influences its stability. An active form of CIPK26 is more stable compared to wild type CIPK26 and a kinase dead CIPK26 form. (A) Western blot analysis showing the protein abundance of GFP-CIPK26, GFP-CIPK26^{KR}, GFP-CIPK26^{TD} in transiently transformed *N. benthamiana* leaves. treated with (+) or (-) 50 µM MG132 for 16 hours. (B) Protein abundance of CIPK26-YFP-HA, CIPK26^{KR}-YFP-HA, CIPK26^{TD}-YFP-HA in transgenic Arabidopsis lines treated (+) or (-) 50 µM MG132 for 16 hours. (C) Quantification of B.

CIPK26^{TD}-YFP-HA. Two independent lines were identified for each transgene. We found that following treatment with MG132, CIPK26^{KR}-YFP-HA accumulated to the greatest extent while CIPK26^{TD}-YFP-HA accumulated the least (Figure 21B, C). These results further suggest that CIPK26^{TD}, or an activated form of CIPK26, is not effectively targeted for degradation through the 26S proteasome. In contrast, a version of CIPK26 that cannot become active, CIPK^{KR}, is rapidly turned over by the 26S proteasome.

We then used a cell-free degradation assay to compare the protein stability of CIPK26-YFP-HA, CIPK26^{KR}-YFP-HA, and CIPK26-YFP^{TD}-HA. Protein extracts were prepared from 4 day old *35S:CIPK26-YFP-HA, 35S:CIPK26^{KR}-YFP-HA,* and *35S:CIPK26^{TD}-YFP-HA* (all line 1) transgenic seedlings. Adenosine triphosphate (ATP), which is required for 26S proteasome activity, was added to the protein extracts at time zero. Samples were collected at the time points indicated and the level of CIPK26-YFP-HA was monitored using an HA-antibodies. Consistent with the above results, levels of CIPK26^{KR}-YFP-HA decreased more rapidly than CIPK26-YFP-HA and CIPK26^{TD}-YFP-HA. In addition, CIPK26^{TD}-YFP-HA was found to be degraded the slowest with a substantial amount of protein still remaining after 30 minutes (Figure 22, panel 3). These results further suggest that the inactive kinase is rapidly degraded through the 26S proteasome. However, when CIPK26 is active and can self-phosphorylate or phosphorylate its targets it is stable and is no longer an efficient substrate for ubiquitin-mediated degradation.

CIPK26 can phosphorylate KEG in vitro

Because KEG has been shown to be phosphorylated *in planta* (Liu and Stone, 2010), we used an *in vitro* kinase assay to determine if CIPK26 can phosphorylate KEG. The N-terminal kinase domain of CIPK26 (CIPK26N) was purified as a recombinant protein, 6XHistidine-Flag-CIPK26N (His-Flag-CIPK26N). Full-length CIPK26 is insoluble and sufficient protein was not able to be purified for the assay. Likewise, KEG is a large 178KDa protein and only the RING, and kinase (RK) segment of KEG (His-Flag-RK) was purified and used in the assay.

We found that His-Flag-CIPK26N displayed self-phosphorylation activity when incubated with *in vitro* kinase assay components alone (Figure 23, lane 1) and
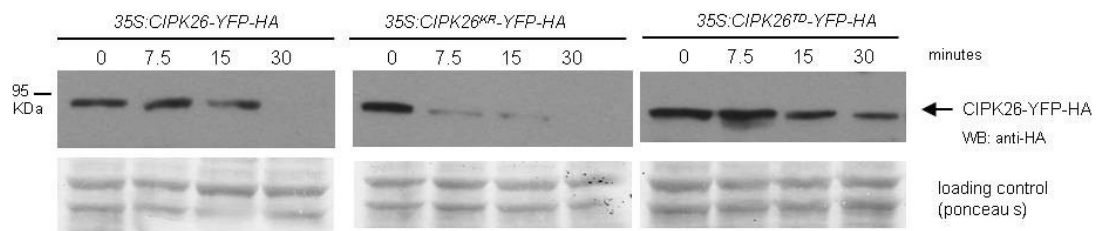
Figure 22. A constitutively active version of CIPK26 is more stable than a kinase dead version. Cell-free degradation assay using 4 day-old 35S:*CIPK26-YFP-HA, 35S:CIPK26^KR-YFP-HA*, and *35S: CIPK26^TD*-YFP-HA (line 1) transgenic seedlings.
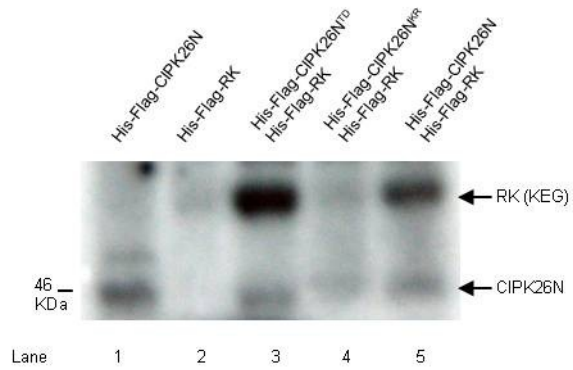
Figure 23. CIPK26 can phosphorylate KEG *in vitro*. *P*hosphorylation assays using His-Flag-CIPK26N and His-Flag-RK (KEG), or inactive His-Flag-CIPK26N^{KR}, or constitutively active His-CIPK26N^{TD}. Autoradiogram shows self- phosphorylation of CIPK26N and phosphorylation of RK (KEG) by CIPK26N and CIPK26N^{TD}.

His-Flag-CIPK26N was also able to phosphorylate the KEG (Figure 23, lane 5). When the kinase dead version of CIPK26 (His-Flag-CIPK26N^{KR}) was used in the assay no self-phosphorylation or KEG phosphorylation was observed (Figure 23, lane 4). However, a constitutive active version of CIPK26 (His-Flag-CIPK26N^{TD}) was able to self-phosphorylate and phosphorylate KEG (Figure 23, lane 3). As expected, phosphorylation of the His-Flag-RK was significantly greater in assays using His-Flag-CIPK26N^{TD} compared to His-Flag-CIPK26N. These results show that CIPK26N is an active kinase that can phosphorylate KEG, with potential phosphorylation sites located within the region of the RING and kinase domain.

KEG phosphorylation pattern changes in the presence of ABA

We have previously provided evidence that CIPK26 positively regulates ABA signalling; overexpression of *CIPK26* leads to ABA hypersensitivity, CIPK26 interacts with negative regulatory phosphatases ABI1/2, and CIPK26 can interact with and phosphorylate ABI5 (Lyzenga *et al.,* 2013). This evidence suggests that CIPK26 is activated in response to ABA. If CIPK26 is phosphorylating KEG in response to ABA, then KEG should have specific residues that are phosphorylated in the presence of ABA. To examine KEG's phosphorylation profile we gathered experimentally determined phosphorylation sites by mass spectrometry from the Plant Protein Phosphorylation Database (Gao *et al*., 2009). Sugiyama *et al*. (2008), Nakagami *et al*. (2010) and Reiland *et al*. (2009) identified the following phosphorylation sites in KEG: kinase domain: S-195, linker region: S-437, S-439, S-441 and Herc2-like repeats: S-974 (Figure 24). To investigate the phosphorylation pattern of KEG in the presence of ABA we immunoprecipitated HA-KEG from ABA and MG132 treated *35S:HA-KEG* seedlings. Liquid chromatography–tandem mass spectrometry (LC/MS/MS) was used to identify phosphorylation sites. This analysis identified the following sites: kinase domain: S-321, Y-324 linker region: S-437, S-439, Ankyrin repeats: S-502 or S-513, Herc2-like repeats S-953 or S-974 and Y-1134 (Figure 24). The following sites were unique to ABA treated sample: S-321, Y-324, S-502 or S513, S-953 or S974 and Y-1134. A few sites were phosphorylated in both samples: S-437, S-449 and possibly S974. This data correlates with previous reports showing that KEG is phosphorylated in the presence and absence of ABA (Liu and Stone, 2010). Here we show that the phosphorylation pattern of KEG

changes in the presence of ABA suggesting that phosphorylation plays a role in regulating KEG activity. This ABA-dependent phosphorylation pattern may account for the phosphorylation event required for ABA-dependent KEG degradation (Liu and Stone, 2010).

CIPK26 kinase activity promotes KEG degradation

KEG ubiquitination and subsequent degradation has been shown to be an important regulation mechanism of KEG activity (Liu and Stone, 2010). ABA induces KEG proteasomal-dependent degradation and the ABA-mediated turnover is phosphorylation dependent (Liu and Stone, 2010). We used a modified cell-free degradation assay to investigate if the kinase activity of CIPK26 can influence KEG turnover. Protein extracts were prepared from 6-day old *35S:HA-KEG* transgenic Arabidopsis seedlings. At time zero ATP was added to the extract along with purified recombinant His-Flag-CIPK26N^{TD}, or His-Flag-CIPK26N^{KR}, samples were collected at the time points indicated and the levels of HA-KEG were monitored using HA-antibodies. We found that the abundance of HA-KEG decreased overtime when *35S:HA-KEG* protein extracts were incubated with recombinant His-Flag-CIPK26N^{TD} (Figure 25 panel 2). This decrease in HA-KEG abundance was not observed in samples lacking CIPK26 (control) and samples incubated with His-Flag-CIPK26N^{KR} (Figure 25, panels 1 and 4). The decrease in HA-KEG protein levels in the presence of His-Flag-CIPK26N^{TD} was blocked when MG132 was included in the degradation assay. These results suggest that the kinase activity of CIPK26 promote the degradation of KEG through the 26S proteasome.

Discussion

Previous work has shown that KEG, a RING-type E3 ligase negatively regulates ABA signalling by targeting both ABI5 and CIPK26 for degradation (Stone *et al.*, 2006; Liu and Stone, 2010; Lyzenga *et al.,* 2013). KEG promotes the degradation of ABI5 and CIPK26 in the absence of ABA; in the presence of ABA a phosphorylation event promotes KEG ubiquitination and subsequent degradation (Liu and Stone, 2010). Conditions that affect the stability of CIPK26 have not been investigated and upstream signalling events that promote KEG degradation have not been established. In this report
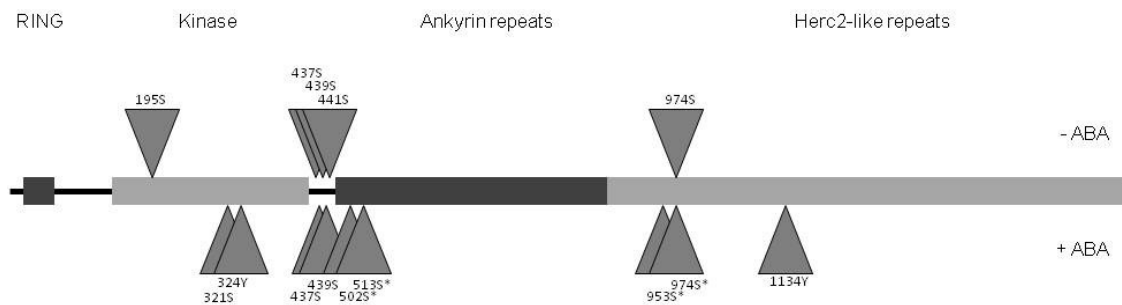
Figure 24. Schematic of KEG phosphorylation sites in the presence and absence of ABA. The Plant Protein Phosphorylation Database (P3DB) was used to search phosphorylation sites from untreated samples. Phosphorylation sites from ABA treatment were identified through liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis of immunoprecipitated HA-KEG from 7-day old ABA treated *35S:HA-KEG* seedlings. *indicates one of the two sites are phosphorylated.
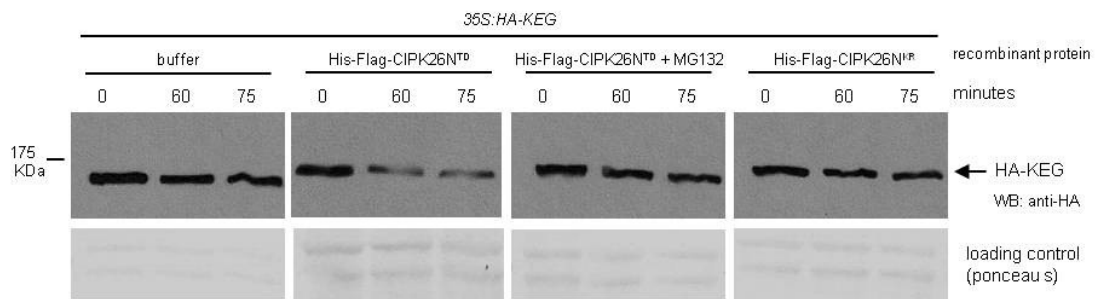
Figure 25. The kinase activity of CIPK26 promotes the degradation of KEG in a modified cell-free degradation assay. Recombinant His-Flag-CIPK26N^{TD}, or His-Flag-CIPK26N^{KR}, or recombinant protein buffer was added to protein extracts prepared from 8-day old *35S:HA-KEG* seedlings. KEG protein levels were determined by western blotting using HA antibodies. Ponceau S stain was used to confirm equal loading.

we show that the kinase activity of CIPK26 promotes the stabilization of CIPK26 and promotes the degradation of KEG.

The interaction between CIPK26 and KEG can have various consequences. KEG may regulate the stability of CIPK26 through ubiquitin mediated degradation and CIPK26 may phosphorylate KEG to regulate KEG activity. We have previously shown that KEG promotes the degradation of CIPK26 through the 26S proteasome in the absence of ABA (Lyzenga *et al.*, 2013). In this report we address how the kinase activity of CIPK26 influences KEG stability. Protein phosphorylation can influence ubiquitination in a variety of ways (de Bie and Ciechanover, 2011). Phosphorylation of a substrate and/or E3 ligases can influence cellular location thereby regulating interaction of E3 and target. There are also many examples of target phosphorylation generating a phosphodegron thereby promoting the degradation of a target. Phosphorylation of E3 ligases can also influence the activity of the E3 ligase. Many RING-type E3 ligases are negatively regulated through phospho-dependent self-ubiquitination and examples from mammalian systems include Mdm2, and Cbl E3 ligases (Weissman *et al.*, 2011). Evidence presented in this and previous work demonstrates that KEG is also an example of a RING-type E3 ligase that is negatively regulated through phospo-dependent ubiquitination (Liu and Stone, 2010).

Previous work has shown that a general kinase inhibitor prevents the ABA-induced degradation of KEG, and that a pre-phosphorylated KEG has increased self-ubiquitination activity *in vitro* than a non-pre-phosphorylated form (Liu and Stone, 2010). KEG also contains a kinase domain. A kinase dead version of KEG is more stable and less ubiquitinated in the presence of ABA than the wild type version of KEG (Liu and Stone, 2010). In addition, this reports shows that specific residues of KEG change their phosphorylation status in the presence of ABA. This evidence suggests that phosphorylation plays an important role in ABA-dependent proteasomal degradation of KEG. In this report, we show that CIPK26 can phosphorylate KEG *in vitro*. Using a modified cell free degradation assay we show that the kinase activity of CIPK26 promotes the degradation of KEG through the 26S proteasome. Based on these findings we suggest a model where activated CIPK26 phosphorylates KEG to negatively regulate KEG activity and promote the degradation of KEG. CIPK26 may phosphorylate KEG to

promote KEG self-ubiquitination or to promote ubiquitination by another E3 ligase. This model provides a link between the activity of KEG and the perception of ABA since CIPK26 can interact with ABI1/2 which act as part of the PYR/PYL/RCAR ABA receptor (Lyzenga *et al*., 2013; Weiner *et al* 2010).

Our model suggests that when CIPK26 is active, KEG is ubiquitinated and degraded through the proteasome (Figure 26). Consistent with our model is our observation that a constitutively active version of CIPK26 is stable. The wild type version of CIPK26 is unstable and degraded through the proteasome, but it is more stable than the kinase dead version of CIPK26. According to our model, in the absence of a stimulus wild type CIPK26 should be inactive and therefore have a similar stability to the kinase dead version. This unexpected observation may be explained by suggestion that over expression of *CIPK26* leads to limited escape from ABI1/2 mediated dephosphorylation and subsequent activation in the absence of a stimulus.

Our observation that an activated form of CIPK26 is stable as compared to wild type or a kinase dead version is uncommon. In other study system many activated kinases are degraded through the proteasome as a form of down regulation (reviewed in Lu and Hunter, 2009). The CIPK family is also referred to as SnRK3s and are part of the same classification as SnRK2s and SnRK1s. While CIPK/SnRK3s and SnRK2s are restricted to the plant kingdom SnRK1 is related to mammalian AMPK and SNF1 from yeast (Hrabak *et al*., 2003; Kolukisaoglu *et al*., 2004). AMPK and SNF1 are both regulated through ubiquitin-mediated degradation (Crozet *et al*., 2014). There is also evidence that both α-SnRK1 subunits in Arabidopsis, AKIN10 and AKIN11, are regulated through ubiquitin-mediated degradation. Phosphate starvation reduces levels of AKIN11 via an unknown E3 ligases and AKIN10, is targeted for degradation via the CUL4-DDB1-E3 ligase receptor, PRL1 (Fragoso *et al*., 2009; Lee *et al*., 2008). Interestingly, Coello *et al*. (2011) provided evidence that ABA promotes the degradation of SnRK1-related kinase in wheat but that an active form is stable. The authors suggest that an inactive pool of SnRK1 is degraded in the presence of ABA to prevent an undesirable increase in SnRK1 activity due to PP2C inhibition. The constitutively active form of CIPK26 may be stable
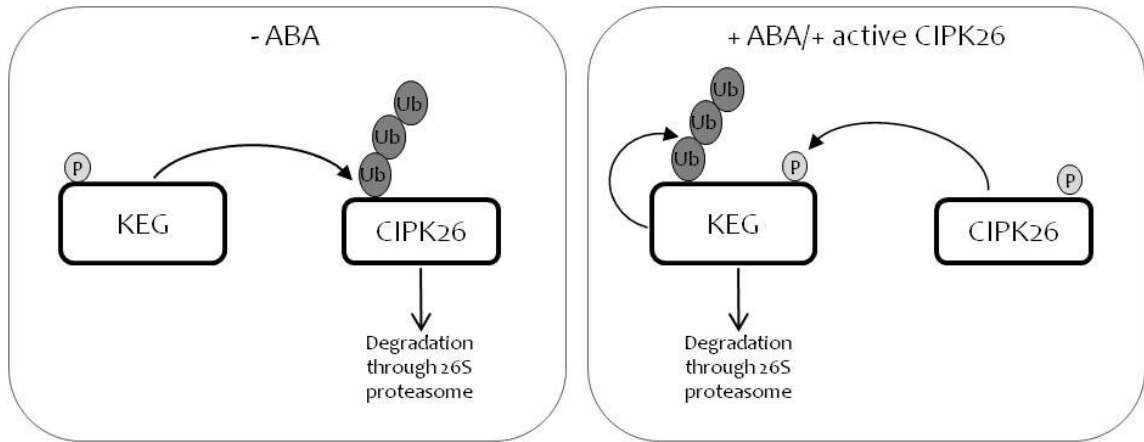
Figure 26. Model of KEG and CIPK26 reciprocal regulation. In the absence of ABA, KEG ubiquitinates CIPK26 and CIPK26 is degraded through the 26S proteasome. In the presence of ABA or when CIPK26 is active, CIPK26 phosphorylates KEG which promotes the ubiquitination of KEG and subsequent degradation through the 26S proteasome.

because of multiple factors. CIPK26 may self-phosphorylate thereby disrupting a degron sequence or CIPK26 may phosphorylate and negatively regulate E3 ligases that promote the degradation of CIPK26. In this report we provide evidence for the later; we suggest that active CIPK26 phosphorylates KEG and promotes the degradation of the E3 thereby allowing accumulation of KEG`s substrate and promotion of ABA responses.

Acknowledgements

CHAPTER 5:
CONCLUSION

E3 ubiquitin ligases play diverse roles in plant development and response to the environment. More specifically, E3 ligases coordinate plant hormone activity through regulation of signalling and hormone biosynthesis. The specificity of protein ubiquitination is largely controlled by E3 ligases and the most described outcome of ubiquitination is target degradation through the 26S proteasome (Vierstra, 2009). This thesis explored the molecular role of two E3 ligases, KEG and XBAT32.

In this thesis I document the novel finding that CIPK26, a member of the SnRK3/CIPK family of kinases, is regulated through ubiquitin-mediated degradation. I explore KEG, an E3 ligase, as a regulator of CIPK26 abundance and I expand on KEG's role as a regulator of ABA signalling (Chapter 3). In this thesis, I explore the reciprocal relationship between E3 ligases and kinases and the interplay between ubiquitination and phosphorylation. These results suggest that CIPK26, a target of KEG's E3 ligase activity, also functions to regulate KEG's stability (Chapter 4). Chapter 2 investigates the role of the E3 ligase XBAT32 with regards to ethylene biosynthesis. I provide an unexpected finding that ethylene biosynthesis protein, ACS7, is regulated through ubiquitin-mediated degradation and demonstrate that XBAT32 contributes to the turnover of both ACS4 and ACS7.

This body of work contributes to knowledge of E3 ligases and protein stability in many ways. With regards to the XBAT32 study, I used a cell free assay, an *in planta* cycloheximide assay, and phenotypic analysis of transgenic plants to demonstrate the relationship between an E3 ligase and its target. Our observation that lysine to arginine mutations in the C-terminal tail of both ACS7 and ACS4 led to decreased stability highlights the importance of protein charge on stability. Our results show that a target, ACS4, can have multiple E3's regulating its abundance as the E3 ligase, ETO1/EOL1/2, also regulates ACS4 abundance (Christians et al., 2009). Investigation into the details concerning how and when XBAT32 and ETO1/EOL1/2 act on ACS4 will likely provide a detailed understanding on how ethylene production is finely tuned during different developmental processes. The main result out of this work is that XBAT32 targets ACS7 for degradation even though ACS7 lacks a regulatory C-terminal extension.

With regards to Chapter 3, I used a cell-free assay and an *in planta* cycloheximide assay to demonstrate that CIPK26 is degraded through the 26S proteasome. CIPK26 is the first example of proteasomal regulation of a CIPK family. Other CIPKS are likely regulated in this fashion and how ubiquitin-mediated degradation ties into other levels of kinase regulation is an unexplored area of research. I used an *in planta* cycloheximide assay with double transgenic plants expressing both a target and the E3 ligase. Because the E3 ligase was on an inducible promoter, I could examine the relationship between an E3 ligase and its target. This is a relatively novel method and is an example of how researchers can investigate the relationship between an E3 ligase and target without using knockout lines. The discovery of the interaction between CIPK26 and KEG also provides a molecular link between KEG and the perception of ABA. CIPK26 interacts with ABI1 and ABI2, which form a complex with the ABA receptor PYR/PYL/RCAR (Weiner *et al*., 2010).

Our model suggests that KEG targets CIPK26 and a phosphorylation target of CIPK26, the ABA-responsive transcription factor ABI5, for degradation. We suggest that KEG targets both CIPK26 and ABI5 to negatively regulate the ABA response. ABI5 influences the expression of more than 100 genes and has a significant impact on plant development (Reeves *et al*., 2011). Accordingly, the regulation of ABI5 is controlled though transcription, stability, and phosphorylation (Lopez-Molina *et al*., 2001; 2002). KEG mediated degradation of both ABI5 and CIPK26 represents multi-layered regulation of ABI5 and highlights the importance of tightly controlled ABI5 activity.

In Chapter 4, our model of CIPK26 and KEG reciprocal regulation explores the interplay between ubiquitination and phosphorylation. We suggest that phosphorylation of KEG by CIPK26 promotes KEG degradation and accounts for the highly stable active version of CIPK26. Many examples from mammalian systems demonstrate that upon activation, receptor kinases and non-receptor kinases are degraded through the proteasome as feedback inhibition (Lu and Hunter, 2009). In plant systems, examples of proteasomal regulation of kinases are limited and the CIPK family of kinases is specific to the plant kingdom. However, in yeast, mammalian study systems, and plant study systems, the SNF1/AMPK/SnRK1 related kinases have also been shown to be regulated through proteasomal degradation (Crozet et al., 2014).

Examples of phosphorylation regulating ubiquitination and E3 ligase activity is widespread (de Bie and Ciechanover, 2011). Phosphorylation can influence the interaction between E3 and target by promoting or preventing interaction and by influencing the cellular localization of E3 and target. The activity of E3 ligases can also be allosterically influenced by phosphorylation. In this thesis, I explore phosphorylation as a mechanism to regulate the stability of KEG as many RING-type E3 ligases are themselves regulated through ubiquitin mediated degradation. We suggest that CIPK26 phosphorylates KEG to promote KEG degradation; KEG ubiquitination may be facilitated through self-ubiquitination or via another E3 ligase. Future work should address conditions that lead to CIPK26 stabilization and if other members of the CIPK family are also regulated through proteasomal degradation

The work in this thesis on the E3 ligases, XBAT32 and KEG, along with their ubiquitination targets highlights the importance of ubiquitin-mediate degradation with regards to hormone signalling. Equipped with the knowledge that many proteins are specifically ubiquitinated and degraded through the proteasome, the plant biotechnology industry can address current problems and solutions.

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Abiotic stress tolerance mediated by protein ubiquitination

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Contributions:

WJL: Wrote the manuscript and participated in revisions.

SLS. Prepared all figures and participated in revisions.

Abstract

Plant growth and development is largely influenced by ubiquitin-mediated regulation of protein stability. Specificity of the ubiquitination pathway is controlled mainly by the substrate-recruiting E3 ubiquitin ligases, and consequently, E3 ligases control numerous cellular processes. Recent evidence that ubiquitination plays a critical role in regulating plant responses to abiotic stresses has launched intensive efforts to identify E3 ligases that mediate plant tolerance of adverse environmental conditions. Most stress-related E3 ligases identified to date facilitate responses to environmental stimuli by modulating the abundance of key downstream stress-responsive transcription factors. In this review, the regulatory roles of ubiquitin during the plant's response to abiotic stress are summarized and highlighted.

Introduction

Ubiquitination serves as a versatile post-translational modification that mediates growth and development of all eukaryotic species. Ubiquitin is a stable, highly conserved, and universally expressed protein. The covalent attachment of ubiquitin to a lysine residue of select proteins can regulate stability, activity, and trafficking. Genome sequencing has revealed the extent to which plants rely on protein ubiquitination to regulate organismal processes. For example, over 6% of *Arabidopsis thaliana* protein-coding genes are dedicated to the ubiquitin 26S proteasome system (UPS) (Vierstra, 2009). In plant species, the UPS regulates fundamental processes such as embryogenesis, photomorphogenesis, and organ development (Thomann *et al.*, 2005; Sonoda *et al.*, 2009;

Pokhilko *et al.*, 2011). In addition to regulating these fundamental processes the UPS has recently emerged as a major player in plant responses to abiotic stresses.

Plants are consistently exposed to unfavourable growth conditions throughout their life cycle. Abiotic stresses such as drought, temperature fluctuations, high salinity, radiation, and nutrient deprivation adversely affect growth, development, and productivity. To ensure survival plants must effectively and efficiently sense, respond, and adapt to their ever-changing environment. Following the perception of environmental stimuli, plants adjust their physiology to mitigate any adverse effects that may result from exposure to abiotic stresses. This is accomplished via signal transduction events leading to changes in gene expression which facilitates various cellular responses. Understanding the molecular basis of abiotic stress perception and signal transduction is of great interest to plant researchers and is an intensely studied area of plant biology. Recent reports in this field have identified ubiquitin conjugation as a major regulator of stress-responsive transcription factors and other regulatory proteins. By modulating the amount and activity of regulatory proteins, ubiquitination plays a central role in regulating the transcriptional changes required for adaption to abiotic stresses. In this review, recent advances made in our understanding of the role the UPS plays during plant responses to various abiotic stresses are discussed.

The ubiquitination enzymes

Ubiquitin is attached to selected proteins through a conjugation cascade consisting of the following three enzymes: the ubiquitin-activating (UBA; E1) enzyme, ubiquitin-conjugating (UBC; E2) enzyme, and ubiquitin ligase (E3) (Figure 27). Ubiquitin is first activated in an ATP-dependent reaction by the E1. A conserved E1 catalytic cysteine is used to form an E1-ubiquitin (E1-Ub) thioester linked intermediate. The E1-Ub interacts with the E2 and the activated ubiquitin is transferred to the active-site cysteine of the E2 forming a thioester linked E2-ubiquitin (E2-Ub) intermediate. Transfer of ubiquitin to the target protein is facilitated by the E3 which interacts with both the E2-Ub and the target protein. There are three major types of E3s: Really Interesting New Gene (RING)-type, Homology to E6-Associated Carboxyl-Terminus (HECT)-type or U-box-type (Figure 28). The RING-type and U-box-type E3s mediate transfer of ubiquitin directly from the E2-Ub to the target protein. HECT-type E3s are unique in that they form an E3-Ub

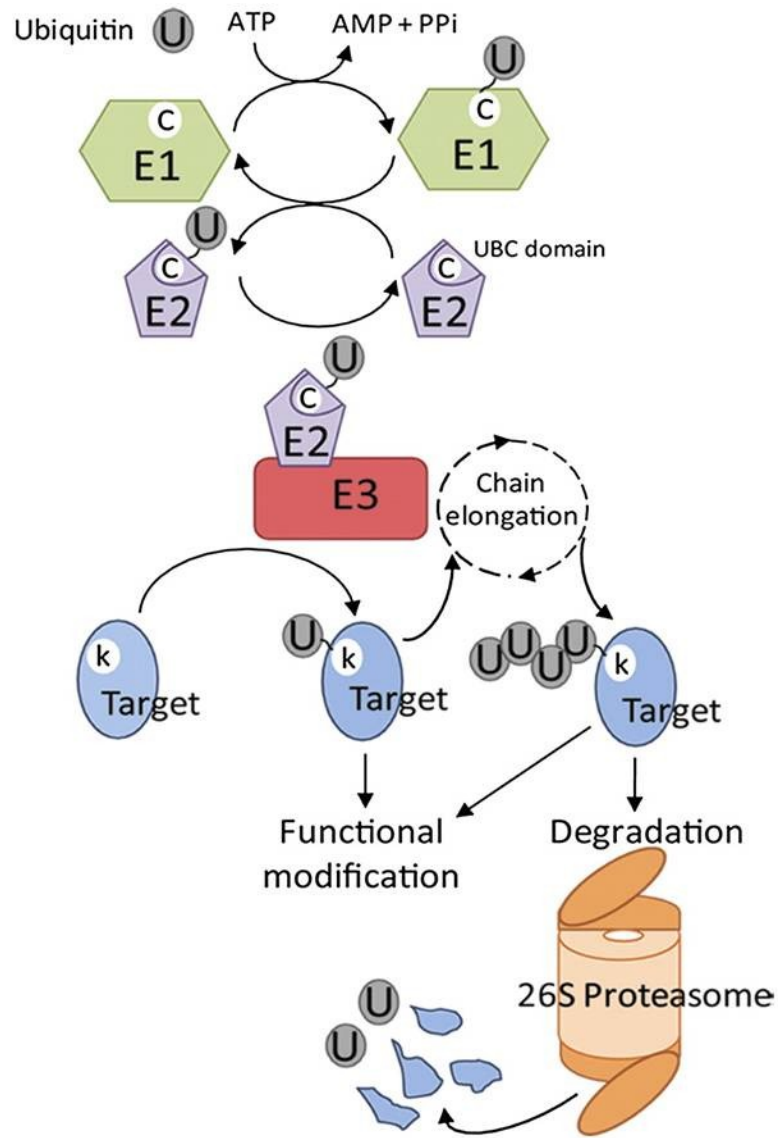Figure 27. The ubiquitination pathway. Ubiquitin is activated by the E1 and then transferred to a conserved cysteine residue on the E2 forming an E2-ubiquitin intermediate. Ubiquitin is transferred from the E2-ubiqiutin intermediate to an internal lysine of a target protein bound to the E3 (mono-ubiquitination). Additional ubiquitin molecules can be added to the mono-ubiquitinated target (polyubiquitination).

intermediate prior to the transfer of ubiquitin to the target protein (Scheffner *et al.*, 1995) (Figure 28). Both mechanisms attach ubiquitin through an isopeptide bond using the carboxyl terminal glycine of ubiquitin and a lysine residue on the target protein.

The attachment of the initial ubiquitin molecule is followed by the assembly of different types of polyubiquitin chains. The first ubiquitin on the target protein acts as an 'acceptor' to which additional molecules are attached during repeated cycles (Figure 27). Although various models have been proposed, the exact mechanism of chain assembly is not very well understood (Hochstrasser, 2006; Deshaies and Joazeiro, 2009). During chain elongation, ubiquitin molecules may be added sequentially to the growing chain on the target protein or the ubiquitin chain may be pre-assembled upon the E2 and then transferred as a whole to the substrate (Wang and Pickart, 2005; Li *et al.*, 2007; Kim and Huibregtse, 2009; Maspero *et al.*, 2011). Ubiquitin contains seven conserved lysine (Lys) residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) all of which can be used to produce structurally diverse polyubiquitin chains (Kirkpatrick *et al.*, 2006; Kim *et al.*, 2007). The topology of the attached polyubiquitin chain (polyubiquitination) determines the fate of the modified protein. A Lys48 linked ubiquitin chain serves as a signal for degradation by the 26S proteasome. By contrast, Lys63 linked ubiquitin chains have been implicated in endocytosis, protein activation, and intracellular trafficking (Pickart and Fushman, 2004). Another aspect of ubiquitination is the attachment of a single (mono-ubiquitination) or multiple mono-ubiquitins (multi-ubiquitination) to a target protein. These types of modifications serve as a signal for membrane protein internalization, vesicle sorting, DNA repair, and gene silencing (Mukhopadhyay and Riezman, 2007). In the case of RING-type and U-box-type E3s, the E2 enzymes mainly determine the specificity of chain assembly (Kim *et al.*, 2007; Rodrigo-Brenni *et al.*, 2010). However,
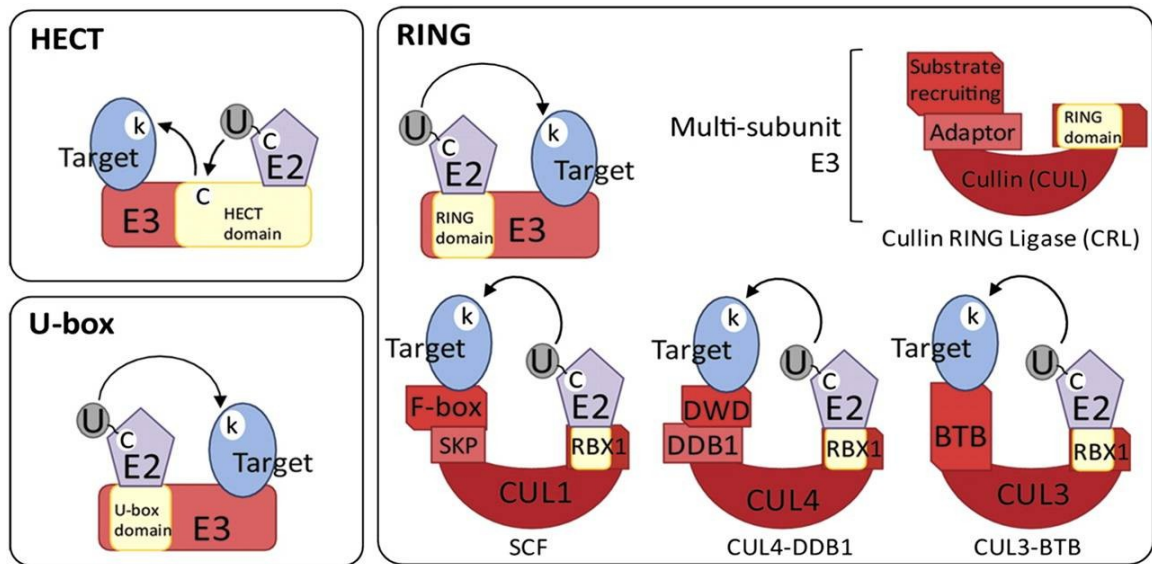
Figure 28. E3 ubiquitin ligases. E3s are categorized based on the presence of a RING, HECT or U-box E2 binding domain. The RING-type E3s are subdivided into groups depending on whether the E2 and substrate-binding functions are found in the same protein (monomeric E3s) or on different proteins (multi-subunit E3s; CRLs). The multi-subunit CRL uses a CUL protein as a scaffold to interact with the E2 binding RING protein and a substrate-recruiting protein. The CUL3-based CRLs utilize the BTB substrate-recruiting proteins. CUL1- and CUL4-based CRLs use SKP and DDB1 adaptor proteins, respectively, to bind the F-box and DWD substrate-recruiting proteins.

there are some cases where the E2–E3 combination plays a role in determining the topology of the chain (Kim *et al.,* 2007; Deshaies and Joazeiro, 2009). By contrast, the HECT-type E3 alone determines lysine specificity during ubiquitin chain synthesis (Wang and Pickart, 2005; Maspero *et al.*, 2011).

Eukaryotes usually possess one or two E1s, tens of E2s, and hundreds of E3 ligases. Analysis of the *Arabidopsis* genome identified two E1s, 37 E2s, and over 1300 E3 encoding genes (Kraft *et al.*, 2005; Craig *et al.*, 2009). *Arabidopsis* E1 isoforms, *AtUBA1* and *AtUBA2*, are encoded by two distinct genes. They share 81% amino acid sequence identity, have similar expression patterns, and they have almost identical E2 interaction specificity (Hatfield *et al.*, 1997). E2 enzymes are defined by the presence of a 140 amino acid UBC domain that contains the conserved cysteinyl residue required for accepting the ubiquitin molecule from the E1-Ub (Wu *et al.*, 2003; Kraft *et al.*, 2005) (Figure 27). The UBC domain also mediates interaction between the E3 and the E2-Ub intermediate (Kalchman *et al.*, 1996; Wu *et al.*, 2003; Kraft *et al.*, 2005).

The RING-type E3s are the most abundant in the predicted *Arabidopsis* proteome followed by the U-box-type and HECT-type E3s. The canonical RING domain is defined by an octet of metal-binding cysteine and histidine residues that co-ordinate two zinc ions in a cross brace globular structure (Freemont, 1993). The spacing between the cysteine and histidine residues is also well conserved. The structure of the RING domain is essential for E2 binding and ubiquitin ligase activity (Lorick *et al.*, 1999). However, the domain does allow for some variability utilizing less conserved amino acids and changes in spacing between key metal binding residues, without loss of E3 ligase activity (Kosarev *et al.*, 2002; Stone *et al.*, 2005). Eleven percent of the predicted *Arabidopsis* RING domain-containing proteins (RING proteins) contain a modified RING domain (Stone *et al.*, 2005). Despite these differences, proteins containing a modified RING domain are capable of mediating ubiquitin conjugation (Stone *et al.*, 2005). Plant genomes contain significantly more RING protein encoding genes than that found in other eukaryotes. *Arabidopsis*, rice, and poplar contain 469, 378, and 399 RING-type E3 encoding genes, respectively, compared with 300 human and 47 *Saccharomyces cerevisiae*genes (Kraft *et al.*, 2005; Li *et al.*, 2008; Du *et al.*, 2009). Bioinformatic analysis of the *Arabidopsis* RING proteins identified a number of additional domains

including protein–protein interaction, transmembrane, kinase, and DNA and RNA binding domains. Based on the presence and organization of these additional domains, the *Arabidopsis* RING family can be subdivided into 39 distinct groups (Stone *et al.*, 2005).

While the majority of RING proteins are predicted to function as monomeric E3s, RING proteins also participate in multiple subunit Cullin RING E3 ligases (CRLs) (Figure 28). In the CRL family, functional E3s are composed of four or five different protein subunits. A Cullin (CUL) protein, CUL1, CUL3a/3b or CUL4, act as a scaffold to bring together the E2-Ub binding RING protein RING Box 1 (RBX1/ROC1/HRT1) and the substrate-recruiting protein (Schwechheimer and Villalobos, 2004; Hotton and Callis, 2008) (Figure 28). The substrate-recruiting subunit can either bind directly to the CUL protein or via an adaptor protein (Figure 28). CUL1 uses the adaptor protein S-Phase Kinase-associated Protein (SKP or ASK for *Arabidopsis*) to bind to substrate-recruiting F-box proteins. CUL4 uses DNA-damage Binding (DDB1) as an adaptor to bind substrate-recruiting DDB1 binding WD40 (DWD) proteins (Bai *et al.*, 1996; Lechner *et al.*, 2006; Lee *et al.*, 2008). The substrate-recruiting Broad complex Tramtrack Bric-a-Brac (BTB) proteins bind directly to CUL3a/b (Gingerich *et al.*, 2005). Because CRLs can be composed of one of three CULs and one of numerous substrate-recruiting proteins, they are the most diverse and numerous families of E3 ligases. For example, an *Arabidopsis* CUL1-based Skp-Cullin-F-box (SCF)-type E3 complex may be assembled using any of 700 substrate-recruiting F-box proteins (Figure 28) (Lechner *et al.*, 2006). The CUL3 scaffold can probably associate with the 80 predicted BTB proteins and CUL4-DDB1 can potentially interact with 85 predicted DWD proteins (Gingerich *et al.*, 2005; Lee *et al.*, 2008).

The U-box domain forms a scaffold structure similar to the RING domain. However, the U-box structure is stabilized via salt bridges and hydrogen bonds instead of metal binding residues (Aravind and Koonin, 2000). Compared with other eukaryotic species there are significantly more U-box protein-encoding genes in plant genomes. The *Arabidopsis* and rice genome contains 64 and 77 U-box-type E3 encoding genes, respectively, compared with eight human and two *Saccharomyces cerevisiae* genes (Li *et al.*, 2008; Yee and Goring, 2009). The 64 members of the *Arabidopsis* plant U-box

(PUB) E3 family can be placed into 13 groups based on the presence or organization of additional domains (Azevedo *et al.*, 2001;Mudgil *et al.*, 2004; Wiborg *et al.*, 2008; Yee and Goring, 2009). The vast majority of PUB proteins (64%) contain armadillo repeats as a potential substrate-binding domain. This is in contrast to the RING proteins that contain a variety of protein–protein interaction domains including, ankyrin repeats, WD40, BRCT, and VWA (Stone *et al.*, 2005). In addition, only a single RING domain-containing protein contains a kinase domain compared with 23% of PUB proteins (Stone *et al.*, 2005, 2006; Wiborg *et al.*, 2008; Yee and Goring, 2009).

HECT-type E3s proteins are distinguished by the presence of a conserved catalytic HECT domain that contains the invariant cysteinyl residue used to form the E3-Ub intermediate (Figure 28) (Huibregtse *et al.*, 1995). Among E3s, the HECT-type family is usually the smallest across all plant species with only seven and eight members found in the *Arabidopsis* and rice genomes, respectively (Downes *et al.*, 2003).

The UPS is essential for plant response to abiotic stresses

The UPS functions within the cytoplasm and nucleus to modulate the levels of regulatory proteins and to remove misfolded or damaged proteins that may accumulate as a result of exposure to abiotic stress. One of the first indications that the UPS was involved in regulating plant stress tolerance was the observation that expression of polyubiquitin genes is stress-regulated (Christensen *et al.*, 1992; Genschik *et al.*, 1992; Sun and Callis, 1997). Ubiquitin is encoded by multiple polyubiquitin genes (*UBQ3, UBQ4,UBQIO, UBQ11*, and *UBQ14*) that contain 3–6 ubiquitin-coding regions in tandem (Callis *et al.*, 1995). Following translation, nascent polyubiquitin proteins are proteolytically processed into ubiquitin monomers (Vierstra, 1996). The pool of free ubiquitin molecules is regulated through differential expression of the polyubiquitin genes (Christensen *et al.*, 1992; Genschik *et al.*, 1992; Sun and Callis, 1997). Specifically, transcript abundance of *Arabidopsis UBQ14* is increased during heat stress (Sun and Callis, 1997). Similarly, high temperatures also induce the expression of multiple polyubiquitin genes in tobacco, potato, and maize (Christensen *et al.*, 1992; Garbarino *et al.*, 1992; Genschik *et al.*, 1992). In fact, over-expression of a single mono-ubiquitin gene enhances tolerance to multiple stresses without adversely affecting growth and development under favourable conditions (Guo *et al.*, 2008). Transgenic tobacco

over-expressing a wheat polyubiquitin gene, containing a single ubiquitin repeat, were more tolerant of cold, high salinity, and drought conditions compared with control plants. The stress-induced expression of polyubiquitin genes is consistent with the role of the UPS in turning over damaged proteins to mitigate the negative effects of environmental stress.

Defects in 26S proteasome function also alter plant tolerance to various environmental stresses. The 26S proteasome is an ATP-dependent protease complex consisting of a proteolytic 20S complex capped on one or both ends by a 19S regulatory particle (RP). Access to the active sites of the 20S complex is regulated by the RP that mediates substrate recruiting, unfolding, translocation into the proteolytic chamber of the 20S, and recycling of ubiquitin molecules (Strickland *et al.*, 2000; Navon and Goldberg, 2001). The RP is composed of two subcomplexes referred to as the Base and the Lid. The Base sits directly on the 20S and contains six AAA-ATPases (RPTs) and three non-ATPase (RPNs) subunits. The Lid subcomplex contains an additional eight RPNs (Fu *et al.*, 2001). Mutations of RP subunits that affect 26S proteasome function can decrease complex accumulation, reduce the rate of ubiquitin-dependent proteolysis, and also alter plant response to abiotic stresses (Smalle *et al.*, 2003; Smalle and Vierstra, 2004; Ueda *et al.*, 2004; Kurepa *et al.*, 2008). *Arabidopsis rpn10-1*, *rpn1a-4*, and *rpn1a-5* plants are less tolerant of salt stress (Smalle *et al.*, 2003; Wang *et al.*, 2009). *rpn10-1* plants are also hypersensitive to UV radiation and DNA damaging agents (Smalle *et al.*, 2003). *rpn1a-4*, *rpn1a-5, rpn10-1, rpn12a-1*, and *rpt2a-2* all exhibit decreased heat shock tolerance (Kurepa *et al.*, 2008; Wang *et al.*, 2009). The sensitivity of RP mutants to various abiotic stresses suggest that the 26S proteasome plays a crucial and general role during plant responses to adverse growth conditions.

With the identification of a growing number of E3 ligases that regulate abiotic stress responses, the mechanisms of E3 mode of action during stress signalling is becoming more defined (Figure 29; Table 2). E3 ligases may function by suppressing the stress signalling pathway during favourable growth conditions, by eliminating negative regulators of the stress signalling pathway in response to a stimulus, or by attenuating the signalling pathway to allow for further growth once conditions have improved (Figure 29). E3 ligases may also function within a positive feedback loop to enhance stress
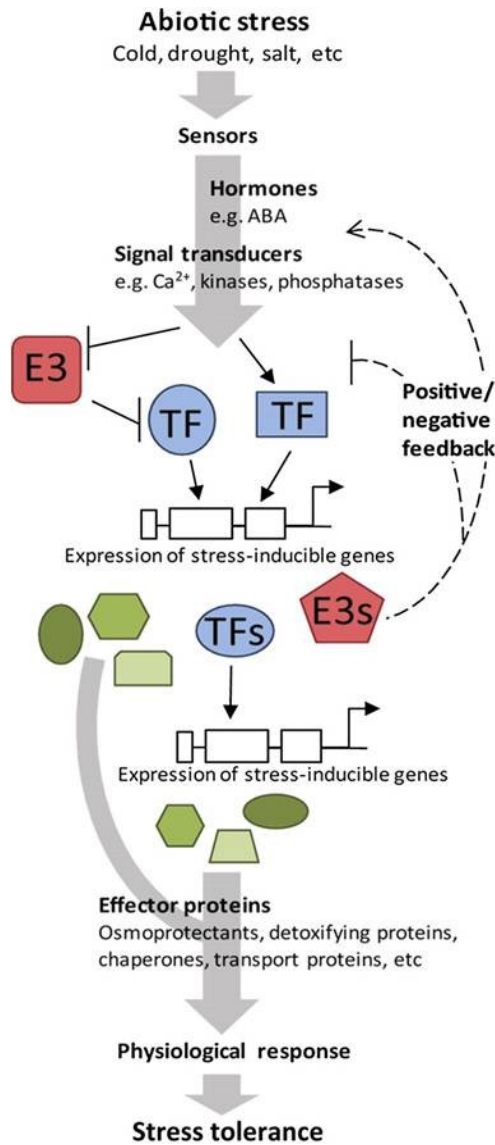
Figure 29. Regulation of abiotic stress signalling by E3 ligases. Plant perceive stress signal via sensors (unknown) and the signal is transduced via plant hormones, secondary messengers, and transcriptional regulators. The expression of stress-inducible genes is facilitated by transcription factors (TF) many of which are stress-regulated. E3 ligases tend to regulate components of the signalling pathway, mainly stress-responsive TFs. In the absence of a stress signal, E3 ligases may suppress the signalling pathway by, for example, promoting the degradation of a TF. E3 ligases may function within a feedback mechanism to enhance or attenuate the stress signal.

Table 2. Ubiquitination enzymes with known or predicted roles in ABA-dependent or independent stress tolerance.

Enzyme	Name	Species*	Biological Function	Target	References
E3					
RING	AIP2	*At*	ABA signalling	ABI3	Zhang *et al.*, 2005
	AIRP1	*At*	ABA-dependent drought tolerance		Ryu *et al.*, 2010
	BIRF1	*Os*	Drought and oxidative stress tolerance		Liu *et al.*, 2008
	BTS	*At*	Iron deficiency response	ILR-3?	Long *et al.*, 2010
	ATL31	*At*	Carbon and nitrogen stress		Sato *et al.*, 2009
	DRIP1/2	*At*	Drought stress tolerance	DREB2A	Qin *et al.*, 2008
	DSG1	*Os*	ABA signalling	ABI3	Park *et al.*, 2010
	HOS1	*At*	Cold stress tolerance	ICE1	Dong *et al.*, 2006
	KEG	*At*	ABA signalling	ABI5	Stone *et al.*, 2006
	NLA	*At*	Nitrogen deficiency stress		Peng *et al.*, 2007
	RFP1	*Ca*	Osmotic stress tolerance		Hong *et al.*, 2007
	RFP1	*Gm*	Cold, salinity and drought tolerance		Du *et al.*,2009
	RHA2a	*At*	ABA signalling		Bu *et al.*, 2009
	RING-1	*Os*	Drought and heat stress tolerance		Meng *et al.*, 2009
	Rma1	*At*	Drought stress tolerance	PIP1;2	Lee *et al.*, 2009
	Rma1H1	*Ca*	Drought stress tolerance	PIP1;2	Lee *et al.*, 2006
	SAP5	*At*	Drought and salinity stress tolerance		Kang *et al.*, 2011
	SDIR1	*At*	Drought and salinity tolerance, ABA signalling		Zhang *et al.*, 2007
	XERICO	*At*	Drought stress tolerance, ABA biosynthesis		Ko *et al.*, 2006
	ZF1	*Zm*	Drought and salinity stress tolerance		Huai *et al.*, 2009
	ZFP1	*Ad*	Drought stress tolerance		Yang *et al.*, 2008
CRL	DDB1	*At*	UV radiation tolerance	DDB2	Molinier *et al.*, 2008; Castells *et al.*, 2011
	FBP7	*At*	Cold temperature tolerance		Calderón-Villalobos *et al.*, 2007
	DWA1/2	*At*	ABA signalling	ABI5	Lee *et al.*, 2010
	DOR	*At*	Drought stress tolerance		Zhang *et al.*, 2008
U-box	CHIP	*At*	Temperature fluctuation tolerance	PP2A	Luo *et al.*, 2006
	PUB1	*Ca*	Drought and salinity stress tolerance	RPN6	Cho *et al.*, 2006
	PUB9	*At*	ABA signalling		Samuels *et al.*, 2008
	PUB15	*Os*	Oxidative stress tolerance		Park *et al.*, 2011
	PUB22/23	*At*	Drought and salinity stress tolerance	RPN12a	Cho *et al.*, 2008
E2	UBC2	*Ah*	Drought stress tolerance		Wan *et al.*, 2010
	UBC2	*Gm*	Drought and salinity stress tolerance		Zhou *et al.*, 2010
	UBC13	*At*	Iron deficiency response		Li *et al.*, 2010

* Species: *Ad - Artemisia desertorum*; *Ah - Arachis Hypogaea (*peanut*)*; *At - Arabidopsis thaliana*; *Ca - Capsicum annuum* (hot pepper); *Gm - Glycine max* (soybean); *Os - Oryza sativa* (rice); *Zm - Zea mays* (maize).

signalling (Figure 29). Not much is known about how abiotic stress signalling regulates the activity of these E3 ligases. In some cases, the expression and cellular localization of E3 ligases is stress-regulated (Ko *et al.*, 2006; Zhang *et al.*, 2007; Molinier *et al.*, 2008).

Known targets of the E3 ligases include many transcriptional regulators (Table 2). A typical example are the *Arabidopsis* DELLA proteins that repress gibberellin (GA) responses in the absence of the growth hormone. In the presence of bioactive GA, DELLA proteins are targeted for proteasomal degradation by the SCF$^{SLY/GID2}$ E3 ligase complex (Dill *et al.*, 2004). The level of bioactive GA is regulated by environmental conditions, suggesting that plants may utilize GA signalling to modulate DELLA protein stability and growth in response to abiotic stresses (Yamauchi *et al.*, 2004; Achard *et al.*, 2006). Other potential targets of E3 ligases may include stress hormone biosynthesis enzymes and effector proteins that mediate tolerance of abiotic stresses. Proteome analysis of the UPS system from *Arabidopsis* identified a number of different types of stress related proteins (Manzano *et al.*, 2008; Igawa *et al.*, 2009). In fact, the majority of ubiquitinated proteins that were isolated in each study were functionally categorized as stress response or abiotic stress proteins. The fact that plants place such an emphasis on the UPS to facilitate abiotic stress response is not surprising. The UPS allows for rapid and efficient responses to abiotic stresses by regulating stress hormone biosynthesis and the abundance of regulatory proteins.

Regulation of abscisic acid-dependent stress signalling requires multiple E3s

The phytohormone abscisic acid (ABA) functions during adaptive response to environmental stresses. ABA regulates seed maturation and prolongs dormancy to ensure that seeds germinate under conditions favourable to growth and development. Immediately following germination, ABA suspends the growth of young seedlings exposed to abiotic stresses such as salinity or drought. Seedling development is slowed until better environmental conditions arise. In adult plants, ABA mediates various protective responses that help to alleviate stress-induced damage (Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003). A well-studied ABA-mediated event is the regulation of stomatal closure in response to drought stress. During times of water scarcity ABA prevents transpirational water loss by promoting stomatal closure (Hetherington, 2001).

Perception of environmental stimuli leads to increased biosynthesis and accumulation of ABA (Cutler and Krochko, 1999; Taylor *et al.*, 2000). ABA triggers intracellular signalling which culminates in the expression of ABA-responsive genes. Transcriptional analyses of ABA-responsive genes identified hundreds of genes that are either up- or down-regulated in response to ABA (Hoth *et al.*, 2002; Seki *et al.*, 2002). Changes in gene expression generated by drought and high salinity are mediated by ABA-responsive transcription factors such as the basic leucine zipper (bZIP) transcriptional activators. These transcriptional activators interact with the ABA-regulatory elements (ABRE) found in the promoter of stress-responsive genes (Hattori *et al.*, 2002; Narusaka *et al.*, 2003). The UPS regulates ABA-responsive transcription by modulating the abundance of these transcription factors.

The observation that ABA promotes the accumulation of the short-lived bZIP transcription factor ABSCISIC ACID INSENSITIVE 5 (ABI5) provided evidence for UPS involvement in regulating ABA signalling (Uno *et al.*, 2000; Lopez-Molina *et al.*, 2003; Smalle *et al.*, 2003). Ubiquitinated ABI5 accumulates in seedlings treated with proteasome inhibitors and ABI5 is stabilized in *rpn10-1* (Lopez-Molina *et al.*, 2003; Smalle *et al.*, 2003). The ABA-dependent stabilization of ABI5 is proposed to serve as an early developmental checkpoint to delay growth during adverse environmental conditions (Lopez-Molina *et al.*, 2001). This proposal is based on the fact that ABA is able to induce ABI5 protein accumulation and seedling growth arrest only within a short period of time following germination (Lopez-Molina *et al.*, 2001). In addition, ABI5 protein accumulation is also induced by salt and drought stress. These observations also support the notion that under favourable growth conditions the UPS is required to maintain low levels of ABI5 and thus permits growth.

Significant strides have been made in understanding the role of ubiquitination in regulating ABI5 function. E3 ligases Keep on Going (KEG), DWD hypersensitive to ABA 1 (DWA1) and DWA2 have been implicated in modulating ABI5 protein abundance (Figure 30) (Stone *et al.*, 2006; Liu and Stone, 2010; Lee *et al.*, 2010). KEG is a large multi-domain protein that contains functional RING and kinase domains followed by a series of ankyrin and HERC2-like repeats that facilitate protein–protein interactions (Stone *et al.*, 2006; Gu and Innes, 2011). KEG is a negative regulator of ABA signalling
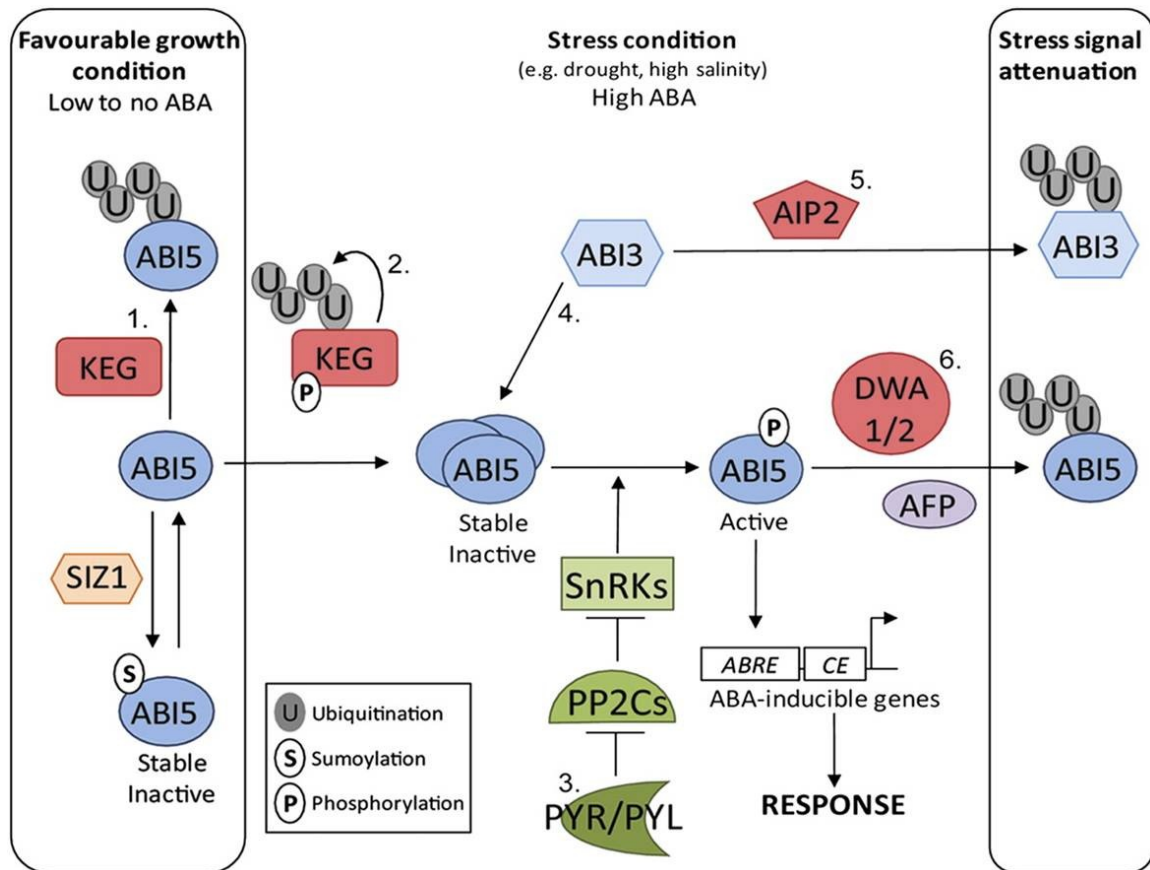
Figure 30. Ubiquitin-mediated regulation of the ABA signalling. Under normal growth conditions (in the absence of stress) KEG is involved in preventing ABI5 accumulation while SIZ1 sumoylation maintains a small pool of inactive ABI5 (1). Under stressed conditions ABA levels increase. ABA promotes the self-ubiquitination and degradation of KEG. Reduction in KEG protein levels assist in the accumulation of ABI5 protein levels (2). ABA binds to its receptor (PYR/PYL/RCAR) which inactivates PP2C resulting in SnRK activation and phosphorylation of ABA-responsive transcription factors such as ABI5 (3). Activated ABI5 promotes the expression of ABA-inducible genes which mediate various responses including post-germinative growth arrest. ABI3 function upstream of ABI5 (4). ABA induces expression of AIP2 which promote the degradation of ABI3 (5). ABI5 is turned over via ubiquitination by DWA1/2 (6). AFP may also be required for the degradation of ABI5. The ubiquitin-mediated degradation of ABI3 and ABI5 attenuate the ABA signal.

and is required for maintaining low levels of ABI5 in the absence of ABA (Stone *et al.*, 2006; Liu and Stone, 2010). *KEG* mutants (*keg-1/2/3*) are hypersensitive to ABA, accumulate extremely high levels of ABI5 and undergo growth arrest shortly after germination. KEG mediates ABI5 ubiquitination *in vitro* and the reduction of ABI5 protein levels in *keg* mutants is dependent on the presence of a functional KEG RING domain (Liu and Stone, 2010). The fact that ABI5 accumulates in *keg* seedlings without ABA treatment suggests that KEG targets ABI5 for degradation to suppress ABI5-dependent post-germinative growth arrest in the absence of the hormone.

The mechanism of ABA-dependent stabilization of ABI5 is not very well understood. However, a recent study by Liu and Stone (2010) has proposed a possible mechanism. As observed with many E3 ligases, KEG is capable of autoubiquitination (Stone *et al.*, 2006). The autocatalytic process can serve as a negative regulatory mechanism leading to the down-regulation of the E3 via degradation by the 26S proteasome (Fang *et al.*, 2000). ABA may manipulate this intrinsic ability of KEG to promote autoubiquitination and subsequent degradation. In the presence of ABA the turnover of KEG protein increases significantly (Liu and Stone, 2010). Mutations in KEG's RING domain and inhibition of 26S proteasome activity prohibit ABA-induced degradation of KEG (Liu and Stone, 2010). These results suggest that ABA promotes the accumulation of ABI5 by reducing KEG protein levels via self-ubiquitination and degradation by the 26S proteasome (Figure 30). The mechanism wherein ABA directs KEG towards self-ubiquitination over substrate ubiquitination remains to be determined. Mutations within KEG's kinase domain or treatments with kinase inhibitors also inhibit ABA-induced ubiquitination and degradation of KEG suggesting that phosphorylation may be involved in this process. Phosphorylation has been shown to regulate E3 ligase activity via modification of the substrate or the E3 ligase itself. In some cases, phosphorylation of E3 ligases, such as Parkin, promotes enzyme activation (Sha *et al.*, 2010). In contrast, phosphorylation of other E3s, such as Mdm2, leads to down-regulation of the E3 and substrate accumulation (Cheng *et al.*, 2009).

DWA1 and DWA2 both function as the substrate recruiting component of a CUL4 based CRL (Lee *et al.*, 2008). DWA1 and DWA2 are also responsible for targeting ABI5 for proteasome-dependent degradation and may do so to attenuate the stress

signalling pathway (Figure 30). ABI5 is more stable in ABA treated *dwa1 dwa2* seedlings compared with the wild type and *DWA1 DWA2* mutants display hypersensitivity to ABA. This is consistent with the model of DWA1 and DWA2 acting as negative regulators of ABA signalling (Lee *et al.*, 2010). Interestingly, ABI5 does not accumulate in *dwa1 dwa2* in the absence of ABA. This is in contrast to *keg* mutants which accumulates extremely high levels of ABI5 without the application of ABA. KEG may function to maintain low levels of ABI5 in the absence of ABA and abiotic stress, while DWA1 and DWA2 may function to attenuate ABA signalling so that plants can readily re-establish growth once environmental conditions improve (Figure 30).

Adding to the complexity of the ubiquitin-mediated regulation of ABI5 is the ABI5 binding protein (AFP). Upon ABA treatment, an increase in AFP protein levels closely follows that of ABI5 (Lopez-Molina *et al.*, 2003). Co-expression of AFP with ABI5 promotes the localization of both proteins to nuclear bodies. Even though AFP is not an E3 ligase it has been proposed to promote the proteasomal degradation of ABI5 (Lopez-Molina *et al.*, 2003). DWA1 and DWA2 interact with each other in the nucleus, although not in nuclear bodies (Lee *et al.*, 2010). It is possible that AFP may facilitate DWA1/2-mediated degradation of ABI5 (Figure 30). Recently, the relationship between ABI5 and AFP has been proposed to be at the level of transcription. The AFP family of proteins (AFP1–4) are similar in domain organization to the adaptor protein Novel Interactor of JAZ (NINJA) that represses expression of jasmonoyl-isoleucine responsive genes by facilitating interactions between jasmonate ZIM-domain (JAZ) repressor proteins and the co-repressor TOPLESS (TPL) (Pauwels *et al.*, 2010). Similarly, AFP interacts with TPL and may function to recruit TPL to ABI5 and generate a transcriptional complex that represses the expression of ABA-responsive genes (Pauwels *et al.*, 2010).

Other ABA-responsive transcription factors are also regulated by the UPS. ABI3, a B3-type transcription factor, functions upstream of ABI5 to mediate ABA-dependent processes (Finkelstein and Lynch, 2000; Lopez-Molina *et al.*, 2002). ABI3 protein is unstable in most stages of plant development but does accumulate during specific developmental windows (Lopez-Molina *et al.*, 2001, 2002; Zhang *et al.*, 2005). The RING-type E3 ABI3-Interacting Protein 2 (AIP2) is required for the ubiquitin-mediated

degradation of ABI3 (Figure 30). Zhang *et al.*, (2005) demonstrated that ABA promotes the expression of AIP2 which results in a reduction in ABI3 protein levels. Similar to DWA1/2, AIP2-mediated degradation of ABI3 may function to attenuate ABA signalling.

Although there is no direct evidence of ubiquitination, studies suggests that ABA-responsive transcription factors ABI4, ABRE Binding Factor 2 (ABF2) and ABF3 are also regulated by the UPS. ABI4 has long been known to regulate ABA signalling (Finkelstein, 1994). However, evidence that ABI4 may be regulated by the UPS has only recently emerged. The abundance of ABI4 was observed by examining the activity of the *β-glucuronidase* (*GUS*) reporter in plants over-expressing a *ABI4–GUS* transgene. The activity of GUS (and therefore ABI4 protein level) increased after treatment with proteasome inhibitors (Finkelstein *et al.*, 2011).

Arm Protein Repeat Interacting with ABF2 (ARIA), a BTB protein which may function as a component of a CRL, interacts with ABF2 and both share a similar gene expression pattern (Kim *et al.*, 2004). Consistent with the hypothesis that ARIA regulates ABF2 and, therefore, ABA responses, *ARIA* over-expressing plants displayed hypersensitivity to ABA and *ARIA* mutants are insensitive to ABA (Kim *et al.*, 2004).

The ABF3 protein levels are stabilized by the application of exogenous ABA or the inhibition of proteasome activity. Phosphorylation of ABF3 by Open Stomata 1 (OST1) is involved in the ABA-mediated stabilization of ABF3 (Sirichandra *et al.*, 2010). OST1 is a member of the Suc non-fermenting1-related protein kinase subfamily 2 (SnRK2) (Yoshida *et al.*, 2002). SnRK2s along with the ABA receptor family, Pyrabactin resistance 1(PYR1)/PYR1-like (PYL)/Regulatory component of ABA receptor (RCAR), and clade A Protein Phosphatase type 2Cs (PP2Cs) represent the core regulatory network of the ABA signalling pathway (Weiner *et al.*, 2010) (Figure 30). Under non-stress conditions SnRK2s are inhibited by PPC2 driven dephosphorylation. An increase in ABA levels result in ABA-bound PYR/PYL/RCAR receptors binding to and inhibiting the activity of PP2Cs leading to the activation of SnRK2s (Weiner *et al.*, 2010) (Figure 30). The ABA-activated SnRK2s phosphorylate transcription factors and possibly other regulatory proteins that regulate the expression of ABA-responsive genes. In response to ABA, OST1 phosphorylates ABF3 within a 14-3-3 protein binding motif found in most

ABF proteins (Sirichandra *et al.*, 2010). Mutant ABF3 protein lacking the 14-3-3 phosphorylation site is only detected after plants are treated with proteasome inhibitors (Sirichandra *et al.*, 2010). This study suggests that phosphorylation by the ABA-activated SnRK2 is required for stabilization of ABF3 and this may be accomplished via binding of a 14-3-3 protein. More importantly, this study demonstrates that the role of ABA-activated kinases is not limited to the activation of transcription factors, but they may also be required for stability.

In addition to the above mentioned ubiquitin ligases there is a growing list of E3s that function in response to ABA but targets remain to be identified (Table 2). These E3 ligases were isolated via efforts to identify stress-responsive genes. Some E3 ligases have received attention because their mRNA transcript abundance is regulated by stress and/or ABA. Other E3s have surfaced in screens for mutants with aberrant ABA-related phenotypes. Examples of E3 ligases in these categories are Salt and Drought Induced RING Finger 1 (SDIR1), *Arabidopsis thaliana* ABA-insensitive RING protein 1(AtAIRP), RING-H2 E3 ligase (RHA) 2a, RHA2b, Drought tolerance repressor (DOR), and XERICO.

RING-type E3 ligases SDIR1, AtAIRP1, RHA2a, and RHA2b are positive regulators of ABA signalling. Plants lacking these RING-type E3 encoding genes are insensitive to ABA while transgenic over-expressing plants are hypersensitive to the effects of ABA (Zhang *et al.*, 2007; Bu *et al.*, 2009;Ryu *et al.*, 2010; Li *et al.*, 2011). Over-expression of *SDIR1*, *AtAIRP1*, or*RHA2b* enhances drought tolerance via an increase in ABA-induced stomatal closure (Zhang *et al.*, 2007; Ryu *et al.*, 2010; Li *et al.*, 2011). SDIR1 is a salt and drought stress-regulated membrane bound protein that functions upstream of ABA-responsive transcription factors (Zhang *et al.*, 2007). Expression of *AtAIRP* is induced by ABA, cold, salt, and drought stresses (Ryu *et al.*, 2010). Interestingly, ABA-induced expression of *AtAIRP* does not occur in the SnRK2 triple mutant, *srk2d/snrk2.2 srk2e/snrk2.6/ost1 srk2i/snrk2.3*, suggesting that expression of *AtAIRP* in response to stress is regulated via the ABA-activated protein kinases (Fujita *et al.*, 2009; Ryu *et al.*, 2010). AtAIRP is also suggested to act upstream of ABA-responsive transcription factors (Ryu *et al.*, 2010). RHA2a and RHA2b function

redundantly and parallel to ABA-responsive transcription factors such as ABI3 and ABI5 (Bu *et al.*, 2009; Li *et al.*, 2011).

The F-box protein DOR functions as a negative regulator of ABA-mediated stomata closure (Zhang *et al.*, 2008). *DOR* mutant plants display enhanced drought tolerance and accumulate higher levels of ABA than the wild type in response to drought. DOR can associate with, two CRL subunits, ASK14 and CUL1. Transcriptome analysis of *dor* plants under drought stress revealed that a variety of ABA biosynthesis genes and ABA-responsive genes were up-regulated compared with wild type. In particular, a key enzyme in ABA biosynthesis, *9-cis-epoxycarotenoid dioxygenase 3*(NCED3), is significantly up-regulated in the *DOR* mutants. RING-type E3 XERICO is another ubiquitin ligase with links to ABA biosynthesis. Over-expression of *XERICO* resulted in drought-tolerant plants that were hypersensitive to ABA and accumulated more cellular ABA than wild type (Ko *et al.*, 2006). Interestingly, the accumulation of ABA in *XERICO* over-expressing plants occurs without a concomitant increase in the expression of ABA biosynthetic genes such as *NCED3* (Ko *et al.*, 2006). In addition, compared with wild type, a stronger, more sustained, expression of *NCED3* was observed in *XERICO* over-expressing plants following ABA treatment. This suggests that XERICO acts post-translationally to regulate ABA biosynthesis.

E3 ligase function during drought and salt stress

The abundance of the drought-responsive transcription factor Dehydration-responsive Element Binding Protein 2A (DREB2A) is regulated by two RING-type E3 ligases, DREB2A Interacting Protein 1 (DRIP1) and DRIP2 (Qin *et al.*, 2008) (Figure 31). DREB2A is usually unstable but accumulates during dehydration stress suggesting regulation by the UPS (Sakuma *et al.*, 2006*a, b*). DRIP1 mediates DREB2A ubiquitination *in vitro* and DREB2A protein levels are more stable in *drip1-1* plants compared with wild-type (Qin *et al.*, 2008). In addition, DREB2A accumulates upon inhibition of the 26S proteasome activity. *DRIP1 DRIP2* double mutants displayed enhanced drought tolerance which coincided with a significant increase in the expression of a number of drought-inducible genes specifically genes regulated by DREB2A (Qin *et al.*, 2008). In the absence of stress stimuli, DRIP1 and DRIP2 function redundantly to suppress drought signalling via the ubiquitin-mediated proteolysis of DREB2A.
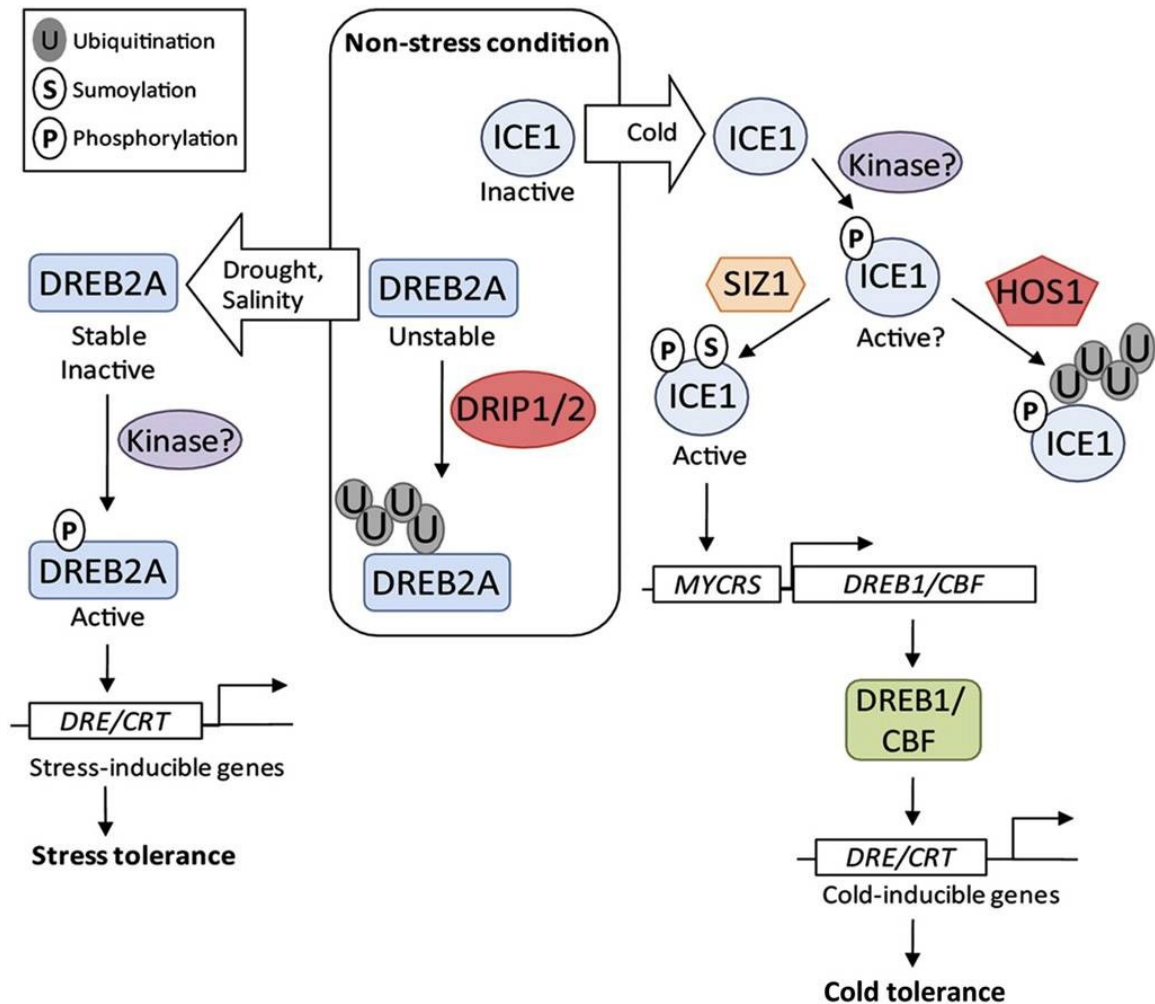
Figure 31. Ubiquitin-mediated regulation of ABA-independent responses to drought, high salinity, and cold stresses. Under normal growth conditions, the DREB2A transcription factor is unstable due to DRIP1/2-mediated ubiquitination. During drought and salinity stress, DREB2A is stabilized, activated via phosphorylation, and initiates transcription of stress-inducible genes. Expression of cold-responsive genes is mediated by the ICE transcription factor. Activation of ICE under cold conditions requires phosphorylation and SIZ1-mediated sumolyation. Under cold conditions ICE1 is targeted for degradation via HOS1-mediated ubiquitination. Ubiquitin-mediated degradation of ICE1 may serve to attenuate the signal to ensure the transient expression of cold-responsive genes.

DREB2A instability is due to a serine and threonine-rich 30–amino acid negative regulatory domain (Sakuma *et al.*, 2006*a*, *b*). Deletion of the negative regulatory domain stabilized DRBE2A indicating the presence of a degron. A degron is an amino acid sequence that serves as a signal for ubiquitin-mediated degradation (Varshavsky, 1991). The DREB2A degron may facilitate degradation of DREB2A under favourable growth conditions while it would be made unavailable to the degradation machinery under stress conditions. DREB2A protein would then accumulate and regulate the expression of stress-responsive genes. Another interesting untested possibility is that since stress conditions do not affect DRIP1/2 transcript levels, DREB2A accumulation may occur as a result of drought-induced relocalization of DRIP1/2.

The RING membrane-anchor 1 homologue 1 (Rma1H1) was originally identified as a dehydration-regulated gene in *Capsicum annuum* (hot pepper; Park *et al.*, 2003). Correspondingly, over-expression of *Rma1H1*in *Arabidopsis* enhanced drought tolerance (Lee *et al.*, 2009). A potential target for Rma1H1 is the plasma membrane aquaporin PIP2;1. Aquaporins have been suggested to enhance symplastic water transport which has a negative impact on plants during water stress (Jang *et al.*, 2004;Alexandersson *et al.*, 2005). In protoplasts co-transformed with *PIP2;1*and *Rma1H1* the protein level of PIP2;1 was lower than when PIP2;1 was transformed alone. The reduction of PIP2;1 protein levels could be blocked by treatment with proteasome inhibitors suggesting Rma1H1-mediated degradation of PIP2;1 via the 26S proteasome (Lee *et al.*, 2009). *Rma1H1*has three *Arabidopsis* homologues, *Rma1*, *Rma2*, and *Rma3* (Lee *et al.*, 2009). One striking difference between hot pepper Rma1H1 and its *Arabidopsis* counterparts is that while Rma1H1 was relatively stable in transgenic plants Rma1 is only detectable after inhibition of the 26S proteasome, suggesting that Rma1 is itself regulated by the UPS (Lee *et al.*, 2009). Similar to Rma1H1, *Rma1* over-expression reduced PIP2;1 levels in co-transfected protoplasts. Lee *et al.* (2009) propose a model in which Rma1H1 and Rma1 promote dehydration tolerance by mediating the degradation of aquaporins that may promote symplastic water transport.

Another hot pepper drought stress-inducible E3 encoding gene is *CaPUB1*.Unlike Rma1H1, over-expression of *CaPUB1* renders transgenic *Arabidopsis* plants more sensitive to salt and drought stress (Cho *et al.*, 2006). Comparison of the protein profiles

of wild type to *CaPUB1* over-expressing plants identified RPN6 as a potential substrate for the U-box-type E3. Subsequent experiments revealed that CaPUB1 was able to interact with and ubiquitinate RPN6. The significance of RPN6 ubiquitination by CaPUB1 is still unclear. One proposal is that ubiquitin-dependent regulation of RPN6 may regulate the activity of the 26S proteasome during drought stress response (Kurepa *et al.*, 2009). *Arabidopsis* PUB22 and PUB23 are homologues of CaPUB1 (Cho *et al.*, 2008). Similar to CaPUB1, over-expression of *PUB22* or *PUB23* rendered transgenic plants more sensitive to drought and salt stress, while *pub22 pub23* were very tolerant of drought and salt stress. PUB22 and PUB23 interact with and ubiquitinate RPN12a (Cho *et al.*, 2008). The importance of this interaction is unknown but it is possible that, similar to the ubiquitination of RPN6 by CaPUB1, ubiquitination of RPN12a may influence the properties of the 26S proteasome (Kurepa *et al.*, 2009). In *PUB22* or *PUB23* over-expressing transgenic plants RPN12a associates with a wide range of protein complexes (200 kDa to 900 kDa) (Cho *et al.*, 2008). In wild-type plants, RPN12a is only found within a specific protein complex (800 kDa to 900 kDa) that is consistent with the size of the RP. Interestingly, in drought-stressed plants, RPN12a associate with complexes that are similar in size to those of the *PUB22* or *PUB23* over-expressing plants (Cho *et al.*, 2008). During drought stress or increased expression of PUB22 or PUB23, the subunit composition of the PR seems to change and this may somehow influence the activity of the 26S proteasome.

Response to temperature fluctuations is mediated by ubiquitination

Inducer of CBF Expression 1 (ICE1), a MYC transcription factor, controls the expression of cold-responsive transcription factor CBF3/DREB1A, that regulates the transcription of numerous cold-responsive genes (Figure 31). The expression of *ICE1*, which is normally constitutive, is up-regulated in response to cold temperatures. Over-expression of *ICE* leads to increased expression of its target genes but only under cold conditions (Chinnusamy *et al.*, 2003). This implies that cold signalling not only increases *ICE* expression but also activates the protein (Figure 31). Another consequence of cold exposure is the reduction in ICE protein levels. The cold-mediated decrease in ICE abundance can be blocked by proteasome inhibitors which implicates the UPS (Dong *et al.*, 2006). Direct regulation of cold signalling by the UPS was confirmed by the

identification of RING-type E3 High Expression of Osmotically Responsive Gene 1 (HOS1) as a mediator of ICE1 ubiquitination and subsequent degradation (Figure 31). Consistent with a role in regulating ICE protein abundance *HOS1* over-expression results in reduced tolerance of freezing conditions as well as a decrease in the expression levels of ICE1 target genes. (Xiong *et al.*, 2002; Dong *et al.*, 2006). Although HOS1 contains a variant RING domain, it is capable of catalysing ICE1 ubiquitination *in vitro* and *in vivo* (Lee *et al.*, 2001; Stone*et al.*, 2005; Dong *et al.*, 2006). Degradation of nuclear localized ICE1 is facilitated by cold-induced relocalization of HOS1 from the cytoplasm to the nucleus (Lee *et al.*, 2001; Dong *et al.*, 2006). The HOS1-mediated degradation of ICE1 in response to cold may seem contradictory at first but cold-responsive genes are only transiently induced by cold treatment (Chinnusamy *et al.*, 2003). Phosphorylation provides another level of regulation of ICE1 activity. Mutation of a potential phosphorylation site, Serine 403, increased transactivational activity, prohibited cold-induced degradation of ICE1 and enhanced freezing tolerance (Miura *et al.*, 2011). Stabilization of the ICE1 mutant against cold-induced degradation is due to inhibition of polyubiquitination (Miura *et al.*, 2011). Surprisingly, the mutation does not hinder HOS1-mediated ubiquitination of ICE1 *in vitro*. Phosphorylation seems to indirectly affect ICE1 stability possibly through another post-translational mechanism that modulates UPS regulation of ICE1. In any event, phosphorylation of ICE1 is involved in regulating protein activation and stabilization (Figure 31).

Arabidopsis thaliana Carboxyl Terminus of Hsc70-Interacting Protein (AtCHIP) is a U-box-type E3 ligase named for its sequence similarity to mammalian co-chaperone CHIP which targets non-native or damaged proteins for degradation by the 26S proteasome (Meacham *et al.*, 2001;Murata *et al.*, 2001). Since cold and heat stress induce expression of*AtCHIP* one would predict that the E3 facilitates stress tolerance by targeting denatured and damaged proteins for degradation. On the contrary, over-expression of *AtCHIP* actually renders plants more sensitive to temperature stress (Yan *et al.*, 2003). An explanation put forward byYan *et al.* (2003) is that high levels of AtCHIP protein facilitate the rapid turnover of misfolded proteins that could otherwise be refolded into functional proteins by the chaperone system. Identified AtCHIP substrates include A3 and RCN1, which are subunits of Protein Phosphatase 2A (PP2A) (Luo *et al.*, 2006).

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A3 and RCN1 protein levels are not altered in *AtCHIP* over-expressing plants, which is consistent with AtCHIP mono-ubiquitination of both proteins *in vitro*. Under cold temperatures, higher phosphatase activity was observed in *AtCHIP* over-expressing plants, which further suggest that AtCHIP-mediated ubiquitination of PP2A may serve a non-proteolytic function.

UV stress tolerance requires a Cullin RING ligase

Plants benefit from and require sunlight for photosynthesis but, at the same time, they must also protect themselves from damage caused by ultraviolet (UV) radiation. Two basic mechanisms are used by plants to repair DNA damage, photoreactivation and nucleotide excision repair (NER) (Tuteja *et al.*, 2009). Repair of UV-induced damaged DNA through the NER pathway involves a CUL4-DDB1 CRL (Groisman *et al.*, 2003; Wittschieben *et al.*, 2005). DDB2, which is turned over after UV exposure, is a target of CUL4-DDB1 E3 ligase activity (Molinier *et al.*, 2008). DDB2 is localized to the nucleus where it binds to bulky DNA lesions caused by UV radiation and presumably recruits NER machinery to the lesions (Luijsterburg *et al.*, 2007; Molinier *et al.*, 2008). Under non-stress conditions DDB1 is localized to the cytoplasm (Molinier *et al.*, 2008). Following UV radiation DDB1 is recruited into the nucleus where it promotes the degradation of DDB2. The reduction in DDB2 protein levels does not occur if components of the CRL, CUL4 or DDB1, are non-functional. CUL4-DDB1 mediated removal of DDB2 from the DNA lesion may be required to permit access of the NER machinery to the lesion. UV-induced degradation of DDB2 by CUL4-DDB1 is facilitated by the Ataxia Telangiectasia-mutated and Rad3-related (ATR) protein kinase that transmits DNA damage signal and by De-etiolated 1 (DET1). Following UV exposure, ATR promotes the nuclear localization of DDB1, which is a prerequisite for DDB2 degradation (Molinier *et al.*, 2008). DDB2 is not degraded in UV-treated *det1* plants (Castells *et al.*, 2011). Adding to the complexity, UV-induced CUL4-DDB1-dependent degradation of DET1 occurs along with DDB2. The purpose for DET1 degradation and DET1 involvement in DDB2 degradation is not clear.

Ubiquitination and plant response to nutrient deprivation

Nitrogen is an essential macronutrient that contributes to plant biomass and influences various aspects of plant development. Plants adapt to low nitrogen availability by redistributing nitrogen from mature to younger actively growing organs and increasing accumulation of anthocyanin (Miller *et al.*, 2007). The RING-type E3 Nitrogen Limitation Adaptation (NLA) is a positive regulator of adaptive response to low nitrogen (Peng *et al.*, 2007). *NLA* mutants are hypersensitive to the effects of low nitrogen conditions and senesce much earlier than the wild type under the same conditions. Metabolite profiling suggests that *nla* plants are able to acquire nitrogen but fail to adapt to low nitrogen conditions by redirecting nitrogen from old to new tissue or by accumulating anthocyanin (Peng *et al.*, 2007).

After germination, nutrient availability determines if the seedling transits through the post-germinative developmental checkpoint described byLopez-Molina *et al.* (2001). A high level of glucose stalls development while an increase in nitrogen and glucose permits growth. This demonstrates the importance of the ratio between carbon and nitrogen during this stage and the characterized carbon/nitrogen (C/N) response (Coruzzi and Zhou, 2001). *Arabidopsis* plants grown under high concentrations of glucose and low concentrations of nitrogen (severe C/N stress) arrest growth post-germination and do not survive (Martin *et al.*, 2002). Over-expression of RING-type E3 *ATL31/*Carbon-Nitrogen Insensitive 1-dominant (*CNI1*) rendered plants insensitive to C/N stress and these transgenic plants were able to pass through the early checkpoint despite the stress conditions (Sato *et al.*, 2009). Conversely, *ATL31* mutants grown under C/N stress are unable to progress through the post-germinative checkpoint. Similarly, mutations in the closely related *ATL31* genes, *ATL2* and *ATL6*, also produce hypersensitivity to C/N stress (Sato *et al.*, 2009). ATL31 is a functional E3 ligase and may function to reduce the level of proteins that stall growth during this checkpoint.

Iron is an essential nutrient facilitating photosynthesis, chlorophyll biosynthesis and a variety of redox reactions. *Arabidopsis* responds to iron-limiting conditions by up-regulating the expression of bHLH transcription factors such as Fer-like Iron Deficiency-Induced Transcription Factor (FIT), Popeye (PYE), and PYE homologue IAA-Leu Resistant-3 (ILR-3). These transcription factors induce expression of genes required for

increasing iron availability and maintaining iron homeostasis (Colangelo and Guerinot, 2004; Rampey *et al.*, 2006; Yuan *et al.*, 2008; Long *et al.*, 2010; Lingam *et al.*, 2011) A recent study demonstrating a link between ethylene signalling and the iron-deficient stress response provided evidence for the involvement of the UPS in regulating FIT protein levels (Lingam *et al.*, 2011). FIT protein accumulates in response to iron-deficiency stress. Inhibition of ethylene biosynthesis via aminoethoxyvinylglycine (AVG) treatment during iron-deficiency stress prohibits FIT accumulation suggesting that ethylene signalling is required for the stabilization of FIT protein (Lingam *et al.*, 2011).

Although ubiquitin ligase activity remains to be experimentally demonstrated, the RING type E3 ligase Brutus (BTS) is proposed to regulate the abundance of ILR-3 (Long *et al.*, 2010). *BTS* along with *PYE* were identified in cell type specific transcription profiling as genes induced during iron deficiency stress. Unlike *pye-1*, *BTS* partial loss of function mutation rendered plants more tolerant to iron deficiency, suggesting that BTS is a positive regulator of the iron-deficient stress response. It is also worth noting that complete loss of function of *BTS* is lethal under normal growth conditions suggesting that BTS function is not limited to iron homeostasis (McElver *et al.*, 2001). The opposing effects of *pye* and *bts-1* during iron-deficient stress suggest that BTS may regulate the abundance of PYE (Long *et al.*, 2010). Surprisingly, BTS does not interact with PYE but it does interact with ILR3, a potential dimerizing partner of PYE. BTS may influence the stability of ILR-3 during iron-deficient stress and therefore indirectly affect the activity of PYE (Long *et al.*, 2010).

E2 ubiquitin conjugating enzymes and abiotic stress tolerance

Research into plant ubiquitination has focused mainly on E3 ligases and therefore considerably less is known about the biological relevance of the E1 and E2 enzymes during abiotic stress tolerance. Recent analysis of E2 function demonstrated a requirement for these enzymes during abiotic stress response. Over-expression of E2 enzymes from *Glycine max* (soybean; *GmUBC2*) and *Arachis hypogaea* (peanut; *AhUBC2*) in *Arabidopsis* enhanced tolerance of drought stress (Wan *et al.*, 2010; Zhou *et al.*, 2010). In addition, *GmUBC2* is up-regulated in response to drought and salt stress and *AhUBC2* is up-regulated during drought conditions. *Cucumis sativus* UBC13

(cucumber; *CsUBC13*) accumulates under iron-deficient conditions (Li and Schmidt, 2010). Plants respond to iron scarcity by increasing root surface area. *Arabidopsis* plants respond by forming branched root hairs and over-expression of *CsUBC13* in *Arabidopsis* plants enhanced this response (Li and Schmidt, 2010). Conversely, plants carrying mutations in the *Arabidopsis* orthologues, *UBC13A* and *UBC13B*[also referred to as UBC35 and UBC36, respectively (Kraft *et al.*, 2005)]*,* did not produce branched root hairs in response to iron-deficient conditions.

Ubiquitin-like proteins in abiotic stress tolerance

Eukaryotic cells employ a variety of small polypeptides as post-translational regulators of protein function. In addition to ubiquitin, plants utilize a number of ubiquitin-like proteins such as Related to ubiquitin 1 (RUB1)*,* Small ubiquitin-like modifier (SUMO) and Ubiquitin fold modifier (UFM), and Homology to ubiquitin (HUB) (Miura and Hasegawa, 2010). In contrast to ubiquitin, where the major function is to facilitate protein degradation, the ubiquitin-like proteins function as modifiers regulating protein activity, subcellular localization, and protein–protein interactions.

The conjugation of SUMO to target proteins increases dramatically in response to various stresses including cold, drought, heat, metal toxicity (copper) and nutrient deprivation (Miura *et al.*, 2005; Catala *et al.*, 2007;Saracco *et al.*, 2007; Chen *et al.*, 2011). Mutations which impair SUMO conjugation decrease tolerance of these stresses (Miura *et al.*, 2005; Chen *et al.*, 2011). Sumolyation is similar to ubiquitination in that it also utilizes the sequential action of three enzymes, E1, E2, and E3, to attach SUMO to an internal lysine of target proteins. In *Arabidopsis*, the pathway is initiated by an E1 heterodimer, SUMO-activating enzyme 1(SAE1), and SAE2 which together is equivalent to the ubiquitin E1, UBA1 (Miura and Hasegawa, 2010). Unlike ubiquitination, only a single E2 SUMO-conjugating enzyme (SCE1) is used by the pathway (Saracco *et al.*, 2007). Few SUMO E3s have been identified to date including SIZ1 [for SAP (scaffold attachment factor, acinus, protein inhibitor of activated signal transducer and activator of transcription) and Miz1 (Ms×2-interacting zinc finger) domain] and High Ploidy2 (HPY2)/Methyl Methane Sulphonate Sensitivity21 (MMS21) (Miura *et al.*, 2005; Ishida *et al.*, 2009; Huang *et al.*, 2009). Substrates identified for *Arabidopsis* SIZ1 include transcription factors phosphate starvation response 1 (PHR1) (phosphate starvation),

ABI5 (ABA signalling), ICE1 (cold signalling), and flowering locus D (FLD) (flowering time) (Miura *et al.*, 2005, 2009, 2011; Jin *et al.*, 2008). Conjugation of SUMO to ABI5 by SIZ1 prohibits ABI5 turnover (Miura *et al.*, 2009). Substitution of ABI5 Lys319 for arginine blocks sumolyation and further destabilizes ABI5 indicating that sumoylated ABI5 is not a suitable substrate for ubiquitination. Miura *et al.* (2009) suggests that sumoylation results in the accumulation of an inactive form of ABI5. Desumolyation of ABI5 provides a readily available pool of ABI5 that can be activated by phosphorylation upon initiation of ABA signalling (Figure 30). Conjugation of SUMO to ICE1 seems to be required for transcriptional activity repressing ubiquitination and enhancing stability (Figure 31) (Miura *et al.*, 2011). Blocking ICE1 sumolyation via a Lys393 mutation decreased freezing tolerance and reduced cold-induced expression of ICE1 target genes. Instances of sumolyation machinery competing with ubiquitination for the same lysine residue to direct substrate stabilization or degradation has been described (Ulrich, 2005). It is not clear if a similar mechanism is used to regulate the abundance of ICE as well as ABI5.

Future perspectives

Plant tolerance of adverse growth conditions such as cold, drought, and high salinity involves developmental, physiological, and biochemical changes, which limit damage, re-establish homeostasis, and facilitate repair of damaged systems. Adaptability to the changing environment influences development, growth and yield. Thus it is important to understand the regulatory mechanisms involved in stress tolerance. The identification of E3 ubiquitin ligases which play a regulatory role in abiotic stress responses, establishes a direct link between the UPS and plant stress tolerance. Only a very small number of the over 1300 *Arabidopsis* E3 ligases have defined roles in abiotic stress tolerance. The fact that the expression of many E3-encoding genes is stress-regulated and numerous stress-related proteins have been identified in searches for ubiquitinated proteins ensures that other E3 ligases that are essential for plant adaptation to abiotic stress will be encountered.

Our understanding of the regulatory role of the E3s during plant responses to abiotic stress is hindered by the lack of substrate identity. The function of E3 ligase depends on the nature of their target protein. That is, whether or not the target proteins

are positive or negative regulators of the stress response. Although substrate identification is essential for determining biological function, it is also very important for understanding the biochemical function of the E3 enzymes. Once a substrate is identified, the mechanism of regulation by ubiquitination can be determined. Currently, very little is known about how plant E3 ligases are regulated specifically in response to external stimuli. Understanding the mechanism of stress signal-mediated up or down-regulation of E3 ligase activity will broaden our knowledge of cellular changes required for adaptation to adverse environmental conditions.

Acknowledgements

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APPENDIX B

Regulation of ethylene biosynthesis through protein degradation

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Contributions:

WJL: Wrote the manuscript and participated in revisions.

SLS. Prepared all figures and participated in revisions.

Abstract

The function of hormones during plant growth, development and response to environmental stresses relies heavily upon the actions of the ubiquitin proteasome system (UPS), which selectively degrades numerous proteins. Synthesis of ethylene, a growth and stress hormone, is regulated in part by the ubiquitin-dependent degradation of the rate-limiting enzymatic protein aminocyclopropane-1-carboxylic acid synthase (ACS). Regulation of ACS protein stability, and therefore ethylene production, is mediated by non-catalytic sequences within the C-terminal extension of many ACS proteins. In this review we provide a brief overview of the E3 ligases that target ACS proteins for degradation and discuss how post-translational modification of the C-terminal extensions influence protein stability.

Introduction

The selective degradation of proteins through the ubiquitin 26S proteasome system (UPS) is required for many cellular processes and subsequently has a major impact on plant physiology. The UPS mediates responses to both abiotic and biotic stress and it facilitates normal growth and development of plants. In many cases, the UPS functions to regulate hormone biosynthesis, perception and/or signal transduction. Similar to ABA, auxin and gibberellins, ethylene biosynthesis and signal transduction is heavily regulated through ubiquitin-dependent degradation (Santner *et al.*, 2010). Across plant species, members of the ethylene biosynthetic gene family, aminocyclopropane-1-carboxylic acid synthase (ACS), have been shown to be subject to ubiquitin-dependent degradation (Argueso *et al.*, 2007). ACS proteins catalyze the rate limiting step in

ethylene biosynthesis; the conversion of S-adenosylmethionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang *et al*., 1984; Wang *et al*., 2002). Members of the ACS family share a high degree of sequence similarity in their catalytic domain and contain variable C-terminal extensions (Yamagami *et al*., 2003). The ACS C-terminal sequences contribute to the stability of the ACS enzymes. In this review we discuss the regulation of ACS proteins through ubiquitin-dependent degradation.

Ubiquitination

Ubiquitin is covalently attached to target proteins through the sequential actions of three enzymes; E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase). The conjugation process begins with the ATP-dependent activation of ubiquitin by the E1 enzymes. Following activation, the ubiquitin molecule is transferred to the E2 forming a thioester linked E2-ubiquitin intermediate. The E3 binds to both the E2-ubiquitin intermediate and the target protein. Both the E2 and E3 enzymes coordinate the transfer of ubiquitin onto a lysine residue of the selected target. This process can be repeated creating a chain of ubiquitin molecules on the target. The outcome of ubiquitination varies depending upon the number of ubiquitin molecules and the topology of the ubiquitin chain attached to the target. Ubiquitination can influence protein function by directing cellular localization, modulating activation and controlling abundance (Mukhopadhyay *et al*., 2007; Smalle and Vierstra, 2004). The attachment of a ubiquitin chain formed using lysine 48 linkages marks the target protein for degradation by the 26S proteasome.

Ubiquitin ligases determine substrate specificity of the ubiquitination process. This important role is reflected in the large number of E3 ligases encoded by plant genomes compared with the smaller number of E1 and E2s. For example, Arabidopsis has only two genes that encode for E1s, 37 genes that encode for E2s and over 1,300 genes that encode for E3 ligases (Stone *et al*., 2005; Vierstra, 2009). Ubiquitin ligases can be categorized into three broad groups, HECT-, U box- or RING-type, based on the domain used to interact with the E2-ubiquitin intermediate. RING- and U box-type E3s function by facilitating the transfer of ubiquitin directly from the E2 to the target protein. The HECT-type E3s are unique because they form a E3-ubiquitin intermediate prior to transfer of the ubiquitin to the target protein (Scheffner *et al*., 1995). RING-type E3

ligases function as monomeric enzymes, which contain both the E2 and substrate binding domain on the same polypeptide, or as multi-subunit E3s with the E2 and substrate binding functions found on separate proteins. The multi-subunit E3 ligases contain a cullin (CUL) protein, an E2 binding RING Box1 (RBX1a/b) protein and a substrate binding protein (Schwechheimer *et al*., 2004). The broad complex/tramtrack/bric-a-brac (BTB) proteins are examples of a substrate recruiting proteins that form an E3 ligase complex with CUL3a/b (Gingerich *et al*., 2005). Some cullin-based E3 ligases require an additional adaptor protein to mediate interaction between the CUL protein and the substrate binding protein. For example, CUL1 uses the adaptor protein S-Phase Kinase-associated Protein (SKP) to bind to the substrate-recruiting F-box proteins (Lechner *et al*., 2006).

Both E3 ligases and targets of ubiquitination are regulated such that degradation only occurs under specific conditions. During most stages of plant development ethylene production is kept low in part through ubiquitination and degradation of ACS proteins (Argueso *et al*., 2007; Chae and Kieber, 2005). In response to specific developmental or environmental cues, such as wounding or pathogen attack, the ubiquitin-dependent degradation of specific ACS proteins is slowed or prevented such that ACS proteins can accumulate and contribute to a burst of ethylene production. The resulting ethylene mediates relevant changes in plant physiology.

The factors that influence the stability of ACS proteins from Arabidopsis, tomato (*Lycopersicon esculentum*) and other species have been widely studied. It appears that while the catalytic domain of these proteins is highly conserved, the C-terminal extensions are highly variable and facilitate the specific degradation patterns of the different ACS isoforms. Arabidopsis ACS proteins fall into three groups depending on the presence of phosphorylation sites in their C-terminal extension (Figure 32 A) (Argueso *et al*, 2007)**.** Type 1 ACS proteins (ACS1, 2 and 6) have the longest extensions and contain three mitogen-activated protein kinase (MAPK) phosphorylation sites and one calcium-dependent protein kinase (CDPK) phosphorylation site. Type 2 ACS proteins (ACS4, 5, 8, 9 and 11) contain a CDPK phosphorylation site embedded within a specific sequence (referred to as a Target Of ETO1 or TOE) required for interaction with
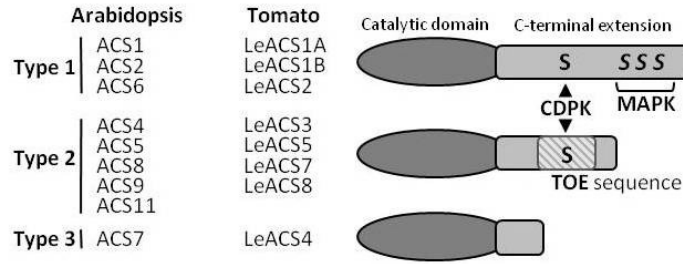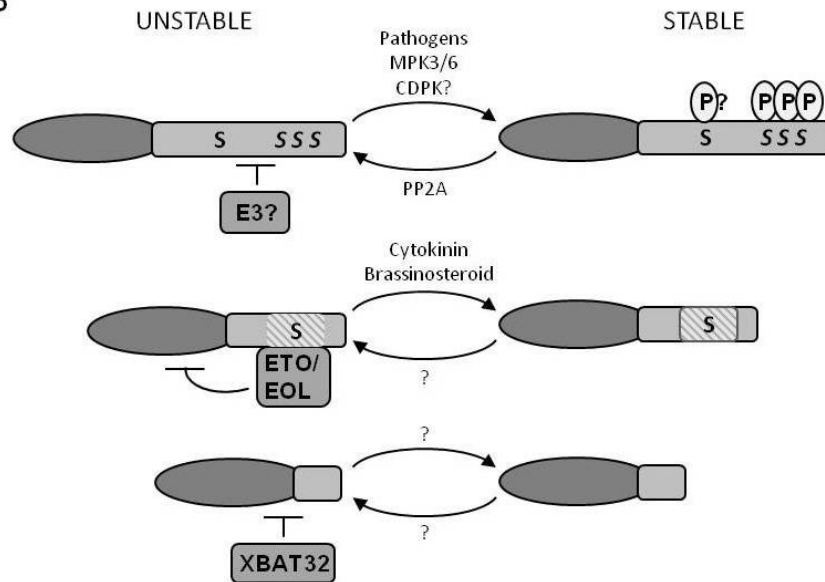
Figure 32. A. Classification of Arabidopsis and tomato (*Lycopersicon esculentum*) ACS proteins based on phosphorylation sites within the C-terminal extension. Type 1 ACS proteins contain one CDPK and three MAPK phosphorylation sites. Type 2 ACS proteins contain one CDPK phosphorylation site and a target of ETO1 (TOE) sequence. Type 3 ACS proteins contain neither phosphorylation sites nor TOE sequence. B. Factors that influence the stability of Arabidopsis ACS proteins. Type 1 ACS protein are phosphorylated by MPK3/6 in response to external stimuli such as *Botrytis cinerea* infection and are possibly phosphorylated by CDPKs. Phosphorylated type 1 ACS proteins are more stable than unphosphorylated forms. Dephosphorylation by PP2A renders type 1 ACS proteins unstable. Type 2 ACS proteins are ubiquitinated by ETO1 and EOL1/2 ubiquitin ligases and are stabilized by cytokinin or brassinosteroid treatment. It is unclear how CDPK phosphorylation affects protein stability. The type 3 ACS protein is ubiquitinated and targeted for degradation by the E3 ligase XBAT32. Factors that influence the stability of type 3 ACS proteins are unknown. P, phosphate group; S, serine residue

ethylene-overproducer1 (ETO1) (Yoshida *et al.*, 2006). Type 3 ACS proteins are interesting because they have a truncated C-terminal extension containing neither phosphorylation sites or TOE sequence. There is only one type 3 ACS protein in Arabidopsis (ACS7) but type 3 ACS proteins are common among many plant species including cucumber (*Cucumis sativus*) and poplar (*Populus trichocarpa*) (Lyzenga *et al.*, 2012).

E3 Ligases that Target ACS Proteins

The stability of type 2 and type 3 ACS proteins is dependent upon the activity of RING-type E3 ligases (Figure 32 B). ETO1 is a BTB protein that functions as the substrate recruiting component of a CUL3a/b based E3 ligase complex (Gingerich *et al.*, 200; Wang *et al.*, 2004). The *eto1* loss of function plants overproduce ethylene and dark-grown seedlings display a constitutive triple response phenotype which can be partially rescued by loss of *ACS5* (Chae *et al.*, 2003; Vogel *et al.*, 1998). ETO1-like1 and ETO1-like2 (EOL1 and EOL2) are homologous to ETO1 and all three are thought to contribute to the ubiquitin-dependent degradation of the type 2 ACS proteins (Christians *et al.*, 2009). ETO1, EOL1 and EOL2 bind to the type 2 ACS proteins through the C-terminal TOE sequence (Yoshida *et al.*, 2006). *eto2* and *eto3* are dominant negative mutations in the TOE sequence of ACS5 and ACS9, respectively, which interrupts binding to ETO1/EOL1 and reduces degradation of the ACSs. The resulting accumulation of either ACS5^{eto2} or ACS9^{eto3} accounts for the overproduction of ethylene observed in *eto* mutants (Chae *et al.*, 2003; Vogel *et al.*, 1998).

Ethylene production can be induced in Arabidopsis by treatment with cytokinin and brassinosteroid hormones (Chae *et al.*, 2003; Hansen *et al.*, 2009). Both hormones appear to induce ethylene biosynthesis through stabilization of ACS5 and ACS9 (Chae *et al.*, 2003; Hansen *et al.*, 2009). The mechanism by which ACS5 and ACS9 are protected from degradation is unclear because both ACS5^{eto2} and ACS9^{eto3} mutations, which have compromised binding to ETO1/EOL1, are further stabilized by cytokinin and brassinosteroid. It is suggested that ACS5 and ACS9 binding to ETO1/EOL1 is only partially disrupted by the *eto2* and *eto3* mutations and that cytokinin and brassinosteroid further prohibits this interaction (Chae *et al.*, 2003; Hansen *et al.*, 2009). Another possibility is that additional E3s contribute to the degradation of type 2 ACS proteins and

cytokinin and brassinosteroid affect the activity of these ubiquitin ligases. The Arabidopsis type 3 ACS (ACS7) does not interact with ETO1 or EOL1 (Christians *et al.*, 2009). Similarly, a type 3 ACS protein from tomato (LeACS4) does not interact with the ETO1 (Yoshida *et al.*, 2005). This suggests that type 3 ACSs are not regulated through ETO1-dependent degradation and may be regulated through a different mechanism.

XBAT32 is a monomeric RING-type E3 ligase involved in ethylene homeostasis. Recently, XBAT32 was shown to be required for the degradation of ACS4 and ACS7 (Lyzenga *et al.*, 2012). *XBAT32* mutants overproduces ethylene and displays a number of ethylene-related phenotypes including a mild triple response in dark-grown seedlings, reduced plant height and a decrease in the number of lateral roots. XBAT32 interacts with and ubiquitinates ACS4 in vitro (Prasad *et al.*, 2010). The half-life of ACS4 is extended in *xbat32* plants compared with wild type suggesting that, in addition to ETO1 and EOL1/2, XBAT32 also contributes to the ubiquitin-dependent turnover of ACS4 (Lyzenga *et al.*, 2012). The role of XBAT32 in regulating the abundance of type 2 ACS protein may be limited because the E3 does not interact with ACS11 (Prasad *et al.*, 2010). However, its ability to interact with or affect the stability of ACS5 or ACS9 has not yet been addressed.

Type 3 ACS proteins lack known points of regulation in their C-terminal extension (Figure 32 A). Whether or not these proteins are regulated through ubiquitin-dependent degradation was unclear. It was suggested that regulation of ACS7 abundance occurs mainly through transcriptional control since external stimuli, such as *Botytis cinerea* infection, induce expression of *ACS7* (Li *et al.*, 2012). In our recent paper we demonstrated that ACS7 is turned over in a proteasome-dependent manner (Lyzenga *et al.*, 2012). XBAT32 can interact with and ubiquitinate ACS7 in vitro (Prasad *et al.*, 2010). The stability of ACS7 has been examined *in planta* in both *xbat32* and wild type plants. While ACS7 is turned over in wild type plants, protein levels are stable in the *xbat32* plants (Lyzenga *et al.*, 2012). Thus, despite the lack of a C-terminal extension, type 3 ACS is regulated by the UPS.

Phosphorylation and ACS Protein Stability

Post-translational modifications through the addition of phosphate groups can influence protein activity by inducing changes in conformation, promoting specific

protein-protein interactions and modulating abundance. Much of our understanding of the effect that phosphorylation has the on the stability of ACS proteins has been revealed by studying type 1 ACS proteins. A variety of evidence points to a model in which unphosphorylated type 1 ACS are rapidly degraded by the 26S proteasome under normal growth conditions thereby maintaining low ethylene levels (Figure 32 B) (Chae *et al.*, 2005). However, under certain conditions such as wounding or pathogen attack the type 1 ACS proteins are phosphorylated, become stabilized, and contribute to increased ethylene biosynthesis (Argueso *et al.*, 2007; Chae *et al.*, 2005; Liu *et al.*, 2004; Joo *et al.*, 2008; Kamiyoshihara *et al.*, 2010; Han *et al.*, 2010). Evidence to support this model comes from the observation that transgenic plants expressing the *ACS6* gene under the control of the constitutively active 35S promoter does not overly produce ethylene (Liu *et al.*, 2004; Joo *et al.*, 2008). This is due to the turnover of ACS6 by the 26S proteasome. The C-terminal extension of ACS6 is enough to target a GFP-reporter protein for proteasome-dependent degradation (Joo *et al.*, 2008). Mutations of the three MAPK phosphorylation sites on the C-terminal extension of ACS6 to phosphomimic mutation (Serine to Aspartic acid, ACS6^{DDD}) rendered ACS6 stable and resulted in ethylene related phenotypes (Liu *et al.*, 2004; Joo *et al.*, 2008). Transgenic plants overexpressing ACS6^{DDD} displayed hairy roots, inhibition of primary root length and dwarfism.

Similar results have also been found in other species including tomato *(Lycopersicon esculentum, Le)*, banana (*Musa acuminate, Ma*) and cotton (*Gossypium hirsutum, Gh)* (Kamiyoshihara *et al.*, 2010; Choudhury *et al.*, 2012; Wang *et al.*, 2011). Treatment with kinase inhibitors decreased the phosphorylation and abundance of tomato ACS2 (LeACS2), while the phosphatase inhibitor okadaic acid produced the opposite effect (Kamiyoshihara *et al.*, 2010). Interestingly, both a MAP Kinase kinase (MAPKK) inhibitor (U0126) and a CDPK inhibitor (CMZ) were able to reduce the accumulation of LeACS2. This suggests that phosphorylation by both MAPKs and CDPKs contribute to the stabilization of type 1 ACS proteins. The stability of banana ACS1 (MA-ACS1), which is induced in response to ripening signals, appears to also be regulated through phosphorylation. Kinase inhibitor treatments decreased the level of phosphorylated MA-ACS1 and decreased the relative protein abundance (Choudhury *et al.*, 2012). Similar to type 1 ACS proteins from Arabidopsis and tomato, MA-ACS1 is phosphorylated in the

C-terminal extension and a MAPKKK-like protein kinase (MA-MAPKKK) may be responsible for this phosphorylation. The cotton CDPK, GhCPK1, was also found to phosphorylate GhACS2 and phosphorylated GhACS2 may contribute to elevated ethylene biosynthesis during fast fiber elongation (Wang *et al*., 2011).

The addition of phosphate groups to the C-terminal extension increases the negative charge of the extension and may promote or prevent the binding of additional proteins. The C-terminal extension of ACS6, and other type 1 ACS proteins, contains a pair of aspartic acids which contribute to the overall charge of the extension. Interestingly, mutation of these aspartic acids to positively charged arginines reversed the stability of ACS6^{DDD}. While ACS6^{DDD} is stable, ACS6^{DDD} with the pair of aspartic acids mutated to arginines (ACS6^{RR-DDD}) is unstable (Joo *et al*., 2008). The C-terminal extension of ACS6 also contains two clusters of positively charged lysines. Increasing the negative charge of the extension, due to the mutations of these lysines to aspartic acid, was enough to stabilize ACS6 without the need for phosphomimic mutations at the MAPK sites (Joo *et al*., 2008). The overall charge of the C-terminal extension of ACS7 and ACS4 also appears to influence their degradation rate. A mutation of the only lysine in the C-terminal extension of ACS7 to arginine, a modest increase in positive charge, was enough to increase the degradation rate of ACS7 (Lyzenga *et al*., 2012). Similarly, mutations of four lysines on the C-terminal extension of ACS4 to arginines also increased its degradation rate (Lyzenga *et al*., 2012). It appears that the stability of ACS proteins is commonly influenced by the C-terminal extension. These results suggest a model whereby the C-terminal extension of type 1 ACS proteins is a key point of regulation and a more negatively charge extension is stable whereas a more positively charged extension in unstable.

The role of the single CDPK phosphorylation site found on type 2 ACS proteins is still under investigation. ACS5 proteins containing CDPK phosphomimic or phospho-null mutations are still able to interact with ETO1 or EOL1 (Christians *et al*., 2009). However, the valine to aspartic acid mutation found on ACS9^{eto3}, may mimic phosphorylation (Chae *et al*., 2003). ACS9^{eto3} is more stable that ACS9, but it is not known if the mutation represents a relevant physiological change on ACS9 that prevents binding to ETO1 or EOL1 under specific conditions. There is some evidence which

suggests that phosphorylation may play a more complicated role in the stability of type 2 ACS proteins. Unlike type 1 ACS proteins, ACS5 is not stabilized by phosphatase inhibitors (Skottke *et al*., 2011). ACS5 was actually more unstable following treatment with the protein phosphatase inhibitor cantharidin. By inhibiting phosphatase activities, cantharidin may be increasing the phosphorylation status of ACS5 which results in increased degradation. Alternatively, cantharidin may influence the phosphorylation of other proteins that affect ACS5 such as ETO1, EOL1 and EOL2 and thereby indirectly influencing the turnover of ACS5.

While type 3 ACS proteins lack these phosphorylation sites, there is some indirect evidence that ACS7 is phosphorylated. ACS7 was found to interact with 14-3-3 proteins which usually require a phosphorylated recognition motif for interaction (Chang *et al*., 2009). It is possible that ACS7 is phosphorylated somewhere within the catalytic domain. The effect of kinase or phosphatase inhibitors on the stability of ACS7 has not been addressed. Another possibility is that regulation of its corresponding E3 ligase, XBAT32, contributes to the modulation of ACS7 protein stability.

Kinases and Phosphatases that Regulate ACS Protein Stability

The three tiered MAP kinase cascade is activated in response to various stimuli and elicits a relevant physiological response including an increase in ethylene production. Tobacco and Arabidopsis MAPKs, SIPK/WIPK and MPK6/3, are activated under a variety of conditions that induce ethylene production including infection by the fungi *B. cinerea* (Li *et al*., 2012; Han *et al*., 2010; Ichimura *et al*., 2002; Kim *et al*., 2003; Colcombet *et al*., 2008). Activated MPK6 and MPK3 can phosphorylate ACS6 and ACS2 in vitro (Han *et al.,* 2010). In a conditionally rescued *mpk3/mpk6* double mutant the level of ACS6 activity is severely reduced in response to infection (Han *et al*., 2010). Correspondingly, the MPK3/6 phosphorylation sites on ACS6 are also required for induction of ACS6 activity in response to infection. These results are consistent with a model in which *B. cinerea* and/or other environmental conditions induce ethylene production through activation of MPK3/6 and subsequent phosphorylation of ACS2 and ACS6 leading to enzyme stabilization and subsequent increased ethylene production (Han *et al*., 2010).

In opposition to the effect of MPK3/6 phosphorylation, it appears that protein phosphatase 2A interacts with ACS6 and dephosphorylates the C-terminal extension (Skottke *et al*., 2011). ACS6 is stabilized in the *rcn1–6* line, which lacks a protein phosphatase 2A (PP2A) subunit and has reduced phosphatase activity. The stabilization of ACS6 in *rcn1–6,* due to reduced dephosphorylation of the enzyme, may contribute to the ethylene over-production observed in the *RCN1* mutants (Skottke *et al*., 2011; Larsen *et al*., 2003).

Conclusions

The UPS plays significant roles in the regulation of ethylene biosynthesis. The three categories of ACS proteins, as defined by their C-terminal extension, reflects different mechanisms of regulation. Despite these differences, the abundance and hence activity of the enzymes from all three categories are regulated by the UPS. The role of phosphorylation in determining the stability of type 1 ACS proteins has been somewhat defined, however the E3 ligase(s) responsible for ubiquitination remain unknown. E3 ligases that target type 2 and type 3 ACS have been identified but the mechanisms that modulate stability under different physiological conditions remains poorly understood. Continued research on the turnover of ACS proteins will provide further insights into ethylene production and its role in plant growth, development and response to environmental stresses.

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