

**Regulation of *Arabidopsis thaliana* Calcineurin B-like Interacting Protein Kinases  
(CIPKs) by the Ubiquitin-Proteasome System**

by

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*This thesis is the result of a long and great adventure. It started in 2012 when my husband left his life behind him just to support me. With love, I dedicate this work to my husband, Abdullah Alotaibi, with great appreciation for your support and love. I am really thankful to Allah for having you in my life. Also, this work is dedicated to my lovely children, Rayan, Yazan, and Malak, you mean the world to me and I will face every challenge just for you.*

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## ABSTRACT

Plants are usually exposed to a wide array of environmental stresses, such as drought, increased salinity, temperature changes and nutrient deprivation. To maximize their chances of survival under stressful conditions plants utilize various cellular response mechanisms, including changes in gene expression and the production of protective proteins. In addition to mounting a stress response, plants must have the correct response mechanism only when required and occurs only for the duration of the stress. Plants utilize the ubiquitin-proteasome system (UPS) to regulate stress responses such as signal transduction pathways that are activated by stress-related hormones or kinases. The UPS regulates the abundance of proteins by first attaching ubiquitin molecules and then targeting the modified protein by the 26S proteasome for degradation. The ubiquitin ligases (E3) are the central enzymes of the UPS as they are responsible for selecting substrates for ubiquitination. Recently, the *Arabidopsis thaliana* E3 Keep on Going (KEG) was shown to negatively regulate the activity of the stress hormone abscisic acid (ABA) by ubiquitinating components of the hormone signaling network, including Calcineurin B-Like Interacting Protein Kinase 26 (CIPK26). This revealed a possible role for ubiquitin-dependent proteolysis in regulating the activity of members of the CIPK family of stress-related protein kinases. CIPKs activate downstream signaling proteins via phosphorylation, which leads to changes in gene expression that mediate responses to diverse environmental stimuli. With this work, we investigate the role of the UPS in regulating members of the *Arabidopsis thaliana* CIPK family, specifically CIPK3, CIPK8, CIPK20, and CIPK24. I examine ubiquitination and the proteasome-dependent degradation of the selected CIPKs in the plant cell using transient protein expression systems. All the examined CIPKs were found to be ubiquitinated in plant cells. Interestingly, the outcome of the ubiquitination in the CIPK proteins are different. I found that CIPK3, CIPK8, and CIPK20 are targeted for degradation by 26S proteasome, while CIPK24 remains stable when expressed in plant cells. These findings expand our understanding of how the UPS regulates these essential proteins under normal growth condition. The significance of the regulation of the CIPK proteins by the UPS will ideally lead to improving our understanding of plant responses to environmental stresses. This knowledge could be applied to the generation of stress tolerant plants with improved crop quality and yield maintenance during growth under adverse environmental conditions.

## LIST OF ABBREVIATIONS USED

ABA	Abscisic Acid
ABF3	Abscisic Acid-Responsive Element-Binding (ABRE-binding) Factor 3
ABI1	Abscisic Acid Insensitive 1
ABI2	Abscisic Acid Insensitive 2
ABI5	Abscisic Acid Insensitive 5
ABRC	Arabidopsis Biological Resource Center
ATP	Adenosine triphosphate
AtUBC32	<i>Arabidopsis thaliana</i> Ubiquitin-conjugating enzyme E2 32
CBL	Calcineurin B-like protein family
cDNA	Complimentary DNA
CHYR1	Arabidopsis CHY ZINC-FINGER and RING protein1
CIPK	Calcineurin B-like Interacting Protein Kinase
CP	Core Particle
DTT	DL-Dithiothreitol
DUB	Deubiquitinating Enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin Ligase
ECL	Enhanced Chemiluminescence



EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
GFP	Green Fluorescent Protein
HA	Hemagglutinin
HECT	Homologous to E6-associated protein carboxyl-terminus
IP	Immunoprecipitation
KA1	kinases-associated 1 domain
kDa	Kilodalton
KEG	Keep on Going
Lys	Lysine
$\mu\text{M}$	Micro Molar
MES	2-(N-morpholino) ethane sulfonic acid
MG123	26S proteasome inhibitor
mM	Millimolar
MS	Murashige and Skoog
NAF	Conserved asparagine (N), alanine (A)and phenylalanine (F)
OD	Optical Density
PP2C	Clade A type 2C protein phosphatases
PPI	Protein phosphatase interaction
PVDF	Polyvinylidene fluoride
PYR	Pyrabactin resistance

RCAR	Regulatory components of ABA receptor
RING	Really Interesting New Gene
RP	Regulatory Particle
Rpt	Regulatory Particle AAA+ ATPase subunits
SCF	Skp1-Cullin-F-box
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Skp1	S-phase kinase-associated protein 1
SNF	Sucrose non-fermenting
SDIR1	Salt- And Drought-Induced Ring Finger1
SnRK	Sucrose non-fermenting-related kinases
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline and Tween 20
Ub	Ubiquitin
UPS	Ubiquitin Proteasome System
V	Volts
WB	Western Blot
YFP	Yellow fluorescent protein

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## CHAPTER ONE: INTRODUCTION

### 1.1. Ubiquitin Proteasome System (UPS)

The Ubiquitin-Proteasome System (UPS) is a complex regulatory pathway that is involved in modulating the activities of numerous proteins in eukaryotic cells (Hershko et al., 1998). The UPS works mainly to control the degradation of unnecessary, mis-folded and short-lived regulatory proteins (Ciechanover, 1998; Goldberg, 2003). The pathway consists of two distinct sequential steps, starting with ubiquitination of a particular substrate followed by degradation of the modified substrate by the 26S proteasome (Figure 1) (Vierstra, 2003). The 26S proteasome is an ATP-dependent protease, which functions in the cytoplasm and the endoplasmic reticulum (Rivett et al., 1992; Pickart & Cohen, 2004). The UPS controls many cellular processes that are essential for growth and development, including protein signaling, cell cycle progression, and transcription (Vierstra, 2009; Lander et al., 2012). In plants, the UPS functions as one of the major regulatory systems to control the activity of intercellular proteins to regulate growth, development and responses to biotic and abiotic stresses (Smalle et al., 2003; Kurepa & Smalle, 2008).

The structure of the 26S proteasome is comprised of 33 different subunits forming two sub-complexes, the Core Particle (CP) or 20S proteasome and the 19S Regulatory Particle (RP) (Figure 1C) (Coux et al., 1996; Smalle & Vierstra, 2004; Finley, 2009). The CP is assembled from four heptameric rings, consisting of two peripheral and two central rings (Figure 1C). The related peripheral rings are composed of seven alpha ( $\alpha$ ) subunits and the central rings are composed of seven beta ( $\beta$ ) subunits (Glickman & Ciechanover, 2002). The RP is located on both ends of the CP and makes up the lid and base subcomplexes (Figure 1C). The RP serves as a gate that recognizes and control access of the ubiquitinates substrate into the interior of the

CP. The lid subcomplex contains ubiquitin receptors that work to recognize polyubiquitinated proteins with four attached ubiquitin moieties (Yao & Cohen, 2002). The base subcomplex directly interacts with the  $\alpha$ -subunits of the CP and contains six distinct AAA+ ATPases (named Rpt1 to Rpt6) that work to unfold the substrate protein and translocate the polypeptides into the CP (Glickman & Ciechanover, 2002). The CP is responsible for cleaving the substrate allowing the short peptides to be recycled in the cell. The proteolytic  $\beta$ -subunits,  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5, of each ring work to breakdown the substrate into short peptides of 3-25 residues in length (Kisselev et al., 1999; Callis, 2014). The degradation of a particular protein by the 26S proteasome require at least four ubiquitin molecules attached as a linear polyubiquitin chain to the target protein (Bhattacharyya et al., 2014).

### *1.1.1. Ubiquitination*

The covalent attachment of ubiquitin molecule(s) to a selected substrate is known as ubiquitination. Ubiquitin (Ub) is a small, 8.5 kD, regulatory protein that is widely conserved in all eukaryotic cells (Craig et al., 2009; Zuin et al., 2014). Examination of the ubiquitin amino acid sequence across eukaryotes has shown that ubiquitin is highly conserved between human, yeast and plant species (Komander & Rape, 2012; Zuin et al., 2014). Only three to four amino acids variation is observed between eukaryotes when comparing ubiquitin across eukaryotes (Zuin et al., 2014). Ubiquitin consist of 76 amino acids and contains seven lysine (K) residues at positions K6, K11, K27, K29, K31, K48 and K63, each of which can be used to create ubiquitin-ubiquitin linkages on a target substrate (Sun & Chen, 2004).

The process of ubiquitination is accomplished by the sequential action of three enzymes E1 (ubiquitin-activating enzyme, UBA), E2 (ubiquitin-conjugating enzyme, UBC) and E3 (ubiquitin-protein ligase) (Glickman and Ciechanover, 2002). Ubiquitination begins when the ubiquitin molecule is being activated through an ATP-dependent reaction by the E1 (Figure 1A) (Vierstra, 2003). The activated ubiquitin is then transferred from E1 to the E2 forming an E2-Ub intermediate. The E2 is involved in regulating the topology of the polyubiquitin chain in that it assists in determining which lysine is used for ubiquitin-ubiquitin linkages (Van Wijk & Timmers, 2010; Valimberti et al., 2015). In the final step of the ubiquitination cascade, an E3 ligase mediates the transfer of ubiquitin from the E2-Ub intermediate to a lysine residue on the substrate (Figure 1B) (Spratt et al., 2012).

A single ubiquitin molecule can be attached to a lysine residue on the substrate, referred to as monoubiquitination. Alternatively, a chain of ubiquitin molecules can be attached to the lysine residue and this is called polyubiquitination (Hunter, 2007; Sadowski et al., 2012). The attachment of one or more ubiquitin molecules to a target protein may have varied outcomes including regulation of protein activity, trafficking, localization as well as changes in abundance (Komander & Rape, 2012; Stone, 2014). The lysine residue used to assemble the ubiquitin chain also determines the fate of the modified substrate (Sadanandom et al., 2012). For example, K63-linked ubiquitin chains primarily function by regulating protein activation and endocytosis (Sun & Chen, 2004; Yang et al., 2010). Whereas, a polyubiquitin chain that is assembled using K48 linkages is involved in targeting modified proteins for degradation via the 26S proteasome (Verma et al., 2004).

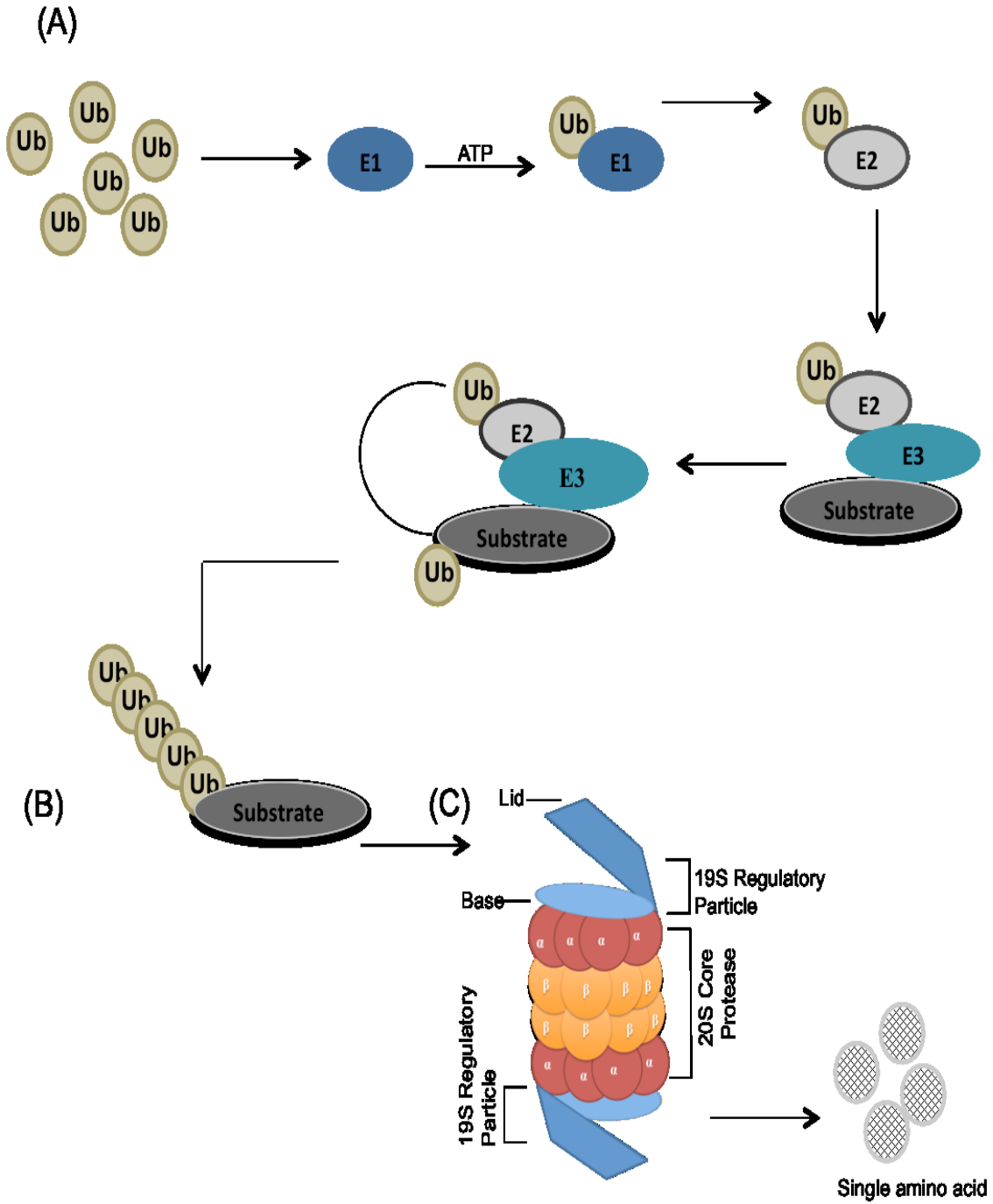
## **Figure 1. The Ubiquitin Proteasome System**

The Ubiquitin Proteasome System is a multi-enzyme pathway that uses the ubiquitin moiety as a signal to target a substrate for degradation by the 26S proteasome.

(A) Ubiquitination pathway. The process of ubiquitination is controlled by a cascade of three enzymes known as ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3 (Glickman and Ciechanover, 2002). The pathway begins with the activation of an ubiquitin molecule by the E1 enzyme. The activated Ub is then transferred to the E2 enzyme. The E3 enzyme interacts with the E2-Ub intermediate and the substrate and facilitates the transfer of ubiquitin from the E2 to the substrate.

(B) The process of ubiquitination is repeated to generate a chain of ubiquitin molecules, called polyubiquitination. The polyubiquitinated protein modified with a chain containing at least four ubiquitin molecules and is sent for degradation by the 26S proteasome.

(C) The 26S proteasome mediates substrate degradation. The polyubiquitin chain enables substrate recognition by the 19S RP, which recognizes and unfolds the polyubiquitinated substrates. The unfolded protein is fed into the central chamber of the 20S CP where it is broken down into short peptides by proteases. Ubiquitin is recycled into the cell and is available for reuse.





### 1.1.2. Ubiquitin Ligases

The capability of the ubiquitin pathway to regulate protein abundance is dependent on the large variety of ubiquitin ligases (Stone et al., 2005; Deshaies & Joazeiro, 2009). The *Arabidopsis thaliana* (*Arabidopsis*) genome contains more than 1400 genes that encode for E3 ubiquitin ligases (Smalle & Vierstra, 2004). E3 ubiquitin ligases can be classified based on the type of domain used to interact with the E2-Ub intermediate including the Homology to E6-Associated Carboxy-Terminus (HECT), U-box, and Really Interesting New Gene (RING) domains (Moon et al., 2004) (Figure 2A). The *Arabidopsis* genome encodes for 7 HECT-type, 64 U-box-type, and 470 RING-type E3 ligases (Stone et al., 2005; Mazzucotelli et al., 2006). In addition to the E2 binding domain, E3 ligases utilize different types of protein-protein interaction domains to interact with various substrate proteins (Pickart, 2001; Smalle & Vierstra, 2004). The HECT-type E3s form an isopeptide bond with ubiquitin before transferring the molecule to the target substrate (Figure 2A) (Kim & Huibregtse, 2009). In contrast to HECT type E3s, U-box-type and RING-type E3s function by bringing the target substrate and the E2-Ub intermediate into close proximity in order to mediate the transfer of ubiquitin to the substrate (Figure 2A) (Azevedo et al., 2001; Stone et al., 2005).

RING-type E3s are the most abundant class of ubiquitin ligases in the *Arabidopsis* proteome (Moon et al., 2004). They are characterized by a RING domain that contains eight cysteine and histidine residues that coordinate two zinc ions forming a cross-braced structure (Freemont, 1993; Pickart, 2001). RING-type E3 ligases can be further divided into single or multi-subunit enzymes (Figure 2B). Single subunit RING-type E3s contains both the E2 and substrate binding domains within a single polypeptide (Berndsen & Wolberger, 2014). The multi-subunit RING-type E3 ligase utilizes different proteins within the complex to bind the E2

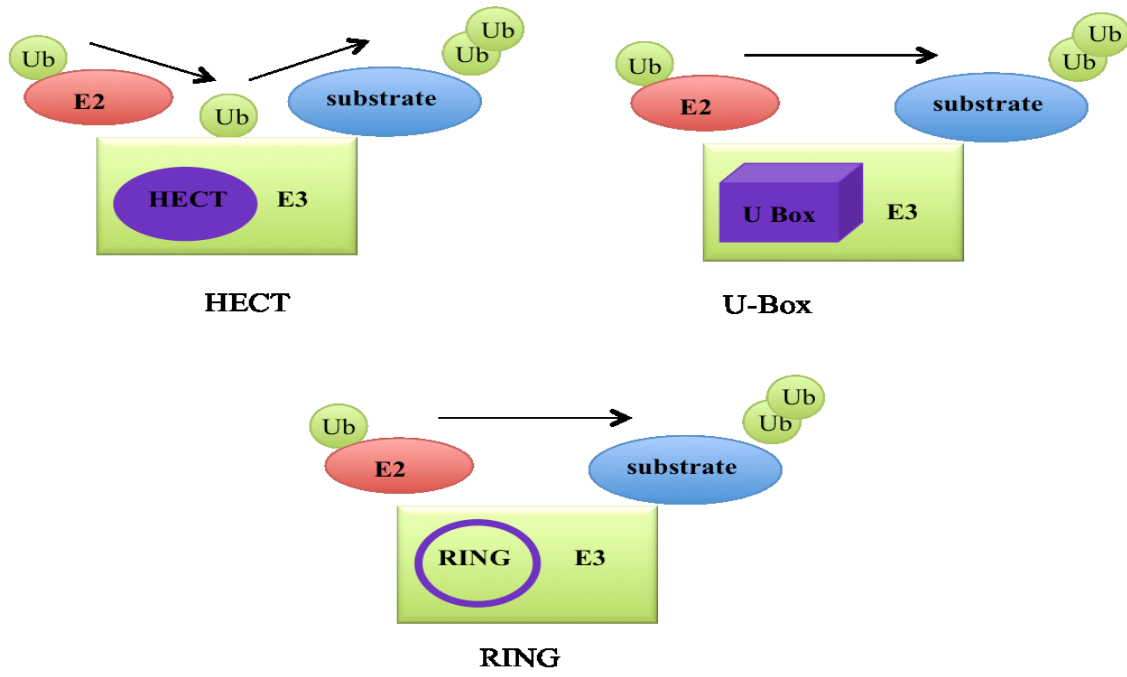
and substrate (Berndsen & Wolberger, 2014). The multi-subunit RING E3s ligases, known as Cullin-based RING E3 (CRLs) are comprised of the scaffold protein Cullin, which interacts with the E2-binding RING-box 1 (RBX1) protein and the substrate binding protein. The substrate recognition proteins can bind directly to the Cullin or indirectly through adapter proteins (Hotton & Callis, 2008; Berndsen & Wolberger, 2014) (Figure 2B).

## **Figure 2. The Different Types of E3 Ubiquitin Ligases.**

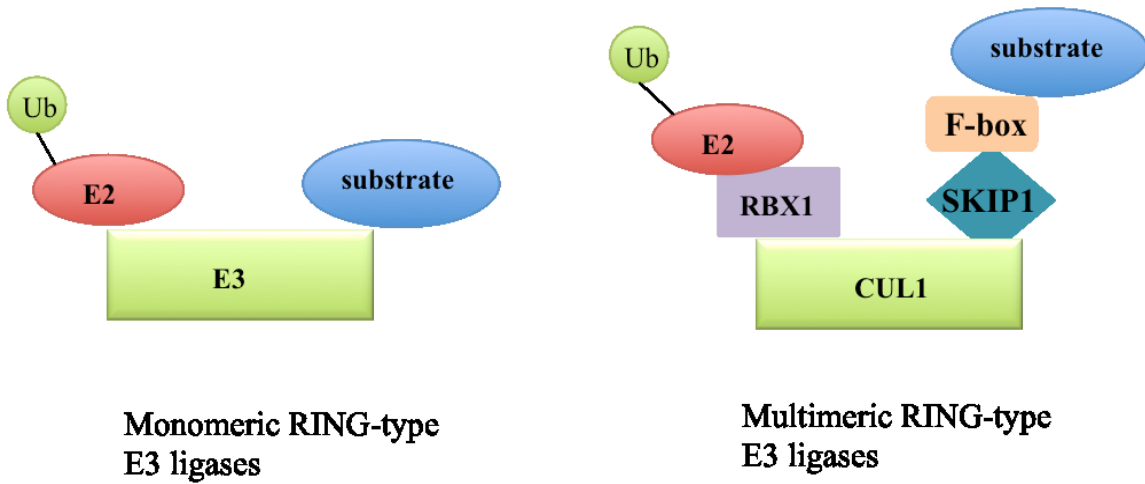
A) Schematic representation of the three major groups of E3 ubiquitin ligases, which are characterized based on the domains that bind to the E2-Ub. Homologous to E6-AP carboxyl terminus (HECT)-type E3s play a direct role in catalyzing the transfer of ubiquitin to the substrate by first forming a E3-Ub intermediate prior to transfer of ubiquitin to the substrate. U-box-type and Really Interesting New Gene (RING)-type E3s function by bringing the E2-Ub and substrate into close proximity in order to promote the transfer of ubiquitin directly to the substrate.

B) RING type E3 ligases can be assembled as monomeric or multimeric enzymes. Monomeric E3s contain the E2-Ub and substrate binding domains within the same protein, whereas the multimeric E3 ligases use different subunits to interact with the E2-Ub and the substrate. An example of the complex multi-subunit E3 ligases is the SKP-Cullin-F-box (SCF) CRL which contains CUL1 that interacts with the substrate recognition F-box protein through an adaptor called Arabidopsis S-phase kinase-associated protein 1-like (ASK1) (Hotton & Callis, 2008; Deshaies & Joazeiro, 2009)

(A)



(B)



### 1.1.3. The Role of UPS in Plant Responses to Environmental Stresses

Plants are exposed to a wide array of environmental changes, such as drought, increased salinity, and temperature changes, which negatively affect their growth and development. Plants have established number of defense mechanisms to enable survival during environmental changes. The ubiquitination pathway can influence plant responses to particular environmental stresses by modulating the abundance of stress-responsive proteins (Zhou et al., 2010; Stone, 2014). During stress the *ubiquitin* gene expression observed to increased during the exposure to high temperature, salinity, and drought which indicate the important of the ubiquitination pathway in plant responses to stress (Sun & Callis, 1997; Guo et al., 2008). The E2 enzyme, *Arabidopsis thaliana Ubiquitin-conjugating enzyme 32 (AtUBC32)*, is a stress-inducible gene that is up regulated in response to drought and salt stresses (Callis et al., 1995; Zhou et al., 2010). The overexpression of *AtUBC32* is able plant to be less tolerance to drought stress, whereas, the loss of *AtUBC32* results in higher tolerance of the stress (Cui et al., 2012).

E3 ligases have also been shown to play important roles in stress responses. Recent studies have identified significant roles for E3 ligase in regulating synthesis and signal transduction of plant hormones, including the stress-related hormone abscisic acid (ABA) (Vierstra, 2009; Stone, 2014). ABA is an essential plant hormone that regulates plant responses to abiotic stresses such as drought and salinity (Nakashima et al., 2013). ABA functions by regulating maturation and prolongs dormancy to ensure that seedling growth and development occurs during favorable growth conditions (Finkelstein et al., 2013). The single subunit RING domain-containing E3 ligases Salt- and Drought-Induced Ring Finger1 (SDIR1), is function to up regulating ABA signalling (Ryu et al., 2010; Stone, 2014). SDIR1 is expressed in all *Arabidopsis* tissues and its expression is increased by high salinity and drought stress (Zhang et

al., 2007). Overexpression of *SDIR1* causes hypersensitivity to ABA, high salinity, and drought conditions (Zhang et al., 2007). *SDIR1* acts as a positive regulator in the ABA signaling by upregulating expression of ABA-responsive transcription factors including *Abscisic Acid Insensitive 5 (ABI5)*, *Abscisic Acid-Responsive Element-Binding (ABRE-binding) Factor 3 (ABF3)*, and *ABF4* (Zhang et al., 2007). The single subunit RING-type E3 Keep on Going (KEG) mediates the degradation of *ABI5*, *ABF3*, and *ABF1* (Finkelstein et al., 2005; Stone et al., 2006; Chen et al., 2013).

During favorable growth conditions, KEG has been shown to negatively regulate ABA signaling by targeting *ABI5* for degradation using the 26S proteasome to preserve low levels of *ABI5*, enabling seedling establishment (Stone et al., 2006). During stress growth conditions, the level of ABA increases and promote KEG self-ubiquitination leading to degradation of the E3 by the 26S proteasome (Liu & Stone, 2010). The decrease in KEG abundance results in the accumulation of ABA-responsive transcription factors that mediate changes in gene expression required for minimizing the negative effects of stress on growth and development (Liu & Stone, 2010). KEG is also involved in regulating the abundance of the Calcineurin B-like Interacting Protein Kinase 26 (*CIPK26*) (Lyzenga et al., 2013). KEG has been demonstrated to interact with and target *CIPK26* for degradation via the 26S proteasome (Lyzenga et al., 2013). By targeting both the kinase (e.g. *CIPK26*) and transcription factors (e.g. *ABI5*) for degradation, KEG is able to effectively inhibit ABA signaling and dampen ABA responses in the absence of stress.

## 1.2. Calcineurin B-like Interacting Protein Kinase (CIPK) Family

Sucrose non-fermenting 1 (SNF1) is a protein kinase originally found in *Saccharomyces cerevisiae* (yeast) (Alderson et al., 1991). SNF1 proteins control many important cellular activities including cell division and responses to external environmental stimuli (Hunter, 1987). Plants contain a large group of protein kinases related to SNF1 known as SNF1-related kinases (SnRKs) (Hunter, 1987). The SnRK family has been categorized into three subfamilies: SnRK1, SnRK2 and SnRK3 (Figure 3) (Xue-Fei et al., 2012). SnRKs are serine/threonine (Ser/Thr) protein kinases that participate in various cellular processes (Xue-Fei et al., 2012). SnRK1s regulates glucose metabolism, hormonal signaling, such as Auxin and ABA, and generation of alternative energy sources including sucrose, starch, amino acids and lipids (Ruben et al., 2011). The SnRK2 and SnRK3 subfamilies are involved in abiotic stress responses and regulating abscisic acid (ABA) signaling (Kulik et al., 2011; Xue-Fei et al., 2012).

SnRK3s, also known as Calcineurin B-like Interacting Protein Kinases (CIPKs), are located in the nucleus and cytoplasm of plant cells (Batistic et al., 2010). CIPKs interact with intercellular calcium ( $\text{Ca}^{2+}$ ) binding Calcineurin B-like (CBL) proteins (Shi et al., 1999; Kim et al., 2000). The primary role of the CIPKs is to induce the required cellular changes in response to environmental stress in order to maintain nutrient homeostasis and promote cell survival (Ruben et al., 2011; Xue-Fei et al., 2012). The numbers of CIPK proteins differ among plant species; for instance, *Arabidopsis* contains 26 genes that encode for CIPKs while the rice (*Oryza sativa*) genome encodes for 31 CIPK proteins (Table 1) (Kolukisaoglu et al., 2004; Chen et al., 2011). The table number 1 presents number of CBL-CIPK obtained from bioinformatics research reports that used comparative genomic analyses to identify CBL-CIPK protein family members in different plant species. According to comparative genomic analyses, CBL-CIPK

networks are found in a large number of plant species, that provides evidence for the importance of these proteins in plants (Weinl & Kudla, 2009; Kanwar et al., 2014).



**Table 1. Numbers of CBL and CIPK proteins in different plant species.**

<b>Species</b>	<b>CBL-CIPK</b>	<b>Evident</b>
<i>Arabidopsis thaliana</i>	10 CBLs and 26 CIPKs	Kolukisaoglu et al., 2004
<i>Populus trichocarpa</i>	10 CBLs and 31 CIPKs	Zhang et al., 2008
<i>Oryza sativa</i>	10 CBLs and 31 CIPKs	Chen et al., 2011
<i>Zea mays</i>	8 CBLs and 43 CIPKs	Chen et al., 2011
<i>Vitis vinifera</i>	8 CBLs and 21 CIPKs	Weinl & Kudla, 2009
<i>Sorghum bicolor</i>	6 CBLs and 32 CIPKs	Weinl & Kudla, 2009

### *1.2.1. Structure and Regulation of SnRK Proteins*

All SnRK proteins consist of an amino-terminal Ser/Thr kinase domain, a variable domain followed by a carboxyl-terminal auto-regulatory domain (Figure 3) (Jiang & Carlson, 1996; Hashimoto et al., 2012). Although the SnRK subfamilies are similar in structure, each subfamily has unique structural characteristics (Figure 3). SnRK1 members have a ubiquitin-associated (UBA) domain and a kinases-associated 1 (KA1) domain (Figure 3A). The UBA domain is used to mediate the interaction with ubiquitinated proteins (Farras et al., 2001). The KA1 domain is responsible for the interaction with the clade A type 2C protein phosphatases (PP2C), which dephosphorylates and inactivate the SnRK1s (Rodrigues et al., 2013). The SnRK2 carboxyl-terminal regulatory domain is divided into two domains, DI and DII (Figure 3B). DII has an interaction region for recognition of the PP2C phosphatases (Yoshida et al., 2002). Interactions with PP2C inhibit the activity of SnRK2 proteins in ABA- signaling responses (Fujii & Zhu, 2009). The DI domain contains an autophosphorylation site, which enables SnRK2s to gain full activity independent of ABA (Belin et al., 2006). The SnRK3/CIPK carboxyl-terminal regulatory domain has an auto-inhibitory NAF domain and a protein phosphatase interaction (PPI) domain (Figure 3C) (Albrecht et al., 2001; Weinl & Kudla, 2009). The 24 amino acid NAF domain, named for the presence of conserved amino acids asparagine (N), alanine (A) and phenylalanine (F), is required for mediating the interaction with calcium binding CBLs, which activates the kinase (Guo et al., 2001; Chaves-Sanjuan et al., 2014). The protein phosphatase interaction (PPI) domain is responsible for the interaction with PP2C such as the ABA-responsive ABA-insensitive 1 (ABI1) or ABA-insensitive 2 (ABI2) (Ohta et al., 2003).

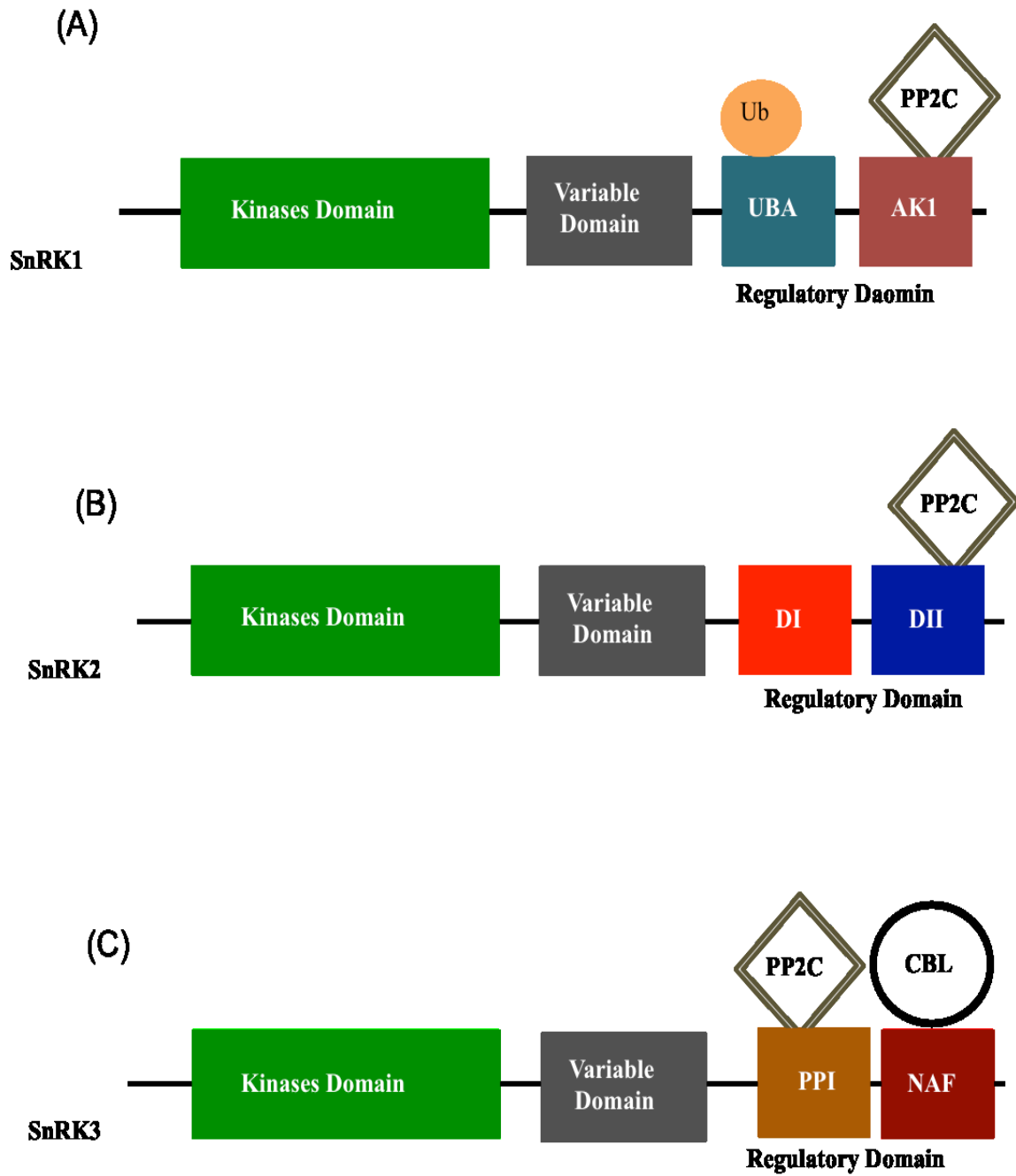
### **Figure 3. Comparison of Domain Structure of SnRK Subfamilies**

Schematic representation of the general structure of the SnRK1, SnRK2, and SnRK3 sub families.

A) Common structure of SnRK1 proteins. SnRK1 contains a kinase domain (green), variable domain (gray) and regulatory domain that include the ubiquitin (Ub)-associated (UBA) domain and the kinases-associated 1 (KA1) domain. The UBA domain is used to interact with ubiquitinated proteins and KA1 is responsible for the interaction with clade A type 2C protein phosphatases (PP2C).

B) SnRK2 protein structure showing the regulatory domain, which is divided into DI and DII. DI is essential for kinase activity and is responsible for ABA-independent activation. DII has a region for recognition of the PP2Cs.

C) The regulatory domain of SnRK3/CIPK subfamily protein consists of an auto-inhibitory NAF domain and a PPI domain, which interacts with PP2Cs.



### *1.2.2. CIPK Dependent Signaling*

Plant CIPKs and their activators, the calcium-binding CBLs, act as signaling mechanisms that decode intracellular calcium levels and function to regulate cellular responses to different environmental stimuli (Batistic & Kudla, 2012). In the absence of stress, CIPKs have little kinase activity (Gong et al., 2002a). PP2Cs that bind to CIPK function to inhibit activity by dephosphorylating the activation loop of the kinases domain (Figure 4A) (Ohta et al., 2003). During exposure to stresses, the level of cellular calcium increases and generates a specific and temporary unique oscillation inside the cell. The increase in calcium levels results in activation of CBL allowing for interaction with other proteins (Figure 4B) (Luan et al., 2002; Batistic et al., 2008). The interaction between CBLs and CIPKs disrupt the auto-inhibitory effect of the NAF domain to allow activation of the kinase domain. The activated CIPK then phosphorylates the appropriate target proteins to mediate stress responses (Figure 4B) (Guo et al., 2001; Gong et al., 2002b; Li et al., 2006).

The physiological role of the CBL-CIPK network was first identified in the Salt Over Sensitive (SOS) pathway during the study of plant response to high salt stress (Qiu et al., 2002; Mao et al., 2016). The SOS pathway contributes to plant salt stress tolerance, which consists of two signaling components, changes in intracellular  $\text{Ca}^{2+}$  levels and the interaction between CBL and CIPK (Zhu et al., 1998; Gong et al., 2004). Under high salt conditions, CBL4 interacts with CIPK24 to maintain the transport of sodium ions across the plasma membrane (Shi et al., 2002; Gong et al., 2004). In addition to response to salt stress, the CBL-CIPK pathway has been found to play a major role in plant adaptation to the changing environment, by regulating the uptake of ions, including potassium (Li et al., 2006), and magnesium (Tang et al., 2015; Mao et al., 2016). For example, in order to regulate potassium uptake from the environment CBL9 and CBL1 interact

with CIPK23 in response to low potassium levels. The interaction leads to increases in potassium ion uptake from the environment into the cell (Li et al., 2006; Hashimoto et al., 2012). CBL-CIPK pathways also targeting in the vacuolar membrane in order to help plants avoid toxicity due to harmful ions such as high level of magnesium ( $Mg^{2+}$ ) (Kim et al., 2007; Tang et al., 2015). CIPK3 interaction with CBL2 is involved in protecting plants from high  $Mg^{2+}$  by sequestering it into the vacuole (Tang et al., 2015; Mao et al., 2016).

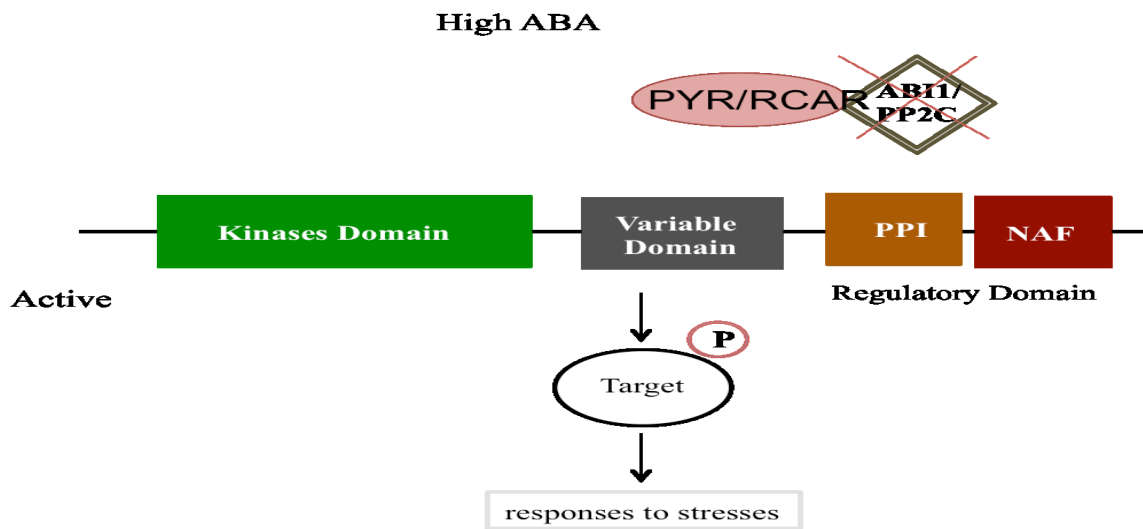
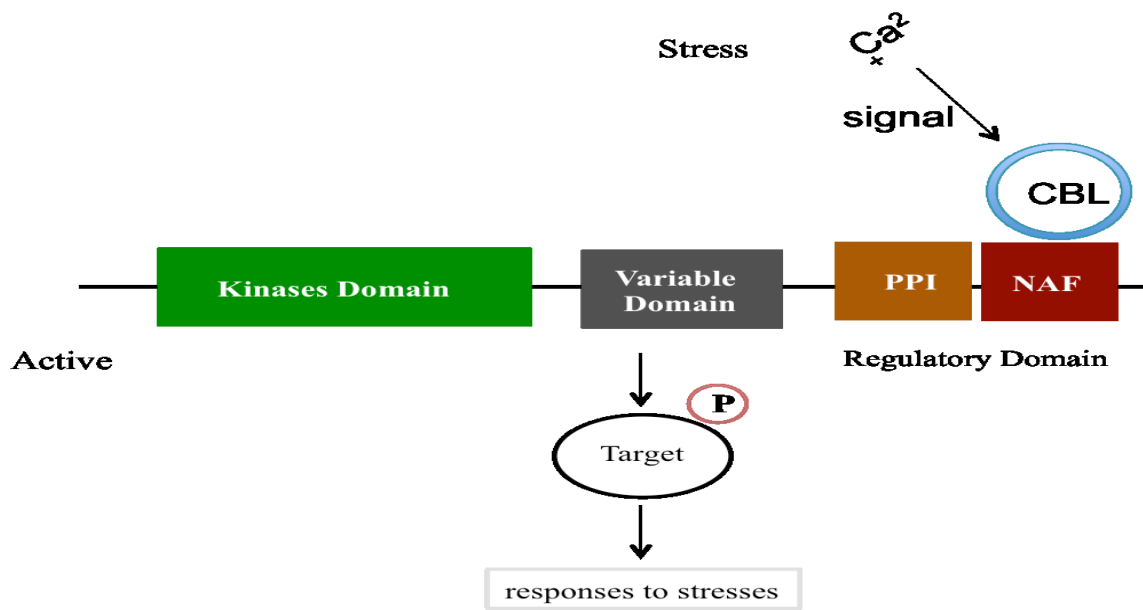
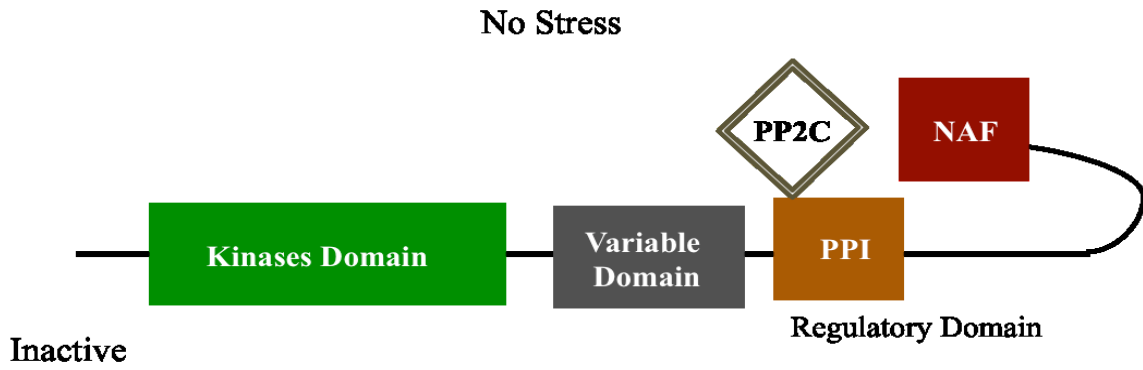
Some members of the CIPK family are responsive to changes in ABA levels during environmental stress (Sanchez Barrena et al., 2013). ABA is an essential plant hormone that regulates plant responses to stresses such as drought and salinity (Nakashima et al., 2013). In the absence of ABA, the clade A type 2C protein phosphatases (PP2Cs) interact with several CIPKs to inhibit activity through dephosphorylation of the kinase (Quintero et al., 2011; Sanchez Barrena et al., 2013). In response to stress and an increase in ABA levels, PP2C activity is inhibited, which allows for phosphorylation of CIPKs and activation of ABA signal transduction pathway (Figure 4C). The activated CIPKs phosphorylate ABA-responsive transcription factors that mediate changes in gene expression required for plant responses to stresses such as drought and salinity (Pandey et al., 2008). In addition, CBLs interact with CIPKs to regulate ABA signaling. The interaction between CIPK3 and CBL9 has been shown to negatively regulate ABA signal transduction (Kim et al., 2003; Pandey et al., 2008).

#### **Figure 4. Regulation of Calcineurin B-like Interacting Protein Kinase (CIPK) Activity**

A) In the absence of stress CIPK is inactive. The auto-inhibitory NAF domain (red) and interaction between the PP2C protein and PPI negatively regulate CIPK proteins by inhibiting kinase activity.

B) During stress, the level of cellular  $\text{Ca}^{2+}$  increases and this leads to CBL interaction with the NAF domain, which disrupts the inhibitory effect of the domain and activates the kinase. The activated kinase then phosphorylates target proteins required for stress responses.

C) In response to stress ABA levels increase leading the activation of the ABA receptors (PYR/RCAR), which interact with PP2Cs to disrupt the interaction between PP2C and CIPKs. Subsequently, CIPK is activated and available to phosphorylate a specific target protein to promote stress responses.





### *1.2.3. CIPK and the Ubiquitin-Proteasome System*

The SnRKs subfamily proteins are involved in ABA signaling and were recently shown to be regulated by the UPS (Farrás, 2001; Lyzenga et al., 2013). CIPK26 a member of the interact with the ABA-responsive protein phosphatases, ABI1 and ABI2, as well as the transcription factor ABI5 (Lyzenga et al., 2013). These interactions leads to inhabit the kinases activites of CIPK26. In fact, overexpression of CIPK26 renders plants sensitive to the inhibitory effects of ABA on germination and root growth (Lyzenga et al., 2013). Protein-protein interaction assays also demonstrated interactions between CIPK26 and the RING-type E3 ligase, KEG (Lyzenga et al., 2013). CIPK26 was shown to be ubiquitinated and degraded by the 26S proteasome, and KEG is involved in regulating the proteasome-dependent turnover of the kinase (Lyzenga et al., 2013). CIPK26 is the only SnRK3 subfamily member that regulated by the UPS. In contrast, the members of the SnRK1 subfamily have been shown to be regulated by the UPS. The abundance of SnRK1 family members, AKIN10 and AKIN11, are modulated by SKP-Cullin-F-box E3 complex in Arabidopsis (Farrás, 2001). SnRK2.6 is also a target for Arabidopsis CHY ZINC-FINGER AND RING PROTEIN1 (CHYR1) E3 ligase (Ding et al., 2015). Interaction between SnRK2.6 and CHYR1 positively regulates plant responses to ABA and drought stress (Ding et al., 2015).

### **1.3. Purpose of Study**

The UPS is involved in every aspect of plant growth and development including plant responses to biotic and abiotic stresses. E3 ligase mediates the selection of specific substrates for ubiquitination, thus regulating protein activity and abundance changes in the cell. The importance of E3 ligases has been well established; however, the identities of many ubiquitination substrates remain unknown. This project investigates the regulation of the CIPK family members by the UPS, specifically CIPK3, CIPK8, CIPK20, and CIPK24. This goal will be achieved by 1) determining if CIPK proteins are ubiquitinated in plant cells and 2) demonstrating proteasome-dependent degradation of the selected CIPKs. Studying the regulation of CIPK by the UPS will provide a better understanding of how plants regulate these essential kinases. The significance of UPS and CIPK interactions will ideally lead to an improved understanding of how plants respond to environmental stresses. This knowledge could be useful in the generation of stress tolerant plants with improved crop quality and yield maintenance during growth under adverse environmental conditions.

## CHAPTER TWO: MATERIALS AND METHODS

### 2.1. Plant Materials and Growth Conditions

Tobacco (*Nicotiana tabacum*) seedling were grown in the soil for 6 weeks in a photoperiod of 16 h light, and 8 h dark at 22°C in a growth chamber. Plants were watered twice weekly with a 1g/L of plant fertilizer (Plant-Prod, product number 10529). The *Arabidopsis thaliana* ecotype Columbia (Col-0) seeds were sterilized with sterilization solution which includes 50% (v/v) bleach and 0.1% Triton X-100, then grown on solid ½ Murashige and Skoog (MS) medium (0.8% agar and 1% sucrose). Seeds were subjected to a cold treatment at 4°C for 48 hours. After cold treatment, seeds were incubated under continuous light at 22°C for 10 days and seedlings transferred to soil then grown under the same photoperiod as described above.

### 2.2. Transient Protein Expression in *Nicotiana tabacum*

Transient expression of fusion proteins in tobacco plants was carried out as previously described in Sparkes et al., 2006. Full-length cDNAs of CIPK3 (At2g26980), CIPK8 (At4g24400), CIPK20 (At5g45820), and CIPK24 (At5g35410) were previously cloned into the plant transformation vector pEarleyGate 201 (Earley et al., 2006), which allows for the production of CIPK as a fusion protein with the Yellow fluorescent protein (YFP) and hemagglutinin (HA) tag (YFP-HA-CIPK). The plasmid was then introduced into *Agrobacterium tumefaciens* (Agrobacterium) strain GV3101. The transformed Agrobacterium was incubated overnight in Luria-Bertani Broth (LB) medium with appropriate selection antibiotics at 27 °C. The Agrobacterium cultures were then harvested and resuspended in an infiltration solution containing 5 mg/ml D-glucose, 50 mM MES, two mM Na<sub>3</sub>PO<sub>4</sub>, and 0.1 mM acetosyringone. The resuspended Agrobacterium were adjusted to an optical density (OD) of OD<sub>600</sub> ≈ 0.8 with additional

infiltration solution. The prepared Agrobacterium were then injected into the underside of tobacco leaves using a 1 ml needleless syringe. The infiltrated plants were placed in a growth cabinet for 48h. The infiltrated tobacco leaves were then collected and frozen in liquid nitrogen and stored at -80°C.

### **2.3. Transient Protein Expression in *Arabidopsis thaliana***

Transient expression of fusion proteins in Arabidopsis plants was carried out as described in Lee and Yang, 2006. Agrobacterium was transformed with plant transformation vector as described above in section 2.2. Transformed Agrobacterium were incubated overnight in LB medium with appropriate selection antibiotics at 27 °C. The incubated Agrobacterium cultures were collected through centrifugation (3000g for 5 min). Agrobacterium cultures were suspended in 4 ml of induction medium (10.5 g/L K<sub>2</sub>HPO<sub>4</sub>, 4.5 g/L KH<sub>2</sub>PO<sub>4</sub>, 1 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g/L NaCitate, 1 g/L glucose, 1 g/L fructose, 4 g/L glycerol, 1 mM MgSO<sub>4</sub>, 10 mM 2 methanesulfonic acid (MES) and 100 µM acetosyringone) supplemented with 100 µM acetosyringone and appropriate antibiotics. The suspension Agrobacterium was grown at 27°C for 6h. After incubation, Agrobacterium cultures were collected by centrifugation (3000g for 5 min) then re-suspend in the infiltration medium (10 mM MgSO<sub>4</sub>, 10 mM MES, and 200 µM acetosyringone). Agrobacterium were adjusted to an OD of 0.4 at 600 nm. The bacterial suspension was infiltrated into 4-week-old leaves using a 1 ml needleless syringe. After infiltration, Arabidopsis plants were placed in the growth room for 48h. The infiltrated tissues were collected and frozen in liquid nitrogen then stored at -80°C.

## 2.4. Protein Extraction and Western Blot Analysis

For extraction, infiltrated tissue was homogenized in the protein extraction buffer containing of 20mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM PMSF, 10mM DTT, 30 $\mu$ M MG132, 6% glycerol, and protease inhibitor cocktail (Sigma-Aldrich). The samples were centrifuged for 10 minutes at 14, 000 g at 4°C to pellet plant debris. The supernatant of plant homogenate was transferred to a fresh 1.5 ml tube. 5x Sodium dodecyl sulfate (SDS) loading buffer (300 mM Tris-HCl pH 6.8, 30% glycerol, 12% SDS, 5%  $\beta$ -mercaptoethanol, 0.6% Bromophenol Blue) was added to samples and boiled for 5 minute followed by centrifugation at 10,000g for 1 minute. Samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred from the SDS-PAGE gel to polyvinylidene fluoride (PVDF) membrane using a semi-dry electro-transfer unit (Thermo Scientific). After transfer, membranes were blocked in a TBST (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.05% Tween 20) with 5% milk for 1 hour at room temperature, followed by incubation with primary antibodies for 1 hour (Table 2). Membranes were washed three times for 10 minutes with TBST. Secondary antibodies (Table 2) were then applied for 1 hour. Antibodies were diluted in TBST with 0.5% milk and used according to manufacturer's instructions (Table 2). Table 1 shows the list of primary and secondary antibodies that we used to detect YFP-HA-CIPK fusion protein. GFP antibody was used in immunoblot analysis for protein extraction and cell free degradation assays to detect the presence of the protein. Ubiquitin antibody was used to detect the ubiquitination in CIPK proteins. After the incubation with the secondary antibody, membranes were washed three times, 10 minutes each wash, with TBS-T before visualization with an Enhanced Chemiluminescence (ECL) Western Blotting Substrate kit according to manufacturer's

instructions (Thermo Scientific). Ponceau S (1g/L Ponceau S, 3% (v/v) acetic acid) was used to visualize total protein on the membrane.

**Table 2. List of primary and secondary antibodies used**

Protein Tag	Primary Antibody	Secondary Antibody	Dilution Factor
Ubiquitin	Mouse anti-Ubiquitin (Sigma-Aldrich)	Anti-Mouse (Sigma-Aldrich)	1:5000
GFP	Rabbit anti- GFP (Sigma-Aldrich)	Anti- Rabbit (Sigma-Aldrich)	1:5000

## **2.5. Cell-free Degradation Assays**

Cell-free degradation assays were performed as previously described by Wang et al. (2010). Briefly, plant tissue was collected from infiltrated tobacco and Arabidopsis tissue expressing YFP-HA-CIPK and protein extraction was carried out as described in section 2.4. Total protein extract was divided equally into two treatments; one treated with the 30 $\mu$ M of the 26S proteasome inhibitor MG132 and the other with an equivalent volume of extraction buffer. The reaction mixtures were incubated for 30 minutes at 25°C before adding 10mM adenosine triphosphate (ATP) to activate the reaction, which marked time point zero. Equal volume of each reaction mixture was removed at the indicated time points, and 6x SDS loading buffer was added to stop the reaction. Samples were subjected to SDS-PAGE, and the abundance of each CIPK protein was detected by Western blot analysis with YFP antibodies as described in section 2.4.

## **2.6. Post-Translational Modification Prediction, Sequence Alignment and Generation of the Sequence Similarity Tree**

Sequences for all 26 CIPK proteins were obtained from The Arabidopsis Information Resource (TAIR) database (<https://www.arabidopsis.org>). For posttranslation modification predictions, the ubiquitin prediction software UbiProber (Chen et al., 2013) was utilized to predict the ubiquitination sites (<http://bioinfo.ncu.edu.cn/UbiProber.aspx>). Sequence alignments were done using The European Bioinformatics Institutes (EBI) Clustal Omega protein sequence alignment program (McWilliam et al., 2013) (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). A sequence similarity tree comparing CIPKs was constructed using complete FASTA formatted amino acid sequences and the Phylogeny.fr website (<http://www.phylogeny.fr>) (Dereeper et al., 2010).



## **2.7. Immunoprecipitation**

Total protein extracts, prepared as described above, from un-infiltrated (control) and infiltrated tobacco tissue expressing YFP-HA-CIPK were incubated with EZview Red HA-Agarose beads (HA-beads) (Sigma-Aldrich). HA-beads were prepared according to manufacturer's instructions and incubated with protein extracts at 4°C for 2 hours. After incubation, the beads were collected through centrifugation at 8, 200g for 30 seconds, followed by two washes with the TBS for ten minutes each (50 mM Tris-HCl, pH7.5, 150 mM NaCl), then 30µL of 1X SDS was added and the sample was boiled for 5 minutes, followed by 3 minutes in a centrifuge at 10 000g. Samples were loaded onto the SDS-PAGE gels, followed by western blot analysis with ubiquitin antibody to detect the ubiquitinated protein and GFP antibody to detect the isolation of the protein (Table 2).

## **2.8. P62-Agarose Pull Down Assay**

Total protein extracts were prepared from un-infiltrated (control) and infiltrated tobacco tissue expressing YFP-HA-CIPK using protein extraction buffer containing (20mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 6% glycerol, and protease inhibitor cocktail (Sigma-Aldrich). P62 Agarose (Sigma-Aldrich) beads were prepared according to manufacturer's instructions and 25µm of the beads were incubated with protein extract at 4 °C for 2 hours. After incubation, the beads were collected through centrifugation at 8, 200g for 30 seconds, followed by two 10 minute washes with the TBS washing buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl) then 30µL of 1X SDS was added and boiled for 5 minutes, followed by 3 minutes of centrifugation at 10 000g. Samples were loaded onto the SDS-PAGE gels, followed by immunoblot analysis with GFP antibodies to detect the isolation of the protein.

## 2.9. Pixel Intensity Analysis

Pixel intensity of western blot samples was quantified using ImageJ software (<http://imagej.nih.gov/ij/>) (Abramoff et al., 2004). Exposed films that were obtained from cell free degradation assays were scanned and imported into ImageJ. The mean value for each protein bands was quantified using 16-bit grey scale. The pixel intensity values of were inverted to percent to determine the percentage remaining of each protein abundance over 240 minutes. The average of three trails for the remaining percent of each protein was used to compare protein abundance in + and - MG132 treatment. Standard Error were obtained to show the difference of protein abundance at each time point. One-way a nova test were used to determine the significant deference of the overtime point for protein abundance in + and - MG132 treatment.

## 2.10. Determining Protein Half-life

Protein half-life for each protein was determined using the half-life calculator (<http://www.calculator.net/half-life-calculator.html>). The decay process is computed by the flowing formulas:  $N_t = N_0(1/2)^{t/t_{1/2}}$ ,  $t_{1/2} = \ln(2) \tau = \ln(2)/\lambda$ ; where,  $N_0$  is the initial quantity,  $N_t$  is the quantity that still remains after a time  $t$ ,  $t_{1/2}$  is the half-life,  $\tau$  is the mean lifetime, and  $\lambda$  is the decay constant (calculator.net, 2017).

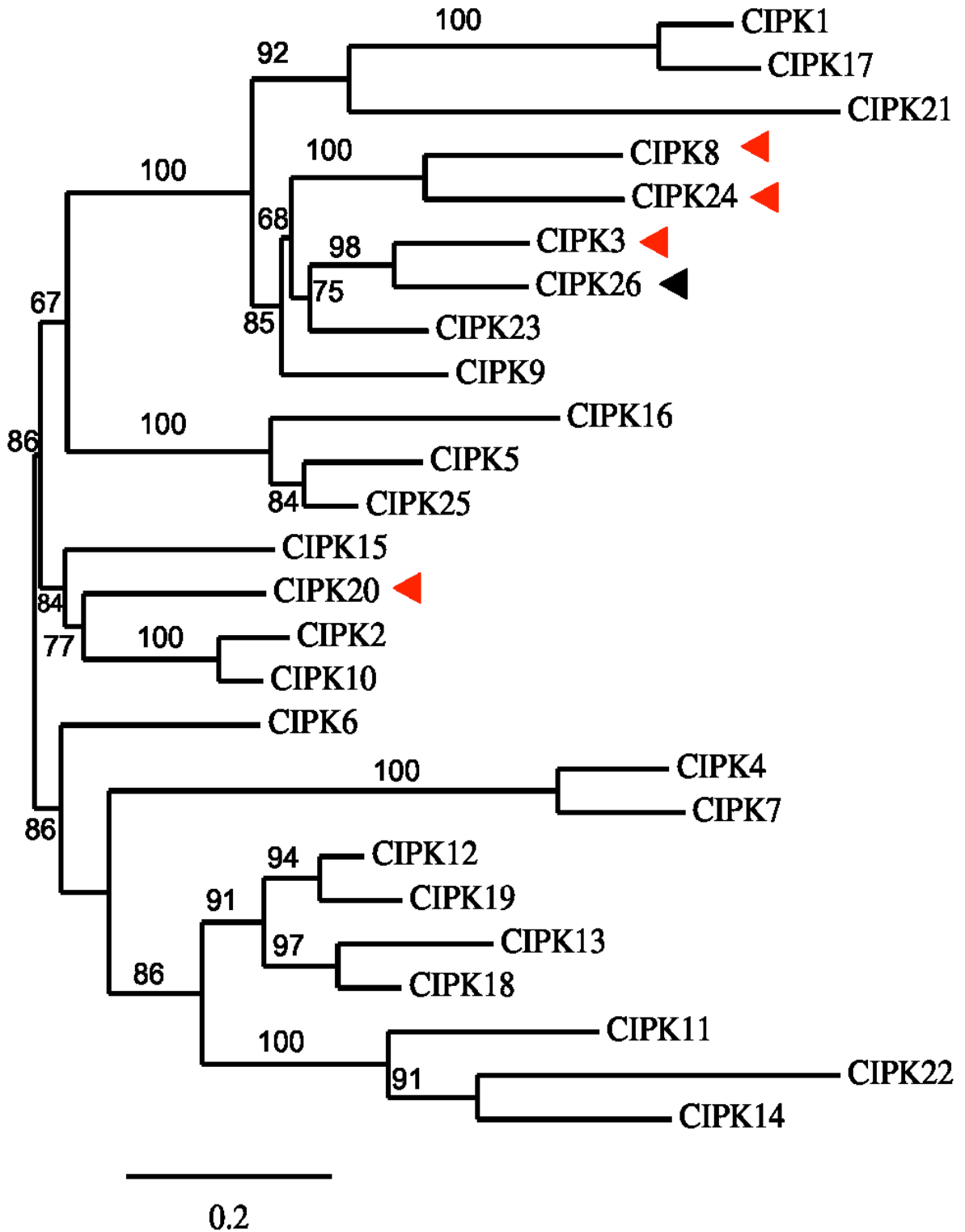
## CHAPTER THREE: RESULTS

### 3.1. Selection of CIPK Protein Family Members for Analysis

Recently the UPS was shown to regulate the abundance of CIPK26 (Lyzenga et al., 2013), suggesting possible regulation of the CIPK proteins family by the ubiquitination pathway. The main purpose of this study is to continue this investigation to determine if regulation by the UPS is common in the CIPK family. To accomplish this, members of the CIPK family were selected for this study based on their amino acid sequence similarity to CIPK26. A phylogenetic tree was generated to demonstrate relationships between CIPK26 and other members of the CIPK family (Figure 5). CIPK3 was included in our study as it is the most closely related to CIPK26 (Figure 5). CIPK3 was found to share 77 percent amino acid sequence similarity with CIPK26 (Table 3). CIPK8 and CIPK24 were also utilized in this study as slimier members; they each shared 62 percent sequence similarity and belong to the same clade of CIPK26 (Figure 5 and Table 3). The fourth selected member was CIPK20, which is in a different clade in the sequence similarity tree (Figure 5) and shows only 53 percent sequence similarity to CIPK26 (Table 3). In addition, two of the selected CIPKs were predicted to interact with various RING-type E3 ligases (Table 4). Information on predicted interactions were obtained from Bio-Analytic Resource Plant Biology (BAR), which provides evidence for CIPK8 interaction with two RING E3 ligases (At2g16090 and At5g06420) and CIPK24 is predicted to interact with one RING E3 ligase (At1g30860). This analysis served as the basis for utilizing CIPK3, CIPK8, CIPK20, and CIPK24 as representative proteins to study the possible regulation of the CIPK family proteins by the UPS. The selected proteins are not an extensive representation of the family as members that are not closely related (> 50% Amino acid sequence similarity) to CIPK26 are not included in the analysis.

**Figure 5. Phylogenetic Relationships between *Arabidopsis thaliana* CIPK Family Members**

Sequence similarity tree for the 26 members of the CIPK family generated using full length amino acid sequences. The red arrows indicate the four CIPKs that were chosen for this research. The black arrow indicates CIPK26 the only family member known to be regulated by the UPS. Numbers represent bootstrap values.



**Table 3: Amino acid sequence similarity between CIPK3, CIPK8, CIPK20, CIPK24, and CIPK26.**

	CIPK3	CIPK8	CIPK20	CIPK24	CIPK26
CIPK3	100%	57%	53%	58%	77%
CIPK8	_____	100%	49%	52%	56%
CIPK20	_____	_____	100%	47%	53%
CIPK24	_____	_____	_____	100%	58%
CIPK26	_____	_____	_____	_____	1005

**Table 4: Known and predicted CIPK-interacting RING-type E3**

Calcineurin B-like Interacting Protein kinases			Interacting RING E3 *	Evidence for Interaction
Loci	Name	Function	Loci	References
At2g26980	<b>CIPK3</b>	ABA signaling Response to cold stress	Not known	Pandey et al., 2008
At5g45820	<b>CIPK20</b>	ABA signaling	Not known	Gong et al., 2002
At4g24400	<b>CIPK8</b>	Nitrate sensing	At2g16090	Geisler-Lee et al., 2007
At4g24400	<b>CIPK8</b>		At5g06420	Leran et al., 2015
At5g35410	<b>CIPK24</b>	Salt tolerance	At1g30860	Geisler-Lee et al., 2007 Qiu et al., 2002; Gong et al., 2004
At5g21326	CIPK26	ABA signaling	At5g13530 (KEG)	Lyzenga et al., 2013

\* Prediction interactions are based on information obtained from Bio-Analytic Resource for Plant Biology (BAR), <http://bar.utoronto.ca/welcome.htm>, using the Arabidopsis Interactions Viewer tool.

### 3.2. Transient Expression of Selected CIPKs in Tobacco and Arabidopsis Leaves

The transient protein expression system provides an efficient and rapid approach for exploring protein function in the plant cells and represents an advantageous alternative to the time consuming of generation of transgenic plants (Sparkes et al., 2006). The use of the transient expression approach will allow for post-translational modifications (PTM) of the CIPKs, which may regulate their activity and abundance within the cell. Before the objectives of the study were carried out, we first had to determine if the selected CIPKs could be efficiently expressed in the transient expression systems. Plant transformation vectors allowing the expression of yellow fluorescence protein (YFP) and hemagglutinin (HA) tagged CIPK (YFP-HA-CIPK) were introduced into the epidermal cells of *Nicotiana tabacum* (tobacco) leaves using *Agrobacterium* mediated infiltration.

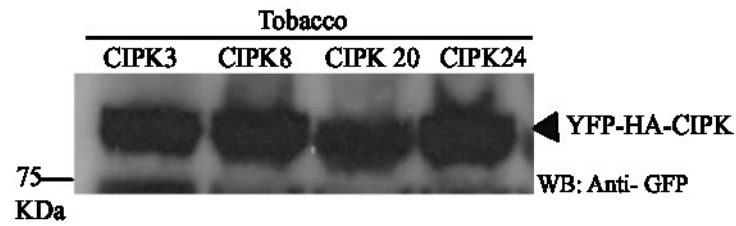
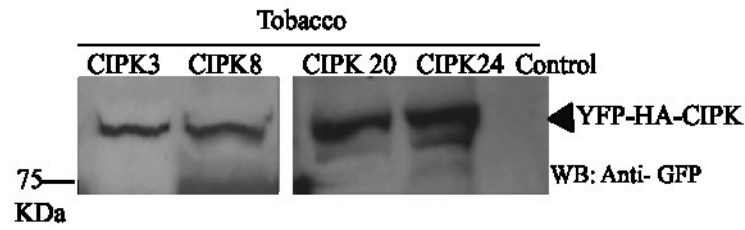
After forty eight hours, the infiltrated tissues were collected, protein extracts were prepared and subjected to western blot analysis using GFP antibodies to detect the presence of YFP-HA-CIPK. As shown in Figure 8A, YFP-HA-CIPK3, YFP-HA-CIPK8, YFP-HA-CIPK20, and YFP-HA-CIPK24 were successfully expressed in tobacco leaves. The CIPK proteins are from *Arabidopsis thaliana*, therefore the results obtained using the transient tobacco system were confirmed using an Arabidopsis based transient expression system (Lee & Yang 2006). Infiltrated Arabidopsis tissues were collected and protein extracts were prepared and subjected to western blot analysis using GFP antibodies to detect the presence of YFP-HA-CIPK. Results in Figure 8B shows that YFP-HA-CIPK3, YFP-HA-CIPK8, YFP-HA-CIPK20, and YFP-HA-CIPK24 are all expressed in transiently transformed Arabidopsis leaves.



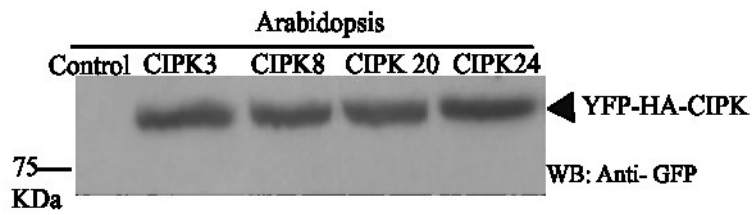
**Figure 6. Expression of CIPK3, CIPK8, CIPK20, and CIPK24 in Transient Expression Systems**

Transient expression of YFP-HA-CIPK3, YFP-HA-CIPK8, YFP-HA-CIPK20 and YFP-HA-CIPK24 in tobacco (A) and Arabidopsis (B) leaves. Protein extracts prepared from infiltrated and un-infiltrated (control) tissue were subjected to Western blot (WB) analysis with GFP antibodies that recognize the YFP tag on the YFP-HA-CIPK fusion proteins.

(A)



(B)



### **3.3. CIPKs Contain Multiple Predicted Ubiquitination Sites**

To begin investigating whether the Arabidopsis CIPK proteins undergo regulation by UPS, we examined the extent to which CIPK3, CIPK8, CIPK20, and CIPK24 may be ubiquitinated. Possible sites for ubiquitination were identified using the UbiProber prediction software (Chen et al., 2013). The prediction software identified ten lysine residues for CIPK3, five lysine residues for CIPK8, seven lysine residues for CIPK20, and six lysine residues for CIPK24 as possible sites for ubiquitination (Figure 7 and Supplemental Figure 1).

We then determined if the predicted ubiquitination sites are also found in other members of the CIPK family. A multiple sequence alignment was generated using the complete amino acid sequence of all 26 CIPKs (Figure 7). The alignment showed that three ubiquitination sites were conserved across all members of the CIPK family including the selected CIPKs (CIPK3, CIPK8, CIPK20, and CIPK24) (Figure 7). The conserved ubiquitination sites were found in the kinases domain of all CIPK proteins. The identification of these three conserved sites suggests that the ubiquitination may regulate CIPK function.

**Figure 7. Amino Acids Sequence Alignment for the CIPK Family Highlighting Predicted Ubiquitination Sites for CIPK3, CIPK8, CIPK20, and CIPK24**

Predicted ubiquitination sites for CIPK3, CIPK8, CIPK20, and CIPK24 are highlighted in blue and black boxes. Three predicted sites (blue boxes) are conserved across all CIPK proteins. The period (.) indicates amino acids that have weakly similar properties, the colon (:) shows amino acids that have highly similar properties, the asterisk (\*) indicates amino acids that are conserved.

CIPK3	-----MLTPNK-----KLRE	10
CIPK8	-----	0
CIPK20	-----	0
CIPK24	-----	0
CIPK1	-----MVRROE	6
CIPK2	-----	0
CIPK4	-----MESPYPK	7
CIPK5	-----	0
CIPK6	-----MVGAKPVENG	10
CIPK7	-----MESLPQPQNS	11
CIPK9	-----MSGSR	5
CIPK10	-----	0
CIPK11	-----MPEIEIA	7
CIPK12	-----MAEKITRETS-----LP	12
CIPK13	MAQVL-----STPLAIPGPTPIQFMAGLLARIVTKNTNK-ETSTPESPR	43
CIPK14	-----MVSDPVE	8
CIPK15	-----	0
CIPK16	-----M	1
CIPK17	-----	0
CIPK18	MAQALAQPPLVVTTVPPDPPPPPPHPKPYALRYMADLLGRIGIMDTDKDGNIS PQSPR	60
CIPK19	-----MADLLRKVKSIIK-----KK	14
CIPK21	-----	0
CIPK22	-----MSFTIPRLFLFTLFFCSVFFVCLLMAEDSNSESIIVN	38
CIPK23	-----MAS-----RTPSRST--PSRSTP	17
CIPK25	MGSKLKLYPL-----LNHSSVFHPDSRY--QSAPTM	29
CIPK26	-----	0

**Kinases Domain**

CIPK3	MNRROQVKRRVGKYEVGRTIGEGTFAKVKFARNSE-TGEPVALKILLK <sup>Q</sup> EKVL-KHKMAEQ	68
CIPK8	----MVRKVK <sup>Q</sup> YELGRTIGEGTFAKV <sup>K</sup> FAQNTS-TGESVAMKIVDRSTII-KRKMVDQ	53
CIPK20	--MDKNGIVLMRKYELGRLLGQGTFAK <sup>V</sup> VYHARNIK-TGESVAIKVIDKQKVA-KVGLIDQ	56
CIPK24	--MTKKMRRVGKYEVGRTIGEGTFAK <sup>V</sup> KFARNTD-TGDNVAIKIMAKSTIL-KNRMVDQ	55
CIPK1	EEKKA <sup>E</sup> KGMRLGKYELGRTLGE <sup>G</sup> NFGK <sup>V</sup> KFAKDTV-SGHSFAVKIIDKSRIA-DLNFSLQ	64
CIPK2	--MENKPSVLT <sup>E</sup> RYEVGRLLGQGTFAK <sup>V</sup> VYFGRSNH-TNESVAIKMIDKDKVM-RVGLSQQ	56
CIPK4	SPEKITGTVLLGKYELGRLLGSGSFAK <sup>V</sup> VHVAR <sup>S</sup> IS-TGELVAIKIIDKQKTI-DSGMEPR	65
CIPK5	--MEEERRVLF <sup>G</sup> KYEMGRLLGKGTFAK <sup>V</sup> VY <sup>G</sup> KEII-GGECVAIKVINKDQVMKRPGMMEQ	57
CIPK6	SDGGSSTGLLHGRYELGRLLGHGTFAK <sup>V</sup> VYHARNIQ-TGKSVAMKVVGKEKVV-KVGMVDQ	68
CIPK7	SPATTPAKILLGKYELGRLLGSGSFAK <sup>V</sup> VHLARSIE-SDELVAIKIIEKKKTI-ESGMEPR	69
CIPK9	RKATPASRTRVGN <sup>E</sup> MGRTLGEGSFAK <sup>V</sup> VYAKNTV-TGDQAAIKIILDREKVF-RHKMVEQ	63
CIPK10	--MENKPSVLT <sup>D</sup> KYDVGRLLGQGTFAK <sup>V</sup> VYGRSIL-TNQSVAIKVIDKEKVM-KVGLIEQ	56
CIPK11	AGSGDNDALF <sup>G</sup> KYELGKLLGCGAF <sup>A</sup> KVFHARDRE-TGQSVAIKILNKKKLLTNPALANN	66
CIPK12	KERSSPQALILGRYEMGKLLGHGTFAK <sup>V</sup> VYLARNVK-TNESVAIKVIDKEKVL-KGGLIAH	70
CIPK13	SPRTPQGSILMDKYEIGKLLGHGTFAK <sup>V</sup> VYLARNIH-SGEDVAIKVIDKEKIV-KSGLAGH	101
CIPK14	FPPENRRGQLF <sup>G</sup> KYEVGKLVGCGAF <sup>A</sup> KVYHGRSTA-TGQSVAIKVVSQKRL-QKGLNGN	66
CIPK15	--MEKKGSVLMLRYEVGKFLGQGTFAK <sup>V</sup> VYHARHLK-TGDSVAIKVIDKERIL-KVGMTEQ	56
CIPK16	EESNRSSSTVLF <sup>D</sup> KYNIGRLLGTGNFAK <sup>V</sup> VYHGTEIS-TGDDVAIKVIK <sup>D</sup> HVF <sup>K</sup> RRGMMEQ	60
CIPK17	---MVIKGMRVGKYELGRTLGE <sup>G</sup> NSAK <sup>V</sup> VKFAIDTL-TGESFAIKIIEKSCIT-RLNVSFQ	55
CIPK18	SPRSPRNNILM <sup>G</sup> KYELGKLLGHGTFAK <sup>V</sup> VYLAQNIK-SGDKVAIKVIDKEKIM-KSGLVAH	118
CIPK19	QDQSNHQALILGKYEMGRLLGHGTFAK <sup>V</sup> VYLARNAQ-SGESVAIKVIDKEKVL-KSGLIAH	72
CIPK21	--MGLFGTKKIGKYEIGRTIGEGNFAK <sup>V</sup> VKLG <sup>Y</sup> DTT-NGTYVAVKIIDKALVI-QKGLSQ	56
CIPK22	VTGDDNKSALF <sup>G</sup> KYDLGKLLGSGAF <sup>A</sup> KVYQAEDLNGGSEVAIKVVQKRL--KDGLTAH	96
CIPK23	SGSSSGRTRV <sup>G</sup> KYELGRTLGE <sup>G</sup> TFAK <sup>V</sup> VK <sup>F</sup> ARNVE-NGDNVAIKVIDKEKVL-KNKMIAQ	75
CIPK25	EEEQ <sup>Q</sup> QLRVLF <sup>A</sup> KYEMGRLLGKGT <sup>F</sup> GK <sup>V</sup> VY <sup>G</sup> KEIT-TGESVAIKIINKDQV-KREGMMEQ	87
CIPK26	-MNRPKVQRRVGKYEVGKTLGQGTFAK <sup>V</sup> RCAVNTE-TGERVALKVIDKEKVL-KHKMAEQ	57

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Kinases Domain

CIPK3	IRREIATMKLIK-HPNVVQLYEVMSKTKIFIIILEYVTGGELFDKIVNDGRMKEDEARRY	127
CIPK8	IKREISIMKLVR-HPCVVRLYEVLASRTKIYIILEYITGGELFDKIVRNGRLSESEARKY	112
CIPK20	IKREISVMRLVR-HPHVVFLHEVMSKTKIYFAMEYVKGGELFDKIVS-KGKLNENIARKY	114
CIPK24	IKREISIMKIVR-HPNIVRLYEVLASPSKIYIVLEFVTGGELFDRIVHKGRLEESESRKY	114
CIPK1	IKREIRTLKMLK-HPHIVRLHEVLASKTKINVMELVTGGELFDRIVSNGLTETDGRKM	123
CIPK2	IKREISVMRIAK-HPNVVLELYEVMATKSRIYFVIEYCKGGELFNKVA-KGKLKEDVAWKY	114
CIPK4	IIREIEAMRRLHHPNVLKIHVEMATKSKIYLVVEYAAGGELFTKLIRFGRLNESAARRY	125
CIPK5	IKREISIMKLVR-HPNIVELKEVMATKTKIFFVMEFVKGGELFCKIS-KGKLHEDAARRY	115
CIPK6	IKREISVMRMVK-HPNIVELHEVMASKKIYFAMELVRGGELFAKVA-KGRLREDVARVY	126
CIPK7	IIREIDAMRRLRHHPNILKIHVEMATKSKIYLVMEVLAAGGELFSKVLRRGRLPESTARRY	129
CIPK9	LKREISTMKLIK-HPNVVEIIEVMSKTKIYIVLELVNGGELFDKIAQQGRKLEDEARRY	122
CIPK10	IKREISVMRIAR-HPNVVLELYEVMATKTRIFVMEYCKGGELFNKVA-KGKLRRDDVAWKY	114
CIPK11	IKREISIMRRLS-HPNIVKLHEVMATKSKIFFAMEFVKGGELFNKISKHGRLSEDLRRY	125
CIPK12	IKREISILRRVR-HPNIVQLFEVMATKAKIYFVMEYVRGGELFNKVA-KGRLKEEVARKY	128
CIPK13	IKREISILRRVR-HPYIVHLFEVMATKTKIYFVMEYVRGGELYNTVA-KGRLPEETARRY	159
CIPK14	IQREIAIMHRLR-HPSIVRLFVSLATKSKIFFVMEFAKGGELFAKVS-KGRFCEDLSRRY	124
CIPK15	IKREISAMRLLR-HPNIVELHEVMATKSKIYFVMEHVKGGGELFNKVS-TGKLREDVARKY	114
CIPK16	IEREIAVMRLLR-HPNVVLELREVMATKTKIFFVMEYVNGGELFEMIDRDGKLPEDLARKY	119
CIPK17	IKREIRTLKVLK-HPNIVRLHEVLASKTKIYVLECVTGGDLFDRIVSKGKLSETQGRKM	114
CIPK18	IKREISILRRVR-HPYIVHLFEVMATKSKIYFVMEYVGGELFNTVA-KGRLPEETARRY	176
CIPK19	IKREISILRRVR-HPNIVQLFEVMATKSKIYFVMEYVKGGELFNKVA-KGRLKEEMARKY	130
CIPK21	VKREIRTMKLLN-HPNIVQIHEVIGTKTKICIVMEYVSGGQLSDRLGR-QKMKESDARKL	114
CIPK22	VKREISVMRRLR-HPHIVLLSEVLATKTKIYFVMEVLAAGGELFSRVT-SNRFTELSRKY	154
CIPK23	IKREISTMKLIK-HPNVIRMFEVMSKTKIYFVLEFVTGGELFDKISSNGRLKEDEARVY	134
CIPK25	IKREISIMRLVR-HPNIVELKEVMATKTKIFFIMEYVKGGELFSKIV-KGKLKEDSARKY	145
CIPK26	IRREICTMKLIN-HPNVVRLYEVLASKTKIYIVLEFVTGGELFDKIVHDGRLKEENARKY	116

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Kinases Domain

CIPK3	FQQLIHAVDYCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSALSQQ-----VRDDGL	180
CIPK8	FHQLIDGVYCHSKGVYHRDLKPENLLLDSEGNLKI SDFGLSALPEQ-----GVTI	163
CIPK20	FQQLIGAIIDYCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSALRES-----KQDGL	167
CIPK24	FQQLVDAVAHCHCKGVYHRDLKPENLLLDSEGNLKI SDFGLSALPQE-----GVEL	165
CIPK1	FQQLIDGYSYCHSKGVYHRDLKLENVLLDAKGHIKITDFGLSALPQH-----FRDDGL	176
CIPK2	FYQLISAVDFCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSALADC-----KRQDGL	167
CIPK4	FQQLASALSFCCHRDGIAHRDVKPQNLLLDSEGNLKI SDFGLSALPEH-----RSNNGL	178
CIPK5	FQQLISAVDYCHSRGVSHRDLKPENLLLDSEGNLKI SDFGLSALPEQ-----ILQDGL	168
CIPK6	FQQLISAVDFCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSAFTEH-----LKQDGL	179
CIPK7	FQQLASALRFSSHQDGVHRDVKPQNLLLDSEGNLKI SDFGLSALPEH-----L-QNGL	181
CIPK9	FQQLINAVDYCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSAFSRQ-----VREDGL	175
CIPK10	FYQLINAVDFCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSALADC-----KRQDGL	167
CIPK11	FQQLISAVGYCHARGVYHRDLKPENLLLDSEGNLKI SDFGLSALTDQ-----IRPDGL	178
CIPK12	FQQLISAVTFCHARGVYHRDLKPENLLLDSEGNLKI SDFGLSAVSDQ-----IRQDGL	181
CIPK13	FQQLISSVAFCHSRGVYHRDLKLENVLLDDKGNLKI SDFGLSVVSEQ-----LKQEGI	212
CIPK14	FQQLISAVGYCHSRGIFHRDLKPENLLLDSEGNLKI SDFGLSALTDQ-----IRPDGL	177
CIPK15	FQQLVRAVDCHSRGVCHRDVKPENLLLDSEGNLKI SDFGLSALSDDS-----RRQDGL	167
CIPK16	FQQLISAVDFCHSRGVFHRDLKPENLLLDSEGNLKI SDFGLSALMMPPEGLGRRGSSDDL	179
CIPK17	FQQLIDGVSYCHNKGVYHRDLKLENVLLDAKGHIKITDFGLSALSQH-----YREDGL	167
CIPK18	FQQLISSVAFCHGRGVYHRDLKPENLLLDSEGNLKI SDFGLSAVAEQ-----LRQDGL	229
CIPK19	FQQLISAVSFCCHRGVYHRDLKPENLLLDSEGNLKI SDFGLSAVSDQ-----IRQDGL	183
CIPK21	FQQLIDAVDYCHNRGVYHRDLKPENLLLDSEGNLKI SDFGLSAVPKS-----GDM	164
CIPK22	FRQLISAVRYCHARGVYHRDLKPENLLLDSEGNLKI SDFGLSAMKEQ-----IHPDGM	207
CIPK23	FQQLINAVDYCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSALPQQ-----VREDGL	187
CIPK25	FQQLISAVDFCHSRGVSHRDLKPENLLLDSEGNLKI SDFGLSALPEQ-----ILQDGL	198
CIPK26	FQQLINAVDYCHSRGVYHRDLKPENLLLDSEGNLKI SDFGLSALSRSQ-----VRDGL	169

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### Kinases Domain

CIPK3	LHTSCGTPNYVAPEVLNDRGYDGATADMWSCGVVLYVLLAGYLPFDDSNLMNLYKIKISSG	240
CIPK8	LKTTGCTPNYVAPEVLSHKGYNGAVADIWSCGVILYVLMAGYLPFDEMDLPTLYSKIDKA	223
CIPK20	LHTTCGTPAYVAPEVIGKGYDGAKADVWSCGVVLYVLLAGFLPFHEQNLVEMYRKITKG	227
CIPK24	LRTTCGTPNYVAPEVLSGQGYDGSAAADIWSCGVILFVILAGYLPFSETDLPGLYRKINAA	225
CIPK1	LHTTCGSPNYVAPEVLNRGYDGAASDIWSCGVILYVILTGLCLPFDDRNLAFLYQKICKG	236
CIPK2	LHTTCGTPAYVAPEVINRKGYEGTKADIWSCGVVLFVLLAGYLPFHDTNLMEMYRKIGKA	227
CIPK4	LHTACGTPAYTAPEVIAQRGYDGAKADAWSCGVVLFVLLAGYVPFDDANIVAMYRKIHKR	238
CIPK5	LHTQCGTPAYVAPEVLKKKGYDGAKADIWSCGVVLYVLLAGCLPFQDENLMNMYRKIFRA	228
CIPK6	LHTTCGTPAYVAPEVILKKGYDGAKADLWSCGVILFVLLAGYLPFQDDNLVNMRYKIYRG	239
CIPK7	LHTACGTPAYTAPEVISRRGYDGAKADAWSCGVILFVLLVGDVFPFDDSNIAAMYRKIHRR	241
CIPK9	LHTACGTPNYVAPEVLSDKGYDGAADVWSCGVILFVLMAGYLPFDEPNLMTLYKRICKA	235
CIPK10	LHTTCGTPAYVAPEVINRKGYDGTKADIWSCGVVLFVLLAGYLPFHDSNLMEMYRKIGKA	227
CIPK11	LHTLCGTPAYVAPEILSKKGYEGAKADVWSCGVILFVLLVAGYLPFNDPNVMNMYKKIYKG	238
CIPK12	FHTFCGTPAYVAPEVLARKGYDAAKVDIWSCGVILFVLMAGYLPFHDRNVMAMYKKIYRG	241
CIPK13	CQTFCGTPAYLAPEVLTRKGYEGAKADIWSCGVILFVLMAGYLPFDDKNILVMYTKIYKG	272
CIPK14	LHTLCGTPAYVAPEVLAKKGYDGAKIDIWSCGIIILFVLNAGYLPFNDHNLVMYRKIYKG	237
CIPK15	LHTLCGTPAYVAPEVLLKKGYDGSKADIWSCGVVLFVLLAGYLPFRDNLMELYKKIGKA	227
CIPK16	LHTRCGTPAYVAPEVLRNKGYDGAMADIWSCGVILYVLLAGFLPFIDENVMTLYTKIFKA	239
CIPK17	LHTTCGSPNYVAPEVLANEYDGAASDIWSCGVILYVILTGLCLPFDDANLAVICRKIFKG	227
CIPK18	CHTFCGTPAYIAPEVLTRKGYDAAKADVWSCGVILFVLMAGHIPFYDKNIMVYKKIYKG	289
CIPK19	FHTFCGTPAYVAPEVLARKGYDGAKVDIWSCGVILFVLMAGFLPFHNRNVMAMYKKIYRG	243
CIPK21	LSTACGSPCYIAPELIMNKGYSGAAVDWSCGVILFELLAGYPPFDDHTLPVLYKKILRA	224
CIPK22	LHTLCGTPAYVAPEVLLKKGYDGSKADIWSCGVVLFVLLAGYLPFRDNLMELYKKIYKA	267
CIPK23	LHTTCGTPNYVAPEVINNKGYDGAKADLWSCGVILFVLMAGYLPFEDSNLTSLYKKIFKA	247
CIPK25	LHTQCGTPAYVAPEVLRKKGYDGAKDIWSCGIIILYVLLAGFLPFQDENLMKMYRKIFKS	258
CIPK26	LHTACGTPNYAAPEVLNDQGYDGATADLWSCGVILFVLLAGYLPFEDSNLMTLYKKIYAG	229

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### Kinases Domain

CIPK3	EFNCPWLSLGMKLIIRLDPNPMTTRVTPQEV-FEDEFKDYKPPVFEERD-DSNMDD	298
CIPK8	EFSCPSYFALGAKSLINRILDPNPETRITIAEI-RKDEWFLKDYTPVQLIDY-EHVNLD	281
CIPK20	EFKCPNWFPEVKLLSRILDPNPNRSRIKIEKI-MENSWFQKGFKKIETPKSPEHQID-	285
CIPK24	EFSCPPWFSAEVKFLIHRILDPNPKTRIQIQGI-KKDPWFRNLNYVPIRAREE-EEVNLDD	283
CIPK1	DPPIPRWLSPGARTMIKRMLDPNPVTRITVGI-KASEWFKLEYIPIPSIPDDDD-EEVD	294
CIPK2	DFKCPSWFAPEVKRLLCKMLDPNHETRITIAKI-KESSWFRKGLHLKQKMKMEKQOQVR	286
CIPK4	DYRFPWSISKPARSIYKLLDPNPETRMSIEAV-MGTWVWFQKLEISEFQSSVF-----	291
CIPK5	DFEFPWFSPPEARLISKLLVDPDRRISIPAI-MRTPWLRKNFTPLAFKIDEPICS-Q	286
CIPK6	DFKCPGWLSSDARRLVTKLLDPNPTRITIEKV-MDSPWFKKQATRNRNEPVAATITIT--	296
CIPK7	DYRFPWSISKQAKSIYQMLDPNPVTRMSIETV-MKTNWFKKSLTSEFHRNVF-----	294
CIPK9	EFSCPPWFSGAKRVIKRILEPNPITRISIAEL-LEDEFKKGKPPSPFQDDEEDITIDD	294
CIPK10	DFKAPSWFAPEVRRLLCKMLDPNPETRITIAI-RESSWFRKGLHMKQKMKMEKRVKEI--	284
CIPK11	EYRFPWMSPDLKRFRVSRLLDINPETRITIDEI-LKDPWFVRRGGFKQIK-FHDE--I-	292
CIPK12	EFRCPRWFSTELTRLLSKLLETNPEKRFTPEI-MENSWFKKGFHKIKFYVEDD-KLC-N	298
CIPK13	QFKCPKWFSPELARLVTRMLDTPDTRITIEPI-MKHRWFKKGFHKVIFYIEND-KLC-R	329
CIPK14	EFRIKPTWSPDLRRLRLTRLDTPNPQTRITIEEI-IHDPWFKQGYDDRMSKFLHEDSDM--	294
CIPK15	EVKFPNWLAPGAKRLLKRIILDPNPTRVSTEKI-MKSSWFRKGLQEEVKEVSEEEETE--	284
CIPK16	ECEFPWFWSLESKELLSRLLVDPPEQRISMSEI-KMIPWFRKNFTPSVAFSIDETIPS-P	297
CIPK17	DPPIPRWISLGAKTMIKRMLDPNPVTRVTIAGI-KAHDWFKHDYTPSNYDDDD-DVYLI-	284
CIPK18	EFRCPRWSSDLVRLRLTRLDTPNPTRITIEPI-MKNRWFKKGFHKVIFYIEDD-KLC-R	346
CIPK19	DFRCPRWFPVEINRLLIRMLETKPERRFTMPDI-METSWFKKGFHKIKFYVEDDHLQCL-N	301
CIPK22	QYKLPDWTSSDLRLLRRLLEPNPELRITVEEI-LKDPWFNHGVDPEI-----I--	316
CIPK23	EFTCPWFWSASAKLIKRIILDPNPATRITFAEV-IENEWFKKGYKAPKFENA--DVSLDD	304
CIPK25	EFEYPPWFSPESKRLLSKLLVDPNKRISIPAI-MRTPWFRKNINSPIEFKIDELEIQ-N	316
CIPK26	EYHCPWLSPGAKNLIVRILDPNPMTTRITIEV-LGDAWFKKNYKPAVFEEKE-EANLDD	287

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	Regulatory Domain	NAF	
CIPK3	IDAVFKDS-----EEH--LVTEK---REEQPAAINAFEII-SMSRGLN		335
CIPK8	VYAAFDDP-----EEQ--TYAQD-GTRDTGPLTLNAFDLI-ILSQGLN		320
CIPK20	SLIS-----DVHAAFVSVKPMYSYNAFDLISLSQGF		316
CIPK24	IRAVFDGI-----EGS--YVAENVERNDEGPLMMNAFEMI-TLSQGLN		323
CIPK1	DDDAFSIQ-----ELG----SEEGKGSDSPTIINAFQLI-GMSFGLD		331
CIPK2	EATNPMEAGSGQEN-----GE----NHEPPRLATLNAFDII-ALSTGFG		327
CIPK4	-----ELDRFLEKEAKSSNAITAFDLI-SLSSGLD		320
CIPK5	SSKNN-----EEEEEDGCENQTEPI SPKFFNAFEFISMSGGFD		326
CIPK6	-----TEEDVDVLVHK SKEETETLNAFHII-ALSEGFD		328
CIPK7	-----DSEVEM---KSSVNSITAFDLI-SLSSGLD		320
CIPK9	VDAAFSNS-----KEC--LVT----EKKEKPVSMNAFELI-SSSEFS		330
CIPK10	---NSVEAGTAGTNEENGAGPSENGAGPSENGDRVTEENHTDEPTNLNAFDLI-ALSAGFD		340
CIPK11	-----EDQ-KVESLEAVKSLNAFDLI-SYSSGLD		320
CIPK12	VVDD-DEL-ESDSVESDRDSAAS-ESEIEYLEPRRRVGG LPRPASLNAFDII-SFSQGF		354
CIPK13	EDDD-NDDDDSSLSGSRSTAS-EGDAEFDIKRV--DSMPRPASLNAFDIL-SFS---D		381
CIPK14	-----KLPADETDSEM GARRMNAFDII-SGSPGFN		323
CIPK15	-----DAEA-----EGNASAEKKEKRCINLNAFEII-SLSTGFD		317
CIPK16	PEPPT-----KK-KKKDLNEKEDDGASPRSFNAFQFITMSGGFD		336
CIPK17	QEDVFMK-----E-----YEEKSPDSPTIINAFQLI-GMSFGLD		319
CIPK18	EDED-EEEEA---SSSGRSSTVS-ESDAEFDVKRMGIGSMRPSSLNAFDII-SFSSGFD		400
CIPK19	VADD-DEI-ESIESVSGRSSTVSEPEDFE SFDGRRRGSMRPASLNAFDLI-SFSPGFD		358
CIPK21	V-----AEINAATASSNFINAFQII-AMSSDLD		309
CIPK22	-----GIQADDYDLENGKILNAFDLI-SSASSN		345
CIPK23	VDAIFDDS-----GESKNLVVERREEGLKTPVTMNAFELI-STSQGLN		346
CIPK25	VED-E-----TPTTTATTATTTTTTPVSPKFFNAFEFISMSGGFD		355
CIPK26	VDAVFKDS-----EEH--HVTEK---KEEQPTSMNAFELI-SMSRALD		324

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	NAF	Regulatory Domain	PPI	
CIPK3	LENLFDPEQE-FKRETRITLRRGGANEIIEKIEEAKKPLGFDV--Q-KKNYKMRLENVKAG			391
CIPK8	LATLFDRGKDSMKHQTRFI SHK PANVVLSSMEVVSQSMGFKT--H-IRNYKMRVEGLSAN			377
CIPK20	LSGLFEK---ERSESKFTTKDAKEIVSKFEEIATSSERFN--LTKSDVGVKMEDKREG			371
CIPK24	LSALFDRRQDFVKRQTRFVSRREPEIIANIEAVANSMGFKS--H-TRNFKTRLEGLSSI			380
CIPK1	LSGFFEQEN-VSERRIRFTSNSSAKDLLEIETAVTEMGFSV--Q-KKHAKLRVQGEERN			387
CIPK2	LAGLFGDV--YDKRESRFASQXPASEIISKLVEVAKCLKLKI--RKQAGLFLKLERVKEG			383
CIPK4	LSGLFERR---KRKEKRFTARVSAERVVEKAGMIGEKLGFRVEKK-----EETKVVG			369
CIPK5	LSSLFESK---RKVQSVFTSRSSATEVMEK IETVTKEMNMKV--KRTKDFKVKMEGKTEG			381
CIPK6	LSPLFEEKKKEEKREMRFATSRPASSVISSLEEAARVGNKFD--VRKSESRVRIEGKQNG			386
CIPK7	LSGLFEAK---KKKERRFTAKVSGVEVEEKAKMIGEKLG YVVKKKM---MKKEGEVQVVG			374
CIPK9	LENLFEKQAQLVKKETRFTSQRSASEIMSKMEETAKPLGFNV--R-KDNYKIKMKGDKSG			387
CIPK10	LAGLFGDD---NKRESRFTSQPASVII SKLEEVAQRLKLSI--RKREAGLFLKLERLKEG			395
CIPK11	LSGLFAGCSNSSGESERFLSEKSPEMPLAEVEGFAREENLRMCKKKEEYGFEMEG----			376
CIPK12	LSGLFD-D---DGEGRFVSGAPVSKII SKLEEIAKVVVSFTV--RKKD-CRVSLEGSROG			407
CIPK13	LSGLFE-E---GGQGARFVSAAPMTKII SKLEEIAKEVKFMV--RKKD-WSVRLEGCREG			434
CIPK14	LSGLFGDARK-YDRVERFVSAWTAERVVERLEEIVSAENLTV--AKKETWGMKIEG----			376
CIPK15	LSGLFEKG--EEKEEMRFTSNREASEITEKLV EIGKDLKMKV--RKKEHEWRV---K-M			368
CIPK16	LSNLF EIK---RKP KRMFTSKFPAKSVKERLETAAREMDMRV--KHVKDCMKLQRRTEG			391
CIPK17	LSGFFETEK-LSERQIRFTSNLAKDLLEN IETIFTMGGFCL--Q-KKHAKLKAIKEEST			375
CIPK18	LSGLFEEE---GGEGRFVSGAPVSKII SKLEEIAKIVSFTV--RKKE-WSLRLEGCREG			454
CIPK19	LSGLFE-D---DGEGRFVSGAPVQII SKLEE IARIVSFTV--RKKD-CKVSLEGSREG			411
CIPK21	LSGLFEENDD-KRYKTRIGSKNTAQETIKKIEAAATYVSLSV--ERIKHFVKVIQPK EIR			366
CIPK22	LSGLFGNFVT-PDHCDQFVSDSTAVIMRKVEEVAKQLNLR I--AKKERAIKLEG----			398
CIPK23	LGSLFEKQMGVLVKKRTRFTSKSSANEIVTKIEAAAAPMGFDV--K-TNNYKMKLTGEKSG			403
CIPK25	LSSLFESK---RKLRSMTFSRWSASEIMGKLE GIGKEMNMKV--KRTKDFKVKLFGKTEG			410
CIPK26	LGNLFEEEEG-FKRETRFAAKGAANDLVQKIEEASKPLGFDI--Q-KKNYKMRLENVTAG			380

\* : \*



**PPI**                      **Regulatory Domain**

CIPK3	-RKGN--LNVATEIFQVAPSLHMQVSKSGGDT--LEFHK---FYKLLSNSLEQVWVTN	442
CIPK8	-KTSH--FSVILEVFKVAPSLMVDIQNAAGDA--EAYLK----FYKTFCSKLDIIWKP	428
CIPK20	-RKGH--LAIDVEIFEVTNSFHMVEFKKSGGDT--MEYKQFCDR---ELRPSLKDIVWKK	423
CIPK24	-KAGQ--LAVVIEIYEVAPSLFMVDVRKAAGET--LEYHK----FYKLLCSKLENIWRA	431
CIPK1	-QKGQVGLSVTAEVFEIKPSLNVVELRKSNGGDT--CLYRQLYERLLKDVGTSSPE-----	439
CIPK2	-KNGI--LTMDAEIFQVTPFHLVEVKKCNGDT--MEYQKLVVE---DLRPAADIVVWV	435
CIPK4	LGKGR--TAVVVEVVEFAEGLVVADVQVVEGEEEEVEVSHWS---ELIVELEEIVLSW	424
CIPK5	-RKGR--LSMTAEVFEVAPEISVVEFCKSAGDT--LEYDRLYEE---EVRPALNDIVWSW	433
CIPK6	-RKGK--LAVEAEIFAVAPSFVVVEVKKDHGDT--LEYNNFCST---ALRPALKDIFWTS	438
CIPK7	LGRGR--TVIVVEAVELTDVVVVEVVKVVE-----GEEDSRWS---DLITELEDIVLSW	424
CIPK9	-RKGQ--LSVATEVFEVAPSLHVVELRKTGGDT--LEFHKVCDSFYKFNFSGLKDVVWNT	442
CIPK10	-RKGI--LSMDAEIFQVTPNFHLVEVKKSGGDT--LEYQKLVAE---DLRPAALSDIVVWV	447
CIPK11	-QNGK--FGIGICISRLNDLVVVEARRRGGDG--DCYKEMWNG---KLRVQLIRVCDQT	428
CIPK12	-VKGK--LTIAAEIFELTPSLVVVEVKKGGDK--TEYEDFCNN---ELKPKLQNLTADD	459
CIPK13	-AKGP--LTIRVEIFELTPSLVVVEVKKGGNI---EYEEFCNK---ELRPQLEKLMHYQ	486
CIPK14	-QKGN--FAMVVEINQLTDELVMIEVRKRQRAA--ASGRDLWTD---TLRPFVVELVHES	428
CIPK15	-SAEA--TVVEAEVFEIAPSYHMVVLKSGGDT--AEYKRVKME---SIRPALIDFVLAW	420
CIPK16	-RKGR--LSVTAEVFEVAPEVSVVEFCKTSGDT--LEYLFCED---DVRPALKDIVWSW	443
CIPK17	-QKRQCGLSVTAEVFEIAPSLNVVELRKSNGGDT--SLYKQLYERLLNELGSSSQV-----	427
CIPK18	-AKGP--LTIAAEIFELTPSLVVVEVKKGGDR--EYEEFCNK---ELRPELEKLIHEE	506
CIPK19	SMKGP--LSIAAEIFELTPALVVVEVKKGGDK--MEYDFCNK---ELKPKLQNLSEN	464
CIPK21	-SRSS-YDLLSAEVIEVTPNCVIEISKAGEL--RLYMEFCQSLSSLTAEVS-----	416
CIPK22	-PHGV--ANVVVKVRRRLTNELVVMEMKNQRDV--G---LVWAD---ALRQKLRRLINQP	447
CIPK23	-RKGQ--LAVATEVQVAPSLYVMEVMRKSGGDT--LEFHK---FYKNLTTGLKDIVWKT	454
CIPK25	-RKGQ--IAVTAEVFEVAPEVAVVELCKSAGDT--LEYNRLYEE---HVRPALEEIVWSW	462
CIPK26	-RKGN--LRVATEIFQVSPSLHMIEVRKTKGDT--LEFHK----FYKLLSTSLNDVVWKS	431

CIPK3	NEVKKETAK-----	451
CIPK8	PDASMRNRVTKAKSKRR-----	445
CIPK20	QGNNNSNNEKLEVIH-----	439
CIPK24	TEGIPKSEILRTITF-----	446
CIPK1	-----QEIVT-----	444
CIPK2	QGEKEKEEQLLQDEQ-----GE-QEPE-----	456
CIPK4	HN-----	426
CIPK5	HGDNNNTSSEDC-----	445
CIPK6	TPA-----	441
CIPK7	HNDIM-----	429
CIPK9	DAAAEEQKQ-----	451
CIPK10	QGEKDELTSQOETEYQOQQOQEQQEQEPLKF-----	479
CIPK11	SSTNAAI-----	435
CIPK12	VVAEPVAVSAVDETAI-----PNSPTISFLPSDTE-	489
CIPK13	ADEVVEVM-----CLPPEIEQ	502
CIPK14	DQTDPEPTQVHTTS-----	442
CIPK15	H-----	421
CIPK16	QGDDDEDDVTNDNVDTND-NKINNVS-----	469
CIPK17	-----QELLA-----	432
CIPK18	VV-VEEAL-----YLPSDTE-	520
CIPK19	GQRVSGS-----RSLPSFLLSDTD-	483
CIPK21	-----	416
CIPK22	VYKVPDKP-----	455
CIPK23	IDEEKEEGTDGGGTNGAMANRTIAKQST-----	482
CIPK25	HGDNHNHNNIVKSNNGNYVSDENSGSDC-----	488
CIPK26	GESSGLSK-----	439

### 3.4. Arabidopsis CIPKs are Ubiquitinated in Plant Cells

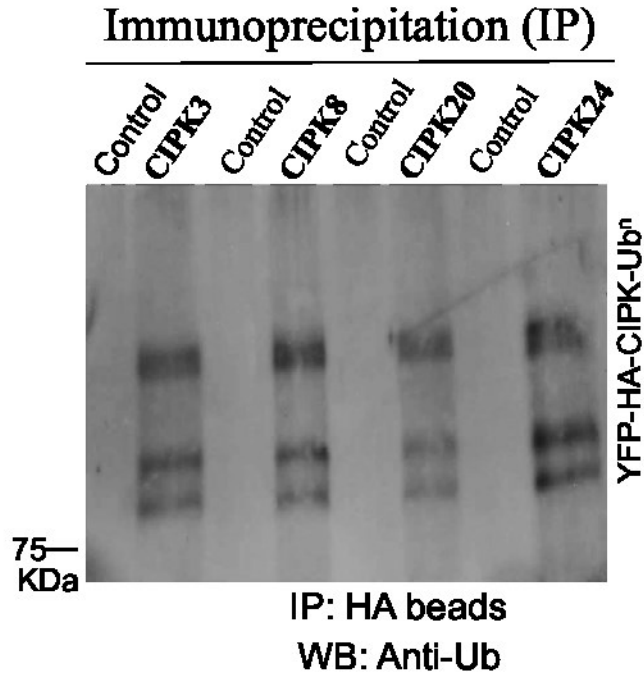
The above results suggest that CIPK3, CIPK8, CIPK20, and CIPK24 may be ubiquitinated in plant cells. To demonstrate experimentally the ubiquitination of CIPK3, CIPK8, CIPK20, and CIPK24 *in planta*, we performed an immunoprecipitation assay to isolate YFP-HA-CIPK followed by western blot analysis with ubiquitin antibodies to determine if the isolated protein is modified with ubiquitin. To isolate each CIPK, HA-beads were incubated with total protein extract prepared from infiltrated tobacco tissue transiently expressing YFP-HA-CIPK. The isolated proteins were then assessed by western blot analysis with ubiquitin antibodies in order to detect the ubiquitinated form of YFP-HA-CIPK (Figure 8A and Supplemental Figure 2) and GFP antibodies to ensure that YFP-HA-CIPK was bound to the HA-beads (Figure 8B and Supplemental Figure 2). The result in Figure 8A and Supplemental Figure 2 shows the ubiquitination of all four YFP-HA-CIPKs, which is indicated by the presence of high molecular weight versions of the proteins.

To provide further support for ubiquitination of CIPKs in plant cells, pull-down assays was performed using p62-agarose beads. The agarose beads utilize the ubiquitin-binding ubiquitin-associated (UBA) domain of p62 to interact with and isolate ubiquitinated proteins (Kong et al., 2015). Total protein extract prepared from tobacco tissue transiently expressing YFP-HA-CIPK8 was incubated with the p62-agarose beads. If YFP-HA-CIPK8 is ubiquitinated *in planta* then western blots using GFP antibody should be able to detect the presence of YFP-HA-CIPK8 among the isolated proteins. As shown in Figure 9, YFP-HA-CIPK8 was detected among the ubiquitinated proteins.

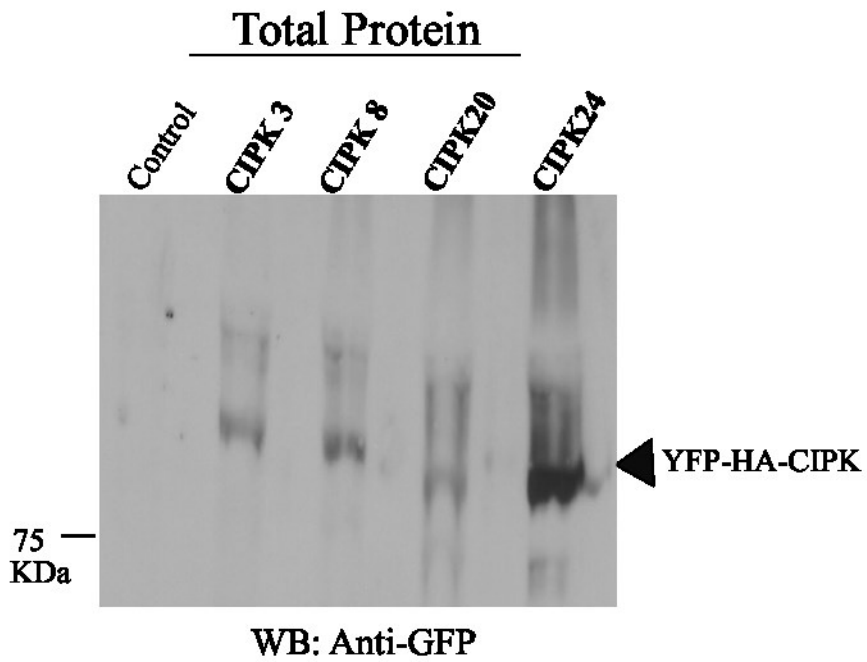
**Figure 8. CIPK3, CIPK8, CIPK20 and CIPK24 are Ubiquitinated in Plant Cells**

Total protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK were incubated with HA-beads to immunoprecipitate (IP) YFP-HA-CIPK. Isolated proteins were subjected to Western blot (WB) analysis with ubiquitin (Ub) antibodies to detect the ubiquitinated YFP-HA-CIPK (A) and GFP antibodies to detect the presence of YFP-HA-CIPK (B). Protein extracts prepared from untransformed tobacco tissue was used as a control.

(A)



(B)

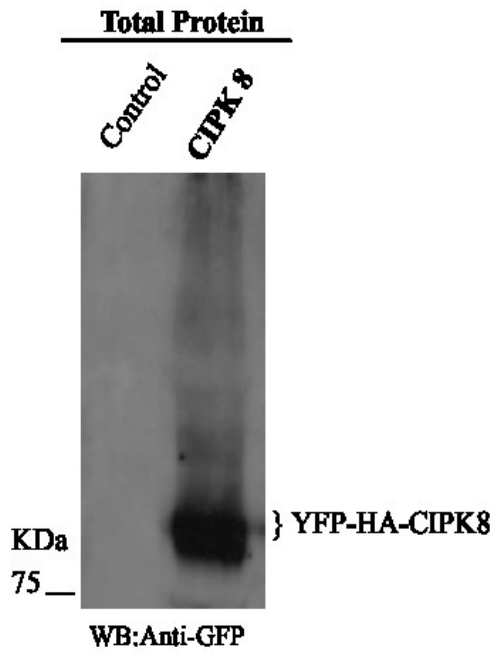


**Figure 9. Ubiquitin Pull-Down Assay Showing Ubiquitination of CIPK8 in Plant Cells**

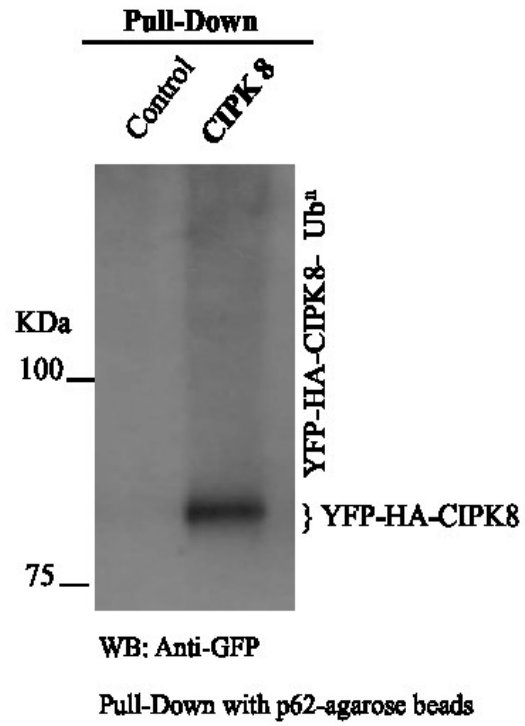
A) Total protein extract prepared from uninfiltreated (control) and infiltrated tobacco tissue transiently expressing YFP-HA-CIPK8 was subjected to Western blot (WB) analysis with GFP antibodies to demonstrate expression of YFP-HA-CIPK8.

B) Total protein extract prepared from un-infiltrated (control) and infiltrated tobacco tissue transiently expressing YFP-HA-CIPK8 was incubated with ubiquitin-beads (p62 agarose) to pull down ubiquitinated proteins. The isolated proteins were then subjected to Western blot (WB) analysis with GFP antibodies to detect the presence of YFP-HA-CIPK8.

(A)



(B)



### 3.5. 26S Proteasome Dependent Degradation of CIPK Proteins

The above results indicate that all the four CIPK family members are ubiquitinated *in vivo*. Therefore, it is worth determining if the outcome of ubiquitination is proteasomal degradation. As previously discussed, CIPK26 is degraded by the 26S proteasome, therefore other CIPK family members may also be regulated in a similar manner (Lyzenga, 2013). Cell-free degradation assays were performed to determine if the CIPKs were similarly targeted to the 26S proteasome for degradation (Wang et al., 2010). Protein extracts obtained from infiltrated tobacco tissue transiently expressing each YFP-HA-CIPK were used in the degradation assays. In addition, protein extracts were treated with proteasome inhibitor (MG132) to demonstrate that the observed degradation of each protein was dependent on the activity of the 26S proteasome.

The results in Figures 10A and 11A, show that the abundance of CIPK3 and CIPK20 reduced significantly within four hours during the assays. Whereas, the abundance of CIPK3 and CIPK20 remain relatively constant over the indicated time in assays that included proteasome inhibitor (Figures 10B and 11B). The figure 10C and 11C represent the average percent remaining of CIPK3 and CIPK20 over time from two separate trials, respectively (see also Supplemental Figures 3-4 for CIPK 3 and 5-6 for CIPK20). The proteasome-dependent turnover of CIPK8 was also observed in the cell free degradation assays. The abundance of CIPK8 decreases significantly over time in the absence of proteasome inhibitor (Figure 12A; Supplemental Figure 8). Whereas in the presence of proteasome inhibitor the abundance of CIPK8 remains relatively stable (Figure 12B). The graph indicates the percent remaining of CIPK8 over 90 minutes (Figure 12; Supplemental Figure 7).

The half-life for each protein was calculated using a protein half-life calculator. The half-life is the amount of time it takes for the abundance of each protein to be decreased by fifty percent (Figure 13). CIPK3 and CIPK20 each have a half-life of approximately 2.5 hours, whereas CIPK8 has a shorter half-life of approximately 30 minutes (Figure 13).

CIPK24 is ubiquitinated *in vivo*, however the abundance of CIPK24 remained relatively unchanged over time in all cell free degradation assays (Figure 16A and 16B; Supplemental Figure 9 and 10). The figure 16C is the average percent remaining of CIPK24 abundance over 240 minutes. These results suggest that ubiquitinated CIPK24 is not targeted to the 26S proteasome for degradation.

To provide further evidence for proteasome dependent turnover of the CIPKs, cell free degradation assays were repeated using protein extracts obtained from Arabidopsis leaf tissue transiently expressing each YFP-HA-CIPK. The results show that, similar to tobacco, CIPK3, CIPK8, and CIPK20 are degraded by the 26S proteasome, whereas CIPK24 levels remain relatively stable over the indicated timepoints (Figure 15). Taking these results together CIPK3, CIPK8, and CIPK20 are regulated by the UPS as shown in tobacco and Arabidopsis, while CIPK 24 is ubiquitinated but not degraded by the 26S proteasome.



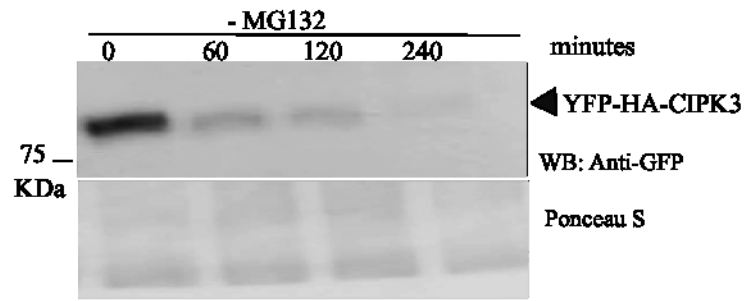
### **Figure10. CIPK3 is Degraded by the 26S Proteasome**

Cell free degradation assays were performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK3. Assays were without (-) (A), or with 50  $\mu$ M (+) (B) of the proteasome inhibitor, Mg132. The abundance of YFP-HA-CIPK3 at the indicated time points was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The figure (C) indicates the average percent remaining for YFP-HA-CIPK3 at each time point for two replicates. Error bars represent standard error. The asterisk (\*) indicates a statistically significant variation between mean of + and - MG132 treatment within indicated time points.

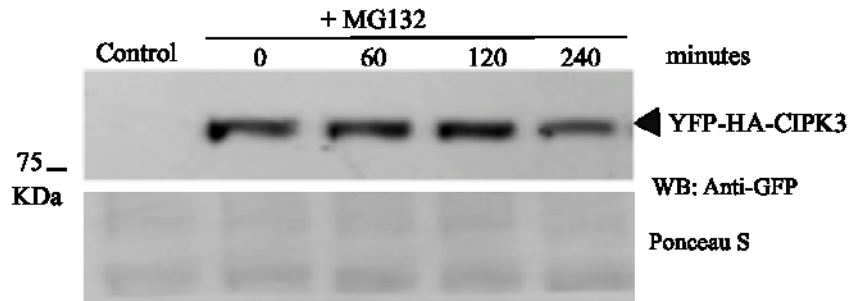
\* P < 0.01

\*\* P < 0.001

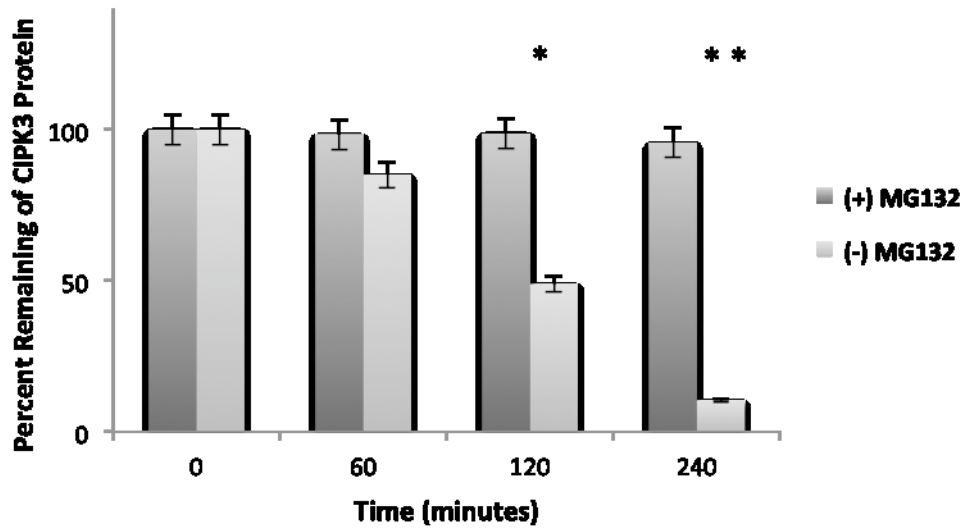
(A)



(B)



(C)



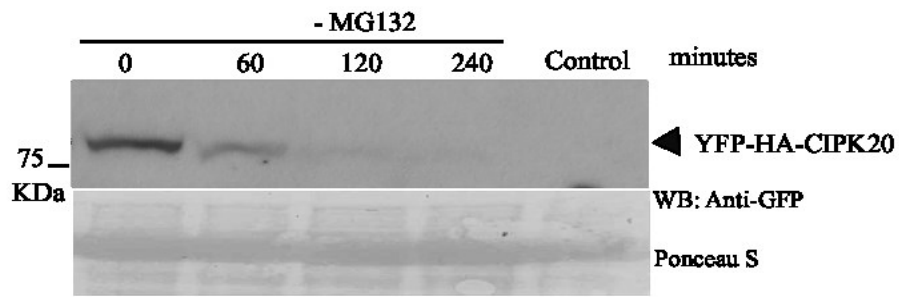
### **Figure11. Proteasome Dependent Degradation of CIPK20**

Cell free degradation assays were performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK20. Assays were without (-) (A), or with 50  $\mu$ M (+) (B) of the proteasome inhibitor, Mg132. The abundance of YFP-HA-CIPK20 at the indicated time points was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The figure (C) indicates the average percent remaining for YFP-HA-CIPK20 at each time point for two replicates. Error bars represent standard error. The asterisk (\*) indicates a statistically significant variation between mean of + and – MG132 treatment within indicated time points.

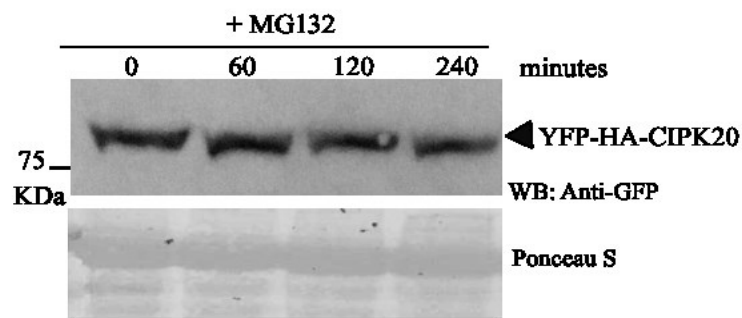
\* P < 0.01

\*\* P < 0.001

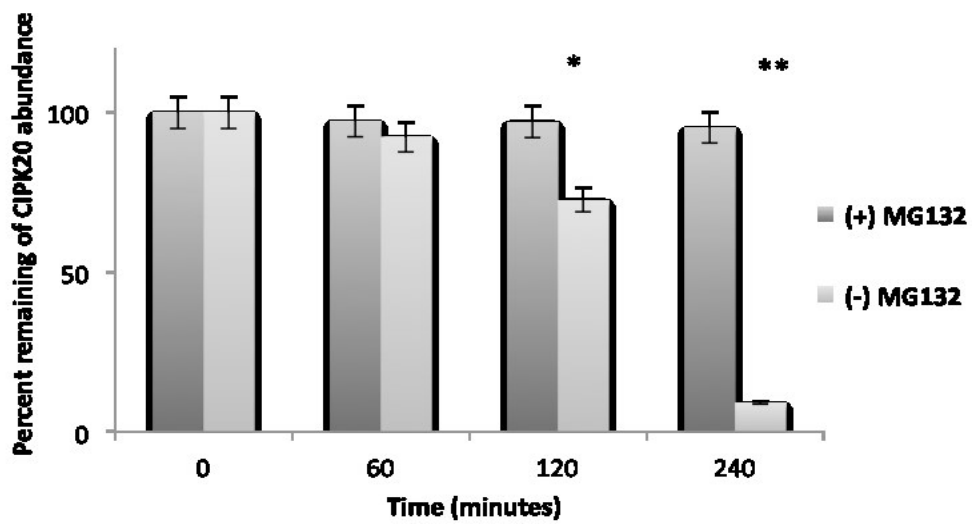
(A)



(B)



(C)

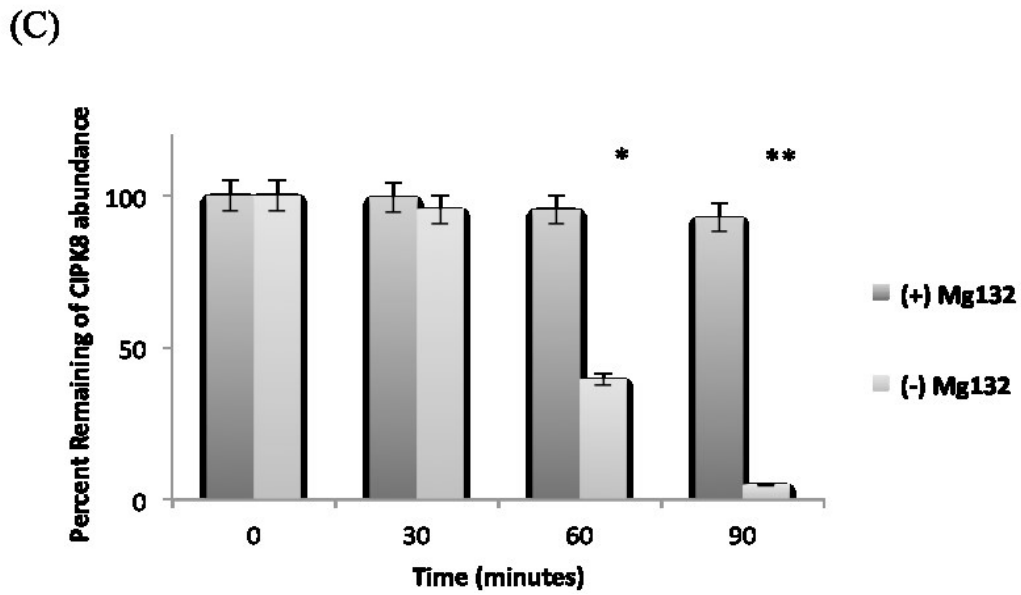
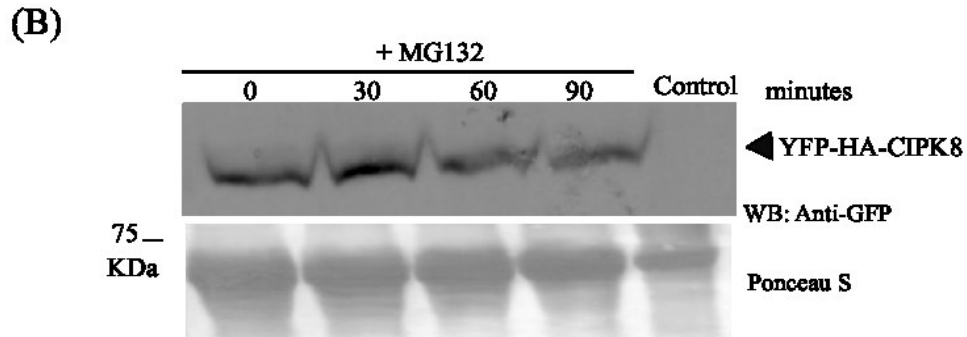
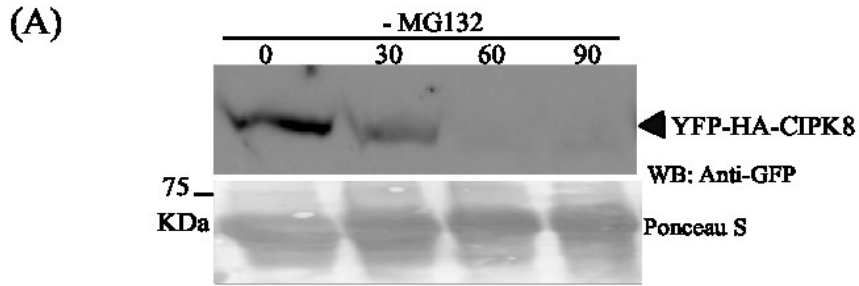


### **Figure 12. CIPK8 is Degraded by the 26S Proteasome in Tobacco Cells**

Cell free degradation assays were performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK8. Assays were without (-) (A), or with (+) (B) 50  $\mu$ M of the proteasome inhibitor, Mg132. The abundance of YFP-HA-CIPK8 at the indicated time points was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The figure (C) indicates the average percent remaining for YFP-HA-CIPK8 at each time point for two replicates. Error bars represent standard error. The asterisk (\*) indicates a statistically significant variation between mean of + and - MG132 treatment within indicated time points.

. \* P < 0.01

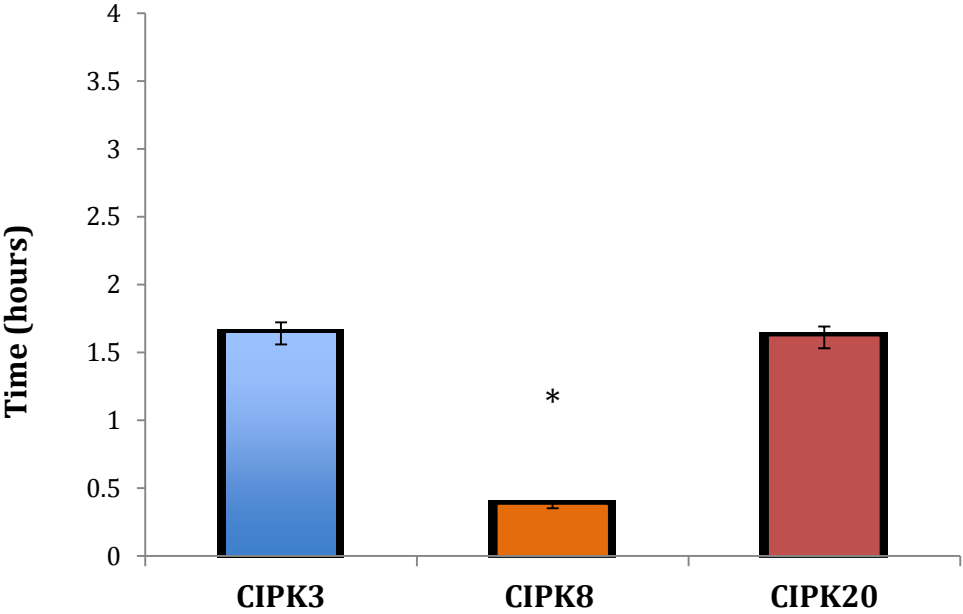
\*\* P < 0.001



**Figure13. Calculated Half-life for CIPK3, CIPK8 and CIPK20.**

The figure presents the calculated half life of CIPK3, CIPK8 and CIPK20. The blue and red bars represent CIPK3 and CIPK20 half-life, which is approximately 1.5 hours during four hours trials. The orange bar represents CIPK8, which has a short half-life of approximately 30 minutes over one and half hour trials. The asterisk (\*) indicates a statistical significant ( $p < 0.005$ ) variation within calculated half-life for CIPK3, CIPK8 and CIPK20.

**Half-life for CIPK3,  
CIPK8 and CIPK20**

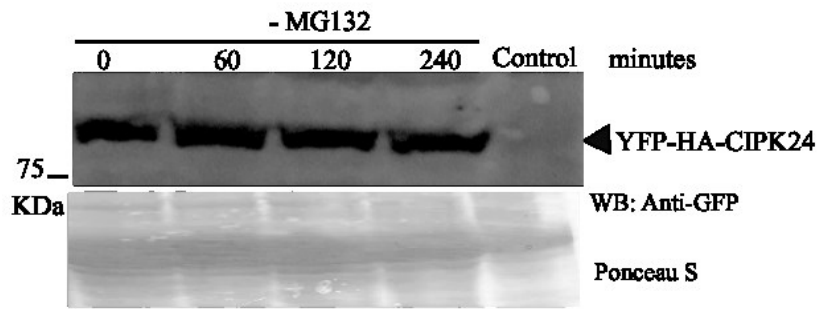




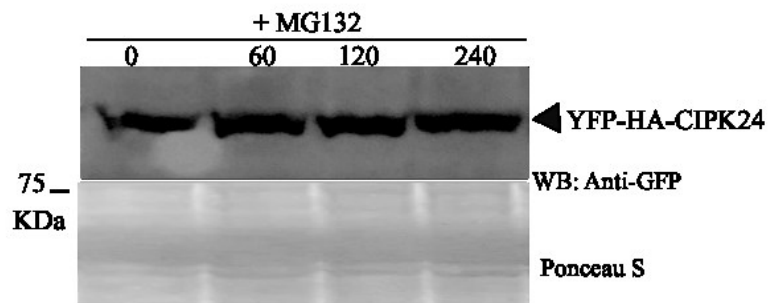
**Figure14. CIPK24 is not Degraded in Plant Cells.**

Cell free degradation assays were performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK24. Assays were performed without (-) (A) or with (+) (B) 50  $\mu$ M of the proteasome inhibitor, Mg132. The abundance of YFP-HA-CIPK8 (arrow) at the indicated time points was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The figure (C) indicates the average percent remaining for YFP-HA-CIPK24 at each time point for two replicates. Error bars represent standard error.

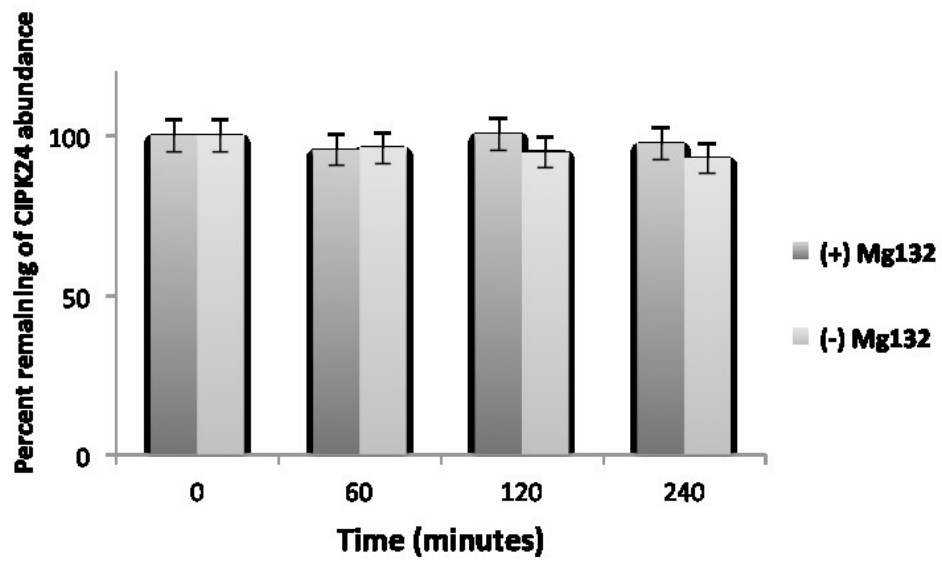
(A)



(B)



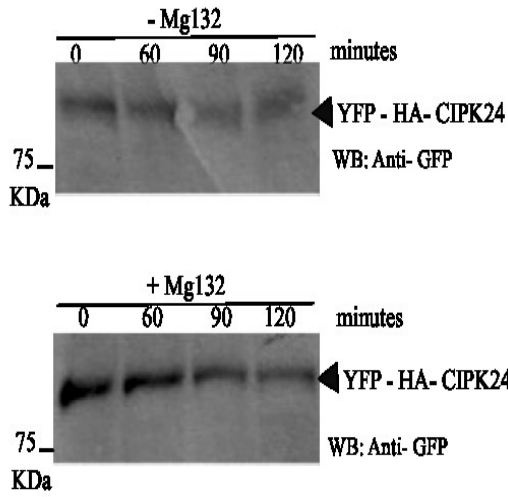
(C)



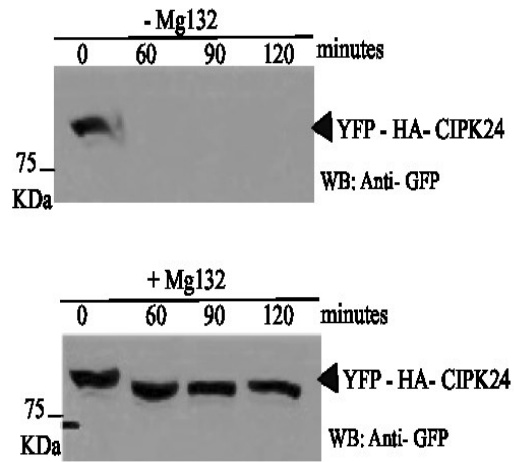
**Figure 15: Proteasome-Dependent Degradation of CIPK3, CIPK8 and CIPK20, and Stability of CIPK24 in *Arabidopsis thaliana*.**

Cell free degradation assays were performed using protein extracts prepared from Arabidopsis tissue transiently expressing YFP-HA-CIPK3, YFP-HA-CIPK8, YFP-HA-CIPK20 or YFP-HA-CIPK24. Assays were performed without (-) (A) or with (+) (B) 50  $\mu$ M of the proteasome inhibitor, Mg132. The abundance of YFP-HA-CIPK3 (A), YFP-HA-CIPK8 (B), YFP-HA-CIPK20 (C) or YFP-HA-CIPK24 (D) at the indicated time points was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading.

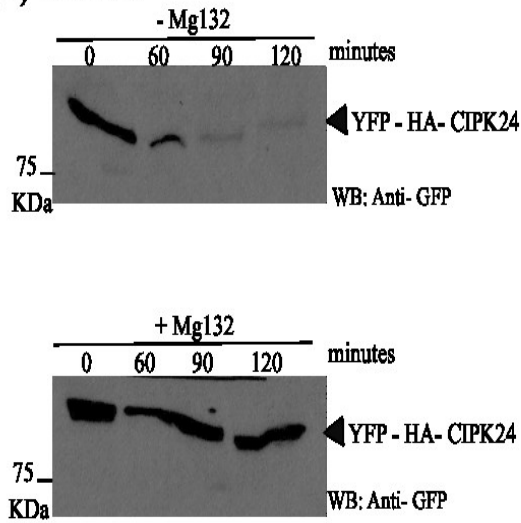
**(A) CIPK3**



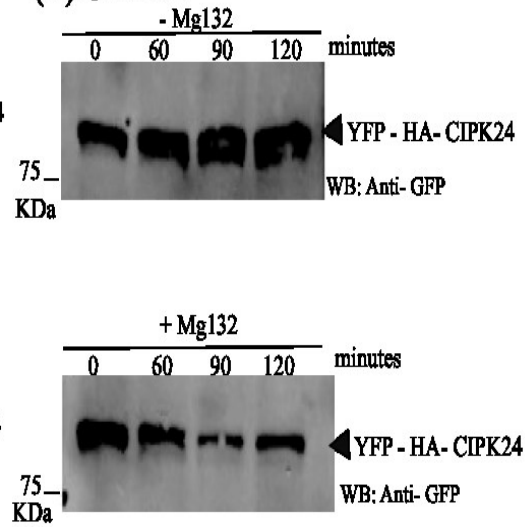
**(B) CIPK8**



**(C) CIPK20**



**(D) CIPK24**



## CHAPTER FOUR: DISCUSSION

### 4.1. The Ubiquitination of CIPK Protein Family

The purpose of this study was to determine if regulation by the UPS is a common occurrence in the CIPK family. The analysis demonstrated ubiquitination of all family members that were investigated in the study. However, proteasome dependent degradation was only observed for three of the four kinases examined. Ubiquitination of CIPK3, CIPK8, CIPK20, and CIPK24 contributes an additional layer of regulation in CIPK function. CIPK proteins are regulated by CBL proteins, which bind to the NAF domain to relieve inhibition and activate the kinase in response to stress-induced increases in calcium levels (Weinl & Kudla, 2009). ABA responsive protein phosphatases, ABI1 and ABI2, play a critical role in regulating CIPK's activities through dephosphorylation of the kinase in the absence of stress. Here we provide convincing evidence that CIPKs are also ubiquitinated in plant cells by using immunoprecipitation and ubiquitin pulldown assays coupled with Western Blot analysis (Figures 10 and 11). The experimental results are complemented by prediction analysis, which identified a number of potential ubiquitination sites in each CIPK (Figure 9). Of the identified predicted sites, three were conserved within the kinase domain across all 26 members of the family (Figure 9). Regulation of function by the UPS may be more common in the CIPK family than previously reported. A literature search of high-throughput mass spectrometry studies centering on the Arabidopsis ubiquitome, identified SnRK1.1 and SnRK2.4 as the ubiquitinated proteins (Kim et al., 2013). Thus, ubiquitination regulates the function of members from all three SnRK subfamilies.

## 4.2. Ubiquitin-mediated Degradation of CIPKs

Ubiquitination of a substrate is thought to have different outcomes, most notably targeting the modified protein to the 26S proteasome for degradation. All four CIPKs examined in this study are ubiquitinated in plant cells. The outcome of the ubiquitination of CIPK3, CIPK8, and CIPK20 seems to be degraded by the 26S proteasome. The degradation of these CIPKs indicates extensive involvement of the UPS in regulating the activity of SnRK subfamily members. Ubiquitination and proteasomal degradation of CIPK3, CIPK8, and CIPK20 are observed under non-stress conditions suggesting that the UPS is required for maintaining low abundance of the kinases during favourable growth conditions. Previous reports support this hypothesis, as CIPK26 degradation mediated by KEG also occurs during non-stress conditions (Lyzenga et al., 2013). In response to stress, ubiquitination of CIPK could be inhibited allowing the abundance of the kinase to increase. The accumulated and activated CIPK would then mediate the required stress response. Ubiquitin-dependent proteolysis has been implicated in controlling the abundance of various kinases and in providing the proper response to particular stresses. The mammalian AMP-activated protein kinase distantly related to SNF1 proteins, is subject to ubiquitin-dependent degradation mediated by the E3 ligase Cell Death-Inducing DFF45-like Effector  $\alpha$  in order to balance the consumption of energy in plant cell (Qi et al., 2008). In Arabidopsis, the SnRK1.2/AKIN11 abundance is degraded through the 26S proteasome in response to phosphate starvation (Fragoso et al., 2009). The results of this study strongly suggest that the UPS regulates CIPK function to maintain low abundance to inhibit activity until the plant perceives stress.

### **4.3. Ubiquitin-dependent, Proteasome-independent Regulation of CIPK24**

Most ubiquitinated substrate proteins are targeted for degradation by the 26S proteasome. However, degradation is not the only outcome of ubiquitination; other ubiquitin substrates are regulated in different ways (Erales & Coffino, 2014). Ubiquitination may regulate protein-protein interaction, protein activation, or localization within the cell (Katzmann et al., 2002). Proteasome independent outcomes of ubiquitination are usually mediated by monoubiquitination and polyubiquitination that generates non- K48 linked ubiquitin chains (Schnell & Hicke, 2003). For example, ubiquitin chains built using K63 are linked to protein relocalization (Moreno et al., 2010). As demonstrated by mass spectrometry analysis of the Arabidopsis proteome, the most abundant linkages of the polyubiquitin chain are K48, K63, and, K11 (Kim et al., 2013). In this study CIPK24 is polyubiquitinated; however, the kinase was not turned over in cell free degradation assays. This strongly suggests that CIPK24 is stable in plant cells and not subjected to proteasomal degradation. Unlike the other family members studied, ubiquitination of CIPK24 may involve the assembly of non-K48 linked ubiquitin chains such as K63 or K11 ubiquitin-ubiquitin linkages. Modification of CIPK24 may result in changes in localization of the kinases or promote interaction with other proteins. Alternatively, CIPK24 may have a long half-life and any decrease in abundance may only be apparent during cell-free degradation assays carried out for longer than the time (four hours) used in this study. Future study of CIPK24 could determine the lysine is used to make ubiquitin-ubiquitin linkages in the polyubiquitin chain. This analysis could provide insights into the outcome of ubiquitination and the observed stability of CIPK24 in this study.

#### **4.4. Summary**

CIPK3, CIPK8, CIPK20, and CIPK24 are regulated by the ubiquitination pathway. The ubiquitination of CIPK3, CIPK8, and CIPK20 results in degradation via the 26S proteasome, possibly to maintain the kinases at low levels to avoid an unnecessary activation of stress responses during favourable growth conditions. CIPK24 is ubiquitinated in plant cells; however, the outcome of ubiquitination of CIPK24 does not result in degradation and remains to be determined. These findings help to demonstrate the extent to which the CIPK family is regulated by the UPS.



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## **APPENDIX**

The following section includes supplement figures (S-1 to S-6) represent replicates, different time points for the previous data displayed in the results section.

**Supplemental figure 1. Post-Translational Modification Predictions of Arabidopsis CIPK3 At2g26980, CIPK8 At4g24400, CIPK20 At5g45820, and CIPK24 At5g35410.** Protein sequence of CIPK3 (A), CIPK8 (B), CIPK20 (C), and CIPK24 (D) with predicted ubiquitination site in lysine (K) residue, red box (Chen et al., 2013a) and phosphorylation sites, which presented in the serine (S), threonine (T) and tyrosine (Y), green box (Blom et al., 1999).

A)

MLIPNKKLREMNRROQVKRRVVGKYEVGRTIGEGTFAKVKFARNSETGEPVALKILDKEKV	60
LKHKMAEQIRREIATMMLIKHPNVVQLYEVMASKTKIFIILEYVTGGELFDKIVNDGRMK	120
EDEARRYFQQLIHAVDYCHSRGVYHRDLKPENLLLDSYGNLKI SDFGLSALSQQVRDDGL	180
LHTSCGTPNYVAPEVLNDRGYDGATADMWSSCGVVLYVLLAGYLPFDDSNLMNLYKKS SSG	240
EFNCPFWLSLGAMKLITRILDPNPMTRVTPQEVFEDEWFKKDYKPPVFEERDDSNMDDID	300
AVFKDSEEHLVTEKREEQPAAINAFEIISM SRGLNLENLFDPEQEFKRETRITLRGGANE	360
IEKIEEAAKPLGFDVQKKNYKMRLLENVKAGRKGNLNVATEIFQVAPSLHMVQVSKSKGD	420
TLEFHKFYKKL SNSLEQVWWTNNEVKKETAK	451

B)

MVVRKVGKYELGRTIGEGTFAKVKFAQNTETGESVAMKIVDRSTIIKRKMVDQIKREISI	60
MKLVRHPCVVRLYEVLASRTKIYIILEYITGGELFDKIVRNGRLSESEARKYFHQLIDGV	120
DYCHSKGVYHRDLKPENLLLDSQGNLKI SDFGLSALPEQGVITLKTTCGTPNYVAPEVLS	180
HKGYNAGAVADIWSCGVILYVLMAGYLPFDEMPLTLYSKIDKAEFSCPSYFALGAKSLIN	240
RILDPNPETRIITIAEIRKDEWFLKDYTPVQLIDYEHVNLDDVYAAFDDPEEQTYAQDGTR	300
DTGPLTLNAFDLIILSQGLNLATLFDRGKDSMKHQTRFISHKPANVVLSSMEVVSSMGF	360
KTHIRNYKMRVEGLSANKTSHFSVILEVFKVAPSI L MVDIQNAAGDAEELKFKYKTFCSK	420
LDDI IWKPPDASMRNRVT KAKSKRR	445

C)

MDKNGIVLMRKYEGLRLLGQGTFAKVVYHARNIKTGESVAIKVIDKQKVAKVGLIDQIKRE	60
ISVMRLVRHPHVFLHEVMASKTKIYFAMEYVKGELFDKVS K GK LKENIARKYFQQLIG	120
AIDYCHSRGVYHRDLKPENLLLDENGDLKISDFGLSALRESKQODGLLHTTCGTPAVVAP	180
EVIGKGYDGAKADVWSSCGVVLYVLLAGFLPFHEQNLVEMYRKITKGEFKCPNWFPPPEVK	240
KLLSRILDPNPNSRIKIEKIMENSWFQKGFKKIEIPKSPESHQIDSLISDVHAAF SVKPM	300
SYNAFDLISSLSQGFDSLGLFEKEERSSESKFTTKKDAKEIVSKFEEIATSSERFNLTKSD	360
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WKWQGNNNNSNNEKIEVIH	439

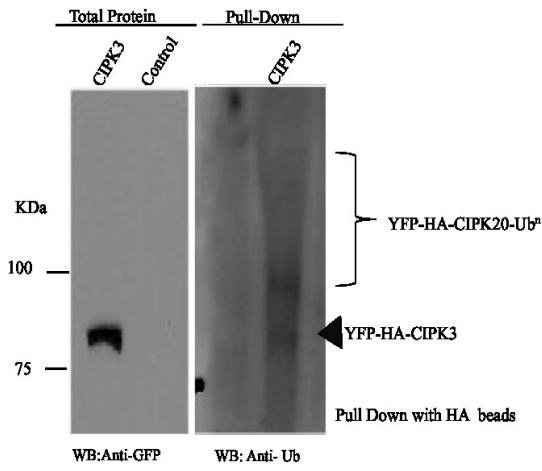
D)

MTKKMRRVGVYEVGRTIGEGTFAKVKFARNITDIGNVAIKIMAKSTILK NRMVDQIKREI	60
SIMKIVRHPNIVRLYEVLASPSKIYIVLEFVTGGELFDRIVHKGRLEEESRKYFQQLVD	120
AVAHCHCKGVYHRDLKPENLLLDTNGNLKVSDFGLSALPQEGVELLRTTCGTPNYVAPEV	180
LSGQGYDGSAAIWSCGVILFVILAGYLPFSETDLPGLYRKINAAEFSCPPWFSAEVKFL	240
IHRILDPNPKTRI IQIGIKKDPWFRNLNVP IRAREEEVNLD DIRAVFDGIEGSYVAENV	300
ERNDEGPLMMNAFEMITLSQGLNLSALFDRRQDFVKRQTRFVSRREPSEIANIEAVANS	360
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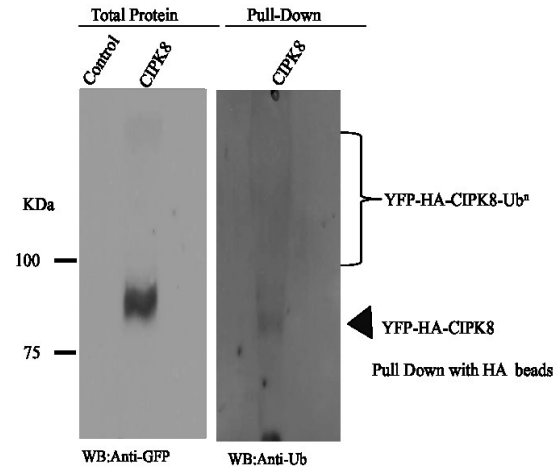
**Supplemental figure 2. CIPK 3, and CIPK8, CIPK 20, and CIPK24 are Ubiquinated in plant cell.**

Figure A-E present the second replicate (A to D) and third replicate (E) of the immunoprecipitate (IP) of CIPK3, CIPK8, CIPK20, and CIPK24. Total protein extracts from tobacco tissue transiently expressing YFP-HA-CIPK were incubated with HA-beads to immunoprecipitate (IP) YFP-HA-CIPK. Isolated proteins were subjected to Western blot (WB) analysis with ubiquitin (Ub) antibodies to detect the ubiquitinated YFP-HA-CIPK (labeled as Pull-Down) and GFP antibodies to detect the presence of YFP-HA-CIPK (labeled as Total protein). Untransformed tobacco tissue was used as a control in the experiments.

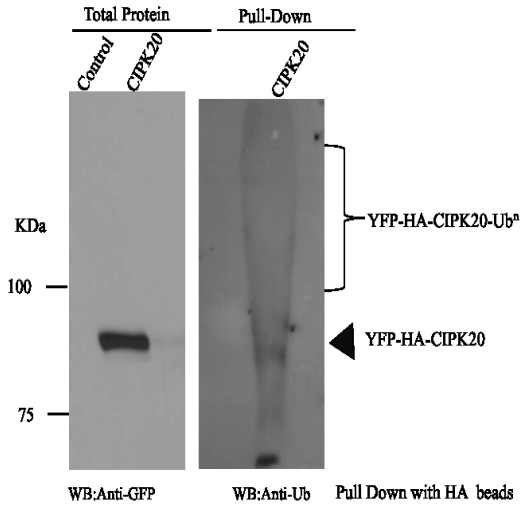
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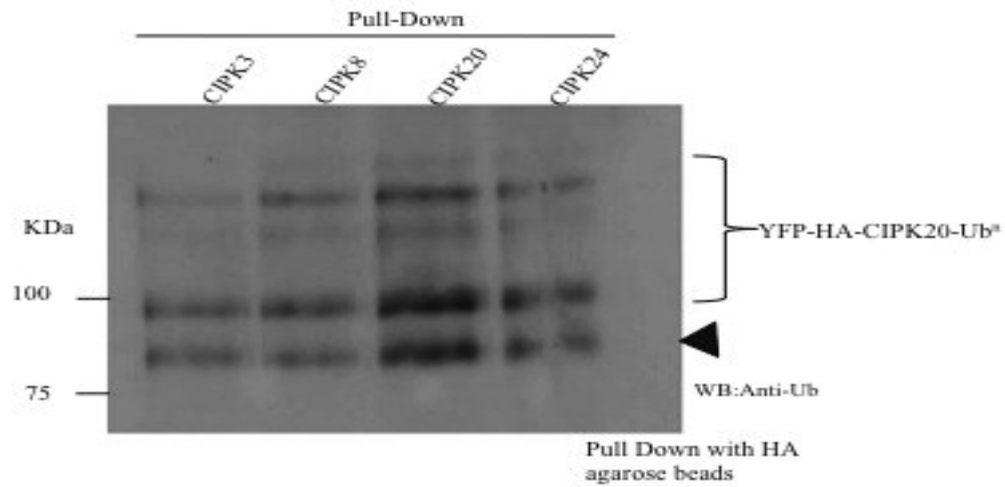
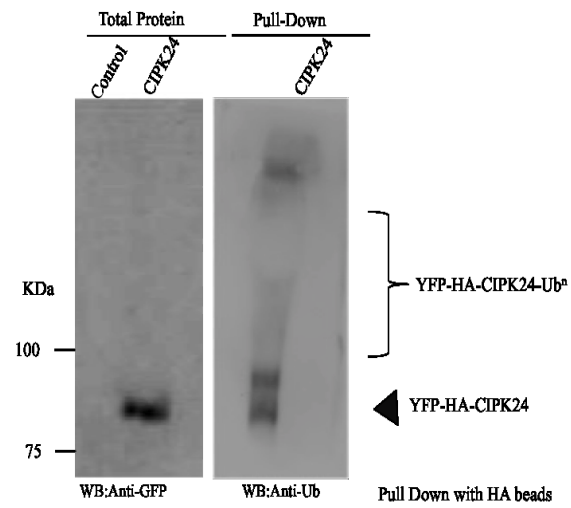
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(C)



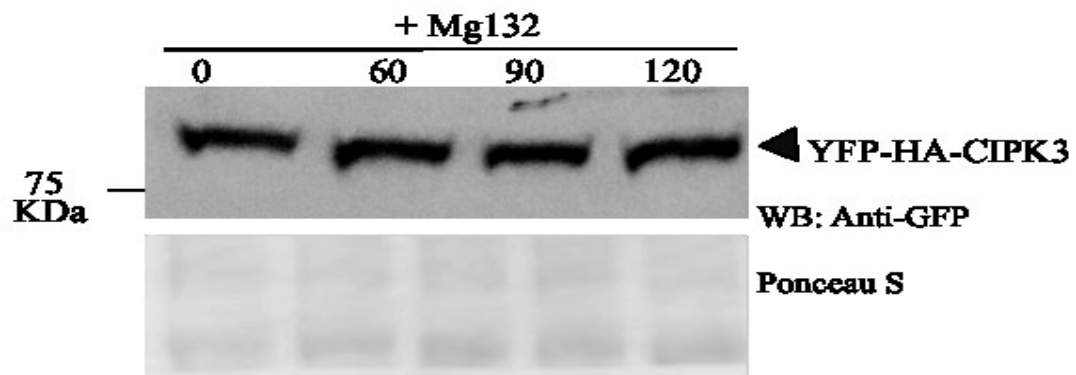
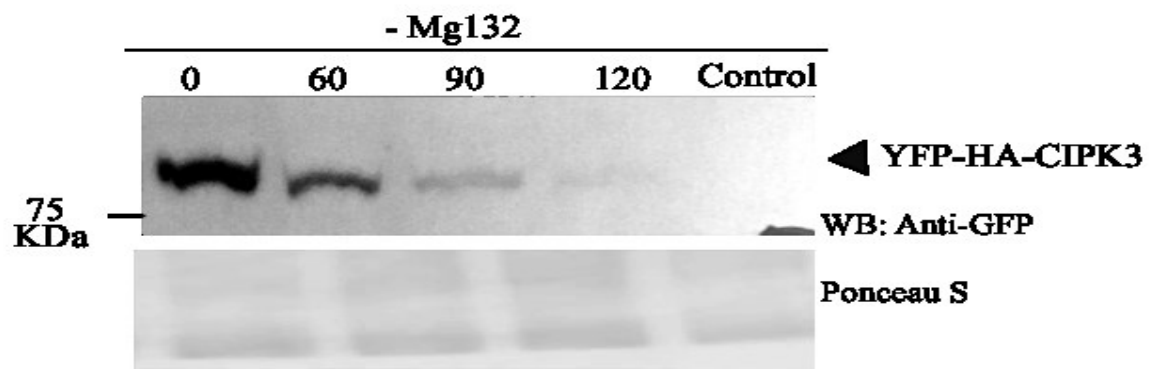
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**Supplemental figure 3. CIPK3 is degraded by the 26S proteasome.**

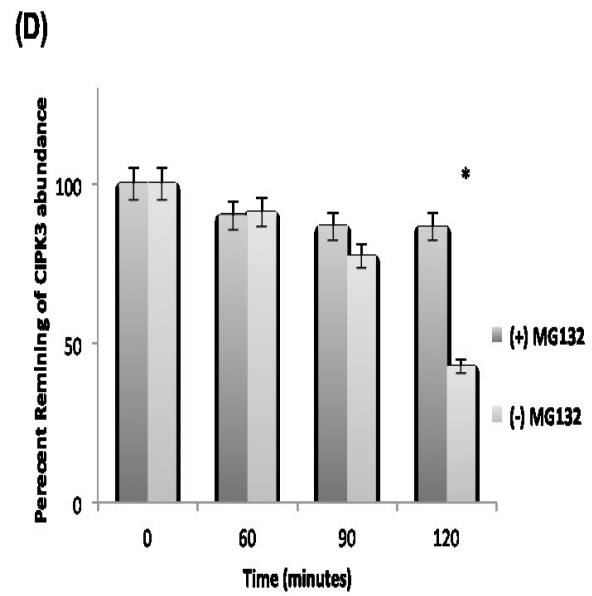
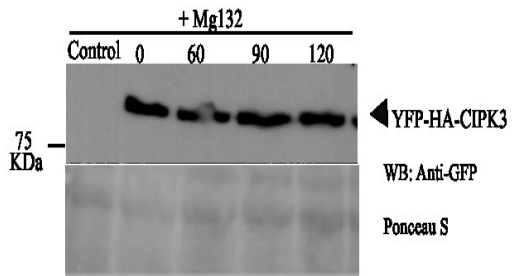
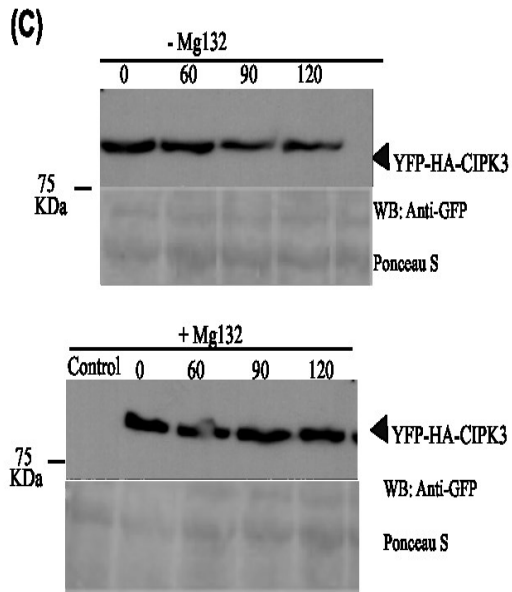
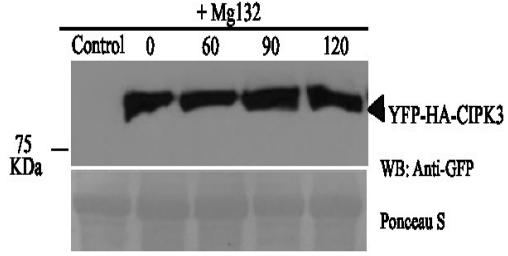
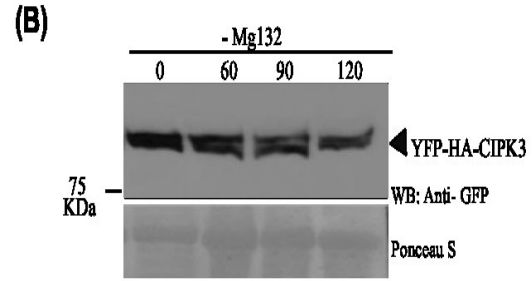
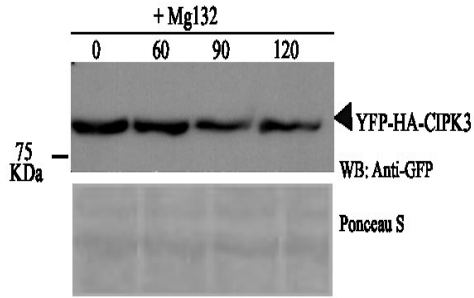
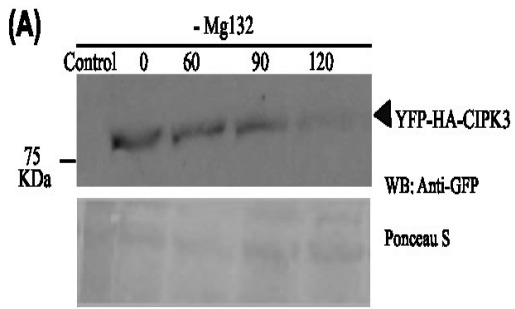
Cell free degradation assay (A) was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK3. Assay represents a replicate that shown in Figure 10. Assay was carried out without (-) or with (+) 50  $\mu$ M (+) proteasome inhibitor, Mg132 for 240 minutes. The abundance of YFP-HA-CIPK3 at each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading.





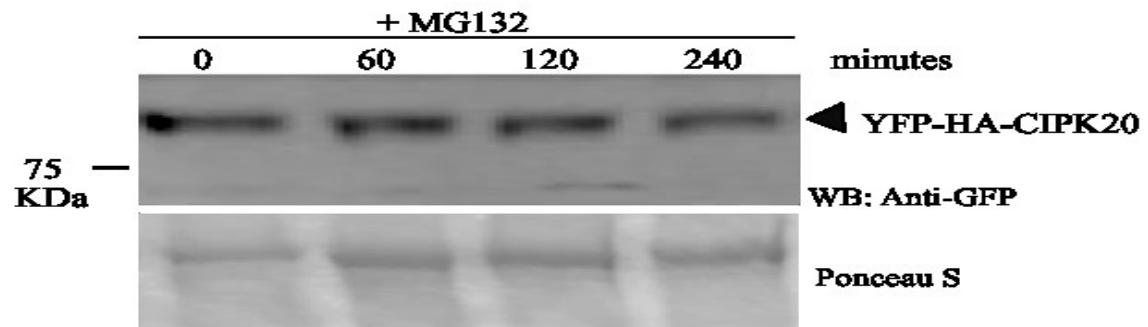
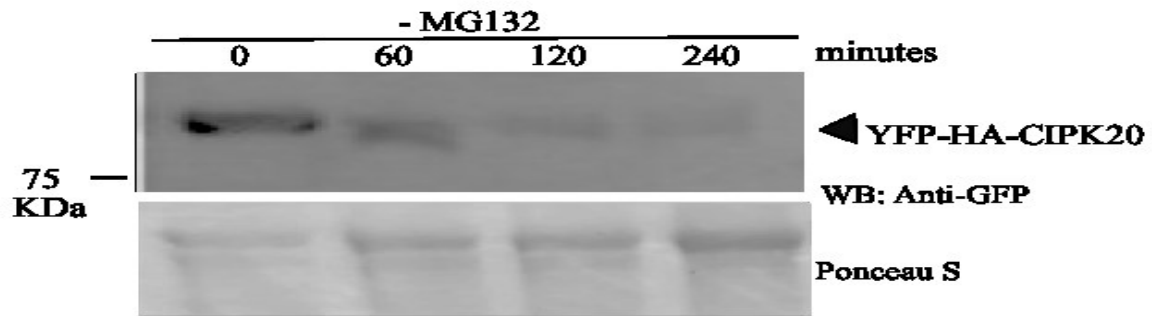
**Supplemental figure 4. CIPK3 is degraded by the 26S proteasome.**

Cell free degradation assay was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK3. Assays (A-C) represent three replicates carried out without (-) or with (+) 50  $\mu$ M proteasome inhibitor, Mg132 for 120 minutes. The abundance of YFP-HA-CIPK3 in each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The graph (D) indicates the average percent remaining for YFP-HA-CIPK3 at each time point for all three replicates. Error bars represent standard error. The asterisk (\*) indicates a statistically significant ( $p < 0.001$ ) variation between mean of + and - MG132 treatment within indicated time points.



**Supplemental figure 5. CIPK20 is degraded in plant cell.**

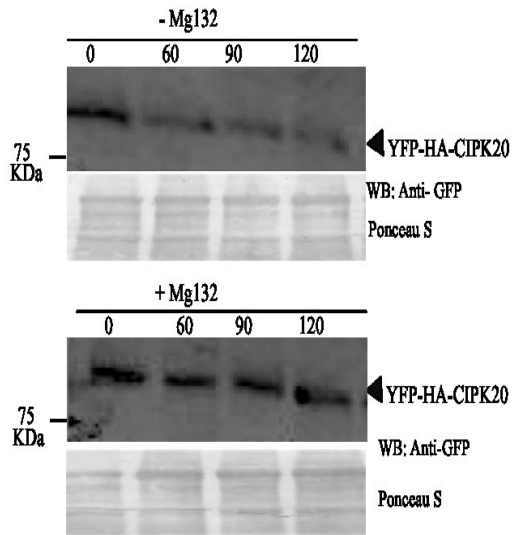
Cell free degradation assay (A) was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK20. Assay represents a replicate that shown in Figure 11. Assay was carried out without (-) or with (+) 50  $\mu$ M (+) proteasome inhibitor, Mg132 for 240 minutes. The abundance of YFP-HA-CIPK20 at each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading.



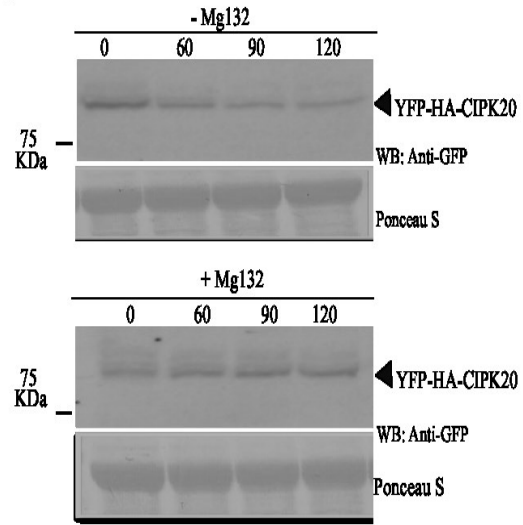
**Supplemental figure 6. CIPK20 is degraded in plant cell.**

Cell free degradation assay was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK20. Assays (A-C) represent three replicates carried out without (-) or with (+) 50  $\mu$ M proteasome inhibitor, Mg132 for 120 minutes. The abundance of YFP-HA-CIPK20 in each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The graph (D) indicates the average percent remaining for YFP-HA-CIPK20 at each time point for all three replicates. Error bars represent standard error. The asterisk (\*) indicates a statistically significant ( $p < 0.001$ ) variation between mean of + and - MG132 treatment within indicated time points.

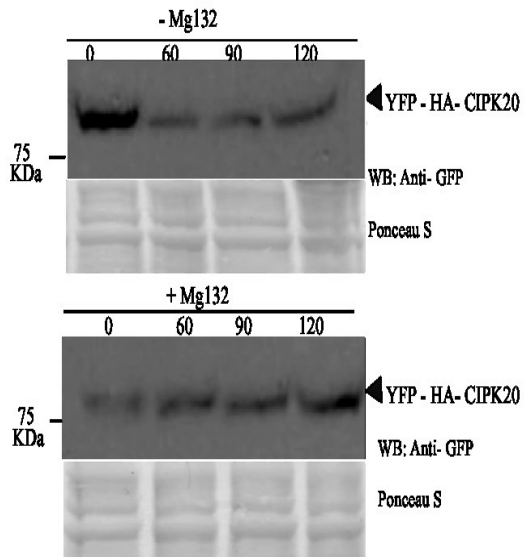
(A)



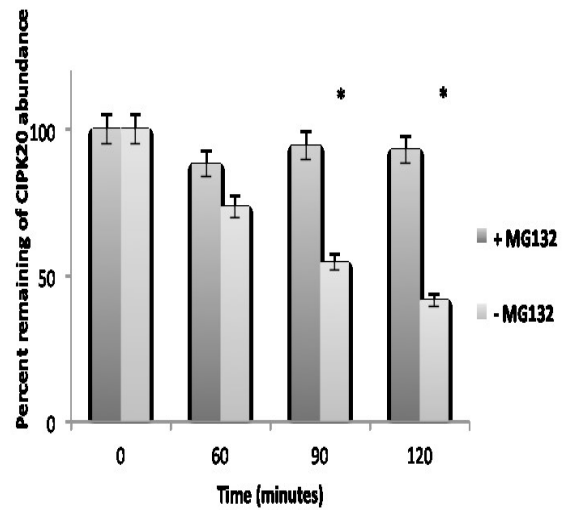
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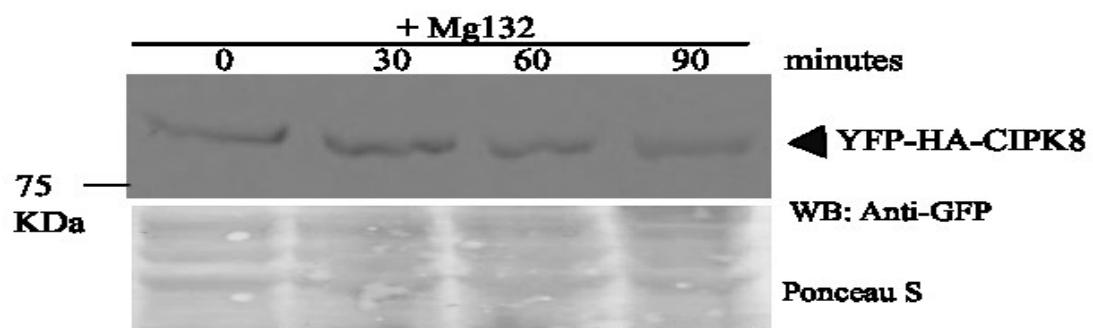
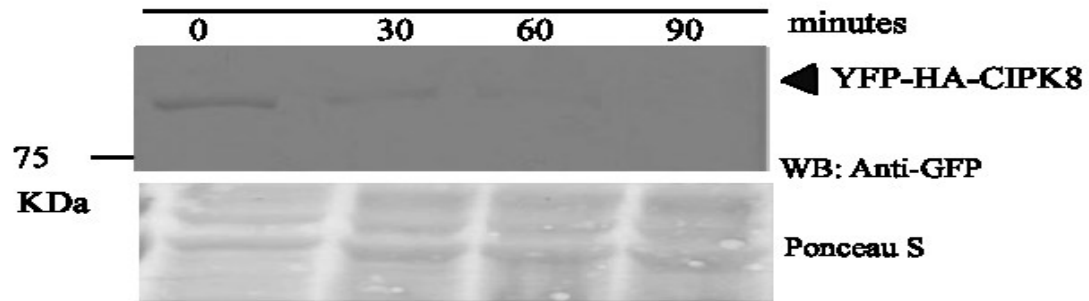
(D)



**Supplemental figure 7. CIPK8 observed to have short half live to be targeted for degradation by the 26S proteasome.**

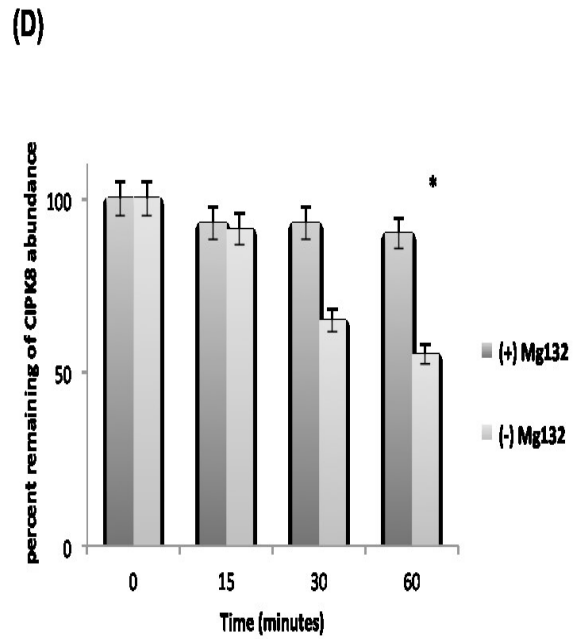
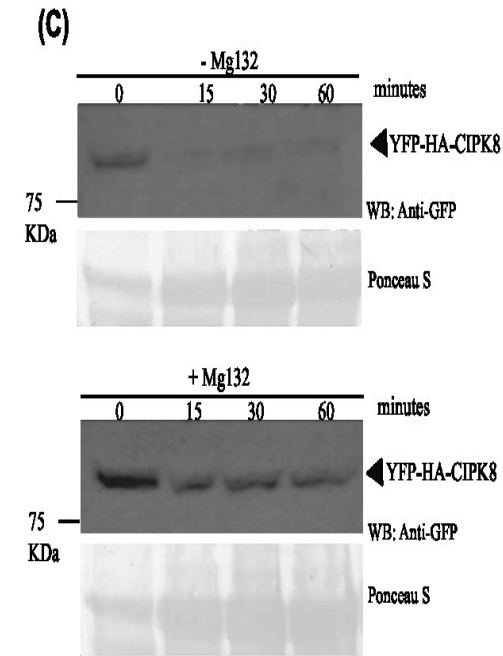
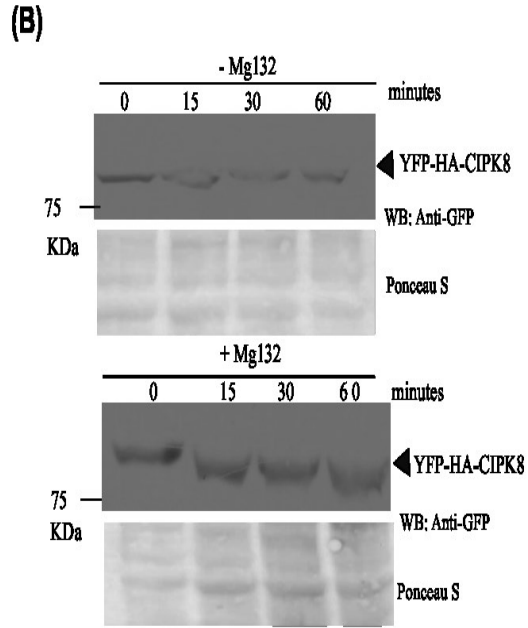
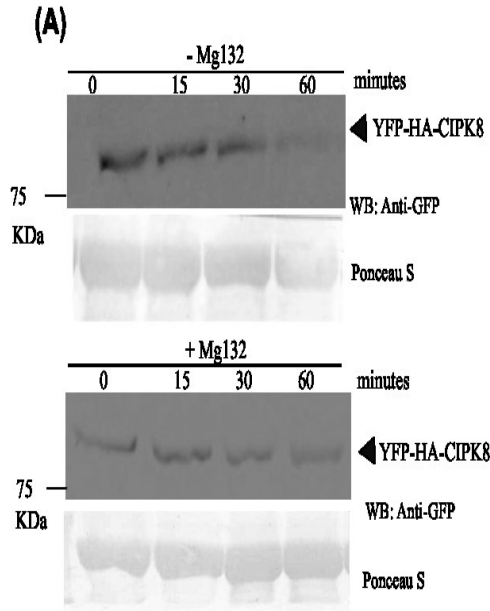
Cell free degradation assay (A) was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK8. Assay represents a replicate that shown in Figure 12. Assay was carried out without (-) or with (+) 50  $\mu$ M (+) proteasome inhibitor, Mg132 for 90 minutes. The abundance of YFP-HA-CIPK8 at each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading.





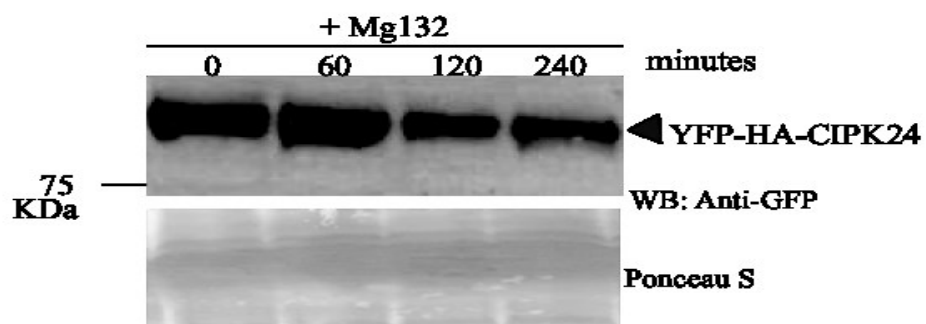
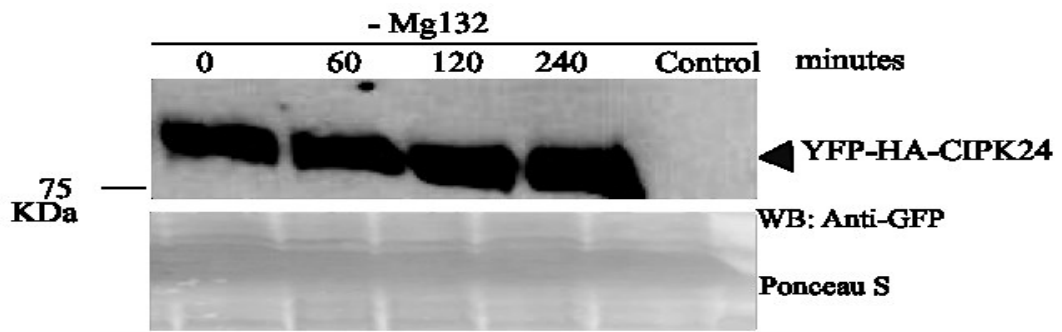
**Supplemental figure 8. CIPK8 observed to have short half live to be targeted for degradation by the 26S proteasome.**

Cell free degradation assay was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK8. Assays (A-C) represent three replicates carried out without (-) or with (+) 50  $\mu$ M proteasome inhibitor, Mgl32 for 60 minutes. The abundance of YFP-HA-CIPK8 in each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The graph (D) indicates the average percent remaining for YFP-HA-CIPK8 at each time point for all three replicates. Error bars represent standard deviation. The asterisk (\*) indicates a statistically significant ( $p < 0.001$ ) variation between mean of + and – MG132 treatment within indicated time points.



**Supplemental figure 9. CIPK24 is stable in plant cell.**

Cell free degradation assay (A) was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK24. Assay represents a replicate that shown in Figure 13. Assay was carried out without (-) or with (+) 50  $\mu$ M (+) proteasome inhibitor, Mg132 for 120 minutes. The abundance of YFP-HA-CIPK24 at each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading.



**Supplemental figure 10. CIPK24 is stable in plant cell.**

Cell free degradation assay was performed using protein extracts prepared from tobacco tissue transiently expressing YFP-HA-CIPK24. Assays (A-C) represent three replicates carried out without (-) or with (+) 50  $\mu$ M proteasome inhibitor, Mg132 for 120 minutes. The abundance of YFP-HA-CIPK24 in each replicate was determined by Western blot (WB) analysis with GFP antibodies. Ponceau S staining shows protein loading. The graph (D) indicates the average percent remaining for YFP-HA-CIPK24 at each time point for all three replicates. Error bars represent standard error. The asterisk (\*) indicates a statistically significant ( $p < 0.001$ ) variation between mean of + and - MG132 treatment within indicated time points.

