THE ROLE OF N-TERMINAL REGIONS IN REGULATING THE LEVEL OF PLANT ICK CYCLIN-DEPENDENT KINASE INHIBITORS

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ABSTRACT

Plants have a family of cyclin-dependent kinase (CDK) inhibitors named ICKs (interactor/inhibitor of CDK), which are important cell cycle regulators and can modulate CDK activity through direct binding. The model plant *Arabidopsis thaliana* has seven members in the ICK family.

Previous results show that changing the levels of ICKs by either overexpression or down-regulation affects plant growth and plant morphology, and the effects depend on ICK expression levels, indicating that the function of ICKs is dosage-dependent. Thus, it is important to understand how the ICK levels are regulated. Several independent studies indicated that the ubiquitin proteasome system is involved in the degradation of ICKs. However, the functional sequence signals in ICKs for regulating their levels remain largely unknown.

In this study, to identify sequences involved in regulating the levels of ICKs, the N-terminal regions of ICKs were fused to the green fluorescence protein (GFP) and expressed in Arabidopsis. Results showed that the presence of these N-terminal regions can dramatically reduce the GFP protein level. Since previous work showed that a motif as short as 10 amino acid residues could greatly affect the level of GFP fusion protein, 10-residue fragments of ICK4 and ICK6 were analyzed by fusing to GFP. Results showed that ICK6$^{66-75}$ led to a much reduced level of the fusion protein. Interestingly, this fragment belongs to a conserved motif in ICKs and the corresponding sequence of ICK1 has been shown to dramatically reduce GFP fusion protein expression. Mapping ICK2, ICK3, ICK4 and ICK6 using fragments about 20 residues in length identified several other sequences that could reduce the GFP expression level in Arabidopsis. Since all the ICK3 fragments were capable of dramatically decreasing the level of a reporter GFP protein, they were further analyzed in yeast and *E. coli*. ICK3$^{38-60}$ and ICK3$^{61-83}$ could reduce the level of GFP in both yeast and *E. coli*, implying a ubiquitin-independent mechanism. ICK3$^{21-37}$ could reduce the GFP level only in yeast suggesting that it functions differently from the other two sequences. Furthermore, ICK3$^{21-37}$

belongs to another conserved motif among ICKs.

Results from this study provide new understanding regarding the role of N-terminal regions in regulating the level of ICKs. They also raise new questions for future investigation on this family of plant cell cycle regulators.

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LIST OF ABBREVIATIONS

ACT8	Actin 8
AGO1	ARGONAUTE1
AMP	Adenosine monophosphate
APC/C	Anaphase-promoting complex/cyclosome
ATG	Autophagy-related gene
ATP	Adenosine triphosphate
BY-2	Bright Yellow-2
CAK	CDK-activating kinases
CDK	Cyclin-dependent kinase
CKI	CDK inhibitor
CP	Core protease
CYC	Cyclin
D-box	Destruction box
DEPC	Diethylpyrocarbonate
DP	Dimerization partner
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E. coli	Escherichia coli
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E2F	E2 Promoter-binding factor
E3	Ubiquitin-ligase
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescent protein
HECT	Homology to E6-AP C-Terminus

ICK	Inhibitor of CDK
KRP	Kip-related protein
LB	Luria Broth
LIR	LC3-interacting region
mNBR1	Mammalian neighbor of BRCA1 gene 1
MS	Murashige and Skoog salts
NLS	Nuclear localization signal
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEST	Proline-, glutamic acid-, serine-, and threonine-rich
PPB	Preprophase band
PRT1	PROTEOLYSIS 1
PVDF	Polyvinylidene fluoride
RB	Retinoblastoma
RBR	RB-related
RKP	Related to KPC1
RING	Really Interesting New Gene
RP	Regulatory particle
RT-PCR	Reverse Transcriptase-Mediated PCR
SD	Synthetic dextrose
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SD-Ura$^-$	SD plates lacking uracil
SIM	SIAMESE
SV40	Simian virus 40
THP	Tris(hydroxypropyl)phosphine
TOR	Target of Rapamycin

Ub	Ubiquitin
UPS	Ubiquitin-proteasome System
YPD	Yeast peptone dextrose

1. LITERATURE REVIEW AND INTRODUCTION

1.1 Plant Cell Cycle and Relevant Regulators

The cell cycle is one of the most crucial biological processes, especially given its importance to growth, development and regulation in all organisms. Studies on fungi, plants, animals and humans have successfully drawn a picture of the key components of the basic cell cycle machinery. It is generally accepted that the cell cycle is conserved in eukaryotes (Scofield et al., 2014). Nevertheless, the basic machinery has been modified during evolution to cope with the specific developmental and environmental challenges of each organism (Inze and De Veylder, 2006).

Plants possess some unique characteristics concerning the cell cycle. For instance, plant development is mostly post-embryonic, involving the life-long initiation, differentiation and growth of new organs such as roots, stems, leaves, and flowers. These organs are derived from cell proliferation occurring at particular zones termed meristems. Another interesting point is that many differentiated plant cells have the ability to dedifferentiate and obtain pluripotentiality, an important property that confers to plants considerable developmental plasticity (Grafi and Avivi, 2004). The rigid plant cell walls prevent cytokinesis through constriction as it occurs in animal cells. Instead, plants have evolved a specific mechanism to generate two daughter cells that involves two unique cytoskeletal arrays, known as the preprophase band (PPB) and the phragmoplast (Inze and De Veylder, 2006; Smith, 2001). Furthermore, plant cells usually do not produce tumors unless as responses to some pathogens (Doonan and Hunt, 1996).

1.1.1 General Review of Plant Cell Cycle

As with other eukaryotes, the general plant cell cycle comprises four sequential ordered phases: G1, S, G2 and M (Figure1.1). The G_1 phase (the first gap) separates the mitosis (M phase) of the previous cycle and S phase (DNA replication) of the new cycle and G_2 phase separates the S phase and subsequent M phase (Dewitte and Murray, 2003). However, there

are exceptions in specialized cell types or situations such as the endoreduplication of many plant cells. In this mode, cells undergo iterative DNA replications without subsequent cytokinesis, resulting in increased ploidy levels (Sugimoto-Shirasu and Roberts, 2003).

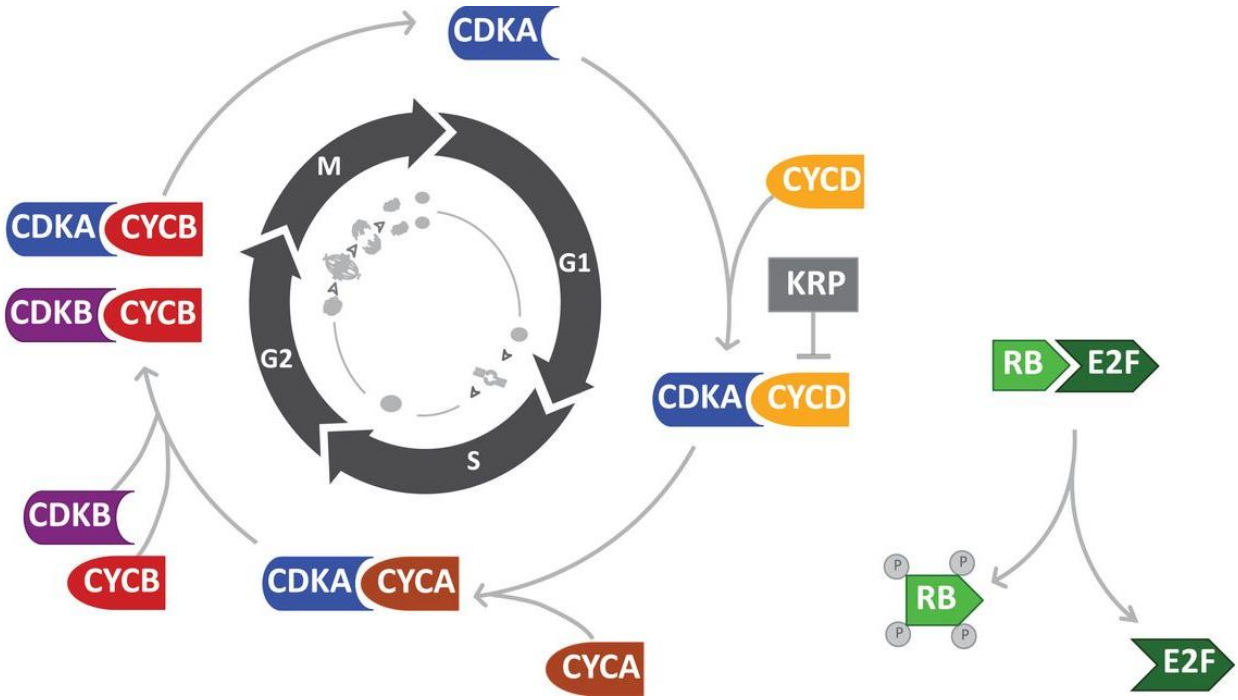


Figure 1.1 Simplified view of the plant cell cycle. During the G1/S transition, RB/RBR is phosphorylated by CDKA-CYCD complex and thus releases E2F to allow transcription of the target genes. ICK/KRPs can directly bind to both CDKA and CYCD subunits and inhibit the activity of the complex. CYCA contributes to CDKA activity during S-phase. CDKA, mitosis-specific CDKB and CYCB are required for the G_2/M checkpoint. This figure is adapted from (Scofield *et al.*, 2014) with permission.

During the cell cycle, the gap phases provide the operation of controls that ensure the proper progression and completion of one phase before the next one starts, and not surprisingly the main regulatory points occur at the G_1/S and G_2/M transitions. During G_1, cells will not initiate DNA replication until obligatory conditions have been met and signals integrated, implying not only the completion of the last cycle, but also the commitment to S phase. After the duplication of the genetic material, the separation of the chromosomes is authorized by multiple cellular processes starting from early G_2 phase. Many regulatory mechanisms and

molecular players are utilized throughout the cell cycle, particularly at these two important checkpoints. In the following section, important plant cell cycle regulators and their underlying mechanisms will be reviewed.

1.1.2 Relevant Plant Cell Cycle Regulators

Many of the cell regulators are conserved in eukaryotes, with the cyclin-dependent kinase (CDK) as the central component. CDK controls the progression of the cell cycle and coordinates other cellular processes that must be balanced to achieve DNA replication and segregation. A CDK requires the association with a cyclin and itself being phosphorylated for full activity. Multiple CDK-cyclin complexes phosphorylate various substrates, controlling transitions from one phase to another. The CDK subunit is responsible for the catalytic activity and able to recognize the target motif (a serine or threonine followed by a proline) in substrate proteins, whereas the cyclin subunit plays a regulatory role and also recognizes distinct protein substrates. This working mechanism of CDK provides different possible ways for regulation, such as regulated synthesis and destruction of the cyclin protein, assembly and activation of CDK-cyclin complex, and the binding of inhibitory proteins. In general, the cell cycle can be regarded as an oscillator of CDK activity, with an ebb in the G_1 phase and a peak during the M phase (Coudreuse and Nurse, 2010). This oscillator is driven by regulated synthesis and timely proteolysis of proteins at specific points in the cycle (Genschik *et al.*, 2013a) .

1.1.2.1 CDKs

All eukaryotic species possess at least one ‘universal’ CDK, characterized by a conserved amino acid sequence motif “PSTAIRE” in their cyclin-binding domain. There is strong conservation in the function of PSTAIRE CDKs as shown by their ability to complement the yeast *cdc2* mutant deficient in the PSTAIRE-type CDK (Ferreira *et al.*, 1991). In plants, this type of CDK is named as CDKA. Generally, CDKA proteins remain at constant levels

throughout the cell cycle and play a crucial role at both the G_1/S and G_2/M transitions (Sorrell *et al.*, 2001) (Figure 1.1). Tobacco plants with the CDKA activity down-regulated by overexpressing a dominant negative mutant showed reductions in both the cell division rate and plant size (Hemerly *et al.*, 1995). However, the G_1/G_2 ratio remained unchanged, consistent with the observation that CDKA is essential at both checkpoints (Joubes *et al.*, 2004). In Arabidopsis, extremely dwarfed homozygous *cdka* mutants have been identified, which have increased cell sizes in some organs (Nowack *et al.*, 2012).

No homologues of the mammalian G_1/S-specific *CDK4* or *CDK6* genes are found in plants. Hence, CDKA seems to be the only CDK active at the G_1 and S phases in plants (Figure 1.1), whereas the G_2/M transition is controlled by multiple CDKs (Inze and De Veylder, 2006). Plants possess another type of CDK, the CDKB that shows no clearly detectable homology to the CDKs in non-plant species (Joubès *et al.*, 2000). CDKBs are characterized by either the PPTALRE or PPTTLRE motif, reflecting two sub-types, namely CDKB1 and CDKB2 (Vandepoele *et al.*, 2002). The two sub-types are found in both monocotyledonous and dicotyledonous organisms, implying a conserved function for each type in the cell cycle. The *CDKB* genes are expressed only in mitotic cells, from the S-phase to the M-phase (Scofield *et al.*, 2014). In Arabidopsis, the *CDKB1* transcripts accumulate from S phase and peak in G_2, whereas the *CDKB2* genes are expressed during G_2 and M phases (Menges *et al.*, 2005). During the cell cycle, the CDKB protein level matches their transcription pattern, and the kinase activity peaks during mitosis (Inze and De Veylder, 2006). Arabidopsis has two CDKB1 (CDKB1;1 and CDKB1;2) and two CDKB2 (CDKB2;1 and CDKB2;2) members. Overexpression of a dominant negative mutant of *CDKB1;1* resulted in lower kinase activity and an increased 4C/2C ratio because of a block at the G_2/M transition (Boudolf *et al.*, 2004; Porceddu *et al.*, 2001). When the two *CDKB2* genes were downregulated simultaneously by an artificial microRNA (amiRNA) targeting both genes, plants were dwarfed (Andersen *et al.*, 2008). These results demonstrate the requirement for CDKB activity to progress through the cell cycle, especially the G_2/M transition (Figure 1.1).

In addition to CDKA and CDKB, other CDKs also play a role in the cell cycle and their mis-expression impacts plant growth as well. The CDKD and CDKF class proteins are known as CDK-activating kinases (CAKs) and modulate the activity of other CDKs by phosphorylating their T-loop domains (Tank and Thaker, 2011). The phosphorylation induces a conformational change in the CDKs, allowing them to recognize their substrates. Arabidopsis contains four CAK-encoding genes, divided into the two classes (Umeda *et al.*, 2005). The two CAK classes differ in their substrate specificity and cyclin dependence. Only CDKDs phosphorylate RNA polymerase II and require the association with the H-type cyclin (CYCH) (Yamaguchi *et al.*, 2003), whereas CDKF is able to phosphorylate and activate CDKDs in Arabidopsis (Shimotohno et al., 2004). Single *cdkd* knockout mutants had no growth phenotype (Hajheidari *et al.*, 2012). However, when two or all three *CDKD* genes were mutated, the plants were dwarfed and produced curly, serrated leaves (Hajheidari *et al.*, 2012). T-DNA insertion lines of *CDKF;1* displayed severe dwarfism and retarded development due to a decrease in cell number, cell size and DNA content (Hajheidari *et al.*, 2012; Takatsuka *et al.*, 2009). These data show that CAKs have a significant function in regulating the growth rate of plants through supervising the overall CDK activity.

It has been shown that CAKs respond to endogenous hormone signals, supported by results from *in vitro* callus induction work. Normally, undifferentiated callus cells can be induced from differentiated leaf tissues when auxin and cytokinin are present in the culture medium. The explants overproducing a rice CDKD could form calli without cytokinin (Yamaguchi *et al.*, 2003).

Although not directly involved in cell cycle control, CDKC and CDKE proteins have a role in plant development (Cui *et al.*, 2007; Wang and Chen, 2004). Double *cdkc;1/2* mutants were small and showed delayed flowering (Cui et al., 2007). Phenotypic characterization of *cdke;1* mutants has illustrated that CDKE participates in cell expansion in leaves and cell-fate specification in floral organs (Wang and Chen, 2004). In addition, both CDKC and CDKE are implicated in transcription elongation by phosphorylating RNA polymerase II (Fulop *et al.*,

2005; Wang and Chen, 2004).

1.1.2.2 Cyclins

Cyclins provide the primary mechanism for regulating CDK activity because the CDK subunit is inactive unless bound to an appropriate cyclin. Cyclins are a diverse group of proteins with a low degree of overall homology except for a conserved region called the cyclin core responsible for the interactions with a CDK. Compared with CDKs, less is known on plant cyclins. This problem to a large extent is due to the fact that plants possess many more cyclins than other organisms (Vandepoele *et al.*, 2002). For example, the angiosperm genomes generally contain about 50 to 60 cyclin genes in approximately 10 types (Hu *et al.*, 2010; La *et al.*, 2006; Ma *et al.*, 2013; Wang *et al.*, 2004). The classification is mainly based on the sequence similarity among the plant cyclins as well as to their mammalian counterparts. The large number of cyclins in plants may reflect the high developmental plasticity of sessile plants to respond to both intrinsic developmental signals and extrinsic environmental cues (Inze and De Veylder, 2006). Many of these cyclin types have putative roles in cell cycle progression; however, some of them may function in other processes instead of being directly involved in cell cycle regulation. Different types of cyclins function in different stages of the cell cycle, providing specific regulation by targeting CDK-cyclin complexes to different substrates. Generally, the majority of D-type cyclins are involved in the regulation of the G_1/S checkpoint; A-type cyclins in S-phase and the G_2/M transition; and B-type cyclins in G_2/M transition and intra-mitotic control (Inze and De Veylder, 2006). However, some deviations from this general assignment exist.

D-type cyclins exhibit high diversity in sequence and were originally identified by the functional complementation of a yeast strain deficient for G_1 cyclins (Dahl *et al.*, 1995; Soni *et al.*, 1995). All higher plants have seven conserved subgroups of D-type cyclins, named CYCD1-CYCD7 (Menges *et al.*, 2007). Different plants have different numbers of genes in each subgroup. In a broad sense, D-type cyclins bind to CDKA and control the G_1/S transition

(Van Leene *et al.*, 2010) (Figure 1.1). Some members of this family have been characterized for their effects on plant growth. In general, overexpression of the Arabidopsis *CYCD2;1* gene increases the rate of cell proliferation through faster progression through the G_1 phase (Blomme *et al.*, 2014; Qi and John, 2007). When *CYCD3;1* was overexpressed in Arabidopsis, the number of cells in G1 phase was decreased (Dewitte *et al.*, 2003). Downregulation of *CYCD5;1* through an amiRNA led to a decrease in cell number and DNA content (Sterken *et al.*, 2012). In contrast to animals, some evidence suggests an additional function of plant D-type cyclins at the G_2/M checkpoint. For instance, Arabidopsis CYCD4;1 associates and activates G_2/M-specific CDKB2;1 *in vitro* (Kono *et al.*, 2003). Induced overexpression of the tobacco *CYCD3;3* and the snapdragon *CYCD1;1* in tobacco Bright Yellow-2 (BY-2) cell promoted both S phase and M phase entry (Koroleva *et al.*, 2004; Nakagami *et al.*, 2002). Furthermore, some D-type cyclins exhibit a transcriptional peak at the G_2/M transition (Menges *et al.*, 2005; Meszaros *et al.*, 2000; Sorrell *et al.*, 1999).

As in animals, the expression of plant D-type cyclin genes has been shown to respond to external signals, both hormonal and developmental (Scofield *et al.*, 2014). For example, sucrose concentration plays a major role during the cell cycle in controlling the expression of *CYCD2;1*, *CYCD3;1* and *CYCD4;1* in Arabidopsis (Nieuwland *et al.*, 2009; Riou-Khamlichi *et al.*, 2000). Expression of Arabidopsis *CYCD3;1* mediates responses to the phytohormone cytokinin in the cell cycle (Dewitte *et al.*, 2007; Riou-Khamlichi *et al.*, 1999). Other D-type cyclin genes are shown to be modulated by various plant growth factors, such as auxins, brassinosteroids and gibberellins (Gaudin *et al.*, 2000; Hu *et al.*, 2000; Richard *et al.*, 2002; Sauter *et al.*, 1995). Some D-type cyclin genes are directly regulated by certain transcription factors with important developmental functions. For instance, Arabidopsis *CYCD6;1* is regulated by the transcription factor *SHORTROOT*, which controls formative divisions in generating the root ground tissue (Sozzani *et al.*, 2010a). The larger number of cyclins in plants compared with other species may reflect a more flexible regulatory network required by the tremendous plasticity of the plant cell cycle.

As for D-type cyclins, no definite conclusion could be drawn yet on the function of A-type cyclins although they are generally regarded as mitotic cyclins which function in the G2/M transition. However, CYCA has been reported to contribute to cell cycle-specific kinase activity also at the entry to the S phase (Roudier *et al.*, 2000) (Figure 1.1). Phenotypes have also been reported for *CYCA* mutants. The Arabidopsis *cyca2;3* knockout plants were similar to the wild type plants, but had a slightly increased ploidy level (Imai *et al.*, 2006). A triple *cyca2;2/3/4* mutant displayed acute increases in DNA content and cell size for the first true leaves; while the mature leaves contained about 50% fewer cells compared with the wild type due to a lower cell division rate (Vanneste *et al.*, 2011). Furthermore, overexpression of tobacco *CYCA3;2* cyclin in Arabidopsis led to ectopic cell divisions and delayed differentiation, along with increases in the expression of S phase-specific genes and CYCA3;2-associated CDK activity (Yu *et al.*, 2003), suggesting that CDK activity needs to be down-regulated for cell differentiation.

The B-type cyclins are known to regulate the G_2/M transition (Doerner *et al.*, 1996) (Figure 1.1). In addition, it has been reported that CYCB1;1 can associate and activate both the CDKA and the CDKB *in vitro* (Weingartner *et al.*, 2004) (Figure 1.1).

T-type cyclins are regulatory subunits for CDKCs and are involved in transcription elongation (Cui *et al.*, 2007). As a result, transgenic lines with a loss of function of *CYCT1;4* and *CYCT1;5* were smaller, similar to *CDKC* double mutants (Cui *et al.*, 2007).

1.1.2.3 CDK Inhibitors

In eukaryotic species, the activity of CDK-cyclin complexes can be regulated by the interaction with regulatory proteins. Direct binding of the complex by a group of proteins called CDK inhibitors (CKIs) leads to interference with the kinase activity. In fission yeast (*Schizosaccharomyces pombe*), only one CKI, called Rum1, is known to control mitotic CDK complexes. The situation in budding yeast (*Saccharomyces cerevisiae*) is more complex because it has three CKIs: Far1p regulates G_1 CDK activity; Sic1p controls S-phase entry

through inhibiting G_1/S CDK-cyclin complexes; and Pho81p inactivates another CDK-cyclin complex that plays a role in a signal transduction pathway sensing phosphate limitation (Inze and De Veylder, 2006). Mammals have seven CKIs, which are subdivided into two classes based on their sequence similarity and CDK specificity. The INK4 family members [$p16^{INK4a}$ (Cdkn2a), $p15^{INK4b}$ (Cdkn2b), $p18^{INK4c}$ (Cdkn2c) and $p19^{INK4d}$ (Cdkn2d)] contain an ankyrin repeat and primarily inhibit the G_1 CDKs. The Cip/Kip family members [$p21^{Cip1}$ (Cdkn1a), $p27^{Kip1}$ (Cdkn1b) and $p57^{Kip2}$ (Cdkn1c)] share an N-terminal conserved domain which is required for CDK inhibition. Furthermore, the Cip/Kip CKIs are more promiscuous and inhibit a board range of CDK-cyclin complexes involved in the control of both G_1/S and G_2/M transitions (Nakayama and Nakayama, 1998; Sherr and Roberts, 1999).

Plants also have two classes of CDK inhibitors, the first being inhibitors of CDK (ICKs) or Kip-related proteins (KRPs). To avoid confusion, the name ICK will be used exclusively for the rest of the thesis. The second class is known as the SIAMESE (SIM) family (Peres *et al.*, 2007). The Arabidopsis genome encodes seven ICKs, ICK1-ICK7, and at least 13 SIMs (Komaki and Sugimoto, 2012).

In Arabidopsis, the first two ICKs (ICK1 and ICK2) were identified in a yeast two-hybrid as interactors of CDKA (Lui *et al.*, 2000; Wang *et al.*, 1997). These as well as the other five members (ICK3-ICK7) of the family (Coelho *et al.*, 2005; De Veylder *et al.*, 2001) share a C-terminally located 31-amino-acid domain (motif 1 in Figure1.2). This conserved domain is involved in binding CDKs and thus essential for the CDK inhibition function (Coelho *et al.*, 2005; De Veylder *et al.*, 2001; Jasinski *et al.*, 2002a; Jasinski *et al.*, 2002b; Lui *et al.*, 2000; Schnittger *et al.*, 2003; Wang *et al.*, 1997; Wang *et al.*, 1998; Zhou *et al.*, 2002a; Zhou *et al.*, 2003a; Zhou *et al.*, 2003b). Interestingly, this domain is similar to the N-terminally located CDK inhibition domain of the mammalian Cip/Kip proteins and present in ICKs of other plant species such as tobacco (Jasinski *et al.*, 2003; Jasinski *et al.*, 2002b), alfalfa (Pettkó-Szandtner *et al.*, 2006), maize (Coelho *et al.*, 2005), rice (Barroco *et al.*, 2006) and tomato (Bisbis *et al.*, 2006). The plant ICKs also have a second and shorter conserved motif adjacent to the

conserved C-terminal domain (Zhou *et al.*, 2002b) (motif 2 in Figure 1.2). Results show that this region is important for the interaction with cyclin (Wang *et al.*, 1998). Apart from these two regions, plant ICKs are very different from the mammalian CKIs and also show considerable sequence variability among themselves, although some other conserved domains and motifs have been identified through protein sequence analysis, including nuclear localization signals (NLS), protein degradation signals, a coiled-coil domain and CDK phosphorylation sites (Figure 1.2) (Torres Acosta *et al.*, 2011; Wang *et al.*, 2008). Identification of the conserved sequences provides useful hints for further functional characterization and determining differences among individual ICKs.

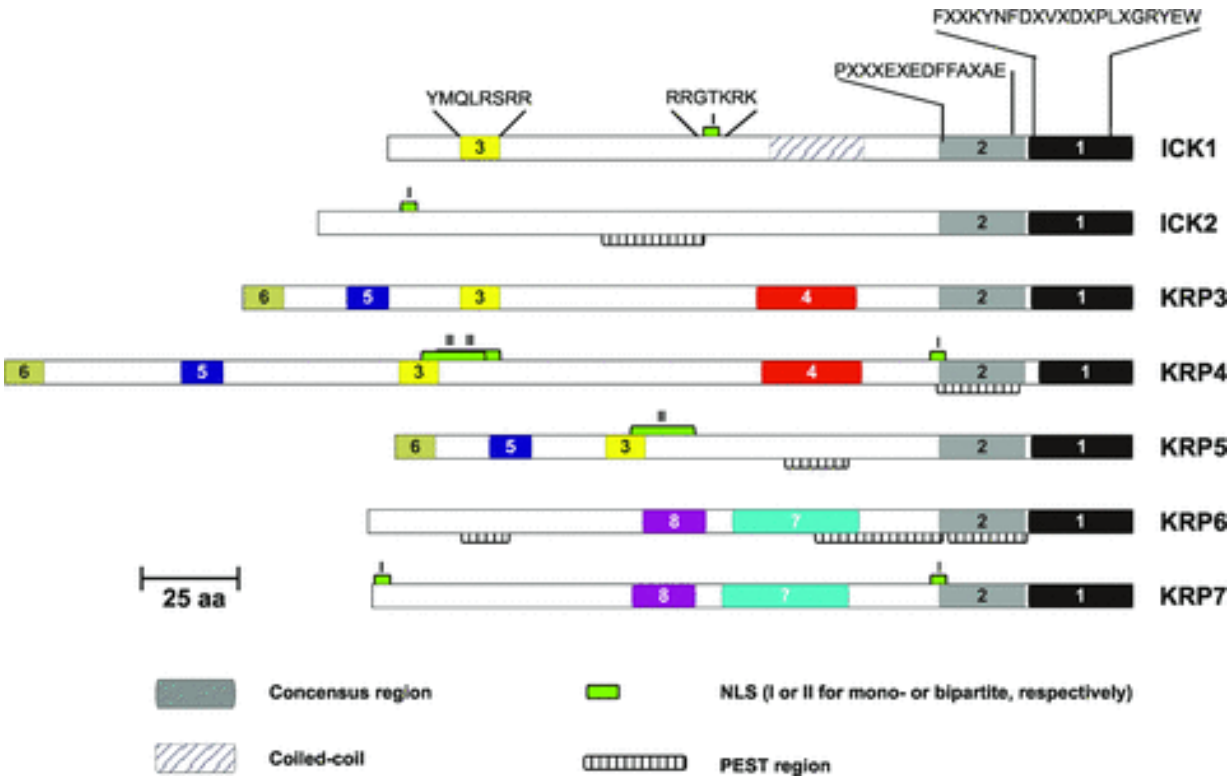


Figure 1.2 Schematic presentation of consensus sequences and functional motifs in seven Arabidopsis ICK/KRP CDK inhibitors. Conserved sequences are numbered starting from the two C-terminal motifs separately interacting with CDKA and CYCD. Some other conserved domains are also shown, implying putative functions such as nuclear localization signal (NLS), protein degradation signal (PEST) and coiled-coil domain. This figure is adapted from (Wang *et al.*, 2008) with permission.

Localization to specific cellular compartments is an important mechanism for cell cycle regulation. Results from the GFP protein fusion experiments show that all seven Arabidopsis ICKs are exclusively localized in the nucleus (Bird *et al.*, 2007). Detailed analysis shows that each ICK contains multiple independent sequences for nuclear localization (Bird *et al.*, 2007; Jakoby *et al.*, 2006; Zhou *et al.*, 2006). Furthermore, results indicate that ICK1 can transport CDKA from the cytoplasm into the nucleus and that this transportation capability depends on the C-terminal CDK interacting domain (Zhou *et al.*, 2006).

Proteomic analyses in Arabidopsis have showed that all seven ICKs co-purify with CYCDs and CDKA (Van Leene *et al.*, 2011), confirming the suggestion that ICKs mainly inhibit the activity of the CDKA-CYCD complex (Figure 1.1). This conclusion is consistent with previous data based on the yeast two-hybrid assay (De Veylder *et al.*, 2001; Lui *et al.*, 2000; Verkest *et al.*, 2005b; Wang *et al.*, 1998; Zhou *et al.*, 2002a). Further supporting this conclusion, it has been shown that the inhibitory effects on cell proliferation and plant growth from *ICK* overexpression can be reversed by co-overexpression of a *CYCD* (Jasinski *et al.*, 2002b; Schnittger *et al.*, 2003; Zhou *et al.*, 2003b). Furthermore, studies using Arabidopsis and alfalfa ICKs demonstrate that ICKs can also inhibit the activity of the CDKB-CYCD complex (Nakai *et al.*, 2006; Pettko-Szandtner *et al.*, 2006). Some *in vitro* analysis suggests that different ICKs may differ in their ability to inhibit CDK activity (Nakai *et al.*, 2006; Zhou *et al.*, 2002a); however, it is not known whether these differences exist *in planta*.

In general, Arabidopsis *ICKs* are expressed at low levels but show preferential expression in specific tissues and developmental conditions. The seven Arabidopsis *ICKs* display overlapping but some distinct expression patterns (Barroco *et al.*, 2006; De Veylder *et al.*, 2001; Jain *et al.*, 2007; Lui *et al.*, 2000; Menges *et al.*, 2005; Ormenese *et al.*, 2004; Torres Acosta *et al.*, 2011; Wang *et al.*, 1998). For example, in the shoot apex, *ICK3* and *ICK7* are predominantly expressed in dividing cells, whereas strong expression of *ICK1* and *ICK2* is observed in differentiating cells. Meanwhile, *ICK4*, *ICK5* and *ICK6* are expressed in both dividing and differentiating cells (Ormenese *et al.*, 2004). Specific temporal and spatial

expression patterns during cell cycle progression and plant development point to possible functional differences among different ICKs.

To survive during frequent and sometimes drastic environmental changes, plants need to modify their growth behaviors. As expected, the expression of *ICKs* is regulated by external cues. In Arabidopsis, it has been shown that *ICK1* expression in cultured cells is induced by sucrose starvation (Menges and Murray, 2002). Moreover, *ICK1* expression can also be induced by abscisic acid, low-temperature treatment and salt stress (Ruggiero *et al.*, 2004; Wang *et al.*, 1998). Additionally, auxin signaling is translated into modifying the *ICK* expression through PROPORZ1, which is a component of the chromatin-remodeling complex required for histone acetylation in response to auxin (Anzola *et al.*, 2010). Another study shows that gibberellin signaling controls cell proliferation by modulating the activity of CDK-cyclin complexes and also partly through regulating the *ICK* expression (Achard *et al.*, 2009).

The effects of ICKs on plant growth and development have been studied by overexpressing *ICKs* in plants. When *ICK1, -2, -4, -6* was overexpressed in Arabidopsis, the small leaves of the transgenic plants had a characteristic serrated phenotype (De Veylder *et al.*, 2001; Jun *et al.*, 2013; Liu *et al.*, 2008; Wang *et al.*, 2000). Leaves of plants overexpressing *ICK3* were also serrated but were similar in size to wild type plants (Jegu *et al.*, 2013). Some other common phenotypes observed in *ICK*-overexpressing plants include a reduced plant size, reduced cell number and enlarged cells (Barroco *et al.*, 2006; Bemis and Torii, 2007; De Veylder *et al.*, 2001; Jasinski *et al.*, 2003; Jasinski *et al.*, 2002b; Jegu *et al.*, 2013; Jun *et al.*, 2013; Kang *et al.*, 2007; Liu *et al.*, 2008; Vieira *et al.*, 2014; Wang *et al.*, 2000; Zhou *et al.*, 2002a). Generally, mitosis is hampered in plants overexpressing *ICKs*, leading to a drastic decrease in cell number, which is partially compensated by an increase in cell size (De Veylder *et al.*, 2011). The phenotypic changes associated with *ICK1* and *ICK2* overexpression are correlated with the level of overexpression (Verkest *et al.*, 2005b; Wang *et al.*, 2000; Weinl *et al.*, 2005). Particularly, strong overexpression of *ICK1* or *ICK2* inhibits the cell cycle

progression at both G1/S and G2/M transitions, leading to a complete cell cycle arrest, while weak overexpression of *ICK1* or *ICK2* preferentially inhibits mitosis and promotes the entry into endoreduplication (Verkest *et al.*, 2005b; Wang *et al.*, 2000; Weinl *et al.*, 2005). These results therefore suggest that different levels of ICKs have different effects in the cell cycle control.

Some ICKs likely have redundant functions since single loss-of-function mutants of *ICKs* did not display visible phenotypes (Komaki and Sugimoto, 2012). Down-regulation of multiple *ICK* genes in Arabidopsis severely compromised organ growth and led to the formation of callus-like tissues from the shoot apical meristem (Anzola *et al.*, 2010). In another down-regulation study using T-DNA insertion mutants, quadruple (*ick1/2/6/7*) and quintuple (*ick1/2/5/6/7*) mutants had longer leaves, which were narrow and curled downwards (Cheng *et al.*, 2013). An increase in organ size and fresh and dry weight was observed for these higher-order mutants, attributed to increased cell proliferation accompanied by a decrease in cell size (Cheng *et al.*, 2013). These results strongly suggest that ICKs act as a negative regulator of cell proliferation. In addition, a trend of gradual changes was observed from single to higher-order *ICK* mutants, suggesting a dosage-dependent sensitivity of CDKA to ICKs (Cheng *et al.*, 2013). Surprisingly, in contrast to its supposed role as cell cycle inhibitor, ICK4 overexpression leads to accelerated G_1/S and G_2/M transitions in Arabidopsis cell cultures and root cells (Vieira *et al.*, 2014).

Recently, ICKs have been demonstrated to be involved in different physiological processes. ICK4 and ICK5 have been linked to the control of Arabidopsis male gametogenesis (Kim *et al.*, 2008a). Recombinant ICK4 and ICK5 proteins can be phosphorylated by SNF1-Related protein Kinase-1, a sensor that maintains cellular energy homeostasis via control of anabolism/catabolism balance, providing a possible connection between energy sensing and cell proliferation (Guerinier *et al.*, 2013). ICK2 influences lateral root density in an auxin-dependent manner, whereas ICK3 appears to be limiting for primary root growth (Sanz *et al.*, 2011; Wen *et al.*, 2013). Additionally, Arabidopsis transcription factor JAGGED links

floral organ patterning to tissue growth by repressing *ICK2* and I*CK7* (Schiessl *et al.*, 2014). Most recently, studies have shown that ICK1, ICK2, ICK4 and ICK7 are involved in the establishment and maintenance of the nematode feeding site caused by the infection of plant-parasitic root-knot nematodes (Vieira *et al.*, 2014; Vieira *et al.*, 2013a; Vieira *et al.*, 2013b). In addition to their role in CDK inhibition, some ICKs may have other functions. ICK3 has been reported to function in regulating gene transcription during cell wall organization (Jegu *et al.*, 2013).

The second family of plant CDK inhibitors is known as the SIAMESE (SIM) family (Peres *et al.*, 2007), which is found only in plants. The founder member is SIM, a nuclear localized protein that contains a cyclin-binding motif also presents in ICKs (Churchman *et al.*, 2006). Other SIM-related (SMR) proteins have also been identified (Peres *et al.*, 2007). In Arabidopsis, *Sim* mutants developed multicellular trichomes instead of the wild type unicellular trichomes (Walker *et al.*, 2000), because SIM is required to repress the mitotic cell cycle in trichomes through inhibiting the CYCD-CDKA complex (Churchman *et al.*, 2006; Peres *et al.*, 2007). Moreover, plants had a dramatic reduction in size when the *SIM* gene was overexpressed (Churchman *et al.*, 2006). Another member of the SIM family, SMR1/LGO, is implicated in the control of the cell cycle in sepals through coordinating the mitotic cell division and endoreduplication (Roeder *et al.*, 2010). A recent *in vivo* protein interaction study revealed that most SIM family proteins co-purify with CDKA while some of them interact with CDKB (Van Leene *et al.*, 2010). SIM and related SMR proteins are proposed to regulate the cell cycle in response to biotic and abiotic stresses (Peres *et al.*, 2007).

1.1.2.4 The RBR/E2F/DP Pathway

In both plants and animals, gene expression that supports the progression from G_1 to S phase is generally controlled by the E2F-dimerization partner (DP)-retinoblastoma (RB) pathway (Komaki and Sugimoto, 2012). *RB* is a tumour suppressor gene originally discovered in humans. The RB protein is a member of a small family of three proteins that share a

so-called pocket domain and that have different roles in suppressing cell division and advancing both differentiation and quiescence in diverse circumstances (Dick and Rubin, 2013). The *RB-related (RBR)* gene in plants is the orthologue of the animal *RB* gene, and several of the known functions of the RB protein are conserved in plants as well (Gutzat *et al.*, 2012). The fundamental functional property is the ability to interact with a class of transcription factors known as E2F (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991; Magyar *et al.*, 2012; Uemukai *et al.*, 2005). Many E2F proteins associate with another DP family of transcription factors to form heterodimeric complexes that promote the expression of genes required for cell cycle process from G_1 to S phase (van den Heuvel and Dyson, 2008). The binding of E2F by RB protein through the pocket domain inhibits the transcriptional activity of E2F. During the G1/S transition, RB is phosphorylated by CDKs-CYCD, targeted to the pocket domain by an LXCXE motif of CYCD, resulting in the release of E2F which can then activate genes required for entry into the S phase (van den Heuvel and Dyson, 2008).

DNA viruses use this machinery to oblige host DNA polymerases to replicate the viral genomes (Scofield *et al.*, 2014). Certain mammalian DNA tumor viruses such as adenovirus, simian virus 40 (SV40), and human papillomavirus (causing cervical cancer) contain a type of proteins with an LXCXE motif that can inactivate RB to promote entry of cells into S phase and proliferation (Giacinti and Giordano, 2006). Similarly, the plant gemini viruses also have proteins that carry the LXCXE motifs to inactivate RBR and drive cells into S-phase (Hanley-Bowdoin *et al.*, 2013). A number of these viruses have critical agricultural impacts, especially in tropical areas.

The Arabidopsis genome encodes three typical E2F transcription factors E2Fa, E2Fb and E2Fc, which form dimers with DPa or DPb proteins to bind to specific sites in the promoters of the target genes (Heckmann *et al.*, 2011; Kuwabara and Gruissem, 2014). E2F-responsive promoters usually contain at least one consensus E2F-bingding sequence (TTTCCCGC), identical to that occurring in animal cells (Gutierrez, 2009). Both E2Fa and E2Fb act as transcriptional activators to promote the G_1/S transition and, accordingly, their putative direct

target genes include those required for DNA replication, DNA repair and chromatin maintenance (De Veylder *et al.*, 2002; del Pozo *et al.*, 2002; Magyar *et al.*, 2005; Mariconti *et al.*, 2002; Naouar *et al.*, 2009; Ramirez-Parra *et al.*, 2003; Takahashi *et al.*, 2008; Vandepoele *et al.*, 2005; Vlieghe *et al.*, 2003). During the G_1 phase, the CDKA-CYCD complex phosphorylates RBR and thus releases E2Fa-DPa and E2Fb-DPa to allow transcription of their target genes (Boniotti and Gutierrez, 2001; Nowack *et al.*, 2012) (Figure 1.1). On the other hand, E2Fc-DPb dimers function as transcriptional repressors and, although their direct target genes have not been described, they appear to repress cell cycle progression through an RBR-independent mechanism (de Jager *et al.*, 2009; del Pozo *et al.*, 2002; del Pozo *et al.*, 2006). During G_1/S transition, the E2Fc-DPb is phosphorylated and targeted for destruction (Inze and De Veylder, 2006).

Three atypical E2Fs, E2Fe/DEL1, E2Fd/DEL2 and E2Ff/DEL3, also function as transcriptional repressors in Arabidopsis, but so far none of them has been shown to control the expression of cell cycle genes directly (Komaki and Sugimoto, 2012). These atypical E2Fs can bind to the target promoters as monomers instead of complexes with the DP proteins (Gutierrez, 2009). In contrast to the E2Fa-c proteins, the atypical E2F/DEL proteins possess two DNA-binding domains (Gutierrez, 2009), but lack other conserved domains and the RBR-binding motif (Mariconti *et al.*, 2002). Some E2Fe-f responsive genes have been experimentally demonstrated. The E2Fe/DEL1 protein binds to the promoter of the *CCS52A2* gene, an activator of the plant anaphase-promoting complex (APC), and represses the entry into the endoreduplication (Lammens *et al.*, 2008). Based on both gain-of-function and loss-of-function studies, the E2Fd/DEL2 protein is implicated in the control of cell proliferation but its direct target genes have not been identified (Sozzani *et al.*, 2010b). The E2Ff/DEL3 protein directly represses the expression of several cell wall biosynthesis genes, including three expansin genes and a UDP-glucose-glycosyl transferase gene, through direct binding to their promoters, to control cell elongation (Ramirez-Parra *et al.*, 2004).

In addition to the G_1/S transition, increasing evidence suggests that RB and RBR are also

required during the progression through G_2 and for the transition to M phase (Henley and Dick, 2012; Kuwabara and Gruissem, 2014). Hence, in plants, RBR plays key roles in cell proliferation control. Recently, some new functions of RB and RBR have emerged. For example, RBR has been shown to participate in various cellular processes such as endoreduplication, chromatin remodeling, cell growth, stem cell biology, differentiation and asymmetric cell division (Desvoyes *et al.*, 2014). These and other results support the idea that RBR is used as a protein-docking platform for a plethora of cellular proteins and complexes to control various aspects of cell physiology and plant development (Desvoyes *et al.*, 2014; Kuwabara and Gruissem, 2014).

This overview presents a simplified view of the plant cell cycle, focusing primarily on the main cell cycle regulators to highlight their mechanisms. Increasingly, studies of these regulators have focused not only on the cell cycle processes but also on the impacts on plant growth and development. It is important to understand how cell proliferation and organogenesis are coordinated during the post-embryonic development of plants.

In most cases to date studies on the expression of cell cycle regulators have focused on the transcriptional level. Although transcriptional regulation is a necessary component for the resulting protein expression, other levels of regulation including posttranslational modifications, specific proteolytic degradation and subcellular localization need also to be elucidated. As mentioned before, the cell cycle can be regarded as an oscillator of the CDK activity, and this oscillator is driven by regulated synthesis as well as by timely proteolysis of key components through the protein degradation system (Genschik *et al.*, 2013a). In the next section, the intracellular protein degradation mechanisms in plant cells will be reviewed.

1.2 Intracellular Protein Degradation Mechanisms in Plant Cells

Protein degradation is indispensable for protein homeostasis and cell survival, because it recharges the amino acid pool for the synthesis of new proteins. Reused amino acids are also

utilized by the central carbon metabolism to generate adenosine triphosphate (ATP), and serve as substrates for gluconeogenesis (Schreiber and Peter, 2014). Moreover, the timely- and spatially-controlled degradation of key regulatory proteins is crucial for processes like cell cycle progression, DNA replication and repair, cell differentiation and apoptosis (Schreiber and Peter, 2014).

While the protein degradation machinery in prokaryotes has a low complexity, eukaryotes possess a more complex 26S proteasome plus a specialized intracellular compartment called the lysosome (or vacuole in yeast and plants) (Schreiber and Peter, 2014). In plants, the degradation of proteins occurs through the action of the 26S proteasome and also through different protease classes (Nelson *et al.*, 2014).

1.2.1 The Ubiquitin-proteasome System (UPS)

The ubiquitin-proteasome system is responsible for clearance of abnormal, denatured or in general damaged proteins as well as for the regulated degradation of short-lived proteins. It is actually composed of two systems, the ubiquitin system and the proteasome system. The ubiquitin system is responsible for the recognition of a substrate protein to be degraded and its "tagging" with ubiquitin, while the proteasome system is the downstream player that performs the actual hydrolysis of the protein.

1.2.1.1 The Ubiquitin System

Cellular proteins are targeted for proteasomal degradation almost exclusively through ubiquitination (Schreiber and Peter, 2014). Ubiquitin (Ub) is a small protein (76 residues) found in all eukaryotes and its sequence is highly conserved with only three residues differences among yeast, humans and plants (Sadanandom *et al.*, 2012). Ubiquitin is the prototypical member of a family of proteins (ubiquitin like proteins) that covalently modify target proteins to mediate a diverse range of cellular functions (Jentsch and Pyrowolakis, 2000). Ubiquitin is covalently attached to substrate proteins via an isopeptide bond formed between

the C-terminal glycine (G76) of ubiquitin and the ε-amino group of a lysine within the target molecule or ubiquitin itself (Schreiber and Peter, 2014). Ubiquitin contains seven lysines (K6, K11, K27, K29, K31, K48 and K63). While linkages through each of the seven lysines have been identified *in vivo*, ubiquitin chains (poly-Ub) connected through lysine 48 (K48) predominate and function in the cell as a proteasome targeting signal (Pickart and Fushman, 2004). By and large, poly-Ub chains of no less than four ubiquitin moieties (tetra-ubiquitin) are required to give an efficient proteasome recognition signal (Thrower *et al.*, 2000). More recently, other ubiquitin chain linkages and even monoubiquitination have been found to represent proteasomal degradation signals (Amm *et al.*, 2014). Further, ubiquitination on residues (cysteine, serine, threonine) other than lysine of a protein can also serve as proteasomal degradation signals (Kravtsova-Ivantsiv *et al.*, 2013). Although ubiquitination was initially identified in the context of proteolysis, it is now known that ubiquitination can have different consequences other than degradation of the substrates (Hicke, 2001; Pickart and Fushman, 2004).

Connection of free Ub moieties to appropriate substrates is accomplished by an ATP dependent E1-E2-E3 enzyme conjugation cascade (Figure 1.3). This cascade starts with E1 (Ub activating enzyme), which catalyzes the synthesis of an acyl phosphoanhydride bond between the AMP (adenosine monophosphate) of ATP and the C-terminal glycine carboxyl group of Ub. Activated Ub then forms a stable intermediate by binding directly to an E1 cysteine via a thioester linkage. The Ub is then transferred to E2 (Ub conjugating enzyme) by transesterification. The E2-Ub intermediate transfers Ub to an acceptor lysine on a substrate using an E3 (Ub ligase). Generally, the E3 ligase confers substrate recognition, and either promotes direct transfer of Ub to the substrate from E2 or forms an E3-Ub intermediate before the transfer. After connection of the initial Ub moiety to a substrate, extra Ubs can be ligated to the first Ub to form poly-Ub chains. Currently, it is not clear whether chains are extended by adding preassembled Ubs or by iterative rounds of ligation utilizing the E3 or other factors. The poly-Ub chains are removed by deubiquitinating enzymes from ubiquitinated proteins

before unfolding, import and proteolysis (Hartmann-Petersen *et al.*, 2003).

The E1 enzymes start the Ub conjugation process. In plants, it is a single polypeptide contains a conserved cysteine that binds activated Ub and a nucleotide interacting motif that binds to either ATP or AMP-Ub intermediates (Hatfield *et al.*, 1997). In Arabidopsis, there are two E1 isoforms and one of them is nucleus-localized (Hatfield *et al.*, 1997).

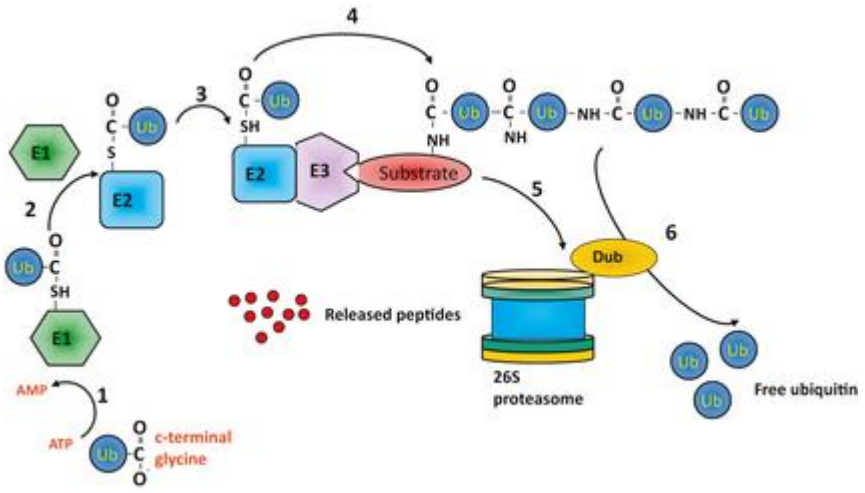


Figure 1.3 Ubiquitin–proteasome cascade. (1) E1 catalyzes the synthesis of an acyl phosphoanhydride bond between the adenosine monophosphate (AMP) of ATP and the C-terminal glycine carboxyl group of Ub. Activated Ub then structures a stable intermediate by binding directly to a conserved E1 cysteine via a thioester linkage on the carboxyl group of its terminal glycine. (2) Transfer of the activated Ub to E2 by transesterification, forming an E2-Ub thioester linkage. (3) E3 confers substrate recognition and transfers the Ub from the E2 to the target protein, usually by forming an intermediate complex (E2, E3 and the target). (4) Initial ubiquitination forms an isopeptide bond between the Ub and the protein. Extra Ubs are ligated to the first Ub to form a poly-Ub chain. (5) Poly-Ub chains tagged proteins are targeted to the 26S proteasome. (6) A proteasome associated deubiquitinating enzymes (DUB) disassembles the Poly-Ub chains. Target proteins are then unfolded, imported and degraded. This figure is adapted from (Sadanandom *et al.*, 2012) with permission.

The E2 enzymes contain a conserved 150-residue catalytic core that encompasses an active site cysteine. Using this characteristic region, 37 E2 isoforms have been identified in the Arabidopsis genome (Vierstra, 1996). In addition to the core E2 domain, different E2 isoforms may contain different N- and C-terminal expansions that are believed to affect target

recognition and localization (Hamilton *et al.*, 2001). Sequence analysis has grouped Arabidopsis E2s into 12 subfamilies (Vierstra, 1996), which show different interaction preferences for E3 enzymes (Sadanandom *et al.*, 2012). This property of E2s is supposed to play a role in the distribution of activated Ub to the diverse E3s.

The E3s are the most diverse proteins in the ubiquitination cascade to confer selectivity for a broad scope of substrates. Seven types of E3 ubiquitin ligases are known and belong to two large basic groups depending on the existence of either a 'Homology to E6-AP C-Terminus' (HECT) or 'Really Interesting New Gene' (RING)/U-box domain (Figure 1.4). RING E3s can function as single subunits or in multi-subunit complexes. The E3s all have an E2 interaction domain and a substrate recognition domain. Approximately 1406 putative E3 genes have been identified in Arabidopsis (Vierstra, 2009).

A particular case of substrate recognition is the 'N-end rule', which states that the half-life of a protein is affected by its N-terminal residues (Varshavsky, 1996). N-terminal residues are grouped by their ability to diminish protein half-life and are named as N-degrons (Varshavsky, 1996). Specific E3s have been connected to the N-end rule, the best examples of which in Arabidopsis are PROTEOLYSIS 1 (PRT1) and PRT6 (Stary *et al.*, 2003). PRT1 targets aromatic amino acids at the amino-terminal, while PRT6 ubiquitinates proteins with arginine at the N-end (Garzon *et al.*, 2007).

HECT E3s are single subunit proteins sharing a conserved 350-residue region (the HECT domain). HECT E3s are unique as they frame a thioester linkage with Ub on a conserved cysteine in the C-terminal region of the HECT domain (Huibregtse *et al.*, 1995). Substrate recognition and localization are assumed to be accomplished by protein-protein interaction domains upstream of the HECT region (Smalle and Vierstra, 2004). In Arabidopsis, seven HECT E3s (UPL1–7) have been identified by genome analysis (Vierstra, 2009).

The RING/U-Box E3s are a loosely defined collection of polypeptides bearing either a signature RING finger motif or a structurally related derivative called the U-Box. Sequence analyses in plants have identified large families of each type. The Arabidopsis genome

encodes approximately 480 RING finger-containing proteins and 64 proteins with a U-Box motif (Azevedo *et al.*, 2001; Smalle and Vierstra, 2004). For the RING E3s, the 70-amino acid finger is a cross brace formed by an octet of cysteines and histidines that bind zinc in either a C3H2C3 (RING-H2) or a C3H1C4 (RING-HC) configuration (Yanagisawa *et al.*, 2003). The U-Box exploits electrostatic interactions instead of metal ion chelation to stabilize a RING finger-like structure (Melchior, 2000). The RING/U-Box serves as a Ub-E2 docking site that allosterically activates transfer of the Ub to substrate lysine. Numerous other motifs are present, some of which likely confer target specificity (Kim *et al.*, 2003; Woo *et al.*, 2001).

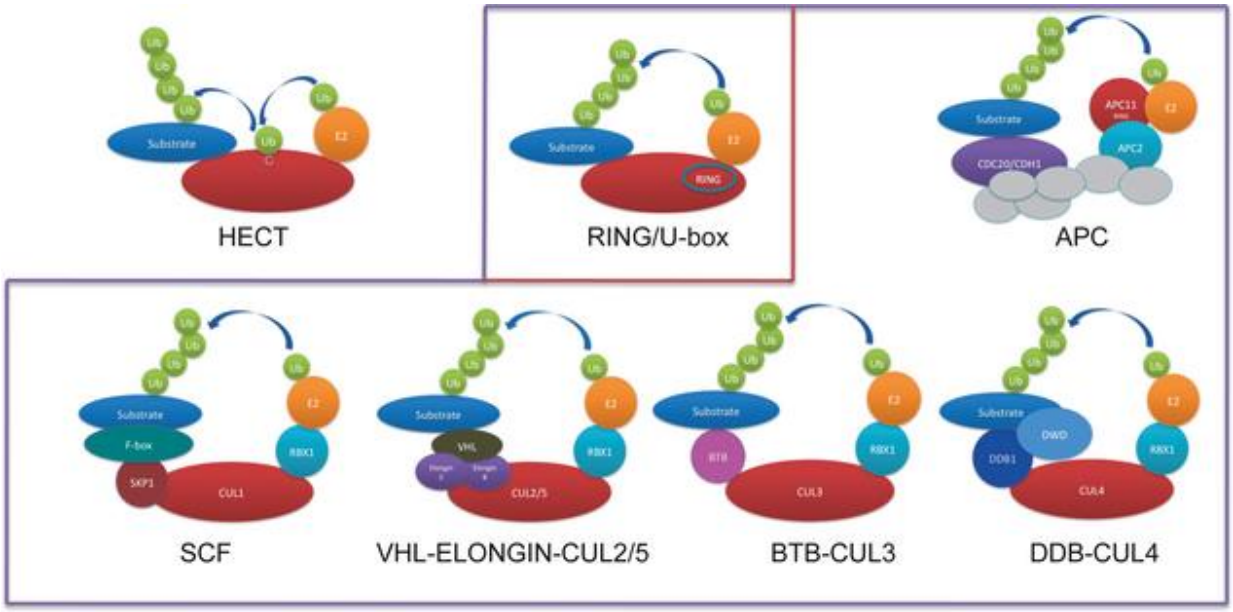


Figure 1.4 Types of E3 ligases. Seven types of E3s can be divided into two groups dependent on the existence of either a HECT or a RING domain (purple box). The E3s all share a typical requirement for an E2 interaction domain and a substrate recognition domain. HECT E3s are single subunit and have an additional ubiquitin acceptor function. RING domain E3s have two subgroups, (1) single-molecule RING/U-box domain E3s (red box) normally contain a substrate interaction motif and (2) multi-subunit E3s (purple box) have RING domain containing RBX1 subunit (APC11 provides the RING domain in APC), Cullins act as a molecular assembly platform. In both cases, the RING is responsible for the E2 recognition and interaction. Different multi-subunit E3 ligases utilize different substrate recognition subunits. This figure is adapted from (Sadanandom *et al.*, 2012) with permission.

Multisubunit E3s are based on Cullin (CUL) and RING finger components. The RING

part is responsible for E2 and adaptor molecule recognition. Substrate specificity is achieved by the adaptor molecule. This design of multiple components with an adaptor allows wider substrate scope as distinctive adaptors can interact with diverse RING fingers. The two modules associate through Cullin, which acts as a molecular assembly platform.

SCF (Skp, Cullin, F-box containing) E3s are heterotetrameric ligases with the subunits named after their founding members: SKP1 (S-phase kinase-associated protein 1), CDC53 (Cell division control protein 53) (or CUL1), RBX1 (Ring-box 1) and an F-box protein (Deshaies, 1999). The RBX1 component interacts with E2-Ub via its RING domain and is a part of the Cullin-RBX1-SKP subcomplex which possesses Ub transferase activity (Deshaies, 1999). Substrate specificity is provided by the F-box subunit, which anchored to SKP via an N-terminal F-box motif (Kipreos and Pagano, 2000) and interacts with targets proteins via the C-terminal substrate recognition domain. F-box proteins have the largest single protein family in Arabidopsis (about 700 members) (Gagne *et al.*, 2002), demonstrating a more prominent divergence than their mammalian counterparts (Vierstra, 2009). The diversity of F-box proteins coupled with Cullin1, two RBX1s and 21 possible SKPs (termed ASKs in Arabidopsis) could potentially generate over 100,000 distinct SCF complexes in Arabidopsis (Smalle and Vierstra, 2004). In many cases, substrate phosphorylation is a prerequisite for recognition by F-box proteins, implicating that many plant kinases are involved in the regulation of proteolysis (Deshaies, 1999).

CUL3-BTB E3 ligases are similiar to SCF E3 ligases and based on three components: CUL3, BTB /POZ (bric-à-brac tramtrack broad complex/pox viruses and zinc fingers) and an RBX1 subunit. In this model, BTB appears to be the functional analog to the SKP and F-box components of SCF E3s.

CUL4 is the molecular scaffold of another E3 class which contains RBX1 and DDB1 (DNA damage-binding protein 1) proteins. CUL4 has only recently been linked to E3 ligase activity in Arabidopsis. Based on the sequence of human orthologue, two highly related Arabidopsis genes *DDB1a* and *DDB1b* are identified. CUL4-DDB1 is considered to be

sufficient for E3 activity but may sometimes require additional factors, many of which contain a DWD (DDB1 binding WD40) motif that functions to interact with DDB1 (Lee *et al.*, 2010).

The Anaphase Promoting Complex (APC) is the most complicated E3 ligase, comprising of 11 subunits (APC1–11). The APC was initially identified in a yeast mutant unable to degrade the mitotic cyclin Clb2 (Wasch and Cross, 2002). Subsequently, the role of APC in degrading other pivotal cell cycle regulators was discovered in Arabidopsis (Capron *et al.*, 2003). The APC2 and APC11 subunits are functionally analogous to SCF subunits CUL1 and RBX1 respectively.

The ligation of Ub to substrates is reversible and the Ub linkages can be efficiently cleaved by deubiquitinating enzymes (DUBs). DUB enzymes maintain the pool of free ubiquitins in the cell through processing precursor ubiquitins from translation products and through recycling poly-Ub chains bound to the proteasome regulatory particle (RP) (Amerik and Hochstrasser, 2004). DUBs also negatively regulate protein degradation. The of ubiquitinated substrates to proteasomal degradation can be reversed by DUBs, altering the half-life of specific targets in response to signaling events (Amerik and Hochstrasser, 2004). In other words, DUB enzymes are proposed to function as a final proof-reading for protein degradation (Lam *et al.*, 1997). In addition, DUBs are able to edit the polyubiquitin signal on protein substrates to change the fate of the protein (Wertz *et al.*, 2004). More specifically, deubiquitination of parts of a polyubiquitin chain allows for modified ubiquitin signal on the protein substrate (Ndubaku and Tsui, 2015).

1.2.1.2 The Proteasome System

The 26S proteasome is a 2.4 MDa ATP-dependent proteolysis complex that degrades ubiquitin tagged substrates. The 26S proteasome is highly abundant both in the nucleus and cytosol where it rapidly degrades predominantly short-lived regulatory proteins and thus governs central cellular signaling processes (Schreiber and Peter, 2014). Studies indicate a similar design for the complex in yeast, mammals and plants (Sadanandom *et al.*, 2012) (Figure

1.5a). The 26S proteasome comprises 31 subunits separated into two subcomplexes: the 20S core protease (CP) and 19S regulatory particle (RP).

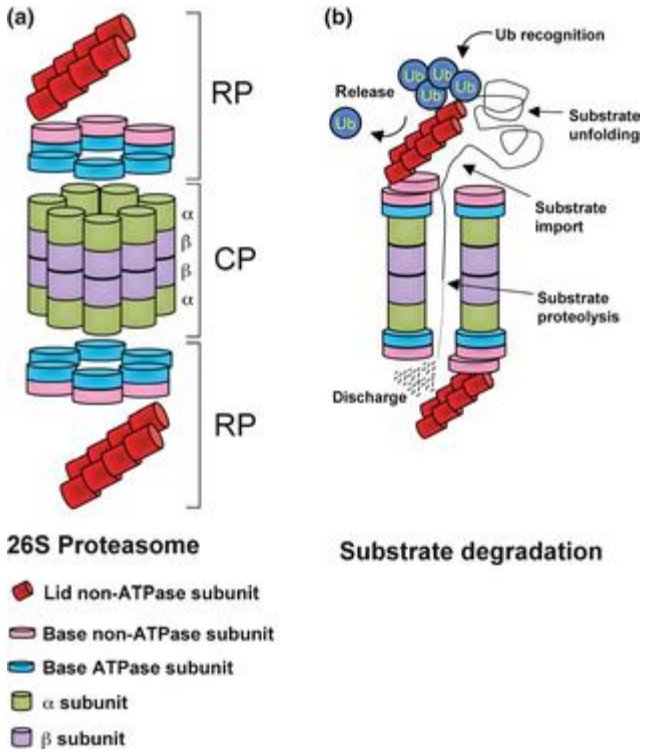


Figure 1.5 Organization and structure of the 26S proteasome. (a) The 26S proteasome comprises 31 subunits separated into two subcomplexes: the 20S core protease (CP) and 19S regulatory particle (RP). (b) Known and predicted activities of various subunits of 26S proteasome during the substrate degradation. This figure is adapted from (Sadanandom *et al.*, 2012) with permission.

The CP complex acts as a nonspecific ATP- and Ub-independent protease which is assumed to be a cylindrical structure including four heptameric rings. The peripheral rings are assembled from seven α-subunits and the central rings from seven β-subunits, presenting a $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ configuration (Wolf and Hilt, 2004). The protease activities which cleave most peptide bonds are contributed by the β1, β2 and β5 subunits (Wolf and Hilt, 2004). These three subunits possess peptidylglutamyl, trypsin-like and chymotrypsin-like activities (Wolf and Hilt, 2004). The peripheral ring forms a narrow pore to restrict the entry into the CP chamber, requiring entering proteins to be unfolded (Hartmann-Petersen *et al.*, 2003).

The 19S RP connects with both ends of the CP (Figure 1.5a) and provides poly-Ub recognition for the proteasome. The RP is made out of 17 subunits which form two subcomplexes termed Lid and Base. The Base associates directly with the CP and consists of a ring of six ATPase subunits (RPT1–6) and three non-ATPase subunits (RPN1, 2 and 10). The Lid associates with the Base via RPN10 and contains the remaining non-ATPase subunits (RPN 3, 5–9 and 11–12) (Fu *et al.*, 1998). The 19S RP mediates the recognition of K48 linked poly-Ub chains, recycling of Ub moieties, unfolding of substrates, pore gating and import of substrates to the proteasome (Figure 1.5b). K48 poly-Ub recognition by RPN10 has been identified, but is nonessential in yeast and Arabidopsis, implying that it is not the only poly-Ub-binding determinant (Hartmann-Petersen and Gordon, 2004). RPN11 confers a deubiquitinating activity that disassembles Ub chains (Dunand-Sauthier *et al.*, 2002). ATPase subunits in the Base contact the CP α-subunits and are presumed to facilitate substrate unfolding and pore opening (Sadanandom *et al.*, 2012). Other subunits have been proposed to function as distinct receptors for different substrate proteins (Hartmann-Petersen and Gordon, 2004).

In addition to the 19S RP, there are additional regulators of the proteasome in eukaryotes, with the 11S complex being the best characterized (Stadtmueller and Hill, 2011). These factors are not as conserved as the 19S complex, and their substrates and biological functions are less clear. These complexes are believed to be involved in ubiquitin-independent degradation pathways, since the ubiquitin/polyubiquitin-binding property has not yet been attributed to any of these complexes (Chondrogianni *et al.*, 2014). Moreover, these complexes do not possess ATPase activity, suggesting that their substrates are small peptides or proteins with unstructured regions for which active unfolding is not needed (Chondrogianni *et al.*, 2014).

The UPS in plants, like in other eukaryotes, degrades numerous intracellular regulators and thus modulates almost every aspect of growth and development. Genetic and cell biological studies have uncovered that the UPS is involved in nearly all processes in plants,

including the cell cycle (Capron *et al.*, 2003), plant development (Samach *et al.*, 1999), plant hormone pathways (Sullivan *et al.*, 2003) and responses to abiotic and biotic stimuli (Smalle and Vierstra, 2004).

1.2.2 Selective Autophagy in Plant Cells

1.2.2.1 General Introduction of Plant Autophagy

Macroautophagy (hereafter referred to as autophagy) is a conserved eukaryotic mechanism, which is classically defined as the degradation of cytoplasmic constituents in the lytic organelle (vacuoles in yeast and plants; lysosomes in mammals) (Xie and Klionsky, 2007). The general targets of autophagy vary from long-lived proteins to protein complexes and even organelles (Kelekar, 2005; Reumann *et al.*, 2010). Morphologically, autophagy begins with the formation of a cup-shaped double membrane, which expands to form the autophagosome engulfing the cargo (Bassham, 2007) (Figure 1.6). The autophagosome is then trafficked to the vacuole where the outer membrane fuses with the tonoplast, creating a single-membrane vesicle inside the vacuole, termed the autophagic body (Bassham, 2009) . The autophagic bodies and their contents are then degraded inside the vacuole by lytic enzymes, making materials available to the cell for re-use (Liu and Bassham, 2012).

The genes participating in the autophagy (termed *autophagy-related* or *ATG* genes) were initially identified in yeast (*Saccharomyces cerevisiae*) (Xie and Klionsky, 2007). Homologs of the yeast *ATG* genes have been discovered in many organisms including mammals and plants (Reumann *et al.*, 2010; Tanida, 2011). Autophagy is largely regulated by the Target of Rapamycin (TOR) pathway and its downstream target, the ATG1/ATG13 complex (Floyd *et al.*, 2012; John *et al.*, 2011; Liu and Bassham, 2010; Suttangkakul *et al.*, 2011). The TOR pathway regulates protein synthesis, cell proliferation and autophagy in response to nutrient conditions in a variety of eukaryotic species including yeast, algae, plants, and animals (Floyd *et al.*, 2012; Liu and Bassham, 2010; Perez-Perez *et al.*, 2010).

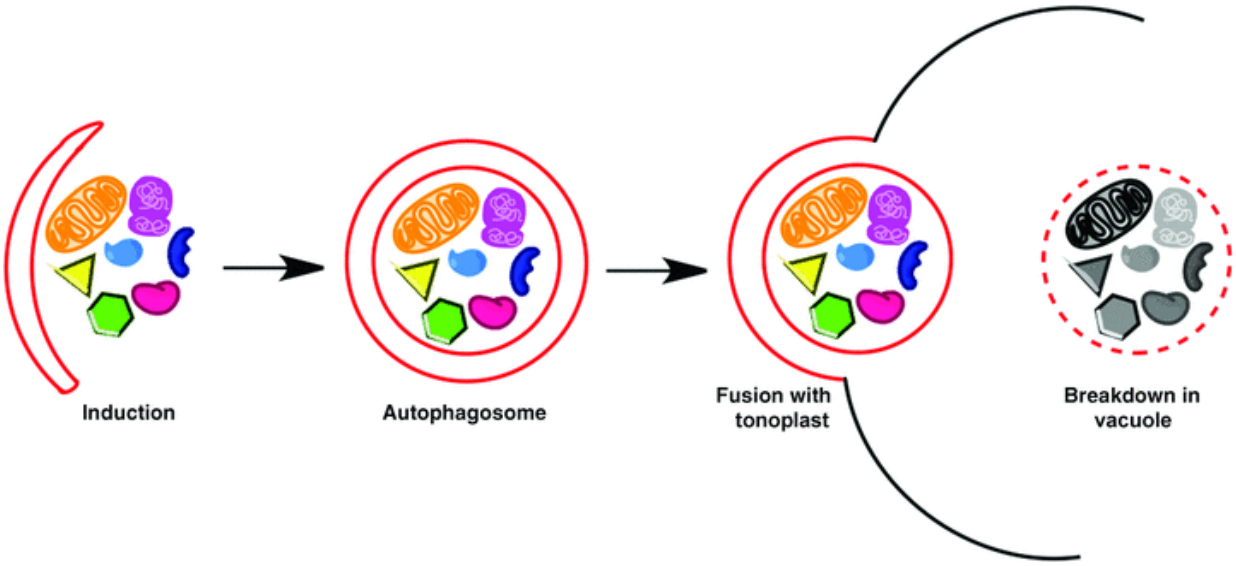


Figure 1.6 Autophagosome biogenesis in plants. (1) Autophagy begins in the formation of a cup-shaped double membrane structure near the cargo. (2) The double membrane expands to form the autophagosome (or vesicle) engulfing the cargo. (3) The autophagosome is trafficked to the vacuole, the outer membrane fuses with the tonoplast, creating a single-membrane vesicle inside the vacuole, which is degraded by lytic enzymes. This figure is adapted from (Floyd *et al.*, 2012) with permission.

In plants, basal autophagy takes part in the turnover of cellular components and serves as a quality control mechanism (Inoue *et al.*, 2006). However, autophagy additionally participates in some processes such as senescence, cell death and stress responses (Avin-Wittenberg *et al.*, 2012; Liu and Bassham, 2012). In addition, autophagy plays a role in plant-pathogen interactions (Avin-Wittenberg *et al.*, 2012; Cacas, 2010).

Autophagy was originally considered to be a non-selective process, mediating the bulk degradation of cytosolic components. However, in recent years, autophagy has also been shown to operate as a selective process, termed selective autophagy (Michaeli and Galili, 2014; Suzuki, 2013; Svenning *et al.*, 2011). Signaling molecules, protein aggregates, mitochondria, peroxisomes, and ribosomes have been identified to be substrates of selective autophagy in yeast and mammals (Floyd *et al.*, 2012). Although the systematic mechanism of selective autophagy is not clear, growing evidence reveals an important role for ATG8 homologs and

ubiquitin in this process in all eukaryotes (Noda *et al.*, 2010; Reggiori *et al.*, 2012; Svenning *et al.*, 2011).

ATG8 is an autophagosomal membrane protein that is needed for autophagosome biogenesis in both non-selective and selective autophagy (Floyd *et al.*, 2012; Perez-Perez and Crespo, 2010; Yoshimoto *et al.*, 2004). While yeast has only one ATG8 protein, humans have seven ATG8 proteins separated into two subfamilies, and Arabidopsis has nine (Doelling *et al.*, 2002). ATG8s are processed before the fusion into the autophagosome membrane (Kirisako *et al.*, 2000). Afterwards, ATG8 may cooperate with adaptors to engulf cargo into the autophagosome selectively (Noda *et al.*, 2010). This review will highlight current knowledge of the selective autophagy of proteins and protein aggregates in plants.

1.2.2.2 Selective Autophagy of Proteins and Protein Aggregates by Autophagic Adaptors

Selective autophagy is regulated by the adaptor proteins that link a targeted cargo and the biosynthesis of autophagosome (Johansen and Lamark, 2011). Proteins labeled with Ub are normally degraded by the 26S proteasome system. However, under certain conditions (such as increased abiotic stress), Ub-tagged proteins can form protein aggregates hydrophobically, making degradation by the 26S proteasome less effective (Lasch *et al.*, 2001; Riley *et al.*, 2010). The degradation of Ub-tagged protein aggregates by autophagic adaptor proteins has been demonstrated in various eukaryotes (Floyd *et al.*, 2012).

The mNBR1 (mammalian neighbor of BRCA1 gene 1) is an autophagic adaptor with a role in targeting ubiquitinated protein aggregates to the autophagy system (Kirkin *et al.*, 2009; Waters *et al.*, 2009). Homology of mNBR1 has been described in plants. The Arabidopsis homolog, AtNBR1 is selectively targeted by the autophagy machinery (Svenning *et al.*, 2011). Similar to its animal homolog, AtNBR1 is able to bind both to ATG8s and to Ub (Svenning *et al.*, 2011). This protein has the following functional regions: an LC3-interacting region (LIR) that binds multiple ATG8-family proteins, duplicated UBA domains that bind Ub, and a PB1 domain essential for homopolymerization (Svenning *et al.*, 2011). The homopolymerization

of AtNBR1 makes the interaction between AtNBR1 and ATG8 isoforms more efficient (Svenning *et al.*, 2011). Most recently, it has been shown that AtNBR1-mediated selective autophagy can target insoluble ubiquitinated protein aggregates in Arabidopsis (Zhou *et al.*, 2013).

Another potential autophagic adaptor, Joka2, has been identified in tobacco (*Nicotiana tabacum*) and contains conserved domains of mNBR1 (Zientara-Rytter *et al.*, 2011). In tobacco, Joka2 is transferred from the nucleus to acidic structures, similar to another mammalian adaptor protein p62 (Ponpuak *et al.*, 2010; Zientara-Rytter *et al.*, 2011).

A few cases exist in which autophagy is responsible for the degradation of proteins tagged by Ub, bypassing the 26S proteasome (Baumberger and Baulcombe, 2005; Filimonenko *et al.*, 2010; Pankiv *et al.*, 2007). In plants, an example is the autophagic degradation of ARGONAUTE1 (AGO1) (Derrien *et al.*, 2012).

The collection of targets for selective autophagy is expanding quite rapidly, and so is the list of individual autophagic adaptors involved in their recognition. However, at present, where and how selective autophagosomes are initiated in the plant cell is still not clear. Ubiquitination of the target appears to be a conserved precondition in many types of selective autophagy; however, non-ubiquitinated cargoes are also selectively degraded by this pathway in plants (Veljanovski and Batoko, 2014). The ubiquitin-independent selective autophagic pathway also involves adaptor proteins linking the cargo to the autophagic machinery (Veljanovski and Batoko, 2014). Further characterization of the regulatory and targeting mechanisms is needed to elucidate the basis of the selective autophagy.

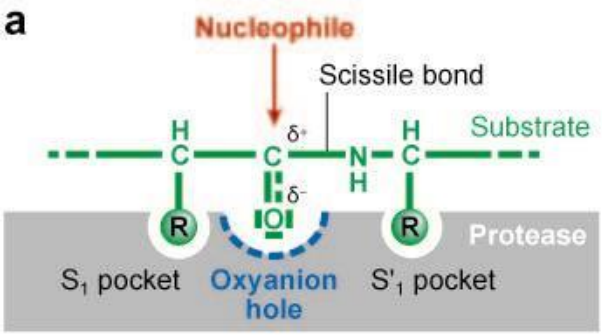
1.2.3 Plant Proteases

Proteases hydrolyze peptide bonds and lead to the fragmentation of their target proteins. These enzymes may act on terminal or internal amino acids of proteins and are respectively called exo- or endopeptidases. All proteases polarize the carbonyl group of the substrate peptide bond by stabilizing the oxygen in an oxyanion hole, making the carbon atom more

vulnerable for attack by an activated nucleophile (van der Hoorn, 2008) (Figure 1.7a). Proteases can do this in four major ways, which give the names to four major catalytic classes: cysteine proteases, serine proteases, metalloproteases, and aspartic proteases (van der Hoorn, 2008) (Figure 1.7b). The amino acid on the N-terminal side of the hydrolyzed peptide bond is referred to as P1, whereas the amino acid on the C-terminal side is referred to as P1'. Similarly, the site on the proteolytic enzyme binding the P1 residue is referred to as S1, and the site recognizing the P1' residue is referred to as S1'.

Proteolysis is a prominent post- and co-translational modification with diverse functional implications. It is employed either to destroy proteins (protein catabolism), to mature precursor proteins (limited and very often specific) or to remove methionine by methionine aminopeptidases after new proteins are synthesized (Tsiatsiani *et al.*, 2012). As key regulators, proteases govern several important processes at the cell and tissue levels of organisms (van der Hoorn, 2008). Particularly in plants, proteases have been shown to be involved in meiosis; suspensor formation; xylem formation; embryo cuticle deposition; seed maturation; meristem size; epidermal cell fate; stomata development; plastid development; nutrient mobilization; responses to environmental stimuli and virulence factors; flowering time; branching and senescence (Chichkova *et al.*, 2010; Coll *et al.*, 2011; Kato and Sakamoto, 2010; Nixon *et al.*, 2010; Tsiatsiani *et al.*, 2012; van der Hoorn and Jones, 2004; van der Hoorn, 2008).

Proteases in the MEROPS protease database have been subdivided into families and clans on the basis of evolutionary relationships (http://merops.sanger.ac.uk) (Rawlings *et al.*, 2014). A protease clan refers to proteases derived from a single common ancestor, and clans are subdivided into families. A protease family refers to a subgroup of proteases that share sequence similarity, either throughout the entire protein sequence or only within the catalytic domain. The *Arabidopsis thaliana* genome encodes 879 known and putative proteases, corresponding to approximately 3.2% of all Arabidopsis protein-coding genes (The Arabidopsis Information Resource). These proteases are distributed over 60 families that belong to around 30 different clans in the MEROPS protease database.



b

Catalytic class	Nucleophile	Oxyanion stabilizer
Cysteine proteases	Cys-His	-NH-(2x)
Serine proteases	Ser-His	-NH-(2x)
Metalloproteases	H_2O-Me^{2+}	Me^{2+}
Aspartic proteases	H_2O-Asp	H^+-Asp

Figure 1.7 Cleavage mechanisms of the four major catalytic classes of proteases. (a) The protein (green) binds to the substrate (S) pockets of the protease (gray) through amino acid residues (R). Proteases polarize the carbonyl group of the peptide bond by stabilizing the oxygen in an oxyanion hole (blue), which makes the carbon atom more vulnerable for attack by an activated nucleophile. (b) The nucleophile and the oxyanion stabilizer are the main differences among the four catalytic classes. This figure is adapted from (van der Hoorn, 2008) with permission.

Proteases are crucial for plants. Protease mutations are frequently lethal, and many result in severe fitness-reducing phenotypes (van der Hoorn, 2008). Despite the large number of proteases and important functions they play in plants, only a few natural substrates have been identified to date (reviewed in Tsiatsiani *et al.*, 2012). The scarcity of identified substrates for the proteases is the major bottleneck for protease research because it hampers further progress in understanding the molecular basis of how these proteases function.

Different proteases accumulate in different subcellular compartments. In plants, proteases have been extensively studied in vacuoles, mitochondria, and chloroplasts (Ariizumi *et al.*, 2011; Hatsugai *et al.*, 2015; Huang *et al.*, 2009; Richter *et al.*, 2005; Shipman and Inoue,

2009; Zhang *et al.*, 2010; Zybailov *et al.*, 2008). There is also evidence for the proteolysis by proteases in other organelles, such as the peroxisome, the Golgi and the endoplasmic reticulum (Ge *et al.*, 2005; Helm *et al.*, 2007; Schuhmann *et al.*, 2008; Wolf *et al.*, 2009). In addition, nuclear and cytosolic proteases have been shown to play a number of essential roles in plants (Adam, 2013; Tsiatsiani *et al.*, 2012; van der Hoorn, 2008).

1.3 Proteolysis of CKIs

The activity of plant cell cycle regulators is also controlled through proteolytic mechanisms and, amongst others, the ubiquitin proteasome pathway is the most prominent for the timely degradation of cell cycle proteins. The UPS ensures the timely progression of the cell cycle by triggering the rapid degradation of target proteins, thus providing an irreversible mechanism that drives forward the cycle. Two kinds of E3s, the SCF and the APC play prominent roles during the cell cycle in all eukaryotes (Heyman and De Veylder, 2012; Marrocco *et al.*, 2010; Mocciaro and Rape, 2012; Pesin and Orr-Weaver, 2008). They target many different substrates through short destruction motifs, mainly the D-box and the KEN-box. For example, in all eukaryotes, the D-box in cyclins and other cell cycle regulators is a conserved motif of 9 amino acid residues (RxxLxxIxN). Deletion or point mutations in this motif inhibit proteolysis (Genschik *et al.*, 2014). In addition to the SCF and APC, other families of E3s also contribute to the cell cycle regulation, such as monomeric RING, BTB-CUL3 and DDB-CUL4 (Genschik *et al.*, 2013b; Marrocco *et al.*, 2010).

In fungi and mammals, the G_1/S transition requires the degradation of CKIs to release CDK activity, permitting the phosphorylation of proteins needed to enter S phase. In budding yeast, one CKI, Sic1p, is degraded after being phosphorylated by G1 CDK-cyclin complex and then ubiquitinated by SCF^{CDC4} (Feldman *et al.*, 1997; Schwob *et al.*, 1994). Mammals have a comparative mechanism, and $p27^{Kip1}$ is degraded after the phosphorylation by the CDK2-cyclin E complex and ubiquitination by SCF^{SKP2} (Starostina and Kipreos, 2012). SCF^{SKP2} additionally targets other cell cycle regulators such as $p21^{Cip1}$, another CKI (Frescas and Pagano,

2008). Other E3 ligases controlling mammalian CKI protein levels have also been identified (Starostina and Kipreos, 2012). For instance, KPC1 (a RING-finger protein) promotes the degradation of $p27^{Kip1}$ in the cytoplasm through a phosphorylation-independent pathway during G1 phase (Kamura *et al.*, 2004), and the $CRL4^{CDT2}$ E3 ligase is responsible for the turnover of $p21^{Cip1}$ during S phase as well as after DNA damage (Abbas *et al.*, 2008; Kim *et al.*, 2008b). Notably, a physical connection of $p21^{Cip1}$ with proliferating cell nuclear antigen is required for its destruction, mediated by the PIP box degron in $p21^{Cip1}$ (Havens and Walter, 2011). Together, these findings indicate that ubiquitination of CKIs often requires post-translational modifications, normally phosphorylation, and specific E3 ligases.

The understanding of proteolysis at the G_1/S transition in plants is still limited. However, protein levels of plant CKIs must be tightly regulated due to their significant roles during the cell cycle. While the degradation of SIM proteins has not yet been investigated, it is known that ICKs are UPS substrates (Marrocco *et al.*, 2010). Treatment of Arabidopsis plants with MG132, an inhibitor of 26S proteasome, increased the levels of ICK1, ICK2, ICK5 and ICK6, suggesting the involvement of 26S proteasome in their degradation (Anzola *et al.*, 2010; Jakoby *et al.*, 2006; Jun *et al.*, 2013; Verkest *et al.*, 2005a). It was also shown that the proteasomal degradation of ICK2 requires the CDK-dependent phosphorylation, a situation similar to mammalian $p27^{Kip1}$ SCF^{SKP2}-dependent degradation (Verkest *et al.*, 2005a).

Several E3s promoting ICKs degradation have been found in Arabidopsis. SKP2B (a F-box protein) recognizes diverse substrates, most likely including ICK1 (Ren *et al.*, 2008). The leaf serration phenotype caused by *ICK1* overexpression was suppressed by *SKP2B* overexpression in Arabidopsis (Ren *et al.*, 2008). Based on the similarity with the mammalian E3 KPC1, an Arabidopsis E3 protein RKP (Related to KPC1) was found and suspected to be responsible for ICK1 proteasomal degradation (Ren *et al.*, 2008). However, ICKs did not accumulate to higher levels in a *skp2b rkp* mutant, possibly due to the presence of other E3s (Ren *et al.*, 2008).

Indeed, other Arabidopsis E3 ligases have been suggested to be involved in the

degradation of ICKs. RHF1a and RHF2a are two closely related RING-finger E3 ligases, and the *rhf1a rhf2a* double mutant was defective in mitotic cell divisions in female and male gametogenesis (Liu *et al.*, 2008). The findings that RHF1a and RHF2a interacted with ICK4, ICK4 protein level accumulated in the *rhf1a rhf2a* double mutant, and down-regulation of *ICK4* partially suppressed the *rhf1a rhf2a* mutant phenotype suggest that these E3s target ICK4 for degradation (Liu *et al.*, 2008). Similar results have been obtained with an F-box protein FBL17, which plays an important role during male gametogenesis, and the knockout *fbl17* mutants failed to undergo pollen mitosis II (Gusti *et al.*, 2009; Kim *et al.*, 2008a). It has been shown that FBL17 interacted with ICK4 and ICK5, ICK4 level increased in the *fbl17* knockout mutant, and the pollen defect phenotype of the mutants was partially rescued by *ICK* loss-of-function mutations, supporting the notion that FBL17 targets ICK4 and ICK5 for degradation (Kim *et al.*, 2008a, Gusti *et al.*, 2009; Zhao *et al.*, 2012).

The results above show that the UPS is a general mechanism regulating the stability of ICKs. However, little is known regarding the specific sequences that confer instability to ICKs. Sequence analysis suggests that putative PEST regions involved in protein degradation are present in some of the ICK proteins (Figure 1.2) (Wang *et al.*, 2008). However, none of these PEST sequences has been verified experimentally (Wang *et al.*, 2008).

When the N-terminal 108 amino acids were remove from ICK1, the mutant protein showed stronger interactions with CDKA and CYCD3;1 in the yeast two-hybrid system (Wang *et al.*, 1998), indicating that the N-terminal region has a negative effect on ICK1 function. This negative regulatory impact was confirmed *in planta* by the finding that tissue-specific expression of the mutant ICK1 in trichomes of Arabidopsis resulted in stronger phenotypes with further decreased number of branched trichomes and reduced nuclear DNA content (Schnittger *et al.*, 2003). The mechanism for this negative effect was elucidated when a set of GFP tagged ICK1 deletion mutants was expressed in transgenic plants. Results indicated that the level of GFP-ICK1$^{109\text{-}191}$ protein was much higher compared with wild type GFP-ICK1 and two other GFP-ICK1 mutants even though the transcript levels remained similar, suggesting that the

N-terminal region confers instability to wild type ICK1 protein (Zhou *et al.*, 2003a). However, it is not clear whether the N-terminus of ICK1 renders protein instability through the UPS-mediated protein degradation pathway.

1.4 Research Hypotheses and Objectives

As plant cell cycle regulators, the levels of ICKs in plant cells are critical and understandably are controlled both transcriptionally and post-translationally. Several studies have shown that the degradation of ICK proteins is through the ubiquitin-26S proteasome pathway. However, the specific sequence signals involved in are not known. Unpublished data from our lab indicate that the N-terminal 40 amino acid region was critical for ICK1 instability and a similar observation was made in yeast using HA-tagged ICK1 lacking the N-terminal region, suggesting that the mechanism by which the N-terminal region makes ICK1 unstable is conserved in plants and yeast (Li *et al.*, manuscript submitted). Our results also indicate that ICK1 degradation is likely through both SCF-dependent and SCF-independent pathways (Li *et al.*, manuscript submitted). Therefore, we hypothesize that the N-terminal 40 amino acid region of ICK1 contains a specific sequence signal that can strongly decrease the protein level.

Since a short region of ICK1 (1-40) can regulate the protein level, we further hypothesize that motifs with this property also exist in the N-terminal regions of other ICKs. If so, since the N-terminal sequences are diverse among plant ICKs, different sequence signals responsible for regulating the protein levels may be present in other ICKs. The main aim of this project is to understand how the levels of ICK1 and other ICK proteins are regulated. More specifically, the objectives of this research are:

(1) to identify the specific sequence motif in the N-terminal region of ICK1 with a strong ability to decrease the protein level;

(2) to determine the roles of the N-terminal regions of other ICKs in regulating the protein levels;

(3) to identify additional sequence motifs involved in regulating the levels of other ICKs.

2. MATERIALS AND METHODS

2.1 Plasmid Construction

2.1.1 General Cloning Techniques

Target DNA fragments were amplified by polymerase chain reaction (PCR). A single PCR was set up in a total volume of 50 μL, containing *pfu* DNA polymerase (0.04 U/μL) with reaction buffer, dNTPs (200 μM each), sequence-specific oligonucleotide primer pairs (0.25 μM each), template DNA (generally 25-100 ng), and nuclease-free water. The thermal cycle conditions consisted of 3 minutes initial denaturation at 95℃, followed by 25 cycles (30 seconds at 95℃, 30 seconds at annealing temperature and appropriate extension time at 72℃), and 5 minutes final extension at 72℃. PCR products were purified using the PCR Product Purification Kit (Bio Basic Inc.) according to the manufacturer's instructions. Vectors and inserts were both digested with restriction enzymes according to the manufacturer's instructions, and analyzed by electrophoresis in 1% (w/v) agarose gel. The inserts and linearized vectors were then extracted from the agarose gel using Bio Basic DNA Gel Extraction Kit following the manufacturer's instructions.

Primers used in this study were synthesized by Integrated DNA Technologies (IDT). The restriction enzymes and the corresponding enzyme buffers were purchased from New England Biolabs (NEB) or Life Technologies. Primers are listed in Table 2.1.

The purified vectors (generally 10-15 ng) and inserts (generally 2-5 ng) at a molar ratio of 1:3 were ligated in the ligation reaction mixture (10μL) containing T4 ligase buffer and T4 DNA ligase (0.1 U/μL) according to the manufacturer's instructions (Life Technologies). The ligation mixture was incubated at room temperature for two hours.

For bacterial transformation, the ligation mixture (0.5 μL) was added to 20 μL of *Escherichia coli* (*E. coli*) DH10β competent cells and subjected to a high voltage charge of 1.8 kV using an electroporator, and 200 μL of pre-chilled SOC (super optimal broth) medium were added to the cuvette immediately after the electroporation. Electroporated cells were then

collected and incubated at 37℃ for 1 hour without shaking. Cells were plated onto Luria Broth (LB) agar plates (20 g/L LB broth and 1.5% (w/v) agar) supplemented with an appropriate antibiotic (100 μg/mL ampicillin or 50 μg/mL kanamycin) and plates were incubated at 37℃ overnight. Single colonies were picked up and used to inoculate 15 ml culture tubes containing LB medium (20 g/L LB broth) supplemented with an appropriate antibiotic. After overnight growth at 37℃, bacterial cells were collected and plasmid DNA was isolated using the Plasmid DNA MiniPrep Kit (Promega). All constructs used in this study were verified by PCR screening and DNA sequencing (Eurofins Scientific).

Table 2.1 List of primers used for generating constructs.

Primer ID	Purpose	Primer sequence
855	ICK2, forward	GAC TGT CGA CAG AAA ATA ACG GTG ACG ATC G
939	ICK2, reverse	CAG TGC GGC CGC TAA TCA GAC CTA GAT ATT CTC T
1057	ICK4 10-aa fusion	CTG TCG ACA ATG AGC GAG AGA AAG CGA GAG CTT GCA GAA AGT AAA GGA GAA GAA CTT T
1058	ICK4 10-aa fusion	CTG TCG ACA ATG GAA GCT TCA AGC ACA AGC TTC TCA CCA CTG AGT AAA GGA GAA GAA CTT
1059	ICK4 10-aa fusion	CTG TCG ACA ATG AAG AAA ACG AAG CTT AAT GAT TCT TCT GAT AGT AAA GGA GAA GAA CTT
1060	ICK4 10-aa fusion	CTG TCG ACA ATG TCA TCA CCG GAC TCT CAT GAC GTC ATC GTC AGT AAA GGA GAA GAA CTT
1061	ICK4 10-aa fusion	CTG TCG ACA ATG TTC GCG GTT TCA TCT TCT TCC GTT GCT TCG AGT AAA GGA GAA GAA CTT
1062	ICK4 10-aa fusion	CTG TCG ACA ATG TCG GCG GCT TTA GCG TCT GAT GAA TGT TCC AGT AAA GGA GAA GAA CTT
1063	ICK4 10-aa fusion	CTG TCG ACA ATG GTT ACC ATC GGT GGA GAA GAA AGT GAT CAG AGT AAA GGA GAA GAA CTT
1064	ICK4 10-aa fusion	CTG TCG ACA ATG TCC TCG AGT ATC AGC TCC GGT TGT TTC ACC AGT AAA GGA GAA GAA CTT
1065	ICK6 10-aa fusion	CTG TCG ACA ATG GGG AAA TAC ATG AAG AAA TCA AAG ATA AGT AAA GGA GAA GAA CTT T

1066	ICK6 fusion	10-aa	CTG TCG ACA ATG ACT GGC GAT ATC AGC GTC ATG GAA GTC TCT AGT AAA GGA GAA GAA CTT
1067	ICK6 fusion	10-aa	CTG TCG ACA ATG AAA GCA ACA GCT CCA AGT CCA GGT GTT CGA AGT AAA GGA GAA GAA CTT
1068	ICK6 fusion	10-aa	CTG TCG ACA ATG ACC AGA GCC GCT AAA ACC CTA GCC TTG AAG AGT AAA GGA GAA GAA CTT
1069	ICK6 fusion	10-aa	CTG TCG ACA ATG CGG CTT AAT TCC TCC GCC GCT GAT TCA GCT AGT AAA GGA GAA GAA CTT
1070	ICK6 fusion	10-aa	CTG TCG ACA ATG CTA CCT AAC GAC TCT TCT TGC TAT CTT CAG AGT AAA GGA GAA GAA CTT
1071	ICK6 fusion	10-aa	CTG TCG ACA ATG CTC CGT AGC CGC CGT CTC GAG AAA CCC TCT AGT AAA GGA GAA GAA CTT
1072	ICK6 fusion	10-aa	CTG TCG ACA ATG TCG CTG ATT GAA CCG AAA CAG CCG CCG AGA AGT AAA GGA GAA GAA CTT
1073	ICK6 fusion	10-aa	CTG TCG ACA ATG GTT CAC AGA TCG GGA ATT AAA GAG TCT GGT AGT AAA GGA GAA GAA CTT
1074	ICK6 fusion	10-aa	CTG TCG ACA ATG TCC AGG TCT CGC GTT GAC TCG GTT AAC TCG AGT AAA GGA GAA GAA CTT
1079	ICK4 fusion	10-aa	CTG TCG ACA ATG CGA GAG CTT GCA GAA GAA GCT TCA AGC ACA AGT AAA GGA GAA GAA CTT
1080	ICK4 fusion	10-aa	CTG TCG ACA ATG AGC TTC TCA CCA CTG AAG AAA ACG AAG CTT AGT AAA GGA GAA GAA CTT
1081	ICK4 fusion	10-aa	CTG TCG ACA ATG AAT GAT TCT TCT GAT TCA TCA CCG GAC TCT AGT AAA GGA GAA GAA CTT
1082	ICK4 fusion	10-aa	CTG TCG ACA ATG CAT GAC GTC ATC GTC TTC GCG GTT TCA TCT AGT AAA GGA GAA GAA CTT
1083	ICK4 fusion	10-aa	CTG TCG ACA ATG TCT TCC GTT GCT TCG TCG GCG GCT TTA GCG AGT AAA GGA GAA GAA CTT
1084	ICK4 fusion	10-aa	CTG TCG ACA ATG TCT GAT GAA TGT TCC GTT ACC ATC GGT GGA AGT AAA GGA GAA GAA CTT
1085	ICK4 fusion	10-aa	CTG TCG ACA ATG GAA GAA AGT GAT CAG TCC TCG AGT ATC AGC AGT AAA GGA GAA GAA CTT
1124	ICK6 fusion	10-aa	CAT GTC GAA ATG AAG AAA TCA AAG ATA ACT GGC GAT ATC AGC AGT AAA GGA GAA GAA CT
1125	ICK6 fusion	10-aa	CAT GTC GAC AAT GGT CAT GGA AGT CTC TAA AGC AAC AGC TCC AAG TAA AGG AGA AGA ACT

1126	ICK6 fusion	10-aa	CAT GTC GAC AAT GAG TCC AGG TGT TCG AAC CAG AGC CGC TAA AAG TAA AGG AGA AGA ACT
1127	ICK6 fusion	10-aa	CAT GTC GAC AAT GAC CCT AGC CTT GAA GCG GCT TAA TTC CTC CAG TAA AGG AGA AGA ACT
1128	ICK6 fusion	10-aa	CAT GTC GAC AAT GGC CGC TGA TTC AGC TCT ACC TAA CGA CTC TAG TAA AGG AGA AGA ACT
1129	ICK6 fusion	10-aa	CAT GTC GAC AAT GTC TTG CTA TCT TCA GCT CCG TAG CCG CCG TAG TAA AGG AGA AGA ACT
1130	ICK6 fusion	10-aa	CAT GTC GAC AAT GCT CGA GAA ACC CTC TTC GCT GAT TGA ACC GAG TAA AGG AGA AGA ACT
1131	ICK6 fusion	10-aa	CAT GTC GAC AAT GAA ACA GCC GCC GAG AGT TCA CAG ATC GGG AAG TAA AGG AGA AGA ACT
1132	ICK6 fusion	10-aa	CAT GTC GAC AAT GAT TAA AGA GTC TGG TTC CAG GTC TCG CGT TAG TAA AGG AGA AGA ACT
1133	~ 20-aa fragments	ICK2	CAG TGC GGC CGC TCA CGT CGT CGT CGT AAC TC
1134	~ 20-aa fragments	ICK2	CAG GTC GAC AAT GGT GAA ACG AAG GAA GAT GG
1135	~ 20-aa fragments	ICK2	CAG TGC GGC CGC TCA AAC ACT CGT CTC CGA CG
1136	~ 20-aa fragments	ICK2	CAG GTC GAC AAT GGT TAT AGT ACG ACG GCG A
1137	~ 20-aa fragments	ICK2	CAG TGC GGC CGC TCA CTC AAG ATC TAC AAA TTC GA
1138	~ 20-aa fragments	ICK3	CAG TGC GGC CGC TCA AGA TTT ATC CTT AAC GGA AAC
1139	~ 20-aa fragments	ICK3	CAG GTC GAC AAT GCA CCC ACC CGC TCT AGG
1140	~ 20-aa fragments	ICK3	CAG TGC GGC CGC TCA GCG GCT CCG GAG CTG GA
1141	~ 20-aa fragments	ICK3	CAG GTC GAC AAT GCG TCT CGT GAA GCT TCC A
1142	~ 20-aa fragments	ICK4	CAG TGC GGC CGC TCA CAG TGG TGA GAA GCT TGT
1143	~ 20-aa fragments	ICK4	CAG GTC GAC AAT GAA GAA AAC GAA GCT TAA TG

1144	~ 20-aa ICK4 fragments	CAG TGC GGC CGC TCA GGA ACA TTC ATC AGA CGC
1145	~ 20-aa ICK4 fragments	CAG GTC GAC AAT GGT TAC CAT CGG TGG AGA AG
1146	~ 20-aa ICK6 fragments	CAG TGC GGC CGC TCA AGA GAC TTC CAT GAC GCT G
1147	~ 20-aa ICK6 fragments	CAG GTC GAC AAT GAA AGC AAC AGC TCC AAG TC
1148	~ 20-aa ICK6 fragments	CAG TGC GGC CGC TCA CTG AAG ATA GCA AGA AGA GT
1149	~ 20-aa ICK6 fragments	CAG GTC GAC AAT GCT CCG TAG CCG CCG TCT
1271	ACT8, forward	CAG TCT CGA GAA TGG CCG ATG CTG ATG ACA
1272	ACT8, reverse	CAG TGC GGC CGC TTA GAA GCA TTT TCT GTG GA
1275	ACT8-ICK3$^{21-37}$	CAT GCG GCC GCT CAG AGG TTT TTA GCG GCG GCG GCT CTG GTA CGG AAA CCT AGA GCG GGT GGG TGG AAG CAT TTT CTG TGG ACA A

2.1.2 Protein Expression Constructs

For protein expression in Arabidopsis, the target gene was cloned into the plant expression vector pBI121 (Clontech) behind the CaMV 35S promoter. For protein expression in yeast, the fragments containing the target genes were cloned into the yeast expression vector pYES2 (Stratagene). For protein expression in *E. coli*, the target DNA fragment was cloned into expression vector pET-28a-c (+) (Novagen) downstream of the T7 promoter.

2.2 Arabidopsis Lines and Plant Transformation

The pBI121-based constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation as described above (high voltage charge of 1.44 kV). Electroporated cells were then collected and plated onto 2×YT agar plates (1.6% (w/v) peptone,

0.5% (w/v) yeast extract, 0.5% (w/v) NaCl and 1.5% (w/v) agar) supplemented with kanamycin (50 μg/mL) and gentamycin (25 μg/mL) and incubated at 30 ℃ for two days. Then, the *Agrobacterium tumefaciens* cells containing the plasmids of interest were harvested and suspended in 200 mL solution containing 1/2 Murashige and Skoog medium (Sigma) and 5% (w/v) sucrose. Surfactant Silwet-77 was added into the suspension to a final concentration of 0.02% (v/v) before the infiltration.

Arabidopsis *thaliana* ecotype "Col-0" was used in this study. Arabidopsis plants were grown in 10-cm square pots in a growth room with a density of about 9 plants per pot, at 20 ℃ and with 16/8 h (day/night) photoperiod. The inflorescence of 5-6 week-old Arabidopsis plants was submerged into *Agrobacterium tumefaciens* suspension and subsequently placed in a vacuum chamber. A vacuum of 600 to 700 mm Hg was applied for 2 minutes. Infiltrated plants were recovered and grown in the growth room until the seeds (T_1) were harvested.

2.3 Selection of Arabidopsis Transformants and Protein Extraction

Arabidopsis T_1 seeds were plated on the plates of 1/2-strength Murashige and Skoog agar plates (2.2 g/L Murashige and Skoog salts, 1% (w/v) sucrose, 0.7% (w/v) agar and pH 5.7) supplemented with 50 mg/L kanamycin and 300 mg/L Timentin (referred to as 1/2 MSTK plates hereafter). Seeds were sterilized in 20% (v/v) bleach for 15 minutes and rinsed with sterile distilled water at least three times. Seeds were plated on the 1/2 MSTK plates, which were then placed in a tissue culture chamber at with 20 ℃ with continuous lighting. After 10 days, kanamycin-resistant transformants were transferred from the initial screening plates onto fresh selection plates, with each plate having 30 transformants. After one week on the new plate, the 30 transformants in the plate were pooled and used for protein extraction. Each protein sample was prepared from a group of independent Arabidopsis transformants in a plate and thus represented the average protein level for these independent Arabidopsis transformants.

To extract proteins from the Arabidopsis seedlings, plant materials were placed in 1.5 mL microfuge tubes and the weights of the plant materials were measured. Ice-cold extraction

buffer (250 mM NaCl, 25 mM Tris-HCl (pH 8.0), 2.5 mM EDTA (ethylenediaminetetraacetic acid), 10 mM DTT (dithiothreitol), 1% (v/v) Triton X-100 and Protease Inhibitor Cocktail (Sigma)) was added into each sample at a ratio of 1 mL buffer per gram of fresh tissue. Plant material was ground in the tube with a plastic pestle until the mixture was homogeneous. Once all the extracts were ground, the tubes were centrifuged at 15,000 ×g at 4 ℃ for 10 minutes and the supernatant was transferred to a new tube. The fresh tubes were then centrifuged again at 15,000 ×g at 4 ℃ for 10 minutes. The supernatant was transferred to a new microfuge tube and stored at -80 ℃.

2.4 Yeast Strain and Transformation

Yeast strain MaV203 was used for protein expression in this study. MaV203 was originally purchased from GIBCO/BRL Life Technologies (presently Life Technologies) with the following genotype: MATa, *leu*2-3,112, *try*1-901, *his*3D200, *ade*2-101, *gal*4D, *gal*80D, *SPAL*10::*URA*3, *GAL*1::*lac*Z, *HIS*3$_{\text{UAS GAL1}}$::*HIS*3@*LYS*2, *can*1^{R}, *cyh*2^{R}.

Yeast cells were transformed using a modified lithium acetate method (Elble, 1992). Briefly, yeast cells were inoculated in the non-selective yeast peptone dextrose (YPD) medium (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose) at 30 ℃ overnight to saturation. On the day of the transformation, 1 mL of cells was transferred into a 1.5 mL microfuge tube and centrifuged at 15,000 ×g for 5 s at 4 ℃. The supernatant was decanted with approximately 50 μL liquid remaining. The following reagents were added into the tube: 10 μg of denatured salmon sperm DNA, 0.5 μL of plasmid (300 ng/μL), 10 μl of 1.0 M DTT and 250 μL of PLATE mixture (consisted of sterile 45% (w/v) polyethylene glycol 4000, 1 M lithium acetate, 1 M Tris-Cl pH 7.5, and 0.5 M EDTA). Then, the cell pellet was resuspended. The mixture was left at room temperature overnight without shaking. On the following day, this mixture was subjected to a heat shock at 42 ℃ for 10 minutes, and then plated onto selective SD plate (synthetic dextrose: 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, appropriate amino acid components and 1.5% (w/v) agar) to screen the yeast

auxotrophs. Specifically, MaV203 cells transformed with pYES2-based constructs were selected on SD plates without uracil (SD-Ura$^-$).

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2.5 Protein Expression in Yeast

pYES2-based constructs were used to transform MaV203 cells. Due to the presence of the *GAL1* promoter in pYES2, the protein expression in these transformants can be induced by galactose. Single colonies picked from the selective plates (SD-Ura$^-$) were used to inoculate into culture tubes each with 3 mL of SD-Ura$^-$ medium. The cultures were incubated at 30 ℃ in a shaking incubator (250 rpm) overnight to reach an optical density at 600 nm (OD_{600}) of 1.0-1.2. The cells were harvested from each culture by centrifuging at 800 ×g for 5 min at room temperature and decanting the supernatant. Cells were then washed with 2 mL sterile water and pelleted again at 800 ×g for 5 min to remove any remaining SD-Ura$^-$ medium.

Following the removal of the SD-Ura$^-$ medium, the cell pellet was resuspended in 3 mL of YPD-galactose (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) galactose, and pH 6.5) medium to induce expression. The culture was incubated with shaking at 30 ℃ for 4 h. Subsequently, 1 mL of culture was transferred to a 1.5 mL microfuge tube and centrifuged at 15,000 ×g for 15 s. The supernatant was discarded, and the yeast pellet was resuspended in 50 µL of ice-cold extraction buffer (Yeastbuster Protein Extraction Reagent (Novagen) with 1 × tris(hydroxypropyl)phosphine (THP) solution and 2 × protease inhibitor cocktail (Roche)). Two 0.5 mm glass beads were added to the mixture, which was then incubated at 25 ℃ with gentle shaking for 20 min. After centrifugation at 16,000 × g at 4 ℃ for 20 min, the supernatant was transferred to a fresh tube and stored at -80 ℃.

2.6 Protein Expression in *E. coli*

For bacterial transformation, 50 ng of the plasmid containing the target gene were added into 50 µL *E. coli* BL21 (DE3) competent cells and the mixture was incubated on ice for half an hour. They were then heat-shocked at 42 ℃ for 2 min and placed on ice before 500 µL

antibiotic-free SOC medium was added into the mixture. The mixture was incubated in a shaker at 37℃ for 1 h. Following centrifugation at 4,000 × g for 30 seconds, 450 µL of the supernatant was removed. The remaining cells were resuspended by a pulse vortex, and plated on LB agar plates supplemented with 50 µg/mL kanamycin. The plates were placed in a 37℃ incubator overnight for the colonies to grow.

A single colony was picked up and grown at 37℃ for 12-16 h, and 100 µL of cell suspension was sub-cultured into 4 mL LB media (20 g/L LB broth and 50 µg/mL kanamycin). When the OD_{600} reached about 0.6, IPTG was added to a final concentration of 1 mM and the culture was incubated at 37℃ with shaking for 4 hours to induce the expression of the target protein. For protein extraction, 1 mL of cell suspension was transferred to a 1.5 mL microfuge tube and cells were pelleted by centrifugation at 15,000×g for 1 minute. The harvested cells were re-suspended in 50 µL Sigma CelLytic Reagent supplemented with 2× protease inhibitor cocktail (Roche), and incubated at room temperature for 3 min. The cell lysate was then centrifuged at 18,000×g for 5 min, and the supernatant was transferred to a fresh microfuge tube and stored at -80℃.

2.7 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Protein concentration was measured using the Bradford method (Bradford, 1976). The protein extracts were then diluted with sterile water and sample buffer (6× buffer consisted of 0.25 M Tris-HCl pH 6.5, 15% (w/v) sodium dodecyl sulfate (SDS), 50% (v/v) glycerol, 25% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue) to the concentration of 1 µg/µL, and denatured at 95℃ for 10 min. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Normally, in this study a 12% separation gel was used. The 12% gel was composed of 375 mM Tris-HCl (pH 8.8), 12% (v/v) acrylamide/bis-acrylamide (29:1), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate (APS) and 0.1% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED), and the 5% stacking gel consisted of 125 mM Tris-HCl (pH 6.8), 5% (v/v) acrylamide/bis-acrylamide (29:1), 0.1% (w/v)

SDS, 0.1% (w/v) APS and 0.1% (v/v) TEMED. Preheated samples and protein marker were loaded when gel polymerization was completed. The gel was run at 120 V for 90 min in 1×running buffer (0.1% (w/v) SDS, 25 mM Tris, 192 mM glycine). After completion of electrophoresis, the gel was removed, stained with a solution containing 25% (v/v) isopropanol, 10% (v/v) acetic acid and 0.05% (w/v) Coomassie brilliant blue R-250 for 30 min, and destained with a solution containing 10% (v/v) acetic acid for 90 min. After destaining, the gel was scanned.

2.8 Western Blotting Analysis

Following SDS-PAGE, the gel was washed with double distiller H_2O and then transfer buffer (1.0 L transfer buffer consisted of 14.41 g glycine, 3.03 g Tris Base and 200 mL methanol). A stack was assemble in the order of (cathode to anode) 2 sheets of filter paper (pre-soaked with transfer buffer), polyvinylidene fluoride (PVDF) membrane (pre-soaked in methanol for 5 min and washed with transfer buffer), gel, another 2 sheets of filter paper (pre-soaked with transfer buffer). Air bubbles were removed. For transferring proteins to the membrane, the prepared transfer stack was placed in a Mini Trans-blot electrophoretic transfer apparatus (Bio-Rad) with transfer buffer, and then a constant voltage of 100 V was applied for 60 minutes at 4 ℃.

The membrane was then removed from the apparatus, washed with PBST (0.1% (v/v) Tween-20 in phosphate buffered saline (PBS)), and then incubated in the blocking solution (5% (w/v) skim milk in PBST) at room temperature for 1 h. It was incubated with the primary antibody in blocking solution at 4 ℃ for 16 h, washed with PBST three times for 8 min each time, and then incubated with the secondary antibody conjugated with the enzyme horseradish peroxidase in blocking solution for 1 h, followed by washes with PBST three times (10 min each time) and PBS once (10 min). A monoclonal antibody against GFP was purchased from Santa Cruz Biotechnology. ECL Western blotting detection reagent mixture (GE Healthcare) was applied to membrane according to the manufacturer's instructions. After 2 min

incubation with ECL solution, the membrane was placed on X-ray film, which was then developed using X-ray film developer.

2.9 RNA Isolation from Arabidopsis Plants and Analysis by Reverse Transcriptase-Mediated PCR (RT-PCR)

Arabidopsis T_1 seeds were grown as described in section 2.3. Each RNA sample was prepared from a group of independent Arabidopsis transformants (30 seedlings) in a plate and thus represented the average level of these transformants. Total RNAs from Arabidopsis seedlings were extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The final RNA was dissolved in diethylpyrocarbonate (DEPC) treated water and stored at -80 ℃.

For the reverse transcriptase-mediated PCR (RT-PCR) analysis, 1 μg of total RNAs was used as the template for each cDNA synthesis reaction. The RT-PCR was performed using the ThermoScript RT-PCR System (Invitrogen) following manufacturer's instructions. The cDNA was diluted (1/3 dilution) and used as the template for amplifying the target gene by PCR. For each reaction, 1 μL of the diluted cDNA along with gene-specific primer pairs were used. The thermal cycle conditions consisted of 3 minutes initial denaturation at 95 ℃, followed by 28 cycles (30 seconds at 95 ℃, 30 seconds at 55 ℃, and 1 min at 72 ℃), and 5 minutes final extension at 72 ℃. Arabidopsis *At4g33380* was amplified in the same PCR reaction as the internal control, since it is one of the most uniformly expressed genes in Arabidopsis (Czechowski *et al.*, 2005).

3. EXPERIMENTAL RESULTS

3.1 The Role of the N-terminal 40 Amino Acid Region of ICK1 in Regulating Protein Level

Previous work has shown that the residues 21-40 of ICK1 are sufficient to dramatically decrease the level of reporter GFP protein and the effect is through posttranscriptional regulation (Li *et al*., manuscript submitted). Further, unpublished data from our lab indicated that the N-terminal 20 amino acid region of ICK1 was also able to decrease the level of reporter GFP when expressed in yeast. To confirm the observation in plants, as well as to map the sequence within the 1-40 region, transgenic Arabidopsis plants constitutively expressing GFP-tagged *ICK1*$^{1-10}$, *ICK1*$^{1-20}$, *ICK1*$^{1-30}$, *or ICK1*$^{1-40}$ under the control of the CaMV 35S promoter were generated.

Arabidopsis T_1 transformants were grown, and proteins were extracted from a pool of 30 seedlings and quantified. Twenty micrograms of proteins from each sample were loaded into each lane of an SDS-PAGE gel and subjected to electrophoresis. Proteins were transferred to a PVDF membrane and detected with an antibody against GFP.

Each protein sample was prepared from a group of independent transformants and thus represents the average level of protein for these independent transformants. As shown in Figure 3.1, low levels of proteins were detected for GFP- ICK1$^{1-40}$ and GFP- ICK1$^{1-30}$, suggesting that the ICK1 fragments when fused with GFP decreased the GFP protein level. Although a protein band was observed for GFP-ICK1$^{1-20}$, the level was clearly lower than those of GFP and GFP- ICK1$^{1-10}$, suggesting that the 1-20 region could also decrease the level of GFP, consistent with the previous observation when the fusion protein was expressed in yeast (unpublished data from Dr. Wang's lab). Since the above results were obtained with ICK1 fragments fused to the C-terminus of GFP, a set of constructs with the ICK1 fragments fused to the N-terminus of GFP was also tested. Similar results were obtained (Figure 3.1), suggesting that these fragments exerted the same effects regardless whether they are fused to either C-terminus or N-terminus of GFP.

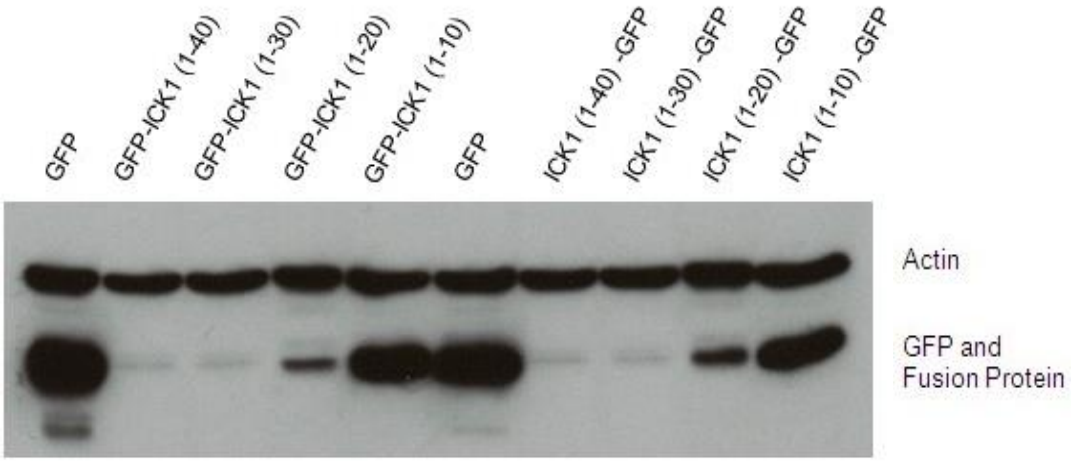


Figure 3.1 The protein levels in 17-day old transgenic plants. Arabidopsis T_1 (first generation and independent) transformants of GFP, GFP-tagged ICK1$^{1-10}$ (GFP-ICK1$^{1-10}$ or ICK1$^{1-10}$-GFP), ICK1$^{1-20}$ (GFP-ICK1$^{1-20}$ or ICK1$^{1-20}$-GFP), ICK1$^{1-30}$ (GFP-ICK1$^{1-30}$ or ICK1$^{1-30}$-GFP), or ICK1$^{1-40}$ (GFP-ICK1$^{1-40}$ or ICK1$^{1-40}$-GFP) were selected on medium plates containing kanamycin and timentin. Thirty transformants from the initial screening were transferred to a fresh plate. After one week on the new plate, seedlings were used to determine the levels of GFP and GFP-tagged proteins. For each sample, all seedlings in one plate were pooled for protein extraction. Twenty micrograms of protein samples for each construct were used in SDS-PAGE gel electrophoresis. GFP and GFP fusion proteins were detected by Western analysis using an anti-GFP antibody, and actin was detected as a loading control.

To further reveal the sequence within the first 40 amino-acid region of ICK1 that can dramatically lower the protein expression, transgenic Arabidopsis plants constitutively expressing GFP fused to a 10 amino-acid ICK1 fragment *(ICK1$^{1-10}$, ICK1$^{11-20}$, ICK1$^{21-30}$, or ICK1$^{31-40}$)* under the control of the CaMV 35S promoter were generated. As shown in Figure 3.2A, under the conditions used, little protein was detected for GFP- ICK1$^{21-30}$, suggesting that this fragment when fused with GFP can dramatically decrease the level of GFP. Although the 1-20 region can also reduce the level of GFP (Figure 3.1), neither the 1-10 region nor the 11-20 region had this ability.

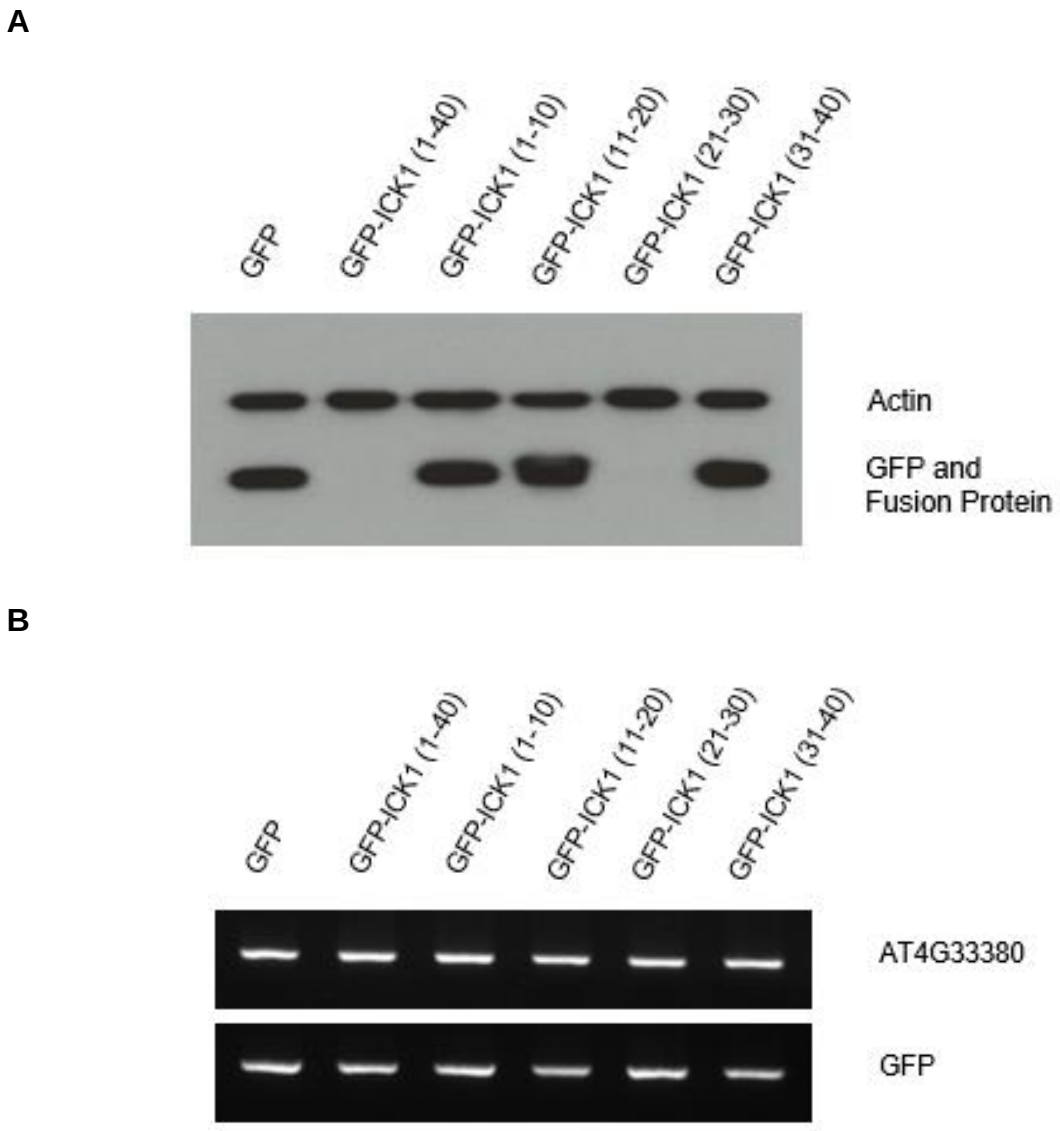


Figure 3.2 The protein levels and gene expression in 17-day old transgenic plants. Arabidopsis T_1 (first generation and independent) transformants of GFP, GFP-tagged $ICK1^{1-10}$ (GFP- $ICK1^{1-10}$), $ICK1^{11-20}$ (GFP-$ICK1^{11-20}$), $ICK1^{21-30}$ (GFP- $ICK1^{21-30}$), $ICK1^{31-40}$ (GFP-$ICK1^{31-40}$), or $ICK1^{1-40}$ (GFP- $ICK1^{1-40}$) were selected on medium plates containing kanamycin and timentin. Thirty transformants from the initial screening were transferred to a fresh plate. After one week on the new plate, seedlings were used to determine the protein and mRNA levels. For each sample, all seedlings in one plate were pooled for protein or RNA extraction. (A) Western detection of GFP and GFP-tagged proteins. Twenty micrograms of protein from each sample were used in SDS-PAGE gel electrophoresis and Western analysis with an anti-GFP antibody. Actin was detected as a loading control. (B) Detection of transcript levels by RT-PCR. cDNA was synthesized from the total RNA and was used in PCR for detecting transcripts of *GFP* and *At4g33380* (control).

It remains possible that the lower level of GFP-ICK1$^{21-30}$ compared to GFP alone is due to a difference in transcript level. Therefore, the transcript levels were analyzed by reverse transcriptase-mediated PCR (RT-PCR) using total RNA samples, which were isolated from transgenic seedlings grown in the same experiment of the Western analysis. The mRNA levels of GFP were similar in these transgenic lines (Figure 3.2B). Thus, these results indicate that the dramatic differences at the protein level were not due to differences at the transcript level.

3.2 Role of the 41-108 Region of ICK1 in Regulating Protein Level

Unpublished data from our lab suggest that the sequence responsible for ICK1 instability resides in the N-terminal 1-40 region (Li *et al*., manuscript submitted), but the role of the 41-108 region is not clear. To compare the influence of different regions in 1-108 residues of ICK1, Arabidopsis transformants were obtained using a set of constructs: GFP- ICK1 (full length), GFP-ICK1$^{1-40}$, GFP-ICK1$^{41-77}$, GFP-ICK1$^{78-108}$ and control GFP. Protein samples were extracted from these transgenic seedlings and analyzed by Western blotting as described above.

As shown in Figure 3.3, GFP-ICK1$^{41-77}$ and GFP-ICK1$^{78-108}$ were highly expressed in the transgenic seedlings. The level of GFP-ICK1$^{41-77}$ protein was similar to that of control GFP, while the level of GFP-ICK1$^{78-108}$ protein was lower than the control GFP.

These results indicate that the regions of 41-77 and 78-108 residues do not dramatically reduce the level of GFP and also exclude the possibility that the effect of N-terminal 1-40 region is due to a non-specific effect of the fusion with GFP. It is noted that the level of GFP-ICK1$^{78-108}$ was lower than that of GFP control, suggesting that 78-108 residues can moderately decrease the level of reporter GFP. However, the effect is much less than that of 1-40 residues indicating that this region plays a major role in regulating ICK1 protein level.

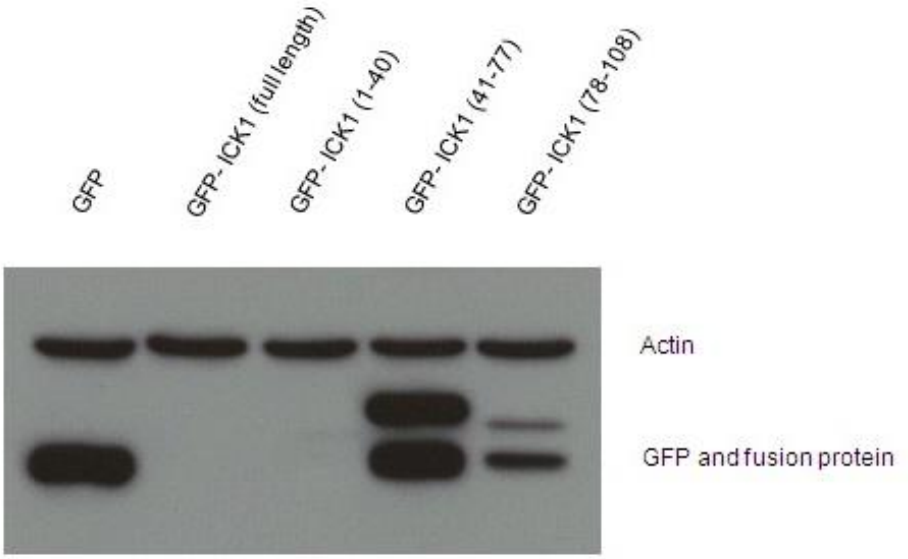


Figure 3.3 The protein levels in 17-day old transgenic plants. Arabidopsis T_1 (first generation and independent) transformants of GFP, GFP-tagged ICK1 (GFP-ICK1), $ICK1^{1-40}$ (GFP-$ICK1^{1-40}$), $ICK1^{41-77}$ (GFP- $ICK1^{41-77}$), or $ICK1^{78-108}$ (GFP- $ICK1^{78-108}$) were selected on medium plates containing kanamycin and timentin. Thirty transformants from the initial screening were transferred to a fresh plate. After one week on the new plate, seedlings were used to determine the levels of GFP and GFP-tagged proteins. For each sample, all seedlings in one plate were pooled for protein extraction. Twenty micrograms of each protein sample were used in SDS-PAGE gel electrophoresis and Western analysis with an anti-GFP antibody. Actin was detected as a loading control.

3.3 Role of N-terminal Regions of Other ICKs in Regulating Protein Level

It is not known whether the N-terminal regions of other ICKs have a similar role in negatively regulating the protein levels of ICKs as in the case of ICK1. If so, since N-terminal sequences are diverse among ICKs, we can hypothesize that different sequence signals may be involved in protein degradation, analogous to the diversity of sequences for the nuclear localization of different ICKs (Bird *et al*., 2007).

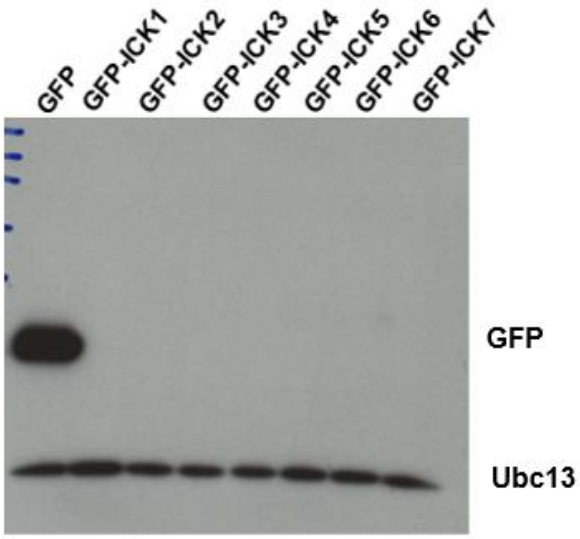
Thus, we analyzed the expression of GFP-ICK fusion proteins in Arabidopsis. Arabidopsis transformants for each of the seven GFP tagged Arabidopsis ICKs (ICK1, ICK2, ICK3, ICK4, ICK5, ICK6, and ICK7) under the control of the CaMV 35S promoter were generated previously in our lab and the expression of GFP-ICK fusion proteins was determined

with Western blotting. Each protein sample was prepared from a pool of 25 or more non-selected independent transformants, to represent an average expression level for the particular construct. As shown in Figure 3.4, while a strong GFP protein band was detected for plants transformed with the GFP construct, no GFP-ICK band was detected for any of the seven GFP-ICK constructs, implying that fusion of any of the ICKs dramatically reduced the GFP protein level.

Since no GFP-ICK fusion proteins could be detected using pools of independent transformants, to determine the presence of the GFP fusion proteins, we used selected lines that showed strong ICK overexpression phenotypes. Protein samples were extracted from these transgenic lines and analyzed by Western blotting as described. As shown in Figure 3.5A, GFP-ICK proteins were detected for almost all the selected lines with strong phenotypes, but at a low level compared with the control.

We next investigated whether N-terminal regions of other ICKs regulated protein levels. Accordingly, the N-terminal half of six other ICKs (ICK2 to ICK7) were fused to GFP. These ICK-GFP fusion constructs were used to transform Arabidopsis plants. Protein samples were extracted from a pool of at least 30 transformants for each construct and analyzed. The levels of these ICK-GFP proteins were very low compared with the GFP control (Figure 3.5B). This observation indicates that the N-terminal regions of other ICKs can also decrease the protein levels.

A



B

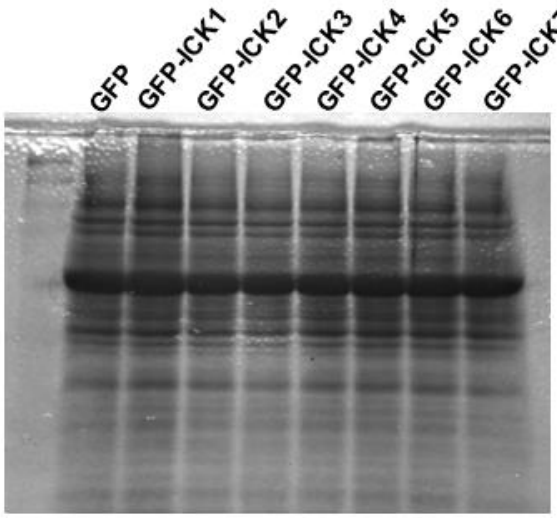
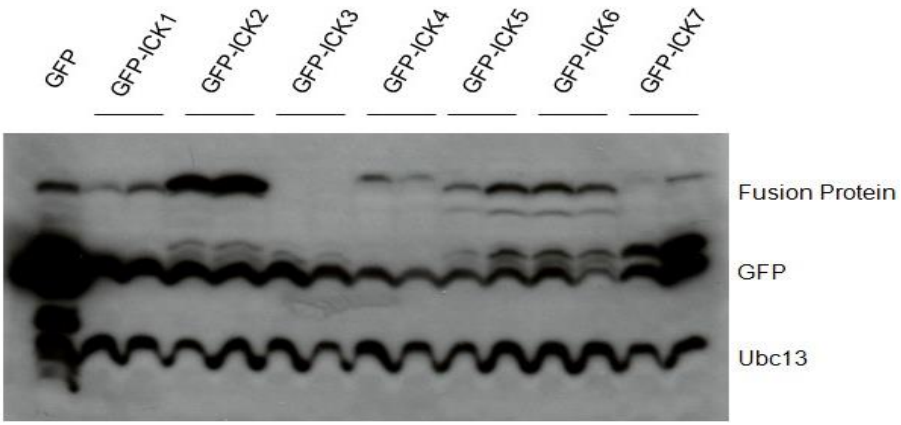


Figure 3.4. Survey of the expression of GFP-ICK proteins. (A) Each protein sample was prepared from a pool of 25 or more non-selected independent transformants, to represent an average expression level for the particular construct. The protein samples were used for electrophoresis and Western blotting analysis, using an anti-GFP antibody. Transformants carrying the GFP construct alone were used as the positive control and the UBC13 protein that showed stable level expression was used as a loading reference. (B) After protein transfer, the same gel was stained with Comassie Blue to show the consistency in the amount of proteins loaded.

A



B

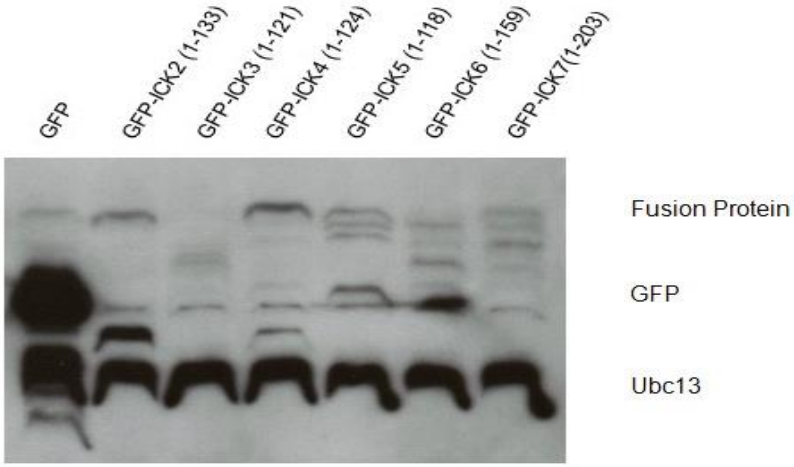


Figure 3.5 The protein levels in transgenic plants. (A) Expression of GFP and GFP tagged Arabidopsis ICKs (GFP-ICK1, GFP-ICK2, GFP-ICK3, GFP-ICK4, GFP-ICK5, GFP-ICK6, and GFP-ICK7). Arabidopsis transformants with strong ICK overexpression phenotypes were selected. Each protein sample was prepared from 25 or more seedlings to represent the average expression for a construct, and 25 μg proteins from each sample were used in SDS-PAGE gel electrophoresis. GFP and GFP fusion proteins were detected by Western blotting using a GFP antibody, and UBC13 was detected as a loading control. For each GFP-ICK construct, two independent protein samples were used. (B) GFP fusion protein levels in 17-day old transgenic plants. Arabidopsis T_1 (first generation and independent) transformants of GFP and GFP-tagged N-terminal half of ICKs (GFP-ICK2$^{1-133}$, GFP-ICK3$^{1-121}$, GFP- ICK4$^{1-124}$, GFP- ICK5$^{1-118}$, GFP- ICK6$^{1-159}$, GFP- ICK7$^{1-203}$) were selected on medium plates containing kanamycin and timentin. Thirty transformants from the initial screening were transferred to a fresh plate. After one week on the new plate, all seedlings in one plate were pooled for protein extraction. Twenty micrograms of protein samples for each construct were used in SDS-PAGE gel electrophoresis. GFP and GFP fusion proteins were detected by Western analysis using an anti-GFP antibody and UBC13 was detected as a loading control.

3.4 Mapping Specific Protein-Destabilizing Sequences in Plants

We have observed that the N-terminal regions of other ICKs (ICK2 to ICK7) have a similar property in dramatically reducing the level of GFP fusion proteins. Thus, further experimental work was performed to identify the protein destabilizing sequences in these Arabidopsis ICKs.

Based on sequence similarity and gene exon-intron organization, plant ICK genes and proteins have been classified into three subgroups (Torres Acosta *et al.*, 2011). For the seven Arabidopsis ICKs, ICK1 and ICK2 belong to group 1; ICK3, ICK6 and ICK7 to group 2 with ICK3 less related to the other two; and ICK4 and ICK5 to group 3. Since ICK1 of group 1 has been relatively well studied, we selected ICK4 and ICK6 to represent the other two groups in this study.

Since a short motif consisted of 10 amino acids in ICK1 has been found to be sufficient in dramatically reducing the level of GFP fusion protein, we would like to identify the potential short sequence motifs in other ICKs that can decrease protein levels. For that purpose, a series of constructs consisting of a 10-amino-acid fragment of ICK4 or ICK6 fused to the N-terminus of GFP were prepared. For instance, the ICK4 constructs are (the number in superscripts indicates the positions of amino acid residues): ICK4$^{1\text{-}10}$-GFP, ICK4$^{11\text{-}20}$-GFP, ICK4$^{21\text{-}30}$-GFP, ICK4$^{31\text{-}40}$-GFP, ICK4$^{41\text{-}50}$-GFP, ICK4$^{51\text{-}60}$-GFP, ICK4$^{61\text{-}70}$-GFP, ICK4$^{71\text{-}80}$-GFP; ICK4$^{6\text{-}15}$-GFP, ICK4$^{16\text{-}25}$-GFP, ICK4$^{26\text{-}35}$-GFP, ICK4$^{36\text{-}45}$-GFP, ICK4$^{46\text{-}55}$-GFP, ICK4$^{56\text{-}65}$-GFP, ICK4$^{66\text{-}75}$-GFP (Figure 3.6A). These constructs were used to transform Arabidopsis plants. Protein samples were prepared from pools of thirty independent transfromants from each construct and analyzed by Western blotting for ICK fragment-tagged GFP expression.

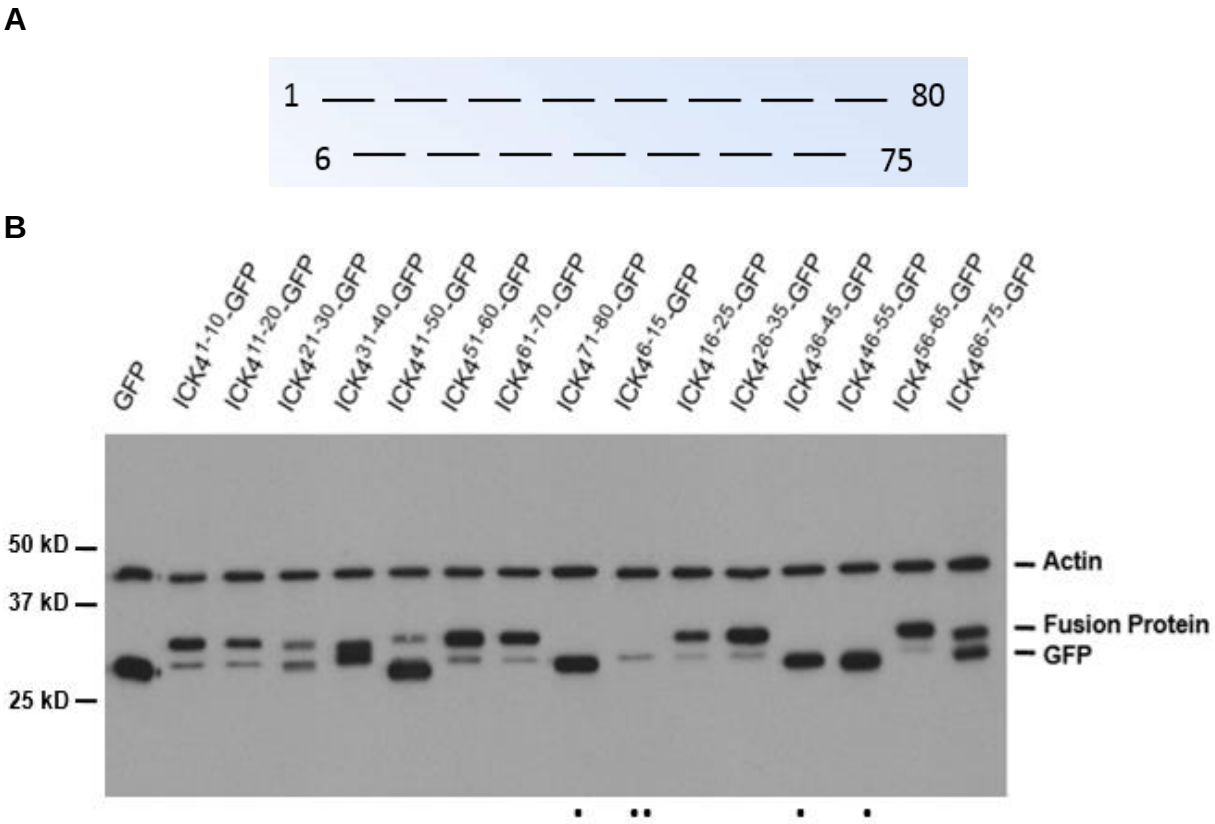


Figure 3.6 Mapping specific protein-destabilizing sequences in ICK4. (A) Schematic presentation of the 10-amino-acid ICK4 fragments used in the analysis. Each was fused with GFP and the construct introduced into Arabidopsis. (B) Immunodetection of GFP and ICK fragment-tagged GFP in 17-day old transgenic Arabidopsis plants. Arabidopsis T_1 (first generation and independent) transformants were selected on medium plates containing kanamycin and timentin. Thirty transformants from the initial screening were transferred to a fresh plate. After one week on the new plate, all seedlings in one plate were pooled for protein extraction. Twenty micrograms of each sample were used in SDS-PAGE gel electrophoresis. GFP and ICK-GFP fusion proteins were analyzed by Western blotting using an anti-GFP antibody. Actin was detected as a loading control. Samples marked by "." had little GFP fusion protein, but showed a prominent GFP band. Samples marked by ".." had little of either GFP or GFP fusion protein.

The results are shown in Figure 3.6 and Figure 3.7. If the ICK short fragment does not have much effect on the protein expression, the ICK-GFP fusion protein would show a similar level to the control GFP. In general, four different expression patterns could be identified involving the relative amounts of ICK-GFP fusion protein and GFP protein alone: type I – a prominent ICK-GFP band with relatively weak GFP band; type II – similar abundance of ICK-GFP and GFP bands; type III – a prominent GFP band with a relatively weak ICK-GFP

band, and type IV – no ICK-GFP band and only a weak GFP band. About half or more of the ICK4 (Figure 3.6) and ICK6 (Figure 3.7) constructs showed the type-I pattern with a prominent ICK-GFP fusion protein band, indicating that these fusion proteins were fairly stable. Some constructs (marked by "." at the bottom of the lane) showed the type-III with a prominent GFP band, but little or no ICK-GFP fusion protein, indicating that the short ICK fragment was not stable and removed from the fusion protein resulting in mostly only GFP protein. For two constructs, ICK4$^{6\text{-}15}$-GFP and ICK6$^{66\text{-}75}$-GFP, no fusion protein and only little GFP protein was detected. Short motifs such as the two are the primary interest to us. One possibility for the observed reduction in GFP fusion or GFP protein is that the short ICK motif reduces the level of GFP through protein degradation. The different patterns from type-I to type-IV reflect increased reduction of the ICK-GFP protein. In the type-I pattern, the ICK fragment had little impact on GFP expression level; in the type-III pattern, the ICK fragment was mostly degraded but it had little effect on the GFP expression level; and in type-IV, the ICK fragment likely caused the degradation of the whole ICK-GFP fusion protein and as a result little GFP was observed.

Sequencing of the constructs indicated that the ICK4$^{6\text{-}15}$ fragment in ICK4$^{6\text{-}15}$-GFP construct had one point mutation (E6A) due to primer synthesis. The ICK6$^{66\text{-}75}$-GFP sequence was correct. Interestingly, ICK6$^{66\text{-}75}$ contains part of a conserved motif (motif 7 in Figure 3.8) in ICKs. In ICK1, motif 7 is the sequence spanning amino acid residues 20-33 and ICK1$^{21\text{-}30}$ has been found to be able to greatly reduce GFP expression level (Figure 3.2). Thus, the ability of ICK6$^{66\text{-}75}$ to reduce the GFP level is likely due to this conserved motif as well. The approach of short fragment mapping as described above allowed us to confirm one known and one possible unknown sequence motif able to decrease the GFP protein level.

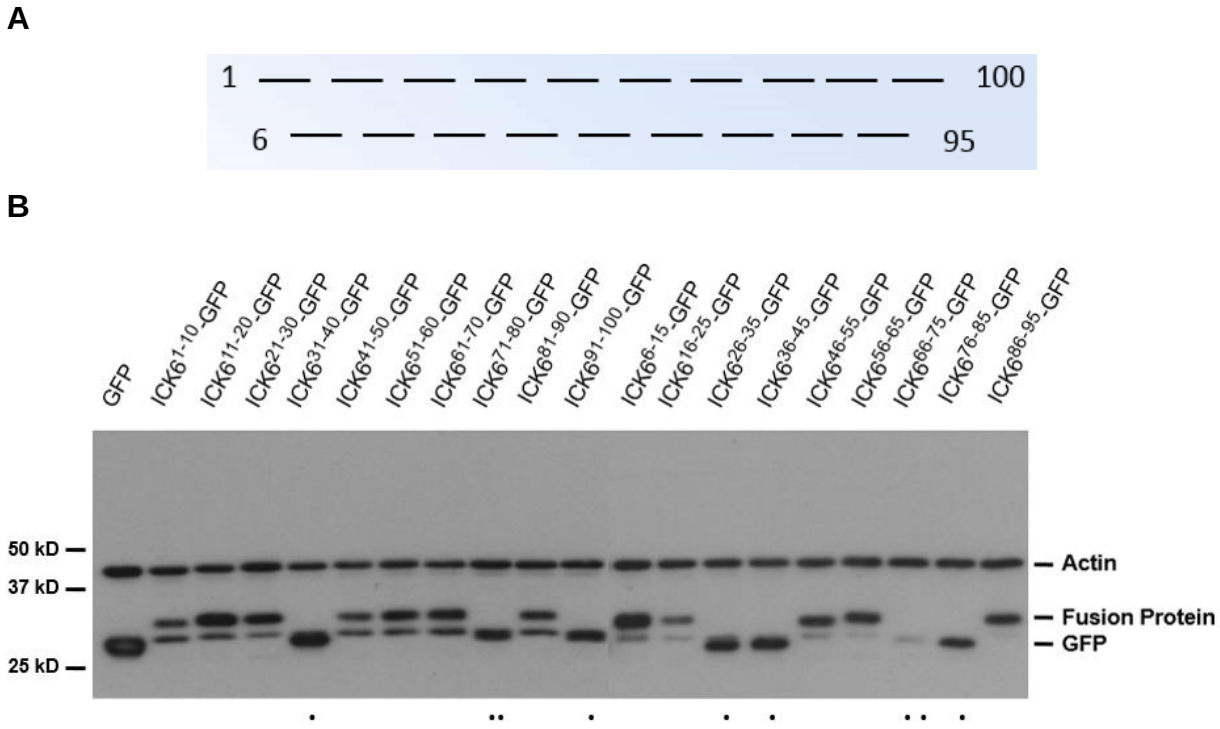


Figure 3.7 Mapping specific protein-destabilizing sequences in ICK6. (A) Schematic presentation of the 10-amino-acid ICK6 fragments used in the analysis. Each was fused with GFP and the construct introduced into Arabidopsis. (B) Immunodetection of GFP and GFP fusion proteins in 17-day old transgenic Arabidopsis plants. Arabidopsis T_1 (first generation and independent) transformants were selected on medium plates containing kanamycin and timentin. Thirty transformants from the initial screening were transferred to a fresh plate. After one week on the new plate, all seedlings in one plate were pooled for protein extraction. Twenty micrograms from each sample were used in SDS-PAGE gel electrophoresis. GFP and GFP tagged ICK6 fusion proteins were analyzed by Western blotting using an anti-GFP antibody. Actin was detected as a loading control. Samples marked by "." had little GFP fusion protein, but showed a prominent GFP band. Samples marked by ".." had little of either GFP or GFP fusion protein.

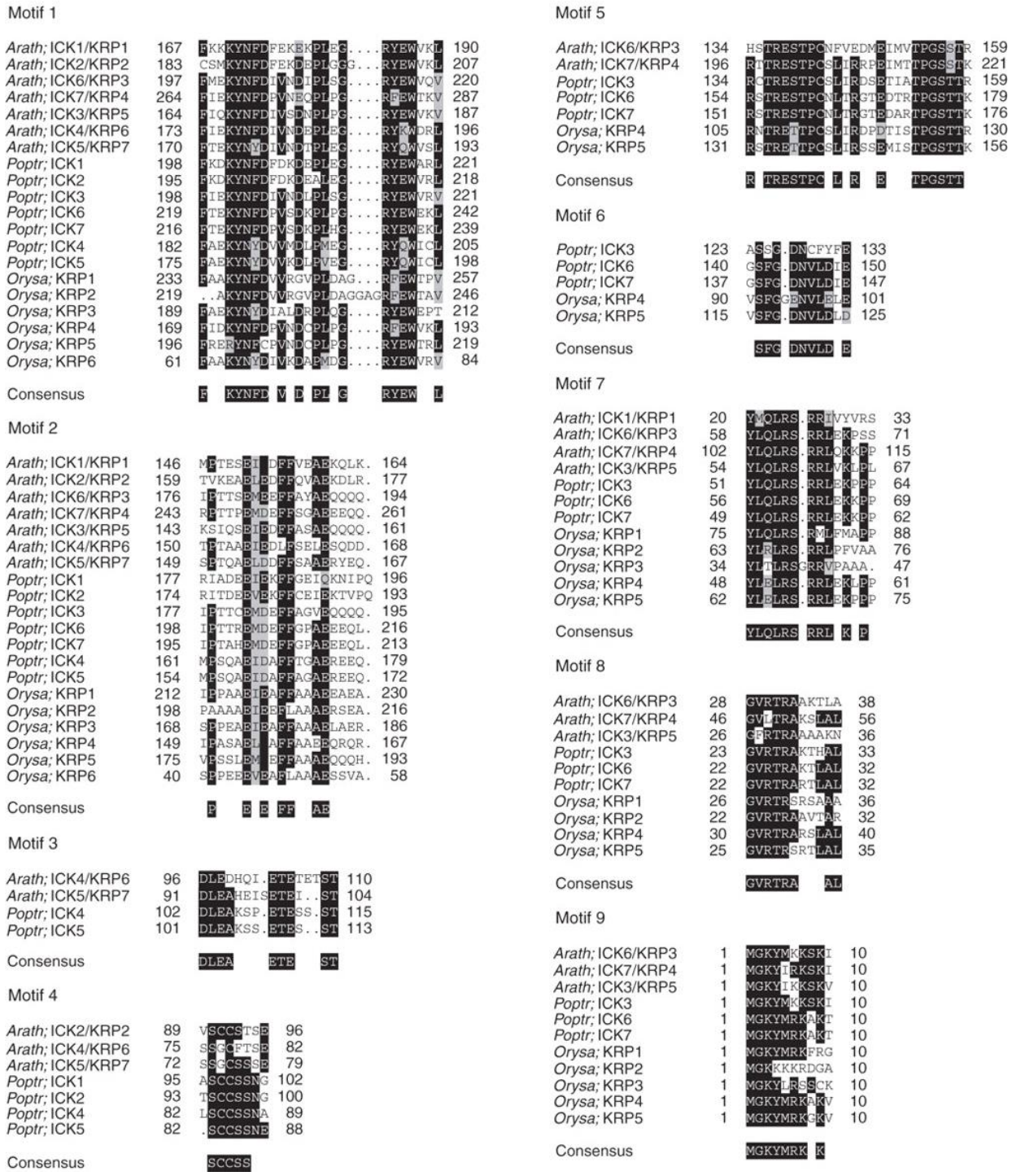


Figure 3.8 Conserved motifs in ICK proteins of Arabidopsis, rice and poplar. Amino acid residues with at least 40% identity are shaded. This figure is adapted from (Torres Acosta et al., 2011) with permission.

Considering that there was little expression of ICK-GFP proteins when the much longer

N-terminal ICK regions were used (Figure 3.5B), we reasoned that the 10-amino-acid fragment mapping might not be able to identify longer sequences that are needed to reduce the level of protein expression. This suggestion is supported by the observation that the N-terminal 1-20 region of ICK1 reduces GFP protein level while neither 1-10 residues nor 11-20 residues has this ability (Figure 3.1 and 3.2). Therefore, the N-terminal regions of selected ICKs from group 1 (ICK2) group 2 (ICK3 and ICK7) and group 3 (ICK4) were divided into multiple fragments (about 20 amino acids) based on our previous analysis of ICK1 and fused to GFP, resulting in a set of constructs, as shown in Figure 3.9. Arabidopsis transformants were obtained and protein samples were extracted from pools of at least 30 transformants for each construct and analyzed.

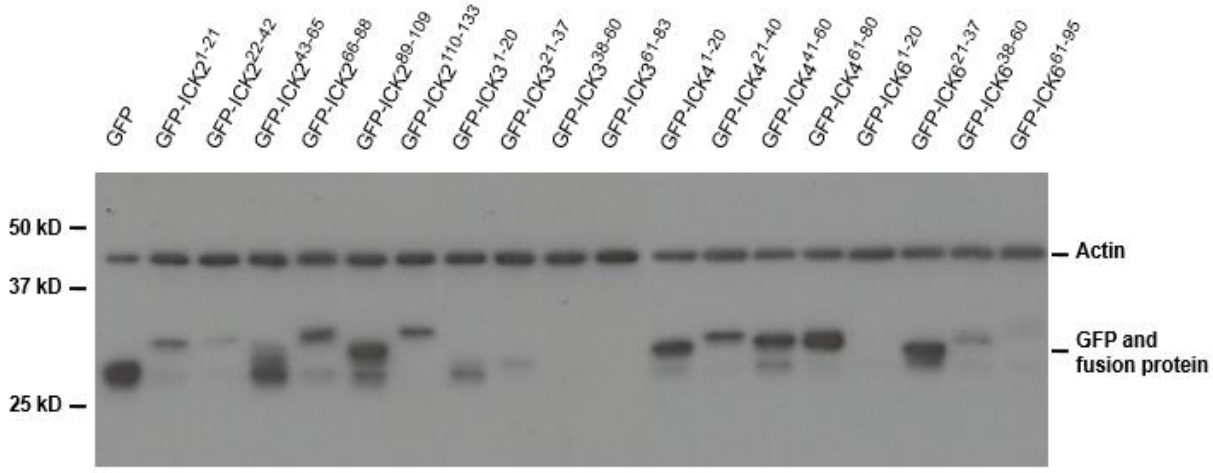


Figure 3.9 Immunodetection of proteins in 17-day old transgenic Arabidopsis plants. Arabidopsis T_1 (first generation and independent) transformants were obtained to express GFP or GFP-ICK fusion constructs containing ICK fragments (1-21, 22-42, 43-65, 66-88, 89-109, 110-133 regions for ICK2; 1-20, 21-37, 38-60, 61-83 regions for ICK3; 1-20, 21-40, 41-60, 61-80 regions for ICK4; 1-20, 21-37, 38-60, 61-95 regions for ICK6). Transformants were selected on medium plates containing kanamycin and timentin. Thirty transformants from the initial screening were transferred to a fresh plate. After one week on the new plate, all seedlings in one plate were pooled for protein extraction. Twenty micrograms from each sample were used in SDS-PAGE gel electrophoresis and Western analysis with an anti-GFP antibody. Actin detected as a loading control.

As shown in Figure 3.9, Western blotting demonstrated that fusion of these fragments lead to various levels of reduction of GFP protein expression. While four out of four GFP-ICK4

constructs had a type-I pattern with a prominent GFP-ICK fusion protein band, four out of four GFP-ICK3 and three out of four GFP-ICK6 constructs had the type-IV pattern with little or no fusion protein detected. In addition, three out of six ICK2 constructs showed a much lower level of GFP fusion protein than the control GFP. These results indicate that the sequences from ICK3 and ICK6 have a stronger ability than ICK4 sequences in reducing the GFP fusion protein level, which is consistent with the result of the fusions with the N-terminal half of the ICK proteins showing that GFP-ICK4$^{1-124}$ level was higher than the levels of GFP-ICK3$^{1-121}$ and GFP-ICK6$^{1-159}$ (Figure 3.5B).

Thus, we have identified short sequences from Arabidopsis ICKs that are capable of dramatically reducing the level of a reporter GFP protein. However, further analyses are needed to identify the specific residues and more importantly to understand the mechanisms by which these sequences reduce protein expression levels.

3.5 Analysis of Specific ICK3 Sequences that Can Reduce Protein Expression in Yeast and *E. coli*

Previous work in our lab (Li *et al*., manuscript submitted) showed that the levels of expression for some ICK1 deletion mutants in yeast are similar to those in Arabidopsis. Particularly, the N-terminal 21-40 region in ICK1 could greatly reduce the reporter GFP protein level in both Arabidopsis and yeast. These results suggest that some sequences may mediate the degradation of ICKs in both yeast and plants. Since all of the ICK3 fragments showed a clear effect in reducing the level of GFP fusion proteins in Arabidopsis (Figure 3.9), the ICK3 sequences were selected for further analysis in yeast. Accordingly, the same ICK3 sequences as shown in Figure 3.9 were cloned as GFP fusions in a yeast vector (modified pYES2 vector).

Yeast (strain, MAV203) cells were transformed using a modified lithium acetate method. Induction of expression and protein extraction were subsequently performed. GFP fusion proteins were detected with Western blotting. As shown in Figure 3.10, the pattern of GFP fusion proteins with the ICK3 fragments is very similar to what was observed in Arabidopsis. There was a clear GFP band for GFP-ICK3$^{1-20}$, but little GFP protein for GFP-ICK3$^{21-37}$, GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$, suggesting that these sequences lower the levels of GPP

fusion proteins in both yeast and plants, likely through protein degradation.

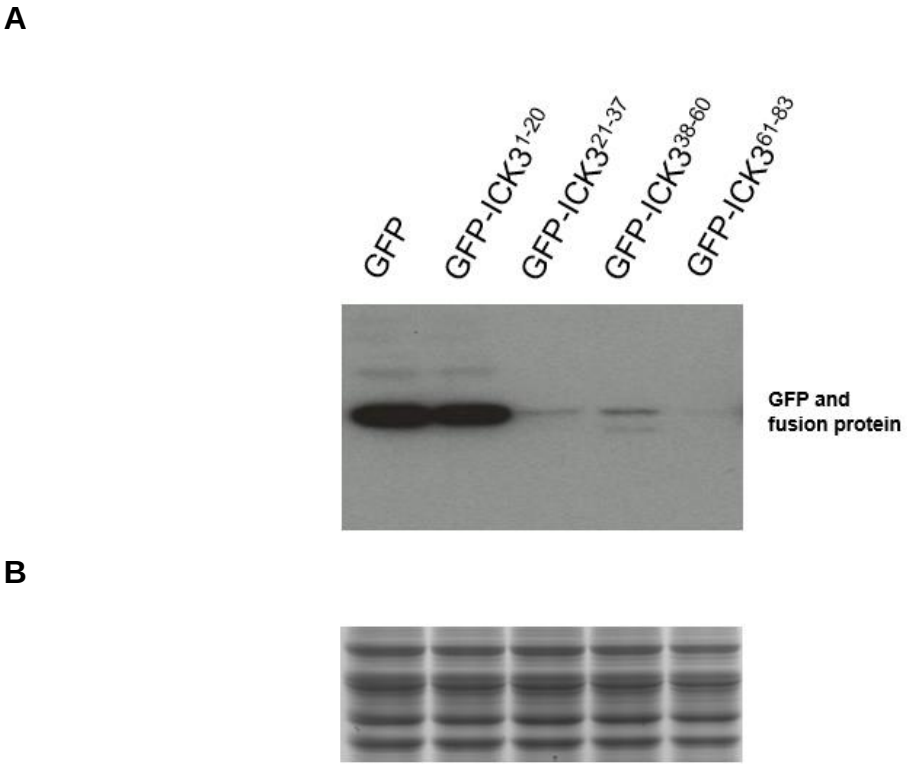


Figure 3.10 Expression of GFP, GFP-ICK3$^{1-20}$, GFP-ICK3$^{21-37}$, GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$ in yeast strain MaV203. Yeast transformants carrying one of the constructs were induced with galactose for 4 h before protein extraction. Extracted protein samples were used in SDS-PAGE and Western blotting, with 15 μg of proteins loaded per lane. The yeast cell lines expressing different constructs are indicated above the lanes. (A) Western blotting of GFP and GFP fusion proteins using an anti-GFP antibody. (B) Non-specific protein bands on the Coomassie brilliant blue stained gel serve as a loading control.

Results so far have shown that the UPS is involved in the degradation of ICK proteins (Gusti *et al.*, 2009; Kim *et al.*, 2008; Liu *et al.*, 2008; Ren *et al.*, 2008). However, results from our lab suggested that ICK1 degradation is likely through both UPS-dependent and UPS-independent pathways (Li Q *et al*., manuscript submitted). Based on this preliminary conclusion, we tested the four short ICK3 sequences to determine whether they could reduce GFP levels in *E. coli*. To this end, *E. coli* (BL21(DE3)) cell lines each with a GFP tagged specific fragment (GFP-ICK3$^{1-20}$, GFP-ICK3$^{21-37}$, GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$) under the

control of the T7 promoter were generated. The GFP-tagged proteins were analyzed by SDS-PAGE gel electrophoresis. Our data showed decreased fusion protein levels in GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$ cells (Figure 3.11), suggesting that ubiquitination is not critical to the ability of these short sequences to reduce the levels of fusion proteins, since *E. coli* does not have a UPS. This observation, consistent with what was observed in ICK1, supports that at least two separate pathways, *i.e.* ubiquitin-dependent and ubiquitin-independent pathways, are involved in ICK degradation.

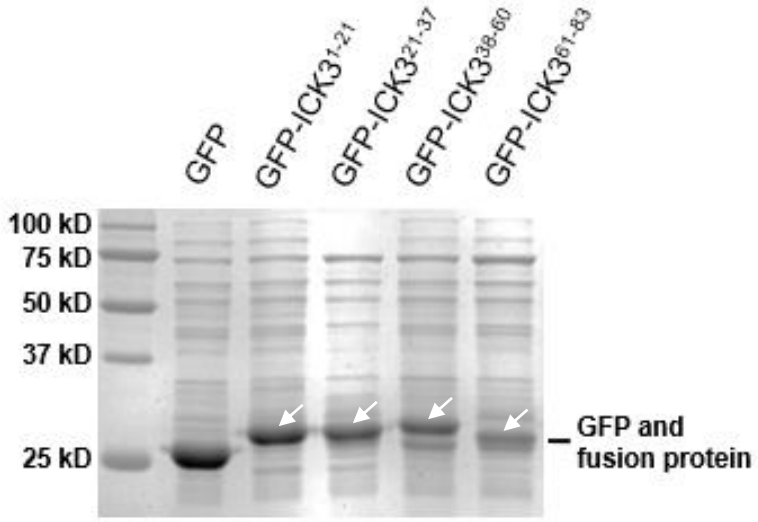


Figure 3.11 Expression of GFP, GFP-ICK3$^{1-20}$, GFP-ICK3$^{21-37}$, GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$ in *E. coli* (BL21(DE3)). Extracted protein samples were subjected to electrophoresis and Coomassie brilliant blue staining. In each lane, 10 μg of proteins were loaded. The *E. coli* cell lines expressing different constructs are indicated above the lanes. The arrows indicate the perspective fusion proteins.

The present results have identified four ICK3 fragments with the potential functional property to lower the level of protein expression. The ICK3 protein sequence is shown below (Figure 3.12). Among the four short ICK3 sequences tested, there was little expression of GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$ in plants, yeast and *E. coli*. A conserved sequence, motif 7

in Figure 3.8, is present between residues 54-67. This sequence in ICK1 has been shown to be critical for reducing GFP fusion protein levels in plants and yeast likely through a non-ubiquitin-mediated mechanism (Li Q et al., manuscript submitted). Thus, it is likely that this sequence is responsible for the much reduced GFP fusion expression of GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$.

```
  1 MGKYIKKSKV AGAVSVKDKS HPPALGFRTR AAAAKNLALH RLRSHSDEAD
 51 SFNYLQLRSR RLVKLPLLTN TRKQQKQQLI PSVNQCQTKN PRASSGPAKK
101 LEPDTTTEEA CGDNERISRS DCNFGDKGFD LESENRSMIS DSKSIQSEIE
151 DFFASAEQQQ QRFFIQKYNF DIVSDNPLPG RYEWVKVMP
```

Figure 3.12 Amino acid sequence of Arabidopsis ICK3. A sequence motif conserved among some plant ICKs (motif 7 in Figure 3.8) and shown to lower protein expression level is bolded.

For the other two sequences, ICK3$^{21-37}$ has a stronger effect of decreasing the GFP level in both plants and yeast. For ICK3$^{1-20}$, in plants there was a GFP band but at a much reduced level than that of control GFP, while in yeast the GFP band for the GFP-ICK3$^{1-20}$ construct was similar to control GFP. We hypothesize that the difference in the effect of ICK3$^{1-20}$ between plants and yeast might be the difference in protein induction and expression. In yeast, the strong induction during a short period of time may allow more protein to accumulate coupled with the slower degradation of GFP, resulting in a prominent GFP band. Nevertheless, results from both plants and yeast indicate that ICK3$^{21-37}$ is able to drastically reduce the level of GFP protein. They thus have likely identified another novel sequence motif with the property of lowering protein level.

4. DISCUSSION

As core plant cell cycle regulators, the ICK family of CDK inhibitors is attracting increasing attention. Detailed understanding of their expression, function and regulation will lead to better understanding of plant cell cycle control and unravel the unique aspects of cell cycle regulation in the context of plant development. Previous studies on ICKs have provided a number of important contributions, including transcriptional regulation, protein-protein interactions, cellular localization and functions in some aspects of plant development. Research has also shown that, like other cell cycle regulators, ICKs are regulated at the post-translational level. However, the mechanisms responsible for the degradation of plant ICKs remain largely unknown.

4.1 ICK Proteins Are Expressed at Low Levels in Plants

As cell cycle regulators, the ICK proteins are expected to be important for plant growth and development, although their functional roles in plants are not fully understood. On the other hand, the ICK proteins inhibit the activity of plant CDKs by direct binding, targeting mainly CDKA-CYCD complexes, but too much ICK expression would inhibit cell proliferation and a range other aspects of plants. This functional property determines that ICK levels is important for their biological roles. Consistent with this notion, the phenotypes from overexpressing an *ICK* gene, including smaller plants and serrated leaves, vary greatly depending on the expression levels (Wang *et al.*, 2000; Zhou *et al.*, 2002a). Studies of the T-DNA knockout *ick* mutants revealed a trend of gradual changes from single to higher order mutants in CDK activity, seedling growth, cotyledon size, leaf size, and seed size suggesting that ICKs function redundantly (Cheng *et al.*, 2013). Furthermore, different levels of *ICK* expression can also have different effects on endoreduplication and ploidy levels of Arabidopsis (Verkest *et al.*, 2005; Weinl *et al.*, 2005). These data clearly indicate that functions of ICKs are dosage-dependent and thus protein levels of plant ICKs must be tightly regulated.

Understandably, the expression of ICKs is controlled at both transcriptional and post-translational levels.

At the transcript level, differential expression of *ICK*s in specific tissues and developmental stages has been observed in different studies (Wang *et al.*, 1998; Lui *et al.*, 2000; De Veylder *et al.*, 2001; Jasinski *et al.*, 2002a; Pettko-Szandtner *et al.*, 2006). Analysis also showed differences in expression for the seven Arabidopsis *ICK* genes (Ormenese *et al.*, 2004; Torres Acosta *et al.*, 2011). In general, the transcript abundance of Arabidopsis *ICKs* is relatively low in the tissues surveyed (Torres Acosta *et al.*, 2011).

The transition from G1 to S phase is considered as a key step in cell cycle regulation. Various mechanisms exist to integrate endogenous information (such as nutrient status and hormonal signals) and exogenous environmental conditions into the control over the G1/S transition. In both fungi and metazoans, the G1/S transition requires the degradation of CKIs to release CDK activity, which in turn allows the phosphorylation of regulatory proteins required to enter S phase. The level of CKIs could contribute to a low threshold which a cell needs to overcome before entering the cell cycle. In contrast to the much better understood G1/S transition in the mammalian cells, our understanding of proteolytic events at the G1/S transition in plants is still limited. The Arabidopsis CDKA;1, which is required for both S-phase entry and mitosis, is negatively regulated by ICKs. Constitutive overexpression in transgenic Arabidopsis plants of all ICKs tested so far can block both M and S phases, leading not only to growth retardation, including a reduction in cell number and organ size, but also to changes in plant morphology, such as leaf serration (Verkest *et al.*, 2005b). Hence, protein levels of plant ICKs need to be tightly regulated at the post-translational level in a timely fashion.

In this study, we confirmed that all ICKs are expressed at a very low level in Arabidopsis. Western blotting result showed that the recombinant GFP-ICK fusion proteins could only be detected at a low level in transgenic overexpression lines that showed strong phenotypes, suggesting that the full-length ICKs are degraded quickly together with the fused GFP in plants.

Currently information is very limited regarding how ICKs are regulated at the protein level. Studies by different laboratories have reported the roles of UPS in the degradation of ICKs, with several E3s or E3 components implicated in the degradation of ICKs in Arabidopsis. However, little is known regarding the specific sequences that negatively regulate ICK protein levels. Our previous studies have shown that removal of the N-terminal 108 residues of ICK1 leads to a much higher level of ICK1$^{109\text{-}191}$ compared with ICK1, suggesting the importance of this region in regulating ICK protein level (Zhou *et al.*, 2003). Furthermore, unpublished data from our lab show that the N-terminal 40 amino acid region was critical for negatively regulating ICK1 level and a similar observation was made in yeast using HA-tagged ICK1 lacking the N-terminal region, suggesting that the mechanism by which the N-terminal region negatively regulates ICK1 protein level is conserved in plants and yeast (Li Q *et al*., manuscript submitted).

4.2 Role of N-terminal Regions in Different ICKs

In this study, the role of N-terminal regions of ICKs in protein regulation is compared. Our Western results have shown that each of the N-terminal regions of ICKs dramatically reduces GFP fusion protein level in plants, suggesting that the N-terminal regions of other ICKs also negatively regulate ICK expression, likely through protein degradation. Since the N-terminal sequences differ among ICKs, different sequence signals may be involved in protein degradation. Also, since the N-terminal sequences of ICKs do not share any similarity with the mammalian CDK inhibitors, the sequence signals involved in the degradation of ICKs may be different from those in the mammalian CDK inhibitors.

Accumulating evidence suggests that different ICKs may function and be regulated differently. It is reasonable to propose that differential controls of ICK degradation may be also a regulatory means to achieve their temporal or spatial specificity. This resembles the situation in other cell cycle regulators, such as cyclins. However, ICKs may also share common mechanisms for regulating protein stability. Despite the overall dissimilarity in the

N-terminal region of ICKs, several short sequence elements shared by different ICK members have been identified (Figure 3.8) (Torres Acosta *et al.*, 2011).

4.3 Use of GFP Fusion to Identify ICK Sequence Motifs Involving in Regulating Protein Levels

To identify the specific sequence motifs in ICKs that function to reduce the protein level, GFP was used as a reporter since its size (27 kDa) is close to the molecular masses of ICKs and it could be conveniently detected with Western blotting using a monoclonal antibody. Constructs with short ICK fragments fused to GFP were prepared and introduced in Arabidopsis plants. Their expression was driven by a strong and constitutive 35S promoter. If the ICK short fragment does not have much effect on the protein expression, the ICK-GFP fusion protein should show a similar level to that of the GFP control. The majority of these constructs showed a prominent ICK-GFP fusion protein band, indicating that these fusion proteins were fairly stable. For others, the fusion with a short ICK sequence resulted in the removal of the ICK sequence (type-III) but not the GFP or almost complete removal of the ICK-GFP fusion protein and the GFP (type-IV). It is puzzling why in some cases the whole fusion protein appeared to be degraded, while in other cases only the short ICK fragment was removed from the fusion protein, resulting in a prominent GFP protein band. One possibility is that the differences may be due to a difference in the ability of the short fragment to degrade a protein. If the ability is weaker, the process is slower. While the short ICK1 fragment is removed, the GFP protein remains. Nevertheless, these short motifs that lead to the degradation of the whole fusion protein are the primary interest to us and the underlying mechanisms will be the subject of further investigation. One possibility is that these short ICK motifs are recognized by a protein degradation system, leading to the degradation of the fusion proteins. Using this approach we are able to identify sequence motifs that can reduce the level of a reporter protein.

As shown previously, the N-terminal 40 amino-acid region of ICK1 can dramatically

reduce the GFP protein level. This region of ICK1 was thus further dissected to identify the specific sequence involved in protein instability control. The analysis of protein and transcript levels on a set of fusion constructs in plants showed that the critical sequences conferring instability of ICK1 are mapped to the regions of 1-20 and 21-30 residues. There is a possibility that a short sequence 30-60 nucleotides (for 10-20 residues) may affect translation efficiency. In such a case, the short sequence fused to the C-terminus of GFP would be much less effective in blocking the translation of GFP. The observation that these fragments exert similar effects regardless whether they are fused to C-terminus or N-terminus of GFP indicates that the reduced protein levels are unlikely be due to a reduction in translation efficiency.

It is worth noting that the region of residues 21 to 30 in ICK1 contains a motif YM/LQLRSRR (residues 20 to 27) that is conserved in ICK1, ICK3, ICK6 and ICK7. The view that this conserved motif may be a critical element responsible for negatively regulating the level of ICKs is further supported by the observation that other sequences containing this conserved motif or part of it ($ICK6^{66-75}$, $ICK3^{38-60}$ and $ICK3^{61-83}$) are also able to reduce the GFP level.

In addition to this conversed motif, other potential sequence motifs that can negatively regulate fusion protein levels were also identified in different ICKs. Since these motifs do not share significantly sequence similarity, we can speculate that the negative regulation of ICK protein levels involves different sequence signals, or even different regulatory mechanisms. More intriguingly, for some ICKs (e.g. ICK3), more than one such motif were found in their N-terminal regions. Although these motifs still need to be assessed in further research, this observation implies that multiple motifs in a single ICK protein may function independently to control the expression level.

4.4 Identification of Sequence Motifs in ICK3

Previous work in our lab showed that some motifs of ICKs might mediate the degradation of ICKs in both yeast and plants (Li Q *et al*., manuscript submitted). Hence, analysis of the

specific sequences in yeast may provide a good method for the further assessment of these motifs. Since all the four ICK3 fragments exerted a clear effect in reducing the level of GFP fusion proteins in Arabidopsis, the ICK3 fragments were selected for this analysis (ICK3$^{1-20}$, ICK3$^{21-37}$, ICK3$^{38-60}$ and ICK3$^{61-83}$). The results showed that GFP-ICK3$^{21-37}$, GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$ accumulated to a much lower level than GFP control, suggesting that the mechanism responsible for degrading these proteins is shared between yeast and plants and also that these motifs likely mediate the degradation of ICK3. In this regard, it is interesting to note that the conserved D-box in cyclins functions similarly in yeast, plants and animals to target the cyclins for degradation (Genschik *et al.*, 2014).

As discussed earlier, the conserved motif (ICK1$^{20-33}$) is present between residues 54-67 in ICK3. Thus, it is likely that this sequence may be responsible for the much reduced GFP fusion expression of GFP-ICK3$^{38-60}$ and GFP-ICK3$^{61-83}$. For the other two sequences, ICK3$^{21-37}$ has a stronger effect in lowering the GFP level in both plants and yeast.

It is interesting to observe that the ICK3$^{21-37}$ sequence is conserved in ICK3, ICK6, and ICK7 (motif 8 in Figure 3.8). Thus, it is very likely that we have identified another conserved motif which plays a critical role in controlling the level of ICKs. Since the N-terminal regions are diverse among ICKs, there are very few conserved motifs among these sequences. However, it is quite possible that these two identified motifs function to regulate the levels of ICKs, likely through protein degradation.

4.5 Possible Mechanisms Involved in Regulating ICK Protein Levels

Results so far have shown that the UPS is involved in the degradation of ICK proteins. However, previous results from our lab suggest that ICK1 degradation is likely through both UPS-dependent and UPS-independent pathways (Li Q *et al*., manuscript submitted). More specifically, the 21-30 region of ICK1 may function to reduce the protein level through a non-ubiquitin-mediated mechanism in both yeast and plants (Li Q *et al*., manuscript submitted).

In this study, the fusion of ICK3$^{38-60}$ and ICK3$^{61-83}$ fragments to GFP decreased the GFP

proteins levels in *E. coli*, which together with previous result in ICK1 suggest that the conserved motif YM/LQLRSRR (motif 7 in Figure 3.8) decreases the protein level through a ubiquitin-independent mechanism.

For the ICK3$^{21-37}$ region, in *E. coli*, the GFP-ICK3$^{21-37}$ level was similar to control GFP, indicating that this motif does not reduce the protein level in *E. coli* as ICK3$^{38-60}$ and ICK3$^{61-83}$. Thus, this region can negatively regulate the level of reporter GFP in Arabidopsis and yeast, but likely through a different mechanism from that used by ICK1$^{21-30}$. It is very likely that this conserved motif has the property of reducing protein expression level. Since this motif has the consensus sequence of "GVRTRAXXXAL", we could speculate that if the motif increases protein degradation through ubiquitinaiton, an atypical residue instead of a lysine residue would be ubiquitinated. The short length of this motif should allow the identification of the critical residues by site-directed mutagenesis.

4.6 Future Work

The present study examines the role of the N-terminal regions of ICKs in regulating their expression levels and identifies some specific potential sequences responsible for dramatically reducing reporter GFP expression in Arabidopsis. Furthermore, some of these sequences are also able to decrease the reporter protein level in yeast. Results also point to a possibility that a ubiquitin-independent mechanism may play a role, adding another layer of complexity to the regulation of ICKs.

In this study, GFP has been used as the reporter to identify the sequence motifs with a strong ability to lower the protein level. To verify that the function of these motifs is not dependent of a particular protein, other reporters need to be tested. They should be soluble and can be expressed at relatively high levels without a major effect on plant growth and development. In addition, good antibodies should be available for detecting the fusion proteins. We are planning to use ACT8 (Actin 8), which is a component of the cytoskeleton. Accordingly, plant expression plasmids, with ACT8 fused to the sequences of interest, will be

prepared and used for transformation of plants. Another possible candidate is GUS (β-glucuronidase) which has been widely used in plants as a reporter. A drawback is that the protein is much larger than ICK proteins.

One interesting line of future work is to compare the two conserved motifs (motif 7 and motif 8 in Figure 3.8), which have been shown to greatly reduce the protein level, in order to understand the molecular mechanisms, since these two sequence motifs seem to function differently. The motifs could be analyzed by site-direct mutagenesis to determine the critical specific amino acid residues. Once confirmed, the sequence could be used as a tool to further probe the mechanism involved. The sequence could also be used as a bait to identify potential interacting proteins through the yeast two-hybrid or another protein-protein interaction approach. If positive results are obtained, they could provide new insights regarding how these sequence motifs negatively regulate the levels of ICK proteins.

Accumulating evidence suggests that ICKs are subjected to very complex and fine regulation at the protein level. It appears that multiple sequences are present in ICKs that control their expression levels. In this regard, it will be interesting to identify additional sequences involved.

So far, most of the studies on protein degradation focus on the ubiquitination-mediated 26S proteasome pathway. From this study and the results by Li *et al.*, it seems that some sequences in ICKs may regulate protein level through a Ub-independent pathway. Unlocking the mechanism behind this observation will help expand our knowledge on the regulation of protein levels. Furthermore, the knowledge gained with ICKs can help us to understand how other plant proteins may be regulated, particularly those that are fast degraded.

5. REFERENCES

Abbas, T., Sivaprasad, U., Terai, K., Amador, V., Pagano, M., and Dutta, A. (2008). PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdt2 ubiquitin ligase complex. Genes Dev. *22*, 2496-2506.

Achard, P., Gusti, A., Cheminant, S., Alioua, M., Dhondt, S., Coppens, F., Beemster, G.T., and Genschik, P. (2009). Gibberellin signaling controls cell proliferation rate in Arabidopsis. Curr. Biol. *19*, 1188-1193.

Adam, Z. (2013). Emerging roles for diverse intramembrane proteases in plant biology. BBA - Biomembranes *1828*, 2933-2936.

Amerik, A.Y., and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. BBA - Mol. Cell Res. *1695*, 189-207.

Amm, I., Sommer, T., and Wolf, D.H. (2014). Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. BBA - Mol. Cell Res. *1843*, 182-196.

Andersen, S.U., Buechel, S., Zhao, Z., Ljung, K., Novak, O., Busch, W., Schuster, C., and Lohmann, J.U. (2008). Requirement of B2-type cyclin-dependent kinases for meristem integrity in Arabidopsis thaliana. Plant Cell *20*, 88-100.

Anzola, J.M., Sieberer, T., Ortbauer, M., Butt, H., Korbei, B., Weinhofer, I., Mullner, A.E., and Luschnig, C. (2010). Putative Arabidopsis transcriptional adaptor protein (PROPORZ1) is required to modulate histone acetylation in response to auxin. Proc. Natl. Acad. Sci. U. S. A. *107*, 10308-10313.

Ariizumi, T., Higuchi, K., Arakaki, S., Sano, T., Asamizu, E., and Ezura, H. (2011). Genetic suppression analysis in novel vacuolar processing enzymes reveals their roles in controlling sugar accumulation in tomato fruits. J. Exp. Bot. *62*, 2773-2786.

Avin-Wittenberg, T., Honig, A., and Galili, G. (2012). Variations on a theme: plant autophagy in comparison to yeast and mammals. Protoplasma *249*, 285-299.

Azevedo, C., Santos-Rosa, M.J., and Shirasu, K. (2001). The U-box protein family in plants. Trends Plant Sci. *6*, 354-358.

Bandara, L.R., and La Thangue, N.B. (1991). Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature *351*, 494-497.

Barroco, R.M., Peres, A., Droual, A.M., De Veylder, L., Nguyen le, S.L., De Wolf, J., Mironov, V., Peerbolte, R., Beemster, G.T., Inze, D., *et al.* (2006). The cyclin-dependent kinase inhibitor Orysa;KRP1 plays an important role in seed development of rice. Plant Physiol. *142*, 1053-1064.

Bassham, D.C. (2007). Plant autophagy--more than a starvation response. Curr. Opin. Plant Biol. *10*, 587-593.

Bassham, D.C. (2009). Function and regulation of macroautophagy in plants. BBA - Mol. Cell Res. *1793*, 1397-1403.

Baumberger, N., and Baulcombe, D.C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. Proc. Natl. Acad. Sci. U. S. A.

102, 11928-11933.
Bemis, S.M., and Torii, K.U. (2007). Autonomy of cell proliferation and developmental programs during Arabidopsis aboveground organ morphogenesis. Dev. Biol. *304*, 367-381.
Bird, D.A., Buruiana, M.M., Zhou, Y., Fowke, L.C., and Wang, H. (2007). Arabidopsis cyclin-dependent kinase inhibitors are nuclear-localized and show different localization patterns within the nucleoplasm. Plant Cell Rep. *26*, 861-872.
Bisbis, B., Delmas, F., Joubes, J., Sicard, A., Hernould, M., Inze, D., Mouras, A., and Chevalier, C. (2006). Cyclin-dependent kinase (CDK) inhibitors regulate the CDK-cyclin complex activities in endoreduplicating cells of developing tomato fruit. J. Biol. Chem. *281*, 7374-7383.
Blomme, J., Inze, D., and Gonzalez, N. (2014). The cell-cycle interactome: a source of growth regulators? J. Exp. Bot. *65*, 2715-2730.
Boniotti, M.B., and Gutierrez, C. (2001). A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in Arabidopsis, a CDKA/cyclin D complex. Plant J. *28*, 341-350.
Boudolf, V., Barroco, R., Engler Jde, A., Verkest, A., Beeckman, T., Naudts, M., Inze, D., and De Veylder, L. (2004). B1-type cyclin-dependent kinases are essential for the formation of stomatal complexes in Arabidopsis thaliana. Plant Cell *16*, 945-955.
Bradford, M.M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.
Cacas, J.L. (2010). Devil inside: does plant programmed cell death involve the endomembrane system? Plant Cell Environ. *33*, 1453-1473.
Capron, A., Okresz, L., and Genschik, P. (2003). First glance at the plant APC/C, a highly conserved ubiquitin-protein ligase. Trends Plant Sci. *8*, 83-89.
Chellappan, S.P., Hiebert, S., Mudryj, M., Horowitz, J.M., and Nevins, J.R. (1991). The E2F transcription factor is a cellular target for the RB protein. Cell *65*, 1053-1061.
Cheng, Y., Cao, L., Wang, S., Li, Y., Shi, X., Liu, H., Li, L., Zhang, Z., Fowke, L.C., Wang, H., *et al.* (2013). Downregulation of multiple CDK inhibitor ICK/KRP genes upregulates the E2F pathway and increases cell proliferation, and organ and seed sizes in Arabidopsis. Plant J. *75*, 642-655.
Chichkova, N.V., Shaw, J., Galiullina, R.A., Drury, G.E., Tuzhikov, A.I., Kim, S.H., Kalkum, M., Hong, T.B., Gorshkova, E.N., Torrance, L., *et al.* (2010). Phytaspase, a relocalisable cell death promoting plant protease with caspase specificity. EMBO J. *29*, 1149-1161.
Chittenden, T., Livingston, D.M., and Kaelin, W.G., Jr. (1991). The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell *65*, 1073-1082.
Chondrogianni, N., Petropoulos, I., Grimm, S., Georgila, K., Catalgol, B., Friguet, B., Grune, T., and Gonos, E.S. (2014). Protein damage, repair and proteolysis. Mol. Aspects Med. *35*, 1-71.
Churchman, M.L., Brown, M.L., Kato, N., Kirik, V., Hulskamp, M., Inze, D., De Veylder, L., Walker, J.D., Zheng, Z., Oppenheimer, D.G., *et al.* (2006). SIAMESE, a plant-specific cell cycle regulator, controls endoreplication onset in Arabidopsis thaliana. Plant Cell *18*, 3145-3157.

Coelho, C.M., Dante, R.A., Sabelli, P.A., Sun, Y., Dilkes, B.P., Gordon-Kamm, W.J., and Larkins, B.A. (2005). Cyclin-dependent kinase inhibitors in maize endosperm and their potential role in endoreduplication. Plant Physiol. *138*, 2323-2336.
Coll, N.S., Epple, P., and Dangl, J.L. (2011). Programmed cell death in the plant immune system. Cell Death Differ. *18*, 1247-1256.
Coudreuse, D., and Nurse, P. (2010). Driving the cell cycle with a minimal CDK control network. Nature *468*, 1074-1079.
Cui, X., Fan, B., Scholz, J., and Chen, Z. (2007). Roles of Arabidopsis cyclin-dependent kinase C complexes in cauliflower mosaic virus infection, plant growth, and development. Plant Cell *19*, 1388-1402.
Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol *139*, 5-17.
Dahl, M., Meskiene, I., Bogre, L., Ha, D.T., Swoboda, I., Hubmann, R., Hirt, H., and Heberle-Bors, E. (1995). The D-type alfalfa cyclin gene cycMs4 complements G1 cyclin-deficient yeast and is induced in the G1 phase of the cell cycle. Plant Cell *7*, 1847-1857.
de Jager, S.M., Scofield, S., Huntley, R.P., Robinson, A.S., den Boer, B.G., and Murray, J.A. (2009). Dissecting regulatory pathways of G1/S control in Arabidopsis: common and distinct targets of CYCD3;1, E2Fa and E2Fc. Plant Mol. Biol. *71*, 345-365.
De Veylder, L., Beeckman, T., Beemster, G.T., de Almeida Engler, J., Ormenese, S., Maes, S., Naudts, M., Van Der Schueren, E., Jacqmard, A., Engler, G., *et al.* (2002). Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. EMBO J. *21*, 1360-1368.
De Veylder, L., Beeckman, T., Beemster, G.T., Krols, L., Terras, F., Landrieu, I., van der Schueren, E., Maes, S., Naudts, M., and Inze, D. (2001). Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell *13*, 1653-1668.
De Veylder, L., Larkin, J.C., and Schnittger, A. (2011). Molecular control and function of endoreplication in development and physiology. Trends Plant Sci. *16*, 624-634.
del Pozo, J.C., Boniotti, M.B., and Gutierrez, C. (2002). Arabidopsis E2Fc functions in cell division and is degraded by the ubiquitin-SCF(AtSKP2) pathway in response to light. Plant Cell *14*, 3057-3071.
del Pozo, J.C., Diaz-Trivino, S., Cisneros, N., and Gutierrez, C. (2006). The balance between cell division and endoreplication depends on E2FC-DPB, transcription factors regulated by the ubiquitin-SCFSKP2A pathway in Arabidopsis. Plant Cell *18*, 2224-2235.
Derrien, B., Baumberger, N., Schepetilnikov, M., Viotti, C., De Cillia, J., Ziegler-Graff, V., Isono, E., Schumacher, K., and Genschik, P. (2012). Degradation of the antiviral component ARGONAUTE1 by the autophagy pathway. Proc. Natl. Acad. Sci. U. S. A. *109*, 15942-15946.
Deshaies, R.J. (1999). SCF and Cullin/Ring H2-Based Ubiquitin Ligases. Annu. Rev. Cell Dev. Biol. *15*, 435-467.
Desvoyes, B., de Mendoza, A., Ruiz-Trillo, I., and Gutierrez, C. (2014). Novel roles of plant RETINOBLASTOMA-RELATED (RBR) protein in cell proliferation and asymmetric cell

division. J. Exp. Bot. *65*, 2657-2666.
Dewitte, W., and Murray, J.A.H. (2003). The Plant Cell Cycle. Annu. Rev. Plant Biol. *54*, 235-264.
Dewitte, W., Riou-Khamlichi, C., Scofield, S., Healy, J.M., Jacqmard, A., Kilby, N.J., and Murray, J.A. (2003). Altered cell cycle distribution, hyperplasia, and inhibited differentiation in Arabidopsis caused by the D-type cyclin CYCD3. Plant Cell *15*, 79-92.
Dewitte, W., Scofield, S., Alcasabas, A.A., Maughan, S.C., Menges, M., Braun, N., Collins, C., Nieuwland, J., Prinsen, E., Sundaresan, V., *et al.* (2007). Arabidopsis CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc. Natl. Acad. Sci. U. S. A. *104*, 14537-14542.
Dick, F.A., and Rubin, S.M. (2013). Molecular mechanisms underlying RB protein function. Nat. Rev. Mol. Cell Biol. *14*, 297-306.
Doelling, J.H., Walker, J.M., Friedman, E.M., Thompson, A.R., and Vierstra, R.D. (2002). The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in Arabidopsis thaliana. J. Biol. Chem. *277*, 33105-33114.
Doerner, P., Jorgensen, J.E., You, R., Steppuhn, J., and Lamb, C. (1996). Control of root growth and development by cyclin expression. Nature *380*, 520-523.
Doonan, J., and Hunt, T. (1996). Why don't plants get cancer? Nature *380*, 481-482.
Dunand-Sauthier, I., Walker, C., Wilkinson, C., Gordon, C., Crane, R., Norbury, C., and Humphrey, T. (2002). Sum1, a component of the fission yeast eIF3 translation initiation complex, is rapidly relocalized during environmental stress and interacts with components of the 26S proteasome. Mol. Biol. Cell *13*, 1626-1640.
Elble, R. (1992). A simple and efficient procedure for transformation of yeasts. Biotechniques *13*, 18-20.
Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.
Ferreira, P.C., Hemerly, A.S., Villarroel, R., Van Montagu, M., and Inze, D. (1991). The Arabidopsis functional homolog of the p34cdc2 protein kinase. Plant Cell *3*, 531-540.
Filimonenko, M., Isakson, P., Finley, K.D., Anderson, M., Jeong, H., Melia, T.J., Bartlett, B.J., Myers, K.M., Birkeland, H.C., Lamark, T., *et al.* (2010). The selective macroautophagic degradation of aggregated proteins requires the PI3P-binding protein Alfy. Mol. Cell *38*, 265-279.
Floyd, B.E., Morriss, S.C., Macintosh, G.C., and Bassham, D.C. (2012). What to eat: evidence for selective autophagy in plants. J. Integr. Plant Biol. *54*, 907-920.
Frescas, D., and Pagano, M. (2008). Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat. Rev. Cancer *8*, 438-449.
Fu, H., Doelling, J.H., Arendt, C.S., Hochstrasser, M., and Vierstra, R.D. (1998). Molecular organization of the 20S proteasome gene family from Arabidopsis thaliana. Genetics *149*, 677-692.
Fulop, K., Pettko-Szandtner, A., Magyar, Z., Miskolczi, P., Kondorosi, E., Dudits, D., and Bako,

L. (2005). The Medicago CDKC;1-CYCLINT;1 kinase complex phosphorylates the carboxy-terminal domain of RNA polymerase II and promotes transcription. Plant J. *42*, 810-820.
Gagne, J.M., Downes, B.P., Shiu, S.-H., Durski, A.M., and Vierstra, R.D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. *99*, 11519-11524.
Garzon, M., Eifler, K., Faust, A., Scheel, H., Hofmann, K., Koncz, C., Yephremov, A., and Bachmair, A. (2007). PRT6/At5g02310 encodes an Arabidopsis ubiquitin ligase of the N-end rule pathway with arginine specificity and is not the CER3 locus. FEBS Lett. *581*, 3189-3196.
Gaudin, V., Lunness, P.A., Fobert, P.R., Towers, M., Riou-Khamlichi, C., Murray, J.A., Coen, E., and Doonan, J.H. (2000). The expression of D-cyclin genes defines distinct developmental zones in snapdragon apical meristems and is locally regulated by the Cycloidea gene. Plant Physiol. *122*, 1137-1148.
Ge, X., Dietrich, C., Matsuno, M., Li, G., Berg, H., and Xia, Y. (2005). An Arabidopsis aspartic protease functions as an anti-cell-death component in reproduction and embryogenesis. EMBO Rep. *6*, 282-288.
Genschik, P., Marrocco, K., Bach, L., and Noir, S. (2013a). Selective protein degradation: a rheostat to modulate cell-cycle phase transitions. J. Exp. Bot.
Genschik, P., Marrocco, K., Bach, L., Noir, S., and Criqui, M.C. (2014). Selective protein degradation: a rheostat to modulate cell-cycle phase transitions. J. Exp. Bot. *65*, 2603-2615.
Genschik, P., Sumara, I., and Lechner, E. (2013b). The emerging family of CULLIN3-RING ubiquitin ligases (CRL3s): cellular functions and disease implications. EMBO J. *32*, 2307-2320.
Giacinti, C., and Giordano, A. (2006). RB and cell cycle progression. Oncogene *25*, 5220-5227.
Grafi, G., and Avivi, Y. (2004). Stem cells: a lesson from dedifferentiation. Trends Biotechnol. *22*, 388-389.
Guerinier, T., Millan, L., Crozet, P., Oury, C., Rey, F., Valot, B., Mathieu, C., Vidal, J., Hodges, M., Thomas, M., *et al.* (2013). Phosphorylation of p27(KIP1) homologs KRP6 and 7 by SNF1-related protein kinase-1 links plant energy homeostasis and cell proliferation. Plant J. *75*, 515-525.
Gusti, A., Baumberger, N., Nowack, M., Pusch, S., Eisler, H., Potuschak, T., De Veylder, L., Schnittger, A., and Genschik, P. (2009). The Arabidopsis thaliana F-box protein FBL17 is essential for progression through the second mitosis during pollen development. PLoS One *4*, e4780.
Gutierrez, C. (2009). The Arabidopsis Cell Division Cycle. The Arabidopsis Book / American Society of Plant Biologists *7*, e0120.
Gutzat, R., Borghi, L., and Gruissem, W. (2012). Emerging roles of RETINOBLASTOMA-RELATED proteins in evolution and plant development. Trends Plant Sci. *17*, 139-148.
Hajheidari, M., Farrona, S., Huettel, B., Koncz, Z., and Koncz, C. (2012). CDKF;1 and CDKD protein kinases regulate phosphorylation of serine residues in the C-terminal domain of Arabidopsis RNA polymerase II. Plant Cell *24*, 1626-1642.

Hamilton, K.S., Ellison, M.J., Barber, K.R., Williams, R.S., Huzil, J.T., McKenna, S., Ptak, C., Glover, M., and Shaw, G.S. (2001). Structure of a Conjugating Enzyme-Ubiquitin Thiolester Intermediate Reveals a Novel Role for the Ubiquitin Tail. Structure *9*, 897-904.
Hanley-Bowdoin, L., Bejarano, E.R., Robertson, D., and Mansoor, S. (2013). Geminiviruses: masters at redirecting and reprogramming plant processes. Nat. Rev. Microbiol. *11*, 777-788.
Hartmann-Petersen, R., and Gordon, C. (2004). Protein degradation: recognition of ubiquitinylated substrates. Curr. Biol. *14*, R754-756.
Hartmann-Petersen, R., Seeger, M., and Gordon, C. (2003). Transferring substrates to the 26S proteasome. Trends Biochem. Sci. *28*, 26-31.
Hatfield, P.M., Gosink, M.M., Carpenter, T.B., and Vierstra, R.D. (1997). The ubiquitin-activating enzyme (E1) gene family in Arabidopsis thaliana. Plant J. *11*, 213-226.
Hatsugai, N., Yamada, K., Goto-Yamada, S., and Hara-Nishimura, I. (2015). Vacuolar processing enzyme in plant programmed cell death. Front. Plant Sci. *6*, 234.
Havens, C.G., and Walter, J.C. (2011). Mechanism of CRL4(Cdt2), a PCNA-dependent E3 ubiquitin ligase. Genes Dev. *25*, 1568-1582.
Heckmann, S., Lermontova, I., Berckmans, B., De Veylder, L., Bäumlein, H., and Schubert, I. (2011). The E2F transcription factor family regulates CENH3 expression in Arabidopsis thaliana. Plant J. *68*, 646-656.
Helm, M., Luck, C., Prestele, J., Hierl, G., Huesgen, P.F., Frohlich, T., Arnold, G.J., Adamska, I., Gorg, A., Lottspeich, F., *et al.* (2007). Dual specificities of the glyoxysomal/peroxisomal processing protease Deg15 in higher plants. Proc. Natl. Acad. Sci. U. S. A. *104*, 11501-11506.
Hemerly, A., Engler, d.J.A., Bergounioux, C., Montagu, M., Engler, G., Inzé, D., and Ferreira, P. (1995). Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. EMBO J. *14*, 3925.
Henley, S.A., and Dick, F.A. (2012). The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. Cell Div. *7*, 10.
Heyman, J., and De Veylder, L. (2012). The anaphase-promoting complex/cyclosome in control of plant development. Mol. Plant *5*, 1182-1194.
Hicke, L. (2001). Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell Biol. *2*, 195-201.
Hu, X., Cheng, X., Jiang, H., Zhu, S., Cheng, B., and Xiang, Y. (2010). Genome-wide analysis of cyclins in maize (Zea mays). Genet. Mol. Res. *9*, 1490-1503.
Hu, Y., Bao, F., and Li, J. (2000). Promotive effect of brassinosteroids on cell division involves a distinct CycD3-induction pathway in Arabidopsis. Plant J. *24*, 693-701.
Huang, S., Taylor, N.L., Whelan, J., and Millar, A.H. (2009). Refining the definition of plant mitochondrial presequences through analysis of sorting signals, N-terminal modifications, and cleavage motifs. Plant Physiol. *150*, 1272-1285.
Huibregtse, J.M., Scheffner, M., Beaudenon, S., and Howley, P.M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. U. S. A. *92*, 5249.
Imai, K.K., Ohashi, Y., Tsuge, T., Yoshizumi, T., Matsui, M., Oka, A., and Aoyama, T. (2006). The A-type cyclin CYCA2;3 is a key regulator of ploidy levels in Arabidopsis

endoreduplication. Plant Cell *18*, 382-396.
Inoue, Y., Suzuki, T., Hattori, M., Yoshimoto, K., Ohsumi, Y., and Moriyasu, Y. (2006). AtATG genes, homologs of yeast autophagy genes, are involved in constitutive autophagy in Arabidopsis root tip cells. Plant Cell Physiol. *47*, 1641-1652.
Inze, D., and De Veylder, L. (2006). Cell cycle regulation in plant development. Annu. Rev. Genet. *40*, 77-105.
Jain, M., Nijhawan, A., Arora, R., Agarwal, P., Ray, S., Sharma, P., Kapoor, S., Tyagi, A.K., and Khurana, J.P. (2007). F-box proteins in rice. Genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. Plant Physiol. *143*, 1467-1483.
Jakoby, M.J., Weinl, C., Pusch, S., Kuijt, S.J., Merkle, T., Dissmeyer, N., and Schnittger, A. (2006). Analysis of the subcellular localization, function, and proteolytic control of the Arabidopsis cyclin-dependent kinase inhibitor ICK1/KRP1. Plant Physiol. *141*, 1293-1305.
Jasinski, S., Leite, C.S., Domenichini, S., Stevens, R., Raynaud, C., Perennes, C., Bergounioux, C., and Glab, N. (2003). NtKIS2, a novel tobacco cyclin-dependent kinase inhibitor is differentially expressed during the cell cycle and plant development. Plant Physiol. Bioch. *41*, 667-676.
Jasinski, S., Perennes, C., Bergounioux, C., and Glab, N. (2002a). Comparative molecular and functional analyses of the tobacco cyclin-dependent kinase inhibitor NtKIS1a and its spliced variant NtKIS1b. Plant Physiol. *130*, 1871-1882.
Jasinski, S., Riou-Khamlichi, C., Roche, O., Perennes, C., Bergounioux, C., and Glab, N. (2002b). The CDK inhibitor NtKIS1a is involved in plant development, endoreduplication and restores normal development of cyclin D3; 1-overexpressing plants. J. Cell Sci. *115*, 973-982.
Jegu, T., Latrasse, D., Delarue, M., Mazubert, C., Bourge, M., Hudik, E., Blanchet, S., Soler, M.N., Charon, C., De Veylder, L., *et al.* (2013). Multiple functions of Kip-related protein5 connect endoreduplication and cell elongation. Plant Physiol. *161*, 1694-1705.
Jentsch, S., and Pyrowolakis, G. (2000). Ubiquitin and its kin: how close are the family ties? Trends Cell Biol. *10*, 335-342.
Johansen, T., and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. Autophagy *7*, 279-296.
John, F., Roffler, S., Wicker, T., and Ringli, C. (2011). Plant TOR signaling components. Plant Signal Behav. *6*, 1700-1705.
Joubès, J., Chevalier, C., Dudits, D., Heberle-Bors, E., Inzé, D., Umeda, M., and Renaudin, J.-P. (2000). CDK-related protein kinases in plants. Plant Mol. Biol. *43*, 607-620.
Joubes, J., De Schutter, K., Verkest, A., Inze, D., and De Veylder, L. (2004). Conditional, recombinase-mediated expression of genes in plant cell cultures. Plant J. *37*, 889-896.
Jun, S.E., Okushima, Y., Nam, J., Umeda, M., and Kim, G.T. (2013). Kip-related protein 3 is required for control of endoreduplication in the shoot apical meristem and leaves of Arabidopsis. Mol. Cells *35*, 47-53.
Kamura, T., Hara, T., Matsumoto, M., Ishida, N., Okumura, F., Hatakeyama, S., Yoshida, M., Nakayama, K., and Nakayama, K.I. (2004). Cytoplasmic ubiquitin ligase KPC regulates

proteolysis of p27(Kip1) at G1 phase. Nat. Cell Biol. *6*, 1229-1235.
Kang, J., Mizukami, Y., Wang, H., Fowke, L., and Dengler, N.G. (2007). Modification of cell proliferation patterns alters leaf vein architecture in Arabidopsis thaliana. Planta *226*, 1207-1218.
Kato, Y., and Sakamoto, W. (2010). New insights into the types and function of proteases in plastids. Int. Rev. Cell. Mol. Biol. *280*, 185-218.
Kelekar, A. (2005). Autophagy. Ann. N. Y. Acad. Sci. *1066*, 259-271.
Kim, H.J., Oh, S.A., Brownfield, L., Hong, S.H., Ryu, H., Hwang, I., Twell, D., and Nam, H.G. (2008a). Control of plant germline proliferation by SCF(FBL17) degradation of cell cycle inhibitors. Nature *455*, 1134-1137.
Kim, M., Ahn, J.W., Jin, U.H., Choi, D., Paek, K.H., and Pai, H.S. (2003). Activation of the programmed cell death pathway by inhibition of proteasome function in plants. J. Biol. Chem. *278*, 19406-19415.
Kim, Y., Starostina, N.G., and Kipreos, E.T. (2008b). The CRL4Cdt2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing. Genes Dev. *22*, 2507-2519.
Kipreos, E.T., and Pagano, M. (2000). The F-box protein family. Genome Biol. *1*, Reviews3002.
Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000). The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J. Cell Biol. *151*, 263-276.
Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P., *et al.* (2009). A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol. Cell *33*, 505-516.
Komaki, S., and Sugimoto, K. (2012). Control of the plant cell cycle by developmental and environmental cues. Plant Cell Physiol. *53*, 953-964.
Kono, A., Umeda-Hara, C., Lee, J., Ito, M., Uchimiya, H., and Umeda, M. (2003). Arabidopsis D-type cyclin CYCD4;1 is a novel cyclin partner of B2-type cyclin-dependent kinase. Plant Physiol. *132*, 1315-1321.
Koroleva, O.A., Tomlinson, M., Parinyapong, P., Sakvarelidze, L., Leader, D., Shaw, P., and Doonan, J.H. (2004). CycD1, a putative G1 cyclin from Antirrhinum majus, accelerates the cell cycle in cultured tobacco BY-2 cells by enhancing both G1/S entry and progression through S and G2 phases. Plant Cell *16*, 2364-2379.
Kravtsova-Ivantsiv, Y., Sommer, T., and Ciechanover, A. (2013). The lysine48-based polyubiquitin chain proteasomal signal: not a single child anymore. Angew. Chem. Int. Ed. Engl. *52*, 192-198.
Kruger, N. (1994). The Bradford Method for Protein Quantitation. In Basic Protein and Peptide Protocols, J. Walker, ed. (Humana Press), pp. 9-15.
Kuwabara, A., and Gruissem, W. (2014). Arabidopsis Retinoblastoma-related and Polycomb group proteins: cooperation during plant cell differentiation and development. J. Exp. Bot. *65*, 2667-2676.

La, H., Li, J., Ji, Z., Cheng, Y., Li, X., Jiang, S., Venkatesh, P.N., and Ramachandran, S. (2006). Genome-wide analysis of cyclin family in rice (Oryza Sativa L.). Mol. Genet. Genomics *275*, 374-386.
Lam, Y.A., Xu, W., DeMartino, G.N., and Cohen, R.E. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature *385*, 737-740.
Lammens, T., Boudolf, V., Kheibarshekan, L., Zalmas, L.P., Gaamouche, T., Maes, S., Vanstraelen, M., Kondorosi, E., La Thangue, N.B., Govaerts, W., *et al.* (2008). Atypical E2F activity restrains APC/CCCS52A2 function obligatory for endocycle onset. Proc. Natl. Acad. Sci. U. S. A. *105*, 14721-14726.
Lasch, P., Petras, T., Ullrich, O., Backmann, J., Naumann, D., and Grune, T. (2001). Hydrogen peroxide-induced structural alterations of RNAse A. J. Biol. Chem. *276*, 9492-9502.
Lee, J.H., Yoon, H.J., Terzaghi, W., Martinez, C., Dai, M., Li, J., Byun, M.O., and Deng, X.W. (2010). DWA1 and DWA2, two Arabidopsis DWD protein components of CUL4-based E3 ligases, act together as negative regulators in ABA signal transduction. Plant Cell *22*, 1716-1732.
Liu, J., Zhang, Y., Qin, G., Tsuge, T., Sakaguchi, N., Luo, G., Sun, K., Shi, D., Aki, S., Zheng, N., *et al.* (2008). Targeted degradation of the cyclin-dependent kinase inhibitor ICK4/KRP6 by RING-type E3 ligases is essential for mitotic cell cycle progression during Arabidopsis gametogenesis. Plant Cell *20*, 1538-1554.
Liu, Y., and Bassham, D.C. (2010). TOR is a negative regulator of autophagy in Arabidopsis thaliana. PLoS One *5*, e11883.
Liu, Y., and Bassham, D.C. (2012). Autophagy: pathways for self-eating in plant cells. Annu. Rev. Plant Biol. *63*, 215-237.
Lui, H., Wang, H., Delong, C., Fowke, L.C., Crosby, W.L., and Fobert, P.R. (2000). The Arabidopsis Cdc2a-interacting protein ICK2 is structurally related to ICK1 and is a potent inhibitor of cyclin-dependent kinase activity in vitro. Plant J. *21*, 379-385.
Ma, Z., Wu, Y., Jin, J., Yan, J., Kuang, S., Zhou, M., Zhang, Y., and Guo, A.Y. (2013). Phylogenetic analysis reveals the evolution and diversification of cyclins in eukaryotes. Mol. Phylogenet. Evol. *66*, 1002-1010.
Magyar, Z., De Veylder, L., Atanassova, A., Bako, L., Inze, D., and Bogre, L. (2005). The role of the Arabidopsis E2FB transcription factor in regulating auxin-dependent cell division. Plant Cell *17*, 2527-2541.
Magyar, Z., Horvath, B., Khan, S., Mohammed, B., Henriques, R., De Veylder, L., Bako, L., Scheres, B., and Bogre, L. (2012). Arabidopsis E2FA stimulates proliferation and endocycle separately through RBR-bound and RBR-free complexes. EMBO J. *31*, 1480-1493.
Mariconti, L., Pellegrini, B., Cantoni, R., Stevens, R., Bergounioux, C., Cella, R., and Albani, D. (2002). The E2F Family of Transcription Factors from Arabidopsis thaliana : NOVEL AND CONSERVED COMPONENTS OF THE RETINOBLASTOMA/E2F PATHWAY IN PLANTS. J. Biol. Chem. *277*, 9911-9919.
Marrocco, K., Bergdoll, M., Achard, P., Criqui, M.C., and Genschik, P. (2010). Selective proteolysis sets the tempo of the cell cycle. Curr. Opin. Plant Biol. *13*, 631-639.

Melchior, F. (2000). SUMO--nonclassical ubiquitin. Annu. Rev. Cell Dev. Biol. *16*, 591-626.
Menges, M., de Jager, S.M., Gruissem, W., and Murray, J.A. (2005). Global analysis of the core cell cycle regulators of Arabidopsis identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. Plant J. *41*, 546-566.
Menges, M., and Murray, J.A. (2002). Synchronous Arabidopsis suspension cultures for analysis of cell-cycle gene activity. Plant J. *30*, 203-212.
Menges, M., Pavesi, G., Morandini, P., Bogre, L., and Murray, J.A. (2007). Genomic organization and evolutionary conservation of plant D-type cyclins. Plant Physiol. *145*, 1558-1576.
Meszaros, T., Miskolczi, P., Ayaydin, F., Pettko-Szandtner, A., Peres, A., Magyar, Z., Horvath, G.V., Bako, L., Feher, A., and Dudits, D. (2000). Multiple cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells. Plant Mol. Biol. *43*, 595-605.
Michaeli, S., and Galili, G. (2014). Degradation of organelles or specific organelle components via selective autophagy in plant cells. Int. J. Mol. Sci. *15*, 7624-7638.
Mocciaro, A., and Rape, M. (2012). Emerging regulatory mechanisms in ubiquitin-dependent cell cycle control. J. Cell Sci. *125*, 255-263.
Nakagami, H., Kawamura, K., Sugisaka, K., Sekine, M., and Shinmyo, A. (2002). Phosphorylation of retinoblastoma-related protein by the cyclin D/cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. Plant Cell *14*, 1847-1857.
Nakai, T., Kato, K., Shinmyo, A., and Sekine, M. (2006). Arabidopsis KRPs have distinct inhibitory activity toward cyclin D2-associated kinases, including plant-specific B-type cyclin-dependent kinase. FEBS Lett. *580*, 336-340.
Nakayama, K., and Nakayama, K. (1998). Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. Bioessays *20*, 1020-1029.
Naouar, N., Vandepoele, K., Lammens, T., Casneuf, T., Zeller, G., Van Hummelen, P., Weigel, D., Rätsch, G., Inzé, D., Kuiper, M., *et al.* (2009). Quantitative RNA expression analysis with Affymetrix Tiling 1.0R arrays identifies new E2F target genes. Plant J. *57*, 184-194.
Ndubaku, C., and Tsui, V. (2015). Inhibiting the deubiquitinating enzymes (DUBs). J. Med. Chem. *58*, 1581-1595.
Nelson, C.J., Li, L., and Millar, A.H. (2014). Quantitative analysis of protein turnover in plants. Proteomics *14*, 579-592.
Nieuwland, J., Maughan, S., Dewitte, W., Scofield, S., Sanz, L., and Murray, J.A. (2009). The D-type cyclin CYCD4;1 modulates lateral root density in Arabidopsis by affecting the basal meristem region. Proc. Natl. Acad. Sci. U. S. A. *106*, 22528-22533.
Nixon, P.J., Michoux, F., Yu, J., Boehm, M., and Komenda, J. (2010). Recent advances in understanding the assembly and repair of photosystem II. Ann. Bot. *106*, 1-16.
Noda, N.N., Ohsumi, Y., and Inagaki, F. (2010). Atg8-family interacting motif crucial for selective autophagy. FEBS Lett. *584*, 1379-1385.
Nowack, M.K., Harashima, H., Dissmeyer, N., Zhao, X., Bouyer, D., Weimer, A.K., De Winter, F., Yang, F., and Schnittger, A. (2012). Genetic framework of cyclin-dependent kinase function

in Arabidopsis. Dev. Cell *22*, 1030-1040.
Ormenese, S., de Almeida Engler, J., De Groodt, R., De Veylder, L., Inze, D., and Jacqmard, A. (2004). Analysis of the spatial expression pattern of seven Kip related proteins (KRPs) in the shoot apex of Arabidopsis thaliana. Ann. Bot. *93*, 575-580.
Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. *282*, 24131-24145.
Peres, A., Churchman, M.L., Hariharan, S., Himanen, K., Verkest, A., Vandepoele, K., Magyar, Z., Hatzfeld, Y., Van Der Schueren, E., Beemster, G.T., *et al.* (2007). Novel plant-specific cyclin-dependent kinase inhibitors induced by biotic and abiotic stresses. J. Biol. Chem. *282*, 25588-25596.
Perez-Perez, M.E., and Crespo, J.L. (2010). Autophagy in the model alga Chlamydomonas reinhardtii. Autophagy *6*, 562-563.
Perez-Perez, M.E., Florencio, F.J., and Crespo, J.L. (2010). Inhibition of target of rapamycin signaling and stress activate autophagy in Chlamydomonas reinhardtii. Plant Physiol. *152*, 1874-1888.
Pesin, J.A., and Orr-Weaver, T.L. (2008). Regulation of APC/C activators in mitosis and meiosis. Annu. Rev. Cell Dev. Biol. *24*, 475-499.
Pettkó-Szandtner, A., Mészáros, T., Horváth, G.V., Bakó, L., Csordás-Tóth, É., Blastyák, A., Zhiponova, M., Miskolczi, P., and Dudits, D. (2006). Activation of an alfalfa cyclin-dependent kinase inhibitor by calmodulin-like domain protein kinase. Plant J. *46*, 111-123.
Pettko-Szandtner, A., Meszaros, T., Horvath, G.V., Bako, L., Csordas-Toth, E., Blastyak, A., Zhiponova, M., Miskolczi, P., and Dudits, D. (2006). Activation of an alfalfa cyclin-dependent kinase inhibitor by calmodulin-like domain protein kinase. Plant J. *46*, 111-123.
Pickart, C.M., and Fushman, D. (2004). Polyubiquitin chains: polymeric protein signals. Curr. Opin. Chem. Biol. *8*, 610-616.
Ponpuak, M., Davis, A.S., Roberts, E.A., Delgado, M.A., Dinkins, C., Zhao, Z., Virgin, H.W.t., Kyei, G.B., Johansen, T., Vergne, I., *et al.* (2010). Delivery of cytosolic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. Immunity *32*, 329-341.
Porceddu, A., Stals, H., Reichheld, J.P., Segers, G., De Veylder, L., Barroco, R.P., Casteels, P., Van Montagu, M., Inze, D., and Mironov, V. (2001). A plant-specific cyclin-dependent kinase is involved in the control of G2/M progression in plants. J. Biol. Chem. *276*, 36354-36360.
Qi, R., and John, P.C. (2007). Expression of genomic AtCYCD2;1 in Arabidopsis induces cell division at smaller cell sizes: implications for the control of plant growth. Plant Physiol. *144*, 1587-1597.
Ramirez-Parra, E., Frundt, C., and Gutierrez, C. (2003). A genome-wide identification of E2F-regulated genes in Arabidopsis. Plant J. *33*, 801-811.
Ramirez-Parra, E., López-Matas, M.A., Fründt, C., and Gutierrez, C. (2004). Role of an Atypical E2F Transcription Factor in the Control of Arabidopsis Cell Growth and

Differentiation. Plant Cell *16*, 2350-2363.
Rawlings, N.D., Waller, M., Barrett, A.J., and Bateman, A. (2014). MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. *42*, D503-509.
Reggiori, F., Komatsu, M., Finley, K., and Simonsen, A. (2012). Autophagy: more than a nonselective pathway. Int. J. Cell Biol. *2012*, 219625.
Ren, H., Santner, A., del Pozo, J.C., Murray, J.A., and Estelle, M. (2008). Degradation of the cyclin-dependent kinase inhibitor KRP1 is regulated by two different ubiquitin E3 ligases. Plant J. *53*, 705-716.
Reumann, S., Voitsekhovskaja, O., and Lillo, C. (2010). From signal transduction to autophagy of plant cell organelles: lessons from yeast and mammals and plant-specific features. Protoplasma *247*, 233-256.
Richard, C., Lescot, M., Inzé, D., and De Veylder, L. (2002). Effect of auxin, cytokinin, and sucrose on cell cycle gene expression in Arabidopsis thaliana cell suspension cultures. Plant Cell Tiss. Org. *69*, 167-176.
Richter, S., Zhong, R., and Lamppa, G. (2005). Function of the stromal processing peptidase in the chloroplast import pathway. Physiol. Plant. *123*, 362-368.
Riley, B.E., Kaiser, S.E., Shaler, T.A., Ng, A.C., Hara, T., Hipp, M.S., Lage, K., Xavier, R.J., Ryu, K.Y., Taguchi, K., *et al.* (2010). Ubiquitin accumulation in autophagy-deficient mice is dependent on the Nrf2-mediated stress response pathway: a potential role for protein aggregation in autophagic substrate selection. J. Cell Biol. *191*, 537-552.
Riou-Khamlichi, C., Huntley, R., Jacqmard, A., and Murray, J.A. (1999). Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science *283*, 1541-1544.
Riou-Khamlichi, C., Menges, M., Healy, J.M., and Murray, J.A. (2000). Sugar control of the plant cell cycle: differential regulation of Arabidopsis D-type cyclin gene expression. Mol. Cell Biol. *20*, 4513-4521.
Roeder, A.H., Chickarmane, V., Cunha, A., Obara, B., Manjunath, B.S., and Meyerowitz, E.M. (2010). Variability in the control of cell division underlies sepal epidermal patterning in Arabidopsis thaliana. PLoS Biol. *8*, e1000367.
Roudier, F., Fedorova, E., Gyorgyey, J., Feher, A., Brown, S., Kondorosi, A., and Kondorosi, E. (2000). Cell cycle function of a Medicago sativa A2-type cyclin interacting with a PSTAIRE-type cyclin-dependent kinase and a retinoblastoma protein. Plant J. *23*, 73-83.
Ruggiero, B., Koiwa, H., Manabe, Y., Quist, T.M., Inan, G., Saccardo, F., Joly, R.J., Hasegawa, P.M., Bressan, R.A., and Maggio, A. (2004). Uncoupling the effects of abscisic acid on plant growth and water relations. Analysis of sto1/nced3, an abscisic acid-deficient but salt stress-tolerant mutant in Arabidopsis. Plant Physiol. *136*, 3134-3147.
Sadanandom, A., Bailey, M., Ewan, R., Lee, J., and Nelis, S. (2012). The ubiquitin-proteasome system: central modifier of plant signalling. New Phytol. *196*, 13-28.
Samach, A., Klenz, J.E., Kohalmi, S.E., Risseeuw, E., Haughn, G.W., and Crosby, W.L. (1999). The UNUSUAL FLORAL ORGANS gene of Arabidopsis thaliana is an F-box protein required for normal patterning and growth in the floral meristem. Plant J. *20*, 433-445.
Sanz, L., Dewitte, W., Forzani, C., Patell, F., Nieuwland, J., Wen, B., Quelhas, P., De Jager, S.,

Titmus, C., Campilho, A., *et al.* (2011). The Arabidopsis D-type cyclin CYCD2;1 and the inhibitor ICK2/KRP2 modulate auxin-induced lateral root formation. Plant Cell *23*, 641-660.
Sauter, M., Mekhedov, S.L., and Kende, H. (1995). Gibberellin promotes histone H1 kinase activity and the expression of cdc2 and cyclin genes during the induction of rapid growth in deepwater rice internodes. Plant J. *7*, 623-632.
Schiessl, K., Muino, J.M., and Sablowski, R. (2014). Arabidopsis JAGGED links floral organ patterning to tissue growth by repressing Kip-related cell cycle inhibitors. Proc. Natl. Acad. Sci. U. S. A. *111*, 2830-2835.
Schnittger, A., Weinl, C., Bouyer, D., Schobinger, U., and Hulskamp, M. (2003). Misexpression of the cyclin-dependent kinase inhibitor ICK1/KRP1 in single-celled Arabidopsis trichomes reduces endoreduplication and cell size and induces cell death. Plant Cell *15*, 303-315.
Schreiber, A., and Peter, M. (2014). Substrate recognition in selective autophagy and the ubiquitin–proteasome system. BBA - Mol. Cell Res. *1843*, 163-181.
Schuhmann, H., Huesgen, P.F., Gietl, C., and Adamska, I. (2008). The DEG15 serine protease cleaves peroxisomal targeting signal 2-containing proteins in Arabidopsis. Plant Physiol. *148*, 1847-1856.
Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. Cell *79*, 233-244.
Scofield, S., Jones, A., and Murray, J.A.H. (2014). The plant cell cycle in context. J. Exp. Bot. *65*, 2557-2562.
Sherr, C.J., and Roberts, J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501-1512.
Shimotohno, A., Umeda-Hara, C., Bisova, K., Uchimiya, H., and Umeda, M. (2004). The plant-specific kinase CDKF;1 is involved in activating phosphorylation of cyclin-dependent kinase-activating kinases in Arabidopsis. Plant Cell *16*, 2954-2966.
Shipman, R.L., and Inoue, K. (2009). Suborganellar localization of plastidic type I signal peptidase 1 depends on chloroplast development. FEBS Lett. *583*, 938-942.
Smalle, J., and Vierstra, R.D. (2004). The ubiquitin 26S proteasome proteolytic pathway. Annu. Rev. Plant Biol. *55*, 555-590.
Smith, L.G. (2001). Plant cell division: building walls in the right places. Nat. Rev. Mol. Cell Biol. *2*, 33-39.
Soni, R., Carmichael, J.P., Shah, Z.H., and Murray, J.A. (1995). A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell *7*, 85-103.
Sorrell, D.A., Combettes, B., Chaubet-Gigot, N., Gigot, C., and Murray, J.A. (1999). Distinct cyclin D genes show mitotic accumulation or constant levels of transcripts in tobacco bright yellow-2 cells. Plant Physiol. *119*, 343-352.
Sorrell, D.A., Menges, M., Healy, J.M., Deveaux, Y., Amano, C., Su, Y., Nakagami, H., Shinmyo, A., Doonan, J.H., Sekine, M., *et al.* (2001). Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar Bright Yellow-2 cells. Plant Physiol. *126*, 1214-1223.
Sozzani, R., Cui, H., Moreno-Risueno, M.A., Busch, W., Van Norman, J.M., Vernoux, T., Brady,

S.M., Dewitte, W., Murray, J.A., and Benfey, P.N. (2010a). Spatiotemporal regulation of cell-cycle genes by SHORTROOT links patterning and growth. Nature *466*, 128-132.
Sozzani, R., Maggio, C., Giordo, R., Umana, E., Ascencio-Ibanez, J.T., Hanley-Bowdoin, L., Bergounioux, C., Cella, R., and Albani, D. (2010b). The E2FD/DEL2 factor is a component of a regulatory network controlling cell proliferation and development in Arabidopsis. Plant Mol. Biol. *72*, 381-395.
Stadtmueller, B.M., and Hill, C.P. (2011). Proteasome activators. Mol. Cell *41*, 8-19.
Starostina, N.G., and Kipreos, E.T. (2012). Multiple degradation pathways regulate versatile CIP/KIP CDK inhibitors. Trends Cell Biol. *22*, 33-41.
Stary, S., Yin, X.J., Potuschak, T., Schlogelhofer, P., Nizhynska, V., and Bachmair, A. (2003). PRT1 of Arabidopsis is a ubiquitin protein ligase of the plant N-end rule pathway with specificity for aromatic amino-terminal residues. Plant Physiol. *133*, 1360-1366.
Sterken, R., Kiekens, R., Boruc, J., Zhang, F., Vercauteren, A., Vercauteren, I., De Smet, L., Dhondt, S., Inze, D., De Veylder, L., *et al.* (2012). Combined linkage and association mapping reveals CYCD5;1 as a quantitative trait gene for endoreduplication in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. *109*, 4678-4683.
Sugimoto-Shirasu, K., and Roberts, K. (2003). "Big it up": endoreduplication and cell-size control in plants. Curr. Opin. Plant Biol. *6*, 544-553.
Sullivan, J.A., Shirasu, K., and Deng, X.W. (2003). The diverse roles of ubiquitin and the 26S proteasome in the life of plants. Nat. Rev. Genet. *4*, 948-958.
Suttangkakul, A., Li, F., Chung, T., and Vierstra, R.D. (2011). The ATG1/ATG13 protein kinase complex is both a regulator and a target of autophagic recycling in Arabidopsis. Plant Cell *23*, 3761-3779.
Suzuki, K. (2013). Selective autophagy in budding yeast. Cell Death Differ. *20*, 43-48.
Svenning, S., Lamark, T., Krause, K., and Johansen, T. (2011). Plant NBR1 is a selective autophagy substrate and a functional hybrid of the mammalian autophagic adapters NBR1 and p62/SQSTM1. Autophagy *7*, 993-1010.
Takahashi, N., Lammens, T., Boudolf, V., Maes, S., Yoshizumi, T., De Jaeger, G., Witters, E., Inze, D., and De Veylder, L. (2008). The DNA replication checkpoint aids survival of plants deficient in the novel replisome factor ETG1. EMBO J. *27*, 1840-1851.
Takatsuka, H., Ohno, R., and Umeda, M. (2009). The Arabidopsis cyclin-dependent kinase-activating kinase CDKF;1 is a major regulator of cell proliferation and cell expansion but is dispensable for CDKA activation. Plant J. *59*, 475-487.
Tanida, I. (2011). Autophagosome formation and molecular mechanism of autophagy. Antioxid Redox Signal *14*, 2201-2214.
Tank, J.G., and Thaker, V.S. (2011). Cyclin dependent kinases and their role in regulation of plant cell cycle. Biologia Plantarum *55*, 201-212.
Thrower, J.S., Hoffman, L., Rechsteiner, M., and Pickart, C.M. (2000). Recognition of the polyubiquitin proteolytic signal, Vol 19.
Torres Acosta, J.A., Fowke, L.C., and Wang, H. (2011). Analyses of phylogeny, evolution, conserved sequences and genome-wide expression of the ICK/KRP family of plant CDK

inhibitors. Annals of Botany *107*, 1141-1157.
Tsiatsiani, L., Gevaert, K., and Van Breusegem, F. (2012). Natural substrates of plant proteases: how can protease degradomics extend our knowledge? Physiol. Plant. *145*, 28-40.
Uemukai, K., Iwakawa, H., Kosugi, S., de Uemukai, S., Kato, K., Kondorosi, E., Murray, J.A., Ito, M., Shinmyo, A., and Sekine, M. (2005). Transcriptional activation of tobacco E2F is repressed by co-transfection with the retinoblastoma-related protein: cyclin D expression overcomes this repressor activity. Plant Mol. Biol. *57*, 83-100.
Umeda, M., Shimotohno, A., and Yamaguchi, M. (2005). Control of cell division and transcription by cyclin-dependent kinase-activating kinases in plants. Plant Cell Physiol. *46*, 1437-1442.
van den Heuvel, S., and Dyson, N.J. (2008). Conserved functions of the pRB and E2F families. Nat. Rev. Mol. Cell Biol. *9*, 713-724.
van der Hoorn, R.A., and Jones, J.D. (2004). The plant proteolytic machinery and its role in defence. Curr. Opin. Plant Biol. *7*, 400-407.
van der Hoorn, R.A.L. (2008). Plant Proteases: From Phenotypes to Molecular Mechanisms. Annu. Rev. Plant Biol. *59*, 191-223.
Van Leene, J., Boruc, J., De Jaeger, G., Russinova, E., and De Veylder, L. (2011). A kaleidoscopic view of the Arabidopsis core cell cycle interactome. Trends Plant Sci. *16*, 141-150.
Van Leene, J., Hollunder, J., Eeckhout, D., Persiau, G., Van De Slijke, E., Stals, H., Van Isterdael, G., Verkest, A., Neirynck, S., Buffel, Y., *et al.* (2010). Targeted interactomics reveals a complex core cell cycle machinery in Arabidopsis thaliana. Mol. Syst. Biol. *6*, 397.
Vandepoele, K., Raes, J., De Veylder, L., Rouze, P., Rombauts, S., and Inze, D. (2002). Genome-wide analysis of core cell cycle genes in Arabidopsis. Plant Cell *14*, 903-916.
Vandepoele, K., Vlieghe, K., Florquin, K., Hennig, L., Beemster, G.T., Gruissem, W., Van de Peer, Y., Inze, D., and De Veylder, L. (2005). Genome-wide identification of potential plant E2F target genes. Plant Physiol. *139*, 316-328.
Vanneste, S., Coppens, F., Lee, E., Donner, T.J., Xie, Z., Van Isterdael, G., Dhondt, S., De Winter, F., De Rybel, B., Vuylsteke, M., *et al.* (2011). Developmental regulation of CYCA2s contributes to tissue‐specific proliferation in Arabidopsis, Vol 30.
Varshavsky, A. (1996). The N-end rule: functions, mysteries, uses. Proc. Natl. Acad. Sci. U. S. A. *93*, 12142-12149.
Veljanovski, V., and Batoko, H. (2014). Selective autophagy of non-ubiquitylated targets in plants: looking for cognate receptor/adaptor proteins. Front. Plant Sci. *5*.
Verkest, A., Manes, C.-L.d.O., Vercruysse, S., Maes, S., Van Der Schueren, E., Beeckman, T., Genschik, P., Kuiper, M., Inzé, D., and De Veylder, L. (2005a). The Cyclin-Dependent Kinase Inhibitor KRP2 Controls the Onset of the Endoreduplication Cycle during Arabidopsis Leaf Development through Inhibition of Mitotic CDKA;1 Kinase Complexes. Plant Cell *17*, 1723-1736.
Verkest, A., Weinl, C., Inze, D., De Veylder, L., and Schnittger, A. (2005b). Switching the cell cycle. Kip-related proteins in plant cell cycle control. Plant Physiol. *139*, 1099-1106.

Vieira, P., De Clercq, A., Stals, H., Van Leene, J., Van De Slijke, E., Van Isterdael, G., Eeckhout, D., Persiau, G., Van Damme, D., Verkest, A., *et al.* (2014). The Cyclin-Dependent Kinase Inhibitor KRP6 Induces Mitosis and Impairs Cytokinesis in Giant Cells Induced by Plant-Parasitic Nematodes in Arabidopsis. Plant Cell *26*, 2633-2647.
Vieira, P., Engler, G., and de Almeida Engler, J. (2013a). Enhanced levels of plant cell cycle inhibitors hamper root-knot nematode-induced feeding site development. Plant Signal Behav. *8*, e26409.
Vieira, P., Escudero, C., Rodiuc, N., Boruc, J., Russinova, E., Glab, N., Mota, M., De Veylder, L., Abad, P., Engler, G., *et al.* (2013b). Ectopic expression of Kip-related proteins restrains root-knot nematode-feeding site expansion. New Phytol. *199*, 505-519.
Vierstra, R. (1996). Proteolysis in plants: mechanisms and functions. Plant Mol. Biol. *32*, 275-302.
Vierstra, R.D. (2009). The ubiquitin-26S proteasome system at the nexus of plant biology. Nat. Rev. Mol. Cell Biol. *10*, 385-397.
Vlieghe, K., Vuylsteke, M., Florquin, K., Rombauts, S., Maes, S., Ormenese, S., Van Hummelen, P., Van de Peer, Y., Inze, D., and De Veylder, L. (2003). Microarray analysis of E2Fa-DPa-overexpressing plants uncovers a cross-talking genetic network between DNA replication and nitrogen assimilation. J. Cell Sci. *116*, 4249-4259.
Walker, J.D., Oppenheimer, D.G., Concienne, J., and Larkin, J.C. (2000). SIAMESE, a gene controlling the endoreduplication cell cycle in Arabidopsis thaliana trichomes. Development *127*, 3931-3940.
Wang, G., Kong, H., Sun, Y., Zhang, X., Zhang, W., Altman, N., DePamphilis, C.W., and Ma, H. (2004). Genome-wide analysis of the cyclin family in Arabidopsis and comparative phylogenetic analysis of plant cyclin-like proteins. Plant Physiol. *135*, 1084-1099.
Wang, H., Fowke, L.C., and Crosby, W.L. (1997). A plant cyclin-dependent kinase inhibitor gene. Nature *386*, 451-452.
Wang, H., Qi, Q., Schorr, P., Cutler, A.J., Crosby, W.L., and Fowke, L.C. (1998). ICK1, a cyclin-dependent protein kinase inhibitor from Arabidopsis thaliana interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. Plant J. *15*, 501-510.
Wang, H., Zhou, Y., Bird, D.A., and Fowke, L.C. (2008). Functions, regulation and cellular localization of plant cyclin-dependent kinase inhibitors. J. Microsc. *231*, 234-246.
Wang, H., Zhou, Y., Gilmer, S., Whitwill, S., and Fowke, L.C. (2000). Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. Plant J. *24*, 613-623.
Wang, W., and Chen, X. (2004). HUA ENHANCER3 reveals a role for a cyclin-dependent protein kinase in the specification of floral organ identity in Arabidopsis. Development *131*, 3147-3156.
Wasch, R., and Cross, F.R. (2002). APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit. Nature *418*, 556-562.
Waters, S., Marchbank, K., Solomon, E., Whitehouse, C., and Gautel, M. (2009). Interactions with LC3 and polyubiquitin chains link nbr1 to autophagic protein turnover. FEBS Lett. *583*,

1846-1852.
Weingartner, M., Criqui, M.C., Meszaros, T., Binarova, P., Schmit, A.C., Helfer, A., Derevier, A., Erhardt, M., Bogre, L., and Genschik, P. (2004). Expression of a nondegradable cyclin B1 affects plant development and leads to endomitosis by inhibiting the formation of a phragmoplast. Plant Cell *16*, 643-657.
Weinl, C., Marquardt, S., Kuijt, S.J., Nowack, M.K., Jakoby, M.J., Hulskamp, M., and Schnittger, A. (2005). Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis. Plant Cell *17*, 1704-1722.
Wen, B., Nieuwland, J., and Murray, J.A. (2013). The Arabidopsis CDK inhibitor ICK3/KRP5 is rate limiting for primary root growth and promotes growth through cell elongation and endoreduplication. J. Exp. Bot. *64*, 1135-1144.
Wertz, I.E., O'Rourke, K.M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D.L., *et al.* (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature *430*, 694-699.
Wolf, D.H., and Hilt, W. (2004). The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. BBA - Mol. Cell Res. *1695*, 19-31.
Wolf, S., Rausch, T., and Greiner, S. (2009). The N-terminal pro region mediates retention of unprocessed type-I PME in the Golgi apparatus. Plant J. *58*, 361-375.
Woo, H.R., Chung, K.M., Park, J.H., Oh, S.A., Ahn, T., Hong, S.H., Jang, S.K., and Nam, H.G. (2001). ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. Plant Cell *13*, 1779-1790.
Xie, Z., and Klionsky, D.J. (2007). Autophagosome formation: core machinery and adaptations. Nat. Cell Biol. *9*, 1102-1109.
Yamaguchi, M., Kato, H., Yoshida, S., Yamamura, S., Uchimiya, H., and Umeda, M. (2003). Control of in vitro organogenesis by cyclin-dependent kinase activities in plants. Proc. Natl. Acad. Sci. U. S. A. *100*, 8019-8023.
Yanagisawa, S., Yoo, S.-D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature *425*, 521-525.
Yoshimoto, K., Hanaoka, H., Sato, S., Kato, T., Tabata, S., Noda, T., and Ohsumi, Y. (2004). Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell *16*, 2967-2983.
Yu, Y., Steinmetz, A., Meyer, D., Brown, S., and Shen, W.-H. (2003). The Tobacco A-Type Cyclin, Nicta;CYCA3;2, at the Nexus of Cell Division and Differentiation. Plant Cell *15*, 2763-2777.
Zhang, H., Dong, S., Wang, M., Wang, W., Song, W., Dou, X., Zheng, X., and Zhang, Z. (2010). The role of vacuolar processing enzyme (VPE) from Nicotiana benthamiana in the elicitor-triggered hypersensitive response and stomatal closure. J. Exp. Bot. *61*, 3799-3812.
Zhao, X., Harashima, H., Dissmeyer, N., Pusch, S., Weimer, A.K., Bramsiepe, J., Bouyer, D., Rademacher, S., Nowack, M.K., Novak, B., *et al.* (2012). A general G1/S-phase cell-cycle control module in the flowering plant Arabidopsis thaliana. PLoS Genet. *8*, e1002847.
Zhou, J., Wang, J., Cheng, Y., Chi, Y.J., Fan, B., Yu, J.Q., and Chen, Z. (2013). NBR1-mediated

selective autophagy targets insoluble ubiquitinated protein aggregates in plant stress responses. PLoS Genet. *9*, e1003196.
Zhou, Y., Fowke, L., and Wang, H. (2002a). Plant CDK inhibitors: studies of interactions with cell cycle regulators in the yeast two-hybrid system and functional comparisons in transgenic Arabidopsis plants. Plant Cell Rep. *20*, 967-975.
Zhou, Y., Li, G., Brandizzi, F., Fowke, L.C., and Wang, H. (2003a). The plant cyclin-dependent kinase inhibitor ICK1 has distinct functional domains for in vivo kinase inhibition, protein instability and nuclear localization. Plant J. *35*, 476-489.
Zhou, Y., Niu, H., Brandizzi, F., Fowke, L.C., and Wang, H. (2006). Molecular control of nuclear and subnuclear targeting of the plant CDK inhibitor ICK1 and ICK1-mediated nuclear transport of CDKA. Plant Mol. Biol. *62*, 261-278.
Zhou, Y., Wang, H., Gilmer, S., Whitwill, S., and Fowke, L.C. (2003b). Effects of co-expressing the plant CDK inhibitor ICK1 and D-type cyclin genes on plant growth, cell size and ploidy in Arabidopsis thaliana. Planta *216*, 604-613.
Zhou, Y., Wang, H., Gilmer, S., Whitwill, S., Keller, W., and Fowke, L.C. (2002b). Control of petal and pollen development by the plant cyclin-dependent kinase inhibitor ICK1 in transgenic Brassica plants. Planta *215*, 248-257.
Zientara-Rytter, K., Lukomska, J., Moniuszko, G., Gwozdecki, R., Surowiecki, P., Lewandowska, M., Liszewska, F., Wawrzynska, A., and Sirko, A. (2011). Identification and functional analysis of Joka2, a tobacco member of the family of selective autophagy cargo receptors. Autophagy *7*, 1145-1158.
Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., and van Wijk, K.J. (2008). Sorting Signals, N-Terminal Modifications and Abundance of the Chloroplast Proteome. PLoS One *3*, e1994.