Studies of *Arabidopsis* Cyclin-Dependent Kinase Inhibitors: Protein-Protein Interactions, Phosphorylation and Stability

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Ron Chan

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ABSTRACT

The cyclin-dependent kinase (CDK) inhibitors have been demonstrated to be an important component in the regulation of plant cell cycle. Although they share a conserved CDK inhibitory region with a family of CDK inhibitors in mammals, the plant CDK inhibitors are very different from the animal and yeast CDK inhibitors. Thus studies of the plant CDK inhibitors could provide insight on the molecular mechanisms regulating the cell cycle in plants as well as the differences between plants and animals. The research described in this thesis investigated the seven *Arabidopsis* CDK inhibitors ICKs in terms of transgenic expression, phosphorylation, stability and interactions with other proteins.

ICKs were expressed in transgenic *Arabidopsis* plants as fusion proteins with the green fluorescent protein (GFP). Consistent with the previous studies on ICK1, ICK2 and ICK4, overexpression of all seven ICKs inhibited plant growth and resulted in plants with serrated leaves and flowers with altered morphology. A Survey based on large a number of independent transformants showed that GFP-ICK3 and GFP-ICK4 had weaker phenotypic effects compared to other GFP-ICKs. The Western blotting results showed that all GFP-ICKs were expressed at a low level in general. The levels of GFP-ICK3 and GFP-ICK4 were the lowest, suggesting that the weaker effects for ICK3 and ICK4 may partly be due to low protein levels. Treatments with MG132, an inhibitor of the proteasome, resulted in moderate but clear accumulation of fusion proteins for ICK1, ICK5, ICK6 and ICK7 in plants, suggesting that the proteasome is involved in the degradation of these proteins.

To study the state of protein phosphorylation, the proteins extracted from the plants were treated with calf intestinal phosphatase (CIP). The CIP treatment caused a faster migration of the GFP fusion protein for ICK1, ICK2, ICK5, ICK6 and ICK7, while the effect was not observed for control GFP and other non-specific proteins, indicating that these proteins can be phosphorylated in plants. The shift also differed among ICKs. Interestingly, dephosphorylation of ICK7 might have rendered it less stable. The protein pulldown experiments using $p13^{Suc1}$-conjugated agarose beads showed that GFP-ICK4, GFP-ICK5 and GFP-ICK6 could associate with the CDK complex, similar to what has been shown for ICK1 and ICK2. CIP treatments of the $p13^{Suc1}$ affinity-purified proteins also showed that ICK1, ICK2, ICK5 and ICK6 associated with the CDK complex were phosphorylated.

Attempts were also made to isolate peptide aptamers that are able to interact with ICKs for the purpose for expressing such an aptamer in plants. However, an aptamer that has a strong ability to interact with ICKs in two of yeast two-hybrid systems was not identified. In addition, the analysis of *Arabidopsis* CYCD3;1 for its interaction with ICK1 using a series of deletion mutants showed that the removal of both the N-terminal and C-terminal regions of CYCD3;1 greatly reduced or abolished the interaction with ICK1.

In summary, transgenic *Arabidopsis* plants have been obtained for expressing each of the seven *Arabidopsis* CDK inhibitors fused to GFP. The results confirmed and extended previous finding that overexpression of a CDK inhibitor inhibits plant growth as well as changes plant morphology. The observation that the ICK fusion proteins were generally at low and often undetectable levels, in comparison to much higher levels of the GFP protein, suggests that ICKs are unstable in the cell. Results from the MG132 experiments indicate that the 26S proteasome may play a role in the degradation of ICK1, ICK5, ICK6 and ICK7. Results from CIP treatments further show that most ICKs, particularly ICK1, ICK2, ICK5, ICK6 and ICK7, can be phosphorylated *in vivo*. Interestingly, ICK7 stability may depend on the status of protein phosphorylation. This study provides new understanding on how the family of proteins is regulated at the post-transcriptional level and the differences among *Arabidopsis* CDK inhibitors.

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AD	DNA transactivation domain
AP3	APETALA3
BD	DNA binding domain
CAK	CDK-activating kinase
CAKAK	CAK-activating kinase
CDKs	cyclin dependent kinases
CIP	calf intestinal phosphatase
CKIs	cyclin-dependent kinase inhibitors
CTD	C-terminal domain
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E. coli	*Escherichia coli*
E. coli TrxA	*Escherichia coli* thioredoxin
Gal	galactose
Glu	glucose
GFP	green fluorescent protein
His	histidine
ICKs	interactors/inhibitors of cyclin-dependent kinase
KRPs	Kip-related proteins
Leu	leucine
LB	Luria Bertani
MG132	carbobenzoxyl-leucinyl-leucinyl-leucinal
MSA	M-specific activator

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Importance of studying cell cycle regulation in plants

The regulation of the cell cycle in eukaryotes has been studied extensively throughout history due to its fundamental nature and its influence over many other processes such as differentiation, apoptosis, growth and development. When one or more of these processes are not regulated precisely, the consequences can be dire for the organism in question. Traditionally, most of the research and subsequent understanding of the cell cycle regulation is derived from animals and yeast. Recently, more attention is being paid to the cell cycle regulation in plants. Why do we need to study cell cycle regulation in plants?

Plants have characteristics that are very different from animals in cell cycle regulation. For instance, plants develop mainly post-embryonically. A group of stem cells referred to as meristems, are where new cells are added to produce the organs of the plant. Post-embryonic growth allows the plant to have greater flexibility to change in terms of organ number and size. Since body size is a function of the number and size of cells, plants are well suited for studying the effects of cell number and size on growth and development.

Another characteristic of plants that is remarkably different from animals is the presence of the cell wall. The cell wall provides strong structural support to the cell but in return restricts mobility or migration that occurs often in animals. In addition, plants can not move, and thus must adapt and withstand adverse environmental conditions. To successfully adapt to these environmental conditions and ensure survival, plants may have specific regulatory circuits that make the cell cycle regulation and cell proliferation sensitive to changes in environmental conditions.

The ability of plants to continuously produce organs indeterminately makes them an interesting and useful system to study cell cycle regulation. Plants normally do not show cancerous growth, except in some unique situations. *Agrobacterium tumefaciens,* a bacterium that can insert a very large plasmid (Ti plasmid) into plants cells, can cause tumors that are

commonly referred to as crown galls in plants. The T-DNA inserted by *Agrobacterium tumefaciens* encodes genes for plant hormones which can induce tumor formation in the plant. Although plant tissues are not immune to cancerous condition, as evident by *Agrobacterium tumefaciens* infection, the prevalence of tumor formation in plants is remarkably less than that of mammalian cells. The ability of plants to control its cell cycle to ensure normal growth is of great interest to many researchers. Understanding the control of cell cycle in plants is the first step in uncovering this mystery.

1.2 Overview of cell cycle regulation in plants

Regulation of the cell division cycle in plants is essential to ensure proper growth and development. Plant cell cycle is controlled by an evolutionarily conserved family of serine-threonine protein kinases, referred to as cyclin-dependent kinases (CDKs) (reviewed by Morgan 1997; Joubes *et al.* 2000). CDK activation requires the presence and binding of a regulatory partner, cyclin. Upon binding to its cyclin partner, CDKs will phosphorylate a series of downstream targets including the retinoblastoma protein (Rb). When Rb becomes phosphorylated, it dissociates itself from the E2F transcription factor (reviewed in de Jager and Murray 1999). This allows E2F to activate transcription of various S phase-related genes and promoting the entry into a new cell cycle.

To make the cell cycle control more complicated, the CDK-cyclin complex is further regulated by a group of CDK inhibitors (Sherr and Roberts 1999). In plants, ICKs (Interactors/inhibitors of Cdc2 Kinase) or KRPs (Kip related proteins) were the first plant CDK inhibitors discovered by the yeast two-hybrid technique using *Arabidopsis* CDKA; 1 as bait (Wang *et al.* 1997; Lui *et al.* 2000; De Veylder *et al.* 2001; Zhou *et al.* 2002a). This family of proteins share only limited sequence similarity to the animal Kip/Cip family of CDK inhibitors. Thus, ICKs have provided us with a good tool and opportunity to further our understanding of the cell cycle regulation in plants. This family of proteins is the focus of this thesis and background information of ICKs as well as the other major cell cycle regulators (CDKs, cyclins, Rb, E2F) will be discussed in the following sections.

1.3 Core regulators of the cell cycle

1.3.1 Cyclin-dependent kinase (CDKs)

CDKs play a central role in governing the cell cycle in eukaryotes, and their kinase activity generally depends on the association with cyclins (Pines 1995). The first CDK was discovered as Cdc2 in fission yeast *Schizosaccharomyces pombe* (Hindley and Phear 1984) and as CDC28 (a Cdc2 homolog) (Lorincz and Reed 1984) in budding yeast *Saccharomyces cerevisiae*. While only one CDK plays an essential role in regulating the cell cycle in yeast, multiple CDKs are involved in cell cycle regulation of plants and animals. In animals, the nomenclature of CDK1 to CDK7 has been used (Pines 1995) while in plants, CDKs are classified into CDKA to CDKF types based upon sequence similarity (Joubes *et al.* 2000; Vandepoele *et al.* 2002). Of the six types of plant CDKs, CDKA and CDKB are believed to be involved in regulating the cell cycle progression while CDKD and CDKF play a role in phosphorylating and activating the kinase activity of CDKA and CDKB (Harper and Elledge 1998; Umeda *et al.* 1998).

1.3.1.1 CDKA

Plant CDKA is perhaps the most well known type of the CDK family in plants. CDKA, yeast Cdc2/CDC28 and animals CDK1 all possesses the evolutionarily conserved PSTAIRE (single amino acid code) sequence motif within its cyclin-binding domain. In fact, the sequence homology between plant CDKA and its yeast and mammalian counterpart is closer than between plant CDKA and other plant CDK members (i.e. CDKB). CDKA is the only member of the plant CDK family that can complement yeast *cdc2/cdc28* mutants (Ferreira *et al.* 1991; Hirt *et al.* 1991; Imajuku *et al.* 1992). The CDKA mRNA and protein levels do not change very much throughout the cell cycle (Martinez *et al.* 1992; Hemerly *et al.* 1993). However, the kinase activity of CDKA in *Arabidopsis* is upregulated and remains high from G1 to S phase of the cell cycle, reduced in G2 and finally increases again at the G2/M transition (Menges and Murray 2002). Thus, CDKA seems to be required at both G1/S and G2/M transitions of the cell cycle.

1.3.1.2 CDKB

Plant CDKB seems to be unique to plants since no homologue of this gene has been identified in other species. Contrary to CDKA which has the traditional PSTAIRE hallmark,

CDKB are subdivided into two groups possessing either the PPTALRE (CDKB1) or PPTTLRE (CDKB2) amino acid sequence (Joubes *et al.* 2000). CDKB is an important regulator of the G2/M checkpoint as evident by the high kinase activity during G2/M transition (Magyar *et al.* 1997). Overexpression of a dominant negative CDKB leads to a substantial increase of G2 population, suggesting that the G2/M transition is blocked (Porceddu *et al.* 2001). Interestingly, overexpression of CDKB as well as other CDKs does not induce extra cell divisions (Hermerly *et al.* 1995; Boudolf *et al.* 2004). Thus, the protein levels of CDKs do not seem to be the rate limiting factor for their activity. Cyclins, which are the regulatory partners with CDKs, are most likely the limiting factor in controlling the kinase activity of CDKs (Doerner *et al.* 1996).

1.3.1.3 CAK: CDKD/CDKF

CDK-activating kinase (CAK) plays an important role in cell cycle regulation by phosphorylating and thus activating CDKA and CDKB. In plants, two types of CAK have been identified: CDKD and CDKF. CDKD is the plant homologue to yeast Crk1/Mop1 (*Schizosaccharomyces pombe)*, Kin28p (*Saccharomyces cerevisiae)* and human CDK7. The CDK7 kinase has duel functions as it phosphorylates both CDKs and the C-terminal domain (CTD) of RNA polymerase II (Harper and Elledge 1998). Rice R2, which is the first plant CDKD identified, displays the dual function of CDK7 and is capable of complementing a CAK-deficient mutant of the budding yeast (Hata 1991; Yamaguchi *et al.* 1998). In *Arabidopsis*, three CDKD genes have been identified and are named At;CDKD;1, At;CDKD;2, and AtCDKD;3 respectively. One major structural difference between vertebrate CAK and plant CAK is the presence of an extended C-terminal region of 92 amino acids in plant CDKs. This extended region of amino acids contains a nuclear localization signal (NLS) (Fabian-Marwedel *et al.* 2002). Studies using green fluorescent protein (GFP) fused to the At;CDKDs revealed that At;CDKD;1 and At;CDKD;3 were localized exclusively in nuclei, while At;CDKD2 was localized in both the cytoplasm and nuclei of plant cells (Shimotohno *et al.* 2004).

CDKF is a plant-specific type of CAK-activating kinase isolated initially as a suppressor to CAK mutation in budding yeast (Umeda *et al.* 1998). Unlike Rice R2, the *Arabidopsis* CDKF;1 can't phosphorylate the CTD of RNA polymerase II and only has kinase activity towards At;CDKD;2 and At;CDKD;3. In fission yeast, Csk1 has been shown to be able to phosphorylate CDK (Mcs6) and is commonly referred to as a CAK-activating kinase (CAKAK).

Only At;CDKF;1, but not any of the At;CDKDs, was able to complement fission yeast strain carrying a disrupted *csk1Δ* gene (Shimotohno *et al.* 2004). Adopting the term from yeast, CDKF is a plant specific CDKAK that regulates the activity of At;CDKD;2 and At;CDKD;3.by phosphorylation.

1.3.2 Cyclins

Cyclins were first identified in marine invertebrates as proteins showing a 'cyclical' pattern of accumulation and degradation during each cell cycle (Evans *et al.* 1983). Later it was discovered that cyclins are the regulatory partner of CDKs by binding to and activating CDK kinase activity (Meijer *et al.* 1989). Cyclins are a diverse group of proteins that are classified into several types based upon sequence similarity. The most common types include the A-type, B-type, D-type, and H-type (Renaudin *et al.* 1996). All cyclins possess a large region of homology commonly referred to as the cyclin box. The cyclin box is a stretch of about 100 amino acids composing of 5 helices which are responsible for binding to CDKs (Nobel *et al.* 1997). Different cyclins have been found to interact with different CDKs (Nakagami *et al.* 1999, Healy *et al.* 2001, Yamaguchi *et al.* 2000). In many cases the expression patterns and activity profile of plant cyclins during the cell cycle show similarity to those of the mammalian counterparts.

1.3.2.1 A and B-type cyclins

The functions of A and B-type cyclins appear to overlap and thus are often grouped together when describing them. Both A- and B-type cyclins are considered to be mitotic cyclins, meaning that their protein levels accumulate around the G2/M transition point in the cell. A-type cyclins generally appear before B-type cyclins at around the beginning of S phase and are present until the M phase where both type of cyclins are destroyed (Genschik *et al.* 1998). The levels of A- and B-type cyclins are stringently controlled as both families of proteins contain a 'destruction box' that targets them for degradation by the anaphase-promoting complex.

In plants, A-type cyclins are subdivided into three subclasses: CYCA1, CYCA2, and CYCA3 (Renaudin *et al.* 1996.) The greater diversity of the cyclin class in plants as compared to animals (no subclass) may be related to the heightened level of regulation of the cell cycle due to the sessile characteristics of plants and the need to respond to both intrinsic and environment

cues. One difference between the CYCA subclasses lies in the consensus sequence for the destruction box: A-type (RxA/PLxNL/IxN), B-type (RAV/ILxDxxN) and C-type (RVVLGEL/IxN) (Renaudin *et al.* 1998). This difference suggests that each subclass may have distinct role in the cell cycle regulation as the timing of their expression/degradation pattern may be different.

Similar to animals, B-type cyclins in plants are divided into two groups: CYCB1 and CYCB2. Apart from their differences in amino acid sequence, B-type cyclins distinguish themselves from the A-type cyclins by their later expression in the cell cycle. The regulation of G2/M checkpoint by B-type cyclins has been well demonstrated (Qin *et al.* 1996; Schnittger *et al.* 2002). As well, ectopic expression of CYCB2 stimulates the cells into mitosis from G2 and affects root and shoot development (Weingartner *et al.* 2003). In the promoter region of B-type cyclins, a common *cis*-acting element, called the M-specific activator (MSA) was discovered that is capable of directing G2 to M specific gene expression (Ito *et al.* 1998).

1.3.2.2 D-type cyclins

D-type cyclins are considered to be G1 cyclins and their presence is required for the G1 to S transition. In *Arabidopsis*, D-type cyclins are divided into 3 subclasses: CYCD1, CYCD2, and CYCD3. The overall sequence homology between the plants CYCD to its mammalian counterpart is very low, but they do share an important feature, the Rb protein binding motif, LxCxE (where x can be any amino acid). The LxCxE motif of CYCD is essential for CYCD/CDK complex to bind and phosphorylate Rb (Dowdy *et al.* 1993). In addition to the LxCxE motif, D-type cyclins also possess the PEST (proline, glutamine, serine and threonine rich) sequence. The PEST sequence is known to be associated with unstable, rapidly degrading proteins. *Arabidopsis* CYCD3;1 which possess the PEST sequence were found to be a very unstable protein with a half life estimated at 7 min (Planchais *et al.* 2004). Interestingly, CYCD2;1 which also possesses the PEST sequence turns out to be a rather stable protein (Planchais *et al.* 2004). Similar to mammals, plant D-type cyclins are degraded through a proteasome-dependent mechanism, specifically the ubiquitin/26S proteasome pathway (Planchais *et al.* 2004).

The expression of D-type cyclins has been shown to be modulated by plant growth factors such as cytokinins, brassinosteroids, sucrose and gibberellins (Stals and Inze 2001). It

has been shown that the expression of CYCD2 and CYCD3 genes is induced by sucrose and this induction appears to be mediated by a phosphatase (Riou-Khamlichi *et al.* 2000). Although CYCD2 and CYCD3 appear to function in the G1 to S transition and interact with the same CDK partner, they appear to be regulated differently. While the expression of both CYCD2;1 and CYCD3;1 expression is regulated by sucrose, CYCD3;1 but not CYCD2;1 shows strong response to plant hormones (Riou-Khamlichi *et al.* 2000). As well, the protein level of CYCD3 degrades rapidly upon sucrose removal, a direct correlation to its declining mRNA level. In contrast, the protein level of CYCD2 does not correlate to its mRNA level and the protein is relatively stable upon sucrose removal (Riou-Khamlichi *et al.* 2000). These observations suggest that CYCD2 is regulated post-translationally, whereas CYCD3 is regulated mainly on the transcript level.

1.3.2.3 H-type cyclins

Plant H-type cyclins are the regulatory subunit of CAK (CDKD and CDKF) and shares 40-60% sequence homology with the mammalian counterpart. In rice and poplar, high transcript levels of CYCH were observed with dividing cells (Yamaguchi *et al.* 2000). Yeast two-hybrid analysis reveals that rice CYCH interacts with Rice R2 kinase and not with other rice CDKs (Yamaguchi *et al.* 2000). Recently, CYCH has been shown to form a stable complex with CDKD;2, but not CDKD;3, and its kinase activity is downregulated by WEE1 kinase (Shimotohno *et al.* 2006).

1.3.3 Cyclin-dependent kinase inhibitors (CKIs)

Another component that plays a prominent role in regulating the cell cycle in eukaryotes is the CKI. Binding of a CKI to the CDK/cyclin complex can modulate the CDK activity. CKIs have been studied extensively in humans due to their potential role as tumor suppressors. Plants also have CKIs, however much less is known about them in comparison to the mammalian CKIs.

1.3.3.1 Comparison of mammalian CDK inhibitors vs plant CDK inhibitors

In mammals, there are two families of CKIs, Kip/Cip family and INK4 family, which are different in their mode of action and sequence similarity (Sherr and Roberts, 1999). The Kip/Cip family is composed of 3 members: $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, while the INK4 family is

composed of 4 members: $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. The Kip/Cip family appears to have a broader spectrum of inhibitory effects as it can form complexes with CDK4/6-cyclin D, CDK2/cyclin E, CDK1/2-cyclin A and CDK1/cyclin B (Toysoshima and Hunter 1994; Lee *et al.* 1995). This observation suggests that Kip/Cip plays a role in both G1 to S and G2 to M checkpoints in the cell cycle. In contrast the INK4 family strictly targets G1 CDKs, CDK4 and CDK6. The mechanism by which Kip/Cip and INK4 inhibit the kinase activity of CDK/cyclin complex is also different. The INK4 protein prevents cyclin binding to CDK by causing an allosteric change in the region where the two proteins bind (Pavletich 1999). In contrast, when Kip/Cip protein binds to CDK, it does not dissociate or prevent cyclin binding. It inhibits the kinase activity by distorting the catalytic ATP-binding center of the CDK subunit (Pavletich 1999).

The first plant CDK inhibitors were isolated using the yeast two-hybrid technique using *Arabidopsis* CDKA;1 as the bait (Wang *et al.* 1997). This family of proteins was thus named ICKs (inhibitors or interactors of CDK). Another common name for this family of proteins is called KRPs (Kip-related proteins). This name was given by a group of researchers who discovered the remaining members of the ICKs family by data mining the *Arabidopsis* genomic databases (De Veylder *et al.* 2001). To avoid confusion, the name ICKs will be used exclusively for the rest of the thesis.

In *Arabidopsis*, there are a total of seven ICK members in the family. These ICKs have a region conserved among themselves as well as with the mammalian counterparts. The region of similarity between ICKs and mammalian CKIs involves a stretch of 31 amino acids residues that are found in both ICKs and $p27^{Kip1}$. This region of amino acids appears to be essential for the inhibitory function of both ICKs and mammalian $p27^{Kip1}$. Apart from this region, ICKs differ greatly among themselves and also with the mammalian CDK inhibitors. Thus far, no plant CKIs similar to the mammalian INK4 family of inhibitor have been found.

1.3.3.2 Targets of ICKs: CDKA/CYCD complex

Despite their low sequence similarity to mammalian CKIs, the ICKs have been shown to be potent inhibitors of CDK activity *in vitro* (Wang *et al.* 1997; Lui *et al.* 2000) and *in vivo* (Wang *et al.* 2000; De Veylder *et al.* 2001). ICK1 exerts its inhibitory effect by binding to and interacting with both CDKA and D-type cyclins (Wang *et al.* 1998). All ICKs from *Arabidopsis*

have been shown to be capable of interacting with D-type cyclins (De Veylder *et al.* 2001; Jasinski *et al.* 2002a; Zhou *et al.* 2002a) but not with mitotic cyclins (Zhou *et al.* 2002a) in the yeast two-hybrid system. Furthermore, the phenotypic changes of transgenic plants overexpressing ICK1 can be partially rescued by co-overexpressing CYCD2 and CYCD3 (Zhou *et al.* 2003a), indicating that ICK1 also interacts with the D-type cyclins in plants.

Besides ICK1, most of the other members of the ICK family appear to be able to also interact with CDKA. In one yeast two-hybrid experiment, all ICKs except for ICK3 were able to interact with *Arabidopsis* CDKA (De Veylder *et al.* 2001). Although in another yeast two-hybrid experiment, ICK4 and ICK5 were not able to interact with the same CDKA (Zhou *et al.* 2002a). As suggest by Wang *et al.* (2006) this differences in results may be related to the sensitivity of different yeast two-hybrid systems used. Additional and different techniques may be required to resolve the differences observed. One consensus that was reached with all the yeast two-hybrid experiments is that none of the ICKs interact with CDKB;1 (De Veylder *et al.* 2001; Jasinski *et al.* 2002a; Zhou *et al.* 2002a). This observation is further supported biochemically by the observation that ICK2 overexpressed in transgenic plants only inhibits the activity of A-type CDK, but not that of B-type CDK (Verkest *et al.* 2005). Summarizing the results from literature to date, it appears that plant CDK inhibitors most likely interact with CDKA and cyclin D and suppress the kinase activity of the CDKA/cyclin D complex.

1.3.3.3 Expression patterns of *ICKs* at the transcript level

Transcriptional regulation of genes is an important means to control the abundance and activity of the proteins. Not surprisingly, the transcript levels of *ICKs* have been studied to see when, where and how much of the transcripts and proteins are present in plants. Northern analysis of *ICK1* and *ICK2* has shown that the transcript levels of both genes are relatively low among the tissues tested: root, inflorescence, flower and seedlings (Lui *et al.* 2000). Using reverse transcriptase-PCR (RT-PCR) to study the expression of *ICK* genes (De Veylder *et al.* 2001) it was observed that *ICK1* and *ICK4* were present ubiquitously in all the tissues tested: root, inflorescence, flower, leaves and cell suspension. While *ICK3*, *ICK5* and *ICK7* were also detected in all tissues, the level of expression seems to be higher with tissues that have higher mitotic activity (i.e. flowers, suspension cultures). *ICK2* was most abundant in flowers while *ICK6* was only observed in cell suspension cultures.

The expression of *ICKs* has also been analyzed using *in situ* hybridization (Ormenese *et al.* 2004). *ICK1* and *ICK2* expression is restricted to endoreduplicating tissues, suggesting they may specifically be involved in transition between mitotic cycle to endoreduplication cycle. *ICK3* and *ICK7* were expressed in all tissues where mitotic divisions occur, indicating that the expression of these genes may play a role in regulating the progression of the mitotic cell cycle. *ICK4, ICK5 and ICK6* were all present in both mitotically dividing cells and endoreduplicating cells (Ormense *et al.* 2004). This study provides evidence that *ICKs* exhibit differential expression patterns.

1.3.3.4 Regulation of ICKs at post-transcriptional level

Similar to its mammalian counterpart, ICKs are regulated at the post-transcriptional level to control the abundance of the protein. In transgenic *Arabidopsis* plants overexpressing *ICK1*, the fusion protein level was low even though the mRNA level was high (Zhou *et al.* 2003b). The N-terminal region of ICK1 appears to be important in regulating the stability of the protein. The deletion mutant ICK1$^{109\text{-}191}$ (amino acid 1-108 deleted) has been shown to interact with both CDKA and CYCD3;1 much stronger than full length ICK1 in the yeast two-hybrid system (Wang *et al.* 1998). When the deletion mutant is overexpressed in transgenic plants, higher protein expression level was detected for the mutant than the full length ICK1. Furthermore, the phenotypic effects of overexpression of the ICK1$^{109\text{-}191}$ deletion mutant in transgenic *Arabidopsis* plants appear much more severe (i.e. smaller plant size and stronger leaf serrations) than the full length ICK1 (Zhou *et al.* 2003b).

Post-transcriptional modification such as phosphorylation is one of many important regulatory mechanisms in eukaryotes. The phosphorylation state of a protein often determines its cellular function and destiny in cells. Quite often, the phosphorylation of a protein signals its degradation. One way by which proteins are degraded is through ubiquitin-proteasome complex. The ubiquitin-proteasome pathway plays a critical role in controlling the proper protein level in eukaryotic cells. Proteins that are targeted for degradation are marked by the covalent attachment of multiple ubiquitin molecules, a process called ubiquitylation (Review by Roos-Mattjus 2004). The ubiquitylated proteins are recognized and eventually degraded by the 26S proteasome complex.

Mammalian and yeast CDK inhibitors have been shown to be phosphorylated by CDKs and subsequently targeted for degradation by the proteasome (Vlach *et al.* 1997; Tomoda *et al.* 1999). A recent study suggests that plant CDK inhibitors may also share this mode of regulation with the mammalian and yeast counterparts. In one study, ICK2 was found to be phosphorylated by both CDKA;1 and CDKB;1 *in vitro* and that the level of recombinant ICK2 protein accumulates when a proteasome inhibitor, MG132 was added (Verkest *et al.* 2005). Also, the proteolysis of ICK2 protein appears to be dependent on CDKB;1 kinase activity, since no destruction was observed in extracts incubated with olomoucine, a strong and specific inhibitor of CDK activity (Verkest *et al.* 2005). Even though ICK2 appears to be subjected to proteolysis in a phosphorylation dependent manner, more work needs to be conducted to determine whether other ICKs behave in a similar manner. Since ICKs appear to have different roles in regulating the cell cycle, it will be interesting to understand how their protein levels are regulated. So far, only limited data are available mostly from *in vitro* experiments.

1.3.3.5 Effects of ICK overexpression on plant growth and development

The question of whether cell cycle regulators play a significant role in plant growth and development has always been a heavily debated subject. Traditionally, the thinking was that cell division plays a minor role in morphogenesis of plants (Review by Kaplan and Hagemann 1991). Recently, more evidence suggests that cell division and plant development may be more intrinsically linked than previously thought (Review by Doonan 2000). The overexpression of ICKs has been shown to severely affect cell cycle progression, but what effect does it have on the growth and development of plants? Transgenic plants overexpressing *ICK1, ICK2, ICK4, NtKIS1a* (a tobacco ICK-related gene), and *ICKCr* (a Chenopodium ICK1-related gene) all display similar phenotypes, in that the leaves of the transgenic plants are serrated and the overall size of the plant is smaller compared to wild type plants (Wang *et al.* 2000; De Veylder *et al.* 2001; Jasinski it al. 2002b; Zhou *et al.* 2002a). It is easy to understand why the plants would be smaller due to inhibition of the cell cycle, but why are the leaves serrated? Perhaps the strong reduction in cell number due to cell division inhibition also contributes to this phenotype in the more sensitive 'teeth' region of the leaves. Nevertheless it appears that manipulation of ICK protein level can affect organ morphogenesis. This idea is further reinforced by the study of targeting ICK1 expression in petal primordial of *Brassica* plants using the *Arabidopsis* AP3

(APETALA3) promoter (Zhou *et al.* 2002b). Some of the petals in the transgenic plants were severely altered into tubular shapes (Zhou *et al.* 2002b). ICK1 has also been expressed in pollen using a pollen specific promoter. While no phenotypic changes can be observed, the pollen viability decreased resulting in reduced fertility indicating that ICK1 expressions play a role in affecting nuclear division during pollen development (Zhou *et al.* 2002b).

The above results regarding the effects of ICKs are obtained with transgenic overexpression. It is not clear at present about the physiological concentrations of ICKs and their effects in wild type *Arabidopsis* plants. One approach to determine the functions of ICKs in normal plants is to study T-DNA insertion mutants in which the expression of gene of interest is knocked out. However, no phenotype has been reported with any of the ICKs when its function is disrupted (Wang *et al*. 2006).

1.3.3.6 Level of ICKs controls cell cycle progression, endoreduplication and cell cycle exit

Cell division in eukaryotes is divided into four sequential ordered phases: S phase (DNA replication), M phase (mitotic division) and in between them are two gap phases (G1 and G2) that are important to ensure that the previous phase has been accurately and fully completed before proceeding to the next phase. Once the cells finish DNA replication, they pause in G2 phase to ensure proper criteria are met before embarking on mitotic division. It is interesting to note that in some cases, cells after finishing DNA replication will not proceed to the mitotic division phase, but rather repeat the replication of DNA. This process is called endoreduplication, simply defined as duplication of genomic DNA without mitosis (Joubes and Chevalier 2000). In plants, endoreduplication is widespread and in *Arabidopsis*, the ploidy levels can be up to 32C in the final stages of leaf development (Galbraith *et al.* 1991).

The decision of the cell to undergo mitotic division or endoreduplication is under the control of cell cycle regulators. In *Arabidopsis*, overexpression of an *ICK* inhibits endoreduplication in leaves (De Veylder *et al.* 2001; Jasinski *et al.* 2002b; Zhou *et al.* 2002a). Since ICKs target CDKA and D-type cyclins, CDKA and D-type cyclins may be important for endoreduplication. Consistent with this idea, it was observed that a dominant negative mutant of CDKA reduces endoreduplication in maize endosperm (Leiva-Neto *et al.* 2004). As well, CYCD3 expression level increases in endoreduplicating maturing tomato tissues, suggesting that

some D-type cyclins are involved in endoreduplication by promoting the G1 to S transition (Joubes and Chevalier 2000).

Recently it was observed that different expression levels of ICKs may be critical in determining whether a cell undergoes cell cycle progression, endoreduplication or cell cycle arrest. In wild type plants where the protein level of ICKs are relatively low, cell cycle progression is permitted. In transgenic plants weakly overexpressing ICK2, entry into mitosis is blocked but progression through S-phase is still allowed resulting in an increase in DNA ploidy levels (Verkest *et al.* 2005). Comparatively, in transgenic plants strongly overexpressing ICK2, CDK activity is inhibited in both mitotically dividing and endoreduplicating leaf tissues, and the ploidy level was lower than transgenic plants weakly expressing ICK2 (Verkest *et al.* 2005). The contradicting effects of ICKs on ploidy level may be due to two possibilities (Wang *et al.* 2006). Either higher levels of CDK/cyclin activity are required for entry into mitosis than for entry into S-phase or ICK2 has a higher affinity for an inhibitory effect on mitotic cyclin/CDK complexes. Thus it appears that the level of ICKs dictates which cell cycle pathway the cell takes, mitotic versus endoreduplication. A low level of ICK inhibits mitotic cycles but permits endocycles, whereas a higher level of ICK inhibits the endocycle resulting in lower ploidy levels.

1.4 G1 to S transition.

One critical stage for the regulation of the cell cycle in eukaryotes is G1 to S transition. G1 to S entry is initiated by the synthesis of D-type cyclins upon mitogenic stimulation. In plants, the protein level of CYCD3 is induced in response to sucrose and hormones and form active CDKA/cyclin complex (Figure 1.1). Furthermore, the enzymatic activity of CDKA/cyclin D is also regulated by being phosphorylated by CDKD/cyclin H complex. The active CDKA/cyclin D complex then phosphorylate the Rb protein, preventing its association with the E2F transcription factor, thus allowing transcription of genes responsible for cell cycle progression. In the process described above, CYCD3 appears to be the rate-limiting factor, as overexpression of this gene stimulates cells to exit the G1 phase (Nakagami *et al.* 2002). Conversely, CDK inhibitors or ICKs function to negatively regulate the cell cycle progression by inactivating the CDKA/cyclin D3 complex. There is preliminary evidence to show that ICK2 appears to be regulated by CDKB through a phosphorylation mechanism and targeted to the proteasome for protein degradation (Verkest *et al.* 2005). Thus, the control of G1 to S cell cycle

progression involves a complex mix of positive and negative regulators, the expression of which are controlled spatially and temporally to ensure proper regulation of the cell cycle.

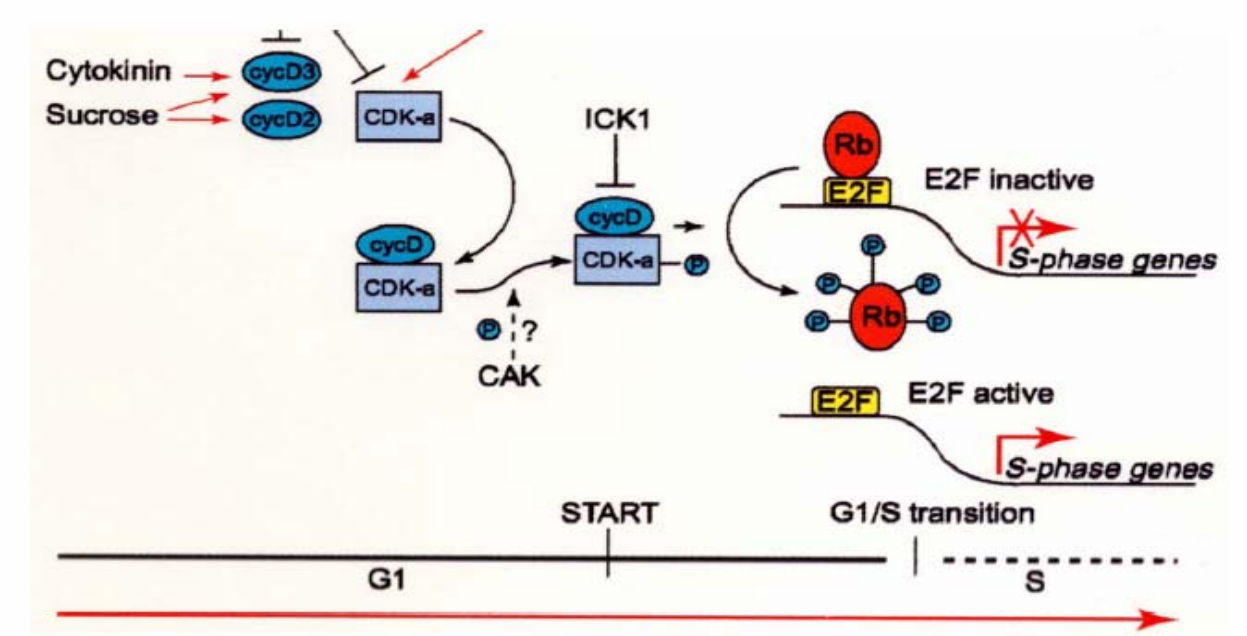

Figure 1.1. Model for the control of G1 to S transition in plants. D-type cyclins (CYCDs) are induced by cytokinin and sucrose and form complexes with CDKA. The CDKA/CYCD complex is further regulated positively by CAK phosphorylation and negatively by CDK inhibitors such as ICK1. In the absence of a negative regulator, active CDKA/CYCD complex phosphorylates Rb protein, effectively dissociating Rb from the E2F transcription factor. The unbound E2F becomes active and activates the transcription of various S-phase related genes required for cell cycle progression. The above figure is taken from den Boer and Murray (2000).

1.5 G2 to M transition

In plants, both CDKA and CDKB appear to be important for the G2 to M transition by interacting with both A and B type cyclins. CDKB probably regulates plant-specific features of cell cycle since no homologue of this gene is found in other species. CDKA/B complexed with cyclin A/B is also regulated through phosphorylation by the CDKD/cyclin H complex as well as WEE1 kinase (Shimotohno *et al.* 2006). WEE1 kinase phosphorylates CDKs on both Tyr15 and Thr14 residues to inhibit ATP fixation and blocks substrate binding. CDKs phosphorylated at these residues are thus inactive. A homolog to WEE1 has been characterized in maize and *Arabidopsis* (Sun *et al.* 1999; Sorrell *et al.* 2002). In animals, a phosphatase CDC25

dephosphorylates the Tyr15 and Thr14 residues to activate the CDK/cyclin H complex. In plants, a CDC25-like protein has been identified. However, it lacks a regulatory domain (Landrieu *et al.* 2004) and its function as the typical CDC25 is questioned (Boudolf *et al.* 2006). All together, a complicated network of interactions between multiple proteins is required to drive the cell to and through the mitotic divisions.

1.6 Yeast two-hybrid system

Specific interactions between proteins form the basis of many essential biological processes. The yeast two-hybrid system, a technique that was developed in 1989, has revolutionarized how researchers study protein-protein interactions (Fields and Song 1989). One advantage of using the yeast two-hybrid system is the ability to identify novel proteins that interact with a known, well-characterized protein (Hoppe-Seyler *et al.* 2001). As well, this system can be used to study the interaction of known binding partners in detail, such as defining, by mutational analysis, the protein domains required for binding. The premise behind the yeast two-hybrid system is that the domains of transcription factors, DNA-activation and DNA-binding domains, are modular and can function in close proximity to each other. Thus, even when the DNA-activation and DNA-binding domains are split apart, they remain functional when brought together by two interacting proteins. The protein of interest, 'X' is often fused to the DNA-binding domain (BD) and expressed as 'bait' in the yeast strain. The other protein, 'Y' or a cDNA library is fused to the DNA-activation domain (AD) and referred to as the 'prey'. Neither the bait nor the prey can activate transcription by itself. However, if the bait and prey interact, the DNA-activation and DNA-binding domains are bridged together by the interacting proteins. This transcription factor is reconstituted and thus able to activate the promoter of a marker gene which can be monitored by colorimetric assay or by growth selection.

1.6.1 GAL4-based yeast two-hybrid system vs LexA-based yeast two-hybrid system

Two most common yeast two-hybrid systems employ the yeast GAL4 or *Escherichia coli* (*E. coli)* LexA transcription factors. Both two-hybrid systems function similarly in theory, with the main differences being the different activation domains, origins of replication, and reporter system used. The GAL4-based yeast two-hybrid system uses both the DNA-binding domain and DNA-activation domain from the yeast GAL4 transcription factor. The LexA yeast two-hybrid

uses the DNA-binding domain from the LexA protein and uses the activation domain from VP16, a herpes simplex virus protein or a weaker activation domain derived from bacteria, called B42. Different yeast two-hybrid vectors may have the ARS4/CENS origin of replication that has a low copy number in yeast, or the "2μ" origin of replication that has a relatively high copy number. Both systems use the lacZ reporter gene, which when activated, will turn the yeast colony into a blue color due to the cleavage of X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) by beta-galactosidase. In addition, use of multiple reporter genes (i.e. nutrient marker like His-) help eliminate some of the false positives commonly observed with both yeast-two hybrid systems. One of the advantages of using the LexA-based yeast two-hybrid system is that fusion proteins can be expressed inducibly under the control of the GAL1 promoter, allowing high levels of expression in the presence of galactose and repression in the presence of glucose. This regulated expression allows rapid elimination of background colonies due to strain mutations. However, both systems have been proven to be successful in studying protein-protein interactions.

1.6.2 Peptide aptamers

Peptide aptamers represent a novel approach in identifying molecules which can disrupt protein-protein interactions *in vivo* (Geyer and Brent 2000). Peptide aptamers are molecules in which a variable peptide with affinity for a target protein is displayed from a scaffold protein. They function under intracellular conditions and can block the function of the target proteins upon binding to them. Peptides with randomized amino acids of 20 residues are synthesized and displayed in the active site of *E. coli* thioredoxin (TrxA). The length of 20mer is an ideal size since it is long enough for the aptamers to fold into a diverse patterns of shape and charge, but short enough so that the number of stop codons introduced into the encoding oligonucleotides is not overwhelming. Peptide aptamer libraries are screened against bait proteins using the LexA based yeast two-hybrid assay to select for aptamers that bind chosen baits. Peptide aptamers that are selected to bind a target protein can be further analyzed for their ability to interfere with the functions of the target. Thus, these aptamers can be used as trans-dominant agents to control and inhibit protein-protein interactions.

It has been shown that peptide aptamers could disrupt the functions of a number of proteins, including two important cell cycle regulatiors: CDK2 and E2F (Colas *et al.* 1996;

Fabbrizio *et al.* 1999). Peptide aptamers generated against CDK2 were able to interfere with CDK2-mediated phosphorylation of histone H1, but not with CDK2-mediated phosphoryation of pRb (Colas *et al.* 1996). This is due to the non-overlapping regions on the CDK2 molecule for H1 and pRb interactions. If the target protein has non-overlapping functional domains, peptide aptamers may inhibit one specific function of the protein without affecting the others depending on where the peptide aptamer binds. This functional feature of peptide aptamers is very attractive, if it is desirable to block one specific function, but not other functions, of a target protein.

1.7 Research objectives

The research objectives for this master thesis are as follows: (1) Isolate and characterize peptide aptamers that interact with ICK1. This project was conducted in collaboration with Dr. Ron Geyer. (2) Identify regions of CYCD3 responsible for interacting with ICK1 using the yeast two-hybrid system. (3) To determine the association and interaction of ICKs with the CDK complex *in vivo* and the effects of transgenic expression in plants. (4) To determine whether ICKs are regulated by protein phosphorylation and further whether the protein level of ICKs is controlled through the ubiquitin-proteasome pathway.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial transformation and plasmid isolation

2.1.1 Plasmid isolation

A 5 ml culture of bacterial cells was grown overnight at 37^{o}C in LB medium containing an appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin). The next day, bacterial cells were collected by centrifugation and the pellet was re-suspended in 200 μl of solution 1 (25 mM Tris-HCl (pH 9.0), 10 mM EDTA and 50 mM glucose). The cells were then mixed with 250 μl of solution 2 (0.2 N NaOH and 1% SDS) and following incubation on ice for 5 minutes, 250 μl of solution 3 (3 M KOAc, pH 5.2) was added. The solutions were mixed thoroughly and 1/10th volume of 1 M $MgCl_2$ was added. The cell lysate was incubated for 10 minutes at -20^{o}C, and centrifuged. The upper liquid layer was transferred to a new 1.5 ml microtube. One volume of 2-propanol was added and the tube was incubated for 10 minutes at room temperature. The tube was centrifuged and the pellet was washed with 70% ethanol. The pellet was then air-dried and resuspended in 30 μl of H_20 with 10 μg/ml RNAase.

2.1.2 Bacterial transformation

Competent *E. coli* DH10 cells for electro-transformation were prepared as described (Shi *et al.* 2003). Plasmid DNA was added to 25 μl of competent cells and subjected to a high voltage charge of 1.6 kV using an electroporator (Eppendorf Electroporator 2510). Electroporated cells were collected with 1 ml of pre-chilled LB medium added to the cuvette immediately after the electroporation and incubated at 37^{o}C for 1 hr without shaking. Cells were plated onto LB plates containing an appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) and plates were incubated at 37^{o}C overnight.

2.2 LexA-based yeast two-hybrid system

2.2.1 Yeast manipulations

YPD (yeast peptone dextrose: 1% yeast extract, 2% peptone and 2% glucose) was used as the medium for non-selective growth of yeast. SD medium (synthetic dextrose: 0.67% yeast nitrogen base without amino acids and with addition of any necessary auxotrophic supplements at recommended concentrations) was used for selective growth of yeast auxotrophs. Minimal dropout media are designated by the component that is left out (e.g. His-Trp-Leu- medium lacks histidine, tryptophan, and leucine). Each minimal dropout medium contains either 2% glucose (Glu) or 2% galactose plus 1% sucrose (Gal/Suc). X-Gal minimal drop-out plates contain X-gal and phosphate buffer at pH 7.0 (Geyer and Brent 2000). Yeast cells were transformed by the modified lithium acetate method described by Elble (1992). Yeast cultures were inoculated overnight at 30^{o}C. The following day, the 1 ml of cell cultures were spun down for 5 seconds. The supernatant was removed, leaving approximately 100 µl solution at the bottom of the tube with the cells. Ten micrograms of carrier DNA, 0.5 µg of plasmid, 250 µl of PLATE mixture (sterile 45% PEG 4000, 1 M LiOAc, 1 M Tris-Cl (pH 7.5), 0.5 M EDTA) and 10 µl of 1.0 M DTT (dithiothreitol) were added to the cell suspension. The cell culture was incubated at room temperature overnight and subjected to heat shock treatment (10 minutes at 42^{o}C) the next day. Yeast cells were plated on appropriate amino acid dropout plates. Yeast plasmid minipreps were prepared by the 'smash and grab' procedure of Hoffman and Winston (1987). The *E. coli* strain MC1061 (Invitrogen) was used as competent cells for the transformation.

2.2.2 Isolation of peptide aptamers

The 20mer random peptide aptamer libraries used in this study were constructed in Dr. Ron Geyer's laboratory as described (Geyer and Brent 2000). *Saccharomyces cerevisiae* strains EGY48 (Matα his3 trp1 ura3-52 leu2::LexA6op-LEU2) and EGY42 (Mata his3 trp1 ura3 leu2) were used for the library screen. The LexA fusion protein was expressed from the *ADH1* promoter on the bait expression vector pEG202 (Geyer and Brent 2000). Aptamers fused to the B42 DNA-activation domain were conditionally expressed from the *GAL1* promoter on the plasmid pJM-1 (Colas *et al.* 1996), a derivative of pJG4-4 (Gyuris *et al.* 1993). The pJM-1-derived peptide library in EGY42 were mated with the bait in EGY48. The mated cells were plated onto YPD plates and incubated at 30^{o}C for 3 days. Yeast colonies were collected by

scraping the plates and the collected cells were stored at -80^{o}C as frozen stock. Aliquots of the frozen stock were plated onto Glu His-Trp- plates and grown at 30^{o}C to select for colonies that contained both the bait and prey plasmids. Colonies from these plates were replica-plated using sterile velvet onto Gal/Suc His-Trp-Leu-Ade- X-gal plates for determining reporter activity and visualization of interaction between the bait and prey (Geyer and Brent 2000). The pJM-1 plasmids containing the selected aptamers were recovered by selecting the yeast colonies and culturing them in SD Trp- medium. The plasmids were isolated and used to transform the *E. coli* strain MC1061. The recovered pJM-1 plasmids were re-transformed into the yeast strains containing the bait constructs to confirm interactions.

2.3 GAL4-based yeast two-hybrid system

2.3.1 Transfer of peptide aptamers and yeast two-hybrid analysis

The yeast strain used for all GAL4-based yeast two-hybrid experiments was MaV203 (Matα, leu2-3, 112, trp1-901, his3Δ200, ade2-101, gal4Δ, gal80Δ, SPAL10::URA3, GAL1::lacZ, $HIS3U_{ASGAL1}$::HIS3@LYS2, $can1^{R}$, $cyh2^{R}$) (Vidal 1997; cell line was from GIBCO/BRL Life Technologies). The DNA-binding domain vector (bait vector) was pDBLeu, a derivative of pPC62 (Chevray and Nathans 1992) and the DNA-activation domain vector (prey vector) used was pPC86 (both vectors were from GIBCO BRL/Life Technologies). Primers were designed to amplify the 20 amino acid peptide aptamers from the pJM-1 plasmid and the PCR fragments were purified using the Qiagen PCR purification kit (Qiagen, Canada) according to the manufacturer's instructions. The recovered fragments were cloned into the bait vector pDBleu (and later into the prey vector pPC86 for domain swapping) and used to transform competent *E. coli* strain DH10 cells. All clones were verified by PCR. The constructs were used to transform MaV203 yeast strain carrying ICK1, cloned in the prey vector (bait vector for domain swapping). Interactions in the yeast two-hybrid system were analyzed by X-gal filter assay (Chevray and Nathans 1992). Sterilized filter paper (Whatman) was used to lift yeast cells from plate and the filter was submerged in liquid nitrogen to permeabilize the cells. The filter paper was then placed (cell side up) in a Petri dish covered with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$) plus 2-mercaptoethanol (2.7 ml/L) and X-gal (50 mg/ml). The plates were incubated at 30^{o}C and checked at 2 hr and 24 hr for visualization of blue colonies.

2.3.2 Construction of CYCD3 deletion mutants and yeast two-hybrid analysis

To analyze the interaction of *Arabidopsis* CYCD3;1 (referred to as CYCD3 hereafter; Soni *et al.* 1995), both the N and C-terminal deletion mutant constructs were prepared by PCR using *Pfu* DNA polymerase with sequence-specific primers (See Table 2.1). The PCR fragments were amplified and cut with SalI and NotI and purified following agarose gel electrophoresis. The resulting DNA fragments were cloned into the bait vector pDBLeu (or into the prey vector for domain swapping). The desired deletion clones were verified by PCR and DNA sequencing. The constructs were used to transform the yeast strain MaV203 harboring ICK1, cloned into the prey vector (bait vector for domain swapping) and grown on selective SD medium with the appropriate amino acids dropped out according to the manufacturer's instructions (GIBCO BRL/Life Technologies). Three or more independent transformants were tested for interactions for each construct using X-gal filter assay as described above.

2.4 Construct preparation and plant transformation

ICKs (ICK1 to ICK7) cDNAs were first cloned into the cloning vector pWZ851 (pZ31C) (Zhou *et al.* 2003b). The *BamH*I-*EcoR*I fragments from pWZ851 were then cloned separately and linked transcriptionally behind the CaMV 35S promoter in the plant expression vector pBI121 (Clontech, USA). For this study, the GFP-ICK1 construct was from Dr. Yongming Zhou; GFP-ICK2, GFP-ICK3, GFP-ICK6 and GFP-ICK7 constructs were prepared by Dr. Xianzong Shi; and GFP-ICK4 and GFP-ICK5 constructs were prepared by my self using the same cloning strategy. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 (An *et al.* 1998).

Transformation of *Arabidopsis thaliana* ecotype Columbia was performed as described (Wang *et al.* 2000). Plants were grown in 10-cm pots in a growth room (20-22^{o}C constant temperature, 16/8 h (day/night) photoperiod). Seeds (T_1) from infiltrated plants were harvested and selected on half strength MS basal medium (Sigma-Aldrich) containing 50 mg/l kanamycin and 300 mg/l timentin (½ MSTK). Seeds were sterilized in 20% bleach and 0.02% Triton X-100 for 15 minutes and rinsed with sterile distilled H_2O three times. Kanamycin-resistant seedlings were transferred to soil in pots and grown in a growth room. Seeds (T_2) from selected lines were collected and used for subsequent experiments.

Table 2.1. Primers used for preparing *Arabidopsis* CYCD3 mutants.

Name	Sequence	Used for
#90	5′-CAGTGCGGCCGCTTTCGATTATGGAGTGGC	N-terminal deletion mutants
#206	5′-CAGTGTCGACAATGTCTTCTTCTTCTTCTCC	ΔN42 mutant, with primer #90
#207	5′-CAGTGTCGACAATGCTTTCCACGGATCGAAA	ΔN82 mutant, with primer #90
#208	5′-CAGTGTCGACAATGAGCTTACAGAGAGACAA	ΔN122 mutant, with primer #90
#209	5′-CAGTGTCGACAATGAAGTATGTGTTTGAAGC	ΔN162 mutant, with primer #90
#210	5′-CAGTGTCGACAATGGGACTTAAGAACAATGC	ΔN202 mutant, with primer #90
#89	5′-CAGTGTCGACAATGGCGATTCGGAAGG	C-terminal deletion mutants
#211	5′-CAGTGCGGCCGCTTAGTTTGAGCTTTCGTCGC	ΔC49 mutant, with primer #89
#212	5′-CAGTGCGGCCGCTTATAGTTGGAGGATTAGA	ΔC94 mutant, with primer #89
#213	5′-CAGTGCGGCCGCTTACATGGTAGCTGCCGC	ΔC134 mutant, with primer #89
#214	5′-CAGTGCGGCCGCTTATCTCCTGATAATGTGGTC	ΔC175 mutant, with primer #89
#401	5′-CAGTGCGGCCGCTTATGTCTCCTCCTCTTGAA	ΔC214 mutant, with primer #89
#402	5′-CAGTGCGGCCGCTTAGTAGCTACAGATGAACT	ΔC254 mutant, with primer #89
#403	5′-CAGTGCGGCCGCTTAATAAACATCATCGAGAC	ΔC304 mutant, with primer #89
#404	5′-CAGTGCGGCCGCTTACAAGGAAGAGTTTTCTT	ΔC344 mutant, with primer #89

2.5 Plant phenotype surveys

Approximately 100 kanamycin-resistant plant seedlings for each construct growing on ½ MSTK plates for three weeks were transferred to soil (½ Sunshine mix and ½ ReadyEarth; purchased from Phytotron Plant Facilities, Agriculture Building, University of Saskatchewan). Plants freshly transferred to soil in 10-cm pots were covered with plastic lids to prevent plants from drying out. The lid was gradually opened until it was completely removed after one week. The sizes and phenotypes of transgenic plants were surveyed in two weeks after the transfer to soil. The diameter (the greatest width from leaf to leaf at the base) of the each plant was

measured. Plants were surveyed for leaf serration and flower morphology. Plants were categorized as having strong serrations if they had obvious and severe serrations in all of the leaves and were categorized as having moderate serrations if they had obvious but not severe serrations in most of the leaves. For flower phenotype, plants were categorized as having strong flower abnormality if short pedicel, smaller petals and clustering of flower buds were observed. Plants were categorized as having moderate flower abnormality if moderate reduction in petal size and moderate clustering of flower buds were observed.

2.6 Protein extraction and Western blotting analysis

Protein samples were extracted from either seedlings or floral and leaf tissues of 3-4 week old plants with the extraction buffer (25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 1 mM beta-glycerophosphate, 2.5 mM EDTA and Sigma protease inhibitor cocktail (Sigma # P9599)) as described (Wang *et al.* 1998). For Western blotting analysis of GFP fusion proteins, 25 µg of total protein was used for each sample and separated by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% resolving gel). The SDS-PAGE sample buffer (6X buffer consisted of 0.25 M Tris-HCl pH 6.5, 15% SDS, 50% glycerol, 25% ß-mercaptoethanol, 0.01% bromophenol blue) was added to the protein sample, followed by heating at 95^{o}C for 10 minutes. The sample was used in electrophoresis. Following electrophoresis, proteins were transferred onto a piece of PVDF membrane (Bio-Rad, USA) using Bio-Rad Mini-protein apparatus. The blot was washed with distilled H_2O briefly, then PBS buffer and blocked in 5% skimmed milk in PBS-T (PBS with 0.1% Tween 20) for either 1 h at room temperature or overnight at 4^{o}C. The membrane was probed for 1 hr with a monoclonal antibody against GFP (Clontech, USA; with a dilution of 1:3000) in PBS-T containing 2.5% skimmed milk and rinsed with PBS-T twice (5 minutes each). The blot was then incubated with an anti-mouse horseradish (IgG) peroxidase conjugate (Bio-Rad; dilution 1:4000 in PBS-T containing 2.5% skimmed milk) for 1 hr and washed four times with PBS-T. Probed proteins on blots were visualized using ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotech) following the manufacturer's instructions.

2.7 Affinity purification of proteins with p13^{Suc1}-agarose beads.

In all experiments involving proteins pulled down with p13^{Suc1}-agarose beads, 200 µg of proteins were mixed with 6 µl suspension of p13^{Suc1}-agarose beads. The mixture in a tube was incubated, tumbling on a rotator at 4^{o}C for 3 hrs followed by three washes with WB2 buffer (50 mM Tris-HCL (pH 7.4), 250 mM NaCl, 0.1 % NP-40, 2.5 mM EDTA, and Sigma protease inhibitor cocktail). The purified proteins were resolved by SDS-PAGE and subjected to Western detection using a GFP antibody as described above.

2.8 Calf intestine phosphatase treatment of total proteins and proteins purified with p13^{Suc1}-agarose beads

For calf intestine phosphatase (CIP) treatments, 4 units or 20 units of CIP (New England Biolabs) or were used to treat 25 µg of total proteins or proteins affinity-purified with p13^{suc1}-agarose beads from 200 µg total proteins. CIP treated samples were incubated at either 37^{o}C (total proteins) or room temperature (protein samples pulled down with p13^{Suc1}-agarose beads) for 20 minutes. The enzymatic activity of CIP was halted by adding the SDS-PAGE sample buffer, and treated at 95^{o}C for 10 minutes. Samples were analyzed by Western detection as described above.

2.9 Treatment of seedlings with proteasome inhibitor, MG132

Twelve- to fourteen-day old T_2 seedlings were treated with 100 µM of carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) or equal volume of dimethyl sulfoxide (DMSO, control) dissolved in ½ MS media. Seedlings were removed from incubation at 0, 4 and 8 hr, and used for protein extraction (Wang *et al.* 1998). Proteins were extracted from the seedlings and 25 µg of protein was separated by SDS-PAGE and analyzed by Western blotting as described above.

2.10 Bioinformatics analysis

Primary sequences of ICKs were entered into three different internet based programs, Netphos1 (http://www.cbs.dtu.dk/services/NetPhosK/) , Netphos2 (http://www.cbs.dtu.dk/services/NetPhos/) and Disphos (http://core.ist.temple.edu/pred/pred.html) to predict possible amino acid residues (serine, threonine and tyrosine) that can be phosphorylated (Blom *et al.* 1999; Iakoucheva *et al.* 2004). Due to the vast amount of 'hits' for

each program, the stringency of each program was controlled. For Netphos1, only residues that were predicted by the program to be phosphorylated by Cdc2 were included. For Netphos2, an artificial neural network computation method is used to predict how likely a particular residue is phosphorylated (Blom *et al*. 1999). The score ranges from 0 to 1. The higher the score, the more likely a residue is phosphorylated. A cutoff value of 0.8 was used in this study. Disphos relies on position-specific amino acid frequencies and disorder information for the predictions (Iakoucheva *et al*. 2004). The score generally approximates probability that the residue is phosphorylated. A cutoff value of 0.5 was used in this study. The amino acids that were predicted by all three programs were deemed the 'consensus' residues. Only the results for serine and threonine residues predicted to be phosphorylated were presented in this study.

CHAPTER 3: ISOLATION OF PEPTIDE APTAMERS INTERACTING WITH ICK1 AND CHARACTERIZATION USING TWO YEAST-TWO HYBRID SYSTEMS

3.1 Results

3.1.1 Isolation of peptide aptamers in a LexA-based yeast two-hybrid system

The *Arabidopsis* CDK inhibitor ICK1 and ICK1$^{109\text{-}191}$ (a truncated ICK1 containing a C-terminal CDK inhibitory region conserved among ICKs) were used as the bait to screen two different peptide aptamer libraries. Peptide aptamers were either fused to a scaffold protein, the *Escherichia coli* thioredoxin (*E. coli* TrxA) or an intein. The intein library is a cyclic peptide yeast two-hybrid library with 7 amino acids designed by a graduate student, Kris Barreto in Dr. Ron Geyer's lab. A total of 6.5 x 10^8 and 1.0 x 10^7 yeast transformants for the *E. coli* TrxA and intein library respectively were screened by the LexA-based version of the yeast two-hybrid system. The yeast transformants were plated onto Gal/Suc His-Trp-Leu-Ade- X-gal plates to allow selection and visualization of blue colonies that indicated an interaction between the bait (ICK1) and prey (peptide aptamers). The color intensity of colonies on X-gal plates was rated (data not shown). Since the color intensity is correlated to the strength of reporter gene expression, aptamers that had a stronger interaction with ICK1 would be expected to have a stronger color. For the purpose of this study, only aptamers that have a strong interaction with ICK1 were selected. To ensure that a single prey plasmid, not multiple prey plasmids, was responsible for the interaction with the bait, individual blue colonies were selected and cultured in SD Trp- media to select only for the prey plasmid. To recover the plasmid, yeast DNA mini-prep was obtained and used to transform *E. coli* cells (MC1061). Transformants were selected in LB medium containing ampicillin. The plasmid was then purified using an alkaline mini-prep method and subsequently used to transform the yeast strain EGY48 carrying the bait plasmid. Transformants were plated onto Gal/Suc His-Trp-Leu-Ade- X-gal and if a single prey plasmid was responsible for interaction with the bait, all of the colonies on the plate should turn blue. If the population of yeast transformants were not all blue, multiple prey plasmids were most likely

isolated in the initial screen, with one or more of the plasmids having no interaction with the bait. In such cases a single blue colony from the population was isolated and the verification process was repeated. In the end, 16 aptamers were isolated from the ICK1-TrxA screen (referred to as ICK1-TrxA aptamers), 8 aptamers from ICK1-Intein screen (ICK1-Intein aptamers), 17 aptamers from ICK1$^{109-191}$-TrxA screen (ICK1$^{109-191}$-TrxA aptamers) and 0 aptamers from ICK1$^{109-191}$-Intein screen.

3.1.2 Characterization of peptide aptamers in a GAL4-based yeast two-hybrid system

3.1.2.1 Analysis of interactions with the aptamers in the prey vector

Peptide aptamers isolated from the LexA yeast two-hybrid screens were re-cloned into the GAL4-based yeast two-hybrid system for further characterization. Aptamers were re-cloned into the DNA-binding domain vector, pDBleu (from now on referred to as the bait vector) and checked for interaction with ICK1 previously cloned in the activation-domain vector, pPC86 (from now on referred to as the prey vector) in the yeast strain MaV203. Yeast transformants were plated onto SD Trp-Leu- plates to select for the bait and prey plasmids. Interactions between the aptamers and ICK1 were analyzed using the beta-galactosidase assay. In this assay, if the aptamers and ICK1 interact with each other, the yeast colonies on the filter membrane will turn blue. The level of reporter activity or the strength of the interaction was rated by observing the blue color intensity of the colonies. Interestingly, not all of the aptamers that were positive in the LexA system in the initial screens were positive in the GAL4-based yeast two-hybrid. As shown in Table 3.1, only 8 out of 16 ICK1-TrxA aptamers, 2 out of 17 ICK$^{109-191}$-TrxA aptamers and 5 out of 8 ICK1-Intein aptamers showed varying levels of interactions. Thus, interactions that were observed between the bait and prey in the system for isolating the aptamers might not show similar interactions in the GAL4-based yeast two-hybrid system.

For the aptamers that showed an interaction with ICK1 in the GAL4-based yeast two-hybrid system, further work was performed to determine whether these aptamers could interact with a truncated ICK1 containing a shorter C-terminal region important for the ICK1 interaction with CDK. Aptamers in the bait vector were transformed into yeast cells containing ICK1$^{137-191}$ (an ICK1 deletion construct with 136 amino acid residues deleted from the N-terminus) in the prey vector or the prey vector alone. Interactions between aptamers and ICK1$^{137-191}$ were analyzed once again by beta-galactosidase assay. In terms of interaction with ICK1$^{137-191}$, 7 of 8

ICK1-TrxA, 1 of 2 ICK1$^{109-191}$-TrxA and 5 of ICK1-Intein aptamers showed varying degrees of reporter activities (Table 3.2). However, many of them also showed reporter activity with the prey vector alone, indicating that some of the aptamers, when they are fused with the GAL4 DNA-binding domain, could activate the *lacZ* gene without interacting with ICK1. Of all the aptamers tested in this experiment, only 3 of the ICK1-TrxA aptamers showed interactions with both versions of ICK1 (full length and ICK1$^{137-191}$) without activating the *lacZ* reporter gene on their own (Table 3.2). However, it should be noted that for most of the aptamers, the intensity of the color or strength of reporter activity with the vector alone was considerably less than the intensity observed for interactions with ICK1 or ICK1$^{137-191}$.

Table 3.1. Initial analysis of interactions between aptamers and ICK1 in a GAL4-based yeast two-hybrid system. Aptamers isolated from a LexA-based yeast two-hybrid system were re-tested in a GAL4-based system.

Aptamers	Number of aptamers tested	Interaction with ICK1	Strong Interaction ****/*****	Medium Interaction ***	Weak Interaction **/*
ICK1 TrxA	16	8	3	3	2
ICK1$^{109-191}$ TrxA	17	2	0	1	1
ICK1 Intein	8	5	4	0	1
Total	37	15	7	4	4

(1) Strength of interactions between aptamers and ICK1 was determined by observing the intensity of the colonies' color ranging from strong (*****) to weak (*).
(2) To ensure that the beta-galactosidase filter assay worked, a positive control (a yeast transformant with CYCD3-ICK1 interaction) was plated along with the aptamers. After two hours the intensity of the positive control was rated as (**), and after 24 hours the intensity was rated as (*****).

Table 3.2. The analysis of interactions between selected aptamers and ICK1 or ICK1$^{137-191}$ in a GAL4-based yeast two-hybrid system

Aptamers	Control (pPC86)		ICKI full length		ICK1$^{137-191}$	
ICK1$^{109-191}$ TrxA	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
21.2	/	/	/	**	/	*
ICKI TrxA	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
12.1	*	***	***	****	**	*****
38.1	*	***	**	****	**	*****
49.3	/	/	/	***	/	***
32.1	/	/	/	***	/	**
35.4	/	*	/	***	/	***
8.2	/	*	/	****	*	****
38.3	/	/	/	**	/	**
ICKI Intein	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
20.1	/	**	*	****	/	**
13.1	*	***	***	*****	/	**
11.2	*	***	**	****	/	*
19.1	/	*	/	**	/	*
12.1	**	***	**	*****	*	****

(1) To ensure that the beta-galactosidase filter assay worked, a positive control (a yeast transformant with CYCD3-ICK1 interaction) was plated along with the aptamers. After two hours the intensity of the positive control was rated as (**), and after 24 hours the intensity was rated as (*****).

Eight aptamers (6 ICK1-TrxA, 1 ICK1$^{109-191}$-TrxA, and 1 ICK1-Intein) that showed an interaction with both ICK1 and ICK1$^{137-191}$, but not with or less with the prey vector pPC86 alone were further selected to test for their interactions with other members of the ICK families. As summarized in Table 3.3, most of the eight aptamers showed interactions with several ICKs.

Table 3.3. The analysis of interactions between selected aptamers and ICKs in a GAL4-based yeast two-hybrid system

	Control (pPC86)		ICKI full length		$ICK1^{137-191}$		ICK2		ICK3	
Aptamers	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24h
Control (pLeu)	/	/	/	/	/	/	/	/	/	/
$ICK1^{109-191}$ TrxA										
21-2	/	/	/	**	/	*	/	/	/	/
ICK1 TrxA										
12-1	*	***	***	****	**	*****	**	***	**	***
49-3	/	/	/	***	/	***	/	**	/	*
32-1	/	/	/	***	/	**	/	**	/	*
35-4	/	*	/	***	/	***	*	***	/	*
8-2	/	*	/	****	*	****	*	**	/	**
38-3	/	/	/	**	/	**	/	*	/	**
ICK1 Intein	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24h
12-1	**	***	**	*****	*	****	***	*****	***	*****

Table 3.3. (continued)

	ICK4		ICK5		ICK6		ICK7	
Aptamers	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
Control (pLeu)	/	/	/	/	/	/	/	/
ICKBD TrxA								
21-2	/	/	/	/	/	/	/	/
ICK1 TrxA								
12-1	**	**	*	**	**	***	/	*
49-3	/	*	/	/	/	*	/	/
32-1	/	**	/	*	/	*	/	/
35-4	/	**	/	*	*	*	/	/
8-2	*	**	*	**	*	**	/	*
38-3	/	**	/	*	/	*	/	/
ICK1 Intein	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
12-1	**	*****	*	*****	**	*****	/	****

(1) To ensure that the beta-galactosidase filter assay worked, a positive control (a yeast transformant with CYCD3-ICK1 interaction) was plated along with the aptamers. After two hours the intensity of the positive control was rated as (**), and after 24 hours the intensity was rated as (*****).

3.1.2.2 Analysis of interactions with the aptamers in the bait vector

Since some peptide aptamers when fused with the GAL4 DNA-binding domain in the bait vector could activate reporter activity with the empty prey vector, we wanted to know (1) whether swapping the bait and prey into the 'opposite' vector could eliminate this self activation and (2) whether aptamers still retain the interaction with ICKs when they are expressed from the prey vector. The aptamers were re-cloned into the prey vector while ICK1 was cloned into the bait vector. The presence of the cloned fragment for both the aptamers and ICK1 was verified by PCR. Analyses of interactions between aptamers and ICK1 in this context were measured using beta-galactosidase filter assay on yeast transformants. Surprisingly, none of the peptide aptamers in the prey vector retained its interaction with ICK1.

To clarify the issue regarding why interactions between aptamers and ICK1 were observed in one context, but not in the other, the aptamers in the initial LexA-based prey vector (pJG4-5) were used to determine their interactions with ICK1 in the GAL4-based bait vector. Since the DNA-binding domain of GAL4 and activation domain of LexA are expected to function modularly, I determined whether the aptamers in a LexA-based prey (activation-domain) vector could interact with ICK1 in a GAL4-based bait (binding-domain) vector. Yeast transformants were plated onto SD Trp-Leu- plate and three independent colonies were streaked onto a Gal/Suc Trp-Leu- plate. Galactose was used to activate the GAL1 promoter of the aptamer plasmid. Interactions between the aptamers and ICK1 were determined using the beta-galactosidase filter assay. Seven out of the eight aptamers showed no interaction with ICK1 in this test. One of the aptamers (one of three independent colonies) showed an interaction with ICK1.

3.2 Discussion

Peptide aptamers have been shown to be able to bind to specific proteins and be used as transdominant agents to control and inhibit protein-protein interactions (Colas *et al.* 1996; Fabbrizio *et al.* 1999; Butz *et al.* 2001; Xu and Zhijun 2002; Schmidt *et al.* 2002). Due to its simplistic nature and effectiveness in affecting protein-protein interactions *in vivo*, they have been used for studying the biochemical pathways within living cells and also considered as possibly useful tools for drug therapy (reviewed by Crawford *et al.* 2003). As peptide aptamers are becoming an increasingly popular technique to study protein-protein interactions, we would like to use peptide aptamers to study the role of ICKs in plants. Our goal was to (1) isolate aptamers that are able to interact with ICK1 and (2) express these aptamers in plants to modulate the protein-protein interactions and to assess the effects of the aptamer on plants.

A total of forty-one aptamers were initially isolated with a LexA-based yeast-two hybrid system. Selected aptamers were re-cloned into a different system for further analyses mainly due to the following two reasons. (1) The family of ICK proteins has been studied in a GAL4-based yeast two-hybrid system. The aptamers with the scaffold protein thus need to be expressed in a compatible vector with the ICKs. (2) The interaction of these aptamers needed to be verified. Since the subsequent objective is to express them in plants, it is beneficial to verify the interaction when the aptamers are expressed in a slightly different cellular context. If the interaction is not retained in a different yeast two-hybrid system, it is not very likely that the interaction will be retained when the aptamer is expressed in the plant.

Of the forty-one aptamers isolated initially from the screens in the LexA-based yeast two-hybrid system, only fifteen of them showed an interaction with ICK1 after the aptamers were cloned into the GAL4-based yeast-two hybrid bait vector. The observed difference in terms of the aptamer interaction with ICK1 between the system used for isolating the aptamers and the system for characterizing the interaction can be due to several technical differences between the two systems e.g. different origins of replication for the plasmids, and different activation domains used for the two-hybrid interaction. Generally speaking, the LexA system used for aptamer isolation is more sensitive, but gives rise to higher backgrounds, than the GAL4 system used in this study. More sensitivity could allow the identification of weaker interacting aptamers. Thus, interactions that were observed between the bait and prey in the system for isolating the aptamers might not show an interaction in the GAL4-based yeast two-hybrid system.

In the GAL4-based yeast two-hybrid system, eight aptamers that were in the bait vector showed an interaction with ICK1. However, they did not show an interaction with ICK1 when they were expressed in the prey vector. There may be several possible reasons for this observation. One possible reason could be that the interactions between ICK1 and the aptamers in the GAL4-based yeast two-hybrid system are also context-dependent. Swapping the bait and prey might have altered the context within which an interaction between the bait and prey occurred initially. Similarly, the observation that no interactions were observed between aptamers in the LexA-based prey vector and ICK1 in the GAL4-based bait vector also indicates that the bait and prey interactions are affected by the proteins with which they are fused. To ensure that the negative interaction observed in the GAL4-based system was not due to the conformation change of ICK1 cloned in the bait vector, CYCD3, a known interacting partner of ICK1 (Wang *et al.*1998), was cloned into the prey vector and tested for interaction with ICK1. A strong interaction between CYCD3 and ICK1 in this context was observed, indicating that ICK1 cloned in the bait vector likely folded correctly and was properly targeted to the nucleus.

Another possible reason could be that weak interactions might not be detected due to the low levels of fusion proteins in the GAL4-based system. The plasmids of the LexA-based yeast two-hybrid system used in the present study contain the 2μ origin of replication, while the plasmids of the GAL4-based system use CEN6 origin of replication (Chevray and Nathans 1992). Since the 2μ plasmids usually have much higher copy numbers in yeast than the CEN-based plasmids (Gerbaud and Guerineau 1980; Bonneaud *et al.* 1991), the protein levels produced from CEN-based plasmids are expected to be lower. Thus, two proteins that show a weak interaction in the LexA-based system used may not show an interaction when tested in the GAL4-based system. In addition, the binding affinity of the GAL4 and LexA binding domains to DNA may also be different, contributing to the differences observed between the two systems.

CHAPTER 4: IDENTIFICATION OF REGIONS IN CYCD3 INTERACTING WITH ICK1

4.1 Results

4.1.1 Interactions between CYCD3 deletion mutants in bait vector and ICK1 in prey vector

Since the approach to isolate aptamers able to interact with ICK1 produced only limited results, and D-type cyclins all interact with ICK1, it was decided that it would be interesting and fitting to identify regions or motifs in CYCD3 responsible for interacting with ICK1. To that end, a series of deletion mutants were prepared and cloned into the GAL4-based bait vector pDBLeu. In total, five CYCD3 N-terminal deletion mutants (ΔN42, ΔN82, ΔN122, ΔN162, and ΔN202 with the number following the letter "N" indicating the number of amino acid residues deleted from the N-terminus) and eight CYCD3 C-terminal deletion mutants (ΔC49, ΔC94, ΔC134, ΔC175, ΔC214, ΔC254, ΔC304, ΔC344) were prepared. The deletion mutants were designed so that sequence fragments of similar lengths (40-50 amino acid residues) were removed stepwise from either the N-terminus or C-terminus. These constructs were confirmed by DNA sequencing and used for determining the interaction with ICK1 in the prey vector. The full length CYCD3 (376 amino acids) was used as a positive control. As shown in Table 4.1, full length CYCD3 was able to interact with ICK1, which resulted in a strong activity of the reporter beta-galactosidase. Interestingly, deletion of 42 amino acids in the N-terminal region of CYCD3 abolished most of the ability of CYCD3 to interact with ICK1. Further deletion (i.e. deletion of N-terminal 82 residues) completely abolished CYCD3 interaction with ICK1. For the C-terminal deletion mutants, deletion of 49 and 94 amino acids from the C-terminal region of CYCD3 resulted in a weaker interaction with ICK1 compared to the full-length CYCD3 (Table 4.1). Surprisingly, ΔC175 showed a weak interaction with ICK1 while ΔC134 did not show an interaction with ICK1. To confirm this observation, this experiment was repeated and similar results were observed. Furthermore, ΔC304 also showed an interaction with ICK1 (Table 4.1). However, this interaction appeared to be false, since ΔC304 could activate the reporter with the

prey vector pPC86 alone. All other deletion mutants that are not mentioned above showed no interaction with ICK1 (Table 4.1).

Table 4.1. Determination of interactions between CYCD3 deletion mutants in the bait vector pDBLeu and ICK1 in the prey vector pPC86

	pPC86 (prey vector)	ICK1
pLeu (bait vector)	/	/
CYCD3 full length	/	****
ΔN42	/	*
ΔN82	/	/
ΔN122	/	/
ΔN162	/	/
ΔN202	/	/
ΔC49	/	*
ΔC94	/	**
ΔC134	/	/
ΔC175	/	**
ΔC214	/	/
ΔC254	/	/
ΔC304	****	****
ΔC344	/	/

Number of (*) denotes the strength of interaction as indicated by the blue color intensity observed in the beta-galactosidase assay, with (****) representing a strong interaction and (/) representing no interaction.

4.1.2 Interactions between CYCD3 deletion mutants in prey vector and ICK1 in bait vector

To verify the results observed on the interactions of CYCD3 deletion mutants with ICK1, the fusion proteins were swapped between the bait and prey vectors so that the CYCD3 deletion mutants were in the prey vector pPC86 while ICK1 was in the bait vector pDBLeu. The full-length CYCD3 in the prey vector was used as a positive control and showed a strong interaction with ICK1 in the bait vector (Table 4.2). In contrast to previous observations that ΔN42 deletion mutant showed a weak interaction with ICK1, none of the N-terminal deletion mutants showed an interaction with ICK1 in this context. As for the C-terminal deletion mutants, the ΔC49 and

Δ94 deletion mutants in the prey vector, as in the bait vector, still showed an interaction with ICK1 (Table 4.2). In contrast, the ΔC175 and ΔC304 deletion mutants in the prey vector showed no interaction with ICK1.

Table 4.2. Determination of interactions between CYCD3 deletion mutants in the prey vector pPC86 and ICK1 in the bait vector pDBLeu

	pDBLeu (bait vector)	ICK1
pPC86 (prey vector)	/	/
CYCD3 full length	/	****
ΔN42	/	/
ΔN82	/	/
ΔN122	/	/
ΔN162	/	/
ΔN202	/	/
ΔC49	/	***
ΔC94	/	***
ΔC134	/	/
ΔC175	/	/
ΔC214	/	/
ΔC254	/	/
ΔC304	/	/
ΔC344	/	/

Number of (*) denotes the strength of interaction as indicated by the blue color intensity observed in the beta-galactosidase assay. With (****) representing strong interaction and (/) representing no interaction.

4.2 Discussion

ICK1 has been shown to interact with both CYCD3 and CDKA;1 (Wang *et al.* 1998). However, the regions in both CYCD3 and CDKA;1 responsible for interacting with ICK1 remain to be identified. Interestingly, ICK1 overexpression in *Arabidopsis* plants leads to an increase in transcript levels of *CYCD2* and *CYCD3*, indicating a possible feedback regulation system (Zhou *et al.* 2003a). It is believed that cell division and phenotype of plants depends on the interactions of different positive (i.e. CYCD3) and negative regulators (i.e. ICK1) of CDKs. A series of CYCD3 deletion constructs were prepared and the interactions of these CYCD3 deletion mutants with ICK1 were analyzed using the GAL4-based yeast two-hybrid system along with beta-galactosidase filter assay.

To analyze the interaction between CYCD3 and ICK1, CYCD3 deletion mutants were cloned into the bait vector pDBLeu and ICK1 into the prey vector pPC86. To confirm the results observed in this combination, the fusion proteins were swapped to the opposite vectors and again tested for yeast two-hybrid interactions. In contrast to what was observed for ICK1, the results from a series of deletion mutants of CYCD3 suggest that both the N-terminal and C-terminal regions of CYCD3 are needed for CYCD3 to interact with ICK1 in the yeast two-hybrid system, although a slightly longer C-terminal region appears to be dispensable.

There are two possibilities for this observation. Firstly, the residues involved in the interaction of CYCD3 with ICK1 may be scattered in the linear sequence but are brought together when the protein is folded so that deletion of either the C-terminal or the N-terminal region demolishes the interaction. Secondly, the protein expression level or structural stability may be affected by the deletions. In this regard, it is worth noting that CYCD3 (with 376 amino acids) is a highly unstable protein (Planchais *et al.* 2004). The primary sequence of CYCD3 suggests that the protein possess two PEST sequences that may confer instability to the protein (Soni *et al.* 1995). One of the PEST sequences is located in the N-terminal region (amino acid 28-69) of the protein, while the other is located in the C-terminal (302-351). In the deletion mutants of CYCD3 (ΔN42, ΔC49, ΔC94), one of the PEST sequences was removed or partially removed. In these mutants, the ability for interacting with ICK1 was retained. The inability of other mutants, with further deletions to interact with ICK1, could not be attributed to the removal of the PEST sequences, since if the PEST sequences are functional, their removal would increase the accumulation of the mutant proteins.

The primary sequence of CYCD3 also has a region of amino acids (86-191) designated as the cyclin box which appears to be evolutionarily conserved among diverse species (Soni *et al.* 1995). The cyclin box is the region responsible for binding with CDKs. Thus, it is more likely that the inability of the CYCD3 mutants with a deletion beyond the N-terminal 42 amino acids or C-terminal 94 amino acids to interact with ICK1 is due to possible alterations of structures required for the interaction. This suggestion could be further determined by analyzing the protein expression levels of the various deletion mutants. However, based on the above rationale and considering that we could not map the sequence in CYCD3 responsible for interacting with ICK1 to a narrow region, we decided not to proceed with the work of determining protein levels. Instead, I proceeded with the work described in the next two chapters.

Different results were observed for the ΔC175 deletion mutant. When it was in the prey vector, ΔC175 showed an interaction with ICK1, but it did not show such an interaction when it was in the bait vector. Both experiments (with ΔC175 mutant and ICK1 in opposite vectors) were performed twice and the results were consistent. It is possible that the ΔC175 deletion mutant could activate the reporter expression when fused with the GAL4 DNA-binding domain. However, in this case, the activation of reporter should also be expected when the ΔC175 deletion mutant was expressed with the vector alone, but it was not observed. It is also possible that the interaction of the ΔC175 deletion mutant might be more sensitive to the context change (switching from the prey vector to the bait vector) than the full-length protein. Thus, it remains to be further clarified whether ΔC175 deletion mutant could interact with ICK1.

The ΔC304 deletion mutant in the bait vector could activate reporter activity with the prey vector alone. However, it could not activate the reporter expression when expressed in the prey vector which contains a GAL4 DNA-activation domain, suggesting that the ΔC304 mutant might function as a transcription activation domain when fused with the DNA binding domain in the bait vector in the yeast two-hybrid system. This observation is surprising and interesting, indicating that this CYCD3 deletion mutant could function as a transcription-activating domain. However, the observation that the full-length CYCD3 showed little activity of transcription activation suggests that the strong activity of transcription activation for the truncated CYCD3 may more likely be incidental rather than a biologically relevant function of CYCD3.

CHAPTER 5: STUDIES OF ICK PROTEIN EXPRESSION, INTERACTION WITH CDK/CYCLIN COMPLEX, AND STABILITY IN TRANSGENIC PLANTS

5.1 Results

5.1.1 Plant transformation and phenotypic effects of ICK expression

Previous studies have shown that transgenic overexpression of ICK1, ICK2, ICK4, NtKIS1a (a tobacco ICK-related gene), and ICKCr (a *Chenopodium rubrum* ICK-related CDK inhibitor) results in changes to plant growth and morphology (Wang *et al.* 2000; De Veylder *et al.* 2001; Jasinski *et al.* 2002b; Zhou *et al.* 2002a). These transgenic plants are often smaller in size, and have serrated leaves and altered flower morphology compared to wild type plants (Wang *et al.* 2000). However, the phenotypic effects from overexpression of other ICKs (ICK3, ICK5, ICK6 and ICK7) are not known and it will be interesting to see whether those ICKs exert similar phenotypic effects on transgenic plants. A number of independent transgenic plants were obtained with each of the ICK constructs (ICK1 to ICK7 linked to GFP) plus the GFP control. The 35S promoter was used to drive the expression of the respective gene. A survey of the GFP-ICK and control (GFP-only) transgenic plants on the size of seedlings, leaf serrations and flower morphology was conducted. Transgenic GFP-ICK plants showed a greater variation in terms of size, likely due to various levels of GFP-ICK expression. The diameter (the span from rosette leaf to rosette leaf) of each transgenic plant was measured in two weeks after transfer and the average for each construct was obtained. The averages for the seven GFP-ICK constructs ranged from 32.7 mm to 55.0 mm and were all lower than the average for the control GFP construct (61.1 mm). The trend for GFP-ICK plants to be smaller than the control GFP plants was clear, although the size varied from plant to plant. The inhibition of plant growth, defined simply as reduction in the physical size of plants, could be better observed in the progeny plants of the GFP-ICK lines when they were compared to the GFP control plants under identical conditions (Figure 5.1). These results provide evidence that similar to ICK1, ICK2 and ICK4 reported

previously, overexpression of ICK3, ICK5, ICK6 and ICK7 are all capable of inhibiting growth of transgenic *Arabidopsis* plants.

Some transgenic plants (Table 5.1) showed the typical phenotype of serrated leaves for all ICKs (Figure 5.2). The percentages of transgenic plants showing moderate and strong serrations were obtained from the survey for each ICK construct (Table 5.1). Since weak serrations were occasionally observed for GFP-control transgenic lines, only moderate and strongly serrated leafs were considered to be true phenotypic effects of ICK overexpression. The percentages of plants with moderate to strong leaf serrations were comparable for GFP-ICK1 (7.5%), GFP-ICK2 (9.8%), GFP-ICK3 (10.3%), GFP-ICK6 (10.2%) and GFP-ICK7 (9.7%) (Table 5.1). The percentages were lower for GFP-ICK4 (3.1%) and GFP-ICK5 (4.4%). For the GFP control, no plant with moderate to strong serrations of leaves was observed. Furthermore, there were differences in terms of the extent of leaf serrations among different ICKs. Strongly serrated leaves were observed in the transgenic plants of GFP-ICK1, GFP-ICK2, GFP-ICK5, GFP-ICK6, and GFP-ICK7, but not in GFP-ICK3 and GFP-ICK4 plants (Table 5.1; Figure 5.2). Since the survey was based on 59-104 independent transformants for each construct, these results suggest that although all ICK members are capable of causing phenotypic changes to the leaves of transgenic plants, some ICK members may be stronger inhibitors and can cause stronger phenotypic changes than others.

The flower morphology of the transgenic plants was also surveyed. Again, guidelines were established to aid the categorization of the degree of flower abnormality. Transgenic lines displaying shorter pedicel, smaller petals and compact inflorescence are categorized as having a strong phenotype. Transgenic lines displaying intermediate clustering of inflorescence and reduction in petal size are categorized as having a moderate phenotype. Similar to the leaf serration phenotype, strong changes in floral morphology were only observed in transgenic plants of GFP-ICK1, GFP-ICK2, GFP-ICK5, GFP-ICK6 and GFP-ICK7, not in the plants of GFP-ICK3 and GFP-ICK4.

Table 5.1 Phenotypic effects of overexpressing GFP or GFP-ICKs on transgenic *Arabidopsis* plants.

	Number of plants transferred and survived		Percentage (%) of plants with leaf serrations			Percentage (%) of plants with changes in flower morphology		
	Trans.	Surv.	Moderate	Strong	Total	Moderate	Strong	Total
GFP	100	92	0.0	0.0	0.0	0.0	0.0	0
GFP-ICK1	112	94	1.1	6.4	7.5	8.5	3.2	11.7
GFP-ICK2	110	102	4.9	4.9	9.8	2.0	3.9	5.9
GFP-ICK3	88	78	10.3	0.0	10.3	3.8	0.0	3.8
GFP-ICK4	101	98	3.1	0.0	3.1	5.1	0.0	5.1
GFP-ICK5	108	92	1.1	3.3	4.4	4.3	2.2	6.5
GFP-ICK6	100	59	3.4	6.8	10.2	1.7	6.8	8.5
GFP-ICK7	120	104	1.0	8.7	9.7	3.8	5.8	9.6

Primary transformants (T1) were selected on Petri plates and after three weeks transferred to soil in pots. Two weeks after the transfer, a survey of the GFP and GFP-ICK transgenic plants measuring size of seedlings, leaf serrations and flower abnormality were performed. Leaf serration of transgenic plants were categorized as either strong (strong leaf serrations in all leaves) or moderate (obvious but not severe serrations in majority of leaves). Surveys were conducted at 3- and 4-weeks after the transfer on flower morphology. Transgenic plants were categorized as either strong (shorter pedicel, smaller petals and strong clustering of floral buds) or moderate (with moderate changes).

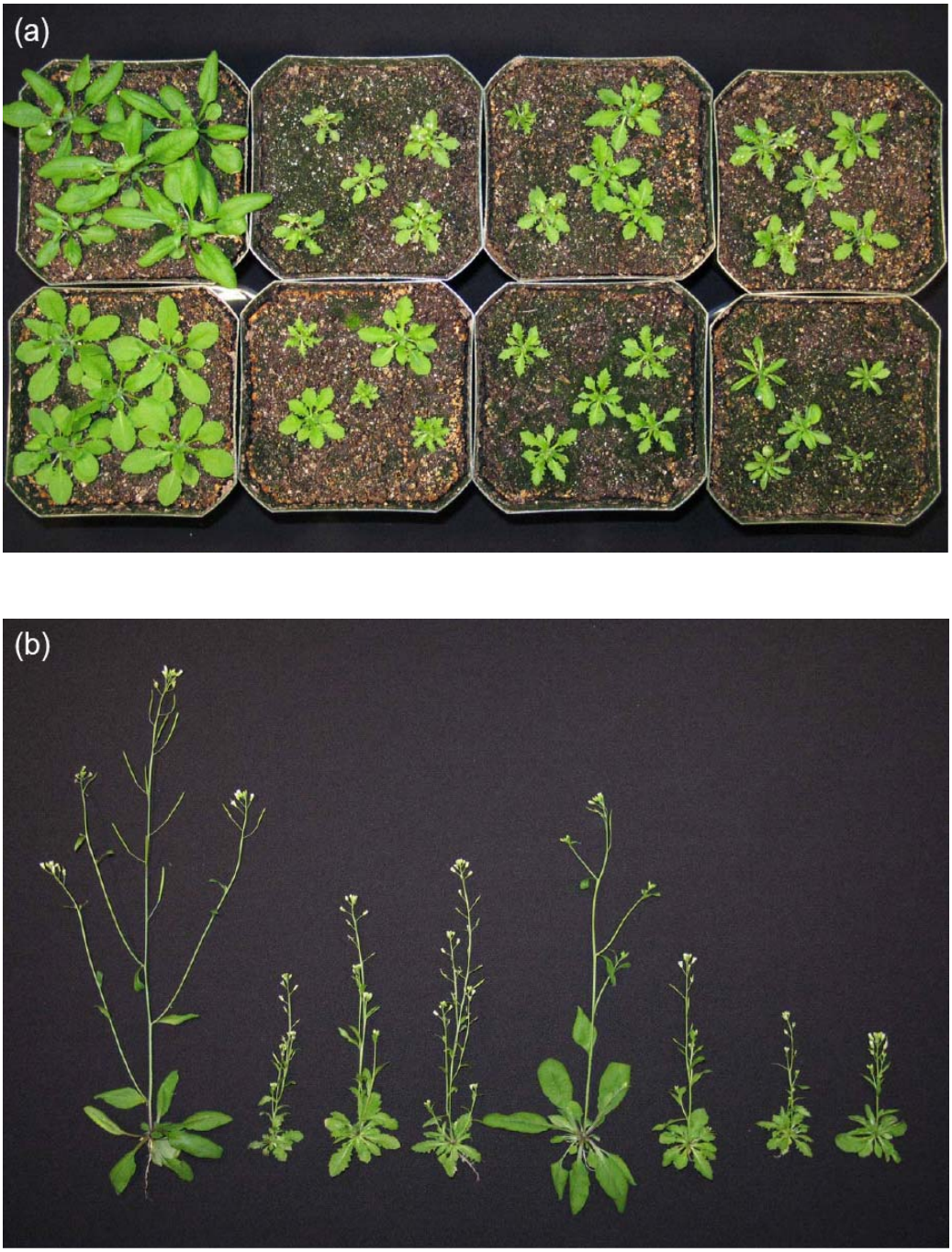

Figure 5.1. Phenotypes of transgenic GFP-ICK plants. **(a)** Seedlings were grown in soil and pictures were taken when plants was 23 days old. The top four pots from left to right are: GFP, GFP-ICK1, GFP-ICK2 and GFP-ICK3. The bottom four pots from left to right are: GFP-ICK4, GFP-ICK5, GFP-ICK6 and GFP-ICK7. **(b)** Representative plants were removed from pots and being photographed together so that the relative plant size could be better compared. Plants from left to right: GFP, GFP-ICK1, GFP-ICK2, GFP-ICK3, GFP-ICK4, GFP-ICK5, GFP-ICK6, GFP-ICK7.

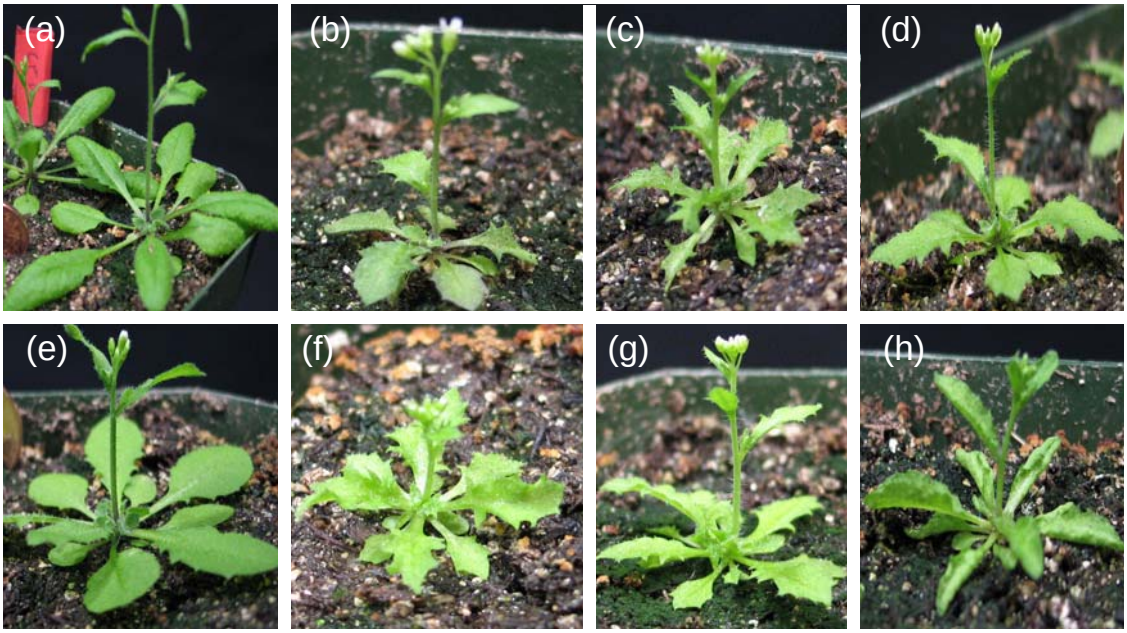

Figure 5.2. Individual transgenic plants. GFP **(a)**, GFP-ICK1 **(b)**, GFP-ICK2 **(c)**, GFP-ICK3 **(d)**, GFP-ICK4 **(e)**, GFP-ICK5 **(f)**, GFP-ICK6 **(g)** and GFP-ICK7 **(h)**. Note serrated leaves with transgenic GFP-ICK plants.

5.1.2 Expression of GFP-ICK fusion proteins in transgenic *Arabidopsis* plants

Since ICKs were expressed as GFP-fusion proteins, Western blotting using an anti-GFP antibody provided a good way to determine the presence and level of fusion protein in transgenic plants. Preliminary analysis revealed that GFP-ICK1 recombinant proteins were expressed at a low level in general, consistent with the observation on GFP-ICK1 (Zhou *et al.* 2003b). Thus, only transgenic plants with strongest phenotypes (leaf serrations and modified flowers) along with control plants were used to determine presence of the fusion proteins. Three independent lines for each GFP-ICK construct and one proven transgenic line for the GFP control were selected for the analysis.

Results are shown in Figure 5.3. Fusion proteins were detected in at least one line for each of the GFP-ICK constructs at around the 62 kDa mark except for the GFP-ICK3 construct. For the GFP-control transgenic line, no fusion protein is detected, however a high level of GFP with a molecular weight of 28 kDa was detected in the Western blot. For GFP-ICK1, GFP-ICK2 and GFP-ICK5, fusion proteins were detectable for all three transgenic lines tested (Figure 5.3). For GFP-ICK3, no fusion protein was detected for the three transgenic lines tested.

Subsequently, several other transgenic lines of GFP-ICK3 were also tested and again none showed a detectable level of the fusion protein. For GFP-ICK4, GFP-ICK6 and GFP-ICK7, only one out of three transgenic lines tested showed a detectable level of fusion protein. Thus, despite the generally low level of expression, fusion proteins were detected in at least one line for each construct.

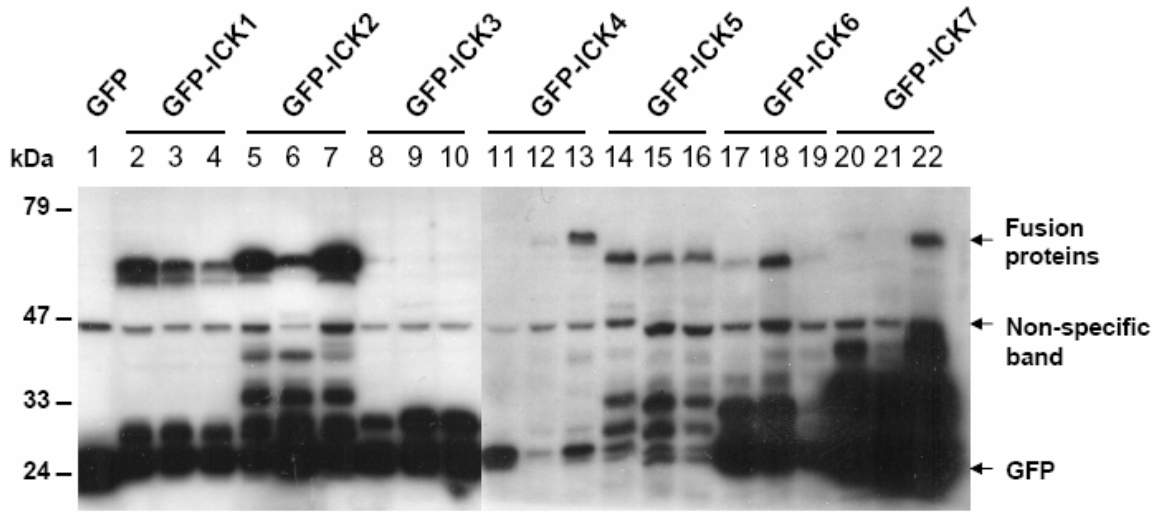

Figure 5.3. Western analysis of GFP and GFP-ICK fusion proteins in transgenic *Arabidopsis* plants. Three independent lines for each GFP-ICK construct were selected and protein samples were extracted from the floral buds and the leaves. For each sample, 25 μg of protein for each sample were separated by SDS-PAGE and analyzed on a Western blot. The type of transgenic plant is indicated above the lane. A non-specific band was observed for all transgenic lines at around 45 kDa.

5.1.3 Effects of a proteasome inhibitor, MG132

The mammalian CDK inhibitor $p27^{Kip1}$ has been shown to be targeted for degradation through the proteasome upon phosphorylation by CDK2 (Vlach *et al.* 1997). A recent study shows that ICK2 protein can be phosphorylated by *Arabidopsis* CDKB *in vitro* and ICK2 protein level increases in the *cdkb* mutant plant (Verkest *et al.* 2005). To determine whether ICKs are degraded through the proteasome pathway *in vivo*, a proteasome inhibitor MG132 was used to treat transgenic GFP-ICK as well as GFP plants. Twelve to fourteen day-old seedlings for each

ICK construct were treated with either 100 μM MG132 (a peptide proteasome inhibitor) or an equal volume of DMSO (control). For GFP-ICK1, GFP-ICK5, GFP-ICK6 and GFP-ICK7, the results were consistent showing an increase in protein level following MG132 treatments (Figure 5.4). For GFP-ICK2 and GFP-ICK4, no clear increase was observed under the conditions used, indicated that MG132 had no effect.

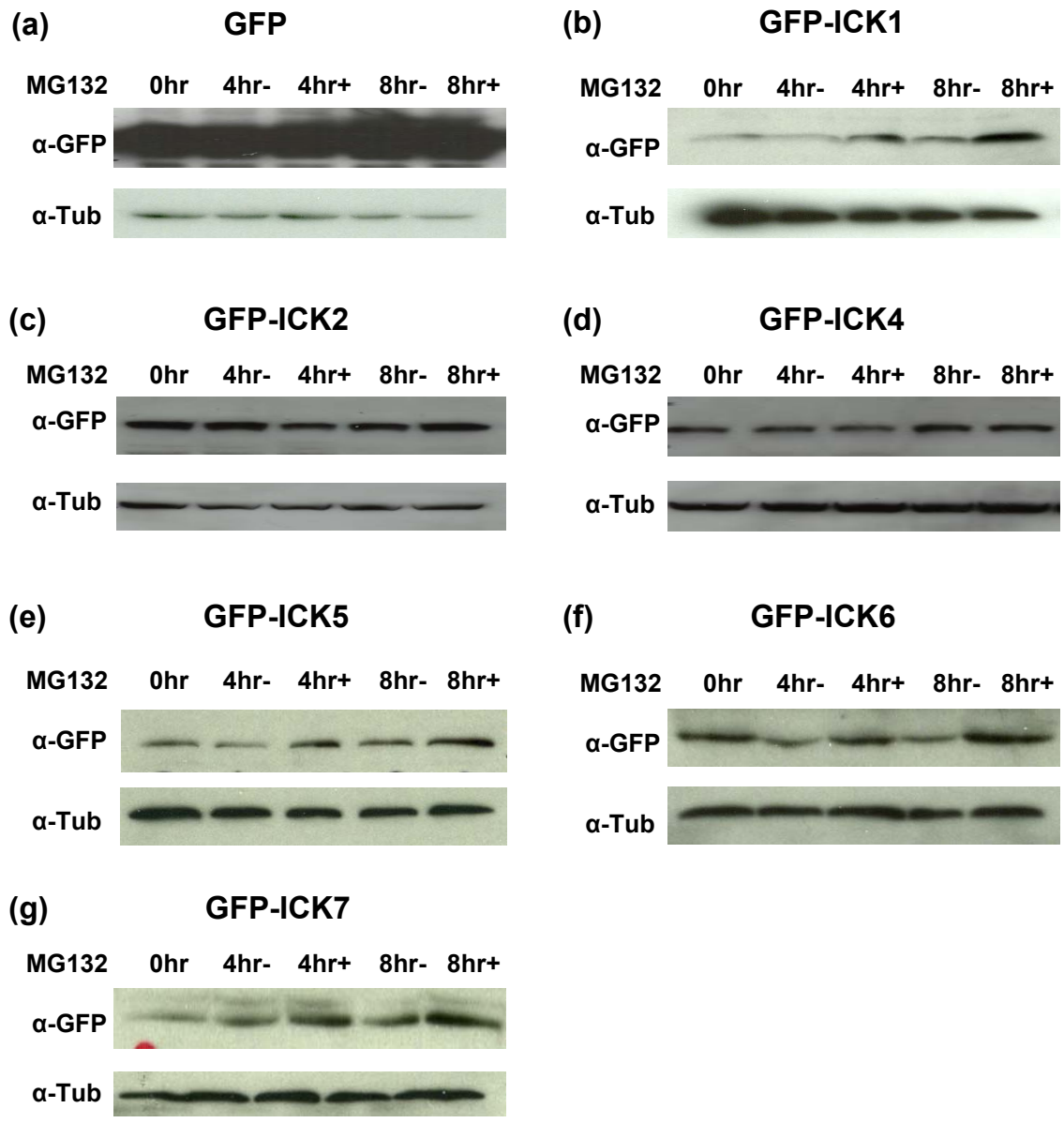

Figure 5.4. Effect of MG132 treatments on the accumulation of GFP-ICKs in transgenic plants. Twelve to fourteen day-old seedlings were subjected to the treatment of either 100 μM MG132 or equal volume of DMSO (control) for up to 8 hrs. Seedlings were removed from the treatment at specific time points (0, 4 or 8 hrs) and proteins extracted. Twenty five micrograms of proteins were resolved on SDS-PAGE and Western blot analysis was performed using an anti-GFP antibody. Blots were stripped and re-probed with an antibody against tubulin as a loading control. **(a)** GFP, **(b)** GFP-ICK1, **(c)** GFP-ICK2, **(d)** GFP-ICK4, **(e)** GFP-ICK5, **(f)** GFP-ICK6 and **(g)** GFP-ICK7.

5.2 Discussion

Previous studies have demonstrated that overexpression of ICK1, ICK2 and ICK4 in plants results in inhibition of growth, leaf serrations and modifications of flower morphology (Wang *et al.* 2000; De Veylder *et al.* 2001; Zhou *et al.* 2002a). The present results indicate that all ICKs exert similar phenotypic effects on *Arabidopsis* plants, although the extent of the phenotypic effect appears different among members of the ICK family. This study shows that all ICK members, to a varying degree, are capable of inhibiting the growth of plants.

For all ICK constructs, the frequency of plants showing clear leaf serrations and changes in flower morphology was low. There are two major reasons for this observation. First, the morphological changes were only observed in plants where the cell division and cell number were reduced to a great extent (Wang *et al*. 2000). Second, GFP-ICK fusion proteins were only detected in a small proportion of transgenic plants, indicating that ICKs were expressed in general at a low level. Thus, only when the ICK1 fusion protein is expressed at a sufficient level, do plants show the morphological changes.

The study further shows that only moderate leaf serration and floral changes were observed for ICK3 and ICK4 transgenic lines, while the other ICKs had at least one or more lines from 59-104 primary transformants displaying strongly serrated leafs and floral changes. The observation on the frequency and severity of the phenotype in the independent transformants (Table 5.1) indicates that ICK3 and ICK4 have a weaker phenotypic effect on *Arabidopsis* than other ICK members. One explanation for the differences in phenotypic effect observed between different ICKs may be related to the ability of ICK to interact with cyclin and CDKA. A previous study had shown that overexpression of ICK1 has greater phenotypic effects on transgenic plants than ICK4 (Zhou *et al.* 2002a). It was suggested that the stronger effect for ICK1 may be due to the ability of ICK1 to interact with both D-type cyclins and CDKA while ICK4 can only interact with D-type cyclins, making ICK1 a more potent inhibitor. Consistent with the suggestion, ICK3 has also been shown to be unable to interact with CDKA (De Veylder *et al.* 2001). Thus, the differences in inhibition strength between different ICKs may depend on their ability to interact with CDKA. The ICK members that are able to interact with both CDKA and cyclins appear to have the greater phenotypic effects on transgenic plants.

Alternatively, the weaker phenotypic effects by ICK3 and ICK4 could be due to lower levels of protein accumulation in plant cells. It has been shown that when the level of ICK1

increases, the phenotypic effect in *Arabidopsis* plants is stronger (Zhou *et al.* 2003b). Since the strongest three independent lines for each GFP-ICK construct were selected from 80-100 transformants, they could be considered to represent the group of high expressors for a particular GFP-ICK construct. Also, since the non-specific band (Figure 5.3) was much more similar among different samples, most of the differences in the level of fusion proteins could not be attributed to the variation in the amount of protein loaded in the gel. Instead, most of the differences should be attributed to the differences in the level of fusion proteins expressed in the plants. The results indicate that GFP-ICK3 was expressed at the lowest level among all ICKs, while GFP-ICK4 was also at a relatively low level. Taken together, both a lower level of protein and the lack of interaction with CDKA may contribute to the weaker phenotypic effect exhibited by ICK3 and ICK4.

The control of protein abundance in a cell is essential to ensure proper functioning of all cellular processes. Environmental or intracellular cues perceived by cells may lead to more synthesis or degradation of a particular protein, to maintain an appropriate protein level. In the present study, the expression of GFP-ICK constructs was driven by the 35S promoter, which is a strong and widely used promoter. Despite this strong promoter, GFP-ICK fusion proteins were only detected in a small proportion of transgenic plants. The transgenic lines used for analyzing protein expression were selected from 80 to 100 independent transformants for each construct based on their phenotypes (Table 5.1), although a few lines for some constructs could not produce seeds due to the severity of the phenotype. The observations that only a low level of fusion protein was observed for all ICKs and in the case of ICK3 no fusion protein was detected clearly indicates that the fusion proteins for all ICKs were expressed at a low level. This low level of protein expression should not be due to the GFP protein used for the protein fusion. First, the 35S-GFP plants showed a very high level of GFP protein. Second, in GFP-ICK plants a large amount of degraded fusion protein at various sizes and particularly at the size to GFP protein alone were observed (Figure 5.3). Thus, the low levels of GFP-ICK proteins are likely due to the inherent instability of ICK proteins.

There are two main mechanisms by which proteins are degraded in cells, the proteolytic enzymes within the lysosome and the ubiquitin-proteasome pathway (review by Myung *et al.* 2001). Many cell cycle regulators, including A, B and D-type cyclins as well as CDKB are degraded by the 26S proteasome, which is mediated by protein ubiquitylation (Genscik *et al.*

1998; Planchais *et al.* 2004, Weingartner *et al.* 2003). The mammalian CDK inhibitor $p27^{Kip1}$ is shown to be degraded by the proteasome upon phosphorylation on the Thr187 residue (Vlach *et al.* 1997). A recent study has also provided preliminary evidence that the plant CDK inhibitor ICK2 may be targeted for degradation through the ubiquitin-proteasome pathway (Verkest *et al.* 2005). Thus, we are interested in finding out whether all other ICKs are subjected to degradation through the ubiquitin-proteasome pathway *in vivo*.

The mode of degradation for ICK *in vivo* was addressed in this study by using a proteasome inhibitor MG132 to inhibit the function of the 26S proteasome. If the ICK proteins are degraded by the proteasome, the fusion protein level of transgenic plants will be expected to accumulate to a higher level in the presence of MG132 than the control. As shown in Figure 5.4, the level of fusion protein when treated with MG132 was noticeably higher for GFP-ICK1, GFP-ICK5, GFP-ICK6 and GFP-ICK7 compared to the control at both the 4-hr and 8-hr time points, indicating the involvement of proteasome in the degradation of these ICK proteins. However, no clear effect by MG132 was observed on the protein level of GFP-ICK2 and GFP-ICK4, suggesting that the proteasome may not play a significant role in the degradation of those two proteins. In Western blotting experiments, variation among samples could be introduced due to several factors including protein concentration determination and sample loading. Precautions were taken to ensure that an observed effect was reproducible. For instance, to ensure the differences in fusion protein level observed was not due to unequal amount of protein loaded, the PVDF blots were stripped and re-probed with an antibody against tubulin. The experiments were repeated two to three times independently. Taking the possible loading variation into consideration, the results suggest that, if there is an effect, the effect on ICK2 and ICK4 by MG132 would be less compared to other ICKs.

The observation that MG132 treatment did not have an effect on the stability of ICK2 protein is different from that observed by Verkest *et al.* (2005). The specific reason for this difference is yet to be determined. One possible reason might be seedling age. While 5-day old seedlings were used in the study by Verkest *et al.* (2005), 14-day old seedlings were used in the present study. Other experimental conditions might also be responsible for the difference. However, since all treatments were performed under the same conditions in this study, the present results indicate that the proteasome affects the degradation of some ICKs more than

others. A clear effect was observed for ICK1, ICK5, ICK6 and ICK7, but not for ICK2 and ICK4.

Although an effect on protein accumulation by MG132 treatment was observed for ICK1, the increase was modest. In contrast, a great amount of change in protein level was observed when the N-terminal region of ICK1 was removed (Zhou *et al.* 2003b). The regulation through ubiquitin-mediated pathway implicated by the effect of MG132 treatment may contribute to the increased protein stability after the N-terminal removal. However the mild increase following MG132 treatment may be difficult to explain all the change in protein level after the removal of N-terminal region. Further work is needed to determine whether another mechanism exists that regulates the stability of ICK1 as well as other ICKs. In this regard, Jakoby *et al.* (2006) observed that MG132 treatment also resulted in the accumulation of ICK1$^{109\text{-}191}$, consistent with the suggestion that more than one mechanism may be involved in regulating the stability of ICK1.

CHAPTER 6: PHOSPHORYLATION STUDIES OF ICKS IN TRANSGENIC *ARABIDOPSIS* PLANTS

6.1 Results

6.1.1 Analysis of putative phosphorylation sites in ICKs

Since post-transcriptional modifications such as phosphorylation can critically affect protein-protein interactions and other protein functions, we were interested in determining whether ICKs undergo phosphorylation. Bioinformatics analysis was performed to obtain information on putative phosphorylation sites in ICKs. The primary sequences of ICKs were inputted into Netphos1, Netphos2 and Disphos (Blom *et al.* 1999; Iakoucheva *et al.* 2004). Both Netphos2 and Disphos are bioinformatics programs that predict potential phosphorylation sites on serine, threonine and tyrosine residues while Netphos1 predicts possible phosphorylation sites by Cdc2 (a yeast homologue of CDK). The stringency of the software was controlled to limit the number of hits. For example, in the case of Netphos2, a score of >0.8 was needed in order for the residue to be considered a 'hit'. Even with such stringency, each software still produced numerous hits of possible phosphorylated residues. Thus, only the residues that were a hit with all three softwares were counted as the 'consensus' residues for the purpose of this study. Since CDKs are known serine/threonine kinases and ICKs are most likely phosphorylated by them, I used the number of serine/threonine residues that were predicted to be potential phosphorylated sites as an indicator for how likely an ICK protein is phosphorylated.

As shown in Table 6.1, no residues were predicted to be phosphorylated for ICK1, ICK2 and ICK3. Three residues were predicted to be phosphorylated for ICK4, seven residues for ICK5, three residues for ICK6 and four residues for ICK7. These predictions were similar to those predicted by Wang *et al.* (2007) who predict the residues of ICKs that can be phosphorylated by CDKA based on the motif sequence [TS]-P-x-[RKQSL]. Using this approach, no residues are predicted for ICK1, ICK2 and ICK3, while three residues for ICK4, two residues for ICK5, one residue for ICK6 and four residues for ICK7 are predicted.

Table 6.1. Analysis of potential phosphorylation sites in ICKs using three programs

Protein	Predicted potential serine/threonine residues			
	Netphos1	Netphos2	Disphos	Consensus
ICK1	4	11	6	0
ICK2	2	13	20	0
ICK3	4	13	6	0
ICK4	6	21	17	3
ICK5	12	29	30	7
ICK6	7	19	10	3
ICK7	8	26	15	4

Number of potential phosphorylation sites of serine/threonine in ICKs predicted using bioinformatics programs Netphos1, Netphos2 and Disphos. The score of >0.8 and >0.5 is required for residues to be counted as a possible phosphorylation site for Netphos2 and Disphos respectively. Residues that are predicted to be phosphorylated by all three programs are grouped under 'consensus'.

6.1.2 Determining the conditions for using CIP to study phosphorylation of ICKs

One technique to study the phosphorylation status of ICKs is to treat proteins or protein extracts with CIP. CIP is a phosphatase that can remove 5' phosphate groups from DNA, RNA, nucleotides and proteins. To determine whether CIP is a useful tool to study the phosphorylation status of ICKs, GFP-ICK1 total protein extracts were treated with increasing amount of CIP and for the control samples without CIP. The samples were subjected to electrophoresis and Western blotting analysis. As shown in Figure 6.1a, the GFP-ICK1 fusion protein band showed a gradual downward shift with increasing amount of CIP added, indicating that the molecular weight was decreasing with increased amount of CIP. CIP treatment appears to specifically affect GFP-ICK1 since no 'shift' is observed for the non-specific band in the gel. CIP was also used to treat the proteins pulled down using the $p13^{Suc1}$ –conjugated agarose beads. As shown in Figure 6.1b, GFP-ICK1 proteins of CIP-treated samples showed a downward shift compared to the non-CIP treated samples. This observation confirms the feasibility of using CIP to study phosphorylation state of ICK1 and provides evidence that at least a proportion of ICK1 is phosphorylated in the cell and can be dephosphorylated.

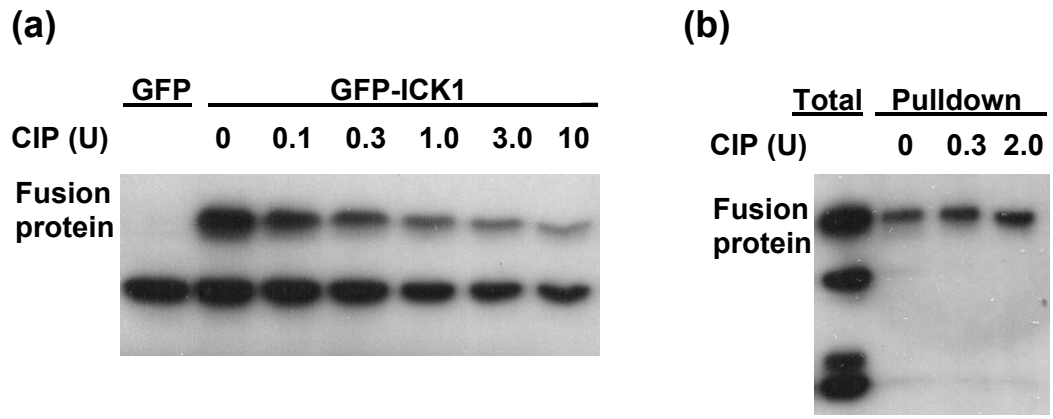

Figure 6.1. Test of CIP treatments for detecting phosphorylated GFP-ICK1 in total protein extract and CDK complex. **(a)** Effect on GFP-ICK1 in total protein extract. Twenty five micrograms of total protein extract from GFP-ICK1 were treated with increase amount (units) of CIP as indicated for 20 minutes. The same amount of total protein from GFP plant was also loaded as the control. The samples were analyzed by Western detection using an antibody to GFP. **(b)** Effect on GFP-ICK1 in the CDK complex pulled down with $p13^{Suc1}$-conjugated agarose beads. A total of 200 μg protein extract from GFP-ICK1 plants was used in the protein pulldown and the pulldown proteins were treated with 0, 0.3 and 2 units of CIP as indicated for 20 minutes. Twenty five micrograms of total protein (first lane) were also used as a reference. Western detection was performed as in **(a).**

6.1.3 Effect of CIP treatment on ICKs

Since the initial experiments with GFP-ICK1 showed that CIP could be used to treat the total protein extract and the GFP-ICK1 fusion protein showed increased migration in the gel, the effect of CIP treatment on other GFP-ICK proteins was determined similarly. One transgenic line for each of the GFP-ICK constructs (with the exception of GFP-ICK3) that showed a detectable level of fusion protein under the current conditions was used and the total protein extract was treated with CIP. CIP-treated samples and non-CIP-treated samples were compared on Western blot to determine whether a change in the migration of the fusion protein occurred. Samples treated with CIP showed a clear downward shift (increased migration) for GFP-ICK1, GFP-ICK2, GFP-ICK5, and GFP-ICK6 compared to their non-CIP treated counterparts (Figure 6.2). The CIP treated GFP-ICK4 sample did not show a clear shift compared to the non-treated sample. Comparison of the migration rate between the CIP-treated GFP-ICK7 and non-CIP-treated GFP-ICK7 could not be done since the CIP-treated GFP-ICK7 fusion protein band disappeared on the Western blot. The increased migration by CIP treatment was specific only to

several GFP-ICK fusion proteins and was not observed for the GFP and other protein bands (Figure 6.2), suggesting that the effect of CIP treatment could not be due to non-specific protein degradation. More likely, this increased migration of the GFP-ICK fusion proteins is due to dephosphorylation by CIP treatment. These results indicate that ICK1, ICK2, ICK5 and ICK6 undergo phosphorylation in plant cells.

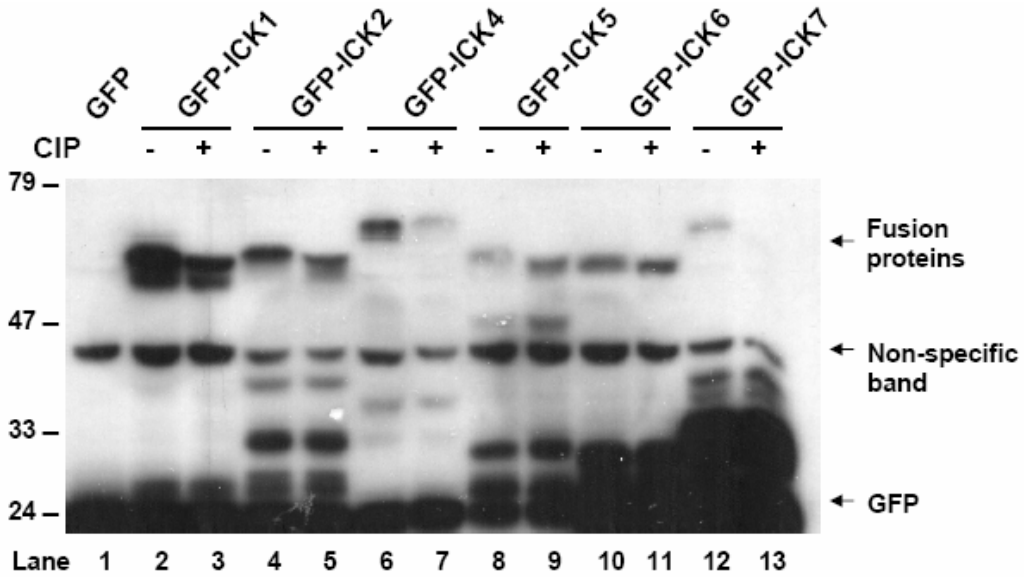

Figure 6.2. Effects of CIP treatment on GFP-ICK fusion proteins extracted from transgenic plants. Total protein extract (25 μg for each sample) from one line for each of the GFP-ICK constructs was treated with 2.5 units of CIP or water for control for 30 minutes. The CIP-treated (indicated by "+" sign) and untreated samples (indicated by "-" sign) were run on a SDS-PAGE gel and analyzed by Western blotting. The type of transgenic plant is indicated above the lane.

The above observation suggests that GFP-ICK7 is less stable following the CIP-treatment. To confirm this observation, a time course experiment was conducted to compare the stability of GFP-ICK1 and GFP-ICK7. The total protein extracts were treated with CIP and removed at 0, 10, 20 and 30 minutes. The samples were subject to Western analysis. For both GFP-ICK1 and GFP-ICK7, as observed previously, a shift in fusion protein band position was detected in CIP-

treated samples compared to the non-CIP-treated samples (Figure 6.3). Further, the level of fusion protein gradually decreased with the increasing time of CIP treatment. Interestingly, this decrease in the amount of fusion protein was more evident for GFP-ICK7 than for GFP-ICK1 (Figure 6.3), indicating that GFP-ICK7 is less stable following CIP treatment.

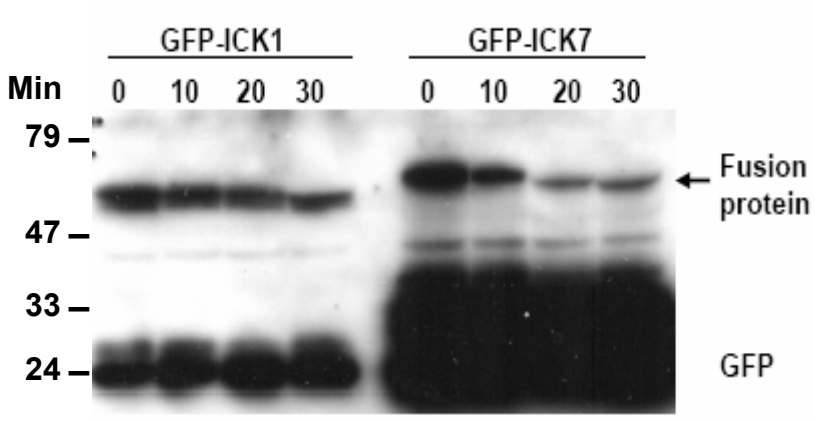

Figure 6.3. A time course experiment of CIP treatment on GFP-ICK1 and GFP-ICK7
Total protein extracts of GFP-ICK1 and GFP-ICK7 were treated with CIP at 37°C. Samples were removed at specific time points (0, 10, 20 and 30 min) and 25 μg of each sample were loaded onto a SDS-PAGE, followed by Western blotting.

6.1.4 Interaction of ICKs with CDK/Cyclin complex *in vivo* and effect of CIP treatment on ICKs associated with the complex

The association of the inhibitors with CDK complex *in vivo* was demonstrated only for ICK1 (Zhou *et al.* 2003b) and ICK2 (Verkest *et al.* 2005). It is interesting to determine whether other ICK members also interact with the CDK complex *in vivo*. In this experiment, one transgenic line for each of the GFP-ICK construct (with the exception of GFP-ICK3) that has shown a detectable level of fusion protein was used and CDK complex of associated proteins were pulled down with $p13^{Suc1}$-conjugated agarose beads. The $p13^{Suc1}$ beads can bind to the CDK complex of eukaryotic species (De Veylder *et al.* 1997; Pines 1996) and thus pull down proteins associated with the CDK complex. If ICK is able to interact with the CDK complex, it

will be pulled down along with the CDK/cyclin complex by the $p13^{suc1}$ beads. The fusion protein can then be detected by Western blotting using anti-GFP antibody. As shown in the Western blot in Figure 6.4, fusion proteins pulled down with $p13^{Suc1}$ beads were detected for each of the ICK construct except for the GFP control and GFP-ICK7. Fusion proteins for GFP-ICK1, GFP-ICK2, GFP-ICK4, GFP-ICK5 and GFP-ICK6 were all detected. Unfortunately, several GFP-ICK7 primary transformants with a strong phenotype did not produce seeds and among the GFP-ICK7 lines obtained, only one line showed sufficient GFP-ICK7 protein for this type of experiments. Thus we were not able to use another line to test whether ICK7 is able to interact with the CDK complex *in vivo*. Nevertheless, the present results suggest that among the six ICKs examined, five are shown to be able to interact with the CDK complex *in vivo*, with ICK7 to be further determined.

To determine whether ICKs bound with the CDK complex are also phosphorylated, the $p13^{Suc1}$-affinity purified proteins, instead of total proteins, were treated with CIP and subjected to Western analysis. The fusion proteins of GFP-ICK1, GFP-ICK2, GFP-ICK5, and GFP-ICK6 treated with CIP showed a clear shift compared to non-CIP-treated proteins (Figure 6.4). This observation is consistent with the results on the effect of CIP treatment on total protein extract. For GFP-ICK4, the CIP-treated fusion protein appeared to migrate faster. However, the fusion protein for both the CIP-treated and non-CIP-treated samples showed smearing and a doublet band (Figure 6.4). The observed smearing of the fusion protein and diffused bands suggest either that GFP-ICK4 fusion protein is prone to degradation or that some of GFP-ICK4 fusion protein may also be in phosphorylated form. As for GFP-ICK7, no fusion protein was detected in the CIP treated and non-CIP-treated samples.

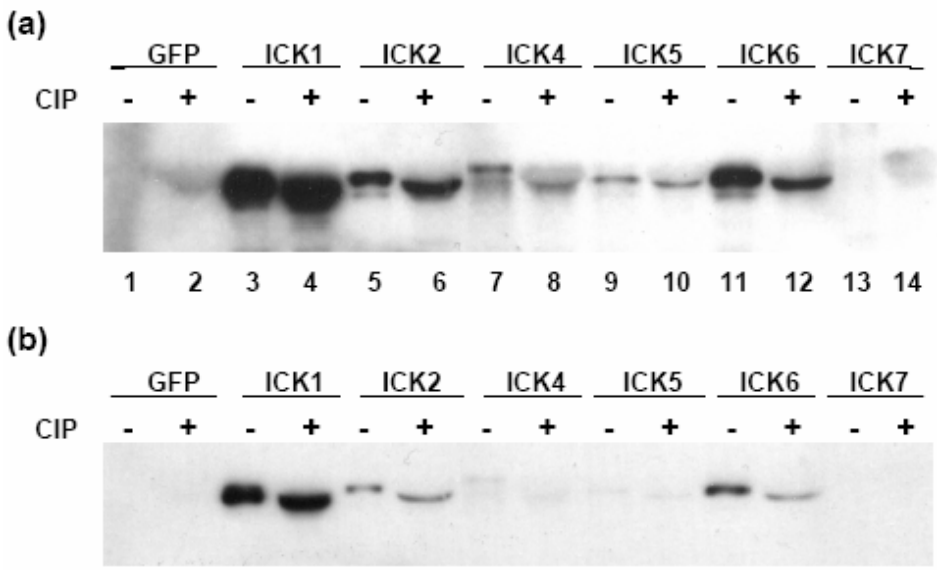

Figure 6.4. Western analysis of GFP-ICK fusion protein pulled down with $p13^{suc1}$-agarose conjugate beads with CIP treatment. For each sample, 200 μg of proteins from seedlings was used for affinity purification with $p13^{Suc1}$-conjugated agarose beads and then treated with CIP or for control without CIP. Samples were resolved by SDS-PAGE, following by Western analysis. Samples for non-CIP treated (indicated by a "-" sign) and CIP treated (indicated by a "+" sign) were loaded side by side to detect possible band shift on the Western blot. **(a)** and **(b)** are images of the same gel at different exposure times: 5 minutes **(a)** and 1 minute **(b).** With the shorter exposure, the shifts for GFP-ICK1, GFP-ICK2 and GFP-ICK6 can be better seen.

6.2 Discussion

Phosphorylation is one of the most fundamental mechanisms in eukaryotes to modify proteins, through which their functional activities, abundance and cellular localization can be regulated. Since mammalian and yeast CDK inhibitors have been shown to undergo phosphorylation (Vlach *et al.* 1997; Tomoda *et al.* 1999), it is our interest to see whether plant CDK inhibitors also possess this mode of regulation. The putative phosphorylation sites of serine and threonine residues were predicted using three software programs. The sites predicted by Netphos2 program were too numerous and difficult to interpret. The Netphos1 and Disphos software programs predict some sites on all ICKs. The results from CIP treatment experiments however show that ICK1, ICK2, ICK5, and ICK6 are likely more phosphorylated than ICK4. This profile is not consistent with profiles predicted by the Netphos1 and Disphos programs or with the "consensus" profile generated by the three prediction software programs. Thus, for the plant CDK inhibitors, the putative phosphorylation sites predicted by the current software programs should not be viewed as the true phosphorylation sites or an indication for the extent of phosphorylation of ICKs. Actual experimental work is required to determine the phosphorylation status of ICKs.

To test whether ICKs undergo phosphorylation *in vivo*, an alkaline phosphatase CIP was used to treat protein samples extracted from 10 to 14 day old seedlings. Since CIP has been shown to be capable of effectively releasing phosphate groups from phosphorylated tyrosine, serine and threonine residues in proteins, it has been used by researchers to study the phosphorylation state of proteins (Su *et al.* 2006). Phosphorylated proteins often display a downward shift in band positions after CIP treatment on the gel compared to untreated proteins. One possible reason for this observation may be that CIP treatment of a protein dephosphorylates it, making it less bulky and lower mass compared to the phosphorylated form. As a result the dephosphorylated form travels faster in the SDS-PAGE gel. Another reason for the observed differences in mobility may be due to the changes in protein structure after CIP treatment. The application of SDS theoretically eliminates and linearizes the secondary and tertiary structures of a protein. However, the secondary structures may not be completely disrupted by SDS treatment. Dephosphorylation of the protein by CIP treatment may result in a conformation change and a faster migration on the SDS-PAGE gel. It has been shown that phosphorylation may make a protein longer and more rigid (Hagestedt *et al*. 1989).

In this experiment, when total protein extracts or proteins affinity-purified with $p13^{Suc1}$-beads (containing CDK complexes) were treated with CIP, GFP-ICK1, GFP-ICK2, GFP-ICK5, and GFP-ICK6 fusion proteins all showed a migratory shift on the Western blot. This result provides the first evidence that ICK1, ICK2, ICK5 and ICK6 proteins can be dephosphorylated *in vivo*. The observation that no shift is detected for the non-specific bands indicates that the migratory shift observed for the fusion protein was not due to protein degradation or a non-specific effect. The data also suggest that perhaps not all ICK members undergo the same level of phosphorylation in plants. GFP-ICK4, compared to other GFP-ICKs showed no shift following CIP treatment in the case of total protein extract and showed a not very visible shift in the case of protein sample affinity-purified with $p13^{suc1}$-beads. Thus more work is needed to clarify whether ICK4 undergoes phosphorylation *in vivo*.

The degree of shift from non-CIP treated samples to CIP treated samples also appear to differ among ICKs members, although it was difficult to objectively quantify the amount of shift observed. Since protein with more phosphorylation sites will be expected to show a greater shift than proteins with fewer phosphorylation sites after being subjected to CIP treatment, the greater shift may be indicative of multiple phosphorylation sites in these proteins. In this study, GFP was used to aid the detection of ICKs, since the expression system for the fusion proteins and detection techniques were proven to be working in the laboratory. The migration shift of the ICK proteins following CIP treatments may be better studied when they are tagged with a short epitope tag.

Another possible approach to study protein phosphorylation is to use Pro-Q phosphoprotein reagent (Invitrogen) that is said to bind the phosphate groups in phosphoproteins, allowing in-gel visualization of phosphoproteins. Pro-Q staining was thus tested using total protein extracts and proteins purified with $p13^{Suc1}$-conjugated beads. In the case of total proteins, many bands appeared on the gel after staining. With $p13^{Suc1}$ affinity-purified proteins, only very faint bands could be observed, indicating that the amount of proteins was too low for good visualization using this method. Further optimization may be needed.

It was observed that the fusion protein of GFP-ICK7 was present in the non-CIP-treated sample, but was absent in the CIP-treated sample (Figure 6.2). The possibility that the GFP-ICK7 band in the CIP-treated sample was not transferred properly from the SDS-PAGE gel to the nitrocellulose membrane was low since other bands in the lane were transferred and evident.

The amount of protein loaded, as judged by the non-specific band was also similar between the CIP treated and non-CIP treated samples. A more likely and attractive explanation for the absence of the fusion protein band is that the dephosphorylated form of GFP-ICK7 is less stable than the phosphorylated form. In a time course experiment in which the total protein extract was treated with CIP for different lengths of time, it was revealed that the longer the protein extract was treated with CIP, the less abundant of fusion protein was detected on the Western blot (Figure 6.3). This decrease in protein level was more drastic for GFP-ICK7 than for GFP-ICK1. These results suggest that dephosphorylation of ICK7 protein by CIP treatment renders the protein less stable. It is known that mammalian and yeast CDK inhibitors are targeted for degradation after being phosphorylated by CDKs (Vlach *et al.*1997; Tomoda *et al.* 1999). The present results indicate that the stability of at least one member of the ICK family may also be subject to regulation by protein phosphorylation. It will be interesting to verify this observation and determine how phosphorylation may affect the stability of ICK7.

Yeast two-hybrid analyses have revealed that there are likely two groups of plant CDK inhibitors. The A-group inhibitors ICK1, ICK2, ICK6 and ICK7 interact with CDKA;1 and three D-type cyclins (D1, D2, D3), while the B-group inhibitors ICK4, ICK5 and ICKCr interact with D-type cyclins but not with *Arabidopsis* CDKA;1 (Zhou *et al.* 2002a). Thus far, only ICK1 and ICK2 have been shown to be able to interact with the CDK complex *in vivo*. The present results confirm the previous observation that ICK1 and ICK2 are able to interact with the CDK complex *in vivo* (Wang *et al.* 2000; De Veylder *et al.* 2001). These results also show that ICK4, ICK5 and ICK6 are able to interact with the CDK complex in *vivo*. As for ICK7, it is surprising that no GFP-ICK7 was pulled down by $p13^{Suc1}$-affinity pull down assay. Considering that ICK7 can interact with both CDKA;1 and D-type cyclins in the yeast two-hybrid system, this lack of binding to the CDK complex may not be due to a lack of interaction, but more likely due to a low level of stability and rapid degradation of ICK7 *in vivo* and also during the assay. This suggestion is supported by the observation that fusion protein of GFP-ICK7 was comparably less stable than other GFP-ICK proteins.

The results from CIP treatment of $p13^{Suc1}$-affinity purified proteins further show that some proteins of ICK1, ICK2, ICK5, and ICK6 associated with CDK complexes are phosphorylated. This observation suggests that these ICKs when phosphorylated can still interact with the CDK complex. For GFP-ICK4, the observed smearing of the fusion protein and

diffused bands suggest either that GFP-ICK4 fusion protein is prone to degradation or that GFP-ICK fusion protein exists in phosphorylated and dephosphorylated forms. Due to the apparent instability, this observation requires further investigation.

CHAPTER 7: GENERAL DISCUSSION, CONCLUDING REMARKS AND FUTURE DIRECTIONS

In all eukaryotes, the cell cycle is controlled by the CDK/cyclin complex and thus also by the positive and negative regulators of the CDK/cyclin complex. CDK inhibitors can bind and inhibit the activities of CDKs. In plants, a family of CDK inhibitors (referred to as ICKs or KRPs) has been identified. Since the plant CDK inhibitors are very different from the mammalian CDK inhibitors, understanding the functions and regulation of the plant CDK inhibitors could provide new insight on the molecular mechanisms regulating the cell cycle in plants and the differences between plants and animals. Since the initial discovery in 1997, substantial progress has been made on many aspects of plant CDK inhibitors particularly with ICK1, including the expression, protein-protein interactions, cellular localization, role in cell division and endoreduplication, and effect on plant growth (Wang *et al.* 2006).

Despite the progress, there are still many questions about the plant CDK inhibitors as they are related to the cell cycle directly as well as to other processes in plants. In *Arabidopsis*, the ICK family has seven members. One general question is why *Arabidopsis* needs seven CDK inhibitors and what the differences are among different CDK inhibitors. A fuller understanding to this question will undoubtedly be achieved from studies examining various aspects of the ICK expression, function and regulation. To that end, this thesis project attempted to investigate mainly two aspects of ICKs: their interactions with other proteins and their posttranslational modification by phosphorylation.

All of the seven *Arabidopsis* ICKs can interact with D-type cyclins. Preliminary evidence indicates that the majority of them can also interact with CDKA, while ICK4 and ICK5 may not interact with CDKA. It is important to understand the basis for ICK interactions with CDKA and D-type cyclins and functional roles for the interactions. Since peptide aptamers have been shown to be able to bind known proteins and used as trans-dominant agents to perturb protein-protein interactions, we attempted first to isolate peptide aptamers that could interact

with ICKs, particularly with the conserved CDK inhibitory region. If such an aptamer could be obtained, we could express it with a scaffold protein in plants to disrupt the protein-protein interaction between ICKs and CDKA, and to study the biological effect. Following the initial screen, a set of aptamers were obtained. After subjecting to vigorous tests in two yeast two-hybrid systems, we did not obtain an aptamer that possesses a strong interaction with ICKs in both systems.

Following the protein-protein interaction theme, work was then performed to analyze the interaction between D-type cyclins and ICKs. We felt that it might be possible to identify a region in *Arabidopsis* CYCD3 and further specific motifs or amino acid residues responsible for the interaction. However, results from this study show that removal of a relatively short region from either the N-terminal or the C-terminal region of CYCD3 weakened or completely abolished the interaction. This observation suggests that the residues involved in the interaction of CYCD3 with ICK1 may be scattered in the linear sequence but are brought together when the protein is folded. Although this observation provides a helpful clue on how CYCD3 interacts with ICK1, it may be difficult to identify a short region in CYCD3 that still retains the ability to interact with ICKs.

Since limited results were obtained from the work on aptamer and CYCD3 interactions with ICKs, I then examined the expression, stability and possible phosphorylation of ICKs in plants, which forms the major part of my thesis. All seven ICKs were expressed as GFP fusion proteins in transgenic plants. The effects on plants, stability, interaction with the CDK complex and protein phosphorylation of the GFP-ICK proteins were investigated. The present results show that all seven ICKs can produce similar phenotypic effects, including smaller plant size, serrated leaves and altered floral morphology, when they are overexpressed in plants. The levels of ICK fusion proteins are generally low. For some transgenic plants, even though they displayed obvious ICK overexpression phenotypes, the fusion protein was not detectable, suggesting that ICK proteins are generally quite unstable. These results raise a question regarding the underlying mechanism for the instability of ICKs.

Previously, it has been shown that the N-terminal region of ICK1 has a major role in its instability. Since the ubiquitin-proteasome pathway is important for actively degrading certain proteins and this pathway has been shown to be important for cell cycle regulation, it is interesting to know whether ICK fusion proteins are degraded through the ubiquitin-proteasome

pathway. MG132, a proteasome inhibitor, was used. Following MG132 treatments, the levels of GFP-ICK1, GFP-ICK5, GFP-ICK6 and GFP-ICK7 showed an increase compared to the controls, indicating that the proteasome may be involved in the observed low protein levels for these proteins. It is interesting that the effect was not ubiquitously observed for all ICKs. Further, compared to the large amount of protein accumulation observed when the N-terminal region is removed from ICK1, the accumulation of GFP-ICK1 following MG132 treatment is modest. It will be interesting to examine the possible differences among ICKs and why only moderate effects are observed following MG132 treatments considering that ICK proteins appear to be very unstable.

Since protein phosphorylation may affect protein stability as well as protein-protein interactions and cellular localization, the possible phosphorylation of GFP-ICK fusion proteins was also analyzed in this study. CIP was used to treat total protein extracts, and also proteins associated with the CDK complex after $p13^{Suc1}$-affinity purification. The results provide the first evidence that ICK1, ICK2, ICK5 and ICK6 are phosphorylated *in vivo*.

It is clear that more work will be needed to better understand how plant CDK inhibitors are regulated at the post-transcriptional level. In one aspect, other techniques in addition to the CIP approach are needed to confirm that ICKs indeed undergo phosphorylation. Techniques such as Western detection using phosphor-specific antibodies or mass spectrometry can be used since the usefulness of both techniques have been demonstrated in other studies. The specific amino acid of ICK1 that is phosphorylated is also of interest to us. As phosphorylation or dephosphorylation of a protein is linked to cellular functions and often to a cascade event, the discovery of the specific amino acids important for phosphorylation should provide us more insight on the regions of a protein important for its cellular functions. In this regard, a series of HA-tag ICK1 deletion mutants have been constructed and transformed into *Arabidopsis*. This series of constructs may aid us in narrowing down the region of ICK1 that undergoes phosphorylation.

The observation that ICK7 protein is less stable after CIP treatment provides some evidence that phosphorylation status of ICK proteins may play a role in the stability of the protein. The decreased level of ICK7 following CIP treatment may be due to rapid degradation of the protein through the ubiquitin-proteasome pathway. More work will need to be conducted in the future to establish the link between ICK phosphorylation/dephosphorylation and

subsequent stability of the protein. Although we have gained some important understanding on functions and regulation of the plant CDK inhibitors, further studies on the questions discussed above and related questions should provide a fuller picture on this family of plant cell cycle regulars, particularly in terms of posttranscriptional regulation.

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