Role of SIAMESE and its Post-translational Regulation in *Arabidopsis thaliana*

1. ABSTRACT

Cell differentiation is coordinated with cell cycle progression. Before complete withdrawal from the cell cycle and concomitant with differentiation, some cells switch from a mitotic cell cycle to an endoreduplication cycle. During this cycle, the cells duplicate their genome in the absence of chromosome segregation and cytokinesis. A putative plant-specific cyclin-dependent kinase (CDK) inhibitor, SIAMESE (*SIM*) is required to suppress mitosis as a part of the switch to endoreduplication in *Arabidopsis* trichomes. SIM and its homologs are induced under a diverse set of biotic and abiotic stress conditions. Little is yet known about the molecular mechanism by which SIM regulates cell cycle progression. I therefore propose to identify the function of SIM in cell cycle progression and its post-translational regulation. First, I will examine if SIM has CDK inhibitory activity and identify the regions involved in protein-protein interactions. Secondly, the phosphorylation sites of SIM will be identified and the regulatory role of phosphorylation in the function of SIM will be determined through mutagenesis. Thirdly, the role of the PEST domain in SIM protein instability will be determined. The studies proposed here will contribute to the understanding of the role of this putative CDK inhibitor in cell cycle progression and will also shed light on the molecular links between cell cycle regulation and stress responses in plants.

SPECIFIC AIMS

**Aim 1: Determine if SIM has CDK inhibitory activity and identify its protein-protein interaction Domains**

(a) *in vitro*/*in vivo* inhibition assays  
(b) Identification of CDK- and cyclin-binding domains of SIM  
(c) *in vitro*/*in vivo* inhibition assays of binding domain mutants  
(d) Complementation analysis in planta using mutant forms of sim

**Aim 2: Determine the role of phosphorylation in the function of SIM**

(a) Phosphorylation assays and identification of phosphorylation sites  
(b) Generate phosphomimetic and non-phosphorylatable mutants  
(c) *in vitro* inhibition assays of phosphomimetic and non-phosphorylatable mutants  
(d) Complementation analysis in planta using mutant forms of sim

**Aim 3: Determine the role of proteasome-mediated degradation in the function of SIM**

(a) Generate PEST-domain mutants  
(b) *in vitro*/*in vivo* turnover assays of sim mutants  
(c) Complementation analysis in planta using mutant forms of sim

2. BACKGROUND AND SIGNIFICANCE

2.1 Cell cycle regulation

Plants share the basic phases of the cell cycle with all other eukaryotes\(^\text{19,31}\) (Fig 1). The progression of the cell cycle in eukaryotes is regulated by a special class of serine-threonine protein kinases known as cyclin-dependent kinases (CDKs). The kinase activity of CDKs depends on their association with a regulatory protein known as a cyclin (CYC). Cell cycle progression is regulated by cyclic expression of different cyclins and their ubiquitin-mediated proteolysis and by the phosphorylation of a variety of substrates at the key G1/S and G2/M transition points by cyclin/CDK complexes.

The *Arabidopsis* CDK family has 12 members in 6 classes\(^\text{33,67}\). The functions of CDKA and CDKB involved in cell cycle regulation are the best

![Figure 1. Plant Cell Cycle](image)
documented. CDKA is related to the yeast (Cdc2 and Cdc28) and mammalian (CDK1 and CDK2) CDKs and contains the PSTAIRE motif in the cyclin-binding domain. In Arabidopsis, there is only one gene for CDKA (designated AtCDKA;1) and its protein level remains constant throughout the cell cycle. Kinase activity of CDKA is upregulated from late G1 phase to the end of mitosis, suggesting a role for this special CDK at both the G1/S and G2/M transition points. CDKB is unique in plants and, unlike CDKA, its expression is controlled by the cell cycle. Arabidopsis CDKB is further subdivided into two subclasses, each with two members. CDKB1 transcripts accumulate during S, G2, and M phases, whereas CDKB2 expression is specific to G2 and M phases. CDKB proteins follow their transcription pattern, and display a peak of kinase activity at the G2/M transition only. Arabidopsis has 49 cyclins in 10 classes; at least 32 cyclins have putative roles in cell cycle progression. Cyclins with primary cell cycle roles are represented by the classes of A-, B-, and D-type cyclins. The G1/S transition is regulated by a CDKA/CYC complex. The G2/M transition most likely requires both CDKA and CDKB, as well as CYCA, CYCB and probably CYCD proteins, to form mitotic CDK/CYC complexes.

2.2 CDK inhibitors

Cell cycle progression is also regulated by CDK inhibitors (CKIs). In yeast and animals, CKIs play an essential role in orchestrating cell proliferation and differentiation and are regulated at the post-translational level. In budding yeast, the CKI p40Sic1 inhibits S-phase CDK complexes Cdc28/Cln5-6 until it is destroyed by ubiquitin-dependent proteolytic degradation initiated by G1-phase CDK-dependent phosphorylation. Interestingly, p40Sic1 is involved in keeping Cdc28/Cln5-6 complexes inactive in response to stress and nutrient starvation. Furthermore, p40Sic1 can regulate the exit from mitosis by inhibition of mitotic CDK activity. In mammals, CKI p27kip1 is phosphorylated on Ser-10 at G1 phase and exported from the nucleus. In the cytoplasm, p27kip1 is ubiquitinated and then degraded by the proteasome. During late G1 and S phase, p27kip1 is phosphorylated on Thr-187 by CDK2-cyclE, ubiquitinated in the nucleus by the ubiquitin ligase, and degraded by the proteasome.

CKIs have been identified in a number of plant species. All of them share a short sequence motif with mammalian CDK inhibitor p27kip1 in their CDK- and cyclin-binding site and are therefore named Kip Related Proteins (KRP) or Interactors of Cdc2 Kinase (ICK). ICKs/KRPs interact with CDKA;1 and D-type cyclins and inhibit CDK activity in vitro and in vivo. Arabidopsis ICK/KRP genes are regulated by hormones and show developmental- and tissue-specific expression patterns. Little is known on how these inhibitors are regulated at the translational level and/or by post-translational modification. Only ICK2/KRP2 has been demonstrated to be controlled by proteasome-mediated degradation which depends on phosphorylation of ICK2/KRP2 by CKIs.

2.3 Control of mitosis-endoreduplication transition

Exit from the mitotic cycle and initiation of cell differentiation frequently correlates with the onset of endoreduplication, a modified cell cycle during which cells undergo DNA replications without any subsequent cytokinesis. Although the physiological role of endoreduplication is still unresolved, it has been postulated to control a variety of biological processes, such as cell differentiation, cell expansion, metabolic activity and resistance against irradiation. Not only is the functional role of endoreduplication unclear, but also the molecular mechanisms responsible for this specialized cell cycle remain elusive. Recent studies in animal and plant systems suggest that endoreduplication requires elimination of mitotic CDK activity and oscillations in the activity of S-phase CDKs. Various strategies have been proposed by which mitotic CDK activity could be reduced. CCS52 protein activates the anaphase-promoting complex and thereby triggers the destruction of mitotic cyclins. In alfalfa (Medicago sativa), CCS52 expression correlates with the onset of endoreduplication. Mitotic CDK activity could also be negatively regulated by WEE1 kinase, because WEE1 transcript levels increase during the endoreduplication process in maize endosperm. A likely candidate for a mitotic CDK that regulates the mitosis-endoreduplication transition is CDKB1;1. CDKB1;1 is highly expressed in dividing cells and is down-regulated at the onset of endoreduplication. The absence of CDKB1;1 transcripts in polyploid tissues suggests a mechanism that operates to suppress the transcriptional activation of CDKB1;1 in endoreduplicating cells.

In animals, expression of CKI genes is associated with endoreduplication. The accumulation of
mammalian CKI p57Kip2 in endoreduplicating trophoblasts causes a drop in CDK activity after each S phase, which is required for correct re-initiation of origins of replication. In Drosophila, the CKI Dacapo also oscillates in endoreduplicating nurse cells, indicating that oscillation of Cip/Kip proteins may be a common feature of endoreduplication in different organisms. Recently, the ICKs/KRPs have been shown to contribute to the control of the mitosis-endoreduplication transition in plants. Expression of ICK1/KRP1 and ICK2/KRP2 is restricted to endoreduplicating tissues and overexpression of ICK2/KRP2 inhibits the mitotic CDKA complex only, resulting in a premature onset of endoreduplication. However, the mechanisms of ICKs/KRPs in triggering the onset of endoreduplication and in maintaining low CDK activity remain elusive.

Recently, a family of putative plant CDK inhibitors was found, distantly related to the ICKs/KRPs family, known as SIAMESE (SIM). SIM and its homologs contain a putative cyclin binding motif (motif 3) and a motif (motif 4) which is similar to the D-type cyclin binding domain of ICKs/KRPs. SIM interacts with CDKA;1 and D-type cyclins and its overproduction results in slow-growing plants with narrow leaves containing enlarged epidermal cells with an increased DNA content. Recessive mutations in the SIM gene have multicellular trichomes, the individual nuclei of which have reduced levels of the endoreduplication. These observations suggest that SIM plays an integral role in regulation of endoreduplication. In addition, the expression of SIM was induced under a diverse set of biotic and abiotic stress conditions. Both biotic and abiotic stress stimuli negatively affect plant growth through the inhibition of the cell cycle machinery, but the molecular mechanisms that link the stress perception directly to the cell cycle machinery is lacking. This observation implies that SIM family proteins might coordinate stress perception and cell cycle progression.

2.4 Significance

The basic cell cycle mechanisms are remarkably conserved in all eukaryotes. However, plant genomes have significantly larger gene families for the cell cycle components, suggesting that the plant cell cycle may have additional complexity. Study of SIM may give additional insights into the diversity of cell cycle progression and the mechanisms of endoreduplication. In this proposal, we attempt to identify the role of SIM in cell cycle progression and its post-translational regulation. The results from these studies will shed light on: (1) Regulation of the cell cycle (2) The role of endoreduplication (3) Stress responses in plants.

(1) Regulation of the cell cycle

Numerous studies have shown that CDK inhibitors (CKIs) are essential in orchestrating cell proliferation and differentiation. Progress in understanding the differential functions of CKIs will help us to elucidate their roles during cell cycle progression and in linking cell proliferation, differentiation, and other cellular processes.

(2) The role of endoreduplication

Endoreduplication occurs in arthropods and mammals, but it is particularly common in a wide variety of cell types in angiosperms, including agriculturally important tissues. Although the mechanisms of endoreduplication are still poorly understood, it has been postulated that endoreduplication controls a variety of biological processes. Since CKIs are also involved in controlling endoreduplication, understanding the regulation of CKIs during cell cycle progression will help us elucidate the molecular mechanisms of endoreduplication. The experiments proposed here will contribute not only to our understanding of endoreduplication but also to further researches on how cell cycle machinery communicates with intrinsic developmental signals.

(3) Stress responses in plants

Because of the sessile life style, plants have probably developed mechanisms that allow them to modulate their cell cycle progression in response to environmental cues. The perception of biotic and abiotic stress signals involves activation of signaling cascades that trigger an impaired G1/S transition, a prolonged S phase, and/or delayed entry into mitosis. However, the molecular links between cell cycle regulation and stress responses in plants are mostly unknown. Due to the stress-induced expression feature, SIM might have a major role in connecting cell-cycle progression with stress perception. The results from this proposal will provide information for further elucidation of stress responses in plants.
3. RESEARCH DESIGN AND METHODS

3.1. Aim-1: Determine if SIM has CDK inhibitory activity and identify its protein-protein interaction domains

3.1.1 Rationale

SIM interacts with CDKA and D-type cyclins and its overproduction results in a strong inhibition of cell division activity. However, biochemical proof of its inhibitory activity is still lacking. Recently, Peres et al. showed that a SIM related protein of rice, OsEL2 can bind to CDKA1;1 and D-type cyclins. They found that recombinant OsEL2 abolished CDKA1;1 kinase activity by in vitro inhibition assay. Hence, I hypothesize that SIM inhibits CDK kinase activity and this inhibitory activity is through direct interaction with CDKA;1 and/or D-type cyclins. I will first use inhibition assays to examine its CDK inhibitory activity and then identify its CDKA;1- and cyclin-binding domains. The inhibition assays with mutations in the CDKA;1-binding domain and/or cyclin-binding domains will be performed to determine if SIM can inhibit CDKA;1 kinase activity through direct interaction. The complementation analysis will be performed to examine the effects of the interaction between SIM and CDK complexes in planta.

3.1.2 Experimental Design

(a) in vitro/in vivo inhibition assays

To determine the role of SIM as a CDK inhibitor, an in vitro inhibition assay will be performed. To obtain enough SIM protein for in vitro assays, I will first generate a functional His$_6$–tagged SIM fusion protein by inducible expression in E. coli. This expression method has been used to produce functional ICKs/KRPs or OsEL2 for inhibition assays. Secondly, immunoprecipitations will be performed to obtain CDKA;1 and CDKB1;1 complexes from synchronized Arabidopsis cell suspension culture MM1 by anti-CDKA;1 and anti-CDKB1;1 antibodies, respectively. The MM1 cell line will be synchronized by applying an aphidicolin block/release and cell cycle phases will be examined by flow cytometry analysis as described by Menges et al. For inhibition assays, CDK complexes will be incubated with histone H1 as a CDK substrate in the presence or absence of the recombinant SIM. The histone phosphorylation reaction will be initiated by adding ATP and $^{32}\text{P}-\gamma$-ATP, and stopped by adding SDS-PAGE sample buffer. Samples will be resolved by SDS-PAGE and CDK activity can be detected through autoradiography according to the kinase assays described by Wang.

Since recombinant OsEL2 abolished CDKA1;1 kinase activity in the in vitro inhibition assays, I predict that lower CDKA;1 activity will be detected in the reaction with the recombinant SIM. On the other hand, Churchman et al. have shown that SIM protein does not interact with CDKB1;1 as well as A- and B-type cyclins by FRET. Recently, Peres et al. also showed that recombinant OsEL2 specifically inhibited CDKA;1, but not CDKB1;1 activity. Therefore, I predict that CDKB1;1 activity will not be inhibited significantly after incubating with recombinant SIM.

Although functional ICKs/KRPs have been generated by inducible expression in E. coli, it is possible that SIM may not be functional by this expression method. In such case, in vitro transcription/translation performed by rabbit reticulocyte lysate will be used as an alternative method to produce a functional His$_6$–SIM fusion protein. It is also possible that CDK activity may be blocked by other CDK inhibitors from Arabidopsis cells. Nakai et al. have produced Flag-tagged CDK complexes from insect cells; these complexes can phosphorylate histone H1 in vitro. By using the insect cell expression system, I can eliminate the effects of other CDK inhibitors from Arabidopsis cells.

Because the inhibition of CDK kinase activity through SIM may be regulated by other molecules which do not exist in vitro, it is possible that the inhibitory activity is unable to be detected through in vitro assays. Overexpression of ICKs/KRPs has been demonstrated to result in a decrease in CDK activity. Thus, an in vivo inhibition assay as a complement to the in vitro assay will be carried out. To perform in vivo inhibition assays, transgenic plants expressing wild-type SIM ectopically under the cauliflower mosaic virus 35S promoter need to be produced first. The resulting construct 35S:SIM will be introduced into Agrobacterium tumefaciens by transformation and subsequently into wild-type Arabidopsis plants (Columbia...
ecotype) via the floral dip method. Plants overexpressing SIM are slow-growing and have narrow leaves, but sufficient tissue samples can be obtained by collecting more seedlings for in vivo assays. Inhibition of CDK activity by SIM will be determined by comparison of immunoprecipitated CDK-associated kinase activity from an untransformed control line to a SIM overexpression line. Immunoprecipitations of CDKA;1 and CDKB1;1 complexes from wild-type and SIM overexpression plants and kinase assays will be performed as previously described in the in vitro inhibition assays. The predicted result of the in vivo inhibition assays is that SIM overexpression lines will show lower CDKA;1 activity in comparison with wild-type lines, whereas no significant inhibition of CDKB1;1 activity will be observed. Even though CDKA;1 or CDKB1;1 complexes are purified from seedlings at the same age, cells may be at different cell cycle phases with different CDK activity. To overcome this obstacle, a cell suspension culture MM1 can be used for in vivo assays. To generate overexpressing lines for in vivo inhibitory assays, a modified Agrobacterium-mediated transformation method will be performed as described by Menges et al.

(b) Identification of CDK- and cyclin-binding domains of SIM

Although it has been demonstrated that SIM interacts with CDKA;1 and D-type cyclins by FRET analysis, the identification of CDK and cyclin-binding domains is still lacking. An alignment of SIM and other related plant proteins (Fig 2B) shows that motif 4 of the SIM family is similar to the cyclin-binding domain of ICKs/KRPs and that motif 3 of SIM family is a putative cyclin binding motif. However, there is no conserved CDK binding domain predicted by this alignment.

To identify the CDK binding domain, I will use the yeast two-hybrid system to test different truncations of SIM. The truncations of SIM will be generated by PCR amplification and then fused to the GAL4 activation domain (GAD). The CDKA;1 coding region will be cloned as translational fusions to the GAL4 DNA binding domain (GBD). The full-length SIM fused with GAD will serve as a positive growth control and the empty vector with GAD domain will serve as a negative growth control. For pairwise two-hybrid interactions, different combinations between GAD and GBD constructs will be transformed into yeast cells. Cells transformed with truncated sim which can not interact with CDKA;1 will not be able to grow under histidine-deficient minimal media. The strength of each mutation will be quantified using a liquid β-galactosidase (β-gal) assay. To confirm the CDKA;1 binding region of SIM, alanine-scanning mutagenesis will be applied to the region predicted by the yeast two-hybrid assays. The mutant sim construct will be fused to GAD and then examined by yeast two-hybrid assays as described previously. Since SIM has a consensus D-type cyclin-binding motif, mutant sim protein which are impaired in their ability to interact with D-type cyclins can also be generated by alanine-scanning mutagenesis. I will use in vitro pull-down assays to confirm the mutations with impaired ability to interact with CDKA;1 or D-type cyclins. A glutathione S-tranferase (GST) will be fused to wild-type SIM coding region or sim mutants and these GST-fusion proteins will be expressed in E. coli and purified by using glutathione-Sepharose beads. Total protein extracted from wild-type Arabidopsis will then be incubated with GST-fusion protein tethered to glutathione-Sepharose. Proteins will be eluted from beads by incubation in SDS-PAGE loading buffer, resolved in SDS-PAGE and detected by western blotting with anti-CDKA;1 antibody or anti-D-type cyclins antisera.

Peres et al. have demonstrated that mutations in the cyclin-binding motif of OsEL2 decreased the binding affinity to CDKs, indicating that besides a direct interaction, OsEL2 also partially interacts with CDKs indirectly through the association of cyclins. Accordingly, I predict that sim mutants with mutations in the CDK-binding motif (simCDKA) and cyclin-binding motif (simCYC) will not be able to pull down CDKA;1 and D-type cyclins respectively and also the simCYC mutant will show reduced affinity for CDKA;1.

Due to the high occurrence of false-positives, a complement to the yeast two-hybrid screen, Bimolecular Fluorescence Complementation (BiFC) method will be used to identify the CDKA;1- or D-type cyclin-binding domains. Many reports on the use of BiFC in planta have been published, and this system is becoming a routinely used approach to study protein-protein interactions in living plant cells. The mutant sim will be fused to nYFP (N-terminal YFP fragment) and CDKA;1 or D-type cyclin genes will be fused to cYFP (C-terminal YFP fragment). Both nYFP and cYFP fusion proteins will be transiently coexpressed in...
the leaf epidermal cells of Arabidopsis through agroinfiltration. A functional YFP is reconstituted if the fusion proteins interact with each other. Thus, by BiFC, the interaction status of SIM and CDKA;1 or D-type cyclins will be examined via fluorescence emission upon excitation with a suitable wavelength. However, BiFC methods only determine the close physical proximity of two proteins in vivo, the direct interaction of these two proteins needs to be confirmed by in vitro assays, such as in vitro pull-down assays.

(c) in vitro/in vivo inhibition assays of binding domain mutants

CDK activity is inhibited by binding of CKIs. To determine if the binding of SIM to the CDK complex is required for inhibiting CDK activity, simCDKA and simCYC mutants will be used for in vitro/in vivo inhibition assays. For in vitro inhibition assays, His6-tagged wild-type SIM or mutant sim will be expressed in E.coli and subjected to in vitro inhibition assays as described in Aim 1(a). For in vivo assays, transgenic lines overexpressing wild-type SIM or binding-domain mutant sim will be generated by Agrobacterium-mediated transformation into the SIM knock-out background (sim-1). These transgenic lines will then be used for in vivo inhibition assays as described in Aim 1(a). I will examine the function of different types of sim mutations: (1) 35S:SIM (2) 35S:simCYC (3) 35S: simCDKA (4) 35S: simCDKA/CYC (sim with CDKA;1- and cyclin-binding domain mutations).

If the binding of SIM to the CDK complexes is required for inhibiting CDK activity, the kinase activity of CDKA;1 will be (4)>(3)>(2)>(1). Because SIM might indirectly interact with CDKA;1 through the binding of cyclins, I predict that the kinase activity of (4) will be higher than (3).

(d) Complementation analysis in planta using mutant forms of sim

To examine the effects of interaction between SIM and CDK complexes in planta, complementation analysis will be performed. On wild-type leaves, trichomes are unicellular and most of them occur singly. Instances where two or more adjacent trichomes occur with no intervening cells have been termed trichome clusters. Multicellular trichomes and trichome clusters are observed in sim-1 leaves. Individual nuclei of sim-1 trichomes have about one-third the DNA content of wild-type trichome nuclei. Constructs of wild-type or mutant SIM under a trichome-specific GL2 promoter will be introduced into the sim-1 background for complementation analysis. Four constructs will be used for complementation analysis: (1) pGL2:SIM (2) pGL2: simCYC (3) pGL2: simCDKA (4) pGL2: simCDKA/CYC. Complementation will be quantified by counting the trichome cluster frequency and by measuring endoreduplication levels of trichomes. The endoreduplication levels of trichomes can be determined by measuring relative DNA content of DAPI-stained trichomes as described by Szymanski et al. It has been shown that pGL2:SIM can completely complement sim-1. Thus, I predict that construct (2) and construct (3) will partially rescue the trichome endoreduplication levels and clustering phenotype, whereas no significant complementation occurs with construct (4).

3.2. Specific Aim-2 : Determine the role of phosphorylation in the function of SIM

3.2.1 Rationale

Mammalian and yeast CKIs are phosphorylated by CDKs, after which they are recognized and degraded by the proteasome. It has been demonstrated that phosphorylation of ICKs/KRPs by CDK complexes or other kinases can result in either degradation of ICKs/KRPs or enhancement of CDK inhibitory activity. In addition, 35S:GFP:SIM lines showed strong GFP expression in nondividing tissues of the root, but expression was absent in the root tip. This strong selection against SIM expression in dividing tissues suggests that high CDKB activity in dividing cells may induce SIM protein degradation. Apart from phosphorylation by CDK complexes, CDK inhibitors can also be controlled through phosphorylation by other kinases. Pettkó-Szandtner et al. have found that phosphorylation of alfalfa KRPMt by the recombinant MsCPK3 resulted in enhanced inhibition of CDK function. CPK3 belongs to the group of calmodulin-like domain protein kinases that function in the transmission of stress and hormonal signals in different plant species. By using NetPhosK 1.0 server, 14 phosphorylation sites of SIM were predicted, including three putative CDK phosphorylation sites (Fig 2A). Therefore, I hypothesize that SIM is regulated through phosphorylation by CDK complexes or other kinases. I will first determine if SIM is a phospho-protein by phosphorylation assays and then identify the phosphorylation sites by mass spectrometry.
Phosphomimetic and non-phosphorylatable SIM mutants will be generated for inhibition assays. The significance of SIM phosphorylation will then be examined by complementation analysis.

3.2.2 Experimental Design

(a) Phosphorylation assays and identification of phosphorylation sites

To determine if SIM is a phospho-protein, an in vitro phosphorylation assay will be performed. Verkest et al. have used this method to illustrate that ICK2/KRP2 is phosphorylated by CDKB1;1. Purification of His$_6$-tagged SIM fusion protein expressed in E. coli and CDKA;1 or CDKB1;1 complexes will be performed as described in Aim 1(a). Incubation of His$_6$-SIM fusion protein with CDKA;1 or CDKB1;1 complexes will be performed in the presence of radioactively labeled ATP and proteins will then be separated by SDS-PAGE. Phosphorylation of SIM by CDK complexes will be determined by autoradiography.

For identification of the phosphorylation sites, in vitro phosphorylation assays will be performed in the presence of ATP rather than radioactively labeled ATP. SDS-PAGE will be stained with a fluorescent phosphosensor dye, Pro-Q Diamond and then the phosphorylated His$_6$-SIM band will be excised. In-gel digestion with trypsin will be performed and the digested mixture will then be subjected to ion trap liquid chromatography-tandem mass spectrometry (LC/MS/MS) to identify the precise location of the phosphorylation sites as described by Wang et al. This method has been used for identifying phosphorylation sites of phosphoproteins in Arabidopsis.

Since CDKs are serine-threonine kinases, I predict that the CDK phosphorylation sites of SIM will be on the serine or threonine residues. However, in vitro phosphorylation has limitations in that phosphorylation sites may differ from what might take place in vivo. As a consequence, the phosphorylation sites must be confirmed by in vivo assays.

To identify non-CDK mediated and CDK mediated phosphorylation sites in vivo, transgenic Arabidopsis MM1 suspension cells expressing His$_6$-SIM fusion protein will be generated by a modified Agrobacterium-mediated transformation. These transgenic cells will be treated with/without olomoucine, a specific inhibitor of CDKs. The effectiveness of olomoucine can be examined by detecting CDK kinase activity as described in Aim 1(a). His$_6$-SIM fusion protein will be purified in the presence of phosphatase inhibitors. Proteins will then be separated by SDS-PAGE and processed for LC/MS/MS as described previously. It is possible that SIM protein in plants will not be present in sufficient amounts to be identified by LC/MS/MS. To overcome this limitation, I can generate phospho-protein by incubating Arabidopsis cell lysates with ATP and His$_6$-SIM fusion protein expressed by E. coli.

The phosphorylation sites, especially those at conserved regions, will be further examined by generating phosphomimetic or non-phosphorylatable mutations for in vitro and in vivo assays.

(b) Generate phosphomimetic and non-phosphorylatable mutants

The functional significance of each of the phosphorylation sites identified by LC/MS/MS will be assessed by site-directed mutagenesis. I will use wild-type pDEST15 SIM-GST or pEarleygate 202 SIM-Flag constructs as templates for site-directed mutagenesis with the Quick Change II site-directed mutagenesis kit (Stratagene). The primers will be designed according to the phosphorylation sites identified by mass spectrometry and the amino acid residues at phosphorylation sites will be mutated to either Glu for phosphomimetic mutations or Ala for non-phosphorylatable mutations. In yeast, multisite phosphorylation of CKI Sic1 sets a threshold in regulating protein-protein interaction and degradation, suggesting that a single phosphorylation mutation may not have any effect on SIM protein activity or stability. Thus, phosphorylation sites of SIM will be mutagenized either singly or in various combinations. The resulting constructs from pDEST15 SIM-GST will be introduced into E. coli for in vitro assays. The resulting constructs from pEarleygate202 SIM-Flag will be introduced into Agrobacterium tumefaciens for in vivo assays.

It has been shown that phosphorylation on CDK inhibitors can result in either degradation of ICKs/KRPs or enhancement of CDK inhibitory activity. To determine the phosphorylation effects on SIM degradation, protein turnover experiments will be performed in Aim 3(b). The regulation of CDK inhibitory activity by phosphorylation of SIM will be determined by inhibition assays in Aim 2(c). In addition, I will examine the significance of phosphorylation in plants by complementation analysis.
Qualifying Exam                                                                       Yi-Hsuan Chiang

(c) **in vitro** inhibition assays of phosphomimetic and non-phosphorylatable mutants

Phosphomimetic or non-phosphorylatable mutant sim will be expressed and purified from *E. coli*. The **in vitro** inhibition assays will be performed to determine CDK kinase activity as described in Aim 1(a). If the phosphorylation of SIM can enhance its CDK inhibitory activity, the reaction with the non-phosphorylatable mutant sim will have higher CDK activity than the reaction with the phosphomimetic mutant sim. If the phosphorylation of SIM decreases its CDK inhibitory activity, I will detect higher CDK activity in the reaction with the phosphomimetic mutant sim. The decrease of CDK inhibitory activity may result from phosphorylation-mediated degradation or conformational change of SIM. This part will be further confirmed by protein turnover assays in Aim 3(b).

(d) **Complementation analysis in planta** using mutant forms of sim

It is possible that differences in CDK activity between phosphomimetic and non-phosphorylatable mutants will not be detected by inhibition assays. I will therefore examine the effects in planta. **Multicellular trichomes and trichome clusters** are observed in *sim-1* leaves. Individual nuclei of *sim-1* trichomes have about one-third the DNA content of wild-type trichome nuclei. Since mutant sim may still have basal function, it will be hard to investigate the effects of phosphorylation by expressing mutant sim under *GL2* promoter. However, in sharp contrast with *pGL2:SIM, 35S:SIM* only partially complements *sim-1*. This partial complementation of *sim-1* by *35S:SIM* can be used for examining the effects of SIM phosphorylation. Constructs of wild-type or mutant SIM under a 35S promoter will be introduced into the *sim-1* background for complementation analysis. Complementation will be quantified as describe in Aim 1(d). Four constructs will be used for complementation analysis: (1) *35S:SIM* (2) *35S:phosphomimetic sim* (3) *35S:non-phosphorylatable sim*.

If the phosphorylation of SIM enhances its function, the number of multicellular trichomes and trichome clusters in *sim-1* plants transformed with different constructs will be (3)>(1)>(2) and the endoreduplication levels of trichomes will be (2)>(1)>(3).

In Aim 3(b), I will determine if the phosphorylation of SIM promotes protein degradation. If phosphorylation by CDK complexes promotes SIM degradation, the number of multicellular trichomes and trichome clusters in *sim-1* plants transformed by different constructs will be (2)>(1)>(3) and the endoreduplication levels of trichomes will be (3)>(1)>(2).

### 3.3. **Specific Aim-3** : Determine the role of proteasome-mediated degradation in function of SIM

#### 3.3.1 Rationale

Cell cycle regulators such as cyclins and E2F transcription factors are controlled by proteasome-mediated pathways. In *Arabidopsis*, some ICKs/KRPs are controlled by proteasome-mediated degradation through the PEST domains which serve as a proteolytic signal. SIM, SIAMESE-Related 2 (SMR2), and SMR3 all have putative PEST domains. In addition, phosphorylation of ICKs/KRPs by CDK complexes leads to the degradation of ICKs/KRPs by proteasome-mediated pathways as mentioned in Aim 2. Therefore, I hypothesize that the degradation of SIM is mediated by the proteasome through its PEST domain and through phosphorylation by CDK complexes. I will generate mutant sim with mutations at the PEST domain to examine protein stability through **in vitro/in vivo** turnover assays. Transgenic plants expressing mutant sim with mutations at the PEST domain will be generated and complementation analysis will be performed. Protein stability of SIM with mutations at CDK phosphorylation sites from Aim 2(b) will also be examined.

#### 3.3.2 Experimental Design

(a) **Generate PEST-domain mutants**

It has been shown that SIM contains a putative PEST domain (amino acids 26 to 40). To determine if the protein instability of SIM is mediated by the PEST domain, this domain will be mutated into a stretch of alanine residues by alanine-scanning mutagenesis, resulting into the mutant simPEST protein. The amino acid sequence of mutant sim will be subjected to PESTfind to confirm that no PEST domain is predicted in mutant sim. Mutant will then be used for **in vitro/in vivo** turnover assays or complementation analysis.

It is possible that the putative PEST domain is not involved in SIM protein turnover. In such case, I
will generate truncated SIM proteins to identify domains which are responsible for protein instability. PEST domains may also be involved in protein-protein interactions through phosphorylation. It is possible that the PEST domain mediates the interaction between SIM and CDK complexes. This alternative function of the PEST domain in protein-protein interactions can also be further examined by yeast two-hybrid with simPEST as described in Aim 1(b). If the PEST domain of SIM is involved in binding to CDK complexes, yeast cells transformed with simPEST which can not interact with CDK or cyclins will not be able to grow under histidine-deficient minimal media.

(b) \textit{in vitro/in vivo} turnover assays

The phosphorylation mutant sim-GST (from Aim 2) and simPEST-GST fusion proteins will be expressed and purified from \textit{E. coli}. Wild-type \textit{Arabidopsis} cell extracts will be incubated with each of the purified mutant fusion proteins. At each time point, protein samples will be removed from the reactions and will then be subjected to SDS-PAGE and immunoblotting by anti-GST antibody. The 26S proteasome inhibitor MG132 or DMSO will be used to test if SIM protein turnover is mediated by the 26S proteasome. Verkest \textit{et al.} have used this \textit{in vitro} assay to examine ICK2/KRP2 stability in the presence or absence of MG132.

Planchais \textit{et al.} have performed an \textit{in vivo} turnover assay to examine the stability of \textit{Arabidopsis} CYCD3;1 in the presence or absence of MG132. To examine the protein turnover of SIM \textit{in vivo}, transgenic \textit{Arabidopsis} MM1 suspension cells expressing SIM-Flag or simPEST-Flag will be generated by a modified \textit{Agrobacterium}-mediated transformation method. Transgenic cells with phosphomimetic or non-phosphorylatable mutant sim will also be generated for \textit{in vivo} turnover assays. Synchronized transgenic cells will be treated with cycloheximide to block de novo protein synthesis. Samples will be collected at different time points for protein extraction. Protein extracts will be subjected to SDS-PAGE followed by immunoblotting with anti-Flag antibody. MG132 or DMSO will be used to test if SIM protein turnover is mediated by the 26S proteasome.

PEST domains contribute to protein instability. Hence, I predict that simPEST will be more stable than wild-type SIM. Because the ubiquitin/26S proteasome proteolytic pathway regulates a wide variety processes in eukaryotes to selectively remove specific proteins, and also the degradation of CKIs is regulated by this pathway, I expect that the turnover of SIM protein is also regulated by this pathway. It has been shown that phosphorylation of CKIs by CDK complexes leads to protein degradation by proteasome. Therefore, I predict that sim with the phosphomimetic mutations at CDK phosphorylation sites is less stable than the non-phosphorylatable mutant.

(c) Complementation analysis in planta using mutant forms of sim

To examine the function of the PEST domain in SIM, complementation analysis of sim-1 with wild-type SIM or simPEST will be performed as described in Aim 2(d). If the protein stability of SIM is regulated by the PEST domain, sim-1 plants transformed by 35S:simPEST will have fewer multicellular trichomes and trichome clusters than sim-1 plants transformed by 35S:SIM. In addition, sim-1 plants transformed by 35S:simPEST will have higher endoreduplication levels of trichomes than sim-1 plants transformed by 35S:SIM.

4. TIMELINE

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<th>Year 1</th>
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<tr>
<td>Aim1</td>
<td>(1) inhibition assays. (2) Identify CDKA;1- or cyclin-binding site mutants</td>
<td>Generate CDKA;1- and cyclin-binding site mutants</td>
<td>Examination of CDKA;1- and cyclin-binding site mutants.</td>
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<tr>
<td>Aim2</td>
<td>Identification of phosphorylation sites</td>
<td>Generate phosphorylation mutants</td>
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<td>Aim3</td>
<td>Generate PEST-domain mutations</td>
<td>\textit{in vitro/in vivo} turnover assays Complementation analysis</td>
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Figure 2 (Adapted from Churchman et al. 8)

(A) Alignment of conceptual translation of SIM reading frame and related plant proteins. The regions numbered 1 to 5 denote conserved domains. Sl, Solanum lycopersicum; St, Solanum tuberosum; Zm, Zea mays; Os, Oryza sativa; Pt, Populus tremula; Gm, Glycine max. ▲: putative CDK phosphorylation site. *: putative phosphorylation site.

(B) Similarity between SIM and D-type cyclin binding domain of ICKs/KRPs.

5. REFERENCES


Qualifying Exam


22 Fobert, P.R., V. Gaudin, P. Lunness, E.S. Coen, and J.H. Doonan. 1996. Distinct classes of cdc2-related genes are differentially expressed during the cell division cycle in plants. *Plant Cell* 8: 1465-1476.


