Analysis of Plant Homeodomain Proteins and the Inhibitor of Growth Family Proteins in
Arabidopsis thaliana

Natasha Marie Safaee

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Biochemistry

Glenda E. Gillaspy
Erin Dolan
James Mahaney
James Tokuhisa

August 18, 2009
Blacksburg, Virginia

Keywords: Plant Homeodomain, Inhibitor of Growth, H3K4me3, *Arabidopsis thaliana*,
Oryza sativa, chromatin remodeling

Analysis of Plant Homeodomain Proteins and the Inhibitor of Growth Family Proteins in
Arabidopsis thaliana

Natasha Marie Safaee

ABSTRACT

Eukaryotic organisms require the ability to respond to their environments. They do so by utilizing signal transduction pathways that allow for signals to effect final biological responses. Many times, these final responses require new gene expression events that have been stimulated or repressed within the nucleus. Thus, much of the understanding of signal transduction pathways converges on the understanding of how signaling affects gene expression alterations (Kumar et al., 2004). The regulation of gene expression involves the modification of chromatin between condensed (closed, silent) and expanded (open, active) states. Histone modifications, such as acetylation, can determine the open versus closed status of chromatin.

The PHD (Plant HomeoDomain) finger is a structural domain primarily found in nuclear proteins across eukaryotes. This domain specifically recognizes the epigenetic marks H3K4me2 and H3K4me3, which are di- and tri-methylated lysine 4 residues of Histone H3 (Loewith et al., 2000; Kuzmichev et al., 2002; Vieyra et al. 2002; Shiseki et al., 2003; Pedeux et al., 2005, Doyon et al., 2006). It is estimated that there are ~150 proteins that contain the PHD finger in humans (Solimon and Riabowol, 2007). The PHD finger is conserved in yeast and plants, however an analysis of this domain has only been performed done in *Arabidopsis thaliana* (Lee et al., 2009). The work presented in this report aims to extend the analysis of this domain in plants by identifying the PHD fingers of the crop species *Oryza sativa* (rice). In addition, a phylogenetic analysis of all PHD fingers in Arabidopsis and rice was undertaken. From these analyses, it was determined that there are 78 PHD fingers in Arabidopsis and 70 in rice. In addition, these domains can be categorized into classes and groups by defining features within the conserved motif.

In a separate study, I investigated the function of two of the PHD finger proteins from Arabidopsis, ING1 (INhibitor of Growth1) and ING2. In humans, these proteins can be found in complexes associated with both open and closed chromatin. They facilitate chromatin remodeling by recruiting histone acetyltransferases and histone deacetylases to chromatin (Doyon et al., 20

Pena et al., 2006). In addition, these proteins recognize H3K4me2/3 marks and are believed to be "interpreters" of the histone code (Pena et al., 2006, Shi et al., 2006). To understand the function of ING proteins in plants, I took a reverse genetics approach and characterized *ing1* and *ing2* mutants. My analysis revealed that these mutants are altered in time of flowering, as well as their response to nutrient and stress conditions. Lastly, I was able to show that ING2 protein interacts *in vitro* with SnRK1.1, a nutrient/stress sensor (Baena-Gonzalez et al., 2007). These results indicate a novel function for PHD proteins in plant growth, development and stress response.

Acknowledgements

Do you not know?
Have you not heard?
Has it not been told you from the beginning?
Have you not understood since the earth was founded?

He sits enthroned above the circle of the earth,
and its people are like grasshoppers.
He stretches out the heavens like a canopy,
and spreads them out like a tent to live in.

He brings princes to naught
and reduces the rulers of this world to nothing.
No sooner are they planted,
no sooner are they sown,
no sooner do they take root in the ground,
than he blows on them and they wither,
and a whirlwind sweeps them away like chaff.

"To whom will you compare me?
Or who is my equal?" says the Holy One.
Lift your eyes and look to the heavens:
Who created all these?
He who brings out the starry host one by one,
and calls them each by name.
Because of his great power and mighty strength,
not one of them is missing.

Why do you say, O Jacob,
and complain, O Israel,
"My way is hidden from the LORD;
my cause is disregarded by my God"?

Do you not know?
Have you not heard?
The LORD is the everlasting God,
the Creator of the ends of the earth.
He will not grow tired or weary,
and his understanding no one can fathom.

He gives strength to the weary
and increases the power of the weak.
Even youths grow tired and weary,
and young men stumble and fall;
but those who hope in the LORD
will renew their strength.
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint.

Isaiah 40: 21-31

iv

Table of Contents

List of Figures

List of Tables

Chapter 2

List of Abbreviations

5-PTase	*myo*-inositol polyphosphate 5-phosphatase
ABA	abscisic acid
DAG	diacylglycerol
FT	flowering time
GUS	β-glucuronidase
H3K4me3	trimethylated histone 3 at lysine 4
HAT	histone acetyl transferase
HDAC	histone deacetylase
HMT	histone methyltransferase
Ins	*myo*-inositol
InsPs	inositol phosphates
Ins(1,4,5)P$_3$	*myo*-inositol (1,4,5)-trisphosphate
IP	immunoprecipitation
MIOX4	*myo*-inositol oxygenase
MS	Murashige & Skoog (plant culture salts)
NaCl	sodium chloride
PHD	plant homeodomain
PBR	polybasic region domain
PLC	phospholipase C
PRL1	pleiotropic regulator locus 1
PtdIns	phosphatidylinositol
PtdInsPs	phosphatidylinositol phosphates
qPCR	quantitative PCR
SnRK	sucrose non fermenting 1-related kinase
WT	wild type

Chapter I

<p style="text-align:center">Introduction and Objective</p>

Epigenetics and the Histone Code

In eukaryotes, gene transcription from genomic DNA is a highly-regulated process. There are many protein factors that repress, activate and coordinate the conversion of genomic information into an mRNA transcript. Chromatin remodeling complexes play an important role in the regulation of active gene transcription in the nucleus (Feil, 2008). These complexes add post-translational modifying elements to histone proteins in chromatin and are commonly called epigenetic marks. These epigenetic modifications lead to changes in chromatin structure, making gene promoters more or less accessible to transcription factors. In some cases, these modifications act as a signal, which recruits other histone modifying complexes, increasing the level of regulation (Jenuwein and Allis, 2001). The addition of chemical marks such as acetyl or phosphate groups to specific residues on histone proteins is widely viewed as a code, namely a "histone code." The formation of the code, as well as the reading of the histone code, adds to the intricacy of gene transcription regulation (Loidl, 2004).

Chromatin Remodeling

Changes in gene expression involve the modification of chromatin structure (Eberharter and Becker, 2002). Active transcription is associated with open, non-condensed chromatin. Closed, condensed chromatin, on the other hand, is associated with repressed transcription. One of the factors that controls the opened vs. closed status of chromatin is the modification state of histone proteins within chromatin (Pfluger and Wagner, 2007). Histone proteins can be modified at specific amino acid residues post-translationally (Fletcher and Hansen, 1996). Residues such as lysine and arginine can be methylated, while serine and tyrosine residues can be phosphorylated. In addition to being methylated, lysine can also be acetylated (Fischle et al. 2003). Hyperacetylation of histone residues is commonly identified with open chromatin, while

<p style="text-align:center">1</p>

deacetylation is characteristic of closed chromatin. This is mainly due to the positive charge of the acetyl groups, which, in the presence of other acetylated lysines, will cause repulsion and force open chromatin.

As mentioned above, methylation is a component of the "histone code" (Fischle et al., 2003). This "code" refers to the idea that the instructions for the transcriptional regulation of a given promoter comes in the form of a chemical mark, or combination of marks on a histone tail residue or combination of residues. For example, histone 3 (H3) can be acetylated at lysine residue 9 (K9), K14, K18 and K23. In addition, H3 can be methlyated (mono-, di- or tri-) at K4, K9, K27, K36 and K79. Each modification and each combination of modifications conveys a message about the way a particular gene should be transcribed. A H3K4me0 (lysine 4 on histone H3) is associated with silent chromatin, however, H3K4me3 (trimethylated lysine 4 on histone H3) is known to be a mark associated with at active chromatin.

Plant HomeoDomain (PHD)

The PHD (Plant HomeoDomain) finger is considered is a protein domain that reads the histone code because PHD fingers bind to H3K4me3 and other marks (Martin et al., 2006; Pena et al., 2006; Shi et al., 2006; Taverna et al., 2006; Wysocka et al., 2006; Matthews et al., 2007; Champagne et al., 2008; van Ingen et al., 2008; Palacios et al., 2008; Lee et al., 2009; de la Paz Sanchez et al., 2009). The PHD finger is a 50-80 residue cysteine-rich zinc-finger with a Cys4-His-Cys3 motif. These core residues coordinate two zinc ions and are separated by three loop regions (Schindler et al., 1993). PHD fingers are conserved throughout eukaryotes and are mostly found in nuclear proteins, specifically transcription factors (Aasland et al., 1995; Bienz, 2006).

Mammalian INhibitor of Growth (ING)

The mammalian protein, INhibitor of Growth (ING) contains a PHD finger and is involved in chromatin remodeling (He et al., 2005). These proteins recruit histone acetyltransferases (HATs) and histone deacetylases (HDACs) to chromatin (Doyon et al., 2006). There are five human INGs. All of these proteins have been shown to assoicate with the epigenetic marks, H3K4me3 and H3K4me2 (Pena et al. and Shi et al., 2006). Because ING proteins can associate with epigenetic marks and interact with chromatin remodeling complexes, they are considered

2

interpreters of the histone code, or code readers (Lee et al., 2009). ING functions in the regulation of apopotosis and cell proliferation, cellular senescence, contact inhibition, DNA damage repair and angiogenesis. (Garkavtsev et al.,1996). These findings are consistent with the status of this family as class II tumor suppressors, as INGs are mis-regulated in many types of cancers (He et al., 2005).

ING2 activity has been linked to p53 induction of apoptosis (Coles et al., 2007; Unoki et al., 2008). Like p53, ING2 initiates prosurvival responses to a variety of cellular stresses, such as DNA damage (Nagashima et al., 2001; Soliman and Riabowol, 2007). It has recently been shown that p53 directly associates with two elements of the ING2 promoter (Kumamoto et al., 2009). This association down-regulates ING promoter activity. Interestingly, cellular senescence can be induced by knocking-down ING2 and by overexpression of ING2 (Pedeux et al., 2005; Soliman and Riabowol, 2007). Senescence induced by overexpression is p53-independent, while senescence induced by ING2 repression is p53-dependent and presumably is due to p53-regulation of the ING2 promoter (Kumamoto et al. 2009).

In addition, to associations with chromatin, ING2 has been shown to interact with phosphatidyl inositol phosphates (PtdInsPs; Gozani et al., 2003, Kaadige and Ayer, 2006). Specifically, ING2 associates with PtdIns(3)P, PtdIns(4)P and PtdIns(5)P (Gozani et al., 2003). This binding ability was initially credited to the PHD finger, however it was later determined that an adjacent domain, the polybasic region domain (PBR) was responsible for PtdInsP binding (Kaadige and Ayer, 2006). Although the function of this interaction is unknown, it is speculated that this interaction allows ING2 protein to integrate chromatin and phosphoinositide signaling resulting in the appropriate regulation of cell cycle progression and growth in human cells (Gozani et al., 2003; Soliman and Riabowol, 2007).

Role of Inositol in signal transduction pathways
Inositol based signal transduction is central to a variety of developmental and physical processes (Erneux et al., 1998 and Astle et al., 2007). PtdInsP are components of lipid bilayers and provide membrane structure. PtdInsPs, however, are also precursors to signaling molecules (Figure 1). In the $InsP_3$ signaling pathway, $PtdInsP_2$ is hydrolyzed by phospholipase C (PLC) yielding two

signaling molecules, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP$_3$). In plants InsP$_3$ is utilized in response to ABA (Lee et al., 2007; Sanchez and Chua, 2001; Burnette et al., 2003; Gunesekera et al., 2007), gravity (Perera et al., 2001, 2006), salt stress (DeWald et al., 2001; Takahashi et al., 2001) and pathogen response (Ortega and Perez, 2001; Andersson et al., 2006). An increase in cytosolic InsP$_3$ activates the release of Ca^{2+} from intracellular stores resulting in a change in stomatal physiology (Burnette et al., 2003). InsP$_3$ signaling is a transient process that can be terminated by *myo*-inositol polyphosphate 5-phosphatases (5PTases; EC 3.1.3.56). 5-PTases remove the 5-phosphate from the inositol ring, thus changing the structure of the InsP$_3$ (now InsP$_2$) and terminating the signal (Stevenson and Perera, 2000).

It has become apparent in the last 20 years that inositol signaling may not be restricted to the plasma membrane and cytoplasm (Irvine, 2002; Maraldi, 2008). Although less is known about inositol cycling and signaling in the nucleus, what is becoming clear is that inositol signaling may play a large role in gene transcription events (Capitani et al., 1991 and Irvine, 2002). In mammalian cells, nuclear PLC can be activated by a variety of cellular processes (Cocco et al., 1996; Cocco et al., 2009). In addition, the transition to erythrocyte development during cellular differentiation is speculated to involve PtdIns signaling, as these lipids increase in abundance in the nucleus during this phenomenon (Martelli et al., 1995). In Arabidopsis, the inositol polyphosphate kinase, AtIpk2β, catalyzes the production of Ins(1,4,5,6)P$_4$, and Ins(1,3,4,5,6)P$_5$ and is predominantly localized to the nucleus. This enzyme is considered to play a role in transcriptional control in plants (Xia et al., 2003). In addition, the Arabidopsis PtdIns 3-kinase has been shown to localize to the nucleus and specifically localizes to sites of active transcription within the nucleus (Bunney et al., 2000). These data, and that of ING, support the idea of inositol based signal transduction mechanisms within the nucleus.
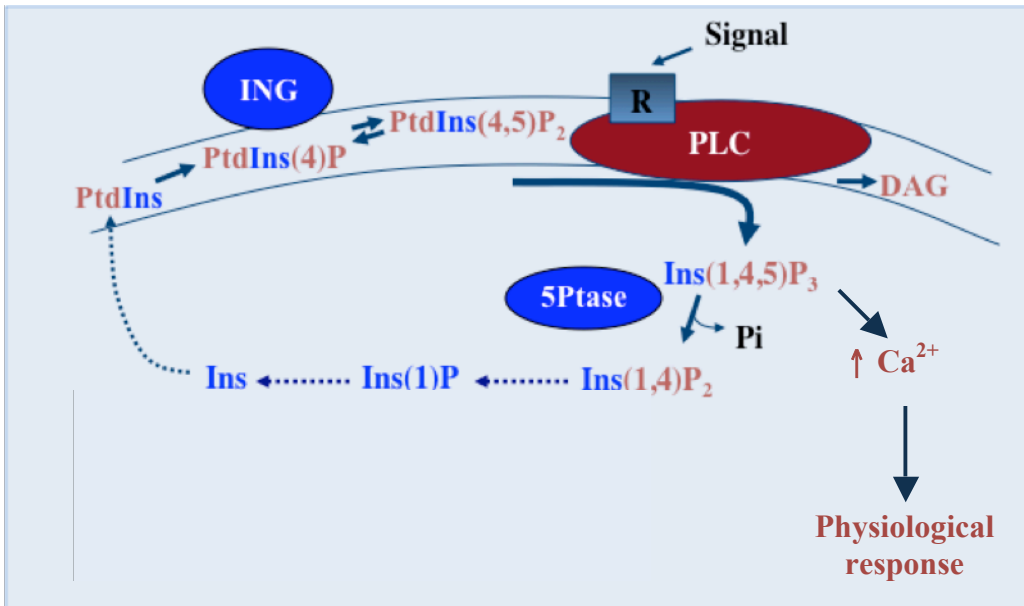
Figure 1.1. Inositol cycling and signaling. Inositol based signaling is initiated by the activation of PLC by an extracellular signal, such as ABA. PLC will cleave $PtdInsP_2$ producing DAG and $InsP_3$. $InsP_3$ causes an increase in cytosolic calcium stores, which leads to down stream physiological effects. This signal can be terminated by 5PTases, which remove the 5-phosphate. Subsequent phosphatases degrade $InsP_2$ and $InsP$ to *myo*-Ins, which can be incorporated into the plasma membrane as PtdIns. PtdIns-kinases phosphorylate these lipids, restoring $PtdInsP_2$ levels.

Role of SnRK Protein in the Global Regulation of Gene Transcription in the Response to Nutrient Status

Plants, like other eukaryotes, encounter a variety of stresses during a life cycle. A variety of factors, environmental as well as biological, can be perceived as stress. Stress responses in plants involve the modulation of gene transcription. In some cases, this change in transcriptional regulation can have global effects (Beana-Gonzalez and Sheen, 2008). The lack of nutrients can be perceived as a stress. Recent findings regarding the enzyme, SnRK (Sucrose nonfermenting 1-related kinase) show that plants will initiate an extensive transcriptional reprogramming in response to "low nutrient" status. This response involves the activation of energy redistributing processes such as amino acid catabolism and sucrose hydrolysis. Under low nutrient conditions, SnRK also initiates the repression of biosynthetic processes while activating catabolic pathways. For this reason, SnRK is considered a central regulator of transcriptional response to multiple nutrient and stress signals (Baena-Gonzalez et al., 2007).

Ananieva et al. (2008) showed a relationship between 5PTase13 and SnRK1.1. Arabidopsis 5PTases catalyze the removal of the fifth position phosphate of the inositol ring of the cytosolic signaling molecules, $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ and the lipid soluble molecules, $PtdIns(4,5)P_2$, $PtdIns(3,5)P_2$ and $PtdIns(3,4,5)P_3$ (Erneux et al., 2003, Astle et al., 2006 and 2007). Ananieva et al. (2008) showed that 5PTase13 and SnRK1.1 recombinant protein form a protein complex. In addition, this interaction regulates SnRK1.1 activity in a nutrient-dependent manner. This change in SnRK activity is due to 5PTase mediated protection of SnRK1.1 from proteasomal degradation. These data indicate a novel mechanism for SnRK1.1 protein stability, as well as a novel role for 5Ptases in sugar signaling (Ananieva et al., 2008).

In yeast, Snf1 kinase, a SnRK ortholog, regulates the transcription of a variety of genes by phosphorylating histone 3 at specific promoters (Polge and Thomas, 2006). One such promoter is that of INO1, a *myo*-inositol phosphate synthase, which is involved in inositol phosphate anabolism (Shirra et al., 2005). In addition to the direct modification of histones, Snf1 regulates the ability of TATA binding protein (TBP) and GCN5, an H3 acetyl transferase, to associate with

select promoters. GCN5 is also post-translationally modified directly by Snf1 kinase, indicating that Snf kinase activity facilitates a variety of regulatory mechanisms (Polge and Thomas, 2006).

OBJECTIVES

The goal of this research is to understand the function of PHD containing proteins in plant growth and development. My objectives are as follows:

Objective 1. Analysis PHD fingers in Arabidopsis and Rice
    A. Analysis of Phylogenetic Relationships
    B. Analysis of H3K4me3 and PtdInsP Binding Potential

Objective 2. Characterization of ING Mutants
    A. Phenotypic Characterization of *ing1* and *ing2* Mutants
    B. Analysis of ING2 protein interactions

Chapter II: Phylogenetic Analysis of PHD finger domains in *Arabidopsis thaliana* and *Oryza sativa*.

This chapter is adapted from a manuscript in preparation entitled: "Classification of PHD finger domains in *Arabidopsis thaliana* and *Oryza sativa*"

Natasha M. Safaee, Jonathan O. Watkinson, Glenda E. Gillaspy

NMS completed the PtdInsP and H3K4me3 binding analysis, contributed to sequence retrieval and analysis, prepared phylogenetic data and alignments, and prepared the final draft. JOW performed the initial sequence retrieval, analysis, phylogenetics and produced the first draft. GEG assisted in sequence retrieval and phylogenetic analyses and edited the final draft.

Abstract

Since its discovery in Arabidopsis proteins, the plant homeodomain (PHD) finger has been implicated in a large number of processes involving the regulation of chromatin structure and transcription. The PHD finger was first identified as a cysteine-rich domain that coordinates two zinc ions. The majority of PHD finger-containing proteins are nuclear, and participate in protein-protein interactions that frequently involve histones and proteins that are associated with histones. Recent data indicates that some PHD finger proteins interact with specific epigenetic marks on chromatin. In addition, some PHD fingers contain an adjacent polybasic region domain (PBR), which interacts with phosphatidylinositol phosphates. To further our understanding of the diverse functions of PHD finger proteins in plants, we have undertaken an analysis of the PHD finger proteins in Arabidopsis and rice. Our analysis showed that Arabidopsis encodes 78 PHD fingers in 75 different proteins. Rice encodes 70 PHD fingers in 65 proteins. In addition, many of these domains have putative PBRs, suggesting that they can bind to phosphatidylinositol phosphates and may be involved in signaling. The PHD fingers of Arabidopsis and rice can be divided into subgroups of a larger family that may correspond to function. From these data we conclude that the PHD finger domain is relatively abundant in both Arabidopsis and rice. In addition, we predict that a subset of PHD fingers are involved in not only protein interactions, but in phosphatidylinositol phosphate binding as well.

Introduction

The PHD finger is a zinc finger domain of 50-80 amino acids first identified in Arabidopsis as a cysteine-rich motif in the HAT3.1 protein (Histone acetyltransferase 1, Schindler, 1993; For reviews see Adams-Cioaba and Min, 2009; Mellor, 2006). The PHD finger has since been found in numerous eukaryotic proteins, and most of these proteins have been demonstrated or predicted to have a role in modifying chromatin structure (Bienz, 2006). This association suggests that the PHD finger is crucial for proper maintenance of the chromatin state during transcription. Indeed, mutations in some mammalian PHD finger proteins can lead to proliferative cell growth and cancer (Garkavstev et al, 1996; Russel et al., 2006; Cai et al., 2009), while mutations in yeast and plant PHD fingers are known to result in developmental aberrations (Dul and Walworth, 2007; Yang et al., 2003; Wilson et al., 2001; Saiga et al., 2008).

The PHD finger is similar to the well-characterized RING (Really Interesting New Gene) finger domain that interacts with ubiquitin E2 ligases and functions in targeted protein turnover (for review see Deshaies et al., 2009). Both the PHD and RING finger domains form zinc coordinating, cross-brace structures containing loop regions (Figure 1, Garkavstev et al, 1996; Borden and Freemont, 1996). Although similar in nature to the RING finger, the major function of PHD fingers is chromatin regulation as opposed to protein turnover processes (Scheel and Hofmann, 2003). Binding studies from a selection of PHD fingers have shown interactions with phosphatidylinositol phosphates (PtdInsPs) and methylated histones (Gozani et al., 2003; Kaadige and Ayer, 2006; Wysocka et al., 2006; Lee et al., 2009; Zhao et al., 2009). Accordingly, the PHD finger domain has been suggested as a specific interaction domain for these substrates. Binding to PtdInsPs, for example, is suggested as a mechanism for localizing chromatin-remodeling complexes to nuclear membranes, or as a receptor for nuclear PtdInsP-mediated signaling (Gozani et al., 2003; Soliman and Riabowol, 2007). Binding to methylated histones, on the other hand, is hypothesized to locate enzymatic function of chromatin remodeling complexes to specific histone marks (Shi et al., 2006; De Lucia et al., 2008).

In the recent publication from *Lee et al.*(2009), the characterization of PHD fingers in Arabidopsis was presented. Based on Pfam annotation and SMART (smart.embl-heidelberg.de) searches, the authors determined that there are 83 PHD fingers encoded by the Arabidopsis

genome (Lee et al., 2009). We have expanded this analysis to the crop species *Oryza sativa* (rice). This analysis includes the consideration of potential PtdInsP binding by the determining the presence of polybasic regions (PBRs) in Arabidopsis and rice PHD finger proteins. In addition, we have examined the potential of rice PHD fingers to bind methylated histones. Finally, a phylogenetic analysis of PHD fingers in Arabidopsis and rice was performed. Our novel examination of evolutionary relationships between all PHD fingers of two plant species indicates that the plant PHD fingers contain key conserved elements that could impart functionality.

A.

Loop 3 (10-20)

N

C1 C5
Zn
C2 H1

C4 C6
Zn
C3 C7

Polybasic region

C

Loop 1 (8-18)

Loop 2 (4-5)

B.

Conserved tryptophan
of Class I

Conserved aromatic
of loop 3

VYCKCEMPYNPDDLMVOCEGCKDWYHPACVGMTIEEAKKLDHFVCAECSS

Loop 1   Loop 2   Loop3

Cys4   His   Cys3
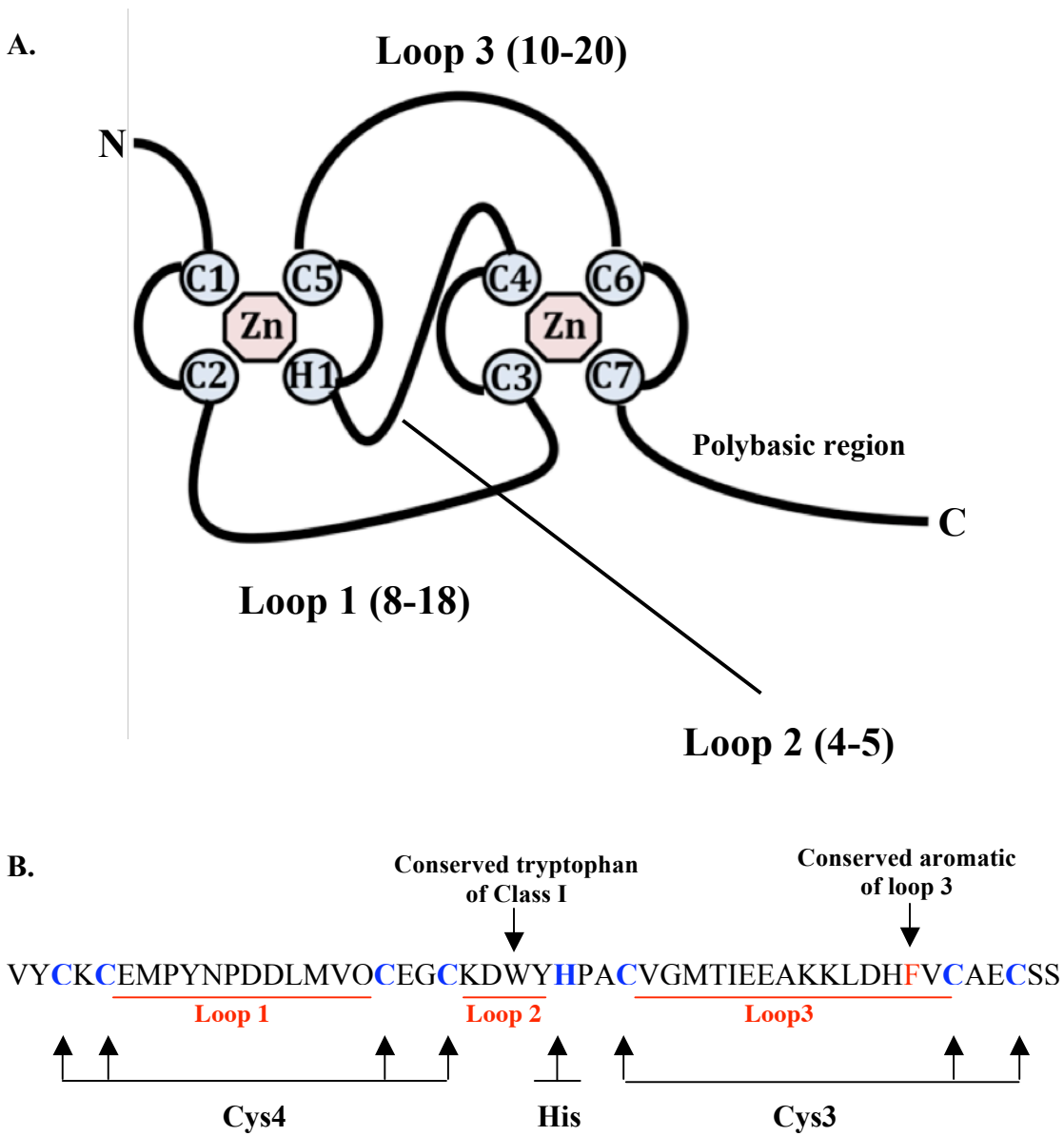
Figure 2.1: Schematic of PHD structure. A) Metal binding cysteines (C) and histidine (H) are represented in blue. The order of metal binding ligands is represented by a number following the single letter amino acid code. Coordinated zinc residues are shown as pink hexagons in the middle of each metal binding tetrad. Loop regions are represented as lines with the number of residues in each loop indicated. N-terminal and C-terminal ends of the domain are represented as N and C respectively. B) Key residues that distinguish the two Classes are indicated.

Methods

Sequence Searches

An initial list of PHD finger proteins in Arabidopsis was compiled by searching the literature for PHD finger proteins of known function and by searching for homologues using BlastP. A Perl script was also used to identify 'PHD fingers' within the annotations of all Arabidopsis proteins. These genes were manually scanned and the PHD finger domain identified. PHD finger domains were stored in a text file and included 2 residues upstream of the initial cysteine residue and 10 residues downstream of the final cysteine residue in the C4HC3 motif. To find additional PHD domains, hidden markov models (HMM) were used through the web interface for HMMer at http://bioweb.pasteur.fr/seqanal/motif/hmmer-uk.html. This program uses a text file of protein sequences to build a model representing conserved primary structure and uses that model to search sequence databases for matches. The output is a text file with a list of matches and the score for each match. The HMMer search was limited to the plant database and returned matches from Arabidopsis, rice, maize and other crops. As the goal of our analysis focused solely on the identification of Arabidopsis and rice PHD fingers, matches from these two species were used for subsequent analyses. The file was manually parsed for matches to Arabidopsis and unique PHD finger domains were manually curated to the Arabidopsis PHD finger file. An E-value of 0.15 or lower was chosen as it became apparent that with higher E-values HMMer was identifying closely related zinc finger domains such as RING. PHD finger domains were also identified for rice and a separate file was created for them.

Naming of Sequences

Protein names listed in tables were derived from annotation from the TAIR and NCBI websites for Arabidopsis (www.arabidopsis.org, www.ncbi.nlm.nih.gov). In addition the naming of AtING1 (At1g54390) and AtING2 (At3g24010) reflects the homology of these proteins with the human ortholog, HsING2. This is the opposite annotation as that of Lee *et al*. (2009) as they have named At1g54390 as AtING2 and At3g24010 as AtING1.

Multiple Sequence Alignments and Domain Identification

Alignments of the PHD finger domains were generated using ClustalX. The resulting sequence alignments generated were used to develop phylogenetic trees using Mobyl@Pasteur (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=clustalw-multialign). Phylogenetic trees were also generated using PhyML, a maximum likelihood algorithm, hosted at Mobyle@Pasteur. Trees were viewed using the Interactive Tree of Life (http://itol.embl.de/). Domain structure was determined by submitting each protein sequence to pFAM and searching using gLocal and Local alignments.

Lipid binding prediction analysis

PHD finger domains were stored in a text file and included four residues upstream of the initial cysteine residue and 27 residues downstream of the final cysteine residue in the C4HC3 motif. The PBR, i.e., the 27 residues adjacent to the final conserved cysteine motif, was manually inspected for the presence of lysine and arginine residues. A PBR was judged as positive with respect to potential PtdInsP binding if it contained a minimum of 15% lysine and/or arginine residues (4 out of 27 residues). This criteria was based on experimental data presented by Kaadige and Ayer (2006).

Results and Discussion

Identification of Evolutionary Relationship Between PHD fingers in *A. thaliana* and *O. sativa.*

Since the identification of the first PHD finger, many groups have identified novel PHD finger containing proteins, however, a specific definition for characterization has not been established. In an effort to define the PHD finger, we have inspected over 100 PHD fingers from Arabidopsis and rice. Based on our analysis, we define the PHD finger domain as a domain of 50-80 amino acids, which includes a motif containing four cysteines (C4), one histidine (H) and four more conserved cysteines (C3), i.e. C4HC3. The arrangement of cysteines and histidine forms four zinc-coordinating pairs separated by three variable loop regions. The first loop ranges from 8-18 residues in length; the second from 4-5 residues, and the third is 10-20 residues in length (Figure 1). The spacing between each metal-coordinating residue of a particular pair is always one to two residues long, with a few exceptions where it stretches to four residues. In addition to the eight

13

conserved metal coordinating residues, there is a conserved aromatic residue that is in loop 3. This residue is predominantly a tryptophan, but can also be a phenylalanine or tyrosine (Figure 1). Although it is not a metal coordinating residue, this position in loop 3 is always occupied by an aromatic amino acid throughout all PHD finger domains in Arabidopsis and rice (Figure 1). Initial phylogenetic analyses indicated that the PHD fingers of Arabidopsis and rice clustered into two classes with Class I fingers having a tyrosine or phenylalanine at the loop 3 conserved aromatic residue position, and Class II containing a tryptophan at this same position (Figure 1). Although bootstrap values did not provide good statistical support for these classes, we propose that there is merit in defining these classes for two reasons. First, manual inspection of the alignments generated during the analysis revealed that specific regions with one class would be conserved, while this same region would be highly variable in the other class, and vice versa; and second, each class can be further differentiated into groups with good statistical support using PhyML. We describe these groups and their attributes in the following sections.

Class I PHD finger domains

The Class I PHD finger domains contain phenylalanine or tyrosine as the conserved aromatic residue in loop 3 (Figure 1). When Class I PHD fingers were subjected to phylogenetic analyses we found evidence for distinct clades or groups, as supported by bootstrap analysis (Figure 2). Most of these groups (called groups A-F) appear to be comprised of small gene families as the proteins within these groups have conserved domain structure and similar exon number (Table I). Another important characteristic of the Class I PHD fingers is the presence of tryptophan as the third residue in loop 2 (see consensus sequences in Figure 3). The presence of tryptophan at this position has been demonstrated as essential for binding of the PHD finger of Drosophila NURF301 to trimethylated lysine 4 of histone 3 (H3K4Me3; Li et al, 2006). The complete conservation of this residue in Class I PHD finger domains suggests that it is critical to their function.

Class I Group A

Group A contains six Arabidopsis and five rice proteins (Figure 2; Table I). The consensus sequence for Group A includes a DDGXXMb motif in loop 1, where XX is usually ER and b is a basic residue (Figure 3). Loop 2 contains a conserved threonine after the metal binding histidine

14

and the conserved aromatic residue at the end of loop 3 is phenylalanine. Some PHD fingers in this group are known or predicted to function in the development of male reproductive tissues, including the Male Meiocyte Death (MMD) gene product (Yang et al., 2003) (Table 1). Plants deficient in MMD exhibit meiotic effects and are male sterile (Yang et al., 2003). MMD is a nuclear protein that is implicated in meiosis checkpoint control by regulating transcriptional events (Yang et al., 2003). This group also includes, Male Sterility1 (MS1). Studies with ms1 loss-of-function mutants demonstrate that MS1 is expressed in the anther and is involved in pollen and tapetum development (Wilson et al., 2001; Ito et al., 2007). A mutation in the PHD finger of MS1 leads to plants with non-viable pollen (Wilson et al., 2001). Microarray analysis of *ms1* anther tissue revealed that MS1 potentially acts as a transcriptional regulator of genes involved in pollen and tapetum development (Ito et al., 2007). The results from MMD and MS1 indicate that members of this group potentially regulate the development of male reproductive tissues in Arabidopsis and rice. The PHD fingers in Group A are all similar in exonic and domain structure (Table I and not shown), thus it is possible that the proteins of unknown function in this group participate in similar developmental programs.

Class I Group B

Group B contains five Arabidopsis and two rice proteins (Figure 2). This group has four resides between the second and third metal-binding ligands, which is unusual given that almost all other plant PHD fingers contain only two residues at this position (Figure 3, 5). Other hallmarks include a conserved glutamate residue immediately following C3 (Figure 3). Three of the four Group B proteins are SUMO E3 ligases. One member in particular, At5g60410 (SIZ1), is a known negative regulator of ABI5 (ABA insensitive 5), a transcription factor involved in the signaling of the plant hormone, ABA (Garcia-Dominguez et al., 2008; Miura et al., 2009). ABI5 is directly sumoylated by SIZ1, negatively affecting ABA signaling (Miura et al., 2009). SIZ1 is interesting because its known function is not direct interaction with chromatin, but to facilitate the binding of SIZ1 to E2 enzymes (Garcia-Dominguez et al., 2008).

Also in this group are the Oberon proteins, OBE1 and OBE2. OBE proteins function in the establishment and maintenance of both root and shoot apical meristems, as seen by abnormal root

and shoot meristem development in obe2 mutants (Saiga et al., 2008). OBE genes have similar expression patterns and genetic analysis of double mutants indicates that these proteins have redundant function. The double mutant of these genes exhibits defects in the shoot and root meristems. In addition, OBE proteins are proposed to regulate the expression of meristem genes WUS (Leibfried et al., 2005), PLT1 (Aida et al., 2004) and PLT2 (Dello Ioio et al., 2007). This regulation is presumed to be mediated by the involvement of the OBE PHD fingers in chromatin remodeling (Saiga et al., 2008).

Class I Group C

Group C contains four Arabdidopsis and four rice proteins (Table I.C, Figure 2). The conserved aromatic residue is a phenylalanine. There is an acidic residue and an aromatic residue that flank the conserved tryptophan of loop 2. There is a conserved proline after the conserved histidine. Within loop 1 there is a conserved EMPYNPD and an AKK in loop 3 (Figure 2, 3A). There is also a conserved tyrosine before the C1 and a conserved methionine three residues after C3 (Fig. 2 and 3C). Of particular interest is the presence of a JmjC (Jumonji C) domain protein in this group. JmjC domain-containing proteins are known to be involved in chromatin remodeling. Specifically, these proteins often participate in the removal of methyl groups from histones (Tsukada et al., 2006, Klose et al., 2006). ELF6 (Early Flowering 6) and REF6 (Relative of Early Flowering 6) are jumonji C containing proteins in Arabidopsis. Genetic studies of *elf6* and *ref6* mutants indicate that ELF6 and REF6 function in the regulation of flowering time. It was determined that ELF6 acts as a repressor upstream of the photoperiodic floral regulatory pathway, while REF6 acts as a repressor of FLC, a repressor of flowering time (Noh et al., 2004).

Class I Group D

Group D contains seven Arabidopsis and eight rice proteins. The conserved aromatic residue of this group is tyrosine. Within loop 1 is a semi-conserved tyrosine (sometimes substituted by aspartic acid), and an EFWI immediately prior to the C3. Loop 2 has an EXXWAr sequence, where Ar is an aromatic residue. Loop 3 has a conserved V[R/K]ITPA[R/K]A after C5 and a YK prior to C6 (Figure. 2). A conserved proline immediately follows C6. Conserved glycines occur after the first, second and fifth metal ligands (Figure. 2 and 3D). This group contains the entire Arabidopsis Alfin-like protein family. These nuclear proteins are involved in chromatin

remodeling and have been shown to associate with methylated histone proteins (Lee et al., 2009). Although not much more is known about Alfin proteins in Arabidopsis, the Alfin1 protein in Alfalfa is expressed in root tissue. In addition, these proteins bind to promoters elements in genes that are salt-inducible (Bastola et al., 1998; Winicov and Bastola, 1999; Winicov, 2000; Winicov et al, 2004). Considering the Alfalfa data, it is possible that the Arabidopsis proteins occupy the promoters of genes involved in salt tolerance. It is likely that the rice PHD fingers in this group are also Alfin-like orthologs.

Class I Group E.

Group E contains three Arabidopsis and one rice protein (Fig. 2 and 3E). The conserved aromatic residue is tyrosine. Within loop 1 is a conserved K [K/r] I, a conserved aspartate in the middle of the loop and a WV[R/C] at the end. Loop 2 contains the conserved tryptophan. Loop 3 begins with a conserved aspartate, and is followed by another acidic residue. This loop also ends in an aromatic residue. There is a proline directly following C6. The PHD fingers in this group are annotated as SET (Suppressor of variegation 3-9, Enhancer of zeste, trithorax) domain containing proteins of the Trithorax (TrxG) family (ATX3, ATX4, ATX5). The SET domain has protein lysine methyltransferase activity. Arabidopsis TrxG proteins are orthologs to the Drosophila TrxG proteins (Alverez-Venegas et al., 2003). TrxG proteins regulate the expression of genes that control organ identity and patterning (homeotic genes). Trithorax family proteins methylate chromatin at lysine residues on histone tails, activating gene transcription (Pien et al., 2007). PHD fingers from other ATX proteins are classified as Class II Group C fingers. It is possible that the difference in finger structure between groups has an impact on the role these proteins play in Arabidopsis development.

Class I Group F.

This group consists of two PHD finger domains from Arabidopsis and one from rice. Tyrosine is the conserved aromatic residue. There are two residues between C1 and C2, PV, and a conserved NY immediately prior to the first metal ligand. Loop 1 contains a LKVYRDSE consensus, as well as a conserved VC. Loop 2 has the conserved W followed by a V. Loop3 has a DGISAcAcKY (Figures 2 and 3F). The members of the group have unknown function and the majority have only

17

one PHD finger. Interestingly, all group members are predicted to associate with both PtdInsPs and with H3K4me3.
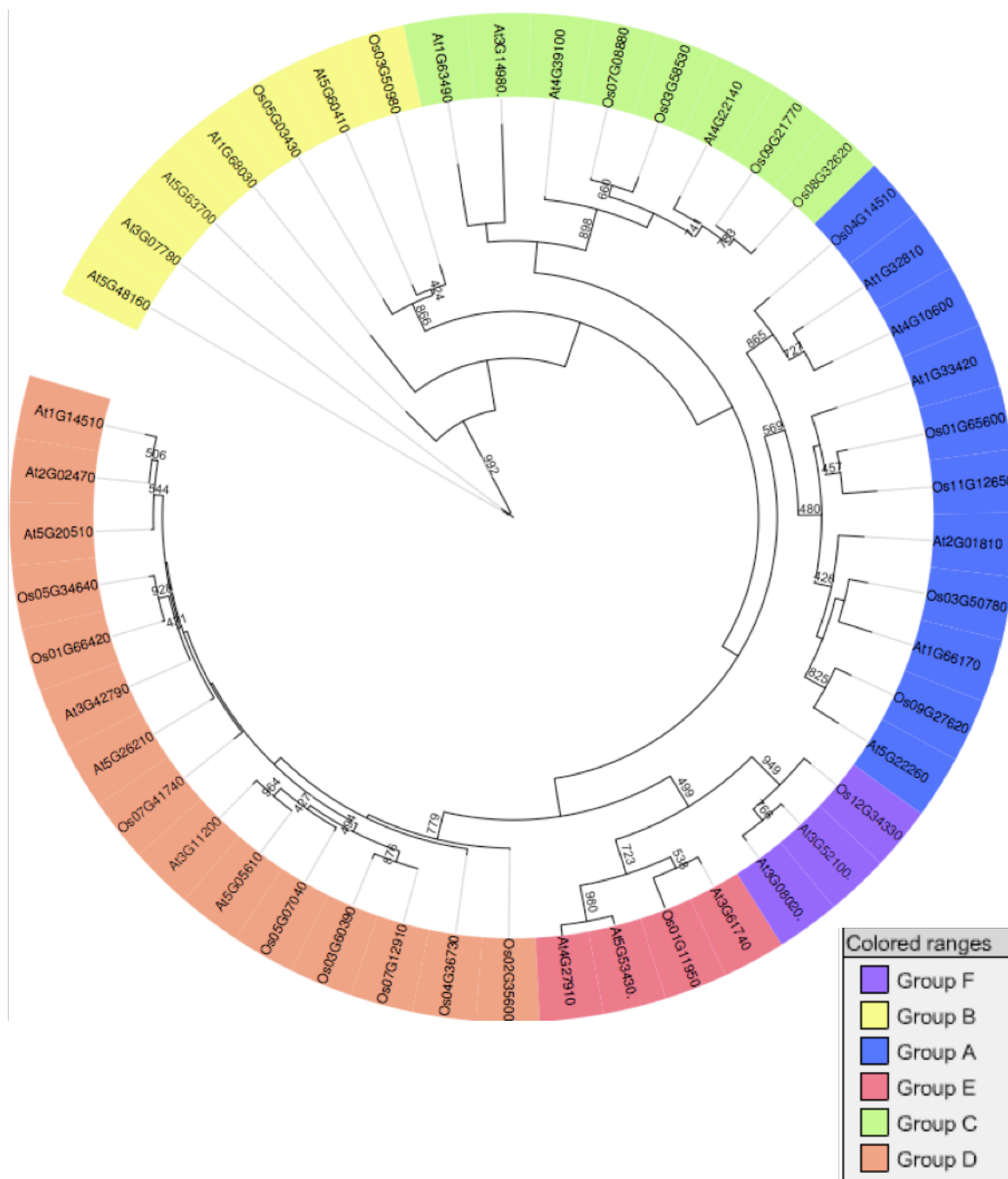
Figure 2.2: Cladogram of Class I PHD fingers from Arabidopsis and Rice. Phylogenetic tree was created using the PhyML function at Mobyle@Pastuer with 1000 bootstraps. The resulting files were sent to ITOL for tree visualization and modification. Class I PHD fingers form 6 major clades which represent the 6 groups, A-F. These groups were also supported by sequence alignment analysis. Group A is shown in blue, group B in yellow, group C in green, group D in orange, group E in red and group F in purple. Bootstrap values of >400 are displayed.

Table I. **Class I PHD finger proteins of *Arabidopsis thaliana* and *Oryza sativa*.** This table lists all Class I PHD fingers grouped by family (A-F). Also listed are each gene's identifier, functional annotation, histone and PtdInsP binding prediction, number of exons and domain structure. A + indicates a predicted binding for the indicated molecule.

Family	Gene Number	Annotation	Predicted Binding H3K4me3	PtdInsP	Exon	Domain Structure
A	Os01g65600	PHD-finger family protein	-	+	05	PHD
	At1g33420	PHD finger family protein (similar to MMD1)	-	+	03	PHD
	Os03g50780	PHD-finger family protein	-	-	03	PHD
	At2g01810	PHD finger family protein similar to MMD1	-	+	03	PHD
	Os09g27620	PHD-finger family protein	-	-	03	PHD
	At1g66170	Male Meiotic chromosome organization protein (MMD1/DUET)	-	-	03	PHD
	At5g22260	Male Sterility Protein 1	-	-	03	PHD
	Os11g12650	PHD-finger family protein	-	+	05	PHD
	At4g10600	PHD finger family protein	-	-	01	PHD
	At1g32800	PHD finger protein-related	-	-	02	PHD
	Os04g14510	PHD-finger family protein	-	-	06	PHD
B	At5g60410	DNA-binding protein	-	-	17	SAP, PHD, zf-MIZ
	Os03g50980	Sumoylation ligase E3	-	-	16	SAP, PHD, zf-MIZ,
	Os05g03430	Sumoylation ligase E3	-	-	16	SAP, PHD, zf-MIZ
	At1g68030	Cytochrome P450	-	+	04	PHD
	At5g63700	C3HC4 type RING finger	-	-	08	PHD, SWIB, Plus-3
	At3g07780	Oberon1 (OBE1)	-	-	02	PHD
	At5g48160	Oberon2 (OBE2)	-	-	02	PHD
C	Os03g58530	ES43	-	+	05	BAH, PHD
	Os07g08880	ES43	-	+	05	PHD
	Os08g32620	BAH domain containing protein	-	+	05	BAH, PHD
	Os09g21770	receptor like protein	+	+	05	BAH, PHD
	At4g22140	PHD finger family protein /BAH domain-containing protein	+	+	03	BAH, PHD
	At4g39100	PHD finger family protein /BAH domain-containing protein	-	+	05	BAH, PHD
	At3g14980.b	SIZ1; DNA binding / SUMO ligase	-	-	08	PHD, zf-FCS
	At1g63490	jumonji (jmjC) domain-containing protein	-	+	25	JmjC, zf-C5HC2, PLU-1, PHD
D	At1g14510	PHD finger family protein	-	-	05	PHD
	At2g02470	PHD finger family protein	-	-	05	PHD
	At5g20510	PHD finger family protein	-	-	05	PHD
	Os01g66420	PHD finger protein	-	-	05	PHD

	At3g42790	PHD finger family protein	-	-	05	PHD
	At5g26210	PHD finger family protein	-	-	05	PHD
	Os07g41740	PHD finger family protein	-	-	04	PHD
	Os04g36730	PHD finger protein	-	-	05	PHD
	At5g05610	PHD finger family protein	-	-	05	PHD
	Os02g35600	PHD finger protein	-	-	04	PHD
	At3g11200	PHD finger family protein	-	-	05	PHD
	Os05g07040	PHD finger protein	-	-	05	PHD
	Os03g60390	PHD finger protein	+	-	05	PHD
	Os07g12910	PHD finger protein	-	-	05	PHD
	Os05g34640	PHD finger protein	-	-	06	PHD, AB Hydrolase-1
E	At5g53430.a	Trithorax like protein /PHDcontaining (TX5)	-	+	23	PWWP, PHD, PHD, SET
	At4g27910	Trithorax like protein/PHD containing (ATX4)	-	+	23	PWWP, PHD, PHD, SET
	Os01g11950	PHD finger protein	+	+	23	PWWP, PHD, PHD, SET
	At3g61740.a	Trithorax like protein/PHD containing (ATX3)	+	-	20	PWWP, PHD, PHD
F	At3g52100.b	PHD-finger family protein	+	+	13	PHD
	At3g08020.b	PHD finger protein-related	+	+	13	PHD
	Os12g34330.a	PHD-finger family protein	+	+	10	zf-C3HC4, PHD, PHD

A. VK**C**I**C**RARD**DDGXXMb**S**C**DV**C**EV**WQHTR**C**CGIDDSDTLPPL**FV**C**SN**C**CEEFAEQRKVL

Loop 1 (11-14aa)   Loop 2 (4aa)   Loop3 (15aa)

B. VR**C**V**C**GNSLETDSMIQ**C**E**DPR**C**HV**WQH**VG**C**VILPDKPMDGNPPLPES**FY**C**EI**C**RLTRADPFWV

Loop 1 (11aa)   Loop 2 (4aa)   Loop3 (14-20aa)

C. V**Y**C**K**C**EMPYNPD**DL**M**VQ**C**EG**C**KD**WY**H**PA**C**VGMTIEE**AKK**LDH**FV**C**AE**C**SSSQ

Loop 1 (12aa)   Loop 2 (4aa)   Loop3 (11-14aa)

D. AV**C**GA**C**GDNYGGD**EFWIC**C**DA**C**EXXWArH**GK**C**V**[R/K]**ITPA**[R/K]**A**EHIKH**YKC**PS**C**TT

Loop 1 (12aa)   Loop 2 (4aa)   Loop3 (15aa)

E. QY**C**GI**C**K**[K/R]**I**WHPS**D**DGD**WV**[R/C]**C**DG**C**DV**WV**H**AE**C**DN**ITNERFKELEHNN**YYC**P**D**C**KS

Loop 1 (14aa)   Loop 2 (4aa)   Loop3 (16/17aa)

F. **NYC**PV**C**LKVYRDSEA**TPM**VC**C**DFCQR**WVH**CQ**C**DGISAcAcKY**MQFQVDGNLQYK**C**ST**C**RGE

Loop 1 (12/14aa)   Loop 2 (4aa)   Loop3 (20aa)

Figure 2.3. Consensus sequences for Class I. The PHD motif residues are colored blue. The loop regions are also indicated. Key residues that define each group within each group are indicated by bold and underlined text. **A)** Group A; **B)** Group B; **C)** Group C; **D)** Group D; **E)** Group E and **F)** Group F. Abbreviations: X, any residue; b, basic; /, or; Ac, acidic residue; Ar, aromatic.

Class II PHD finger domains

The Class II PHD finger domains contain tryptophan as the conserved aromatic residue in loop 3 (Figure 1). When Class II PHD fingers were subjected to phylogenetic analyses we found evidence for distinct clades or groups (called groups A-I), as supported by bootstrap analysis (Figure 4). Interestingly, a large number of the proteins in this Class have more than one PHD finger (Table II). Overall, the PHD fingers in Class II are better conserved as a group as compared to those in Class I.

Class II Group A

Group A contains 15 Arabidopsis and 11 rice proteins. Loop 2 has a Px(T/A)(F/Y) motif. There is a conserved glycine in loop 1 and proline in the loop 3. Loop 3 also begins with a hydrophobic residue (valine, leucine or isoleucine). Although this is a large group, the majority of PHD fingers in this group have unknown function. One of the rice domains, Os03g5330, is annotated as a GNAT (GCN5-related N-acetyltransferase) family acetyltransferases. In Arabidopsis, the GNAT mutant *tpl* (topless) forms apical roots in place of apical shoots in embryos under specific temperatures (Long et al., 2002). In conjunction with this, genes specific for root formation are found in the shoot apex instead of apical markers (Long et al., 2002). The TPL protein functions as a repressor of transcription under the regulation of auxin, a hormone that regulates apical dominance (Szemenyei et al., 2008).

Class II Group B

Group B has seven Arabidopsis and 10 rice proteins. There is a conserved proline in loop 3. There is a conserved tyrosine between the conserved H and C5. The conserved proline in loop 3 is preceded by conserved valine or isoleucine and followed by a semi-conserved acidic residue. Like Group A, Group B PHD fingers belong to proteins that, for the most part, are without known function. However, At3g01460, named MBD9, has known function in associations with DNA, methyl-CpG binding. In Arabidopsis, loss of MBD9 function leads to alterations in flowering time and shoot development (Peng et al., 2006). MBD9 is a nuclear protein that directly modifies chromatin by acetylating histones. In addition, this protein participates in the regulation of flowering time by physically associating with the promoter of FLC (Yaish et al., 2009).

23

Class II Group C

Group C contains eight Arabidopsis and seven rice proteins. There is a conserved valine after C1, a valine before the conserved histidine and a tyrosine immediately following C5. This group, like Class I Group E, contains ATX proteins, as well as rice trithorax (TrxG) group proteins. In Arabidopsis, ATX1 activates flower homeotic genes and thus regulates floral organ development (Alverez-Venegas et al., 2003). ATX proteins carry a SET domain, which has methyltransferase activity. ATX1 associates with the promoter of FLC and establishes an active epigenetic mark, H3K4me3. This mark promotes FLC expression and prevents repression (Pien et al., 2008). Interestingly, not all ATX proteins function in the same way. While ATX1 produces a trimethylated H3K4 mark, ATX2 only dimethylates histones (Saleh, 2009). Both ATX1 and ATX2 are Class II group C proteins.

Class II Group D

Group D contains eight Arabidopsis and nine rice proteins. There is a conserved LXP motif to start loop 3 (Figure. 2). There is a conserved hyrdophobic residue at the end of loop 1. It is predominantly leucine, though valine and isoleucine can be substituted. One member of this group, (PKL) is annotated as PICKLE , an ATP-dependent chromatin remodeler that generally represses genes involved in development. PKL represses seed-associated genes in Arabidopsis seedlings and adult plants by establishing the repressive epigenetic mark, H3K27me3 (Zhang et al., 2008).

Class II Group E

This group contains the PHD fingers from the plant homologs of the mammalian Inhibitor of Growth (ING) proteins (At1g54390, At3g24010, Os03g04980, and Os03g53700). These proteins all have a C-terminal PHD finger and are relatively short. The proteins show homology to human ING1 and ING2. This group is marked by a single amino acid between the first and second metal binding cysteines and atypical spacing of four residues between the second and third metal binding cysteines. Loop 2 also has an unusual number of residues (5-6) with a conserved tryptophan and phenylalanine immediately before the metal binding histidine. There is also a conserved tryptophan and tyrosine prior to C6 and a conserved proline immediately after the sixth cysteine. These PHD fingers occur at the C-terminus of the protein, which ends approximately 7-

10 residues after the final metal-binding cysteine. In addition, all members have a conserved tyrosine that precedes C1. This residue is crucial for the association of Human ING2 with H3K4me3 (Shi et al. 2006, Pena et al. 2006). Recent findings from Lee *et al*. show that the Arabidopsis ING proteins associate with H3K4me3 as well (Lee et al., 2009). The Arabidopsis At1g54390 has four splice variants listed, all of which occur in the 3' end of the gene. One of these splice variants results in a truncated PHD finger domain, whereas the other appears to have an alternate start site. The human ING2 facilitates a switch from active to inactive expression of target genes. ING2 is a member of the HDAC acitive complex, mSin3, which is involved in inactivating gene expression by the removal of acetyl groups from chromatin (Doyon et al., 2006). ING2 also recruits histone methyltransferase (HMT) complexes to histone 3 and histone 1. This novel interaction is another mechanism for ING2 repression of gene expression (Goeman et al., 2008).

To address the nomenclature of the Arabidopsis ING proteins, we have performed an amino acid sequence analyses with the PHD fingers of both ING genes and the human ING2 protein (Figure 6). The PHD finger of At3g24010 is 70.5% identical to the PHD finger of Human ING2. At1g54390, however, is only 61.7% identical to HsING2. In addition, the PHD finger of At3g24010 is more similar to the HsING2 PHD finger than it is to AtING1, with only 61.7% identity to At1g54390. For this reason, we suggest the naming of At3g24010 as AtING2 as it is similar in sequence to that of Human ING2, thus At1g54390 should be named AtING1.

Class II Group F
Group F has two Arabidopsis and four rice proteins. This group has a conserved glutamine prior to C4 and a conserved tyrosine prior to the metal-binding histidine (Figure 2). Loop 1 is longer than most other PHD-finger domains with 16-18 residues. There are three residues between the final pair of metal coordinating ligands, a spacing not seen in any other PHD finger domain. This group contains the second PHD fingers of Group A proteins. One rice protein is annotated as a GNAT family protein, however none of the other fingers have been explored. For a summary of GNAT protein function, see Group A.

Class II Group G

Group G contains four Arabidopsis and one rice proteins. There is a tryptophan near the start of loop3. Tryptophan is not found in this location in any other PHD-finger domains, suggesting a potential role for this residue. This group contains one member that is annotated as a SREBP (sterol regulatory element binding proteins). Mammalian SREBPs are bHLH (basic helix-loop-helix) type transcription factors. These proteins are typically found in the endoplasmic reticulum until they are activated by sterol deprivation and then they are translocated to the nucleus (Liu et al., 2008). In plants, SREBP-like proteins respond to stress signals (e.g., salt, drought; Liu et al., 2008).

Class II Group H

Group H has 2 Arabidopsis and 2 rice proteins. This group has a conserved glutamine between the C1 and C2 and a conserved DA between C3 and C4 (Figure 2). Loop 2 starts with a glutamate and there is a conserved LK between the C4 and histidine. There is also a conserved lysine in loop 3. Like the majority of the proteins in this class, Group H fingers belong to proteins of unknown function.

Class II Group I

Group I has 3 Arabidopsis and 3 rice proteins. This group includes PHD fingers that cannot be classified into any group and include two PHD finger domains (At1g77250, Os08g01050) representing the first PHD fingers from proteins containing three adjacent PHD domains. The third protein (At2g19260) is from the ELM protein of Arabidopsis, which has a C-terminal ELM domain in addition to the PHD finger domain. The ELM domain was first identified in a transcription factor in *Caenorhabditis elegans*. This domain recruits an HDAC chromatin remodeler (Ding et al., 2003). This domain has not yet been characterized in plants. Another Arabidopsis PHD finger that cannot be grouped is the first PHD finger from the methyl CpG binding protein (At3g01460) as well as the PHD family proteins, Os01g66070 and Os06g51490. The second finger from At3g01460 is a group D finger. According to phylogenetic data, the group that holds the closest potential relatives to Group I is Group B.
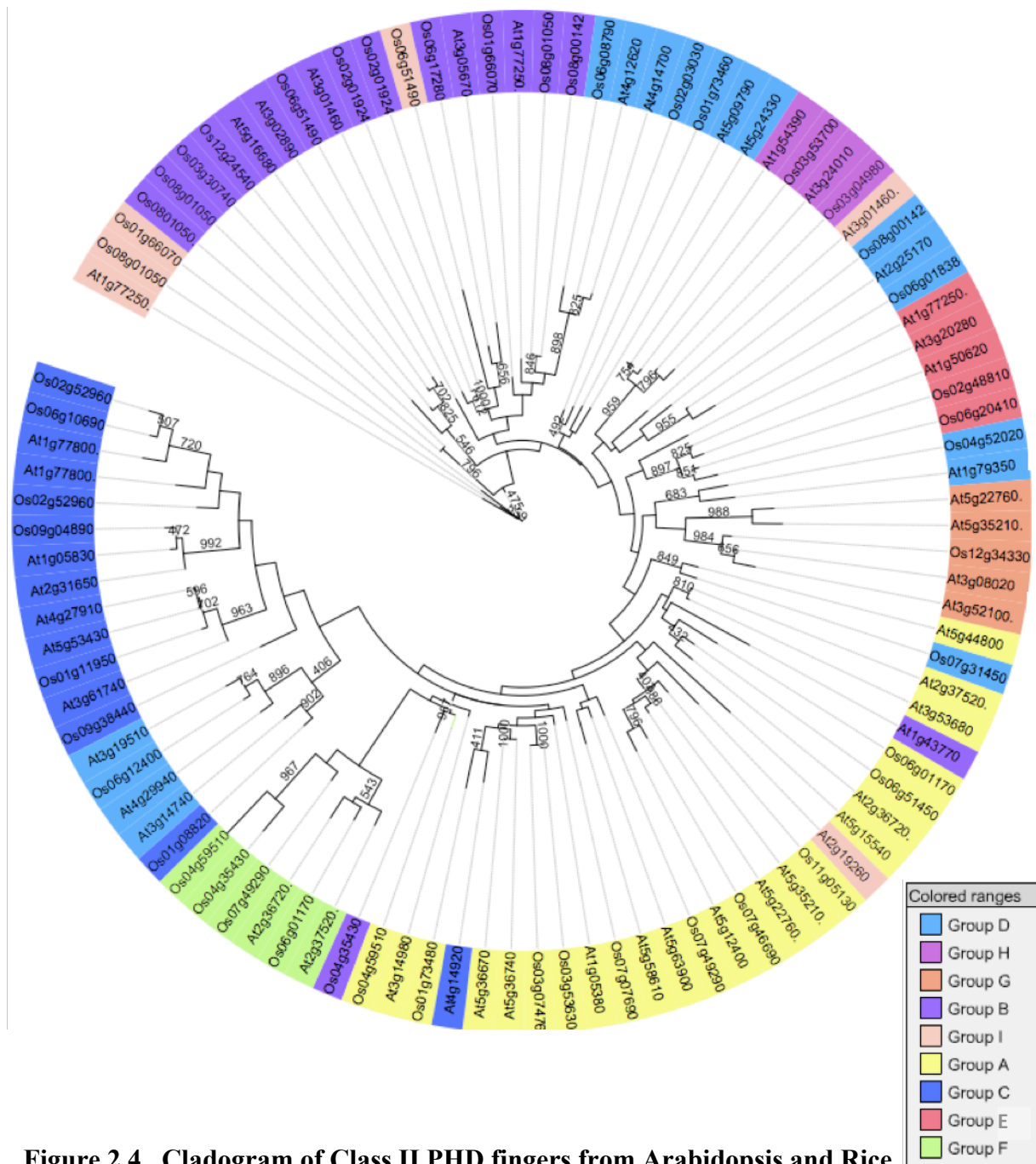
Figure 2.4. Cladogram of Class II PHD fingers from Arabidopsis and Rice.
Phylogenetic tree was created using the PhyML function at Mobyle@Pasteur with 1000
bootstraps. The resulting files were sent to ITOL for tree visualization and modification.
Class II PHD fingers form 6 major clades, which correspond to the nine groups
determined by sequence alignments, A-I. These groups were also supported by sequence
alignment analysis. Group A is shown in yellow, group B in purple, group C in blue,
group D in teal, group E in red, group F in green, group G in orange, group H in light
purple and group I in beige. Bootstrap values of >400 are displayed. The four fingers that
are not colored did not fit into a group.

Table II. Class II PHD finger proteins of *Arabidopsis thaliana* and *Oryza sativa*. This table lists all Class I PHD fingers grouped by family (A-I). Also listed are each gene's identifier, functional annotation, histone and PtdInsP binding prediction, number of exons and domain structure. A "+" indicates a predicted binding for the indicated molecule.

Family	Gene Number	Annotation	Predicted Binding H3K4me3	PtdInsP	Exon	Domain Structure
A	At5g44800	chromodomain-helicase-DNA-binding family protein	-	+	10	PHD, SNF N, Helicase C, chromo, chromo
	At2g36720.a	PHD finger transcription factor	-	-	20	PHD,PHD
	Os06g51450	PHD-finger family protein	-	+	16	PHD
	Os06g01170.a	PHD-finger family protein	-	+	17	PHD,PHD
	At2g37520.a	PHD finger family protein	-	+	19	PHD
	At3g53680	PHD finger transcription factor	-	-	19	PHD
	At5g12400	PHD finger transcription factor	-	+	08	DTT, PHD, AT-HOOK, AT-HOOK
	Os07g46690	PHD finger family protein	-	+	09	DTT, PHD, PHD
	At5g22760.a	PHD finger family protein	-	+	09	DTT, PHD, PHD
	At5g35210.a	peptidase M50 family protein	-	+	10	DTT, PHD, peptidase M50, PHD
	Os11g05130	PHD finger family protein	-	+	09	DTT, PHD, PHD
	At5g63900	PHD finger family protein	-	-	01	PHD, acetyltransferase
	At5g58610	PHD finger transcription factor	+	+	09	Agenet,PHD, acetyltransferase
	At3g14980	PHD finger transcription factor	-	-	08	PHD, acetyltransferase
	Os04g59510.a	PHD finger transcription factor	-	-	13	PHD, acetyltransferase
	Os04g35430.a	PHD finger family protein	-	-	08	PHD
	Os07g07690	PHD finger family protein	-	+	10	PHD
	At5g36670	PHD finger family protein	-	+	09	PHD
	Os03g53630	acetyltransferase, GNAT family protein	-	-	09	AT-HOOK, AT-HOOK, AT-HOOK, AT-HOOK, PHD, acetyltransferase
	At1g05380	PHD finger transcription factor	-	-	07	PHD
	At4g14920	PHD finger transcription factor	-	-	08	PHD
	Os07g49290.a	acetyltransferase, GNAT family protein	-	-	05	PHD, acetyltransferase
	At5g36740	PHD finger family protein	-	+	08	AT-HOOK, AT-HOOK, AT-HOOK, AT-HOOK, PHD
	Os01g73480	acetyltransferase, GNAT family protein	-	+	08	Acetyltransferase. PHD
	At5g15540	Adherin SCC2	-	+	28	PHD
	Os03g07476	Hypothetical protein	-	-	09	PHD
B	At1g77250.b	PHD finger family protein	-	+	05	PHD, PHD,PHD
	Os08g01050.c	PHD finger family protein	-	+	10	PHD, PHD,PHD
	Os01g66070.b	PHD finger family protein	-	+	01	PHD, PHD
	At3g01460.b	Methyl-CpG binding (MBD9)	-	+	10	PHD, MBD, FYRN, FYRC, PHD
	At3g05670	PHD finger family protein	-	-	04	zf-C3HC4,PHD
	Os06g17280	PHD finger family protein	-	-	13	PHD
	At3g02890	PHD finger protein-related	-	+	11	PHD
	At5g16680	PHD finger protein-related	-	+	12	PHD
	Os12g24540	PHD finger family protein	-	+	14	PHD
	At1g43770	PHD finger family protein	-	+	05	PHD

	Os03g30740	expressed protein	-	-	06	C1 3, PHD, U Box, porin 3
	Os02g09920	PHD finger family protein	-	+	07	PHD, PHD
	Os02g01924	Hypothetical protein	-	+	04	PHD
	Os08g01050.b	PHD finger family protein	-	-	10	PHD, PHD,PHD
	Os06g51490	Hypothetical protein				
	At1g77250.c	PHD finger family protein	-	+	05	PHD, PHD,PHD
	Os08g00142.b	Hypothetical protein	-	+	03	PHD, PHD, RING
C	At1g05830	trithorax protein, putative / PHD finger family protein / SET domain-containing protein	-	-	25	PWWP, FYRN, FYRC, PHD, SET
	At2g31650	trithorax 1 (ATX-1) (TRX1)	-	-	24	PWWP, FYRN, FYRC
	Os09g04890	trithorax 1	+	+	25	PHD
	At3g14740	PHD finger family protein	-	+	02	PHD
	Os01g08820	PHD finger family protein	-	+	02	PHD
	At4g27910.b	PHD finger protein-related / SET domain-containing protein (TX4)	-	+	23	PWWP, PHD, PHD, SET
	At5g53430.b	PHD finger family protein	-	+	23	PWWP, PHD, PHD, SET
	Os01g11952.b	PHD finger protein	-	+	23	PWWP, PHD, PHD, SET
	At3g61740.b	PHD finger family protein (ATX3)	-	-	20	PWWP, PHD, PHD
	Os09g38440	SET domain containing protein	-	-	21	SET, PHD
	Os02g52960.a	PHD finger family protein	-	+	19	PHD, PHD
	At1g77800.a	PHD finger family protein	-	+	19	PHD, PHD
	Os02g52960.b	PHD finger family protein	+	-	19	PHD, PHD
	Os06g10690	C1-like domain containing protein	-	-	17	PHD, PHD
	At1g77800.b	PHD finger family protein	-	-	19	PHD, PHD
D	At2g25170	chromatin remodeling factor CHD3 (PICKLE)	-	-	30	PHD, chromo, chromo, RES III, SNF2 N, Helicase C, DUF1087, DUF1096
	Os06g01838	Hypothetical Protein	-	-	30	PHD, chromo, Res III, SNF 2N, helicase C, DUF1087, DUF 1086
	At3g19510	homeobox protein (HAT 3.1)	+	-	09	PHD, homeobox
	Os06g12400	PHD finger family protein	-	-	09	PHD, homeobox
	At4g29940	pathogenesis-related homeodomain protein (PRHA)	+	-	15	PHD, homeobox
	At4g12620	replication control protein	-	+	01	PHD, BAH, AAA
	At4g14700	replication control protein	-	+	01	PHD, BAH, AAA
	Os06g08790	origin recognition complex subunit 1	-	+	17	PHD, BAH, AAA
	Os02g03030	PHD finger family protein	-	+	04	PHD, SET
	Os01g73460	PHD finger family protein	-	+	06	PHD, SET
	At5g24330	PHD finger family protein / SET domain-containing protein	-	+	06	PHD, SET
	At5g09790	PHD finger family protein / SET domain-containing protein	-	+	05	PHD, SET
	Os06g51490.a	PHD finger family protein	-	+	06	JmjC, ARID, PHD, JmjC, zf-C5HC2, PHD
	Os07g31450	SNF2 family N-terminal domain containing protein	-	+	11	PHD, Snf2N, helicase C, chromo, chromo
	At1g79350	DNA-binding protein	-	+	31	PHD
	Os04g52020	DNA-binding protein	-	+	14	PHD
	Os08g00142.a	Hypothetical protein	-	-	03	PHD, PHD, RING

29

E	At3g24010	Inhibitor of Growth (ING)	+	+	07	PHD
	Os03g04980	PHD finger family protein	+	+	07	PHD
	At1g54390	Inhibitor of Growth (ING)	+	-	06	PHD
	Os03g53700	PHD finger family protein	+	-	08	PHD
F	Os06g01170.b	PHD finger family protein	-	+	17	PHD
	At2g36720.b	PHD finger transcription factor	-	-	20	PHD, PHD
	At2g37520.b	PHD finger family protein	-	-	19	PHD, PHD
	Os04g59510.b	PHD finger transcription factor	-	-	13	PHD, acetyltransferase
	Os4g35430.b	PHD finger family protein	-	-	08	PHD
	Os07g49290.b	acetyltransferase, GNAT family protein	-	+	05	PHD, acetyltransferase
G	Os12g34330.a	PHD finger family protein	-	+	10	zf-C3HC4, PHD, PHD
	At3g08020.a	PHD finger protein-related	-	+	13	PHD, PHD
	At3g52100.a	PHD finger family protein	-	+	13	PHD, PHD
	At5g22760.b	PHD finger family protein	-	-	09	DTT, PHD, PHD
	At5g35210.b	peptidase M50 family protein / sterol-regulatory element binding protein (SREBP) site 2 protease family protein	-	+	10	DTT, PHD, peptidase M50, PHD
H	Os02g48810	PHD finger family protein	-	+	02	PHD
	Os06g20410	BAH domain containing protein	-	+	06	BAH, PHD
	At3g20280	PHD finger family protein	-	+	04	PHD
	At1g50620	PHD finger family protein	-	+	04	PHD
I	Os08g01050.a	PHD finger family protein	-	-	10	PHD, PHD,PHD
	At1g77250.a	PHD finger family protein	-	+	05	PHD, PHD,PHD
	At2g19260	ELM2 domain-containing protein / PHD finger family protein	-	+	07	PHD, ELMZ
	At3g01460.a	PHD finger family protein / methyl-CpG binding domain-containing protein	-	+	10	PHD, MBD, FYRN, FYRC, PHD
	Os06g51490.b	PHD finger family protein	-	+	06	PHD
	Os01g66070.a	PHD finger family protein	+	-	01	PHD, PHD

A. FECVICDL**GG**DLLCCDSC**PRTYH**TAC**L**NPPLKRIPNGKWICPKCSP

Loop 1 (8aa)   Loop 2 (4aa)   Loop3 (11-15aa)

B. IICTECHQGDDDGLMLLCDLCDSSA**HT**Y**C**VGLGRE**VPE**GN**W**YCEGCRP

Loop 1 (11aa)   Loop 2 (4aa)   Loop3 (11-15aa)

C. IMCA**V**CQSTDGDPLNPIVFCDGCDLM**VH**ASCYGNPLVKA**IPE**GD**W**FCRQC

Loop 1 (9-14aa)   Loop 2 (4aa)   Loop3 (12-15aa)

D. EDCQICFKSDTNIM**I**ECDDCLGGF**H**LKC**LXP**PLKEVPEGD**W**ICQFCEV

Loop 1 (8-13aa)   Loop 2 (4aa)   Loop3 (13-18aa)

E. TY**CV**CHQVSFGDMIACDNENCQGGE**WF**HYTCVGLTPETRFKGK**WY**C**P**TCRL

Loop 1 (8-13aa)   Loop 2 (5-6aa)   Loop3 (13-18aa)

F. NGCVLCSGSDFCRSGFGPRTIIICD**Q**CEKE**YH**IGCLSSQNIVDLKELPKGN**WF**C**SMD**CTR

Loop 1 (16-18aa)   Loop 2 (4aa)   Loop3 (17-18aa)

G. ITCHMCYLVEVGKSERAKMLSCKCCGKKY**H**RNCVKS**W**AQHRDLFNWSSWACPSCRI

Loop 1 (10/15aa)   Loop 2 (4aa)   Loop3 (12-17aa)

H. MTC**QI**CQGTINEIETVLIC**DA**CEKGY**HLK**CLHAHNIKGVP**K**SE**W**HCSRCVQ

Loop 1 (12aa)   Loop 2 (4aa)   Loop3 (15-16aa)

Figure 2.5. Consensus sequences for Class II. The PHD motif residues are colored blue. The loop regions are also indicated. Key residues that define each group within group II are indicated by bold and underlined text. **A)** Group A; **B)** Group B; **C)** Group C; **D)** Group D; **E)** Group E; **F)** Group F; **G)** Group G and **H)** Group H.

```
HsING2       CLCNQVSYGEMIGCDNEQCP-IEWFHFSCVSLTYKP--KGKWYCPKC
At3g24010    CICNQVSFGEMVACDNNACK-IEWFHFGCVGLKEQP--KGKWYCPEC
At1g54390    CVCHQVSFGDMIACDNENCQGGEWFHYTCVGLTPETRFKGKWYCPTC
             *:*:***:*:*:.***: *   ****: **.*. :.  ******* *
```

Figure 2.6. Alignment of AtING PHD with HsING2 PHD. Alignments of the PHD fingers of At3g24010, At1g54390 and HsING2 were generated using ClustalW and percent identity was determined using the Align tool on Biology Workbench (workbench.sdsc.edu). Conserved residues are colored blue. Residues of conservative substitution are colored green.

PHD finger structural variants

In their analysis, Lee *et al*. (2009) included a subset of zinc fingers that do not follow the structural definition shown by the majority of the PHD fingers in Arabidopsis and rice. These fingers either deviate in loop length, in the spacing of zinc-coordinating residues, or do not contain the conserved aromatic residue of loop 3. These proteins may be variant PHD finger domains, listed in Table III. As mentioned previously, the loop regions, although variable in length, remain consistent within a range. Loop ranges from 8-18 residues in length; the second from 4-5 residues, and the third is 10-20 residues in length (Figure 1). In the case of At1g67220 and At3g12980 loop 1 is 36 amino acids in length, which is at minimum twice as long as seen in the majority of PHD fingers. In the same way, At2g18090 and At3g51120 have a loop 2 with 26 amino acids in length, while At2g40770 has a loop 3 that is 40 amino acids long. These elongated loops do not conform to the majority of PHD fingers, however the full length PHD fingers retain all other key factors. The putative PHD fingers of At2g31650, At5g55390 and At4g10940 also show abnormalities in the spacing of key domain features. In the case of At2g31650, C6 is separated from C7 by 7 amino acids, while the normal spacing is 1-2 residues. Ten amino acids separate H1 from C5 in At5g55390. In addition, At4g10940, a partial PHD finger, has an elongated stretch of amino acids between C1 and C2, which potentially disrupts this metal coordinating pair.

Table III: Variant PHD fingers. Putative PHD fingers are shown with description of variation and prediction of PHD finger authenticity. PHD fingers sequences are also shown with motif residues displayed in blue. The variant region of each finger is underlined.

Gene ID	Variation
At1g67220	Elongated loop 1
At2g18090	Elongated loop 2
At2g31650	Pairing of C6 and C7 lost; lacking aromatic residue of loop 3
At2g40770	Elongated loop 3
At3g12980	Elongated loop 1
At3g51120	Elongated loop 2
At4g10940	Pairing of C1 and C2 lost
At5g55390	Pairing of H1 and C5 lost

At1g67220 SPCHSRCKTKFPLCGVFIDKHKMLKRSNFDNADTEEWVQCESCEKWQHQICGLYNKLKDEDKTAEYICPTCLL

At2g18090 DVCFVCFDGGSLVLCDRRGCPKAYHPACVKRTEAFFRSRSKWNCGWHICTTCQKDSFYMCYTCPY

At2g31650 LMCTICGVSYGACIQCSNNSCRVAYHPLCARAAGLCVELENDMSVEGEEADQCIRMLSFCKRH

At2g40770 VECICGAVSESHKYKGVWVQCDLCDAWQHADCVGYSPKGKGKKDSQHIDEKASQKKSKKDATEIIVREGEYICQMCSE

At3g12980 YVCIPCYNEARANTVSVDGTPVPKSRFEKKKNDEEVEESWVQCDKCQAWQHQICALFNGRRNHGQAEYTCPNCYI

At3g51120 DVCFICFDGGDLVLCDRRNCPKAYHPACIKRDEAFFRTTAKWNCGWHICGTCQKASSYMCYTCTF

At4g10940 VVCLDGDLCKIRNTFSYIEGDSNLDTSIACDSCDMWYHAICVGFDVENASEDTWVCPSKDTLYQF

At5g55390 SVCAICDNGGEILCCEGSCLRSFHATKKDGEDSLCDSLGFNKMQVEAIQKYFCPNCEH

PtdInsP binding predictions

Human ING proteins have been shown to have multiple binding partners. One key interaction involved the phosphoinositide PtdIns(3)P (Gozani et al., 2003; Huang et al., 2007). PtdInsPs are key members of inositol-mediated signaling pathways (Meijer and Munnik, 2003; Berridge, 2009). In mammalian cells, it is thought that the ability of HsING1 and HsING2 to associate with PtdInsPs may be a mechanism for regulating gene transcription in response to stress by acting as nuclear PtdInsP receptors (Soliman and Riabowol, 2007). The specificity of HsING interaction with PtdInsPs is attributed to a highly basic domain adjacent to the PHD finger, called the polybasic region or PBR (Kaadige and Ayer, 2006). This domain is found in ING1, ING2 and Pf1 (PHD zinc finger 1) and is C-terminal to the PHD finger. On its own, the PBR is sufficient for specific PtdInsP binding. By exchanging the PBRs between different PHD fingers Kaadige and Ayer (2006) showed that a PBR containing 7-10 basic residues can bind PtdInsP, however, a PBR with only 3 basic residues cannot, thus PtdInsP binding is correlated with a high percentage of basic residues in the PBR. To determine whether plant PHD finger proteins have the potential to act as PtdInsP receptors, we inspected the amino acid composition of the PBRs of all PHD fingers from Arabidopsis and rice. The criteria used for judging whether a PBR is likely to bind PtdInsPs is the amount of lysine and arginine residues contained in the PBR. Proteins were judged likely to bind to PtdInsPs if they contain 4 lysine or arginine residues out of the 27 amino acids contained in the PBR.

Our analysis revealed that a large portion of PHD fingers from Arabidopsis and rice contain PBRs that we predict bind to PtdInsPs. This was a surprising result, as few groups have commented on potential PtdInsP binding for known PHD finger proteins. Of the 78 PHD fingers in Arabidopsis, 44 have a putative PBR. In rice, 36 of 70 have lipid-binding potential (Table I and Table II). These results indicate a potential for a large variety of PHD finger proteins to interact with PtdInsPs and highlights the potential significance of PtdInsP interactions with plant PHD fingers.

H3K4me3 binding predictions

As mentioned previously, the PHD finger of Drosophila BPTF, a NURF301 complex member, is able to bind to H3K4me3. A conserved tryptophan in loop 2 was found to be essential for this

association. In this interaction, the K4me3 groups align in a binding pocket that contains the essential tryptophan, as well as three tyrosines. One of these tyrosines in particular, Y23, is critical for K4me3 recognition. (Li et al., 2006). The human ING2 proteins have also been shown to bind H3K4me3 (Pena et al., 2006; Shi et al., 2006). ING2 requires a conserved tyrosine that precedes the first cysteine in the PHD motif. This tyrosine (Y215) and a structurally adjacent tryptophan (W238 of loop 2) have been shown by x-ray crystollography to recognize the trimethyl group of K4 (Pena et al., 2006). Thus it appears that the conservation and juxtaposition of W residue and Y23 in BPTF, and Y215 and W238 in ING2 are likely to be critical for H3K4me3 binding (Li et al., 2006).

We also evaluated whether the PHD fingers in Arabidopsis and rice are likely to bind with H3K4me3 by identifying the W and/or Y residues in candidates that correspond to those found in ING2 and BPTF as described above. Specifically, the trypotophan of loop 2 and tyrosine that preceeds C1. Every member of Class I has the conserved tryptophan in loop 2 (Figure 3), however, not all contain the conserved tyrosine before C1. Only eight of the 46 PHD fingers of Class I contain both residues and thus are predicted to bind methylated histones (Table I). For the most part, Class II PHD fingers lack a tryptophan in loop 2. The plant ING family Class II Group E and the single rice finger were exceptions. Although this group lacks the conserved W, it is worth mentioning the members that have the conserved tyrosine. Of the Class II PHD fingers, 10 fingers have a tyrosine preceding C1. These have been annotated on Table II as putative H3K4me3 binding domains.

Of note are the recent findings that the Class I Group D members (Alfin-like) and Class II Group D members (ORC1) associate with methylated histone proteins in binding assays (Lee et al, 2009; de la Paz et al., 2009). These proteins are not predicted to bind according to our analysis and indicate that there is a limitation to our criteria. None of these PHD fingers contain the conserved tyrosine and the two ORC1 PHD fingers do not contain the conserved tryptophan in loop 2 either. Preliminary evidence indicates that a phenylalanine in loop 2 may substitute for the key tryptophan (de la Paz et al., 2009). Of the 87 Class II PHD fingers, 32 have a loop 2 phenylalanine. Twenty one others have a loop 2 aromatic residue (mainly tyrosine). These attributes may allow members to associate with H3K4me3, however the data presented by de la

Paz *et al*. is not based on structural experiments, as is the case with human ING2 and BPTF (de la Paz et al., 2009; Pena et al., 2006; Li et al., 2006). According to data presented by Lee *et al*., the Alfin-like PHD fingers form a putative, unique aromatic cage that contains a conserved loop 1 tryptophan. Of the PHD fingers in Arabidopsis and rice, 19 have a loop 1 tyrosine (excluding the alfin-likes of Class I Group D).

Conclusion

PHD finger proteins are numerous in both the model organisms *Arabidopsis thaliana* and *Oryza sativa*. These fingers are found in a variety of proteins, the majority of which are predicted to be nuclear localized. In this study, we have refined the characterization of PHD fingers in Arabidopsis. In addition PHD finger proteins of rice have been identified and subsequent phylogenetic analyses has elucidated the evolutionary relationships between these proteins. Of the 78 PHD fingers from Arabidopsis and 70 of rice, 80 fingers contain an adjacent PBR domain. These findings indicate that many PHD finger proteins have the potential to function as nuclear PtdInsP receptors. The PHD fingers can be clearly separated into two Classes, which have different properties. Each Class has distinct groups. Class I has fewer members, though they are more similar overall and very similar within groups. Class II PHD fingers have more members, though the fingers are less similar overall, even within groups. Class II PHD fingers also are more likely to occur in proteins with an additional PHD finger domain, an association which seems to have allowed divergence among these additional domains. Though the PHD finger is most often associated with other domains involved in chromatin remodeling, it also occurs in proteins containing no other domains, suggesting that the PHD finger may function as a scaffold for chromatin remodeling complexes.

Chapter III: Characterization of *ing1* and *ing2* mutants

Introduction

The PHD (Plant HomeoDomain) finger domain is a nuclear structural domain present in many proteins involved chromatin remodeling (Bienz, 2006). The PHD finger contains a C4HC3 zinc-coordinating motif (Schindler et al., 1993). In humans, the loss of PHD function can lead to the development of disorders such as autoimmune diseases (Villasenor et al., 2005) and cancer (Russel et al., 2006; Cai et al., 2009). This is most likely due to the fact that these proteins play critical roles in regulating gene transcription in a diverse set of cellular pathways (Bienz, 2006).

The human ING (INhibitor of Growth) protein family has been linked to a variety of cancers and some members can function as tumor suppressors (for review see Soliman and Riabowol, 2007). The INGs contain PHD fingers and the majority of human ING proteins have been shown to interact with histones within actively transcribed chromatin (Gozani et al., 2003; Pena et al., 2006). Specifically, the ING PHD fingers bind to histone H3, which carries a trimethylated lysine 4 residue (H3K4me3; Pena et al., 2006; Shi et al., 2006). This H3K4me3 is considered an epigenetic mark that provides information on the transcriptional status of the chromatin. In addition, ING proteins have been shown to associate with proteins that "remodel" chromatin from active to inactive states or vice versa, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs). INGs can recruit such proteins to active promoters (Loewith et al., 2000; Kuzmichev et al., 2002; Vieyra et al. 2002; Shiseki et al., 2003; Pedeux et al., 2005, Doyon et al., 2006). This combination of histone mark recognition and protein complex recruitment allows ING proteins to play an integral role in epigenetic mechanisms in mammalian cells.

Although there are over 50 ING protein isoforms identified in eukaryotes, only seven are plant INGs (He et al., 2005). Both rice and Arabidopsis have two ING genes (At1g54390 and At3g24010). Arabidopsis ING1 has four putative alternatively spliced isoforms, while ING2 has only one predicted transcript (Lee et al., 2009). Like their mammalian orthologs, Arabidopsis INGs are nuclear proteins and associate with methylated histone residues *in vitro* (Lee *et al*., 2009). Although we are beginning to understand ING properties, little is known about how the

function of these proteins impact plant physiology and development. As mentioned previously, the loss of PHD finger function in mammalian systems leads to disease and developmental disorders (Villasenor et al., 2005; Russel et al., 2006; Cai et al., 2009). Since the Arabidopsis ING proteins show conservation in amino acid sequence and domain organization to the mammalian INGs (Chapter II), it is likely that their impact on plant growth and development is equally as important.

To further understand the role of ING proteins in Arabidopsis growth and development, I have examined Arabidopsis *ing* mutant plants. In this analysis, I have observed developmental phenotypes, such as alterations in time to flowering. In addition, I have observed physiological phenotypes, such as altered responses to nutrients and stress. Lastly, I have determined that ING1 has the potential to form a protein complex with a key regulator of nutrient-dependent transcriptional programming, called SnRK1.1. Interestingly, *ing1* and *ing2* mutants often had opposite phenotypes, suggesting that their roles in plant development and physiology are complementary.

MATERIALS AND METHODS

Plant growth and germination experiments

Arabidopsis thaliana ecotype Columbia plants were used for all experiments. For flowering time experiments, WT and mutant seeds were placed on pre-wetted Pro Mix potting soil and grown under l6 hours of light in a growth room set at 22°C and 24°C night and day temperature and watered every other day. Light was provided using a mixture of fluorescent and incandescent lamps or fluorescent lamps only at 100-200 µE of light in a 16 h day/8 hour night cycle. Low light conditions were performed at 20-60 µE in a 16 h day/8 hr night cycle. For root growth experiments, seeds were surface-sterilized with dilute bleach and plated on 0.8% agar medium, 0.5X MS + 0.8% ultra-pure agar (Fluka, Sigma-Aldrich) and 0.5x MS + 0.8% agar medium + 3-11% glucose. Each agar plate was divided into sections, and >10 seeds of WT or mutant type were plated per section. Plates were placed at 4°C for three days before moving to room temperature. Plates were oriented vertically to allow measurements of root length. Root length experiments were performed in triplicate or more. For seed germination experiments, age-

matched WT and mutant seeds were harvested on the same day from plants grown in parallel on the same shelf of a growth rack and were stored at 23°C in the dark for at least 30 days before germination. For ABA studies, WT and mutant seeds were surface-sterilized and plated on 0.5x MS salts (pH 5.8) and 0.8% agar containing 0, 1, 2, or 3 µM ABA. Each agar plate was divided into sections, and >50 seeds of WT or mutant type were plated per section. Plates were stratified in the dark at 4°C for at least 3 days then transferred to 23°C under continuous light. A seed was scored as germinated when the radicle protruded through the seed coat. For hydroponic nutrient deprivation experiments, age matched seeds were surface sterilized and spotted on nylon mesh strips on 0.5X MS + 1% sucrose ultra-pure agar plates. Seedlings were allowed to germinate and grow on plates for seven days and were then transferred to flasks containing 30-50 mL of 0.5X MS + 1% sucrose medium. Seedlings shook in liquid culture for two weeks before transferring to 0.5X MS medium. Seedlings were exposed to ~10 µE light during liquid growth.

Mutant Isolation

Potential *ing1* and *ing2* mutants were identified from the Salk T-DNA lines (Alonso *et al.*, 2003) through the analysis of the SiGnAL database (http://www.signal.salk.edu/cgi-bin/tbnaexpress). Seeds for *ing1* (SALK_026592) and *ing2* (Salk_009598) mutants were obtained from the Ohio State University Arabidopsis Biological Resource Center. Both lines are available as homozygous seed stocks where the T-DNA has been sequenced to map the location of insertion. However, we obtained heterozygous lines and screened these lines by using PCR. Genomic DNA from segregating plants was isolated and used in PCR reactions. A primer specific to the left border (LB) of the SALK T-DNA was used in combination with *ING1* promoter-specific primers and the Ing2 gene-specific primers. A product formed by amplification with a T-DNA specific primer and promoter or gene specific primer indicates the presence of the T-DNA within the gene (or promoter) of interest. To analyze the *ing1* line, annealing at 60°C was performed with the LB primer and the following forward and reverse primers: *1-pfor*, 5'-CCGGTCTAGCTAGAGATCC-3' and *1-prev*, 5'-CTTTGATCTCTGATGAACTGGA-3'. The line SALK_009598 was previously analyzed for homozygosity by Dr. Jonathan Watkinson. Briefly, the primers 2-pfor and SALK LB were used to amplify a 700 bp fragment.

RT-PCR

Total RNA was extracted from 100 mg flash-frozen mature leaves from WT and mutant using an RNeasy kit (QIAGEN Inc plants., Valencia, CA). Extracted RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, LLC, Wilmington, DE). cDNA was synthesized from 1 μg of RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). For *ING1* gene-specific amplification, the *1-for* primer and *1-rev* primer set was used in 35 cycles of PCR amplification (1 min 94°C, 1.5 min 60°C, 2.0 min 72°C) resulting in a 915 bp product. For *ING2* gene-specific amplification, the *2-for* primer and *2-rev* primer were used in 30 cycles of PCR amplification (1 min 94°C, 1 min 56°C, 1 min 72°C) resulting in a 706 bp product. The amplification of actin has been described and generated a 425 bp product (Berdy *et al*., 2001).

Flowering Time Assays

WT and mutant plants were grown as described under long-day (16 hrs) or short-day (8 hrs) conditions and/or with cold treatment (13°C). Careful attention was given to growing plants side-by-side or in the same pot for comparison. Plants were examined at the point of inflorescence emergence. Plants were removed from soil, inverted, and rosette leaves were removed in developmental order to facilitate counting. Fifteen or more plants per variant were examined.

Immunoprecipitation (IP)

For IP experiments, 30-70 ng uL^{-1} of purified and dialyzed proteins were used. The IP reactions were carried out in a total of 400 uL of IP buffer consisting of 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM MgCl$_2$, 0.05% Triton-X 100. Proteins were mixed with IP buffer and incubated at room temperature for two hours on a rocking platform. The resulting mixture of two interacting protein was further incubated with anti-V5:protein A-Sepharose beads, for SnRK-V5 and Ni-NTA agarose beads, for ING2-Xpress at room temperature for two hours on a rocking platform. The beads were washed three to five times with 1-2 mL IP buffer. Protein A beads carrying the protein complexes were collected after each wash by brief centrifugation. The washed beads were resuspended in 25-50 μL 2X SDS-PAGE loading buffer, separated on 10-12% SDS-PAGE gels and transferred to a nitrocellulose membrane. Western blot conditions were as follows: the nitrocellulose membranes were incubated in blocking solution: 5% non-fat milk in 1X TBST (50 mM Tris pH 7.5, 0.9% NaCl and 0.01% Tween-20) buffer for 30-60 mins at room temperature.

For detection of V5-tagged or X-press tagged proteins, a 1:5000 dilution of a mouse anti-V5 or mouse anti-Xpress monoclonal antibody (Invitrogen, CA) was used. This step was followed by three to five washes in 1X-TBST and an incubation with a 1:10,000 dilution of goat anti-mouse horseradish peroxidase-conjugated antibody (BioRad Laboratories, Hercules, CA). After washing, the membranes were activated with the ECL Plus detection kit (Amersham-Pharmacia Biotech) according to the manufacturer's instruction. The nitrocellulose blots were exposed to X-ray film for 1-30 minute exposure times.

RESULTS

Characterization of *ing* T-DNA mutant lines

To gain an idea of the endogenous levels of *ING* gene expression, I utilized the online resource, Genevestigator (Zimmermann *et al.*, 2004). Microarray data from Genevestigator indicates that both *ING1* and *ING2* mRNAs are expressed throughout development in a variety of tissues (Figure 3.1). Along with the similarity in amino acid sequence, this similarity in expression suggests that the encoded proteins could have redundant functions. One could expect therefore, that a single mutation in either gene may lead to minimal defects in physiology and development. However, there seems to be a variation in amount of *ING1* versus *ING2* transcript in specific tissues; for example, reproductive tissues, shoot apex and senescent leaves. Thus, I focused on these tissues for my phenotypic analysis of mutants.

To determine the physiological function of *ING1* and *ING2* genes, I isolated T-DNA insertion mutants for these genes from the Salk collection (Alonso *et al.*, 2003). A single T-DNA mutant line for each gene was obtained from the Arabidopsis Biological Resource Center. I obtained the SALK_026592 line for *ING1* and the line SALK_009598 for *ING2*. The T-DNA insertion in the *ING1* gene was confirmed by PCR using a T-DNA left border primer and ING1 promoter-specific primers (*1-pfor* and *1-1rev*), resulting in the amplification of a 1162 bp fragment (*1-pfor + LB*) and a 137 bp fragment (*1-prev + LB*) in *ing1* mutants (Figure 3.2A). The presence of the 137 bp amplification product indicates that a second T-DNA was present in tandem in the *ING1* promoter.

The characterization of the *ing2* mutant involved the use of a T-DNA left border primer and *ING2* gene-specific primers (*2-pfor*). Amplification resulted in a 700 bp fragment in *ing2* mutants (Figure 3.1B). T-DNA sequence information provided by SIGnal (http://signal.salk.edu/) and TAIR (www.arabidopsis .org) indicates that the insertion for *ing1* mutant line is in the promoter, while the T-DNA insertion in *ing2* mutant line is in the third inron.

To determine whether these T-DNA insertions resulted in decreased ING gene expression, I examined RNA levels in the mutant lines as compared to WT plants. I used RNA isolated from mature leaf tissue for both ING1 and ING2 transcript analysis, and semi-quantitative RT-PCR to determine whether *ing1* and *ing2* mutants retained gene expression. Primers specific for an Actin gene (ACT8) were used as a positive control. Using primers specific for *ING1* (*1- for* and *1-rev*), I detected a 922 bp PCR product in wildtype plants corresponding to the predicted Ing1 full-length transcript. Using primers *2-for* and *2-rev* I detected a 706 bp product in wildtype plants corresponding to the predicted *ING2* full-length transcript. In *ing1* leaf tissue, I was unable to detect a full-length transcript for *ING1*, however, *ING2* was expressed at wildtype levels. I conclude that the *ing1* mutant is a loss-of-function.

When I analyzed ING expression in the *ing2* mutant I found no evidence for expression of ING1, and the presence of a smaller ING2 transcript of approximately 650 bp (Figure 3.2C). I sequenced this PCR product and found 672 bp with identity to *ING2*. Within this 672 bp fragment, there is a deletion after the second codon, thus would result in a frame-shift in the peptide sequence. The protein product of this transcript is predicted to be 64 amino acids in length. I used this as a query in a BLASTp analysis and found no matches to the Arabidopsis proteome (http://blast.ncbi.nlm.nih.gov). Searches via SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk) did not recover any matches to potential protein domains, thus I conclude that the *ING2* transcript in *ing2* mutants cannot encode a full-length, functional ING2 protein. In addition, this smaller *ING2* transcript is not as abundant as the wildtype *ING2* transcript. From these data, I conclude that the *ing2* mutant is a partial loss-of-function.

One limitation of RT-PCR is that it is only semi-quantitative. It is possible that there are full-length *ING2* transcripts in the *ing2* mutant that are present but below the level of detection by

agarose gel electrophoresis. A western blot analysis with a primary antibody specific for ING1 or ING2 would clarify the size and amount of ING protein that is made in these mutants.
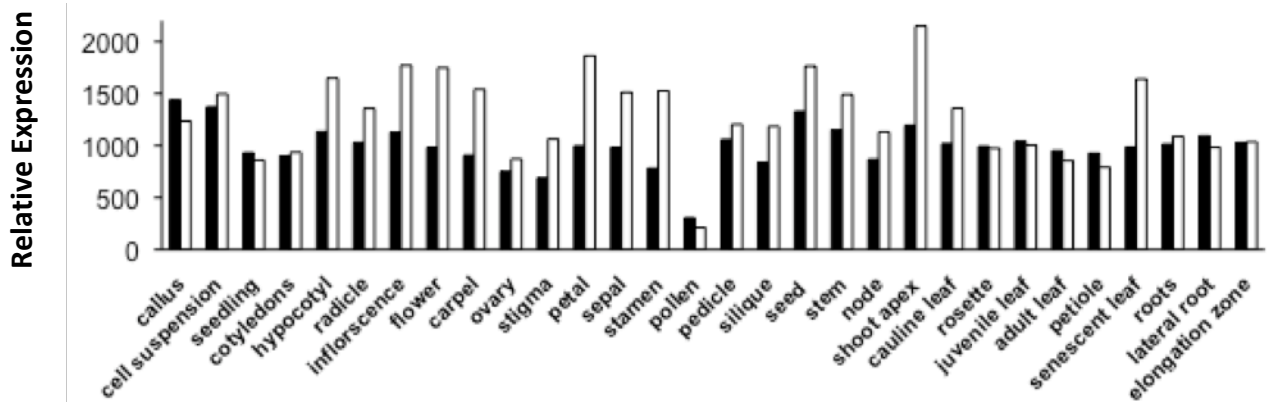
Figure 3.1. Expression data for ING1 and ING2 from Genevestigator. Microarray data from Genevestigator from the ATH1:22K showed consistent expression in most tissues of both Ing1 (black) and Ing2 (white). Expression values are supplied for various developmental tissues.
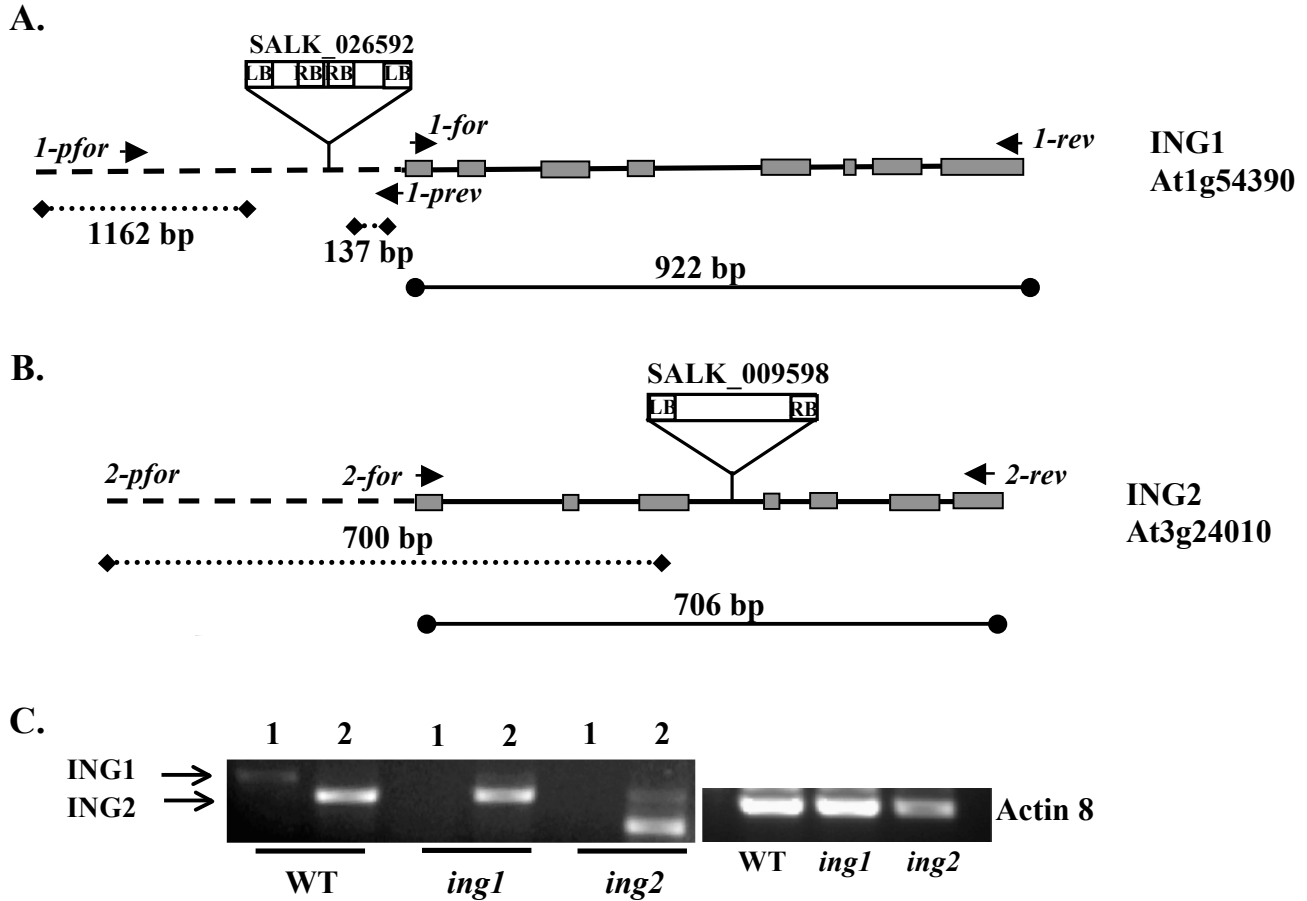
A.

B.

C.

Figure 3.2. T-DNA insertions and gene expression in *ing* mutant lines. T-DNA insertions in the ING1 and ING2 genes. Exons are dark gray boxes; black arrows indicate positions of gene-specific primers. **A)** T-DNA line obtained for At1g54390. PCR products for genetic screening are indicated as dotted lines. Amplification products for transcript identification are shown as solid lines. **B)** T-DNA line obtained for At3g24010. **C)** 1-2 μg of RNA was isolated from WT, *ing1* and *ing2* leaf tissue and used to generate cDNA. Semi-quantitative RT–PCR reactions were performed with the indicated primers. 1. Amplification with Ing1 specific primers. 2. Amplification with Ing2 specific primers.

Developmental Analysis of *ing* mutants

Both *ing* mutants show little deviation from WT in growth and development; however, Jonathan Watkinson noticed that both mutants have differences in time to flowering. To quantify these differences, I performed flowering time assays. WT, *ing1* and *ing2* seeds were sown on pre-wetted soil and allowed to stratify at 4°C in the dark for 3-5 days. Pots were moved to long-day growth conditions where seeds were allowed to germinate and plants were grown to the flowering stage. To determine time to flowering, leaves of plants that had visible inflorescence tissue were removed and counted. Mutant *ing2* plants produce fewer leaves at the point of floral transition than WT plants (Figure 3.3.C, left). Conversely, *ing1* plants develop more leaves than WT plants (Figure 3.3C, lower). *ing2* plants also show an early flowering phenotype under short-day conditions (8 hrs light), however this difference is not statistically significant (data not shown). In addition, plants also produce fewer leaves before inflorescence initiation under cold treatments (Figure 3.3C, right). It is important to mention that although WT leaf number can vary under the same temperature and light treatment, the mutant trend is always the same. I conclude, that the loss of full-length ING transcripts results in an alteration in the timing of floral transition in mutant plants. In the case of *ing2*, this alteration is independent of growth temperature and/or day length. These conditions were not tested for *ing1* mutants.
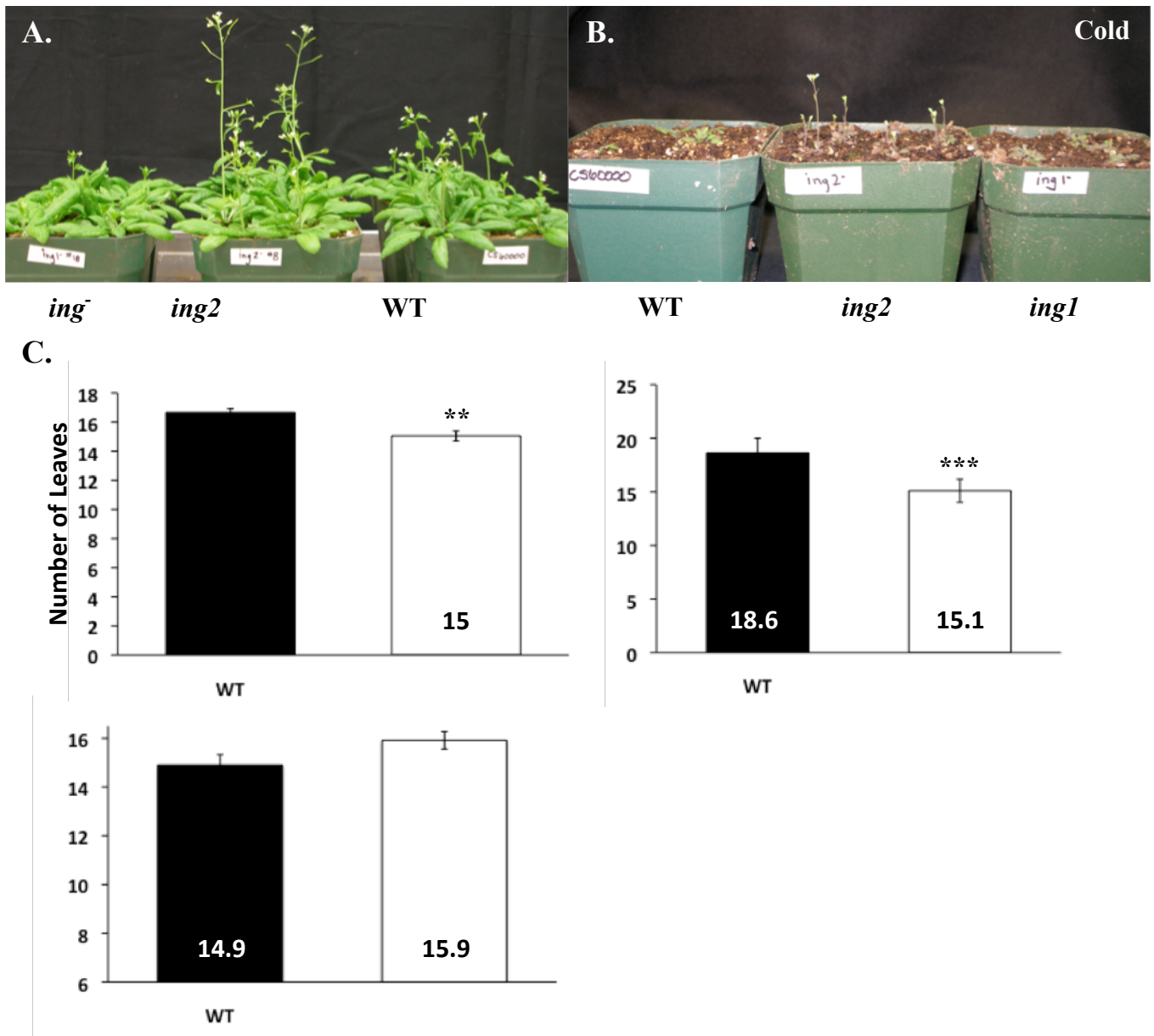
Figure 3.3: ING are altered in time to flowering. WT, *ing1*, and *ing2* mutant seeds were sown on pre-wetted soil, stratified at 4°C for 3 days, and then placed in a growth chamber under long day (16hrs light) and at A,C) normal temperature (23°C) or B,C) under cold treatment (13°C) conditions. Rosette leaves that developed prior to the inflorescence were counted. Values represent the mean ±SE where n=15-38 plants. * indicates a p value <0.09, ** indicates a p value <0.002, and *** indicates a p value <0.0002

Physiological Responses of ing Mutants

To determine whether ING proteins are required for physiological responses, I examined responses to varying nutrients and to different stresses. To examine nutrient status sensing and/or response, I performed root length assays in the presence of various nutrient conditions (Figure 3.4). Conditions for these assays were based on previous work by Elitsa Ananieva et al. (2008) who have investigated the nutrient sensing signaling pathway. In addition, work from Baena-Gonzalez et al. (2007) on nutrients and transcriptional reprogramming used these root length assays and found that a loss- or gain-of-function in the low energy/stress sensor called SnRK1.1 results in altered root growth.

Wild type, *ing1*, *ing2*, L*er* (Landsberg *erecta*) and SnRK1.1 overexpressor (*Ler* background) seeds were surface sterilized and plated on medium containing no exogenous nutrients (H$_2$O), 0.5X strength MS (Murashige and Skoog) salts, 0.5X MS + 3 % sucrose or 0.5X MS + 11 % sucrose. *Ing1* mutants have less root growth at both low nutrient conditions (H$_2$O and 0.5X MS), however, when sucrose is present (3 or 11%) root growth is comparable to wildtype. I conclude that *ing1* mutants are hypersensitive to low nutrient conditions. The *ing2* mutant, overall, has slightly decreased root growth under all conditions. This response is most consistent with an overall decrease in root growth potential, rather than a nutrient-sensing defect.

I also examined germination responses of *ing* mutants when 3% sucrose and 6% glucose were present. The rationale for this analysis is that nutrient and/or signal transduction mutants with altered responses to sugar can be easily indentified. For example, mutants that are sugar-insensitive will exhibit higher germination rates than wildtype when 6% glucose is present (Laby et al., 2000). My results indicate that *ing1* mutant seed are less sensitive to sugar in this assay (Figure 3.4). Specifically, *ing1* mutant seed germinate faster in the presence of both 3% sucrose and 6% glucose as compared to wildtype seed. In contrast, *ing2* mutant seed are hypersensitive to both 3% sucrose and 6% glucose in the seed germination assays. This suggests that *ing2* mutant seed are sugar hypersensitive, which is the opposite phenotype of the *ing1* mutants. It should be noted that I also compared germination responses of the SnRK1.1 gain of function seed, which exhibited hypersensitivity to glucose in this assay. Together, these data suggest that *ing2* and

49

SnRK1.1 seed share the phenotype of sugar hypersensitivity, which is consistent with an increase in SnRK1.1 sensor function, while *ing1* mutant seed are sugar-insensitive, which is consistent with lack of SnRK1.1 sensor function.

SnRK1.1 gain of function seedlings and plants exhibit a delayed time to senescence. In seedlings, this delayed senescence can be visualized as continuous growth in culture under nutrient deprivation conditions (Baena-Gonzalez et al., 2007). I have performed a similar nutrient deprivation experiment with the *ing* mutants and the SnRK1.1 overexpressor. In this assay, seeds are germinated on agar medium containing 1% sucrose and 0.1X MS. After 2 weeks of growth these seedlings are transferred to liquid medium containing 1% sucrose and allowed to grow for 1 week under low-light. The seedlings are then transferred to medium without any sucrose (0.1X MS). *Ing2* seedlings grown under low-light, low-nutrient and no-carbon conditions showed an early senescence phenotype, which was apparent by the loss of green color (Figure 3.4E). This is the opposite of SnRK1.1 seedlings that remain green in color long after wildtype begins to senesce. Data from *ing1* seedlings were inconclusive.

Response of *ing* Mutants to Abscisic Acid

To determine whether ABA mediated stress signaling is altered in the *ing* mutants, I performed preliminary germination experiments with various concentrations of ABA. For these experiments, seeds were germinated on plates containing 0.5X MS salts + 0-3uM ABA + ultra-pure agar. At higher levels of ABA (2-3 uM), *ing1* mutants exhibited insensitivity to ABA (Figure 3.5A). In addition, *ing1* is also insensitive to the osmolyte mannitol at a concentration of 6% (Figure 3.5B). Preliminary germination experiments on salt-rich media showed that *ing1* is insensitive to increasing concentrations of NaCl (50 mM-100 mM, data not shown). Together, these data suggest that *ing1* is insensitive to several stress signals, which could be indicative of a lack of SnRK1.1 nutrient/stress sensor function.

In contrast, *ing2* does not exhibit an altered response to ABA (Figure 3.5A), nor salt (data not shown). However, *ing2* does show hypersensitivity to 6% mannitol at early germination time points. From this data, I conclude that *ing2* mutants do not have greatly altered responses to

several stress stimuli, however these seedlings are hypersensitive to sugars and mannitol in seed germination assays.
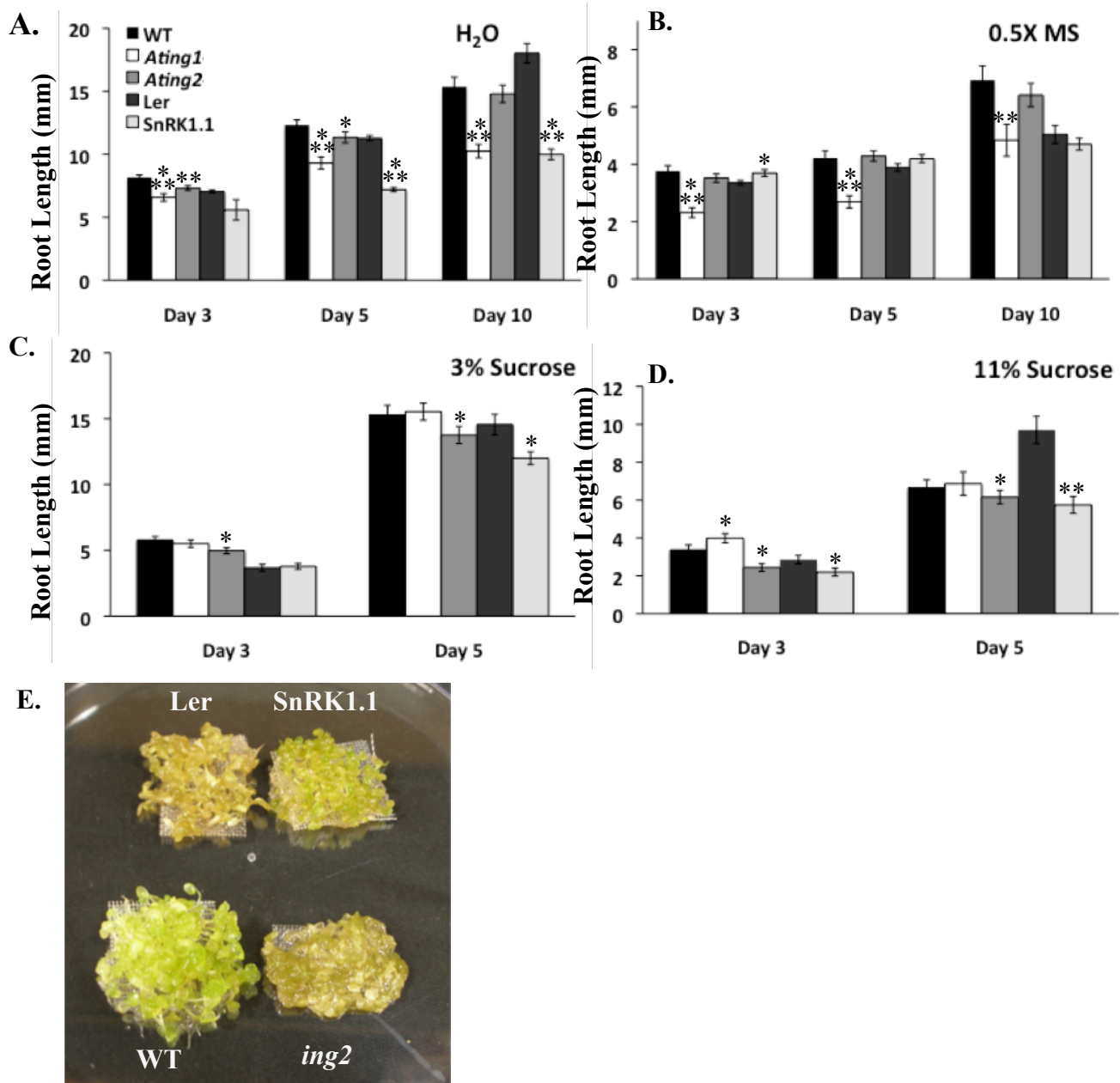
Figure 3.4: *Ing* Mutant Response to Nutrient Status and ABA. WT, mutant and overexpressor seeds were grown on medium containing **A)** water + agar, **B)** 0.5x MS salts + agar, **C)** 0.5X MS salts + 3% sucrose + agar, and **D)** 0.5X MS salts + 11% sucrose + agar. Root length was measured every other day. Bars represent the mean ±SE where n = 40-60, with each replicate containing 10-15 seedlings. .   *** indicates p-value ≤0.0009, ** indicates p-value ≤0.009, and * indicates p-value ≤0.09. E) ing2-, SnRK1.1 overexpressor and respective WT seedlings were grown in limited nutrient medium in liquid culture. Seeds were plated on 0.1X MS + 1% sucrose. 7-day old seedlings were transferred to liquid medium containing 0.1X MS + 1% sucrose. After 2-weeks of growth, the medium was replaced with a 0.1X MS salts medium. Seedlings grown in culture were continuously shaken, under low-light conditions (~10 uE). Ultra-pure agar was utilized in all nutrient related experiments involving agar plates.
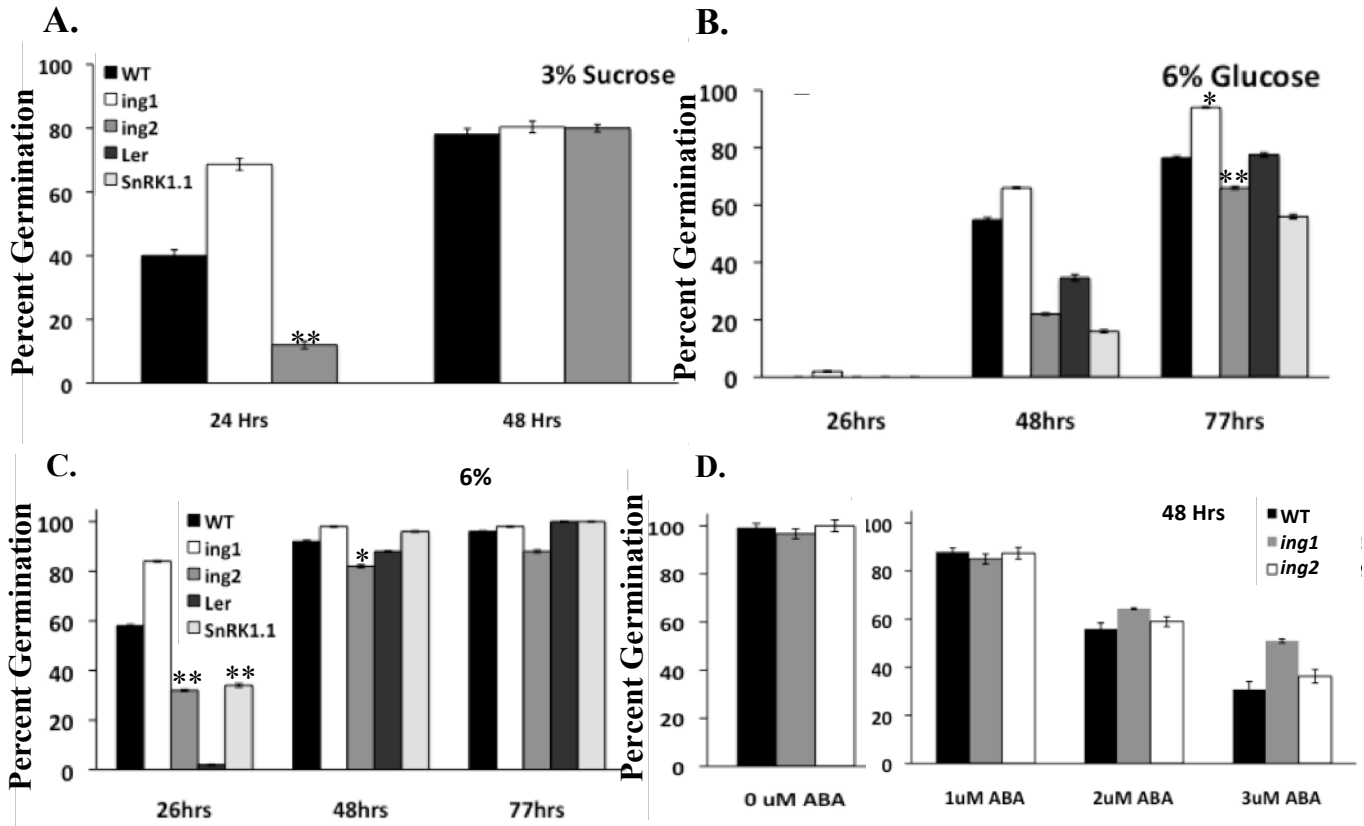
A.

B.

C.

D.

Figure 3.5. Analysis of ABA Response of *ing* Mutant Seeds. WT, *ing1* and *ing2* seeds were allowed to germinate on ultra-pure agar plates containing 0.5X MS and either sucrose, glucose, mannitl or ABA. Seeds were placed at 23°C under continuous light (~60uE). Germination was scored at the indicated time points. **A)** Seeds plated on 0.5X MS salts and 3% sucrose were allowed to germinate on regular long-day, low-light conditions. **B)** Seeds plated on 0.5X MS salts and 6% glucose were allowed to germinate on regular long-day, low-light conditions. **C)** Seeds plated on 0.5X MS salts and 6% mannitol were allowed to germinate on regular long-day, low-light conditions. **D)** Percent germination of WT, *ing1⁻* and *ing2⁻* seeds on 0-3uM ABA at 48 hours. Bars represent the mean ±SE where n = 40-60, with each replicate containing 10-15 seedlings. ** indicates p-value ≤0.004, and * indicates p-value ≤0.04.

Recombinant ING2 protein complexes

Arabiodipsis SnRK1.1 is a master regulator of nutrient response transcriptional programming (Baena-Gonzalez et al., 2007). SnRK1.1 controls the transcriptional activation and repression of a variety of genes involved in metabolism. To determine if INGs interact with SnRK1.1, I performed immunoprecipitation (IP) experiments. In these assays, I incubated recombinant ING2-Xpress (40 kDa) and SnRK1.1-V5 (62 kDa) tagged proteins together and immunoprecipitated the complex with an antibody that recognizes the V5 epitope tag. My results show that ING2-Xpress protein can be immunoprecipitated with an anti-V5 antibody, only if SnRK:V5 is present. This indicates that SnRK1.1-V5 associates with ING2-Xpress protein in these experiments (Figure 3.6 bound, ING2+SnRK lane). When I incubated α–V5 agarose with ING2-Xpress protein alone, as expected, I saw no ING2 protein immunoprecipitation (Figure 3.6 bound, ING2 lane). This control shows that ING2-Xpress is not pulled-down by the anti-V5 agarose alone. I conclude that SnRK1.1 and ING2 recombinant proteins can interact *in vitro*.
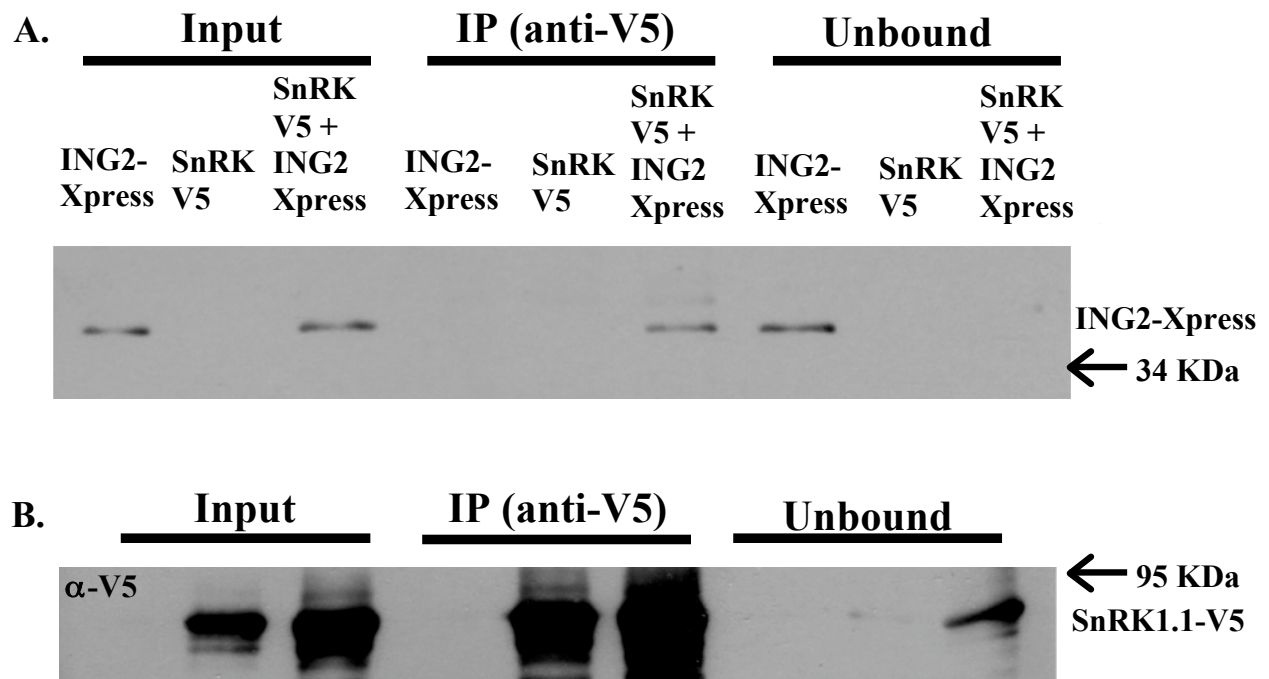
Figure 3.6. Immunoprecipitation (IP) of ING2 and SnRK1.1 *in vitro* complex. A) IP using anti-V5 antibody bound agarose (Invitrogen, USA). Purified ING2-Xpress SnRK1.1-V5 were incubated for 2 hours in IP buffer before transferring to anti-V5 agarose slurry in IP buffer. The first three lanes are "input" fractions of ING2-Xpress, SnRK-V5 and ING2-Xpress + SnRKV5. Lanes 3, 4, 5 are "bound "fraction of ING2-Xpress, SnRKV5, and both proteins incubated with anti-V5 agarose, respectively. Lanes 7, 8 and 9 are the "unbound" fractions showing proteins that did not bind to the anti-V5 agarose. All lanes were probed with anti-Xpress primary antibody for the detection of X-press-tagged proteins. Purified ING2-Xpress protein is present in the IP lane of sample ING2-Xpress + SnRKV5 but not in the IP lane where ING2 protein was incubated with anti-V5 agarose alone. B) Lanes probed with anti-V5 primary antibody for the detection of V5-tagged proteins.

Discussion:

***ING1* and *ING2* are consistently expressed throughout development and alterations in this expression pattern promotes changes in Arabidopsis flowering time**

Microarray data indicates that both *ING1* and *ING2* are constitutively expressed throughout various tissues and throughout development. Both genes are abundantly expressed in tissues ranging from seedlings to mature seeds (Figure 3.1). Along with the amino acid similarity of these proteins, this expression pattern suggests that these genes perform redundant functions and that a knock-out of one or the other would provide minimal phenotypic changes. Indeed, it has been previously shown by Lee *et al*. (2009) that both ING1 and ING2 proteins are nuclear localized and associate with methylated histones. This binding ability might allow ING1 and ING2 to perform similar functions in chromatin remodeling (Lee et al., 2009). The human *ING* genes, however, encode five ING isoforms that provide similar molecular functions (i.e, recruitment of transcription factors to the epigenetic mark H3K4me3; Pena et al., 2006), yet their activity impacts diverse cellular events (cell cycle progression, DNA repair, etc; Soliman and Riobowol, 2007). The diversity of cellular processes impacted by ING function in human cells can be attributed in part to the diversity of protein domains found within ING isoforms (Solimon and Riabowol, 2007). In a similar way, the Arabidopsis INGs are predicted to have different structural domains (Chapter II, Table II). ING2 contains a putative PBR domain, while no such domain can be identified in ING1. This, in part, may account for differences in phenotypes between mutants deficient in these proteins.

Altered *ING* expression causes varied responses to environmental stimuli

Overall, *ing1* mutants exhibit nutrient responses consistent with a lack of function in the SnRK1.1 low nutrient/stressor. Specifically, *ing1* mutant root growth is compromised under low nutrient conditions, in a similar manner as that reported for the SnRK1.1 knockout mutant mutants (Baena-Gonzalez et al., 2007). In addition, *ing1* mutant roots grow similar to wildtype when media is supplemented with glucose, as do SnRK1.1 mutants (Baena-Gonzalez et al., 2007). SnRK1.1 mutants have not been tested for germination responses in the presence of sugars. However, I tested the gain of function SnRK1.1 seed for response to glucose and found that SnRK1.1 overexpressors are hypersensitive to 6% glucose. Thus the *ing1* mutant glucose

56

insensitivity is consistent with a lack of proper sensing of sugars as would occur with a lack of SnRK1.1 sensor function. In addition, a separate SnRK1.1 gain of function line in the Columbia background has recently been reported to have ABA hypersensitivity (Jossier et al., 2009), which fits with my data from the SnRK1.1 (Ler) plants containing sugar hypersensitivity. Since sugars stimulate ABA production, most ABA hypersensitive mutants are also hypersensitive to sugar (Pourtau et al., 2004). Together, these data indicate that the responses I measured in *ing1* mutants could be due to lack of SnRK1.1 function.

Ing2 mutants, in contrast to *ing1* mutants, show nutrient phenotypes that are consistent with SnRK1.1 gain of function at the seed stage (i.e., hypersensitivity to sugar, ABA). Seed hypersensitivity to glucose is shared by both SnRK1.1 gain of function seed and *ing2* seed. These data indicate that SnRK1.1 sensor function could be increased in *ing2* seed. At the seedling stage, however, *ing2* mutants exhibit a general decrease in root growth potential despite the nutrient status, which may indicate an uncoupling of ING2 and SnRK1.1 activity. In addition, nutrient deprivation experiments, where carbon is supplied and then withheld, showed an opposing phenotype between SnRK1.1 gain of function and *ing2* mutants. These data suggest that ING2 may participate in different cellular processes in different developmental stages. However, at the seed stage, it is possible that ING2 acts as a negative regulator of SnRK1.1 activity, as a loss of ING2 function mimicks a gain of SnRK1.1 function.

ING2 recombinant protein interacts with SnRK1.1 in vitro

In vitro, recombinant ING2 and SnRK1.1 protein interact. These findings, in addition to the findings of the nutrient response studies indicated that these two proteins may be involved in similar processes at early developmental stages. For example, it is possible that ING2 protein negatively regulates SnRK1.1 by a physical interaction. This interaction may inhibit SnRK1.1 from binding to other protein partners or possibly inhibit its kinase activity. There is a known negative regulator of SnRK1.1, PRL1 (Pleotropic Regulator Locus1). PRL1 inhibits SnRK1.1 activity by forming a complex with SnRK1.1 and recruiting it to the proteasome for degradation Lee et al., 2008). Although, a role for ING proteins in SnRK1.1 is still speculative, the data presented here is preliminary evidence that there may be many roles for ING proteins in plant cells.

Model of ING impact on SnRK1.1 mediated signaling

Upon consideration of the data presented, it is possible to produce a model of ING and SnRK1.1 interactions (Figure 3.7). ING1 and ING2 proteins have been shown to associate with the epigenetic mark, H3K4me3 (Lee et al., 2009). In mammals, this is coupled with the recruitment of chromatin remodeling complexes, HATs and HDACs (Loewith et al., 2000; Kuzmichev et al., 2002; Vieyra et al. 2002; Shiseki et al., 2003; Pedeux et al., 2005, Doyon et al., 2006). In a similar way, AtING1 could associate with the promoter of SnRK1.1 and subsequently recruit a HAT complex to promote SnRK1.1 gene expression (Figure 3.7A). Alternatively, ING1 could stabilize SnRK1.1 protein or increase it's kinase activity (Figure 3.7B). Both of these models fit my observed data which suggests that ING1 positively affects SnRK1.1 function. Conversely, data from *ing2* mutants indicates that ING2 may be a negative regulator of SnRK1.1 function in seeds. In addition, we know that ING2 interacts with SnRK1.1 *in vitro*. It is possible; therefore, that ING2 could regulate the SnRK1.1 promoter by recruiting an HDAC to repress SnRK1.1 transcription (Figure 3.7C). Alternatively, ING2 could bind to SnRK1.1 and negatively regulate its stability or kinase activity (Figure 3.7D). Unlike ING1, ING2 is predicted to bind PtdInsPs. This interaction could result in the regulation of SnRKactivity as a response to nuclear PtdInsP signaling.
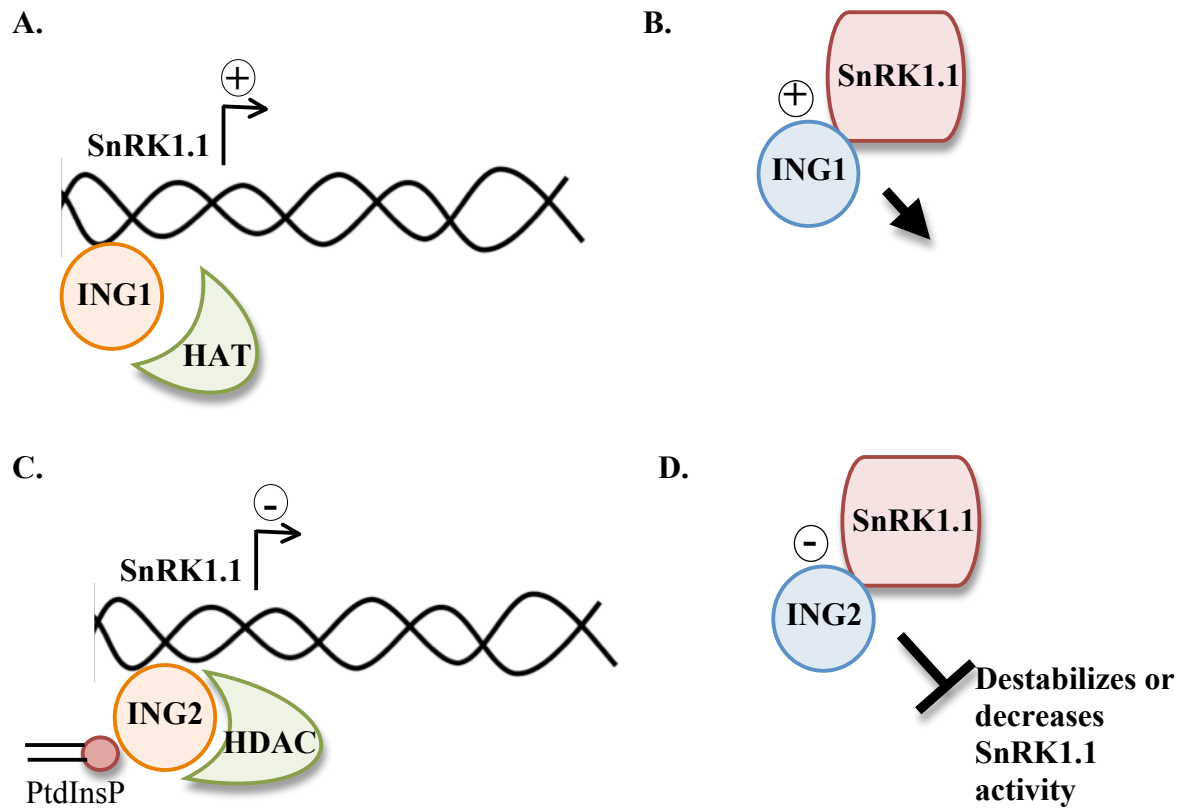
Figure 3.7. Model of ING-SnRK1.1 relationship. A-B) Promotion of SnRK1.1 activity. A) ING1 recruitment of a HAT complex to the promoter of SnRK1.1 activates gene expression. B) ING1 interaction promotes SnRK1.1 activity. C-D) Repression of SnRK1.1 activity. C) ING2 recruitment of an HDAC complex to the promoter of SnRK1.1 represses gene expression. ING2 binding to PtdInsPs. D) ING2 association with SnRK1.1 inhibits binding of SnRK1.1 protein partners, preventing SnRK1.1 from performing its kinase activity.

Chapter IV: Future Directions

Future Directions

The work presented was performed in an effort to understand the relevance of ING protein function in plant growth and development. I have shown that *ing* mutants exhibit alterations in time to flowering and responses to nutrient and stress signals. In addition, recombinant ING2 protein interacts *in vitro* with the sugar/stress sensor, SnRK1.1. To further our understanding of ING impact on plant physiology, I would recommend a series of experiments to address the following questions:

1. Does ING function affect SnRK1.1 activity and if so, how is this alteration in activity regulated?
2. With what protein complexes do ING proteins interact at the chromatin level?
3. What are the physiological consequences of complete ING loss-of-function or the concurrent loss of ING and SnRK function in Arabidopsis plants?

As discussed in Chapter III, *ing1* and *ing2* mutants displayed different phenotypes when grown on varying concentrations of carbon nutrient. I suggested that the *ing1* mutant response could be attributed to a lack of SnRK1.1 function. To investigate this hypothesis, I would measure SnRK1.1 transcript and protein levels in the *ing1* mutant. In addition, I would perform a SnRK1.1 kinase assay to establish the activity of this protein in *ing* mutants. Elitsa Ananieva has previously performed these experiments with *5ptase13* mutants. Her results revealed the role of 5PTase 13 in the positive regulation of SnRK1.1 in Arabidopsis (Ananieva et al., 2008). In addition, to identify whether this regulation occurs at the level of chromatin, I would perform chromatin immunoprecipitation (ChIP) assays. This type of experiment identifies what promoters are occupied by ING protein. It is also possible to determine whether ING1 interacts with chromatin remodeling complexes by initiating a Yeast-2-Hybrid. Although this type of technique can yield false negatives and positives, it is an unbiased means to determining candidate protein interactors. This type of analysis would dramatically broaden the scope of this project, as it would reveal the ING protein partners, as well as ING promoter targets.

To further understand the impacts of ING function on plant physiology, I would generate double mutants of *ing1* and *ing2*. A homozygous line of the double mutant will be deficient in both ING1 and ING2. Such a mutation may reveal other phenotypes and expose other potential roles for ING protein in Arabidopsis physiology. In addition, a double mutant deficient in *ING* and *SnRK1.1* would be useful in elucidating the role of ING involvement in SnRK1.1 function. Previously, a SnRK1.1 mutant was generated using RNAi (Beana-Gonzalez et al., 2007). The development of an *ing-snrk1.1* double mutant would require the use of RNAi in an *ing* mutant background.

REFERENCES

Aasland R, Gibson TJ, Stewart AF (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation.Trends Biochem Sci 20: 56-59

Adams-Cioaba MA, Min J (2009) Structure and function of histone methylation binding proteins. Biochem Cell Biol. 87: 93-105

Aida M, Beis D, Heidstra R, Willemsen V, Blilou I, Galinha C, Nussaume L, Noh YS, Amasino R, Scheres B. (2004) The PLETHORA genes mediate patterning of the Arabidopsis root stem cell niche. Cell.119: 109-120

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301: 653–657196–203

Alvarez-Venegas R, Pien S, Sadder M, Witmer X, Grossniklaus U, Avramova Z (2003) ATX-1, an Arabidopsis homolog of trithorax, activates flower homeotic genes. Curr Biol.13: 627-637.

Andersson MX, Kourtchenko O, Dangl JL, Mackey D, Ellerstrom M (2006) Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*. Plant J 47: 947–959

Ananieva EA, Gillaspy GE, Ely A, Burnette RN, Erickson FL. (2008) Interaction of the WD40 domain of a myoinositol polyphosphate 5-phosphatase with SnRK1 links inositol, sugar, and stress signaling. Plant Physiol. 148:1868-1882

Astle MV, Horan KA, Ooms LM, Mitchell CA. (2007) The inositol polyphosphate 5-phosphatases: traffic controllers, waistline watchers and tumour suppressors? Biochem Soc Symp: 161-81

Baena-González E, Rolland F, Thevelein JM, Sheen J. A central integrator of transcription networks in plant stress and energy signaling (2007) Nature 448: 938-942

Baena-González E, Sheen J. (2008) Converent energy and stress signaling. Trends Plant Sci. 13: 474-482

Bastola, D.R., Pethe, V.V. and Winicov, I. (1998) Alfin1, a novel zinc-finger protein in alfalfa roots that binds to promoter elements in the salt-inducible MsPRP2 gene. Plant Mol. Biol. 38: 1123–1135.

Berdy SE, Kudla J, Gruissem W, Gillaspy GE (2001) Molecular characterization of At5PTase1, an inositol phosphatase capable of terminating inositol trisphosphate signaling. Plant Physiol 126: 801-810

Berridge MJ (2009) Inositol trisphosphate and calcium signalling mechanisms.Biochim Biophys Acta. 1793: 933-940.

Bienz M (2006) The PHD finger, a nuclear protein-interaction domain. Trends Biochem Sci. 31: 35-40

Bunney TD, Watkins PA, Beven AF, Shaw PJ, Hernandez LE, Lomonossoff GP, Shanks M, Peart J, Drobak BK. (2000) Association of phosphatidylinositol 3-kinase with nuclear transcription sites in higher plants. Plant Cell. 12:1679-1688.

Burnette RN, Gunesekera BM, Gillaspy GE (2003) An Arabidopsis inositol 5-phosphatase gain-of-function alters abscisic acid signaling. Plant Physiol. 132: 1011-1019

Cai L, Li X, Zheng S, Wang Y, Wang Y, Li H, Yang J, Sun J. (2009)Inhibitor of growth 4 is involved in melanomagenesis and induces growth suppression and apoptosis in melanoma cell line M14.

Capitani S, Cocco L, Maraldi NM, Mazzotti G, Barnabei O, Manzoli FA. (1991) Inositol lipid phosphorylation in the cell nucleus. Adv Enzyme Regul. 31:399-416.

Champagne KS, Saksouk N, Peña PV, Johnson K, Ullah M, Yang XJ, Côté J, Kutateladze TG (2008)The crystal structure of the ING5 PHD finger in complex with an H3K4me3 histone peptide. Proteins.72: 1371-1376

Cocco L, Capitani S, Maraldi NM, Mazzotti G, Barnabei O, Gilmour RS, Manzoli FA. (1996) Inositol lipid cycle and autonomous nuclear signalling. Adv Enzyme Regul. 36:101-114.

Cocco L, Faenza I, Follo MY, Billi AM, Ramazzotti G, Papa V, Martelli AM, Manzoli L (2009) Nuclear inositides: PI-PLC signaling in cell growth, differentiation and pathology. Adv Enzyme Regul. 49: 2-10

Coles AH, Liang H, Zhu Z, Marfella CG, Kang J, Imbalzano AN, Jones SN (2007) Deletion of p37Ing1 in mice reveals a p53-independent role for Ing1 in the suppression of cell proliferation, apoptosis, and tumorigenesis. Cancer Res. 67 2054–2061

de la Paz Sanchez M, Gutierrez C (2009)Arabidopsis ORC1 is a PHD-containing H3K4me3 effector that regulates transcription. PNAS 106: 2065-2070

Dello Ioio R, Linhares FS, Scacchi E, Casamitjana-Martinez E, Heidstra R, Costantino P, Sabatini S. (2007) Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation.Curr Biol. 17: 678-682

Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. Annu Rev Biochem. 78: 399-434

DeWald DB, Torabinejad J, Jones CA, Shope JC, Cangelosi AR, Thompson JE, Prestwich GD, Hama H (2001) Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed Arabidopsis. Plant Physiol 126: 759–769

Ding Z, Gillespie LL, Paterno GD (2003) Human MI-ER1 alpha and beta function as transcriptional repressors by recruitment of histone deacetylase 1 to their conserved ELM2 domain. Mol Cell Biol. 23: 250-258

Doyon Y, Cayrou C, Ullah M, Landry AJ, Côté V, Selleck W, Lane WS, Tan S, Yang XJ, Côté J (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation require for genome expression and perpetuation. Mol Cell 21: 51-64

Dul BE, Walworth NC (2007) The plant homeodomain fingers of fission yeast Msc1 exhibit E3 ubiquitin ligase activity. J Biol Chem. 282:18397-18406

Erneux C, Govaerts C, Communi D, Pesesse X. (1998) The diversity and possible functions of the inositol polyphosphate 5-phosphatases. Biochim Biophys Acta 1436:185-99

Eberharter A, Becker PB (2002) Histone acetylation: a switch between repressive and permissive chromatin. EMBO Rep. 3: 224-229

Feil R (2008) Epigenetics, an emerging discipline with broad implications. C R Biol. 331: 837-843

Fischle W, Wang Y, Allis CD (2003) Histone and chromatin cross-talk. Curr Opin Cell 15: 172-183

Fletcher TM, Hansen JC (1996) The nucleosomal array: structure/function relationships. Crit Rev Eukaryot Gene Expr. 6:149-88

Garcia-Dominguez M, March-Diaz R, Reyes JC (2008) The PHD domain of plant PIAS proteins mediates sumoylation of bromodomain GTE proteins..J Biol Chem. 283: 21469-21477

Garkavtsev I, Kazarov A, Gudkov A, Riabowol K (1996) Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. Nat Genet 14: 415-420

Gozani O, Karuman P, Jones DR, Ivanov D, Cha J, Lugovskoy AA, Baird CL, Zhu H, Field SJ, Lessnick SL, Villasenor J, Mehrotra B, Chen J, Rao VR, Brugge JS, Ferguson CG, Payrastre B, Myszka DG, Cantley LC, Wagner G, Divecha N, Prestwich GD, Yuan J (2003) The PHD finger of the chromatin- associated protein ING2 function as a nuclear phosphoinositide receptor. Cell 114: 99-111

Gunesekera B, Torabinejad J, Robinson J, Gillaspy GE (2007) Inositol polyphosphate 5-

phosphatases 1 and 2 are required for regulating seedling growth. Plant Physiol **143:** 1408–1417

He GH, Helbing CC, Wagner MJ, Sensen CW, Riabowol K (2005) Phylogenetic analysis of the ING family of PHD finger proteins. Mol Biol Evol 22:104-116

Ito T, Nagata N, Yoshiba Y, Ohme-Takagi M, Ma H, Shinozaki K (2007) Arabidopsis MALE STERILITY1 encodes a PHD-type transcription factor and regulates pollen and tapetum development. Plant Cell. 19: 3549-62

Irvine, RF (2003) Nuclear lipid signaling. Nat Rev Mol Cell Biol 4: 349-360

Jenuwein T, Allis CD. (2001) Translating the histone code. Science. 239: 1074-1080
    Kaadige and ayer

Jossier M, Bouly JP, Meimoun P, Arjmand A, Lessard P, Hawley S, Grahame Hardie D, Thomas M. SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in *Arabidopsis thaliana*. Plant J. 59: 316-328

Kumamoto K, Spillare EA, Fujita K, Horikawa I, Yamashita T, Appella E, Nagashima M, Takenoshita S, Yokota J, Harris CC. (2008) Nutlin-3a activates p53 to both down-regulate inhibitor of growth 2 and up-regulate mir-34a, mir-34b, and mir-34c expression, and induce senescence.Cancer Res. 68: 3193-3203

Kumar P, Miller AI, Polverini PJ (2004) p38 MAPK mediates gamma-irradiation-induced endothelial cell apoptosis, and vascular endothelial growth factor protects endothelial cells through the phosphoinositide 3- kinase-Akt-Bcl-2 pathway. J Biol Chem 279: 43352-43360

Kuzmichev A, Zhang Y, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33(ING1). Mol Cell Biol 22: 835-848

Laby RJ, Kincaid S, Kim D, Gibson S (2000) 1The Arabidopsis sugar-insensitive mutants sis4 and sis5 are defective in abscisic acid synthesis and response. Plant J. 23: 587-596

Lee WY, Lee D, Chung WI, Kwon CS. (2009) Arabidopsis ING and Alfin1-like protein families localize to the nucleus and bind to H3K4me3/2 via plant homeodomain fingers. Plant J. 58:511-524

Lee Y, Kim YW, Jeon BW, Park KY, Suh SJ, Seo J, Kwak JM, Martinoia E, Hwang I (2007) Phosphatidylinositol 4,5-bisphosphate is important for stomatal opening. Plant J **5:** 803– 816

Lee JH, Terzaghi W, Gusmaroli G, Charron JB, Yoon HJ, Chen H, He YJ, Xiong Y,

Deng XW (2008) Characterization of Arabidopsis and rice DWD proteins and their roles as substrate receptors for CUL4-RING E3 ubiquitin ligases. Plant Cell 20: 152–167

Leibfried A, To JP, Busch W, Stehling S, Kehle A, Demar M, Kieber JJ, Lohmann JU (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature. 438:1172-1175

Li H, Ilin S, Wang W, Duncan EM, Wysocka J, Allis CD, Patel DJ (2006) Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. 442: 91-95

Liu JX, Srivastava R, Che P, Howell SH (2008) **S**alt stress signaling in *Arabidopsis thaliana* involves a membrane-bound transcription factor AtbZIP17 as a signal transducer. Plant Signal Behav. 3: 56–57

Loewith R, Meijer M, Lees-Miller P, Riabowol K, Young D (2000) Three yeast proteins related to the human candidate tumour suppressor p33(ING1) are associated with histone acetyltransferase activities. Mol. Cell. Biol. 20, 3807–3816

Loidl P. (2004) A plant dialect of the histone language. Trends Plant Sci. 9:84-90

Long JA, Woody S, Poethig S, Meyerowitz EM, Barton MK (2002) Transformation of shoots into roots in *Arabidopsis* embryos mutant at the *TOPLESS* locus. *Development.* 129: 2797–2806

Maraldi, N. "A Lipid-based Code in Nuclear Signalling." The Codes of Life: The Rules of Macroevolution. Marcello Barbieri, Jesper Hoffmeyer. Springer: Netherlands, 2008. 207-221

Martelli AM, Cataldi A, Manzoli L, Billi AM, Rubbini S, Gilmour RS, Cocco L. (1995) Inositides in nuclei of Friend cells: changes of polyphosphoinositide and diacylglycerol levels accompany cell differentiation. Cell Signal. 7:53-56

Martin DG, Baetz K, Shi X, Walter KL, MacDonald VE, Wlodarski MJ, Gozani O, Hieter P, Howe L. (2006) The Yng1p plant homeodomain finger is a methyl-histone binding module that recognizes lysine 4-methylated histone H3. Mol Cell Biol. 21:7871-7879

Matthews AG, Kuo AJ, Ramón-Maiques S, Han S, Champagne KS, Ivanov D, Gallardo M, Carney D, Cheung P, Ciccone DN, Walter KL, Utz PJ, Shi Y, Kutateladze TG, Yang W, Gozani O, Oettinger MA (2007) RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination. Nature. 450: 1106-1110

Meijer HJ, Munnik T (2003) Phospholipid-based signaling in plants. Annu Rev Plant Biol. 54: 265-306

Mellor J (2006) It takes a PHD to read the histone code. Cell. 126: 22-24

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM (2009) Sumoylation of ABI5 by the Arabidopsis SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. PNAS. 106: 5418-5423

Nagashima M, Shiseki M, Miura K, Hagiwara K, Linke SP, Pedeux R, Wang XW, Yokota J, Riabowol K, Harris CC (2001) DNA damage-inducible gene p33ING2 negatively regulates cell proliferation through acetylation of p53. PNAS. 98: 9671-9676

Noh B, Lee SH, Kim HJ, Yi G, Shin EA, Lee M, Jung KJ, Doyle MR, Amasino RM, Noh YS (2004) Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. Plant Cel. 16:2601-2613

Ortega X, Perez LM (2001) Participation of the phosphoinositide metabolism in the hypersensitive response of Citrus limon against Alternaria alternata. Biol Res 34: 43-50

Palacios A, Muñoz IG, Pantoja-Uceda D, Marcaida MJ, Torres D, Martín-García JM, Luque I, Montoya G, Blanco FJ (2008) Molecular basis of histone H3K4me3 recognition by ING4. J Biol Chem. 283: 15956-15964

Pedeux R, Sengupta S, Shen JC, Demidov ON, Saito S, Onogi H, Kumamoto K, Wincovitch S, Garfield SH, McMenamin M, Nagashima M, Grossman SR, Appella E, Harris CC (2005) ING2 regulates the onset of replicative senescence by induction of p300-dependent p53 acetylation. Mol Cell Biol 25: 6639-6648

Peña PV, Davrazou F, Shi X, Walter KL, Verkhusha VV, Gozani O, Zhao R, Kutateladze TG (2006) Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. Nature 442: 100-103

Peng M, Cui Y, Bi YM, Rothstein SJ (2006) AtMBD9: a protein with a methyl-CpG-binding domain regulates flowering time and shoot branching in Arabidopsis Plant J. 46: 282-296

Perera IY, Heilmann I, Chang SC, Boss WF, Kaufman PB (2001) A role for inositol 1,4,5-trisphosphate in gravitropic signaling and the retention of cold-perceived gravistimulation of oat shoot pulvini. Plant Physiol 125: 1499–1507

Perera IY, Hung CY, Brady S, Muday GK, Boss WF (2006) A universal role for inositol 1,4,5-trisphosphate-mediated signaling in plant gravitropism. Plant Physiol 140: 746–760inositol 1,4,5-trisphosphate independent of abscisic acid in Arabidopsis cell culture. Plant Cell Physiol 42: 214–222

Pfluger J, Wagner D (2007) Histone modification and dynamic regulation of genome

accessibility in plants. Curr Opin Plant Biol 10: 645-652

Pien S, Fleury D, Mylne JS, Crevillen P, Inzé D, Avramova Z, Dean C, Grossniklausa U (2008) ARABIDOPSIS TRITHORAX1 Dynamically Regulates FLOWERING LOCUS C Activation via Histone 3 Lysine 4 Trimethylation. Plant Cell. 20: 580–588.

Polge C, Thomas M. (2006) SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? Trends Plant Sci. 12:20-28

Pourtau N, Mares M, Purdy S, Quentin N, Ruel A, Wingler A (2004) Interactions of abscisic acid and sugar signalling in the regulation of leaf senescence. Planta vol. 219: 765-772

Russell M, Berardi P, Gong W, Riabowol K (2006) Grow-ING, Age-ING and Die-ING: ING proteins ling cancer, senescence and apoptosis. Exp Cell Res 312: 951-961

Saiga S, Furumizu C, Yokoyama R, Kurata T, Sato S, Kato T, Tabata S, Suzuki M, Komeda Y (2008) The Arabidopsis OBERON1 and OBERON2 genes encode plant homeodomain finger proteins and are required for apical meristem maintenance. Development. 135: 1751-1959

Saleh A, Al-Abdallat A, Ndamukong I, Alvarez-Venegas R, Avramova Z. (2007) The Arabidopsis homologs of trithorax (ATX1) and enhancer of zeste (CLF) establish 'bivalent chromatin marks' at the silent AGAMOUS locus Nucleic Acids Res. 35: 6290-6296

Sanchez JP, Chua NH (2001) *Arabidopsis* plc1 is required for secondary responses to abscisic acid signals. Plant Cell **13:** 1143–1154

Scheel H, Hofmann K (2003) No evidence for PHD fingers as ubiquitin ligases.Trends Cell Biol. 13: 285-287

Schindler U, Beckmann H, Cashmore AR (1993) HAT3.1, a novel Arabidopsis homeodomain protein 16 containing a conserved cystein-rich region. Plant J 4: 137-150

Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, Carney D, Peña P, Lan F, Kaadige MR, Lacoste N, Cayrou C, Davrazou F, Saha A, Cairns BR, Ayer DE, Kutateladze TG, Shi Y, Côté J, Chua KF, Gozani O (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature 442: 96-99

Shirra MK, Rogers SE, Alexander DE, Arndt KM. (2005) The Snf1 protein kinase and Sit4 protein phosphatase have opposing functions in regulating TATA-binding protein association with the Saccharomyces cerevisiae INO1 promoter. Genetics. 169:1957-1972

Shiseki M, Nagashima M, Pedeux RM, Kitahama-Shiseki M, Miura K, Okamura S,

Onogi H, Higashimoto Y, Appella E, Yokota J, Harris CC (2003) p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity. Cancer Res. 63, 2373–2378

Soliman MA, Riabowol K (2007) After a decade of study-ING, a PHD for a versatile family of proteins. Tends Biochem Sci 32: 509-519

Stevenson JM, Perera IY, Heilmann I I, Persson S, Boss WF. (2000) Inositol signaling and plant growth. Trends Plant Sci. 5:357

Szemenyei H, Hannon M, Long JA (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science. 319:1384-1386

Takahashi S, Katagiri T, Hirayama T, Yamaguchi-Shinozaki K, Shinozaki K (2001) Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in Arabidopsis cell culture. Plant Cell Physiol 42: 214–222

Taverna SD, Ilin S, Rogers RS, Tanny JC, Lavender H, Li H, Baker L, Boyle J, Blair LP, Chait BT, Patel DJ, Aitchison JD, Tackett AJ, Allis CD (2006)Yng1 PHD finger binding to H3 trimethylated at K4 promotes NuA3 HAT activity at K14 of H3 and transcription at a subset of targeted ORFs. Mol Cell. 24: 785-796

Unoki M, Kumamoto K, Robles AI, Shen JC, Zheng ZM, Harris CC (2008)A novel ING2 isoform, ING2b, synergizes with ING2a to prevent cell cycle arrest and apoptosis. FEBS Lett. 582: 3868-3874

van Ingen H, van Schaik FM, Wienk H, Ballering J, Rehmann H, Dechesne AC, Kruijzer JA, Liskamp RM, Timmers HT, Boelens R (2008) Structural insight into the recognition of the H3K4me3 mark by the TFIID subunit TAF3. Structure. 16: 1245-1256

Vieyra D, Loewith R, Scott M, Bonnefin P, Boisvert FM, Cheema P, Pastyryeva S, Meijer M, Johnston RN, Bazett-Jones DP, McMahon S, Cole MD, Young D, Riabowol K (2002) Human ING1 proteins differentially regulate histone acetylation. J Biol Chem 277: 29832-29839

Villasenor J, Benoist C, Mathis D (2005) AIRE and APECED: molecular insights into an autoimmune disease. Immunol. Rev. 204, 156–164

Wilson ZA, Morroll SM, Dawson J, Swarup R, Tighe PJ (2001) The Arabidopsis MALE STERILITY1 (MS1) gene is a transcriptional regulator of male gametogenesis, with homology to the PHD-finger family of transcription factors. Plant J. 28: 27-39

Winicov, I. (2000) Alfin1 transcription factor overexpression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. Planta, 210, 416– 422

Winicov II, Bastola DR (1999) Transgenic overexpression of the transcription factor
Alfin1 enhances expression of the endogenous MsPRP2 gene in alfalfa and improves
salinity tolerance of the plants. Plant Physiol. 120, 473–480

Winicov I, Valliyodan B, Xue L, Hoober JK (2004) The MsPRP2 promoter enables
strong heterologous gene expression in a root-specific manner and is enhanced by
overexpression of Alfin 1 Planta. 219: 925-935

**Wysocka J, Swigut T, Xiao H, Milne TA, Kwon SY, Landry J, Kauer M, Tackett AJ,
Chait BT, Badenhorst P, Wu C, Allis CD** (2006) A PHD finger of NURF couples histone
H3 lysine 4 trimethylation with chromatin remodelling. Nature. 442: 86-90

Xia HJ, Brearley C, Elge S, Kaplan B, Fromm H, Mueller-Roeber B. (2003)
Arabidopsis inositol polyphosphate 6-/3-kinase is a nuclear protein that complements a
yeast mutant lacking a functional ArgR-Mcm1 transcription complex. Plant Cell. 15:229-
263

Yang X, Makaroff CA, Ma H (2003) The Arabidopsis MALE MEIOCYTE DEATH1
Gene Encodes a PHD-Finger Protein That Is Required for Male Meiosis. Plant Cel. 15:
1281-1295

Yaish MW, Peng M, Rothstein SJ (2008) AtMBD9 modulates Arabidopsis
development through the dual epigenetic pathways of DNA methylation and histone
acetylation. Plant J. 59: 123-135

**Zhang H, Rider SD Jr, Henderson JT, Fountain M, Chuang K, Kandachar V,
Simons A, Edenberg HJ, Romero-Severson J, Muir WM, Ogas J.** (2008) The CHD3
remodeler PICKLE promotes trimethylation of histone H3 lysine 27. J Biol Chem. 283:
22637-2248.

**Zhao Q, Rank G, Tan YT, Li H, Moritz RL, Simpson RJ, Cerruti L, Curtis DJ, Patel DJ,
Allis CD, Cunningham JM, Jane SM** (2009) PRMT5-mediated methylation of histone
H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. Nat
Struct Mol Biol. 16:304-311