Molecular Characterization of *Arabidopsis thaliana* Snf1-Related Kinase 1

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ABSTRACT

Plants have molecular mechanisms for nutrient-related stress responses; however, their exact regulation remains unclear. For example, the integral *myo*-inositol (inositol) signal transduction pathway allows *Arabidopsis thaliana* to sense and respond to changes in environmental stimuli, such as water, light availability, and nutrient stress. The inositol signaling pathway relies on dynamic changes in second messenger levels of inositol(1,4,5)P_3 ($InsP_3$) and is regulated by *myo*-inositol polyphosphate 5-phosphatases (5PTases). The 5PTses keep balance between $InsP_3$ signal transduction and termination. Previous work has identified the Sucrose non-fermenting (Snf) 1-related kinase (SnRK1.1) as a binding partner to 5PTase13, a potential $InsP_3$ regulator, and a novel protein called P80, a predicted component of the Cullin4 (CUL4) E3 Ubiquitin ligase complex. In plants, SnRK1.1 is a central integrator of metabolism, stress responses, and developmental signals. Moreover, SnRK1.1 is conserved with the eukaryotic AMP-activated protein (AMPK) and Snf1 kinases—enzymes fundamental to transcriptional regulation and metabolic balance. Studying SnRK1.1 regulation may reveal mechanisms for agricultural sustainability and may offer valuable links to understanding metabolic diseases and lifespan in humans.

Therefore, the research presented here centered on characterizing the regulation of SnRK1 gene expression and steady-state protein levels in plants. I show developmental and nutrient-related regulation of spatial expression patterns of SnRK1 genes and SnRK1.1 protein. Further, I present a model for regulation of SnRK1.1 protein stability *in vivo* based on SnRK1.1

steady-state protein levels in *p80* and *cul4 co-suppressed (cs)* mutants. My results indicate SnRK1.1 regulation is dynamic, and dependent on the timing of particular cues from development and the environment.

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LIST OF ABBREVIATIONS

λPP	lamba phosphatase enzyme
5PTases	*myo*-inositol polyphosphate 5-phosphatase
ABA	abscisic acid
AMPK	AMP-activated protein kinase
AREBP	ABA-response element-binding protein
CUL4	cullin 4 E3 ubiquitin ligase
DWD	aspartic acid-tryptophan-aspartic acid conserved sequence
GFP	green fluorescent protein
GUS	β-glucuronidase
IMAC	ion-metal affinity chromatography
Ins	*myo*-inositol
InsP	inositol phosphate
Ins(1,4,5)P_3	*myo*-inositol-(1,4,5)-triphosphate
IP	immunoprecipitation
LB	luria broth
NPA	naphthyphthalamic acid
P80	arabidopsis homolog of the human p80 gene/protein
PBP1	pinoid binding protein 1
PLC	phospholipase C
PtdIns	phosphatidylinositol
PTM	post-translational modification
SEC	size exclusion chromatography

Snf1	sucrose non-fermenting 1
SnRK1	snf-related kinase 1
WD	tryptophan-aspartic acid dipeptide

CHAPTER I

LITERATURE REVIEW

Introduction

One important aspect of living organisms is their ability to survive. Responses and adaptation to the environment can not only be necessary for immediate survival, but can be essential for reproduction and the passage of genetic material to progeny. Plants, static in nature, have adapted survival strategies independent of locomotion (Dehesh and Liu, 2010; Walley and Dehesh, 2010). Thus, plants have unique response mechanisms for defense and nutrient acquisition that are highly dependent on their immediate environment. Understanding the mechanism and regulation of nutrient and stress responses in *Arabidopsis thaliana* will help elucidate novel ways to increase plant fitness and survival (Baena-Gonzalez et al., 2007; Smeekens et al., 2010).

Overview of Inositol Signaling

Inositol signaling is integral to all eukaryotes, and functions through changes in inositol phosphate (InsP) levels (Munnik and Testerink, 2009). InsPs are derived from a carbon ring polyol called *myo*-inositol, and have unique phosphorylation patterns on ring hydroxyl groups (Michell, 2008). In eukaryotes, InsPs are important second messengers in a vast array of biological responses (Michell, 2008; Munnik and Testerink, 2009; Tsui and York, 2010).

In plants, the inositol signaling pathway is essential in how plants respond to their environment (Munnik et al., 1998). For example, the inositol transduction cascade allows *Arabidopsis thaliana* to sense and respond to changes in water (Munnik and Vermeer, 2010) and light availability (Salinas-Mondragon et al., 2010), as well as stress (Darwish et al., 2009; Meijer

et al., 2001; Munnik and Vermeer, 2010; Zonia and Munnik, 2006). Although the exact mechanism of inositol signaling in plants remains unclear, there is speculation on how inositol signaling may function in plants (Krinke et al., 2007; Munnik and Testerink, 2009). Briefly, inositol signal transduction begins with an environmental signal, such as water loss, change in light availability, or nutrient stress, that activates phospholipase C (PLC) and, in turn, generates inositol (1,4,5) P_3 ($InsP_3$) from phosphatidylinositol (4,5)P_2 ($PtdInsP_2$) (Figure 1). $InsP_3$ most likely interacts with intracellular receptors to release Ca^{2+} from intracellular stores, thus, initiating calcium-dependent, downstream biological responses (Munnik et al., 1998; Trewavas and Knight, 1994). As $InsP_3$ is catalyzed to $InsP_2$, intracellular calcium concentration decreases (Trewavas and Knight, 1994), and signal transduction of biological responses is terminated (Figure 1).

Similar to other eukaryotic species, plants regulate the amount of $InsP_3$ by a family of enzymes called the *myo*-inositol polyphosphate 5-phosphatases (5PTases). Specifically, the 5PTases catalyze the removal of a 5-phosphate from $InsP_3$, resulting in $InsP_2$, and also catalyze the reaction converting $PtdInsP_2$ to phosphatidylinositol (4)P (PtdInsP) (Ooms et al., 2009; Trewavas and Knight, 1994) (Figure 1).

The Role of the 5PTases in *Arabidopsis thaliana*

The 5PTases in Arabidopsis have been characterized more than in any other plant species (Berdy et al., 2001; Burnette et al., 2003; Carland and Nelson, 2004; Chen et al., 2008; Ercetin et al., 2008; Ercetin and Gillaspy, 2004; Gunesekera et al., 2007; Zhong et al., 2004). In fact, 5PTases from other plant species still remain poorly understood and characterized (DePass et al., 2001).

In the Arabidopsis genome there are fifteen distinct 5PTase genes that encode for 15 different 5PTase proteins (Berdy et al., 2001). Phylogenetically, they are divided into Group A (5PTase 1-11) and Group B (5PTase 12-15). Unlike Group A 5PTases, Group B 5PTases contain unique N-terminal WD40 repeats (Zhong and Ye, 2004). Briefly, the WD40 repeat has been implicated as a scaffold for protein:protein interactions in many eukaryotic processes such as signal transduction, pre-mRNA processing, and cell cycle control mechanisms (Li and Roberts, 2001; Smith, 2008; Smith et al., 1999). Structurally, the WD40 domain adopts a β-propeller conformation and in proteins exists as several repeated units (Smith et al., 1999). The name stems from the conserved tryptophan-aspartic acid (W-D) dipeptide at the end of an approximately 40 amino acid protein sequence (Smith et al., 1999). The presence of the WD40 repeat in Group B 5PTases may implicate these proteins as important molecules in cell-signaling mechanisms necessary for protein:protein interactions.

The 5PTase enzymes are important for many aspects of plant growth and development. For example Gunesekera et al. (2007) found that both 5PTase1 (At1g34120) and 5PTase2 (At4g18010) have roles in seed germination and early seed development. Seedlings with mutations in the 5PTase1 and 5PTase2 genes germinated faster than wildtype seedlings. Moreover, the mutant seedlings displayed an increase in $InsP_3$ levels and a decrease in PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 levels (Gunesekera et al., 2007). Other work showed 5PTase11 (At1g47510) is also essential for seedling growth, as *5ptase11* mutants germinate slower when compared to wildtype seedlings, and have increased levels of $InsP_3$ and an $InsP_2$ species that may be Ins(4,5)P_2 (Ercetin et al., 2008). Together, these data suggest that 5PTase1, 5PTase2, and 5PTase11 are important for the regulation of $InsP_3$ in seed germination and early seedling development. Interestingly, several groups reported 5PTase13 plays a role in auxin and light

signaling (Chen et al., 2008; Lin et al., 2005). Lin et al. (2005) found that *5ptase13* mutants had deficiencies in the expression of auxin-regulated genes and levels of auxin, suggesting that 5PTase13 is important for auxin homeostasis. Experiments by Chen et al. (2008) suggest that 5PTase13 plays a role in Phototropin1 signaling via crosstalk between 5PTase13 and PHOT1 (Chen et al., 2008). Further, 5PTases have also been implicated in mechanisms regulating the vascular pattern of cotyledons (5PTase6) (Carland and Nelson, 2004). Given that the 5PTases are important for many aspects of plant life, a better understanding of these enzymes will exploit how plants respond to environmental cues, like nutrient availability and stress.

Using the yeast two-hybrid (Y2H) system, the Gillaspy lab identified the Snf-Related Kinase 1 (SnRK1.1), a known nutrient, energy, and stress sensor (Coello et al., 2011; Halford et al., 2003; Jossier et al., 2009), as a binding partner to the WD40 repeats of one particular 5PTase, 5PTase13 (Ananieva et al., 2008). This interaction points to a potential interplay between inositol signaling, and nutrient and stress responses. The next section details knowledge of the SnRK1 sensors, which could help delineate how plants respond to nutrients and, or stress.

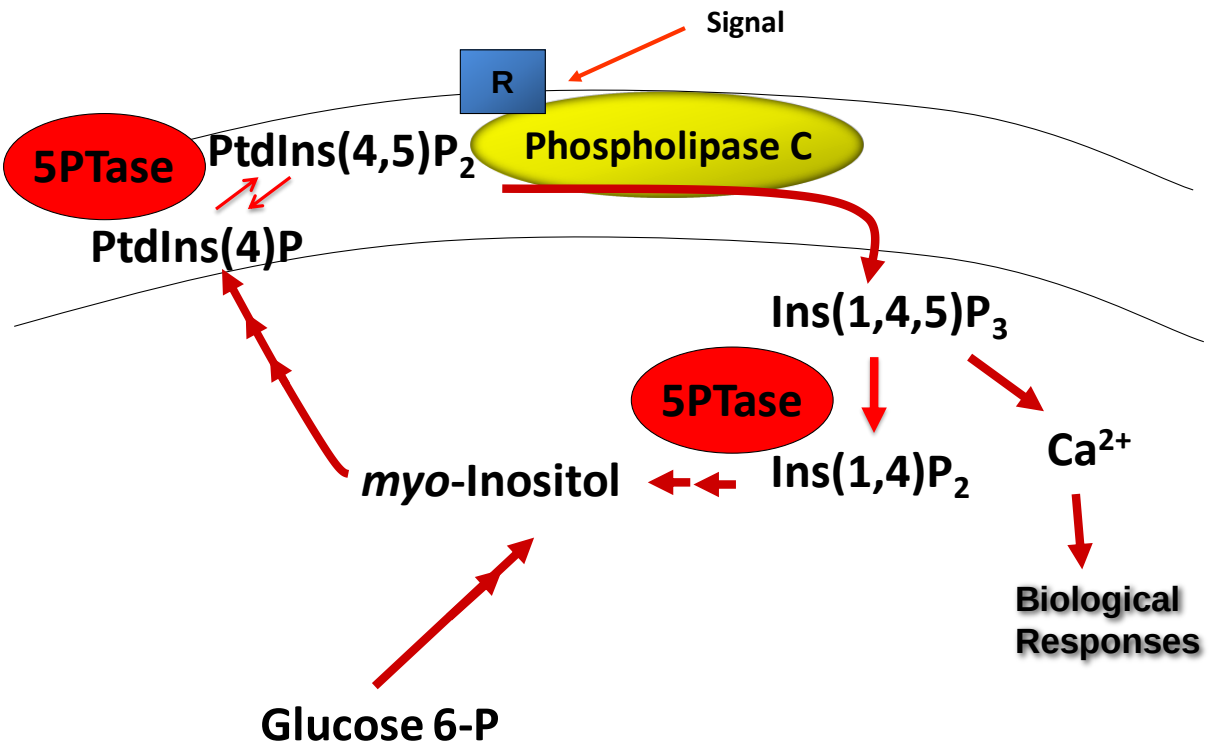


Figure 1. General Mechanism of Inositol Signaling in *Arabidopsis thaliana*. Inositol signal transduction begins with an environmental signal, which activates membrane receptors (R) that bind phospholipase C (PLC) and, in turn, generates inositol (1,4,5) P_3 ($InsP_3$). $InsP_3$ most likely interacts with intracellular receptors to release Ca^{2+} from intracellular stores, which initiate biological responses. The 5PTases (red circles) regulate inositol signaling. Arrows indicate enzymatic reactions.

The SnRKs and the SnRK1 Sub-Family

In plants, the SnRK family of proteins functions as serine/threonine kinases, and is comprised of SnRK1, SnRK2, and SnRK sub-families (Halford and Hey, 2009). As kinases, SnRKs are important for phosphorylation events in signal transduction pathways (Halford and Hey, 2009). Signals can be transferred and amplified from one step in a pathway to the next via protein kinase cascades. Interestingly, protein kinase cascades may be a cornerstone to understanding how certain pathways are linked and how cells may use reaction networks for pathways to converge and emerge (Halfrod and Hey, 2009). Using SnRK proteins as a model would strengthen our current understanding of the dynamics between phosphorylation and dephosphorylation in many essential biological response pathways, and elucidate how protein kinases are important for signaling networks in plant species.

As a family, SnRKs show no evidence of redundancy (Halford and Hey, 2009). In fact, examination of transgenic plant lines reveals neither SnRK2 nor SnRK3 can compensate for the role of SnRK1 (McKibbin et al., 2006; Zhang et al., 2001). However, even though little is known biochemically about SnRK2 or SnRK3 when compared to our understanding of SnRK1, some have reported these kinases may utilize similar substrates (Halford and Hey, 2009). For example, SnRK2 phosphorylates ABA-response element-binding proteins (AREBP) from both Arabidopsis and rice (Furihata et al., 2006; Kobayashi et al., 2005), and is important in ABA stress responses. Even though SnRK2 phosphorylates AREBP, SnRK1 and SnRK3 have recently been shown to phosphorylate the same protein (Zhang et al., 2008). Not only does this bring confidence to the possibility of SnRKs having a connected role, but may be conserved and act on similar substrates. Even though as a family of proteins SnRKs are becoming a focus for stress

and nutrient signaling, it is really the SnRK1 subfamily that directly ties into signaling and metabolism, described later.

There are three SnRK1 genes in Arabidopsis, SnRK1.1, SnRK1.2, and SnRK1.3. Both SnRK1.1 and SnRK1.2 undergo differential splicing, producing three splice variants, whereas predicated cDNA show SnRK1.3, a widely accepted pseudogene, only has one (Figure 2). The intron-exon maps (Figure 2) indicate there could be differences in the translated products of SnRK1.1 and SnRK1.2 genes. For example, At3g01090.2 has an extra N-terminal exon the other SnRK1.1 splice variants do not have (Figure 2). Moreover, a SnRK1.2 splice variant, At3g29160.3, is missing two exons when compared to the other possible differential splicing forms (Figure 2).

Examination of SnRK1 protein sequences (Figure 3) shows all SnRK1s share strong sequence homology. However, the domain map (Figure 4) does indicate some differences between SnRK1 proteins. For example, SnRK1 proteins predicted from the splice variants of SnRK1.1 (At3g01090.1, At3g01090.2, and At3g01090.3) have the protein kinase, kinase-associated 1 (KA1), and ubiquitin-associated (UBA) domains. This is not the case for SnRK1.2 and SnRK1.3. Two of the reported splice variants of SnR1.2 (At3g29160.1 and At3g29160.2) have the protein kinase, KA1, and UBA domains. The third SnRK1.2 splice variant, At3g29160.3, does not have the KA1 domain (Figure 4). Lastly, SnRK1.3 does not have the UBA domain (Figure 4). Data suggest that SnRK1.3 is never expressed (Baena-Gonzalez et al., 2007).

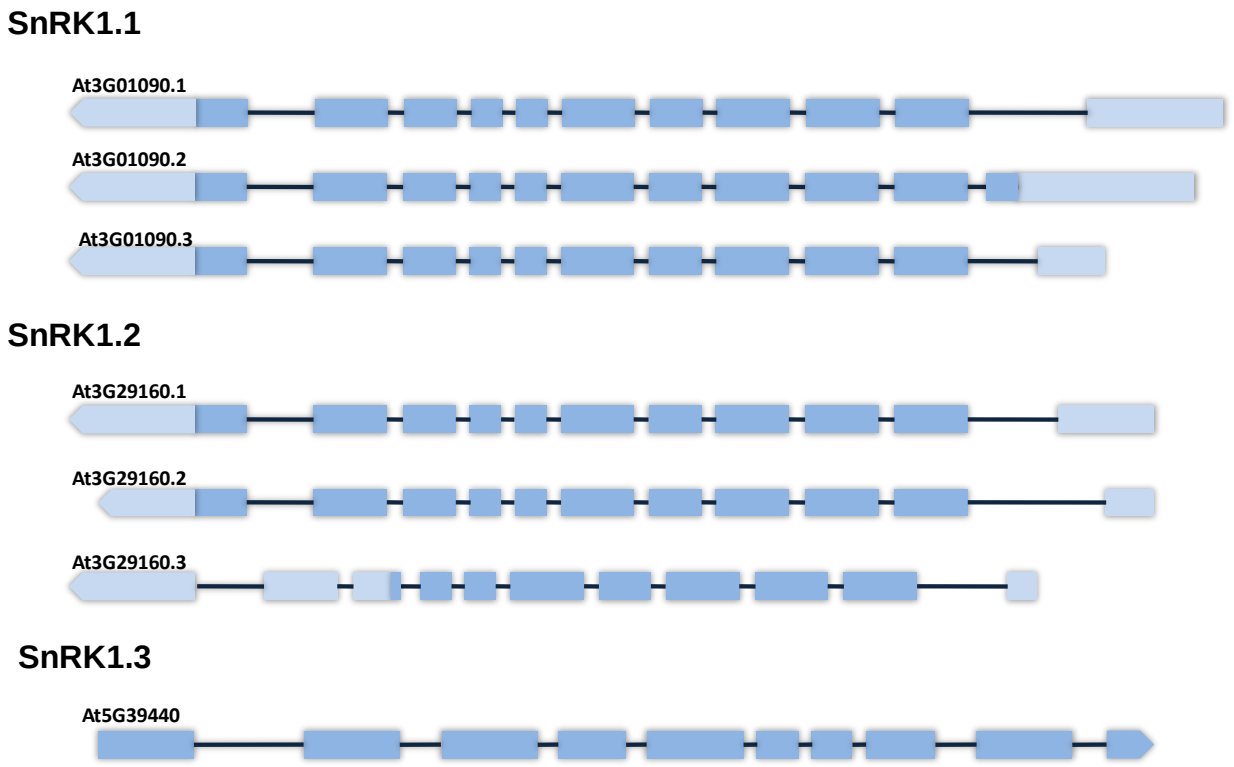


Figure 2. SnRK1 Intron-Exon Maps. The reported intron-exon maps of SnRK1 genes. SnRK1.1 and SnRK1.2 have three splicing variants, while SnRK1.3 (a pseudogene), only has one. Dark blue boxes denote exons, light blue boxes denote 5' and 3' UTRs.

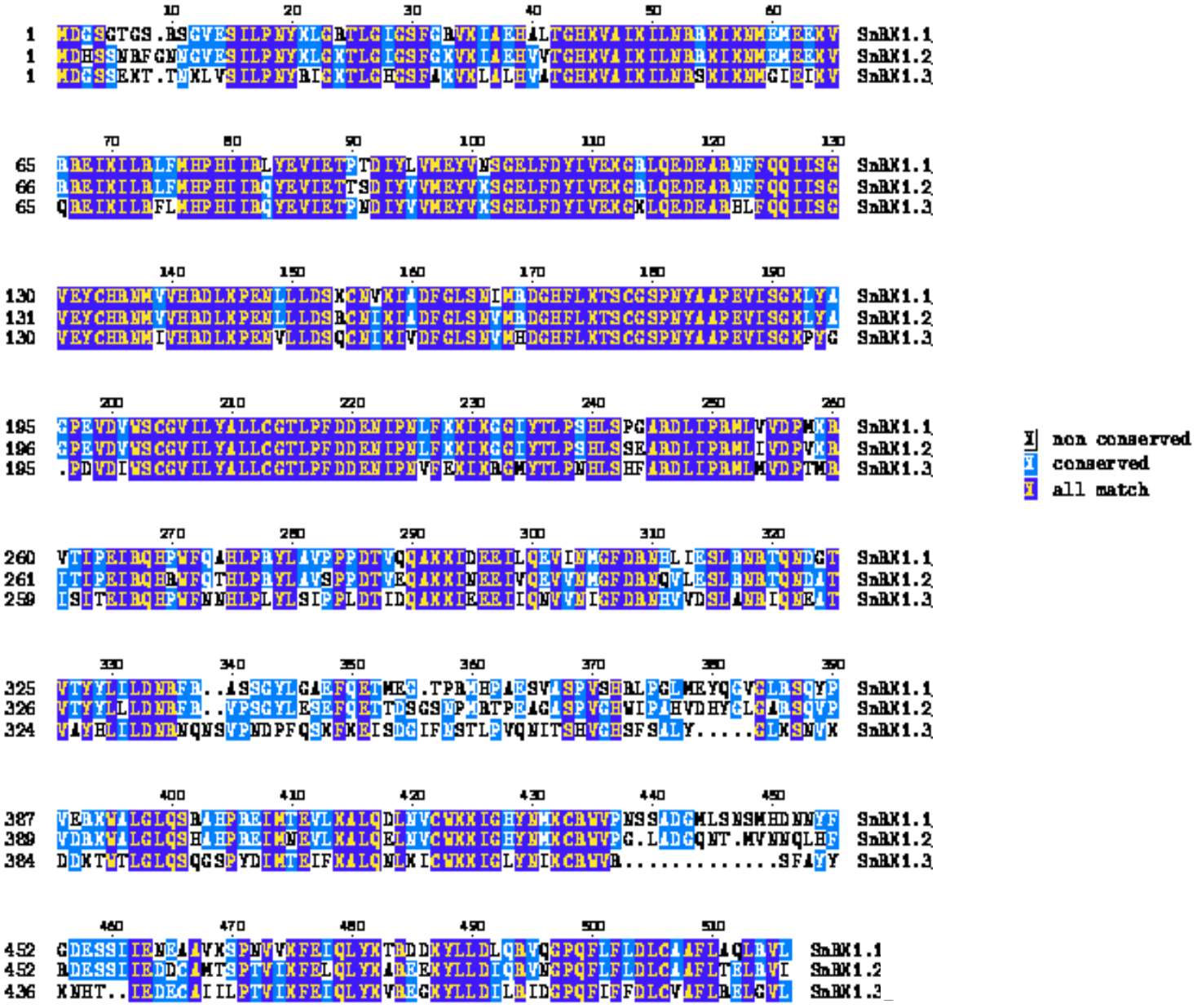
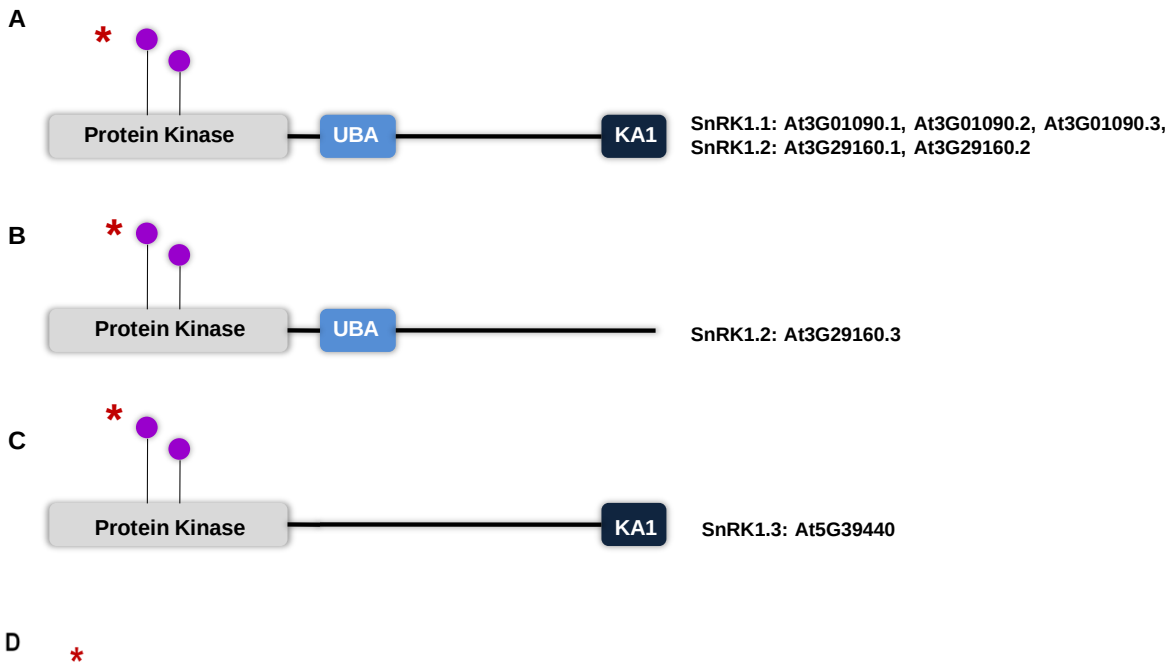


Figure 3. SnRK1 Protein Alignments. CLUSTALW was used to align all predicted SnRK1 proteins. Analysis shows that SnRK1.1 and SnRK1.2 share 80% homology, while SnRK1.1 and SnRK1.3 share 67%. SnRK1.2 and SnRK1.3 protein sequences are 69% homologous.



Protein	Predicted Active Site Residues
At3G01090.1, At3G01090.3, At5G39440	160, 142
At3G29160.1, At3G29160.2, At3G29160.3	161, 143
At3G01090.2	183, 165

Figure 4. SnRK1 Domain Structure. (A-C) Domain structure of SnRK1 kinases containing the Protein Kinase, UBA (Ubiquitin Associated), and KA1 (Kinase Associated 1). The SnRK1.2 splice variant, At3g29160.3, is missing the KA1 domain (B) and, SnRK1.3 is missing the UBA domain (C). (D) Location of active site residues in the Protein Kinase domain (purple circles). Red asterisk refers to table in (D).

The Role of SnRK1.1 in Metabolism

The first indication of SnRK1's connection to metabolism stems from phylogenetic analysis (Halford and Hey, 2009). The SnRK1 subfamily of serine/threonine kinases has strong homology with mammalian AMP-activated kinase, AMPK, and the yeast sucrose non-fermenting kinase 1, (Snf1) (Halford and Hey, 2009). AMPK is activated by adenosine monophosphate (AMP) to stimulate essential functions for mammals involved in metabolic homeostasis such as cell growth and development, calcium release, and glucose absorption (Hardie, 2008). Similarly, yeast Snf1 is key to the response to glucose (or carbon) availability (Hardie, 2007). As the glucose availability decreases, yeast cells undergo glucose starvation. Snf1 becomes active and promotes the transcription of genes repressed by glucose (Celenza and Carlson, 1986; Jiang and Carlson, 1996). The Snf1 gene was identified in a screen for mutants that could not activate SUC2, an invertase gene, in response to low glucose conditions (Celenza and Carlson, 1986). However, mutant complementation restored Snf1 function, and the cells' ability to survive glucose starvation. Interestingly, SnRK1.1 from *Arabidopsis* was found to complement the *snf1* mutation in yeast (Alderson et al., 1991). Given that the plant SnRK1.1 is closely related to enzymes from other organisms that serve as important regulators of metabolism, and in some cases can even complement them (Alderson et al., 1991), SnRK1 may have a similar function in Arabidopsis.

In order to test this hypothesis, overexpression and knock-down lines of SnRK1 were produced (Baena-Gonzalez et al., 2007). When SnRK1.1 was overexpressed, plants displayed increased time to flowering and senescence, which indicate increased lifespan (Baena-Gonzalez et al., 2007). In addition, the Gillaspy lab has shown SnRK1.1 overexpressors have increased vegetative biomass. These phenotypes imply that an abundance of SnRK1.1 alters metabolism and development. RNAi lines of SnRK1.1 have subtle growth defects, and double RNAi

SnRK1.1/1.2 plants are not viable (Baena-Gonzalez et al., 2007). These data suggest SnRK1.1 and SnRK1.2 are partially redundant and SnRK1 function is important for survival. Since SnRK1.1 RNAi seedlings are viable, they were tested for nutrient/energy-associated phenotypes at the seedling stage. Seedlings were grown under very low energy or low nutrient conditions (i.e., low light to limit photosynthesis) as compared to optimal energy conditions (plus 3% sucrose). Under these conditions SnRK1.1 RNAi seedlings have decreased root growth in low nutrients, and greater root growth under optimal conditions (Baena-Gonzalez et al., 2007). SnRK1.1 overexpressors exhibit opposite responses with increased root growth under low nutrients, and decreased root growth with optimal nutrients (Baena-Gonzalez et al., 2007). These data support a role for SnRK1.1 as a sugar sensor and that SnRK1.1 is required for optimal plant growth. The role of SnRK1.2 in energy-sensing and nutrient responses is less clear. SnRK1.2 RNAi plants were reported to have no phenotypes (Baena-Gonzalez et al., 2007). In addition, recent studies report SnRK1.1 is the predominate SnRK1 that can be immunoprecipitated from plant extracts in *Arabidopsis thaliana* (Jossier et al., 2009). Together, these data support a major role for SnRK1.1 in plants, and a partially redundant function for SnRK1.2.

The Role of SnRK1.1 in Signaling

The molecules that interact with SnRK1.1 may affect its function as a carbon sensor. Yeast two-hybrid screens performed by Dr. Les Erickson, a collaborator of the Gillaspy lab, revealed SnRK1.1 interacts with the WD40 repeat region of 5PTase13 as mentioned earlier, and a second messenger regulator of the $InsP_3$ pathway (Ananieva et al., 2008). SnRK1.1 also interacts with AtP80 (P80), a protein predicted to interact with the Cullin 4 E3 Ubiquitin Ligase complex (CUL4 complex). The same two-hybrid screen elucidated interactions between

5PTase13 and P80, and the C-termini of P80 and Pinoid-Binding Protein 1 (PBP1), a protein predicted to function in auxin efflux signaling (Benjamins et al., 2003; Benjamins et al., 2001).

Regulation of SnRK1.1 by 5PTase13

To understand the role of 5PTase13:SnRK1.1 and 5PTase13:P80 complexes in nutrient and energy sensing in plants, *5ptase13* mutants were examined. *5ptase13* mutants have overall lower SnRK1 activity when grown on low nutrients as compared to wildtype plants when grown on low nutrients (Ananieva et al., 2008). Further, SnRK1.1 degradation by the proteasome is greater in *5ptase13* mutant extracts as compared to wildtype extracts, indicating that 5PTase13 acts to protect SnRK1.1 from proteasomal degradation under low nutrient conditions (Ananieva et al., 2008).

Regulation of SnRK1.1 by P80 and the Proteasome

The AtP80 gene encodes a protein containing WD40 repeats at its N-terminus. One of these repeats has an additional conserved aspartic acid residue, giving it the hallmark of a so-called DWD domain. DWD domains have been found in proteins that act as substrate receptors of the CUL4 complex (Lee et al., 2008). Thus P80, which interacts with 5PTase13 and SnRK1.1, may also interact with the CUL4 complex. As mentioned previously, the CUL4 complex has E3 ubiquitin ligase activity and functions in the 26S ubiquitin-mediated proteasomal degradation pathway (Higa and Zhang, 2007). Eukaryotes use ubiquitin-mediated proteasomal degradation as a way to turnover proteins no longer needed by the cell (Higa and Zhang, 2007). Proteins targeted to the proteasome are polyubiquitinated at lysine residues by three enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). E3 ligases

serve as a platform for ubiquitin transfer from E2s to target protein substrates. Once a protein is tagged with ubiquitin, it is trafficked to the 26S proteasome for disassembly. P80's predicted interaction with CUL4 suggests that this complex functions to regulate the stability of SnRK1. Previous data have shown 5PTase13 can act to protect SnRK1 from degradation (Ananieva et al., 2008). The role of AtP80 in SnRK1 stabilization remains unclear, yet it seems likely that AtP80 acts to regulate SnRK1 stability.

Atp80 loss-of-function mutant plants show dramatic phenotypes—premature leaf senescence, decreased leaf biomass, and decreased root growth under low nutrient conditions (Ananieva, 2009). These phenotypes, in conjunction with the P80:SnRK1.1 interaction, suggest that AtP80 may play a novel role in nutrient and inositol signaling (Ananieva, 2009) and it also suggests P80 may regulate SnRK1.1 stability. However, the P80:SnRK1.1 complex has not been shown *in vivo*, only Y2H analysis shows *in vitro* interactions. Figure 5 illustrates a model of the physical interactions between P80, SnRK1.1, and 5PTase13 *in vitro* and how these interactions may impact nutrient sensing. Elucidating the stability of SnRK1.1 in *p80* mutants, which have not been reported, would provide a stronger understanding of this signal transduction cascade in plants. Ultimately, this may aid in engineering plants with desired metabolic traits such as increased lifespan and stress-tolerance.

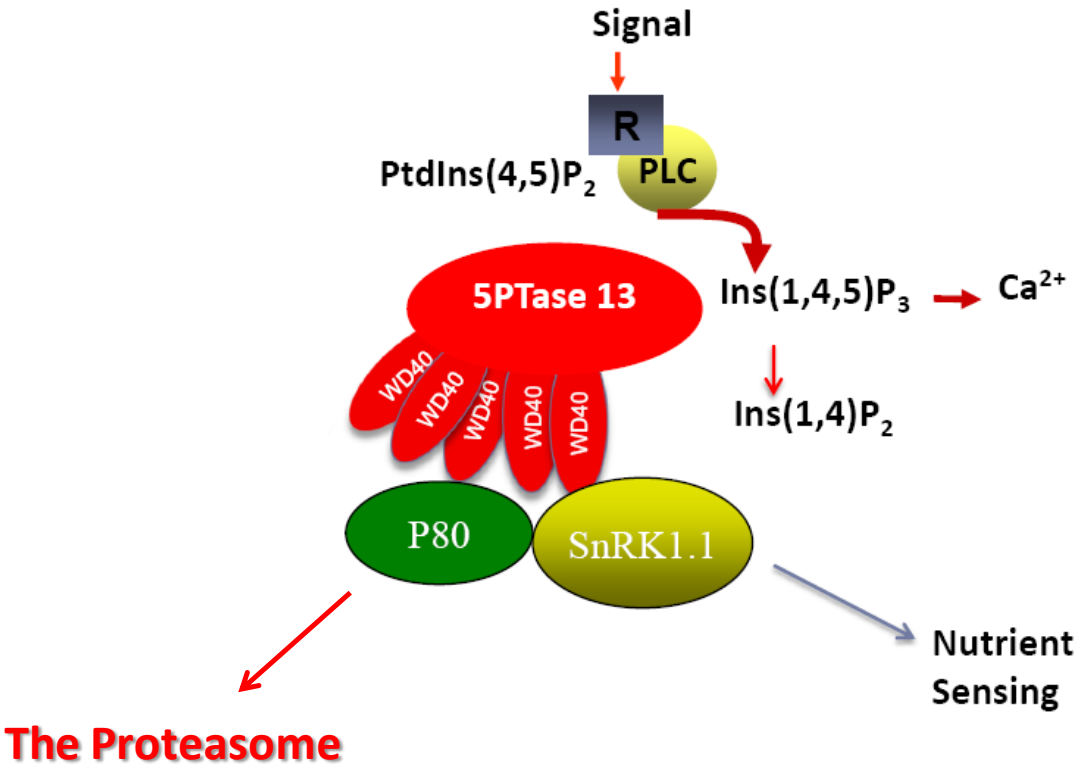


Figure 5. Proposed Model for Protein:Protein Interactions. Shows 5PTase13 (red circle) interaction with P80 (green circle) and SnRK1.1 (yellow circle) and its relationship to inositol signaling. These protein:protein interactions may have function in downstream nutrient sensing responses and proteasomal regulation.

RATIONALE

Despite the importance of SnRK1.1 and SnRK1.2 in metabolism, there is no information on how these genes are regulated. This information could aid in understanding how SnRK1 genes and proteins function in nutrient and stress sensing. Previous work reveals a connection between SnRK1.1 and metabolism via phylogentic similarities to other eukaryotic molecules paramount in growth and development, sugar sensing, calcium-release responses, and glucose-stress responses (Halford et al., 2003; Hardie, 2008). SnRK1.1 transgenic lines have nutrient-related phenotypes. Yet, there is debate in the literature about SnRK1.1's role in nutrient and energy sensing. Some data show SnRK1.1 is required for optimal growth in low nutrient and low energy conditions (Baena-Gonzalez et al., 2007). Other data support the idea that SnRK1.1 activity is activated by sugar or high energy conditions (Halford et al., 2003; Halford and Hey, 2009; Jossier et al., 2009). Alternatively, SnRK1.1 may be functioning not strictly as either a low or high nutrient sensor, but functioning to sense the change or difference in nutrient status, acting as a metabolic reprogammer, converging nutrient and stress responses (Baena-Gonzalez and Sheen, 2008). Moreover, the ties of SnRK1.1 to key signaling molecules in the inositol signaling and the proteasomal degredation pathways (Ananieva et al., 2008) position SnRK1.1 as a potential nexus for coordinating both signaling and metabolic cascades. However, there is little data on SnRK1.1 regulation by either inositol signaling or the proteasome.

Thus, my thesis will focus on studying the developmental and nutrient regulation of SnRK1.1 at the level of transcription and translation. Further, my work will address how SnRK1.1 is regulated by molecules like P80 and CUL4, and whether any post-translational modifications (phosphorylation) are occurring. Specifically, my research focuses on revealing the spatial expression patterns of SnRK1.1 and SnRK1.2 genes, determining whether a SnRK1.1

antibody can be used to characterize steady-state SnRK1.1 protein levels, and how and where SnRK1.1 is regulated *in vivo*.

OBJECTIVES

In order to help address my main research questions, I have developed the following objectives:

I. To Characterize SnRK1.1 and SnRK1.2 Spatial Expression Patterns Using Promoter:Gene Reporter Transgenic Plants

II. To Develop and Characterize a SnRK1.1 Antibody

III. To Characterize How and Where SnRK1.1 is Regulated *in vivo*

CHAPTER II

OBJECTIVE I: To Characterize SnRK1.1 and SnRK1.2 Spatial Expression Patterns Using Promoter:Gene Reporter Transgenic Plants

INTRODUCTION

In this study I explore how SnRK1.1 and SnRK1.2 genes are regulated. Currently, there is little known about transcriptional regulation of SnRK1 genes and whether SnRK1.1 and SnRK1.2 genes are differentially regulated. I utilized promoter:gene reporter transgenic plants to observe and qualitatively address spatial expressions patterns of SnRK1.1p:β-Glucuronidase (GUS) and SnRK1.2p:GUS *in vivo*. Through analysis of SnRK1.1p:GUS and SnRK1.2p:GUS promoters, I found evidence that *cis*-acting regulatory elements involved in sugar homeostasis, development, auxin, light, and abscisic acid may have a role in the regulation of both genes. To test this, I performed GUS assays on seedlings and mature plants. My results show SnRK1.1p:GUS is regulated during development and in response to exogenous sugar. Further, SnRK1.2p:GUS transgenic plants have restricted spatial expression patterns independent of development and sugar availability. Preliminary data suggest SnRK1.1p:GUS and SnRK1.2:pGUS expression patterns change in response to naphthylphthalamic acid, a polar auxin transport inhibitor, and light intensity. Additively, the spatial expression patterns provide evidence to support SnRK1.1 is important for developmental and sugar-related responses, and SnRK1.2, in contrast, has a restricted role in these processes.

RESULTS

The structure of SnRK1.1p:GUS and SnRK1.2p:GUS constructs

In order to characterize how SnRK1.1 and SnRK1.2 genes are regulated, promoter:gene reporter transgenic plants were made. The GUS reporter system uses a histochemical reaction to monitor the expression patterns of a gene's native promoter that drives GUS expression. The enzyme (β-glucuronidase), removes glucuronic acid from the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), to form 5,5'-dibromo-4, 4'-dichlor-indigo, a blue colored product (Jefferson, 1989). Portions of the native, intergenic regions of SnRK1.1 and SnRK1.2 genes were fused to the open reading frame of the GUS gene (Figure 6). Approximately 0.8 kB and 4.3 kB of the SnRK1.1 and SnRK1.2 promoters were used, respectively. The schematic illustrates the GUS constructs carry a BASTA resistance marker, which was used to select transgenic plants containing the construct (Figure 6).

In collaboration with other lab members, both SnRK1.1 and SnRK1.2 GUS constructs were inserted into Arabidopsis using Agrobacterium transformation (Bechtold et al., 1993; Bechtold and Pelletier, 1998). I was able to generate homozygous lines for SnRK1.1p:GUS and heterozygous lines for SnRK1.2p:GUS through BASTA resistance screening of each plant generation.

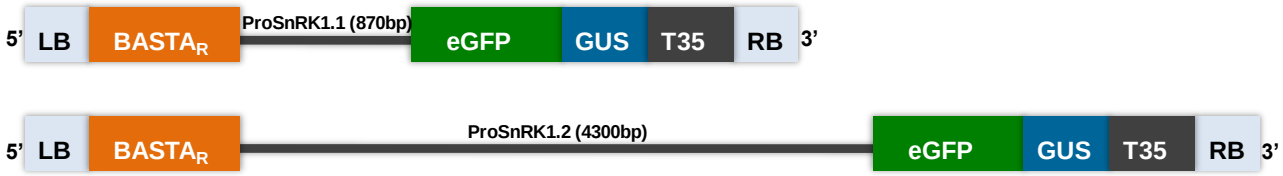


Figure 6. SnRK1.1 and SnRK1.2 Promoter GUS Constructs. The GUS reporter system uses a histochemical reaction to monitor the expression pattern of a gene. The figure shows SnRK1.1 and SnRK1.2 promoter GUS construct maps. Portions of each of the native promoters were fused to the enhanced green fluorescent protein (eGFP)/GUS fusion transgene, followed by the 35S CaMV termination element (T35). LB and RB are left and right borders, respectively. The Basta resistance gene ($BASTA_R$) allows for selections of transgenic plants with the herbicide Basta.

SnRK1.1 and SnRK1.2 promoters may be regulated by sugar, development, light, and hormones

Using the tool, Plant Cis-Acting Regulatory Element (PLACE; http://www.dna.affrc.go.jp/PLACE), I examined elements that may regulate SnRK1.1 and SnRK1.2 promoters. I was primarily interested in elements important for sugar-sensing and responses to stress, given the implications of SnRK1.1 as a nutrient and stress sensor (Baena-Gonzalez et al., 2007; Jossier et al., 2009). Moreover, my selection of elements had to fit two main criteria: elements were encoded by at least 5 nucleotide bases and their proximity to the TATA box was within 1000 bp. I compiled a list of elements fitting these criteria and generated a map (Figure 7). I found the SnRK1.1 promoter may be regulated by sugar (Lu et al., 2002; Lu et al., 1998; Lu et al., 2007), light (Terzaghi and Cashmore, 1995), water and ABA stress (Busk and Pages, 1998; Marcotte et al., 1992; Urao et al., 1993), and auxin (Despres et al., 2003), as I found *cis*-elements related to these responses in the SnRK1.1 promoter region (Figure 7). Similarly, the SnRK1.2 promoter region contains predicted elements important for developmental (Michaels et al., 2003), light (Terzaghi and Cashmore, 1995), and ABA and auxin responses (Hagen and Guilfoyle, 2002; Hobo et al., 1999) (Figure 7). However, the SnRK1.2 promoter also contains elements predicted to be regulated by gibberellic acid (GA), phosphate-starvation, and cold temperature (Rubio et al., 2001) (Figure 7). Along with evidence on SnRK1's role as a nutrient and energy sensor, these predicted transcriptional elements prompted me to address whether spatial expression patterns of SnRK1.1 or SnRK1.2 genes change during development, in the presence of exogenous sugar, auxin, or light.

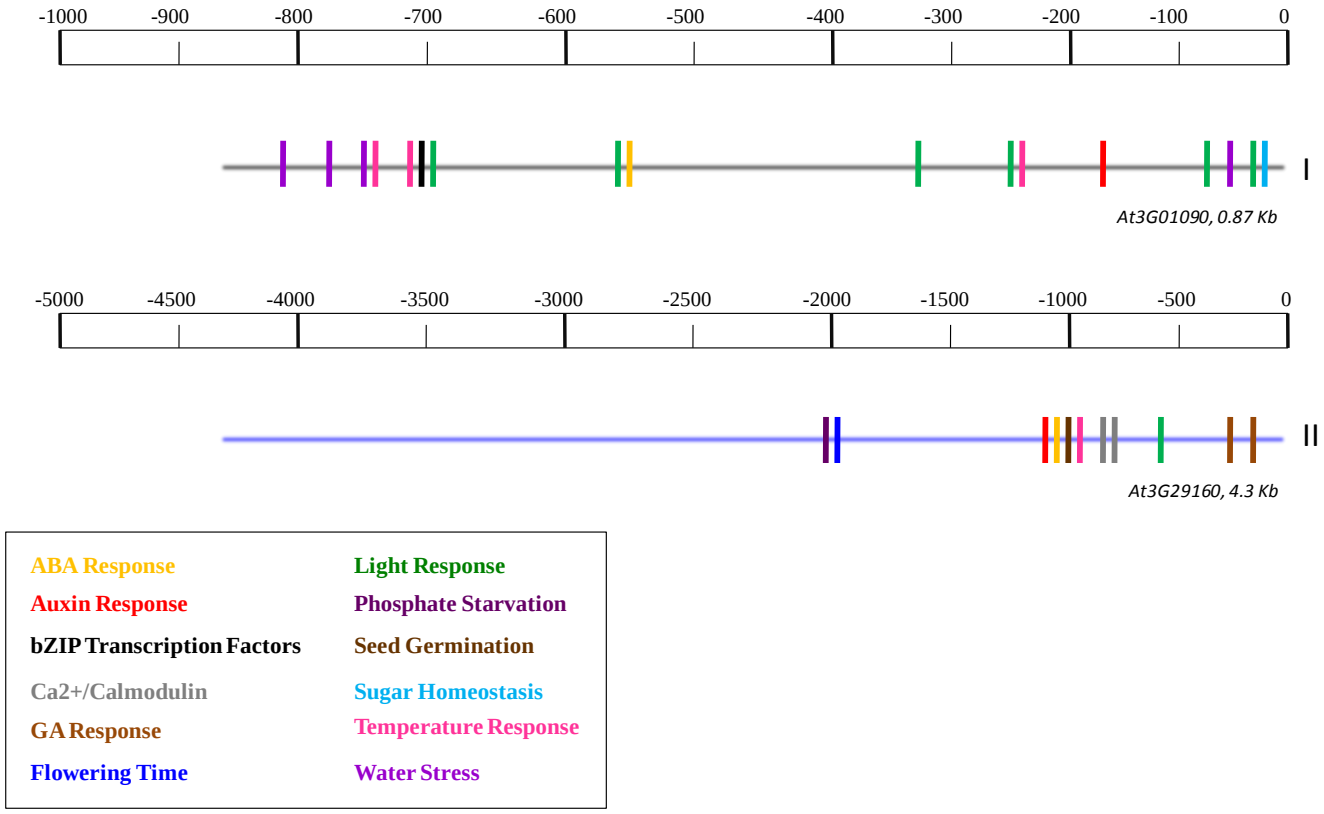


Figure 7. SnRK1.1 and SnRK1.2 Promoters. Schematic map showing promoter regions and predicted *cis*-acting regulatory elements. Black bar denotes native SnRK1.1 (At3g01090) promoter (I), blue bar denotes native SnRK1.2 (At3g29160) promoter (II). Promoter sequences analyzed for each gene were used in promoter:gene reporter constructs (Figure 1). SnRK1.1 promoter region is 0.87 kb, SnRK1.2 promoter region is approximately 4.3 kb. Colored tic marks along the promoter schematic correspond to response elements defined in the lower box.

The role of SnRK1.1 and SnRK1.2 in seedling development

I wanted to determine where SnRK1.1 and SnRK1.2 genes are expressed during seedling development. Using SnRK1.1p:GUS and SnRK1.2p:GUS transgenic plants I established spatial expression profiles based on GUS patterns from seedlings grown on agar plates containing 0.5x Murashige and Skoog (MS) salts and low light (low nutrients) (Figure 8). Figure 3 illustrates SnRK1.1p:GUS and SnRK1.2p:GUS expression patterns of both the shoot and root structures in early seedling development (4 to 15 days). SnRK1.1 expression is very strong in 4-day-old seedlings (Figure 3A) and expression decreases as the seedling ages to 15 days (Figure 8B, C, and D). At 4 days, the expression is in both vascular and non-vascular tissues in the cotyledons (Figure 8A). However, as the seedling matures, expression decreases in non-vascular tissues in the cotyledon at 7 days (Figure 8B), with a vascular-specific pattern in 10-day and 15-day-old seedlings (Figure 8C and D). As true leaves begin to develop SnRK1.1 expression is evident in these structures (Figure 8C and D), as well as in the true leaf primordia in younger seedlings (Figure 8A and B). Overall, SnRK1.1p:GUS expression is found in the cotyledon with the highest expression seen at 4 days, and expression becoming restricted to vascular structures and true leaves by 15 days (Figure 8).

SnRK1.2p:GUS expression, however, is vastly different from the expression pattern of SnRK1.1 in cotyledons (Figure 8E, F, and G). SnRK1.2 has minimal cotyledon expression in 4-day-old through 15-day-old seedlings (Figure 8E, F, and G). Expression is only seen in true leaf primordia and hydathodes (Figure 8E, F, and G). Even as true leaves expand between 10 and 15 days, SnRK1.2p:GUS expression remains restricted to primordial, hydathodes, and the proximal portion of the leaves.

SnRK1.1 and SnRK1.2 expression in root structures closely resembled shoot patterning. SnRK1.1 is expressed in the root and root tips of 4-day (Figure 8H), 7-day (Figure 8I), 10-day (Figure 8J), and 15-day-old (Figure 8K) seedlings. Interestingly, SnRK1.1p:GUS has high expression in 4-day-old seedlings (Figure 8H) and expression decreases at 7 days (Figure 8I), and is mainly in the root tip. However, as the root ages to 10 days, expression also increases (Figure 8J), with even more expression in 15-day-old roots and root tips (Figure 8K). The SnRK1.2p:GUS expression pattern in root structures is minimal (Figure 8L, M and N). There is no visible expression in 4-day-old roots (Figure 8L), and expression in 10-day and 15-day-old roots is seen in lateral root primordia (Figure 8M and N, respectively), expected areas of lateral root formation.

My results indicate SnRK1.1p:GUS expression is vascular and non-vascular in both shoot and root tissues, depending on the developmental stage. Further, cotyledon expression is greatest in the beginning of development and decreases as the seedling ages, while root expression increases with age, with the exception of 7-day-old roots. SnRK1.2p:GUS expression in cotyledons is limited to areas of growth; leaf primordia, and specific vascular structures, called hydathodes. In the root expression of SnRK1.2 is minimal and restricted to lateral root primordia. I conclude SnRK1.1p:GUS gene expression is regulated by development in a broad range of tissues, while SnRK1.2p:GUS gene expression is strictly limited to the same tissues over the course of seedling development.

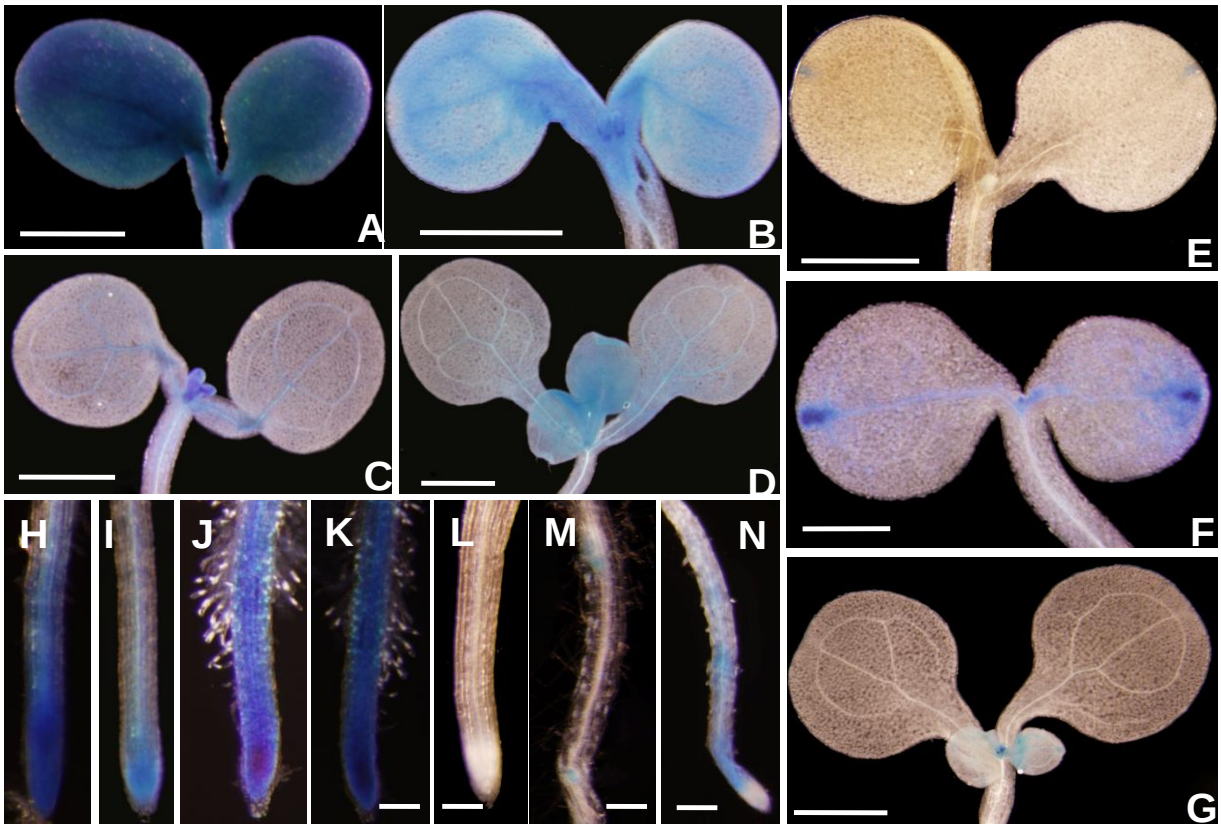


Figure 8. SnRK1.1p:GUS and SnRK1.2p:GUS Shoot and Root Developmental Spatial Expression Patterns in Seedlings. All seedlings were grown on 0.5x MS under low light. (A)-(D) and (H)-(K) are SnRK1.1p:GUS 4-, 7-, 10-, and 15-day-old cotyledons, and 4-, 7-, 10-, and 15-day-old roots, respectively. (E)-(G) and (L)-(N) represent SnRK1.2p:GUS 4-, 10-, and 15-day-old cotyledons, and 4-, 10-, and 15-day-old roots, respectively. Scale bars = 1 mm for (A-G) and 500 μm for (H-N).

The role of SnRK1.1p:GUS and SnRK1.2p:GUS in mature plant tissues

To address how spatial expression patterns are regulated in mature plant tissues—rosette leaves, cauline leaves, and reproductive organs—I grew SnRK1.1p:GUS and SnRK1.2p:GUS transgenic plants on soil under low light (30-40 µE) (Figure 9).

SnRK1.1 and SnRK1.2 are both expressed in rosette leaves (Figure 9A and B, respectively). SnRK1.1p:GUS expression in the rosette is varied, with cotyledons having the highest expression, the youngest leaves having little to no expression, and mature leaves having an array of patterns ranging from a strictly vascular to a diffuse pattern (Figure 9A). The SnRK1.2p:GUS expression pattern is vascular and limited to hydathodes and primordia of the cotyledons and youngest leaves in the rosette (Figure 9B). Moreover, my data provide evidence that SnRK1.1 and SnRK1.2 are expressed in mature root and lateral structures, respectively (data not shown).

My results show SnRK1.1 is expressed in the proximal end of cauline leaves (Figure 9C). However, this expression could be caused by wounding when it was removed from the plant. In contrast, SnRK1.2p:GUS does not show any expression in the cauline leaf (Figure 9D). SnRK1.1 is expressed in stamens (Figure 9E) and immature pistols, while SnRK1.2 is expressed in vascular structures associated with floral buds and sepals, and within developing pistols (Figure 9F). Lastly, SnRK1.1 and SnRK1.2 show opposite expression patterns in siliques and seeds (Figure 9G, H, I, and J). SnRK1.1p:GUS is expressed in both mature siliques and seeds (Figure 9G and I), however, SnRK1.2p:GUS is not expressed in these organs (Figure 9H and J). There are areas within the silique that appear to be devoid of expression of SnRK1.1 (Figure 9G). The differential staining pattern is likely due to the inaccessibility of the X-Gluc substrate to all seeds

within the silique. In addition, there are some areas where seeds are missing from the silique, and thus, can explain the differential staining pattern seen in the silique also.

Qualitatively, I can conclude SnRK1.1 is expressed in rosettes, male and female reproductive organs, siliques, and seeds. Moreover, SnRK1.2 expression in mature plants is still limited to primordia structures and hydathodes, and is present in developing floral tissues. In conjunction with my previous data, I conclude the SnRK1.1 gene is more highly expressed and less spatially restricted than the SnRK1.2 gene throughout development. Further, SnRK1.2 appears to be restricted to lateral roots and specific vascular tissues throughout development.

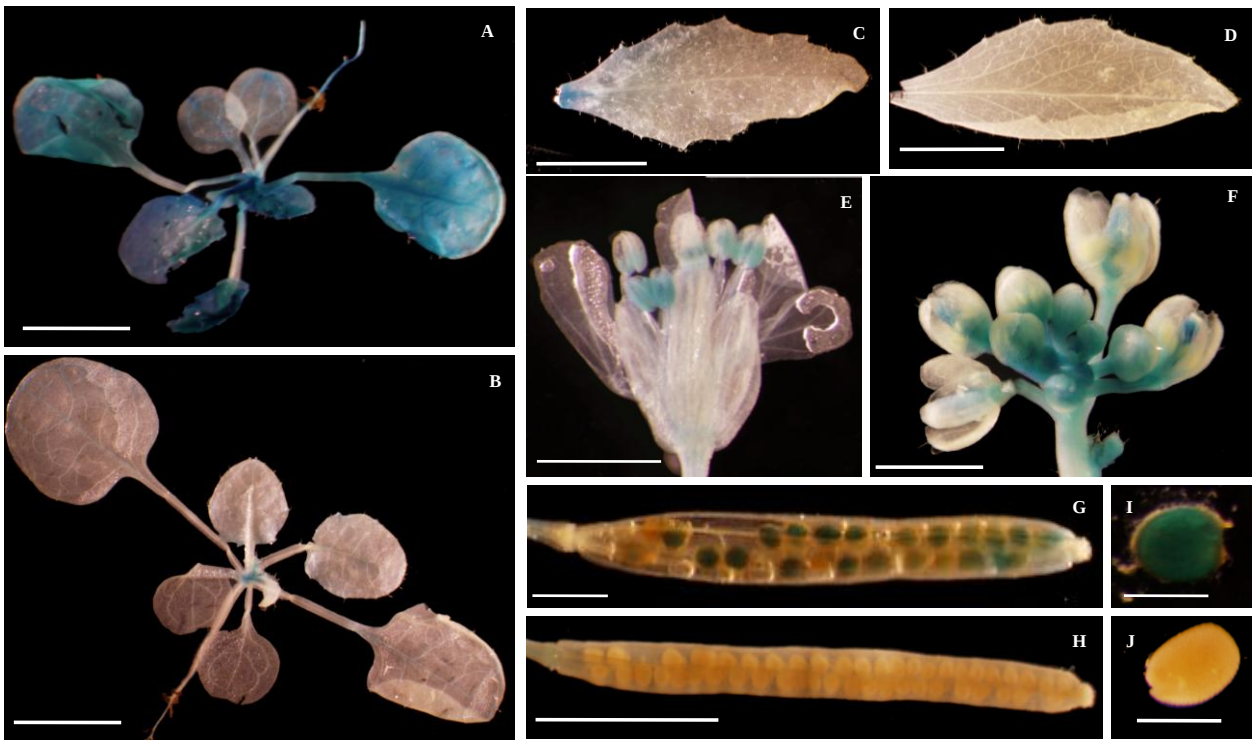


Figure 9. SnRK1.1p:GUS and SnRK1.2p:GUS Spatial Expression in Mature Plant Tissues. SnRK1.1 and SnRK1.2 are expressed in rosettes (A and B), cauline leaves (C and D), flowers (E and F), siliques (G and H), and seeds (I and J), respectively. Scale bars = (A and B) 5 mm, (C and D) 1 cm, (E and F) 1 mm, and (G-J) 500 µm.

The Effect of Sugar, Light, and Auxin on SnRK1.1 and SnRK1.2

Based on results from my analysis of of SnRK1.1 and SnRK1.2 gene promoters and current evidence in the literature linking SnRK1 to nutrient and stress responses (Baena-Gonzalez et al., 2007; Coello et al., 2011; Jossier et al., 2009), I wanted to investigate whether SnRK1.1p:GUS and SnRK1.2p:GUS expression patterns changed in response to sugar, light, and auxin. This approach could aid our understanding of how SnRK1 genes are transcriptionally regulated.

Sugar

To study the effect of sugar on spatial expression (Figure 10), I grew seedlings on MS media plus/minus 3% sucrose under low light (Figure 10). It was important to grow these plants under low light to reduce the rate of photosynthesis and in turn, reduce native production of sugars as an energy source. Based on my developmental analysis of SnRK1.1p:GUS and SnRK1.2p:GUS expression patterns, I chose to focus on the response of 7-day-old seedlings, as 4-day-old seedlings have high expression of SnRK1.1 under these conditions (Figure 8A). As well, I tested 10-day-old and 15-day-old seedlings grown on low nutrients for comparison, and 10-day-old SnRK1.2p:GUS seedlings, as these will contain true leaves and lateral root primordia.

Figure 10 illustrates sugar induces SnRK1.1p:GUS expression in both shoot and root tissues, and increases expression in non-vascular areas as well (Figure 10B). Sugar does not increase expression of SnRK1.2p:GUS in either shoots or roots (Figure 10D) when compared to seedlings grown on low nutrients (Figure 10C). However, sugar does promote SnRK1.2 expression in nascent lateral roots (Figure 10H). This increase in SnRK1.2 expression is most likely due to developmental differences, as the extra sugar in the media promotes lateral root

initiation. Therefore, I conclude SnRK1.1 gene expression is activated by exogenous sugar in young seedlings, while SnRK1.2 does not appear to be affected by exogenous sugar.

Light intensity

To study the effect of light intensity on SnRK1.1p:GUS and SnRK1.2p:GUS spatial expression patterns, I grew plants in soil under varying light conditions. Both SnRK1.1 and SnRK1.2 spatial patterns are influenced by light intensities (Figure 11). Under low light SnRK1.1 has a variety of expression patterns within the leaf which appears to be dependent on the age of the leaf in the rosette (Figure 9A). However, optimal light (90-110 µE) does not appear to increase expression (Figure 11B), rather, expression remains the same within the rosette leaves (Figure 11B). Rosette leaves grown under regular light do show more leaves having little to no expression when compared to rosette leaves grown under low light conditions (Figure 11A and B). High light (200-210 µE) actually decreases expression when compared to both low light and regular light conditions, with almost a complete loss in overall SnRK1.1p:GUS expression (Figure 11C).

SnRK1.2p:GUS expression (Figure 11D-F) shows an interesting difference from SnRK1.1p:GUS. Regular light (Figure 11E) increases gene expression in vascular tissues in most, if not all leaves in the rosette, a noticeable difference from low light (Figure 11D). Increasing the light intensity decreases SnRK1.2p:GUS expression, similar to results seen with SnRK1.1 expression (Figure 11F). I consider these data to be preliminary, as I have only performed this experiment two times.

Auxin

If SnRK1.1 and SnRK1.2 genes are regulated by auxin, I hypothesized that spatial expression patterns of each gene would change in response to NPA, a polar auxin transport inhibitor. I germinated seedlings on 0.5x MS supplemented with 40 µM NPA, and assayed the seedlings at 7 days. My results show both SnRK1.1p:GUS and SnRK1.2p:GUS are impacted by NPA (Figure 12). For both constructs, inhibiting auxin transport via NPA increases expression in cotyledons and alters expression in the root (Figure 12). SnRK1.1p:GUS seedlings without NPA treatment have high levels of expression in the root and root tip. This changes in the presence of NPA, as SnRK1.1p:GUS root tips show decreased expression and expression appears to build-up right above the root tip (Figure 12). SnRK1.2p:GUS roots without NPA treatment show no visible expression in root or root tip. Adding NPA increases SnRK1.2 expression in the root tip (Figure 12). It is apparent NPA is altering the expression pattern in both GUS lines, and this could indicate these genes are regulated by auxin. These results are only preliminary, as I have performed this experiment two times. However, this result is important because it links the expression of a major nutrient and stress sensor to the major growth-regulating hormone, auxin. Further investigation is needed to generate more confidence that NPA is effecting spatial gene expression.

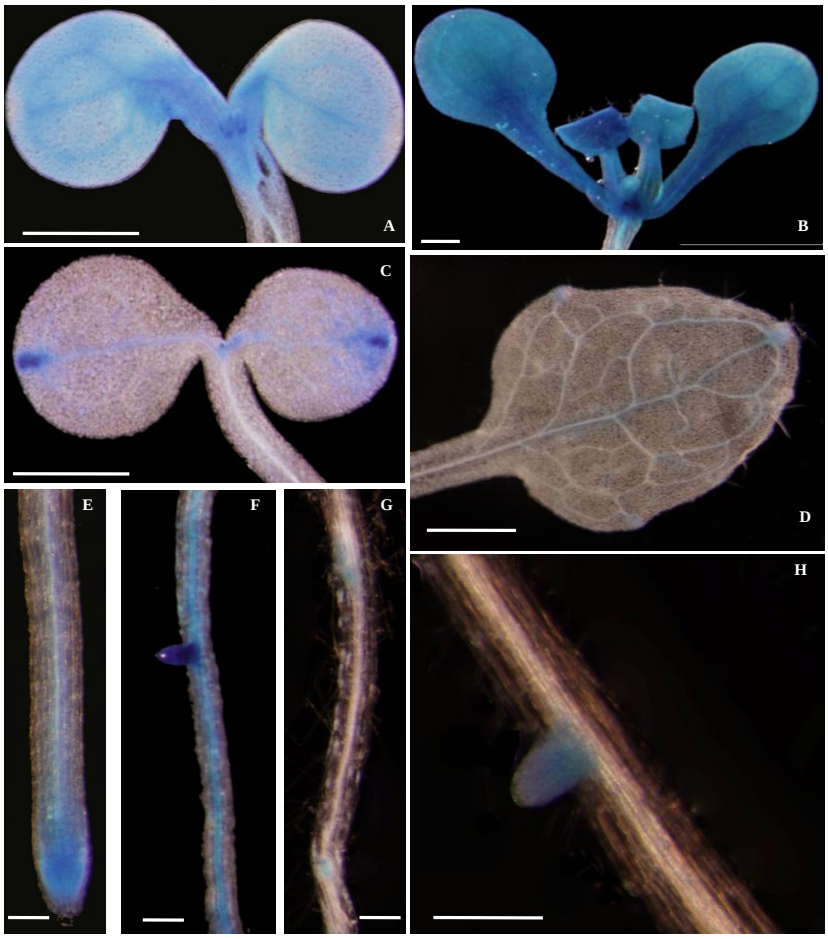


Figure 10. The Effect of Carbon on SnRK1.1p:GUS and SnRK1.2p:GUS Spatial Expression Patterns. To determine if SnRK1.1 or SnRK1.2 genes are regulated by sugar, seedlings were grown on 0.5x MS ± 3% sucrose under low light. (A and B) 7-old SnRK1.1p:GUS shoots and (E and F) roots. (C and D) 10-day-old SnRK1.2p:GUS shoots and (G and H) roots. Seedlings grown on 0.5x MS are shown in (A, C, E, and G). Seedlings grown on 0.5x MS + 3% sucrose are shown in (B, D, F, and H). Scale bars= (A, C, and D) 1 mm, (B and H) 500 µm, and (E, F, and G) 125 µm.

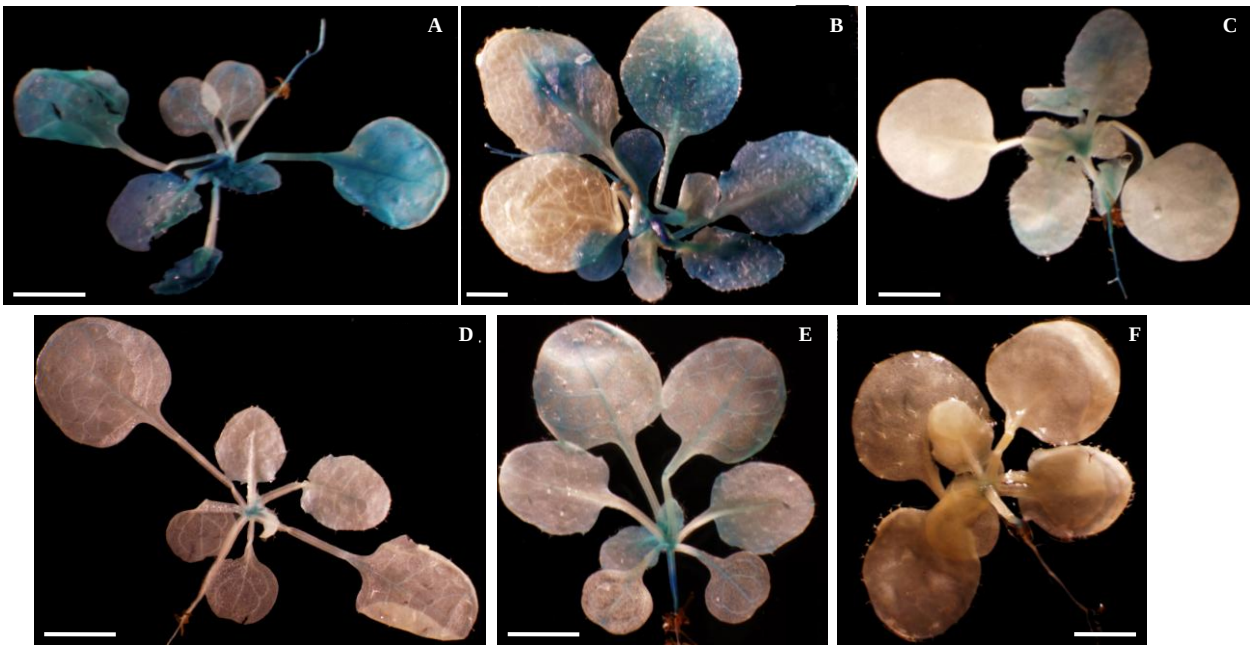


Figure 11. The Effect of Light on SnRK1.1 and SnRK1.2p:GUS Spatial Expression. 15-day-old SnRK1.1p:GUS and SnRK1.2p:GUS rosettes were grown under different light intensities. (A) SnRK1.1p:GUS rosette grown under constant low light, (B) regular light, and (C) high light. (D) SnRK1.2p:GUS rosette grown under low light, (E) regular light, and (F) high light. Light intensity was defined as 30-40 µE for low light, 90-120 µE for regular light, and 200-210 µE for high light. Scale bars= 2.5 mm

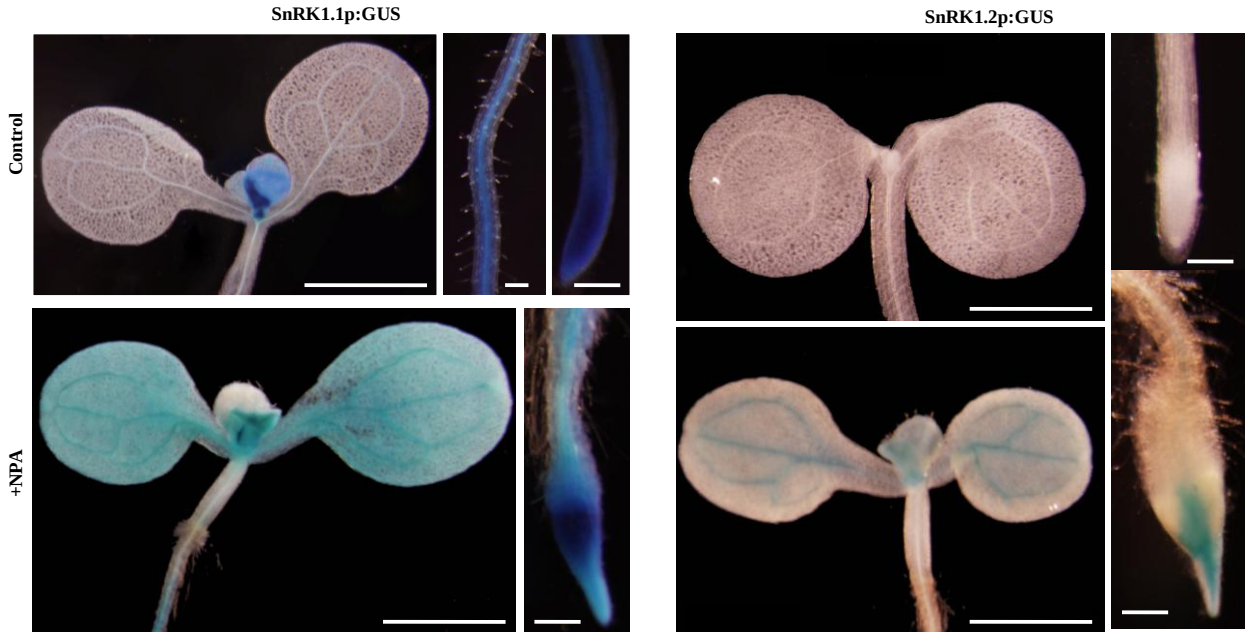


Figure 12. The Effect of NPA on SnRK1.1 and SnRK1.2 Spatial Expression Patterns. 7-day-old SnRK1.1p:GUS and SnRK1.2p:GUS seedlings grown on 0.5x MS ± 40 µM Naphthylphthalamic acid (NPA), a polar-auxin transport inhibitor. Scale bars = 1 mm.

DISCUSSION

There is no question SnRK1 is important for sugar sensing, stress responses, and metabolic reprogramming (Baena-Gonzalez et al., 2007; Coello et al., 2011; Jossier et al., 2009). Yet, currently there are still missing pieces in understanding the regulation of this vital sensor. It is necessary to establish a foundation for how SnRK1 genes are transcriptionally regulated in order to understand how SnRK1 functions in plant sensing mechanisms.

SnRK1 genes are developmentally regulated (Figure 9 and 10). Through my study, it is reasonable to conclude SnRK1.1 plays a more predominant role in development than SnRK1.2. SnRK1.1 is expressed in more tissues, with greater levels of expression throughout the entire developmental spectrum. However, this does not discredit SnRK1.2 as having an important role in development considering it is expressed in very specialized plant structures important for growth and vascular architecture, like hydathodes and primordia, and in reproductive tissues (Figure 9 and 10). Interestingly, hydathodes and leaf and lateral root pirmordia have all been found to have high levels of auxin (Mattsson et al., 2003). This suggests that auxin regulates expression of the SnRK1.2 gene. The restricted pattern of SnRK1.2 suggests its expression may be needed for a specialized role in specific times and places during plant development. Thus, my data provide transcriptional evidence supporting SnRK1.1 as the major SnRK1 kinase in plants (Jossier et al., 2009), and a specialized and potentially redundant role for SnRK1.2. Much emphasis is placed on SnRK1.1 as the major energy sensor for plants (Baena-Gonzalez et al., 2007; Halford et al., 2003), but at the transcriptional level it is unknown whether sugar regulates expression. I have used exogenous sucrose as a way test the effect of sugar on SnRK1 gene regulation (Figure 10). My data fits with previously described functional implications of SnRK1, in that SnRK1.1 expression does increase with added sucrose, however SnRK1.2 does not. This

result correlates with the presence of a predicted sugar homeostasis response element in the SnRK1.1 promoter that is not found in the SnRK1.2 promoter. Thus, SnRK1.1 may be the only SnRK1 gene regulated by sugar, which is consistent with its role as a nutrient and energy sensor.

Even though, SnRK1.2 is not noticeably regulated by sugar, preliminary data suggests it is regulated by light intensity and auxin. The SnRK1.2 promoter has a predicted auxin and several light-response elements, and so does SnRK1.1. The expression patterns of both genes in the light experiment (Figure 6) and auxin-transport inhibition experiment (Figure 12) show SnRK1.1 and SnRK1.2 may be responding and regulated by light and auxin differently, even though their promoters contain response elements for both stimuli. For example, even though both genes have light response elements (Figure 2) it is possible they are regulated by different types of light cues; SnRK1.2 may be regulated by the actual intensity of light (Figure 6), as seen through an expression increase under regular light conditions. This increase was not seen with SnRK1.1p:GUS transgenic plants, and therefore, I speculate that the SnRK1.1 gene is regulated by light in a different manner, and could be more strongly regulated by the absence of light completely. Total darkness could trigger stress-response programs, which could turn on SnRK1.1 gene expression. This is not an unreasonable speculation considering SnRK1.1 is the predominant stress sensor. In this case, SnRK1.1 expression may be more critical than SnRK1.2 because it is an integral stress sensor and extreme changes in light trigger stress-response programs (Terzaghi and Cashmore, 1995). Moreover, SnRK1.2's role in light regulation could be important on a smaller-scale, for less severe changes in light, or forms of stress. The absence of SnRK1.1 and SnRK1.2 gene expression in rosette leaves grown under high light was unexpected, however it suggests that both genes are down-regulated by the stressful intensity of light.

The idea that SnRK1.1 and SnRK1.2 genes may be regulated in some instances similarly,

but in others differently is supported by auxin-transport inhibition (Figure 12). As mentioned previously, both promoters contain predicted auxin response elements, suggesting that SnRK1.1 and SnRK1.2 may be regulated by auxin-transport, and further, may be important for auxin signaling in general. However, this is another case where SnRK1.1 and SnRK1.2 are responding to the same stimuli but in different ways. The NPA treatment (Figure 12) increased expression of SnRK1.1 and SnRK1.2 as compared to seedlings grown without NPA. This suggests as auxin transport is inhibited, expression of both genes is increased, particularly in shoot vascular tissues for SnRK1.2 and shoot non-vascular tissues for SnRK1.1. Root patterning also changes in response to NPA, however differently for each gene. SnRK1.2 spatial expression is limited to the root tip, while SnRK1.1 exhibits a build-up above the root up and decreases in areas of the root closer to the hypocotyl. This result suggests that the change in auxin transport has impacted gene expression of both SnRK1.1 and SnRK1.2. Yet, the patterns also suggest the genes may be impacted differently, and that plants use SnRK1.1 and SnRK1.2 function in tissues where auxin is transported.

In conclusion, the analysis of spatial gene expression patterns provides a fundamental understanding of SnRK1 gene regulation. SnRK1 genes are regulated at the transcriptional level by development, sugar, light, and auxin. Establishing this transcriptional profile adds the needed information to strengthen and support what is currently known about SnRK1.

CHAPTER III

OBJECTIVE II. To Develop and Characterize an anti-SnRK1.1 Antibody

INTRODUCTION

Despite the evidence of SnRK1.1's role in both cell signaling (Ananieva et al., 2008) and nutrient and stress sensing (Baena-Gonzalez et al., 2007; Jossier et al., 2009), there is no current understanding on how it is regulated *in vivo* or precisely how SnRK1.1 is functioning. Therefore, in order to study SnRK1.1 *in vivo*, I needed a way to monitor and detect endogenous SnRK1.1 from plant extracts. Thus, developing an antibody was necessary to establish a developmental profile of SnRK1.1 protein levels, to test whether SnRK1.1 changes in response to sugar (low and optimal nutrients), and to test whether SnRK1.1 changes in response to mutations in genes such as P80 or CUL4. In this study I purified recombinant SnRK1.1 from BL21-DE3pLysS competent cells using Ni^{2+} ion-metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). I found using an autoinduction media coupled with both IMAC and size exclusion yielded sufficient pure protein for antibody production. I later characterized the SnRK1.1 antibody using western blot analysis. The antibody was able to detect both recombinant and endogenous SnRK1.1 protein, as well as SnRK1.1:HA and SnRK1.1:GFP fusion proteins. Further, I show the antibody has immunoreactivity to post-translationally modified forms of endogenous SnRK1.1 protein. My results show that the SnRK1.1 antibody is a valuable tool for detecting recombinant, endogenous, and modified SnRK1.1 protein, as well as, SnRK1.1 fusion proteins. The SnRK1.1 antibody is an important molecular tool to study how and where SnRK1.1 is regulated *in vivo*

RESULTS

Recombinant SnRK1.1 protein can be purified

To express recombinant, His-tagged SnRK1.1 protein, I transformed SnRK1.1 plasmid DNA into BL21-DE3 pLysS competent cells. The plasmid contains an Ampicillin resistant marker and 6x-Histadine (His)-tag for isolation. Previous results (unpublished data) indicated poor expression and induction of SnRK1.1 using Luria broth (LB) and Isopropyl β-D-1-thiogalactopyranoside (IPTG). Therefore, I explored other options of protein expression and induction that would produce higher densities of bacterial cells. In collaboration with the Sobrado Laboratory of Virginia Polytechnic and State University, I decided to use autoinduction media to induce expression of SnRK1.1. Autoinduction media stimulates lactose-inducible promoters from plasmid DNA when bacterial cells are forced to use lactose, instead of glucose, as a carbon source for metabolism (Studier, 2005). Using ratios of low carbon to high lactose, in the media, induces expression of the T7 Polymerase without the addition of IPTG and, subsequently the desired recombinant protein is expressed (Studier, 2005).

I prepared an overnight starter culture containing SnRK1.1 BL21-DE3 pLysS and Ampicillin in LB. Autoinduction media was inoculated with 10 mls of overnight culture. After 24 hours, I harvested the cells and prepared the complete lysate for purification over a column packed with Ni^{2+}-NTA agarose resin. The Ni^{2+}-NTA resin was used to bind SnRK1.1:6x His in the supernatant. A 100 to 500 mM imidazole elution gradient was applied to the resin to elute SnRK1.1 from the column. SnRK1.1 eluted from the column between 100 mM and 200 mM of imidazole (Figure 13, lane 2 and 3). The expected size of SnRK1.1 with a 6x-His-tag is approximately 60 kD. Endogenous SnRK1.1 protein has a molecular weight of approximately 59 kD, and the tag adds another 1 kD. Initial results indicated successful expression and induction

of SnRK1.1 protein, however, higher and lower molecular weight containments were still present in the 100 mM elution fraction (Figure 13, lane 1).

Size exclusion chromatography (SEC) was used to eliminate the contaminants from the SnRK1.1 protein sample. The Sobrado laboratory helped with this procedure, supplying a S200 Superdex size exclusion column. I pooled eluates together, concentrated the protein sample and began SEC. Elution fractions with the largest peaks in the chromatagraph were mixed together and re-concentrated for SDS-PAGE analysis. The concentrated protein sample was analyzed and contained purified SnRK1.1 protein (Figure 14, lane 1). Through a Bradford Assay, I determined 500 µg of SnRK1.1 was isolated.

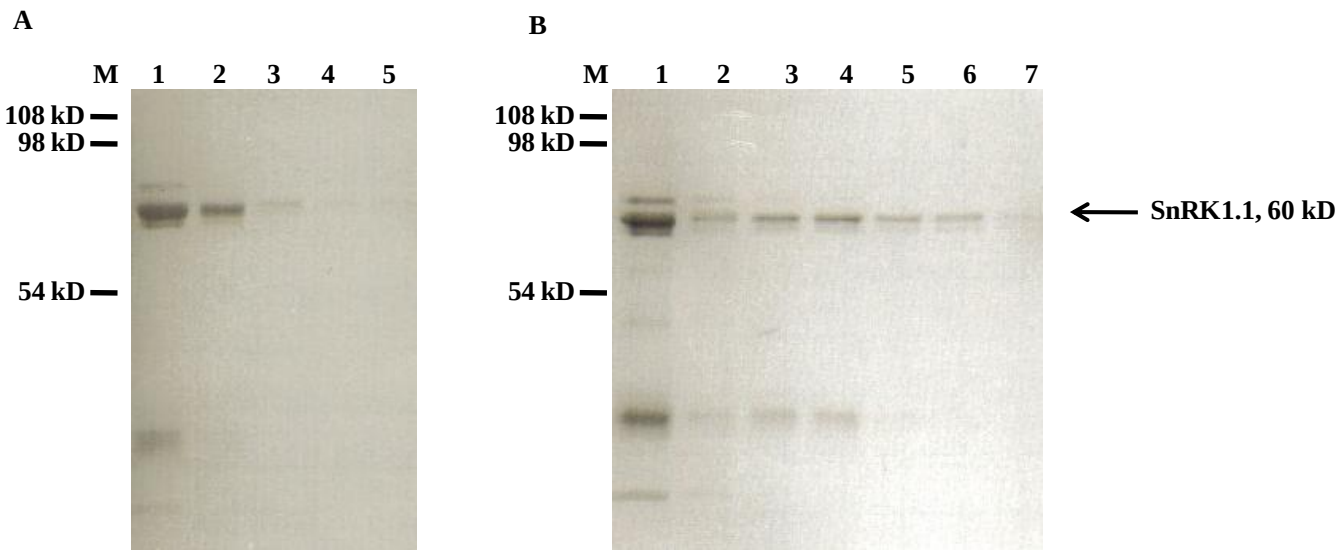


Figure 13. SnRK1.1 Protein Purification Elution Gradients. SnRK1.1 recombinant protein, expected 60 kD molecular mass, was purified by Ni^{2+}-ion-metal affinity chromatography (IMAC). (A) SnRK1.1 protein was eluted by a 100 mM to 500 mM imidazole elution gradient. Most SnRK1.1 eluted between 100 mM (Lane 1) and 200 mM (Lane 2) imidazole, with small amounts eluting at higher concentrations of imidazole, 300 mM, 400 mM, and 500 mM, respectively (Lanes 3, 4, and 5). (B) The 100 mM fraction from (A) was dialyzed, applied to Ni^{2+}, and eluted an imidazole gradient. Lane 1, SnRK1.1 protein before elution, Lanes 2-7 represent, 25, 50, 75, 100, 200, and 500 mM imidazole elution fractions, respectively.

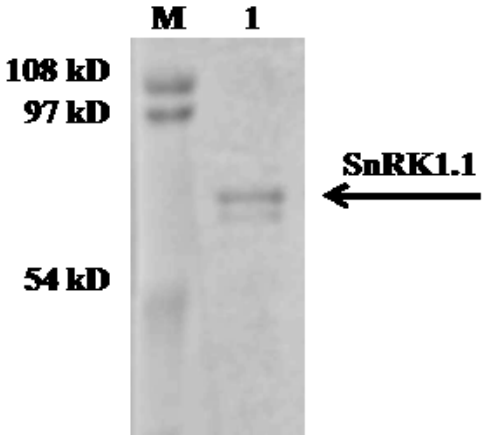


Figure 14. Purified Recombinant SnRK1.1 Protein. SnRK1.1 was expressed and purified by ion-metal affinity (IMAC) and size exclusion chromatographies. Lanes 1 contains the dialyzed and concentrated fraction after size exclusion chromatography. The fraction in Lane 1 was sent for antiserum production. Expected size of recombinant SnRK1.1 is 60 kD.

Anti-SnRK1.1 detects recombinant, endogenous, and SnRK1.1 fusion proteins

The purified SnRK1.1 protein was injected into a rabbit (by CoCalico Laboratory Inc.), resulting in an anti-SnRK1.1 polyclonal antibody. The antiserum was characterized by western blot analysis. I performed several western blots with different concentrations of anti-SnRK1.1 in blots containing recombinant and endogenous, native SnRK1.1 protein. The crude anti-serum was best at a 1:10,000 dilution. However, over time the antibody began to degrade, and needed to be purified. Initially, I tried purifying the crude-antiserum by affinity chromatography using purified, recombinant SnRK1.1 protein. But, this approach resulted in a loss of sensitivity to endogenous, native SnRK1.1.

In collaboration with Janet Donahue, lab technician, anti-SnRK1.1 was purified on protein blots containing recombinant SnRK1.1 (described Harlow and Lane, 1988). After this procedure the antiserum detected endogenous protein from wildtype and SnRK1.1:HA plant extracts at a dilution of 1:1,500 (Figure 15A, lanes 1 and 2). However, no cross-reactivity was seen from previously characterized SnRK1.1 RNAi knock-down lines, *10-1* and *10-2* (Figure 15A, lanes 3 and 4) (Baena-Gonzalez et al., 2007). The transgenic plant line, SnRK:HA, which overexpresses SnRK1.1 protein fused to a HA-epitope tag was used as a positive control. The expected molecular weight of SnRK1.1:HA is 60 kD. I used the anti-HA antibody to detect SnRK1.1:HA protein from the SnRK:HA plant extract (Figure 15B), and compared the results to my anti-SnRK1.1 blot. The western shows anti-SnRK1.1 and anti-HA are detecting similar-sized proteins around the expected mass of 60 kD. Interestingly, SnRK1.1:HA protein displays a characteristic shift-up (Baena-Gonzalez et al., 2007) when compared to wildtype endogenous SnRK1.1 protein. This result is consistent with previously described results (Baena-Gonzalez et al., 2007).

I wanted to characterize the anti-SnRK1.1 antibody further and explore if it could detect other SnRK1.1 fusion proteins. I made extracts from wildtype and transgenic plants expressing SnRK1.1:GFP. I probed the extracts with both anti-GFP and anti-SnRK1.1 antibodies (Figure 16). My results show that anti-GFP only detects SnRK1.1 fused to the GFP-tag with a immunoreative band at the expected 86 kD molecular weight (Figure 16A, lane 2). However, anti-SnRK1.1 detects both SnRK1.1:GFP around 86 kD and native SnRK1.1 protein around its expected molecular mass (59 kD) (Figure 16B, lane 2).

My results confirm the anti-SnRK1.1 antiserum is a usable tool to detect both recombinant SnRK1.1 protein, as well as endogenous and SnRK1.1 fusion proteins. This will be useful to analyze SnRK1.1 steady-state protein levels *in vivo*.

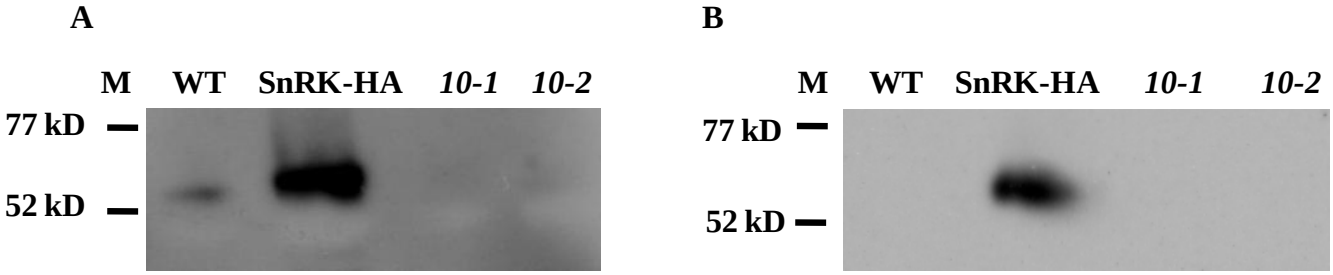


Figure 15. Immunoreactivity of anti-SnRK1.1 with native SnRK1.1. Plant extracts were made from WT LER, SnRK-HA, and RNAi knock-down lines *10-1* and *10-2* plants. Protein blots were probed with 1:1,500 purified anti-SnRK1.1 or 1:2,500 anti-HA. The SnRK-HA transgenic plant line overexpresses SnRK-HA tagged protein and was used as a positive control. *10-1 and 10-2* extracts were negative controls. (A) Shows detection of endogenous SnRK1.1 proteins probed with anti-SnRK1.1. (B) Shows detection of HA-tagged SnRK1.1 with the anti-HA antibody.

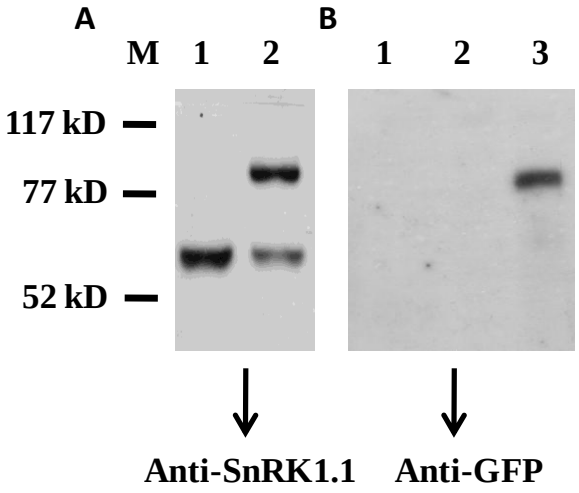


Figure 16. Immunoreactivity of anti-SnRK1.1 with GFP fusion proteins. Wildtype and transgenic plants expressing SnRK1.1:GFP were probed with anti-SnRK1.1 and anti-GFP. (A) Anti-SnRK1.1 was used in a protein blot containing wildtype (Lane 1) and transgenic (Lane 2) plant extracts. (B) Anti-GFP probed wildtype (Lane 1), SnRK-HA (Lane 2), and SnRK1.1:GFP (Lane 3) plant extracts.

Anti-SnRK1.1 detects post-translationally modified forms of SnRK1.1

In order to establish if SnRK1.1 is or can be post-translationally modified (PTM). To address this, I probed plant extracts with anti-pAMPK (Thr172) used by Jossier et al. and Baena-Gonzalez et al., and the anti-SnRK1.1 antibody. Using these antibodies I hypothesized I could detect whether SnRK1.1 could be phosphorylated *in vivo*, and if the anti-SnRK1.1 antibody could detect these forms of SnRK1.1 protein.

First, I incubated a wildtype plant extract with lambda phosphatase (λPP) to remove phosphate groups from SnRK1.1. I probed the extract with the anti-pAMPK (Thr172) antibody to determine the phosphorylation patterns before and after λPP incubation (Figure 17A). The anti-pAMPK antibody is used to detect phosphorylated forms of the mammalian AMPK (Hawley et al., 1996). Due to phylogenetic homology between AMPK and SnRK1.1, the antibody can be used to detect phosphorylated SnRK1.1 from *Arabidopsis* on threonine residue 175 (Jossier et al., 2009). The expected size of phosphorylated SnRK1.1 is approximately 59 kD. The blot shows immunoreactive bands in the wildtype extract (Figure 17B, lane 1) before λPP incubation, and a decrease of the same band after incubation. This result shows that native SnRK1.1 can be phoshorylated, as these immunoreactive bands are similar to bands seen when extracts are probed with anti-SnRK1.1 (data not shown). However, there are two bands present in the protein blot. These bands could be two different SnRK1.1 splice variants, or SnRK1.2 protein that is phosphorylated. From my data, I cannot definitively identify each band, only that they are SnRK1 proteins.

My results show SnRK1.1 is post-translationally modified *in vivo*. I found I could detect phosphorylated SnRK1.1, and confirmed these results using the anti-SnRK1.1 antibody as a

control. This result provides a foundation for addressing how SnRK1.1 is regulated at the translational level *in vivo*.

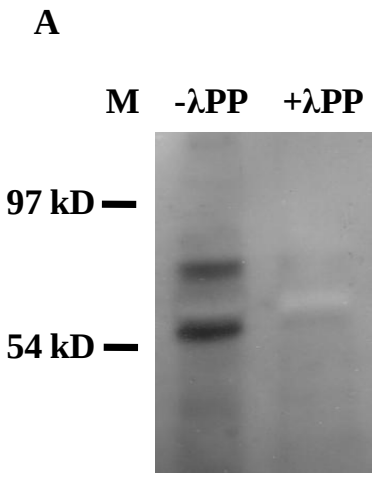


Figure 17. Lambda Phosphatase Assay. Wildtype plant extracts were incubated with (Lane 2) or without (Lane 1) lambda phosphatase enzyme. The protein blot was probed with 1:500 anti-pAMPK (Thr 172).

DISCUSSION

SnRK1.1 belongs to a sub-family of serine/threonine protein kinases that are evolutionarily conserved to mammalian AMPK and yeast Snf1 (Halford and Hey, 2009). This relationship provided the first insight to SnRK1.1's role in nutrient and stress sensing in plants (Baena-Gonzalez et al., 2007; Jossier et al., 2009). However, despite its link to nutrient and stress responses, and metabolic reprogramming, there is little data showing where and how SnRK1.1 is regulated.

I purified SnRK1.1 in order to help me obtain a useful tool to study SnRK1.1 *in vivo*. I conclude the SnRK1.1 antibody is a valuable tool to detect endogenous and fusion proteins of SnRK1.1 proteins *in vivo*. I also believe it can be used to confirm any post-translational modifications. Using the anti-pAMPK(Thr172) antibody revealed SnRK1.1 is phosphorylated. Which is not unlikely considering SnRK1.1 has been implicated as an important molecule for networks of cell signaling reactions dependent on phosphorylation and dephosphorylation reactions (Halford and Hey, 2009), especially those in response to nutrient availability and stress (Baena-Gonzalez et al., 2007; Halford et al., 2003; Jossier et al., 2009).

The anti-Ub antibody indicated SnRK1.1 may be ubquitinated as well (data not shown). While the ubiquitination results are preliminary, it is likely SnRK1.1 can be ubiquinated because previous studies show SnRK1.1 is regulated by the proteasome (Ananieva et al., 2008). If SnRK1.1 is regulated by the proteasome, I would expect it can be ubquitinated. However, more work still needs to be done to address this aspect. Specifically, there is no evidence supporting an *in vivo* complex containing SnRK1.1 and any member of the CUL4 complex and, or proteasome. However, there are *in vitro* interactions (Ananieva, 2009)which support the investigation of an *in*

vivo complex. Further, using antibodies to show steady-state protein levels of SnRK1.1 in the presence of proteasome inhibitors, like MG132, would offer valuable data.

CHAPTER IV

OBJECTIVE III: To Characterize How and Where SnRK1.1 is Regulated *in vivo*

INTRODUCTION

The AMP-activated protein kinase (AMPK) and sucrose non-fermenting kinase 1 (Snf1) are essential for metabolic balance in mammals and yeast, respectively. For mammals, AMPK also regulates signaling cascades fundamental to growth and development, intracellular calcium release, cell-cycle control, and apoptosis (Bright et al., 2009; Hardie, 2008). Similarly, plants have SnRK1 kinases, which are strongly homologous to both AMPK and Snf1 (Halford and Hey, 2009), and have been shown to be just as important for cell signaling and metabolism (Baena-Gonzalez et al., 2007; Jossier et al., 2009). For example, plants overexpressing SnRK1 have increased biomass and lifespan, correlating the function of SnRK1 to metabolic phenotypes. However, little is known on how or where SnRK1 proteins are regulated *in vivo*. But, there are implications that one SnRK1 protein, SnRK1.1, could interact with members of the CUL4 complex and, or the proteasome; providing some insight on SnRK1.1 *in vivo* regulation (Ananieva, 2009).

Previous data show a novel Arabidopsis protein, P80, is homologous to mammalian proteins involved in the proteasomal degredation. Specifically, P80 proteins contain a DWD domain which is speculated to interact with Cullin4 E3 Ubiquitin Ligase complex (CUL4 complex), as proteins containing DWD domains have been found to act as substrate receptors in the CUL4 complex (Higa et al., 2006; Higa and Zhang, 2007; Lee et al., 2008). Additionally, P80 is also homologous to mammalian proteins that form complexes with deubquitinating enzymes, like the factor UAF1 (Cohn et al., 2009). Deubiquinating enzymes remove ubiquitin modifications on proteins, protecting them from proteasomal degradation (Hurley and Stenmark,

2010; Kessler and Edelmann, 2011). How P80 precisely functions to either target or protect proteins from the proteasome is unclear. Keeping in mind its relationship to 5PTase13 and inositol signaling (Ananieva et al., 2008), P80 might function to regulate proteins via the proteasome in responses to nutrients and stress. Given SnRK1.1 is paramount in these types of responses and interacts with P80 *in vitro* (Ananieva, 2009), investigating SnRK1.1 steady-state protein levels in *p80* and co-suppressed *cul4* mutants (*cul4(cs)*) mutants plants would accomplish two major goals: one, characterize how SnRK1.1 is regulated at the translational level *in vivo* and two, bring more insight to how key signaling molecules, like P80, may function in response to nutrients or stress. While, my focus is on SnRK1.1, my research is still fundamental to understanding the P80 component.

Using the anti-SnRK1.1 antibody, I show SnRK1.1 steady-state protein levels change during development, in response to sugar, and in *p80* and *cul4(cs)* mutant plants. My data also identifies where SnRK1.1 is regulated in wildtype and *p80* plants using 35S:SnRK1.1:GFP fusion proteins.

RESULTS

Mutant and RNAi knock-down plants have smaller biomass

I used several plant mutant and transgenic lines to investigate phenotypic changes. These plants are *p80* and *cul4(cs)* mutants, SnRK1.1 RNAi knock-down plants (*10-1* and *10-2),* and the SnRK1.1 overexpresser (SnRK:HA) (Ananieva, 2009; Baena-Gonzalez et al., 2007; Zhang et al., 2008). Previous work reported that SnRK1.1 overexpressers have increased life-span (Baena-Gonzalez et al., 2007) and increased biomass as compared to wildtype plants (unpublished data, Gillaspy Lab). I scored growth phenotypes from plants grown on soil (Figure 18) and agar plates, supplemented with 0.5x MS or 0.5x MS + 3% sucrose (Figure 19), under low light.

At 7 and 15 days, *p80* and *cul4(cs)* mutants are smaller than wildtype plants (Figure 18A and B). This trend continues as the mutant plants mature, and show decreased biomass compared to wildtype in mature rosettes (Figure 18C). My results indicate light may also impact the phenotypes of the mutant lines, as previous data shows increased senescence and altered leaf development under regular light conditions (Ananieva, 2009). SnRK1.1 knock-down lines are also smaller than wildtype at 7 and 15 days, as well as in mature plants (Figure 18). However, unexpectedly SnRK:HA plants have decreased biomass as compared to wildtype plants at earlier stages of development (Figure 18A and B). But, as development continues, mature SnRK:HA rosettes have more biomass than wildtype (Figure 18C).

Seedlings grown on plates, however, presented different phenotypes dependent on sugar. Both *p80* and *cul4(cs)* mutant seedlings have altered leaf development, color, and biomass (Figure 19) on plates only supplemented with 0.5x MS, or low nutrients. These phenotypes are consistent with phenotypes seen in soil-grown plants (Figure 18). But mutant seedlings

germinated on 0.5x MS + 3% sucrose (optimal nutrients) did not exhibit the same dramatic phenotypes as seedlings grown on low nutrients (Figure 19). In fact, these seedlings looked very similar to wildtype plants (Figure 19).

My observations of these phenotypes in both soil grown plants and seedlings germinated on plates may indicate changes in SnRK1.1 function and regulation within *p80* and *cul4(cs)* mutants. Seedlings grown on plates containing sucrose may have rescued phenotypes because of changes in SnRK1.1, a known sugar and energy sensor in plants, which is activated by sugars (Halford et al., 2003; Jossier et al., 2009).

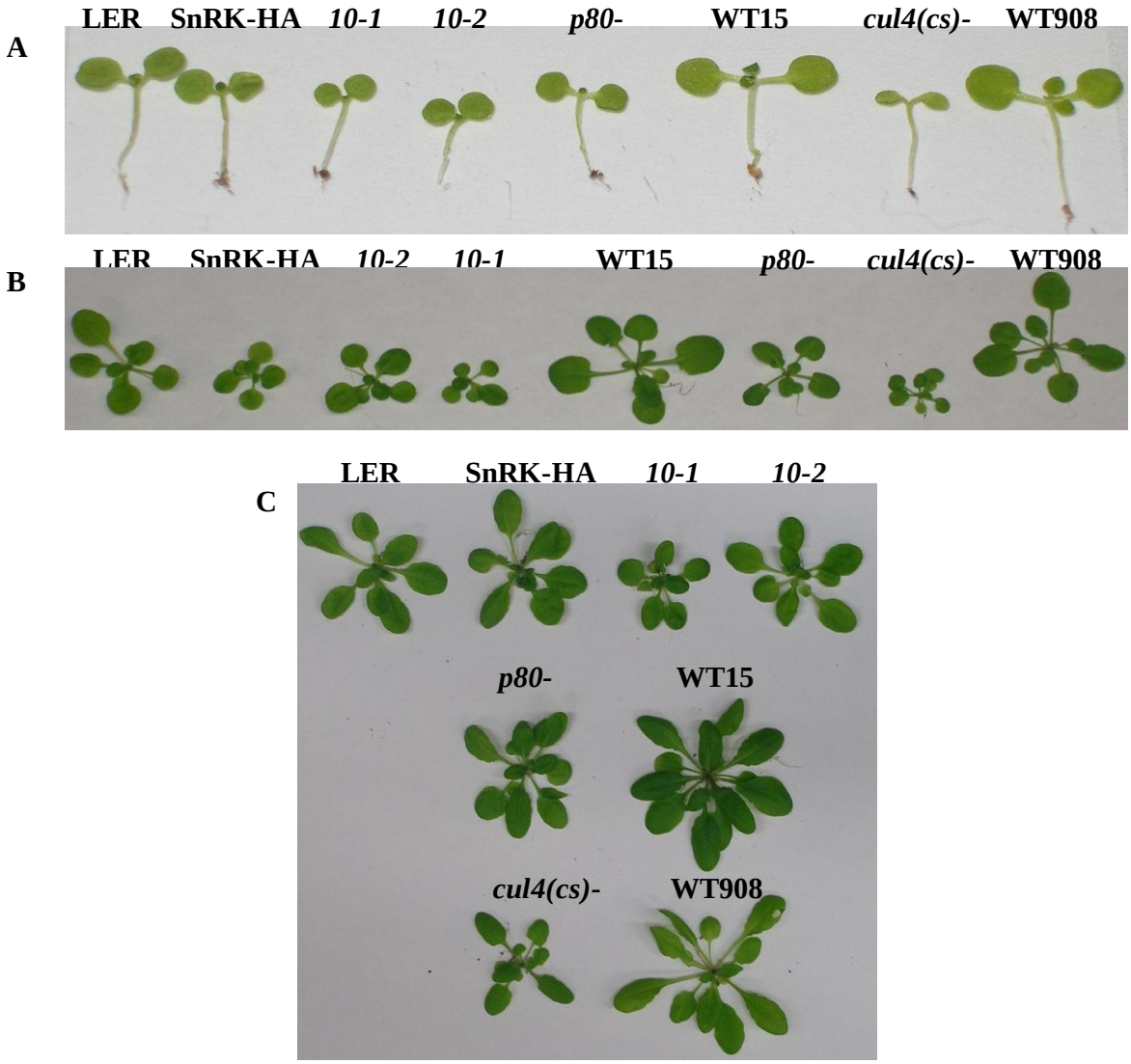


Figure 18. Plant Phenotypes. (A) Illustrates differences in biomass phenotypes of 7-day-old seedlings, (B) 15-day-old seedlings, and (C) mature plants in soil grown under low light. *Landsberg erecta* (LER), and *Columbia* WT15 and WT908 are wildtype plants, SnRK-HA overexpresses SnRK:HA protein, *10-1* and *10-2* are SnRK1.1 RNAi knock-down lines, and *p80* and *cul4(cs)* are mutant plants.

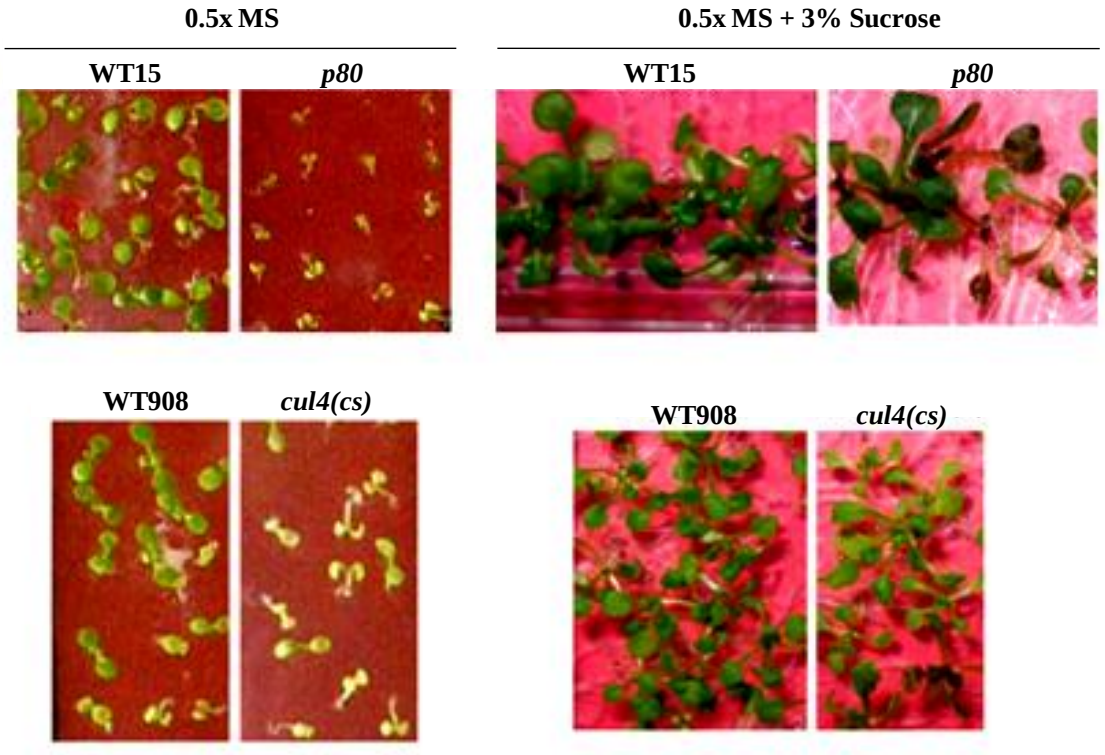


Figure 19. The Effect of Sucrose on Wildtype and Mutant Phenotypes. Wildtype and *p80* and *cul4(cs)* mutant seedlings were grown on 0.5x MS or 0.5x MS + 3% sucrose for 15 days. WT15 and WT908 are age-matched with *p80* and *cul4(cs)*, respectively.

SnRK1.1 steady-state protein in development

Before characterizing how SnRK1.1 may be altered in mutant plants, I wanted to establish a profile in wildtype plants to see how steady-state protein levels were inherently changing over development. I grew wildtype plants in soil and harvested material at different stages in development. Using western blotting and the anti-SnRK1.1 antibody, I was able to characterize how steady-state protein levels change as a result of development (Figure 20A). Moreover, I correlated this with the presence of phosphorylated SnRK1.1 by using the phospho-specific AMPK antibody (p-AMPK) (Figure 20B).

SnRK1.1 protein is expressed at all stages of development except for senescent leaves and siliques (Figure 20A), where SnRK1.1 protein was not detected. To show if sugar increases steady-state protein levels, I included a 15-day-old extract from seedlings grown on 0.5x MS + 3% sucrose. In comparison to 15-day-old seedlings grown in soil, I detected more SnRK1.1 protein in seedlings germinated on this added sugar source (Figure 20A). SnRK1.1 protein levels were highest in bolts, flowers, and seeds, and comparable in 7-, 15-, and 21 day-old plants. For some points in development, two bands appeared on the blot. These occurred at 7 days, 21 days, bolts, and flowers, and when 15-day seedlings were grown with 3% sucrose. The extra band could result from a noval SnRK1.1 isoform (a different splice variant of the SnRK1.1 gene), SnRK1.2, or a post-translationally modified form of either (Appendix, Table 2).

The phosphorylation state of SnRK1.1 changes in response to development as well. Using anti-pAMPK I probed the same plant extracts to establish how SnRK1.1 protein may be post-translationally modified (Figure 20B). My results show that there is detectable phosphorylation of SnRK1.1 proteins in most plant tissues, except for senescent leaves (Figure

20B). Further, most phosphorylation occurs at 15 days in seedlings grown on sucrose, 21 days and in seeds (Figure 20B).

I conclude that SnRK1.1 steady-state levels change as the plant ages, and energy demands are shifted from tissue to tissue. Also, the phosphorylation state of SnRK1.1 is changing in response to developmental regulation.

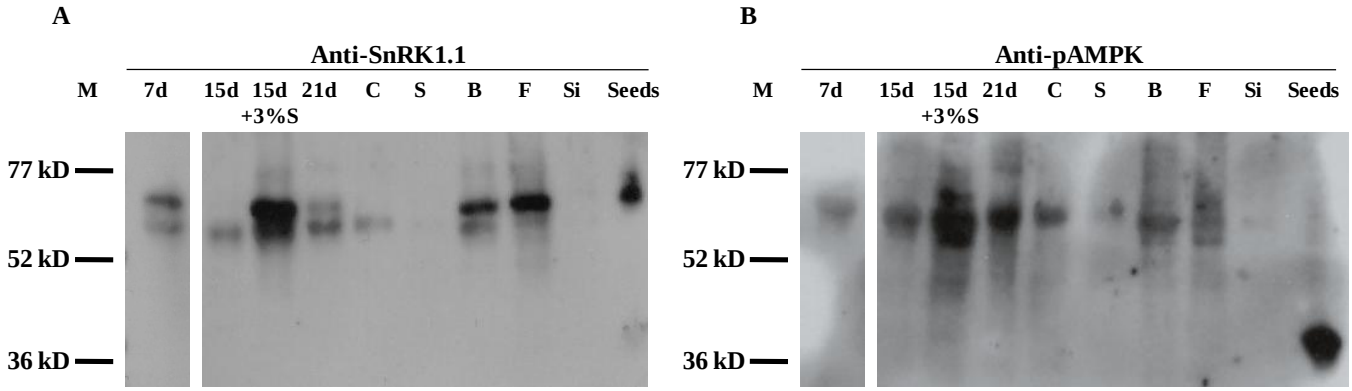


Figure 20. Developmental Steady-State SnRK1.1 Protein and Phosphorylation. (A) Protein blot containing wildtype plant extracts were probed with 1:1,500 anti-SnRK1.1 (B) Protein blot containing wildtype plant extracts were probed with 1:500 anti-pAMPK. (A and B) Wildtype plants were grown in soil under low light and harvested at 7 days (Lane 2), 15 days (Lane 3 and 4)*, and 21 days (Lane 5). Cauline leaves (Lane 6), senescent leaves (Lane 7), bolts (Lane 8), flowers (Lane 9), immature siliques (Lane 10), and seeds (Lane 11) were also collected for analysis. (*) Lane 4 contains 15-day-old seedlings grown on agar plates supplemented with 0.5x MS + 3% sucrose.

SnRK1.1 steady-state protein in p80 and cul4(cs) mutants

Both *p80* and *cul4(cs)* mutant phenotypes could be explained by changes in SnRK1.1 or SnRK1.1 availability. First, I hypothesized if P80 and CUL4 proteins regulate SnRK1.1 via the proteasome, then *p80* or *cul4(cs)* mutant plants should have increased steady-state levels of SnRK1.1 protein, as SnRK1.1 protein would be unable to turn over via the proteasome. Second, I wanted to see if nutrient availability altered SnRK1.1 protein levels, in either mutant, because phenotypically *p80* and *cul4(cs)* mutant biomass becomes similar to wildtype in the presence of 3% sucrose (Figure 19).

I examined SnRK1.1 steady-state protein levels in 7- (Figure 21A) and 15-day-old (Figure 21B) in wildtype, *p80*, and *cul4(cs)* mutant seedlings germinated on either 0.5x MS or 0.5x MS + 3% sucrose. At both 7 and 15 days, wildtype seedlings grown on 0.5x MS (low nutrients) have more SnRK1.1 protein than either mutant (Figure 21A and B). Specifically, in these developmental and nutrient conditions, SnRK1.1 is decreased in both *p80* and *cul4(cs)* seedlings as compared to wildtype. This result is consistent with phenotypic data (Figure 18) and the hypothesis that SnRK1.1 controls factors leading to biomass accumulation. However, results under low nutrient conditions were inconsistent with the idea that P80 and CUL4 target SnRK1.1 for proteasomal degredation in that SnRK1.1 protein was not elevated in these mutants under these conditions. In contrast, I did find elevated SnRK1.1 protein in 7-day-old *cul4(cs)* seedlings grown on 3% sucrose, with smaller increase in *p80* mutants (Figure 21A). At 15 days, extra sugar does not change the levels of SnRK1.1 between wildtype and mutant seedling (Figure 21B), as SnRK1.1 protein levels are equal among mutant and wildtype extracts. I conclude that SnRK1.1 protein accumulates in 7-day-old *p80* and *cul4(cs)* seedlings grown on sucrose, but not

on low nutrients. Further, at 15 days sucrose has no effect on SnRK1.1 protein accumulation in either mutant, and on low nutrients mutants have a decrease in SnRK1.1 protein.

The phosphorylation of SnRK1.1 is different in wildtype and mutant seedlings; however, this is dependent on nutrient availability. In both 7-day and 15-day-old seedlings, wildtype plant extracts have more phosphorylated SnRK1.1 than either *p80* or *cul4(cs)* mutants (21A and B) grown on low nutrients. This is also seen in 15-day-old seedlings grown on optimal nutrients (Figure 21B). But it does not appears sucrose has an effect on the phosphorylation of SnRK1.1 in 7-day-old seedlings, as levels of phosphorylated SnRK1.1 are comparable to total levels of SnRK1.1 in all seedling extracts (Figure 21A). I conclude SnRK1.1 is phosphorylated differently in wildtype and mutant plants under low nutrient conditions in both 7 and 15-day-old seedlings, but the pattern and degree of phosphorylation is the same between wildtype and mutant plants under optimal nutrients (Figure 21A and B). Thus, for the most part, the phosphorylated SnRK1.1 detected with the anti-pAMPK antibody mirrors the pattern of SnRK1.1 detected with the anti-SnRK1.1 antibody.

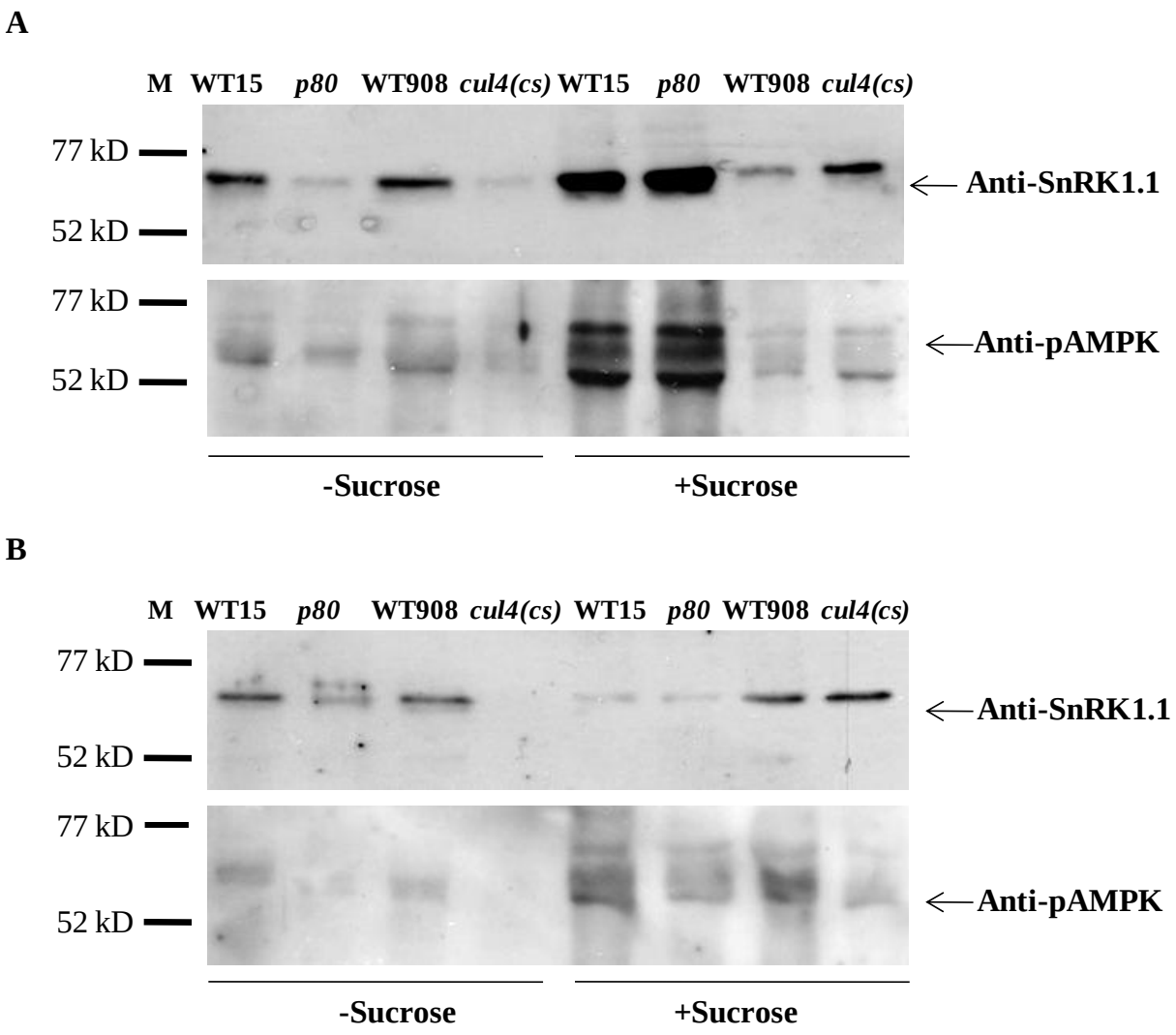


Figure 21. SnRK1.1 steady-state protein levels and phosphorylation in wild-type and mutant seedlings. (A) **7-** or (B)15-day-old wildtype (WT15 and WT908) and *p80* or *cul4(cs)* mutant seedlings grown on 0.5x MS (-Sucrose) or 0.5x MS + 3% sucrose (+Sucrose). (A and B) Extracts were probed with anti-SnRK1.1 (upper blot) or anti-pAMPK (lower blot). WY15 is a wild-type line age-matched and contains the same ecotype background as *p80* mutant plants. Similarly, WT908 is matched with the *cul4(cs)* mutants.

SnRK1.1:GFP localization in p80 mutant plants

In order to better understand where SnRK1.1 is regulated within seedlings, I examined 35S:SnRK1.1:GFP fluorescence in wildtype, as well as *p80* mutant backgrounds. If P80 helps target SnRK1.1 for proteasomal destruction, then I expected I would see in increase in SnRK1.1:GFP fluorescence in a *p80* mutant seedling *in vivo*. And, that fluorescence intensity would change depending on nutrients. For this experiment, I used transgenic plants constructed and characterized by other members of the Gillaspy Lab.

I grew seedlings on agar plates supplemented with 0.5x MS (low nutrients) or 0.5x MS + 3% sucrose (optimal nutrients). Using a Zeiss microscope equipped with fluorescence optics, I was able to detect increased SnRK1.1:GFP fluorescence in *p80* seedlings as compared to wildtype in seedlings grown on low or optimal nutrients. I probed extracts from these seedlings with anti-GFP to confirm SnRK1.1:GFP expression in both transgenic lines (Figure 22B). The blot revealed SnRK1.1:GFP protein is present in both transgenic lines and that SnRK1.1:GFP protein accumulates about 100 fold in SnRK1.1:GFP/*p80* seedlings (Figure 22B). The accumulation of SnRK1.1:GFP protein in the mutant background was consistant with the increased fluorescence I saw using microscopy.

I collaborated with another lab member to use confocal imaging to refine my results. Confocal imaging revealed SnRK1.1:GFP fluorescence is greater in the *p80* mutant background (Figure 22A). SnRK1.1:GFP is elevated in cotyledons, root vascular tissue, and root tip cells of *p80* mutants (Figure 22A). In the wildtype background, fluorescence is restricted to certain areas of the vascular structure in the root and the root tip (Figure 22A), and fluorescence is less intense when compared to the fluorescence in *p80* mutants (Figure 22A). However, I was unable to

conclude from confocal microscopy whether the intensity of fluorescence or subcellular localization of SnRK1.1:GFP changes in response to nutrients.

In preliminary work, I have tested whether native SnRK1.1 protein changes in the SnRK1.1:GFP transgenic plants or if SnRK1.1:GFP accumulates differently depending on nutrients. I incubated protein blots containing extracts of SnRK1.1:GFP in the wildtype and *p80* mutant backgrounds with anti-SnRK1.1 and anti-GFP (Figure 23). As in previous western blots, I detected native SnRK1.1 with anti-SnRK1.1, which migrated slightly above the 52 kD marker (Figure 23). Interestingly, I also detected a band whose migration is consistent with the predicated molecular mass of SnRK1.1:GFP (87 kD) (Table 2). The SnRK1.1:GFP was only present when SnRK1.1:GFP was expressed in the wildtype background. To confirm the identity of this band, I also incubated a similar blot with an anti-GFP antibody (Figure 23, left panel). The anti-GFP antibody cross-reacted with a protein consistent with being SnRK1.1:GFP. The SnRK1.1:GFP was greatly elevated in *p80* mutants as compared to wildtype. Thus, my anti-SnRK1.1 antibody appears to be incapable of recognizing SnRK1.1:GFP when expressed in the *p80* mutant background. In addition, SnRK1.1:GFP protein expression may be elevated when seedlings are grown on sucrose (Figure 23).

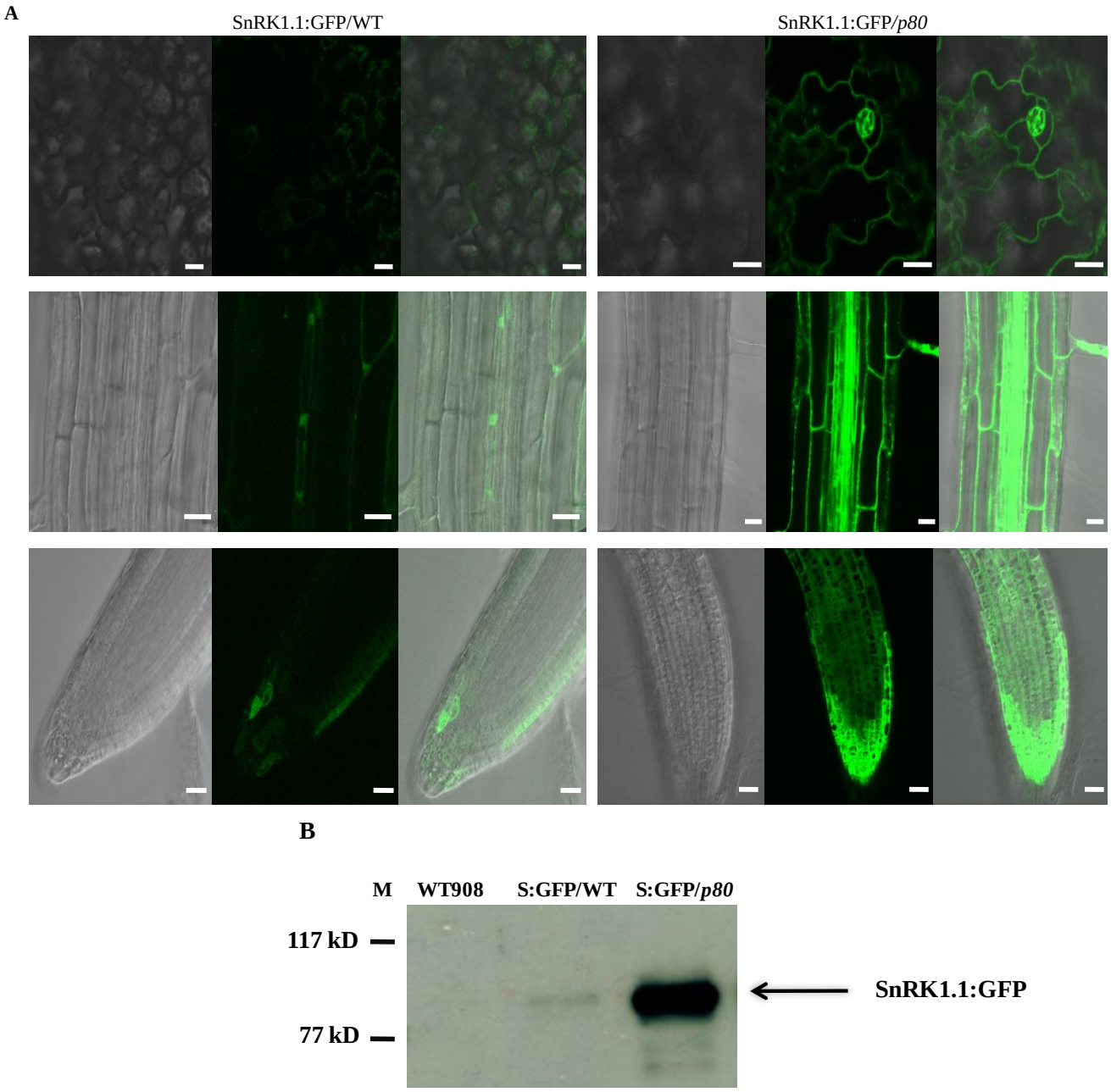


Figure 22. SnRK1.1:GFP Fluorescence and SnRK1.1:GFP Protein accumulation *in vivo*. (A) Confocal imaging of SnRK1.1:GFP fluorescence in wildtype and *p80* mutant backgrounds. (B) SnRK1.1:GFP protein in wildtype and *p80* mutant plant extracts. WT908 plant extracts contain no SnRK1.1GFP fusion proteins. Top panels represent fluorescence in cotyledons, middle panels in roots, and lower panels in root tips. Expected size of SnRK1.1:GFP is approximately 86 kD. Scale bars = 200 µm

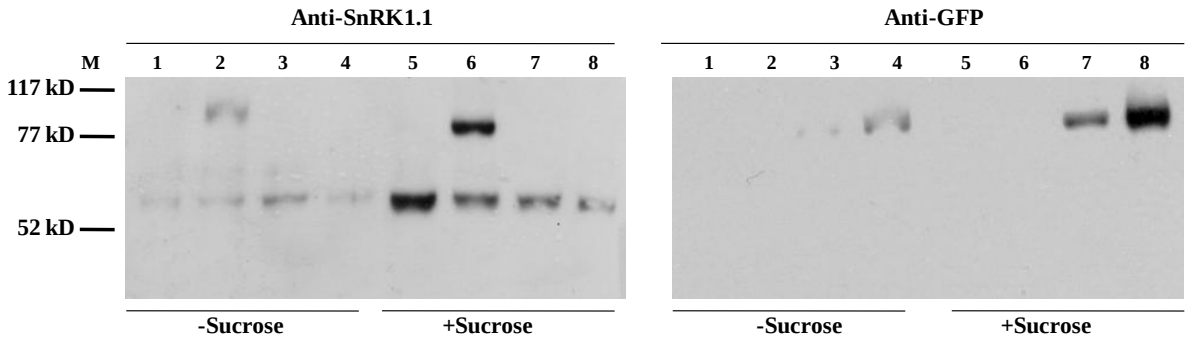


Figure 23. Native SnRK1.1 versus SnRK1.1:GFP detection by anti-SnRK1.1 and anti-GFP antibodes. (Left panel) Wildtype and *p80* plant extracts grown ± 3% sucrose were probed with anti-SnRK1.1. (Right panel) The same extracts were incubated with the anti-GFP antibody. Expected sizes of native SnRK1.1 and SnRK1.1:GFP protein are 59 and 86 kD, respectively.

DISCUSSION

Addressing how steady-state SnRK1.1 protein levels are regulated is important to understand SnRK1 function in nutrient and stress sensing. Changes in SnRK1.1 have been linked to changes in biomass of plants, so one goal of this study was to determine whether specific mutants, with altered growth and biomass phenotypes, had SnRK1.1 alterations.

My data suggest SnRK1.1 protein may be regulated by developmental cues from the plant. It is also likely that as the plant ages, the energy-demands of tissues change (Rolland et al., 2006), and this could be reflected by changes in SnRK1.1 protein accumulation. For example, at the time reproductive bolts and flowers were harvested, the plant probably has put most of its energy into reproduction. Therefore, those tissues may have a higher energy status and consequently, higher amounts of SnRK1.1 protein. This is consistent with reports defining SnRK1.1 as being activated by sugars (Jossier et al., 2009).

One of the most interesting conclusions was the nutrient dependent differences in SnRK1.1 protein accumulation in both *p80* and *cul4(cs)* mutants (Figure 21). It appears under different nutrient conditions, that SnRK1.1 is regulated by P80 and CUL4 proteins differently. SnRK1.1 protein increased in mutant seedlings as compared to wildtype when the seedlings were young and grown on optimal nutrients. However, this was not the case when the seedlings did not have extra sugar as a nutrient source. This nutrient regulation of steady-state SnRK1.1 protein levels helps shed light on the mechanisms that regulate SnRK1.1 *in vivo*. For example, under optimal nutrients SnRK1.1 may be activated by exogenous sucrose (Jossier et al., 2009). In this situation, the plant may produce more SnRK1.1 protein than is needed, and therefore, molecules important for proteasome regulation (P80 and CUL4) degrade SnRK1.1. This idea is consistent with steady-state protein levels of mutants grown on optimal nutrients. In the absence

of these genes, SnRK1.1 protein is accumulating (Figure 21). But, under low nutrient conditions, the correlation between the compromised growth and biosmass of *p80* and *cul4(cs)* mutants and reduction in SnRK1.1 protein, suggest plants use SnRK1.1 as a nutrient and stress sensor to increase growth.

I propose that there are two different mechanisms to regulate SnRK1.1, one for low nutrients and one for optimal nutrients. In addition, I propose that these mechanisms change based on the developmental age of the plant. Thus, regulation of steady-state SnRK1.1 protein levels may be dependent on the exact developmental state and the nutrient status of that tissue and the overall plant.

CHAPTER V

FUTURE DIRECTIONS

My results complement the current view of SnRK1.1 as a high energy sensor (Halford et al., 2003; Jossier et al., 2009), and offers a stronger understanding of its regulation at the level of transcription and translation. My data suggest SnRK1.1 and SnRK1.2 genes are regulated by development, sugar, light, and auxin. Further, I have shown that SnRK1.1 steady-state protein levels are regulated by development, nutrient status, and by signaling molecules such as P80 and CUL4. In conclusion, I propose SnRK1.1 regulation is dynamic, and dependent on the timing of particular cues from development and the environment. However, as with much research, many new questions are unanswered. The following questions could be addressed using the knowledge gained and tools developed from my research.

What is the doublet seen on western blots?

In some of my western blots there is a protein doublet around the expected size of SnRK1 proteins (CHAPTER III and IV). I have speculated that the identity of these bands could either be SnRK1.1 and SnRK1.2 protein, different SnRK1.1 proteins (from differential splicing variants), or post-translationally modified forms of SnRK1.1 protein. I think it would be very important to determine the identity of these bands. Ideally, it would be best to have SnRK1.2 knock-down or knockout plants. Probing either of these plant extracts would hopefully determine if either of the bands are SnRK1.2 protein. From the tools available now, I would use anti-SnRK1.1 to immunoprecipitate (IP) SnRK1 proteins from plant extracts. Run these plant extracts on a gel and use mass spectrometry to identify the protein bands. Also, I think it would be interesting to determine the steady-state SnRK1.1 protein in either *10-1* or *10-2* plants. Plant

from different tissues in development could be probed with anti-SnRK1.1 (like I did for wildtype plants in CHAPTER IV). This result could be compared with the developmental western (CHAPTER IV). Theoretically, the *10-1* or *10-2* plant extracts should not contain SnRK1.1 protein and my results indicate that anti-SnRK1.1 does not cross react with any SnRK1.1 protein in these extracts (CHAPTER III). However, if the bands in the doublet are only SnRK1.1, then the developmental western on the knock-down plants should not contain any bands. But, if the doublet contains SnRK1.2 protein, then I would expect only one band to remain in the doublet.

How does auxin polar transport regulate SnRK1.1 and SnRK1.2?

The spatial expression patterns of SnRK1.1p:GUS and SnRK1.2p:GUS in response to NPA is interesting (CHAPTER II). This result implicates a new role for SnRK1, and would be a new perspective on the SnRK1 story. I would repeat GUS assays on seedlings to look at spatial gene expression patterns of both SnRK1.1 and SnRK1.2 in response to auxin and NPA. I also think it would be interesting to grow seedlings on plates without treatment, and transfer them to plates with either NPA or auxin to identify how the patterns change during a short term stimulus. Quantifying these responses using Q-PCR would be useful as well.

The presence of auxin response elements in the SnRK1 promoters suggests that the SnRK1 protein may be important for auxin signaling in plants. To address this, I would want to further characterize SnRK:HA, *10-1*, and *10-2* plant phenotypes in response to auxin, such as changes in root length or biomass. These experiments could address how the gain or loss of the SnRK1 gene impacts auxin signaling. I would grow seedlings on plates supplemented with either auxin or NPA to assess for these phenotypes.

To study SnRK1.1 protein in response to auxin, I would investigate SnRK1.1 activity and turn-over in the presence of auxin or NPA and establish how the steady-state protein levels

change in response to auxin or NPA treatment. Doing this would establish if SnRK1.1 protein is more or less active with exogenous auxin or NPA. Also, looking at the steady-state protein levels would address how SnRK1.1 protein might be regulated by auxin. I would address where in the seedling SnRK1.1 protein is regulated looking at GFP fluorescence when seedlings are grown on auxin or NPA, to see if either of these treatments change the subcellular location of SnRK1.1:GFP.

Does P80 protect SnRK1.1 under low nutrients and target SnRK1.1 under high nutrients?

At the end of Chapter IV, I speculate there may be two mechanisms for how P80 and CUL4 impact SnRK1.1 stability. I think testing this hypothesis would be beneficial to continuing to address how SnRK1.1 stability is affected by P80 and CUL4, but also to identify a protein complex containing SnRK1.1. I would use turnover assays to test SnRK1.1 stability in wildtype and mutant plants, and in varying nutrient conditions. Similarly, I would do kinase assays in these same conditions to see how SnRK1.1 activity changes. Based on my results, I propose at 7 days under optimal nutrient conditions (or plus sucrose), SnRK1.1 specific activity would be higher in both *p80* and *cul4*(*cs)* mutants. Looking for protein:protein interactions between SnRK1.1, P80, CUL4 and novel proteins might be facilitated by use of the SnRK1.1 antibody and 7-day-old *p80* and *cul4(cs)* mutant seedlings, as the complex could be very abundant under these conditions (CHAPTER IV).

I have begun to address whether these molecules interact i*n vivo*. My preliminary data (not shown) indicates an *in vivo* complex between SnRK1.1 and P80. In this experiment I used the anti-SnRK1.1 antibody to IP P80:GFP from plant extracts containing P80:GFP. I probed the IP sample with anti-GFP in order to detect P80:GFP. From the IP sample, there was a protein band around 107 kD, which is the expected size of P80:GFP. This result needs to be repeated,

and using MG132 to inhibit the proteasome may increase the chances of identifying this complex *in vivo*.

Does SnRK1.1 rescue other mutants with decreased biomass?

SnRK:HA plants have increased biomass and lifespan (Baena-Gonzalez et al., 2007), and preliminary data from the Gillaspy lab suggest that SnRK1.1:GFP can rescue the lack of growth in *p80* mutants. It would be interesting to test if adding SnRK1.1 to other mutants with biomass and lifespan deficiencies (early senescence) rescues those mutants as well. Several mutants in the Gillaspy lab, like the *mips1* (Donahue et al., 2010), *impl-2* (Nourbahksk, 2011; Torabinejad et al., 2009), and *cul4* mutants (Bernhardt et al., 2006; Zhang et al., 2008), have decreased biomass and increased time to senescence when compared to wildtype plants. I would like to see if the SnRK1.1:GFP construct can rescue these mutants. If it could, it would point directly to SnRK1.1's ability to act as a master regulator to increase biomass and lifespan in several different pathways.

How is SnRK1.2 regulated at the level of translation?

Most of the current research on SnRK1 has been centered on SnRK1.1. This may be because SnRK1.1 is speculated to be the major SnRK1.1 gene in plants (Jossier et al., 2009). However my data suggest that under certain situations the SnRK1.2 gene may have a specialized role to complement the role of SnRK1.1. Therefore, I think looking at SnRK1.2 protein levels would be important for understanding why there are two SnRK1 genes. I would want to analyze steady-state protein levels of SnRK1.2 over development, and in response to nutrient status. My GUS data suggest that SnRK1.2 gene is not regulated by sugar as is SnRK1.1, but it would still be interesting to test whether the SnRK1.2 protein changes when exogenous sugar is added.

Further, establishing the steady-state protein levels of SnRK1.2 in response to light intensity or varying other growth conditions, like phosphate availability, gibberellic acid or auxin would be interesting, given the presence of predicted response elements in the SnRK1.2 promoter. In order to establish this information on SnRK1.2 steady-state protein levels, I would generate an anti-SnRK1.2 antibody.

Testing SnRK1.2 protein levels in either *p80* or *cul4(cs)* mutants would indicate if P80 and CUL4 regulate SnRK1.2 stability and would elucidate whether SnRK1.2 have similar protein binding partners. Also, testing SnRK1.2 protein activity or turnover would be useful in these mutants as well, as it would suggest if P80 or CUL4 regulate SnRK1.2. Much of the role of SnRK1.2 in plant biology and signaling remains unexplored, and I feel there is a lot of potential here for further investigation. For example, there is no successful SnRK1.2 RNAi or SnRK1.2 mutant lines. Generating either of these would allow plant phenotypes to be characterized, and allude to how SnRK1.2 is important in plant phenotypes, much like the analysis of phenotypes in SnRK1.1 RNAi or overexpressing lines.

CHAPTER VI

MATERIALS AND METHODS

Promoter Analysis

The promoter sequences of SnRK1.1 (At3g01090) and SnRK1.2 (At3g29160) were analyzed using tools available from the web site Plant Cis-Acting Regulatory Element (P.L.A.C.E.).

Plant Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col) and Lansdsberg erecta (Ler) were used for experiments. All seedlings and plants were grown in a controlled growth chamber at 22°-24°C under 16 hours of day/light (long day). Visible radiation was provided with fluorescent/incandescent lamps. Low light conditions were 30-40 µE. while regular-light and high-light conditions were 90-120 µE and 200-210 µE, respectively. Soil-grown plants were maintained on Sunshine Mix #1 and watered with Miracle-Gro Liquid Houseplant Food (8-7-6: 8% total nitrogen, 7% available phosphate, P_2O_5, 6% soluble potash, K_2O, 0.1% Iron, Fe; Scotts Miracle-Gro Products, Inc.). Seed for plate-grown seedlings were sterilized with 33% Chlorox, rinsed, and germinated on 0.5x Murashige and Skoog Basal Salt Mixture (MS Salts) (Sigma) ± 3% sucrose for 4, 7, 10, and 15 days. All plant materials were harvested between 11 AM and 2 PM.

GUS Assays

Samples of 4- to 15-day-old tissues grown on 0.5x MS ± 3% sucrose under low light conditions were immersed in GUS assay solution (0.025 µM ferrocyanide, 0.025 µM ferrous cyanide, 0.1 mM sodium phosphate buffer pH 7.0, 0.01% Triton X-100, 0.5 µg/mL 5-bromo-4-

chloro-3-indoyl-β-D-glucuronic acid), vacuum infiltrated for 25 minutes, and incubated overnight at 37°C (Styer et al., 2004). After incubation, the assay solution was removed and replaced by 70% ethanol. Tissues were stored at 4°C. Samples were later imaged using an Olympus SZX16 microscope.

Expression and Purification of SnRK1.1

To express recombinant, His-tagged SnRK1.1, I used a previously described (Ananieva et al., 2008) construct regulated by the pCR®T7/CT-TOPO vector. The SnRK1.1 plasmid (pSnRK1V5) was used to transform BL21-DE3 pLysS competent cells. Transformants grew on a Luria Broth (LB) agar plate supplemented with 100 µg/mL Ampicilliin and a colony was selected for inoculation of a starter culture. One liter of auto-induction media (0.17 M KH_2PO_4/0.72 M K_2HPO_4, 2 mM $MgSO_4$, 30x 80155 solution, and 0.375% succinic acid) was inoculated with 10 mLs of pSnRK1V5 BL21-DE3 pLysS cells and incubated at 37°C for 9h and 25°C for 12h. Cells were harvested through centrifugation and resuspended in 50 mM KH_2PO_4/K_2HPO_4, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole pH 7.6. The cells were incubated with 100 mg of lysozyme and a protease inhibitor mix reconstituted in dimethyl sulfoxide (DMSO), ruptured through sonication, and centrifuged at 4°C and 11,000 rpm for 30 minutes. The supernatant was applied to a Ni-NTA agarose column containing 500 µl of packed resin. The agarose beads were washed with 50 mM KH_2PO_4/0.72 M K_2HPO_4, 400 mM NaCl, 100 mM KCl, pH 7.6 (Wash Buffer A). SnRK1.1 was eluted from the beads using a 100-500 mM imidazole gradient. The complete lysate, insoluble pellet, supernatant, each eluate, and column beads were analyzed by Bradford Assay and SDS-PAGE. All usable eluates were pooled and concentrated using a 5,000 MWC Millipore centrifugation system and the protein in the resulting fraction was quantified by Bradford Assay. The protein

was dialyzed in a phosphate-buffered saline (PBS) buffer and re-concentrated before size exclusion chromatography. The final concentration of the buffer was 25 mM KH_2PO_4/0.72 M K_2HPO_4, 200 mM NaCl, 50 mM KCl, pH 7.6. Approximately 500 µg of protein was injected into a Superdex S200 size exclusion column. Fractions were collected, re-concentrated in PBS, and analyzed by SDS-PAGE.

Anti-SnRK1.1 Antisera Characterization and Purification

Recombinant SnRK1.1 protein was sent to Cocalico Biologicals Inc. for injection into a rabbit. The sera from the pre-bleed, first and second test bleeds, and production (final) bleed were analyzed by Western blot analysis for reactivity to recombinant protein and endogenous SnRK1.1 protein from plant extracts. An optimal concentration of crude α-SnRK1.1 was determined. Anti-SnRK1.1 was purified as described (Harlow and Lane, 1988). Approximately 50 µg of SnRK1.1 protein was loaded into multiple lanes of a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane (described in Western Blot Analysis) for 30 minutes at 15V. The strip of membrane containing only the SnRK1.1 protein was cut and blocked in 5% non-fat dry milk in 1x TBST (50 mM Tris-HCl pH 7.5, 0.9% [w,v] NaCl, and 0.01% [v,v] Tween-20) buffer for 2hrs at room temperature. The blot was cut further into smaller pieces and incubated in 1 mL of the anti-SnRK1.1 production bleed and 1 mL of 5% non-fat dry milk in 1xTBST rocking overnight at 4°C. The cut membranes were washed three times for 20 minutes in 1x TBST and incubated with 750 µL of 0.1 M glycine pH 2.5 for 10 minutes to elute the SnRK1.1 antibody. Membrane pieces were removed and 75 µL of 1 M Tris pH 8.5 and 0.1% Sodium azide was added, and the antiserum was stored at 4°C. After western blot analysis (described in Western Blot Analysis) the purified anti-SnRK1.1 is optimal at a 1:1,500 dilution in 2.5% non-fat dry milk.

Plant Extracts

Plant tissues were frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. Samples were homogenized in an extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.05% Triton X-100, 10% glycerol, 1 mM Dithiothreitol (DTT), and Protease Inhibitor Cocktail for plant extracts, Sigma-Aldrich) and centrifuged at 4°C for two minutes at 13.2 rpm on a table-top microcentrifuge. The supernatant was retained, quantified by Bradford Assay, and mixed with protein loading buffer (100 mM Tris-HCl pH 6.8, 4% Sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, and 20% glycerol), and boiled for 10 minutes. Samples were centrifuged and used for Western blot analysis or stored at -20°C.

Lambda Phosphatase (λPP) Assay

Proteins from sample tissues were extracted as previously described (Plant Extracts) without the addition of protein loading buffer. Lambda phosphatase reactions were performed according to manufacturer's instructions (New England BioLabs Inc.). For experimental reactions, 25 µL of a plant extract was combined with 5 µL of 10x NEBuffer for Protein MetalloPhosphates (PMP), 5 µL of 10x $MnCl_2$ (10 mM), 14.75 µL of deionized water, and 0.25 µL of 100U λPP enzyme. Reactions were placed in a 30°C water bath for 2hrs. After incubation, 2x PLB was added and the sample was boiled for 10 minutes, centrifuged, and analyzed by western blot analysis with anti-pAMPKα1/2 (Thr 172) (Santa Cruz Biotechnology, CA) and anti-SnRK1.1.

Western Blot Analysis

Proteins were electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose membranes were incubated in blocking solution: 5% non-fat dry milk in deionized water at 4°C overnight. For detection of GFP or HA tags, a 1:5,000 dilution of the rabbit anti-GFP (Invitrogen, CA) or anti-HA antibody (Santa Cruz Biotechnology Inc., CA) was used. For detection of SnRK1 proteins, a 1:1,500 dilution of rabbit anti-SnRK1.1 (Cocalico Biologicals Inc., PA) was used. For detection of post-translational phosphorylation of SnRK1.1 protein, a 1:500 to 1:100 dilution of anti-pAMPKα1/2 (Thr 172) (Santa Cruz Biotechnology, CA) was used. All membranes were probed with a 1:2,500 dilution of goat anti-rabbit horseradish peroxidase-conjugated (H + L) antibody (Bio-Rad Laboratories, Hercules, CA). All antibody solutions were in 2.5% non-fat dry milk in deionized water. Primary antibodies were incubated overnight at 4°C, while secondary antibodies were incubated for at least 1hr at room temperature. The nitrocellulose membranes were wasted three times for 20 minutes with 1X TBST (50mM Tris-HCl pH 7.5, 0.9% [w,v] NaCl, and 0.01% [v,v] Tween-20) buffer before and after application of the secondary antibody. Membranes were activated with the Amersham ECL Plus Western Blotting Detection kit (GE Healthcare, UK) and exposed to X-ray film for signal detection. To ensure equal loading of proteins, Ponceau S filters were used.

GFP Localization and Imaging

The 1.6kB coding region of the SnRK1.1 open reading frame was amplified by high-fidelity PCR (Velocity Enzyme, Bio-Rad Laboratories, Hercules, CA) (Supplemental Materials Table 1), confirmed by sequencing, cloned into the pENTR/D-TOPO vector (Invitrogen), and recombined via the Gateway system (Invitrogen) using the manufacturer's instructions into

pK7FWG2. The resulting 35S cauliflower mosaic virus promoter:SnRK1.1:GFP construct was transformed into *Agrobacterium tumefaciens* by cold shock and used in the transformation of wildtype and *p80* mutant plants as described (Bechtold et al., 1993). WT/SnRK1.1:GFP and *p80*/SnRK1.1:GFP seedlings were identified on kanamycin plates. Two independent homozygous wildtype lines (WT/SnRK1.1:GFP-1D and WT/SnRK1.1:GFP-2B), two independent heterozygous mutant lines (*p80*/SnRK1.1:GFP-15 and *p80*/SnRK1.1:GFP-105) with detectable GFP expression were used for subcellular localization and western blot analysis. Seedlings were harvested at 4, 7, and 10 days for imaging utilizing a Zeiss Axiovision microscope (Zeiss) equipped with fluorescent optics. For confocal imaging, GFP fluorescence was detected with a Zeiss LSM 510 laser scanning microscope (Carl Zeiss) using excitation with a 488-nm argon laser and a 505- to 550-nm band-pass emission filter. Slides were examined with a ×40 C-Apochromat water immersion objective lens.

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APPENDIX A

Table1. Primers

Gene	Gene Specific Primer
SnRK1.1prom Forward	5'-GATCACCTTTTTACTTGAGCTATTGAAG-3'
SnRK1.1prom Reverse	5'-TAAAAGGGATCCTAATTCTCGCTAAATTCTC-3'
SnRK1.2prom Forward	5'-GACAGATAAAAGCTTGGATTATAGAGATACAG-3'
SnRK1.2prom Reverse	5'-GCAGAACCTCATCTAGGCTCTTAG-3'
SnRK1.1 Forward	5'-CACCATGTTCAAACGAGTAGATGA-3'
SnRK1.1 Reverse	5'-GAGGACTCGGAGCTGAGCAAG-3'

Table 2. SnRK1 Predicted Masses

Protein	Mass (kD)
SnRK1.1:	
At3G01090.1	56.3
*At3G01090.2	58.9
At3G01090.3	56.3
SnRK1.2:	
At3G29160.1	56.3
At3G29160.2	56.3
At3G29160.3	39.5
SnRK1.3:	
At5G39440	54.3

Protein	Mass (kD)
SnRK1.1:	
*SnRK1.1:HA	60.0
*SnRK1.1:GFP	85.5