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The Role of OsWRKY71 and Its Interacting Proteins in Seed Germination and Early Growth of Cereal Grains

Margaret Ja Shin
University of Nevada, Las Vegas, mjshin@unlv.nevada.edu

THE ROLE OF OSWRKY71 AND ITS INTERACTING PROTEINS IN SEED GERMINATION AND EARLY GROWTH OF CEREAL GRAINS

By

Margaret J. Shin

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School of Life Sciences

Jeffery Q. Shen, Ph.D., Committee Chair

Andrew J. Andres, Ph.D., Committee Member

Frank van Breukelen, Ph.D., Committee Member

Paul J. Schulte, Ph.D., Committee Member

Lloyd R. Stark, Ph.D., Committee Member

Ernesto Abel-Santos, Ph.D., Graduate College Representative

Thomas Piechota, Ph.D., Interim Vice President for Research &
Dean of the Graduate College

May 2013

ABSTRACT

The Role of OsWRKY71 and Its Interacting Proteins in Seed Germination and Early Growth of` Cereal Grains

By

Margaret J. Shin

Dr. Jeffery Q. Shen, Examination Committee Chair
Associate Professor of Life Sciences
University of Nevada, Las Vegas

During seed germination and early seedling growth, complex molecular and physiological events occur in rice (*Oryza sativa*) and other cereal grains. As the seed transitions to vegetative tissue, it responds to both favorable and unfavorable environmental conditions and is vulnerable to attack by predation and disease. Although seeds are relatively small and tender in size, extensive and sophisticated molecular networks enables the immobile seed to grow, survive and adapt in its environment. One of the networks I am interested in is in the crosstalk between the gibberellin (GA) and abscisic acid (ABA) signaling pathways. These pathways are interesting because they are largely antagonistic. GA is a hormone that generally promotes germination and growth-related processes while ABA, also a hormone, promotes seed dormancy and represses growth. Although a great deal of research has been dedicated towards understanding these two pathways, the actual mechanism of crosstalk during seed germination is less understood. Any deficiencies in GA and ABA regulation and response may result in altered interpretation of environmental signals and aberrations in seed development and germination, leading to lower grain yields. My research is dedicated towards deciphering the specific role of *Oryza sativa* WRKY71 (OsWRKY71; amino acid W-R-K-Y) and harpin-induced1-like (HIL) members in the crosstalk between GA and ABA in rice, with

the goal that this research will be used to improve cereal grain yield in areas of the world with limited plant productivity.

The crosstalk between GA and ABA directs the synthesis of α-amylase, which is an enzyme that breaks down starch in seeds to provide energy for germination. OsWRKY71 was shown to be a transcriptional regulator of *α-amylase* and was regulated by both GA and ABA in barley. In this study, I have provided a model of the regulation of OsWRKY71 in seed germination in rice. Although it was previously determined that OsWRKY71 negatively regulated *α-amylase*, I show that it positively regulated not only germination but also root growth. To support this, I performed seed germination and root elongation assays using knockout mutants of *OsWRKY71.* Mutant analysis determined that germination in *oswrky71* was delayed for approximately 1 day and was able to recover from the delay. Additionally, after 4 days, *oswrky71* seedling roots were nearly 2 cm shorter than wildtype (wt), suggesting that OsWRKY71 may regulate other aspects of plant development. This is further supported by analysis of β-Glucuronidase (*GUS*) reporter expression of *OsWRKY71p-GUS*, which indicated that *OsWRKY71* was localized to the third node of rice culms. Thus, the function of OsWRKY71 appears to be more complex and versatile than predicted.

To further understand the mechanism of OsWRKY71 regulation in rice seed germination, I investigated the role of one of its interacting partners, *Oryza sativa* harpin-induced1-like 58 (OsHIL58). Using rice aleurone RNA-sequencing data, I found that OsHIL58 was induced upon ABA treatment. Thus, the two proteins may interact during ABA induction. I also annotated the HIL family using *in silico* methods and identified several other HIL members that were differentially and significantly expressed in the

aleurone. One member, OsHIL16, was highly expressed and also coexpressed with an ABA receptor, regulatory component of ABA receptor 9 (RCAR9). Surprisingly, both were repressed by ABA, suggesting that they be involved in the same pathway in the aleurone aside from OsWRKY71 regulation. From this annotation, I also identified and compiled a large family of 104 unique HIL members expressed in various rice tissues. A classification system was designed based on the presence of several conserved amino acid motifs: NPN, RPP, and YQYF. Most HIL members, including OsHIL16 and -58, were Group I members with all three motifs present. These and further analyses suggest that HILs may have multiple roles in plant development, including in seed germination.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

OVERVIEW

Seed Germination

Introduction

Seed germination in angiosperms, or flowering plants, is a complex and diverse process. The process of germination is governed by many factors, including environmental conditions such as light and water availability, physical barriers on the seed, and intrinsic regulation within the seed. Diversity arises due to the distinct combination of these elements each species may require for this process to occur: the amount of water, cold or warm temperature, and light intensity or shade, for instance. I am interested in the endogenous regulation of seeds in response to their environment, in essence, to better understand the molecular control regarding seed germination. To illuminate on this process, I used rice, an important food staple, as a model organism to understand development in cereal crops, in general.

What Is Seed Germination?

Seed germination begins when a quiescent, dry seed responds to favorable conditions, mainly light (Schwechheimer and Willige, 2009; Lau and Deng, 2010); temperature (Heggie and Halliday, 2005; Penfield *et al.*, 2005); and nutrient availability, moisture, and oxygen (Linkies and Leubner-Metzger, 2012). As an initial response, seeds will allow water to pass through previously developed seed barriers, enabling cellular activity to be resumed (Linkies and Leubner-Metzger, 2012). The entrance and uptake of

water, also called imbibition, results in metabolic activity. This includes protein translation of stored mRNAs (Weitbrecht *et al.*, 2011), mobilization of storage proteins (Tan-Wilson and Wilson, 2012), and the transport of stored nutrients and energy reserves from various parts of the seed to provide vigor for cell expansion, division, and development (Bewley 1997; Finkelstein *et al.*, 2008). These activities climactically lead to the growth and transition of the seed to vegetative development (Suzuki and McCarty, 2008). As seed tissues and layers swell due to imbibition, primary tissue from the part of the seed called the embryo will subsequently develop, elongate, and penetrate through the weakened seed coverings. The primary tissue to break through is generally the radicle or embryonic root (Bewley 1997; Finkelstein *et al.*, 2008; Linkies and Leubner-Metzger, 2012), but in some cases, the coleoptile, the tissue that covers the shoot, will emerge first (Kordan 1977). In this respect, it is evident that although metabolic activity occurs, seed germination is, by definition, successful and complete only when a visible tissue is able to emerge from the embryonic axis (Bewley 1997).

The Importance of Seed Dormancy

In many respects, seed germination may not occur unless there is an allotted period of seed dormancy. Seed dormancy normally begins while the seed is still developing on the mother plant, where it will start to accumulate nutrient and protein reserves, and thereafter, initiate processes linked to dehydration (Finkelstein *et al.*, 2008). During this course, regulators will be set in place to ensure that spontaneous germination does not occur while the seed is still maturing on the head of the plant, an agronomic issue known as vivipary or pre-harvest sprouting (Gubler *et al*., 2005). Once the seed has

been developed and released, it will be able to survive for prolonged periods of time even in harsh environments. Lending to its low water content, desiccation tolerance, and robust seed coat structure, seeds may be dispersed far-distance, allow for the persistence of a species in the local habitat (Linkies and Leubner-Metzger, 2012), decrease competition between individuals of the same species, and prevent germination out of season (Finkelstein *et al*., 2008).

By definition, then, seed dormancy is the blockage of seed germination, even under environmental conditions that would otherwise be favorable for growth (Finkelstein *et al*., 2008; Bewley 1997; Linkies and Leubner-Metzger, 2012). This is primarily caused by the presence of dormancy factors, which may need to be removed or perturbed. These factors may include external constraints of the seed or endogenous regulation within the seed, particularly within the embryo or surrounding layers. For instance, the outer layer of the seed, also called the husk or seed coat, may physically prevent water uptake. Seeds may need to undergo scarification or be broken down, which usually occurs due to normal existence in the natural environment. Hence, modification of the seed coat will allow for the penetration of water and nutrients readily into the seed (Finkelstein *et al*., 2008; Bewley 1997). Still, even with these physical changes, germination may be prevented internally, for example, by the synthesis of hormones. Hormones may repress gene expression and molecular processes linked to growth. Therefore, a change in hormone balance is necessary for the release of molecular repression. For instance, a period of cold weather, or stratification, may alter the hormone balance inside of the embryo of the seed, allowing it to survive through the winter and preparing it to germinate in the spring (Kucera *et al*., 2005). Seed germination is,

therefore, not only dependent on favorable environmental conditions but also the release of dormancy, which is dependent on seed maturation and desiccation completion, decomposition of the seed coat, and internal regulatory changes.

Rice as a Model Organism

Rice is an important cereal crop and a food staple for nearly 3 billion people around the globe, close to half of the world's population (Marathi *et al*., 2012). As with other crops, the challenge is to produce rice varieties with higher yield potential and greater yield stability (Khush 2005), especially by virtue of sustainability issues concerning nutrient depletion in the soil, salt increases due to land overuse, and the buildup of insects, weeds, and diseases (Fujisaka *et al.*, 1994).

The rice plant is a member of the grass family, *Poaceae* or *Gramineae.* It is a tropical, annual grass that may grow up to about 3-6 ft, or 1-1.8 m, in height and produce hundreds to thousands of seeds per plant (World Crops Database). The general conditions for growing rice includes the following: a high moisture level, or a relative humidity of about 60-80%; long day conditions, for instance, 14h day/10h nights; and sufficient amounts of water. About 1,432 L of water is needed to produce 1 kg of rice, which is more than wheat, maize (Velázquez 2007), and barley (Bouman 2009). Therefore, rice grows best in paddy fields or submerged in water and is generally tolerant to flooding. On the other hand, a warm atmosphere, 24°C, will generally suffice, and plants may thrive given low levels of oxygen, particularly during seed germination and seedling growth (Magneschi and Perata, 2009).

As a model organism, rice is an ideal plant used to study many aspects of plant pathology and development. In particular, research in rice has contributed to the understanding of mechanisms regarding plant response to abiotic factors such as cold, heat, drought, salinity, waterlogging, soil toxicity, mineral loss, heavy metal accumulation, and herbicide treatment, to name a few (Khong *et al.*, 2008; Witcombe *et al.*, 2008; Santos *et al.*, 2011). It has also been important in uncovering the modes of plant disease recognition, response, and resistance (White *et al.*, 2009; Liu *et al.*, 2010). This is impart supported by research of certain rice diseases such as bacterial blight, a vascular disease caused by *Xanothomonas oryzae* (Yang *et al.*, 2006) and rice blast, fungal lesions in tissues caused by *Magnaporthe grisea* and its related species (Zhu *et al.*, 2000). Both abiotic and biotic stress affects the plant's health, overall height, and production of a favorable number of reproducing stems, also called tillers. It may also increase the chance of pre-harvest spouting, poor seed development, or loss of seed vigor in major cereal crops. These implications result in significant grain loss, for instance, 50% in rice due to biotic factors (Bandyopadhyay and Sanyal, 2011) and up to 100% in severe abiotic stress, 40% in maize due to drought (Castiglioni *et al.*, 2008), and 70% due to abiotic stress in crops, in general (Xiao *et al*., 2007). This has led to considerable progress over the years encompassing advances in generating, breeding, and enhancing resistance in plants (Witcombe *et al.*, 2008; Ahmad *et al.*, 2012). With the availability of the rice genome, many functional genomic resources and global expression profiles (Jiang *et al.*, 2011), and large proteomic resources and protein databases (Kosova *et al.*, 2011), are made available. This makes rice an ideal organism to use to decipher complex networks surrounding plant development and its response to the environment, in hopes to

further improve grain yields in rice and many other cereal crops, including corn, wheat, barley, oats, millet, sorghum, buckwheat, rye, and quinoa.

Hormone Signaling Pathways that Regulate Seed Germination

Introduction

Hormones are one of many chemicals plants use to perceive stimuli from their surroundings. A stimulus may induce the synthesis of hormones or change the concentration of hormones, which then has the potential to regulate multiple signaling pathways. These lead to altered gene and protein expression, metabolism, and other cellular changes. Often called phytohormones, about 13 major classes of non-peptide hormones and growth regulators exist and are synthesized by the plant, all which have distinct and similar roles in plant development; many have been shown to be involved in seed germination. Some of these include abscisic acid (ABA), gibberellic acid (GA), ethylene, jasmonic acid (JA; Linkies and Leubner-Metzger, 2012), auxin (IAA), cytokinin (CK; Chiwocha *et al.*, 2005), nitric oxide (NO; Bethke *et al.*, 2006), salicylic acid (SA; Rajjou *et al.*, 2006), and brassinosteroids (BR; Steber and McCourt, 2001; Chen *et al.*, 2004). Of these phytohormones, the antagonistic role of GA and ABA in seed germination is well known. GA is primarily associated with germination or growth, while ABA is linked to seed dormancy or growth inhibition. Although multiple hormones influence seed germination, I am particularly interested in the crosstalk of the signaling pathways regulated by GA and ABA in rice.

The Roles of GA and ABA in Seed Development and Germination

An important endogenous determinant to the timing of seed germination is the hormonal balance between the amounts of GA and ABA within the seed, notably within the embryo. The ratio of GA to ABA shifts over time and is altered by environmental stimuli.

When seeds are developing on the mother plant, the level of ABA is important. Typically, ABA accumulation is low during the early stages of development, is greatest during mid-development and when storage reserves are being synthesized, and declines as the seed matures and dries (Bewley 1997). ABA is, therefore, a positive regulator of seed dormancy and an inhibitor of seed germination in maturing seeds (Kucera *et al.*, 2005). When seeds are dried for a prolonged state, such as during air-dry storage at ambient temperature, an effect called after-ripening occurs. During this stage, there is a transition of the seed from a dormant state to a non-dormant state, which decreases sensitivity to ABA. This is governed by catabolism of ABA by specific ABA 8'-hydroxylases encoded by the cytochrome P450 monooxygenase (CYP707A) family (Finklestein *et al.*, 2008, Kucera *et al.*, 2005).

A decrease in ABA levels may be required prior to an increase in GA levels and sensitivities. This decrease promotes the accumulation of the GA biosynthetic gene *gibberellin 3-oxidase 2* (*GA3ox2*). Increased synthesis of GA may then promote degradation of ABA, as shown in lettuce seeds (Finklestein *et al.*, 2008). The synthesis of GA may also increase during after-ripening, as the seeds are more sensitive to stimuli such as light and cold. To promote GA response, light signals, for example, may translate into hormones signals via the action of phytochromes, predominately phyA and phyB,

and cryptochromes, such as cry1 and cry2, which can directly sense different qualities of light (Seo *et al.*, 2009; Lau and Deng, 2010). These then regulate transcription factors such as phytochrome-interacting factor 3-like 5 (PIL5)/PIF1, which integrates with GA signaling components, possibly by regulating DELLA degradation (Seo *et al.*, 2009; Lau and Deng, 2010). Therefore, external processes may integrate with the GA pathway and modulate GA response, which will in turn, contribute to seed germination. Specifically, GA may stimulate the weakening of tissue barriers, the mobilization of seed storage reserves, and the expansion of the embryo. GA may also stimulate chromatin-remodeling factors such as PICKLE (PKL), which contributes to embryonic root growth and thereby transitions the seed to vegetative development (Finklestein *et al.*, 2008).

Shortly after the completion of seed germination, the levels of ABA have been shown to increase. In particular, ABA may be used to monitor water availability, respond to stress in the environment, and regulate the rate of seedling growth (Skriver and Mundy, 1990; Molina *et al.*, 2001). The balance between GA and ABA appears, therefore, to be important throughout plant development.

The GA Signaling Pathway

GAs are plant growth hormones that are synthesized and active throughout a plant's life cycle from seed germination, stem elongation, leaf expansion, flowering, and to fruit ripening, the most well known response being an increase in plant height when exogenous GA is applied (Daviere *et al.*, 2008; Schwechheimer 2008; Schwechheimer and Willige, 2009). At the cellular level, GAs may stimulate cell division, differentiation, and elongation (Achard and Genschik, 2009). Hundreds of GAs have been identified and

collectively form a large family of tetracyclic, diterpenoid acids; however, only GA1, GA3, GA4, and GA7 are considered bioactive (Yamaguchi 2008).

GA signaling comprises of several steps: the perception of GA by a receptor, the action of secondary messengers, a change in transcriptional control, and an alteration in the expression of response genes. Two fundamental pathways have been deduced (Figure 3-1). The general pathway begins with an initial perception of GA by a soluble receptor GA-insensitive dwarf 1 (GID1), which is homologous to hormone sensitive lipases in humans (HSLs; Ueguchi-Tanaka *et al.*, 2005). Direct contact leads to interaction with a DELLA protein (conserved amino acid sequence D-E-L-L-A; Sun 2010), which is phosphorylated and targeted for degradation via the E3 ubiquitin ligase, S-phase kinase-associated protein (Skp) Cullin F-box (SCF; Daviere *et al.*, 2008; Schwechheimer 2008; Schwechheimer and Willige, 2009). DELLA degradation is central to this pathway, as this leads to the release of sequestered transcription factors, which can then promote GA response (Daviere *et al.*, 2008; Schwechheimer 2008; Schwechheimer and Willige, 2009). The regulation of DELLAs may also be an important in integrating the GA pathway with other signaling pathways, including crosstalk with ABA (Zentella *et al.*, 2007).

The secondary pathway is hypothesized to occur during seed germination. It involves perception of GA by an uncharacterized plasma membrane receptor (Figure 3-1). It has been reported that G-protein activation may be involved, which may lend to increases in calcium and calmodulin (CaM) activity (Lovegrove and Hooley, 2000). Calmodulin may interact with OsWRKY51 (Dr. Shen, unpublished), which would then lead to GA response.

The ABA Signaling Pathway

ABA is a 15-carbon sesquiterpenoid carboxylic acid, similar in structure and biosynthesis to retinoic acid found in chordates (Xie *et al*., 2005). While GA promotes perpetuates growth and metabolism (Asad and Komatsu, 2006), ABA inhibits growth phase transitions and decelerates metabolism (Finklestein *et al.*, 2008). Also, ABA mediates physiological responses to various stresses in plants including pathogen attack, high salinity, desiccation, hypoxia, cold, and wounding, to name a few (Raghavendra *et al.*, 2005; Finklestein *et al.*, 2008; Hubbard *et al.*, 2010). It was also shown to aid in leaf abscission, to which its name was derived, stomatal closure, embryo development (Hubbar *et al.*, 2010), and the coordination of ion-flux changes (Raghavendra *et al*., 2005). Despite its adverse effects, it has been shown to work synergistically with other hormones to coordinate development, for instance, leaf size (Raghavendra *et al*., 2005).

ABA signaling begins with the perception of ABA by a soluble receptor PYR1/PYL/RCAR (pyrabactin resistance 1/pyrobactin 1-like/regulatory component of ABA receptor), or RCAR for short (Figure 3-1; Raghavendra *et al.*, 2005; Hubbard *et al*., 2010). Other potential receptors, including membrane bound receptors, have been identified (Verslues and Zhu, 2007; McCourt and Creelman, 2008). Generally, the soluble receptor will bind to a co-receptor serine/threonine protein phosphatase type 2Cs (PP2Cs), such as ABA-insensitive 1 (ABI1), ABI2, and Hypersensitive to ABA 1 (HAB1), leading to their deactivation (Raghavendra *et al.*, 2005; Hubbard *et al*., 2010). Inhibition of PP2Cs subsequently activates protein kinases, specifically Open Stomata 1 (OST1)/ sucrose-nonfermenting kinase1 (SNF1)-related protein kinase 2s (SnRK2s), which may phosphorylate and activate ion channels, additional secondary messengers, or

transcription factors such as ABI5 to direct ABA-related responses (Raghavendra *et al.*, 2005; Hubbard *et al*., 2010).

Seed Germination in Rice

The rice seed consists of several basic parts, a covering or seed coat to protect the seed, an endosperm filled with starch to provide energy and nutrition for germination, and an embryo from where the vegetative tissue will grow and develop. Additionally, surrounding the seed but underneath the seed coat is a single layer of cells called the aleurone layer (Hoshikawa 1993), which aids in starch hydrolysis (Figure 1).

In rice, seed germination begins with the uptake of water into living cells by aquaporins, namely OsPIP1;1, OsPIP1;2, OsPIP1;3 and OsPIP2;8, which regulates water flow and was found to be important for normal germination (Liu *et al.*, 2007). Once water enters living cells, metabolic activity will increase or resume. GA will be biosynthesized in the embryo of the rice seed, and from there, it will mobilize to the aleurone layer where it is perceived by a receptor (Kaneko *et al.*, 2002). This will activate the GA signaling pathway and lead to the expression and synthesis of α-amylase, which was previously repressed due to ABA regulation. Accumulated α-amylases will be secreted from the aleurone cells and into the endosperm where they may hydrolyze stored starch. As a result, the sugars may be used to promote vegetative growth of the embryo (Kaneko *et al.*, 2002).

The aleurone cells are also important in producing other hydrolases in addition to α-amylase. These include protease, phytase, phosphatase, lipase, RNase, esterase, peroxidase, catalase, β-glucosidase and α- and β-galactosidase (Palmiano and Juliano,

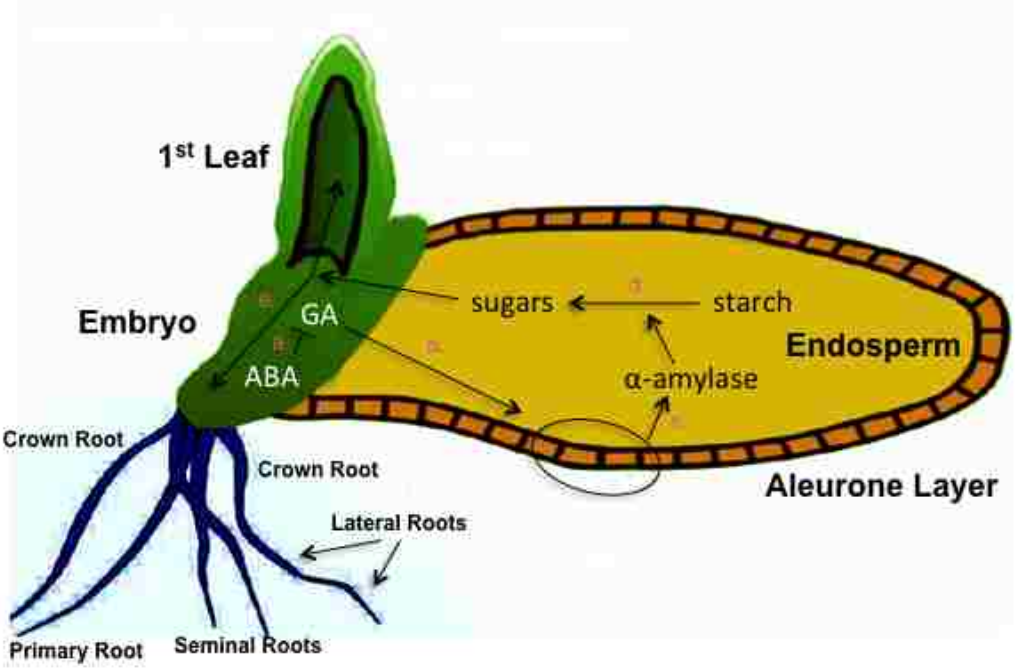


Figure 1-1. A model of seed germination in rice. (a) Before germination, ABA promotes seed dormancy. Under the right set of environmental conditions, including the removal of physical and endogenous dormancy factors, GA is synthesized in the embryo. (b) GA mobilizes to the aleurone layer where it will interact with GA receptors, and thus, initiate the GA signaling pathway. (c) In response, ABA-inhibition of α-amylase is removed and α-amylase is synthesized and secreted into the starchy endosperm, where it will (d) convert starch into sugars to be freely used as energy for embryonic development of (e) the primary root, also called the radicle, and then the coleoptile, from where the first leaf will emerge. During the early growth of the seedling, seminal roots, followed by crown roots will emerge, and additional leaves will begin to differentiate from the nodes of the developing shoot.

1973). Specifically, Palmiano and Juliano showed that phytase levels increased and coincided with oxygen uptake and ATP levels. Furthermore, an increased activity of lipase was present in the aleurone, which also stores lipids. Lipase activity appeared to increase prior to α-amylase activity, indicating that lipid metabolism in the aleurone may occur before the breakdown of the endosperm. Yet, despite the multitude of events that take place in the germinating rice grain, the role of α-amylases are fundamental, for the seed will depend on starch for energy until its depletion by the time the fourth leaf emerges.

Protein Families Involved in This Study

Introduction

A small amount of hormone may initiate a network of downstream signaling cascades to promote global changes in gene regulation and protein expression to generate a particular cellular response. Many regulatory and response processes are due to the action of proteins. I am interested in several proteins in regards to their specific roles in seed germination. These proteins are members of the WRKY or HIL family, both of which have been shown to be involved in diverse physiological responses in plants.

The WRKY Superfamily

The WRKY superfamily is a large transcription factor family with diverse roles in plant development and physiological response. Of the 37 major transcription factor families in plants, the WRKY superfamily is one of the largest and may be found in both higher and lower plant species (Xiong *et al.*, 2005; Riechmann *et al*., 2000). There are

101 members in rice (Rushton *et al.*, 2010) and 75 in Arabidopsis (Ulker and Somssich, 2004). Members differ in sequence at large, but all possess a distinct DNA-binding domain containing amino acids W-R-K-Y-G-Q-K at the N-terminus and a zinc finger motif, CysX4-5CysX22-23HisXHis or CysX7CysX23HisXCys, at the C-terminus (Euglem *et al.*, 2000; Ulker and Somssich, 2004; Ross *et al.*, 2006; Rushton *et al.*, 2010). Generally, WRKY transcription factors may bind to promoters of genes containing the sequence TTGAC(C/T), or the "W-box." The coordination of Zn^{2+} (Xie *et al.,* 2006) stabilizes the binding of the WRKY protein to this sequence (Rushton *et al.*, 2010).

Initially, many WRKY members were shown to respond to biotic stress, including fungal and bacterial pathogens, nematodes, and herbivores (Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Rushton *et al.*, 2010). More recent investigations identified roles of certain WRKY members in abiotic stresses such as salt, heat, osmotic, drought, cold, and high CO_2 and ozone levels (Rushton *et al.*, 2010). In regards to GA and ABA signaling, multiple WRKYs have been implicated in ABA-dependent abiotic and biotic stress signaling; some examples in rice include OsWRKY45, -24, -72, -77, -11, -25, and -33 (Mauch-Mani and Mauch, 2005; Xie *et al.*, 2006; Rushton *et al.*, 2010). Only several WRKYs have been characterized in GA-mediated development, including OsWRKY24, -51, and -71, (Rushton *et al.*, 2010; Xie *et al.*, 2006). WRKY members with roles in seed germination include the following: *Arabidopsis thaliana* WRKY 2 (AtWRKY2; Jiang and Yu, 2009), AtWRKY15, -53, and -70 (Cao *et al.*, 2006), barley or *Hordeum vulgare* WRKY 38 (HvWRKY38; Xie *et al.*, 2007, Salicylic Acid), TTG2 (Johnson *et al.*, 2002), OsWRKY51 and -71 (Xie *et al.*, 2006), ABA overly sensitive mutant (ABO3)/AtWRKY63; Ren *et al.*, 2010), AtWRKY57 (Jiang *et al.*, 2012), wheat or

Triticum aestivum WRKY6 (TaWRKY6; Guo *et al.*, 2011), AtWRKY18, -40, -60 (Chen *et al.*, 2010), OsWRKY72 (Yu *et al.*, 2010), AtWRKY25 (Li *et al.*, 2009), and AtWRKY39 (Li *et al.*, 2010). Specifically, OsWRKY71, ~38 kDa, was found to regulate the expression of *α-amylase* in transient expression assays in barley (Zhang *et al.*, 2004). In the present study, I further investigate the role of OsWRKY71 in plant development, particularly in seed germination and early seedling growth, in Chapters 2 and 3.

The HIL Family

Although I describe this family as the HIL family herein, related proteins have been identified as HIN (harpin-induced; Gopalan and He, 1996), NDR (non-race-specific disease resistance; Dormann *et al.*, 2000), or NHL (NDR1/HIN1-like; Varet *et al.*, 2002) based on their roles in disease resistance. Few publications exist on individual members of this family. These include tobacco or *Nicotiana tabacum HIN1* (*NtHIN1*; Gopalan and He, 1996), and *NtHIN9* and *-18* (Takahashi *et al.*, 2004); *AtNDR1* (Century *et al.*, 1997; Dormann *et al.*, 2000), *AtNHL3* and *-25* (Varet *et al.*, 2002), *AtNHL10* and related genes (Zheng *et al.*, 2004); *OsHIN1* (Kim *et al.*, 2000); and oilseed rape or *Brassica napus NHL18A* (*BnNHL18A*; Lee *et al.*, 2006). In most of these organisms, members were not linked to any particular family except in Arabidopsis, in which a total of 45 members were identified and described as the NHL family (Dormann *et al.*, 2000; Zheng *et al.*, 2004). Since then, no particular reviews were found regarding this family, although references were found acknowledging the presence of a shared domain with a small subgroup of proteins in the late embryogenesis abundant (LEA) family called LEA14 (Ciccarelli and Bork, 2005). In order to compile distinct members of the HIL family in

rice, I performed *in silico* analyses in the present study. Although not shown, I also updated the HIL family in Arabidopsis.

Besides their roles in disease recognition, roles in other processes in plants are limited. HILs were induced by one or more of the following factors: harpin, SA, JA, (a)virulent bacterial and fungal pathogens, or polyamines such as spermine, which specifically induces the production of pathogenesis-related proteins (Takahashi *et al.*, 2004). *NtHIN1*, the first member found, was specifically induced by harpin, an elicitor produced by *Erwinia amylovora* (Wei *et al.*, 1992), which causes fire blight in pear, apple, and other rosaceous plants (Gopalan and He, 1996). This suggested that related genes might also respond to pathogens. Interestingly, in rice, *OsHIN1* was induced by rice blast, a common and potentially detrimental disease caused by *Magnaporthe grisea* (Kim *et al.*, 2000). Further research suggested that *HIL* expression may mediate hypersensitive response or protection against a wide array of pathogens in other plant species, including *Pseudomonas syringae* (Gopalan and He, 1996; Dormann *et al.*, 2000; Varet *et al.*, 2002; Varet *et al.*, 2003), *Peronospora parasitica* (Century *et al.*, 1997), *Cucumber mosaic virus* (Zheng *et al.*, 2004), and *Tobacco mosaic virus* (Takahashi *et al.*, 2004). Although, like WRKYs, members were initially discovered to play a role in biotic stress, it is possible that HILs may be also involved in abiotic stress or plant development. For example, *BnNHL18A* was induced by NaCl, ethephon, an ethylene derivative, and hydrogen peroxide, in addition to being induced by pathogen defense hormones SA and JA (Lee *et al.*, 2006). Furthermore, HILs share a common domain sequence with LEA14 proteins suggesting that they may have similar roles in osmotic stress (Ciccarelli and Bork, 2005). Although HILs were not previously found to mediate

seed germination, in Chapter 5, I show that HILs may be involved in GA- and ABA-mediated seed germination.

Interestingly, despite limited information regarding the exact function of HILs in the cell, members of the HIL family were found to localize in the plasma membrane of cells (Varet *et al.*, 2003; Lee *et al.*, 2005). Protein localization in other parts of the cell were also discovered, for instance, retention in the endoplasmic reticulum (Lee *et al.*, 2006) and translocalization to the chloroplast, although the latter is subject to debate (Zheng *et al.*, 2004). These suggest that a given stressor may alter the localization and function of HILs. A better understanding of the HIL family in its entirety will enable researchers to identify their unique and interesting roles in plant defense, abiotic response, and plant development.

Conclusion

Several key topics were introduced in this section including seed germination, hormone regulation, and the roles of WRKY and HIL members. My goal was to understand the function of one specific WRKY member, OsWRKY71, in GA- and ABA-mediated seed germination. Although OsWRKY71 has been studied in barley, its role in seed germination was not yet investigated in rice (Chapters 2 and 3). Furthermore, OsWRKY71 was linked to a member of the HIL family, from which another project was developed. For this second project, my goal was to annotate the HIL family in rice and then to identify HILs expressed in GA- and ABA-treated aleurone in rice seeds, to investigate their roles during seed germination (Chapter 4).

CHAPTER 2

SELECTION AND VERIFICATION OF OSWRKY71 MUTANTS

Introduction

To further understand the role of the WRKY superfamily in plant development, individual rice WRKY members were investigated such as OsWRKY71. To test whether or not OsWRKY71 had a role in seed germination, Zhang *et al.* (2004) performed transient expression assays in barley seeds. Specifically, *UBI-OsWRKY71* was introduced in barley aleurone cells with GA- and ABA-inducible reporter constructs *Amylase32b-GUS* and *HVA22-GUS*, respectively, using particle bombardment. After GUS quantification, Zhang *et al.* (2004) discovered that OsWRKY71 repressed GA-induced expression of *Amylase32b-GUS* 50-fold but not *HVA22-GUS*. Also, treatment with ABA increased *OsWRKY71* expression, while GA slightly decreased its expression (Zhang *et al.*, 2004; Xie *et al.*, 2006). These suggested that OsWRKY71 might mediate the crosstalk between GA and ABA and have a specific role in the regulation of seed germination by repressing *α-amylase*.

Additional discoveries followed showing that OsWRKY71 was able to regulate α-amylase expression by 1) binding to the dual W-box core sequence in the *Amylase32b* promoter, 2) interrupting and preventing the major transcriptional activator Gibberellic Acid-Induced Myeloblastosis-like protein (GAMYB) from binding to the GA-response element (GARE) in a dosage-dependent manner, and 3) physically interacting with OsWRKY51 to enhance its repressive activity (Zhang *et al.*, 2004; Xie *et al.*, 2006).

Because these findings of OsWRKY71 in seed germination have been performed

using transient expression assays, I further addressed its role in rice mutants. Prior to analysis, rice mutant were obtained and verified. The main focus of Chapter 2 is to report my results regarding the verification of *dSpm* knockout mutants for *OsWRKY71*, which were obtained from Dr. Sundareson's Lab, UC Davis, California (Kumar *et al.*, 2005), and mutants that were produced in collaboration with Dr. Su May Yu's lab in Academia Sinica, Taiwan. These include GUS reporter, overexpression, and dominant negative lines. In order to identify positive transgenic lines, PCR, qPCR, or chemical selection was performed.

Materials and Methods

Plant Materials

Transposon knockout lines for *OsWRKY71*, RdSpm1689 and RdSpm3171A (cv. *Oryza sativa*), were obtained from Dr. Sundareson's Lab, UC Davis, California. Rice seeds were mechanically dehusked and sterilized with 10% commercial bleach (NaOCl) and 80% ethanol. Then seeds were germinated on moist filter paper at 23 ºC in a growth chamber under 14 h light/10 h dark cycles (light intensity: 450 μmol s^{-1} m^{-2}) at a relative humidity (RH) of 60-80%. After 10 days, plants were transplanted in soil and watered daily. Every 5 days, the water was supplemented with 4 drops/L of Schultz Liquid Plant Food (Lowe's, Product # 94652). Pots were rotated once every week to reduce position effects and unplugged for 2 days to cycle water. After a month, the pots were plugged with a rubber stopper and filled with water up to two inches above the soil level.

For hygromycin or Basta selection, seeds were first dehusked and sterilized by rinsing in 80% ethanol for 1 min, sterile water for 1 min, and then in 10% commercial

bleach (NaOCl) and 80% ethanol. The seeds were then agitated on a Rotaflex apparatus for 30 min and rinsed. Finally, seeds were blotted on sterile filter paper before plating on 1% agar plates containing 125 µg/ml Basta or 50 µg/ml hygromycin B in full-strength MS media adjusted to pH 5.6. Selection was finished after nine to ten days, and then the percent survival rates were calculated.

PCR Genotyping

PCR was used to screen for the insertion of *dSpm* in lines 1689 and 3171A. The leaves from 2-week-old seedling were used for genomic DNA preparation from plants using the CTAB/Chloroform:Isoamyl Alcohol Method (Stewart and Via, 1993). For standard PCR reactions, 4% (v/v) DMSO was used in GoTaq Reaction Buffers (Promega, Madison, WI). Primers were designed against gene- and transposon-specific primers and were synthesized by IDT (Integrated DNA Technologies; Table 2-1). PCR was performed using the following program: 94°C for 5 min; 10 cycles of 94°C for 1 min, 64°C for 1 min (-1°C per cycle), and 72°C for 1 min and 20 s; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and 20 s; and 72°C for 5 min. Digestion of the PCR products with EcoRI, PstI, and BamHI was used to confirm the insertion of the transposon. Some of the PCR products were sequenced to further verify the sequence of the inserts.

Quantitative RT-PCR

Mature leaf tissue from rice plants were frozen in liquid nitrogen and ground in a mortar with a pestle. RNA was extracted from the frozen powder with the RNeasy Plant

Primer Description	No.	Sequence	Hybrid Tm	% GC
RdSpm1689-2-3; Right; PCR verification	10043	CGTCTTATCCACTCAAAAACCC	51.3	45.5
RdSpm3171A-2-1; Right; PCR verification	10045	GGAGCAGCAGAAAAGTTTGC	50.4	50
dSpm; Left Border; PCR verification	10050	GAGCGTCCATTTTAGAGTGAC	50.9	47.6
OsWRKY71 1st exon; Forward; qPCR	12045	GCTAGCTAACTAGGTCGTCGG	54.8	57.1
OsWRKY71 1st exon; Reverse; qPCR	12046	ACGAGCACCTTGGGCTTAAC	52.5	55
OsWRKY71 3'-UTR; Forward; qPCR	10009	AGCAAGAACACGATCGACGC	52.5	55
OsWRKY71 3'-UTR; Reverse; qPCR	10010	GATCGTTGGTTGATCAGCCATG	53.1	50

Table 2-1. PCR primers used to verify *dSpm* knockout lines for OsWRKY71.

Mini Kit (Qiagen, Valencia, CA). To remove DNA, the TURBO DNA-free Kit (Applied Biosystems, Foster City, CA) was used. cDNA was synthesized using the standard procedure from the SuperScript III First-Strand Synthesis protocol (Invitrogen, Grand Island, NY).

Primers were designed based on the sequences of the first exon and the 3'-UTR of *OsWRKY71* (Table 2-1). Quantitative RT-PCR (qPCR) was performed using SYBR Green (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The reactions were run on the iCycler Real-Time PCR Thermal Cycler (BioRad, Hercules, CA) using the following conditions: 94°C for 2 min; 40 cycles of 94°C for 15 s, 53°C for 30 s, and 72°C for 30 s. The expression of *OsWRKY71* was normalized to *β-Actin,* and the 2-ΔΔCt method (Livak and Schmittgen, 2001) was used to compare the expression of *OsWRKY71* in *dSpm* knockout lines to wt samples.

GUS Staining

Select rice tissues were harvested and sectioned. Seeds were first imbibed for 18 h in imbibing solution (20mM CaCl2, 20mM succinic acid pH 5.0) and sectioned longitudinally. Sections were transferred into a clean microtiter plate. To each microtiter well, 200µl of GUS stain (0.1 M Phosphate Buffer pH7.2; 20% methanol; 0.5 mM $K_3[Fe(CN)_6]$; 0.5 mM $K_4[Fe(CN)_6]$ x $3H_2O$; 10 mM EDTA pH8.0; 1 mg/ml X-Gluc; 0.5% Triton-X) was added. The tissues were vacuum infiltrated for 5 min to 1 hr and sealed. The plate was covered in foil and was agitated for 48 h at 37°C at 100 rpm. Sections were stored in 80% ethanol.

Protein Extraction and Western Analysis

Rice tissues (500 mg of mature leaf tissue, 300 mg of 8-day old seedlings, and 40 embryos) were ground in liquid nitrogen. For embryos, TCA was used to precipitate and purify total protein from embryos. First, the powdered tissue was suspended in cold 20% TCA (5 volumes of TCA: 1 volume embryo tissue). The suspension was inverted several times to mix and centrifuged at 13,000 rpm for 10 min. The pellet was then washed with cold Tris-buffered acetone (80% acetone in 25 mM Tris-HCl pH7.5) and centrifuged at 13,000 rpm for 10 min twice. Finally, the pellet was gently washed with 100 mM Tris-HCl pH6.8, centrifuged at 13,000 rpm for 5 min, and the supernatant was removed.

For all tissues, two volumes of 2x SDS buffer (20% glycerol; 120 mM DTT; 6% SDS; 80 mM Tris-HCl pH6.8; 0.01% bromophenol blue) were added to the powdered tissue or, in the case of embryos, the resulting pellet from TCA precipitation. The sample was boiled for 1.5 minutes, mixed with another 0.5-1 volume of 2x SDS buffer, and boiled for an additional 2 min. The samples were centrifuged at 13,000 rpm for 2-5 min. Then, the supernatant was collected and centrifuged again at 13,000 rpm for another 2-5 min to remove lipids and other byproducts. Samples were loaded and run on SDS-PAGE gels (90V for 140 minutes).

For Western analysis, protein was electrophoretically transferred (90V, 60 min) onto a PVDF membrane (Bio-Rad). To visualize transferred protein, the membrane was first stained with Ponceau-S solution (0.1% Ponceau-S, 3% acetic acid). Then, the membrane was cut into strips and placed inside an Accutran slotted incubation tray (S&S). The strips were blocked with 3% milk solution (Carnation, non-fat dry milk in 1x PBST) for one hour prior to the addition of primary antibodies. A 1:1,000 dilution of

primary antibody was added to the strips in 1ml of 3% blocking buffer and incubated overnight at 4°C. After incubation, the strips were washed with 1x PBST for 5 min, five times. Lastly, the strips were incubated in 1:20,000 secondary antibody (KPL Reserve AP Anti-Rabbit Conjugate, HRP Anti-Rabbit/Mouse Conjugates). The strips were washed once with 1x PBST and prepared for Western detection.

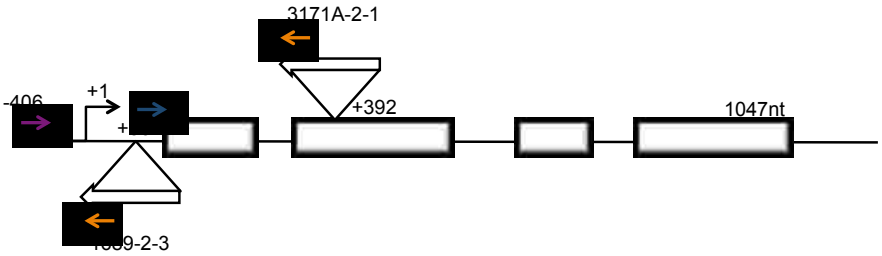
For horseradish peroxidase enhanced chemiluminescence-based Western detection, equal volumes of peroxide and luminol were mixed (WestDura ECL, Thermo Scientific). Strips were placed inside a small kapack pouch and 0.5-1 ml of luminol/ peroxide solution was applied to the strips. Exposures were taken using the UVP BioSpectrum machine using the "ECL" setting. For Western detection, sixteen 30 sec exposures were taken. Every 2, 4, 6, and 8 min, the exposures were extracted, and later compiled and analyzed.

Results and Discussion

Verification of oswrky71 Knockout Rice Lines

PCR was used to screen and genotype *dSpm* insertional knockouts of *OsWRKY71* (Figure 2-1a). Among 11 plants examined for 1689, two were positive for *dSpm*, one homozygous, 1689-2-3, and the other heterozygous, 1689-2-4 (Table 2-2). Among 18 plants screened for the second line, 3171A, a greater number of plants were found to be transgenic. Four plants were homozygous, 3171A-2-1, 2-2, 2-6, and 2-15, while five others were heterozygous for the insertion of *dSpm*, 3171A-1-2, 2-9, 2-10, 2-12, and 2-14. Out of these plants, the lines that were propagated for seed production and used for analyses were the following: 1689-2-3, 1689-2-4, 3171A-2-1, and 3171A-2-15.

a



b

-30 +1

TATATATATTAGGGCTCAGCTCCTCCTCTC**A**CCTCG
TCTCCTCCTCTCCTCTCTTCTCTCA**CTACAAGAAAA**
AAGGCAAGGAGTGTCGGCCAAAACCCCACACTCTTA
CGAAATAAGCCGACACTCTAAGTGAAGAG…
CTTGGCTCAGCTTGACATCGTCGGAGCAGCAGAAAA
GTTTGCTCGATCGAGCTAGCTAACTAGGTCGTCGGA
TCAGTGATAAATCGTTCGCCGATGAAACCCCATCTC
+245

c

+210

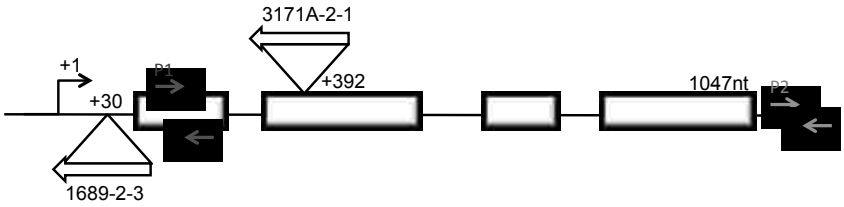
TCAGTGATAAATCGTTCGCCGATGGATCCGTGGATT
AGCACCCAGCCTTCGCTGAGCCTGGACCTCCGCGTC
GGGCTGCCGGCGACGGCGGCCGTCGCCATGGTTAAG
CCCAAGGTGCTCGTCGAGGAGGACTTCTTTCACCAG
CAGCCTCTCAAGAAAGACCCAGAGGTTGCGGCGCTG
GAGGCGGAGCTGAAGCGGATGGGCGCGGAGAACC**CT**
ACAAGAAAAAAGGCAAGGAGTGTCGGCCAAAACCCC
ATCTCTTACTGAAATAA…GCCGACACTTAAACATAA
+410

Figure 2-1. Diagram of *dSpm* knockouts for *OsWRKY71*. (a) Positions of *dSpm* in two different *OsWRKY71* knockout lines, 1689-2-3 and 3171A-2-1, obtained from Dr. Sundareson's lab, UC Davis, California. PCR primers, used to verify insertion of *dSpm* in the *OsWRKY71* gene, are colored. Gold arrows represent transposon-specific primers; the purple arrow was used for 1689-2-3 verification; and the blue arrow was used for 3171A-2-1 verification. Sequencing was used to verify the position of *dSpm* in *OsWRKY71* in (b) 1689-2-3 and in (c) 3171A-2-1. Partial sequence of the left border of *dSpm*, is bolded. Full genomic sequence for *OsWRKY71* may be seen in Appendix A.

dSpm Plant #	Transposon Insertion	Genotype
WT	-	-
1689-1-1	-	-
1689-1-2	-	-
1689-2-2	-	-
1689-2-3	+	homozygous
1689-2-4	+	heterozygous
1689-2-5	-	-
1689-2-6	-	-
1689-2-7	-	-
1689-2-8	-	-
1689-2-9	-	-
1689-2-10	-	-
3171A-1-1	-	-
3171A-1-2	+	heterozygous
3171A-2-1	+	homozygous
3171A-2-2	+	homozygous
3171A-2-3	-	-
3171A-2-4	-	-
3171A-2-5	-	-
3171A-2-6	+	homozygous
3171A-2-7	-	-
3171A-2-8	-	-
3171A-2-9	+	heterozygous
3171A-2-10	+	heterozygous
3171A-2-11	-	-
3171A-2-12	+	heterozygous
3171A-2-13	-	-
3171A-2-14	+	heterozygous
3171A-2-15	+	homozygous

Table 2-2. Screening results for *OsWRKY71* knockout lines. PCR was used to identify positive (+) or (-) lines for the insertion of the *dSpm* transposon. PCR-genotyping also determined lines that were homozygous or heterozygous for the *dSpm*. DNA was obtained from mature leaf tissue from rice. The "1" or "2" represents the number of times a batch of seeds were obtained from Dr. Sundareson's lab at UC Davis. The last numerical value represents the seed number from each batch able to be propagated.

a



b

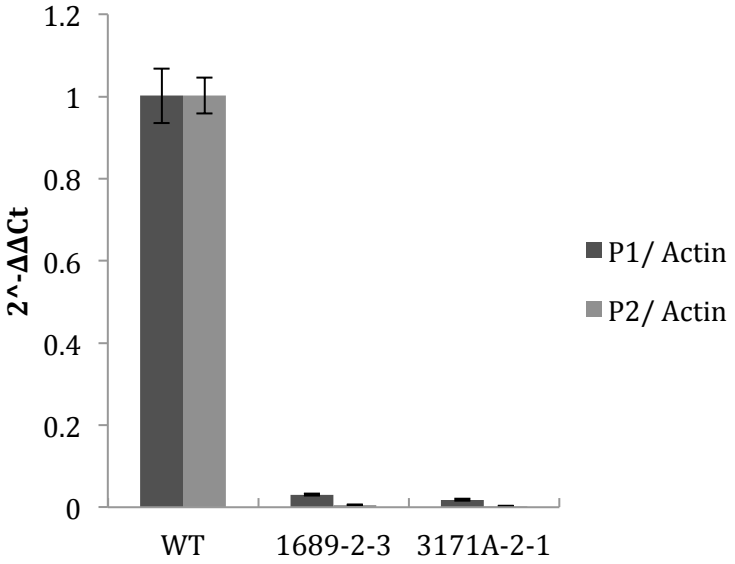


Figure 2-2. qPCR verification of *dSpm* knockout lines for *OsWRKY71*. (a) Position of primer sets against *OsWRKY71*. P1 and P2 are against the first exon and 3'-UTR of *OsWRKY71*, respectively. (b) Relative expression of *OsWRKY71* in 1689-2-3 and 3171A-2-1.

To further verify the knockout lines, sequencing and qPCR were performed. First, the location of *dSpm* within 1689 and 3171A lines were confirmed by sequencing (Figure 2-1b). For 1689-2-3, the *dSpm* transposon was inserted 30 nucleotides downstream from the transcription start site, within the 5'-UTR region of *OsWRKY71*. And for 3171A-2-1, the insertion was 392 nucleotides downstream, within the second exon, producing a truncated protein about 64 of 348 amino acids in length. qPCR was then used to determine the mRNA expression level of *OsWRKY71* in both mutants (Figure 2-2). Compared to wt, the expression levels were dramatically lower in both lines, suggesting that two different knockout *oswrky71* lines were obtained.

Selection of OsWRKY71 GUS-Expressing Rice Plants

Rice plants, transformed with the promoter of *OsWRKY71* fused to a β-glucuronidase (*GUS*) reporter gene, were selected for based on Basta or herbicide resistance (Figure 2-3a). Out of eleven T2 rice plants examined, all were either homozygous or heterozygous, based on their survival rates on media containing 125 μM Basta (Table 2-3). Nine seed batches were highly resistant to Basta treatment, with their survival rates equal to or greater than 75% (in comparison to their survival rates on control media). Based on these percentages, nine lines were considered to be homozygous. Of the nine lines, those with 100% survival rates were the following lines: 13-1-5, 13-1-8, 19-1-6, 20-1-1, 23-1-1, and 24-1-12, which may be used for further analyses. Only two lines, 11-1-1 and 24-1-1, had low survival rates, therefore, presumed to be heterozygous for the insertion. Based on these selection results, it appears that several different transgenic lines for GUS were obtained.

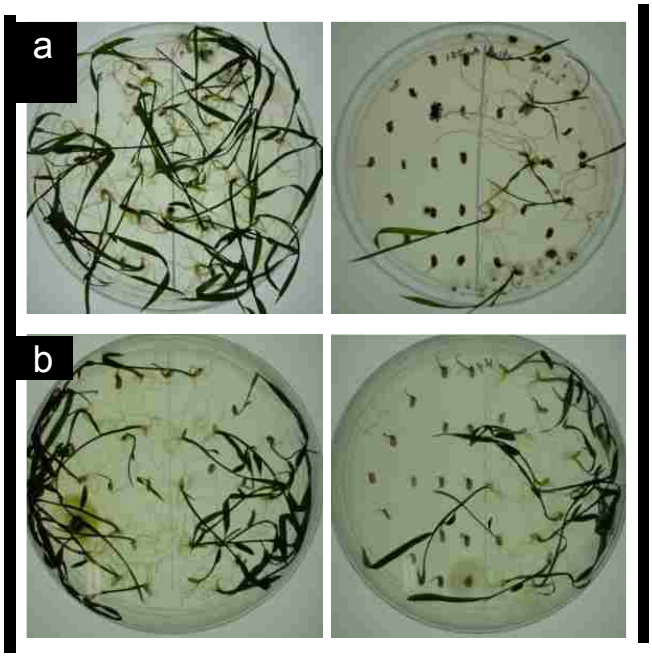


Figure 2-3. Hygomycin and Basta selection photos. (a) Basta selection results after 9 days for wt (left side of plates) and the GUS reporter line 7113-11-1-1 (right side of plates) in the control treatment (left panel) or 125 μM Basta (right panel) (b) Hygromycin selection results after 11 days for wt (left side of plates) and the *OsWRKY71* overexpression line, 7112-25-1-2 (right side of plates) in the control treatment (left panel) or 50μg/ml hygromycin B (right panel). Growth conditions: 14h day/10hr night, 24°C, 60-80% RH.

Plant #	% Survival (No Treatment)	% Survival (Basta)	Genotype
WT	100	0	-
11-1-1	100	56	heterozygous
11-1-5	88	75	homozygous
12-1-2	81	69	homozygous
13-1-5	94	94	homozygous
13-1-8	94	100	homozygous
16-1-8	94	88	homozygous
19-1-6	94	94	homozygous
20-1-1	100	100	homozygous
23-1-1	100	100	homozygous
24-1-1	100	30	heterozygous
24-1-12	94	100	homozygous

Table 2-3. Identification of positive GUS reporter lines for *OsWRKY71*. For selection of *OsWRKY71p-GUS* lines, T2 generation rice seeds were selected for based on resistance against 125 μM Basta. Ratio of percent survival of the experimental treatment to the control was calculated for each line. If the value was over 75%, the seeds from that particular line were considered to be homozygous and heterozygous, if less.

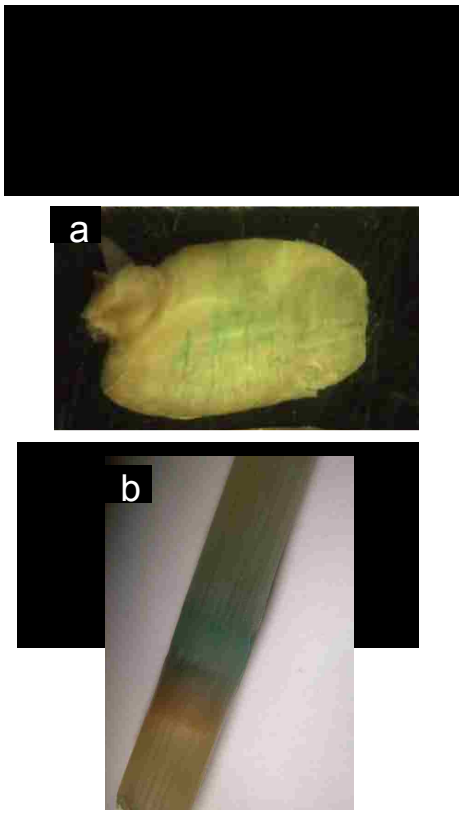


Figure 2-4. Rice tissues with positive GUS staining. GUS was expressed in T1 generation plants in the (a) aleurone layer and (b) third node of mature rice plants.

To check for the expression of GUS, rice tissue from select lines were stained. No evidence of successful GUS staining was found in T2 generation lines. However, staining was observed in T1 lines, particularly in the aleurone layer of seeds, as expected, albeit the degree of staining was faint or low (Figure 2-4a). GUS expression was also localized to the third node of mature rice plants (Figure 2-4b), implying that *OsWRKY71* might be expressed in rice nodes, meristematic regions on rice culms where additional tillers and adventitious roots emerge. Ethylene generally regulates while GA may aid in adventitious root growth, whereas the role of ABA is minimal (Steffens and Sauter, 2005). Furthermore, tiller growth is largely regulated by cytokinin and auxin, but not ABA (Liu *et al*., 2010). Therefore, OsWRKY71 may not only be involved mediating crosstalk between ABA and GA, but it may also be important in ethylene, cytokinin, or auxin signaling.

Selection of OsWRKY71 Overexpression Rice Plants

Several plant lines containing *UBI-OsWRKY71* tagged with a hemagglutinin epitope (*HA*) were selected for based on their resistance to hygromycin (Figure 2-3b, Hiei *et al.*, 1994). Several heterozygous lines were found, 7-1-1, 14-1-1, and 15-1-2, while the remaining lines were homozygous, 12-1-10, 22-1-1, 22-1-7, and 25-1-2 (Table 2-4). Western analysis was used to determine the levels of OsWRKY71 in wt rice tissue. Since analysis using aleurone tissue requires the availability of more seeds, embryos were used instead. An ~42 kDa band was observed in embryos but not in leaf tissue (Figure 2-5a), which is consistent with previous data showing that OsWRKY71 was expressed in embryos (Xie *et al.*, 2006), but was not highly expressed in mature leaves (Liu *et al.*,

Plant #	% Survival (No Treatment)	% Survival (Hygromycin)	Genotype
WT	94	13	-
7-1-1	100	69	heterozygous
12-1-10	94	81	homozygous
14-1-1	85	44	heterozygous
15-1-2	81	50	heterozygous
22-1-1	88	75	homozygous
22-1-7	75	75	homozygous
25-1-2	73	80	homozygous

Table 2-4. Identification of positive overexpression lines for *OsWRKY71*. For hygromycin selection, T2 generation rice seeds were selected for based on resistance against 50μg/ml hygromycin B. Ratio of percent survival of the experimental treatment to the control was calculated for each line. If the value was over 75%, the seeds from that particular line were considered to be homozygous and heterozygous, if less.

a

b

Figure 2-5. Protein detection of OsWRKY71 in rice embryos. (a) OsWRKY71 was detected in rice embryos but not leaf tissue (500 mg). (b) Detection was not observed in seedlings (100 mg). 1:1000 primary antibody and 1:20,000 dilution was used for detection. 15μg of GST-OsWRKY71 was used as an antigen control.

2007). Since *α-amylase* is expressed in the embryo (Umemura *et al.*, 1998), it is possible for the gene to be regulated by OsWRKY71. However, this Western analysis needs to be repeated and confirmed in knockout *oswrky71* embryos. Furthermore, the expression of OsWRKY71 in aleurone tissue should be tested.

Although OsWRKY71 was expressed in stems and young leaves (Liu *et al.*, 2007), no expression was detected in 8-day old seedlings, consisting of both shoot and leaf tissue (Figure 2-5b). The concentration of OsWRKY71 in seedling tissues might have been too low to be detected. Furthermore, there was no detection in overexpression seedlings, T2 line 7112-1-1, when anti-HA was used as the primary antibody. This further suggests that silencing of *OsWRKY71* may be an issue in T2 generation plants. For future analyses, T1 rice lines may be preferred.

Selection of OsWRKY71 Dominant Negative Rice Plants

Dominant negative lines contain *UBI-OsWRKY71x193aa*, which are missing part of the C-terminus of *OsWRKY71*, to retain only 193 out of 348 amino acids. Thus, the WRKY domain was removed to produce a defective OsWRKY71 protein. In order to select for dominant negative lines, 50 μg/ml of hygromycin was used. Of the seven T1 generation lines screened, only one, line 18, survived in hygromycin treatment. Therefore, this line should be propagated for additional seeds.

Conclusion

Knockout, GUS reporter, overexpression, and dominant negative mutants for *OsWRKY71* were selected for using several techniques, including PCR, qPCR, GUS

Plant #	% Survival (No Treatment)	% Survival (Hygromycin)	Genotype
WT	94	0	-
1	0	0	-
8	0	0	-
15	0	0	-
16	0	0	-
17	0	19	-
18	94	81	homozygous
24	0	0	-

Table 2-5. Identification of dominant negative lines for *OsWRKY71*. For hygromycin selection, T2 generation rice seeds were selected for based on resistance against 50µg/ml hygromycin B. Ratio of percent survival of the experimental treatment to the control was calculated. If the value was over 75%, the seeds from that particular line were considered to be homozygous and heterozygous, if less.

staining, and Basta or hygromycin selection. Although some of these mutants were used for immediate physiological tests, others need additional verification. Knockout lines, 1689-2-3 and 3171A-2-1, were the main mutants used for further analyses since the detectable expression levels of *OsWRKY71* were low in both and positive homozygous lines for *dSpm* were obtained; the insertion was also confirmed by sequencing. The seeds of these plants were harvested for subsequent analyses (Chapter 3). On the other hand, the remaining *OsWRKY71* mutants, generated in Taiwan, may need to be further tested. Although select T2 generation rice plants were resistant to Basta or hygromycin, GUS staining and Western analyses were largely unsuccessful, implying that gene silencing might have occurred. From these tests, however, it was hinted that *OsWRKY71* could be expressed in rice nodes, in addition to aleurone cells.

In the future, several additional homozygous and heterozygous 3171A plants, including heterozygous line 1689-2-4, should be propagated and examined. These would aid in verifying any phenotypes observed in 1689-2-3 and 3171A-2-1. One line, 3171A-2-15 has been grown and used for seed weight (Figure 3-3) and germination tests (Figure 3-5), although the expression of *OsWRKY71* has not yet been verified in this mutant. Furthermore, additional mutants should be generated to rescue the knockout lines, for example, by incorporation of a GUS-tagged or overexpression transgene for *OsWRKY71*. A rescue line should be specifically made for 3171A-2-1, which appeared to have the stronger phenotype of the two lines (Chapter 3). Also, to ensure that any phenotypes observed in the two knockout lines indeed come from the same gene, a transheterozygote should be generated by crossing both 1689-2-3 and 3171A-2-1.

CHAPTER 3

PHYSIOLOGICAL FUNCTION OF OSWRKY71 IN SEED GERMINATION AND IN EARLY ROOT DEVELOPMENT

Introduction

Despite the increased understanding of the GA and ABA pathways, the actual mechanism of crosstalk between the two signaling pathways in cereal aleurone cells is still not understood (Figure 3-1). Both GA and ABA appears to coordinate the expression of *α-amylase* genes during seed germination. The two pathways merge at several nodes (Figure 3-1). ABA was shown to inhibit GAMYB, a major transcriptional activator of *α-amylase*, by an SnRK2, ABA-induced Protein Kinase 1 (PKABA1; Gomez-Cadenas *et al.*, 2001) and further inhibited by ABA Response Element Binding Factor 1 (TaABF1; Johnson *et al.*, 2008). On the other hand, GA was shown to activate GAMYB by promoting DELLA degradation, specifically Slender1 (SLR1) in rice (Weiss and Ori, 2007). DELLA control is another point of integration between the two pathways. ABA generally upregulates DELLAs (Penfield *et al.*, 2006), but degradation is promoted by the interaction of GA with a soluble receptor, GID1, or membrane-associated heterotrimeric G protein, Gα/D1 (Hartweck *et al.*, 2006).

As previously mentioned (Chapter 2), ABA-inducible *OsWRKY71*, along with OsWRKY51, competitively inhibited binding of GA-inducible GAMYB to *α-amylase* (Xie *et al.*, 2006). OsWRKY71 could be a third node of communication between ABA and GA. While ABA induced *OsWRKY71* expression, blocking *α-amylase* production, increasing GA levels was shown to degrade OsWRKY71 in transient expression assays in

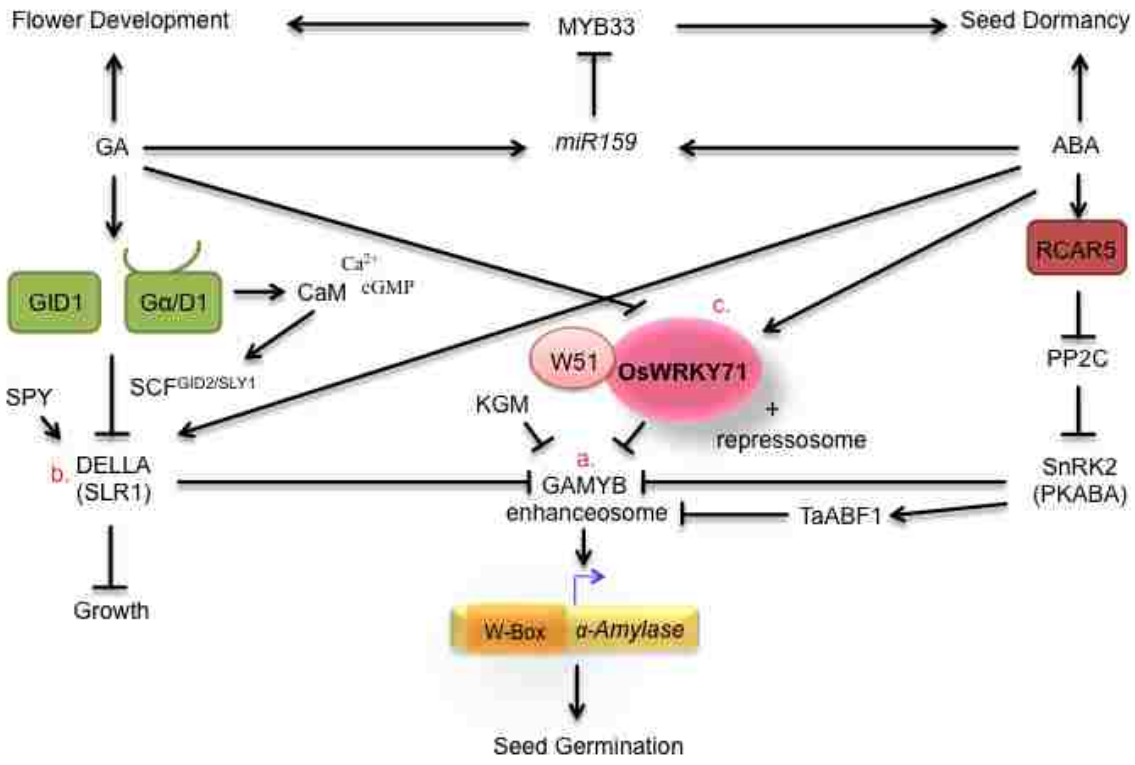


Figure 3-1. Model of crosstalk between GA and ABA signaling pathways in the aleurone of cereal grains. Several nodes exist where the crosstalk between GA and ABA integrate to regulate *α-amylase* expression. (a) GAMYB is repressed by ABA by PKABA1 (Gomez-Cadenas *et al.*, 2001) independently or through TaABF1 (Johnson *et al.*, 2008). GA instead activates GAMYB through SLN1 degradation (Weiss and Ori, 2001). GAMYB may also be repressed independently by interaction with Kinase Associated with GAMYB (KGM; Woodger *et al.*, 2003). (b) DELLA, or SLN1, degradation by the ubiquitin E3 ligase complex (SCFSLY1/GID2) is activated by GA (Hartweck *et al.*, 2006; Sun 2010), but ABA stabilizes the DELLA protein (Penfield *et al.*, 2006). SPINDLY (SPY), an O-linked GlcNAc (O-GlcNAc) transferase (OGT), has been shown to activate DELLA by GlcNAc modification (Sun 2010) (c) Transient expression analyses has shown that *OsWRKY71* is upregulated by ABA but is hypothesized to be degraded by GA (Zhang *et al.*, 2004; Xie *et al.*, 2006). Further note that other transcriptional regulators form a complex with OsWRKY71 or GAMYB to form a repressosome or enhancesome, respectively, to collectively regulate *α-amylase* expression (Rushton *et al.*, 2012). Myeloblastosis 33 (MYB33) is a positive regulator of GA-induced flowering and ABA-induced seed dormancy. Although it is not a node for integration, it depicts one homeostatic mechanism used to desensitize both signaling pathways via accumulation of *miR159* (Weiss and Ori, 2007). The general mechanisms for the GA and ABA pathways are described in Chapter 1. Adapted and modified from Weiss and Ori, (2007).

barley (Dr. Zhonglin Zhang and Angi Liyuan Zhang, unpublished; Zhang *et al.*, 2004). When *UBI -OsWRKY71:GFP* was introduced in barley aleurone cells treated with 100 μM GA, the number of fluorescent spots detected decreased about 50% after 12 h, indicating that OsWRKY71 was degraded. Thus, along with DELLA and GAMYB, the regulation of seed germination by ABA and GA may be mediated by OsWRKY71.

If OsWRKY71 regulates *α-amylase* gene expression in rice and is important in regulating seed germination, then I predicted that knockouts of *OsWRKY71* would result in impairment of germination, specifically by enhancing it. To test this, germination assays were performed. Seedling growth was also monitored to detect for impairments in development after germination. This was carried out using root elongation assays.

Methods and Materials:

Germination and Root Elongation Assays

The following seeds were used for germination and root elongation assays: wt (*Oryza sativa* cv. Nipponbare), 7113-19-1-6 (control for overexpression line 7112-22-1-1), 1689-2-3, 3171A-2-1, and 7112-22-1-1. Seeds were first dehusked and sterilized by rinsing in 80% ethanol for 1 min, sterile water for 1 min, and then in 10% commercial bleach (NaOCl) and 80% ethanol. The seeds were then agitated on a Rotaflex apparatus for 30 min and rinsed. Finally, seeds were blotted on sterile filter paper before plating on 1% agar plates containing water only or full-strength Murashige and Skoog medium (MS) media adjusted to pH 5.6. Additionally, salt plates were supplemented with 100 mM NaCl.

For root elongation assays, 12 seeds were lined up on each square plate, and then they were sealed with UV-sterilized Parafilm. The plates were placed in a growth chamber (25-28° C, 12 h day/12 h night, 60-80% RH) in an upright position on plate racks and monitored for five days. Germination rates were calculated based on radical emergence.

For root length and seed measurements, the plates were scanned at 600dpi. Images were analyzed using ImageJ. To test for statistical significance, a Student's t-test was performed (* = $p < 0.05$, ** = $p < 0.005$).

Results and Discussion

Seed Weight and Measurements for oswrky71 Rice Seeds

Mature wt and knockout rice plants, 1689 and 3171A, did not display any obvious differences in development. Seed production appeared to be normal; the number of seed-bearing panicles were similar between wt and *oswrky71* lines (Table 3-1). The seeds, however, seemed to vary in size or form. From initial inspection, the size of 3171A-2-1 seeds appeared to be slightly larger than wt and 1689-2-3 seeds. But upon measurement, there were no significant differences between the respective lines (Figure 3-2). Still, the mean length for 3171A-2-1 was 7.6 mm, or 0.4 mm longer than wt. There was also more variation in both populations of mutants compared to wt.

A decrease in starch closely parallels with a decrease in seed dry weight (Murata *et al.*, 1968). Both wt and *oswrky71* seeds were weighed with or without the seed coat as an approximate indication of starch abundance. Both 1689-2-3 and 3171A-2-1 seeds (36 seeds per line) weighed ~0.2 g less than wt, which weighed about 1.1 g (Figure 3-3).

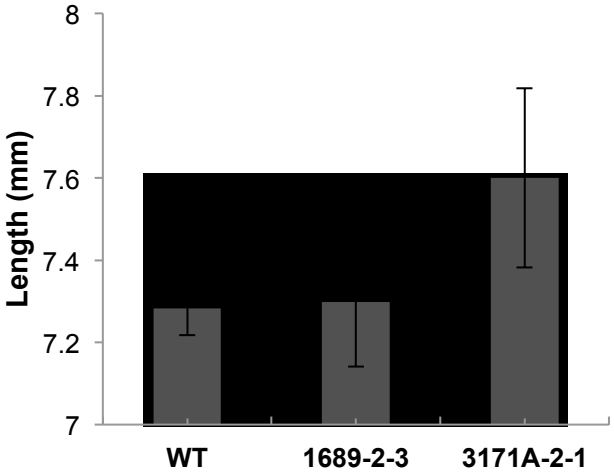


Figure 3-2. Comparison of the length of rice seeds in knockout lines of *OsWRKY71*. Whole seeds were scanned and measured using Image J. One experiment was performed; seed lengths (n=36) were measured for each line, and the average lengths are shown.

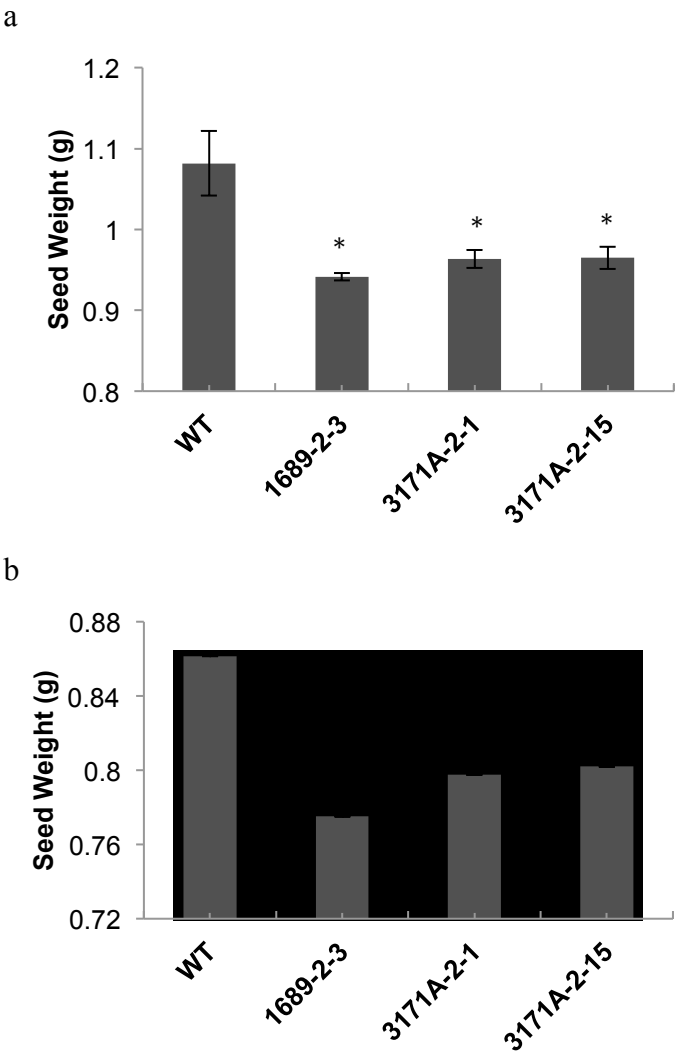


Figure 3-3. Comparison of the weight of rice seeds in knockout lines of *OsWRKY71*. Seeds were weighed (a) with or (b) without the seed coat. For seed measurements with the seed coat, three biological replicates were used. Each replicate consisted of seed weights (n=36; * = $p < 0.05$) for each line. Only one replicate was used for dehusked seeds; the seeds (n=36; * = $p < 0.05$) were weighed several times to obtain error bars. 3171A-2-15 is an additional homozygous 3171A line with *dSpm* localized within the second exon of *OsWRKY71*.

Even when the seed coats were removed, mutants weighed ~0.05-0.1 g less than wt, albeit the differences were much smaller. Wildtype weighed 0.86 g; therefore the seed coat was ~0.25 g. On the other hand, 1689-2-3 and 3171A-2-1 weighed 0.77 g and 0.80 g respectively; thus, the seed coats weighed 0.1-0.15 g. Therefore, it appeared that there might be a difference in the abundance of starch in *oswrky71* seeds and in the weight of the seed coat, although the differences were small. Indeed, if OsWRKY71 could no longer repress *α-amyla*se expression in *oswrky71* seeds, it would be expected that seeds would hydrolyze some starch, causing the seeds to weigh less than wt. The change was not drastic, however, possibly due to redundancy or regulation of *α-amyla*se by other repressors (Rushton *et al.*, 2012). Since seed coat development is also regulated by ABA-mediated dormancy and the balance of GA to ABA (Moise *et al.*, 2005), it is possible that the loss of OsWRKY71 may lead to aberrations in the seed coat as well as in seed germination.

Germination Rate Differences in oswrky71 Rice Seeds

To further understand the role of OsWRKY71 in seed germination, the germination rates between wt and knockout lines, 1689-2-3 and 3171A-2-1 were compared (Figure 3-4). Although ~80% of wt seeds germinated in water alone or in MS media by the first day, germination rates were much lower for both mutants (Figure 3-4a,b). In water, only 10% of 3171A-2-1 seeds germinated on the first day, while 30% more germinated in MS media. 1689A-2-3 seeds germinated slightly better, 30% and 60%, respectively. For both lines, germination rates increased when supplemented with nutrients. When the seeds were sown in 100 mM NaCl, wt seeds were also delayed; only

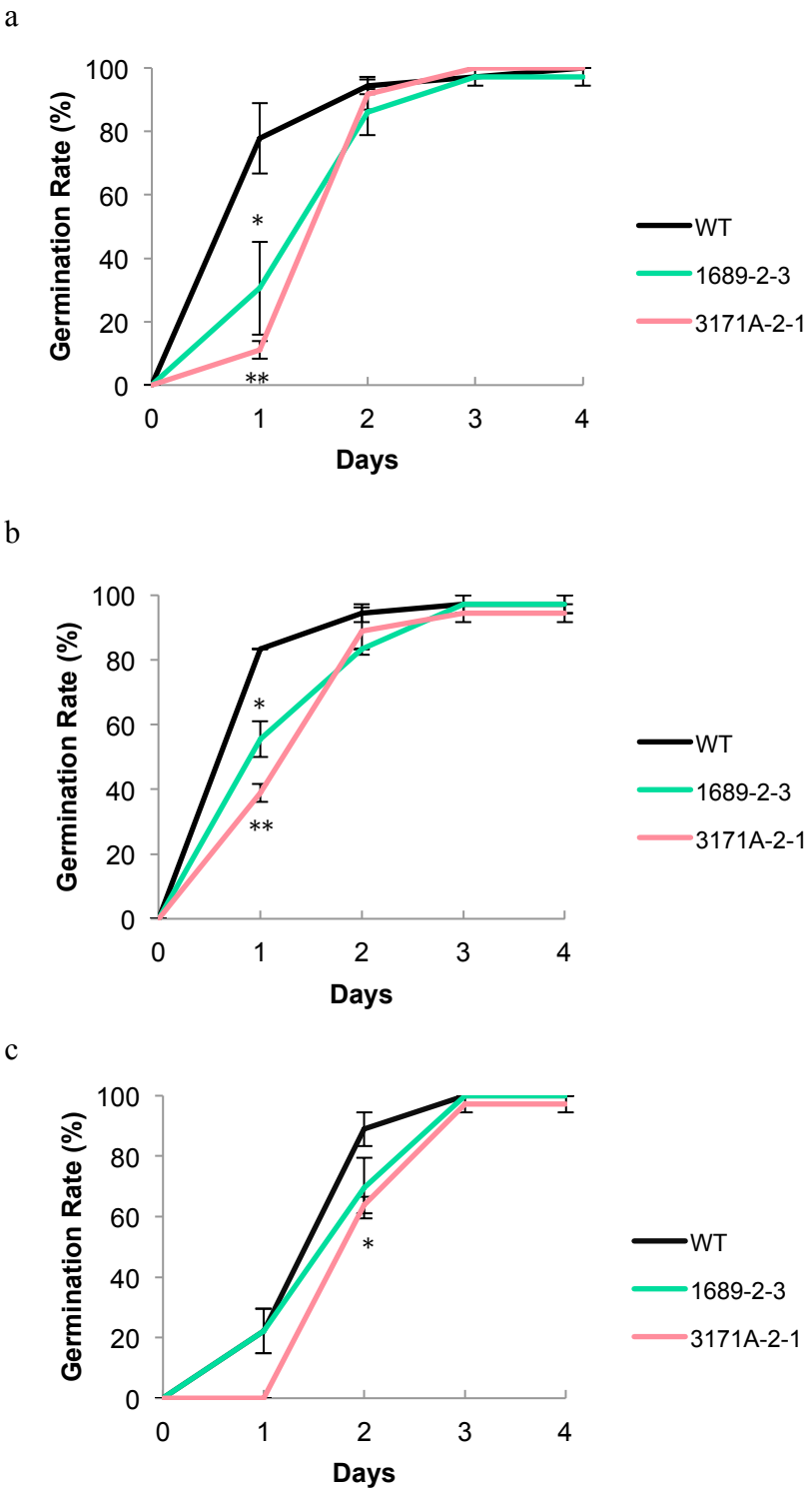


Figure 3-4. Seed germination was delayed in seeds of *dSpm* knockout 3171A-2-1. Germination rates were compared in knockout lines of *OsWRKY71* in (a) water, (b) MS media, and (c) 100 mM NaCl. Three to four biological replicates were used (n=12 seeds per replicate; * = $p < 0.05$, ** = $p < 0.005$). This experiment was repeated three times.

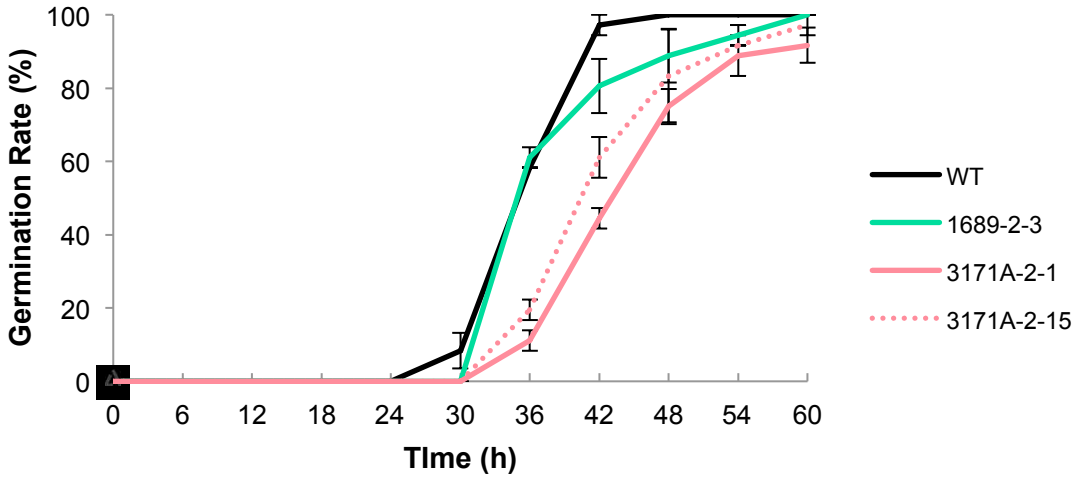


Figure 3-5. Differences in germination rates of *OsWRKY71* knockouts occurring within 6-hour intervals. Germination rates were measured every 6 h for 2.5 days. Three to four biological replicates were used (n=12 seeds per replicate). Germination rates appear to be sigmoidal. This experiment was performed one time.

20% of seeds germinated (Figure 3-4c), comparable to 3171A-2-1 seeds germinating in water alone. Likewise, NaCl caused a delay in both mutants. Percentages for 1689 dropped to 20%, similar to wt, while 3171A-2-1 failed to germinate on the first day.

Although initially delayed, both *oswrky71* lines were able to recover by the second day, with at least 80% of seed germinating in water or MS (Figure 3-4a,b). Steady germination rates proceeded thereafter; nearly 100% of seeds were able to overcome dormancy by the fourth day. The trend was similar in 100mM NaCl (Figure 3-4c). Although the percent germination was lower for the first couple of days, most seeds were able to recover by the third or fourth day. From these results, 3171A-2-1 seeds exhibited a distinctive delay in germination, while germination rates for 1689-2-3 were only slightly delayed with some overlap with wt. Because 1689-2-3 seeds were more variable, this suggested that the position of *dSpm* within *OsWRKY71* might have caused the differences seen in these phenotypes. Since *dSpm* is localized within the 5'-UTR of *OsWRKY71* in 1689-2-3, any leaky expression of *OsWRKY71* might yield functional protein.

When the germination rates were measured within shorter time intervals, every 6 h for 2.5 days, the trend for 3171A-2-1 was more distinct (Figure 3-5). 3171A-2-1 seeds started to germinate around 36 h; about 50% of seeds germinated by the second day and greater than 80 to 90% by 2.5 days. Wildtype seeds germinated about 6 h earlier than 3171A-2-1, and thereafter increased to nearly 50% by 36 h, about the same time 3171A-2-1 seeds started to break dormancy. Most wt seeds germinated by 42 h. This suggested that there could be a delay in germination of about one day for 3171A-2-1 seeds. To further show that environmental differences during seed development had no effect in the

phenotype observed, another line, 3171A-2-15, was tested. Although the seeds were grown in separate pots positioned in different places in the greenhouse, 3171A-2-15 also exhibited a delay in germination.

These results were contrary to my initial prediction. Loss of repression of *α-amyla*se was predicted to increase germination rates, but instead, germination was delayed. Indeed, the loss of *α-amyla*se control could have disrupted the process of dormancy during seed maturation (Gubler *et al*., 2005). Since seeds must complete dormancy in order to successfully germinate (Kucera *et al.*, 2005; Finklestein *et al.*, 2008), the inability to repress *α-amylase* completely during development might have reduced seed vigor.

Root Length Differences in oswrky71 Rice Seedlings

In order to examine the role of OsWRKY71 in seedling growth, root elongation assays were performed. The roots of 3171A-2-1 seedlings were noticeably shorter than wt. This was seen by the second day, and more prominently by the seventh day (Figure 3-6). Although inhibition was also seen in 1689-2-3, the results were variable and not as distinct. Again, my explanation is that the differences in phenotype are attributed to the differences in the positions of *dSpm*, lending to functional protein from leaky expression of *OsWRKY71* in 1689-2-3.

In 3171A-2-3 seedlings, root inhibition was more pronounced in MS media (Figure 3-7), than in water (Figure 3-6). Furthermore, both wt and 3171A-2-1 roots were inhibited by 100 mM NaCl, as expected. To show that differences in light, nutrition, and

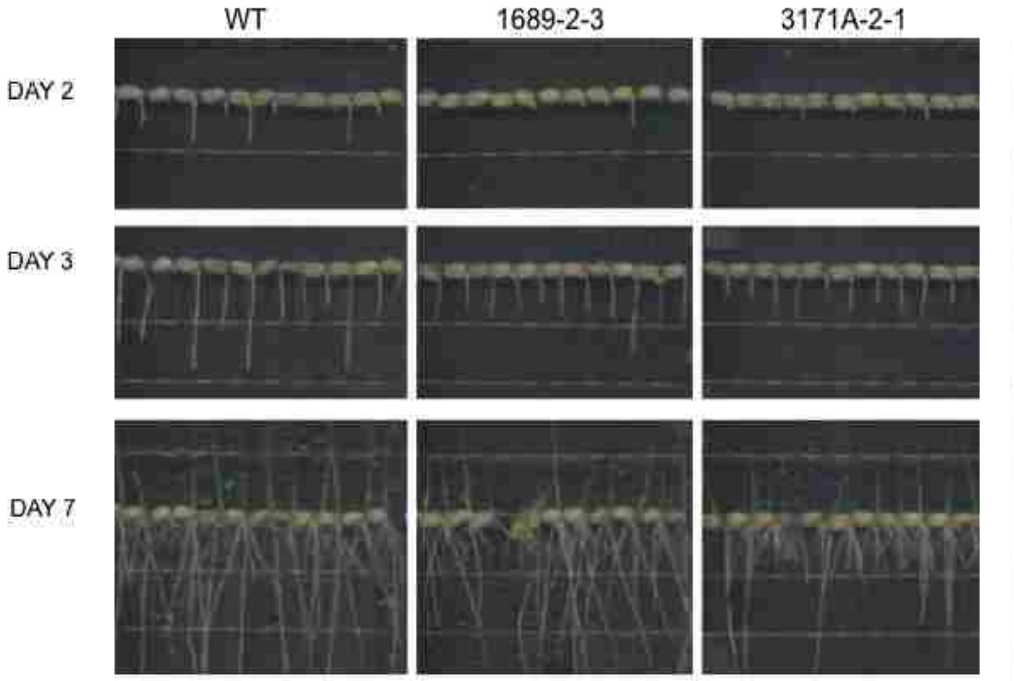


Figure 3-6. Root inhibition was observed in seedlings of *dSpm* knockout 3171A-2-1. Wildtype, 1689-2-3, and 3171A-2-1 root lengths were compared after 2, 3, and 7 days in water. *dSpm* knockout 3171A-2-3 roots were noticeably shorter compared to wt. Three to four biological replicates were used (n=12 seedlings per replicate). The experiment was repeated three times.

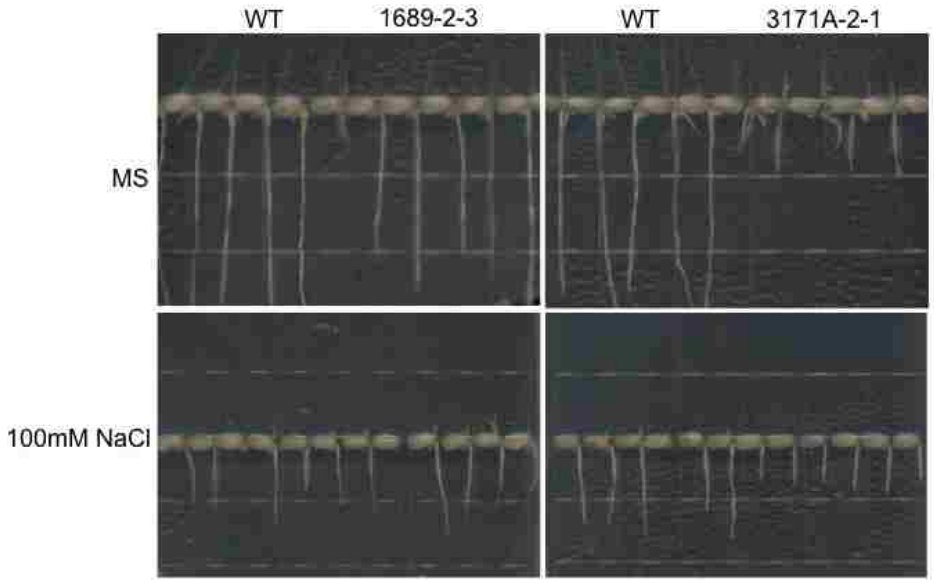


Figure 3-7. Root inhibition in 3171A-2-1 was exhibited in salt treatment. Wildtype, 1689-2-3, and 3171A-2-1 root lengths were compared after 4 days. Roots of 3171A-2-1 were noticeably shorter in MS and in 100mM NaCl. Three to four biological replicates were used (n=12 seedlings per replicate). The experiment was performed one time.

the position of the plates did not influence the phenotype observed, wt and *oswrky71* seeds were also sown side-by-side in each plate.

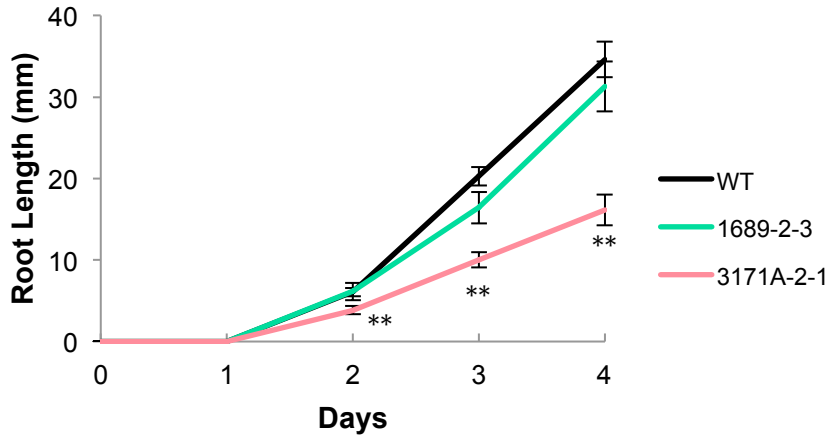
The actual root lengths were measured and graphed (Figure 3-8). By the second day, roots from all lines were visible. Wildtype roots were ~5 mm and thereafter grew, on average, about 15 mm/day. By the fourth day, wt roots were nearly 35 mm. Likewise, 1689-2-3 root lengths were similar to wt, although there was more variation seen among seedlings. 3171A-2-1 seedling roots were ~2.5 mm by the second day, about half of the length of wt. The rate of growth was about 5 mm/day, resulting in roots that were about 2 cm shorter than wt by the fourth day. 3171A-2-1 roots were also significantly shorter than wt in 100 mM NaCl. These data suggest that OsWRKY71 may be necessary for n normal germination and root development in early seedling growth.

Since 3171A-2-1 roots were inhibited, it was predicted that overexpression of *OsWRKY71* might enhance root elongation. However, when T2 overexpression lines, 7112-22-1-1 and -25-1-2, were tested, no differences were observed in either water or 100 mM NaCl. This further verifies that silencing of the transgene has occurred in T2 generation seeds. Or, there may be an optimum level of gene expression of *OsWRKY71* that may affect root elongation.

Conclusion

Mutation of *OsWRKY71* resulted in decreased seed weight, and abnormal seed germination and root growth. These results were based on seed measurements, and germination and root elongation assays. Specifically, knockdown line, 3171A-2-1, exhibited a delay in seed germination and an inhibition in root elongation in water, MS,

a



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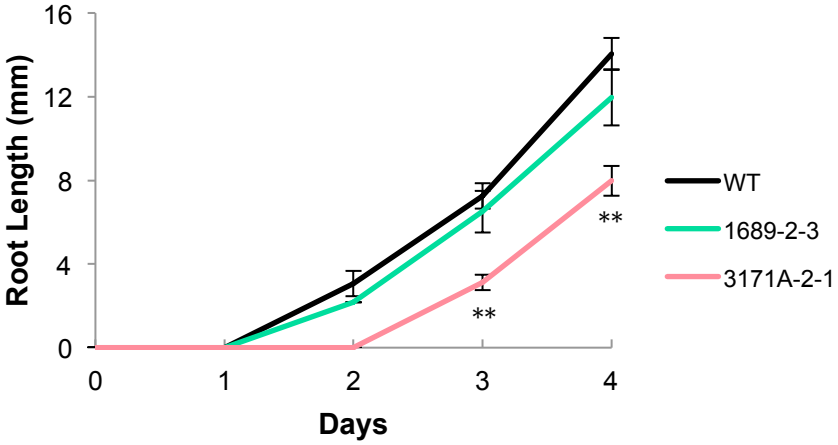
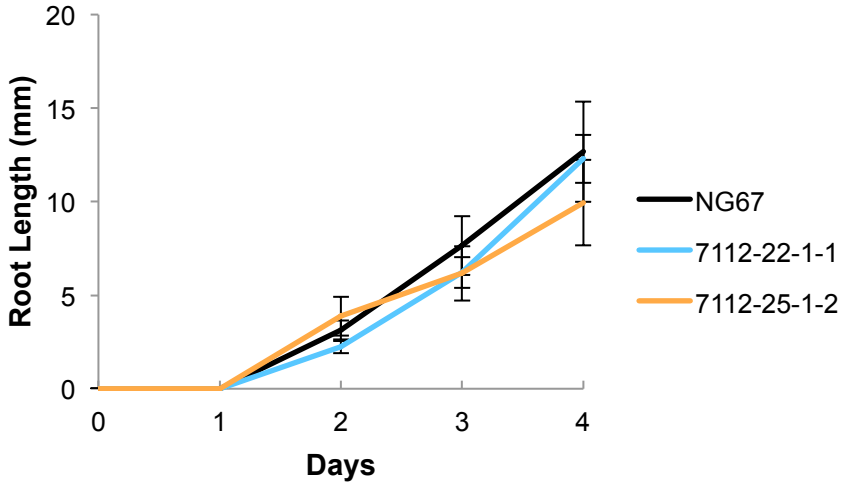


Figure 3-8. Roots of *dSpm* knockout 3171A-2-1 were significantly inhibited. Root lengths in (a) water and (b) 100mM NaCl. Three biological replicates were used (n=12 seedlings per replicate; ** = $p < 0.005$). This experiment was repeated three times.

a



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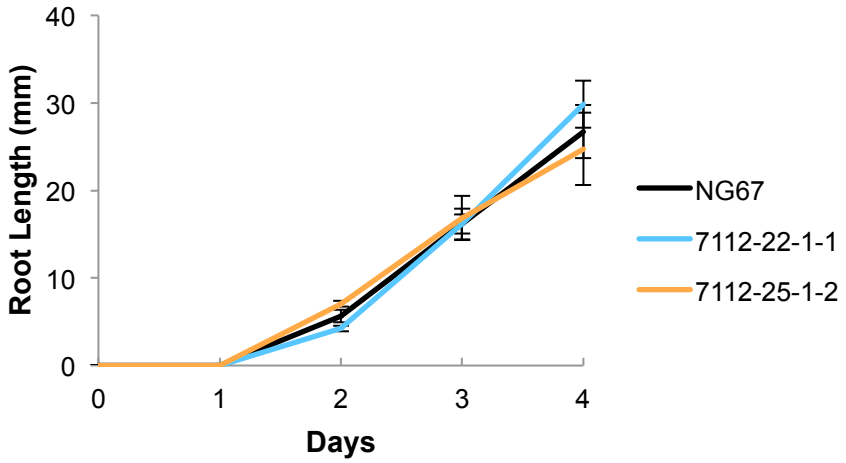


Figure 3-9. Overexpression lines for *OsWRKY71* do not exhibit differences in root length. Roots were measured in (a) water and in (b) 100mM NaCl. Three biological replicates were used (n=12 seedlings per replicate). This experiment was performed one time.

and 100 mM NaCl. Interestingly, the recovery observed in seed germination suggests that additional mechanisms or redundant functions exist during germination. Also, once the process of germination does begin, it appears that germination may not be inhibited but progresses towards completion, suggesting that there is a threshold or all-or nothing event to ensure that germination completes itself, by forth the emergence of the radical (Finklestein *et al.*, 2008). Hence, this ensures survival even if surrounding conditions are unpleasant, since the seed is vulnerable at this stage and cannot revert back to dormancy without facing possibilities of deterioration. Therefore, OsWRKY71 appears to affect the initial start of germination.

Unexpectedly, these observations were contrary to my initial predication, based on initial transient expression assays. It is possible that OsWRKY71 may indeed regulate *α-amylase* but mutation may have resulted in a loss in seed vigor in developing seeds. Or OsWRKY71 may regulate other genes (Chujo *et al.*, 2008), resulting in indirect repression of *α-amylase* or other growth-related genes. From these analyses, it appears that OsWRKY71 is a positive regulator of seed germination and root elongation. Therefore, to confirm that the phenotypes are indeed caused by the disruption of *OsWRKY71*, the germination and root elongation assays should be performed in the near future on rescue or heterozygous lines, particularly for 3171A-2-1. The delay should be lessened or removed in these lines compared to homozygous lines. Also, since there were some differences exhibited in both knockout lines for *OsWRKY71*, the phenotypes should be examined in a transheterozygote of 1689-2-3 and 3171A-2-1. The strong delay in germination and root growth should thus be observed in the transheterozygote, if not emphasized. If the mutations are not on the same gene, then the addition of a wt copy

would rescue the delay or make the phenotype less pronounced. Furthermore, it is possible that the enhancement in the knockout line 3171A-2-1 is overshadowed by a dominant negative function of the N-terminal peptide, about 64 amino acids long, resulting from the transcription of *OsWRKY71* upstream of the *dSpm* insertion at the second exon (Chiu *et al*., 2010). Since *OsWRKY71* shares sequence similarity to *OsWRKY18*, it is possible that the truncated peptide acts as a dominant negative for *OsWRKY18*, which may act as a positive regulator of seed germination. If 3171A-2-1 is indeed a dominant negative, then heterozygotes would still exhibit repression instead of improved growth.

To further attribute low levels of *α-amylase* to the delay in growth, particularly during seed germination, qPCR should be performed to test the level of expression of *α-amylase* in germinating seeds. Furthermore, the expression of *α-amylase* should be measured in developing seeds, during the maturation stage, on the mother plant to associate a possible loss in repression to lower seed weight, incomplete dormancy, and a loss in seed vigor. Finally, because leaky expression of *OsWRKY71* might be one possible explanation for the phenotype differences between 1689-2-3 and 3171A-2-1, the expression of *OsWRKY71* should be examined in germinating seeds and roots. Although the expression was low in mature leaves (Figure 2-2), the level of leaky expression might be higher in other tissues, contributing to differences in phenotype.

CHAPTER 4

ANNOTATION OF THE HIL SUPERFAMILY AND INVESTIGATION OF THEIR EXPRESSION PATTERNS IN RESPONSE TO GA AND ABA IN THE RICE ALEURONE

Introduction

Recently, OsWRKY71 was found to interact with several proteins expressed in the rice aleurone, as suggested by yeast-2-hybrid experiments (Dr. Lingkun Gu, unpublished). One of these proteins (Locus #Os05g45070 or LOC_ Os05g45070) was interesting because it shares some sequence similarity to a harpin-induced protein, *NtHIN1*, but the actual roles in plants has not yet been described. Homologs of this protein in Arabidopsis were identified as a family of NHL proteins, which consisted of 45 members (Dormann *et al.*, 2000; Zheng *et al.*, 2004). However, this family has not been annotated in rice or other plant species. The primary aim for this project is to compile all members of the HIL family in rice using a hidden Markov model (HMM) and to further annotate this family using additional *in silico* applications.

Because "LOC_Os05g45070" was able to interact with OsWRKY71, I hypothesized that it was involved in ABA- and GA-mediated seed germination. HIL members that were previously reported were found to be involved in pathogen stress, some of which were induced by defense hormones SA and JA (Varet *et al.*, 2002; Zheng *et al.*, 2004; Lee *et al.*, 2006). Since SA and JA were shown to contribute to seed germination (Linkies and Leubner-Metzger, 2012), it is not unlikely that other HILs might be expressed in the aleurone cells. Therefore, for the second aim, RNA-sequencing

data (Dr. Shen's lab) was used to identify HILs expressed in the aleurone and when treated with GA or ABA. Other rice tissues were analyzed as well, using the Michigan State University Rice Genome Annotation Project (MSU RGAP) collection of deep sequencing data.

Methods and Materials

Identification and Annotation of the HIL Family in Rice

HIL members were identified from the protein sequence database from MSU RGAP (7th Release) using an HMM approach. First, the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) was used to identify closely related rice paralogs to LOC_Os05g45070. Of these, 10 of the most closely related sequences to LOC_Os05g45070 were used to build the HMM, a probability model, using the HMMER (v2.3.2) software (Sonnhammer *et al.*, 1998). The software was then used to search the MSU RGAP protein sequence database for potential HIL proteins similar to the input at an e-value of 1.0, which is the same e-value used for the Protein Family database (PFAM) HMM. These proteins were used as a new input for another reiteration of HMM, which would build an improved probability model that could then search for additional HIL members. Several reiterations of the HMM model constructions and searches were performed until no other unique protein members were identified. The resulting protein sequences were named according to their MSU annotation identifier.

Protein motifs were identified using MEME software (Bailey et al. 1994). With the resulting motifs, the HIL members were classified into eight groups, based on the

presence or absence of the identified motifs. Multiple alignment was performed to highlight these motifs within representative members using the PRALINE alignment tool (Simossis et al. 2005).

To reconstruct the phylogeny of the HIL family, protein domain sequences (output from HMMER v3.0 searches) were used to construct a bootstrap consensus tree (10,000 bootstrap replications) with MEGA 5.0. Reconstruction was based on amino acid differences, with colored dots used to represent the group to which each member belonged.

A chromosome map was built to visualize the distribution of HIL members in the rice genome. The MSU annotation identifiers of all HIL members were inputted into the map tool of National BioResource Project (NBRP) Oryzabase (http://viewer.shigen.info/oryzavw/maptool/MapTool.do). The map was reproduced to contain the general name and the locus of each member.

Analysis of RNA Expression in Rice Aleurone and Other Tissues

Total RNA was extracted from hormone-treated rice aleurone tissue treated with or without hormone treatment for five hours (1 μM GA, 20 μM ABA, or 1 μM GA and 20 μM ABA). RNA was extracted using the guanidinium sulfate–phenol–chloroform extraction and purified using RNeasy RNA purification columns (Qiagen, Valencia, CA). The mRNA was then purified from the total RNA using Oligo(dT) Dynabeads (Invitrogen, Grand Island, NY). RNA library preparation and sequencing was performed using the Illumina HiSeq 2000 at the Huntsman Cancer Institute (University of Utah). Reads were aligned to the rice genome using Cufflinks and Tophat. RNA expression of

HIL members was analyzed using normalized RPKM (reads per kilobase of exon per million fragments mapped to the genome) values. For identification of HILs in other rice tissues, the entire MSU RGAP (7th Release) RPKM collection of the rice genome was extracted and organized by Patricia Ringler. Then, the expression of specific HIL members in select rice tissues were identified and compiled.

Gene Clustering

Gene clustering experiments were conducted using R programming to generate a heat map. The HIL members and ABA receptors or RCARs were clustered based on similar expression patterns in response to the hormone treatments using aleurone RNA-sequencing data.

Results and Discussion

Identification of the HIL Protein Family in Rice

In order to identify members belonging to the HIL family in rice, HMM was performed using LOC_Os05g45070 and 10 closely related proteins in rice. The HMM search against the rice genome was able to identify 104 unique HIL proteins (Table 4-1) after 4 iterations. The identified members were named in the order of their chromosome and locus number (HIL1-104), to which LOC_Os05g45070 was identified as OsHIL58.

Of the 104 HILs identified, 66 members were also described by PFAM HMM as LEA_2 (PF03168) domain proteins or putative harpin-induced proteins. None of the putative LEA14 proteins, which were also part of PF03168, were identified as part of the

Name	Annotation ID (LOC_)	GenBank Accession	SwissProt ID	cDNA	Group	Name	Annotation ID (LOC_)	GenBank Accession	SwissProt ID	cDNA	Group
OsHIL1	Os01g09880	BAD72207	Q5SMX9	-	I	OsHIL53	Os05g11010	AAV24918	Q60E41	AK069019	II
OsHIL2	Os01g12820	BAF04392	Q5N7Z3	AK119627	I	OsHIL54	Os05g14880	AAU90162	Q60ET9	AK073471	I
OsHIL3	Os01g13340	EAZ11166	A2ZR19	-	I	OsHIL55	Os05g24760	EAY97488	A2Y328	-	I
OsHIL4	Os01g39290	BAB91906	Q8LQJ5	-	I	OsHIL56	Os05g30490	BAF17254	Q0DIR9	-	II
OsHIL5	Os01g46670	-	-	-	III	OsHIL57	Os05g40400	EAY98459	A2Y5U9	-	I
OsHIL6	Os01g46730	BAD72328	Q5SN78	-	III	OsHIL58	Os05g45070	AAU43951	Q65X87	AK108457	I
OsHIL7	Os01g51460	EAZ13298	A2ZX51	-	VII	OsHIL59	Os05g50720	AAS16886	Q75HZ1	AK063149	II
OsHIL8	Os01g51470	BAB39429	Q9ASD0	-	VII	OsHIL60	Os06g02630	-	-	-	II
OsHIL9	Os01g51490	EEE55283	B9EZ39	-	VII	OsHIL61	Os06g02670	BAD68544	Q5VPT1	-	II
OsHIL10	Os01g51500	BAD81537	Q5N8D7	-	III	OsHIL62	Os06g02800	BAD68366	Q5VPR5	-	V
OsHIL11	Os01g51510	BAD81538	Q5N8D6	-	VII	OsHIL63	Os06g02810	BAD68367	Q5VPR4	-	II
OsHIL12	Os01g51520	BAB89116	Q8S0I3	-	III	OsHIL64	Os06g02840	BAD68370	Q5VPR1	-	II*
OsHIL13	Os01g53470	BAB68095	Q942B1	AK111351	I*	OsHIL65	Os06g02930	BAD68376	Q5VPQ5	-	II
OsHIL14	Os01g56500	BAD52727	Q5ZD20	-	VII	OsHIL66	Os06g02940	BAD68377	Q5VPQ4	-	V
OsHIL15	Os01g59680	BAB85244	Q8RUR5	-	I	OsHIL67	Os06g03030	BAD68383	Q5VQ92	-	II
OsHIL16	Os01g64450	BAF06808	Q0JHH0	AK063651	I	OsHIL68	Os06g03040	EAZ35647	A3B7V8	-	V*
OsHIL17	Os01g64470	BAB63815	Q94CU7	AK068115	III	OsHIL69	Os06g03059	BAD68386	Q5VQ85	-	II
OsHIL18	Os01g64480	EAZ14260	A2ZZW3	-	I	OsHIL70	Os06g03070	BAD68387	Q5VQ88	-	II
OsHIL19	Os01g68080	EEE55856	-	-	V	OsHIL71	Os06g03099	Same As *OsHIL69*		-	II
OsHIL20	Os01g68090	BAB92611	Q8LQJ2	-	II	OsHIL72	Os06g03110	BAD68391	Q5VQ84	-	II
OsHIL21	Os02g01060	BAD08179	Q6YU99	AK062483	II	OsHIL73	Os06g06780	BAD67647	Q5WA73	AK060328	I
OsHIL22	Os02g16030	BAF08399	Q0E289	-	II	OsHIL74	Os06g10990	BAD35186	Q69TV0	AK071518	VIII
OsHIL23	Os02g16610	EAZ22508	A3A5B9	-	I	OsHIL75	Os06g32970	EAZ37172	A3BC83	-	I
OsHIL24	Os02g30450	BAD23189	Q6K2G8	-	I*	OsHIL76	Os06g49650	BAD53642	Q5Z9G5	AK062418	II
OsHIL25	Os02g33550	BAD27922	Q6ER87	AK060001	II	OsHIL77	Os07g10610	-	-	-	VII
OsHIL26	Os02g40560	BAD21461	Q6K946	-	VII	OsHIL78	Os07g14660	EAZ39289	-	-	V
OsHIL27	Os02g40590	BAD21556	Q6K945	-	VII	OsHIL79	Os07g14700	BAC20128	Q8GRV7	AK107935	V
OsHIL28	Os02g40600	-	-	-	III	OsHIL80	Os07g14740	BAC20132	Q8H3F6	-	V
OsHIL29	Os02g40610	-	-	-	VII*	OsHIL81	Os07g34040	BAD30741	Q69SA8	AK108285	I
OsHIL30	Os02g40620	BAD21560	Q6K941	-	VII	OsHIL82	Os07g34050	EAZ40060	-	-	I
OsHIL31	Os02g40630	EEE57388	-	-	VII	OsHIL83	Os07g34720	BAC45158	Q8GVN7	AK241202	I
OsHIL32	Os02g40640	BAD21562	Q6K939	-	VII	OsHIL84	Os08g01210	BAD33160	Q69U66	-	III
OsHIL33	Os02g40650	BAD21563	Q6K938	-	VII	OsHIL85	Os08g01220	BAD33162	Q69U64	AK061430	II
OsHIL34	Os02g44670	BAD27780	Q6ESG7	-	I	OsHIL86	Os08g23460	BAD03245	Q6Z0E0	-	I
OsHIL35	Os02g44740	BAD27789	Q6ET94	AK073500	III	OsHIL87	Os08g38580	BAD10024	Q6Z8S6	AK110788	I
OsHIL36	Os03g09160	ABF94405	Q10QM2	-	II	OsHIL88	Os08g44410	BAD13128	Q6YZI9	-	I
OsHIL37	Os03g11710	ABF94647	Q10PY9	-	VI*	OsHIL89	Os09g09460	EAZ43981	A3BWP2	-	I
OsHIL38	Os03g15630	ABF95102	Q10NQ5	AK061109	I	OsHIL90	Os09g26480	BAD36123	Q69PF4	-	III
OsHIL39	Os03g26080	ABF96240	Q10KM6	AK099806	VI	OsHIL91	Os09g36210	BAD46021	Q652J9	-	II
OsHIL40	Os03g48950	NP_001050978	-	-	I*	OsHIL92	Os10g34550	-	-	-	VIII
OsHIL41	Os03g62010	AAR88579	Q75LJ9	AK061174	V	OsHIL93	Os10g34560	AAL59027	Q8W380	-	VIII
OsHIL42	Os03g62020	AAR88575	Q75LK0	AK243622	V	OsHIL94	Os10g39970	ABB47955	Q336V2	AK060129	II
OsHIL43	Os04g02410	EAZ29483	A3AQ94	-	V	OsHIL95	Os11g02730	ABA91198	Q2RB97	AK101778	I
OsHIL44	Os04g33990	BAF14680	Q7X7T9	AK068271	II	OsHIL96	Os11g03600	ABA91314	Q2RB10	-	III
OsHIL45	Os04g35130	CAE03026	Q7XQQ4	-	III	OsHIL97	Os11g05860	AAX92937	Q53NC9	AK070326	I
OsHIL46	Os04g42970	CAE04733	Q7XM96	-	VII	OsHIL98	Os11g05870	ABA91553	Q2RAC3	AK071072	I
OsHIL47	Os04g43519	CAE01916	Q7X8V0	-	VII	OsHIL99	Os11g37680	ABA94463	Q2R1Y2	-	II
OsHIL48	Os04g53650	BAF15873	Q7XN75	-	II	OsHIL100	Os12g02700	ABA95669	Q2QYH1	AK059980	I
OsHIL49	Os04g58090	BAF16177	Q7XKB7	-	I	OsHIL101	Os12g03370	ABA96360	Q2QY99	-	III
OsHIL50	Os04g58850	BAF16231	Q7XPU1	AK070446	I	OsHIL102	Os12g06210	ABA96517	Q2QXF6	-	II
OsHIL51	Os04g58860	BAF16232	Q7XPU0	AK061257	II	OsHIL103	Os12g06220	ABA96518	Q2QXF5	AK107952	II
OsHIL52	Os04g59330	CAE01801	Q7XST6	AK060983	I	OsHIL104	Os12g06260	ABA96522	Q2QXF1	AK070481	I

Table 4-1. List of HIL members in rice. HIL proteins were named based on the consecutive order of their chromosome position. Helpful assession identifiers of gene models were included. Group numbers were based on the presence of the NPN, RPP, and YQYF motifs (Figure 4-2). Members with multiple motifs were identified with astericks.

HIL family. LEA14 is a small, atypical subgroup of the LEA family, a large group of proteins with many functions but particularly recognized for their role as an osmoprotectant during dehydration in embryogenesis (Wise *et al*., 2004; Tunnacliffe and Wise, 2007; Hand *et al*., 2011). Despite the conservation of the LEA_2 domain (Ciccarelli and Bork 2005), the HIL family is distinct from the LEA14 subgroup, as predicted by PFAM HMM (Appendix C). Additionally, 38 HIL proteins were undescribed by the PFAM and considered novel HIL members.

Efforts were made to identify rice homologs of the HIL proteins in other species. The closest homolog of NtHIN1 (Gopalan and He, 1996), the first member discovered and found to be harpin-induced, was OsHIL50, which was previously described as OsHIN1 (Kim *et al.*, 2000). Two other NtHIN1 homologs, NtHIN9 and NtHIN18 (Takahashi *et al.*, 2004), were also homologous to OsHIL50. Additionally, OsHIL97 was discovered to be the homolog of BnNHL18A and -B in rapeseed (*Brassica napus*). The expression of *BnNHL18A* and -*B* was elevated upon treatment with NaCl, H_2O_2, and SA, which induced changes in localization (Lee *et al.,* 2006).

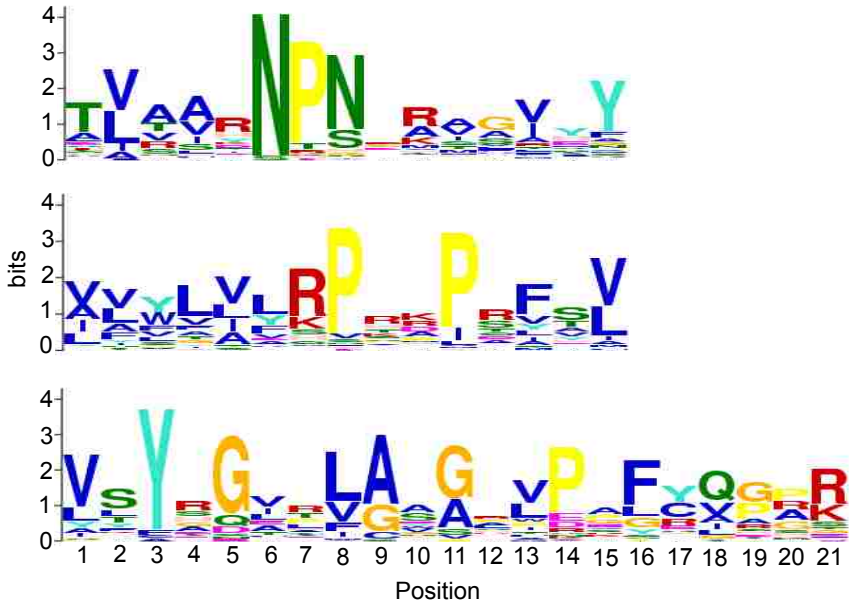
Additional rice homologs were identified for several Arabidopsis members. OsHIL83 was homologous to AtNDR1, the first HIL identified in Arabidopsis found to be required for resistance to bacterial and fungal infection (Century *et al.*, 1997). HILs were also homologous to previously described NHL proteins (Varet *et al.*, 2002; Varet *et al*., 2003, Zheng *et al*., 2004; Lee *et al.*, 2006). AtNHL1, -2, and -3 were differentially expressed in response to pathogen-induced stimuli. OsHIL97 was a homolog of AtNHL1. OsHIL50 was homologous to AtNHL2, which was found to elevate pathogenesis-related 1 (PR-1) gene expression. It was also similar to AtNHL3, which was implicated in

wounding-responsiveness and suppressed by virulent *Pseudomonas syringae* pv. tomato DC3000 during pathogenesis (Dörmann *et al.*, 2000; Varet *et al*., 2002; Varet *et al*., 2003). AtNHL25, which was induced by SA and the pathogen *Peronospora parasitica*, was similar to OsHIL49 (Varet *et al.*, 2002). In addition, Yellow Leaf-Specific-9 (AtNHL10/YLS9), a member induced during hypersensitive response to CMV and senescence in leaf tissue, was found to be homologous to OsHIL17 (Zheng *et al.*, 2004).

Analysis of Motifs within the HIL Family of Proteins

MEME was used to determine the amino acid motifs that were most conserved in all members of the HIL family in rice (Figure 4-1). Based on the results, three motifs were highly conserved throughout all members. The first motif discovered had a highly conserved asparagine, proline, followed by another asparagine, with the consensus sequence T[VL]A[AV]RNPNxRAG[VI]YY (E-value of $2.10e^{-190}$). This first motif was called the NPN motif. It was generally present near the center of the HIL protein sequences. The second most conserved motif was characterized by two highly conserved proline residues preceded by either an arginine or a lysine with the consensus sequence [VA][VL][YW]LV[LY]RPRxP[RS]FS[VL] (E-value of $6.10e^{-126}$). This motif was called the RPP motif and was nested in the predicted transmembrane region towards the N-terminal region; the residues preceding the RPP signature were typically hydrophobic. Third, a stretch of amino acids was identified characterized by a highly conserved tyrosine, followed by a phenylalanine and a tyrosine, and separated by a stretch of hydrophobic residues. The consensus for this motif was determined to be

a

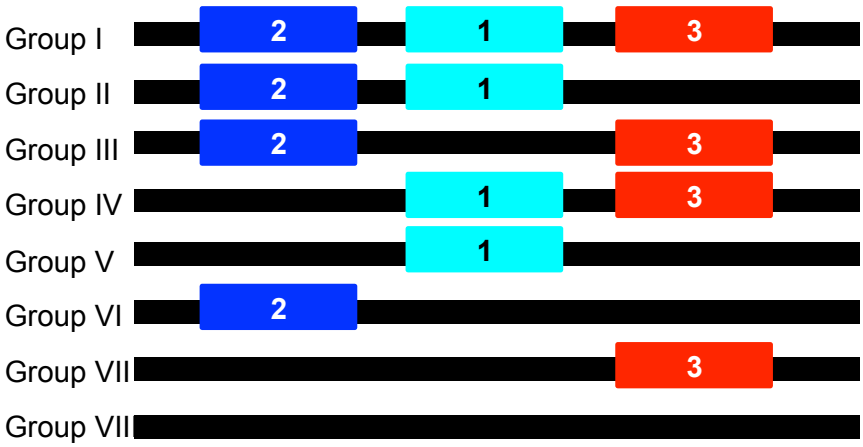


b

Rice Motifs	Sequence	Name	E-Value
1	T[VL]A[AV]R**NPN**xRAG[VI]YY	**NPN**	2.10E-190
2	[VA][VL][YW]LV[LY]**RP**Rx**P**[RS]FS[VL]	**RPP**	6.10E-126
3	VS**Y**RGVR[LV][AG][AS][GA]x[VL]PA**F**[**Y**C]**Q**[GP]P[RK]	**YFYQ**	4.70E-134

Figure 4-1. Motif analysis of proteins in the HIL family. Rice protein sequences were inputted into MEME software (Bailey *et al.*, 1994) and shown are the (a) output results with the most conserved amino acids in large letters. Colors represent physiological properties as determined by MEME. (b) Motifs were named NPN, RPP, and YQYF, according to their respective consensus sequence, which is highlighted in bold.

a



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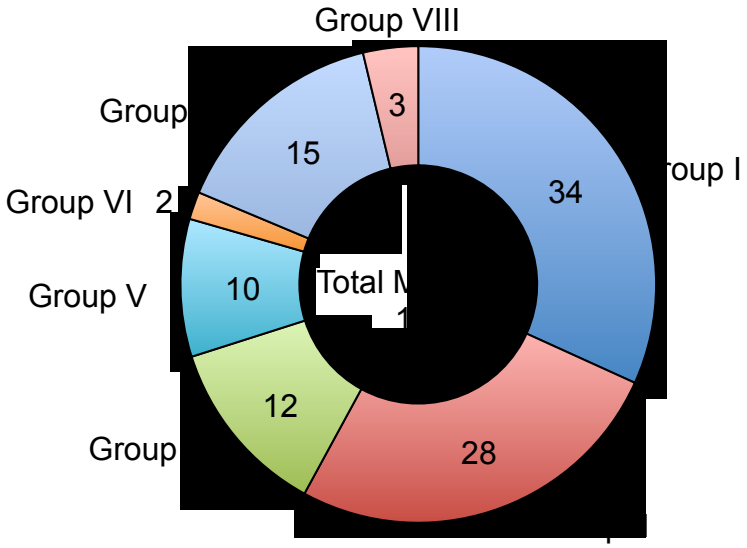


Figure 4-2. Schematic diagram of the subgroups in the HIL family. (a) HIL groups were determined based on the presence or absence of the three major motifs: NPN, RPP, and YQYF. (b) Total number of HIL members within each group.

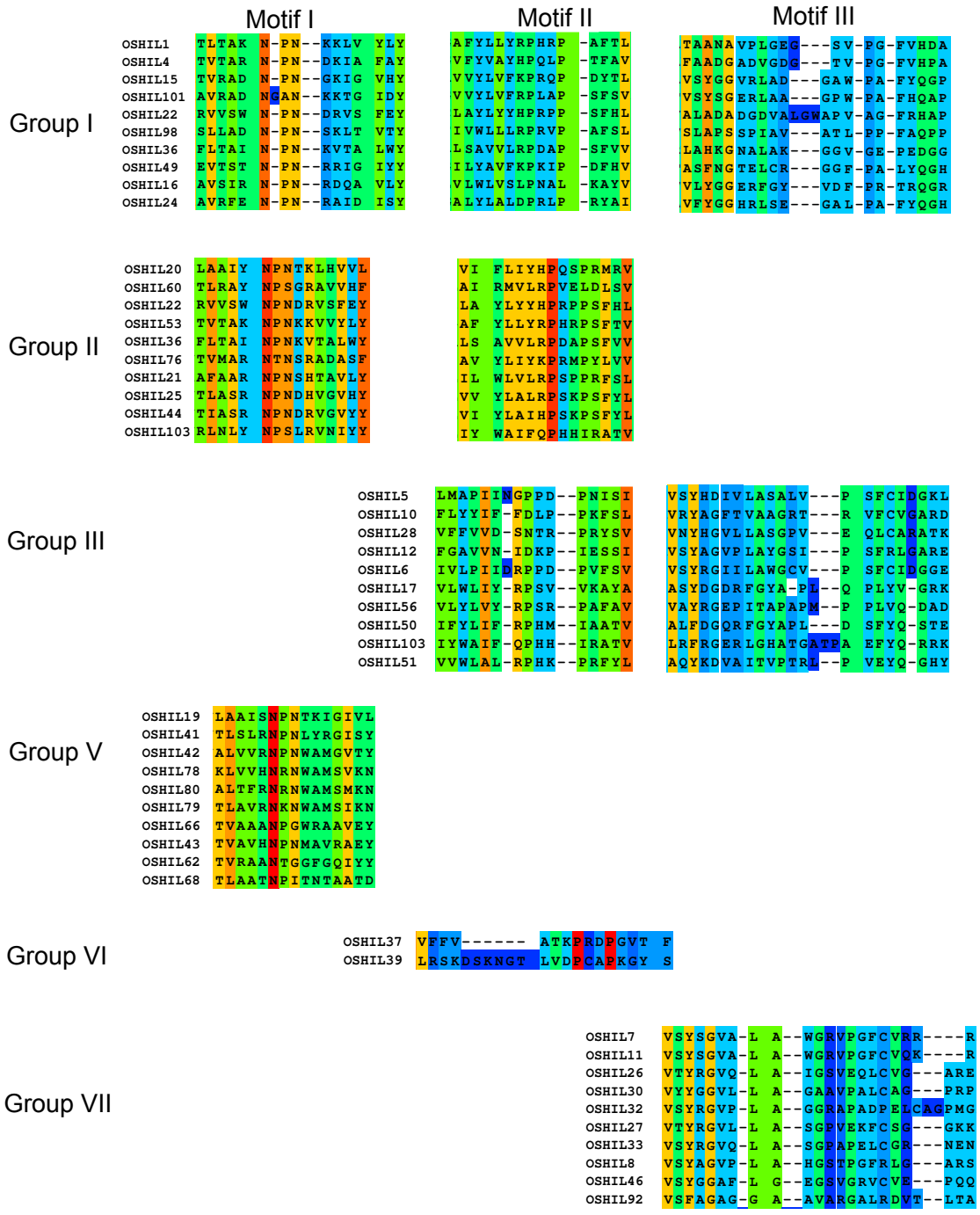


Figure 4-3. Multiple sequence alignment for HIL subgroups I-VII. Alignment was performed using PRALINE (Simossis *et al.*, 2005). Representative HILs from each group are shown.

VSYRGVR[LV][AG][AS][GA]x[VL]PAF[YC]Q[GP]P[RK] (E-value of $4.70e^{-134}$) and was generally found in the C-terminus region. It was given the name YFYQ based on the consensus determined from previous MEME/MAST analysis performed on the NHL members of Arabidopsis (Dörmann *et al.*, 2000).

The HIL members in rice were organized based on the presence or absence of the three key protein motifs (Figure 4-2). Members belonging to Group I have all three of the motifs, NPN, RPP, and YQYF, present. This group encompassed the most members in rice, 34 out of 104, including the OsWRKY71-interacting protein, OsHIL58. Major rice HIL members OsHIL50, the homolog of NtHIN1, AtNHL2 and -3; OsHIL49 (AtNHL25); OsHIL83 (AtNDR1); and OsHIL97 (AtNHL1 and BnNHL18A/B) were classified in Group I (Dörmann *et al.*, 2000; Varet *et al*., 2002; Varet *et al*., 2003; Zheng *et al.*, 2004; Lee *et al*., 2006). Group II-IV had a combination of any two of the motifs, with Group II having the most members of the three, 28 of the 104 members. Described HIL member OsHIL1, a homolog of AtNHL10/YLS9, belonged to Group III, which is classified as having an RPP and YQYF motif but not the highly conserved NPN motif. In rice, no members were classified into Group IV. Members with at least one of the three motifs were classified into Groups V-VII, with Group VII having the most members of the three (15 members out of 104 members). Group VIII HIL proteins were atypical, in that the three motifs were not present in the amino acid sequence. The alignments of reference members from each group may be seen (Figure 4-3).

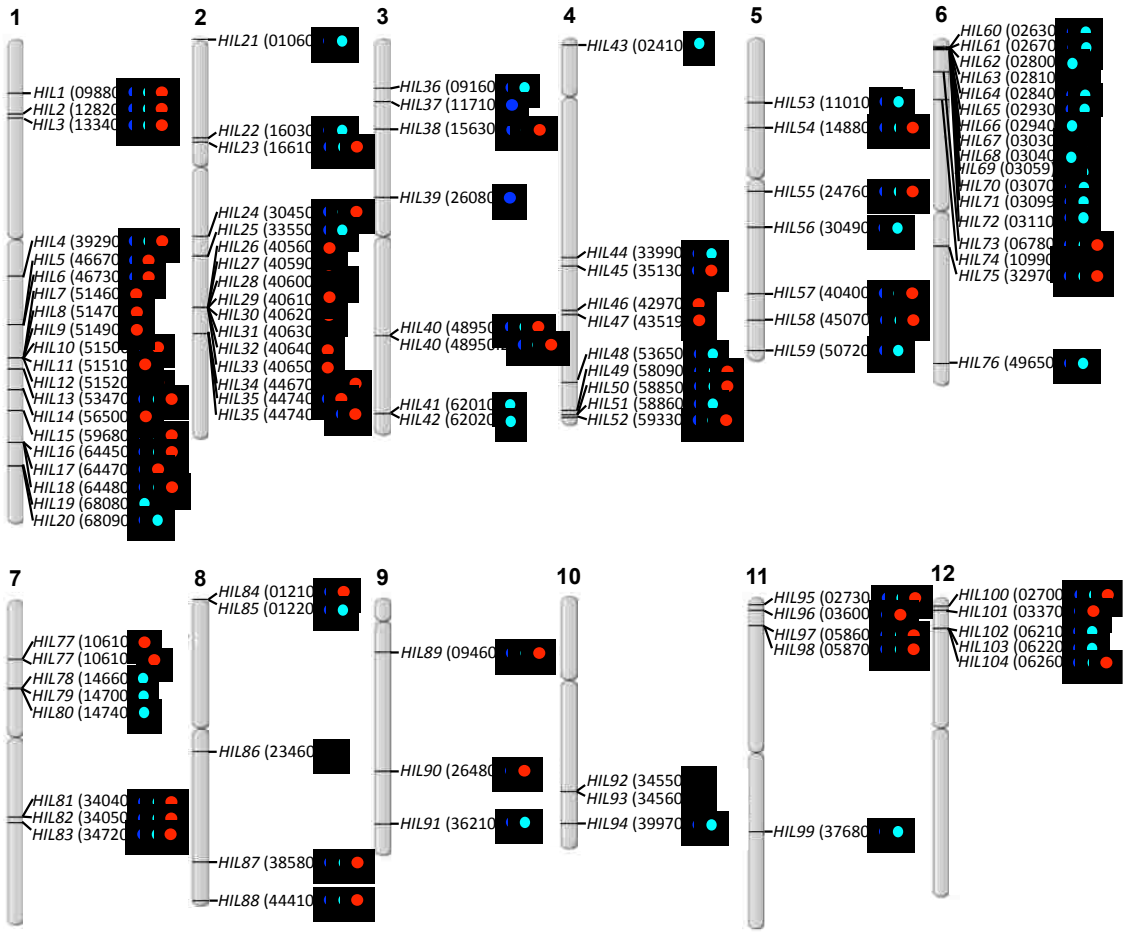


Figure 4-4. Distribution of the HIL family in the rice genome. Rice members were distributed along the 12 chromosomes in rice. NBRP Oryzabase (http://viewer.shigen.info/oryzavw/maptool/MapTool.do) was used to develop the chromosome map. The locus identifiers were provided without the "LOC_Os##" for simplicity. Group identification was designated by the same color codes as those for Figure 4-2. The color codes are: turquoise for motif 1, NPN; blue for motif 2, RPP; and red for motif 3, YQYF. Triangles symbolize the absence of the motifs.

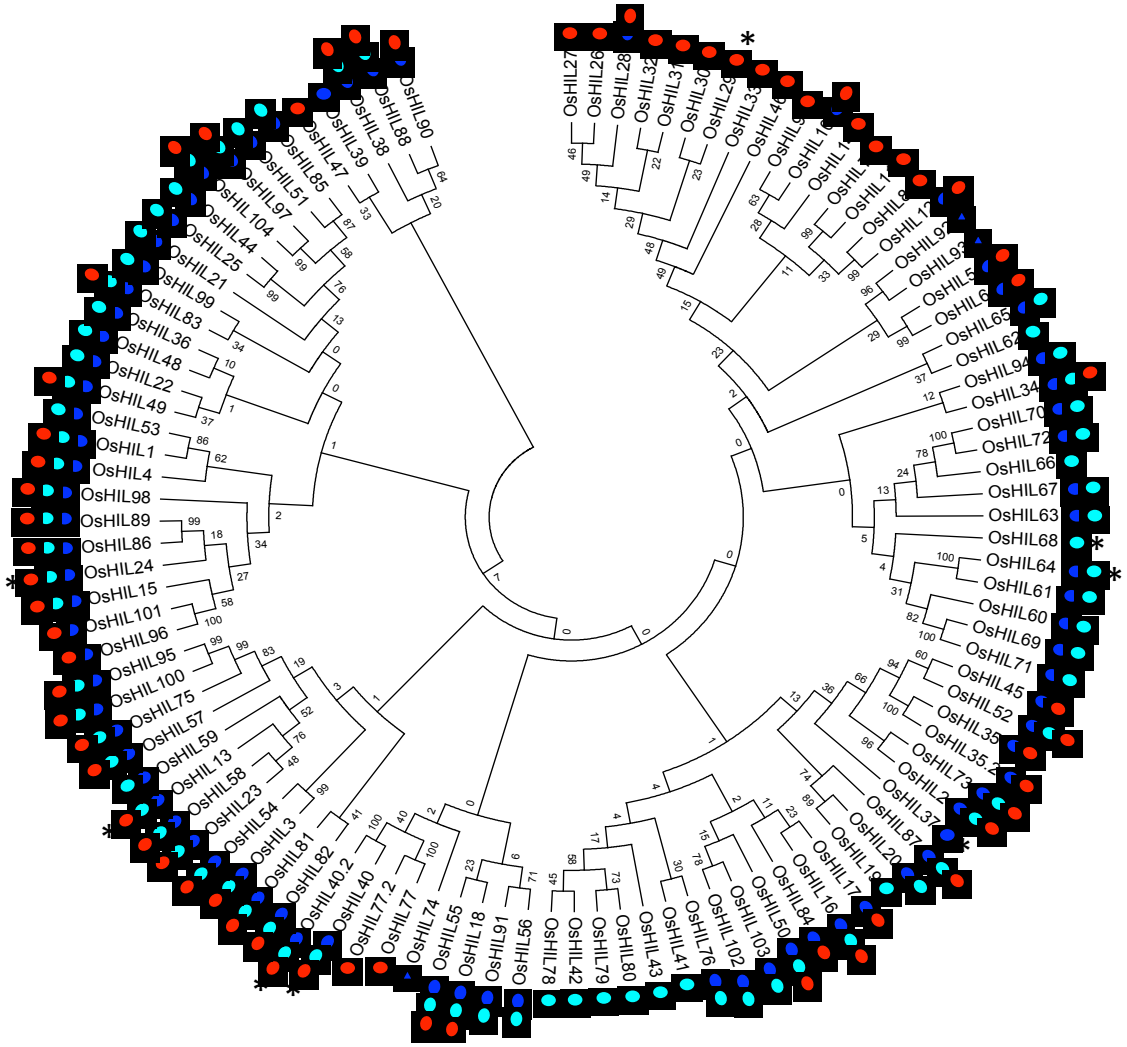


Figure 4-5. Phylogenetic tree of the members in the HIL family. Alignment was performed using the ClustalW algorithm included in the MEGA 5.0 software package and the default parameters (Gap Opening 10, Gap Extension 0.2, Gonnett weight matrix). The tree was constructed with 10,000 bootstraps. Group identification was designated by the same color codes as those seen in Figure 4-2. The color codes are: turquoise for motif 1, NPN; blue for motif 2, RPP; and red for motif 3, YQYF. Triangles symbolize the absence of the motifs.

Distribution of HIL Members in the Rice Genome

The genes encoding the HIL proteins were fairly evenly distributed throughout the rice genome (Figure 4-4). Of the 12 rice chromosomes, chromosomes 1 and 6 encompassed the most HIL genes, 20 and 17, respectively. Large clusters may be found on these chromosomes. Of the eight major groups in the family, Group I HILs were predominant on chromosome 1. The major gene cluster on chromosome 6 comprised of Group II members, suggesting an occurrence of gene duplication events. On chromosome 2, the majority of members in the minor gene cluster of eight members belonged to Group VII; one member was a Group III protein. Chromosomes 9 and 10, the smallest chromosomes, had the fewest members with three HILs on each.

To better understand the relationship of HILs to one another, a phylogenetic tree was created using MEGA 5.0 (Figure 4-5). Members of a particular group were generally clustered together, and those that did were also clustered in the genome (Figure 4-4). For example Group II members shared sequence similarities and were clustered together on chromosome 6. Group VII members were also similar based on phylogenetic analysis and were arranged in a cluster on chromosome 2. This further suggested that gene duplication events might have occurred.

On the other hand, some members, such as those belonging to Group I, were generally dispersed throughout the phylogenetic tree; OsHIL1, -2, and -3 were neighbors on chromosome 1, but their sequences were divergent. OsHIL102, -103, and -104 were clustered on chromosome 12, but OsHIL104 shared sequence similarity to OsHIL97, which was localized on chromosome 11. Furthermore, OsHIL91 and -56 were Group II members with sequence similarity but were found on separate chromosomes. Therefore,

transposition of these duplicated genes might have occurred. The paralog of OsHIL58, OsHIL23, was localized on chromosome 2, whereas OsHIL58 was located on chromosome 4. The tree also identifies OsHIL13 as a closely related protein; thus, OsHIL13 and -23 may share some functional redundancy with OsWRKY71.

Expression of HIL Members in Various Rice Tissues

The expressions of the HIL genes were extracted from different developmental stages and tissues in rice from the MSU RGAP (7th Release) RPKM collection. Of the tissues examined, including seedlings, mature leaves and shoots, and reproductive tissue, most HILs, 50 genes, were expressed in pre-emergence inflorescence, whereas the least number of HILs were expressed in the endosperm (Figure 4-6). Interestingly, *OsHIL50* was a highly expressed HIL in 4 day seedlings (>200 RPKM), leaves (100-200 RPKM), and throughout all tissues examined. The expression is consistent with the strong elicitation of hypersensitive response observed in tobacco leaves in *NtHIN1* (Gopalan and He, 1996). Therefore, *OsHIL50* may be a primary responder during pathogen attack in rice. Other HILs such as *OsHIL44*, *-25*, *-77*, and -97 also had a large number of reads in multiple tissues.

Unlike the HILs mentioned, OsHIL56 may have a more specific role during a specific time in development. The expression of *OsHIL56* was largely restricted to any stage regarding seed development. Specifically, the number of reads in embryos was 100-200 RPKM, and 50-100 RPKM in 5 days after pollination (DAP) and 10 DAP, with an average number of reads in the endosperm after 25 DAP.

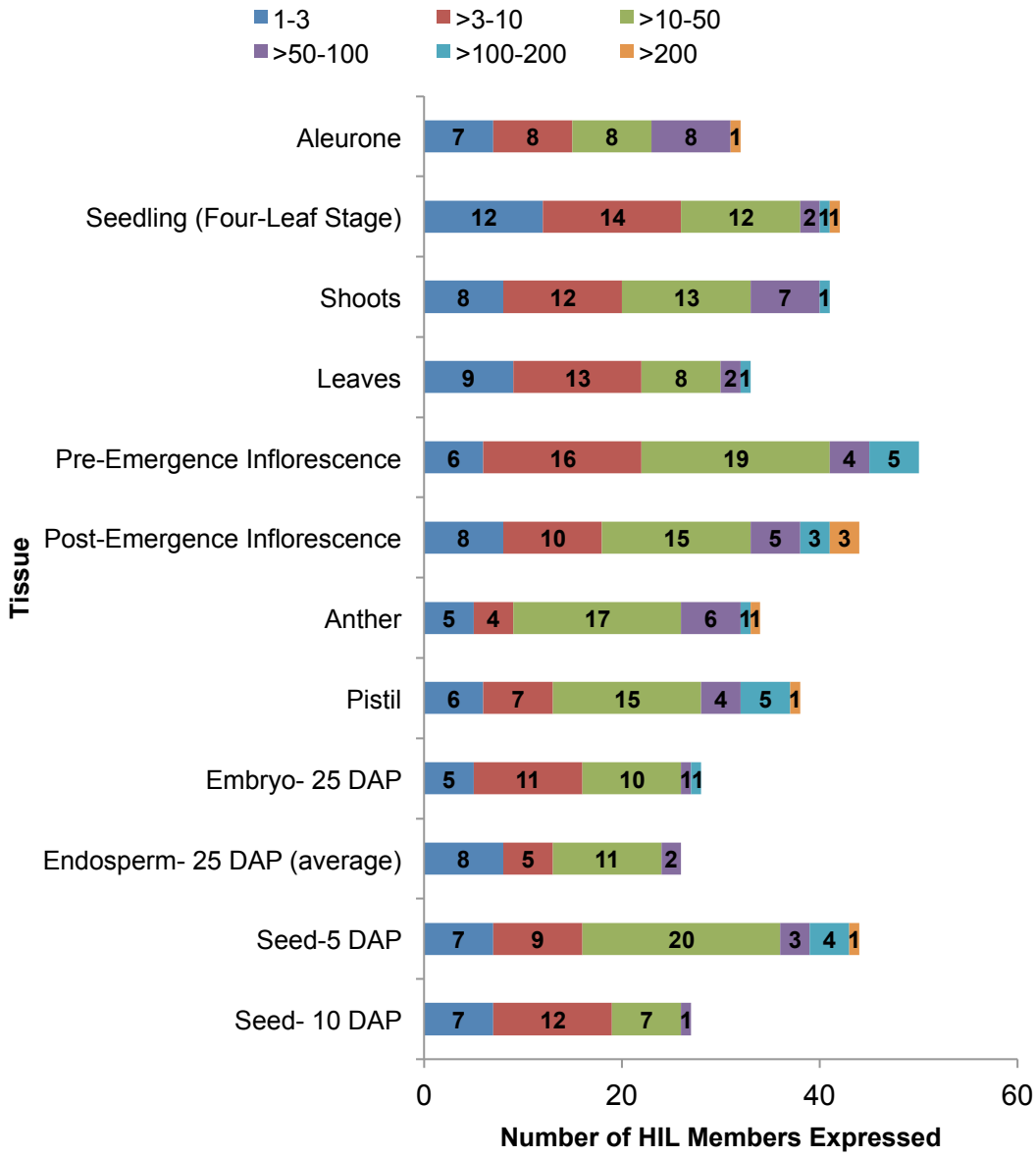


Figure 4-6. Expression of HIL members in respective rice tissues. (a) Number of members expressed within each tissue analyzed ranging from low read count (blue) to high read count (orange). Actual HIL members are listed in Appendix D.

So far, the expression of HILs in the cereal aleurone has not yet been investigated. Based on RNA-sequencing data from libraries prepared with aleurone mRNA, several HILs were highly expressed in the aleurone. *OsHIL16* transcripts were relatively abundant (Log_2RPKM ~8-9), and moreover, was not expressed in any other rice tissue examined. This suggested that OsHIL16 might have specific role in the aleurone and possibly in seed germination. Elucidation of its function may aid in deciphering the unique role that the aleurone has in cereal development. Besides *OsHIL16*, other HILs were highly expressed in the aleurone, including *OsHIL50*. Despite what was predicted, the OsWRKY71-interacting protein, OsHIL58, was not expressed in the control treatment.

Expression of HIL Members in ABA- and GA-treated Aleurone Cells

To identify HILs that may be important in seed germination, RNA-sequencing libraries were prepared from aleurone mRNA treated with 1 uM GA, 20 uM ABA, or both (Dr. Shen's lab). For the most part, HILs that were highly were expressed in the control were also expressed in the experimental treatments (Figures 4-7 thru 4-10). Notably *OsHIL16* was highly expressed under all conditions. HILs with Log_2RPKM values greater than 4, also had high read count in ABA, GA, and ABA+GA treatments. Of the HILs that were expressed, those with low Log_2RPKM values generally had lower read counts throughout all treatments, with few exceptions. Specifically, the Log_2RPKM for *OsHIL96* was around 4 but less reads were present in all experimental treatments.

When the aleurone was treated with ABA or GA, several HILs were induced (Figures 4-11 thru 4-13; Appendix E). ABA induced *OsHIL40, 56, -58, -60, -81, -88, and*

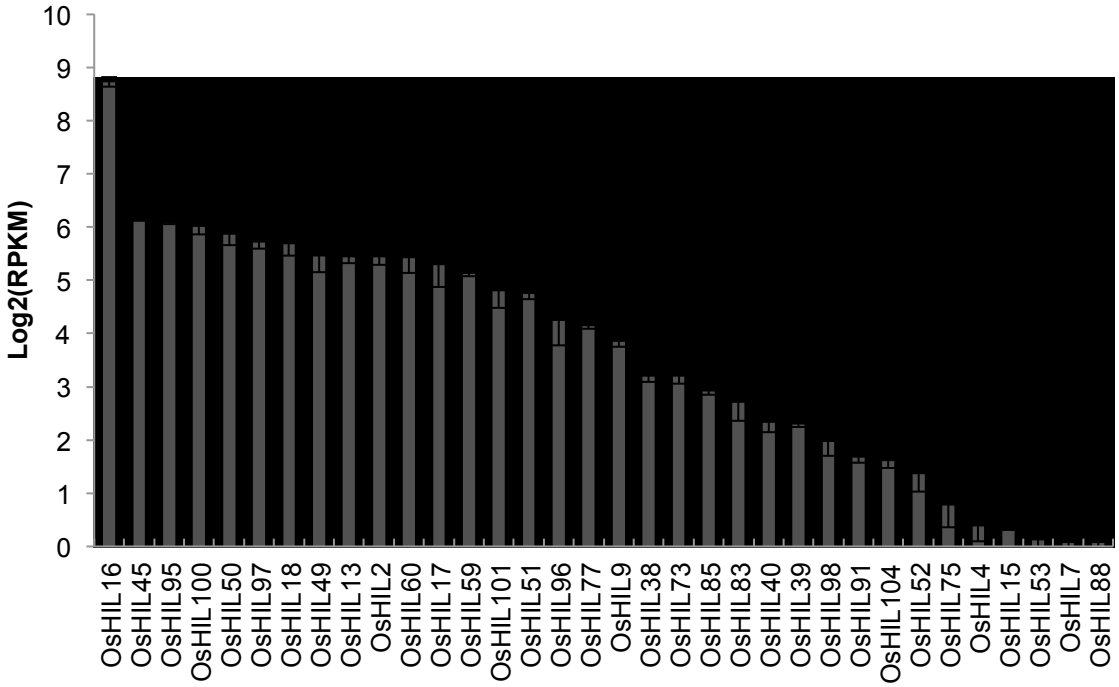


Figure 4-7. Expression of HIL members in the aleurone under control treatments. Total RNA was purified from rice aleurone tissue treated with or without hormone, 1 μM GA, 20 μM ABA, or 1 μM GA and 20 μM ABA, for 5 hours (3 independent biological replicates). Control treatments were imbibed in water. The RNA library was sequenced using the Illumina HiSeq at the Huntsman Cancer Institute, University of Utah. Only HILs significantly expressed are shown (average control RPKM + average experimental RPKM ≥ 1). RPKM=reads per kilobase of exon per million fragments mapped to the genome.

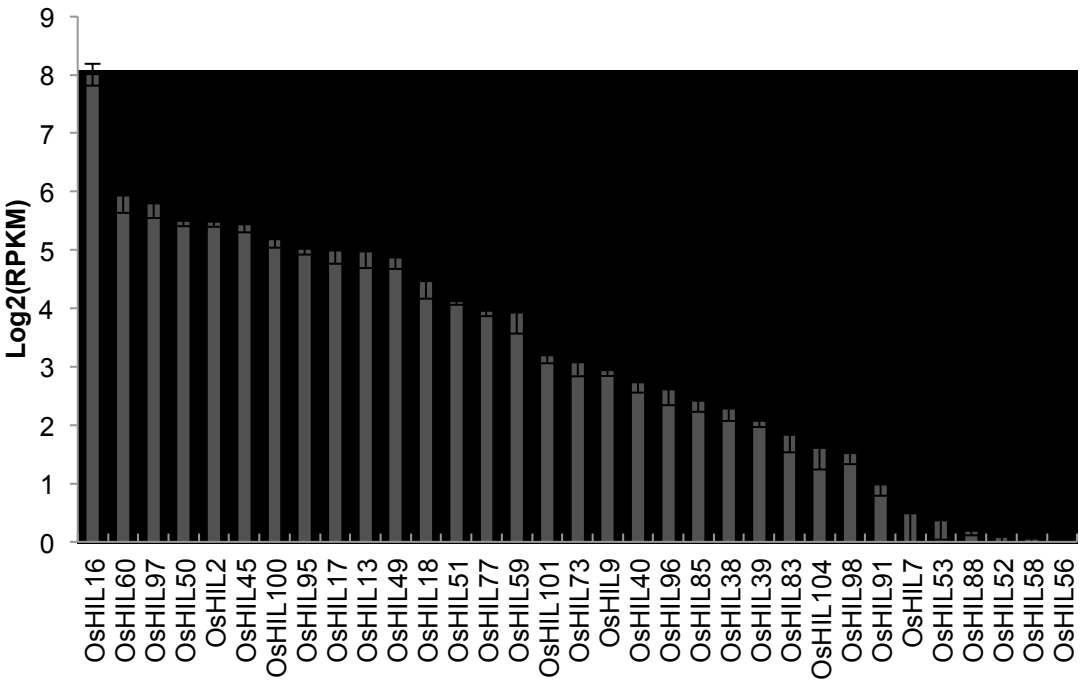


Figure 4-8. Expression of HIL members in ABA-treated aleurone tissue. Total RNA was purified from rice aleurone tissue treated with 20 μM ABA for 5 hours (3 independent biological replicates). The RNA library was sequenced using the Illumina HiSeq at the Huntsman Cancer Institute, University of Utah. Only HILs significantly expressed are shown (average control RPKM + average experimental RPKM ≥ 1). RPKM=reads per kilobase of exon per million fragments mapped to the genome.

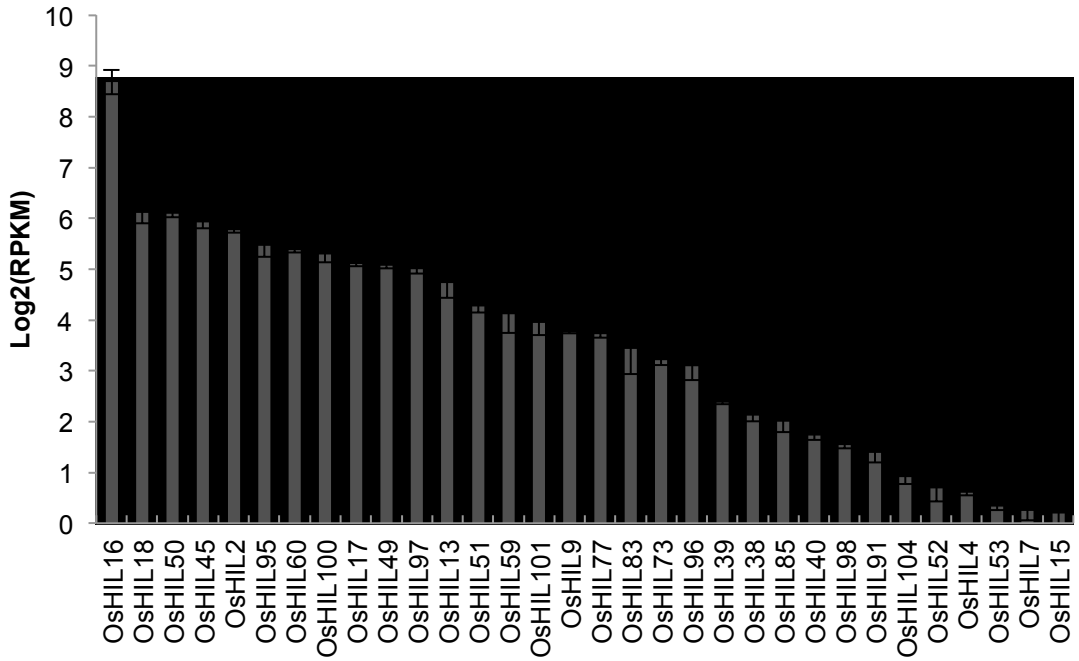


Figure 4-9. Expression of HIL members in GA-treated aleurone tissue. Total RNA was purified from rice aleurone tissue treated with 1 μM GA for 5 hours (3 independent biological replicates). The RNA library was sequenced using the Illumina HiSeq at the Huntsman Cancer Institute, University of Utah. Only HILs significantly expressed are shown (average control RPKM + average experimental RPKM ≥ 1). RPKM=reads per kilobase of exon per million fragments mapped to the genome.

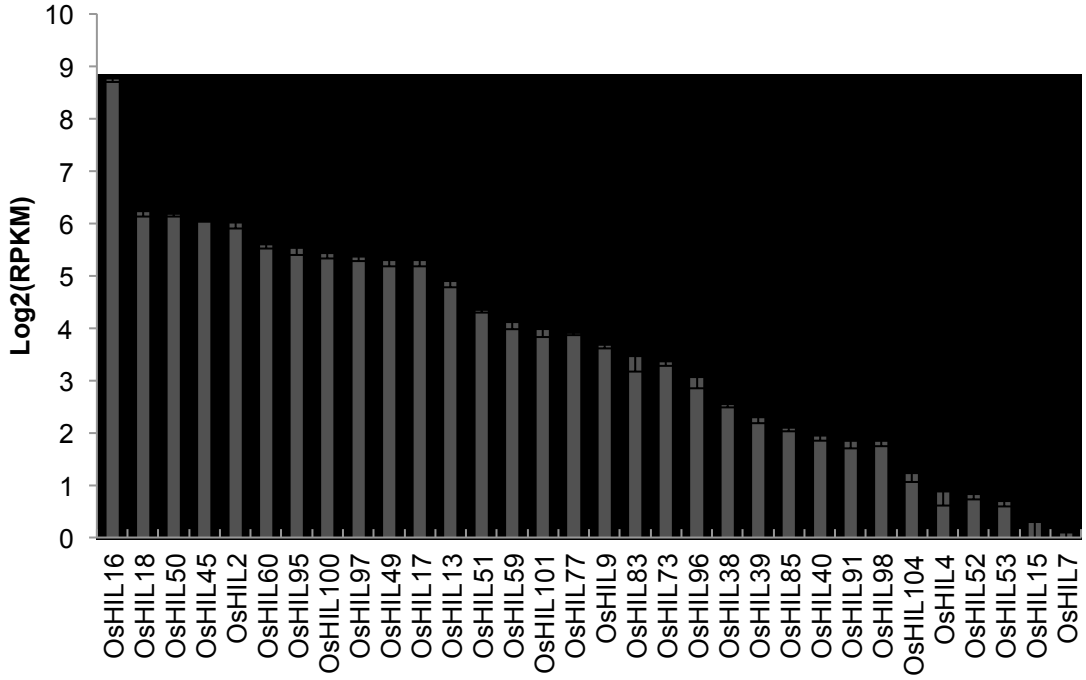


Figure 4-10. Expression of HILs in GA- and ABA-treated rice aleurone tissue. Total RNA was purified from rice aleurone tissue treated with 1 μM GA and 20 μM ABA for 5 hours (3 independent biological replicates). The RNA library was sequenced using the Illumina HiSeq at the Huntsman Cancer Institute, University of Utah. Only HILs significantly expressed are shown (average control RPKM + average experimental RPKM ≥ 1). RPKM=reads per kilobase of exon per million fragments mapped to the genome.

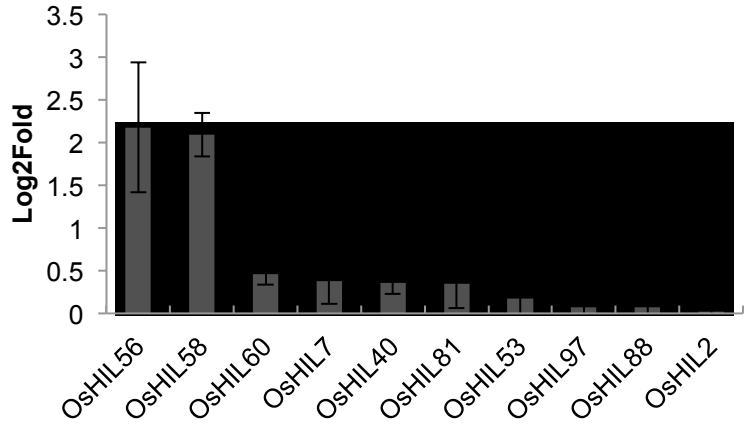
-97, (Figure 4-11a), all of which, except for *OsHIL56*, were also repressed by GA (Figure 4-12b). Although *OsHIL58* was not expressed under normal conditions, it was specifically induced by ABA, similar to *OsWRKY71*. This supports the prediction that the two proteins may interact in the aleurone to regulator seed germination. Particular members that were induced by GA were *OsHIL4, 83, -18, -39, -50, and -73* (Figure 4-12a). And of these, all, except *OsHIL83*, were simultaneously repressed by ABA (Figure 4-11b). HILs induced by both ABA and GA included *OsHIL2, -7, and -53* (Figures 4-11a and 4-12a). However, *OsHIL53* was not induced when compared to ABA+GA treatment; *OsHIL2,* and *-7,* in addition to other HILs, were induced (Figure 4-13a). Lastly, of the remaining HILs that were repressed in experimental treatments, those that were repressed under GA treatment were also repressed by ABA. For example, *OsHIL75* was not induced in any treatment but was highly repressed by ABA and GA (Figure 4-13b). In this respect, HIL members were differentially regulated by ABA and GA.

Coexpression of HIL Members with ABA Receptors

Coexpression analysis of the HIL members with hormone receptors was performed to better associate the function of HILs in hormone signaling pathways. Although GA receptors and those in other hormone pathways would like to be considered eventually, coexpression using ABA receptors were tested first.

Nearly 14 ABA receptors, or RCARs, were identified in Arabidopsis (Hubbard *et al*, 2010), but despite their importance, only one receptor was found to function in rice, RCAR5, while most other receptors have only been recognized based on sequence homology (Kim *et al.*, 2012). Herein, RCARs were coexpressed with several HIL

a



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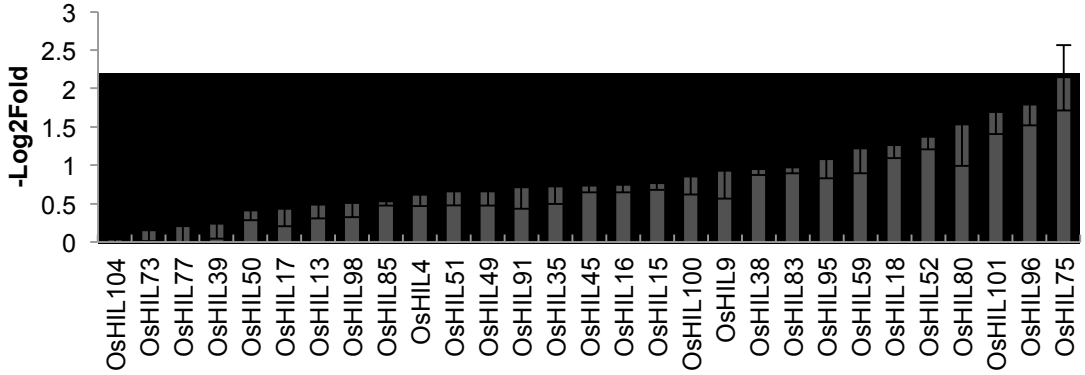
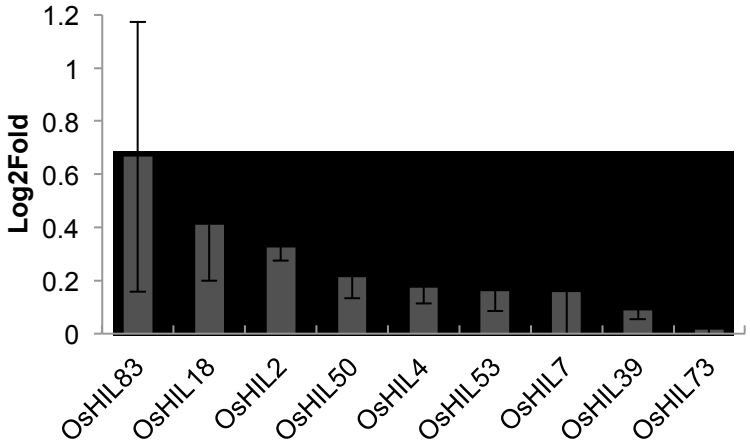


Figure 4-11. ABA-induced and repressed HILs in the rice aleurone. HIL members are shown that were significantly (a) induced or (b) repressed by ABA. Total RNA was purified from rice aleurone tissue treated with 20 μM ABA for 5 hours (3 independent biological replicates). The RNA library was sequenced using the Illumina HiSeq at the Huntsman Cancer Institute, University of Utah. RPKM=reads per kilobase of exon per million fragments mapped to the genome.

a



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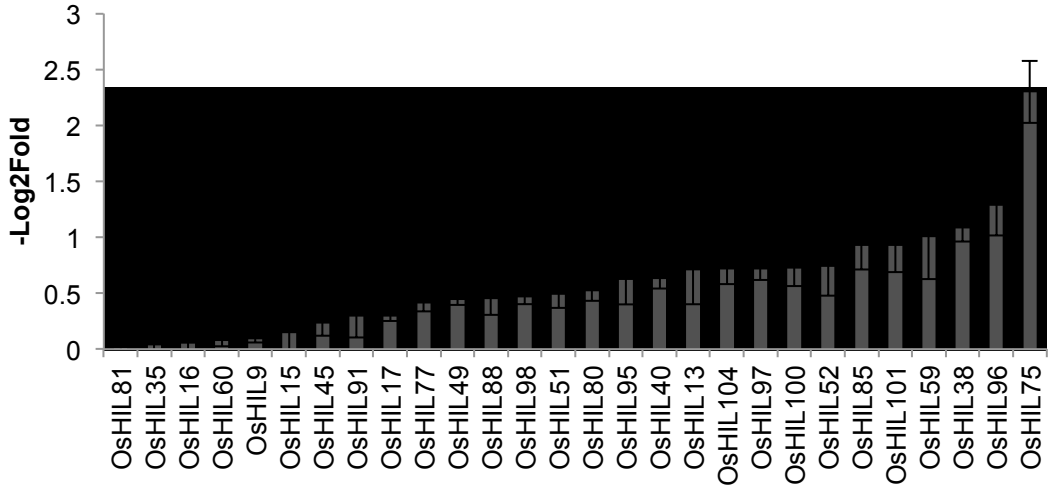


Figure 4-12. GA-induced and repressed HILs in the rice aleurone. HIL members are shown that were significantly (a) induced or (b) repressed by GA. Total RNA was purified from rice aleurone tissue treated with 1 μM GA for 5 hours (3 independent biological replicates). The RNA library was sequenced using the Illumina HiSeq at the Huntsman Cancer Institute, University of Utah. RPKM=reads per kilobase of exon per million fragments mapped to the genome.

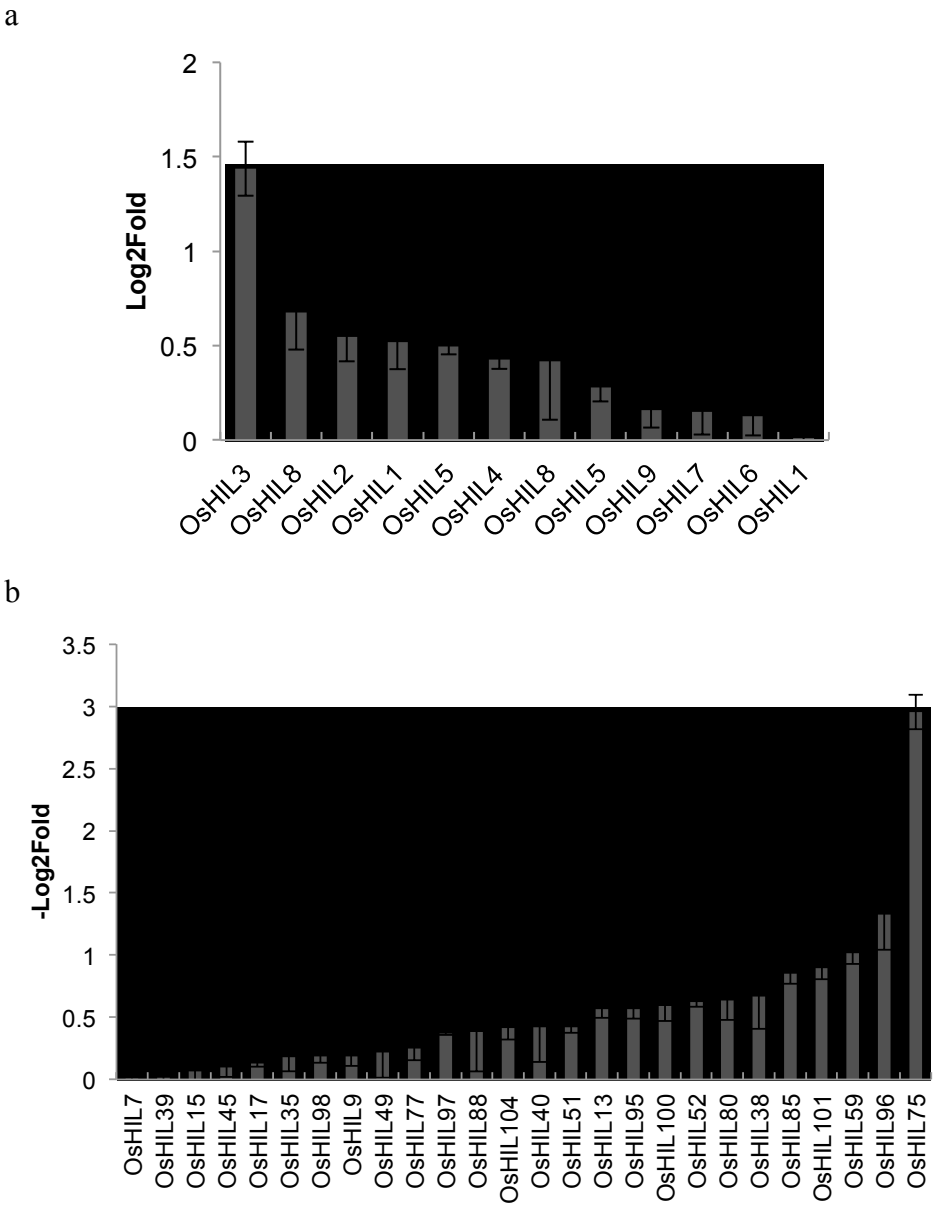


Figure 4-13. GA- and ABA-induced and repressed HILs in the rice aleurone. HIL members are shown that were significantly (a) induced or (b) repressed by ABA + GA. Total RNA was purified from rice aleurone tissue treated 1 μM GA and 20 μM ABA, for 5 hours (3 independent biological replicates). The RNA library was sequenced using the Illumina HiSeq at the Huntsman Cancer Institute, University of Utah. RPKM=reads per kilobase of exon per million fragments mapped to the genome.

members in the rice aleurone, based on hierarchical clustering data. Several distinct expression clusters were identified, with each cluster containing at least one RCAR receptor. *OsHIL2, -7, -53, -60,* and *-81* ($0 \leq \text{Log}_2\text{Fold} \leq 1$) was coexpressed with *RCAR7* and *-8*, which were mildly induced by ABA, forming the first cluster. The second identified cluster contained members associated with *RCAR9* and was typically repressed only in the presence of ABA. These members included *OsHIL4, -9, -15, -16, -18, -35, -39, -45, -50, -73, -83,* and *-91*. The third major cluster consisted of members that were generally repressed in at least two hormone treatments. Within this cluster, *RCAR4, -5, -62, -10,* and *-3* were found to be associated with several HIL members. As a result, respective HIL members were indeed coexpressed with ABA receptors in the aleurone, suggesting that they may mediate or relay ABA response during germination.

Conclusion

In summary, 104 proteins were identified as members of the HIL family in rice. Using *in silico* analyses, several motifs were recognized in these proteins, including the NPN, RPP, and YQYF domains, from which members were further categorized into eight groups. Group I members were characterized with all three motifs were the most prevalent. Members were also evenly distributed throughout the rice genome, although some large and small clusters were found, which suggests functional redundancy and gene duplication events.

The OsWRKY71-interacting protein, LOC_Os05g45070, was named OsHIL58 based on chromosome location. It was identified as a Group I member and was homologous to OsHIL13 and -23. *OsHIL58* was expressed at low levels upon ABA

induction in the aleurone, suggesting possible interaction with OsWRKY71 during seed germination. Although *OsHIL58* was not highly expressed, another HIL member, *OsHIL16*, was highly and specifically expressed in the aleurone and was coexpressed with the ABA receptor, *RCAR9*. Other HILs were also coexpressed with respective RCARs, and some were found be differentially regulated ABA and GA, suggesting possible roles in seed germination. In addition, HILs were identified that were not affected by either ABA or GA in the rice aleurone, as well as in other vegetative and reproductive rice tissues, implying that HILs might have many roles in the physiology of plants.

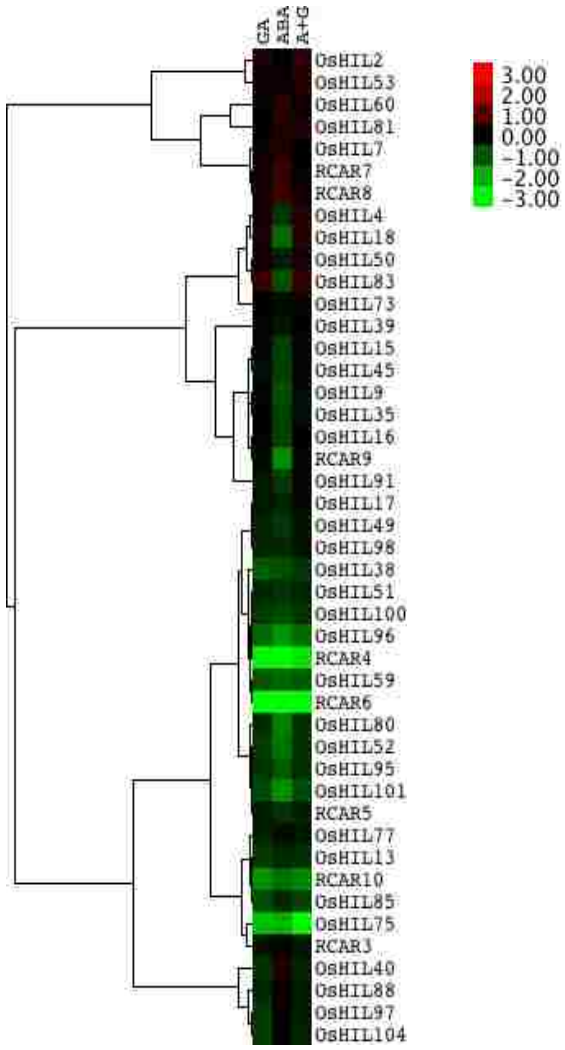


Figure 4-14. Coexpression profile of HIL members differentially expressed with ABA receptors in rice. The HIL members and ABA receptors or RCARs were clustered based on similar expression patterns in response to the hormone treatments used RNA-sequencing data. Total RNA was purified from rice aleurone tissue treated with or without hormone, 1 μM GA, 20 μM ABA, or 1 μM GA and 20 μM ABA, for 5 hours (3 independent biological replicates). Control treatments were imbibed in water. The heat map was generated using R programming. Similar expression patterns suggest possible involvement in the same pathway based on "guilt-by-association."

CHAPTER 5

SUMMARY AND DISCUSSION

Introduction

The aleurone tissue has the primary function of responding to endogenous hormones, namely GA and ABA, during seed germination in cereal grains. It is in the aleurone where hydrolases, such as amylases, are secreted into the endosperm to break down stored compounds such as starch to supply nutrients for the germinating plant. During germination, it is crucial for the seed to sense the environment; GA and ABA may mediate this process and may affect the rate of growth. Whether or not germination proceeds depends on the action of proteins regulated by these hormones. I propose that OsWRKY71 and HIL members may facilitate processes that may be important during GA and ABA signaling and response in the aleurone.

Several questions were addressed in this thesis as follows:

1. Are current mutants for *OsWRKY71* indeed transgenic?
2. In these stable transgenic mutants, does OsWRKY71 mediate seed germination as predicted by transient expression assays in barley?
3. How many members is the HIL family composed of? If OsWRKY71 interacts with OsHIL58, does OsHIL58 and/or other HIL members have roles in mediating seed germination?

To address these questions, I first verified and propagated *oswrky71* mutant. Second, I performed physiological germination and root elongation assays using *oswrky71* mutants to study its role in early plant development. And third, I performed computational analyses to identify and annotate all members of the HIL family in rice. Following this, I using RNA-sequencing of the aleurone in rice seeds treated with GA and ABA to identify potential HILs that may mediate seed germination.

Chapter 2 Summary and Discussion

Identification and Verification of dSpm Knockout Mutants for OsWRKY71

Mutants for *oswrky71* were identified using standard screening techniques. Two different knockout lines were obtained from the Sundareson lab at UC Davis, one containing a transposable element, *dSpm*, within the 5'-UTR, named 1689, and another within the 2nd exon of *OsWRKY71*, labeled 3171A. There are several benefits from using these mutants as described by Kumar *et al.* in (2005). The insertion of *dSpm* is immobile and stable due to the removal of sequences necessary for transposition; this ensures that plants will be stably transgenic throughout subsequence generations. Additionally, *dSpm* insertional mutagenesis adapts a fluorescence-based tagging strategy based on the *En/Spm* transposon, which makes it feasible to distinguish between heterozygous and homozygous plants based on fluorescence intensity. Moreover, once the T-DNA is inserted into the genome, a single copy could be identified, since GFP is not mobile and 25% of the selfed progeny will be GFP negative.

To identify positive *dSpm* insertional mutants, PCR was used to screen plant lines 1689 and 3171A. One homozygous and one heterozygous plant were identified in the

1689 line. In the 3171A line, multiple plants were identified that were positive for the insertion of *dSpm.* These knockout mutants are valuable tools to further uncover the function of OsWRKY71 in rice. This present study is the first mention of using knockout lines for *OsWRKY71* for analyses in plant growth and development; previous research has been performed in overexpression lines in regards to disease resistance (Liu *et al.*, 2007; Chujo *et al.*, 2008).

In addition to knockout *oswrky71* lines, other *OsWRKY71* mutants were generated, transformed, and grown by members of the Shen lab in coordination with members from labs in Taiwan. Select T1 and T2 seeds were screened for the transgene, including *GUS* reporter, overexpression, and dominant negative lines. Several lines were identified to be positive for the insertion of a *GUS* reporter or an overexpression transgene, but only one line was identified among the dominant negative transformants. To further verify the transformants, GUS staining and Western analysis was performed on T2 generation rice tissue. However, it was found that silencing of the transgene could be an issue (Kilby *et al.*, 1992). Hence, for analyses, T1 generation rice plants should be tested and used for future analyses, or the effects of silencing may be released by the application of compounds such as sulfamethazine (Zhang *et al.* 2012).

Expression of OsWRKY71 in Rice Nodes

Preliminary GUS staining results in T1 *OsWRKY71p-GUS* reporter lines suggest that OsWRKY71 may be localized in various parts of the rice plant. In seeds, GUS expression was found, albeit low, in the aleurone tissue and the scutellum of embryos (data not shown), as expected for OsWRKY71. Expression was also found at the base of

shoots in growing seedlings, although this data must too be confirmed due to the low and inconsistent expression in several T1 lines and potential effects of silencing in T2 lines.

However, it is interesting to note that GUS expression was also observed in the nodes of rice culms. The node is generally described as the region on a stem where leaves and branches are attached, but in rice, nodes are distinguishing features. In a single rice culm, over a dozen nodes may exist. Only several of these nodes may actually elongate, while the remaining unelongated nodes reside at the base of the plant with the potential to generate into various tissues via meristematic cell differentiation. If nutrient and space is available, additional reproducing culms or tillers with independent root systems may differentiate from the lower nodes (Li *et al.*, 2003). Under certain circumstances such as submergence, rice has the potential to promote internode elongation at these lower nodes as well, at a growth rate of 20 to 25 cm/d to enable it to tolerate flooded water (Kende *et al.*, 1998). Furthermore, adventitious root growth aside from the main root system is stimulated at these nodes to aid in stabilization of the growing semiaquatic plant (Mergemann and Sauter, 2000). During this process, molecular regulation of internode and root elongation is primarily controlled by ethylene, but crosstalk with GA and ABA and the balance of these hormones is also important (Mergemann and Sauter, 2000; Steffens and Sauter, 2005). Together, these hormones may regulate programmed cell death prior to growth (Steffens and Sauter, 2005) and/or expansion afterwards (Cho and Kende, 1997). In fact, several expansin proteins, which aid in loosening of cell wall material to promote growth, were expressed in the intercalary meristems positioned near the nodal regions of deepwater rice. (Cho and Kend, 1997). It is possible that OsWRKY71 may regulate growth by interacting with expansins in nodes (Figure 5-1a).

This is further supported by positive interaction results of OsWRKY71 and expansins in previous yeast-2-hybrid assays (Dr. Lingkun Gu, unpublished). Since transcription factors have been found to localize and function in other parts of the cell other than the nucleus in plants and other organisms (Hoppe *et al.*, 2000; Kim *et al*., 2006; Slabaugh and Brandizzi, 2011), interaction of OsWRKY71 with non-nuclear expansins may be inevitable. In fact, the homolog of OsWRKY71 in Arabidopsis was found to interact with a cytosolic protein; AtWRKY40 interacted with a chloroplast protein, the magnesium-protoporphyrin IX chelatase H subunit (CHLH/ABAR). The interaction between OsWRKY71 and expansins could be further investigated using co-immunoprecipitation or transient expression in rice protoplasts (Zhang *et al.*, 2011).

Future Aims for the OsWRKY71 Project

One future goal for this project is aimed towards global identification of the promoters of genes regulated by OsWRKY71, which may be identified using chomatin-immunoprecipitation followed by high-throughput sequencing or ChiP-Seq (Libault *et al.*, 2009; Zhang *et al.*, 2009; Zhu *et al.*, 2008; Picardi *et al.*, 2010). This would lend to a more complete understanding of the impact of OsWRKY71 on multiple cellular processes. To perform ChiP-Seq in the future, antibodies that would specifically detect OsWRKY71 is of pertinence. Two antibodies are available, polyclonal anti-OsWRKY71 and anti-HA, which would identify OsWRKY71 in wt rice tissue and overexpression lines tagged with HA, respectively. However, the detection of OsWRKY71 in rice has been challenging, as the detection was not always reproducible. Detection issues may result from the relatively low abundance of OsWRKY71 in tissues, protein modification,

chemical interference due to amino acid composition, low levels of protein despite high levels of mRNA, or rapid turn over rates. To resolve these issues, it may be necessary to focus on one tissue type shown to have a high expression and relative abundance of *OsWRKY71*, such as the aleurone or embryo, instead of on multiple tissues. Tissues considered previously were germinating roots, seedlings, mature leaves, and wounded leaves, while the embryo and aleurone tissues were generally neglected due to difficulty in harvesting tissue and possible degradation. Therefore, protein extraction and other methods may need to be adapted for recalcitrant tissue (Wang *et al.*, 2003), the amount of tissue used for detection may need to be increased, a larger gel device may be helpful for protein separation, and other methods such as immunoprecipitation (Park *et al.*, 2008) should be considered. In summary, protein detection of OsWRKY71 may need to be improved, transgenic lines produced in Taiwan need to be verified further before they can be used for analyses, but knockout *oswrky71* lines are readily available for use in germination and other developmental studies in plants.

Chapter 3 Summary and Discussion

The Role of OsWRKY71 in the Regulation of Seed Germination

OsWRKY71 was shown to be involved in many processes such as disease response (Liu *et al.*, 2007; Chujo *et al.*, 2008), but its role in rice seed germination was not thoroughly investigated. In this study, *OsWRKY71* was discovered to play a role in seed germination and seedling root growth. If *OsWRKY71* represses α-amylase production, then the loss of repression and the "brake" for growth was predicted to enhance germination. However, the germination rates for 3171A seeds were opposite of

what was predicted and were delayed compared to wt and root growth was inhibited. Seed germination and root growth in 1689 seeds were not repressed as in 3171A but was more variable than wt, which may be a result of positional differences of *dSpm* in *OsWRKY71*. Since the insertion of *dSpm* did not affect the coding region of *OsWRKY71* and was instead inserted within the 5'-UTR, any leaky expression of *OsWRKY71* may be able to produce functional protein. Because of the differences in phenotype observed between 1689 and 3171A, a rescue line would need to be developed, primarily for 3171A, to prove that OsWRKY71 is in fact involved in these processes.

Several reasons could explain the unexpected delay in germination that was observed in 3171A seeds. Because OsWRKY71 is a transcriptional regulator, the expression of many other genes besides *α-amylase* may be altered. This could lead to an indirect repression of α-amylase production or in the expression of other metabolic and growth-related genes (Figure 5-1b). Also, OsWRKY71 shares sequence similarity to OsWRKY18 (Ross *et al.*, 2007), thus, redundancy in function could play a role. Furthermore, it is possible that OsWRKY71 may indeed regulate α-amylase production during seed germination, but the loss in repression in *oswrky71* mutants may have also affected seed development (Figure 5-1c). While the seed is maturing on the mother plant and is preparing for dormancy, ABA levels increase and regulators such as OsWRKY71 may repress germination-related genes in order to prevent instances such as pre-harvest sprouting (Gubler *et al*., 2005). Although redundancy may exist and the effects of OsWRKY71 may not be sufficient alone in causing pre-harvest sprouting, there may be some disruption or abnormalities occurring within the developing seed. Activation of α-amylase during seed maturation, especially during dehydration, may reduce the amount

of starch stored in the seed, which is consistent with the slightly reduced seed weights seen in knockout seeds. Loss in seed vigor then, could reduce germination rates. To better understand the function of OsWRKY71, a global expression analysis of wt and 3171A seeds may be performed to reveal the large-scale effect of OsWRKY71 in seed maturation and germination. I predict that multiple genes would be affected in *oswrky71* lines, including genes involved in metabolism, nutrient balance, cell enlargement, and multiple hormone signaling pathways.

The Role of OsWRKY71 in the Regulation of Root Development

Based on root elongation assays, *oswrky71* appeared to not only cause a delay in seed germination but also an inhibition in root growth. However, it was questionable as to whether or not the appearance of shorter roots in 3171 seedlings was caused by the initial delay in germination or if root growth was independent. Yet, even when accounting for a one-day delay in germination, the primary roots appeared to be shorter in 3171A seedlings. For example, 3171A seedlings on the seventh day were overall shorter than wt roots, even when compared to wt roots from the third day. Therefore, OsWRKY71 may not only have a role in seed germination but also in root elongation.

Root growth is directed by the coordination of multiple hormones, including ABA (Chen *et al.*, 2005) and GA (Tanimoto 2005), although the role of auxin is more familiar when considering root architecture (Rahman *et al.*, 2007). ABA has been shown to be specifically involved in primary root growth in rice (Yao *et al.*, 2003) and other cereal crops such as maize (Spollen *et al.*, 2000), as a way to promote water stress avoidance or tolerance. Since ABA was shown to induce OsWRKY71, and ABA and GA

inhibit and promote root growth, respectively (Steffens and Sauter, 2005), it is possible that OsWRKY71 might have disrupted GA and ABA crosstalk. In Arabidopsis, root growth was stimulated by the degradation of specific DELLA repressors RGA (Repressor of Gal-3) and GAI (Gibberellic Acid Insensitive) (Steffens and Sauter, 2005) and LA and CRY in peas (*Pisum sativum*; Weston *et al.* 2008). GA also stimulated response genes such as α -expansins, which were expressed in rice roots (Lee and Kende, 2002) and in primary roots of maize (Wu *et al.*, 2007). Since OsWRKY71 was shown to interact with an expansin protein as previously suggested, it is possible that the loss of OsWRKY71 positively affects cell expansion and cell wall extensibility in root growth (Figure 5-1a).

Although it is predicted that OsWRKY71 may be mediating the crosstalk between ABA and GA, I also hypothesize that OsWRKY71 may be important in auxin-dependent response, since auxin is the major hormone involved in root elongation (Rahman *et al.*, 2007). Auxin is a fundamental determinant to cell fate and patterned root development and is unique in that it is synthesized primarily in meristematic regions at the shoot apex and transported in a polar fashion to the root tip, through Pin-formed (PIN) proteins, which serve as efflux carriers (Sabatini et al., 1999). In rice, specifically, the role of auxin has generally been shown to be involved in lateral root growth. Interestingly, the formation and elongation of lateral roots via auxin was recently shown to be regulated by another WRKY member, OsWRKY31 (Zhang *et al.*, 2008). Because the primary roots of 3171A were unable to elongate during seedling growth, I hypothesize that OsWRKY71 could be regulating proteins such a PIN to prevent auxin transport and response. Several PIN proteins were identified in rice, including OsPIN1, -5, -9, and -10 and were expressed in various locations in the root (Wang *et al.*, 2009). The expression of these

proteins may be analyzed in 3171A roots in future analyses. Additionally, other physical attributes of the rice root system should be analyzed, including the appearance, number, and elongation of crown roots, typically observed in cereal crops (Coudert *et al.*, 2010), and the growth of seminal and lateral roots. These would aid in understanding the precise role of OsWRKY71 in GA, ABA, and possibly auxin signaling and response in root development.

Additional Physiological Tests Used to Study OsWRKY71 Function

To further investigate the role of OsWRKY71 in plant development, other experiments or measurements were carried out including starch plate assays, analyses of mature seedling, preliminary measurements of shoots, and electrolyte leakage tests. Starch plates assays (Zhen *et al.*, 2007) were used to examine the degree of starch degradation in the presence of ABA, GA, or in both hormones. Although 3171A seeds were predicted to have a greater degree of starch hydrolysis than wt, the results were inconsistent (data not shown). However, it is possible that long-term storage of seeds and after-ripening effects could alter the balance of ABA and GA in the seed (Kucera *et al.*, 2005) thereby increasing the chance of observing variation in the results. To remain consistent, then, a set period of time should be considered when performing starch assays. Since 3171A seeds were actually delayed in germination, then it is predicted that wt seeds would exhibit more starch hydrolysis. Therefore, a longer incubation time, for example 4-5 days, may produce noticeable differences between wt and 3171A seeds. Furthermore, since OsWRKY71 was shown to be involved in disease response (Chujo *et*

al., 2008), the use of salicylic acid in starch assays (Xie *et al.*, 2007) may be considered for future analyses.

In addition to starch plate tests, the shoots of mature seedlings were measured after 5 days of growth in MS media or 100mM NaCl (data not shown). There appeared to be no significant difference in shoot lengths between wt and knockout mutants, although this data needs to be confirmed. Interestingly, although the shoots were similar in height, the lengths of the first internodes were surprisingly different. 3171A seedlings had longer internodes that were significantly more resistant to NaCl damage (data not shown). It was expected that 3171A seedlings would experience less damage to NaCl stress, but initial electrolyte leakage tests showed that there was no difference in ion leakage between wt and 3171A (data not shown). Although these experiments need to be repeated, this further demonstrates that OsWRKY71 may regulate internode development at rice nodes or that it may have other roles in plant development.

Chapter 4 Summary and Discussion

The HIL Family as a Large Protein Family

Like the WRKY superfamily, the HIL family was discovered to be a large family in flowering and non-vascular plants, with occurrence in smaller organisms. From this study, 104 members were found in rice, of which 66 were identified as part of the LEA_2 domain family or described as putative harpin-induced proteins, and 38 proteins were never described. Although not shown, annotation was also performed in Arabidopsis. Previously, 45 members were identified; however, using the prescribed HMM, 21 additional members were found for a total of 66 members (data not shown). Several other

dicots were also examined, to which 128 members were discovered in soybean (*Glycine max*), 49 in tomato (*Solanum lycopersicum*), 38 in grape vine (*Vitis vinifera*), and 78 in poplar (*Populus trichocarpa*). Furthermore, two other monocots of the grass family besides rice were analyzed, for which 97 members were identified in sorghum (*Sorghum bicolor*) and 167 in corn or maize (*Zea mays*). Moreover, the HIL family was found in moss (*Physcomitrella patens*), which consisted of 36 members. For these organisms, HIL (Harpin-Induced1 Like), was chosen as a more simplified family name rather than NHL (Ciccarelli and Bork, 2005), while still maintaining credit given to the original HIN that was first identified as being harpin-induced. It is clear that naming a family based on the expression of one member may cause confusion (Wise *et al.*, 2007), as not all members may actually be harpin-induced. However, this name was chosen until the true cellular function of HILs might be revealed, to which a more suitable name could be designated.

To further annotate this family in rice, motif analysis, chromosome distribution, phylogenetic analysis, and multiple alignments were built using *in silico* tools. From these analyses, HILs in rice and Arabidopsis appeared to share conserved motifs (Dormann *et al.*, 2000). Herein, the core sequences were identified and used to name each motif, in particular, NPN, RPP, and YQYF, based on highest to lowest conservation. Despite the presence of the core motif in both organisms, the flanking sequences were variable, although some amino acids shared similar chemical properties, such as neutral, non-polar amino acids valine, alanine, and isoleucine (data not shown). The exact roles of these motifs have yet to be discovered. Interestingly, the RPP motif was localized within a largely hydrophobic, predicted transmembrane region, near the N-terminal region. The conserved role of arginine (R) may be an important marker for the protein, since it is

positively charged and has been shown to exist in non-transmembrane parts of the protein or 'loops', particularly on the cytoplasmic side (Sonnhammer *et al.*, 1998). This supports previous findings that HILs might be localized to the plasma membrane (Varet *et al.*, 2003; Lee *et al.*, 2005), and thus, may function to permit transport of certain substances under specific conditions. Furthermore, the NPN and the YQYF motifs were located towards the C-terminal side in most proteins and were in close approximation to each other. The highly conserved asparagine (N) present in almost all HIL members suggests a critical role in the function of these proteins. Asparagine has been shown to be important in stabilizing an active site in ubiquitin-conjugating enzymes (Berndsen *et al.*, 2013) and promoting catalytic activity in ribonucleotide reductase and autotransporters in bacteria (Kasrayan *et al.*, 2002; Barnard *et al.*, 2012), thus, could possibly be a target for fully understanding HIL function. And whether or not these any of these motifs are important for protein-protein interaction has yet to be determined.

Shedding Light on Interaction between OsHIL58 and OsWRKY71

The protein interaction observed between OsHIL58 and OsWRKY71 (Lingkun Gu, unpublished) lent to further characterization of OsHIL58. Herein, it was identified as a Group I protein, containing all three motifs, and was located on chromosome 5, with neighboring HILs approximately 5 KB away. Based on phylogenetic analysis, OsHIL58 was closest in identity to OsHIL13 and -23, which were located on chromosomes 1 and 2, respectively. The expression of *OsHIL58* was restricted to mature shoots and leaves, and was induced by ABA in the aleurone (Figure 5-1d), albeit in low levels, which was

surprising but not eliminating the possibility that interacting is occurring with OsWRKY71, since it is also upregulated by ABA.

Several hypotheses might explain possible reasons for the interaction of OsWRKY71 with OsHIL58. Although OsWRKY71 is expressed in untreated aleurone, ABA treatment may alter the regulation of OsWRKY71 via expression of OsHIL58. This may result in mobilization or differential regulation of OsWRKY71 during seed development or germination. Since HILs are known for their roles in disease response, perhaps differential regulation of OsWRKY71 would allow it to mediate GA and ABA crosstalk with the SA and JA signaling pathways. Although SA and JA signaling are mostly known for their roles in biotic stress, they also have been shown to mediate seed germination (Linkies and Leubner-Metzger, 2012). Moreover, SA was shown to decrease GA production of α-amylase (Xie *et al.* 2007), and SA, pathogen infection, and wounding induced the expression of *OsWRKY71* (Liu *et al.*, 2007). Therefore, interaction with *HIL* members may not be surprising. This would be feasible for OsWRKY71, since other sequences besides the WRKY domain and nuclear localization signals have been identified (Appendix A). Near the N-terminal region there is an LX motif suggesting a role in ethylene signaling; a leucine zipper, for accommodating protein-protein interactions; a proline-rich region, possibly for osmoprotectant activity; and an alanine-rich region on the C-terminal side, which may aid in activation/repression activity. Proline-rich regions are also characteristic of cell wall proteins in plants (Fowler *et al.*, 1999) and osmotic stress in yeast (Ooms *et al.*, 2000). The expression of a PvPRP1 (Proline-Rich Protein 1), in bean, (Phaseolus vulgaris) appeared to be integrated with the remodeling of the plant cell wall during defense response (Sheng *et al.*, 1991). In yeast,

the proline-rich region was found to be sufficient for stress-mediated localization (Ooms *et al*, 2000). These proline-rich regions may direct OsWRKY71 or even HILs to the membrane under certain circumstances. In addition, predicted sumoylation and ubiquitination sites have been found throughout the OsWRKY71 protein. Attachment of ubiquitin and sumo, may occur on the same or different residue, particularly lysine, leading to either the same effect, antagonistic effect, or multiple effects resulting in protein degradation or stability, subcellular transport, localization, and compartmentalization (Denuc, A. and M. Gemma, 2010). These implications further suggest that OsWRKY71 is able to mobilize to various parts of the cell, including the cell membrane where it may interact with OsHIL58 or other HIL members. This is the first identified interaction between a HIL member and another protein.

Comparison of the HIL Family with the LEA14 Subfamily

To further understand the cellular function of HILs, this family perhaps could be compared to the LEA14 members of the LEA family. LEA14 proteins, also called D-95 (Galau *et al.*, 1993), Group 5C, or Group 7 (He *et al.*, 2012) consists of only five members in rice according to PFAM. Together with respective HILs, the LEA14 proteins share the same domain family LEA_2 (PF03168) in the PFAM database; this domain includes the NPN domain and possibly the YQYF. It was initially predicted that LEA14 members would be identified by HMM analysis, however, despite their sequence similarity, was not detected and thereby distinct. Although subject to further examination, preliminary results suggest that LEA proteins share an NPY instead of an over-represented NPN motif (data not shown). Or, LEA proteins may be missing an RPP

motif, despite the presence of a predicted transmembrane region. Unlike other LEA proteins, which are generally hydrophilic and structurally more ordered upon dessication (Wise *et al*., 2004 reviews; Tunnacliffe and Wise, 2007; Hand *et al*., 2011), LEA14 members are considered atypical because they are hydrophobic, similar to HILs, and generally stable (Singh *et al.*, 2005; Battaglia *et al.*, 2008 review). LEA14 members were shown to be induced by various abiotic stresses: soybean D95 was induced by drought (Maitra and Cushman, 1994); tomato ER5 was stimulated by drought, ABA, and wounding (Zegzouti *et al.*, 1997); hot pepper (*Capsicum annuum*), CaLEA6 (Kim *et al.*, 2005), and sweet potato (*Ipomoea batatas*) IbLEA14 (Park *et al.*, 2011) were upregulated by PEG, ABA, and NaCl; and OsLEA5 from rice enabled tolerance to osmotic stress and NaCl, as well as from heat, freezing, and UV radiation (He *et al.*, 2012). It appears as though HILs may also mediate dehydration stress, as the presence of a LEA_2 domain in certain proteins indicates (Battaglia *et al*., 2008).

In addition to possible overlapping functions, HILs from rice were predicted to adopt a similar protein structure to a LEA14 protein in Arabidopsis, based on Phyre analysis (data not shown). This protein, from gene At1g01470, is the only LEA protein with a solution structure available, possibly due to protein stability (Singh *et al.*, 2005). Singh *et al.* (2005) has shown that LEA14 adopted an α-β-fold consisting of one α -helix and seven β-strands that form two antiparallel β-sheets. The beta-sheets appear to form a rather narrow or flattened barrel, possibly for some kind of molecular transport, chaperoning, molecular shielding, or membrane stability. On the other hand, homology modeling of Arabidopsis NDR1, a HIL protein identified in Arabidopsis, suggested that NDR1 was similar in structure to integrins (Knepper *et al*., 2011). Therefore, it is

possible that HILs may mediate abiotic, biotic, or other chemical signals through the plasma membrane. Such examples provide clues to discovering the physiological function(s) of HIL in plants. And although questions were raised regarding whether or not atypical LEA14 proteins should be considered as part of the LEA family (Tunnacliffe and Wise, 2007; He *et al.*, 2012;), from the analyses in the present study, LEA14 members appear to be quite distinct from the HIL family, although they may share similar attributes.

The Role of HIL Members in Seed Germination and Plant Development

Previous research suggested that HILs had a major role in biotic defense and minor roles in abiotic stress (Lee *et al.*, 2006). Herein, the expressions of HILs were investigated to better understand the role of HILs in plant development. Several HILs, *OsHIL44*, *-50*, and *-97* were highly expressed in almost all rice tissues, thus, appears to be important throughout plant development, while *OsHIL56* was mainly expressed in seeds. Furthermore, several HILs were found to be expressed in the aleurone of rice seeds and were differentially regulated by ABA and GA. Namely, *OsHIL56* and *-58* were induced by ABA, while *OsHIL2*, *-18*, and *-83* may be induced by GA. Several HIL members such as *OsHIL75* and *-96*, and *-101* were repressed by both GA and ABA. In addition, ABA receptors were coexpressed with specific HILs in hormone treated aleurone tissue. In particular, ABA induced ABA receptors *RCAR7* and *-8* (Figure 5-1e), and was coexpressed with HIL members such as *OsHIL60* (not shown in Figure 5-1). Interestingly, *RCAR9* was repressed in ABA-treated aleurone (Figure 5-1f) and was coexpressed with *OsHIL16* (Figure 5-1, star). Although several HILs, including

OsHIL13, *-17*, *-18*, *-49*, *-50*, *-95*, *-97*, and *-100*, were highly expressed in untreated aleurone tissue, OsHIL16 was highly and specifically expressed in the aleurone (Figure 5-1g) and not in other rice tissues investigated (Appendix D). Therefore, OsHIL16 might have a major role in aleurone specificity or in seed germination and may be an interesting target for future research.

Concluding Remarks

Plant physiology and development in its entirety is a unique, intricate, and complicated process, the understanding of which is an immense task. Because of the generally immobile nature characteristic to plants, the study of plant interaction with its environment is of great importance.

Several discoveries were made in this research. Firstly, OsWRKY71 was shown to function in not only seed germination, but also in root elongation and possibly in the meristematic activity of rice nodes. Although the latter has yet to be verified, implications may aid in understanding stem cell regulation, differentiation, and expansion. Also, contrary to what was predicted, OsWRKY71 might be a positive regulator of these processes. Secondly, *OsHIL58*, an OsWRKY71-interacting protein, was induced under ABA treatment in the aleurone, suggesting an interaction with OsWRKY71 during seed germination. Thirdly, *OsHIL16* was highly expressed in the aleurone and coexpressed with *RCAR9*, both of which were repressed in ABA treatment. Fourthly, ABA receptors *RCAR-7* and *-8* were induced by ABA in coexpression studies. And finally, 104 members of the HIL family were identified in rice; 21 additional members were identified in Arabidopsis; and HIL members were also identified in other plant species.

This research facilitates the CSREES (Cooperative State Research, Education, and Extension Service) Strategic Plan Goals 1 and 3: to enhance economic opportunities for agricultural producers and to enhance the protection of the nation's agriculture and food supply. Developmental issues such as pre-harvest sprouting, and abiotic and biotic factors may result in poor grain and flour products, which may increase economic costs for both growers and consumers, in addition to reducing food value by 20% to 50% as emphasized by CSREES. This research will aid in the development of biotech applications in reducing yield loss in not only rice, but in closely related crops and other plants.

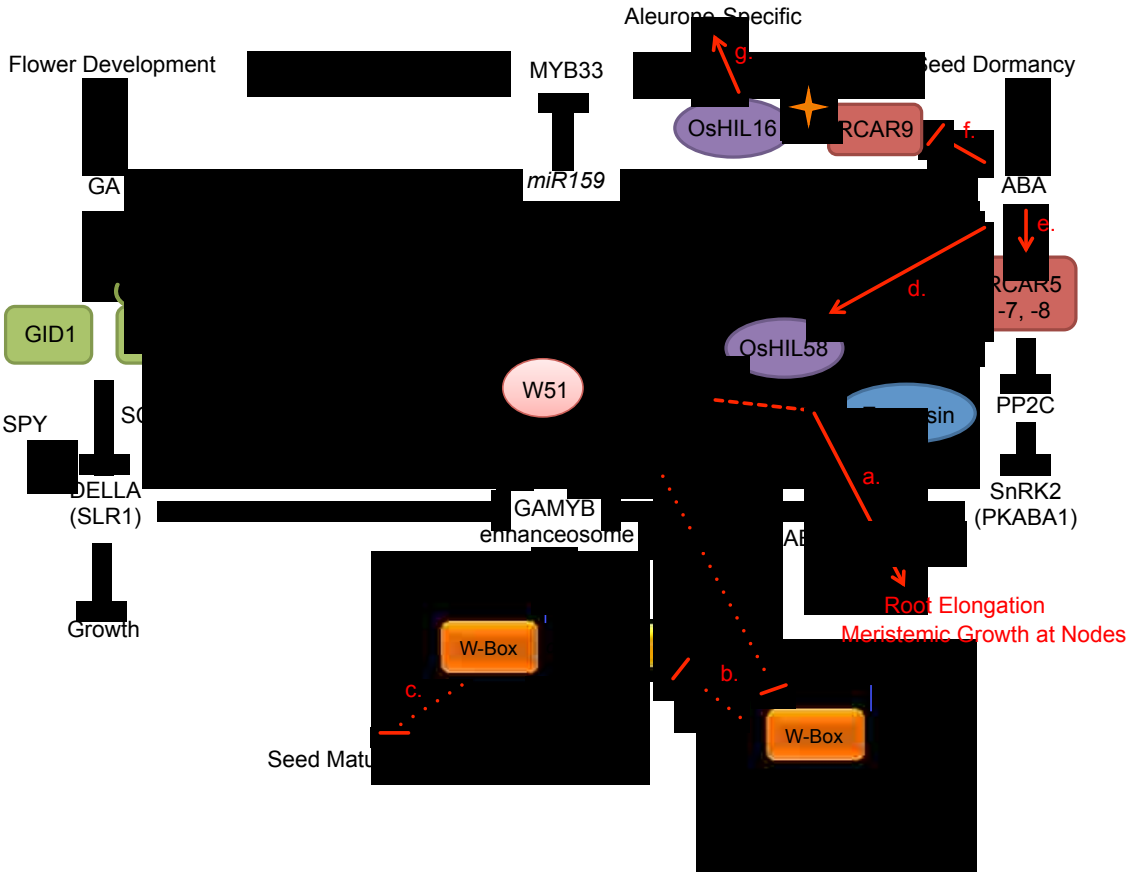
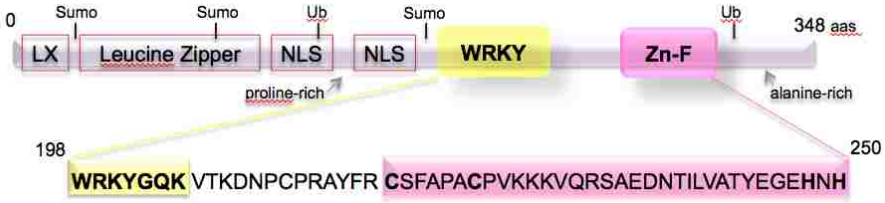


Figure 5-1. Predicted model of OsWRKY71 in regulation of seed germination and rice development. (a) OsWRKY71 may be a positive regulator of root growth as suggested by root elongation studies, and it may also regulate meristematic differentiation in rice nodes, based on GUS expression. This may be mediated by the interaction between OsWRKY71 and expansins based on yeast interaction studies (Dr. Lingkun Gu). (b) Although OsWRKY71 appears to be a positive regulator of growth, it was shown to be a negative regulator of *α-amylase*, suggesting a much more complex network than was originally hypothesized. It is possible that OsWRKY71 may be repressing additional genes that may repress *α-amylase* or (c) OsWRKY71 may be repressing *α-amylase* specifically during seed maturation. Loss of seed vigor, then, may be attributed to immature seed development, resulting in a delay of germination instead of enhancement. (d) Although OsWRKY71 and OsHIN58 were shown to interact (Dr. Lingkun Gu), in this study I show that ABA induced *OsHIN58* in global expression analyses of the aleurone. This further suggests that interaction may be important during seed germination. (e) Furthermore, *RCAR7* and *-8* were induced by ABA in the aleurone, while *RCAR9* was repressed, based on RNA sequencing data. Interestingly, *OsHIN16* was highly and solely expressed in the aleurone and was coexpressed with *RCAR9*; both were repressed by ABA. Red lines or text: novel discovery; dotted red arrows: hypothesized; dotted lines: interaction; star: coexpression. Adapted and modified from Weiss and Ori, (2007).

APPENDIX A

OSWRKY71 STRUCTURE, MOTIFS, AND SEQUENCES



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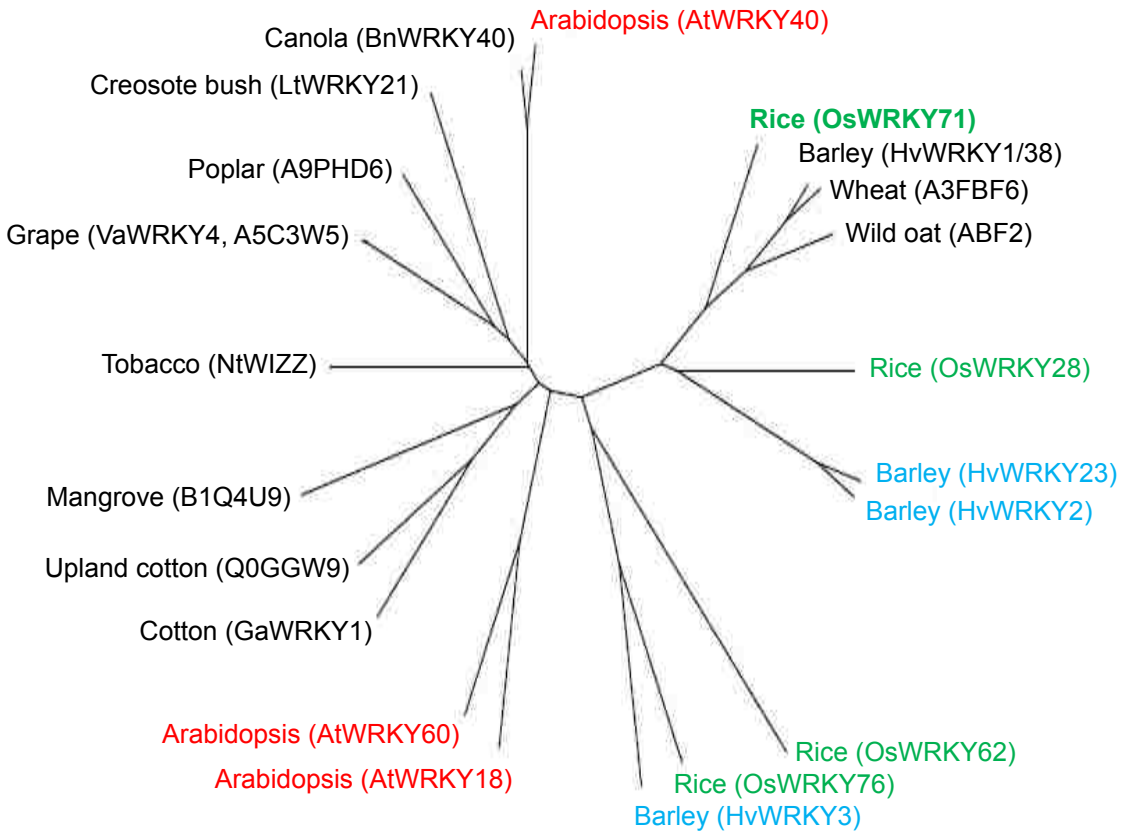
APPENDIX B

OSWRKY71 FAMILY TREE



APPENDIX C

COMPARISON OF HIL MEMBERS WITH PROTEINS LISTED IN THE PFAM DATABASE

Updated HIL (107, 104 unique)		HILs Identified by PFAM (68, 66 unique)	HILs Not Identified by PFAM (39, 38 unique)	PFAM Members Not Identified as HILs (5)*
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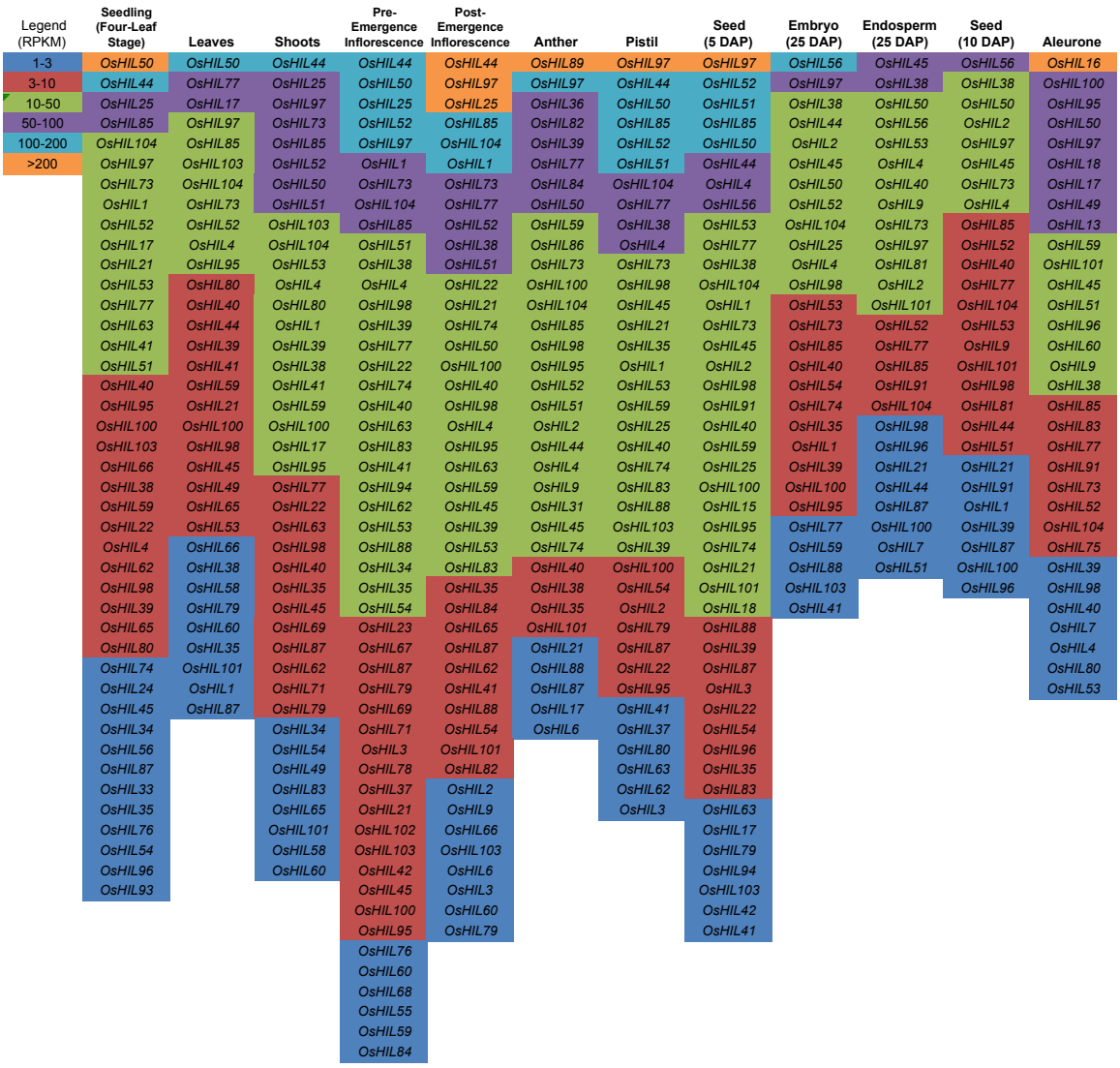
* Putative LEA14 Proteins

APPENDIX D

HILS EXPRESSED IN RICE TISSUES BASED ON RPKM VALUES FROM THE MSU RGAP COLLECTION



Legend (RPKM)	Seedling (Four-Leaf Stage)	Leaves	Shoots	Pre-Emergence Inflorescence	Post-Emergence Inflorescence	Anther	Pistil	Seed (5 DAP)	Embryo (25 DAP)	Endosperm (25 DAP)	Seed (10 DAP)	Aleurone
1-3	OsHIL50	OsHIL50	OsHIL44	OsHIL44	OsHIL44	OsHIL89	OsHIL97	OsHIL97	OsHIL56	OsHIL45	OsHIL56	OsHIL16
3-10	OsHIL44	OsHIL77	OsHIL25	OsHIL50	OsHIL97	OsHIL97	OsHIL44	OsHIL52	OsHIL97	OsHIL38	OsHIL38	OsHIL100
10-50	OsHIL25	OsHIL17	OsHIL97	OsHIL25	OsHIL25	OsHIL36	OsHIL50	OsHIL51	OsHIL38	OsHIL50	OsHIL50	OsHIL95
50-100	OsHIL85	OsHIL97	OsHIL73	OsHIL52	OsHIL85	OsHIL82	OsHIL85	OsHIL85	OsHIL44	OsHIL56	OsHIL2	OsHIL50
100-200	OsHIL104	OsHIL85	OsHIL85	OsHIL97	OsHIL104	OsHIL39	OsHIL52	OsHIL50	OsHIL2	OsHIL53	OsHIL97	OsHIL97
>200	OsHIL97	OsHIL103	OsHIL52	OsHIL1	OsHIL1	OsHIL77	OsHIL51	OsHIL44	OsHIL45	OsHIL4	OsHIL45	OsHIL18
	OsHIL73	OsHIL104	OsHIL50	OsHIL73	OsHIL73	OsHIL84	OsHIL104	OsHIL4	OsHIL50	OsHIL40	OsHIL73	OsHIL17
	OsHIL1	OsHIL73	OsHIL51	OsHIL104	OsHIL77	OsHIL50	OsHIL77	OsHIL56	OsHIL52	OsHIL9	OsHIL4	OsHIL49
	OsHIL52	OsHIL52	OsHIL103	OsHIL85	OsHIL52	OsHIL59	OsHIL38	OsHIL53	OsHIL104	OsHIL73	OsHIL85	OsHIL13
	OsHIL17	OsHIL4	OsHIL104	OsHIL51	OsHIL38	OsHIL86	OsHIL4	OsHIL77	OsHIL25	OsHIL97	OsHIL52	OsHIL59
	OsHIL21	OsHIL95	OsHIL53	OsHIL38	OsHIL51	OsHIL73	OsHIL73	OsHIL38	OsHIL4	OsHIL81	OsHIL40	OsHIL101
	OsHIL53	OsHIL80	OsHIL4	OsHIL4	OsHIL22	OsHIL100	OsHIL98	OsHIL104	OsHIL98	OsHIL2	OsHIL77	OsHIL45
	OsHIL77	OsHIL40	OsHIL80	OsHIL98	OsHIL21	OsHIL104	OsHIL45	OsHIL1	OsHIL53	OsHIL101	OsHIL104	OsHIL51
	OsHIL63	OsHIL44	OsHIL1	OsHIL39	OsHIL74	OsHIL85	OsHIL21	OsHIL73	OsHIL73	OsHIL52	OsHIL53	OsHIL96
	OsHIL41	OsHIL39	OsHIL39	OsHIL77	OsHIL50	OsHIL98	OsHIL35	OsHIL45	OsHIL85	OsHIL77	OsHIL9	OsHIL60
	OsHIL51	OsHIL41	OsHIL38	OsHIL22	OsHIL100	OsHIL95	OsHIL1	OsHIL2	OsHIL40	OsHIL85	OsHIL101	OsHIL9
	OsHIL40	OsHIL59	OsHIL41	OsHIL74	OsHIL40	OsHIL52	OsHIL53	OsHIL98	OsHIL54	OsHIL91	OsHIL98	OsHIL38
	OsHIL95	OsHIL21	OsHIL59	OsHIL40	OsHIL98	OsHIL51	OsHIL59	OsHIL91	OsHIL74	OsHIL104	OsHIL81	OsHIL85
	OsHIL100	OsHIL100	OsHIL100	OsHIL63	OsHIL4	OsHIL2	OsHIL25	OsHIL40	OsHIL35	OsHIL98	OsHIL44	OsHIL83
	OsHIL103	OsHIL98	OsHIL17	OsHIL83	OsHIL95	OsHIL44	OsHIL40	OsHIL59	OsHIL1	OsHIL96	OsHIL51	OsHIL77
	OsHIL66	OsHIL45	OsHIL95	OsHIL41	OsHIL63	OsHIL4	OsHIL74	OsHIL25	OsHIL39	OsHIL21	OsHIL21	OsHIL91
	OsHIL38	OsHIL49	OsHIL77	OsHIL94	OsHIL59	OsHIL9	OsHIL83	OsHIL100	OsHIL100	OsHIL44	OsHIL91	OsHIL73
	OsHIL59	OsHIL65	OsHIL22	OsHIL62	OsHIL45	OsHIL31	OsHIL88	OsHIL15	OsHIL95	OsHIL87	OsHIL1	OsHIL52
	OsHIL22	OsHIL53	OsHIL63	OsHIL53	OsHIL39	OsHIL45	OsHIL103	OsHIL95	OsHIL77	OsHIL100	OsHIL39	OsHIL104
	OsHIL4	OsHIL66	OsHIL98	OsHIL88	OsHIL53	OsHIL74	OsHIL39	OsHIL74	OsHIL59	OsHIL7	OsHIL87	OsHIL75
	OsHIL62	OsHIL38	OsHIL40	OsHIL34	OsHIL83	OsHIL40	OsHIL100	OsHIL21	OsHIL88	OsHIL51	OsHIL100	OsHIL39
	OsHIL98	OsHIL58	OsHIL35	OsHIL35	OsHIL35	OsHIL38	OsHIL54	OsHIL101	OsHIL103		OsHIL96	OsHIL98
	OsHIL39	OsHIL79	OsHIL45	OsHIL54	OsHIL84	OsHIL35	OsHIL2	OsHIL18	OsHIL41			OsHIL40
	OsHIL65	OsHIL60	OsHIL69	OsHIL23	OsHIL65	OsHIL101	OsHIL79	OsHIL88				OsHIL7
	OsHIL80	OsHIL35	OsHIL87	OsHIL67	OsHIL87	OsHIL21	OsHIL87	OsHIL39				OsHIL4
	OsHIL74	OsHIL101	OsHIL62	OsHIL87	OsHIL62	OsHIL88	OsHIL22	OsHIL87				OsHIL80
	OsHIL24	OsHIL1	OsHIL71	OsHIL79	OsHIL41	OsHIL87	OsHIL95	OsHIL3				OsHIL53
	OsHIL45	OsHIL87	OsHIL79	OsHIL69	OsHIL88	OsHIL17	OsHIL41	OsHIL22				
	OsHIL34		OsHIL34	OsHIL71	OsHIL54	OsHIL6	OsHIL37	OsHIL54				
	OsHIL56		OsHIL54	OsHIL3	OsHIL101		OsHIL80	OsHIL96				
	OsHIL87		OsHIL49	OsHIL78	OsHIL82		OsHIL63	OsHIL35				
	OsHIL33		OsHIL83	OsHIL37	OsHIL2		OsHIL62	OsHIL83				
	OsHIL35		OsHIL65	OsHIL21	OsHIL9		OsHIL3	OsHIL63				
	OsHIL76		OsHIL101	OsHIL102	OsHIL66			OsHIL17				
	OsHIL54		OsHIL58	OsHIL103	OsHIL103			OsHIL79				
	OsHIL96		OsHIL60	OsHIL42	OsHIL6			OsHIL94				
	OsHIL93			OsHIL45	OsHIL3			OsHIL103				
				OsHIL100	OsHIL60			OsHIL42				
				OsHIL95	OsHIL79			OsHIL41				
				OsHIL76								
				OsHIL60								
				OsHIL68								
				OsHIL55								
				OsHIL59								
				OsHIL84								

APPENDIX E

LIST OF LOG2 (RPKM) VALUES FOR ALL HIL MEMBERS IN THE RICE ALEURONE TREATED WITH GA OR ABA

Name	Locus	Log2 Fold GA	Log2 Fold ABA	Log2 Fold GA+ABA	Name	Locus	Log2 Fold GA	Log2 Fold ABA	Log2 Fold GA+ABA
OsHIL1	LOC_Os01g09880	1.79769e+308	1.79769e+308	0	*OsHIL53*	LOC_Os05g11010	-0.970859	-1.49093	-1.23503
OsHIL2	LOC_Os01g12820	#N/A	#N/A	#N/A	*OsHIL54*	LOC_Os05g14880	-1.81979	-1.79769e+308	-1.79769e+308
OsHIL3	LOC_Os01g13340	#N/A	#N/A	#N/A	*OsHIL55*	LOC_Os05g24760	0	0	0
OsHIL4	LOC_Os01g39290	1.60976	-1.91543	-1.69606	*OsHIL56*	LOC_Os05g30490	-0.5974	3.67453	6.79949
OsHIL5	LOC_Os01g46670	0.624992	0.00210799	2.10601	*OsHIL57*	LOC_Os05g40400	0	0	0
OsHIL6	LOC_Os01g46730	0	0	0	*OsHIL58*	LOC_Os05g45070	2.08067	-0.734858	5.2879
OsHIL7	LOC_Os01g51460	-0.433901	-0.412929	-1.36793	*OsHIL59*	LOC_Os05g50720	-2.61855	-2.41946	-2.58676
OsHIL8	LOC_Os01g51470	-0.597396	-1.41292	-1.79769e+308	*OsHIL60*	LOC_Os06g02630	#N/A	#N/A	#N/A
OsHIL9	LOC_Os01g51490	-5.09383	-2.29045	-4.26232	*OsHIL61*	LOC_Os06g02670	0	0	0
OsHIL10	LOC_Os01g51500	-4.05682	-1.79769e+308	-3.76846	*OsHIL62*	LOC_Os06g02800	0	0	0
OsHIL11	LOC_Os01g51510	-1.79769e+308	-3.22027	-1.79769e+308	*OsHIL63*	LOC_Os06g02810	0	0	0
OsHIL12	LOC_Os01g51520	-3.59739	-1.41293	-3.30903	*OsHIL64*	LOC_Os06g02840	0	0	0
OsHIL13	LOC_Os01g53470	-0.5974	-1.09705	0.186247	*OsHIL65*	LOC_Os06g02930	0	0	0
OsHIL14	LOC_Os01g56500	0.665634	0.0724973	-1.79769e+308	*OsHIL66*	LOC_Os06g02940	0	0	0
OsHIL15	LOC_Os01g59680	2.03487	0.0724973	0.369039	*OsHIL67*	LOC_Os06g03030	0	0	0
OsHIL16	LOC_Os01g64450	#N/A	#N/A	#N/A	*OsHIL68*	LOC_Os06g03040	0	0	0
OsHIL17	LOC_Os01g64470	3.15224	0.289685	3.52832	*OsHIL69*	LOC_Os06g03059	0	0	0
OsHIL18	LOC_Os01g64480	#N/A	#N/A	#N/A	*OsHIL70*	LOC_Os06g03070	0	0	0
OsHIL19	LOC_Os01g68080	-2.18236	-1.99789	-1.79769e+308	*OsHIL71*	LOC_Os06g03099	0	0	0
OsHIL20	LOC_Os01g68090	0.4026	-0.41293	-1.79769e+308	*OsHIL72*	LOC_Os06g03110	0	0	0
OsHIL21	LOC_Os02g01060	0	0	0	*OsHIL73*	LOC_Os06g06780	0.167471	-0.228054	0.570551
OsHIL22	LOC_Os02g16030	-1.7349	-3.87236	-1.79769e+308	*OsHIL74*	LOC_Os06g10990	0	1.79769e+308	1.79769e+308
OsHIL23	LOC_Os02g16610	0	0	0	*OsHIL75*	LOC_Os06g32970	1.79769e+308	0	0
OsHIL24	LOC_Os02g30450	0	0	0	*OsHIL76*	LOC_Os06g49650	1.79769e+308	0	0
OsHIL25	LOC_Os02g33550	0	0	0	*OsHIL77*	LOC_Os07g10610	-1.41078	-0.547085	-2.27262
OsHIL26	LOC_Os02g40560	0	0	0	*OsHIL78*	LOC_Os07g14660	0	0	0
OsHIL27	LOC_Os02g40590	0	0	0	*OsHIL79*	LOC_Os07g14700	0	1.79769e+308	0
OsHIL28	LOC_Os02g40600	0.4026	-0.41293	-1.79769e+308	*OsHIL80*	LOC_Os07g14740	1.79769e+308	1.79769e+308	0
OsHIL29	LOC_Os02g40610	-1.5974	-1.79769e+308	-1.30903	*OsHIL81*	LOC_Os07g34040	-0.182363	0.58707	0.690968
OsHIL30	LOC_Os02g40620	-2.5974	0.28751	0.77843	*OsHIL82*	LOC_Os07g34050	-0.5974	-1.41293	-1.79769e+308
OsHIL31	LOC_Os02g40630	0	0	0	*OsHIL83*	LOC_Os07g34720	-4.22967	-0.812537	-3.9413
OsHIL32	LOC_Os02g40640	0	0	0	*OsHIL84*	LOC_Os08g01210	0	0	0
OsHIL33	LOC_Os02g40650	0	0	0	*OsHIL85*	LOC_Os08g01220	-1.24329	1.07482	1.35678
OsHIL34	LOC_Os02g44670	0	0	0	*OsHIL86*	LOC_Os08g23460	-0.5974	-1.79769e+308	-0.309032
OsHIL35	LOC_Os02g44740	-0.0966873	-1.14238	-1.99595	*OsHIL87*	LOC_Os08g38580	1.79769e+308	0	1.79769e+308
OsHIL36	LOC_Os03g09160	-1.18236	-1.99789	0.106005	*OsHIL88*	LOC_Os08g44410	-1.79769e+308	-1.79769e+308	-1.79769e+308
OsHIL37	LOC_Os03g11710	1.79769e+308	0	0	*OsHIL89*	LOC_Os09g09460	0	0	0
OsHIL38	LOC_Os03g15630	1.30198	-1.16728	0.165244	*OsHIL90*	LOC_Os09g26480	0	0	0
OsHIL39	LOC_Os03g26080	-1.04486	-0.0177005	-0.940645	*OsHIL91*	LOC_Os09g36210	-0.658801	-2.67596	-0.684542
OsHIL40	LOC_Os03g48950	0.192804	-0.00819362	0.554008	*OsHIL92*	LOC_Os10g34550	0	0	0
OsHIL41	LOC_Os03g62010	0	0	0	*OsHIL93*	LOC_Os10g34560	0	0	0
OsHIL42	LOC_Os03g62020	1.57252	-1.41293	-0.309032	*OsHIL94*	LOC_Os10g39970	0	0	0
OsHIL43	LOC_Os04g02410	0	0	0	*OsHIL95*	LOC_Os11g02730	0.112591	-2.26382	-0.999604
OsHIL44	LOC_Os04g33990	0.572525	-1.79769e+308	-1.30903	*OsHIL96*	LOC_Os11g03600	0.126626	-2.82797	-3.41595
OsHIL45	LOC_Os04g35130	-0.560997	-0.965318	-1.66526	*OsHIL97*	LOC_Os11g05860	-0.0240478	0.118684	1.80047
OsHIL46	LOC_Os04g42970	-1.79769e+308	-1.79769e+308	-1.79769e+308	*OsHIL98*	LOC_Os11g05870	#N/A	#N/A	#N/A
OsHIL47	LOC_Os04g43519	4.3568	-1.41293	1.27593	*OsHIL99*	LOC_Os11g37680	1.79769e+308	0	0
OsHIL48	LOC_Os04g53650	0	0	0	*OsHIL100*	LOC_Os12g02700	0.140371	-1.63622	-0.522942
OsHIL49	LOC_Os04g58090	0.811124	-0.885417	1.3144	*OsHIL101*	LOC_Os12g03370	-0.629173	-2.88527	-3.58873
OsHIL50	LOC_Os04g58850	0.335837	-0.470695	-0.111472	*OsHIL102*	LOC_Os12g06210	0	0	0
OsHIL51	LOC_Os04g58860	0.133652	-1.12781	-0.966443	*OsHIL103*	LOC_Os12g06220	0	0	0
OsHIL52	LOC_Os04g59330	1.8575	-1.68595	-2.50405	*OsHIL104*	LOC_Os12g06260	1.81764	1.00211	2.80644

APPENDIX F

LIST OF ABBREVIATIONS

ABA	abscisic acid
ABI1	aba-insensitive 1
ABO3	aba overly sensitive mutant
anti-	antibody against
AP	alkaline phosphatase
BLAST	basic local alignment search tool
BR	brassinosteroids
CaM	calmodulin or calcium-modulated protein
cDNA	complementary dna
cGMP	cyclic guanidine monophosphate
ChIP-seq	chromatin immunoprecipitation high throughput sequencing
CHLH/ABAR	magnesium-protoporphyrin IX chelatase H subunit/aba receptor
CK	cytokinin
CMV	cucumber mosaic virus
CRY	della clone in peas
cry1	cryptochrome 1
CSREES	cooperative state research, education, and extension service
Ct	cycle threshold
CTAB	cetyltrimethylammonium bromide
cv.	cultivar
CYP707A	cytochrome p450 monooxygenase
D-95	cloned lea gene number
DAP	days after pollination
DELLA	conserved amino acid sequence d-e-l-l-a
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dSpm	transposition-defective suppressor-mutator (spm)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
En/Spm	enhancer/suppressor-mutator
ER5	ethylene-responsive lea-like protein
Gα/D1	alpha subunit of the heterotrimeric G protein/dwarf 1
GA	gibberellic acid, gibberellin
GA3ox2	gibberellin 3-oxidase 2
GAI	gibberellic acid insensitive
GAMYB	gibberellic acid-induced myeloblastosis-like protein
GFP	green fluorescent protein
GID1	ga-insensitive dwarf 1
G protein	guanine nucleotide-binding proteins
GST	glutathione S-transferase
HA	hemagglutinin epitope
HAB1	hypersensitive to aba 1

HIN	harpin-induced
HMM	hidden markov model
HRP	horse radish peroxidase
HSL	hormone sensitive lipases
HVA22	*hordeum vulgare* abscisic acid-induced 22
IAA	auxin
ID	identifier
JA	jasmonic acid
kDa	kilodalton
KGM	kinase associated with gamyb
LA	della clone in peas
LEA	late embryogenesis abundant
LOC	locus number
MAST	multiple alignment and search tool
MEGA	molecular evolutionary genetics analysis
MEME	multiple em for motif elicitation tool
miR159	microrna
mRNA	messenger ribonucleic acid
MS	murashige and skoog medium
MSU RGAP	michigan state university rice genome annotation project
MYB33	myeloblastosis 33
NBRP	national bioresource project
NCBI	national center for biotechnology information
NDR	non-race-specific disease resistance
NHL	ndr1/hin1-like
NO	nitric oxide
OST1	open stomata 1
OsWRKY71	*oryza sativa*; conserved amino acid sequence w-r-k-y 71
P1 or -2	primer set 1 or 2
PBST	phosphate buffered saline + tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFAM	protein family database
phyA	phytochrome A
PIF1	phytochrome-interacting factor 3-like 1; alias of pil5
PIL5	phytochrome-interacting factor 3-like 5
PIN	pin-formed proteins
PIP	plasma membrane intrinsic protein
PKABA1	aba-induced protein kinase
PKL	pickle
PP2C	serine/threonine protein phosphatase type 2Cs
PR-1	pathogenesis-related
Praline	profile alignment application tool
pv.	pathovar
PVDF	polyvinylidene fluoride
PvPRP1	proline-rich protein 1
PYL	pyrobactin 1-like

PYR1	pyrabactin resistance 1
qPCR	quantitative real time polymerase chain reaction
RCAR	regulatory component of aba receptor
RdSpm	red fluorescence dSpm
RGA	repressor of gal-3
RH	relative humidity
RNA	ribonucleic acid
RNAi	rna interference
RPKM	read per kilobase of exon per million fragments mapped to the genome
RT-PCR	quantitative real time polymerase chain reaction
SA	salicylic acid
SCF	s-phase kinase-associated protein (skp) cullin f-box
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLR1	slender1
SLY1	sleepy 1
SnRK2s	sucrose-nonfermenting kinase1 (snf1)-related protein kinase 2s
SPY	spindly 1
T1	first generation transformed plant
T2	second generation transformed plant
TaABF1	*triticum aestivum* aba response element binding factor 1
TCA	trichloroacetic acid
T-DNA	transfer-dna
Tm	temperature
TMV	tobacco mosaic virus
TTG2	transparent testa glabra 2
UBI	ubiquitin
UTR	untranslated region
W51	oswrky51
W-box	wrky box
wt	wildtype
YLS9	yellow leaf-specific-9

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CURRICULUM VITAE

Graduate College
University of Nevada, Las Vegas

Margaret Ja Shin

Degrees

University of Nevada, Las Vegas – Bachelor of Science, Biology – 2006

Publications

Shin MJ, Gu LK, Lam N, Shen QJ. Annotation of the HIL family and their expression patterns in response to ABA and GA. In preparation

Zhang ZL, **Shin, MJ**, Zou, XL, Huang, JZ, Ho THD, Shen QJ. 2009. A negative regulator encoded by a rice WRKY gene represses both abscisic acid an gibberellin signaling in aleurone cells. Plant Molecular Biology 70:139 151.

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Oral Presentations

BIOS Symposium – March 12, 2010 – Regulation of a WRKY transcription factor in rice development.

GPSA – March 29, 2008 – Agamous-like-1, a novel regulator of floral development. (Honorable Mentions)

CMB Club – October 19, 2007 –The genetic basis of Agamous-like-1.

BIOS Symposium – November 17, 2007 – Agamous-like-1, a novel regulator of floral development.

BIOS Symposium – November 18, 2006 – The roles of Arabidopsis WRKY18, -40, and -60 in stress response. (Undergraduate Presentation)

Posters

Plant Biology & Botany Joint Congress (AFS, ASPB, ASPT, BSA) – Summer 2007 – Zhonglin Zhang, **Margaret J. Shin,** Xiaolu Zou, and Qingxi J. Shen. A negative regulator encoded by a rice WRKY gene represses both gibberellin and abscisic acid signaling in aleurone cells.

EPSCOR Undergraduate Research Poster Conference – Summer 2007 – Wendy Seto, **Margaret J. Shin,** and Qingxi J. Shen. The roles of Arabidopsis WRKY18, -40, and -60 in stress response.

Duties

Teaching Assistantship – Fall 2012, Spring 2012, Fall 2011, Spring 2009, Fall 2008, Spring 2008, Fall 2007

BIOS Faculty Representative Spring – Spring 2011, Fall 2010 – School of Life Sciences

Research Assistantship – Fall 2010, Spring 2010, Summer 2009, Summer 2008

Representative for College of Sciences Dean's Grad Council – Fall 2008

Conferences

American Society of Plant Biologists Plant Biology – 2011 – Minneapolis, MN (Attended)

Thesis Title: The Role of OsWRKY71 and Its Interacting Proteins in Seed Germination and Early Growth of` Cereal Grains

Thesis Examination Committee:

Chairperson, Jeffery Q. Shen, Ph.D.
Committee Member, Andrew J. Andres, Ph.D.
Committee Member, Frank van Breukelen, Ph.D.
Committee Member, Paul J. Schulte, Ph.D.
Committee Member, Lloyd R. Stark, Ph.D.
Graduate Faculty Representative, Ernesto Abel-Santos, Ph.D.

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