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The Role of Histone H2A.Z Abundance in Modulating Responses to Phosphorus Deficiency in Rice

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THE ROLE OF HISTONE H2A.Z ABUNDANCE IN MODULATING RESPONSES TO PHOSPHOROUS DEFICIENCY IN RICE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by

Sara Zahraeifard
M.S., Shiraz University Iran, 2007 December 2017
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<tbody>
<tr>
<td>arp6</td>
<td>Actin-Related Protein 6</td>
</tr>
<tr>
<td>arp6C</td>
<td>ARP6-RNAi knock-down line grown under control condition</td>
</tr>
<tr>
<td>arp6P</td>
<td>ARP6-RNAi knock-down line grown under Pi deficiency condition</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>GB</td>
<td>Gene body</td>
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<td>GO</td>
<td>Gene ontology</td>
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<td>P</td>
<td>Phosphorus</td>
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<tr>
<td>PCG</td>
<td>Protein coding gene</td>
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<td>Pi</td>
<td>Phosphate</td>
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<tr>
<td>PG</td>
<td>Pseudogene</td>
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<tr>
<td>RNAi</td>
<td>RNA-interference</td>
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<tr>
<td>TE</td>
<td>Transposable element</td>
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<tr>
<td>TEG</td>
<td>Transposable element-related gene</td>
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<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td>TTS</td>
<td>Transcription terminate site</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type plant</td>
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<tr>
<td>WTC</td>
<td>Wild-type plant grown under control condition</td>
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<tr>
<td>WTP</td>
<td>Wild-type plant grown under Pi deficiency condition</td>
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Abstract

Histone variants contribute to chromatin complexity by creating specialized nucleosomes. Here, to investigate the pattern of H2A.Z histone variant distribution and its role in modulating gene expression in rice (Oryza sativa L.), we performed genome-wide profiling of ARP6-dependent H2A.Z deposition in rice seedlings. We demonstrated that under controlled conditions, the majority of H2A.Z deposition is within protein-coding genes (PCG), and the most highly expressed genes are enriched with H2A.Z at their 5’ ends near the transcription start site (TSS), whereas the medium- to low-expressed genes contain H2A.Z across the entire gene. Based on H2A.Z deposition, we categorized genes into “housekeeping” and “stress-responsive” genes, which reflects their functions. Analysis of 24-hour Pi deficiency showed a significant reduction of H2A.Z at the TSS of housekeeping genes and gene body of stress-responsive genes, with the latter significantly overlapping with Pi deficiency-induced up-regulation of the affected genes. Reciprocally, genes containing an increase in H2A.Z demonstrated a significant correlation with down-regulated genes. These results revealed the negative role of H2A.Z deposition inside the gene body of stress-responsive genes. Also, we found that RNA interference (RNAi)-mediated Knock-down of ACTIN-RELATED PROTEIN 6 (ARP6), which encodes a key component of the SWR1 complex that catalyzes H2A.Z deposition, resulted in a H2A.Z genic pattern that was remarkably similar to that of Pi-deficient wild type (WT). However, the analysis of responses at the H2A.Z level unique to either ARP6 Knock-down or Pi deficiency demonstrated that Knock-down of ARP6 has a larger impact on loss of H2A.Z at the TSS of housekeeping genes associated with plastid functions and translation, whereas Pi deficiency leads to a
greater impact on loss of H2A.Z in the gene bodies of stress-related genes, including many transcription factor genes. Moreover, the analysis of the combined effect of Pi deficiency and ARP6-RNAi Knock-down showed no apparent synergistic effect on changes in H2A.Z deposition or transcript abundance, but resulted in the identification of several categories of genes exhibiting diverse responses to Pi-deficiency, ARP6 Knock-down, or in response to the combined perturbations. Together the results show that a short-term Pi deficiency treatment mimics knock-down of a key determinant of H2A.Z deposition (i.e. ARP6) in terms of differential H2A.Z deposition and changes in gene expression. Because short-term Pi deficiency induces stress components shared among a number of environmental stressors, the results suggest that H2A.Z deposition in the gene body acts as a repressor of many genes that are responsive to diverse stimuli. In addition, deposition of H2A.Z at the TSS may play an important role for the expression of housekeeping genes, particularly those linked to translation and plastid functions.
Chapter 1. Introduction

The Importance of Improving Nutrient Use Efficiency In Plants

According to a United Nations report, the world population is expected to increase to 9.8 billion by 2050 (Gilland, 2002). Such an increase will intensify pressure on the world’s natural resources to achieve higher crop production. Intensive cultivation consistently leads to degradation of land and reduces its fertility and productivity. Many agricultural soils of the world are deficient in one or more essential nutrients to support healthy and productive plant growth (Baligar et al., 2001). Consequently, expensive chemical fertilizers are used to compensate for these deficiencies and to achieve desired crop yields. However, recovery of applied inorganic fertilizers by plants is low in many soils. The estimation of overall efficiencies of these applied fertilizers have been about 50% or lower for nitrogen (N), less than 10% for phosphorus (P), and approximately 40% for potassium (K) (Baligar & Bennett, 1986). These lower efficiencies are attributable to major losses of nutrients by leaching, run-off, gaseous emission and fixation by soil. These losses can potentially contribute to degradation of soil and water quality, and can eventually lead to overall environmental degradation. These are convincing reasons for the need to improve nutrient use efficiency of plants. The nutrient use efficiency of plants is known to be under genetic and physiological control and is also influenced by environmental variables. Thus, there is a need for breeding programs to generate and disseminate nutrient efficient genotypes. Identification of traits involved in nutrient assimilation, utilization, and mobilization in plant cultivars should greatly enhance fertilizer use efficiency. The development of new cultivars with higher nutrient use efficiency will contribute to sustainable agricultural
systems that retain and support soil, water and air quality (Baligar et al., 2001). In this study, I focused on P use efficiency in light of the current and declining shortage of P resources. The long-term application of the study is to contribute to improving the P use efficiency of crop plants to sustain global agriculture.

**Plant Nutrient Acquisition and Utilization**

Plants, like all living organisms, require the right combination of essential nutrients to grow and reproduce. According to Arnon and Stout, an essential nutrient is in fact essential only if a shortage of the specific nutrient inhibits the completion of the vegetative or reproductive phase of the plant (Arnon & Stout, 1939). Essential plant nutrients include 17 elements; depletion of these elements causes various disorders of growth and development. Except for carbon (C), oxygen (O) and hydrogen (H) that are absorbed from the air (non-mineral nutrients), plants take up the remaining 14 elements (mineral nutrients) from the soil. The six macronutrients, N, P, K, calcium (Ca), magnesium (Mg), and sulfur (S) are required by plants in large amounts. The eight micronutrients include boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), molybdenum (Mo), and nickel (Ni), and are required in trace amounts (Marschner, 2011).

Complex interactions involving rock minerals, decaying organic matter, and microbes form inorganic minerals in soil. Roots absorb these mineral nutrients as dissolved ions in soil water (Ahuja, 2006). A variety of factors can adversely affect mineral nutrient levels in plants. Among the factors that can affect critical concentrations are shortages of necessary nutrients as well as inaccessibility of nutrients to the plant in spite of their availability in the soil as a result of suboptimal pH and temperature, water
deficiency, and an excess of another nutrient (Bradley & Hosier, 1999). To combat these issues, higher plants have evolved a sophisticated array of mechanisms to maintain nutrient homeostasis. In the processes related to mineral nutrient homeostasis, plants sense the levels of available nutrients in soil and adjust nutrient uptake or exclusion rates to maintain concentrations within acceptable ranges (Figure 1) (Aibara & Miwa, 2014).

![Figure 1. General scheme of plant growth and yield in response to nutrient availability. This image was taken from a previous publication (Munson, 1998).](image)

Two major strategies plants use to facilitate efficient nutrient acquisition are root system architecture (RSA) modification and expression of transporter proteins, which are specific for each nutrient (Aibara & Miwa, 2014). In response to fluctuations in nutrient availability, root systems can modify their architecture in a number of ways to optimize nutrient acquisition (Aibara & Miwa, 2014). The uptake of many nutrients presents a challenge because of their low concentration in the soil, which leads to their uptake often occurring against a concentration gradient. Depending on their charge, ions may also encounter an electrochemical gradient, which necessitates counter ions and/or a proton gradient, which is built up via proton-ATPases, to achieve uptake. To
meet these challenges, plants have evolved active transport and translocation systems that are regulated in response to changes in environmental and internal conditions. Studies of plant nutrition have revealed several distinct classes of regulated transport activities (Chrispeels et al., 1999). As shown in Figure 2, Epstein (1972) classified these activities into two mechanisms (biphasic kinetics), high- and low-affinity uptake systems. Based on his observation of absorption of potassium by excised barley roots; the high-affinity uptake system activates at a half-maximal rate below 0.02 mM potassium and behaves as a saturable carrier. At higher concentrations, a low-affinity uptake system becomes apparent and displays either linear or multiple saturation kinetics (Figure 2) (Epstein, 1972).

Figure 2. Biphasic kinetics of a potassium transporter from *Arabidopsis*. Under low potassium concentrations (0 mM to 0.2 mM), high-affinity transporter uptake is activated (left curve). Under high potassium concentration (up to 50 mM), a low-affinity system is activated (right curve). This image was taken from a previous publication (Epstein, 1972).
The physiological and biochemical studies such as those described above have set the basis for the molecular and genetic analysis of nutrient uptake. The main challenge has been to characterize the components of these uptake systems and determine their contribution to nutrient uptake and plant growth. The initial conclusions from molecular and genetic investigations are that multiple genes are involved in the uptake of a particular nutrient (Ashley et al., 2005; Rouached et al., 2010; Yang et al., 2015).

**Phosphorus (P) in Plants and its Availability**

P is a pivotal nutrient for all life on Earth. It is vital for plant growth, making up about 0.2% of a plant’s dry weight, and is necessary for a plant to complete its life cycle. P is a recognized essential macronutrient because of the relatively large amounts of P required by plants as a part of several key plant compounds and processes. It forms the sugar-phosphate backbone of DNA and RNA and is also a structural component of phospholipids. In addition, it aids in the conversion of several major biochemical reactions and is important for energy transfer in cells as part of adenosine triphosphate (ATP) (Schachtman et al., 1998).

Despite being a macronutrient in plants, P behaves somewhat like a micronutrient in soils. On average, the total P content of most surface soils is only 0.6% of soil weight. Crops take up P only from the soil solution in the form of inorganic phosphate (Pi) as primary and secondary orthophosphates (H₂PO₄⁻ and HPO₄²⁻). The optimal pH range for maximum Pi availability is 6.0-7.0 and the proportion in which these two forms are absorbed is determined by the soil pH, where at higher soil pH more HPO₄²⁻ is taken up. At a lower pH, when the soil is very acidic, more iron and
aluminum are available to form insoluble Pi compounds and, therefore, less Pi is available. At very high pH, P can react with excess calcium to also form unavailable compounds in the soil. Consequently, the mobility of P in soil is limited and the concentration of soluble Pi in the soil solution is very low (less than 0.6 to 11 µM). Therefore, plant roots can take up Pi only from their immediate surroundings in the rhizosphere (Schachtman et al., 1998).

**Plant root architecture maximizes Pi uptake**

The geometry and morphology of plant roots are important determinants in Pi uptake because ratios of root surface area to volume can regulate the efficiency of exploration of the soil top layer to improve the acquisition of the poorly mobile Pi (Lynch, 1995). By this logic, mycorrhizae also increase Pi acquisition by expanding the absorptive surface of the root system with their hyphae (Smith & Read, 2010). Other Pi starvation adaptations in some species include cluster root formation, which greatly enhance Pi absorption capacity, and release of high amounts of carboxylates, which chelate metal ions and thus release adsorbed P from metal complexes (George et al., 1995; Jost et al., 2015).

In low Pi medium, the changes in root architecture include a decrease in primary root growth and increases in the number and length of root hairs (Jain et al., 2007; Ma et al., 2001; Sánchez-Calderón et al., 2005). The root tip is an important region as a local Pi sensing site. It was shown that physical contact of the primary root tip with low Pi medium was necessary and sufficient to reduce primary root growth by the activation of two multicopper oxidase genes, *LPR1* and *LPR2* (Svistoonoff et al., 2007). Pi deficiency also causes the activation of PDR2, which is responsible for a reduction in cell elongation as well as cell-cycle activity in the root meristem in root tips, leading to
severe reduction in the growth capacity of primary roots (Ticconi et al., 2004). Altogether, these changes have evolved to increase the surface area to volume ratio (Figure 3).

![Diagram](image-url)

Figure 3. Different molecular pathways involved in Pi deficiency responses in plants. Solid lines: connections between Pi-deficiency, regulatory pathways or molecular players. Negative and positive regulatory effects are indicated by flat-ended dashed lines and arrowheads, respectively. Open arrowheads: an increase or decrease in iron and phytohormone levels upon Pi starvation. PSI (Pi starvation-induced genes). This image was taken from a previous publication (Rouached et al., 2010).

**Strategies for the maintenance of intercellular Pi homeostasis in plants**

P in plant tissues is present as either Pi or as organic P esters. In Pi sufficient conditions, the amount of Pi inside the cell is maintained at constant concentrations (5–10 mM) (Lee & Ratcliffe, 1993; Mimura, 1995). When Pi is coming inside the cell at rates that exceeds demand, a number of processes act to avoid the accumulation of toxic Pi levels (Schachtman et al., 1998). The level of metabolically active Pi in the cytoplasm is tightly controlled, and excess Pi is stored in the vacuole in the form of...
organic storage compounds (e.g. phytic acid) (Smith et al., 2015) as well as a drop in the Pi uptake rate from outside (Lee et al., 1990), and Pi loss by efflux (Bieleski & Ferguson, 1983). During short-term Pi deficiency, vacuolar Pi is mobilized to the cytosol and compensates for the deficiency. However, a constant Pi deficiency will eventually deplete the Pi pool and lead to the induction of numerous hydrolases such as nucleases and phosphatases that release Pi from organic P pools (Smith et al., 2015). Also, the coordinated induction of phospholipase genes and galactolipid and sulfolipid biosynthetic genes under Pi deficiency collectively results in the release of Pi from phospholipids and replacement with lipids containing other functional groups (Smith et al., 2015). Relatively speaking, limitation of Pi can cause plants to grow more roots, increase the rate of uptake by roots from the soil, and remobilize Pi from older leaves. In addition, mycorrhizal fungi associations by roots can be initiated or expanded in response to Pi deficiency. These processes are among the general strategies for the maintenance of Pi homeostasis inside the cell (Schachtman et al., 1998).

**Role of phytohormones in Pi deficiency responses**

Many reports have clearly demonstrated the roles of phytohormones in Pi signaling pathways (López-Bucio et al., 2005; Nacry et al., 2005). Previous work has shown that Pi starvation in plants caused a reduction in Gibberellic Acid (GA) levels and accumulation of DELLA proteins. DELLA-mediated signaling contributes to certain aspects of Pi-deficiency responses in roots, i.e. suppression of primary root growth and raise of root hairs (Jiang et al., 2007). Auxin is known to control GA-mediated repression of two growth-repressor DELLA proteins known as RGA and GAI (Fu & Harberd, 2003). Furthermore, it was shown that ethylene and strigolactone biosynthesis is induced in response to Pi deficiency (Nagarajan & Smith, 2011). In addition to their
roles in stimulation of root hair formation, lateral root elongation, and reduction of primary root elongation upon Pi starvation (Jiang et al., 2007), ethylene and strigolactone are likely involved in monitoring and controlling the Pi status of senescing leaves for the remobilization of Pi (Smith et al., 2015). Nevertheless, a comparison of a transcriptome analysis of Pi-deficient Arabidopsis with hormone-related transcript profiling studies revealed that Abscisic Acid (ABA) had more differentially expressed genes in common with Pi deficiency as compared to other hormones (Woo et al., 2012). Future work aimed at understanding the roles of phytohormones in modulating responses to Pi starvation should examine the integrated output of hormonal cross-talk pathways because of the complexity of the effects of phytohormone signaling pathways on each other (Rouached et al., 2010) (Figure 3).

**Phosphate transporters**

Kinetic studies have revealed the presence of two Pi uptake systems in plants, one with high affinity and activity that is either increased or de-repressed by Pi starvation, and one with a lower affinity and activity that is constitutive (Schachtman et al., 1998). Estimates of the $K_m$ for high-affinity uptake range from 2.5 to 12.3 µM, whereas for low-affinity transporters the $K_m$ estimates are more variable, from 50 to 330 µM in several different tissues and plant species (Furihata et al., 1992; McPharlin & Bieleski, 1987; Ullrich-Eberius et al., 1984). In higher plants, four Pi transporter (Pht) families have been characterized (Pht1-Pht4) (Guo et al., 2008; Lin et al., 2009; Nussaume et al., 2011; Poirier & Bucher, 2002; Versaw & Harrison, 2002). Many members of the Pht1 family play important roles in Pi acquisition from soil and root-to-shoot translocation of Pi (Nussaume et al., 2011). The single Pht2 family member, Pht2;1, is a low-affinity Pi transporter localized to the chloroplast envelope where it
plays an important role in photosynthetic activity (Rausch et al., 2004; Versaw & Harrison, 2002). The Pht3 family is comprised of mitochondrial-localized transporters to provide Pi for the oxidative phosphorylation of ADP to ATP (Nakamori et al., 2002; Poirier & Bucher, 2002). Pht4 transporters are low-affinity transporters that localize to plastids and Golgi (Guo et al., 2008). The cooperation among the four Pht families allows plants to maintain Pi homeostasis (Figure 3).

**Regulatory components involved in Pi signaling and Pi homeostasis in plants**

Plants have evolved to react to fluctuations of Pi levels by means of complex responses that tightly control intercellular Pi levels. These responses are initiated and modulated by elaborate signaling networks that maintain Pi homeostasis via global transcriptional changes (Chiou & Lin, 2011). Pi signaling pathways are largely conserved among higher plants (Figure 3). There are two main Pi signaling pathways: (i) local, which depends on external Pi concentrations and correspond mostly to characteristic changes in root system architecture; and (ii) systematic, which is determined by the Pi status of the whole plant and involves long distance signaling (Chiou & Lin, 2011).

In the past decade, major findings of Pi signaling and homeostasis were reported in plants. A MYB transcription factor, PHOSPHATE STARVATION RESPONSE 1 (PHR1), and related transcription factors are known as the main regulators of Pi deficiency responses (Bustos et al., 2010; Ren et al., 2012; Rubio et al., 2001; Wang et al., 2013; Zhou et al., 2008). Also, miRNAs (miR399, miR827, etc.) as mobile signals (Kuo & Chiou, 2011; Pant et al., 2008) and ubiquitin system components, including PHO2 and NLA, are major regulatory components of Pi signaling (Bari et al., 2006;
Huang et al., 2013; Kant et al., 2011; Lin et al., 2013). Also, Pi starvation-induced riboregulators of miRNA activity function via target mimicry (Franco-Zorrilla et al., 2007), which antagonize the miRNAs (Jabnoune et al., 2013). The PHR1-miR399-PHO2 signaling pathway plays a central role in Pi signaling by inducing the expression of a number of genes in response to Pi deficiency (Bustos et al., 2010) (Figure 3). Riboregulators IPS1 and At4, as well as some high affinity phosphate transporters, are among the Pi deficiency response genes (Bustos et al., 2010). IPS1 and At4 function in a feedback mechanism, which fine-tunes Pi deficiency responses by sequestering miR399 (Franco-Zorrilla et al., 2007).

PHR1 activates the expression of numerous downstream loci via a cis-regulatory motif, the PHR1-binding site (P1BS) (Bustos et al., 2010; Rubio et al., 2001). In a recent study of a transcriptomic analysis on a phr1 mutant line of Arabidopsis, 319 potential direct targets of AtPHR1 were identified. The majority of these target loci were also induced by Pi deficiency in wild-type seedlings (Bustos et al., 2010). MYB62, another MYB transcription factor, is also involved in the Pi deprivation response of Arabidopsis. MYB62 expression is induced by Pi starvation (Misson et al., 2005), but only in leaves of young seedlings (Devaiah et al., 2009). MYB62 is likely to be a negative regulator of other Pi starvation-inducible genes, and may moderate their activity during Pi starvation (Yang & Finnegan, 2010) (Figure 3).

Many WRKY transcription factors are also involved in Pi deficiency responses (Chen et al., 2009; Devaiah et al., 2009) (Figure 3). WRKY6 represses expression of PHO1, which is required for normal Pi distribution (Chen et al., 2009). At low-Pi conditions, it appears that WRKY6 is targeted for degradation by an E3 ligase, which
reduces WRKY6 repression of PHO1 (Chen et al., 2009). Another Pi-induced Arabidopsis WRKY is WRKY75, which impacts expression of several Pi starvation response genes. Knock-down of WRKY75 resulted in reduced low-Pi induction of Pht1 transporters and HAD family phosphatases (Devaiah et al., 2007; Devaiah & Raghothama, 2007). Also, in rice, OsWRKY74 was implicated in modulation of Pi homeostasis and potential crosstalk between Pi and Fe deficiencies and cold stress (Dai et al., 2015). Another rice-specific transcription factor linked to Pi signaling is the OsPTF1 basic helix-loop-helix transcriptional activator (Yi et al., 2005). Expression of OsPTF1 is induced in roots during Pi deficiency. However, the function of OsPTF1 in the regulation of Pi response pathways is not fully known.

It has been shown recently that small SPX-domain containing proteins play significant roles in Pi signaling in both rice and Arabidopsis (Rouached et al., 2010; Secco et al., 2012). The SPX superfamily is divided into four classes, SPX, SPX-EXS, SPX-MFS, and SPX-RING. The majority of SPX-containing proteins appear to regulate Pi signaling via negative regulation of Pi-signaling components (Secco et al., 2012). It is known that two plant SPX-domain proteins, SPX1 and SPX3, regulate the expression of multiple downstream targets (Rouached et al., 2010) and cause Pi remobilization during senescence (Smith et al., 2015). Also, SPX4 in rice was shown to repress the transcriptional activator OsPHR2 (Lv et al., 2014), whereas the SPX-RING protein NLA is an E3 ligase that is involved in the degradation of Pht1 transporters (Lin et al., 2013). Furthermore, microRNAs (miRNAs) are another group of regulatory factors in Pi responses. miR827 and miR399 are two important miRNAs targeting NLA and PHO2 transcripts, respectively, for degradation, which leads to a de-repression in Pi
acquisition and translocation (Kant et al., 2011). All these components collectively act in a network to regulate the expression of numerous Pi homeostasis genes, including high-affinity Pi transporters (Pht1s), phosphatases, ribonucleases, and other metabolic genes involved in processes such as lipid and starch biosynthesis and protein turnover.

**The Role of Epigenetic Mechanisms in Regulating Transcriptional Networks**

In eukaryotes, in addition to proven roles of regulatory components, epigenetic mechanisms have emerged as significant factors in conferring environmental adaptability. In spite of the clear role of chromatin structure in controlling gene expression in eukaryotes (Haig, 2004), the relevant mechanisms involved in regulating Pi transcriptional networks has not been fully investigated. In response to environmental signals, epigenetic mechanisms can affect the chromatin landscape by altering its biochemical properties and promote rapid reactivation of numerous stress response genes. An understanding of chromatin structure and the epigenetic mechanisms that modulate DNA and chromatin may help to elucidate the impacts of chromatin level changes in plants under stress conditions, such as Pi deficiency.

The interaction of positively charged histone tails with DNA and the masking of DNA binding sites that face the nucleosome surface present obstacles for the binding of sequence-specific DNA-binding factors. As a result, all DNA-dependent processes, such as transcription, replication, repair and recombination are affected by the position of nucleosomes on regulatory sites (Workman & Buchman, 1993). Distinct epigenetic mechanisms have evolved that dynamically orchestrate responses to exogenous stimuli and developmental signals through changes in chromatin organization (Rosa & Shaw, 2013). This dynamic regulation of gene expression through epigenetic mechanisms is
accomplished via small interfering RNAs (siRNAs), DNA methylation, and through changes in chromatin structure, i.e. post-translational modification of histones, altering the position or eviction of nucleosomes through chromatin remodeling, and replacement of canonical histones with histone variants (Deal & Henikoff, 2011; Sirohi et al., 2016; Weber et al., 2014) (Figure 4).

**Small interfering RNAs (siRNAs)**

siRNAs have been shown to play roles in transmitting epigenetic information (Bäurle et al., 2007; Borsani et al., 2005; Swiezewski et al., 2007), as well as regulating gene expression through DNA methylation (Bao et al., 2004; Chellappan et al., 2010). For example, mutation in two Arabidopsis genes that encode flowering time regulators, FCA and FPA, exhibited defects in flower development due to RNA-mediated chromatin silencing of a range of loci in the genome including the Phytoene Desaturase (PDS) gene. This suppression was accompanied by the asymmetric methylation of the endogenous PDS locus. These data suggest that FCA and FPA regulate chromatin silencing through interaction in a locus-dependent manner with the siRNA-directed DNA methylation pathway to regulate common targets (Bäurle et al., 2007).
Figure 4. Overview of possible epigenetic modifications. These epigenetic modifications can all influence the accessibility of the chromatin structure to the transcriptional machinery. This image was taken from a previous publication (Gräff et al., 2011).

**DNA methylation**

Cytosine DNA methylation is a major epigenetic mark in both plants and animals that tends to generate heterochromatin structure, which leads to gene repression. DNA methylation is not evenly distributed across the genome but rather is enriched at repetitive DNA elements like transposons, whereas most genes have limited methylation or are unmethylated (Zilberman et al., 2007). In plants, three distinct types of methylation occur based on sequence context. These include CG, CHG and CHH site classes, which are established and maintained by separate enzymatic pathways (Bewick et al., 2016). Several studies have shown an impact of environmental perturbation on DNA methylation in plants. In rice, N deficiency led to locus-specific alteration of DNA methylation. Interestingly, 50% of the altered methylation patterns in somatic cells of the stressed plants were present in their progeny, which eventually led
to higher tolerance to N starvation in subsequent generations (Kou et al., 2011). Another study in rice suggested that transposable elements (TEs) in proximity to Pi deficiency-induced genes were silenced via hypermethylation, establishing a temporal hierarchy of transcriptional and epigenomic changes in response to stress (Secco et al., 2015). Furthermore, it was recently reported that Pi starvation resulted in general remodeling of global DNA methylation in Arabidopsis that correlated with changes in transcript abundance of a set of key Pi starvation-induced genes. Interestingly, the expression of genes encoding DNA methyltransferases appeared to be directly controlled by the key regulator PHR1 (Yong-Villalobos et al., 2015).

**Histone modifications**

A main determinant of chromatin structure is the decoration of nucleosomes with post-translational modifications at specific amino acids of their histones (Zhang & Pugh, 2011). Histone modifications are covalent post-translational modifications (PTMs), including acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation (Figure 5). These reversible covalent modifications are catalyzed by the action of a variety of histone modifying enzymes such as SET DOMAIN GROUP 2 (SDG2), an Arabidopsis protein containing a histone lysine methyltransferase domain responsible for deposition of trimethylation on histone 3 lysine 4 (H3K4) (Guo et al., 2010). Consequently, these modifications can activate or suppress transcription by generating open or closed chromatin configurations (Sirohi et al., 2016).

A high-resolution X-ray structure of the nucleosome revealed that highly basic histone amino terminal tails do not significantly contribute to the nucleosome structure and instead can protrude from their own nucleosome and affect inter-nucleosomal and
other protein interactions via modifications, and thus affect the overall chromatin structure (Figure 5).

Figure 5. A nucleosome with the four canonical histones (H2A, H2B, H3 and H4) and the linker histone H1. The covalent PTMs [methylation (Me), acetylation (Ac), ubiquitination (Ub), and phosphorylation (Ph)] are highlighted on the N- and C-terminal tails of each histone. This image was taken from a previous publication (Tollervey & Lunyak, 2012).

Also, histone modifications can recruit remodeling enzymes with specific activities that hydrolyze ATP to shift nucleosomes (Bannister & Kouzarides, 2011; Luger et al., 1997). Furthermore, histone modifications are directly involved in perturbing the overall structure of chromatin, either over short or long distances. For instance, histone acetylation effectively reduces the positive charge of histones, leading to disruption of electrostatic exchanges between histones and DNA. Consequently, the less compact chromatin structure can facilitate DNA access by transcriptional machineries. Thus, in hyper-acetylated regions of the genome, the charge on the histone tails can be
neutralized, which can profoundly alter the chromatin structure (Bannister & Kouzarides, 2011; Kouzarides, 2007). In addition, several studies showed that the histone modifications either positively or negatively regulate the binding of chromatin factors. New proteomic approaches have identified a number of “multivalent” proteins and complexes with specific domains that allow the recognition of more than one modification (Bartke et al., 2010; Vermeulen et al., 2010). For example, the L3MBTL1 protein can simultaneously bind to H4K20 mono/di-methylated and H1K26 mono/di-methylated marks, resulting in compaction of nucleosomal arrays with the two histone modifications (Trojer et al., 2007). A further complexity exists due to crosstalk between different modifications, which helps to fine-tune the overall control. This crosstalk can occur via competitive effect of modifications if more than one modification pathway is directing the same site(s), or the dependence of one modification upon another. Also, cooperation between histone modifications and DNA methylation can be into account. For instance, the protein, UHRF1, binds to H3K9me3-nucleosomes, specifically the nucleosome containing DNA with CpG methylation (Bartke et al., 2010; Vermeulen et al., 2010). Regarding responses to nutrient stress, most investigations of histone modification dynamics have focused on histone methylation. In Arabidopsis, it was shown that symmetric dimethylation of histone H4R3 was involved in iron homeostasis, and mutation in the gene encoding histone modifying enzyme Arginine Methyltransferase 5 (PRMT5) resulted in higher iron accumulation in shoots and greater tolerance to iron deficiency (Fan et al., 2014). The involvement of trimethylated H3K4 in response to Pi starvation was also reported through the identification of the alfin-like 6 (AL6) histone “reader” (Chandrika et al., 2013). AL6 is non-transcriptionally responsive
to Pi starvation, but *al6* mutant plants displayed a pleiotropic phenotype including reduced anthocyanin accumulation and altered root architecture in response to low Pi. Through its Plant Homeodomain (PHD), AL6 might bind to trimethylated H3K4 and affect expression of critical genes involved in root hair elongation (Chandrika et al., 2013). A recent study revealed that histone acetylation was also involved in Pi homeostasis, through histone deacetylase 19 (HD19) as a key player in controlling root cell elongation and regulating a subset of key Pi starvation induced genes, including some SPX genes involved in Pi sensing and signaling (C.-Y. Chen et al., 2015). Furthermore, histone acetylation was recently found to regulate iron homeostasis through the histone acetyltransferase General Control Non-repressed 5 (GCN5) in *Arabidopsis*. GCN5 could directly bind to the promoters of five iron-related genes and modulate their acetylation levels of H3K9 and H3K14, in turn regulating their expression (Xing et al., 2015).

**ATP-dependent chromatin remodelers**

In addition to histone modifications and DNA methylation, chromatin remodeling ATPases and their associated complexes also participate in transcriptional regulation by controlling the positioning, occupancy and composition of nucleosomes (Han et al., 2015; Sudarsanam & Winston, 2000). Chromatin remodelers are large, multi-protein complexes that alter the interaction between DNA and the histone octamer non-covalently (Clapier & Cairns, 2009; Hargreaves & Crabtree, 2011) and assist the transcriptional responses at the expense of ATP (Vignali et al., 2000). In addition to the central ATP hydrolase subunit, each remodeler complex contains unique, specialized proteins that aid in the complex’s activity (Whitehouse et al., 2007). SWR1/INO80 is one of the major chromatin remodeler families. The unique feature of this family is a spacer
to split the ATPase core subunit (Bao & Shen, 2007). The presence of this spacer requires for link with other core complex subunits (Wu et al., 2005). However, INO80 and SWR1 complexes have opposite roles in histone variant exchange, with SWR1 replacing canonical H2A with the H2A.Z, while INO80 catalyzes the opposite reaction, exchange of H2A.Z for H2A (Luk et al., 2010; Mizuguchi et al., 2004; Papamichos-Chronakis et al., 2011).

Nucleosome composition and histone variants

The general composition of individual nucleosomes consists of a histone octamer core around which 147 bp of DNA is wrapped, forming 10 nm beads-on-a-string fibers, the first level of packaging of the eukaryotic chromosome (Sarma & Reinberg, 2005). The core histones, i.e. H2A, H2B, H3 and H4, all contain a conserved carboxyl terminal histone fold domain and unique amino terminal tails. The four core histones interact in pairs via a “handshake motif” with two H3/H4 dimers interacting together to form a tetramer, while the two H2A/H2B dimers associate with the H3/H4 tetramer in the presence of DNA (Kamakaka & Biggins, 2005) (Figure 6A). Canonical histones are deposited in a replication-coupled manner to package the newly replicated genome. Histone chaperones facilitate incorporation of histones on DNA by neutralizing their positive charges, and thus play key roles in regulating nucleosome assembly (Philpott et al., 2000). Nucleosomes are assembled via a stepwise process. First, the H3–H4 tetramer is deposited on DNA, followed by the addition of two H2A–H2B dimers (Smith & Stillman, 1991). This process is reversible to allow disassembly of nucleosomes (Figure 6B). Each of the canonical histones is encoded by multiple genes and most of these genes are organized into clusters throughout the genome to ensure that the
expression of canonical histones during S-phase generates large and equal amounts of all four nucleosome core histone proteins (Albig et al., 1997).

Figure 6. (A) Structure of the nucleosome core particle with (H3/H4)$_2$ at the center of the DNA wrap and (H2A/H2B)$_2$ docked at the edges, near the DNA entry and exit locations. (B) Nucleosomes control access to certain binding sites. Transient DNA unwrapping exposes the binding sites. As nucleosomes unwrap, H2A/H2B dimers can be lost, exposing more DNA, and when the nucleosome is completely unwrapped, (H3/H4)$_2$ is lost, and DNA is completely exposed. This image was taken from a previous publication (Weber & Henikoff, 2014).

One form of altering chromatin structure is through the deposition of histone variants, related protein isoforms encoded by non-allelic paralogous genes of the canonical core histones. These variants may differ in only a few amino acids or may have many differences mainly in their amino or carboxyl terminal tails (Kawashima et al., 2015). Histone variants contribute to chromatin complexity by creating specialized nucleosomes and having species-specific distribution patterns. Unlike canonical histones, the variants are typically encoded by a single (or two) gene(s) located outside
the canonical histone clusters and are not restricted in their expression to the S phase but are expressed throughout the cell cycle (Kamakaka & Biggins, 2005). Furthermore, most histone variant mRNAs are poly-adenylated, and their pre-mRNA can contain introns, giving rise to histone proteins with distinct functional and structural properties (Marzluff et al., 2008). Unlike the canonical histones that function primarily in genome packaging and gene regulation, histone variants have diverse roles including DNA repair, meiotic recombination, chromosome segregation, transcription initiation and termination, and sex chromosome condensation and sperm chromatin packaging (Talbert & Henikoff, 2010). This diversity of roles reflects, at least in part, structural differences from canonical histones that alter nucleosomes from within the core particle (Talbert & Henikoff, 2010). Such changes can profoundly influence the nucleosome stability and biological outcome, where histone variants change the chromatin landscape of cis-regulatory and coding regions in favor of particular transcription programs (Bönisch & Hake, 2012; Weber & Henikoff, 2014). Histone chaperones modulate the dynamics of histone variant deposition into the genome in the right place and at the right time, contributing to the functions of histone variants in chromatin regulation (Jiang & Berger, 2017). Moreover, because histone variant deposition is replication-independent, it is capable of responding to environmental stimuli. Indeed, nearly all histone variants seem to be involved in environmental adaptation (Talbert and Henikoff 2010). In general, H3 and H2A core histone families comprise the most abundant variants. Although they evolved independently in animals and plants, plant H3 and H2A variants developed very similar specialized features as their counterparts in animals, suggesting evolutionary convergence (Jiang & Berger, 2017). Conversely, no
specific sequence variants of histone H4 is known so far (Jiang & Berger, 2017) and to date only a few variants have been described for H2B to have very specific functions including heterochromatin formation and transcription repression, mainly during gametogenesis (Montellier et al., 2013; Yang et al., 2016).

**Histone H3 variants**

The H3 family consists of three major variants: H3.1, centromeric histone variant H3 (CenH3) and H3.3, as well as a plant-specific variant H3.10 (Fig. 7). CenH3 is highly divergent from other H3 variants and is deposited specifically at centromeres for the assembly of kinetochores (Ravi et al., 2011). *Arabidopsis* H3.1 and H3.3 variants have high sequence identity, differing at only four amino acids (Figure 7). The H3.1 variant is predominantly deposited in a replication-coupled manner, while H3.3 incorporation into the nucleosome in replication-independent (Ahmad & Henikoff, 2002). H3.10 differs from conventional H3.1 and H3.3 variants by many amino acid differences especially at its amino terminal tail, which is usually subject to various histone modifications (Fig. 7) (Borg & Berger, 2015).

![Sequence alignment of H3.1, H3.3, H3.10 and CenH3](image)

**Figure 7.** Sequence alignment of H3.1, H3.3, H3.10 and CenH3. Different amino acids between H3.1 and H3.3 variants are marked by asterisks. This image was taken from a previous publication (Jiang & Berger, 2017).

CenH3 is a universal variant with two major functions at centromeres. First, kinetochore formation at active centromeres are localized by CenH3, which implies the
role of CenH3 in chromosome segregation (Ouspenski et al., 2003) (Figure 8A). Also, specific histone modifications aid in propagating centromere identity (Sullivan & Karpen, 2004). CenH3 also targets assembly of the kinetochore protein complex by the protein-binding sites in CenH3 (Kamakaka & Biggins, 2005). In Arabidopsis, the reduction in CENH3 transcript caused a drop in mitosis while an increase in meiotic segregation errors (Lermontova et al., 2011). Also, the cenh3 mutants result in early embryonic lethality (Ravi et al., 2010).

**Figure 8.** Localization of H2A and H3 histone variants across the genome in plants. A) Certain chromatin regions with specific variant enrichment. B) Histone variants at different regions of genes/TEs (e.g. TSS, gene body, TTS) may have different effects on transcription activity. This image was taken from a previous publication (Jiang & Berger, 2017).

H3.3 is present in animals and plants in which it modulates chromatin at transcribed regions and promoters (Figure 8A-B). Based on high similarity between H3.1 and H3.3, two possible roles have been assigned to H3.3 that are linked with gene activity. 1) H3.3 in transcription-coupled deposition could be to compensate for the eviction of nucleosomes due to the progression of the RNA polymerase complex in the
body of highly-transcribed genes (Schwartz & Ahmad, 2006). 2) H3.3 might contribute to a constant process of histone turnover that retains accessibility of regulatory elements to their related factors, which could account for the dynamic memory of an activated state (Henikoff, 2008; Ng & Gurdon, 2008). H3.3 Knock-down in Arabidopsis targeted transcription reduction that mainly affects genes responsive to environmental cues (Wollmann et al., 2017). Furthermore, it was found that DNA methylation and H3.3 enrichment inside gene bodies have a similar dependence on gene transcription levels and are correlated through the inhibition of H3.3 on recruitment of H1. Inhibiting H1 deposition causes the promotion of chromatin folding, which restricts access to DNA methyltransferases responsible for gene body methylation. Thus, gene body methylation is likely initiated by H3.3 dynamics in conjunction with transcriptional activity (Wollmann et al., 2017). On the other hand, while H3.3 presence within transcribed regions is strongly associated with transcriptional activity, H3.3 at promoters, though at a lower level, is often independent of transcription. In particular, promoters with GA repeats carry H3.3 regardless of transcriptional levels (Shu et al., 2014), possibly accounting for a poised state of activity for these genes (Szenker et al., 2011). In addition, genes with promoters enriched in H3.3 tend to associate with higher H3K27 trimethylation across the gene body as compared to other inactive genes (Shu et al., 2014). This suggests a possible connection between H3.3 and complex Polycomb Repressive Complex 2 (PRC2), which catalyzes H3K27 trimethylation in plants and animals (Banaszynski et al., 2013). In addition to its preferential accumulation at sites of active chromatin, H3.3 enrichment is also observed in particular chromosomal landmarks. Elaborate studies on repetitive regions at Arabidopsis centromeres and
telomeres using H3.1 and H3.3 genome-wide mapping data suggested that centromeric repeats are enriched with H3.1, while telomeres are enriched with H3.3 (Wollmann et al., 2017) (Figure 8A), which is consistent with the observations that Arabidopsis telomeres exhibit euchromatic features (Vaquero-Sedas et al., 2010).

**H3.10** is a plant specific histone H3 variant that was identified in Arabidopsis. H3.10 is specifically and abundantly accumulated in sperm cell chromatin and rapidly removed from the zygote after fertilization, indicating a potential role of H3.10 in reprogramming sperm cell chromatin (Okada et al., 2005).

**Histone H2A variants**

H2A variants are the most diverse among all histone variants. In Arabidopsis, four major types of H2A variants have been characterized. Among them, canonical H2A, H2A.X and H2A.Z are variants found in both animals and plants. However, H2A.W is a class of variants restricted to plant species (Kawashima et al., 2015). Interestingly, H2A variants have many amino acid differences and particularly differ from each other at three regions, the L1 loop, the docking domain and the carboxyl terminal tail (Figure 9) (Jiang & Berger, 2017). Amino acids in these three regions play key roles in H2A interaction with other histones within both the same nucleosome and neighboring nucleosomes, and thus affect the stability and compaction of nucleosomes (Kawashima et al., 2015). As seen in Figure 8, canonical H2A and H2A.X variants share common sequences at the L1 loop and docking domain. However, they are divergent at their carboxyl terminal tails, such that canonical H2A ends with a cluster of acidic amino acids, whereas the H2A.X carboxyl terminus harbors a conserved SQEF motif. Both H2A.Z and H2A.W variants contain unique sequences at the L1 loop, the docking
domain and the carboxyl terminal tail. The distinct sequences in the docking domain of H2A.Z are critical for its specific deposition into and exchange from the nucleosome (Jiang & Berger, 2017).

**Figure 9.** Sequence alignment of Arabidopsis H2A variants. Amino acids in the L1 loop, docking domain and C-terminal regions are indicated by brackets. This image was taken from a previous publication (Jiang & Berger, 2017).

**H2A.X.** Genome stability requires the H2A.X variant. This variant is involved in repair of double-strand breaks that occur during replication, recombination, or DNA rearrangement. Similar to canonical H2A in the histone fold domain, H2A.X is distinguished by its carboxyl terminus, which harbors a conserved SQEF motif that is crucial for DNA repair function. This motif can become rapidly phosphorylated on serine, producing the form known as γH2A.X at the sites of DNA breaks when DNA damage occurs. Consequently, γH2A.X helps to recruit and/or maintain DNA repair proteins, histone modifying enzymes and chromatin remodeling complexes (Van Attikum & Gasser, 2009). Although the role of H2A.X has not been shown directly in DNA repair, its suppressing effect on oncogenic translocations and tumor formation was observed in
human (Celeste et al., 2003). Also, microcephalin (MCPH1), a histone variant-specific PTM reader, identifies phosphorylation at carboxyl terminal serine and tyrosine residues in H2A.X during DNA damage. This sensor of DNA damage serves as a mediator between chromatin and the repair machinery (Singh et al., 2012).

**H2A.W.** The plant-specific H2A variant H2A.W is intensely enriched at heterochromatin, TEs and regions enriched in H3K9me2, but is strongly depleted from gene bodies and euchromatic regions (Yelagandula et al., 2014) (Figure 8A-B). The carboxyl terminal KSPKK motif of H2A.W may facilitate the large-scale chromatin condensation of heterochromatin, however, loss of H2A.W does not cause a significant derepression of TEs due to CHG methylation, which plays a compensatory role in maintaining the repression of TEs. Therefore, H2A.W likely acts in conjunction with DNA methylation to silence the expression of transposable elements (Yelagandula et al., 2014).

**H2A.Z.** As one of the most conserved histone variants, H2A.Z constitutes around 15% of the total H2A cellular pool (Jarillo & Piñeiro, 2015). H2A.Z diverged from H2A early in eukaryotic evolution, but has remained extensively preserved across eukaryotes (Talbert & Henikoff, 2010). The crystal structure of a H2A.Z-nucleosome core particle showed similarity to that of a nucleosome structure containing canonical H2A (Figure 10). However, these distinct localized modifications result in slight destabilization of the interaction between the (H2A.Z-H2B) dimer and the (H3-H4)$_2$ tetramer. Moreover, H2A.Z-nucleosomes have a different surface that contains a metal ion (Mn$^{2+}$). This altered surface may cause modifications in higher order structure and could result in the interaction of specific nuclear proteins with H2A.Z (Suto et al., 2000).
Figure 10. Superposition of H2A (gray) and H2A.Z (yellow). The docking domain is boxed. This image was taken from a previous publication (Suto et al., 2000).

Also, a recent study of an H2A.Z/H2A nucleosome structure shows that the H2A.Z L1 loop domain was extremely different without any structural changes of the H2A L1 loop, thus avoiding steric clash. So, the heterotypic H2A.Z/H2A nucleosome is more stable than the homotypic H2A.Z nucleosome (H2A.Z/H2A.Z) (Horikoshi et al., 2016). Consequently, the presence of H2A versus H2A.Z in the histone octamer affects the stability of the nucleosome (Coleman-Derr & Zilberman, 2012a; Jin & Felsenfeld, 2007; Jin et al., 2009; Kumar & Wigge, 2010).

Since its identification, H2A.Z has been extensively studied in yeast, animals and plants. These studies have revealed many contradictory roles of H2A.Z in modulating chromatin dynamics and transcription regulation (Subramanian et al., 2015). The effects of PTMs on H2A.Z nucleosomes may be a reasonable explanation for some of these
contradictions (Ishibashi et al., 2009); the inhibition of H2A.Z nucleosomes in binding linker histone H1 (Thakar et al., 2009); and/or effects on chromatin remodeling complexes (Goldman et al., 2010). In yeast, it is clear that H2A.Z plays an important role in gene activation by modifying promoter chromatin properties (Guillemette & Gaudreau, 2006). In Drosophila, nucleosomes form context-specific barriers to transcription that can be tuned at least in part by incorporation of H2A.Z (Weber et al., 2014). In murine embryonic stem cells, H2A.Z is enriched at active enhancers and promoters and facilitates chromatin accessibility to allow binding of a variety of active and repressive complexes required for self-renewal and differentiation (Hu et al., 2013). Also, transcription-coupled H2A.Z changes may play a role in cancer initiation and progression (Conerly et al., 2010). In human breast cancer cells, H2A.Z acts as an important player for enhancer functions. H2A.Z organizes a chromatin environment required for RNA polymerase II recruitment and enhancer-promoter(s) interactions, all essential features of enhancer activity (Brunelle et al., 2015).

In Arabidopsis, H2A.Z predominantly associates with genes at euchromatic regions (Fig. 8A) (Coleman-Derr & Zilberman, 2012b; Sura et al., 2017; Zilberman et al., 2008). At highly expressed protein coding genes, H2A.Z is intensely enriched around the nucleosome-depleted region (NDR) at transcriptional start sites (TSSs), particularly at the +1 nucleosome (i.e. the first nucleosome downstream of the TSS) (Coleman-Derr & Zilberman, 2012b; Sura et al., 2017; Zhang et al., 2016; Zilberman et al., 2008) (Figure 8B). The +1 nucleosome can act as a barrier to transcriptional elongation by blocking and stalling RNA Polymerase II (Nock et al., 2012; Weber et al., 2014). H2A.Z incorporation moderates the nucleosomal barriers to RNA Polymerase II progression,
likely by facilitating the loss of an H2A.Z/H2B dimer from nucleosomes when encountered by RNA Polymerase II (Weber et al., 2014). Therefore, accumulation of H2A.Z around TSSs promotes transcriptional initiation. In Arabidopsis, H2A.Z is required for activation of Flowering Locus C (FLC), in that way ensuring the proper timing of the transition from vegetative growth to flowering (Deal et al., 2007). It is shown that the TSS of FLC locus is enriched with H2A.Z and its expression is strongly up-regulated by FRIGIDA (FRI). FRI acts as a scaffold protein in a transcription activator complex that binds to the FLC locus and directly associates with the SWR1 complex, suggesting that FLC up-regulation is mediated by direct modulation of H2A.Z deposition by FRI (Choi et al., 2011). Therefore, H2A.Z at the TSS may not directly promote transcription but rather configure the chromatin structure competent for transcription (Jiang & Berger, 2017). On the other hand, it is now clear that the presence of H2A.Z in the Arabidopsis genome contributes to the responsiveness of genes. Transcriptome analysis and genomic mapping of H2A.Z showed that H2A.Z enrichment across gene bodies, rather than at the TSS, is required for maintaining genes poised to respond, and promotes variability in levels and patterns of gene expression (Coleman-Derr & Zilberman, 2012b; Sura et al., 2017; Zilberman et al., 2008). In h2a.z mutants, as well as mutants of key components of the SWR1 complex--pie1 and sef--which mimic h2a.z knock-out mutants, systemic acquired resistance genes are constitutively expressed in the absence of pathogen challenge (March-Díaz et al., 2008). Similarly, mutation of ARP6 (ACTIN-RELATED PROTEIN 6, another key component of SWR1) resulted in several Pi starvation responses despite being grown under normal conditions (Smith et al., 2010). Also, nucleosomes containing H2A.Z
provide thermosensory information that is used to coordinate the ambient temperature transcriptome (Kumar & Wigge, 2010). These results suggested a negative regulation of H2A.Z on hypervariable responsive genes that are transcriptionally activated by biotic or abiotic signals. Overall, these findings were recently confirmed by a comprehensive study in Arabidopsis under osmotic-stress condition, which supports a model where H2A.Z in gene bodies has a strong repressive effect on transcription, whereas in +1 nucleosomes it is important for maintaining the activity of some genes (Sura et al., 2017).

The Role of Epigenetic Mechanisms in Regulating Pi Homeostasis in Plants

Several lines of evidence support a role for epigenetic mechanisms in gene regulation driven by Pi deficiency. Pi deficiency leads to developmental and biochemical flexibility in plants for adaptation, which is followed by a change in patterns of gene expression. Such changes at the molecular level are also affected by altered chromatin structure. In crop plants like wheat, transcriptomic analysis showed the up-regulation of several chromatin remodeling genes under Pi deficiency (Oono et al., 2013), which suggest active chromatin-level alterations. Also, in genome-wide studies, the effects of changes in cytosine DNA methylation patterns have been observed in Arabidopsis (Yong-Villalobos et al., 2015) and rice seedlings (Secco et al., 2015) under Pi deficiency. Pi starvation in Arabidopsis induces extensive remodeling of global DNA methylation and the loss of DNA methylation in specific contexts alters a number of morphological and physiological responses to Pi starvation, which suggest a role for dynamic DNA methylation changes in the modulation of a number of Pi starvation responses in Arabidopsis (Yong-Villalobos et al., 2015). Also, it has been shown that
differential methylation near Pi-responsive cis-acting sequences in the genome correlates with gene expression, suggesting that the methylation status of some regulatory elements could affect the binding capacity of the related transcription factors and hence control transcription (Yong-Villalobos et al., 2016). Interestingly, in rice seedlings, differentially methylated regions were abundant in TEs in close proximity of highly induced Pi stress responsive genes, which indicates that the epigenetic regulation of TEs can also affect the expression of nearby genes, adding complexity to their transcriptional control (Secco et al., 2015). The involvement of histone acetylation was also implicated in Pi homeostasis, through the study of the Arabidopsis histone deacetylase 19 (HD19) (C.-Y. Chen et al., 2015). Through characterization of the Arabidopsis HD19 mutant and over-expression plants, key roles of HD19 in controlling root cell elongation and involvement in regulating a subset of Pi starvation induced genes was observed, including some of the SPX genes involved in Pi sensing and signaling (C.-Y. Chen et al., 2015). The other case study that reveals the involvement of epigenetic mechanisms in Pi homeostasis is in the involvement of Arabidopsis ARP6 for proper H2A.Z deposition at numerous Pi starvation-induced genes. Mutation of ARP6 resulted in the loss of H2A.Z at many phosphate starvation induced genes and led to derepression of these genes under Pi replete conditions (Smith et al., 2010). Interestingly, the involvement of H2A.Z in regulating Pi homeostasis in plants has recently been supported through the study of the role of the Arabidopsis inositol pentakisphosphate 2-kinase coding gene (AtIPK1) that is involved in the biosynthesis of Inositol hexakisphosphate (IP6, the main source of P in the seed). The result of mutation of IPK1 demonstrated numerous phosphate starvation-induced genes being
induced, and correlated with a reduction of histone variant H2A.Z occupation in the chromatin at these loci (Kuo et al., 2014).

Indeed, these studies support an important role of epigenetic mechanisms in modulating responses to Pi deficiency and homeostasis. However, we are still far from understanding the underlying molecular mechanisms and significance of such modifications. Accordingly, thorough understanding of broad epigenetic mechanisms that regulate Pi homeostasis would further advance our understanding of this complex response and serve to provide new opportunities for developing crop plants with improved Pi uptake, Pi use-efficiency, and an enhanced ability to adapt to low Pi conditions. The goal of this study was to investigate the role of H2A.Z abundance in modulating responses to Pi deficiency in the staple crop rice by generating a map of H2A.Z deposition patterns and characterizing the impact of Knock-down of ARP6, as well as 24-hours Pi deficiency, on these patterns. To that end we can determine whether H2A.Z deposition in response to Pi deficiency correlates with differential gene expression.
Chapter 2. Determination of Genome-wide H2A.Z Histone Variant Deposition in Rice and Its Perturbation by Environmental Stress

Introduction

Studies in plants have revealed apparently contradictory roles of H2A.Z in modulating chromatin dynamics and transcriptional regulation in diverse responses and processes, including the thermo-sensory response (Kumar & Wigge, 2010), the regulation of metabolic gene clusters (Nützmann & Osbourn, 2015), tissue development (Zhang et al., 2016), flowering (Deal et al., 2007), immunity (March-Díaz et al., 2008), responses to the diurnal oscillation process (Zhang et al., 2017) and abiotic stress responses (Smith et al., 2010; Sura et al., 2017). In the first genome-wide examination of H2A.Z deposition in Arabidopsis, it was shown that the SWR1 complex deposited H2A.Z preferentially at 5’ ends of genes near the TSS (Zilberman et al., 2008). This study also showed that in contrast to DNA methylation, which was associated with repression of transcriptional initiation, H2A.Z facilitated transcriptional activity (Zilberman et al., 2008). Later, the same group reported a different role for H2A.Z in gene bodies (Coleman-Derr & Zilberman, 2012b), showing a correlation between H2A.Z enrichment across gene bodies and lower transcription levels (and higher gene responsiveness) in genes mainly associated with responses to environmental and developmental stimuli. Also, they proposed that the exclusion of H2A.Z from highly expressed genes is linked to the presence of genic DNA methylation (Coleman-Derr & Zilberman, 2012b). Recently, in another genome-wide examination of H2A.Z localization in Arabidopsis, a dual role for H2A.Z in transcriptional regulation was proposed, specifically for stress response genes (Sura et al., 2017). Their model suggested a repressive effect of H2A.Z on transcription in gene bodies but a positive effect on
maintaining the transcriptional activity of some genes in +1 nucleosomes (Sura et al., 2017). In a recent study in rice, H2A.Z deposition was also shown to co-localize with both active and inactive chromatin marks. H2A.Z deposition was associated with H3K4 trimethylation (active mark) at the 5’ end of expressed genes, whereas its peaks were partially associated with H3K27 trimethylation (inactive mark) (Zhang et al., 2016).

In this study, we used chromatin immuno-precipitation (ChIP) and RNA extraction techniques followed by high-throughput sequencing (ChIPSeq and RNASeq, respectively), to determine genome-wide H2A.Z deposition patterns in rice, a monocot model organism, and examined the relationships among these patterns, gene function and transcriptional activity. We also showed that DNA methylation patterns (using data from a previous study (Secco et al., 2015)) exhibited patterns opposite to the distinct H2A.Z patterns observed in protein-coding genes (PCGs). Furthermore, the effect of a 24-hour Pi deficiency treatment on H2A.Z deposition and gene expression were investigated, which revealed a correlation between changes in H2A.Z localization at genes differentially expressed by Pi deficiency.

Materials and Methods

Generating an OsH2A.Z antibody

The rice genome encodes 13 H2A histones that fall into four major phylogenetic classes. Of these 13 H2As, 3 exhibit strong similarity with Arabidopsis H2A.Z histone variants (HTA705, HTA712, HTA713) (Deal et al., 2007). Based on the amino acid identity among the H2A.Z sequences at the N terminus, the peptide sequence N-AGKGGKGLLLAAKTTAAK-C was synthesized as a fourfold multiple antigenic peptide. Polyclonal antibodies were raised that reacted with the relatively conserved N termini of the rice H2A.Z histone variant subclass proteins HTA705, HTA712 and HTA713, but not
with representatives of the other three H2A subclasses (Figure 11, Results section). The primary injection and three subsequent boosts were done with 250 mg of peptide on rabbits.

**HTA713 transgene construction and expression**

To make the HTA713 recombinant construct, a gBlock (Integrated DNA Technologies) gene fragment of HTA713 was designed with two 5’ restriction sites (XbaI and Ncol) and two 3’ sites (HindIII and BamHI) (ordered from Integrated DNA Technologies) to facilitate cloning into pBluescript II SK+ (pBS-SK), followed by transformation into the pET15b vector for protein expression.

**Cloning HTA713 into pBS-SK**

This is a four steps process:

1) Digest the pBS-SK plasmid as follows:

\[
\begin{align*}
3.4 \mu L & \quad \text{pBS-SK plasmid (1000 ng)} \\
2.0 \mu L & \quad \text{NEBuffer2 (10X)} \\
2.0 \mu L & \quad \text{BSA (10X)} \\
10.6 \mu L & \quad \text{dH}_2\text{O} \\
1.0 \mu L & \quad \text{XbaI} \\
1.0 \mu L & \quad \text{HindIII}
\end{align*}
\]

Reactions were incubated at 37 °C for 2 hours.

2) Dephosphorylate cut-plasmid (linearize the plasmid) as follows:

\[
\begin{align*}
20.0 \mu L & \quad \text{Cut-pBS-SK plasmid} \\
2.0 \mu L & \quad \text{Antarctic Phosphatase reaction buffer (10X)} \\
1.0 \mu L & \quad \text{Antarctic Phosphatase (5 units)}
\end{align*}
\]

Reactions were incubated at 37 °C for 15 minutes following heat inactivation at 65 °C for 5 minutes.
3) Cut gBlock HTA713 as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Contents</th>
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</thead>
<tbody>
<tr>
<td>10.0 μL</td>
<td>HTA713-TE buffer</td>
</tr>
<tr>
<td>2.0 μL</td>
<td>NEBuffer2 (10X)</td>
</tr>
<tr>
<td>2.0 μL</td>
<td>BSA (10X)</td>
</tr>
<tr>
<td>4.0 μL</td>
<td>dH₂O</td>
</tr>
<tr>
<td>1.0 μL</td>
<td>XbaI</td>
</tr>
<tr>
<td>1.0 μL</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

Reactions were incubated at 37 °C for 2 hours following heat inactivation at 65 °C for 15 minutes.

4) Ligation as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 μL</td>
<td>pBS-SK plasmid (50 ng)</td>
</tr>
<tr>
<td>4.0 μL</td>
<td>cut HTA713 (20 ng)</td>
</tr>
<tr>
<td>7.0 μL</td>
<td>dH₂O</td>
</tr>
<tr>
<td>1.5 μL</td>
<td>T₄ ligase buffer (10X)</td>
</tr>
<tr>
<td>1.0 μL</td>
<td>T₄ DNA ligase</td>
</tr>
</tbody>
</table>

Reactions were incubated at 16 °C overnight. 2 μL of the product were used for competent cell transformation.

**Transformation of electro-competent E. coli (DH5α)**

The Eppendorf Electroporator-2510 was used for E. coli DH5α plasmid transformation at 1800 volts. 2 μL of pBS-SK plasmid containing HTA713 gene was used per transformation reaction in 25 μL of electro-competent E. coli (DH5α). Also, an aliquot of 500 μL autoclaved SOC media (20% (w/v) tryptone, 5% (w/v) yeast extract, 2% 5 M NaCl, 2.5% 1 M KCl, 10% 1 M MgCl₂, 10% 1 M MgSO₄, 20% 1 M glucose) was prepared per transformation. The mixture of electro-competent E. coli (DH5α) and pBS-SK plasmid containing HTA713 gene was transferred into a pre-chilled cuvette. The cuvette was placed into the electroporator and electric charge was activated. Immediately following the electric pulse, the SOC media was pipetted into the mixture.
and transferred to 1.5 mL tubes. The mixture was incubated at 37 °C for 1 hour on 150 rpm shaker. 200 µL of the mixture were plate on to LB (5% (w/v) yeast extract, 10% (w/v) tryptone, 10% (w/v) NaCl) plates containing Ampicillin and incubated overnight at 37 °C. Individual colonies were selected from the plates for overnight liquid cultures in preparation for plasmid recovery via miniprep kit.

**pBS-SK-HTA713 plasmid recovery via miniprep kit**

The Qiagen miniprep kit with prepared buffers was used to collect and purify pBS-SK-HTA713 plasmid for downstream sequencing and transferring. The procedure followed the manufacturer’s protocol. Following overnight growth of clones in LB media containing Ampicillin, 5 mL of cell culture was harvested by centrifuging 1 mL aliquots into a 1.5 mL tube at 13,000 g for 30 seconds and discard supernatant (repeat as necessary). The pellet was re-suspended in P1 buffer (Tris, EDTA, glucose and RNaseA) to prevent bursting of cells, prevent DNase damage to the plasmid by chelation of divalent ions, and degrade cellular RNA. Once the pellet is suspended, lysis buffer P2 (NaOH and detergents) was added and inverted gently to solubilize the cell membrane without disrupting DNA bases. N3, a neutralization buffer, was then added and inverted to decrease the alkalinity of the mixture and re-established hydrogen bond in double-stranded DNA. The mixture was centrifuged at 13,000 g for 10 minutes following collected into QIA prep spin columns. After a 30 second centrifuge to bind DNA to the membrane of the column, buffer PE was added to the column and repeat a 30 second spin following a 1-minute spin dry. The spin column was then placed into a 1.5 mL tube and 30 µL of EB was pipetted on the center of the membrane. After at least 1 minute the eluted material was collected. Purified pBS-SK-HTA713 plasmid DNA was quantified and stored at -20 °C.
DNA electrophoresis

An agarose gel was prepared to identify appropriate DNA cuts and clones. A 1% TAE gel was prepared by combining 0.5 g of agarose with 50 mL of TAE buffer (4.84 g Tris Base, 2 mL 0.5 M EDTA, pH 8 and 1.14 mL glacial acetic acid and dH₂O to 1 L) following melting of the agarose and adding 5 µL of ethidium bromide (EtBr) to the gel. After a short cooling, the molten gel was poured into a plate and allowed to solidify with a toothcomb. Gel was placed in TAE buffer and DNAs were loaded using loading dye with a DNA size standard. Gel was run in electrophoresis system at 125 V for 20-25 minutes.

pBS-SK-HTA713 plasmid DNA quantification and sequencing

A spectrophotometer (Shimadzu Biotech BioSpec-mini) was used to measure 1:100 dilution of DNA in quartz cuvettes. pBS-SK-HTA713 plasmid was then sequenced prior to pET15b cloning. The GeneLab facility at LSU School of Veterinary Medicine performed the sequencing analysis using standard sequencing reactions (40 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes) on a PTC 200 Thermalcycler with M13F (-20) primer [GTAAACGACGGCCAG]. 10 µl of purified pBS-SK-HTA713 plasmid was sent in concentration greater than 20 ng/µL. The sequencing result was aligned with template sequence using MultAlin.

PCR amplification of HTA713 for cloning into pET15b

NocI and BamHI containing primers (ordered from Life Technologies) were used as sense [TACGTCCCATGGCGGGAAAGGGAGGTAGGGCTTG] and anti-sense [TAGCTGGGATCCCTCAGTGATGGTGATGGTGATGCTCTTTGGAGGACTTGGTTGATCAGG] primers, respectively, to amplify HTA713 for cloning into pET15b. Primers were prepared to a concentration of 5 µM from a 100 µM stock with dH₂O. PCR reaction was
prepared in standard PCR tube with PFU (BioVision) polymerase. The 25 µL volume was prepared as follows:

1.0 µL pBS-SK-HTA713 plasmid
2.0 µL dNTP
2.5 µL 5 µM sense primer
2.5 µL 5 µM anti-sense primer
2.5 µL PFU (X10) PCR buffer
0.3 µL PFU DNA polymerase
14.2 µL dH₂O

The thermocycler reaction was set as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
<th>Step Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C</td>
<td>2 minutes</td>
<td>initial denaturation</td>
</tr>
<tr>
<td>94 °C</td>
<td>30 seconds</td>
<td>denaturation</td>
</tr>
<tr>
<td>55 °C</td>
<td>1 minute</td>
<td>primer annealing</td>
</tr>
<tr>
<td>72 °C</td>
<td>2 minutes</td>
<td>extension</td>
</tr>
<tr>
<td>72 °C</td>
<td>15 minutes</td>
<td>final extension</td>
</tr>
</tbody>
</table>

(Bold steps cycled 40 times total)

The PCR product (HTA713) was then used to repeat the steps: ligation to pET15b plasmid, transformation of electro-competent E. coli (DH5α), pET15b-HTA713 plasmid recovery via miniprep kit, DNA Electrophoresis, pET15b-HTA713 plasmid DNA quantification and sequencing (for pET15b sequencing, the T7-19 primer [GTAATACGACTCACTATAG] was used). The pET15b-HTA713 plasmid was then used to transform E. coli (BL21), a strain that is generally preferred for recombinant protein expression.

**Transformation of E. coli competent cell (BL21)**

100 µl BL21 competent cells were mixed with β-mercaptoethanol (β-ME) for a final concentration of 25 mM β-ME following incubation on ice for 10 minutes. pET15b-HTA713 plasmid (50 ng) was then added to the reaction and incubated on ice for 30 minutes. The transformation mixture was heat-pulsed in 42 °C water bath for 45
seconds following incubation on ice for 2 minutes. 900 µL preheated (42 °C) SOC medium was added to the transformation reaction and incubated at 37 °C for 1 hour with shaking at 225 rpm. 200 µL of the mixture were plate on to LB (5% (w/v) yeast extract, 10% (w/v) tryptone, 10% (w/v) NaCl) plates containing Kanamycin and incubated overnight at 37 °C. Individual colonies were selected from the plates for overnight liquid cultures in preparation for IPTG induction.

**Induction of target protein (HTA713) using IPTG**

Following overnight growth of clones in LB media containing Kanamycin, 50 µL of cell culture was pipetted into 1mL fresh LB broth following incubation with shaking at 225 rpm at 37 °C for 1.5 hour. 100 µL of the culture was stored at -20 °C to serve as the non-induced control sample. 100 mM IPTG was added to the rest the culture and incubated with shaking at 225 rpm at 37 °C for 4 hours. 100 µL of the culture was then subcultured and stored at -20 °C. The rest of the culture was incubated with shaking at 225 rpm at 37 °C overnight. The non-induced, 4 hours- and overnight-induced cultures were centrifuged for 1 minute at 13,000 g and the supernatant was removed. 50 µL dH₂O was added to each samples and stored at -20 °C to serve as recombinantly expressed HTA713 samples.

**Crude protein extraction of E- coli for SDS-PAGE**

50 µL of sample buffer containing β-ME was added to 50 µL of each sample (The non-induced, 4 hours- and overnight-induced cells). The mixture was heated at 95 °C for 5 minutes following a 10-minute spin to remove cellular debris.
Crude protein extraction of rice and *Arabidopsis* for SDS-PAGE

100 mg of frozen seedlings was ground to a fine powder in liquid nitrogen and sample buffer containing β-ME was added. The mixture was heated at 95 °C for 5 minutes following a 10-minutes spin to remove cellular debris.

SDS-PAGE gel electrophoresis

To separate the proteins and get a separate band for H2A.Z, SDS-PAGE gel electrophoresis was used. 25 µL of each sample and 10 µL of marker were loaded onto Mini-Protein Precast gel (Bio-Rad) and set the power for 120 V for 1 hour. Once the run ended, staining or transferring to a polyvinylidene difluoride (PVDF) membranes (Bio-Rad) were performed on gel. In the staining process, the gel was placed into staining solution (100 mL methanol, 0.1 g R-250 Coomassie Brilliant Blue, 20 mL acetic acid and 80 mL dH2O) for 8 hours followed by washing in de-staining solution (50 mL methanol, 875 mL dH2O and 75 mL acetic acid).

Transferring to PVDF membrane and immuno-blotting

To test the specificity of OsH2A.Z specific antibody, after transferring to a PVDF membrane, the OsH2A.Z antibody serum was used for immune-blotting. The PVDF membrane was soaked in 100% MeOH for about 1 minute following one 5-min wash in dH2O and one 5-min wash in semi-dry transferring buffer (5.81 g Tris Base, 2.93 g Glycine, 4 mL 10%SDS, 200 mL methanol and dH2O to 1 L). Also, the filter paper, fiber pads and gel were soaked and equilibrated in semi-dry transferring buffer for 20 minutes. The gel sandwich was assembled as follows:
The tank was filled to the “Blotting” mark with transfer buffer (6.06 g Tris Base, 28.8 g Glycine and dH$_2$O to 2 L). A stir bar and frozen cooling unit was placed in tank to maintain buffer temperature and ion distribution. The blot was run at 100 V, Constant 350 mA for 1 hour. Upon completion of the run, membrane was blocked in Milk/TBS (5 g dry milk, 4.84 g Tris Base, 58.48 g NaCl and dH$_2$O to 2 L) for 2 hours with shaking at room temperature followed by washing with TTBS (0.5 mL Tween20, 2.42 g Tris Base, 29.24 g NaCl and dH$_2$O to 1 L). The blot was incubated with primary antibody (the OsH2A.Z antibody serum or pre-immune serum) diluted 1:1000 in BTBS (10 g BSA, 2.42 g Tris Base, 29.24 g NaCl and dH$_2$O to 1 L) at room temperature for an overnight. The blot was then washed in TTBS and incubated in secondary antibody (HSP-conjugate goat anti-rabbit) diluted 1:5,000 in TBSB for 4 hours. The blot was then washed in TTBS and incubated for 1 minute in the mixture of SuperSignal West Pico Chemiluminescence Substrate (34077; ThermoFisher Scientific). The signals were then detected by molecular imager ChemiDoc XRS+ (Bio-Rad).

**Plant material and growth conditions**

Rice cultivar Nipponbare (*Oryza sativa* ssp. japonica) seeds were sterilized and pregerminated for 1 day in 37 °C and 2 days in 28 °C. Then seeds were germinated under 12-h light/12-h dark, 30 °C/ 22 °C. Two-week-old seedlings were grown in
Yoshida Rice culture modified solution in a hydroponic system as described, containing 0.513 mM K$_2$SO$_4$, 1.425 mM NH$_4$NO$_3$, 1.643 mM MgSO$_4$, 0.998 mM CaCl$_2$, 0.075 µM (NH$_4$)$_6$Mo$_7$O$_{24}$, 0.25 mM NaSiO$_3$, 0.009 mM MnCl$_2$, 0.019 mM H$_3$BO$_3$, 0.125 mM EDTA-Fe, 0.155 µM CuSO$_4$, 0.323 mM NaH$_2$PO$_4$ and 0.152 µM ZnSO$_4$ (Secco et al., 2013; Yoshida et al., 1971). The solution was renewed every 7 days. After 22 days, shoot tissue of control samples were harvested separately and frozen in liquid nitrogen. The procedure was repeated 3 times to produce 3 replicates.

**Chromatin immuno-precipitation**

The protocol for this procedure was adapted from a previous study (Smith et al., 2010). 1 g of frozen tissue was ground with a mortar and pestle in liquid nitrogen. Before the powder thawed, 25 mL Nuclei Isolation Buffer (NIB) was added and transferred the slurry into 50 mL conical tube to cross-link at room temperature for 10 minutes. The Nuclei Isolation Buffer (NIB) was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM HEPES</td>
<td>250 µL</td>
<td>1 M Heps pH 7.6</td>
</tr>
<tr>
<td>1 M sucrose</td>
<td>21.37 mL</td>
<td>1.17 M Sucrose</td>
</tr>
<tr>
<td>5 mM KCl</td>
<td>125 µL</td>
<td>1 M KCl</td>
</tr>
<tr>
<td>5 mM MgCl$_2$</td>
<td>125 µL</td>
<td>1 M MgCl$_2$</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>250 µL</td>
<td>0.5 M EDTA pH 8.0</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>1.40 mL</td>
<td></td>
</tr>
</tbody>
</table>

To the 25ml NIB the following was added right before starting the assay:
- 1% Formaldehyde; 700 uL of 37% formaldehyde
- 14 mM β-ME (commercial β-ME is about 14M; 25 uL in 25 mL)
- 0.6% Triton X-100 (750 µL filter-sterilized 20% Triton X-100 in 25mL)
- 1/2 of a large protease inhibitor tablet (for 25 mL of buffer)

To terminate cross-linking, 1.7 mL of filter-sterilized 2M glycine was added and incubated at room temperature for 5 minutes following filter lysate through 1 layer of Miracloth into a clean centrifuge tube on ice. The nuclei were then pelleted at 3,000 g at
4 °C for 20 minutes. Next, the pellet was re-suspended in 300 uL cold Nuclear Isolation Buffer (without Triton X-100, β-ME, Formaldehyde and the large tablet). The nuclear suspension was then transferred into a 15% Percoll solution and centrifuged at 3,000 g at 4 °C for 5 minutes to separate the nuclei from carried over chloroplasts. The 15% Percoll solution was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Unit</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percoll</td>
<td>3.75 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM HEPES</td>
<td>250 μL</td>
<td></td>
<td>1 M HEPES, pH 7.6</td>
</tr>
<tr>
<td>1 M sucrose</td>
<td>12.5 mL</td>
<td></td>
<td>2 M Sucrose</td>
</tr>
<tr>
<td>5 mM KCl</td>
<td>125 μL</td>
<td></td>
<td>1 M KCl</td>
</tr>
<tr>
<td>5 mM MgCl₂</td>
<td>125 μL</td>
<td></td>
<td>1 M MgCl₂</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>250 μL</td>
<td></td>
<td>0.5 M EDTA, pH 8.0</td>
</tr>
<tr>
<td>dH₂O</td>
<td>8 mL</td>
<td></td>
<td>dH₂O</td>
</tr>
</tbody>
</table>

To re-suspend the pellet, 600 uL of cold Nuclear Lysis Buffer was added and vortexed vigorously. The Nuclear Lysis Buffer was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Unit</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl</td>
<td>500 μL</td>
<td></td>
<td>0.5 M Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td>1% SDS</td>
<td>250 μL</td>
<td></td>
<td>20% SDS</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>100 μL</td>
<td></td>
<td>0.5 M EDTA, pH 8.0</td>
</tr>
<tr>
<td>dH₂O</td>
<td>4.150 mL</td>
<td></td>
<td>dH₂O</td>
</tr>
<tr>
<td>1/2 of a mini protease inhibitor tablet</td>
<td>(for 5 mL of buffer)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The lysate was then sonicated with a Branson Digital Sonifier 250 at power 5 in 11 cycles of on/off within 10 seconds follow spinning at top speed at 4 °C for 10 minutes to remove debris. Next, 10 μL of sonicated chromatin was stored at -80 °C to serve as the ‘input DNA control’. The rest of sonicated chromatin solution was aliquoted in 3 tubes and 1000 μL ChIP Dilution Buffer was added to each of them. It is essential to dilute the nuclear lysate at least 10 times to inactivate the SDS. If the SDS
concentration is greater than 0.1%, it is likely that the antibody will become denatured.

The ChIP Dilution Buffer was prepared as follows:

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1% Triton X-100</td>
<td>1.1</td>
<td>mL</td>
</tr>
<tr>
<td>1.2 mM EDTA</td>
<td>48</td>
<td>µL</td>
</tr>
<tr>
<td>16.7 mM Tris-HCl</td>
<td>334</td>
<td>µL</td>
</tr>
<tr>
<td>167 mM NaCl</td>
<td>668</td>
<td>µL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>17.85</td>
<td>mL</td>
</tr>
</tbody>
</table>

To reduce background caused by non-specific absorption of irrelevant proteins, 50 µL Protein A-Agarose beads was added and incubated at 4 °C with gentle rotation for 2 hours. Agarose beads were then removed by centrifugation at 12,000 g at 4 °C for 30 seconds. Next, 5 µL of the OsH2A.Z antibody serum was added to one of the tubes, 5 µL of the pre-immune serum to the second tube and 5 µL IgG to the third tube and the mixture was incubated at 4 °C with gentle rotation overnight. The following day, 50 µL Protein A-Agarose beads were added to each tubes and incubated at 4 °C with gentle rotation for 2 hours. For the washing steps, the Protein A-Agarose-antibody-antigen complexes were collected by centrifugation at 3,800 g at 4 °C for 30 seconds. Supernatants were carefully removed and pellets were re-suspend in 1ml Wash Buffer 1 following a 30-second spin beads at 3,800g at 4 °C. Again, supernatants were carefully removed and pellets were re-suspend in 1ml Wash Buffer 1 and agitate at 4 °C for 5 minutes. The Wash Buffer 1 (low salt) was prepared as follows:

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Triton X-100</td>
<td>1</td>
<td>mL</td>
</tr>
<tr>
<td>0.1% SDS</td>
<td>100</td>
<td>µL</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>80</td>
<td>µL</td>
</tr>
<tr>
<td>20 mM Tris-HCl</td>
<td>800</td>
<td>µL</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>600</td>
<td>µL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>17.420</td>
<td>mL</td>
</tr>
</tbody>
</table>

For the second wash:

1. Incubate with gentle rotation at 4 °C overnight.
2. Centrifuge at 3,800 g at 4 °C for 30 seconds.
3. Remove supernatants carefully.
4. Re-suspend in 1 ml Wash Buffer 1 and agitate for 5 minutes.

The Wash Buffer 1 (high salt) was prepared as follows:

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Triton X-100</td>
<td>1</td>
<td>mL</td>
</tr>
<tr>
<td>0.1% SDS</td>
<td>100</td>
<td>µL</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>80</td>
<td>µL</td>
</tr>
<tr>
<td>20 mM Tris-HCl</td>
<td>800</td>
<td>µL</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>600</td>
<td>µL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>17.420</td>
<td>mL</td>
</tr>
</tbody>
</table>

For the third wash:

1. Incubate with gentle rotation at 4 °C overnight.
2. Centrifuge at 3,800 g at 4 °C for 30 seconds.
3. Remove supernatants carefully.
4. Re-suspend in 1 ml Wash Buffer 2 and agitate for 5 minutes.
The Protein A-Agarose-antibody-antigen complexes were collected by centrifugation at 3,800 g at 4 °C for 30 seconds. Supernatants were carefully removed and pellets were re-suspend in 1 mL Wash Buffer 2 following a 30-second spin beads at 3,800 g at 4 °C. Again, supernatants were carefully removed and pellets were re-suspend in 1ml Wash Buffer 2 and agitate at 4 °C for 5 minutes. The Wash Buffer 2 (high salt) was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Units</th>
<th>Component</th>
<th>Volume</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Triton X-100</td>
<td>1 mL</td>
<td>µL</td>
<td>20% Triton X-100</td>
<td>1 µL</td>
<td>mL</td>
</tr>
<tr>
<td>0.1% SDS</td>
<td>100 µL</td>
<td>µL</td>
<td>20% SDS</td>
<td>100 µL</td>
<td>µL</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>80 µL</td>
<td>µL</td>
<td>0.5 M EDTA, pH 8</td>
<td>80 µL</td>
<td>µL</td>
</tr>
<tr>
<td>20 mM Tris-HCl</td>
<td>800 µL</td>
<td>µL</td>
<td>0.5 M Tris-HCl, pH 8</td>
<td>800 µL</td>
<td>µL</td>
</tr>
<tr>
<td>500 mM NaCl</td>
<td>2 mL</td>
<td>µL</td>
<td>5 M NaCl</td>
<td>2 mL</td>
<td>µL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>16.02 mL</td>
<td></td>
<td>dH₂O</td>
<td>16.02 mL</td>
<td></td>
</tr>
</tbody>
</table>

The Protein A-Agarose-antibody-antigen complexes were collected by centrifugation at 3,800 g at 4 °C for 30 seconds. Supernatants were carefully removed and pellets were re-suspend in 1ml Wash Buffer 3 following a 30-second spin beads at 3,800 g at 4 °C. Again, supernatants were carefully removed and pellets were re-suspend in 1ml Wash Buffer 3 and agitate at 4 °C for 5 minutes. The Wash Buffer 3 (LiCl) was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Units</th>
<th>Component</th>
<th>Volume</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Sodium Deoxycholate</td>
<td>4 mL</td>
<td>µL</td>
<td>5% Sodium Deoxycholate</td>
<td>5 mL</td>
<td>µL</td>
</tr>
<tr>
<td>1% IGEPAL CA-630</td>
<td>200 µL</td>
<td>µL</td>
<td>IGEPAL CA-630</td>
<td>200 µL</td>
<td>µL</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>40 µL</td>
<td>µL</td>
<td>0.5 M EDTA, pH 8</td>
<td>40 µL</td>
<td>µL</td>
</tr>
<tr>
<td>10 mM Tris-HCl</td>
<td>400 µL</td>
<td>µL</td>
<td>0.5 M Tris-HCl, pH 8</td>
<td>400 µL</td>
<td>µL</td>
</tr>
<tr>
<td>250 mM LiCl</td>
<td>5 mL</td>
<td>µL</td>
<td>1 M LiCl</td>
<td>5 mL</td>
<td>µL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>10.36 mL</td>
<td></td>
<td>dH₂O</td>
<td>10.36 mL</td>
<td></td>
</tr>
</tbody>
</table>

The Protein A-Agarose-antibody-antigen complexes were collected by centrifugation at 3,800 g at 4 °C for 30 seconds. Supernatants were carefully removed
and pellets were re-suspend in 1 mL Wash Buffer 3 following a 30-second spin beads at 3,800 g at 4 °C. Again, supernatants were carefully removed and pellets were re-suspend in 1ml Wash Buffer 4 and agitated at 4 °C for 5 minutes. The Wash Buffer 4 (TE) was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Unit</th>
<th>Concentration/Ph</th>
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<th>Unit</th>
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<tr>
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<td>40 μL</td>
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<td></td>
<td></td>
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<tr>
<td>10 mM Tris-HCl</td>
<td>400 μL</td>
<td></td>
<td>0.5 M Tris-HCl, pH 8</td>
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<tr>
<td>dH₂O</td>
<td>19.356 mL</td>
<td></td>
<td>dH₂O</td>
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</tbody>
</table>

The Protein A-Agarose-antibody-antigen complexes were collected by centrifugation at 3,800 g at 4 °C for 30 seconds. Supernatants were carefully removed and pellets were re-suspended in 250 μL Elution Buffer to elute the immuno-complexes from the beads. After a brief vortex, the samples were incubated at 4 °C with gentle agitation for 15 minutes following a spin at 3,800 g at room temperature for 2 minutes. Supernatants were then transfered to new tubes. The elution steps were repeated by adding another 250 μL Elution Buffer to the beads and the supernatant were added to the previous ones. Also, at this step, 490 μL Elution Buffer was added to 10 μL 'input DNA control' to make the input DNA control. The Elution Buffer (EB) was prepared as follows:

<table>
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<tr>
<th>Component</th>
<th>Volume</th>
<th>Unit</th>
<th>Concentration/Ph</th>
<th>Volume</th>
<th>Unit</th>
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</thead>
<tbody>
<tr>
<td>1% SDS</td>
<td>1 mL</td>
<td>20% SDS</td>
<td>0.1M NaHCO</td>
<td>2 mL</td>
<td>1 M NaHCO3</td>
</tr>
<tr>
<td>dH₂O</td>
<td>17 mL</td>
<td>dH₂O</td>
<td>1 M NaHCO3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20 μL 5 M NaCl was then added to the samples and incubate at 65 °C for 4 hours to reverse crosslinking. Once crosslinking was reversed, Proteinase K solution
(10 µL 0.5 M EDTA pH 8, 20 µL 1 M Tris-HCl pH 6.5 and 20 µg Proteinase K) was added to each sample and incubate at 50 °C for 2 hours to digest the proteins.

To precipitate the DNA, 520 µL phenol:chloroform solution was added to each sample and incubated on a shaker at room temperature for 5 minutes, then centrifuged at 12,000 g at 4 °C for 15 minutes. 400 µL of the aqueous layer (top layer) was then transferred to a new tube and 50 µL 3 M NaOAc pH 5.2, 2 µL glycogen, and 1 mL cold 100% ethanol were added. The mixture was incubated at -20 °C overnight. The following day, the mixture of each tube was spun at 12,000 g at 4 °C for 20 minutes and the supernatants were removed. The pellets were then re-suspended in 75% ethanol and spun at 12,000 g at 4 °C for 10 minutes then the supernatants were removed to get rid of salts and moisture. After making sure the pellets were dry, the ChIPed-DNAs were dissolved in 30 µL EB containing 60 µg/mL RNaseA and incubated at 37 °C for 30 minutes. The ChIPed DNA was cleaned using DNA clean and Concentrator kit (Zymo Research) and then stored at -80 °C.

**Total RNA extraction**

RNA was extracted and cleaned up from 0.1 g of frozen shoot tissue using RNeasy Plant Mini kit (Qiagen). Tissue was ground with a mortar and pestle in liquid nitrogen. Before the powder thawed, 450 µL Buffer RTL containing βME was added and homogenized. The lysate was pipetted onto a QIAshredder spin column and centrifuged at 17,000 g for 5 minutes. 200 µL 100%ethanol was then added to the supernatant and mixed gently by pipetting. Sample was then transferred to RNeasy mini column and centrifuged at 17,000 g for 15 seconds. 700 µL Buffer RWI was then added to column and centrifuged at 17,000 g for 15 seconds. In a new tube, 700 µL Buffer RPE was
pipetted to the column and centrifuged at 17,000 g for 15 seconds. Another 300 µL of Buffer RPE was pipetted to the column and centrifuged at 17,000 g for 2 minutes to dry. In a new tube, 30 µL RNase-free water was then pipetted directly onto the RNeasy silica-gel membrane and centrifuged at 17,000 g for 1 minute. For RNA cleanup, the sample was adjusted to a volume of 100 µL with RNase-free water and 350 µL Buffer RLT was then added and mixed well. 250 µL 100% ethanol was added to the diluted RNA and transferred the sample to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8,000 g for 15 seconds. At this step, On-Column DNase Digestion was performed with the RNase-Free DNase Set. 350 µL Buffer RW1 was added to the RNeasy spin column and centrifuged at 8,000 g for 15 seconds. Once the membrane was washed, the DNase I incubation mix was pipetted directly to the RNeasy spin column membrane, and placed on the benchtop at room temperature for 15 minutes. 350 µL washing Buffer RW1 was then added to the RNeasy spin column and centrifuged at 8,000 g for 15 seconds. After removed flow-through completely, 500 µL washing Buffer RPE was added to the RNeasy spin column and centrifuged at 8,000 g for 15 seconds following additional 500 µL washing Buffer RPE and centrifuged at 8,000 g for 2 minutes to wash the spin column membrane. The RNeasy spin column was then placed in a new 1.5 ml collection tube and 30 µL RNase-free water was directly added to the spin column membrane and centrifuged at 8,000 g for 1 minute to elute the RNA.

**ChIPed DNA and total RNA quantification for sequencing**

A Qubit 2.0 Fluorometer (Invitrogen) was used for the quantitation of ChIPed DNA and RNA, using the highly sensitive and accurate fluorescence-based Qubit™ quantitation assays. 199 µL Qubit Buffer was mixed with 1 µL Qubit reagent to serve as
working solution for each reaction. Also, two standards were prepared using 10 µL #1 (0 ng/ µL) and #2 (10 ng/ µL ) standard solutions mixed with 190 µL working solution to calibrate the Qubit. For each sample, 1 µL DNA/RNA was added to 199 µL working solution in thin-walled tube and was vortexed vigorously. After 2 minutes standing at room temperature, the reading was taken. The minimum amount of ChIPed DNA and RNA needed to be 5 ng and 100 ng, respectively, to be sent for sequencing.

**ChIPSeq and RNASEq library generation**

The ChIPSeq and RNASEq library generation and sequencing were performed at the biotechnology center at the University of Illinois-Urbana Champaign.

The ChIPSeq libraries were prepared with the Hyper Library Construction Kit from Kapa Biosystems with two modifications: adaptors were diluted 1:20 and adaptored DNA were amplified for 10 cycles. The libraries were quantitated by qPCR and sequenced for 101 cycles from one end of the fragments on a HiSeq2500 using a HiSeq SBS sequencing kit version 4. Fastq files were generated and demultiplexed with the bcl2fastq v2.17.1.14 Conversion Software (Illumina).

The RNASeq libraries were prepared with Illumina's 'TruSeq Stranded mRNAsseq Sample Prep kit' (Illumina). The libraries were quantitated by qPCR and sequenced for 101 cycles from one end of the fragments on a HiSeq2500 using a HiSeq SBS sequencing kit version 4. Fastq files were generated and demultiplexed with the bcl2fastq v2.17.1.14 Conversion Software (Illumina).

**ChIPSeq data analysis**

Sequenced reads were quality-checked using FastQC software (Andrews, 2010). Bowtie was used to uniquely align the reads to the reference genome (MSU Rice Genome Annotation Release 7.1) (Kawahara et al., 2013) with up to two mismatches.
allowed (Langmead, 2010). Regions of H2A.Z enrichment were defined using the SICER software package (Zang et al., 2009), with the input genomic DNA as a background control and pre-immune serum ChIPed DNA as negative control (parameters: \( W = 200; \ G = 200; \ FDR <1E^{-2} \)). Differential enrichment of H2A.Z was determined using SICER-df.sh shell script (parameters: \( W = 200; \ G = 200; \ FDR <1.00E^{-02} \)). The number of reads and peaks obtained for each sample are listed in (Table 1). After the positions of the peaks were determined, genes (including the 250 bp upstream and 250 bp downstream) overlapping the peaks were considered to have the H2A.Z enrichment using Perl parser scripts (Dassanayake et al., 2009). Also, ngs.plot was used to visualize the genome-wide enrichment pattern of H2A.Z through gene body regions using a reference genome (MSU Rice Genome Annotation Release 7.1) (Shen et al., 2014).

**RNASeq data analysis**

RNASeq reads for each sample were mapped to the reference genome (MSU Rice Genome Annotation Release 7.1) (Kawahara et al., 2013) using the Bowtie2 tool (Langmead & Salzberg, 2012). To quantify transcript abundances, the Cuffdiff tool was applied to obtain fragments per kilobase of transcript per million mapped reads (FPKM) values (Trapnell et al., 2012).

For pair-wise comparison, RNASeq reads for each sample were mapped to the reference genome (MSU Rice Genome Annotation Release 7.1) using the Bowtie2 tool(Langmead & Salzberg, 2012). Using an in-house script, the reads for each gene were counted and differential expression was analyzed in the DESeq2 tool to get the pair-wise comparison between WT in control and 24-hours Pi deficiency (Love et al., 2014).
To evaluate the significance of overlap between DEGs and genes contained differential H2A.Z enrichment, an in-house script in FORTRAN was used to pick the same numbers of randomly selected genes as DEGs and examine the overlap to the genes contained differentially H2A.Z enrichment (1000 iterations).

Results

**H2A.Z is enriched at protein-coding genes and is linked to gene expression**

To examine the distribution of H2A.Z across the rice genome, ChIP experiments followed by high-throughput sequencing were performed on aerial tissues of 36-day-old WT seedlings using an OsH2A.Z-specific polyclonal antibody (Figure 11). Unique normalized reads were mapped to the MSU rice genome annotation (Osa1) release 7 (Kawahara et al., 2013). Two biological replicates were examined to ensure reproducibility (r= 0.89, Pearson correlation coefficient, Figure 12). Sicer was used to normalize H2A.Z ChIP sequences to input samples to identify peaks of H2A.Z (Table1).
Figure 11. Determination of OsH2A.Z Antibody Specificity. (a) N-terminal 50 amino acids alignment of the H2A family using online Multalin tool. The red shows the similarity of the position in all aligned sequences, the blue color shows the most frequent amino acid in a certain position in all aligned sequences and the black shows the least frequent amino acid in a certain position in all aligned sequences. The sequence in the yellow box was selected for peptide synthesis. (b) Immunoblot using the OsH2A.Z antibody to test its efficiency to pull down H2A.Z. Lane 1 and 2: Protein extracts from E. coli expressing recombinant HTA713 were made from cultures 4 hours after IPTG induction (Lane 1), and without induction (Lane 2). Lane 3 and 5: Immunoprecipitated proteins, preimmune serum used as a negative control in immunoblot assay (Lane 3) and OsH2A.Z crude serum (Lane 5). Lane 4 and 6: Protein extracts from rice. Preimmune serum used as a negative control in immunoblot assay (Lane 4) and OsH2A.Z crude serum (Lane 6). The expected size for OsH2A.Z is 15 KDa and expected size for IgG presented in the serum is 50 KDa. Lane 7, size standard.
Figure 12. Number of enrichment peaks in each replicate and the overlap between two replicates in (a) WT under control (WTC) or (b) Pi-deficiency conditions (WTP) and (c) ARP6-RNAi under control (arp6C) or (d) Pi-deficiency conditions (arp6P). (This figure is referred to data in chapter 1, 2, and 3.)
Table 1. The number of total reads, mapped reads and enrichment peaks of two replicates. ARP6-RNAi under control (+P) and 24-hours P-deficiency (-P), WT under control (+P) and 24-hours P-deficiency (+P). (This table is referred to data in chapter 1, 2, and 3).

<table>
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<th>H2AZ-Rep1</th>
<th>H2AZ-Rep2</th>
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<tr>
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<td>28346243</td>
</tr>
<tr>
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<td>23721208(83.68)</td>
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<tr>
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</tr>
<tr>
<td><strong>ARP6-RNAi (-P)</strong></td>
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<td></td>
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<tr>
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<td>39813678</td>
</tr>
<tr>
<td>Mapped Reads (%)</td>
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<td>34214172(85.94)</td>
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<tr>
<td>Number of Peaks</td>
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<td><strong>WT (+P)</strong></td>
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<tr>
<td>Mapped Reads (%)</td>
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<tr>
<td><strong>WT (-P)</strong></td>
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</tr>
<tr>
<td>Total Reads</td>
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<td>37529437</td>
</tr>
<tr>
<td>Mapped Reads (%)</td>
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<tr>
<td>Number of Peaks</td>
<td>43123</td>
<td>45016</td>
</tr>
</tbody>
</table>

Based on the high reproducibility, the overlap of the peaks in the two replicates was used to quantify H2A.Z across the genome. Approximately 53% of the total H2A.Z peak area (i.e. enrichment) was within genic regions, whereas 47% was within non-
genic regions (Figure 13.a). Therefore, compared to the rice genome, in which the genic and non-genic regions comprise 44% and 56%, respectively (Kawahara et al., 2013), H2A.Z enrichment is biased in genic regions. This result is consistent with a previous study, which also showed H2A.Z enrichment at genic regions in rice (Zhang et al., 2016).

Figure 13. H2A.Z deposition is biased in genic regions and enriched at protein-coding genes. (a) The comparison of H2A.Z deposition to actual genic and non-genic regions (b) The comparison of H2A.Z deposition in four gene types. (c) The patterns of H2A.Z distribution among the gene types. PCG: protein coding genes, PG: pseudogenes, TE: transposable elements, TEG: transposable element genes.
To observe the genic pattern of H2A.Z, we generated an average H2A.Z profile for all genes for a region 1 kb upstream of the gene start (i.e. transcription start site (TSS) for protein coding genes (PCG)) to 1 kb downstream of the gene end (i.e. transcription termination site (TTS) for PCG). As shown in Figure 14, this profile showed a H2A.Z peak at the 5’ end and a smaller peak at the 3’ end (Figure 14). These results are consistent with previous examinations of H2A.Z genic localization in rice (Zhang et al., 2016), *Arabidopsis* (Zilberman et al., 2008), and other model systems (Adam et al., 2001; Guillemette et al., 2005; Millar et al., 2006; Nekrasov et al., 2012; Santisteban et al., 2000; Tolstorukov et al., 2009; Whittle et al., 2008; Zhang et al., 2005; Zilberman et al., 2008), demonstrating that H2A.Z deposition patterns are conserved among eukaryotes.

![Figure 14. The distribution profile of H2A.Z deposition in rice genome-wide normalized with input control. WTC. WT under control conditions](image-url)
To more specifically examine H2A.Z patterns in rice genes, we first separated all annotated genes into four categories (Kawahara et al., 2013) to compare H2A.Z distribution among gene types: PCGs, pseudogenes (PGs), transposable elements (TEs), and transposable element-related genes (TEGs). The data showed that 93% of the genic H2A.Z enrichments were in PCGs followed by only 5% in TEG, 2% in PGs and 0.1% in TE (Figure 13.b). The patterns of H2A.Z distribution varied among the gene types with PCGs exhibiting a pattern similar to all annotated genes combined, whereas the other three gene types exhibited reductions in H2A.Z deposition, particularly at the 5' and 3' ends (Figure 13.c).

The presence of virtually all rice genic H2A.Z enrichments in PCGs is consistent with a role for H2A.Z deposition in transcriptional regulation. H2A.Z has been shown to be enriched near the TSS which means it likely plays a role in transcription, and work in Drosophila suggests it is involved in RNA Pol II stalling (Weber et al., 2014); also, other work has shown that H2A.Z is involved in transcription elongation and termination (Santisteban et al., 2000; Sura et al., 2017; Weber et al., 2014). To characterize the role of gene expression in H2A.Z deposition patterns of rice genes, PCGs were ordered from highest to lowest of FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values obtained from RNA extraction of the same tissues used for the ChIPSeq experiments described above following high-throughput sequencing (RNASEq) (Figure 15).

All genes with FPKM>0 (n= 27,133) were divided into five equal quintiles (Q₁-Q₅, Q₁ genes with the highest FPKM and Q₅ with the lowest). Also, genes with FPKM=0 ("not expressed", n= 8,893) were combined in Q₀ (Figure 15a-b). As seen in Figure 15a-
b, the highly expressed genes in Q₁ and Q₂ have a prominent peak of H2A.Z deposition at the 5' end near the TSS (Figure 15a-b) and a lesser peak at the 3' end near the TTS.

Figure 15. H2A.Z deposition is correlated with gene expression. (a) Heat map and (b) average profile of H2A.Z deposition in high expressed to low expressed genes (Q₁-Q₀) in WT under control conditions. It showed a 1000bp upstream, TSS (transcription start site), TTS (transcription terminate site) and a 1000bp downstream of the genes. The Q₁ is the highest expressed quintile and Q₅ is the lowest expressed quintile. Q₀ is the genes with no expression (FPKM=0). (c) The correlation between quintiles with H2A.Z deposition patterns (K₁-₃). (d) Two distinct patterns of H2A.Z deposition with no-expressed genes and (e) their correlation with H2A.Z deposition patterns (K₁-₃).
Reciprocally, the low-expressed genes in Q₄ and Q₅ exhibit relatively higher H2A.Z deposition, particularly in the gene body (GB). These results indicate a negative correlation between transcript abundance and H2A.Z. Interestingly genes in Q₀ (i.e. not expressed) can be placed in two divergent sub-groups based on H2A.Z abundance. Almost 43% of these genes lacking detectable transcripts are severely depleted in H2A.Z (Figure 15d). Together these results show a general negative correlation between gene expression and H2A.Z enrichment, except for genes that have severe depletion of H2A.Z, which is consistent with findings in rice (Zhang et al., 2016) and *Arabidopsis* (Coleman-Derr & Zilberman, 2012b; Yelagandula et al., 2014; Zilberman et al., 2008).

**H2A.Z enrichment in rice PCGs exhibits three distinct deposition patterns**

A previous study in *Arabidopsis* indicated different H2A.Z patterns across genes that correlated with gene-responsiveness—that is, the degree to which the gene experiences differences in transcript abundance in response to stimuli or across tissues (Coleman-Derr & Zilberman, 2012b). To investigate a potential correlation between H2A.Z patterns and gene function in rice, we first sought distinct H2A.Z distribution patterns among PCGs via k-means clustering. This revealed three divergent patterns of H2A.Z deposition—two major groups (K₁, n=18,232 and K₂, n=13,128) and a minor group (K₃, n=4,740) (Figure 16). In K₁, H2A.Z deposition showed broad peaks inside the gene body (GB) with three distinct sub-patterns. The first (K₁-a, n=4,010) contained peaks at both the 5’ and 3’ ends; the second (K₁-b, n=9,260) showed a peak across the entire gene, while the third pattern (K₁-c, n=4,962), showed a peak at the 5’ end. Conversely, in K₂, H2A.Z deposition was limited to a sharp peak near the TSS. Finally, in K₃, an explicit depletion of H2A.Z was observed (Figure 16a-b).
Figure 16. H2A.Z deposition patterns in rice PCGs. (a) The heatmap and (b) average profile of three distinct H2A.Z deposition patterns (K_{1-3}). A 1000bp upstream, TSS (transcription start site), TTS (transcription terminate site) and a 1000bp downstream of the meta-gene. The ChIPSeq reads were normalized to control input reads. (c) Average profile of DNA methylation of the K_{1-3} groups.

Gene ontology (GO) enrichment analysis (Du et al., 2010) demonstrated that K_1 genes were enriched with transcription factors and genes related to secondary metabolism and stress-responses (Figure 17), whereas K_2 genes were overrepresented in primary metabolism and biosynthesis GO terms (Figure 18). Genes in the smaller K_3 cluster were not as highly enriched in particular GO terms as clusters K_1 and K_2, and comprised a combination of GO terms enriched in the K_1 and K_2 clusters (Figure 19). However, defense-related “R” genes and other receptor-like kinases were among the most highly enriched gene groups in this cluster.
Figure 1. GO analysis of K_1 group. The color bar shows the significance of overrepresentation.

Comparing our K clusters with expression quintiles showed that K_1 genes tend to be low-expressed, K_2 genes tend to be high-expressed, and K_3 genes tend to exhibit no expression (Figure 15c-e). Based on these results, we assigned the K_1 genes as “stress-responsive” genes and the K_2 genes as “housekeeping” genes. These data indicate correlations between a sharp peak of H2A.Z deposition at the TSS with housekeeping genes, and broad H2A.Z deposition in the gene body with stress-related genes, which is similar to observations in *Arabidopsis* (Coleman-Derr & Zilberman, 2012b).
These results also show that H2A.Z deposition patterns may be generally predictive of gene function in plants.

Furthermore, we examined the DNA methylation data obtained from a previously published study (Secco et al., 2015). The results showed an anti-correlation between H2A.Z deposition and DNA methylation (Figure 16c), which is consistent with antagonistic correlation between H2A.Z deposition and DNA methylation reported in *Arabidopsis* (Zilberman et al., 2008).
Figure 19. GO analysis of $K_3$ group. The color shows the significance of overrepresentation.

**Pi deficiency leads to a redistribution of H2A.Z across rice genes**

Previously, chromatin-level control of Pi starvation responses was shown in which H2A.Z deposition modulates the transcription of a subset of Pi starvation response genes in *Arabidopsis* (Smith et al., 2010). To profile the global distribution of H2A.Z in rice under Pi deficiency, ChIP experiments following high-throughput sequencing were performed on the aerial parts of 36-days-old WT plants grown under control and 24-hour Pi deficiency (PCC= 0.89 and 0.99 for control and 24-hour Pi deficiency two replicates, respectively, Figure 12, Table1). As shown in Figure 20a, Pi deficiency resulted in a reduction of H2A.Z near the TSS and an increase near the TTS.
To facilitate our analyses, we separated PCG H2A.Z deposition into the TSS region (250 bp upstream to 500 bp downstream of the TSS) and gene body (GB) region (500 bp downstream of the TSS to 250 bp downstream of the TTS). As shown in Figure 20c, different ratios of H2A.Z deposition were observed in Pi-starved plants compared to control conditions. There was a 10.3% reduction in H2A.Z deposition in the TSS region in WT under Pi deficiency (WTP) compared to WT under control condition (WTC), whereas a 5% increase in H2A.Z deposition was observed in the GB. These results reflect an apparent “redistribution” of H2A.Z deposition from the TSS to the GB in response to Pi deficiency.

Next we identified changes in H2A.Z deposition at specific genes. A total of 13,989 PCGs contained at least one differential H2A.Z peak between WTP and WTC samples. Approximately 68% of these genes contained a decrease in one or more H2A.Z peaks in WTP relative to WTC, whereas 32% contained an increase in one or more H2A.Z peaks (Figure 20e). A small proportion of genes contained a combination of increased and decreased peaks and were excluded from further analysis. We next separated the genes based on whether the differential H2A.Z peaks were located at the TSS or GB regions (Figure 20g). The majority (67%) of genes containing a decrease in H2A.Z in WTP exhibited the decrease in the TSS region.
Figure 20. Differentially H2A.Z enrichment across rice genes. (a) Average profile of H2A.Z deposition in WT under control (WTC) and 24-hour Pi deficiency (WTP) and (b) in WT (WTC) and ARP6-RNAi (arp6C) under control conditions. (c) H2A.Z distribution in different part of genes in WT under control (WTC) and 24-hour Pi deficiency (WTP) and (d) in WT (WTC) and ARP6-RNAi (arp6C) under control conditions. (e) Distribution of differentially H2A.Z enrichment in different part of genes in WT under control (WTC) and 24-hour Pi deficiency (WTP) and (f) in WT (WTC) and ARP6-RNAi (arp6C) under control conditions. (g) Distribution of differentially H2A.Z enrichment in K1-3 groups in WT under control (WTC) and 24-hour Pi deficiency (WTP) and (h) in WT (WTC) and ARP6-RNAi (arp6C) under control conditions.
These genes were enriched in our K2 group (Figure 20g), and GO term enrichment analysis showed over-representation of translation-related genes (e.g. ribosomal proteins, translation initiation factors, tRNA synthetases, etc.) (Figure 21). Those genes with an H2A.Z deposition decrease in the GB were enriched in cluster K1 (Figure 20g) and were overrepresented with transcription factor families known to be responsive to a variety of signals, including bHLHs, C2H2 zinc finger proteins, homeobox proteins, AP2/ERFs, WRKYs, NAMs, bZIPs, and MYBs (Figure 22).

Figure 21. GO analysis of genes with decreased H2A.Z enrichment at TSS under 24-hour Pi deficiency. The color bar shows the significance of overrepresentation.
Figure 22. GO analysis of genes with decreased H2A.Z enrichment at GB under 24-hour Pi deficiency. The color bar shows the significance of overrepresentation.

Of the genes with a gain of H2A.Z deposition in WTP, around 66% contained the increased H2A.Z peak in the GB (Figure 20e). These genes were more evenly distributed among our K clusters, having the highest proportion of K3 genes (Figure 20g), and were enriched with protein kinases, particularly receptor-like kinases (Figure 23). The smallest proportion of genes containing a differential H2A.Z peak were those containing an increase at the TSS. These genes were somewhat enriched in cluster K2 (Figure 20g), and yielded only one significantly enriched GO term (i.e. plasma membrane), which contained a number of receptor-like kinases (Figure 24).
Figure 23. GO analysis of genes with increased H2A.Z enrichment at GB under 24-hour Pi deficiency. The color bar shows the significance of overrepresentation.
Together these data demonstrate that the major impacts of Pi deficiency on H2A.Z deposition are a reduction in H2A.Z at the TSS region, which correlates with genes related to translation, and an increase in H2A.Z deposition in the GB, which correlates with receptor-like kinase genes. Also, a smaller subset of genes that contained a decrease in H2A.Z in the GB were enriched with responsive transcription factors. These results may reflect a dual role for H2A.Z, which was proposed for *Arabidopsis* (Sura et al., 2017), in which decreases in H2A.Z at the TSS are linked to a decreased gene expression for some genes, possibly those related to translation and other housekeeping functions, whereas a decrease in H2A.Z in the GB is correlated with transcriptional activation of stress-responsive genes.
**Pi deficiency-induced differential gene expression is linked to changes in H2A.Z abundance**

To explore possible correlations between changes in H2A.Z deposition and gene expression in response to 24-hour Pi deficiency, we carried out RNASeq on the same tissues used for ChIPSeq, identified differentially expressed genes (DEGs) between WTP and WTC, and examined whether the DEGs contained differential H2A.Z deposition. The pair-wise comparisons of read counts of RNASeq using Deseq2 showed 805 genes up-regulated and 743 genes down-regulated (FDR <1.0E-3). Response to stimuli and stress were the top significantly enriched GO terms for the up-regulated DEGs (Figure 25), containing a number of WRKY and AP2 transcription factor genes, whereas down-regulated DEGs were enriched in lipid metabolic and cell wall-related GO terms (Figure 26), which included genes related to growth such as several expansin and laccase genes.

![Figure 25. GO analysis of genes with up-regulation under 24-hour Pi deficiency. The color bar shows the significance of overrepresentation.](image_url)
To examine the H2A.Z patterns of the DEGs, we compared the H2A.Z profile of all up or down-regulated DEGs with “all expressed” genes (FPKM >0, n=27,133). As shown in Figure 27, the H2A.Z deposition pattern under Pi deficiency for “all expressed” genes showed a reduction at the 5’ end near the TSS region, but an increase within the GB and at the 3’ end near the TTS region. The differentially expressed genes, whether up- or down-regulated, exhibited higher H2A.Z deposition in comparison to “all expressed” genes in both control and 24-hour Pi deficiency conditions. In up-regulated genes (n=805), a large reduction of H2A.Z deposition through the whole genes was observed under 24-hour Pi deficiency (Figure 27a).

Figure 26. GO analysis of genes with down-regulation under 24-hour Pi deficiency. The color bar shows the significance of overrepresentation.
Figure 27. The average profile of H2A.Z deposition of up-regulated genes in (a) WT under control (C) and 24-hour Pi deficiency (P), (b) WT (WT) and ARP6-RNAi (arp6) under control conditions and down-regulated genes in (c) WT under control (C) and 24-hour Pi deficiency (P), (d) WT (C) and ARP6-RNAi (arp6) under control conditions. allExp: The average profile of H2A.Z deposition in “all expressed” genes, UP: The average profile of H2A.Z deposition of up-regulated genes, DOWN: The average profile of H2A.Z deposition of down-regulated genes.

This result suggests that H2A.Z deposition acts as a repressor on transcriptional regulation of these responsive genes under control conditions. On the other hand, in the down-regulated genes (n=743), we observed a smaller reduction in H2A.Z deposition under 24-hour Pi deficiency at the 5’ end near the TSS region and a marginal increase inside and at the 3’ end near the TTS regions (Figure 27c). Indeed, these data suggest
that the higher H2A.Z deposition inside the genes acts as a hindrance for transcription rate of genes involved in metabolism.

Next we examined the overlap among DEGs with genes containing a differential H2A.Z peak. The proportions of up-regulated DEGs with an H2A.Z decrease at the GB and increase at the TSS were significantly over- and under-represented, respectively, compared to the same numbers of randomly selected genes (p-value=3.34E-9, binomial test, 1000 iterations) (Figure 28a).

Figure 28. The overlap among genes containing a differential H2A.Z peak with (a) up-regulated and (b) down-regulated genes in WT under 24-hour Pi deficiency, (c) up-regulated and (d) down-regulated genes in ARP6-RNAi under control condition.

An opposite trend was observed for down-regulated genes—their proportions were significantly under- and over-represented with genes containing decreased H2A.Z
at the TSS or increased H2A.Z at the GB, respectively (p-value=2.40E-5, binomial test, 1000 iterations). The other significant correlation is increased TSS with DEG_down, but this could be due to overlap with GB increases (Figure 28b). These results reveal that genes differentially expressed in response to a 24-hour Pi-deficiency treatment are negatively correlated with changes in H2A.Z.

**Discussion**

In plants, in addition to proven roles of regulatory components, epigenetic mechanisms have emerged as a significant factor in conferring environmental adaptability. In the past decade, several studies revealed the roles of DNA methylation (Kuo & Chiou, 2011; Secco et al., 2015; Yong-Villalobos et al., 2015), nucleosome positioning (Liu et al., 2015), histone modifications (X. Chen et al., 2015; Du et al., 2013; Mahrez et al., 2016; Zong et al., 2013), and histone variants (Coleman-Derr & Zilberman, 2012a; Du et al., 2010; Shu et al., 2014; Smith et al., 2010; Stroud et al., 2012; Sura et al., 2017; Wollmann et al., 2017; Zhang et al., 2016; Zilberman et al., 2008), on plant gene expression. In spite of the significant role of chromatin structure in controlling gene expression in eukaryotes (Haig, 2004), the relevant mechanisms involved in responses to nutrient deficiency has not been fully understood in plants.

In this study, we found high enrichment of H2A.Z in genic regions specifically at PCGs, which is consistent with its role in transcriptional regulation. In *S. cerevisiae*, H2A.Z is deposited at the flanks of silent heterochromatin to prevent its ectopic spread. However, H2A.Z is favorably associated to narrow regions of promoters, regardless of gene activity, (Guillemette & Gaudreau, 2006; Raisner et al., 2005). This distribution pattern around the TSS is also seen in mammals (Barski et al., 2007; Weber et al.,
2014) and *Drosophila*, but *Drosophila* does not incorporate H2A.Z into the -1 nucleosome (Mavrich et al., 2008). In the case of plants, we observed a peak of H2A.Z at the 5’ end downstream of the TSS (+1 nucleosome) in rice, which is positively correlated with gene expression level. This is consistent with other studies in *Arabidopsis* and rice (Coleman-Derr & Zilberman, 2012b; Zhang et al., 2016). Despite these different observations of transcriptional initiation machinery at the +1 nucleosome across eukaryotes, there is an apparent positive correlation between H2A.Z-containing nucleosomes around the TSS and transcription rate in higher eukaryotes. Following transcription-mediated H2A/H2B dimer loss, the less stable H2A.Z variant is incorporated to genic regions at the TSS, which is produced throughout the cell cycle (Nekrasov et al., 2012; Weber et al., 2010). In light of the inhibitory effects of H2A.Z on intermolecular association of nucleosomes, H2A.Z may generate unstable nucleosomes poised for transcriptional activation (Fan et al., 2002). The pattern in the K2 gene group that we observed with a sharp H2A.Z peak at the 5’ end near the TSS is consistent with transcription rate, as most highly-expressed genes are in this group and genes in this group were enriched in GO terms associated with intercellular and photosynthetic functions. In addition, 82% of a group of rice genes (n= 4,147) considered to be housekeeping in a previous study based on data mining of diverse transcriptome studies were in the K2 gene cluster (Chandran et al., 2016).

In contrast to TSS localization, H2A.Z abundance within gene bodies of plants and animals was reported to have a negative correlation with transcription (Coleman-Derr & Zilberman, 2012b; Latorre et al., 2015; Sura et al., 2017; Zilberman et al., 2008; Zilberman et al., 2007). In *Arabidopsis*, H2A.Z-containing nucleosomes within gene
bodies are correlated with responsiveness of variable genes, which is in contrast to exclusion of H2A.Z at sites of DNA methylation in the bodies of actively transcribed genes (Coleman-Derr & Zilberman, 2012a; Sura et al., 2017; Zilberman et al., 2008; Zilberman et al., 2007). These data demonstrate that the presence of H2A.Z in the gene body may aid in the proper regulation of variable genes but may hinder expression of highly transcribed genes. Consistent with this notion, we also found broad H2A.Z deposition peaks within the gene body of the genes in the K1 group, which are moderate to low expressed and assigned as responsive genes by GO analysis.

Several studies have demonstrated that H2A.Z and DNA methylation are two anti-correlated epigenetic marks (Coleman-Derr & Zilberman, 2012b; Zilberman et al., 2008; Zilberman et al., 2007). In plants, dynamic DNA methylation is believed to target transcriptional regulation by methylation and demethylation at specific sites in gene promoters and gene bodies, and controls many developmental pathways such as the transition from vegetative to reproductive growth (Hafiz et al., 2001) and senescence (Ay et al., 2014), as well as the expression of some plant defense genes (Dowen et al., 2012). It is believed that fully methylated genes act via dynamic reprogramming of chromatin status, switching from methylated heterochromatin to demethylated euchromatin allowing the interaction of transcriptional machinery (Ay et al., 2014). Consistent with these findings, the genes in the K3 group (i.e. genes with almost no H2A.Z deposition) were enriched with DNA methylation, as determined by using data obtained from a previous DNA methylation study in rice (Secco et al., 2015). The genes in this group are associated with receptor-like kinases, and around 26% of resistance genes (n=549) (Luo et al., 2012) were in this group. In plants, receptor-like kinases
control a wide range of processes, including development, disease resistance, hormone perception, and self-incompatibility (Shiu & Bleecker, 2001). Interestingly, around 55% of these genes exhibited no expression suggesting the hyperbolic nature of H2A.Z deposition in correlation with gene expression that the genes with the most and least transcription rate have the least amount of H2A.Z (Coleman-Derr & Zilberman, 2012b; Zhang et al., 2016). Also, the three subgroups of K₁ showed an obvious anti-correlation between DNA methylation and H2A.Z deposition. These results show that H2A.Z deposition patterns may be useful in general prediction of gene function in plants. In addition to the role in transcriptional regulation, DNA methylation also protects genome stability by silencing repeat sequences, such as transposable elements (TEs) (Ay et al., 2014; Secco et al., 2015; Zilberman et al., 2008; Zilberman et al., 2007). Consistent with these data, we detected a depletion of H2A.Z in TE regions, which is explained by the enrichment of DNA methylation on these regions as an antagonistic mark for H2A.Z deposition (Secco et al., 2015; Zilberman et al., 2008).

Next, the effect of short-term Pi deficiency was investigated on H2A.Z patterns. There is not a dramatic change in overall H2A.Z deposition patterns, which suggests that the nucleosomal H2A.Z deposition pattern is a faithful property of genes (Sura et al., 2017). The decrease of H2A.Z deposition at the TSS is the major change present in the K₂ group of genes, which is enriched in genes linked to translation. As mentioned above, H2A.Z at the TSS has a positive effect on transcription rate. The decrease of H2A.Z at the TSS, however, shows no significant correlation with DEGs. Two factors may help reconcile this apparent contradiction, first, maybe the duration of Pi deficiency is too short to observe changes in transcript abundance for the corresponding genes,
and/or second, as these genes are enriched in housekeeping functions, the absence of H2A.Z during Pi deficiency may have an indirect effect on transcription rate and the transcription of these genes may remain high. In other words, these genes with a difference in H2A.Z at TSS do not lose H2A.Z upon transcriptional activation. On the other hand, the decrease of H2A.Z at the GB is correlated with responsive genes, such as transcription factors. The significant overlap of this category with upregulated genes shows a negative correlation between transcription factors and other responsive genes with H2A.Z deposition. The evacuation of H2A.Z at the GB of responsive genes was also reported in a previous study in Arabidopsis subjected to drought-stress conditions (Sura et al., 2017), which suggests a role for H2A.Z in the GB as a repressor of genes that are responsive to multiple stressors (Kumar & Wigge, 2010; Smith et al., 2010; Sura et al., 2017). Accordingly, genes downregulated by Pi deficiency significantly overlap with genes containing an increase in H2A.Z in the GB, further supporting a negative role for H2A.Z inside the GB on transcription rate.
Chapter 3. Knock-down of Rice OsARP6 Perturbs H2A.Z Distribution

Introduction

The SWR1 complex (SWR1c) has been characterized as an evolutionarily conserved ATPase-containing chromatin remodeling complex in eukaryotes with multiple subunits that catalyzes the replacement of H2A by the histone variant H2A.Z in nucleosomes (Kobor et al., 2004; Mizuguchi et al., 2004). In contrast to other organisms, SWR1c and H2A.Z are not essential for viability in Arabidopsis (Coleman-Derr & Zilberman, 2012b). The central subunit of the Arabidopsis SWR1c is the Photoperiod-Independent Early Flowering 1 (PIE1) ATPase, which binds directly to H2A.Z. In several studies, the similarity in misregulation observed in pie1 and h2a.z mutants suggested a link between PIE1 and H2A.Z incorporation (March-Díaz et al., 2007; Martin-Trillo et al., 2006), however, despite the highly pleiotropic phenotypes in mutants defective in SWR1c (Coleman-Derr & Zilberman, 2012b; March-Díaz et al., 2008), some of the h2a.z pleiotropic phenotypes are distinct from pie1 (Coleman-Derr & Zilberman, 2012b), which suggest an H2A.Z-independent role for PIE1. In addition to PIE1, other non-catalytic subunits including ACTIN-RELATED PROTEIN 6 (ARP6) and SWR1 COMPLEX 6 (SWC6) were shown to be crucial for controlling flowering time via deposition of H2A.Z at the locus of the flowering repressor FLC (Choi et al., 2007; Deal et al., 2005; Deal et al., 2007; Noh & Amasino, 2003). Arabidopsis ARP6 was shown to also determine the spatial and temporal patterns of transcription that lead to proper regulation of meiosis during megagametogenesis (Qin et al., 2014). Moreover, a recent study on the regulation of flowering time revealed the role of phosphorylation of H2A at serine 95, a plant-unique site, by MUT9P-Like-Kinase (MLK4) in H2A.Z deposition. The
study demonstrated protein interactions of MLK4, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and YAF9a (a co-subunit of SWR1c and NuA4, histone H4 acetylase) promoted incorporation of H2A.Z into Gigantea (GI), a central gene in flowering time regulation, and consequently increased GI transcription (Su et al., 2017). Further, mutation of ARP6 in Arabidopsis led to decreases in H2A.Z enrichment and increases in expression of a number of responsive genes, which validates the use of ARP6 loss as a means for examining H2A.Z deposition (Berriri et al., 2016; Kumar & Wigge, 2010; Smith et al., 2010). In contrast to Arabidopsis, no mutants impacting H2A.Z deposition have been reported for rice. As in Arabidopsis, rice contains three H2A.Z-encoding genes (Deal et al., 2005), complicating the generation of H2A.Z mutant or knock-down transgenic plants. Herein I targeted OsARP6 with RNA interference as a way to examine perturbation of H2A.Z deposition in rice.

**Materials and Methods**

**Development of OsARP6-RNAi transgenics**

In collaboration with Dr. Niranjan Baisakh’s lab located at the School of Plant, Environmental and Soil Sciences at LSU, the vector pFGC1008 (ABRC) was used for generating the OsARP6-RNAi construct and it was mobilized into Agrobacterium tumefaciens to develop OsARP6-RNAi lines of rice, which show varying degrees of knock-down of the OsARP6 target locus.

**Physiological measurements of WT and OsARP6-RNAi transgenic lines**

WT and 3 transgenic lines of OsARP6-RNAi (#5, #16 and #19) were selected for these studies under controlled conditions along with 6 days of –P deficiency.

One fully expanded leaf per plant was used to extract total chlorophyll and carotenoid (three plants from each of the OsARP6-RNAi lines (#5, #16, #19) and WT
plants following control and –P deficiency) with 80% acetone twice. The chlorophyll and carotenoid concentration were measured following the method described by Joshi et al (Joshi et al., 2014)

Chlorophyll fluorescence was measured at room temperature with a portable fluorometer (PAM-2100; Walz, Germany). The minimal fluorescence level (Fo) with all photosystem (PS) II reaction centers open and Maximal fluorescence level (Fm) with all PSII reaction centers closed was determined (Joshi et al., 2014). Photosynthetic yield was measured as:

\[ \frac{Fv}{Fm} \text{ where } Fv = Fm - Fo \]

Stomata conductance (mM m\(^{-2}\) s) was measured using a Leaf Porometer (Decagon Devices Inc., Pullman, WA). The stomatal conductance of leaves was measured following the method described by Joshi et al (Joshi et al., 2014). The leaf porometer measures the stomatal conductance of leaves by putting the conductance of the leaf in series with two known conductance elements using the following equation:

\[
g_s = \frac{\hat{\rho} D_{\text{vapor}} [h_r e_s(T_a) - h_r e_s(T_a))]}{[e_s(T_a)(1-h_r)]d_2 - [h_r e_s(T_a) - h_r e_s(T_a)]d_1}
\]

where, \(g_s\) = stomatal conductance of the leaf surface, \(D_{\text{vapor}}\) = diffusivity of water vapor, \(d_2\) = distance between the two humidity sensors, \(P_{\text{atm}}\) = atmospheric pressure, \(h_r\) = relative humidity, \(e_s(T_a)\) = saturated vapor pressure at air temperature \(d\) = distance between the leaf surface and the first humidity sensor, 1 and 2 are the readings at the two different sensory nodes of the porometer.

Membrane stability index (MSI) was determined according to Sairam et al. (Sairam, 1994). Leaf samples (100 mg) were heated in 10 ml of double-distilled water at
40 °C for 30 minutes, and the electrical conductivity of the solution (C1) was recorded with multi parameter PCSTestr-35 (Eutech Instruments Pvt. Ltd., Singapore). Again the leaves were boiled at 100 °C on a boiling water bath for 10 minutes, and conductivity (C2) was measured. The membrane stability index (MSI) was calculated as:

$$MSI = [1 - (C1/C2)] * 100$$

Leaf relative water content was determined by collecting middle sections of second-youngest fully expanded leaves from plants 6 days after -P, -Fe, -Fe-P treatments for estimation of RWC according to Joshi et al (Joshi et al., 2014).

Statistical analysis

The data of physiological measurements were analyzed statistically with SPSS-17 statistical software (Norušis, 1990). Means were statistically compared by Duncan’s multiple range tests (DMRT) at p-value<0.05 levels.

Plant material and growth conditions

The OsARP6-RNAi Knock-down#19 seeds were sterilized and grown as described in Chapter 2. For morphological analysis, plants were treated with Pi deficiency for 7 days.

Also, the processes of chromatin immunoprecipitation, total RNA extraction, ChIPed DNA and RNA quantification for sequencing, ChIPed DNA and RNA library generation and ChIPSeq data analysis are done as described in Chapter 2.

RNASeq data analysis

Differential expression genes were analyzed as described in Chapter 2 with DESeq2 tool to get the pair-wise comparison between WT and OsARP6-RNAi#19 (Love et al., 2014).
Results

Knock-down of OsARP6 in rice shows pleiotropic phenotypes in stress responses

To gain insight into the role of H2A.Z on stress responses in rice, we first evaluated OsARP6-RNAi lines for several parameters that can be indicators of a variety of stressors, including stomatal conductance, carotenoid levels, relative water content (RWC), and membrane stability index (Havaux, 1998; Vijayalakshmi et al., 2012). Independent T3 generation OsARP6-RNAi lines showed varying degrees of Knock-down of the OsARP6 target locus (Figure 29a), and three of these lines were selected for analyses (#5,16 and 19). Also, 6-day Pi deficiency treatment was applied to observe the physiological and biochemical level responses.

As shown in Figure 30a-b, all the OsARP6-RNAi lines exhibited decreases in stomatal conductance and total carotenoids relative to WT under control conditions. In response to 6-days of Pi deficiency, stomatal conductance decreased in all genotypes, but remained significantly lower in the OsARP6-RNAi lines. Carotenoid levels also dropped in the WT in response to Pi deficiency, but remained at similar levels in the OsARP6-RNAi lines Figure 30b. In addition, RWC and membrane stability were similar among all genotypes under control conditions. During Pi deficiency, RWC was unchanged in WT but decreased in the OsARP6-RNAi lines Figure 30c, whereas membrane stability decreased in all genotypes, but significantly more in the OsARP6-RNAi lines Figure 30d.
Figure 29. Development of OsARP6-RNAi Transgenics. (a-b) qRT-PCR results of OsARP6-RNAi lines with varying degrees of knock-down of the ARP6 target locus. (c-d) whole seedlings and (e-f) roots of OsARP6-RNAi knock-down compare to wild type (WT) grown under (c-e) control and (d-f) Pi deficiency.
Figure 30. Assessment of physiological parameters in ARP6-RNAi Knock-down lines compared to WT under control and 6-days Pi deficiency. (a-d) stressor indicator parameters. (a) Stomata conductivity, (b) Total carotenoid, (c) Relative water content (RWC), (d) Membrane stability index (MSI). (e-f) Photosynthetic activity. (e) Chlorophyll (Chl) fluorescent, (f) Chlorophyll a (Chla), Chlorophyll b (Chlb) and Total Chlorophyll (TChl). (*) Number of asterisks shows the level of statistically significant difference compare to WT (Duncan test, p.value <0.05)

Because photosynthetic activity is sensitive to many stressors, we next examined chlorophyll content and fluorescence. Under control conditions, a significant reduction was observed in chlorophyll A (chl A), chlorophyll B (chl B), and total chlorophyll (t chl) in all OsARP6-RNAi lines compared to WT (Duncan test, p.value <0.05) (Figure 30f). In response to Pi deficiency, chlorophyll levels decreased in all genotypes, and remained significantly lower in the OsARP6-RNAi lines relative to WT except for the chl B content
Chlorophyll fluorescence as measured by Fv/Fm ratio was similar in all genotypes under control conditions, but showed a larger decrease in the OsARP6-RNAi lines in response to Pi deficiency (Figure 30e). Together these results indicate that knock-down of OsARP6 in rice elicits stress responses, which may reflect upregulation of stress-adaptive mechanisms during control conditions. The results also highlight some differences between WT and the OsARP6-RNAi lines in response to Pi deficiency, suggesting a link between ARP6 and the modulation of Pi deficiency responses. These data demonstrated exacerbation in response to Pi deficiency in some of these parameters (MSI, RWC and chlorophyll fluorescence); similarly, in Arabidopsis under heat stress conditions, architecture responses such as increase in hypocotyl growth and petiole elongation were enhanced in the arp6 background (Kumar & Wigge, 2010).

In Arabidopsis, arp6 mutants display constitutive Pi starvation responses, including root phenotypes (Smith et al., 2010). To gain insight into a similar phenomenon in rice, we quantified several morphological parameters of OsARP6-RNAi under control conditions and in response to a 7-day Pi deficiency treatment. Based on the physiological results and OsARP6 gene expression described above (Figure 29a), the OsARP6-RNAi #19 line was selected for these analyses. Additional RT-qPCR experiments confirmed knock-down of the OsARP6 target locus in OsARP6-RNAi #19 (Figure 29b). Under both control and Pi deficiency conditions, OsARP6-RNAi had less root growth as compared to WT (p-value=2.70724E-05, student’s t-test) (Figures 29c and 31a). In contrast, shoot height and root/shoot ratios were similar between WT and
the OsARP6-RNAi line (Figures 29d and 31b,c). For none of these parameters was there a difference in the genotypes with regard to Pi-deficiency response.

![Figure 31](image)

Figure 31. Morphological parameters in OsARP6-RNAi Knock-down lines compared to WT under control and 7-days Pi deficiency. The average of (a) total root length and (b) total shoot length and (c) root/shoot ratio of nine seedlings. (d) The average of total root hair length of two seedlings. (*) Asterisks show statistically significant differences compared to WT (students t-test).

A classical plant response to Pi deficiency is increased proliferation of root hairs to increase the root surface area for enhanced Pi uptake (Nestler et al., 2016). Therefore, we next compared root hair length among the samples. As shown in Figure 31d, OsARP6-RNAi exhibited longer root hairs than WT under control conditions. In response to Pi deficiency, WT root hair length increased but was unchanged in OsARP6-RNAi (Figure 31d). This is reminiscent of Arabidopsis arp6 mutants, which exhibited constitutive Pi deficiency responses, including increased root hair proliferation (Smith et al., 2010), and supports a role for OsARP6 in repressing Pi starvation responses in plants.

**OsARP6-RNAi knock-down mimics stress-related H2A.Z deposition**

To investigate the impact of OsARP6 knock-down on H2A.Z deposition in rice, we compared the average H2A.Z profile of WT and the OsARP6-RNAi #19 line. Two
biological replicates were examined to ensure reproducibility (PCC= 0.89 and 0.99 for WT and OsARP6-RNAi #19, respectively, Figure 12). Surprisingly, the impact of knock-down of OsARP6 on H2A.Z deposition was remarkably similar to that of Pi deficiency (Figure 20): an apparent redistribution of H2A.Z from the TSS to the gene body (Figure 20b). There was an 8.6% reduction in H2A.Z deposition in the TSS region in OsARP6-RNAi compared to WT, whereas a 7.4% increase in H2A.Z deposition was observed in the GB (Figure 20d), which suggested the role of ARP6 in proper H2A.Z deposition. Also, a total of 13,707 PCGs contained at least one differential H2A.Z peak between OsARP6-RNAi and WT samples. Approximately 68% of these genes contained a decrease in one or more H2A.Z peaks in OsARP6-RNAi relative to WT, whereas 32% contained an increase in one or more H2A.Z peaks. The majority (63%) of genes containing a decrease in H2A.Z in OsARP6-RNAi had the differential peaks at the TSS. These genes were enriched in our K2 group (Figure 20h), and GO term enrichment analysis showed over-representation of genes related to translation, including numerous ribosomal proteins, translation initiation factors, tRNA synthetases, as well as plastid genes (Figure 32).
Figure 32. GO analysis of genes with decreased H2A.Z enrichment at TSS in OsARP6-RNAi Knock-down. The color bar shows the significance of overrepresentation.

Those genes with an H2A.Z deposition decrease in the GB were enriched in clusters K_1 and were overrepresented with transcription factor families known to be responsive to a variety of developmental and environmental signals, including bHLHs, C2H2 zinc finger proteins, homeobox proteins, AP2/ERFs, WRKYs, NAMs, bZIPs, and MYBs (Figure 33).
Figure 33. GO analysis of genes with decreased H2A.Z enrichment at GB in OsARP6-RNAi Knock-down. The color bar shows the significance of overrepresentation.

Of the genes with a gain of H2A.Z deposition in OsARP6-RNAi, around 66% contained the increased H2A.Z peak in the GB. These genes are mostly in the K₁ group and are enriched with disease resistance (R) genes and receptor-like kinases (Figures 20h and 34).
Figure 34. GO analysis of genes with increased H2A.Z enrichment at GB in OsARP6-RNAi Knock-down. The color bar shows the significance of overrepresentation.

Relatively few genes contained an increase in H2A.Z at the TSS in OsARP6-RNAi samples, and these genes were not enriched in any particular K group, nor did they yield any significantly enriched GO terms.

Together these data demonstrate that the major impacts of OsARP6-RNAi knock-down are a reduction in H2A.Z deposition at the TSS region, which correlates with genes related to translation but also other "housekeeping" genes, and an increase in H2A.Z deposition in the GB, which correlates with disease resistance genes but also other biological regulation genes. Also, a smaller subset of genes that contained a decrease in H2A.Z in the GB was enriched with responsive transcription factors.
Changes in H2A.Z deposition by OsARP6 knock-down correlate with transcriptional regulation

We next identified differentially expressed genes between WT and OsARP6-RNAi RNA-Seq samples under control conditions (WTC and arp6C, respectively). The pair-wise comparisons of read counts of RNASeq in OsARP6-RNAi and WT plants using Deseq2 showed 796 genes up-regulated (Figure 35) and 1,473 genes down-regulated (Figure 36) in OsARP6-RNAi compared to WT (FDR<1E-03). As for WTP, we sought the correlation between changes in H2A.Z deposition and changes in gene expression in OsARP6-RNAi. Similar to WTP, OsARP6 down-regulated DEGs were significantly over- and under-represented for H2A.Z increases in the GB (and TSS) and decreases at the TSS, respectively (Figures 27d and 28d). Another similarity was that decreases in the GB were positively correlated to up-regulated DEGs for both arp6C and WTP (Figures 27b and 28c).
Figure 35. GO analysis of genes with up-regulation in OsARP6-RNAi Knock-down. The color bar shows the significance of overrepresentation.

However, unlike WTP, OsARP6-RNAi upregulated genes were under- and over-enriched for H2A.Z decreases at the TSS and increases in the GB, respectively (Figure 28c,d). These results show strong correlations between gene expression and H2A.Z changes in the OsARP6-RNAi plants, some of which differ from the same comparison for WTP.
Figure 36. GO analysis of genes with down-regulation in OsARP6-RNAi Knockdown. The color bar shows the significance of overrepresentation.

Discussion

In previous studies, the pleiotropic phenotype of mutants in H2A.Z and SWR1 components were reported (Berriri et al., 2016; Coleman-Derr & Zilberman, 2012b; Kumar & Wigge, 2010; Smith et al., 2010). In this study, OsARP6-RNAi knock-down exhibited stress-related phenotypes similar to Arabidopsis arp6 mutants, which exhibit a
variety of stress responses, including constitutive heat stress and Pi-starvation responses, as well as increased resistance to pathogen challenge (Berriri et al., 2016; Kumar & Wigge, 2010; Smith et al., 2010). The physiological studies herein indicate that the stress responses in OsARP6-RNAi rice may reflect the activation of stress-adaptive mechanisms during control conditions, and the differences between WT and the OsARP6-RNAi lines in response to Pi deficiency suggest a link between ARP6 and the modulation of Pi deficiency responses. Also, OsARP6-RNAi knock-down lines showed significant root hair proliferation, a sign of Pi deficiency, which is consistent with constitutive Pi starvation responses in Arabidopsis arp6 mutants (Smith et al., 2010).

In the absence of H2A.Z, genes normally harboring H2A.Z within their gene bodies are transcriptionally misregulated (Coleman-Derr & Zilberman, 2012b; Zilberman et al., 2007). In Arabidopsis, it was shown that upon mutation of ARP6, a reduction of H2A.Z deposition was observed at specific genic locations using ChIP-qPCR, regardless of the position in the gene and transcription rate (Sura et al., 2017). In this study, we used a knock-down line of OsARP6 to look at disruption of H2A.Z deposition genome-wide in rice. Based on the role of ARP6 in proper H2A.Z deposition, the OsARP6-RNAi line exhibited a redistribution of H2A.Z from the TSS region to the GB but not a dramatic drop in H2A.Z at a genome level. Several possibilities may help explain the apparent discrepancy between studies on Arabidopsis arp6 mutants and our data. First, in previous studies (Berriri et al., 2016; Kumar & Wigge, 2010; Smith et al., 2010; Sura et al., 2017), the regions related to specific stressors were selected for H2A.Z ChIP analysis using ChIP-qPCR, whereas in our study we looked at H2A.Z deposition genome-wide. Second, we used RNAi to target OsARP6 knock-down, which
is not a complete loss of ARP6. Therefore, it is possible that the amount of ARP6 present in the OsARP6-RNAi knock-down line is sufficient for deposition of H2A.Z in some regions. Finally, the INO80 complex is another chromatin remodeling complex that appears to participate in H2A.Z deposition near the 3' end of genes (Zhang et al., 2015). Therefore, disruption of H2A.Z by SWR1c (via OsARP6 Knock-down), may lead to unbalanced H2A.Z deposition by INO80.

The analyses described above showed that knock-down of OsARP6 leads to changes in both H2A.Z deposition and transcript abundance for a number of housekeeping genes, including many that function in plastids. The majority of these genes are implicated in chloroplast function. For example, two chlorophyll biosynthetic genes are down-regulated by OsARP6 Knock-down and contain a decrease in H2A.Z at the TSS. Consistently, OsARP6-RNAi seedlings have decreases in chlorophyll content relative to WT (Figure 30f). Therefore, in addition to supporting a role for H2A.Z in modulating stress-responsive genes at the gene body, our data also support a role for H2A.Z controlling expression of housekeeping genes, including many related to chloroplast functions, via deposition at the TSS.
Chapter 4. Pi Deficiency and OsARP6 Knock-down Show Common and Specific Impacts on H2A.Z Perturbation

Introduction

The involvement of H2A.Z in transcriptional regulation has been well-documented by extensive studies; however, its roles in environmental stress responses and nutrient status in plants have remained enigmatic. In *Arabidopsis*, it has been shown that upon osmotic stress, H2A.Z is evacuated from induced genes independently of its location in the gene (Sura et al., 2017). In addition, the arp6 mutant exhibited up-regulation of stress-responsive genes with H2A.Z mainly deposited inside the gene body in WT under control conditions and misregulation in response to osmotic stress, which indicated a negative influence of H2A.Z on transcription in nucleosomes located across gene bodies of responsive genes (Sura et al., 2017). This observation also supports the modulation of thermo-sensory responses through temperature-dependent H2A.Z nucleosome dynamics in *Arabidopsis* independent of transcription. In arp6 mutant, the plants demonstrated a prominent constitutive warm temperature transcriptome. These results suggested that H2A.Z-containing nucleosomes provide thermo-sensory information to synchronize the ambient temperature transcriptome (Kumar & Wigge, 2010). Indeed, the modulation of H2A.Z on thermo-sensing in grain development in *Brachypodium distachyon* showed that increase in ambient temperature impacted H2A.Z-nucleosome occupancy in the reproductive tissue of developing grains. The phenotypic responses of H2A.Z perturbation were early maturity and reduction in yield (Boden et al., 2013). Furthermore, a previous study on the role of H2A.Z in responses to Pi deficiency found enrichment of H2A.Z at a number of Pi starvation response (PSR) genes in *Arabidopsis*, which was lost upon Pi deficiency (Smith et al.,
This indicated an apparent repression of these genes by H2A.Z under control conditions. Consistently, H2A.Z deposition at these genes was lost in arp6 mutants, which led to the de-repression (induced transcript abundance) of these PSR genes under control conditions (Smith et al., 2010). These results suggest a chromatin-level control of stress responses in which ARP6-dependent H2A.Z deposition modulates the transcription of responsive genes.

In this study, we examined differential H2A.Z deposition and gene expression in response to 24-hours of Pi deficiency in rice. Interestingly, the data show significant similarity between differential H2A.Z deposition in wild type under 24-hours of Pi deficiency and OsARP6-RNAi knock-down plants under control conditions. In addition, the interaction analysis between OsARP6-RNAi knock-down and 24-hours of Pi deficiency on gene expression showed similar patterns of differential gene expression, particularly of responsive genes. Nonetheless, the data also reveal important differences between Pi deficiency and OsARP6 knock-down, such that Pi deficiency has a larger impact on the de-repression of stress-responsive genes, whereas knock-down of OsARP6 leads to the down-regulation of a greater number of housekeeping genes.

Materials and Methods

Plant material and growth conditions

The WT and OsARP6-RNAi Knock-down#19 seeds were surface sterilized and grown as described in Chapter 2. After 21 days, half of the seedlings were transferred to a solution without NaH$_2$PO$_4$ for a 24-hour P deficiency treatment. Shoot tissue of 24-hour Pi deficiency (-Pi) and control samples of WT and OsARP6-RNAi Knock-down#19 were harvested separately and frozen in liquid nitrogen. The procedure was repeated 3 times to produce 3 replicates.
Also, the processes of chromatin immunoprecipitation, total RNA extraction, ChIPed DNA and RNA quantification for sequencing, ChIPed DNA and RNA library generation and ChIPSeq data analysis were done as described in Chapter 2.

**RNASeq data analysis**

RNASeq reads for each sample were mapped to the reference genome (MSU Rice Genome Annotation Release 7.1) using the Bowtie2 tool (Langmead & Salzberg, 2012). Using an in-house script, the reads for each gene were counted and differential expression was analyzed in DESeq2 tool to get the effect-interaction among WT and OsARP6-RNAi#19 under control and 24-hours Pi deficiency (Love et al., 2014).

**Fuzzy K-means clustering**

The Normalized rLog (regularized Log transformation) values of 2002 selected genes with FDR <0.001 obtained from DESeq2 were used for clustering. The fuzzy K-means clustering was done in the Aerie tool (Gasch & Eisen, 2002) and identified 14 clusters. The ggplot2 in R package was used to make the plots for each cluster.

**Results**

*OsARP6 knock-down and Pi deficiency have partially overlapping effects on H2A.Z deposition*

The majority (70%) of the differential H2A.Z enrichment genes in OsARP6-RNAi are in common with those in 24-hour Pi deficiency (Figures 37a and 38a).
Figure 37. Proportion of common and unique differential H2A.Z deposition genes in OsARP6-RNAi under control (arpC) and WT under 24-hour Pi deficiency (WTP) compared to WT under control conditions (WTC). (a) Number of common and unique genes. (b) Distribution ratio of common and unique genes in the K1-3 gene groups (chapter 2).

To differentiate the impacts of Pi deficiency and OsARP6 knock-down on the H2A.Z patterns of rice genes, we identified genes containing differential H2A.Z peaks that were unique to either OsARP6-RNAi knock-down or Pi starvation (Figure 37a). For genes containing a decrease in H2A.Z at the TSS region, the OsARP6-RNAi unique group were enriched in many of the same GO terms as observed for OsARP6-RNAi or Pi starvation individually, namely those related to plastid functions and translation. In
contrast, Pi starvation-specific genes containing a decrease in H2A.Z at the TSS did not yield any significantly enriched GO terms. On the other hand, for genes containing an H2A.Z decrease in the GB, OsARP6-RNAi-specific genes yielded no enriched terms, whereas those genes unique to Pi starvation were enriched in transcription factors and signaling-related genes. In addition, the OsARP6-RNAi unique genes with a decrease in H2A.Z were enriched in K2 (housekeeping) genes compared to the shared genes, whereas the Pi starvation unique genes with a decrease in H2A.Z were enriched in K1 (responsive) genes (Figure 37b). For genes containing increases in H2A.Z deposition, whether at the TSS or GB, no group of OsARP6-RNAi or Pi-starvation specific genes yielded significantly enriched GO terms (despite OsARP6-RNAi containing more receptor-like kinase genes). Together these results demonstrate that knock-down of OsARP6 has a larger impact on loss of H2A.Z at the TSS relative to Pi starvation, which impacts genes related to plastid functions and translation, whereas Pi starvation leads to a greater impact on loss of H2A.Z at the GB, which impacts responsive transcription factor genes.

Similar to changes in H2A.Z, there was overlap between the DEGs in OsARP6-RNAi and those in WTP, but the overlap was less (40%) (Figure 38b). Of the 796 up-regulated genes in OsARP6-RNAi, 37% (n=297) were also up-regulated in WTP, and 41% (n=607) of the 1,473 down-regulated genes were also down-regulated in WTP (Figure 38b).
Figure 38. The overlap of (a) differential H2A.Z deposition and (b) differentially expressed genes in WT under Pi deficiency (WTP), OsARP6-RNAi under control (arp6C) and Pi deficiency (arp6P) compare to WT under control condition (WTC).

We next looked at significantly enriched GO terms for each group of unique genes. For up-regulated genes, those only up-regulated in OsARP6-RNAi knock-down were enriched in plastid and response to stimulus genes (Figure 39), whereas genes up-regulated only by Pi deficiency were enriched in response to stimulus (Figure 40). On the other hand, genes down-regulated uniquely in OsARP6 were enriched in carbohydrate metabolism, plastid, and cell wall GO terms (Figure 41), whereas Pi deficiency-unique downregulated genes were not enriched in any GO terms. This result
reflects an impact of *OsARP6* knock-down on chloroplast function not only at the H2A.Z level, but also at the transcriptome level.

**Figure 39.** GO enrichment analysis of genes with unique up-regulation in *OsARP6*-RNAi knock-down. The color bar shows the significance of overrepresentation.

**H2A.Z distribution does not change significantly under 24-hour Pi deficiency in *OsARP6*-RNAi plants**

To see how *OsARP6*-RNAi knock-down can affect H2A.Z deposition in response to 24-hour Pi deficiency, the results of differential H2A.Z deposition in *OsARP6*-RNAi under 24-hour Pi deficiency (arp6P) were compared with the differential H2A.Z deposition in *OsARP6*-RNAi knock-down under control conditions (arp6C) (Figure 38a).
In contrast to the comparisons of arp6C to WTC and WTP to WTC, many fewer genes contained a gain or loss of H2A.Z (n= 5202) in OsARP6-RNAi in response to Pi deficiency. This suggested that knock-down of OsARP6 or a 24-hour Pi starvation treatment have dramatic and similar impacts on H2A.Z localization, but that the combined perturbations (i.e. Pi starvation of the OsARP6-RNAi transgenics) led to only a moderate combined/synergistic disruption of H2A.Z localization. To examine this more closely, we compared the overlap in differences in H2A.Z deposition between each of the stress samples to WTC. For each combination there were very similar numbers of H2A.Z decreases and increases, and over 60% overlap of the differences (Figure 38a). This confirms that the impact on H2A.Z deposition in the arp6C, WTP, and arp6P samples is very similar.
Figure 41. GO enrichment analysis of genes with unique in -RNAi. The color bar shows the significance of overrepresentation.

**Clustering of DEGs based on the interaction effect of Pi deficiency and OsARP6-RNAi knock-down on transcription**

Our analyses above showed significant correlations between differential H2A.Z deposition and DEG. To investigate the combined effect of Pi deficiency and OsARP6-RNAi knock-down on gene expression, we used the interaction analysis in Deseq2 software to analyze our RNASeq data (Figure 42).
Figure 42. (a) PCA plot of two replicates of WT under control (WTC) or Pi deficiency (WTP), and OsARP6-RNAi under control (arp6C) or Pi deficiency (arp6P). (b) Differentially expressed genes in both (interaction) and each of WT vs OsARP6-RNAi line (group) under control vs Pi deficiency conditions (treatment) (FDR<1.00E-03).

PCGs with significant differential expression (n=2,002) were selected for downstream clustering (FDR<1.00E-03). 14 distinct clusters were produced using Fuzzy K-means clustering based on rlog transformation of read counts of each of the 2,002 genes (Figure 43).
The majority of DEGs fell into one of 6 key clusters (C1-C6). The largest four clusters each contained between 300 and 400 genes. Genes in clusters C1 (n=356) and C2 (n=376) showed up-regulation in WTP, arp6C, and less so in arp6P as compared to WTC. Significantly enriched GO terms in these clusters were linked to stress-responses and the plasma membrane, including many transporters such as two phosphate transporters (Figure 44).
In contrast, genes in clusters C₃ (n=366) and C₄ (n=308) exhibited an opposite trend: down-regulation in WTP, arp6C, and to a lesser extent in ARP6P compared to WTC. Genes from significantly enriched GO terms in this cluster include those related to carbohydrate and lipid metabolism, secondary metabolism, and the extracellular region, including several expansins (Figure 45). That the majority of DEGs comprise these 4 major clusters confirms that ARP6 and Pi deficiency elicit similar changes to the transcriptome, without an apparent synergistic effect. Many stress-responsive genes are upregulated by either Pi deficiency or OsARP6 knock-down, whereas many metabolic genes are downregulated.
The remaining two major clusters contained approximately 200 genes each. These clusters contain genes whose transcript abundance is altered in response to knock-down of OsARP6 but not Pi deficiency. In cluster C5 (n=182), there is an upregulation of genes in OsARP6-RNAi compared to WT regardless of Pi status. There are no significant GO terms in this cluster, however, 62% of the genes are in the K1 gene group (stress-responsive). In cluster C6, there are 192 genes with an obvious down-regulation in arp6C and arp6P compared to WTC and WTP. The significant GO terms in this cluster are plastid, thylakoid and intracellular membrane-bound organelle (Figure 46). This cluster is more enriched in housekeeping genes.

In addition to the 6 major clusters described above, there were a number of minor clusters. There are two clusters (C9 and C14) which contain genes up and down-regulated by Pi, respectively, but unaffected by ARP6. In contrast to the ARP6-specific genes described above, these Pi specific DEGs are fewer in number and are not significantly enriched in any GO terms. Of the remaining clusters, three yield significant GO terms despite containing small numbers of genes. Genes in C13 are enriched in growth-related GO terms, which include a number of expansins (Figure 46).
Figure 46. GO analysis of genes in clusters C6, C11, C12 and C13. The color bar shows the significance of overrepresentation

The transcript response of this cluster is similar to clusters C1 and C2, in which expansins are also present. Finally, two clusters (C11 and C12) exhibit complex interactions between OsARP6 knock-down and Pi deficiency. Cluster C11 genes, which exhibit down regulation by Pi in WT but not in OsARP6-RNAi, is enriched in response to stimulus. On the other hand, C12 genes are upregulated by both OsARP6 knock-down and Pi deficiency, and their expression is even higher in Pi-starved OsARP6-RNAi. These genes are enriched in transcription factor genes and highlight a unique group of transcription factors that may respond to both Pi and OsARP6 perturbation, but via different mechanisms. These include MYB, bZIP, homeobox, WRKY, and MADS-box transcription factors, which are stress-responsive and/or function in development (e.g. homeobox and MADS-box) (Figure 46).
Discussion

In the previous chapter, we showed stress-related phenotypes in OsARP6-RNAi knock-down plants at the physiological and morphological levels. We also showed a remarkable similarity between arp6C and WTP with regard to both differences in H2A.Z deposition as well as differentially expressed genes. In this chapter, we dissected unique responses to differentiate the impacts of Pi deficiency and OsARP6 knock-down on the H2A.Z patterns of rice genes. The results demonstrate that knock-down of OsARP6 has a larger impact on loss of H2A.Z at the TSS of genes associated with plastid and translation relative to Pi starvation. On the other hand, Pi starvation leads to a greater impact on loss of H2A.Z at the GB of responsive transcription factor genes, which is consistent with the negative effect of H2A.Z deposition at the GB of responsive genes (Sura et al., 2017).

The analysis of the combined effect of Pi deficiency and OsARP6-RNAi knock-down on gene expression also showed remarkable similarity at the transcriptional level in responsive genes without an apparent synergistic effect \((C_{1,4})\). Many stress-responsive genes are upregulated by either Pi deficiency or OsARP6 knock-down, whereas many metabolic genes are downregulated. These results are consistent with a previous study on a subset of PSR genes in Arabidopsis, which demonstrated the derepression of the genes in arp6 mutants under control conditions, and constitutive Pi responses. Interestingly, the combination of OsARP6-RNAi knock-down and Pi deficiency have not shown dramatic exacerbation on H2A.Z and transcription levels. This result suggests the involvement of ARP6 in Pi responses. However, the derepression of responsive genes in the arp6 mutant also observed in heat stress
conditions or pathogen defense suggests that ARP6 is involved in signaling of responses to stimuli generally by determining proper H2A.Z deposition. This notion is strengthened by the observation of the number of plastid genes that are affected by ARP6 knock-down but not Pi deficiency (C6). These photosynthetic genes show down-regulation and decreased H2A.Z at the TSS in OsARP6-RNAi knock-down plants. They are some of the few examples that highlight the role of H2A.Z as controlling transcriptional initiation.
Conclusions

As sessile organisms, plants need to absorb the right combination of mineral nutrients from the soil as well as adapt to many environmental stresses. To overcome the consequences of these challenges, plants have evolved elaborative molecular and biochemical tolerance mechanisms, for which the identification and implementation in breeding programs assists the development of plants with optimal growth and productivity. More recently, the advent of techniques such as chromatin immunoprecipitation and high throughput sequencing enables the investigation of chromatin architecture and epigenetic mechanisms that may modulate responses to stress conditions. Accordingly, thorough understanding of the epigenetic mechanisms that regulate nutrient homeostasis serves to provide new opportunities for developing crop plants with improved nutrient uptake, nutrient use efficiency, and an enhanced ability to adapt to low-nutrient conditions. In this study, the role of H2A.Z histone variant abundance in chromatin as a mechanism involved in Pi homeostasis in rice was examined. Rice provides an ideal system for the study since its complete genome sequence is known, which allows for genome- and epigenome-level analyses, and because it is a staple crop globally.

In the first part of this study, we determined that the genome-wide distribution of H2A.Z in rice is enriched in genic regions with a distinct high peak at the 5’ end and a smaller peak at the 3’ end (Figures 13 and 14). We found particular enrichment of H2A.Z in PCGs (Figure 13), which demonstrated the involvement of H2A.Z in transcriptional regulation. Ranking PCGs based on their expression showed a sharp
enrichment of H2A.Z at the 5’ end near the TSS of highly expressed genes but a broad peak of H2A.Z across the genes of medium to low expressed genes (Figure 15). These findings are consistent with previous results in *Arabidopsis* that showed the deposition of H2A.Z at 5’ ends correlates with housekeeping genes and the deposition of H2A.Z across gene bodies correlates with gene responsiveness (Coleman-Derr & Zilberman, 2012a). Using k-means clustering, two major groups of genes were identified based on H2A.Z deposition patterns. Using GO term enrichment analysis, these gene groups were designated as containing housekeeping or responsiveness genes (Figure 16). Around 13% of PCGs were severely depleted in H2A.Z deposition, comprising a third, minor group. These results can be useful in predicting the function of genes based on their H2A.Z localization pattern. In Arabidopsis, a mutual anticorrelation between H2A.Z and DNA methylation was established (Zilberman et al., 2008). It was shown that the presence of DNA methylation excludes H2A.Z (Zilberman et al., 2008). Interestingly, the analysis of the published data of DNA methylation in rice (Secco et al., 2015) showed an antagonistic correlation between H2A.Z and DNA methylation in the three distinct H2A.Z patterns (Figure 16c). Next, we investigated the effect of 24-hour Pi deficiency on H2A.Z distribution. The results reflected an apparent “redistribution” of H2A.Z deposition from the TSS to the GB in response to Pi deficiency. In other words, the major impacts of Pi deficiency on H2A.Z deposition are a reduction in H2A.Z at the TSS region, which correlates with genes related to translation, and an increase in H2A.Z deposition in the GB, which correlates with receptor-like kinase genes. Also, a smaller subset of genes that contained a decrease in H2A.Z in the GB was enriched with responsive transcription factors (Figure 22). Comparison of the differential H2A.Z peaks with
RNASeq data revealed that changes in H2A.Z deposition under 24-hour Pi deficiency are negatively correlated with transcription (Figure 25 and 26).

In the second part of the study, we investigated the effect of disruption of H2A.Z deposition by OsARP6-RNAi knockdown plants. A number of physiological parameters under control conditions and in response to a 24 hour Pi deficiency treatment showed a reduction in photosynthetic activity in OsARP6-RNAi lines when compared to WT, and for many of the parameters the reductions were more pronounced under Pi deficiency (Figure 30). Also, the morphological study on the relationship between OsARP6-RNAi knockdown and Pi deficiency suggested the importance of OsARP6 in modulating Pi deficiency responses in rice (Figure 31). A comparison of the average H2A.Z deposition profile of WT and OsARP6-RNAi knockdown revealed that the impact of knockdown of ARP6 on H2A.Z deposition was remarkably similar to that of Pi deficiency (Figures 37 and 38). Also, differentially expressed genes between WT and OsARP6 knock-down RNASeq samples under control conditions showed convincing correlations between gene expression and H2A.Z changes in the OsARP6-RNAi plants (Figure 29).

In the last part of the study, we investigated the specific changes in Pi deficiency or OsARP6 knockdown. Analysis of the unique genes in OsARP6-RNAi knockdown and 24-hour Pi deficiency demonstrated that knock-down of ARP6 has a larger impact on loss of H2A.Z at the TSS relative to Pi starvation, which impacts genes related to plastid and translation, whereas Pi starvation leads to a greater impact on loss of H2A.Z at the GB, which impacts responsive transcription factor genes (Figure 37). Also, at the transcriptional level, for upregulated genes, those only upregulated in ARP6-RNAi knockdown were enriched in plastid and response to stimulus genes, whereas genes
upregulated only by Pi deficiency were enriched in response to stimulus (Figures 30 and 40). On the other hand, genes downregulated uniquely by ARP6 knock down were enriched in carbohydrate metabolism, plastid, and cell wall GO terms (Figure 41), whereas Pi deficiency-unique downregulated genes were not enriched in any GO terms. These findings suggest an impact of OsARP6 knockdown on chloroplast function not only at the H2A.Z level, but also at the transcriptional level. The effect of 24-hour Pi deficiency on OsARP6-RNAi knockdown demonstrated many fewer genes contained a gain or loss of H2A.Z, which suggested that knock-down of ARP6 or a 24-hour Pi starvation treatment have dramatic and similar impacts on H2A.Z localization, but that the combined perturbations led to only a moderate combined/synergistic disruption of H2A.Z localization (Figure 38). The combined effect of 24-hour Pi deficiency and ARP6-RNAi knockdown on gene expression were also similar when compared to WT under control conditions (Figure 43).

Together the results support a complex role for H2A.Z in transcriptional regulation. At the TSS, H2A.Z is important for transcription, particularly for highly-expressed house-keeping genes. In contrast, H2A.Z localized to the gene body impacts the responsiveness of genes, and is negatively correlated with the transcription of genes whose expression is influenced by stress. Interestingly, whether by knockdown of OsARP6 or by exposure to a short-term Pi deficiency treatment, H2A.Z deposition is similarly altered genome-wide. This likely reflects the fact that these two types of perturbations illicit similar, general stress responses. Nonetheless, OsARP6-knockdown led to a stronger impact on loss of H2A.Z at the TSS of house-keeping genes, whereas Pi deficiency was more specific to stress-responsive genes dependent on H2A.Z
localization at the gene body. By investigating these contrasting stressors, we revealed key differences between the roles of H2A.Z in poising genes for transcriptional activation and as a repressor of stress-responsive gene expression.
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Vita

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