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Expression analysis of histone acetyltransferases in rice under drought stress

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Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Expression analysis of histone acetyltransferases in rice under drought stress

(Thesis format: Monograph)

by

Hui Fang

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Histone acetylation is one of the vital reversible modifications in eukaryotes. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) maintain the homeostasis of histone acetylation. HATs are associated with genome-wide transcriptional activation and various biological processes in response to various stresses. Drought stress causes a range of physiological and biochemical responses in plants. Eight HATs which belong to four different families (CBP, GNAT, MYST, and TAFII250 family) have been identified in rice. In this research, four OsHATs, one from each family, were chosen based on *in silico* domain and promoter analysis. The real-time qPCR analysis demonstrated that drought stress caused a significant increase in the expression of all four OsHATs (*OsHAC703, OsHAG703, OsHAF701* and *OsHAM701*). Additionally, the western-blot analysis showed that the acetylation level on certain lysine sites of histone H3 (K9, K18 and K27) and H4 (K5) increased accordingly, implicating OsHATs are involved in drought stress responses in rice.

**Key Words:** Histone acetyltransferase, histone acetylation, drought stress, rice.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ABI</td>
<td>abscisic acid (ABA)-insensitive</td>
</tr>
<tr>
<td>ADA</td>
<td>Alteration/Deficiency in Activation</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CAB</td>
<td>chlorophyll a/b-binding protein gene</td>
</tr>
<tr>
<td>CBF</td>
<td>C-repeat binding factor</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP responsive element-binding protein</td>
</tr>
<tr>
<td>CHIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>COR</td>
<td>cold-regulated</td>
</tr>
<tr>
<td>cv</td>
<td>Cultivar</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double-distilled H₂O</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DREB</td>
<td>dehydration-responsive element binding</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eEF</td>
<td>eukaryotic elongation factors</td>
</tr>
<tr>
<td>ERF</td>
<td>ethylene-responsive genes</td>
</tr>
<tr>
<td>ERD</td>
<td>early responsive to dehydration</td>
</tr>
<tr>
<td>Gcn</td>
<td>general control non-repressible</td>
</tr>
<tr>
<td>GNAT</td>
<td>Gcn5-related N-terminal acetyltransferases</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>---------</td>
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</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>K</td>
<td>lysine</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LEA</td>
<td>late embryogenic abundant</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MYST</td>
<td>MOZ, Ybf2/Sas3, Sas2, and Tip60</td>
</tr>
<tr>
<td>PetE</td>
<td>pea plastocyanin gene</td>
</tr>
<tr>
<td>PLT</td>
<td>PLETHORA</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<td>RAB</td>
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<tr>
<td>RBCS</td>
<td>ribulose bisphosphate carboxylase/oxygenase small subunit gene</td>
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<tr>
<td>SAGA</td>
<td>Spt-Ada-Gcn5-Acetyltransferase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SHY</td>
<td>short hypocotyl</td>
</tr>
<tr>
<td>ssp</td>
<td>subspecies</td>
</tr>
<tr>
<td>TAF</td>
<td>TATA-binding protein-associated factor</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start sites</td>
</tr>
<tr>
<td>Ubq</td>
<td>Ubiquitin</td>
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<tr>
<td>VP</td>
<td>viviparous</td>
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Chapter 1: Introduction

1.1 Chromatin in eukaryotes

In eukaryotes, genomic DNA is highly and tightly folded into a complex structure called chromatin, which enables DNA to be constricted into the limited space in the nucleus. In this compaction, four core histones (H2A, H2B, H3, and H4) and one linker histone (H1) play a very important role. First, two H2A and two H2B are formed into two H2A-H2B dimers, whereas two H3 and two H4 are formed into one (H3-H4)$_2$ tetramer. Then the protein octamer, which is maintained by hydrogen bonds, is assembled through connecting two H2A-H2B dimers and one (H3-H4)$_2$ tetramer (Eickbush and Moudrianakis, 1978). The 146 base pairs (bp) of DNA, which are folded into 1.65 turns of flat, left-handed superhelix, are wrapped around the histone octamer (Eickbush and Moudrianakis, 1978). Interactions between histone-fold domains and DNA account for the organization of 121bp of DNA directly. Both histone-fold pairs, H2A-H2B dimer and (H3-H4)$_2$ tetramer, are associated with 27-28bp of DNA, which leaves 4bp linkers between every two units. There are 14 contact points between these histones and DNA in every single nucleosome that consists of the nucleosome core, linker DNA and H1. These contact points generate a very stable DNA-protein connection (Luger et al., 1997). Every nucleosome is linked to another adjacent nucleosome by approximately 50bp of DNA to create a 10-nm fiber, which is also known as the ‘beads on a string’ model (Luger et al., 1997). Finally, with the help of linker
histone H1, this 10-nm fiber is further packaged into a 30-nm fiber which is then further condensed into chromosomes (Luger et al., 1997).

1.2 Chromatin remodeling

The packaging of genomic DNA impedes many biological processes that require access to DNA, such as gene transcription, DNA repair, and replication. In order to control genomic activities, the chromatin structure is dynamically regulated. This remodeling, which happens continuously, is the basic component of genetic and epigenetic regulation of genome expression (Horn and Peterson 2002). DNA methylation, nucleosome remodeling, and covalent modification of histones are three inter-connected processes regulating the chromatin remodeling (Loidl, 2004; Pfluger and Wagner, 2007).

DNA methylation, a common epigenetic modification that usually happens at position five of the pyrimidine ring of cytosine, is mostly associated with heterochromatic, transcriptionally inactive regions in plants (Zemach and Grafi, 2007). Nucleosome remodeling includes both transcription-induced nucleosome movements and nucleosome composition replacement. The nucleosome movements may be caused by nucleosome sliding, histone subunit exchange, complete nucleosome eviction, or histone variant replacement (Rando and Ahmad, 2007), whereas nucleosome composition replacement includes replacement of histone H3, which indicates the eviction and replacement of the whole nucleosome (Thiriet and Hayes, 2005). ATP (adenosine triphosphate)-dependent chromatin remodeling enzymes are one of the major enzyme families that can alter nucleosome positions (Whitehouse and Tsukiyama, 2006).
According to the study of the yeast Isw2, which represses many meiosis-specific genes (Goldmark et al., 2000), the continuous presence of Isw2 is required to position nucleosomes to the target promoter region, which results in transcriptional repression. Removal of Isw2 induces the nucleosome to slide to another position, which allows transcriptional factors to bind to target sites and activates target genes (Whitehouse and Tsukiyama, 2006). The flexibility in nucleosome positioning contributes to not only chromatin remodeling, but also other processes such as gene transcription (Rando and Ahmad, 2007). Histone variants in plants, including CenH3, H3.3 and H2A.Z, are also involved in diverse arrays of nucleosome remodeling. CenH3 plays a vital part in chromosome segregation, whereas H3.3 and H2A.Z are both essential in gene expression regulation (Deal and Henikoff, 2011). Covalent modifications of histone proteins, including acetylation, methylation, ubiquitylation, phosphorylation, sumoylation, deamination, ADP ribosylation, and proline isomerization, take place at specific residues of N-terminal “tail” domains that are sticking out from the surface of the chromatin polymer (Kouzarides, 2007). These modifications may change the interaction between histone and DNA, or, histone and histone to alter chromatin structure that associates with transcriptional activation. Histone acetylation, the most extensively studied histone modification, is associated with both gene transcription and biological processes. The “histone code” hypothesis, which is also referred to as histone “language” that is encoded on specific tail domains, suggests that several histone modifications may act in combination to or sequentially on one or multiple histone tails to result in unique downstream functions (Strahl and Allis, 2000).
1.3 Histone acetylation

Histone acetylation as a regulator of chromosomal activities has been of great scientific interest for decades. It was first discovered in 1964 that acetyl and methyl groups can be introduced to histones and that both of these two modifications happen after the polypeptide chain is assembled (Allfrey et al., 1964). As a reversible regulatory mechanism, the equilibrium of histone acetylation is maintained by histone acetyltransferase (HATs) and histone deacetylases (HDACs) utilizing the acetyl moiety from acetyl CoA (Kuo and Allis, 1998). HATs add the acetyl groups to confined lysine residues in the amino-terminal tails of the core histones, while HDACs remove these acetyl groups from those sites (Figure 1.1). Various lysine residues, such as K9, K14, K18, K23, K27 and K56 of H3, K5, K8, K12, K16 and K20 of H4, K5 of H2A, and K5 of H2B, are target sites for acetylation (Selvi et al., 2010).

Usually, hyperacetylation of histones is linked to transcriptional activation of chromatin, whereas weak acetylation leads to chromatin compaction (Figure 1.2) (Struhl, 1998). There are two different proposals explaining this phenomenon. On one hand, in eukaryotes, lysine and arginine amino acids are both basic components of amino-terminal tails of histones, which are positively charged at their physiological pH. Potentially, this positive charge helps the binding of histones to the DNA backbones which are negatively charged due to the phosphates (Hong et al., 1993). However, the introduction of the acetyl groups to conserved lysine residues neutralizes the positive charge and enhances the hydrophobicity of the core histones, which in turn reduces their affinity for the negatively charged DNA (Luger and Richmond, 1998). On the other hand, the “histone
code” hypothesis proposes that covalent modifications, including acetylation and methylation, could work sequentially and jointly. The variation of concentration and combination of different covalent modifications would change the interaction between chromatin and chromatin-associated proteins and provide signals for recruitment of transcriptional machinery. Consequently, the switch between euchromatic (on) and heterochromatic (off) states is made (Jenuwein and Allis, 2001).
Figure 1.1 HATs and HDACs maintain the equilibrium of histone acetylation.

Acetyl coenzyme A donates the acetyl moiety to histone acetylation, and removed acetyl groups from acetylation are transferred to H$_2$O. HATs add the acetyl moiety to the lysine residues of histones, while histone deacetylases remove the acetyl group from the histones. The activities of HATs and HDACs regulate the equilibrium of histone acetylation. K: Lysine.
Figure 1.2 Histone acetylation regulates DNA accessibilities

The introduction of acetyl groups, catalyzed by HATs, causes a relaxation of chromatin compaction and transcriptional activation. However, the removal of acetyl groups induces the packing of chromatin which reduces the DNA accessibility. The level of histone acetylation (strong/weak) is linked to the switch between euchromatic and heterochromatic states.
1.4 Histone acetyltransferases (HATs)

Studies on HATs started more than 35 years ago. In 1979, Cano and Pestana purified an enzyme with HAT activity from the nuclei of 40h-old Artemia salina larvae, which extensively modified histone H1 (Cano and PastaÑA, 1979). In 1995, Brownell and Allis identified the first nuclear HAT p55, a single polypeptide of 55 kDa, from macronuclei of Tetrahymena thermophile using an acetyltransferase activity gel assay (Brownell and Allis, 1995). Then the discovery that this nuclear HAT p55 is a homologue to the yeast Gcn5p (general control non-repressed) protein, a well-studied positive transcriptional regulator of many genes, established the connection between histone acetylation and gene activation (Brownell et al., 1996). This connection was further confirmed in 1998, when the HAT activity was found necessary for the stimulation of transcription by Gcn5p (Kuo et al., 1998). Since that time, a direct link between HATs and the activation of gene transcription has been built.

Characterization of HATs in Arabidopsis thaliana revealed three distinct families of HAT proteins in plants. The first one is the GNAT (Gcn5-related N-terminal acetyltransferases)-MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) family (Neuwald and Landsman, 1997). The GNAT family, which includes four subfamilies (Gcn5, ELP3, HAT1 and HPA2), is defined by a HAT domain that contains four motifs (A-D), while the MYST proteins possess only one motif(A) in their domains (Pandey et al., 2002). The second family is the p300/CBP (cAMP responsive element-binding protein) co-activator family (Bannister and Kouzarides, 1996). Domains of this family are different
between plants and animals and are absent in fungi (Pandey et al., 2002). The last one is the TAF\(_{11}250\) (TATA-binding protein-associated factor TAF1) family (Mizzen et al., 1996), which is similar among plants, human and \textit{D. melanogaster} (Pandey et al., 2002). A total of 12 HAT genes have been characterized in \textit{Arabidopsis} of which five belong to the GNAT/MYST family, five belong to the CBP family, and two belong to the TAF\(_{11}250\) family (Pandey et al., 2002).

Until now, research on plant HATs is still quite limited and mainly in \textit{Arabidopsis}, which is a model dicotyledonous plant. According to the studies of HATs in \textit{Arabidopsis}, along with other species, HATs play a central role in chromatin modification, which associates with genome-wide gene transcriptional activation, and are involved in various biological processes in response to internal and external signals, such as cell differentiation, growth, development, light, temperature, and abiotic and biotic stresses (Chen and Tian, 2007).

### 1.4.1 HATs and plant growth

In \textit{Arabidopsis}, HATs, such as AtHAG1 which is a member of the Gcn5 subfamily that belongs to the GNAT family, play pivotal parts in plant growth and development. AtGCN5 is similar to yeast Gcn5, a HAT component of both transcription regulatory complex SAGA (Spt-Ada-Gcn5-Acetyltransferase) and ADA (Alteration/Deficiency in Activation), which acetylates histones H3 and H2B in nucleosomes (Vlachonasios et al., 2003). The \textit{athag1} mutant showed pleiotropic phenotypes, such as longer petioles in the first pair of true leaves and serrated leaves after unfolding in the second pair, which starts as folded upward. The palisade of the \textit{gcn5-1}
was smaller in size and the leaves were chlorotic. The mutant plants also displayed shorter stamens and petals and affected development of the inflorescence, which resulted in the dwarf morphology, the loss of apical dominance and reduced fertility (Vlachonasios et al., 2003). In the same year, AtGCN5 was found to be necessary for the floral meristem activity via the WUSCHEL/AGAMOUS pathway. Similar growth defects, such as smaller size of plants and loss of apical dominance were also observed in the mutant plants that have a T-DNA insertion in the 10th intron of AtGCN5 (Bertrand et al., 2003). In contrast, in 2006, it was found that mutation in GCN5 resulted in longer hypocotyls under light conditions, a characteristic indicating light-hyposensitivity (Benhamed et al., 2006). In 2009, Kornet and Scheres discovered that AtHAG1 is necessary for root stem cell niche maintenance through controlling the PLETHORA (PLT) gradient, which switches stem cells from quiescence (high level), division (intermediate level), and differentiation (low level) (Kornet and Scheres, 2009). Additionally, mutants of HAG1, hag1-5 and hag1-6, demonstrated different levels of pleiotropic defects in shoot, root and meristem sizes. The hag1-6 mutant plants can only be maintained as heterozygotes because they are infertile (Kornet and Scheres, 2009).

Other HATs, for instance AtHAC1, AtHAG3, AtHAM1 and AtHAM2, also proved to be important in plant growth and development (Nelissen et al., 2005; Deng et al., 2007; Latrasse et al., 2008).

1.4.2 HATs and stress responses in plants

Plants, as sessile organisms, have to develop a response system against rapid changes in their living environment. The need for the alteration of stress
inducible/repressible gene expression makes the precise control of chromatin modification, which is partially controlled by histone modification, important. A series of studies have shown that histone acetylation is involved in abiotic and biotic stress responses in plants.

1.4.2.1 HATs and abiotic stresses responses in plants

Changes in light signal, including light quantity, quality, periodicity and direction, are vital environmental factors for plants (Franklin and Whitelam, 2004). The light-inducible gene, PetE (pea plastocyanin gene), plays a critical role in the photosynthetic electron transfer chain by transferring electrons from cytochrome to the primary donor P700. It was first discovered in green pea shoots that the increased transcription level of PetE is related to hyperacetylation of histone H3 and H4 at the enhancer/promoter region (Chua et al., 2001). This connection was then further confirmed in transgenic tobacco green shoots. The acetylation of histones has a direct positive effect on the expression of PetE by targeting its promoter and the nearby coding region (Chua et al., 2003). The characterization of an Arabidopsis TAFII250 family mutant, haf2-1, made the association between light signals and HATs more clear. Firstly, the chlorophyll accumulation was reduced. The mRNA levels of both light-induced CAB2 (chlorophyll a/b-binding protein gene) and RBCS-1A (ribulose bisphosphate carboxylase/oxygenase small subunit gene) as well as the promoter activity of CAB2 were significantly inhibited. Secondly, a genetic screen showed that haf2-1 mutants grew longer hypocotyls, an indicator that the ability to perceive continuous far-red, red, and blue light was impaired. Thirdly, the acetylation of histone H3 at the TATA-proximal
promoter region and the expression of light-inducible genes in young leaves in general were both reduced (Bertrand et al., 2005). In the next year, the same lab demonstrated that the mutation of another HAT, GCN5, resulted in similar defects in Arabidopsis, such as longer hypocotyls and reduced expression of light-inducible genes. Moreover, the double mutant gcn5haf2 further decreased the transcript level of light-regulated genes. At the protein level, a cumulative reduction was mainly observed on H3K9 acetylation. However, acetylation of H3K14 was solely dependent on GCN5, while both GCN5 and HAF2 affected H3K9, H3K27, and H4K12 acetylation, suggesting acetylation of specific lysine residues on histone H3 and H4 is required for light-regulated gene expression (Benhamed et al., 2006). Histone acetylation, along with histone methylation, was shown to be involved in light-controlled gene transcription (Guo et al., 2008).

In the natural environment, plants inevitably encounter temperature fluctuations throughout their entire life cycles. The CBF (C-repeat binding factor) is a transcriptional co-activator that binds to the CRT/DRE regulatory element to activate the expression of various cold-regulated (COR) genes. The CBF genes could be induced within minutes after plants are transferred to a low-temperature (~4°C) environment, while the expression of downstream COR genes usually takes two hours (Gilmour et al., 1998). It was found in Arabidopsis that the regulatory activity of CBF1 is dependent on the Gcn5 and the transcriptional adaptors Ada2 and Ada3 (Stockinger et al., 2001). In gcn5 mutant plants, the activation of COR genes was reduced while the CBF genes were normally induced in cold acclimation experiments (Vlachonasios et al., 2003). Overexpression of CBF1 resulted in a constitutive increase in the acetylation of histone H3 at COR gene promoters upon cold acclimation. However, among all tested HATs, including GCN5, no
HAT can solely affect the histone H3 acetylation on the promoter region of COR genes (Pavangadkar et al., 2010).

The opposite of cold stress for plants is heat stress. It was found in tomatoes that the HsfB1, a class B heat stress transcription factor, could be assembled with class A HSFs. This complex can strongly activate heat stress associated gene transcription (Bharti et al., 2004). A histone-like motif, a signal for recruiting the plant CBP ortholog HAC1, was also observed in the C-terminal domain of HsfB1. Later, the discovery of the ternary complexes consisting of HAC1/CBP, HsfA1, and HsfB1 enhanced the efficiency of promoter recognition and transcription activation in plants (Bharti et al., 2004).

1.4.2.2 HATs and hormone responses in plants

The plant hormone ABA (abscisic acid) plays an important role in plant growth, development, and stress responses (Chen et al., 2006). For example, in tobacco and Arabidopsis cells, exogenous ABA treatment caused complicated dynamic response patterns in histone modification, including histone acetylation and phosphorylation, on histone H3 and H4 (Sokol et al., 2007). In 2010, three genes from the GNAT/MYST family, namely HvMYST, HvELP3 and HvGCN5, were demonstrated to be ABA-inducible in barley (Papaefthimiou et al., 2010). During maize seed germination, the expressions of HATs (ZmHAG101 and ZmHAG102) as well as histone acetylation were gradually enhanced. However, upon treatment with 10 μM ABA, the transcription of ZMHATs were repressed, which in turn, delayed the overall histone acetylation (Zhang et al., 2011). In addition, ABA was found to prevent the down-regulation of embryogenesis-related gene viviparous1 (VPI) by inhibiting the histone deacetylation of
its promoter region (Zhang et al., 2011). In *Arabidopsis*, the elongator, a HAT complex that activates RNAPII-mediated gene transcription, was shown to be associated with ABA signaling and plant responses to ABA (Chen et al., 2006; Zhou et al., 2009).

HATs are also involved in plant responses to other hormones. By controlling the histone H3 lysine 14 (H3K14) acetylation at the coding region of the auxin repressor *SHORT HYPOCOTYL 2 (SHY2)/IAA3* and the auxin influx carrier gene *LAX2*, AtHAG3 was shown to play a crucial part in auxin-related gene transcription in *Arabidopsis* (Nelissen et al., 2010). In the *gcn5* mutant plants, the expression of *SHY2/IAA3* was dramatically repressed (Benhamed et al., 2006). In addition, mutations in *AtHAG3* caused a significant increase in the ethylene emanation as well as an enhancement in the jasmonic acid (JA) content. This was further confirmed to be a result of the up-regulation of the ethylene-responsive gene (*AP2/ERF*) and the JA biosynthesis and response genes (*ACX1, AOS, LOX1, LOX2, VSP1, and COR1*) (Nelissen et al., 2010).

### 1.5 Drought stress

Drought stress, which is also called desiccation or water deficit stress, is a very common stress that is caused by water deficit in natural environments. It causes a range of morphological and physiological effects that induce various responses in plants (Farooq et al., 2009). First and foremost, it reduces the water potential and the relative water content (RWC) in leaves, which in turn causes stomatal closure, a decrease in transpiration and eventually an increase in leaf temperature (Siddique et al., 2000). This disturbs water relations and cell turgor in plants severely impairing membrane integrity, cell elongation, cell growth, cell division and cell differentiation (Nonami, 1998). In rice,
plant growth and development will be seriously delayed if drought stress happens during the vegetative phase (Manickavelu et al., 2006). Moreover, water deficit reduces total nutrient absorption by the roots and translocation to/from shoots (Garg, 2003). Last but not least, photosynthesis is also limited under drought stress mainly due to reduced CO2 assimilation of leaves caused by drought-induced stomatal closure (Tezara et al., 1999), disturbed generation and activity of photosynthetic enzymes such as Rubisco (Bota et al., 2004), and impaired ATP synthesis which plays an important role in limiting photosynthesis even under mild drought stress (Lawlor and Cornic, 2002).

1.6 Drought stress responses in plants

In order to acclimate to and survive under drought stress, various physiological and molecular mechanisms are induced in plants (Farooq et al., 2009). Osmotic adjustment, resulting from an accumulation of solutes, increases water influx to help maintain cell turgor. The maintenance of cell turgor, as a premise of water balance and nutrient translocation, helps to minimize and delay the damage caused by drought stress (Subbarao et al., 2000). The integrity and stability of the plasma membrane, which is selectively permeable, is an important physiological characteristic of drought tolerance (Premachandra et al., 1991b). The enhancement of potassium levels in maize, which is associated with the improvement of membrane stability, helps water-stressed plants adjust to water deficit (Premachandra et al., 1991a). Phytohormones regulate physiological processes in plants at very low concentrations (Morgan, 1990). Drought stress usually induces a decrease in the endogenous concentrations of auxins, gibberellins, and cytokinin, while ABA and ethylene normally increase (Nilsen and
Orcutt, 1996). Meanwhile, as a water-saving response to drought stress, the accumulation of abscisic acid or/and the reduction of cytokinin triggers stomatal closure (Turner et al., 2001). On the other hand, compatible solutes, such as proline, are commonly overproduced under various stresses, including drought, for osmotic adjustment, stabilization of macromolecules, and structural protection of proteins (Zhu, 2002).

At the cellular and molecular levels, an assortment of genes, the products of which function in drought stress responses, are induced or repressed when there is a water deficit (Kavar et al., 2008). The establishment of drought stress tolerance is a consequence of the cooperation of many proteins (Cattivelli et al., 2008). Aquaporins, a group of highly conserved membrane proteins, facilitate the movement of water molecules across membranes passively (Tyerman et al., 2002). It has been shown that aquaporins contribute to the total water uptake in root cells (Javot and Maurel, 2002). Drought stress also implicates transcription factors and protective proteins such as DREB (dehydration-responsive element binding) and LEA (late embryogenic abundant). The DREB genes are involved in abiotic stress signaling pathways (Agarwal et al., 2006), whereas LEA proteins, also known as dehydrins, concentrate to form a protective shield for other proteins during cellular dehydration (Gorantla et al., 2007). Since the switch between activation and repression of chromatin plays a central role in gene transcription in general, histone acetylation may be involved in regulating gene expression in response to environmental stresses, including drought stress (Chen and Tian, 2007).
1.7 **HATs play a role in drought stress responses**

Recent studies revealed a connection between histone acetylation and drought stress responses in plants. Three HATs that belong to the GNAT/MYST superfamily in barley, *HvMYST*, *HvELP3* and *HvGCN5*, were shown to be ABA inducible (Papaefthimiou et al., 2010). Later, it was demonstrated that the expression of HATs in rice could be regulated by phytohormones, such as ABA and salicylic acid, as well as abiotic stresses such as salt, cold, and heat (Liu et al., 2012). Also, overexpression of AtHD2C, a histone deacetylase homolog in *Arabidopsis*, in transgenic *Arabidopsis* plants resulted in ABA insensitivity and enhanced tolerance to drought and salt stresses (Sridha and Wu, 2006). In addition, drought-induced expression of drought-inducible genes is associated with an increase in H3K9 acetylation on the promoter regions and H3K23 and H3K27 acetylation on the coding regions (Kim et al., 2008). Comparative analysis of stress-inducible genes in *Arabidopsis* with those in rice revealed a considerable degree of similarity. Among the tested genes, 73 were identified as stress-inducible in rice, of which 51 have already been reported to perform a similar function in *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki, 2007), suggesting that rice shares common drought inducible genes with *Arabidopsis*.

1.8 **Thesis objectives**

In *Arabidopsis*, the association of histone acetylation and drought stress responses has been identified (Sridha and Wu, 2006; Kim et al., 2008). However, information about this direct connection in monocots and economically important species
is still very limited. More importantly, drought stress was the major factor of rice yield loss in Asia since rice seedlings need lots of water for their vegetative growth (Venuprasad et al., 2007). Based on this background, I chose rice, a model monocot species and an economically important crop, as my subject. I hypothesize that drought stress will cause an up-regulation of the expression of OsHATs in rice (Table 2.1). In order to test the expression pattern of different OsHATs families, four OsHATs, OsHAC703, OsHAG703, OsHAF701 and OsHAM701, one from each family, were tested.

My objectives were:

1. To test the change in total mRNA levels of these four OsHATs in response to different dehydration levels.

2. To identify if the acetylation level of total H3, certain lysine sites on H3 (K9, K18 and K27), and H4 (K5) will change if the mRNA levels of these tested OsHATs change.

The detailed knowledge of the role that HATs play in drought responses in rice will contribute to further understanding of molecular mechanisms that control drought stress responses in rice. This will eventually lead to a long-term improvement of drought stress tolerance in other crops.
Chapter 2: Materials and Methods

2.1 HAT cDNA and protein sequences search

In total, there are eight OsHATs in rice (Oryza sativa, japonica cultivar-groups) (Liu et al., 2012). All eight rice HAT cDNA sequences and protein sequences were searched and downloaded from the ChromDB (http://www.chromdb.org) and the UniProt (http://www.uniprot.org) databases (Table 2.1).

Based on the phylogenetic and domain information, these eight HATs (OsHATs) in rice can be grouped into four different families (Pandey et al., 2002). Three of them, OsHAC701, OsHAC703, and OsHAC704, belong to the CBP family. In this CBP family, sequence identity analysis showed that OsHAC703 and OsHAC704 are 80.0% identical in amino acid/nucleotide sequence, while OsHAC701 only shares 46.0% and 42.0% sequence identity with OsHAC703 and OsHAC704, respectively (Liu et al., 2012). Four OsHATs are grouped into the GNAT/MYST superfamily that share the same A motif of the HAT domain (Pandey et al., 2002). More specifically, three of them, OsHAG702, OsHAG703, and OsHAG704, are classified into the GNAT family, whereas OsHAM701 belongs to the MYST family (Liu et al., 2012). Finally, OsHAF701 is grouped into the TAFII250 family (Table 2.1).
Table 2.1 List of rice HAT proteins and their classification based on Pandey et al. (2002) and Liu et al. (2012)

<table>
<thead>
<tr>
<th>Protein Group</th>
<th>ChromDB ID</th>
<th>UniProt ID</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBP family</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAC701</td>
<td>HAC703</td>
<td>HAC704</td>
<td></td>
</tr>
<tr>
<td>Q9XHY7</td>
<td>Q6YXY2</td>
<td>Q5Z8V7</td>
<td>Os01g0246100</td>
</tr>
<tr>
<td>Os02g0137500</td>
<td>Os06g0704800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAFII250 family</td>
<td>HAF701</td>
<td>HAM701</td>
<td></td>
</tr>
<tr>
<td>Q67W65</td>
<td>Q8LI34</td>
<td></td>
<td>Os06g0645700</td>
</tr>
<tr>
<td>MYST family</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAG701</td>
<td>HAG702</td>
<td>HAG703</td>
<td></td>
</tr>
<tr>
<td>Q338B9</td>
<td>Q7X7L3</td>
<td>Q6ES10</td>
<td>Os10g0415900</td>
</tr>
<tr>
<td>Os04g0484900</td>
<td>Os09g0347800</td>
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</tr>
<tr>
<td>GNAT family</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HAG704</td>
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<td></td>
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</tr>
</tbody>
</table>
2.2 Sequence analyses and alignments

Protein domains and functional sites of *OsHAC703*, *OsHAG703*, *OsHAF701* and *OsHAM701* were searched in the ChromDB database. Sequences of these four genes were downloaded from the UniProt database and the protein domains and functions were identified and assigned with InterProScan using the SWISS-MODEL Workspace website (http://swissmodel.expasy.org/workspace/index.php?func=tools_sequencescan1).

2.3 Promoter analyses

Information about the transcription start sites (TSS) and the promoter regions of *OsHAC703*, *OsHAG703*, *OsHAF701* and *OsHAM701* were downloaded from the plant promoter database 3.0 (http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/index.cgi), which provides promoter annotation of rice and *Arabidopsis*. Then cis-elements within 1200 bp upstream of the obtained TSS were searched and analyzed by the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

2.4 Plant growth conditions and drought treatment

Rice (*Oryza sativa* ssp. *japonica* cv. Nipponbare) seeds were originally obtained from the South China Botanical Garden (Chinese Academy of Sciences). The rice seeds were imbibed with distilled water in darkness for 24 hours at 37 ± 1°C and then placed on two filter papers (VWR cat. no. 28320-041) soaked with double-distilled water in a Petri dish at room temperature. After germination in darkness for two days, germinated seeds were then transferred to the light condition, in which the light intensity was 330
After another two days, when the length of the seedling roots were estimated to be 2-3 cm, rice seedlings were planted into clay soil in a growth chamber maintained at 9/15 hours light/dark photoperiod at 29°C.

After seven days, the rice seedlings were at the two-leaf-stage and distributed into two groups. The drought treatment group was subjected to drought stress by withholding water for a total of 33 hours, which is before the permanent wilting point since rice seedlings can recover if re-watered after this 33-hour drought treatment. The control group was watered twice each day during the same period. Based on preliminary results of OsHATs from real-time qPCR, leaves of the rice seedlings were harvested after 24-hour, 29-hour, and 33-hour drought treatment for RWC measurements (Figure A-1), RNA isolation and protein isolation. Since histone acetylation is also involved in light-regulated gene expression in Arabidopsis (Benhamed et al., 2006), it is important to test the expression change of OsHATs in different light conditions at the same time. Therefore, the 33-hour samples were collected after the seedlings grew in darkness for three hours, while the 24-hour and 29-hour samples were collected in the light. To prevent rapid water loss and to retain viability, the plants were covered with a transparent plastic lid after 29 hours for the drought treatment group, while seedlings in the control group were always covered with a plastic lid. For RNA and protein isolation, harvested leaves were immediately frozen in liquid nitrogen and then stored at -80°C.

2.5 RWC measurement

To assess the intensity of the drought stress, the RWC (Barrs and Weatherley 1962) of leaves was measured for each group. Immediately after sampling the leaves of
the drought treatment and the control plants, leaves were excised and immediately weighed to give the fresh weight \((W_{\text{fresh}})\). This leaf was then placed into a 50°C oven for about 24 hours to give the dry weight \((W_{\text{dry}})\). RWC was calculated according to the following equation:

\[
\text{RWC} = \frac{(W_{\text{fresh}} - W_{\text{dry}})}{W_{\text{fresh}}}
\]

where RWC is expressed as a percentage of the water in the fresh plant leaf when it was collected.

### 2.6 RNA isolation and real-time qPCR analyses

Total RNA was extracted from leaves of the two-leaf-stage rice seedlings using a Plant/Fungi Total RNA Purification Kit (Norgen, cat. no. 25800). The quality and quantity of RNA were then measured by a Thermo Scientific NanoDrop™ 1000 spectrophotometer (Wilmington, DE, USA). Before cDNA synthesis, the total RNA was treated with DNaseI (Norgen, cat. no. 25710) for 20-30 min. The first strand cDNA was synthesized from 2 µg RNA with the ThermoScript™ RT-PCR System (Life Technologies, cat. no. 170-8841) with oligo-dT primer. The synthesized cDNA then served as a template for real-time qPCR using SsoFast™ EvaGreen® Supermix Kit (Bio-Rad, cat. no. 172-5202) and data were collected by Bio-Rad C1000™ Thermal Cycler with the CFX96™ Real-Time PCR System.

The real-time qPCR data were expressed as the cycle number necessary to reach a threshold fluorescence value \((Ct)\) and analyzed with the comparative Ct method \((\Delta \Delta Ct)\). The reported values were the means of three biological replicates, and each
biological replicate consisted of three technical replications. *Ubq-1* (AK059011.1, Ubiquitin) was used as a reference gene to normalize the expression data. *OsDREB2A* and *OsLEA3-1*, which are both involved in drought stress responses and drought-inducible in rice (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiao et al., 2007; Mallikarjuna et al., 2011), were selected as positive controls to determine whether the drought treatment was effective in this study. The primers designed for real-time qPCR are listed in Table 2.3. Amplification of efficiency and co-efficiency are shown in the appendix (Table A-2).

All real-time qPCR data were expressed as the mean ± standard deviation. Statistical differences of expression of each *OsHAT* between different treated groups and their corresponding control groups were assessed by the Student’s t-test. Significance was established at p<0.05 or p<0.01. All statistical analyses were performed using Microsoft Excel spread sheet software.

### 2.7 Protein isolation and western-blot analyses

Acid-soluble proteins were extracted following Tariq et al (Tariq et al., 2003), in which a total of 0.3 g fresh rice leaves were crushed in liquid nitrogen and resuspended in 2.25 mL lysis buffer (0.25 N HCl, 10 mM pH 6.8 Tris-HCl, 2 mM EDTA, 20 mM β-mercaptoethanol and 0.2 mM phenylmethlsulfonyl fluoride). The total proteins were homogenized by a Fisher Scientific Model 100 Sonic dismembrator for 2 min and then centrifuged for 15 min (4°C, 20,000 rcf, twice); the supernatant was collected and stored at -80°C. The quantitative analysis of protein was determined by the Micro-Bradford Assay using a Biochrom Novaspce Plus Visible Spectrophotometer before being used for
SDS-PAGE electrophoresis. Precisely 5 µg protein were added to 18.5 mM dithiothreitol (DTT), separated on a 16% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide electrophoretic gel, and transferred to an Immun-Blot\textsuperscript{TM} polyvinylidene fluoride (PVDF) Membrane (Bio-Rad cat. no. 162-0177) using a Trans-Blot Semi-Dry electrophoretic Transfer Cell (15 V, 50 min, Bio-Rad). The N-terminal lysine residues in histones H3 and H4 were detected using commercial antibodies and secondary antibodies from Cell Signaling and Millipore (Table 2.2). Histone H3 was used as an equal loading control. Finally, the bound immune-complexes were detected with ECL Prime Western Blot detection reagents (GE health care Life Sciences, VWR cat. no. CA89168-782) and exposed to Classic Single-Emulsion Autoradiography Film (Mandel Scientific). The films were then automatically developed by an AGFA CP1000 X-Ray Film Processor and scanned with an UMAX Powerlook 1120 scanner.

In order to test another antibody on the same membrane, after exposure and development, the membrane was washed with TBST several times and incubated in a water bath with the western blot stripping buffer [60m M Tris-HCl pH 6.8, 0.7% (v/v) β-mercaptoethanol and 2% SDS (w/v)] at 50°C for 30 min. After being washed with TBST for another five times, the membrane was ready for another blocking.
Table 2.2 Antibodies for western-blot

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Purification</th>
<th>Source</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H3</td>
<td>Monoclonal</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Acetyl-Histone H3</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>Acetyl-Histone H3(Lys9)</td>
<td>Monoclonal</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Acetyl-Histone H3(Lys18)</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Acetyl-Histone H3(Lys27)</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Acetyl-Histone H4(Lys5)</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>N/A</td>
<td>Goat</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>
2.8 Primers

All primers used in this research, unless specifically pointed out, were designed using Primer3 (http://frodo.wi.mit.edu/primer3) software for real-time qPCR. The parameters were set up as follows: primer size was 20-23-25, primer Tm was 57-60-63, and primer GC content was 40-60%, while the product size was 85–250 bp. The primers listed in Table 2.3 are shown in the 5’ to 3’ orientation.
**Table 2.3 Designed primers for real-time qPCR**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ubg-1</em></td>
<td>AACCAGCTGAGGCCCAAGA</td>
<td>ACGATTGATTTAACCAGTCCATGA</td>
</tr>
<tr>
<td><em>HAC701</em></td>
<td>TGGCGGTGCTTGGTTTGCCT</td>
<td>ACGGGCAGGGGTATGACATCGT</td>
</tr>
<tr>
<td><em>HAC703</em></td>
<td>TGTTGAAGAGGTAACGTGGG</td>
<td>GCTTCAACCGTTAAAAAGCGCA</td>
</tr>
<tr>
<td><em>HAC704</em></td>
<td>CAGTGACGAACCAGAGGAGGTG</td>
<td>AGGCATGCGCAACACGTTTT</td>
</tr>
<tr>
<td><em>HAF701</em></td>
<td>ACCAGTGCCGACGATGACGA</td>
<td>TCCGCGAGTCAAAAAGGTGCT</td>
</tr>
<tr>
<td><em>HAG702</em></td>
<td>TGCTTCGKGCGCTTCTTACATGC</td>
<td>CAGCATCTCGGCAATGTTGCTTCA</td>
</tr>
<tr>
<td><em>HAG703</em></td>
<td>TGCTGCAAATGAGGGGCTGGA</td>
<td>CGGCCACATTTTCGCAATCGCA</td>
</tr>
<tr>
<td><em>HAG704</em></td>
<td>AAGCGGCTGGTCCAAATGCC</td>
<td>TTGCGCGGTAGGGTGACGTT</td>
</tr>
<tr>
<td><em>HAM701</em></td>
<td>ACCGGAGCAGCCCCCTTTTCTGAT</td>
<td>AGAACCTTTGGGTCAGCGCA</td>
</tr>
<tr>
<td><em>OSDREB2A</em></td>
<td>GATGGGTTTAGGCTTCTCTCA</td>
<td>CTACAAAGCTTCAGTTGACAGAT</td>
</tr>
<tr>
<td><em>OSLEA3-1</em></td>
<td>TATCGATGCTGATGAGGCTTGA</td>
<td>CGAGAAACTCTGTACGAAAACAAC</td>
</tr>
</tbody>
</table>
Chapter 3 : Results

3.1 Various functional domains present in the four HAT families

The InterProScan analysis identified four domains comprising a CREB binding protein domain, a FYVE/PHD-type zinc finger, a ZZ-type zinc finger, and a TAZ-type zinc finger in OsHAC703 (Figure 3.1). The CREB binding protein domain, as part of the CBP-type HAT domain, is involved in histone acetylation and transcriptional coactivation (Pandey et al., 2002), whereas the FYVE/PHD-type, ZZ-type and TAZ-type zinc fingers are involved in protein recognition and protein–protein interactions (Bienz, 2006; Gamsjaeger et al., 2007; Lallous et al., 2011). The CREB protein binding domain is highly conserved in the plant CBP family. It is the number and species of the zinc-finger domains that varies in the CBP family. The number and species of proteins that these HATs bind to depends on the versatility of these zinc-fingers (Gamsjaeger et al., 2007).

A GCN5-related N-acetyltransferase domain and a HAT ELP3 domain were discovered in OsHAG703 (Figure 3.1). In Arabidopsis, the GNAT family includes three HATs, each belonging to one of the Gcn5, the ELP3, and the HAT1 subfamilies (Pandey et al., 2002). Likewise, in rice, OsHAG702 is classified to the Gcn5 subfamily, OsHAG703 is grouped into the ELP3 subfamily, while OsHAG704 belongs to the HAT1 subfamily (Liu et al., 2012), suggesting that these HATs may form similar complexes for downstream functions in Arabidopsis and rice.
In OsHAF701, three domains, including an Ubiquitin domain, a bromodomain, and a TAF$_{II}$-230 TBP-binding domain, were identified (Figure 3.1). The bromodomains are highly conserved in plants and capable of binding to acetylated lysine residues in histone tails (Marmorstein and Berger, 2001). OsHAM701 contains two domains, a MOZ/SAS-like protein domain and a chromodomain (Figure 3.1). Dehydration stress related cis-acting regulatory elements were also discovered from the promoter analysis of all four OsHATs (Table A-3) using the PLACE database.
Figure 3.1 Domains in four OsHATs in rice.

The structure of the four OsHATs and the locations of domains (boxes in various colours) in rice are shown. The colored boxes and lines represent exons and introns, respectively. The amino acid sequences of the four OsHATs were downloaded from the UniProt database and the protein domains were identified and assigned with InterProScan using the SWISS-MODEL Workspace tool.
3.2 **RWC decreased significantly after drought treatment**

To induce a drought stress, water was withheld from seven-day-old rice seedlings, which were at their two-leaf-stage. Relative water content was used as an indicator of the intensity of the dehydration. As demonstrated in the Figure 3.2, in the treatment groups, the RWC decreased from 55±1.5% to 40±2.3% in the first five hours, while in the last four hours, it decreased from 40±2.3% to 36±2.6%. On the other hand, in the control groups, the RWC remained at similar levels at all three time points (78.3±0.15%, 80.1±3.0%, and 77.9±1.7% respectively) (Figure 3.2).
Figure 3.2 Change in RWC in rice leaves after dehydration treatment

Drought stress was performed by withholding water. Seedlings in the treatment group were covered with lids after drought treatment for 29 hours to prevent water loss, while control groups were regularly watered and always covered with lids. The first two groups (24 and 29 hours) were collected in light conditions; whereas the 33 hour group was sampled after the seedlings had been growing in the dark for three hours. After obtaining the fresh weights and dry weights, RWCs were calculated from \( \frac{W_{\text{fresh}} - W_{\text{dry}}}{W_{\text{fresh}}} \) and expressed as the mean ± SD. The results are the average of three biological replicates, and each biological replicate consisted of three repeats. Two sample t-tests were used for data analysis, and * indicates a significant difference between the treated group and the control group at \( p < 0.01 \).
3.3 Expression of drought-inducible genes *OsDREB2A* and *OsLEA3-1* was induced

The DREB transcription factors bind to the DRE (A/GCCGAC) core cis-acting sequences in the promoter regions of stress-responsive genes to regulate these genes’ expression in an ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 2000). As for LEA proteins, which are ABA-inducible and associated with many stress responses in plants, they independently help prevent protein aggregation due to water loss (Goyal et al., 2005). In rice, both *OsDREB2A* and *OsLEA3-1* are drought-inducible, and the over-expression of both genes enhances drought resistance (Xiao et al., 2007; Cui et al., 2011)

According to the data from the real-time qPCR, the expression of both *OsDREB2A* and *OsLEA3-1* were induced at different levels of drought stress. More specifically, after being treated by drought for 24, 29 and 33 hours, the transcript levels of *OsDREB2A* were increased by 7.8-fold, 9.6-fold, and 7.0-fold, while *OsLEA3-1* transcript was elevated by 1063-fold, 1619-fold, and 221-fold, respectively (Figure 3.3). These data indicated that the drought stress treatment was effective.
Figure 3.3 Expression of drought stress inducible genes, *OsDREB2A* and *OsLEA3-1*, under drought stress conditions.

The expressions of *OsDREB2A* and *OsLEA3-1* were analyzed by real-time qPCR. The relative amounts of mRNA for *OsDREB2A* and *OsLEA3-1* were measured and normalized to those of *Ubg-1* (internal control). The values of treated groups were normalized to their corresponding controls which were defaulted as 1. The data shows the normalized fold change of treatment groups compared to their corresponding controls. Results were confirmed by three biological replicates. Data in this figure were the means of three replicates, while error bars represent their standard deviations. Two sample t-tests were used for significance analyses, ** indicates a significant difference between the treatment and the control at p < 0.01.
3.4 Expression change patterns of *OsHATs* under drought stress

Drought stress, which is considered to be a result of water deficit, is characterized by the reduction of RWC in this study. Real-time qPCR analysis was used to examine the expression of four *OsHATs* (*OsHAC703*, *OsHAG703*, *OsHAF701* and *OsHAM701*) at three different dehydration levels, which were performed by withholding water and assessed by the measurement of RWC (Figure 3.2).

In order to obtain accurate and reliable gene expression data for *OsHATs*, *Ubq* (Ubiquitin) was selected as the reference gene for real-time qPCR (Jain et al., 2006). At the same time, two drought inducible genes in rice, *OsDREB2A* and *OsLEA3-1*, were used as positive controls for the drought stress (Figure 3.3) (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiao et al., 2007; Mallikarjuna et al., 2011).

Different expression patterns were demonstrated among these four *OsHATs* at three different dehydration levels. Firstly, after watering was withheld for 24 hours, the difference in the RWCs between the treatment group (55±1.5%) and the regularly irrigated group (78.3±0.15%) was already statistically significant (Figure 3.2). At this time point, the positive control *OsDREB2A* transcription increased by 7.8-fold compared to the control group, indicating that the drought stress treatment was effective (Figure 3.3). The expression of *OsHAC703* and *OsHAG703* showed a similar expression pattern compare to the positive control as both increased significantly. In contrast, the transcript level of *OsHAM701* did not change and the *OsHAF701* expression showed a significant decrease (Figure 3.4).
For the second dehydration level, which was without watering for 29 hours, the RWC dropped from 55±1.5% to 40±2.3% in the treatment group while no significant water loss was observed in the control group (78.3±0.15% vs. 80.1±3.0%) (Figure 3.2). At this time point, DREB2A transcription increased by 9.6-fold, suggesting that the drought treatment was effective. Meanwhile, the expression levels of OsHAC703, OsHAG703, OsHAM701 and OsHAF701 all increased significantly by 2.7-fold, 2.2-fold, 5.3-fold and 7.7-fold, respectively (Figure 3.4).

At the last dehydration level, the RWC at this time point was 36% (Figure 3.2) and the effectiveness of the dehydration was indicated by the 7.0-fold increase in the transcription level of the DREB2A. The expression levels of OsHAC703, OsHAG703, OsHAM701 and OsHAF701 all increased significantly by 3.8-fold, 4.6-fold, 8.5-fold and 5.2-fold, respectively (Figure 3.4).

Taken all together, among the three different time points (24, 29 and 33 hour), the expressional change pattern of OsHAF701 showed a difference from the other three genes (Figure 3.4). Instead of gradual increase, the increase of the transcript level of OsHAF701 decreased for about 2.5 fold (from 7.7 fold to 5.2 fold) between the last two dehydration levels (Figure 3.4).
Figure 3.4 Expression patterns of OsHATs in rice leaves among different drought treatments.

The expressions of four OsHATs (OsHAC703, OsHAG703, OsHAF701 and OsHAM701) after treatment by drought for 24, 29, and 33 hours were analyzed by real-time qPCR. The relative amounts of mRNA for these four OsHATs were measured and normalized to those of Ubq-1. The data shows the normalized fold change of these four OsHATs transcription of treatment groups compared to their corresponding controls. Results were confirmed by three biological replicates, data in this figure were the means of three repeats, and error bars represent standard deviations.
3.5 **Acetylation on certain lysine residues were increased**

Histone acetylation takes place mainly on lysine residues of histones. Acetylation at certain lysine residues is conserved while for others it is not. Mass spectrometry (MS), together with high performance liquid chromatography (HPLC), identified that K9, K14, K18, K23 and K27 of histone H3 and K5, K8, K12, K16 and K20 of histone H4 can be acetylated in plants (Zhang et al., 2007). In order to examine whether the acetylation levels of different sites in histone H3 (K9, K18 and K27) or H4 (K5) change in response to drought stress, western-blot analysis was performed using antibodies against specific acetylated histone lysine residues (Table 2.2). Bands from the scanned films were quantified with the ImageJ program (http://rsbweb.nih.gov/ij/) (Figure A-4).

After treatment by drought for 24 hours, when the RWC was 55±1.5% (Figure 3.2), the transcription levels of *OsHAC703* and *OsHAG703* in leaves of seven-day-old rice seedlings increased by approximately two-fold (Figure 3.4). With regard to the protein level, western blot analysis showed that the acetylation of histone H3K18, H3K27, and H4K5 were elevated by about 2.8-fold, 3.3-fold, and 2.7-fold compared to the internal reference (Histone H3), while lower increase in the acetylation of histone H3K9 and total H3 was observed (Figure 3.5).

In response to drought treatment for 29 hours, the expression of all four *OsHATs* tested showed significant increases (Figure 3.4), and the RWC from leaves of two-leaf-stage seedlings in the treated group was significantly lower than the control (40±2.3% vs. 80.1±3.0%) (Figure 3.2). The acetylation level of total H3, histone H3K9, H3K18, H3K27, and H4K5 all showed considerable increase (Figure 3.5). Remarkably, the
acetylation level of H3K18 in the treated group was more than 26 fold higher than the control group (Figure 3.5).

After drought treatment for 33 hours, the leaves were sampled in darkness and the RWC fell from 40±2.3% to 36±2.6% (Figure 3.2). The transcript levels of all four *OsHATs* were still significantly higher than their controls (Figure 3.4). Western blot analysis demonstrated that the acetylation of total H3, histone H3K9, H3K27, and H4K5 stayed elevated (8.1, 6.0, 2.2, and 1.8 fold, respectively), whereas no difference in the acetylation of histone H3K18 was found (Figure 3.5).
Figure 3.5 Changes of histone acetylation on lysine residues of histone H3 and H4 in response to drought treatment for 24, 29 and 33 hours in rice leaves.

Proteins were extracted from leaves of two-leaf-stage rice seedlings subjected to drought for 24, 29 and 33 hours or watered regularly. Western blot analysis was performed with specific antibodies (Table 2.2) and representative blots are shown in Figure A-4. The values of treated groups were normalized to their corresponding controls which were defaulted as 1. Histone H3 was used as an internal loading control. The data shows the normalized fold change of acetylated lysine residues on h3 and h4 of treatment groups compared to their corresponding controls. Results in the graph were confirmed by three biological replicates and three repeats and error bars represent standard deviations.
Chapter 4 : Discussion

During this study, four OsHATs, OsHAC703, OsHAG703, OsHAF701 and OsHAM701, one from each HAT family in rice, were evaluated to check whether they are associated with drought stress responses in rice seedlings. They were chosen based on in silico domain and promoter analyses. Different expression patterns were demonstrated among these four genes at different dehydration levels by real-time qPCR. In addition, western-blot analysis showed that the acetylation level on certain lysine residues of H3 (K9, K18 and K27) and H4 (K5) increased accordingly.

4.1 Drought-inducible genes are governed by two pathways

Stress-inducible genes usually contain cis-acting elements in their promoter regions that are associated with stress-responsive gene expression. At cellular and molecular levels, cis-acting elements interact with transcription factors to determine gene transcriptional initiation (Shinozaki et al., 2003). Transcriptome profiling of cis-acting regulatory elements in the promoter regions of drought inducible genes revealed two different regulating pathways in response to drought stress, ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki, 2005). In the ABA-dependent pathway, the expression of the downstream genes is affected by the level of ABA. However, in the ABA independent pathway, the expression of the responsive genes operates regardless of the ABA change.
In dehydration stress responses in plants, there are two main *cis*-acting elements, ABRE (ABA responsive elements) and DRE/CRT, which function in the ABA-dependent and the ABA-independent pathways, respectively. The ABREs were found to be associated with late embryogenesis in wheat seeds (Guiltinan et al., 1990) and expressed in dehydrated rice vegetative tissues (Mundy et al., 1990). The DRE was first identified to be responsible for the rapid response of RD29A to stresses such as dehydration or high salt in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994). The two positive controls, *OsLEA3*-1 and *OsDREB2A*, the expression of which showed significant increases at all three dehydration levels, are related to the ABA-dependent and the ABA-independent pathways, respectively (Shinozaki and Yamaguchi-Shinozaki, 2007).

### 4.2 OsHATs are associated with both pathways

ABA plays central roles in stress responses to abiotic stress such as drought stress as well as seed development and plant growth (Leung and Giraudat, 1998; Finkelstein et al., 2002). Stressors, such as drought, salt, and cold, trigger the biosynthesis and accumulation of ABA (Iuchi et al., 2000), which in turn induces stomatal closure (Schroeder et al., 2001) and global downstream stress related gene transcriptional activation (Yamaguchi-Shinozaki and Shinozaki, 2005). The biosynthesis and function of ABA is regulated by various factors, such as histone acetylation. For instance, in both tobacco and *Arabidopsis*, exogenous ABA treatment causes a dynamic histone H3 and H4 acetylation and phosphorylation change (Sokol et al., 2007). Meanwhile, the expression of constitutively expressed *AtHD2C* (histone deacetylase) is repressed by
ABA in *Arabidopsis* (Sridha and Wu, 2006). However, the overexpression of *AtHD2C* in plants resulted in ABA-insensitivity and enhanced tolerance to salt and drought stresses (Sridha and Wu, 2006). A HAT complex, also known as the elongator, correlates with ABA sensitivity and stomatal closure in *Arabidopsis* (Zhou et al., 2009).

Drought stress causes significant increases in the transcript levels of *OsHAC703*, *OsHAF701*, *OsHAG703*, and *OsHAM701* (Figure 3.4). Additionally, the expressions of *OsHAC703*, *OsHAG703*, and *OsHAM701* showed significant increases after treatment by ABA, whereas no significant difference was observed in *OsHAF701* transcription in rice (Liu et al., 2012). These results indicated that, most likely, *OsHAC703*, *OsHAG703*, and *OsHAM701* are involved in the ABA-dependent response system, while *OsHAF701* is related to the ABA-independent pathway in rice drought-stress responses.

### 4.3 Acetylation level increases after drought treatment

The equilibrium of histone acetylation in plants is a consequence of the regulation of both HATs and HDACs (Kuo and Allis, 1998). The HATs introduce the acetyl groups to confined lysine residues in the amino-terminal tails of the core histones, which cause a relaxation of chromatin compaction and transcriptional activation (Struhl, 1998). On the other hand, HDACs remove the acetyl moiety from core histones and induce chromatin compaction and transcriptional repression (Kuo and Allis, 1998). Among the tested lysine residues, a preference for specific acetylation sites was demonstrated by *OsHAC703* and *OsHAG703* in the early phases of the dehydration process (Figure 3.4 and Figure 3.5).
More specifically, at the first dehydration level, after drought treatment for 24 hours, the RWC was 55±1.5% in the treated group, which is significantly lower than the control group (78.3±0.15%) (Figure 3.2). At this time point, only two of the four OsHATs, OsHAC703 and OsHAG703, showed significantly increased expression. Meanwhile, only the acetylation of H3K18, H3K27, and H4K5 out of five tested sites indicated an enhancement (Figure 3.4 and 3.5). However, the expression of all four OsHATs improved significantly at the second dehydration level (29 hours, 40±2.3%); likewise, all tested residues, including total H3, showed an increase in acetylation level (Figure 3.4 and 3.5).

This correlation was quite different in the last group (33 hours) when the RWC was 36±2.6% and the leaves were sampled in darkness (Figure 3.2). Although the increase in the expression of all four OsHATs stayed significant, increase of acetylation level was only observed on H3K9, H4K5 and total H3 (Figure 3.4 and 3.5). This may be because the switch of light signals affects HDACs expression, which in turn, changes acetylation on H3K18 and H3K27.

4.4 HAT plays a vital role in chromatin remodeling

Hyperacetylation of histones, which is catalyzed by HAT, relaxes the genomic DNA compaction with the neutralization of the positive charge and enhancement of the hydrophobicity of the core histones (Luger and Richmond, 1998), or/and recruitment of the chromatin-associated proteins such as transcriptional factors and RNA polymerase II (Jenuwein and Allis, 2001). In other words, HATs play an important role in the
transcriptional activation of genes, including drought-inducible genes, via chromatin remodeling.

In *Arabidopsis*, the drought-induced increase in the expression of stress-inducible genes *RD29A*, *RD29B*, *RD20* and *RAP2.4*, has been demonstrated to be associated with an increase in H3K9 acetylation of the promoter regions and H3K23 and H3K27 acetylation of the coding regions (Kim et al., 2008). In this research in rice, the expression of two drought-inducible genes, *OsDREB2A* and *OsLEA3-1*, along with four tested *OshATs*, showed significant increases at all dehydration levels (Figure 3.3 and 3.4). Different levels of increase of acetylation on total H3, H3K9, H3K18, H3K27 and H4K5 were also observed (Figure 3.5). Since the transcriptional increase of *OsDREB2A* and *OsLEA3-1* was shown as early as the first sampled group (24 hours), it is highly possible that the transcriptional activation of *OsDREB2A* and *OsLEA3-1* is related to the histone acetylation increase in H3K18, H3K27 and H4K5. In terms of specific regions that are involved in the regulation, for example promoter regions or/and coding regions, further studies such as ChIP (Chromatin Immunoprecipitation) assay may be necessary.
Chapter 5: Perspectives and Long Term Goals

5.1 Perspectives

The expression analysis of the OsHATs under drought stress expands our understanding of the cell and molecular levels of drought stress responses in plants. However, there still remain several outstanding issues/predictions that need to be explored to solidify current observations.

As mentioned above, the direct connection between HATs and the expression of specific regions of drought-inducible genes should be established. More specifically, the acetylation of lysine residues on histone H3 and H4, such as k5, k9, k18, k23, and k27, should be checked to determine whether HATs have a preference in lysine residues to which they add the acetyl moiety. The ChIP assay is one of the most efficient and accurate techniques for such experiments.

In addition, clearer connections between plant stress tolerance, including drought tolerance, and the OsHATs function could be indicated by genetic manipulations, such as over-expression and gene mutation.

Finally, previous studies have reported that ABA correlates with dynamic histone acetylation and HATs expression in plants (Sokol et al., 2007; Papaefthimiou et al., 2010), which suggests that histone acetylation and HATs are involved in the regulation of ABA signaling pathways. However, detailed information about which step within this pathway, such as ABA biosynthesis, ABA translocation, and ABA functioning, that
histone acetylation or HATs play a part in is still unknown. This is very important since ABA plays vital roles in both plant growth and development (Finkelstein et al., 2002) and stress responses (Leung and Giraudat, 1998).

5.2 Long term goals

The final goal of agricultural sciences is to improve the quality and yield of crops to meet the rapidly growing demands from the continually increasing human population. Drought stress, one of the most common stresses in the natural environment, is a global issue that causes crop loss. The main objective for this research is to understand the role that HATs play in drought stress responses in rice, which brings an insight into the molecular mechanisms governing plant drought stress response and tolerance in crop plants. By exploring this and similar ideas, genetic manipulation, such as over-expression of the OsHATs, could be used to improve drought stress tolerance in rice and ultimately lead to enhancement of global rice yields.
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Figure A-1 Drought stress was induced in two-leaf-stage rice seedlings for 24, 29 and 33 hour.

(A) to (C) Rice seeds were germinated on petri dishes and transferred to soil. After about a week, drought stress was induced by withholding water.

(D) to (F) Seedlings looked wilted in 24, 29 and 33 hour treatment groups.

(G) Seedlings look fresh in control groups
Table A-2 Amplification efficiency and co-efficiency for designed primers in real-time qPCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Amplification efficiency</th>
<th>Coefficient($R^2$)</th>
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<td><em>Ubq-1</em></td>
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<td>0.993</td>
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<td><em>OsHAC703</em></td>
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<tr>
<td><em>OsHAM701</em></td>
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<td>0.996</td>
</tr>
<tr>
<td><em>OsHAG703</em></td>
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<td>0.997</td>
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### Table A-3 Drought-related cis-elements in promoter regions of *OsHATs* from the PLACE database

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<th>Factor or Site namea</th>
<th>Site numberb</th>
<th>Signal sequence</th>
<th>Related stresses</th>
<th>HAC703</th>
<th>HAF701</th>
<th>HAG703</th>
<th>HAM701</th>
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a: Factors or sites according to their specific *cis*-acting regulatory elements

b: Unique number for each motif in the PLACE database

Abbreviations: LEA: late embryogenesis abundant; ERD: early responsive to dehydration; ABI: abscisic acid (ABA)-insensitive; DRE/CRT: dehydration-responsive element /C-repeat; RAB: responsive to ABA; RD: responsive to dehydration.
Figure A-4 Change of histone acetylation on lysine residues of histone H3 or H4 in response to drought treatment for 24, 29 and 33 hours in rice leaves.

Proteins were extracted from leaves of two-leaf-stage rice seedlings treated subjected to drought for 29 hours or watered regularly. Western blot analysis was performed with specific antibodies (Table 2.2). The figure shows bands from scanned films demonstrating the amount of protein that antibodies bind to in the western-blot analysis. Histone H3 was used as a loading control.
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</table>
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**Awards and Scholarships**

2011 – 2013 Western Graduate Research Scholarship