Biochemical and Biophysical Research Communications 443 (2014) 400–405

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Expression analysis of histone acetyltransferases in rice under drought stress

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ARTICLE INFO

Article history:
Received 15 November 2013
Available online 2 December 2013

Keywords:
Histone acetyltransferase
Histone acetylation
Rice
Drought stress

ABSTRACT

Histone acetylation is one of the vital reversible modifications of chromatin structure that regulates gene expression in eukaryotes. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) maintain the homeostasis of histone acetylation. Studies in *Arabidopsis* have revealed that HATs are involved in plant responses to various stresses including light, temperature, salt and ABA. Drought stress, a very common environmental stress, could cause a range of physiological and biochemical responses in plants involving HATs. Eight *HATs* in four different families (CBP, GNAT, MYST, and $TAF_{II}250$ family) are known in rice. In this research, four *OsHATs*, one from each family, were chosen based on *in silico* domain and promoter analysis for their response under drought conditions. Drought stress was introduced to two-leaf-stage rice seedlings. The effectiveness of drought treatment was confirmed by the measurement of relative water content (RWC). Real-time quantitative polymerase chain reaction analysis demonstrated that drought stress caused a significant increase in the expression of four *HATs (OsHAC703*, *OsHAG703*, *OsHAF701* and *OsHAM701)* in rice plants. Additionally, the Western-blot analysis showed that the acetylation level on certain lysine sites of H3 (lysine 9, lysine 18 and lysine 27) and H4 (lysine 5) increased with *OsHATs* expression. The significant increase in the transcript levels of *OsHATs* and the acetylation level of lysine residues on Histone H3 and H4 suggest that *OsHATs* are involved in drought stress responses in rice.

1. Introduction

In order to constrict DNA into the limited space in nucleus, DNA is tightly folded into a complex structure called chromatin in eukaryotes [1]. Histone modification, together with DNA methylation and nucleosome remodeling, regulates chromatin remodeling to control DNA accessibility [2,3]. One of the most important histone modifications is acetylation, which is a reversible modification regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [4]. Hyperacetylation of histones induces DNA relaxation and transcriptional activation, whereas weak acetylation leads to chromatin compaction and transcriptional repression [5]. There are two different proposals explaining this phenomenon. The introduction of acetyl groups to conserved lysine residues neutralizes the positive charge and reduces their affinity to the negatively charged DNA [6]. Alternatively, the "histone code" hypothesis proposes that covalent modifications, including acetylation and methylation, could work sequentially and jointly to change the interaction between chromatin and chromatin-associated proteins and provide signals for recruitment of transcriptional machinery [7].

Previous research on plant HATs is mainly on *Arabidopsis* in which 12 OsHATs have been identified that can be classified into four families (CBP, GNAT, MYST, and $TAF_{II}250$ family) [8]. In *Arabidopsis*, HATs, such as AtHAG1 which is a member of the Gcn5 subfamily of the GNAT family, play pivotal parts in plant growth and development [9]. Additionally, studies in *Arabidopsis* revealed that *HATs* are involved in plant responses to various stresses including light stress [10], temperature stress [11,12], salt stress [13] and ABA stress [14,15].

Rice is an economically important monocot that shares common stress inducible genes with *Arabidopsis* [16]. Drought stress, a very common stress that is caused by water deficit, causes a series of physiological and molecular responses in plants [17]. Drought stress has shown to be the major factor of rice yield loss in Asia [18]. Additionally, it has been shown in *Arabidopsis* that

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0006-291X
http://dx.doi.org/10.1016/j.bbrc.2013.11.102

histone acetylation plays an important part in the induction of drought-inducible genes under drought stress [19]. In rice, eight OsHATs have been identified and grouped into the CBP (OsHAC701, OsHAC703, and OsHAC704), $TAF_{II}250$ (OsHAF701), GNAT (OsHAG702, OsHAG703, and OsHAG704), and MYST (OsHAM701) families [20]. However, there as yet is no direct information on the relationship between drought stress and expression of HATs in rice.

To test whether drought stress causes expressional change of *OsHATs* directly, four *OsHATs*, one from each family, were chosen based on *in silico* domain and promoter analysis. Real-time qPCR analysis was performed to test the expression pattern of this four *OsHATs*. Western-blot analysis using different antibodies against total acetylated H3, specific lysine residues on H3 (K9, K18 and K27) and H4 (K5) clarified the acetylation levels.

2. Materials and methods

2.1. Promoter analyses

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

2.2. Plant growth conditions and drought treatment

Rice (*Oryza sativa* ssp. *japonica* cv. Nipponbare) was used in the research. After being imbibed with distilled water in darkness for 24 h at 37 °C, rice seeds were placed on two filter papers soaked with distilled water in a Petri dish at room temperature. Two days after germination in darkness, the germinating seeds were transferred to the light condition with 330 μmoles/m^2/s. After another 2 days, when the length of the seedling roots were 2–3 cm, rice seedlings were planted into clay soil in a growth chamber and plantlets were maintained at 9/15 h light/dark photoperiod at 29/24 °C.

Seven days after germination, the rice seedlings were at their two-leaf stage. Plants were distributed into two groups. The drought treatment group was subjected to drought stress by withholding water, while the control group was watered twice each day. To prevent rapid water loss and to retain viability, the plants were covered with a transparent plastic lid after 29 h for the drought treatment group, while seedlings in the control group were always covered with a plastic lid.

2.3. RWC measurement

To assess the intensity of the drought stress, the relative water content (RWC) [21] of leaves was measured. Immediately after sampling the leaves of the drought treatment and the control plants, leaves were excised and weighed to give the fresh weight (W_{fresh}). This leaf was then placed into a 50 °C oven for 24 h to give the dry weight (W_{dry}). RWC was calculated according to the following equation:

$$RWC = (W_{fresh} - W_{dry})/W_{fresh}$$

2.4. RNA isolation and real-time qPCR analyses

Total RNA was extracted from leaves of rice seedlings using a Plant/Fungi Total RNA Purification Kit (Norgen). The quality and quantity of RNA were measured by a Thermo Scientific NanoDrop™ 1000 spectrophotometer (Wilmington, DE, USA). Before cDNA synthesis, the total RNA was treated with DNaseI (Norgen) for 20 min. The first strand cDNA was synthesized from 2 μg RNA with the ThermoScript™ RT PCR System (Life Technologies) with oligo-dT primer. The synthesized cDNA then served as a template for real-time qPCR using SsoFast™ EvaGreen® Supermix Kit (Bio-Rad) and data were collected in a Bio-Rad C1000™ Thermal Cycler with the CFX96™ Real-time PCR System. *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 [22–24], were selected as positive controls to determine whether the drought treatment was effective. The primers designed for real-time qPCR are listed in Table S1.

2.5. Protein isolation and Western-blot analyses

Acid-soluble proteins were extracted following Tariq et al. [25], in which a total of 0.3 g fresh rice leaves were crushed in liquid nitrogen and suspended 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 phenylmethylsulfonyl fluoride). Total proteins were homogenized by a Fisher Scientific Model 100 Sonic Dismembrator for 2 min and then centrifuged for 15 min (4 °C at 20,000 rcf, twice), and the supernatant was collected and stored at −80 °C. The quantitative analysis of protein was determined by the Micro-Bradford Assay using a Biochrom Novaspec Plus Visible Spectrophotometer before being used for SDS–PAGE electrophoresis. Precisely 5 μg protein were added to 18.5 mM dithiothreitol, separated on a 16% (w/v) sodium dodecyl sulfate polyacrylamide electrophoretic gel, and transferred to an Immun-Blot™ polyvinylidene fluoride Membrane (Bio-Rad) using a Trans-Blot Semi-Dry electrophoretic Transfer Cell (15 V, 50 min, Bio-Rad). The N-terminal lysine residues on histones H3 and H4 were detected using commercial antibodies and secondary antibodies from Cell Signaling and Millipore (Table S2). 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) and exposed to Classic Single-Emulsion Autoradiography Film (Mandel Scientific). The films were then 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 [60 mM 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 the blocking of another antibody test.

3. Results and discussion

3.1. Various drought related cis-elements present in the four OsHATs

cis-acting regulatory elements, which are the usual binding sites for one or more *trans*-acting factors, affect gene expression [26]. A search for *cis*-elements could provide an important index of the involvement of genes in different stress responses. The online database, PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html), was used to analyze the promoter regions of *OsHAC703, OsHAG703, OsHAF701* and *OsHAM701*. Various *cis*-acting regulatory elements were identified in the promoter regions of these four *OsHATs*. The number and function of these *cis*-elements vary. Dehydration stress and ABA related *cis*-acting regulatory elements were both discovered from the promoter analysis of all four

Table 1
Drought-related *cis*-elements in promoter regions of *OsHATs* from the PLACE database.

Factor or site name[a]	Site number[b]	Signal sequence	Related stresses	*HAC703*	*HAF701*	*HAG703*	*HAM701*
ABREA2HVA1	S000140	CCTACGTGGC	ABA, water stress (LEA)	–	–	–	1
ABREATCONSENSUS	S000406	YACGTGGC	ABA	–	–	–	2
ABRELATERD1	S000414	ACGTG	Dehydration (ERD1)	2	1	5	6
ABREZMRAB28	S000133	CCACGTGG	ABA, water stress, cold stress	–	–	–	2
ACGTATERD1	S000415	ACGT	Dehydration (ERD1)	6	4	12	10
DPBFCOREDCDC3	S000292	ACACNNG	ABA (ABI5-LEA)	3	1	2	–
DRECOREZMRAB17	S000401	ACCGAGA	Drought (DRE1-RAB17)	–	–	2	1
DRECRTCOREAT	S000418	RCCGAC	Drought stress (DRE/CRT), cold stress	–	–	2	–
MYB1AT	S000408	WAACCA	Dehydration (MYB-RD22)	3	2	–	3
MYB2CONSENSUSAT	S000409	YAACKG	Dehydration (MYB-RD22)	3	–	–	2
MYBCORE	S000176	CNGTTR	Water stress (MYB)	6	1	–	5
MYCATERD1	S000413	CATGTG	Dehydration (MYC-ERD1)	–	1	–	2
MYCATRD22	S000174	CACATG	Dehydration (MYC-RD22)	–	1	–	2
MYCCONSENSUSAT	S000407	CANNTG	ABA, dehydration stress, cold stress	14	8	4	12

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.
[a] Factors or sites according to their specific *cis*-acting regulatory elements.
[b] Unique number for each motif in the PLACE database.

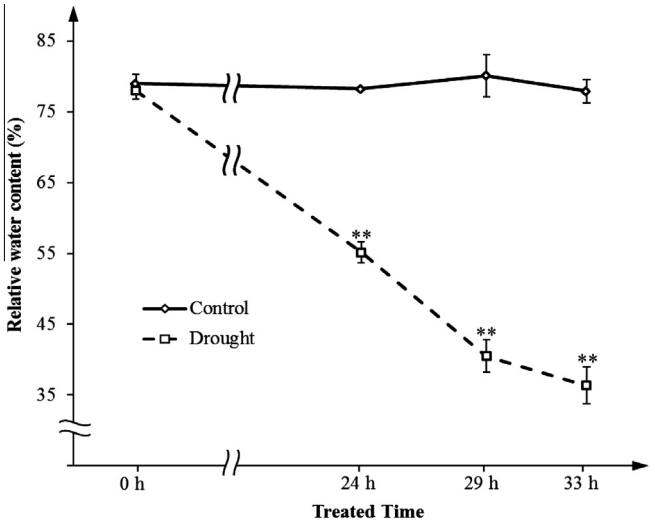

Fig. 1. Change in RWC in rice leaves after dehydration treatment. The results are the means of three replicates ±SD. Two sample t-tests were used for data analysis, and ** indicates a significant difference at $p < 0.01$.

HATs (Table 1). Based on the existence of these drought related *cis*-acting regulatory elements, it is very likely that all four *OsHATs* are involved in drought stress and ABA stress responses in rice.

3.2. RWC decreased significantly after drought treatment

To evaluate the drought stress, RWC was used as an indicator of the intensity of the dehydration. As demonstrated in Fig. 1, in the treatment group, the RWC decreased from 55 ± 1.5% to 40 ± 2.3% in the first 5 h while in the last 4 h, 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) (Fig. 1). Compared to control groups, the RWC in all three dehydration levels decreased significantly. The decrease in RWC indicated that the drought stress was effective.

3.3. Expression of drought-inducible genes OsDREB2A and OsLEA3-1 was induced

The expression of both *OsDREB2A* and *OsLEA3-1* were induced significantly at all dehydration levels (Fig. 2). Transcriptome

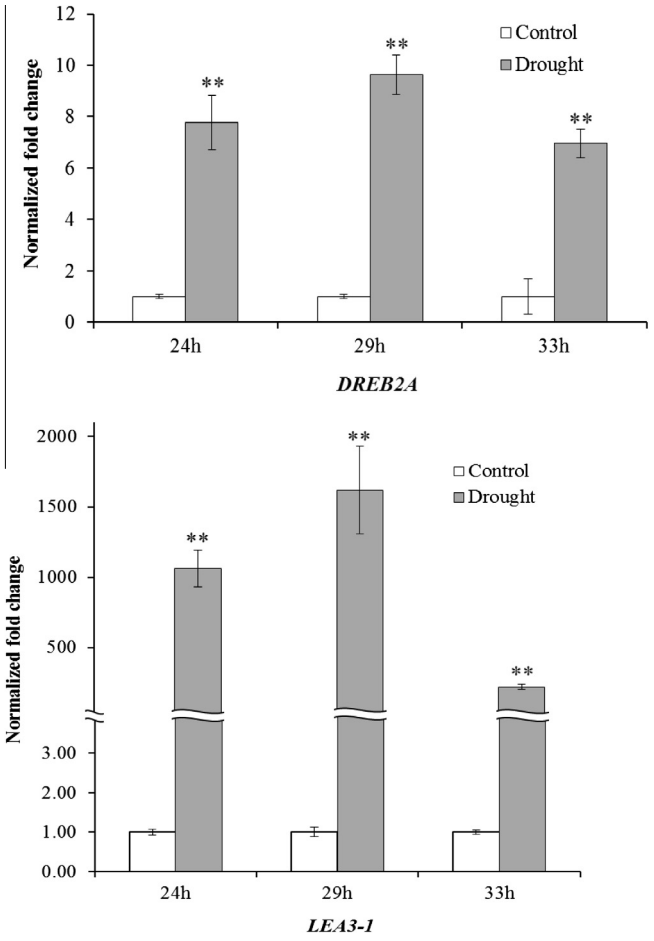

Fig. 2. Expression of drought stress inducible genes, *OsDREB2A* and *OsLEA3-1*, under drought stress conditions. Total RNAs were extracted from leaves of two-leaf-stage rice seedlings treated with drought (grey bars) or without (white bars) for 24, 29 and 33 h. The values of treated groups were normalized to their corresponding controls, which were defaulted as 1. Data in this figure were means of three replicates ±SD. Two sample *t*-tests were used for significance analyses, ** indicates a significant difference at $p < 0.01$.

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 [27]. 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 [22]. 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 [28]. In rice, both *OsDREB2A* and *OsLEA3-1* are drought-inducible, and the over-expression of both genes could significantly enhance the drought stress resistance [24,29]. The induction of both *OsDREB2A* and *OsLEA3-1* further confirmed the effectiveness of the drought stress treatment in this study. In addition, it indicated the induction of both ABA-dependent and ABA-independent drought response after drought treatment.

3.4. Expression change patterns of OsHATs are different under drought stress

Different expression patterns were demonstrated among these four *OsHATs* at three different dehydration levels. Firstly, after watering was withheld for 24 h, the expression of *OsHAC703* and *OsHAG703* firstly showed a similar expression pattern comparing to the positive control as both increased to approximately two times. In contrast, the transcript level of *OsHAM701* did not change and the *OsHAF701* expression showed a significant decrease (Fig. 3). For the second dehydration level, after water was withheld for 29 h, the expression levels of *OsHAC703, OsHAG703, OsHAM701* and *OsHAF701* all increased significantly to 2.7-fold, 2.2-fold, 5.3-fold and 7.7-fold, respectively (Fig. 3). At the last dehydration level, the expression levels of *OsHAC703, OsHAG703*, *OsHAM701* and *OsHAF701* still increased significantly (Fig. 3). Taken all together, the expression of all four *OsHATs* examined was significantly induced after drought treatment. Nonetheless, various change patterns were demonstrated. Firstly, *OsHAC703* and *OsHAG703* showed faster responses to drought treatment than *OsHAM701* and *OsHAF701*. Additionally, among the three different time points (24, 29 and 33 h), the expressional change pattern of *OsHAF701* showed a difference from the other three genes (Fig. 3). Instead of gradual increase among the three dehydration level, 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 (Fig. 3). These results indicated that *OsHAC703, OsHAG703, OsHAM701* and *OsHAF701* are all involved in the drought stress response in rice.

In addition, the expression of *OsHAC703*, *OsHAG703*, and *OsHAM701* showed significant increases after treatment by ABA, whereas no significant difference was observed in *OsHAF701* transcription in rice [20]. ABA plays central roles in stress responses to abiotic stress such as drought stress as well as seed development and plant growth [30,31]. Stressors, such as drought, salt, and cold, trigger the biosynthesis and accumulation of ABA [32], which in turn induces stomatal closure [33] and global downstream stress related gene transcriptional activation [27]. Histone acetylation is regulated by the biosynthesis and function of ABA. For instance, in both tobacco and *Arabidopsis*, exogenous ABA treatment causes a dynamic histone H3 and H4 acetylation and phosphorylation change [13]. Meanwhile, the expression of constitutively expressed *AtHD2C* is repressed by ABA in *Arabidopsis* [34]. These results

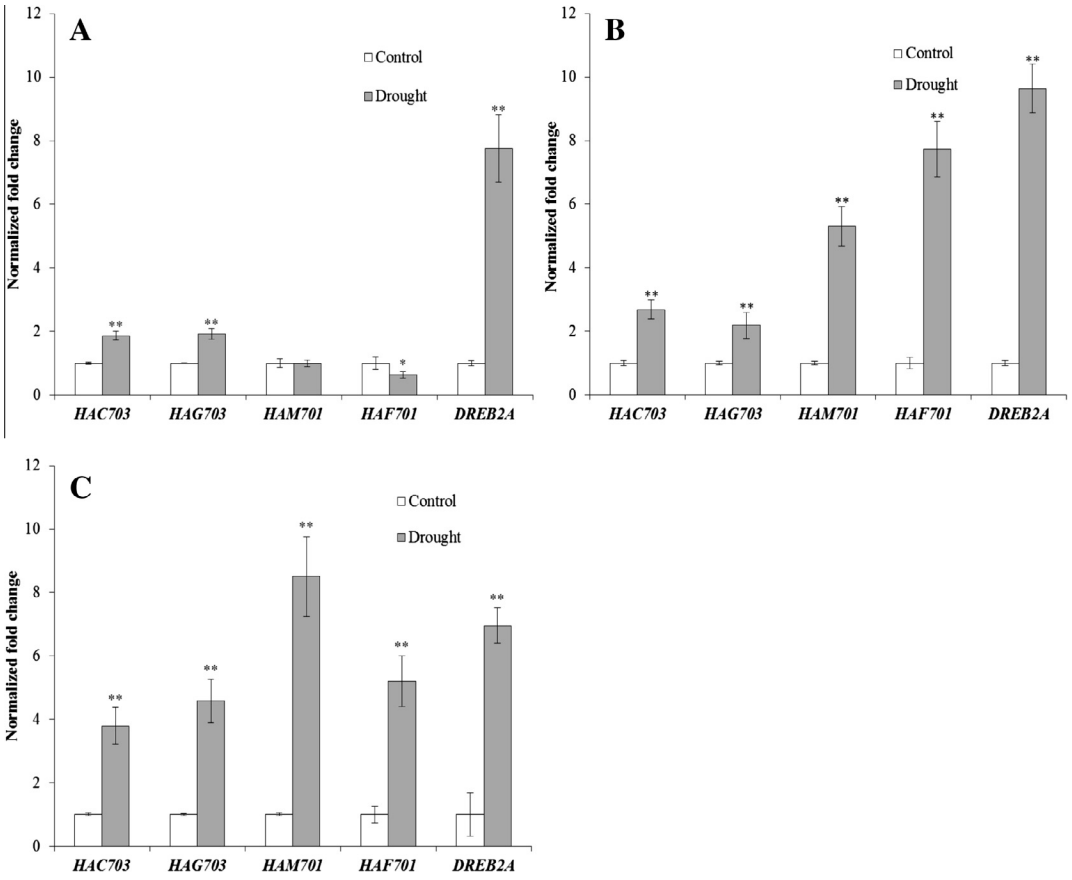

Fig. 3. Expression patterns of *OsHATs* in rice leaves among different drought treatments. Total RNAs were extracted from leaves of two-leaf-stage rice seedlings treated with drought (grey bars) or without (white bars) for 24 h (A), 29 h (B) and 33 h (C). The values of treated groups were normalized to their corresponding controls, which were defaulted as 1. Data in this figure were means of three replicates ±SD. Two sample t-tests were used for significance analyses, $*$ and $**$ indicates a significant difference at $p < 0.05$ and $p < 0.01$, respectively.

demonstrated that *OsHAC703*, *OsHAG703*, and *OsHAM701* are most likely involved in the ABA-dependent response system, while *OsHAF701* is associated with the ABA-independent pathway in rice drought–stress responses.

3.5. Acetylation on certain lysine residues was increased

With regard to the protein level, Western blot analysis showed that the acetylation of histone H3K18, H3K27, and H4K5 was elevated significantly compared to the control group, while no increase in the acetylation level of histone H3K9 and total H3 was observed after treatment by drought for 24 h (Fig. 4). In response to drought treatment for 29 h, the acetylation level of total H3, histone H3K9, H3K18, H3K27, and H4K5 all showed considerable increase (Fig. 4). For the last dehydration level, Western blot analysis demonstrated that the acetylation of total H3, histone H3K9, H3K27, and H4K5 stayed elevated, whereas no difference in the acetylation of histone H3K18 was found (Fig. 4).

Among the tested lysine residues, a preference for specific acetylation sites was demonstrated in the early phases of the dehydration process (Figs. 3 and 4). More specifically, at the first dehydration level, only two of the four *OsHATs*, *OsHAC703* and *OsHAG703*, showed significantly increased expression. Meanwhile, only H3K18, H3K27, and H4K5 showed an acetylation enhancement (Figs. 3 and 4). On the other hand, the expression of all four *OsHATs* increased significantly at the second dehydration level (29 h, 40 ± 2.3%); likewise, all tested residues, including total H3, showed an increase in acetylation level (Figs. 3 and 4). Based on these results, it is highly possible that the acetylation increase of H3K18, H3K27, and H4K5 at the first dehydration level is related to the mRNA increase of *OsHAC703* and *OsHAG703*. However, the equilibrium of histone acetylation is a consequence of the regulation of both HATs and HDACs [4], which means the decrease of HDAC could also contribute to the increase of acetylation.

This study showed that *HATs* are involved in drought stress responses in rice as analyzed by gene expression and acetylation levels. The knowledge advancement on 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. It is hoped that this will eventually lead to a long-term improvement of drought stress tolerance in crops.

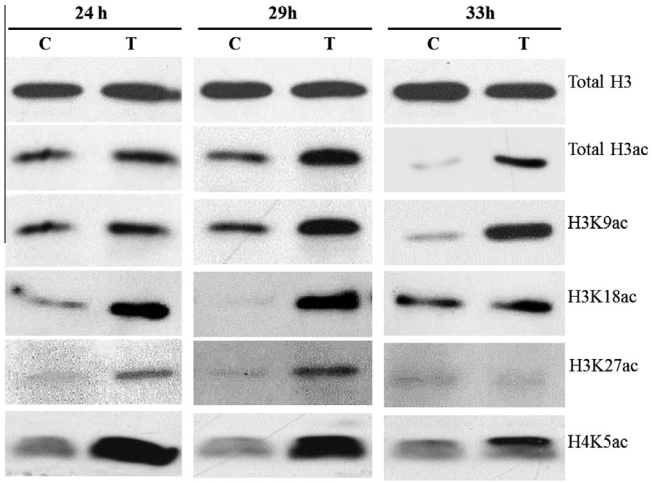

Fig. 4. Change of histone acetylation on lysine residues of histone H3 or H4 in response to drought treatment for 24, 29 and 33 h in rice leaves. Proteins were extracted from leaves of two-leaf-stage rice seedlings treated with drought (grey bars) or without (white bars) for 24, 29 and 33 h. Western blot analysis was performed with specific antibodies (Table S2). 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.

Acknowledgments

We thank Dr. Yanjie Luo, Qingshi Lu, Dr. Gary Tian and Jiang Wang for technical support, Dr.Keqiang Wu, Dr. Norm Hüner and Dr. Frédéric Marsolais for helping with experimental design and Dr. Frédéric Marsolais for providing real-time qPCR equipment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.102.

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