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Expression analysis of histone acetyltransferases in rice under drought stress ☆

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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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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